Université de Montréal

Knockout Mouse Model Generated by CRISPR Technology to Study the Function of BSP Proteins on Male Fertility *in vivo*

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Ce mémoire intitulé :

Modèle de souris knockout généré par la technologie CRISPR pour étudier le rôle des protéines BSP dans la fertilité masculine *in vivo*

Présenté par
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Abstract

Infertility is a worldwide problem that affects 15% of couples, with male and female factors contributing equally to cases of infertility. The cause of male infertility in half of the cases is unknown. A series of coordinated cellular events are responsible for sperm-egg interaction and successful fertilization. Therefore, elucidating the mechanisms involved in sperm production and maturation is critical to facilitate the diagnosis and treatment of male infertility, as well as the regulation of fertility. Over the last three decades, the laboratory of Dr. P. Manjunath has been extensively investigating a particular family of proteins that is expressed specifically in the male reproductive tract, called the Binder of SPerm (BSP) proteins. This protein family was first identified in bovine seminal plasma and subsequently, BSP proteins were identified in other mammalian species such as stallion, boar, ram and goat. Depending on the species, these small ubiquitous proteins are expressed by the seminal vesicles and/or epididymis. Systematic work done by Manjunath's group has demonstrated that these proteins bind to choline phospholipids on the outer leaflet of the sperm membrane upon ejaculation and/or during passage through the epididymis and promote sperm capacitation. In 2007, BSP-homologous genes were identified in human (*BSPH1*) and mouse (*Bsph1* and *Bsph2*) epididymis. Further studies indicated that the interaction of BSP proteins with the sperm membrane results in lipid (phospholipid and cholesterol) alterations and facilitates epididymal sperm maturation. Additional research on BSP proteins suggested that these proteins may play an important role in male fertility. However, the exact role of BSP proteins in fertility has remained largely unknown. In the current study, I generated the first single and double BSP knockout mouse model to investigate whether the absence of BSP proteins could affect male fertility. To target and generate single and double

knockout *Bsph1* and *Bsph2* mice models, the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR/Cas9) approach was utilized. I used RT-PCR, Digital Droplet-PCR (dd-PCR) and Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) techniques to examine *Bsp* gene expression at the mRNA and protein levels and confirm whether BSP proteins were completely eliminated in knockout animals. The knockout BSP mouse lines were used to evaluate sperm parameters of fertility. No obvious abnormalities were observed in the fertilizing ability nor in the number of pups for knockout compared to wild type male mice. Sperm count and motility analyses indicated no significant differences between BSP-null mice compared to control. Interestingly, significant differences in terms of the pup's body weight were observed in double knockout compared to wild type.

Based on these results, we conclude that BSP proteins, individually or together, are not essential for proper sperm function and fertilization in mice, and the absence of these proteins does not affect male fertility. Further studies could provide a better understanding of the mechanisms involved and possible pathways by which BSP proteins could be involved in regulating pup weight.

Keywords: Male infertility, Binder of SPerm (BSP) proteins, Epididymal proteins, Knockout Mice.

Résumé

L'infertilité est un problème mondial qui touche 15% des couples, les facteurs masculins et féminins contribuant de façon égale aux cas d'infertilité. La cause de l'infertilité masculine est inconnue dans la moitié des cas. Une série d'événements cellulaires coordonnés est responsable de l'interaction spermatozoïde-ovocyte et de la fécondation réussie. Par conséquent, l'élucidation des mécanismes impliqués dans la production des spermatozoïdes est essentielle pour faciliter le diagnostic et le traitement de l'infertilité masculine, ainsi que la régulation de la fertilité. Au cours des trois dernières décennies, le laboratoire du Dr P. Manjunath a mené des recherches approfondies sur une famille particulière de protéines exprimées spécifiquement dans l'appareil reproducteur masculin, appelées protéines *Binder of SPerm* (BSP). Cette famille de protéines a d'abord été identifiée dans le plasma séminal bovin, et par la suite, des protéines BSP ont été identifiées chez d'autres espèces de mammifères telles que l'étalon, le verrat, le bélier et la chèvre. Selon les espèces, ces petites protéines ubiquitaires sont exprimées par les vésicules séminales et/ou l'épididyme. Des travaux systématiques effectués par le groupe de Manjunath ont démontré que ces protéines se lient aux phospholipides portant un groupement choline sur le feuillet externe de la membrane des spermatozoïdes lors de l'éjaculation et/ou pendant le passage à travers l'épididyme, et favorisent la capacitation des spermatozoïdes. En 2007, les gènes BSP-homologues ont été identifiés dans l'épididyme humain (*BSPH1*) et de souris (*Bsph1* et *Bsph2*). D'autres études ont montré que l'interaction des protéines BSP avec la membrane des spermatozoïdes entraîne une altération des lipides (phospholipides et cholestérol) et facilite la maturation des spermatozoïdes épididymaires. D'autres recherches sur les protéines BSP ont suggéré que ces protéines pourraient jouer un rôle important dans la fertilité masculine. Cependant, le rôle exact des protéines BSP dans la fertilité est resté largement inconnu. Dans le

cadre de la présente étude, j'ai créé le premier modèle de souris BSP *knockout* simple et double pour déterminer si l'absence de protéines BSP pouvait affecter la fertilité masculine. Pour cibler et générer des modèles de souris *knockout* simple et double *Bsph1* et *Bsph2*, l'approche de courtes répétitions palindromiques regroupées et régulièrement espacées (CRISPR/Cas9) fut utilisée. De plus, les techniques RT-PCR, Digital Droplet-PCR (dd-PCR) et chromatographie liquide – spectrométrie de masse en tandem (LC-MS/MS) ont permis d'examiner l'expression des gènes Bsp au niveau de l'ARNm et des protéines afin de confirmer l'élimination des protéines BSP chez les souris *knockout*. Les lignées de souris BSP *knockout* ont été utilisées pour évaluer les paramètres de fertilité des spermatozoïdes. Aucune anomalie évidente n'a été observée dans la capacité de fertilisation ni dans le nombre de souriceaux engendrés par les mâles *knockout* par rapport au type sauvage. Le décompte du nombre de spermatozoïdes et l'analyse de leur motilité n'ont révélé aucune différence significative entre les souris BSP *knockout* et les souris contrôle. Il est intéressant de noter que des différences significatives en termes de poids corporel des souriceaux ont été observées dans le double *knockout* par rapport au type sauvage.

Sur la base de ces résultats, nous concluons que les protéines BSP, individuellement ou ensemble, ne sont pas essentielles aux fonctions spermatiques et pour la fécondation chez la souris, et que l'absence de ces protéines n'affecte pas la fertilité masculine. D'autres études pourraient permettre de mieux comprendre les mécanismes impliqués et les voies possibles par lesquelles les protéines BSP pourraient être impliquées dans la régulation du poids des souriceaux.

Mots-clés : Infertilité masculine, Protéines *Binder of SPerm Homolog*, épididyme, souris *Knockout*.

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List of acronyms

~ ..... approximately

% ..... percent

e.g. ..... for example

i.e. ..... that is

µl ..... microliter

µg ..... microgram

µM ..... micromolar

°C ..... degrees Celsius

kDa ..... kilodalton

mM ..... millimolar

cm ..... centimeter

mg ..... milligram

List of abbreviations

ADAM..... A disintegrin and metalloprotease

AI ..... Artificial Insemination

AR ..... Acrosome reaction

ART..... Assisted Reproductive Technologies

BSP ..... Binder of SPerm

BSPH..... Binder of SPerm Homolog

Ca^{2+} ..... Calcium

cDNA ..... Complementary DNA

CLU..... Clusterin

CRISP..... Cysteine-rich secretory protein

CRISPR/Cas9 ..... Clustered Regulatory Interspaced Short Palindromic Repeats

DKO ..... Double knockout

DNA ..... Deoxyribonucleic acid

ddPCR ..... Digital droplet PCR

DSB..... Double-strand breaks

ExPASy ..... Expert Protein Analysis System

Fn2 ..... Fibronectin type II domain

GAG..... Glycosaminoglycan

GLB1..... β-galactosidase

GPI ..... Glycosylphosphatidylinositol

GSN..... Gelsolin

HDL ..... High-density lipoprotein

ICSI ..... Intra-cytoplasmic sperm injection

IVF ..... *in vitro* fertilization

KO..... Knock-out

LC/MS..... Liquid chromatography–mass spectrometry

LCN5, E-RAPB ..... lipocalin 5

LBRs ..... Live birth rates

LTF ..... Lactoferrin

Min ..... Minute

NPC2 ..... Niemann–Pick disease type C2

PC ..... Phosphatidylcholine

PCR ..... Polymerase chain reaction

rec-BSPH ..... Recombinant Binder of SPerm Homolog

RT-PCR ..... Reverse transcription PCR

SCA ..... Sperm Class Analyser

SDS-PAGE ..... Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SP ..... Seminal plasma

SSNs ..... Site-Specific Nucleases

s ..... Second

TALENs ..... Transcription activator-like protein nucleases

TF ..... Transferrin

WHO ..... World Health Organization

ZFNs ..... Zinc-finger nucleases

ZP ..... Zona pellucida

ZP3 ..... Zona pellucida glycoprotein 3

<< It makes you more of what you are >> -Rumi

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1. Introduction

Fertilization is a species-specific physio-chemical process, which is defined as a process of fusion between the male and female gametes to produce a new cell called the zygote. The development of the zygote results in a new individual and guarantees the survival of the species [1]. Reproduction is the basis of life and a necessity for the survival of species. Two forms of reproduction exist: asexual and sexual reproduction. In asexual reproduction, offspring arise from a single parent, to which the progeny are genetically identical [2]. Mammals produce offspring through sexual reproduction, which requires two individual parental gametes from the same species and opposite sexes. Each gamete provides half of the genetic material and the combination of the male gamete (sperm) with female gamete (oocyte) results in the creation of a new organism with equal parental genetic contribution [3]. The results of several studies point to an increased male infertility. The sources of male infertility are varied and 30-40% of men presenting to a fertility clinic will receive a diagnosis of idiopathic infertility [4, 5]. It is therefore important to better understand the molecular mechanisms underlying reproductive process to allow the development of appropriate treatments for these men. This thesis will focus only on the male fertility, the spermatozoon, following its path and the mechanisms which, according to the synthesis of sperm maturation and allow them make them capable of fertilizing an egg.

2. Infertility

Disorders in the reproductive system lead to infertility problems. Infertility is a complex and multifactorial problem that has important effects on health and social issues [6]. Based on the World Health Organization (WHO), infertility is defined as observing no pregnancy following

twelve months of regular unprotected intercourse and is considered as a disease of the reproductive system [7]. In recent decades, an alarming decline in human fertility was observed. Indeed, the latest statistics show that in Canada, 15% of couples are affected by infertility, which is about one in six couples [8]. Reproductive problems can be caused by the man or the woman, or sometimes by both. Infertility can be a frustrating issue and has a massive impact on infertile couples. It can affect the relationship and lead to social isolation, emotional pressure, stress, anxiety, depression and other psychological impacts. Research shows that three-quarters of infertile couples are likely to get divorced [9].

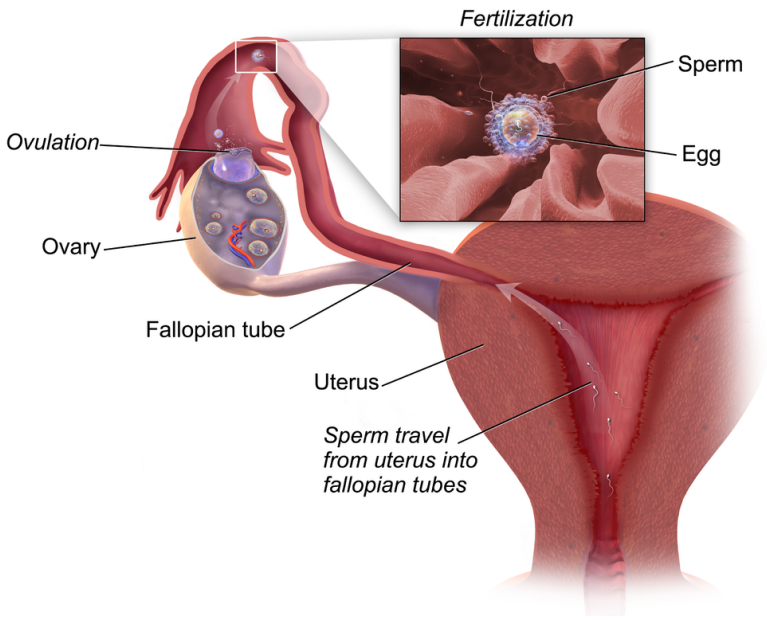

Figure 1: Schematic representation of the fertilization site inside the female reproductive tract. Fertilization usually takes place in the upper third of the fallopian tube. Human sperm are deposited into female reproductive tract, where, sperm swin trought the uterus and uterotubal junctions to reach the fallopian tubes and fertilize the ovulated oocyte oocyte. (Adopted from [10]).

In the industrialized world, enormous changes in social life have led to a remarkable decline in fertility, which has become a worldwide problem [11]. Approximately 15% of couples are suffering from infertility problems, and male and female factors are almost equally responsible for cases of infertility [12]. Primary infertility refers to couples who fail to achieve conception despite at least one year of having sex without using birth control methods. In secondary infertility, the couples have been able to achieve pregnancy at least once, but are subsequently unable to become pregnant [13].

In view of declining fertility rates over the past 20 years, assisted reproductive techniques (ART) have been developed to help couples who are suffering from infertility issues [14]. The first human baby born through *in vitro* fertilization (IVF) is named Louise Brown and was born in 1978 [15]. Thereafter, the number of annual births by ART has been constantly growing.

In order to enhance the success of fertilization, different types of ART have been developed for the different causes of infertility. These include artificial insemination (AI), IVF and intracytoplasmic sperm injection (ICSI) [16]. Couples whose infertility is due to male factors such as low sperm motility or abnormal sperm morphology, may benefit from ICSI, whereas couples diagnosed with female factor infertility may achieve fertilization success with AI or IVF. AI is the least invasive treatment and is used to treat couples who have difficulties with intercourse, or women with normal fallopian tubes but who are unable to conceive due to unknown reasons. In this method, fresh sperm are artificially inserted into the woman's uterus. The most commonly used method in fertility clinics is IVF, which is employed in a variety of circumstances. In IVF, gametes from parents are collected and fertilized *in vitro,* then the fertilized egg is developed and the embryo is transferred into the woman's uterus. ICSI is most often used in cases where the sperm is unable to fertilize the egg for various reasons. In this

treatment, morphologically normal sperm will be selected by a specialist and injected directly into the oocyte. Despite the high cost of ART treatments (~£2,000 per ovulation cycle in the United Kingdom and ~$8,000 in the United States), the use of ART is rising and each year 2 to 3% of births are the result of ART [17]. Several stages of natural selection are bypassed in artificial reproductive treatments, especially in ICSI. Thus, a variety of criteria need to be evaluated to assess the potential risks that could affect the mothers and their offspring in the short and long term [18].

A better understanding of the molecular mechanisms and factors implicated in the male reproductive process could improve existing ART methods and lead to the development of new effective methods of contraception. For all these reasons, this thesis will focus on male fertility in mice and humans.

2.1 Male infertility

Male infertility is a multifunctional disorder and mostly results in low sperm count or no sperm in semen, low sperm motility and high levels of abnormal sperm morphology [19]. Causes for male infertility are classified as being of pre-testicular, testicular or post-testicular origin. Male infertility can be diagnosed by semen analysis, physical examination, medical and reproductive history [20, 21].

Infertility due to pre-testicular factors can be caused by lifestyle as well as genetic history, such as smoking, alcohol intake, nutrition, hormonal abnormalities, toxin exposure, varicocele, medications, and chemotherapy [22]. Physical examination to identify causes of infertility includes an evaluation of the male reproductive system and sex organs. However, semen

analysis is the standard routine diagnostic test in fertility clinics, which assists practitioners in choosing the most appropriate treatment option and is used to predict ART outcome [21].

Despite extensive research on male infertility, the majority of reasons for male-factor infertility are still not clear, and are thus diagnosed as idiopathic male infertility [23]. On a global scale, male factors are responsible for 50% of infertility cases [24]. There exists significantly less accurate information about male compared to female infertility.

Any conditions affecting spermatogenesis such as hypogonadism, genetic defects, radiotherapy, trauma, and testicular cancer, could result in low sperm count or sperm morphological abnormalities. These conditions are classified as testicular problems and can cause male infertility. Most post-testicular problems arise from ejaculatory duct obstruction, infections and retrograde ejaculation [19, 25]. A better understanding of the causes of male infertility would allow the most appropriate treatment to be prescribed. Treatments include [13]:

- Improving sperm quality by making lifestyle changes
- Surgery for duct obstruction, varicocele problem
- IVF for men with low sperm numbers
- ICSI for men with low or no sperm motility

It is therefore important to better understand the molecular mechanisms underlying the reproductive process to allow the development of novel and more effective treatments for male infertility.

3. Male reproductive system

The following sex organs together constitute the male reproductive system [26] (see Fig 2):

- The testicles

- The duct system, which is made up of the epididymis and the vas deferens
- The accessory glands, which include the seminal vesicles and prostate gland
- The penis.

In the seminiferous tubules of the testes, the synthesis of spermatozoa occurs in two main stages; spermatogenesis and spermiogenesis. Spermatozoa originate from germinal stem cells called spermatogonia, which are located in the seminiferous tubules [27]. A continuous complex process comprising mitotic divisions, differentiation, and meiosis leads to haploid cells called spermatids and eventually millions of tiny microscopic spermatozoa, which are stored in the cauda epididymis [27, 28].

A large number of sperm cells are continually produced in the testis by a process named spermatogenesis [29]. During spermatogenesis, the spermatocyte, which originates from the mitotic division of spermatogonial stem cells, undergoes meiotic division and generates two secondary spermatocytes (Meiosis I). Then, each secondary spermatocyte will continue into Meiosis II and divide into two equal haploid spermatid cells. Spermatids mature into spermatozoa in a process referred to as spermiogenesis [27]. The two testicles or testes in sexually mature males also produce testosterone, which is the major male sex hormone and plays a key role in the development and maintenance of the male reproductive organs [30]. The process of spermatogenesis is highly dependent on the activity of two types of cells in testes: Sertoli cells and Leydig cells. These two major cell types line the seminiferous tubules. Sertoli cells provide nourishment and structural support to the developing sperm cells, whereas Leydig cells are responsible for the synthesis of steroid hormones necessary for germ cell differentiation. The testes produce around 95% of testosterone in males, and a small percentage is made by the adrenal glands [31].

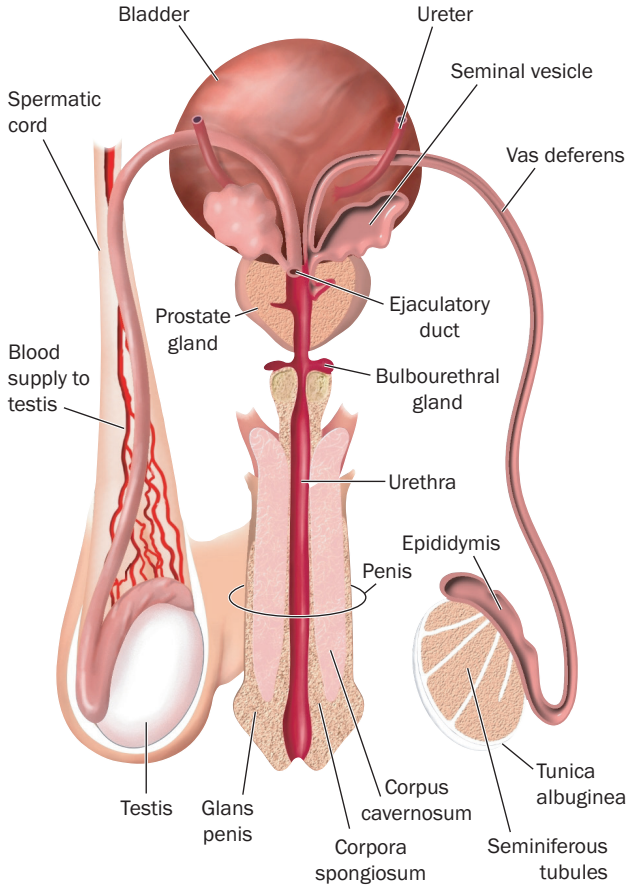

Figure 2: Diagrammatic representation of male reproductive organs in human. Spermatozoa are first form in the testes and become mature by trnsit through the epididymis, they are stroed until ejaculation. (adapted from [13]).

3.1 The epididymis

The epididymis is a narrow and highly segmented organ that serves as a connector tube between the testicles and the vas deferens, as well as providing a milieu for sperm to acquire their motility and fertilizing capacity [32]. It is a tightly-coiled and extremely long organ, with lengths varying from 1 meter in mice to 6-7 meters in human, which is located above the testes [33]. The

epididymis serves not only as a storage organ for viable sperm but also provides an environment in which sperm undergo many biochemical and morphological changes, allowing them to mature gradually in a process collectively called epididymal maturation [34]. Spermatozoa are stored in the cauda segment of the epididymis for one to two weeks. The most important function of this part of the epididymis is to preserve sperm viability by regulating the motility and metabolism of stored sperm [35, 36]. Sperm motility is repressed by several factors in order to save energy, such as reduction in scrotal temperature, the low oxygen content in epididymal fluid and tissue, the absence of glucose, and variations in the ionic composition of epididymal fluid [37]. The epididymal lumen also provides a specific and continually modified environment in which sperm achieve maturation [34, 38]. Thousands of proteins are actively synthesized in the epididymis in response to androgen stimulation [33]. Studies have estimated that the human epididymal proteome contains 7500 proteins, while 2850 proteins have been identified in the murine epididymis[39]. Regardless of species, sperm transit gradually through the epididymis in a journey that lasts approximately 10 days, and undergo a large number of modifications [34]. Transport, storage, and maturation of sperm are the main functions of the epididymis [40]. Anatomically, the mammalian epididymis is constituted of three main parts: the head (caput), body (corpus) and tail (cauda) (Figure 3).

