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Complete List of Authors:	Vallet-Buisan, Montserrat; Oxford University, Nuffield Department of Women's & Reproductive Health Mecca, Rajwa; Oxford University, Nuffield Department of Women's & Reproductive Health Jones, Celine; Oxford University, Nuffield Department of Women's & Reproductive Health Coward, Kevin; Oxford University, Nuffield Department of Women's & Reproductive Health Yeste, Marc; Oxford University, Nuffield Department of Women's & Reproductive Health; University of Girona, Cell Biology
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Contribution of semen to early embryo development: fertilisation and beyond

Running title: Semen and embryo development

Authors

Montserrat Vallet-Buisan[1], Rajwa Mecca[1], Celine Jones[1], Kevin Coward[1], Marc Yeste[2,3,4,*]

Affiliations

[1]Nuffield Department of Women's and Reproductive Health, Level 3, Women's Centre, John Radcliffe Hospital, University of Oxford, OX3 9DU, Oxford, UK.

[2]Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003 Girona, Spain.

[3]Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain.

[4]Catalan Institution for Research and Advanced Studies (ICREA), ES-08010 Barcelona, Spain.

[*]**Corresponding author**

marc.yeste@udg.edu; ORCID: 0000-0002-2209-340X

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Abstract

BACKGROUND: It has long been thought that the factors affecting embryo and foetal development were exclusively maternally derived; hence, if issues regarding fertility and embryo development were to arise, the blame has traditionally been placed solely on the mother. An escalating interest in how paternal factors influence embryo development, however, has begun to prove otherwise. Evidence suggests that both seminal plasma and sperm contribute multiple factors that shape embryogenesis. This review thus focuses on the role that semen has in driving early embryonic development, and describes how paternal factors, such as seminal plasma, sperm centriole, sperm proteins, sperm RNA, sperm DNA and its integrity, together with epigenetics, may influence the female reproductive tract and post-fertilisation events. The important contributions of paternal factors to embryo development highlight the imperative need for further research in this area, which is sure to bring forth breakthroughs leading to improvements in infertility diagnosis and ART as well as reducing the risk of miscarriage.

OBJECTIVE AND RATIONALE: This review provides a comprehensive overview of the role of human semen in development of the early embryo, with the aim of providing a better understanding of the influence of seminal plasma and sperm on early embryonic divisions, gene and protein expression, miscarriage, and congenital diseases.

SEARCH METHODS: PubMed searches were performed using the terms 'sperm structure', 'capacitation', 'acrosome reaction', 'fertilisation', 'oocyte activation', 'PLCζ', 'PAWP', 'sperm-borne oocyte activation factor', 'oocyte activation deficiency', 'sperm centriole', 'sperm transport', 'sperm mitochondria', 'seminal plasma', 'sperm epigenetics', 'sperm histone modifications', 'sperm DNA methylation', 'sperm-derived transcripts', 'sperm-derived proteins', 'sperm DNA fragmentation', 'sperm mRNA', 'sperm miRNAs', 'sperm piRNAs', and 'sperm-derived aneuploidy'. The reviewed articles were restricted to those published in English between 1980 and 2022.

OUTCOMES: The data suggest that male derived factors contribute much more than just the male haploid genome to the early embryo. Evidence indicates that semen contributes multiple factors that help shape the fate of embryogenesis. These male derived factors include contributions from seminal plasma, the paternal centriole, RNA and proteins, and DNA integrity. In addition, epigenetic changes have an impact on the female reproductive tract, fertilisation and early stages of embryo development. For example, recent proteomic and transcriptomic studies have identified several sperm-borne markers that play important roles in oocyte fertilisation and embryogenesis.

WIDER IMPLICATIONS: This review highlights that several male derived factors are required to work in tandem with female counterparts to allow for correct fertilisation and development of the early embryo. A deeper understanding of the contributions of paternal factors that are shuttled over from the sperm cell to the embryo can shed light on how to improve ART from an andrological perspective. Further studies may aid in preventing the passing on of genetic and epigenetic abnormalities of paternal origin, thus decreasing the incidence of male factor infertility. In addition, understanding the exact mechanisms of paternal contribution may assist reproductive scientists and IVF clinicians in determining new causes of recurrent early miscarriage or fertilisation failure.

Key words: male infertility, sperm, early embryo development, sperm DNA fragmentation, assisted reproduction, paternal contribution, seminal plasma, human, sperm-derived transcripts, spermatogenesis

Introduction

Infertility affects 10-15% of couples worldwide (Simon *et al.*, 2017a). Approximately 40-50% of infertility cases are caused by a 'male factor', which refers to the inability of a male to achieve a pregnancy in a clinically sound female. Over the last 40 years, with the introduction of IVF, the field of ART has taken massive strides towards combating fertility-related problems and has granted millions of couples the ability to have children (Niederberger *et al.*, 2018). Despite these achievements, however, far too little attention has been paid to identifying what sperm factors, outside those commonly measured in the conventional spermiogram (concentration, motility, morphology), could be causing these hindrances in embryo development (Colaco and Sakkas, 2018; Bashiri *et al*., 2021; Tarozzi *et al*., 2021). This is because the sperm cell has traditionally been seen as a mere vehicle for delivery of the paternal haploid genome to the oocyte. While, consequently, the role of the father in embryonic development has largely been overlooked, a new focus on the importance of paternal roles in embryogenesis and offspring health has emerged in recent years. This delay in investigation may be because the spermatozoon remains one of the most complex and differentiated mammalian cells. Indeed, it is the only cell that has to leave the body to undertake its function of fertilising an oocyte within the female.

It is now evident that semen, composed of seminal plasma and sperm, delivers much more than just the paternal haploid genome to the oocyte: it interacts with the female genital tract and plays a key role in driving critical aspects of embryogenesis. Thus, this review provides an overview of current knowledge about the paternal contributions to early embryo development and closely examines the function of seminal plasma and sperm in embryogenesis. The interactions between seminal plasma and the female reproductive tract are addressed together with novel findings regarding the effect of sperm production and transport on fertility, and how sperm elements, such as proteins, DNA, RNA, and epigenetic marks contribute to embryo development. Highlighting each of these aspects will help elucidate the overarching and important role of semen.

Methods

A literature review was carried out to identify the most relevant recent publications pertaining to the semen-contributed factors that may affect fertilisation and embryogenesis. The review focuses mainly on research carried out in humans, but also includes data from animal models when relevant. PubMed searches were performed with the terms ‘sperm structure’, ‘capacitation’, ‘acrosome reaction’, ‘fertilisation’, ‘oocyte activation’, ‘oocyte activation deficiency’, ‘PLCζ’, ‘PAWP/WBP2NL’, ‘TMEM95’, ‘IZUMO1’, ‘sperm centriole’, ‘sperm mitochondria’, ‘seminal plasma’, ‘sperm epigenetics’, ‘sperm histone modifications’, ‘sperm DNA methylation’, ‘sperm-derived transcripts’, ‘sperm-derived proteins’, ‘sperm DNA fragmentation’, ‘sperm mRNA’, ‘sperm miRNAs’, ‘sperm piRNAs’, and ‘sperm-derived aneuploidy’. The reviewed articles were restricted to those published in English between 1980 and 2022.

Seminal plasma

Seminal plasma (SP), a complex fluid produced by the male accessory sex organs (prostate, seminal vesicle, and epididymis), has traditionally been regarded as a simple vehicle for sperm to reach the oocyte. New evidence, however, indicates that SP may play a function in embryo development by modulating the endometrial environment, post-fertilisation development, and foetal growth (Bromfield *et al*., 2014; Watkins *et al*., 2018). Studies on vasectomised mice showed that SP modulates the endometrial levels of tumour necrosis factor (TNF), interleukin-1β (IL-1β), interferon-γ (IFN-γ), microphage inflammatory protein 1-α (MIP-1α) and colony stimulating factor 3 (CSF3), and the expression of prostaglandin synthesis and regulatory T-cell genes (Watkins *et al.*, 2018). Other previous findings support that an endometrial cascade of cytokines and chemokines and the recruitment of regulatory T-cells suppresses inflammation, increases maternal tolerance to paternal and foetal antigens, and facilitates embryo implantation in mice (Robertson *et al.*, 1996, 2001; Bromfield *et al.*, 2014), hamsters (Chow *et al.*, 2003), and pigs (Bromfield *et al.*, 2014).

In contrast to the house mouse (*Mus domesticus)*, where the SP is almost directly deposited into the uterus during coitus (Muro *et al*., 2016), humans ejaculate in the vagina. In humans, consequently, it is not known to what extent, or if, the SP reaches the uterus. As such, in a vaginal depositor like the cow it has been hypothesised that SP may use sperm as a vehicle to play its role in priming the maternal environment (Recuero *et al.*, 2020). In addition, in the bovine model, SP alters the protein composition of the periconception environment (Ibrahim *et al*., 2019b) and induces the production of cytokines and chemokines in the maternal tract (Schjenken *et al.*, 2015; Nongbua *et al.*, 2020). Increasing clinical evidence supports the notion that SP in humans plays a crucial function in priming the maternal endometrium for pregnancy (Ibrahim *et al.,* 2019a; George *et al.,* 2020; Ajdary *et al.,* 2021; Szcykutowicz *et al.,* 2021). Exposure of the woman to the partner's SP improves fertility (Crawford *et al*., 2015), increases the pregnancy rate after IVF (von Wolff *et al.*, 2009; Chicea *et al*., 2013; Friedler *et al.*, 2013; Crawford *et al.*, 2015), and may decrease the incidence of pre-eclampsia (Robertson *et al.*, 2003).

The role of seminal plasma extracellular vesicles

Extracellular vesicles (EVs) are membrane-bound particles secreted by cells into the extracellular space and have roles in cell-cell communication (Yáñez-Mó *et al.*, 2015). EVs have been found in prostatic, epididymal, and seminal fluids, and have roles in gametogenesis, fertilisation and embryogenesis. Epididymosomes, which are released by epithelial cells in the epididymis (Thimon *et al.*, 2008), have been observed to fuse with epididymal sperm in mice (Păunescu *et al.*, 2014). This 'fusion' allows for the transfer of micro RNAs (miRNA) and proteins from the epididymis to sperm (Twenter *et al.*, 2020). Proteomic profiling of epididymal EVs in sheep (Gatti *et al.*, 2005), cattle (Girouard *et al.*, 2011), mouse and humans (Barrachina *et al.,* 2022) has revealed that they contain mostly enzymes, transport molecules and have roles in sperm maturation and motility. In mouse, changes in the paternal environment, such as chronic stress, have been reported to alter the proteomic profile of epididymal EVs. These alterations can be transmitted to

sperm and alter foetal development, resulting in offspring with abnormal neurodevelopment (Chan *et al*., 2020).

Prostasomes are produced by prostate epithelial cells (Brody *et al.*, 1983) and have also been shown to transfer proteins and genomic DNA from prostatic cells into human sperm (Ronquist *et al.,* 2011). Prostasomes are particular in their lipid composition, which differs from that of most EVs; they have a high cholesterol:phospholipid ratio (Dubois *et al*., 2015), which affords their membrane more rigidity and viscosity. This characteristic is important because, when internalised by sperm, prostasome-derived lipids decrease the fluidity of sperm membranes and prevent premature acrosome exocytosis (Cross and Mahasreshti, 1997; Brouwers *et al.,* 2013). Prostasomes are also involved in the regulation of sperm motility (Andrews *et al.*, 2015; García-Rodríguez *et al.,* 2018), capacitation (Pons-Rejraji *et al.*, 2011; Aalberts *et al.,* 2013) and in protection from the female immune environment (Milutinović *et al.,* 2019; Paktinat *et al.,* 2019), and have been suggested as potential biomarkers of male infertility (Vickram *et al*., 2022).