In rodents, however, the epididymis can be divided into four regions, compared to the three main anatomical regions of epididymides in other mammals. In rodents, the proximal segment of the caput is distinct and is named the initial segment [33]. The luminal fluid of each segment differs in terms of ion concentration, pH and osmolality. Water absorption actively takes place in the initial and proximal segment [33].

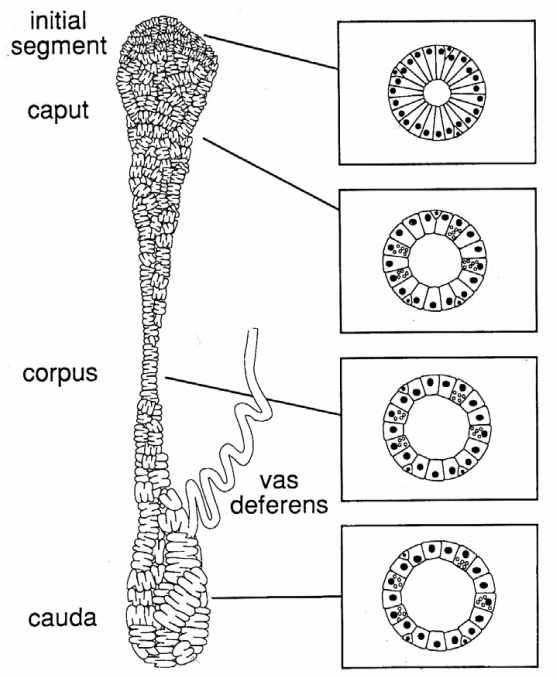

Figure 3. Schematic representation of the different regions of mouse epididymis (left panel) and the right are shown cross sectional of the epididymal duct. The epididymis is an organ located above the testicle. It is a long tube and divided into four major parts: the initial segment, the head (caput), the body (corpus) and the tail (cauda). However, these four parts can differs from one species to another. (adapted from [33]).

3.2 Epididymal maturation

During epididymal transit, mammalian sperm are exposed to the epididymal milieu. During their journey through the epididymis, the sperm plasma membrane undergoes a variety of sequential modifications, which result in the acquisition of sperm motility and fertilization capacity. This process is known as epididymal maturation [41]. The composition of the epididymal milieu varies from one segment to another [42]. During epididymal maturation, the major modifications occurring on the outer leaflet of the sperm plasma membrane allow sperm to acquire motility and the ability to fertilize an oocyte [43]. Before sperm are transferred into the

female reproductive tract upon ejaculation, the epididymal mixture is blended with fluid secreted from the accessory glands (seminal vesicles, ampullae, prostate, and bulbourethral glands). This organic fluid, which is known as semen or seminal plasma (SP), is critical for sperm function. It serves as a source of energy for sperm, allows further sperm maturation, and ensures sperm survival and transport [44]. Semen is a mixture fluid secreted from the testes, the excurrent ducts and the accessory glands, and is constituted of two types of components; cellular and non-cellular. The non-cellular portion is enriched with various proteins that are synthesized and secreted in a region-dependent manner from the male reproductive tract, and contains lipids, carbohydrates, proteins and some minerals [44]. Several biochemical and structural modification events occur during epididymal maturation, rendering sperm fertilization-competent cells and preparing them for the second extra-testicular maturation step that takes place in the female reproductive tract, known as capacitation [45]. The two maturation steps (epididymal maturation and capacitation) alter the concentration and distribution of lipids in the sperm membrane, resulting in a decrease in the cholesterol/phospholipid ratio [46, 47].

In addition to the previously mentioned sperm modifications occurring during epididymal transit, the protein composition of the sperm membrane also undergoes several modifications during this journey. Sperm lose several membrane proteins and acquire others, such as secreted epididymal proteins including decapacitation factors. These decapacitation factors bind to the sperm membrane and protect sperm from premature capacitation [41, 48].

Upon ejaculation, sperm are deposited into the female genital tract along with SP, and decapacitation factors are removed from sperm surface in order for sperm to undergo the second maturation step known as capacitation. [45]. Capacitation includes a number of membrane modifications, the modulation of sperm enzyme activities and sperm hyperactivation [49].

Studies indicate that the isthmus of the oviduct is the region of the female reproductive tract in which the sperm cell surface undergoes the capacitation process, resulting in the full acquisition of fertilizing ability.

3.3 Epididymal proteins

All of the fundamental sperm modifications required to create a fertile sperm occur inside the epididymal tubule. It is therefore important to better understand all the transformations involved in sperm maturation as well as the proteins implicated in these modifications in order to develop appropriate treatments for male infertility [37]. Since the 1970s and 1980s, many investigations have undertaken to elucidate the protein composition of the epididymis. Spermatozoa travel a long distance in the epididymis and undergo multiple sequential changes in each of the epididymal sections, which have different luminal fluid compositions [38]. The majority of epididymal luminal proteins are secreted by the surrounding epithelium. The secretory activity of each epididymal section is different, leading to distinct protein compositions and concentrations in each region of the epididymis [34, 50]. Epididymal fluid consists of a large pool of soluble (hydrophilic) and insoluble (hydrophobic) proteins, most of which are soluble [51]. The proximal region of the epididymis is responsible for secreting 60 to 83% of epididymal proteins [35]. Several of these proteins are epididymal region-specific and are common among different species. Examples of such proteins are glutathione peroxidase (GPX5), prostaglandin D2 synthase (PTGDS) and RNAse10, which have been found in the proximal epididymal regions [38]. Proteins expressed in the middle and distal parts of the epididymis include lactoferrin (LTF), Niemann–Pick disease type C2 (NPC2), several glucosidases and gelsolin (GSN) [38]. Several of these proteins are common to different species, such as Clusterin (CLU),

transferrin (TF), GSN, NPC2, LTF, lipocalin 5 (LCN5), actin, and b-galactosidase (GLB1) [35, 52, 53]. However, However, epididymal proteome is a quite dynamic entity and the proportion of expressed proteins varies from one species to another [54]. In human for example, 77% of the total luminal proteins are represented by albumin (ALB) (43.8%), CLU (7.6%), NPC2 (6%), LTF (5.9%), extracellular matrix protein (ECM1) (3.2%), a1-antitrypsin (SERPINA1 (A1AT)) (2.7%), PTGDS (2.2%, 1.7%), TF (1.3%), and actin (1.2%) [32].

4. Sperm structure

Spermatozoa are highly specialized cells with minimal cytosol and organelles [55]. The mission of these haploid cells with forward motility is to travel towards the ovum in the female reproductive tract and deliver the paternal genome by combining with the ovum to generate a diploid embryo [56]. These small haploid cells possess remarkable features that make them distinguishable from the other cells. Their motility allows them to travel a long distance in the male and female genital tracts, where they undergo multiple modifications to acquire their fertilizing ability, which enables them to accomplish their mission [57]. Sperm cells are composed of four major parts. The first compartment is the head of the sperm, which contains a very condensed cytosol with no organelles such as ribosomes, endoplasmic reticulum or Golgi apparatus. A very large secretory granule known as the acrosome is located on the anterior part of the sperm head [58]. The acrosome contains the hydrolytic enzymes necessary for sperm to penetrate the egg. The nucleus is located behind the acrosome and contains tightly packed DNA. Special proteins named protamines ensure that sperm DNA is tightly condensed in order to stabilize and maintain DNA integrity during sperm transit through the male and female reproductive tracts [59].

The second section is the neck, which connects the head of the sperm to the midpiece, and contains two centrioles for chromatin segmentation [60]. The third section is the midpiece, which contains many spiraled mitochondria that produce ATP and thus provide the energy source for sperm motility [61]. The tail or flagellum is the fourth section, which beats and allows the sperm to move [62] (Figure 4).

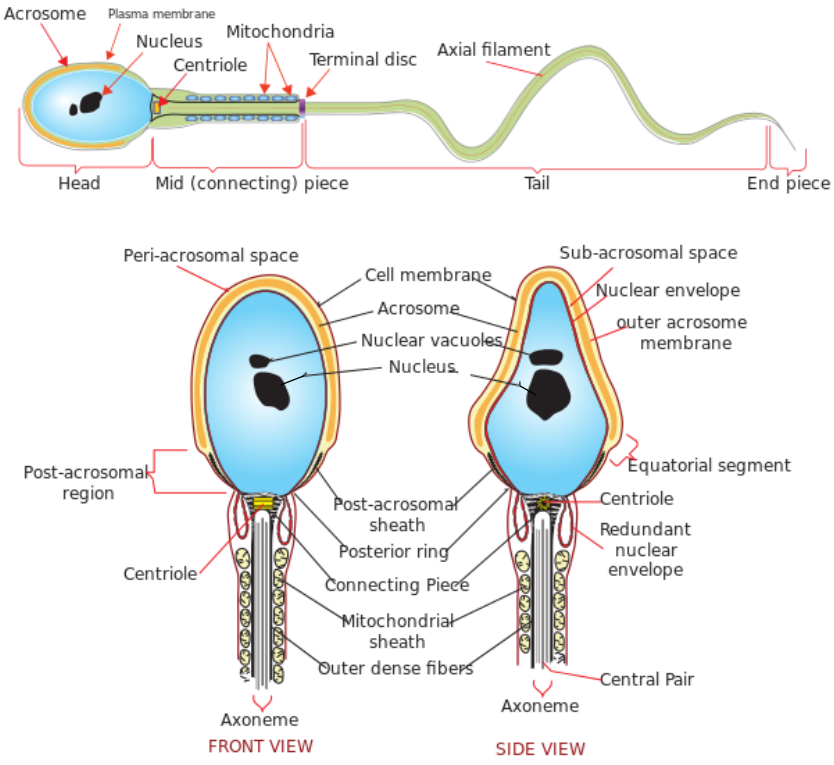

Figure 4. Diagram of the human spermatozoon.

Taken from:

https://fr.wikipedia.org/wiki/Fichier:Complete_diagram_of_a_human_spermatozoa_fr.svg

5. The Binder of SPerm protein family

5.1 Background

Seminal plasma (SP) is a heterogeneous fluid that contains numerous different proteins secreted by the epididymal epithelium, seminal vesicles and other accessory glands. SP induces alterations in the sperm membrane, and mediates the exposure of several sperm membrane proteins and receptors essential for sperm maturation, sperm-egg interaction and fertilization. Despite years of investigations, the function and role of many SP proteins are still unknown.

A highly conserved superfamily of proteins, named Binder of SPerm (BSP) proteins, was identified in the SP of more than 15 different mammalian species. The BSP protein family, which is a ubiquitous superfamily among mammals, has been investigated by our laboratory over the past three decades [63]. Depending on the species, BSP proteins are expressed by the seminal vesicles and/or epididymis [64-66]. The members of this family share many similar characteristics such as their expression site, a common structure, and similar binding properties to various ligands and to the sperm membrane [67-70].

Proteins from the BSP superfamily were first identified and characterized in bovine SP. In the bovine species, three BSP proteins (BSP1, BSP3 and BSP5; previously named BSP-A1/A2 or PDC-109, BSP-A3 and BSP -30K, respectively), constitute more than 60% of total SP proteins, and have been shown to be essential for sperm capacitation [63]. Following this discovery in bovine, BSP genes and proteins were identified in other species such as ram, goat, boar, bison and buffalo [69, 71-73]. In 2006, a study reported the existence of two other BSP-related genes in bovine, which are expressed in the epididymis [74]. In farm animals, BSP proteins are mainly

expressed by seminal vesicles in large quantities, whereas BSP proteins originating from the epididymis are present in very low quantities in SP [75].

Shortly thereafter, BSP homologues expressed in mouse and human epididymis were identified by Lefebvre et al. [74]. The investigations of the mouse and human genomes resulted in the discovery of three and one BSP sequences, respectively [64, 76, 77]. The mouse *Bsp* genes were named mouse Bsp Homologue 1, 2 and 3 (*mBsph1*, *mBsph2* and *mBsph3)*. No mRNA transcripts were identified for *mBsph3*, which appears to not be an active gene and likely represents a pseudogene [76]. The BSP-homologous gene in human was named *hBSPH1* [74]. Important differences exist between mouse/human BSP proteins and bovine BSPs, such as the location of protein expression and the quantity of these proteins in SP. Approximately 60% of bovine SP is constituted by BSP proteins, whereas these proteins form <0.01% of total SP proteins in mice and humans [78].

5.2 BSP protein structure

Most BSP proteins and their homologs are acidic and are relatively small, with molecular masses of 12-30 kDa. The fibronectin type 2 (Fn2) structure is common in this family (function described below) [63]. BSP family proteins possess a conserved secondary structure constituted of a variable N-terminal domain comprising 15 to 71 amino acids, followed by two highly conserved and tandemly arranged Fn2 (Fn2-A and Fn2-B) domains (38-42 amino acids), which are linked by a seven amino acid polypeptide linker as shown in Fig. 5 [77, 79]. A very short variable C-terminal domain (1-5 residues) exists in some members of the BSP family [80-82]. Each Fn2 domain comprises four cysteine residues and two disulfide bonds with connectivities 1-3 and 2-4, to form a hydrophobic pocket [80, 81, 83, 84]. Studies have shown that the two Fn2

domains are responsible for many binding properties of BSP proteins. BSP proteins interact with a wide range of partners including glycosaminoglycans (GAG) such as heparin and chondroitin sulfate B (CBS) [85], choline phospholipids [86], gelatin [87], calmodulin, high-density lipoproteins (HDL) [88], ApoA-I, low density lipoproteins (LDL), insulin-like growth factor II, PLA2, and milk proteins (casein micelles, α-lactalbumin, β-lactoglobulin) [63, 79].

The two active BSP family genes in mice are *mBsph1* and *mBsph2,* which span 24 and 21 kb, respectively. Both genes include 5 exons and 4 introns and are located on chromosome 7. The human *Bsp* gene is located on chromosome 19 and is composed of six exons and five introns [74].

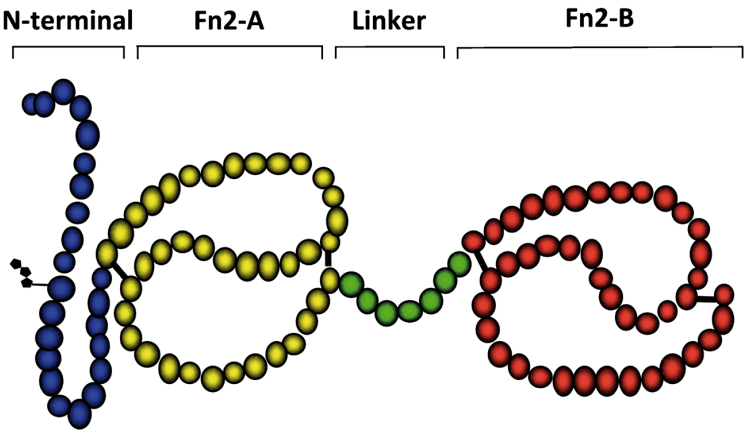

Figure 5. Illustration of the BSP protein structure (adapted from Plante et al. (2016) [67]).

5.3 Biological roles of BSP proteins

Studies have shown that BSP proteins are involved in many stages of fertilization, including epididymal maturation (development of sperm motility), capacitation, formation of the oviductal sperm reservoir and prolonging sperm viability [67].

5.3.1 Role of BSP proteins in the first sperm maturation step (Epididymal maturation)

The primary function associated with BSP proteins is their ability to promote sperm capacitation. This capacitation-inducing role of BSP proteins was shown in bovine by Thérien et al. in 1995 [89]. The binding sites for BSP proteins on the sperm plasma membrane are choline phospholipids. The most predominant choline phospholipid derivatives on the sperm membrane are phosphatidylcholine, plasmalogen and sphingomyelin, which account for 70% of the total phospholipids of the sperm membrane [66, 90]. As sperm pass through the epididymis in mouse and human, BSP proteins expressed by the epididymal epithelium bind to the sperm membrane and remove phospholipids and cholesterol from the membrane, prompting a first cholesterol efflux (Figure 6) [91]. However, in ungulates and other farm animals for whom BSP proteins are expressed in the seminal vesicles and not in the epididymis, the first cholesterol efflux occurs during ejaculation when sperm mix with SP and encounter a high concentration of BSP proteins (Figure 6). As sperm are transferred to the female genital tract and the excess free BSP proteins is removed, the first cholesterol efflux ends [91]. However, in the female tract the interaction of BSP proteins with HDL and GAGs on the surface of sperm induces a second cholesterol efflux, resulting in capacitation (Figure 7) [92, 93].

As sperm transit through the epididymis, the protein, lipid and sugar content of the sperm membrane is changed, and it acquires new proteins [42, 94]. BSP proteins are among the proteins that bind and coat the sperm membrane. Gwathmey et al. showed that BSP proteins maintain sperm viability and motility in the cauda epididymis by preventing excessive membrane alterations (Fig. 6) [91, 95].

Cholesterol and phospholipid efflux from the sperm membrane cannot occur unless free BSP proteins in SP bind to the sperm surface and induce the first cholesterol efflux [96, 97]. In ungulates upon ejaculation, free BSP proteins found in SP are mixed with sperm and interact with the membrane and mid-piece of the flagellum. This interaction induces the first cholesterol efflux, resulting in the activation and/or enhancement of calcium (Ca^{2+})-ATPase (PMCA4) and increase sperm motility following ejaculation (Fig.6) [98-101]. *In vitro* studies have shown that bovine BSP1 binds to the sperm plasma membrane, and this interaction enhances the activity of PMCA4 [88, 102, 103]. The maintenance and regulation of the swimming behavior of spermatozoa is mainly controlled by Ca^{2+} signaling. As Ca^{2+} concentration increases, the sperm flagellar beats increase, thus enabling sperm hyperactivation [56]. As indicated in Fig. 6, in ungulates BSP proteins are expressed by the seminal vesicles and constitute the major proteins of SP (1-50 %). In human and mice, these proteins are expressed in the epididymis and account for a minute amount (0.01%) of epididymal and SP proteins. Remaining unbound BSPs are removed from SP as sperm enter into the female genital tract.

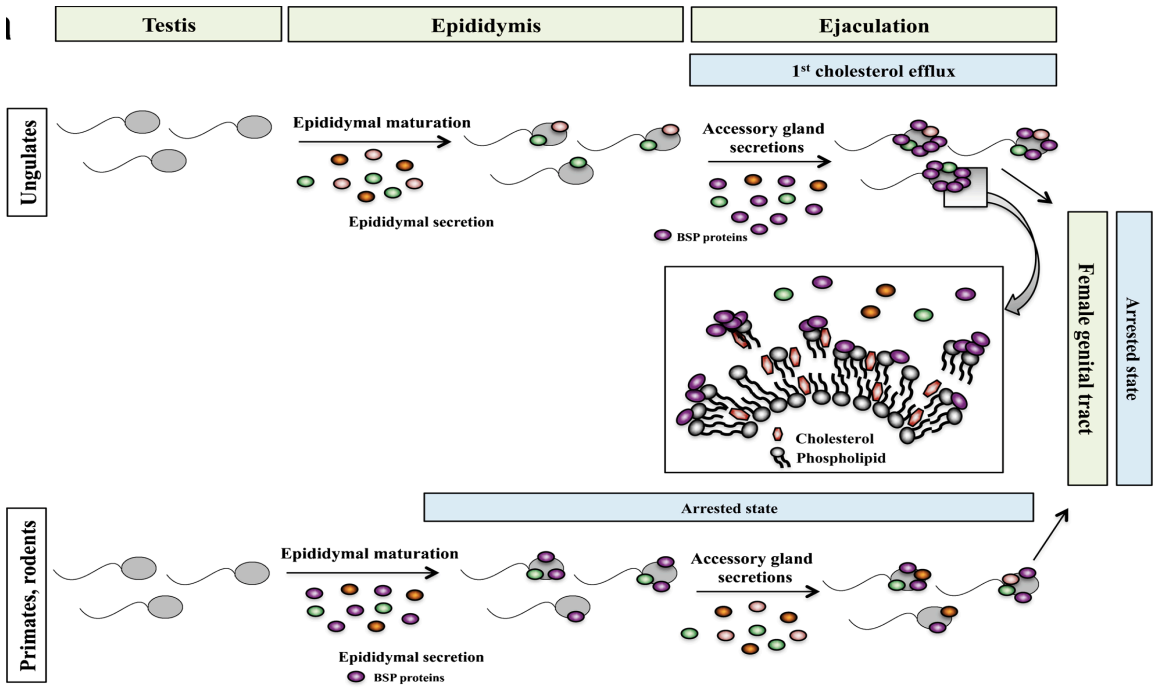

Figure 6. Schematic representation of the differences in the secretion of BSP proteins and their binding to the sperm membrane between ungulates, primates and rodents (adapted from Plante et al. (2016) [67]).

5.3.2 Role of BSP proteins in the second sperm maturation step (Capacitation)

Six decades ago, two investigators (Austin [104] and Chang [105]) demonstrated that when sperm pass through the uterus and oviduct they undergo multiple biochemical and physiological modifications. These modifications are collectively considered as the second sperm maturation step and referred to as capacitation [27]. The efflux of cholesterol initiates capacitation, which includes several changes such as increased intracellular pH, increased permeability to Ca^{2+}, activation of several signaling pathways, increased phosphorylation of tyrosine residues and the

development of hyperactivity of flagellum [106]. The duration of this synchronized event is very short, taking between 50 minutes and 4 hours in human [107-111]. Several studies have demonstrated that one of the main functions of BSP proteins in bull, stallion, ram, and goat is to trigger sperm capacitation [73, 89, 112-114]. The interaction of the three BSP proteins found in bull SP with decapacitation factors such as HDL and/or GAGs results in the removal of cholesterol and phospholipids from the sperm membrane, causing an alteration in membrane permeability (Figure 7) [65, 91, 95, 115]. Studies on bovine spermatozoa indicated that BSP proteins have a dual effect on the sperm plasma membrane [90]. The high concentration of BSP proteins in SP causes the first cholesterol and phospholipid efflux upon ejaculation. As sperm pass through the cervical mucus, the first phospholipid efflux ceases because of removal of excess BSP proteins as SP is left behind. However, the interaction of sperm-bound BSP proteins with follicular and oviductal fluid mediates a second phospholipid and cholesterol efflux, leading to the destabilization of the sperm membrane and the initiation of capacitation [65]. BSP family members showed different levels of affinity in binding to the sperm membrane. However, despite these differences, studies indicate that the BSP proteins play an important role in the preparation of sperm to fertilize the egg [65, 116].

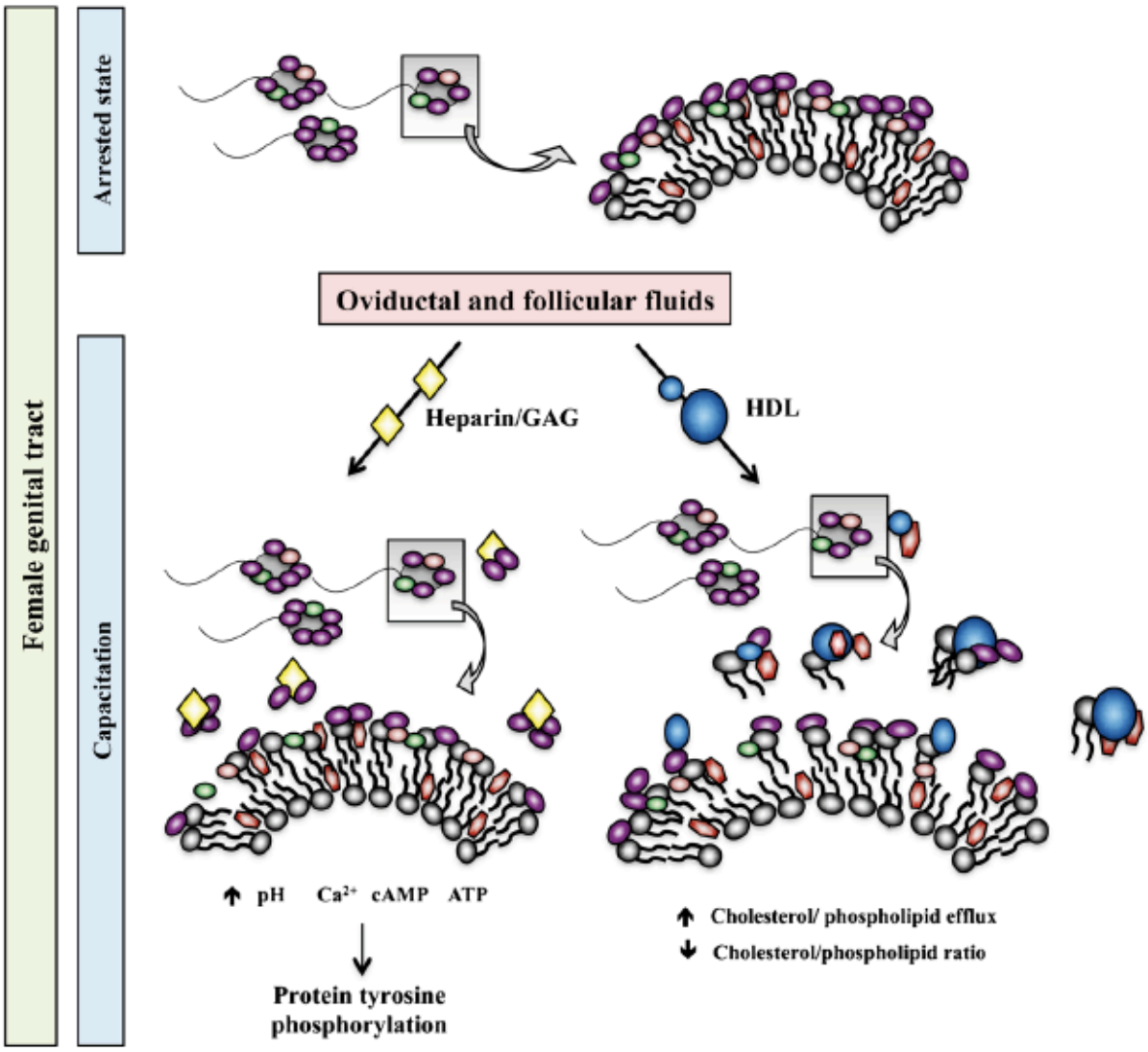

Figure 7. Proposed mechanism of the involvement of BSP proteins in sperm capacitation and the destabilization of the sperm membrane (adapted from Plante and Manjunath (2016) [67]).

5.3.3 Role of BSP proteins in the formation of the oviductal sperm reservoir

Among the hundreds of millions of spermatozoa that enter the female reproductive tract at ejaculation, only a few thousands can reach the fertilization site. Once these sperm reach the oviduct (Fallopian tubes in human), they bind to the oviductal epithelium and form a sperm

reservoir. This contact with the oviductal epithelium preserves the viability and motility of sperm, and prevents their premature capacitation [117, 118]. Sperm liberation from the oviductal reservoir is synchronized with the time of ovulation [119]. Studies have shown that one of the factors implicated in the formation of the sperm reservoir is sperm-bound BSP proteins, which bind to molecules on the epithelial surface [120, 121]. Sperm are released from the reservoir as GAGs in follicular fluid interact with sperm-bound BSP proteins at the reservoir site [122].

6. Application of CRISPR/Cas9 in reproductive research

Recent advances in genome editing is unravelling its benefits in the study of reproductive biology and other wide areas of scientific researches. Nowadays, generating mutant organisms has been widely developed by breakthrough genome editing technology named the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-associated (Cas) system. This system allows modulation of gene expression, targeted gene cleavage using site-specific nucleases (SSNs) and gene editing in a variety of eukaryotic cells with high efficiency outcomes [123]. Over the years, several techniques have been applied to manipulate specific DNA sequences in cells and a variety of animal models, including generation of random mutation by chemical or radiation exposure resulting DNA damage, zinc-finger nucleases (ZFNs), transcription activator-like protein nucleases (TALENs) to create double-strand DNA breaks and RNA interference (RNAi) [124].

Investigation of the adaptive immune system against plasmids and phages in prokaryotes composed of non-contiguous direct repeats interspaced by variable sequences results in the creation of a novel technique called the CRISPR/Cas9 system [125, 126]. This discovery was an important breakthrough in the field of gene editing which led to the identification of novel

factors involved in a variety of cellular pathways. Adapting this recent approach has emerged as the most popular tool for the precise alteration of the genome with dramatically less effort and expenses. CRISPR/Cas9-mediated genome editing requires a short RNA to target its sequence (protospacer) and make double-strand brakes in the genome [127].

Briefly, CRISPR/Cas9 is an adaptive and defence system in Bacteria and archaea, which exploit a short RNA to generate accurate breaks in the specific inserted DNA sequence of bacteriophage and/or invader plasmid to cleave and repel the foreign nucleic acids [128]. CRISPR loci are composed of considerable repeat and non-repeat (spacer-segments derived from cleaved foreign DNA) sequences, while Cas gene is located upstream of these sequences and encode putative nuclease or helicase proteins (Figure 8).

CRISPR-associated nuclease called Cas9 is guided by short single guide RNA (sgRNA) to recognize and cleave a target of 20 nucleotides in the genomic sequence located at downstream of recognition site of Cas9 named Protospacer Adjacent Motif (PAM) sequence [129]. The sgRNA is transcribed from CRISPR loci of incorporated exogenous DNA, which serves as a genetic record and composed around 30 bp sequences. PAM sequence (5′ NGG 3′) is the target binding site for Cas protein and is located immediately upstream of the targeted sequence [129]. The sgRNA and PAM are the two specific and essential features of the CRISPR/Cas9 complex to track and develop an incision at the desired sequence [130]. CRISPR/Cas 9 introduces double-strand breaks (DSBs) at sites of interest approximately three nucleotides upstream of the PAM sequence. The DSBs can repair by either the imprecise non-homologous end joining (NHEJ) or precise homologous recombination (HR) DNA repair pathways. NHEJ rejoins the broken ends of DNA with random insertions or deletions of DNA nucleotides (indels) [131]. Homology-

directed repair, in the presence of a donor DNA sequence, could be harnessed to edit or replace the sequence of any given gene, resulting in gene correction or gene addition [131].

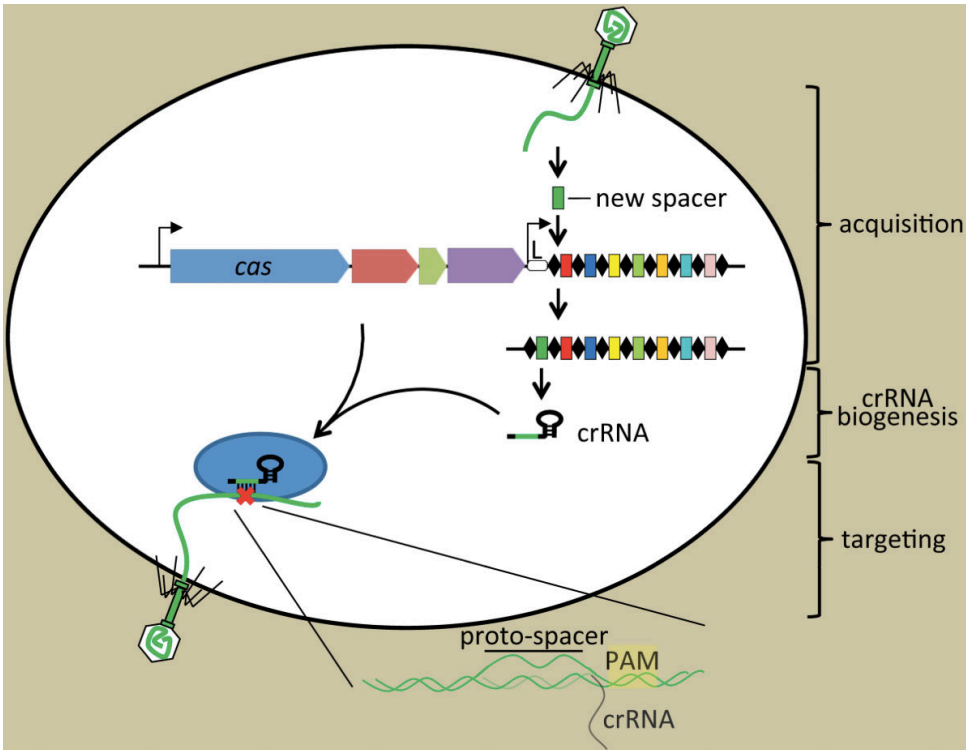

Figure 8. Three stage of defence immune system in Bacteria and archaea. A short DNA sequences of invader is captured and incorporated into the CRISPR loci as new spacers. Upon second infection, the CRISPR locus is transcribed and processed to generate mature CRISPR RNAs, crRNA along with Cas protein guide to target the foreign DNA match with crRNA (adapted from Barrangou and Marraffini (2014) [126]).

Genome editing is used in wide areas of scientific development to modify endogenous genes in a wide variety of cell types and in organisms that have traditionally been challenging to manipulate genetically. The CRISPR/Cas9 system has already been applied to produce genetically modified mice by CRISPR delivery into mouse zygotes to easily and quickly knockout or knock-in specific targeted genes [132]. Nowadays, the CRISPR/Cas9 method has

been widely used in reproductive research to find out the cause of infertility and create therapeutic approach at the molecular level.

In recent years the CRISPR/Cas9 technology is used widely to study several fertility-related genes in males to explore the vital factors of fertility and develop therapeutic approaches. Additionally, the CRISPR/Cas9 systems are used to generate knockout mouse models to study the role of an individual or multiple genes in male reproductive systems *in vivo* to unlock the mechanisms behind fertility and explore the vital genes in reproduction. Due to the rapid reproductive cycle and the high genetic similarity to the human, mice are the ideal models to investigate the essential genes for male fertility. [132]. A combination of a mouse model and new techniques such as CRISPR/Cas 9, can be used to elucidate the function of genes that are similar to those in humans by genetically modifying mice. Human and mouse BSP proteins are orthologues proteins. Mouse BSPH1 shares 56% identity and 78% sequence similarity with human BSP protein. While, mouse BSPH2 shares 40% identity and 55% sequence similarity with Human BSPH [133]. Therefore, the use of the mouse as an animal model for human BSP can help to elucidate the role of epididymal BSP proteins in sperm maturation, as well as the other possible roles of this protein family in fertilization.

7. Thesis Objectives

The main roles that have been assigned to BSP proteins in ungulate species are their interaction with the sperm membrane, their ability to trigger sperm capacitation and prepare to fertilize the ovum [71, 72, 91, 134, 135]. Homologs of BSP proteins were also found in the SP of mouse and human, though in very minute amounts [74]. Studies indicate that these proteins may be involved in the maturation of mouse and human spermatozoa, allowing them to gain fertilizing

ability. However, since BSP proteins are found in negligible quantities in human and mice SP, the isolation of these proteins from SP is not feasible. Consequently, the study of their biological functions *in vitro* has been restricted.

Male fertility has been declining over the last three decades; therefore, understanding the mechanisms underlying fertilization and identifying the key factors required for this process is highly important in order to develop new diagnostic tests and treatments for infertility. The current study thus aimed to gain more insight into the role of BSP proteins in sperm maturation and fertilization in mice. Studies on BSP proteins have shown that these proteins are crucial for fertilization in cattle. The discovery of genes encoding proteins of the same family in humans and mice has opened new doors to the identification of new factors that may have an impact on the fertility of these species. Our working hypothesis for this thesis was that BSPs in humans and mice are added to spermatozoa during epididymal maturation and have roles in sperm functions and fertilization. The overall objective of this thesis was to specifically determine the roles of epididymal *Bsph1* and *Bsph2* in sperm maturation in mice, as well as to determine whether these proteins are essential for fertilization. To answer these questions, the overall objective was divided into six specific objectives:

1. Generation of single (*Bsph1* and *Bsph2* separately) knockout mice. The novel gene-editing technology CRISPR/Cas9 was selected as a method to target and disrupt the *Bsph1* and *Bsph2* individually in mice.
2. Screening for homozygous *Bsph1* and *Bsph2* KOs using different methods. The genotypes of mutant mice were determined by PCR. Homozygous KO mice were confirmed using various methods including Sanger sequencing, quantitative real-time

PCR (qPCR), digital droplet-PCR (ddPCR), reverse transcription PCR (RT-PCR) and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS).

3. Fertility assessment in *Bsph2* single KO mice. To verify whether the KO males are fertile, we mated gene-targeted male mice and WT male controls with WT females.
4. Assessment of sperm functions in *Bsph2* single KO mice. Sperm viability and motility in KO mice was assessed using Sperm Class Analyser (SCA), a computer assisted microscopic analysis system.
5. Fertility assessment in *Bsph1/Bsph2* double knockout (DKO) mice. Two *Bsp* genes were targeted and disrupted simultaneously by CRISPR/Cas 9 in mice.
6. Assessment fertility and sperm functions in *Bsph1/Bsph2* DKO mice. To verify the effects of the absence of BSP proteins on sperm function and fertility *in vivo*, the *Bsph1/Bsph2* DKO were mated with WT females, and as well as sperm viability and motility were assessed.

This work may determine if BSP proteins play a role in sperm maturation and functions in mice. The results of this study will provide insight into the role of mouse BSP proteins in sperm functions and fertilization.

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CRISPR/Cas9-mediated mutation revealed BSPH2 protein is dispensable for male fertility

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Running head: Role of BSPH2 on fertility.

ABSTRACT

Members of the Binder of SPerm (BSP) superfamily have been identified in both human and mouse epididymis. These proteins are known to bind sperm membrane and promote sperm capacitation. Studies suggest that BSPH2 might play a different role in sperm functions from its counterparts; however, the role of BSPH2 remains mainly unexplored. To investigate whether the absence one member of the BSP family could affect fertility, mice lacking *Bsph2* expression were generated using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) associated 9 (Cas9) technology. Knockout (KO) male mice were mated with wild-type (WT) females, and the number and weight of the pups were determined. Sperm motility in WT and KO was assessed using Sperm Class Analyzer (SCA). Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used for protein identification. Fertility analysis of null *Bsph2* mice did not reveal any phenotype. No differences were noticed in average litter size or average pup weight. Normal testis weight and morphology were observed in $Bsph2^{+/-}$ and $Bsph2^{-/-}$ compared to the WT. Quantitative RT-PCR analyses revealed that *Bsph1* mRNA expression was increased in mutant mice, whereas LC-MS/MS analysis displayed no increase in protein expression level. Taken together, we show the existence of redundant function for murine BSPH2 and the lack of BSPH2 itself does not lead to sterility.

Key words: epididymal protein / Binder of SPerm (BSP) proteins / sperm / CRISPR-Cas9 / male fertility.

INTRODUCTION

In recent decades, the prevalence of infertility has increased significantly. Aberrant functioning of male fertility factors contribute to this problem accounting for almost half of the infertility cases [9]. In order to overcome issues of male infertility, it is important to understand all mechanisms related to sperm maturation and fertilization, both *in vitro* and *in vivo* [136]. In mammals, sperm are produced in the testes, with no ability to swim or fertilize the egg unless two consecutive maturation steps, epididymal maturation and capacitation, occur [137]. In epididymal maturation, sperm leave the testes and travel through a convoluted lumen in the epididymis, where they acquire numerous proteins [138, 139]. Multiple lipid/protein modifications on the sperm membrane arise in the male reproductive tract. Sperm are then transferred into the female reproductive tract wherein the late maturation step, capacitation, occurs [118, 140]. Consecutive transformations, such as plasma membrane modifications, ions and pH alterations, occur in the epididymal-matured sperm to make cells that are physiologically and morphologically competent to fertilize the egg and create a zygote [141, 142]. Several studies have been performed illustrating the function of numerous secreted proteins in the epididymis that appear to be key molecules for fertilization [37]. In the past decade, a novel superfamily of protein, named Binder of SPerm (BSP) proteins, which are expressed specifically in epididymis was investigated in our laboratory [143, 144]. Studies in a variety of mammals, including rodents, primates and humans, indicate that BSP proteins are expressed in the epididymis with no expression in female reproductive tract [72, 74, 75, 145-147]. A common structure in BSP proteins is fibronectin type II (Fn-2), which is responsible for all the binding properties in this family [75, 88, 115, 144]. *Bsph2* gene in mouse is located on chromosome 7, spans 16.2 kb and constitute of five exons, and is expressed exclusively in the epididymis [74,

148, 149]. Further studies have indicated that BSPH2 proteins in the male reproductive tract bind loosely to sperm plasma membrane and inhibit premature capacitation, while in the female genital tract, by interacting with components such as high-density lipoprotein (HDL) and glycosaminoglycans (GAGs), promote capacitation [67, 113, 133, 150-152].

Targeted genome editing has been improved significantly in recent years by a novel technique named the CRISPR/Cas9 system [124, 153]. CRISPR-Cas9 introduces double-strand breaks (DSBs) at the site of interest, approximately three nucleotides upstream of the proto-spacer adjacent motif (PAM) sequence [154, 155]. To investigate the role of BSPH2 protein in mice, we generated a mutant *Bsph2* mouse line using the CRISPR/Cas9 genome editing to assess the role of this protein on fertility.

METHODS

Animals

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept in an animal facility under temperature-controlled (22 ± 1°C), light-controlled (cycles of 12 h light/ 12 h dark) and specific pathogen-free (SPF) environment. All protocols for animal studies were approved by the Maisonneuve-Rosemont Hospital Ethics Committee, and experimental work was carried out according to the guidelines of the Canadian Council of Animal Care. Male CRISPR/Cas9 knockout mice were backcrossed with wild-type mice to stabilize the mutation.