SP contains a mixture of epididymosomes and prostasomes. Human SP EVs have the capacity to increase endometrial receptivity *in vitro*, as they can fuse to endometrial stromal cells thereby inducing decidualisation, and increase prolactin secretion (Rodriguez-Caro *et al.*, 2019). Related to this, the SP EVs of men whose partners experience recurrent pregnancy loss have a reduced content of growth differentiation factor 15 (GDF15), which is a member of the transforming growth factor-beta (TGFβ) family. The decreased content of GDF15 could dysregulate its immunomodulatory role in the female reproductive tract, thus impairing decidualisation and leading to pregnancy loss (Jena *et al.*, 2021). Moreover, Lin *et al.* (2019) also studied the proteomic content of human seminal EVs in patients with asthenozoospermia and found a significant reduction of channel protein Transient Receptor Potential channel family Vanilloid subfamily member 6 (TRPV6). All these findings suggest that seminal EVs have important yet unknown functions in fertility regulation, which brings forth the opportunity for their use as putative infertility biomarkers.

Spermatogenesis

The main function of the spermatozoon is to carry a haploid genome to the oocyte. Prior to this, the germ cells must undergo a series of morphological changes within the testes to develop into mature sperm, which can be divided into three parts (head, neck and tail; Alavattam *et al.*, 2019).

Sperm cells develop via a process called spermatogenesis that occurs within the seminiferous tubules of the testis (Fig. 1). It is here that spermatogonium transition from spherical diploid cells into specialised haploid sperm occurs. Spermatogenesis initiates with the division and renewal of spermatogonia via the process of mitosis, following which primary spermatocytes (2n) enter meiosis. Secondary spermatocytes (n) are the result of the first meiotic division, which undergo a second meiotic division, resulting in spermatids (n). Spermatids finally transition from being spherical in nature into elongated testicular sperm (Huang *et al.*, 2021). The process of spermatogenesis is supported by surrounding Sertoli cells via close cell-to-cell interactions. As spermatogenesis is a crucial step in determining the viability and function of sperm cells, it is a highly regulated process.

Changes in organisation of sperm chromatin during spermatogenesis

In somatic cells, chromatin contains histones and DNA, which together form nucleosomes. Each nucleosome comprises just less than two turns of DNA wrapped around a set of eight histones. These histones may be subject to post-translational modifications to control gene expression and transcriptional access to genomic regions (Bartosovic *et al.*, 2021). During spermiogenesis, most of these histones are replaced by protamines, which is followed by the disassembly of nucleosomes and a specialised compaction of chromatin through toroids (Ward and Coffey, 1990; Barral *et al.*, 2017) (Fig. 2). Toroid structures are anchored to the proteinaceous nuclear matrix, known as matrix attachment regions (Narwade *et al.*, 2019). Unlike histones, protamines contain arginine to allow for stronger binding to DNA to form a toroid-like ridged structure (Ward and Coffey, 1991; DeRouchey *et al.*, 2013). Additionally, protamines in some mammalian species, as seen in porcine sperm, have high levels of cysteine residues that are important for the formation of disulphide bonds, which allow for linkage between protamines in toroid regions (Gosálvez *et al.*, 2011; Ribas-Maynou *et al.*, 2021a). Ultimately, this specialised packaging provides a more stable conformation to evade DNA damage (Ni *et al*., 2016; Schneider *et al*., 2020a). Fundamental phosphorylated protamines, such as P1 and P2, typically replace histones H2A, H2B, H3 and H4 during chromatin compaction in spermiogenesis (Wang *et al*., 2019). Yet although most histones are lost during this process, a select few are retained such as linker histone H1, H3 and H4 (Govin *et al.*, 2007; Kasimanickam *et al.*, 2019), which may bear epigenetic marks; this will be discussed in more detail below.

In humans, P1 and P2 are typically found in equal ratios (Sarasa *et al.*, 2020). Should errors arise in relation to the temporal regulation of protamine transcripts, this P1:P2 ratio may be altered. In fact, male infertility has been linked to altered P1:P2 ratios and elevated histone-to-protamine ratios (Aoki *et al.*, 2006b; Rogenhofer *et al.*, 2013). In effect, changes in protamine ratios, measured on the basis of both protein and mRNA,

have been demonstrated to be negatively correlated to IVF success and embryo quality (Rogenhofer *et al.*; 2017; Amor *et al.*, 2019). Additionally, Fournier *et al.* (2018) indicated that histone-protamine ratios may help predict embryo quality at the blastocyst stage, so that their values should lie between 6% and 26% for optimal blastocyst development. Should the histone-to-protamine ratio value exceed this figure, paternal genomic expression could be desynchronised, thus negatively affecting embryonic development until the blastocyst stage.

Epididymal maturation

Once spermatogenesis is complete, sperm cells undergo further maturation during their path to, and throughout, the epididymis. First, sperm detach from Sertoli cells and are released toward the rete testis. Sperm cells then move to the epididymis, where they mature before being transported to the vas deferens. The journey through the epididymis is a crucial step in the correct development of sperm; as they travel through the epididymis, they undergo several alterations affecting nuclear compaction, epigenetics, non-coding RNA (ncRNA) and protein payload, motility, and the cytoskeleton structure.

As sperm cells are first expelled from the rete testis into the epididymis, epithelial cells quickly act to absorb nearly 90% of the fluid, resulting in a great increase in sperm concentration (Zhou *et al.*, 2018). This process is important to keep sperm concentration high in order to maximise the chances of fertilisation. Owing to the high level of metabolic activity produced by epididymal epithelial cells, resulting reactive oxygen species (ROS) remain a threat to the health of sperm (El-Taieb *et al.*, 2009; Schneider *et al.*, 2020b). The epididymis thus releases antioxidant enzymes to neutralise ROS and protect sperm cells (Wu *et al.*, 2020).

One of the most important abilities that sperm cells acquire during their epididymal transit is progressive motility, which allows them to swim through the female reproductive tract once ejaculation occurs (Tourzani *et al*., 2021). Several modifications are carried out on sperm cells; some examples include alterations in plasma membrane composition (Kirchhoff and Hale, 1996; Lu *et al.*, 2018; Shan *et al*., 2020) and phosphorylation of certain sperm proteins (Bhattacharjee *et al.*, 2018; Goswami *et al.*, 2019), all affecting motility in a biochemical manner. Furthermore, sperm accumulate several factors that are important for fertilisation. During epididymal transit, these cells acquire several proteins, such as cytosine-rich secretory protein 1 (CRISP1) (Cohen *et al.*, 2011; Weigel Muñoz *et al.*, 2018), acrosin binding protein (Nagdas *et al.*, 2010; Yin *et al.*, 2018), androgen receptors (Zhang *et al*., 2019) and a disintegrin and metalloproteinase (ADAM) family proteins (Inoue *et al.*, 2005; Nishimura *et al.*, 2007), to name a few. These factors play crucial roles in the binding and penetration of the oocyte cumulus cells and zona pellucida (Maldera *et al.*, 2014). Previous studies showed that epididymal factors released by epithelial cells into the epididymal lumen are responsible for the protection and nourishment of sperm cells; most of these factors are transported via exosomes known as epididymosomes (Thimon *et al.*, 2008; Reilly *et al.*, 2016; Zhou *et al.*, 2017; Barrachina *et al.*, 2022). Additionally, these epididymosomes have been suggested to play an important role in a mode of paternal epigenetic inheritance via small ncRNAs (Zhang *et al.*, 2018; Chan *et al.*, 2020). A recent study in mouse suggested that certain epigenetic changes within sperm coincide with epididymal maturation, which may have lasting effects on progeny. The authors observed large differences in DNA methylation between sperm from the caput and the testis (Chen *et al*., 2022).

Mature sperm cells are finally stored in the epididymal cauda until ejaculation. Proper maturation within the epididymis is important for fertilisation and early embryo development. Early studies showed that oocytes fertilised with sperm derived from the caput (initial area) of the epididymis resulted in defective embryogenesis (Wazzan *et al.*, 1990). In effect, fertilisation rates following IVF were found to plummet when oocytes

were exposed to caput-derived mouse sperm, as they lacked the necessary factors for fertilisation and motility (Wazzan *et al*., 1990). Furthermore, there has been inconsistent evidence regarding the viability of embryos fertilised with immature sperm via ICSI, which bypasses the need for sperm motility and penetration capacity. One research group found 0% survival of embryos and minimal implantation rates (Conine *et al.*, 2018); this was suggested to result from premature ncRNA payloads, which undergo significant changes during the journey through the epididymis (Sharma *et al.*, 2018). Other groups, notwithstanding, were able to generate offspring using caput-derived sperm via ICSI (Suganuma *et al.*, 2005; Fernández-González *et al.,* 2019; Zhou *et al.*, 2019, 2021). Thus, although it is likely that sperm maturation and epididymal factors play a role in fertilisation and early embryogenesis, they may not be crucial to the survival of the embryo. While more research is required to understand the true extent of the importance of these factors, it is reasonable to suggest, based on the current data, that epididymal maturation alterations that ultimately affect epigenetic marks, and mRNA, miRNA and protein payloads, may result in sub/infertility and impaired embryogenesis.

The role of zinc and other trace elements

Sperm and SP in humans and other mammalian species have been found to contain levels of trace elements including selenium (Se), iron (Fe) and zinc (Zn), which are important for sperm function and fertilising capacity. For instance, patients with asthenoteratozoospermia, oligoasthenoteratozoospermia and azoospermia show decreased levels of Zn in their seminal plasma when compared to patients with normozoospermia (Kothari and Chaudhari, 2016). Although the exact role of Zn and how sperm take up this element remain unknown, previous investigations suggested that Zn may play a vital role throughout the lifespan of sperm, from spermatogenesis until fertilisation. This trace element has been shown to affect hormone function in males (Chu *et al., 2016)*, as decreased Zn levels lead to a decline in the production of testosterone in mammalian Leydig cells, where Zn is ordinarily situated (Zhang *et al.*, 2018). In addition, during the initial stages of spermatogenesis in mouse, Zn appears to play a role in maintaining meiosis as it is involved in regulating the stabilisation of sperm chromatin (Ishizuka *et al*., 2016). Once spermatogenesis is complete, Zn is secreted into the seminal fluid via the prostate and acts to protect sperm from oxidative stress (Zhao *et al*., 2016, Zhu *et al*., 2022). Finally, Zn is thought to be involved in sperm capacitation and the acrosome reaction, as low concentrations of Zn are known to aid in the switch to hyperactive sperm motility in the upper female reproductive tract (Allouche-Fitoussi *et al.*, 2019, Matavos-Aramyan *et al.*, 2021, Zigo *et al.*, 2022). Yet Zn can, at high concentrations, act as a chemorepellent to sperm, possibly to prevent polyspermy or remove JUNO, the oocyte membrane receptor that binds the sperm protein IZUMO1 (Guidobaldi *et al.*, 2017, Kerns *et al.*, 2020). Thus, fertility, conception and/or embryonic implantation may be affected by varying Zn levels. While Zn has potential to act as a diagnostic biomarker and/or preventative treatment for male infertility, few studies have investigated the exact role of Zn in human sperm (Zhao *et al.*, 2016).

Other trace elements, such as Se and Fe, have been considered important in male fertility. Selenium has been shown to be an important factor for male infertility, although most of the research has been carried out on animals. This element is known to be involved in antioxidant defence mechanisms, and modulate apoptosis and proliferation of Leydig cells by regulating oxidative stress (Shi *et al.*, 2018). Furthermore, the addition of Se and other trace elements, such as Zn and manganese, to the IVF medium increases the number of sperm cells bound to the zona pellucida of the oocyte when compared to the control (Anchordoquy *et al.*, 2019). Another study showed that Fe and calcium (Ca) levels in the seminal plasma of patients with asthenenoteratozoospermia, teratoleucozoospermia or teratozoospermia are lower than in healthy donors (Ammar *et al*., 2019). High levels of Fe, nevertheless, are positively correlated to several anomalies, including poor sematic quality, impaired superoxide dismutase and induced lipid peroxidation (Marzec-Wròblewska *et al*., 2011) as well as increased seminal leucocyte concentration (Ammar *et al*., 2019), so that one could reasonably posit that an optimal level of this element is required.