Generation of Bsph2-deficient mice

Short, single-stranded gRNA sequences composed of 20 nucleotides followed by a PAM sequence were designed to target region within exon 3, which encodes the first Fn2 domain of

Bsph2 gene. Using the CRISPR design web tool http://crispr.mit.edu, gRNA sequences to target *Bsph2* first Fn2 domain were designed as follows (PAM sequence is underlined):

gRNA1: CCACTACAGTTGTATCTCCCTCC

gRNA2: GTGGCGGTACTGTACAGCACAGG

In order to clone our single-guide RNA (sgRNA) into the humanized *S. pyogenes* Cas9 (D10A) nickase plasmid, the oligos were resuspended in annealing buffer (10 mM Tris pH 7.5-8.0, 50 mM NaCl and 1 mM EDTA) and mixed with equal volume at equimolar concentration (100 μM) in a microcentrifuge tube. Annealing was performed using a thermal cycler with the following profile: 95ºC for 2 minutes and gradually cooled to 25ºC over a period of 45 minutes. DNA was then precipitated using potassium acetate and ethanol. The pX335 plasmid was digested using *BbsI* restriction enzyme (New England Biolabs, Whitby, ON, Canada) and ligated with annealed oligos (gRNAs) containing overhangs of 4 compatible nucleotides using T4 DNA ligase (ThermoFisher Scientific, Waltham, MA, USA).

Pronuclear injection

Gene manipulation techniques were performed at the Transgenic Core Facilities of the Institut de Recherches Cliniques de Montréal by Dr Qinzhang Zhu. Eggs at pronuclear stage were obtained from the ampulla by mating super-ovulated females with males. The pX335 plasmid expressing Cas9 mRNA were injected along with sgRNA into collected fertilized eggs. Embryo at two cell stage were transferred into the oviduct of foster mother [156].

DNA Extraction and Genotyping

Tail snips were used for mice genotyping using the AccuStart II Mouse Genotyping Kit (Quanta Bioscience, Beverly, MA, USA) following the manufacturer's protocol. In order to extract DNA, extraction reagent was added to tail snips and heated to 95ºC for 30 minutes, then equal volume of stabilization buffer was added after cooling the samples at room temperature. PCR genotyping was established using the primer pairs indicated in Table 1 and conditions were as follows: 94ºC for 3 min, followed by 32 cycles of 94ºC for 30 s, 62ºC for 30 s and 72ºC for 30 s.

RNA extraction

Epididymis from six-week-old mice were removed after cervical dislocation and transferred immediately into TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA). Tissues were homogenized using a Polytron homogenizer and RNA extracted according to manufacturer's instructions. Quality of extracted RNA was assessed by Agilent RNA 6000 Nano Kit and samples were stored at –80 °C.

Reverse-transcription PCR (RT-PCR) and quantitative RT-PCR (RT-qPCR)

Reverse transcription was performed using the iScript™ gDNA Clear cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's protocol. In order to synthesize cDNA, 1 µg of extracted RNA was incubated with iScript DNase and iScript DNase Buffer at 25°C for 5 min and 75°C for 5 min, then 4 µl of iScript reverse transcriptase were added and incubated 2 minutes at 25°C, 20 minutes at 46°C and 1 minute at 95°C. Two microliters of cDNA were used for RT-PCR and mouse *Bsph2* gene fragments were amplified using specific sets of primers (Table 1). The program for the RT-PCR assay was as follows: 94°C for 3 min, followed by 30

cycles of 94°C for 45 s, 62°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were visualized on a 1.5% agarose gel containing GreenGlo™ Safe DNA Dye (Denville Scientific Inc., Holliston, MA, USA). RT-qPCR reactions were performed using SsoAdvanced™ Universal SYBR Green Supermix Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's protocol. cDNA and specific primers for mouse *Bsph1* and *Bsph2* were added and run in the Applied Biosystems 7500 fast Real-Time PCR System (Life technologies, Carlsbad, CA, USA). The *Hprt* gene was used as an endogenous control. Results were expressed in relative quantification as a ratio of the mRNA levels of *Bsph1* to the *Hprt* reference gene. Each sample was analyzed in triplicate using primers shown in Table 1. All RT-qPCR runs were done using the following conditions: holding stage 50°C for 2 min and 95°C for 10 min, followed by 33 cycles of 95°C for 15 s and 60°C for 1 min, the melting curve stage include 95°C, 60°C, 95°C and 60°C for 15s, 1 min, 30 s and 15 s, respectively [157]. PCR products were analyzed with the melting curve to confirm specify.

Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS)

To evaluate the absence of BSPH2 protein in knockout animals, total proteins from mouse epididymis were extracted using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Wild-type mice were used as controls. Protein concentration was quantified with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Bremen, Germany). Protein samples (80 μg) were loaded on 18% sodium dodecyl sulfate-polyacrylamide gels and subjected to electrophoresis. Gels were stained with Coomassie Blue, and bands from WT and KO mice were cut at the expected molecular weight region (10-20 KDa). Proteins from the bands were subjected to LC-MS/MS and then data were analyzed using Mascot (Matrix Science,

London, UK; version 2.5.1). The UniProt complete proteome *Mus musculus* Scaffold (version Scaffold_4.8.4, Proteome Software Inc., Portland, OR) was used to interpret MS/MS based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 97 % probability to achieve a false discovery rate (FDR) less than 1 % and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [158]. The Proteomics Platform of the Centre Hospitalier de l'Université de Laval (Québec, Canada) performed the protein digestion and mass spectrometry analyses.

Assessment of fertility

Sexually matured wild-type (WT), *Bsph2*$^{+/-}$ and *Bsph2*$^{-/-}$ male mice (6-8 weeks old) were housed with two WT females (6-8 weeks old) for 2 weeks. Each male was bred at least with six females. Every morning, females were checked for copulatory plug to confirm the mating activity. The fertility of each male was analyzed for average litter size, average pup weight and the time from the beginning of mating to the delivery of the pups. For evaluation of fertility of parents with same genetic background, *Bsph2*$^{-/-}$ male was housed with a *Bsph2*$^{-/-}$ female for 2 weeks, and breeding and analysis were performed as described above.

Sperm Motility Analysis

After cervical dislocation, cauda epididymis was dissected from WT, *Bsph2*$^{+/-}$ and *Bsph2*$^{-/-}$ male mice (6-8 weeks old) and transferred to EmbryoMax M2 (EMD Millipore, Darmstadt, Germany) media. Sperm motility and concentrations were assessed using sperm class analyzer (SCA) system (software version 5.2.0.1.; Barcelona, Spain) with the following parameters: number of frames to analyze, 50; number of frames/s, 50; VCL (rapid), 320 μm/s; VCL

(medium), 193 μm/s; VCL (slow), 80 μm/s; VAP set to 7; Lin rapid, 40% and connectivity, 20. After sperm dispersion, 3 μl of sperm suspension were placed on a prewarmed 20-micron depth Leja slide chamber (Somagen Diagnostic, Edmonton, AB, Canada) and visualized on the heated stage of an Nikon Eclipse 50i microscope equipped with a negative phase contrast objective (10x) (Nikon Canada Inc. Instruments, Mississauga, ON, Canada) and a Basler acA780-75gc camera connected to the SCA system. All motility parameters were evaluated for at least eight random fields for each sample, so that a minimum of 200 sperm trajectories were recorded for each sample.

Statistical Analysis

Data are presented as mean ± SEM for fertility and motility parameters. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test using GraphPad Instat (version 3.05). A P-value of <0.05 was considered significant.

RESULTS

Generation of Bsph2 knockout mice

The CRISPR-Cas9 system was used to establish the knockout male *Bsph2* mice and evaluate the absence of BSPH2 protein on fertility *in vivo*. We cloned two gRNAs into the pX335 DNA plasmid: pX335-Bsph2-gRNA1 and pX335-Bsph2-gRNA2 (Fig 1). Injection of CRISPR-Cas9 constructs along with donor DNA at the zygote stage resulted in either integration of donor DNA by homologous recombination, removing exon 3 and exon 4, or deletion of 520 bp, encompassing exon 3 and a part of the upstream intron (Fig 1). Sequence changes were

confirmed by Sanger sequencing. Then, a mouse line carrying a mutation at the *Bsph2* locus was generated. Genomic DNA was extracted and proved incidence was assessed by PCR amplification of the target region using primers described in Figure 2A and 2B.

In vivo fertility assessment of Bsph2-deficient mice

In total, 8% of founders were targeted by CRISPR-Cas9. After confirmation by genotyping, the generated knockout male was mated with C57BL/6 wild type female to produce the F1 generation. Heterozygous newborns from F1 were then mated together, and PCR was performed on tail snips of F2 progeny to determine their genotype (Fig 2C). The expected Mendelian ratio was observed among the progeny of the F1 generation (WT, 31%; $Bsph2^{+/-}$, 52%; and $Bsph2^{-/-}$, 17%; n= 29), indicating that disruption of *Bsph2* was not lethal at the embryonic stage. DNA products were extracted, purified and confirmed by Sanger sequencing (Fig 3), which indicated that the CRISPR technique had made a cut at the desired site. Expert Protein Analysis System (ExPASy) was used to analyze the sequence and structure of the KO protein, and to compare the KO and WT proteins as shown in Figure 3D (http://www.expasy.org/tools/).

Considering health conditions, body weight and sexual behavior, no discrepancies were observed between mutant and control mice. Mating results showed no significant differences in terms of average litter size and average pups weight between control and knockout groups (Table 2). Therefore, both heterozygous and homozygous groups ($Bsph2^{+/-}$ and $Bsph2^{-/-}$) develop normally with no distinguishable differences from wild type.

Lack of expression of Bsph2 in epididymis of Bsph2-null mice

The expression of the full length *Bsph2* transcript in epididymal tissue from homozygous mutant

mice was not detected by RT-PCR (Fig. 2D). Results show that after deletion of 79 bp of the 5'-extremity of exon 3, no PCR product was observed for *Bsph2* null mice (Fig. 2D; last lane, upper panel). This deletion creates a frameshift, causing a premature termination of transcription (early stop codon); the emerging polypeptide would therefore be incomplete and most likely not functional. The amino acid sequence of the truncated protein was obtained by translating the mutated nucleotide sequence using ExPASy and this sequence was compared with that of the wild-type protein (Fig. 3D). Real-time PCR (RT-qPCR) analysis was carried out to validate the lack of *Bsph2* expression in mice epididymis and results indicated the complete absence of *Bsph2* mRNA in male KO mice (Fig. 4). Heterozygous animals showed a significant reduction (50%) in mRNA levels compared to wild type. Interestingly, when we performed RT-qPCR using specific primers for *Bsph1* (Table 1), we found that *Bsph1* gene expression was increased significantly in *Bsph2*-null animals (Fig. 5).

Mass spectrometry analysis of mice epididymides

Mass spectrometry was carried out to have global insight into the protein content in epididymis and show that BSPH2 protein is not expressed in KO mice. The normalized total spectra were used to compare the presence of proteins between the samples using the protein extract from WT and KO. Sequences results of mass spectrometry detected 2 peptides of BSPH1 (Q3UW26) and 3 peptides of BSPH2 (Q0Q236) in WT mice (Fig 6A). However, no peptides corresponding to BSPH2 proteins were found in KO samples at 97 % level of confidence (or even if level of confidence was decrease to 20 %) (Fig. 6B). Mass spectrometry results further confirmed the successful elimination of BSPH2 protein in our single KO. Since an up-regulation of *Bsph1* transcripts was observed by RT-qPCR in *Bsph2*$^{-/-}$ mice, mass spectrometry was carried out to

verify whether BSPH1 protein level was increased. However, unlike mRNA transcripts, mass spectrometry results showed no enhancement in BSPH1 protein level in *Bsph2*$^{-/-}$ mice (Fig 6B).

Sperm analysis in Bsph2 knockout mice

Examination of testis of *Bsph2*-null mice didn't show any effect on the male reproductive system. No significant differences were observed in testis morphology (Fig 7A) and testis weight (Fig 7B) between *Bsph2* null mice (0.39 ± 0.04) mg and wild type mice (0.52 ± 0.06) mg. No obvious differences were observed in terms of average litter size and average pups weight at 6 days between WT, *Bsph2*$^{+/-}$ and *Bsph2*$^{-/-}$ mice (Table 2). Total motility, progressive motility and hyper-activation of fresh and capacitated epididymal sperm were analyzed by SCA (Fig 8). At both conditions, motility of sperm from heterozygous and homozygous mutant mice did not change significantly compared to WT. Additionally, sperm concentration from all three groups showed similar count (data not shown) indicating that BSPH2 has no effect on sperm parameters.

DISCUSSION

Targeted genome editing has been improved by emergence of a novel technique, called CRISPR/*Cas9*. In this study, we successfully genetically modified *Bsph2* using the CRISPR/Cas9 system to investigate the fertility of male mice *in vivo*. Previous studies have shown that the functional parts of BSP proteins are the fibronectin type II (Fn2) domains, which contain cysteine residues required for proper structure and tyrosine residues with binding properties [74, 75]. Therefore, we specifically targeted these sites using CRISPR/Cas9. Sanger sequencing results confirmed gene modification in knockout animals (Fig 3). In order to confirm

a decrease or absence of *Bsph2* expression, we examined mRNA levels using RT-PCR and RT-qPCR in WT and single *Bsph2* KO mice. However, since we were unable to develop antibodies to discriminate between BSPH1 and BSPH2 antigens, mass spectrometry was applied to confirm the absence of BSPH2 protein in KO animals. Mass spectrometry analysis confirmed the lack of BSPH2 proteins in *Bsph2*$^{-/-}$ mice, indicating that the deletion resulted in complete loss of targeted protein. Mass spectrometry can be used to determine the absolute amount of each protein. Therefore, since an upregulation was observed in mRNA level for *Bsph1* in knockout, mass spectrometry was used to verify whether BSPH1 protein level was increased. It is usually assumed that there is straight correlation between mRNA and protein level. However, no direct correlation was observed between *Bsph1* mRNA transcripts and protein level in knockout, meaning that BSPH1 proteins did not increase as their transcripts. No correlation between *Bsph1* mRNA transcripts and protein level might be because of many complicated and varied post-translational mechanisms involved in turning mRNA into protein.

Numerous knockout studies have been done to characterize the roles of reproductive proteins *in vivo*. Surprisingly, many of the KO were fertile with no obvious effect on fecundity. For example, utilizing CRISPR/Cas9 in more than 54 conserved genes express predominantly in both male mice and human testis were shown to be dispensable for fertility [132]. This may be attributable to that these genes are not individually essential for fertility and sometimes the gene function is covered by the redundancy of other genes or by parallel pathways, which are the big issue in genome research [46, 125, 159]. Sometimes abnormal phenotypes could be observed in KO mice by changing the strain, changing the condition or inactivation of a parallel pathway [160, 161].

Based on numerous studies on the effects of reproductive gene disruption, reproductive genes seem to fall into two categories: those encoding essential and those encoding non-essential fertility proteins. The first group contains proteins with essential roles in sperm maturation, capacitation and fertilization, such as ADAM1, ADAM2, CATSPER1 and IZUMO1. The loss of any one of these proteins could result in an abnormal phenotype and impaired fertility [162, 163]. The BSPH2 protein can be added to the second group, which has little or no impact on fertilization, and already includes other proteins such as RNASE9 [137] and ADAM1b [164, 165]. Our data indicate that BSPH2 protein could not individually affect the fertility and this protein may have redundancy effect and/or cover by the other parallel pathways. Generating a double knockout for *Bsph1* and *Bsph2* in epididymis could provide more insight in regard to the function of these proteins in male fertility.

Declaration of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1. Oligonucleotides used in this study

Oligonucleotides	Sequences (5'—3')	Experimental use
Bsph2-CRISPR-5HAF2*	ACAACTTGGGGAAGGGGATTTGGCT	PCR Genotyping
Bsph2-CRISPR-3HAR2*	CCGTGGAAGGGAGTTTCAGGGGAGA	PCR Genotyping
Bsph2-CRISPR-F2**	AGGCAGCAAGTGACATCAGCTTCAG	PCR Genotyping
Bsph2-CRISPR-R2**	CCTCCTTGGTGCACTTCTTAATGAGC	PCR Genotyping
RT-mBSPH2-E1F	ATGAGCCATCTTGTGCACTGG	RT-PCR
RT-mBSPH2-E3F	TTCCACTACAGTTGTATCTCCCTCC	RT-PCR
RT-mBSPH2-E4R	CCTCCTTGGTGCACTTCTTAATGA	RT-PCR
mBSPH2-F	CCAAAGTGTATTTTCCCTTTCC	RT-PCR
mBSPH2-R	CATTTGGAGGAACATCTGATGTATC	RT-PCR
mHprt-E5E6-F	AGGGATTTGAATCACGTTTGTGT	RT-qPCR
mHprt-E5E6-R	TGCAGATTCAACTTGCGCTC	RT-qPCR
qPCR-Bsph1-F5	GCAGGCAAATGGAAGTGATG	RT-qPCR
qPCR-Bsph1-R5	AGTAGAAATCTCTTGTTCTGGAGG	RT-qPCR
qPCR-Bsph2-F6	GGGTGTTCCTAGCTGTCTAC	RT-qPCR
qPCR-Bsph2-R6	GGAGGGAGATACAACTGTAGTGG	RT-qPCR

* Referred as primers 2F and 2R in Figure 2A

** Referred as primers 1F and 1R in Figure 2A

Table 2. Fertility assessment for *Bsph2*-deficient male mice. Pups weight and litter size were measured for six wild type, four *Bsph2*$^{+/-}$ and six null *Bsph2*$^{-/-}$.

Genotype	Litter size	Pups weight (g)
WT	7.2 ± 1.3	3.7 ± 0.2
Bsph2 $^{+/-}$	6.5 ± 1.2	3.8 ± 0.1
Bsph2 $^{-/-}$	7.7 ± 1.1	3.5 ± 0.7

FIGURE LEGENDS

Figure 1. Generation of *Bsph2* gene knockout mice using CRISPR/Cas9 technology. Schematic diagram of the *Bsph2* gene and modification by NHEJ and/or HDR. Showing alteration of exon 3 of *Bsph2* allele in mice with either deletion (520 bp deletion) or insertion of donor DNA.

Figure 2. Disruption of the mouse *Bsph2* locus. **A)** Schematic representation of WT allele with different primers (arrows) used for PCR. Vertical dashed red lines represent gRNAs. Black and grey boxes are 5'- and 3'-homology arms, respectively. **B)** PCR genotyping on genomic DNA (tail tip) from founder mice with 520-bp deletion (283) and with donor DNA insertion (981) using primer pairs indicated in Figure 2A. No template (NTC) was used as a control. GreenGlo safe DNA-stained 1.5% agarose gel of PCR products show lower fragments for deletion, donor or both in the mutated but not in the wild type (WT) mice. **C)** Subsequent generations were screened using primer pair 2 (referred in 2A), resulting in a 2316 bp-fragment in the WT (+/+) mouse and in a 1796 bp-fragment in the homozygous (-/-) mutated mouse. **D)** RT-PCR analysis using extracted total RNA from epididymis of wild type (+/+), *Bsph2*$^{+/-}$ (+/-) and *Bsph2*$^{-/-}$ (-/-), confirming lack of *Bsph2* mRNA expression in knockout mice. Beta-actin (*Actb*) was used as control.

Figure 3. DNA sequencing results for mouse *Bsph2* deletion. **A)** and **B)** Part of sequence for wild-type (**A**) and mutated (**B**) *Bsph2* genes. Sequences of exon 3 (light grey), nucleotide coding for cysteine residues within the Fn2-A domain (bold italic) and deleted region (520 bp; underlined) are indicated. **C)** Sanger sequencing results of the progeny indicated that 520 bp of *Bsph2* locus is removed by CRISPR/Cas9. **D)** The ExPASy web tool was used to interpret and

compare the function, structure and post-translational of KO protein with wild type protein. Signal peptides are underlined.

Figure 4. Comparison between epididymal *Bsph2* transcript levels of WT, *Bsph2*$^{+/-}$ and *Bsph2*$^{-/-}$ mice. RT-qPCR analysis using cDNA was carried out to validate the lack of *Bsph2* mRNA expression in mouse epididymis. mRNA expression levels were normalized by mouse *Hprt*. Data were analyzed by ordinary one-way ANOVA and Tukey's multiple comparison test of three independent mice for each genotype (**P < 0.01; ***P < 0.001); ****P < 0.0001).

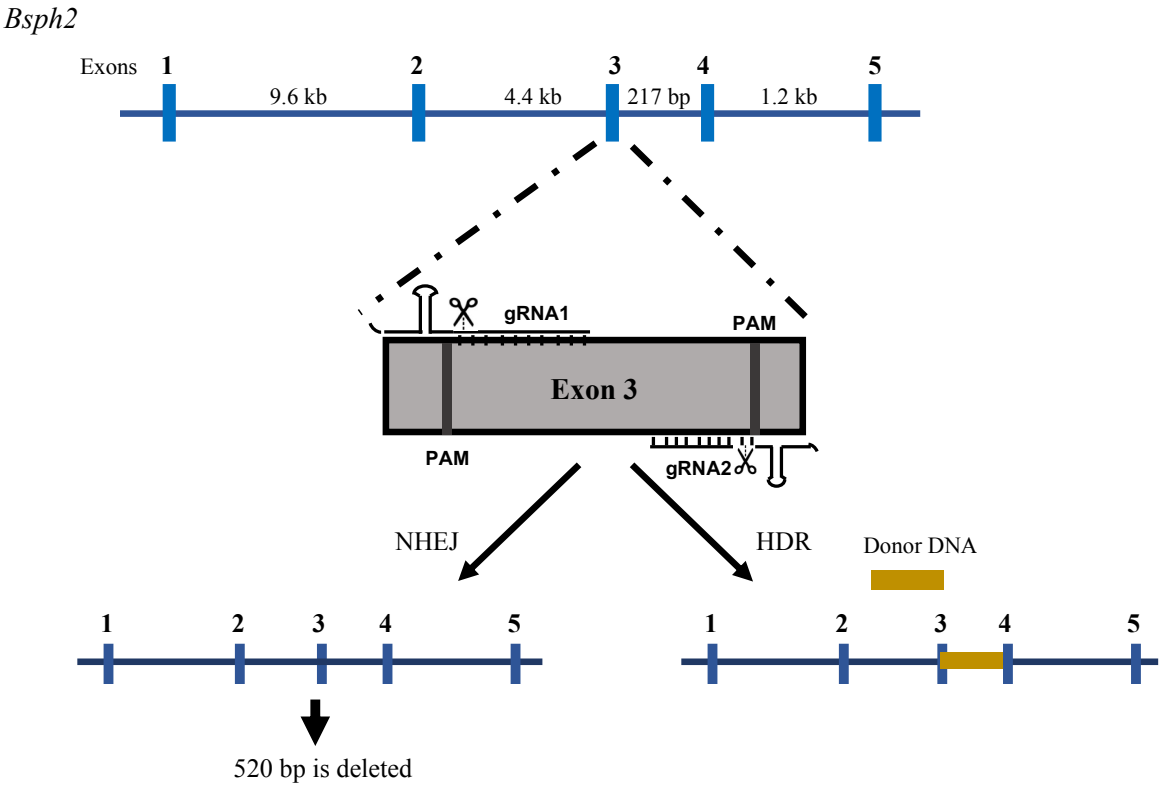
Figure 5. Comparison between epididymal *Bsph1* transcript levels of WT, *Bsph2*$^{+/-}$ and null *Bsph2*. RT-qPCR was used to assess *Bsph1* mRNA levels in three different *Bsph2* genotypes. Results are expressed in relative quantification as a ratio of the mRNA levels of *Bsph1* to *Hprt* reference gene. Data were analyzed by ordinary one-way ANOVA and Tukey's multiple comparison test of three independent mice for each genotype (**P < 0.01).

Figure 6. A) Coomassie-stained SDS–PAGE was performed with epididymal protein extracts from wild-type (WT) and single *Bsph2* knockout (KO) mice. Dashed squares represent bands (#1 and #2) that were cut and subjected for LC-MS/MS analysis. Peptide sequences detected by LC-MS/MS are shown in gray as well as the coverage percentage of the amino acid sequences of BSPH1 and BSPH2. Two peptides of BSPH1 protein (Q3UW26) were found in band #1 of WT and KO, whereas one peptide of BSPH2 (Q0Q236) was found in band #2 of WT and, as expected, nothing in our single KO. **B)** Quantitative analysis of the normalized total spectra from BSPH1 and BSPH2 proteins in WT and KO by LC-MS/MS. The top panel shows BSPH1 which is found in WT and single KO, however as is seen in bottom panel BSPH2 is found only in WT and no BSPH2 is present in single KO.

Figure 7. A) Size and **B)** weight of testes in WT and *Bsph2*$^{-/-}$ male mice. Data are presented as the mean ± SEM of five independent experiments. Differences relative to WT (control) were analyzed by GraphPad Prism 7.

Figure 8. The effect of *Bsph2* KO on mouse sperm motility parameters. Sperm were collected from cauda epididymis of 6-8-week-old mice, and motility assessment was performed in capacitated and non-capacitated sperm using SCA. Data are presented as the mean ± SEM of five independent experiments.

Figure 1


Bsph2
Exons
1
2
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4
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9.6 kb
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217 bp
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gRNA1
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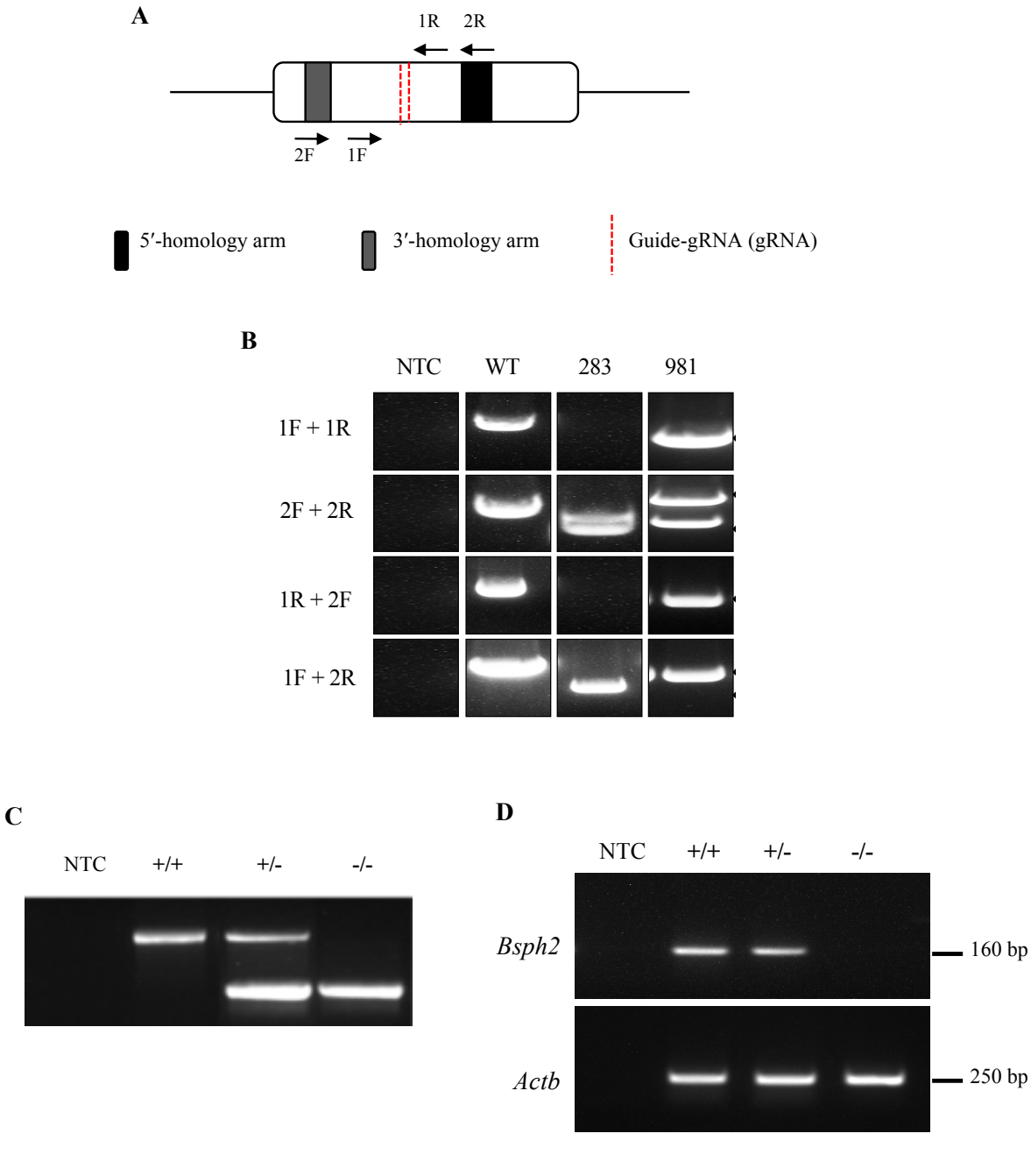
520 bp is deleted

Figure 2


A
1R
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5′-homology arm
3′-homology arm
Guide-gRNA (gRNA)
B
NTC
WT
283
981
1F + 1R
2F + 2R
1R + 2F
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NTC
+/+
+/-
-/-
Bsph2
160 bp
Actb
250 bp

Figure 3

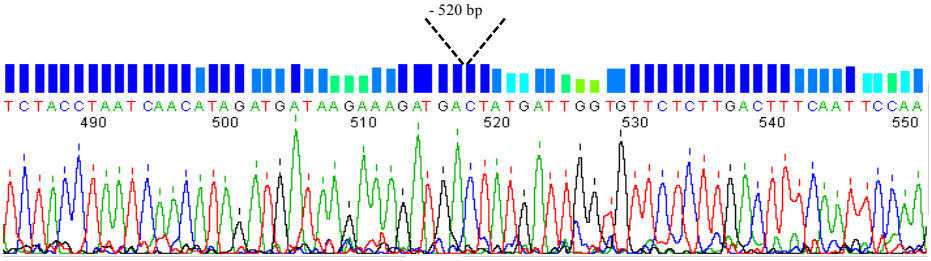
A

…GAAAATACCCTACAGATATGCCCACAGACCAATCTACCTAA
TCAACATAGATGATAAGAAAGATGAAAAAGAGTGTGAACTTT
GATAAATGAGATCCCTCCTTGCTAAAGAACTTTTTATGAGGA
AGCCAGCATCTCCAACCTGAACCCAGCTCTGGGCCAAAATGA
GACTTCCTTGGAAATTTTGCTGAAATTTTGTATTACAAGTGT
GAGAGGCAGCAAGTGACATCAGCTTCAGGTGTATGTGAGGAT
GATGAGAGAAAAATTGTTATGTTGCTGCCAAAATAGGTGTTC
CAAAGTCAAGTCCCAGTTATGCATGACAGCTCAAGCTTCCAA
TCTTAGCACTCACAAAGCTAGACAGGAAAGGTAACATGAGCT
ACAAGCAAGCCCTGGCCTAAGGAGTGAGTCTGAAGTCTAACT
GAGTGAGAACATTTTCAAAGACAAAGCATTTGAAAGTCAGTC
TCCTACATGATCTTATTTTGTATGCTAAAATTTTTCTGTTTT
CCAGAGATTTCTACTGATAGC***TGT***GTTTTTCCATTTGTTTAT
GCTGATGGATTCCACTACAGT***TGT***ATCTCCCTCCACAGTGAC
TATGATTGG***TGT***TCTCTTGACTTTCAATTCCAAGGAAGGTGG
CGGTAC***TGT***ACAGCACAGGGTAAGAATCCTACAAAGGCATAC
ATCAGATGCCAGGAACCAAAAAGAGAGTACA…

B

…GAAAATACCCTACAGATATGCCCACAGACCAATCTACCTAA
TCAACATAGATGATAAGAAAGATGACTATGATTGG***TGT***TCTC
TTGACTTTCAATTCCAAGGAAGGTGGCGGTAC***TGT***ACAGCAC
AGGGTAAGAATCCTACAAAGGCATACATCAGATGCCAGGAAC
CAAAAAGAGAGTACATT…

C



D *Bsph2* protein:

M E V M S H L V H W V F L A V Y M Y E L N A E L I S H L H P P E Q E I S T
D S C V F P F V Y A D G F H Y S C I S L H S D Y D W C S L D F Q F Q G R W
R Y C T A Q D P P K C I F P F Q F K Q K L I K K C T K E G Y I L N R S W C S
L T E N Y N Q D G K W K Q C S P N N F

Bsph2 protein after KO:

M E V M S H L V H W V F L A V Y M Y E L N A E L I S H L H P P E Q V F

Figure 4

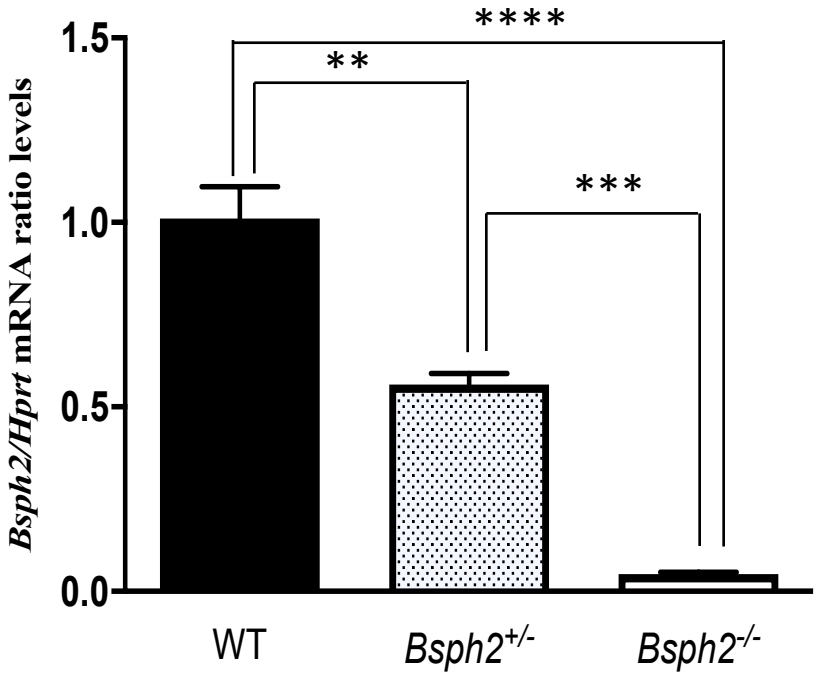


Figure 5

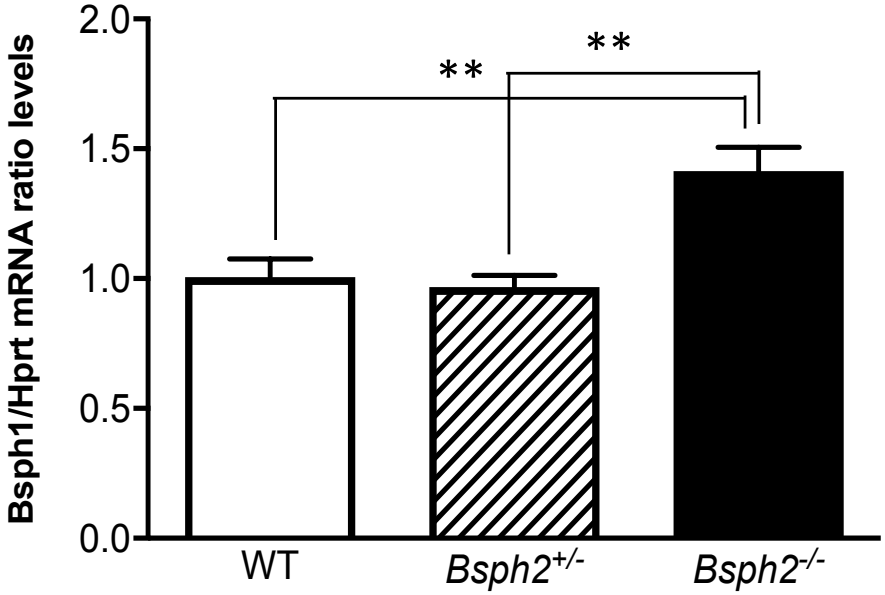
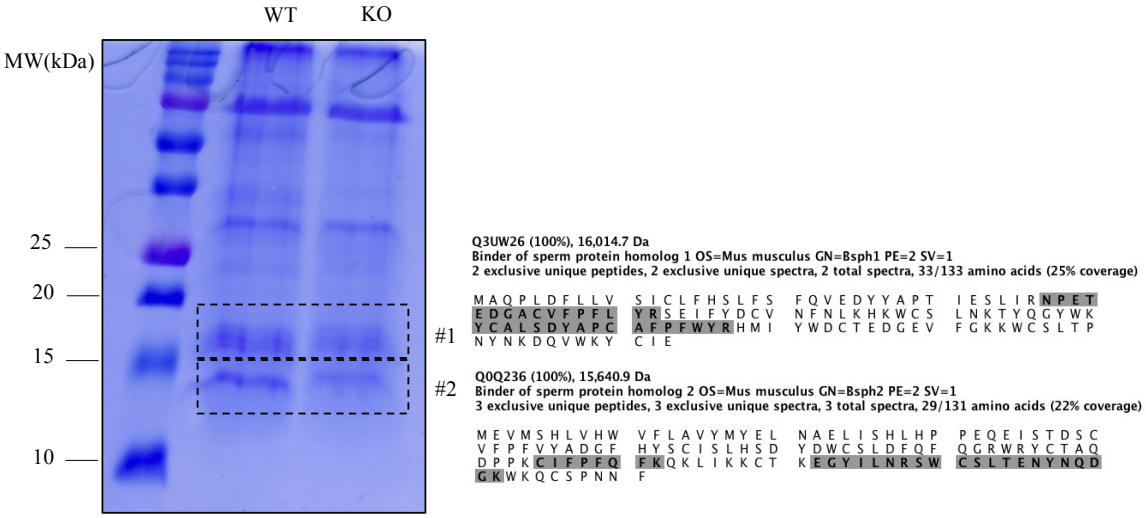


Figure 6

A


WT
KO
MW(kDa)
25
20
15
10
Q3UW26 (100%), 16,014.7 Da
Binder of sperm protein homolog 1 OS=Mus musculus GN=Bsph1 PE=2 SV=1
2 exclusive unique peptides, 2 exclusive unique spectra, 2 total spectra, 33/133 amino acids (25% coverage)
#1
MAQPLDFLLV SICLFHSLFS FQVEDYYAPT IESLIRNPET
EDGACVFPFL YRSEIFYDCV NFNLKHKWCS LNKTYQGYWK
YCALSDYAPC AFPFWYRHMI YWDCTEDGEV FGKKWCSLTP
NYNKDQVWKY CIE
Q0Q236 (100%), 15,640.9 Da
Binder of sperm protein homolog 2 OS=Mus musculus GN=Bsph2 PE=2 SV=1
3 exclusive unique peptides, 3 exclusive unique spectra, 3 total spectra, 29/131 amino acids (22% coverage)
#2
MEVMSHLVHW VFLAVYMYEL NAELISHLHP PEQEISTDSC
VFPFVYADGF HYSCISLHSD YDWCSLDFQF QGRWRYCTAQ
DPPKCIFPFQ FKQKLIKKCT KEGYILNRSW CSLTENYNQD
GKWKQCSPNN F

B

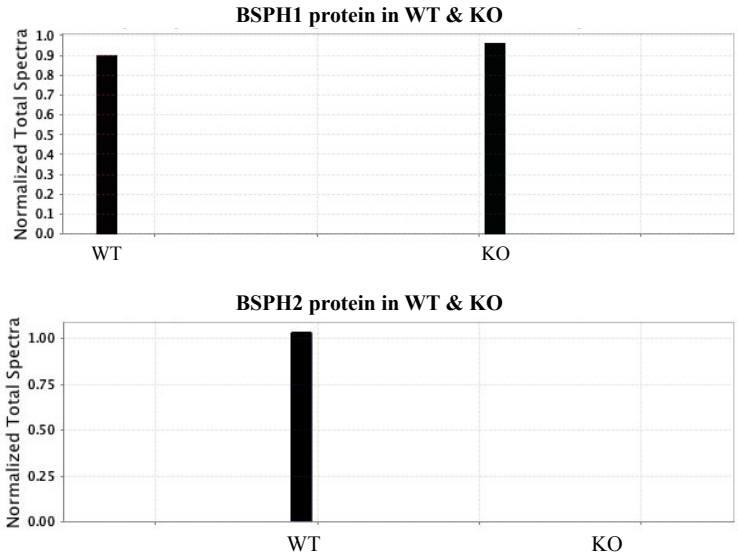
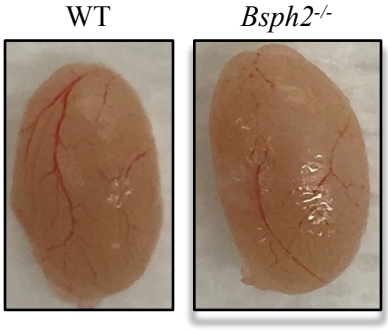

BSPH1 protein in WT & KO
Normalized Total Spectra
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
WT
KO
BSPH2 protein in WT & KO
Normalized Total Spectra
1.00
0.75
0.50
0.25
0.00
WT
KO