Supplementing patients' diet with multi-nutrients including Zn, Se and Fe improves sperm quality (Kopets *et al.*, 2020, Scaruffi *et al.*, 2021). In fact, adding Se to the diet increases sperm motility, viability and mitochondrial membrane potential, and

decreases DNA fragmentation in patients with asthenoteratozoopsermia (Ghafarizadeh *et al.*, 2018). It is thus now apparent that several trace elements, such as Zn, have direct effects on male fertility *in vivo*, and that supplementation with Zn and other trace elements has a beneficial impact on sperm quality and function. In spite of this, further studies to decipher whether these elements are crucial for fertilisation to occur are required.

Sperm transport through the female reproductive tract

Sperm passage along the female reproductive tract is highly regulated to ensure that only the cells with the best morphology and motility parameters reach and fertilise the oocyte. Across the female tract, sperm are exposed to physical stress from ejaculation, oxidative damage and an immune response as well as contractions from the female reproductive tract. Depending on the species, sperm may be deposited in the vagina (as in sheep, cattle, or humans), or the uterus (as in pigs, rodents, or horses) upon ejaculation. The site of sperm deposition conditions the path sperm take through the female reproductive tract. In humans, semen is deposited in the vagina during coitus, and sperm must swim into the cervical canal, where cervical mucus and uterine myometrial contractions push it through the uterine cavity and on to the utero-tubal junction (UTJ) (Ishimoto and Gaffney, 2016). The UTJ has a series of folds that entrap lower quality sperm. Sperm that successfully pass through the UTJ reach the oviduct (also known as Fallopian tube in humans). The oviduct has four different segments: the intramural section, the isthmus, the ampulla, and the infundibulum. In many animal species, it has been observed that, in the isthmus, sperm attach to the epithelial cells and are held in a 'sperm reservoir' (Sharif *et al.*, 2022). This reservoir functions as a temporary sperm storage with the goal of maintaining sperm survival and fertility until ovulation (Pollard *et al.*, 1991; Chian and Sirard, 1995; Teijeiro and Marini, 2012). Such a reservoir has not hitherto been identified in humans. Yet, since: motile sperm bind to the apical surface of the oviductal epithelium *in vitro* (Pacey *et al.*, 1995); the human endosalpingeal epithelium prolongs sperm survival (Ziskind *et al.*, 2000); and pregnancy can be achieved from intercourse that occurred 6 days before ovulation in humans (Wilcox *et al.*, 1995), it is likely that human sperm are stored somewhere in the female reproductive tract after ejaculation, and a reservoir in the Fallopian tubes is a good candidate.

Sperm reservoirs are established through species-specific dynamic biochemical interactions between the female reproductive tract and sperm. Such interactions allow for selection of the most motile and viable sperm for fertilisation, as well as for the elimination of undesired sperm cells (Rath *et al.*, 2008). In most species, sperm-oviduct binding is mediated by oligosaccharides (Liberda *et al.*, 2006; Kadirvel *et al.*, 2012) and female-expressed sperm-binding proteins (Pérez *et al.*, 2006; Teijeiro *et al.*, 2008). For instance, in the pig, lectins on the sperm surface bind to sugar moieties on oviductal epithelial cells to establish a reservoir of high quality sperm in the oviduct (Rath *et al.*, 2016). Likewise, specific proteins in the oviductal cell membrane (annexins) have been hypothesised to be involved in formation of the sperm reservoir in pigs (Teijeiro *et al.*, 2015). In cattle, oviductal trisaccharides also bind sperm to retain them in a reservoir, promote their longevity, and lengthen their fertile life (Dutta *et al.*, 2019). In humans, S100-A9, a protein expressed and secreted by the oviductal epithelium, binds to a subpopulation of migrating sperm and affects capacitation (Massa *et al.*, 2019), which could suggest the possible identification of a human sperm reservoir. Sperm held in the reservoir have also been observed to generate a transcriptomic response from the oviduct by modifying the expression of cytokines, chemokines and growth factors from oviductal cells (Mousavi *et al.*, 2021; Zúñiga *et al.,* 2021), leading to a downregulation of inflammatory molecules and a consequent increase in immunological tolerance towards

sperm. In the same way, sub-par sperm, such as those with high DNA damage, trigger differential expression of growth factors (Mohammadi *et al*., 2022) and an increased inflammatory reaction from the Fallopian tube, generating a pathologic environment for capacitation, fertilisation, and embryo development (Zandieh *et al*., 2019). In the human, *in vitro* experiments have shown that sperm incubated with cultured oviductal epithelial cells remain viable for longer periods of time (Kervancioglu *et al.*, 1994). Studies in pigs (Machado *et al.*, 2019) and cattle (Lamy *et al.*, 2017) indicate that the release of sperm from the oviductal reservoir is triggered by a progesterone increase. Whether and how this occurs in humans remains to be elucidated but, if present, the sperm ability to bind that oviductal epithelium represents a natural selection mechanism that should be considered when developing strategies to improve ART efficiency.

On the other hand, SP and sperm deposition into the female reproductive tract generates early host innate immune reactions in the vagina and the cervix, which involves the migration of female antibodies, complement, and immune cells, such as leukocytes and neutrophils (Fichtner *et al.*, 2020). Polymorphonuclear granulocytes, which are a type of leukocyte, release their DNA into the extracellular environment to generate neutrophil extracellular traps (NETs), which can capture and kill bacteria and sperm via phagocytosis through a process called NETosis (Alghamdi and Foster, 2005; Alghamdi *et al.*, 2009; Katila, 2012). In the human, NETosis is triggered by the appearance of sperm in the female tract in a dose-dependent manner, and has been shown to increase with exposure time (Zambrano *et al.*, 2016). In the donkey, NETosis is sparked by the presence of SP, and not of sperm (Mateo-Otero *et al.*, 2021). Polymorphonuclear granulocytes can also kill pathogens and sperm through the generation of ROS and the release of antimicrobial peptides (Zambrano *et al.*, 2016). Remarkably, NETosis has been shown to remove excess sperm in both humans and other mammalian species and, as the motility of affected sperm diminishes, it has been correlated with decreased fertility (Alghamdi and Foster, 2005; Alghamdi *et al.*, 2009; Katila, 2012; Zambrano *et al.*, 2021). There are, however, components in sperm that may facilitate their survival against female immune responses. Across their surface, sperm are covered by glycoconjugates. One of these is sialic acid which, in humans, is required for sperm to adhere to the oviductal epithelium and penetrate the cervical mucus (Tollner *et al.*, 2012). Sialic acid-binding immunoglobulin-like-lectins (siglecs) are the endogenous ligands of sialic acids, and are expressed in human and mouse endometria (Tecle *et al.*, 2019). In mice, endometrial siglecs interact with sialylated sperm (Tecle *et al.*, 2019) and play a protective role against macrophage-mediated phagocytosis (Ma *et al.*, 2016). To sum up, as sperm travel through the female reproductive tract they are selected according to their morphology and motility parameters by being held in a reservoir in the oviduct and by being exposed to host immune reactions. In turn, sperm have developed measures to counter this, mainly by a targeted suppression of the female immune reaction.

Fertilisation

Fertilisation is the process by which the male and female gametes fuse to create a single diploid cell, the zygote (Georgadaki *et al.*, 2016). Several events must take place in the oviduct to ensure proper fertilisation: sperm capacitation, acrosome reaction, gamete fusion, oocyte activation, and initiation of embryo development. After fertilisation, it is believed that the properties of the sperm cell continue to play various roles as the embryo develops.. It is clear that oocyte fertilisation by a defective sperm cell can lead to hindrance of varying degrees during embryo development prior to implantation (Colombero *et al.*, 1999). In effect, studies in the late 1990s showed that when an oocyte was microinjected with fractions pertaining to the sperm cell, such as the head or tail, the fertilised oocyte was unable to survive past the first few sets of mitotic divisions (Moomjy *et al.*, 1999). This emphasised the importance of structural integrity of the sperm cell to embryonic development. Since then, research has provided cues of the effects of sperm deficiencies on early embryo development, and even of consequences persisting past implantation leading to embryonic and foetal defects. The following sections of this review discuss how the sperm cell contributes to early embryo development within the context of its main components: the centriole, mitochondria, proteins, RNA, DNA and epigenetic marks, and, further, we address how the rise of ART may be affecting the paternal factors involved in embryo development.

Capacitation, acrosome reaction and gamete fusion

Both capacitation and the acrosome reaction are essential processes for a sperm cell to fertilise an oocyte. Capacitation, which only occurs in mammals, consists of several changes in the sperm plasma membrane and intracellular components that sensitise the male gamete to the oocyte (Molina *et al.*, 2018). The acrosome reaction enables sperm to pass through the oocyte vestments and penetrate the zona pellucida (Gupta, 2021). After sperm passage through the zona pellucida, sperm proteins in the equatorial region, such as IZUMO1, participate in the binding of sperm to the oocyte membrane (Inoue *et al.*, 2005; Hirohashi and Yanagimachi, 2018). IZUMO1 interacts with its receptor in the oocyte, JUNO; the gametes' membranes then fuse and the spermatozoon is engulfed by the oocyte cytoplasm (Bianchi *et al.*, 2014; Jean *et al.*, 2019). Other proteins identified on the sperm surface have also been suggested to be involved in oocyte fertilisation. Galactin-3 is a secretory lectin found in SP that is transferred to the sperm surface via extracellular vesicles. In humans, it has been observed to participate in the binding of sperm to the zona pellucida after capacitation (Mei *et al*., 2019). CD151 is a tetraspanin located in the equatorial segment of sperm. In mouse, cattle and humans, it has been proposed to be involved in fertilisation, together with tetraspanins CD9 and CD81 (Jankovicova *et al*., 2020). TMEM95, a sperm membrane protein (Lamas-Toranzo *et al.*, 2020), LYPD4, a mouse homolog of a human acrosome protein (Wang *et al*., 2020b), and epididymal protein 4 (HE4) (Kant *et al*., 2019) have also been found to play important roles in fertilisation in humans and in mouse models. While the mechanisms by which these proteins are involved in fertilisation are still unknown, sperm devoid of them are infertile/subfertile. Further research is thus needed to understand the complex biochemical machinery underlying the fusion of gamete membranes.

Oocyte activation

Oocyte activation occurs upon engulfment of the spermatozoon by the ooplasm and involves resumption of the second meiosis (arrested at metaphase-II) and the subsequent start of embryogenesis (Horner and Wolfner, 2008). It is triggered by a set of periodic intracellular calcium oscillations that are induced by a sperm-specific factor. Three hypotheses have been proposed to explain the calcium release at the onset of gamete fusion: the sperm oocyte-activating factor (SOAF) theory (Dale *et al.*, 1985); the receptor theory (Kline *et al.*, 1988); and the calcium bomb theory (Jaffe, 1983) (Fig. 3A). The SOAF theory gained acceptance when phospoholipase C Zeta (PLCζ), a sperm-specific phospholipase C, was found to trigger calcium oscillations in mouse oocytes (Saunders *et al.*, 2002). Upon gamete membrane fusion, PLCζ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) from the plasma membrane of oocyte vesicles to produce inositol 1,4,5,-triphosphate ($InsP_3$) and diacylglycerol (DAG). DAG remains in the membrane and activates protein kinase C (Fukami *et al.*, 2010). $InsP_3$ binds to its receptors in the endoplasmic reticulum and elicits the release of intracellular calcium in the fertilised oocyte, thus creating a regenerative feedback loop initiated by PLCζ (Fig. 3B; Swann and Yu, 2008; Swann *et al.*, 2012). Sperm that are unable to trigger the normal pattern of calcium oscillations are linked to failed or low fertilisation ability (Heindryckx *et al.*, 2013) and to a higher incidence of recurrent partial hydatidiform moles (Nikiforaki *et al.*, 2014).