Figure 7

A


WT
Bsph2-/-

B

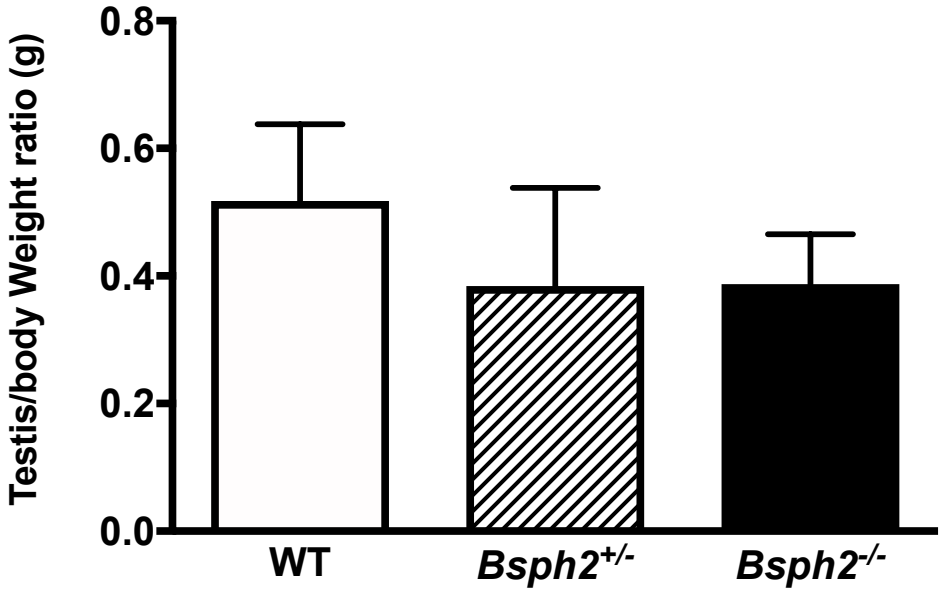
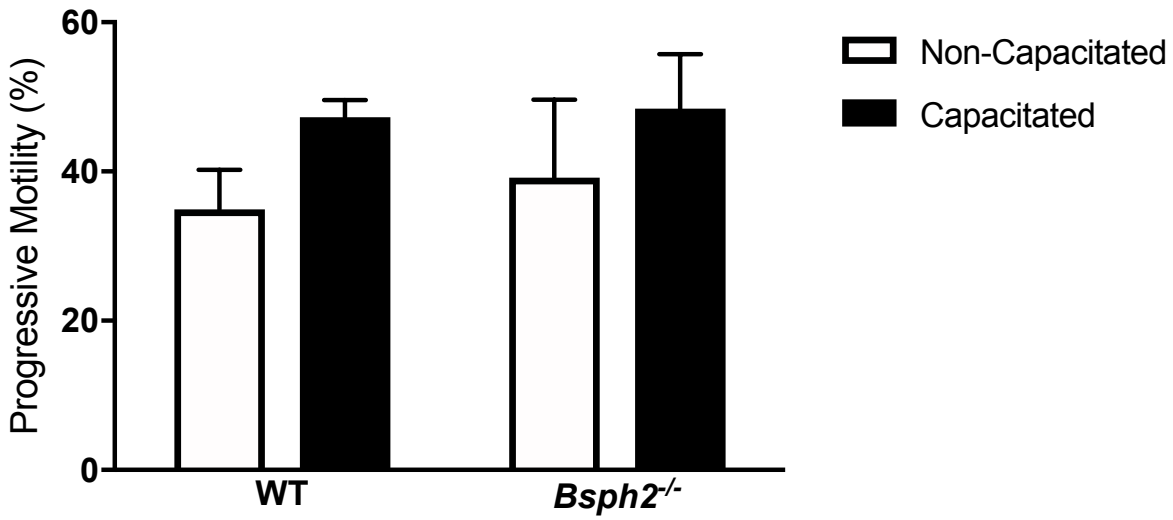

Testis/body Weight ratio (g)
0.8
0.6
0.4
0.2
0.0
WT
Bsph2+/-
Bsph2-/-

Figure 8


Progressive Motility (%)
60
40
20
0
WT
Bsph2-/-
Non-Capacitated
Capacitated

Article 2. Submitted to Scientific Reports (July 2019)

Epididymal proteins Binder of SPerm Homologs 1 and 2 (BSPH1/2) are not essential for male fertility in mice

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Abstract

The binder of sperm family of proteins has been implicated in regulating sperm maturation and capacitation. However, their physiological functions in fertility have only been verified *in vitro*. CRISPR/Cas9 genome editing was utilized to produce a double knockout mouse by simultaneously targeting the two murine binder of sperm genes, *Bsph1* and *Bsph2,* in order to uncover their roles in fertility. To confirm that the homologous genes and proteins were completely eliminated in the double knockout mice, different methods such as reverse transcription polymerase chain reaction, digital droplet-polymerase chain reaction and liquid chromatography tandem mass spectrometry were applied. The *Bsph1*/2 double knockout mouse model was evaluated for fertility and several sperm function parameters. Male *Bsph1/2* null mice*,* lacking BSPH1/2 proteins, showed normal fertility with no differences in sperm motility and sperm count compared to wild type counterparts. *Bsph1/2*-null mice mated naturally and appeared healthy. However, pup weight at 6 and 21 days, and 6 weeks was significantly increased in *Bsph1/2* double knockout animals compared to wild type mice. Taken together, these data indicate that the lack of BSPH1/2 proteins does not affect mouse fertility but their absence may influence normal growth.

Introduction

Mammalian fertilization is a multi-step process that arises from complex and regulated cellular processes, and relies on highly specialized gametes; defects in which can lead to infertility[44]. Despite numerous studies attempting to shed light on the molecular causes leading to human infertility, ambiguity remains. Several studies indicate that male and female factors contribute equally to cases of human infertility[159, 166]. Thus, it is important to assess the causes of infertility in both sexes in order to apply the foremost fertility treatment[13]. Male fertility is affected by numerous factors of environmental, behavioral and genetic origin. However, in comparison to female infertility, male infertility has been investigated less thoroughly and its causes are less accurately documented[13, 17].

In mammals, sperm cells are produced in the testis and subsequently, immotile sperm enter the epididymis, progressively gaining their motility and ability to fertilize the ovum while passing through the lumen of the different sections of this organ [reviewed in [46]. Exposure to secreted proteins in the epididymal microenvironment results in morphological and biochemical alterations in sperm. The enormous diversity of proteins expressed in the epididymis provides the proper milieu for sperm maturation and the acquisition of fertilizing ability[51]. In order to study the role of epididymal proteins in male fertility, numerous genetically-engineered mouse models have been generated and analyzed [reviewed in [46].

The Binder of SPerm (BSP) proteins constitute a conserved and ubiquitous family of mammalian sperm proteins, which are expressed in the seminal vesicles and/or epididymis depending on the species[63, 67, 72]. Two BSP homologs (BSPH1 and BSPH2) are found in mouse, while only one (BSPH1) has been identified in human[74]. The corresponding mouse homologous genes, *Bsph1* and *Bsph2*, are located on chromosome 7 and consist of five exons

and four introns. The human *BSPH1* gene is located on chromosome 19 and encompasses six exons and five introns. The secondary structure of all proteins of the BSP superfamily contains two homologous and tandemly arranged domains, which are similar to the type-II domains present in fibronectin[63]. These fibronectin type-II domains (Fn2) specifically bind to choline phospholipids (PC) present in the sperm membrane, and have been suggested to promote sperm capacitation during sperm progression through the female reproductive tract[67]. Taking into consideration that murine and human BSP proteins share many biochemical and functional characteristics such as the organ in which they are expressed, their binding properties, and a similar structure[143], studying BSP functions in mouse has the potential to provide invaluable information on the role of these proteins in human sperm function and fertility. Due to their co-localization on the same chromosome, generating a mouse model with the *Bsph1*/*Bsph2* double deletion via traditional knockout approaches presents important technical challenges. The development of new gene editing techniques has opened the door to powerful and attractive alternative approaches for investigating *in vivo* gene function. In the present study, we used CRISPR/Cas9 technology to generate a genetically-engineered mouse model in which the *Bsph1* and *Bsph2* genes were simultaneously deleted, and examined the phenotypes of the double knockout (DKO) mice. We studied the effects of the absence of BSP proteins on sperm function and fertility *in vivo*, in *Bsph1/2* DKO mice. We observed that the absence of BSP family proteins in the *Bsph1/2* DKO did not cause male infertility or subfertility. However, the average weight of pups born from mating *Bsph1/2* DKO male mice with WT females was significantly increased compared to the WT. The present study is the first to investigate the functions of BSP proteins and their role in male fertility *in vivo*.

Results

Establishment and characterization of the *Bsph1/2* DKO mouse line

The overall strategy used to generate and analyze *Bsph1*/*2* DKO mice is depicted in Fig. 1. Exon 2 and exon 1 of the *Bsph1* and *Bsph2* loci, respectively were selected for targeting by CRISPR/Cas9 (Fig. 2). The guide RNAs (gRNAs) were designed, synthesized and ligated into the pX330 plasmid to target the early sequence of exons 2 and 1 of the *Bsph1* and *Bsph2* genes, respectively. Before injecting the constructs into mouse embryos, the designed gRNAs were validated in neuroblast N2 cells to ensure that they could target efficiently and cut the desired regions of BSP genes. The neuroblast N2 cells are easy to grow and transfect, are the most commonly used mouse cell line for performing CRISPR/Cas9. After validation, gRNAs along with Cas9 were microinjected into mouse two-cell embryos and transferred into pseudo-pregnant females. Microinjection of plasmid containing the gRNAs and Cas9 to target the *Bsph1* and *Bsph2* genes resulted in 31 newborn pups (12 males and 19 females). Genotyping showed that 8 of 31 founders were targeted by CRISPR/Cas9 for both genes (*Bsph1* and *Bsph2*) on at least one allele. To identify a line containing the *Bsph1/2* double deletion on the same sister chromatid, six founders (2 males and 4 females) were mated with wild-type (WT) animals to establish heterozygous *Bsph1/2* DKO mice. PCR analysis followed by Sanger sequencing showed that the CRISPR/Cas9 technique resulted in the removal of a large portion of the *Bsph1* and *Bsph2* genes, as well as the complete deletion of the 81-Kb linker sequence between these two genes, almost 84 kb of the gene was deleted by CRISPR/Cas9 (Fig. 2).

To investigate gene modifications at the target loci, primers were designed (Table 1) to amplify the respective targeted sites for both *Bsph1* and *Bsph2* (Fig. 3A-D). In order to consider all the probabilities for gene modification, a combination of three primers (listed in Table 1) were used

to screen for *Bsph1*. For the *Bsph1* gene, pairs of forward and reverse primers (1F and 1R) were designed to specifically amplify the gene region targeted by CRISPR. Successful amplification using this primer pair would indicate that the allele is WT (Fig. 3B). However, the third primer (2F) of *Bsph2* assists to recognize if the sequence between two targeted sites has been removed by CRISPR/Cas9. Therefore, the PCR amplicon sizes for WT and DKO alleles were 459 bp and 1.5 kb, respectively (Fig. 3B). To amplify the *Bsph2* gene, a pair of primers (3F and 3R; see Table 1) was used; the expected amplification products are a single 519 bp band for WT and no product for KO as the binding site for the reverse primer is lost (Fig. 3C). PCR products were sequenced by Sanger sequencing to confirm the mutations at the targeted loci. PCR results and Sanger sequencing therefore show that CRISPR/Cas9 editing resulted in elimination of the entire sequence between exon 2 and exon 1 of the *Bsph1* and *Bsph2* genes (Fig 3D). Next, reverse transcription polymerase chain reaction (RT-PCR) was performed using cDNA synthesized from the epididymides of sexually mature male mice to explore the expression pattern of *Bsp* transcripts in the disrupted mice[167]. As expected, *Bsph1* and *Bsph2* transcripts were not detected by RT-PCR in homozygous *Bsph1/2* DKO mice (Fig. 3E). Consistent with the RT-PCR results, digital droplet-polymerase chain reaction (ddPCR) analysis showed that the transcripts of *Bsph1* and *Bsph2* were also eliminated completely in homozygous *Bsph1/2* DKO mice (Fig. 3F).

To further prove that BSP proteins are absent in *Bsph1/2* DKO mice, LC-MS/MS analysis was performed on WT and DKO epididymal protein extracts. The peptides obtained from LC-MS/MS analysis of WT epididymal protein extracts were compared to those obtained from *Bsph1/2* DKO extracts. As shown in Table 2 and Fig. 4, neither BSPH1 nor BSPH2 peptides were detected by LC-MS/MS in epididymal extracts from the *Bsph1/2* DKO mice. We used a

standard 1% false discovery rate on peptides and protein and found two BSPH1 peptides (Q3UW26) and three BSPH2 peptides (Q0Q236) only in epididymal extracts from WT mice. At this level of confidence, no peptides from either BSP protein was found in the *Bsph1/2* DKO samples. No BSP proteins were detected in epididymal protein samples of DKO mice by LC-MS/MS even if the level of confidence was decreased to 20%.

Bsph1/2 DKO male mice have normal sperm parameters

To determine whether the absence of BSP proteins had an effect on sperm parameters, sperm from six-week-old *Bsph1/2* DKO and WT mice were collected and analyzed by Sperm Class Analyzer (SCA) system. No significant differences were observed between WT and *Bsph1/2* DKO mice with regards to sperm motility (Fig. 5A), percentage of progressive motility (Fig. 5B), rapid motility (Fig. 5C), and non-progressive motility (Fig. 5D). No obvious abnormalities were observed in the morphology of *Bsph1/2* DKO sperm (data not shown). Moreover, sperm count was not different between *Bsph1/2* DKO and WT mice. The appearance and weight of the testes of WT and *Bsph1/2* DKO mice were also comparable (Fig. 5E).

Bsph1/2 DKO male mice have increased body weight but exhibit normal fertility

To investigate the effect of the absence of BSP proteins on male fertility, trio mating was performed by either mating one mutant male mouse with two WT female mice, or one WT male with two mutants *Bsph1/2* DKO females. Six-week-old male *Bsph1/2* DKO mice showed normal fertility as assessed by the number of pups born compared to the WT control group (Fig. 5F). The mating experiments resulted in the birth of a comparable number of pups from WT and *Bsph1/2* DKO male mice (Fig. 5F). More matings were carried out using older *Bsph1/2* DKO

male mice with WT females to determine whether the absence of BSP proteins could affect the fertility of aged male mice. However, no significant differences were observed in terms of fertility between the knockout and WT aged mice (data not shown). In addition, gestation time of WT females impregnated by *Bsph1/2* DKO males was comparable to that of WT females impregnated by WT males (data not shown). In our breeding analysis of *Bsph1/2* DKO mice, no obvious abnormalities in pup development were observed when compared to the WT group. No differences were observed between the number and growth rate of male and female in WT and DKO. Interestingly, despite an obvious effect on fertility, the body weight of male and female *Bsph1/2* DKO mice at 6 days, 21 days (weaning) and 6 weeks of age was significantly increased compared to WT mice (Fig. 6).

Discussion

Many molecules and proteins are suspected to play a role in fertility. Determining whether expression of specific genes is required for fertility could help to improve the diagnosis of infertility and select the best option for clinical treatment. Gene knockout (KO) is used to determine whether the absence of one or more genes leads to impacts *in vivo*[168]. In addition, mutation of genes expressed only in reproductive organs often does not affect animal viability. Many *in vitro* studies have characterized BSP proteins and shown that these proteins are present only in the male reproductive tract, suggesting that they could play an important role in sperm function in human and rodents[169]. Gene KO would thus be an effective strategy to determine whether the presence of BSP family proteins is essential for fertility *in vivo*[91]. In this study, we used CRISPR/Cas9 technology to target the two BSP genes in mouse, *Bsph1* and *Bsph2*, in

order to investigate their role in male fertility. Despite their proposed role in sperm maturation and capacitation, we show that the BSP proteins are not required for fertility, at least in mice.

In terms of fertility, our past[169] and current *in vivo* results indicate that BSP proteins, either individually or together, are not essential for sperm maturation and fertilizing ability in mice[169]. More recently, investigation of fertilization related genes by gene disruption technology demonstrated that many of these genes, despite being shown to be essential *in vitro*, were surprisingly found to be dispensable *in vivo* [170]. These include the genes for *Acr* (acrosin)[171], *β4galt1* (GalTase)[172], *Adam1a/b* (Fertilin)[173], *Crisp1*[174] and many other fertilization related factors[170, 175]. However, at present only a handful of fertilization related factors such as IZUMO1[162] and Adam2[176] are known to be essential for fertilization.

A fertility study by Miyata et al. analysed the necessity of 54 conserved testis genes for fertility in mice. Results indicated that despite the evolutionary conservation of the genes, the absence of these genes had no immense impact on male fertility[125]. Hundreds of genes are expressed in the reproductive tract and the potency of gene manipulation techniques now gives us the ability to discover the function and importance of each gene. Despite the lack of an apparent phenotype in some KOs, it is essential to share the phenotypic data to avoid unnecessary redundant experiments and expenditures[125]. Furthermore, a deep systematic analysis of mouse epididymal sperm proteins by Chauvin et al. lead to the identification of 2850 epididymal proteins, which is the most comprehensive and complete proteome in cellular pathways encounter, and some of which might share functions with other epididymal proteins.[177]. Therefore, the absence of one family of proteins such as BSPs might be rescued by other proteins working in parallel, thereby preventing the appearance of any phenotype[169, 177]. The redundancy of the multiple genes involved in male reproduction can compensate for the absence

of the mutated genes in the KO and DKO mice, thus preventing our observation of any infertility phenotype. Therefore, it remains a tedious challenge to discover the genes that are working in parallel to enable fertilization, since many share similar functions and can serve as a backup for one another.

A lack of a male reproductive phenotype in the *Bsph1/2* DKO mice was not unexpected. Data from a study by Fan et al.[75], showed that BSP proteins in certain species such as mice and human are expressed in very low quantities, whereas they constitute around sixty percent of seminal plasma proteins in other species such as bovine. This suggests that BSP proteins may simply play different roles in different species[75, 78]. Based on numerous KO studies, it seems that genetic redundancy also plays an important role in the reproductive system to minimize the effects on fertility of deficiencies and/or mutations in one or more genes. It appears that multiple gene families work in parallel to ensure each other's functions in order to avoid any malfunctions from occurring. This could be the reason why no obvious phenotype was observed in the majority of KO mice produced for epididymal genes such as *Wdr63*[178] and *Dnaic1*[179]. Another possible explanation is gene duplication and multiplicity in gene copy number. In this study, both the *Bsph1* and *Bsph2* genes were deleted and no subfertility or infertility phenotype was observed. This could be attributable to the existence of genes encoding proteins with similar functions to BSP proteins, but with unrelated sequences. Thus, it is possible that for male infertility to arise, mutations or dysregulation must be present in more than one family of genes. It would be fundamental to assess groups of genes in the infertile male to help pinpoint the causes of infertility.

Interestingly, the weight of pups born from WT females fertilized by *Bsph1/2* DKO males was significantly increased compared to those born from WT females fertilized by WT males. This

was an unexpected result that suggests that BSP genes and their encoded proteins could play a role in weight regulation. Alterations in androgen secretion have been shown to be profoundly associated with changes in metabolism and obesity[180]. In fact, a progressive decrease in androgen levels has been shown to result in an increase in body weight[181]. Interestingly, the 81 kb DNA segment between *Bsph1* and *Bsph2* in mice appears to include binding sites for the androgen receptor (AR)[182, 183]. Recent ChIP-sequencing analyses in mice found that the AR binds a DNA region only 500 bp upstream of the *Bsph2* gene, as well as another region located in between *Bsph1* and *Bsph2*[182]. The regulation of the expression of BSP genes is currently not clearly understood, but given the presence of AR binding sites in the mouse BSP genes, androgen-dependent regulation of BSP expression is plausible. Studies in young calves and boars indicated that BSP protein expression increases with age following sexual maturity[184, 185]. In addition, Ekhlasi-Hundrieser et al. showed that the androgen supplementation in castrated adult boar resulted in restored BSP expression[186], whilst in castrated rabbit, androgen treatment was unable to restore BSP expression to normal levels[99]. If androgen levels, body weight, and BSP expression are indeed linked, then an examination of the interaction of AR with the DNA segments within the mouse BSP locus would warrant further investigation.

Conclusion

Our findings demonstrate that epididymal BSP proteins are dispensable for male fertility and do not fulfill critical roles in fertilization in mice. As revealed by recent gene knockout studies [132], many fertilization factors have been shown to be dispensable for fertility. As reported

here, we have found that BSP proteins are also non-essential for fertility in mice; however, they may have an unexpected function in body weight regulation.

Materials and methods

Animals

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a temperature-controlled (22 ± 1°C), light-controlled (a light cycle of 12 h light: 12 h dark), and pathogen-free environment. All mouse experimental work was carried out according to the guidelines of the Canadian Council of Animal Care (CCAC). Mouse protocols were approved by the Maisonneuve-Rosemont Hospital animal care committee (#2017-SE-020).

Generation of *Bsph1*/*Bsph2* DKO mice

A CRISPR design web tool (www.crispor.tefor.net) was first used to identify suitable regions on the mouse genome to target exon 2 and exon 1 of the *Bsph1* and *Bsph2* genes, respectively (Fig. 2). Guide RNA (gRNA) sequences were selected to optimize their predicted low, overall off-target potential. Primers corresponding to the two chosen guide sequences were cloned into the pX330-U6-Chimeric_BBCBh-hSpCas9 (#42230) plasmid was kindly provided by Dr. Qinzhang Zhu. Primers for the *Bsph1*_gRNA were: forward, 5'-caccgATTATTATGCACCAACTAT-3'; reverse, 3'-cTAATAATACGTGGTTGATAcaaa-5'. Primers for the *Bsph2*_gRNA were: forward, 5'-caccgCTCATACATGTAGACAGCT-3'; reverse, 3'-cGAGTATGTACATCTGTCGAcaaa-5'. The SpCas9/chimeric gRNA constructs were first validated in neuroblast Neuro-2a cells to generate the desired deletion in genomic

DNA. The validated guide constructs were then microinjected into fertilized B6C3F1/J mouse eggs using the Microinjection and Transgenesis service of the Institut de Recherches Cliniques de Montréal (IRCM). Genomic DNA was extracted from tail snips using the AccuStart II Mouse Genotyping Kit (Quanta Bioscience, Beverly, MA, USA) following the manufacturer's protocol. PCR genotyping was established using the primer pairs indicated in Table 1 and the following conditions for both *Bsph1* and *Bsph2*: 94ºC for 3 min, followed by 30-35 cycles of 94ºC for 30 s, 62ºC and 72ºC for 30 s each. The guide RNAs along with pX330 plasmid were injected into the pronucleus of a zygote as described previously[169].