PLCζ is widely thought to be the mammalian SOAF. In human sperm, the reduction or absence of PLCζ has been linked to poor oocyte activation and thus to male factor infertility (Heytens *et al.*, 2009; Kashir *et al.*, 2011, 2013, 2018; Yelumalai *et al.*, 2015). Exomic DNA sequencing from patients with ICSI fertilisation failure exposed a point mutation in one of the PLCζ domains (Escoffier *et al.*, 2016). Additionally, Yoon *et al.* (2008) found that sperm pertaining to patients with the inability to fertilise oocytes via ICSI could not induce calcium oscillations when injected into murine oocytes, and that sperm from such patients lacked PLCζ in the equatorial region. The infertile phenotype was only rescued after *Plcz1*-cRNA microinjection into those oocytes, hence indicating the need of PLCζ for oocytes to activate. Other studies discovered that sperm from patients with complete or partial globozoospermia that have reduced levels or absence of PLCζ also cannot fertilise oocytes naturally (Taylor *et al.*, 2010; Kashir *et al.*, 2012; Yassine *et al.*, 2015). Hachem *et al.* (2017) generated *Plcz1* knock-out male mice that, upon fertilisation, failed to trigger calcium oscillations in the oocyte. Their study largely corroborated the role purported for PLCζ. The study had its limitations, however, as some oocytes still managed to initiate embryogenesis. This would indicate that *Plcz1* knock-out males were subfertile rather than infertile, and that PLCζ as the definitive and sole mammalian SOAF has not been completely confirmed.

Other groups have noted that sperm that fail fertilisation after ICSI do not always carry PLCζ alterations (Ferrer-Vaquer *et al.*, 2016). Accordingly, PLCζ might not be the only mammalian SOAF, as other sperm factors have been proposed to be involved in oocyte activation. An example of this is post-acrosomal sheath WW domain-binding protein (PAWP, also known as WBP2NL), a sperm protein located in the post-acrosomal

sheath (Wu *et al.*, 2007) that triggers calcium oscillations in pig, cattle, macaque, *Xenopus* and human oocytes (Wu *et al.*, 2007; Aarabi *et al.*, 2010, 2014; Kennedy *et al.*, 2014). Studies in mouse oocytes, nevertheless, did not report such oscillations (Nomikos *et al.*, 2014, 2015), although it has recently been described as a sperm fertility biomarker in cattle (Kaya et al., 2022). Moreover, in humans, Freour *et al.* (2017) examined transcript and protein (through both immunoblotting and immunofluorescence) levels of WBP2NL/PAWP and found no correlation with fertilisation rate or embryo quality score, nor were they related to pregnancy success following ICSI with donated oocytes. For all these reasons, the role of PAWP in oocyte activation remains controversial.

Contribution of the sperm centriole

In eukaryotic cells, the centrioles are organelles required for cell division as well as cilia and flagella formation, and play a role in arranging the cell cytoskeleton. With the aid of the male pronucleus, centrioles are known to affect human reproduction (Amargant *et al.*, 2021; Avidor-Reiss *et al.*, 2019; 2022). Human sperm contain two centrioles located in the neck region: the proximal centriole (PC) and the distal centriole (DC) (Khanal *et al.*, 2021, Leung *et al.*, 2021). In several species, such as primates, cattle, and pigs, sperm PC is thought to be inherited by the zygote at fertilisation (Schneider *et al.*, 2020a; Amargant *et al.*, 2021). Sperm centrioles, however, are not present in rodent zygotes, as they degenerate at spermatogenesis (Schneider *et al.*, 2020a). This indicates that centrioles may not always be inherited or required for embryo development and suggests that paternal centriolar contributions and functions are species-specific.

As oocyte centrioles are eliminated during human oogenesis (Simerly *et al.*, 2018) (Fig. 4), their paternal counterparts are thought to be essential to ensure appropriate development in human embryos, although this notion is controversial. Turner *et al.* (2021) developed a ratiometric assay, the fluorescence-based ratiometric analysis of sperm centrioles, to assess the quality of centrioles in 33 patients; they found that 79% of higher-grade sperm in men facing infertility had faulty centrioles. They also observed a negative correlation between patient age and tubulin labelling of the sperm distal centriole. This assay could thus provide a reliable method to identify the causes of unknown infertility in couples in the future (Jaiswal *et al.,* 2022). Furthermore, research on abnormal sperm aster formation indicates that sperm centrioles seem to have a function in organising the zygote cytoskeleton to mediate pronuclei migration (Scheffler *et al.*, 2021) and cell cleavage (Rawe *et al.*, 2002). Contrastingly, other data suggest that sperm centrioles are not required for zygotic cleavage divisions but do participate in later stages of embryo development in humans (Sha *et al.*, 2017). A recent bovine study looked at the ultrastructure of individual embryo cells at different stages of development and compared the centrosomal fate of blastomeres. They found that, as in rodent models, bovine embryos are able to develop functional centrioles without the presence of any previous ones; however, unlike mice, centrioles appear at the very early 2-4 cell stage (Uzbekov *et al*., 2022). It should be noted, nevertheless, that the aforementioned studies were conducted with small sample sizes, so caution must be taken when interpreting and extrapolating these findings.

The fate of sperm mitochondria

The main role of sperm mitochondria is to produce ATP through oxidative phosphorylation, thus providing sperm with the energy requirements for motility. Mitochondria are also involved in the regulation of sperm capacitation (Ferreira *et al.*, 2021; Giaccagli *et al.*, 2021). In mammals, sperm mitochondria differ from those in somatic cells in terms of morphology, distribution, and biochemical makeup. During spermiogenesis, sperm mitochondria are tightly wrapped by disulphide bridges (Ursini *et al.*, 1999) and concentrate in the midpiece (Otani *et al.*, 1988). Sperm mitochondria also present specific isoforms for some proteins and enzymes, such as subunit VIb of the cytochrome oxidase (Hüttemann *et al.*, 2003) and creatine kinase (Huszar *et al.*, 2000). Mitochondria are also an important source of ROS, which are needed for some processes like capacitation, but in excess they may contribute to mitochondrial damage through oxidative stress (Aitken *et al.*, 1998; Fernández *et al.*, 2019). Indeed, ROS-mediated damage has been associated with 30-80% of male infertility cases (Ochsendorf *et al.*, 1994; Agarwal *et al.*, 2006; Zandieh *et al.*, 2018).

Distinct features of mitochondria, such as mitochondrial DNA (mtDNA), mitochondrial membrane potential and proteins, can be used to evaluate sperm. In humans, sperm-specific mtDNA mutations and rearrangements have been linked to poor quality sperm and infertility (Holyoake *et al.*, 2001; Talebi *et al.*, 2017). In effect, whereas high mtDNA copy number has been associated to poor sperm quality, lower probability of pregnancy, and longer time-to-pregnancy (May-Panloup *et al.*, 2003; Tiegs *et al.*, 2018; Rosati *et al.*, 2020), low mtDNA copy number has been related to normal semen parameters (Tiegs *et al.*, 2018, 2020). In addition, poor mtDNA integrity has been linked to sperm samples with abnormal or reduced quality (Song and Lewis, 2008; Zhang *et al.*, 2016). Yet other studies have shown that the relative mtDNA copy number in sperm is not prognostic of IVF and ICSI outcomes (Tiegs *et al.*, 2018, 2020). Regarding other parameters, sperm mitochondrial membrane potential has been correlated with normal semen parameters (Zhang *et al.*, 2016), and analysis of sperm-specific proteins has recently unravelled altered mitochondrial function that leads to fertilisation failure in ICSI patients (Torra-Massana *et al.*, 2021). These studies show that mitochondrial biomarkers can be used to predict sperm quality in both the general population and infertile patients.

In mammals, a uni-parental pattern of inheritance is established for mtDNA, where mtDNA is wholly inherited from the mother. This occurs because sperm-borne mtDNA is selectively degraded by the oocyte post-fertilisation through a process known as sperm mitophagy (Giles *et al.*, 1980). Sperm mtDNA is degraded because it tends to acquire deleterious mutations induced by exposure to ROS during the journey to the oocyte (Kumar *et al.*, 2009). As mtDNA is not protected by histones, nor does it have efficient DNA repair mechanisms, ROS-induced mutations and deletions become abundant (Kujoth *et al.*, 2005; Shokolenko *et al.*, 2009). Two hypotheses have been proposed to explain maternal inheritance of mtDNA in mammals: passive dilution (Gyllensten *et al.*, 1991); and active degradation (Song *et al.*, 2016). The first is based on the idea that, as embryonic cells divide throughout development, paternal mitochondria are diluted until

643 they become undetectable. Research using extreme-high depth mtDNA re-sequencing,
644 however, found no evidence of paternal mtDNA being transmitted to offspring in humans
645 (Pyle *et al.*, 2015). Hence, this theory is widely believed to be excluded. As far as the
646 other hypothesis is concerned, Song *et al*. (2016) proposed that post-fertilisation sperm
647 mitophagy in higher mammals occurs by active degradation and is guided by a
648 combination of the ubiquitin-proteasome system and autophagy pathways. Ubiquitin-
649 tagged sperm mitochondrial proteins in fertilised oocytes (Sutovsky *et al.*, 1999, 2003;
650 Song *et al.*, 2021) are recognised by ubiquitin-binding autophagy receptor SQSTM1 and
651 degraded in an autophagosome. Synchronously, a valosin-containing protein presents
652 ubiquitinated mitochondrial membrane proteins to the proteasome for degradation (Song
653 *et al.*, 2016).
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Genetic components

The sperm cell exists to carry the paternal haploid genome to the oocyte; hence, the integrity of its genome is imperative for the survival and quality of life that is passed onto the offspring. Owing to the endogenous machinery governing mitosis within the oocyte post-fertilisation, anomalies from the paternal nucleus may not be detected until the eight-cell stage of embryogenesis (Wong *et al.*, 2010). It is at this point when the expression of paternally derived genes is initiated.

Sperm DNA fragmentation

Sperm DNA fragmentation (SDF) is the accumulation of single- and double-strand DNA breaks in the genome and may be identified in ejaculated human sperm. SDF has been linked to infertility and poor embryo development (Sedó *et al.*, 2017a; Borges *et al.*, 2019; Ribas-Maynou *et al.*, 2021b; 2022a) (Table I), and has been found to detrimentally affect offspring health (Fernández-González *et al.*, 2008). Both extrinsic factors and intrinsic factors can induce SDF in males. Extrinsic factors include lifestyle habits, such as nutrition (Jurewicz *et al.*, 2018), smoking (Cui *et al.*, 2016), ionising radiation (Kumar *et al.*, 2013) and prolonged abstinence periods (Comar *et al.*, 2017); disease states, such as diabetes (Condorelli *et al.*, 2018), obesity (Fullston *et al.*, 2015), cancer (Meseguer *et al.*, 2008), male genital tract infections (Han *et al.*, 2021) and varicocele (Jeremias *et al.*, 2021); and vasectomy (Ribas-Maynou *et al.*, 2022b). Age has also been found to be one of the most important risk factors in increasing SDF in men (Evenson *et al.*, 2020; Vaughan *et al.*, 2020). Intrinsic factors that can induce SDF include defects in the replacement of histones by protamines (Yoshida *et al.*, 2018), defective or sub-optimal chromatin packaging (Tarozzi *et al.*, 2009), nuclease-mediated enzymatic cleavage (Shaman *et al.*, 2006), abortive apoptotic-like changes (Shukla *et al.*, 2012), and oxidative stress (Dorostghoal *et al.*, 2017).

Consequences of sperm DNA fragmentation

Sperm lack some of the key enzymes required for completion of DNA repair after fragmentation occurs (Smith *et al.*, 2013). Upon fertilisation, therefore, the collaboration of the oocyte to repair paternal DNA damage is needed (Shimura *et al*., 2002; Lord and Aitken, 2015). Depending on the extent of SDF, the oocyte might not have sufficient cellular machinery to correct all the damage, and residual DNA aberrations may be passed onto the zygote. Hence, subsequent embryonic DNA replications are likely to increase the embryo mutational load (Lane *et al.*, 2014; Ohno *et al.*, 2014).

Several studies have linked unsuccessfully repaired SDF to a negative effect on early embryo development by altering embryo morphokinetics and genomic stability (Table I). Research has associated SDF with higher incidences of miscarriage in natural pregnancies (Avendaño *et al.*, 2010; Robinson *et al.*, 2012), and meta-analyses (Zhao *et al.*, 2014; Simon *et al.*, 2017b; Ribas-Maynou *et al.*, 2021b) have found a negative correlation between birth rates and SDF. Specifically, Ribas-Maynou *et al*. (2021b) looked at 78 studies involving more than 25,000 cycles of either IVF, ICSI, or both, to

assess the effects of SDF on embryo development; 66% of the studies showed that sperm DNA damage negatively affected rates of fertilisation, embryo quality and blastocyst formation. Other meta-analyses (Li *et al.*, 2006; Collins *et al.*, 2008) as well as the American Society for Reproductive Medicine (ASRM, 2013), however, reported that there were no negative effects from human SDF on clinical pregnancy.

A possible cause for discrepancies among studies may be that the effects of DNA damage differ depending on the type of assay used to measure DNA fragmentation. For instance, the Comet assay exhibits the strongest association between poor embryo quality and DNA damage, as it is the most sensitive assay. Simon *et al.* (2011) used the Comet assay to show that SDF was associated with decreased embryo quality when there was more than 52% SDF present. Other studies observed a negative correlation when the TUNEL assay was employed to assess the effects of DNA damage on blastocyst development and pregnancy rates (Ruvolo *et al.*, 2013; Cankut *et al.*, 2019). Moreover, the meta-analysis performed by Ribas-Maynou *et al*. (2021b) showed differences between the ART techniques used; in effect, few studies reported ICSI to show any significant negative correlation, whereas many showed a negative correlation when IVF was used.

Regarding the degree of DNA fragmentation, high SDF has been correlated to fertilisation failure following IVF and ICSI (Velez de la Calle *et al.*, 2008), low embryo quality (Kim *et al.*, 2019; Ribas-Maynou *et al.*, 2022a), and miscarriage (Haddock *et al.*, 2021). Some studies associated mild to low SDF with embryonic arrest after embryonic genome activation (EGA; Simon *et al.*, 2014), whereas others observed no negative effects on blastulation or pregnancy rates when low SDF is present (Sedó *et al.*, 2017). Overall, the findings regarding the effects of SDF on early embryo dynamics and clinical outcomes remain inconsistent and controversial.

Additionally, an abundance of studies support strong links between SDF and detrimental effects on offspring health (Aitken *et al.,* 2020a). These are instances in which sperm with SDF successfully fertilise the oocyte, but this oocyte in turn is not capable of successfully repairing all of the damage. Insufficient repair of sperm DNA aberrations before the first mitotic division can potentially lead to an increased mutagenic load in the offspring. As such, ART providers should envisage introducing SDF measurements in routine clinical practice to predict fertility, and to avoid the use of sperm with SDF, so as to limit its negative effects on offspring health.

Aneuploidy

The majority of reported embryonic aneuploidy cases are a result of maternal factors, typically meiosis I complications, yet paternal influences may occasionally play a role in sex chromosome trisomy (Oldereid *et al.*, 2018, Wang *et al.*, 2020a). Macrocephalic sperm, sperm with more than one nuclei (Mehdi *et al.*, 2012), teratozoospermia - sperm with poor morphology – (Braham *et al.*, 2019, Nayel *et al.*, 2021), and oligoasthenoteratozoospermia – sperm with poor motility, poor morphology and low count - (Saei *et al.*, 2021) have been shown to be correlated with aneuploidy. Poor sperm motility alone, however, has not been found to cause a difference in aneuploidy rates

(Rodrigo *et al.*, 2019) Thus, increased sperm anomalies have higher chances of leading to embryonic aneuploidy. Furthermore, as expected, high levels of sperm aneuploidy are negatively correlated to implantation and pregnancy rates in ART cycles (Ramasamy *et al.*, 2015).

Epigenetics and paternal RNA

Epigenetics is the regulation of gene expression without any alteration of the DNA sequence. Contrary to the previous belief, it has been shown that both paternal-specific and maternal-specific factors within early embryos are involved in a genome-wide reprogramming of epigenetic marks such as DNA methylation, histone modifications and chromatin accessibility (Smith *et al.*, 2014). The sperm epigenome consists of a combination of DNA-associated proteins, a unique DNA methylation profile, ncRNAs (miRNAs and Piwi-interacting RNAs), non-histone/non-protamine proteins, nucleosome distribution and retained histone modifications.

Histone modifications

Histones undergo several forms of chemical change to regulate gene expression. These changes include methylation, ubiquitination, phosphorylation and acetylation. In order to achieve the necessary levels of nuclear compaction needed for proper sperm function, most sperm histones are replaced by protamines in mammalian spermiogenesis; this is regulated by nuclear transition proteins (Zeyad *et al.*, 2018). The remaining histones – 10-15% in humans and 1-8% in mice (Brykczynska *et al.*, 2010; Jung *et al.*, 2017) – evade the histone-protamine transition and continue to carry epigenetic signatures to the oocyte after fertilisation. These epigenetic marks are subsequently inherited by the embryo to regulate gene expression and help control heterochromatin condensation (Ozturk *et al.*, 2021). Hammoud *et al.* (2009) showed that, in human sperm, maintained histones are predominantly found at loci of key developmental genes, which include HOX gene clusters and promoters of developmental transcription and signalling factors. The retained histone regions may correspond to toroid linker regions (Ward, 2010, Ribas-Maynou *et al.*, 2021a; 2022b). Several studies have correlated mutations in these histones with reproductive problems such as sterility in worms (Katz *et al.*, 2009), impaired EGA in mice (Ihara *et al.*, 2014), and altered embryogenesis in humans (Hammoud *et al.*, 2011; Vieweg *et al.*, 2015; Glanzner *et al.*, 2017; Huang *et al.*, 2019).

More than a dozen different histone modifications have been identified as being important in spermatogenesis and embryogenesis, either independently or in tandem with one another (Schon *et al.*, 2019). For example, if H4K12 is not acetylated appropriately, levels of paternally inherited DNA (post-embryo genome activation) fall and the zygote remains in a state of arrest (Paradowska *et al.*, 2012; Vieweg *et al.*, 2015). Additionally, enzymes such as histone methyltransferase M112 can bear histone modifications. Anomalies of these enzymes have been shown to cause termination of preimplantation embryos, apoptosis (Denissov *et al.*, 2014, Lv *et al.*, 2019), male infertility and poor condensation of chromatin (Kim *et al.*, 2014). Furthermore, sperm epigenetic modifications, such as H3K4me3 (Deng *et al.*, 2020; Lambrot *et al.*, 2021) and H3K27me3 (Sun *et al.*, 2021), play a crucial role in activating the embryonic genome. Levels of H3K27me3 decrease immediately post-fertilisation and remain low until the two-cell stage; following this, they increase and remain high until the blastocyst stage (Liu *et al.*, 2016; Huang *et al.*, 2019). If expression levels of genes that alter H3K4 methylation during genome activation, such as *BRG1* and *KDM1A,* are decreased, the embryo is in danger of arrest (Glanzner *et al.*, 2017).

After fertilisation and before EGA which in humans occurs at the four/eight-cell stage (Yan *et al.*, 2013), the sperm nucleus undergoes reprogramming, involving chromatin decondensation and protamine removal, and recondensation and maternal histone variant addition (Fig. 5A). The resulting nucleosomes contain both inherited paternal and novel maternal histones, whose variants play key functions in early embryogenesis. In effect, a variant of H3, namely H3.3, is maternally deposited to paternal chromatin (Ishiuchi *et al.*, 2021) to switch on pluripotency genes (Wen *et al.*, 2014; Kong *et al.*, 2018). H1FOO, a linker histone variant that regulates zygotic chromatin structure in the mouse (Funaya *et al.*, 2018), is also maternally incorporated onto the paternal genome upon protamine removal (Becker *et al.*, 2005; Funaya et al., 2018). Likewise, oocyte variants of histones TH2A and TH2B (TH2A/B) are laid down onto paternal chromatin and have been observed to induce an open chromatin structure in the paternal genome that is necessary for offspring viability (Shinagawa *et al.*, 2014; Patankar *et al.*, 2021).

DNA methylation

DNA methylation is another epigenetic modification that regulates gene expression. Once transcription occurs, DNA-methyltransferase 1 (DNMT1) catalyses DNA methylation in CpG islands, which are regions of DNA rich in cytosine-phosphate-guanine dinucleotides (CpG) at close proximity to gene promoters. Additionally, novel DNA methylations are regulated by DNMT3a and DNMTb (Kaneda *et al.*, 2004; Yagi *et al*., 2020). The removal and addition of sperm DNA methylations are specifically timed during spermatogenesis and embryo development; remarkably, any deviances have been linked to recurrent miscarriage and poor quality of embryos (Benchaib *et al.*, 2003, 2005; Cao *et al.*, 2020; Richard Albert *et al.*, 2020). For example, a variety of male infertility issues have been associated with defective methylation of the cAMP responsive element modulator (CREM) promoter (Nanassy and Carrell, 2011; Song *et al.*, 2021). Furthermore, overexpression of *DNMT3* (3a, 3b and 3L) has been linked to the development of embryonic carcinomas, which can end in miscarriage (Almstrup *et al.*, 2005; Dahlet *et al.*, 2020).

Global demethylation occurs in the early embryo to establish a pluripotent state in the epiblast (Lee *et al*., 2018). Throughout this process, the maternal and paternal genomes are asymmetrically demethylated (Zhu *et al.*, 2018; Yang *et al*., 2022). Specifically, maternal DNA methylation is mostly passively lost through DNA replication, whereas the paternal genome is subjected to rapid and active demethylation (Guo *et al.*, 2014) (Figure 5B); however, any imprinted genes elude this process (Bar *et al.*, 2021). The mechanisms underlying paternal DNA demethylation in embryogenesis are not currently well understood, but are thought to be driven by the dioxygenase ten-eleven translocation 3 (TET3) enzyme (Cheng *et al*., 2019). TET3 converts 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5mhC). It is predominantly found in the male pronucleus (Gu *et al.*, 2011, Mulholland *et al*., 2020) and demethylates approximately 25% of the paternal genome (Peat *et al.*, 2014; Olszewska *et al*., 2022). The maternal genome is protected from TET3 5mC oxidation via a specific association with developmental pluripotency-association protein 3 (DPPA3) protein (also known as

PGC7 or STELLA), which prevents TET3 binding by interacting with the H3 variant H3K9me2 (Mulholland *et al.*, 2020). On the other hand, the protamine-packed paternal genome is not able to interact with DPPA3 because it lacks H3K9me2 nucleosomes, and is thus vulnerable to TET3 demethylation (Nakamura *et al.*, 2012). In mice, small amounts of DPPA3 are found in the male pronucleus protecting the methylation state of two paternally imprinted gene loci which do have H3K9me2-marked chromatin, *H19* and RAS protein-specific guanine nucleotide-releasing factor 1 (*RASGRF1*) (Nakamura *et al.*, 2007). Imprinting abnormalities have been shown to cause issues regarding embryonic damage, occasionally leading to congenital syndromes, such as Angelman syndrome, Prader-Willi syndrome or Beckwith-Wiedemann syndrome (Kobayashi *et al.*, 2009; Hattori *et al.*, 2019; Inoue *et al.*, 2020). For example, the Beckwith-Wiedemann syndrome has been linked to over methylation of *H19* (Bliek *et al.*, 2001; Kubo *et al.*, 2020) and under methylation of *KvDMR1* (Singh *et al.*, 2017), both alleles being of paternal origin. On the other hand, alterations in the methylation patterns of the retained histone CpG island regions of sperm DNA have not only been associated with patients with oligozoospermia and infertility, but also with normozoospermia, leading to poor embryo quality. Since genes related to embryonic development of these histone-retained CpG regions were found to exhibit decreased methylation in the sperm of men that gave rise to poorly developed embryos, alterations of said genes may contribute to the men's poor fertility (Denomme *et al.*, 2017).