RT-PCR and ddPCR

RNA was extracted from epididymides of six-week-old male mice using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The Agilent RNA 6000 Nano Kit was used to assess the quality of freshly extracted RNA and RNA was stored at –80 °C. The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used to synthesize cDNA from RNA as described previously[169]. Prepared cDNA was subjected to digital droplet-PCR ddPCR using the primers shown in Table 1 to assess *Bsph1* and *Bsph2* gene expression in WT and *Bsph1*/*2* DKO mice. In this study, the ddPCR system included a droplet generator and reader from Bio-Rad, (QX200 Droplet Digital PCR, Bio-Rad, Hercules, California, USA), which fractionates samples into ~20,000 droplets. The ddPCR method was performed according to the manufacturer's instructions with some modifications as described below. Amplification was performed in a 20 µL reaction mixture containing 2 µl of corresponding cDNA diluted 1:100, 800 nM of corresponding primers and 2X ddPCR EvaGreen supermix (Bio-Rad). Samples were subjected to droplet generation by an automated droplet generator and end-point PCR was performed afterwards. Cycling steps for the ddPCR were as

follows: initial enzyme activation at 95°C for 10 min followed by 50 cycles of denaturation and annealing (each cycle at 95°C for 30 s; 58°C for 1 min; 72°C for 30 s) and ending with enzyme deactivation at 98° C for 10 min. Finally, droplets were read on a droplet reader and data were analyzed using QuantaSoft™ Software, which determines the number of droplets that were positive and negative for each fluorophore in each sample. The fraction of positive droplets was then fitted to a Poisson distribution in QuantaSoft™ software to determine the absolute copy number in units of copies/µl. Ratios of *Bsph1* and *Bsph2* were calculated with endogenous control *Hprt*. Primers used are listed in Table 1.

Fertility assessment

Sexually mature *Bsph1/2* DKO male mice (6-8 weeks of age) were paired with two WT female mice for three months. Following pairings, cages were checked every morning for copulatory plugs. Newborn pups were analyzed for numbers and weight at 6 and 21 days, and 6 weeks from birth.

Epididymal sperm analysis

Epididymides from either WT or *Bsph1/2* DKO mice were dissected, and cauda regions were placed in a petri dish containing 300 µl of human tubal fluid medium (HTF) medium. Multiple incisions were made in the separated cauda to squeeze out sperm, which were then incubated at 37°C-5% CO_2 for 10 min. Sperm suspensions (3 µl) were loaded into a Leja slide chamber (20 µm depth) and sperm were analyzed for motility and concentration using Sperm Class Analyzer (SCA) (software version 5.2.0.1.; Barcelona, Spain, MICROPTIC S.L.). The parameters for video utilization were as follows: frames acquired, 50; VCL (rapid), 320 µm/s; VCL (medium), 193 µm/s; VCL (slow), 80 µm/s; VAP set to 7; Lin rapid, 40% and connectivity, 20.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Epididymides from six *Bsph1/2* DKO and WT adult male mice (aged 6-7 weeks) were dissected. Total epididymal protein was extracted using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Protein concentration was quantified with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Bremen, Germany), then 80 µg of each sample was separated by SDS-PAGE on 18% sodium dodecyl sulfate-polyacrylamide gels. Gels were stained with Coomassie Blue and bands corresponding to BSPH1 and BSPH2 (10-20 kDa) were excised and subjected to LC-MS/MS analysis by the Proteomics Core Facility at CHU de l'Université Laval (Québec, Canada), as described previously[169]. The UniProt complete proteome *Mus musculus* Scaffold (version Scaffold_4.8.4, Proteome Software Inc., Portland, OR) was used to interpret MS/MS-based peptide and protein identification.

Statistical analysis

Statistical calculations were performed using Prism 3.0 software (GraphPad Software, La Jolla, CA, USA). Statistical analyses were performed using the Student's t-test and by two-way ANOVA. Differences were considered significant at $P < 0.05$.

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Authors Contributions Statement

M.E.S wrote the main manuscript text and contributed to design, conduct, acquisition of data and analysis all the experiments. B.P contributed to acquisition of data, analysis and design Fig 2. F.B and R.S.V contributed to the conceptual design and analysis of the targeting efficiency of the gRNAs used to generate the DKO mice. P.M contributed to the conception of the experiment. All authors revised the article and approved the final version for publishing.

Competing Interests

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Figure 1. Overall strategy for the design, generation, and analysis of *Bsph1/2* DKO mice.

Figure 2. Schematic representation of the *Bsph1* and *Bsph2* genomic locus in mouse and the CRISPR/Cas9 gene deletion strategy. For each gene, exons are shown (boxes) with untranslated (white) and coding (black) regions. A part of the DNA sequence of the targeted coding exons are shown (highlighted in grey), with the respective selected guide RNA sequences (underlined) associated with the PAM sequence (red underlined). Scissors indicate the theoretical positions where Cas9 creates the double stranded break (3-4 nucleotides upstream of the PAM). The non-homologous end joining (NHEJ) repair pathway creates random insertions or deletions (indels) at the site of cut.

Figure 3. Characterization of the *Bsph1/2* DKO mouse line. Genomic DNA from tail snips of wild type (WT), double heterozygous (HE) and double homozygous (HO) mice was amplified by PCR using primers for *Bsph1*, *Bsph2* and the 81-kb linker between the two genes, as indicated. A) Schematic representation of the *Bsph1/2* gene loci with overlaying primers (arrows) used for PCR amplification of the *Bsph1* and *Bsph2* genes. B) Amplification using *Bsph1* primers resulted in bands of 1.5 kb and 459 bp for the mutated and WT genes, respectively. C) Amplification using *Bsph2* primers generates a 519 bp fragment for WT and no product for *Bsph1/2 DKO.* D) Amplification using 81 kb primers generates a 828-bp fragment for WT and no product for *Bsph1/2* DKO. E) RT-PCR analysis of epididymal RNA from WT and *Bsph1/2* DKO mice using primers for *Bsph1*, *Bsph2*. No template (NTC) was used as a control. F) Expression of *Bsph1* and *Bsph2* genes in WT and *Bsph1/2* DKO epididymis as evaluated by ddPCR. *Bsph1* and *Bsph2* expression in DKO was compared with WT and the

Hprt gene was used as a reference. Data are shown as the mean ±SEM (n= 3). Asterisks indicate a statistically significant difference. (*** $P < 0.001$).

Figure 4. LC-MS/MS analysis of epididymal protein extracts from WT and *Bsph1/2* DKO mice. Peptide sequences for BSPH1 and BSPH2 detected in WT by LC-MS/MS are shown in yellow. No peptide sequences for BSP proteins were detected in protein extracts from DKO mice. As indicated in the circle two peptide of BSPH1 and one of BSPH2 were found in WT1 and WT2. However, no peptide of BSP were found in two Bsph1/2 DKO.

Figure 5. Analysis of *Bsph1/2* DKO and WT epididymal sperm function and fertility **A)** Sperm motility, **B)** Progressive motility, **C)** Rapid motility, **D)** Non-progressive motility, **E)** Ratio of testis to body weight (B.W.) and **F)** Average number of pups born from WT females. The numbers of males analyzed for each parameter studied are indicated in parentheses. Values are presented as the mean ± SEM.

Figure 6. Comparison of the body weight of WT and DKO pups at 6 and 21 days, and 6 weeks. Data are presented as the mean ± SEM of eight independent experiments. Differences relative to WT (control) were analyzed by GraphPad Prism 7. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 1. Oligonucleotides used in this study

Primers	*Sequences (5'—3')*	*Experimental use*
Bsph1-E2-CRISPR-F2[a]	AGGCCACTGGACTAGAGTCAT	PCR
Bsph1-E2-CRISPR-R2[a]	ACAGCAGGCACAAGACCATT	PCR
Bsph2-E1-CRISPR-F[b]	GGCAAGGTATGCTCCTGTGT	PCR
Bsph2-E1-CRISPR-F2[b]	GCAAAACCAAAACCTCCCCA	PCR
Bsph2-E1-CRISPR-F[C]	GGCAAGGTATGCTCCTGTGT	PCR
Bsph2-E1-CRISPR-R[C]	GACCAAGGCTCCGTCATAGG	PCR
R81s-F[D]	CCTGCAATGCAGATTCCAACATATTGCC	PCR
R81s-R[D]	GGTGAGAGCCACATGTATTTGTGGGTCC	PCR
RT-mBsph2-E1F	AGTAGCCATCTTGTGCACTGG	RT-PCR
RT-mBsph2-E4R	CCTCCTTGGTGCACTTCTTAATGA	RT-PCR
mHprt-F	TCCTCCTCAGACCGCTTTT	ddPCR
mHprt-R	CCTGGTTCATCATCGCTAATC	ddPCR
qPCR-Bsph1-F5	AGTAGAAATCTCTTGTTCTGGAGG	ddPCR
qPCR-Bsph1-R5	AGTAGAAATCTCTTGTTCTGGAGG	ddPCR
qPCR-Bsph2-F6	GGGTGTTCCTAGCTGTCTAC	ddPCR
qPCR-Bsph2-R6	GGAGGGAGATACAACTGTAGTGG	ddPCR

[a]Referred to as primers 1F and 1R in Figure 4A.

[b]Referred to as primers 2F in Figure 4A.

[C]Referred to as primers 3F and 3R in Figure 4A.

[D]Referred to as primers 4F and 4R in Figure 4A

Table 2. Peptide sequences found for BSPH1 and BSPH2 in WT and *Bsph1/2* DKO mice.

Band No.	Protein ID's	Mol. wt. (kDa)	Quantitative Value (Norm. total spectra)	Sequence Coverage (%)	Unique Peptide reads	Unique Spectrum matched	Peptide sequence matched
WT # 1	BSPH1	16	2	25	2	2	NPETEDGACVFPFLYR YCALSDYAPCAFPFWYR
WT # 1	BSPH2	15.6	1	6	1	1	CIFPFQFK
WT # 2	BSPH2	15.6	3	22	3	3	CIFPFQFK EGYILNR SWCSLTENYNQDGK
DKO # 1	-	-	0	0	0	0	-
DKO # 2	-	-	0	0	0	0	-

Fig. 1

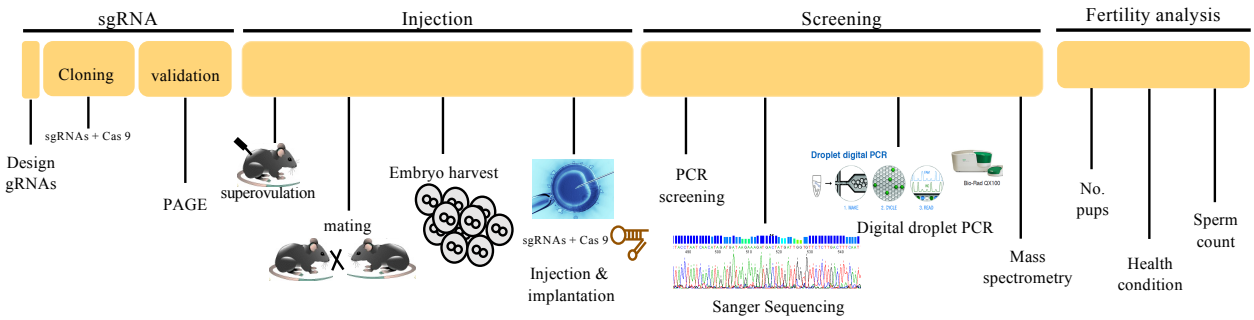
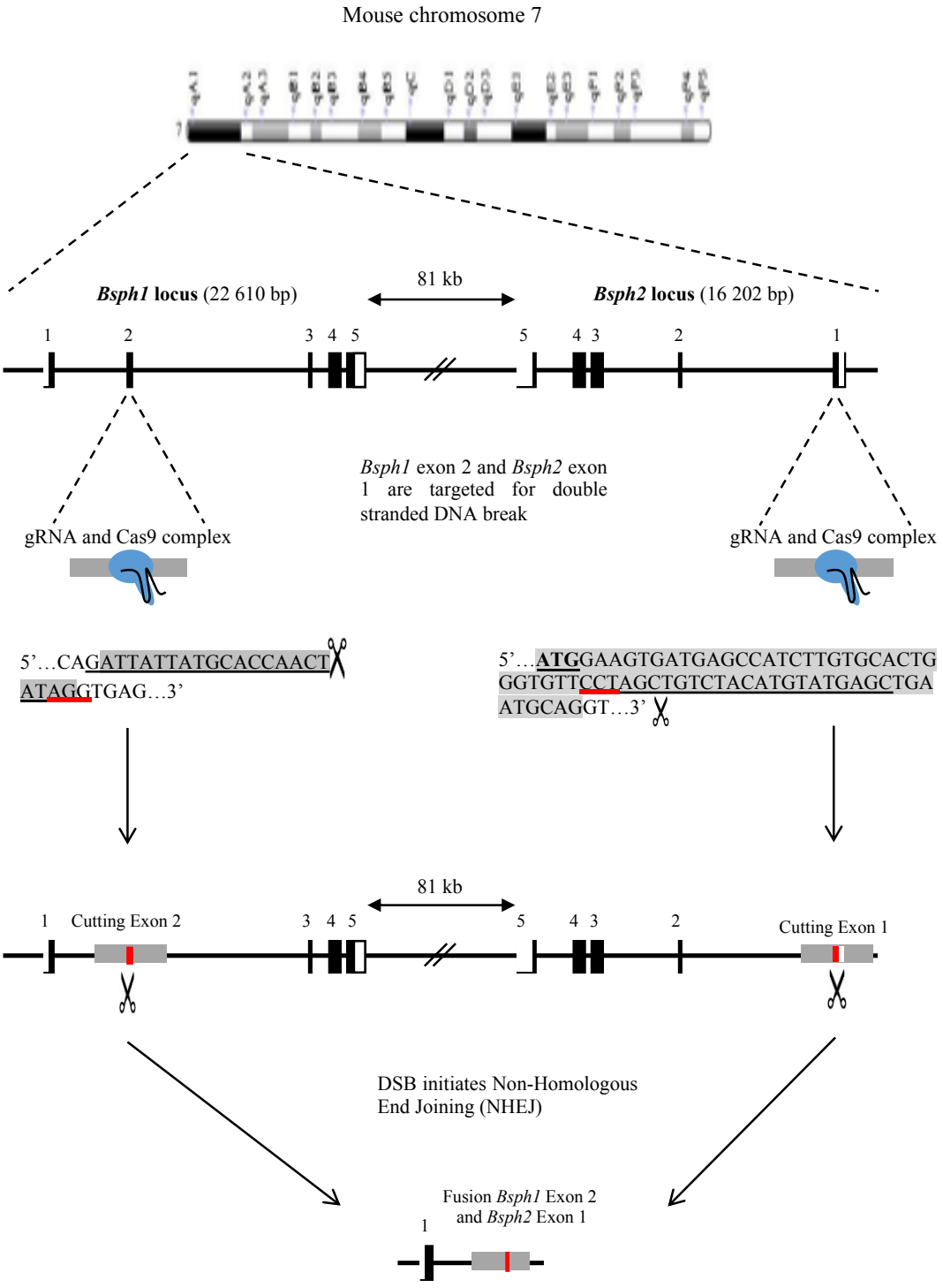
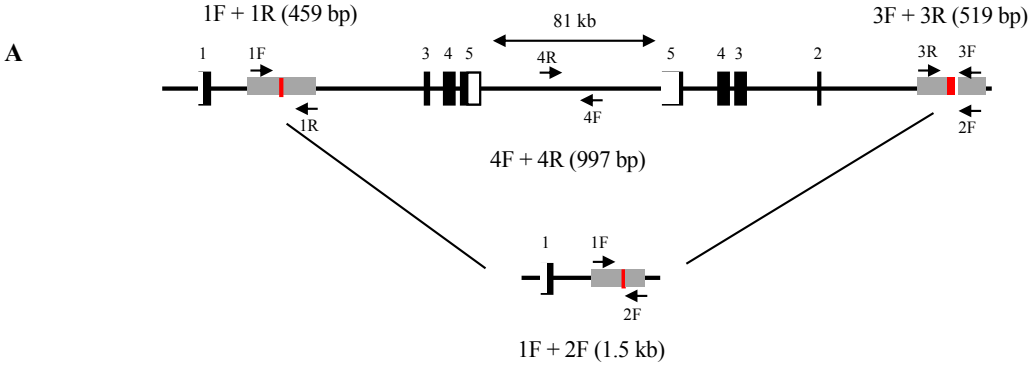

sgRNA
Injection
Screening
Fertility analysis
Cloning
validation
Design gRNAs
sgRNAs + Cas 9
PAGE
superovulation
mating
Embryo harvest
sgRNAs + Cas 9
Injection & implantation
PCR screening
Sanger Sequencing
Droplet digital PCR
Digital droplet PCR
Mass spectrometry
No. pups
Health condition
Sperm count

Fig. 2



***Bsph1* and *Bsph2* knockout**

Fig. 3


A
1F + 1R (459 bp)
81 kb
3F + 3R (519 bp)
1
1F
3 4 5
4R
5
4 3
2
3R
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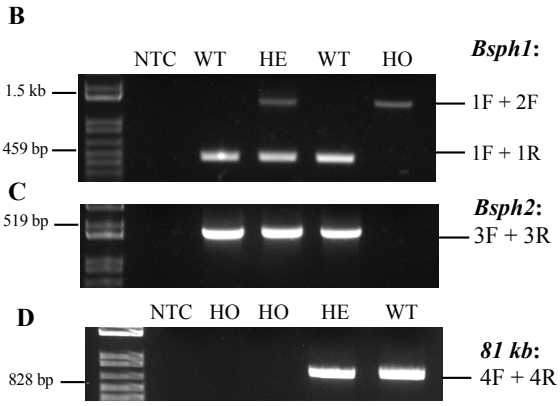
1R
4F
2F
4F + 4R (997 bp)
1
1F
2F
1F + 2F (1.5 kb)


B
NTC WT HE WT HO
Bsph1:
1.5 kb
459 bp
1F + 2F
1F + 1R
C
519 bp
Bsph2:
3F + 3R
D
NTC HO HO HE WT
828 bp
81 kb:
4F + 4R

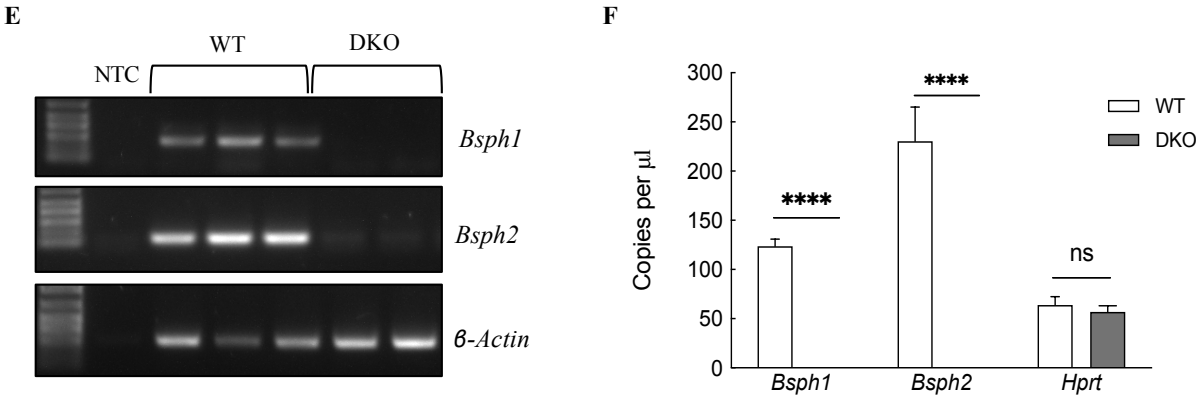
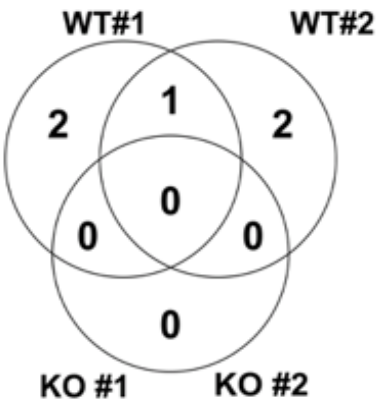

E
WT
DKO
NTC
Bsph1
Bsph2
β-Actin
F
Copies per µl
300
250
200
150
100
50
0
****
****
ns
WT
DKO
Bsph1
Bsph2
Hprt

Fig. 4



Q3UW26 (100 %), 16 014,7 Da
Binder of sperm protein homolog 1 OS=Mus musculus GN=Bsph1 PE=2 SV=1
2 exclusive unique peptides, 2 exclusive unique spectra, 2 total spectra, 33/133 amino acids (25 % coverage)

MAQPLDFLLV SICLFHSLFS FQVEDYYAPT IESLIRNPET EDGACVFPFL
YRSEIFYDCV NFNLKHKWCS LNKTYQGYWK YCALSDYAPC AFPFWYRHMI
YWDCTEDGEV FGKKWCSLTP NYNKDQVWKY CIE

Q0Q236 (100 %), 15 640,9 Da
Binder of sperm protein homolog 2 OS=Mus musculus GN=Bsph2 PE=2 SV=1
3 exclusive unique peptides, 3 exclusive unique spectra, 3 total spectra, 29/131 amino acids (22 % coverage)