Taken together, these data suggest that DNA methylation patterns are critical in establishing proper embryo developmental dynamics. This was especially apparent in a study carried out on 127 men undergoing IVF, where the investigation of over 485,000 DNA methylation sites showed that individuals who gave rise to poorer quality embryos had significantly different DNA methylation patterns when compared to men with normozoospermia who produced embryos of better quality and higher viability (Aston *et al*., 2015).

Sperm-derived transcripts

The replacement of most histones by protamines in sperm leads to a transcriptionally inactive nucleus (Hutchison *et al*., 2017). Over the past decade, advances in RNA-sequencing technologies have allowed the identification, quantification, and characterisation of transcripts that remain in sperm after protamination. These include coding (mRNA) and ncRNA. Within ncRNA, siRNAs, miRNAs, piRNAs, circular (circ) RNAs and lnc (long non-coding)-RNAs have been recognised. Previous research proposed that the paternal RNA contributions may influence fertilisation and embryo development (Ostermeier *et al.*, 2004, Jodar *et al.*, 2015, Bianchi *et al.*, 2018).

Messenger RNA

While human sperm have nearly 600 times fewer mRNAs than somatic cells (Zhao *et al.*, 2006), residual DNA and RNA polymerase activities have been reported in the mammalian sperm nucleus (Bianchi *et al.*, 2018). The function of the mRNAs present in sperm cells is not yet entirely understood but different hypotheses have been raised. It

may be the case that these sperm mRNAs have no function and are simply residual. An alternative possibility is that the presence of mRNA in sperm cells may play a role in late stages of spermatogenesis, particularly during condensation of nuclear chromatin. It is likely that mature sperm typically do not transcribe or translate novel RNA (Ren *et al.*, 2017; Corral-Vazquez *et al.,* 2021); therefore, mRNAs are possibly transcribed before histones pass on chromatin to protamines and are then translated once the male germ cell has matured to an elongated spermatid. If this is the case, these mRNAs could have a function in early embryo development *via* involvement in epigenetic imprinting when the embryo transitions from maternal to embryonic gene transcription (Sendler *et al.*, 2013). A final possibility could be that sperm mRNAs are directly involved in embryogenesis (Ko *et al.*, 2000; Hayashi *et al.*, 2003; Ostermeier *et al.*, 2004; Jodar *et al.*, 2015, Ntostis *et al*., 2017). The sperm-specific mRNA transcripts *PSG1* and *HLA-E* were found to be involved in implantation when an experiment was carried out using human sperm to fertilise hamster oocytes, as these transcripts were found in the hamster oocyte 24 hours post-fertilisation (Avendaño *et al.*, 2008). PSG1 has been linked to T cell proliferation (Motrán *et al.*, 2002; Timganova *et al.*, 2019) indicating that it may be engaged in guarding the embryo against maternal immune cells. When the expression of *PSG1* and *HLA-E* transcripts was compared between infertile and fertile groups of men, the fertile group showed significantly higher expression of these genes (Avendaño *et al.*, 2009). This evidence, therefore, suggests that sperm cells provide mRNAs that are involved in embryonic development prior to EGA (Table II); however, the precise function of many of these transcripts is still unclear and there is a lack of human studies investigating this topic.

It was previously conjectured that mouse sperm transcripts are degraded during the one-cell stage of embryo development, therefore are unable to affect embryogenesis (Hayashi *et al.*, 2003). Other studies, nonetheless, proposed that sperm mRNAs, such as *SSFA2* and *SESN1,* are present until the four-cell stage and are involved in preimplantation embryo development in pigs (Yang *et al.*, 2009; Guo *et al.*, 2017). Additionally, sperm transcripts, such as protamine-2 (*PRM2*) and heat shock protein 90 (*HSP90AA1*) mRNAs, have been implicated in embryo development. Interestingly, the partners of men with lower *PRM2* and higher *HSP90AA1* levels have been shown to have higher rates of recurrent miscarriages (Cho *et al.*, 2003; Hwang *et al.*, 2013; Kause *et al.*, 2019). This may be a result of increased oxidative stress caused by free radicals, thereby leading to fragmentation and damage of blastocysts (Esakky and Moley, 2016). Related to this, another study investigated whether the relative transcript content and activity of glutathione peroxidases 1 and 4, and glutathione reductase (GSR), in sperm affects embryo development (Meseguer *et al*., 2006). While fertilisation rate and pronuclei formation were not correlated to transcript levels of *GPX1*, *GPX4* or *GSR*, the *GPX4*-transcript abundance in sperm was found to be negatively correlated to the proportion of asymmetric embryos. In addition, lower activity of GPX1 in sperm was found to lead to impaired embryo development at 5 days post-fertilisation. This would suggest that the *GPX1*- and *GPX4*-transcripts of paternal origin could play a role during early embryo development (Meseguer *et al*., 2006).

Sadakierska-Chudy *et al.* (2020) investigated further the hypothesis that sperm-borne transcripts are delivered to the oocyte and are involved in embryo development. For this purpose, they compared the levels of transcripts of genes important for fertilisation, oocyte activation and chromatin remodelling between normozoospermic men and men with severe oligozoospermia, and also examined zygotes and embryos. Sperm from the oligozoospermic group showed reduced levels of transcripts related to fertilisation, oocyte activation, chromatin remodelling, and EGA. These transcripts included *AKAP4, PTK7, PLCζ* and *POU5F1,* and were also associated with fertilisation failure and poorer embryo development, thus supporting the involvement of the sperm-borne mRNAs, produced during spermatogenesis, on early development (Sadakierska-Chudy *et al.*, 2020) A recent paper showed a significantly positive correlation between levels of the heat shock protein family D member 1 (HSPD1) transcript and sperm quality/fertilising ability in pigs. Indeed, the expression of *HSPD1* was found to be linked to sperm motility, and groups with greater litter sizes exhibited a higher expression of the HSPD1 marker (Pang *et al.,* 2022).

Next generation sequencing (NGS) technologies have also enabled mRNA sequencing of human sperm to help identify mRNA biomarkers of infertility (Sendler *et al.*, 2013; Jodar *et al.*, 2015; Corral-Vazquez *et al*., 2021). A recent NGS study carried out transcriptome analysis on sperm from 12 men, and a large proportion of transcripts enriched in embryogenesis-related processes was found (Corral-Vazquez et *al.*, 2021). Among these, they identified, in addition to *PRM1* and *PRM2*, which encode protamines 1 and 2; *TNP1*, which encodes transition nuclear proteins that are involved in the replacement of histones to protamines; and *TSSK6*, which is involved in DNA condensation during chromatin remodelling after meiosis. Focusing on *PRM1* and *PRM2*, and as mentioned above, Rogenhofer *et al.* (2017) reported that reduced levels of *PRM1*- and *PRM2*-transcripts, and a lower *PRM1:PRM2* mRNA ratio, are associated with recurrent miscarriage. This supports transcript levels of *PRM1* and *PRM2* in sperm being related to early embryo development, and that sperm fertilizing ability is linked not only to the protamine-1 and protamine-2 protein ratio, but also their transcript levels.

Finally, it is worth noting that many of the aforementioned studies have assumed that sperm-borne mRNA may be involved in embryogenesis purely based on the presence of transcripts. More evidence, however, is required to demonstrate that these mRNA transcripts are functional and that they are specifically translated into proteins involved in embryogenesis.

Non-coding RNA

In addition to protein-coding RNAs, the ncRNAs are involved in the regulation of several physiological processes and include lncRNA, miRNA, piRNA and circRNA. As previously mentioned, various types of ncRNA are thought to be found in human sperm. Evidence suggests they may play a role in fertilisation and preimplantation development of the embryo (Salas-Huetos *et al.*, 2020).

MicroRNAs are small single-stranded RNA molecules that regulate gene expression. They are the best characterised sperm-derived RNAs and modulate

fertilisation and early embryonic development (Yuan *et al.*, 2016). Several miRNAs have been associated with male infertility (Lian *et al.*, 2010; Marcet *et al.*, 2011; Romero *et al.*, 2011; Comazzetto *et al.*, 2014; Gou *et al.*, 2017). The most abundant human sperm-borne miRNA is miR-34c (Salas-Huetos *et al.*, 2014), necessary for the first cleavage division in mice (Liu *et al.*, 2012b). Sperm miR-216b is thought to contribute to the zygote's own miR-216b pool and modulate the expression of KRAS, a protein involved in cell proliferation and differentiation, in two-cell embryos. Levels of zygotic miR-216b are related to those present in sperm, with lower content associated with higher fertilisation rates (Alves *et al.*, 2019). The effect of sperm-borne miRNAs on embryo development has also been observed in other species, including bovine (Wu *et al.*, 2020). For instance, bovine mi-449b is also implicated in zygotic cleavage division, in addition to having functions in embryo epigenetic reprogramming and blastocyst apoptosis (Wang *et al.*, 2017). On the other hand, Dicer is an enzyme involved in the biogenesis of miRNAs and siRNAs. While Yuan *et al.* (2016) found that embryos derived from Dicer conditional-knockout mutant mice were unable to develop normally into the blastocyst stage, they were able to rescue them when the developing embryos were microinjected with purified sperm miRNAs and endo-siRNAs.

Sperm cells found in the cauda epididymis contain a mixture of ncRNAs, comprising miRNAs and fragments of tRNAs. The tRNAs were found to contribute to the metabolism in offspring, as well as epigenetic inheritance (Sharma *et al.*, 2013, 2018; Chen *et al.*, 2016; Zhang *et al.*, 2018). In addition, levels of miRNAs change during maturation in the epididymis. Fertilisation with sperm at different stages of maturation, for example testicular or caudal sperm, show varying effects on development suggesting that miRNAs may be important in the regulation of early embryonic genes (Conine *et al.*, 2018). This is an important factor to consider when carrying out IVF or ICSI with sperm obtained from testicular sperm extraction, testicular sperm aspiration or microsurgical epididymal sperm aspiration.

piRNAs are known to silence transposable elements by destroying their 3'UTR mRNAs (Larriba and del Mazo, 2018) or governing retrotransposon methylator DNMT3L (Itou *et al.*, 2015). Thus, piRNAs have been suggested to protect the embryo from transposable elements that may compromise genome integrity. Further evidence suggests that sperm piRNAs may be involved in transgenerational transmission (Ingerslev *et al.*, 2018) and the regulation of epigenetic states (de Castro Barbosa *et al.*, 2015; Donkin *et al.*, 2016; Tyebji *et al.*, 2020). In mouse, specific pachytene piRNAs from chromosomes 6 and 10 are involved in spermiogenesis, oocyte fertilisation and embryo development (Wu *et al.*, 2020). Similar to miRNAs, sperm-borne lncRNAs have also been associated to embryogenesis and sperm function (Corral-Vazquez *et al.*, 2021); for instance, lncRNAs, such as lnc09522, lnc32058 and lnc98487, have a likely role in the regulation of sperm motility (Zhang *et al.*, 2019).

Finally, circRNAs, circular single-stranded pieces of RNA that are involved in regulating miRNAs, mRNAs and proteins, could also have a function in embryogenesis (Dang *et al.*, 2016, Li *et al.*, 2021). Indeed, Dang *et al.* (2016) reported that certain circRNAs identified in sperm are found in preimplantation embryos but not in oocytes, which would reflect their role in embryogenesis. Moreover, previous research screened

sperm with circRNA microarrays and identified ~20,000 transcripts (Chioccarelli *et al.*, 2019, Manfrevola *et al.*, 2020). Interestingly, circRNA payloads in the sperm of men with poor sperm quality differed from those of normozoospermic men (Chioccarelli *et al.*, 2019; Manfrevola *et al.*, 2020). Another recent study found that the RNA-binding protein FUS plays a crucial role in the circularisation of mRNAs, through an interaction with circCNOT6L and in co-operation with RNA Polymerase II and Quaking (QKI). This study also suggested that both FUS and circCNOT6L are delivered to the oocyte by sperm, and that sperm-borne circCNOT6L takes part in regulation of the transition from zygote to two-cell stage (Chioccarelli *et al.*, 2021).

Defective epigenetic footprints

Aberrant sperm epigenetic footprints include alterations in sperm histone content (Oliva and Ballescà, 2012), DNA methylation patterns (Khosravizadeh et al., 2020), and RNA elements (Jodar *et al.*, 2013; Salas-Huetos *et al.*, 2020). Azpiazu *et al.* (2014) found that six histone variants were significantly downregulated in sperm samples from infertile men, potentially leading to chromatin rearrangements in developmental loci and contributing to failed embryo development. Abnormal sperm methylation patterns, specifically higher levels of hypomethylated DNA, have been associated with low sperm motility and subfertility (Pacheco *et al.*, 2011). With regard to RNA elements, transcriptomics and microarray analysis identified the differential expression of 2,081 mRNAs (Bansal *et al.*, 2015) and 52 miRNAs (Liu *et al.*, 2012a) between fertile individuals and infertile patients. In particular, expression of miR-27a, a miRNA thought to negatively regulate the epididymal sperm-gamete-fusion protein cysteine-rich secretory protein 2 (CRISP2), was found to be increased in sperm from infertile men (Zhou *et al.*, 2017). A clear and strong relation between sperm epigenetic misprints and impaired embryo development has thus been established, which should be considered when evaluating sperm fertility potential.

Proteomics

Despite predictions of sperm proteins playing a role in oocyte fertilisation and embryo development through Gene Ontology annotations and Mouse Genome Informatics databases (reviewed by Cannarella *et al*., 2020), studies focused on each of these proteins are scarce and, hence, more research is needed to address their actual function in oocyte fertilisation and beyond (Table III). The male proteomic contribution to the early embryo remains the subject of active investigation. Given the morphological nature of the sperm cell, determining whether a sperm protein is involved in embryo development or simply a remnant of spermatogenesis is a challenging endeavour. Numerous sperm proteins, nonetheless, have been shown to contribute to embryo development using mass spectrometry (MS-MS) techniques (Amaral *et al.*, 2014; Vandenbrouck *et al.*, 2016; Jodar *et al.*, 2017; Castillo *et al.*, 2018). A meta-analysis carried out by Castillo *et al*. (2018) investigated all known mammalian sperm protein contributions to the early embryo, and identified 103 proteins involved in fertilisation and 93 implied in preimplantation development. Important sperm-borne proteins involved in fertilisation have also been described above. It is worth emphasising that, during preimplantation development, sperm-borne proteins have roles in the first embryonic divisions (up to eight-cell stage; 11 proteins), morula formation (29 proteins) and blastocyst development (19 proteins) (Table III). Remarkably, 59 of the identified human proteins were shown to disrupt preimplantation embryo development when their orthologs were knocked out in mice studies. One of these proteins is transmembrane glycoprotein desmocollin 3 (DSC3), a sperm-derived protein involved in cell adhesion. In mice, it is delivered via the male gamete to the zygote (Den *et al.*, 2006), and in humans it is hypothesised to regulate cell adhesion in blastomeres formed on day 1 of development, before EGA (Castillo *et al.*, 2018).

A sperm-derived protein linked to morula formation is lactosylceramide 1,3-N-acetyl-beta-Dglucosaminyltransferase (B3GNT5), which regulates the formation of lipid membranes. Downregulation of B3GNT5 has been found to alter cell adhesion and signalling processes at the morula stage, resulting in embryo lethality (Biellmann *et al.*, 2008). Choline-phosphate cytidyltransferase A (PCYT1A) is also a sperm-derived protein involved in lipid membrane formation. In mouse models, not only was PCYT1A found to be essential for the generation of blastocysts capable of implantation, but also those lacking PCYT1A failed to proceed past the blastocyst stage to implantation (Wang *et al.*, 2005). Further investigations are, however, required to determine whether the data obtained from mouse models translate to the human sperm contributions to the early embryo.

Another important aspect of proteomics is its potential use for identifying biomarkers for male infertility. Multiple studies have concluded that the sperm proteome of infertile men differs from that of fertile men (e.g., Légaré *et al.*, 2014). Candidate proteins to be used as biomarkers include BAG6 and HIST1H2BA for all types of male factor infertility (Intasqui *et al.*, 2018), and ANXA2, SPA17, and SERPINA5 for unexplained infertility (Panner Selvam *et al.*, 2019). Finally, and in addition to protein content, worthy of note is that low activity of some enzymes in sperm, such as GPX1,

may be related to impaired embryo development at 5 days post-fertilisation (Meseguer *et al.*, 2006).

Relevance of sperm factors for ART

ART has provided us with the opportunity to combat infertility, and the male gamete is a crucial half of this process. Treatment options such as ICSI seem to sidestep the barriers of infertility to provide couples with the long-awaited gift of a child. Yet, by doing this**,** is the integrity of the sperm cell affected? Kobayashi *et al.* (2007) showed the presence of DNA methylation defects in over 20% of embryos derived from ART, 41% of which had anomalies derived from paternally inherited methylation. In addition, syndromes that arise from imprinting errors, such as Angelman Syndrome, have been shown to be more common in children born through IVF, likely related to the sensitivity of gametes to *in vitro* conditions (Cortessis *et al.*, 2018; Hattori *et al.*, 2019). Furthermore, other research has suggested that male children born from ICSI may be at a higher risk of either being sub-fertile themselves or developing other congenital malformations (Esteves *et al.*, 2018; Jwa *et al.*, 2019; Catford *et al.*, 2020). The question, therefore, remains; given what is known about the paternal factors associated with embryo development, is the process of bypassing sperm anomalies to achieve the ultimate outcome of passing on genes truly worth risking the long-term health of the child and possibly further lineages?

Conclusion

This review has explored the functions of semen in the female reproductive tract, at fertilisation, and during early development, and has shown that, in contrast to past beliefs, SP and sperm do much more than merely carry the paternal haploid genome to the oocyte. Recent research has brought forth novel insights into how semen regulates development and implantation via SP, the sperm centriole, proteins and transcripts, and epigenetic marks. Likewise, studies on SDF have shed light on the negative clinical effects of DNA damage and have encouraged the implementation of routine sperm DNA testing for infertility patients. Further findings illustrate how differential mtDNA, proteomic expression and SDF could be used as potential biomarkers for assessing sperm reproductive capacity. Overall, current data highlight a definite need for further research on paternal roles in early embryo development, and a more prominent focus on how paternal factors affect clinical outcomes and their consequences on embryo development and future offspring.

Data availability

There are no new data associated with this article.

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Authors' roles

M.V.B, R.M. and M.Y. wrote the Manuscript. M.Y. supervised the work and critically revised the Manuscript. C.J. and K.C. made a critical revision of the Manuscript. All authors approved the final version.

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Conflict of interest

The authors declare that they have no conflict of interest regarding this review.

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Figure legends

Figure 1 Mammalian spermatogenesis within the testis.
(A) Sperm production occurs within the seminiferous tubules of the testis. Primordial germ cells undergo mitotic division to eventually produce a primary spermatocyte, which enters meiosis and gives rise to four spermatids, which ultimately mature into sperm. Germinal cells are supported by Sertoli cells. Sperm then travel through the lumen of the seminiferous tubules and are stored in the epididymis before leaving during ejaculation. **(B)** The unique structure of the sperm cell helps carry out its function. It has a head that carries the nuclear information and other cytoplasmic components. The acrosome carries digestive enzymes to help penetrate oocyte vestments. As the sperm approaches the oocyte, the sperm plasma membrane fuses with the acrosome membrane to expose the acrosomal digestive enzymes. This process is called the acrosome reaction. The midpiece carries mitochondria that provide ATP through oxidative phosphorylation and play a crucial role during sperm capacitation. The elongated tail allows the sperm cell to swim up the reproductive tract. Figure created with BioRender.com.

Figure 2 The histone to protamine transition in mammals.
During spermatogenesis, histones are replaced by protamines to allow for increased compaction of chromatin within the sperm nucleus. **(A)** During the spermatogonium and primary spermatocyte phases, histones are packed in groups of eight to form a nucleosome. AC represents histone hyperacetylation on the histone tails, which may vary between different histone variants; other epigenetic marks may be applied. **(B)** During the round spermatid phase, remodelling factors, such as transition proteins, help remove chromatin from the nucleosomes and bind it to protamines. (**C**) Protamines form a toroidal loop structure for effective compaction. Figure created with BioRender.com.

Figure 3 The three theories for oocyte activation in humans.
(A) From left to right: sperm oocyte-activating factor (SOAF) theory, receptor theory, calcium bomb theory. **(B)** PLCζ-directed pathway for the generation of calcium (Ca^{2+}) oscillations in the oocyte.
PLCζ= sperm-specific phospholipase C zeta InsP3= inositol 1,4,5 trisphosphate PIP2= phosphatydilinositol-4, 5 bisphosphate
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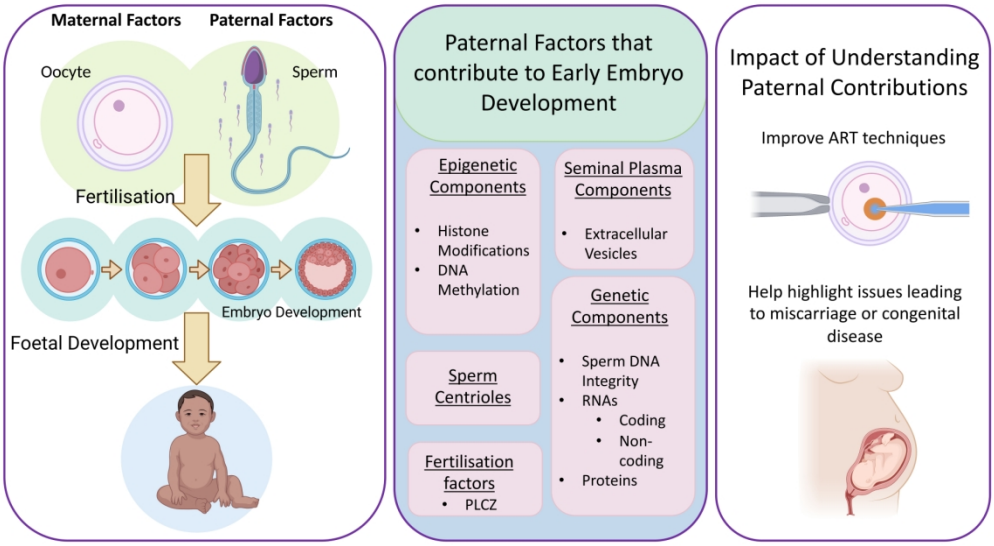
Figure 4 Sperm centrioles and their role at post-fertilisation in humans.
(A) Centriole inheritance in humans from sperm to zygote. **(B-G)**. Centrioles in the zygote post-fertilisation in humans. **(C)** The sperm centrosome forms an aster while the oocyte complete meiosis II and the second polar body is removed. **(D)** Centrioles begin to duplicate to produce two daughter centrioles. **(E)** Zygote centrosomes begin to separate. **(F)** Zygote undergoes mitosis. **(G)** Two blastomeres are formed. Blastomeres alternate between M and S phases and do not form cilia or experience cytoplasmic growth until the blastocyst stage.