MEVMSHLVHW VFLAVYMYEL NAELISHLHP PEQEISTDSC VFPFVYADGF
HYSCISLHSD YDWCSLDFQF QGRWRYCTAQ DPPKCIFPFQ FKQKLIKKCT
KEGYILNRSW CSLTENYNQD GKWKQCSPNN F

Fig. 5

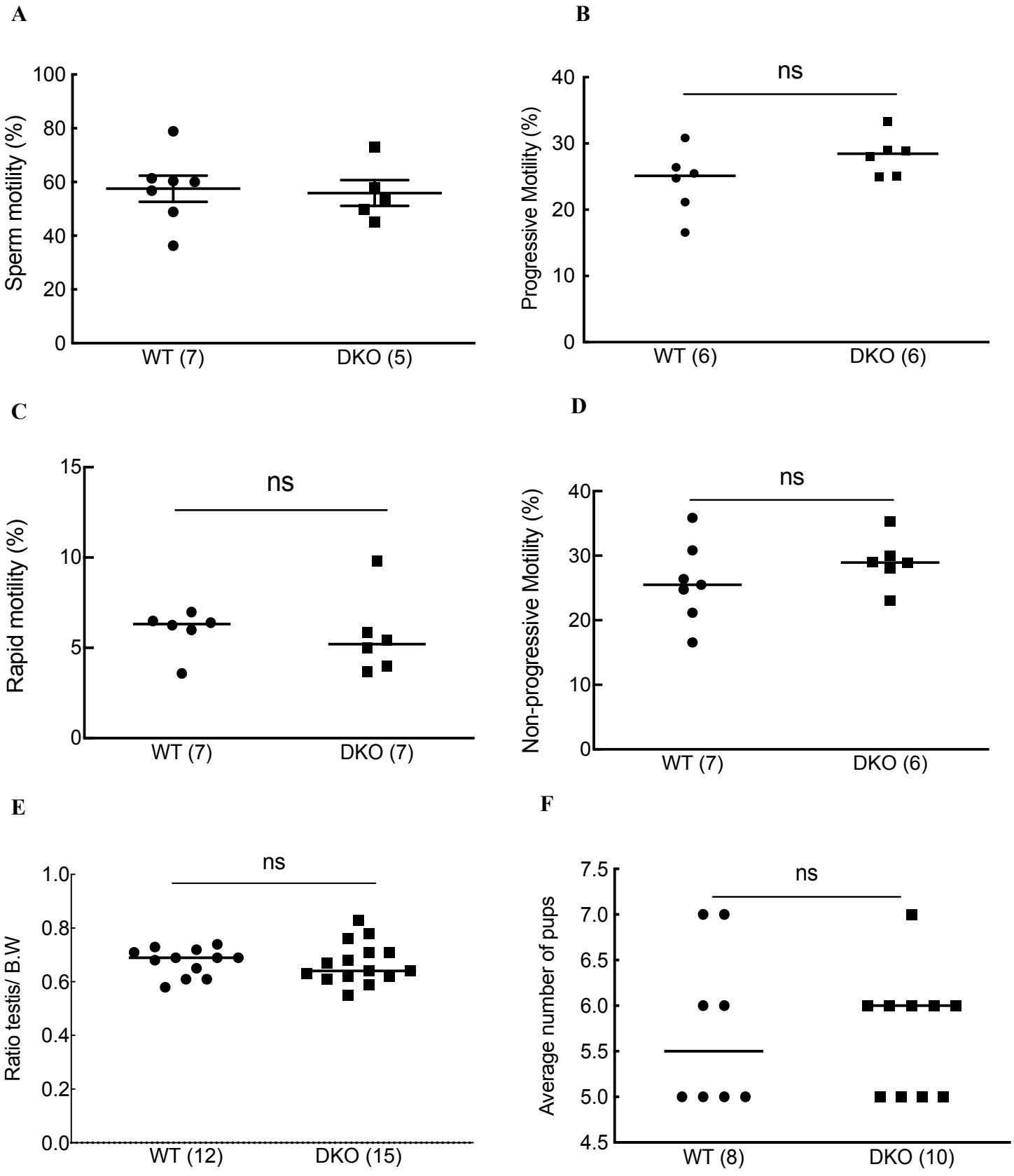
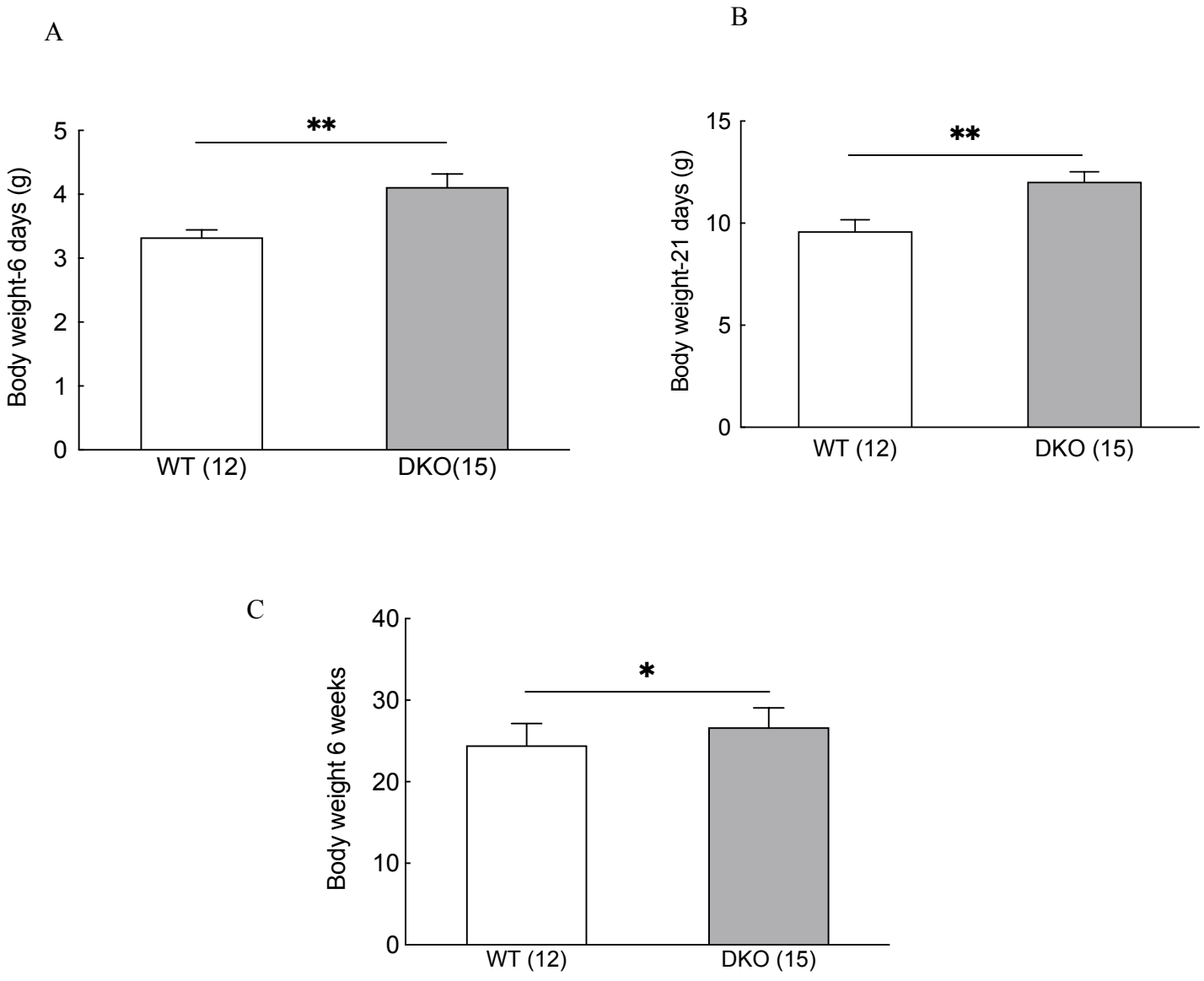

A
Sperm motility (%)
100
80
60
40
20
0
WT (7)
DKO (5)
B
Progressive Motility (%)
40
30
20
10
0
ns
WT (6)
DKO (6)
C
Rapid motility (%)
15
10
5
0
ns
WT (7)
DKO (7)
D
Non-progressive Motility (%)
40
30
20
10
0
ns
WT (7)
DKO (6)
E
Ratio testis/ B.W
1.0
0.8
0.6
0.4
0.2
0.0
ns
WT (12)
DKO (15)
F
Average number of pups
7.5
7.0
6.5
6.0
5.5
5.0
4.5
ns
WT (8)
DKO (10)

Fig. 6


A
Body weight-6 days (g)
5
4
3
2
1
0
**
WT (12)
DKO(15)
B
Body weight-21 days (g)
15
10
5
0
**
WT (12)
DKO (15)
C
Body weight 6 weeks
40
30
20
10
0
*
WT (12)
DKO (15)

8. Discussion

During the sexual life of mammals, the reproductive system of both males and females generate differentiated gametes, sperm and eggs, which each carry half of the genomic materials of the parent individuals. Both are required for the creation of an embryo [187]. Contrarily to the egg, sperm must proceed through a long journey and successfully undergo two maturation processes, epididymal maturation (male reproductive tract) and capacitation (female reproductive tract), in order to become fertilization-competent cells [188-190].

The interaction of sperm with a number of specific molecules secreted in male and female reproductive tracts plays an important role in sperm maturation and fertilization [29]. Since the 1960s, multiple studies have been performed to demonstrate the importance of many different secreted proteins and molecules for sperm maturation, a process that includes the acquisition of progressive motility and fertilizing capacity [191, 192].

Several *in vitro* study demonstrated that proteins in the male reproductive tract, including BSP family proteins, are key players in sperm maturation. In 2006, human and mouse genome analysis was performed and new genes encoding the domain of Fn2 were identified. This led to the identification of two homologous genes from BSP in mice (*Bsph1* and *Bsph2*) and a human gene (BSPH1) which are expressed at the level of the epididymis [74]. As sperm transit through the epididymis, successive biochemical and physiological modifications occur on their plasma membrane, with the assistance of a number of participating epididymal proteins [75, 193]. Thus, it appears obvious that the absence or malfunction of any these molecules could affect the acquisition of sperm fertilizing ability.

The BSP family of proteins is well characterized at the biochemical, structural and molecular levels. BSP proteins secreted by seminal vesicles and /or epididymis are well known for their role in the sperm plasma membrane remodeling events that occur during sperm maturation [68, 184]. Studies indicate that BSP proteins are expressed in males of multiple mammalian species, and originate from the seminal vesicles and/or the epididymides [133, 149]. The exclusive presence of this protein family in the male reproductive tract suggests a possible role for these proteins in the sperm maturation process.

A series of investigations in the bovine species has demonstrated the importance of a family of bovine seminal plasma proteins in capacitation [91]. An extensive study in bovine species by our group has shown that the interaction of BSP proteins with choline phospholipids of the sperm membrane modulates the membrane modification events that lead to sperm maturation and capacitation [64, 112]. In 2007, Lefebvre et al. showed that BSP homologues are expressed in epididymides of mice and human. In contrast to ungulates where BSP proteins are expressed in the seminal vesicles and constitute a major portion of seminal plasma (SP) proteins, these proteins represent a minute proportion of SP proteins in mice and humans [74]. Many *in vitro* studies have been done using native ungulate BSP proteins, which were purified directly by affinity chromatography of SP [151]. Since the BSP protein concentration in mouse and human SP is very low, purifying sufficient quantities of the native proteins is not experimentally feasible with current technologies [76].

To functionally characterize the role of human and mouse BSP proteins in male fertility *in vivo*, the generation of knockout (KO) mice is of fundamental importance. The aim of the present

thesis was to investigate the effects of the absence of BSP proteins (*Bsph1* and *Bsph2*) on sperm functions and male fertilizing ability using knockout mouse models. To our knowledge, this the first report of the generation knockout mice for *Bsph1* and *Bsph2*.

8.1 Efficiency of generating *Bsph2*-KO and *Bsph1/2*-DKO mice

To elucidate the exact function of BSP genes in mice, we utilized the highly efficient CRISPR/Cas9 system to generate individual *Bsph1* and *Bsph2* knockout mice and a *Bsph1/Bsph2* DKO mouse. The two homologues BSP genes in mouse, *Bsph1* and *Bsph2*, are located on chromosome 7. To generate the single KO, we targeted the first Fn2 domain, which is the common structure in BSP proteins responsible for all of their binding properties. To generate the *Bsph1/Bsph2* DKO mouse, the first exons (exon 2 in *Bsph1* and exon 1 in *Bsph2*), were targeted by CRISPR/Cas9. Though we attempted to generate single KO lines for both *Bsph1* and *Bsph2*, the generation of a single *Bsph1* mutant was not successful, and thus the first part of our study was continued only with the single *Bsph2* KO mouse line. Several factors influence CRISPR/Cas9 efficacy. These factors include target DNA site selection, sgRNA design, off-target cutting, incidence of HDR vs. NHEJ, Cas9 activity, and the method of delivery. As delivery remains the major obstacle for use of CRISPR for *in vivo* applications. Furthermore, some guide RNAs with high *in vitro* activity possessed poor mutagenesis activity *in vivo*, suggesting the presence of factors that limit the mutagenesis *in vivo* [194].

To validate nucleotide mutation at the targeted loci, corresponding regions for *Bsph2*-single and *Bsph1/2* DKO were assessed by PCR and sequenced. In both parts of the study, we confirmed that the *Bsph2*-KO mouse line carried mutations at the *Bsph2* locus (520 bp was removed), and

that the *Bsph1/2* DKO mouse line, in which we simultaneously targeted the two BSP homologous genes, carried mutations at both the *Bsph1* and *Bsph2* loci and a large fragment of gene was removed.

To confirm the absence of BSP proteins in KO animals, we first assessed the expression of *Bsp* genes in *Bsph2*-KO and *Bsph1/2* DKO mice at the mRNA level. Interestingly, the RT-qPCR results showed that *Bsph1* gene expression was significantly increased in *Bsph2*-KO mice, suggesting a possible compensation mechanism. To verify whether increased mRNA expression correlated with increased BSPH1 protein expression in single KO mice, mass spectrometry was utilized. Despite the increase in *Bsph1* transcripts, no significant enhancement was observed at the protein level. Gene expression is often interpreted in terms of protein level; however, the correlation can be different because of existing many processes between transcription and translation. Genetic robustness is an important characteristic of a living organisim which is defined as the susceptibility of organism to maintain its viability and fitness despite the genetic perturbations [195]. Genetic compensation in response to a gene knockout has been documented in many number of animal models and is a widespread phenomon. However, the underlying molecular mechanisims remains mainly unexplored [195]. Genetic robustness may arise from redundant gene, whereby the loss of *Bsph2* maybe compensated by *Bsph1* with overlapping function. The BSP proteins in epididymis exist in a minute amount, therefore all *Bsph1* transcripts may not require to translate and therefore no significant enhancement was observed at the protein level as their transcripts. Moreover, there are many complicated and varied post-transcriptional process between transcription and translation and if mRNA is not being engaged onto active translation it becomes accessible for RNases and degradation [195].

The function and structure of the disrupted protein was analyzed by the Expert Protein Analysis System (ExPASy) web tool. Protein sequence and structure analysis by ExPASy indicate that the disrupted BSP proteins in both *Bsph2*-KO and *Bsph1/2* DKO completely lost their function due to lost of Fn2 domains which act as the binding sites, resulting in non-functional proteins and complete loss of their function.

8.2 Normal fertility in *Bsph2*-KO and *Bsph1/2*-DKO mice

All *Bsph2*-KO and *Bsph1/Bsph2* DKO mice were viable and developed normally. The fertility of *Bsph2*-KO and *Bsph1/Bsph2* DKO mutant male mice was examined by performing trio mating; i.e., one mutant male was mated with two WT females. Mating activity was normal as indicated by plug formation. Both the single and double KO mutant male mice showed normal fertility. A comparable number of pups were born from the mating of *Bsph2*-KO, *Bsph1/Bsph2* DKO and WT male mice with WT females. The results of this study indicate that BSP proteins in mice are not essential for sperm function and male fertility. However, in order to determine whether a gene is essential for reproduction, it is important to proceed with further studies on fertility issues.The loss of one family of gene may be compensated by another genes, which are working in the same network and with overlapping functions. Therefore, alterations in expression of other genes, which are working within the same network resulted in genetic compensation by that network. Accordingly, it is fundamental to determine which family of genes are working in parallel with overlapping functions.

Furthermore, a deep systematic analysis of mouse epididymal sperm proteins by Chauvin et al. indicated that sperm cells encounter a very large network of proteins during their journey to the site of fertilization [177]. They identified 2850 proteins in the mouse epididymis, which is the

most comprehensive and complete proteome that can be found in cellular pathways [177]. The redundancy of the multiple genes involved in male reproduction can compensate for the absence of the mutated genes in the KO and DKO mice, thus preventing our observation of any infertility phenotype. Therefore, it remains a tedious challenge to discover the genes that are working in parallel to enable fertilization, since many share similar functions and can cover for one another. The epididymis is responsible not only for sperm maturation, but also plays a role in sperm protection and survival during sperm passage through the epididymis. This protection is critical; hence, the absence of one or more proteins involved in this process can be rescued by other proteins having similar functions, thus preventing the appearance of any phenotype.

8.3 Lack of BSP family proteins leads to increased body weight in mouse pups

Though no differences in pup weight were observed for *Bsph2*-KO mice, the weight of *Bsph1/2* DKO pups was significantly increased at 6, 21 days and 6 weeks compared to WT. ChIP-sequencing analyses in mice indicate that the 81 kb sequence between *Bsph1* and *Bsph2* is the binding domains for androgens [182, 183]. One of the main biological actions of androgens is to regulate metabolism, and low testosterone level have been associated with increased weight [180]. In this study, the observed increased body weight in *Bsph1/2* DKO pups could be the result of the deletion of the 81 kb sequence, which represents an androgen-binding site. Since the androgen binding site is deleted (81 kb sequence), the androgens cannot bind and regulate metabolism. Studies have indicated that the expression of this family of proteins could be regulated by androgens. A study performed by Nixon et al. in rabbit indicated that the elimination of the main source of androgen, castration, resulted in the complete loss of

expression of BSP proteins [99]. The significance of the interaction and regulation of *Bsph1* and *Bsph2* genes by androgens warrants further investigation. AR binding sites play an important role in the development and maintenance of many tissues. To gain further insight into the mechanism of AR action, first of all we need to compare and confirm the deletion of AR binding site in our KO. Thus, chromatin immunoprecipitation coupled with tiling microarray detection of genomic fragments can confirm the deletion of AR binding site in KO. Through the use of KO models we can elucidate how androgen act via the AR bindng sequence.

It seems the effect of BSP proteins differ based on the expression site. Producing KO animals could be provide more information on the role of these proteins in ungulates; however, producing KO in farm animals would need greater investments of time and money.

9. Conclusion and perspectives

Overall, the studies described in this thesis have greatly advanced our knowledge of the epididymal members of the BSP gene and protein family. First, the CRISPR/Cas9 method was used to generate BSP null mice, which in itself is a significant step for further studies of the roles of Binder of SPerm proteins. Second, we demonstrated that the evolutionarily conserved epididymal BSP proteins are not essential for male fertility in mice and it is plausible that BSP proteins have different yet unknown function(s). A study by Chauvin [177] et al. has lead to the identification of 2800 epididymal proteins that are expressed predominantly in the epididymis and still many of them need to be analyzed with the gene editing approach. It is noteworthy to use the knockout animals to identify the impact of the gene on fertility. As several genes such as *Wdr63* [196] and *Dnaic1[179]* were found to be dispensable for male fertility [132]. The application of CRISPR/Cas9 technology to generate gene-disrupted mouse models can facilitate

to determine whether a gene needs further study or not. More recently, an investigation of 54 evolutionary conserved genes in male mice indicates that despite being shown to be essential *in vitro*, these genes are not individually essential for fertility [170]. However, many knockout mice showed no distinct phenotype of infertility or subfertility due to the redundancy of reproductive genes which is a big issue within gene investigation. Third, we observed, for the first time, that the absence of BSP family members and/or the absence of the 81 kb sequence between the 2 genes leads to weight gain and obesity in mouse pups. Evidence from both human and animals suggests that testosterone play an important role in body weight regulation and metabolism. Also, recent ChIP-sequencing analyses in mice indicate that 500 bp upstream of the *Bsph2* gene, as well as another region located in between *Bsph1* and *Bsph2* are AR binding site. Deletion of *Bsph1*, *Bsph2* and 81 Kb, one of the AR binding site, in *Bsph1/2* DKO mice suggests that BSP genes and 81 kb could play a role in weight regulation. Further work is warranted to determine whether the absence of BSP proteins and of the 81 kb androgen binding sequence between the 2 BSP genes in mice might be responsible for increased pup weight. Investigating the molecular mechanisms underlying genetic compensation and gene regulatory networks may help to elucidate why mutations in one or more genes cause no phenotype.

The regulation of BSP protein expression and function, as well as the interaction of this protein family with other proteins has not yet been completely elucidated. Elucidating the mechanisms involved in the regulation of BSP gene expression could shed light on the role of this protein family in epididymal sperm maturation. Much work is still needed to clarify the possible roles of BSP proteins in other biological functions and possibly in body weight regulation in the various animal species.

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