Ax = Axoneme; Ca = Centriole adjunct; DC = Distal centriole; PC = Proximal centriole; PCL = Proximal centriole-like structure; ZdC = Zygotic daughter centriole; PB = Polar body; N = Ploidy. Adapted from Avidor-Reiss and Fishman (2019). Figure created with BioRender.com.

Figure 5 Sperm and zygotic nuclei reprogramming post-fertilisation in humans. **(A)** Protamine and histone variant dynamics in early embryo development (adapted from Yang *et al*., 2015). **(B)** Paternal DNA methylation dynamics in early embryo development. Sperm DNA methylation rapidly decreases; 5mC is converted into 5hmC. 5hmC = 5-hydroxymethylcystosine, 5mC= 5-methylcystosine, PN= pronucleus, H3.3 = histone 3.3, H1FOO= Linker histone H1 FOO, TH2A/B = testis-specific histone H2A/B variants.
Created with BioRender.com.

Graphical Abstract Abbreviated Summary
Understanding the various paternal factors that contribute to embryo development may help address the treatment of male infertility



Graphical Abstract

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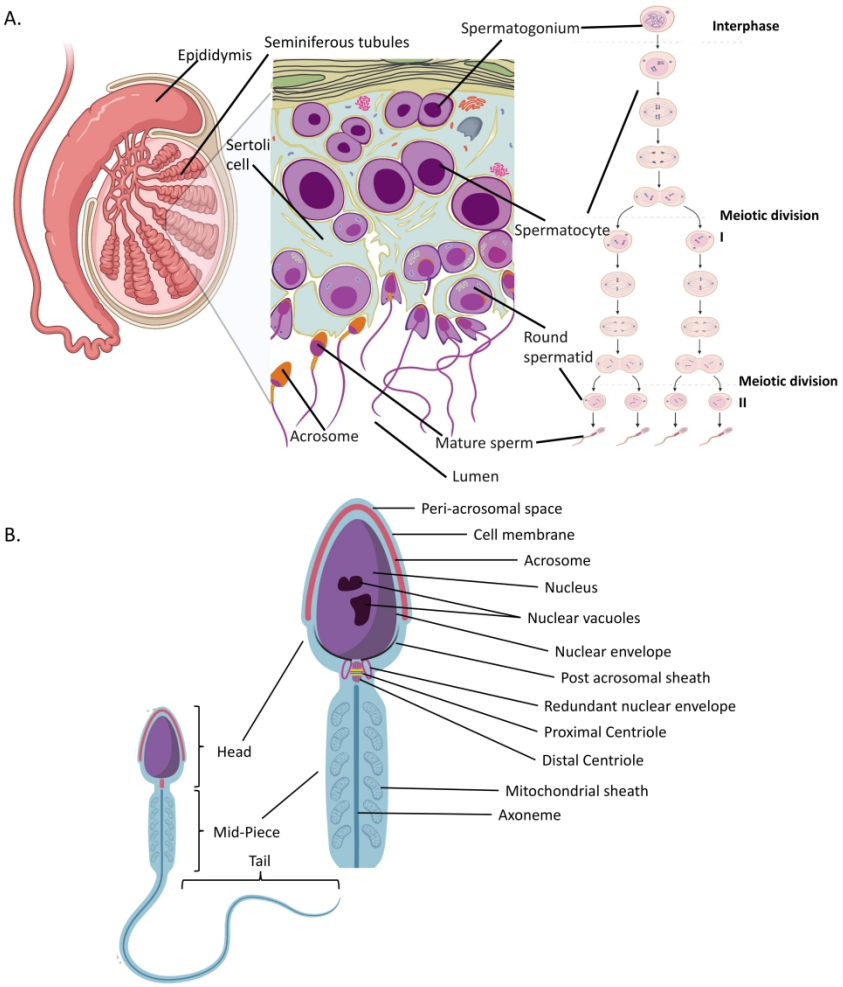

Figure 1

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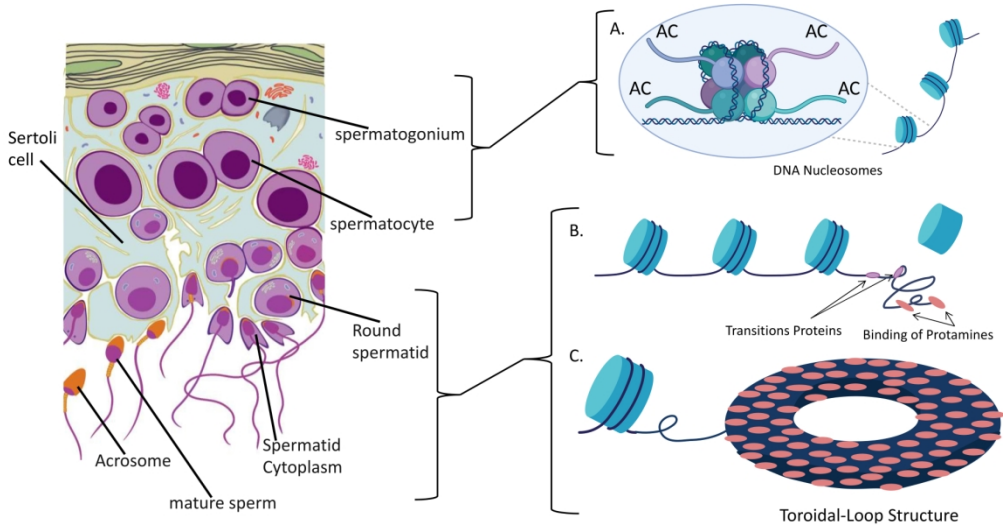

Figure 2

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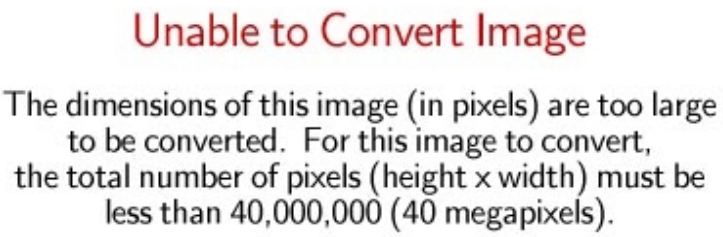
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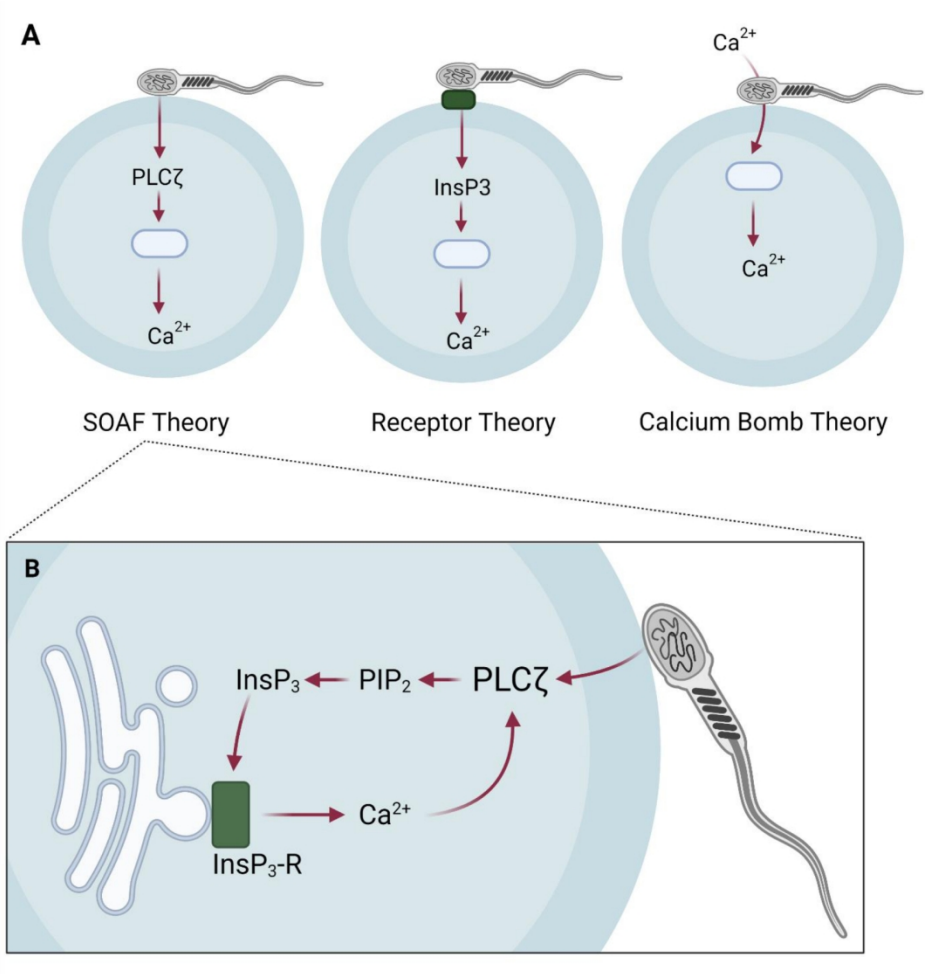
Figure 2



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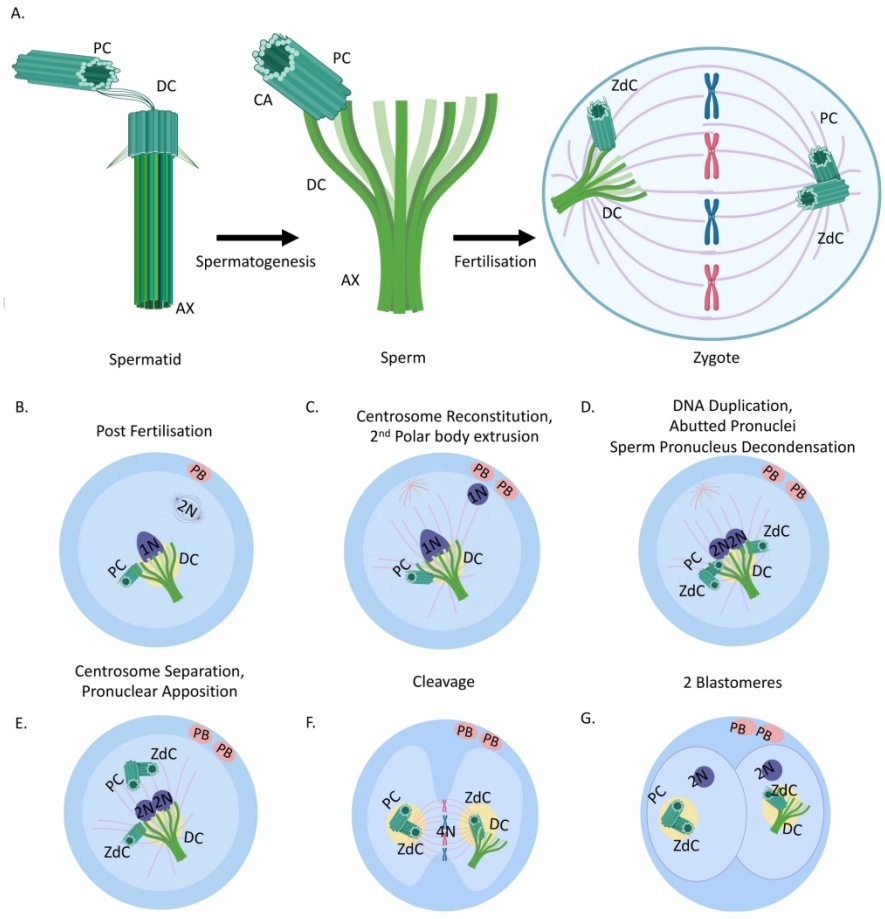

Figure 4 revised

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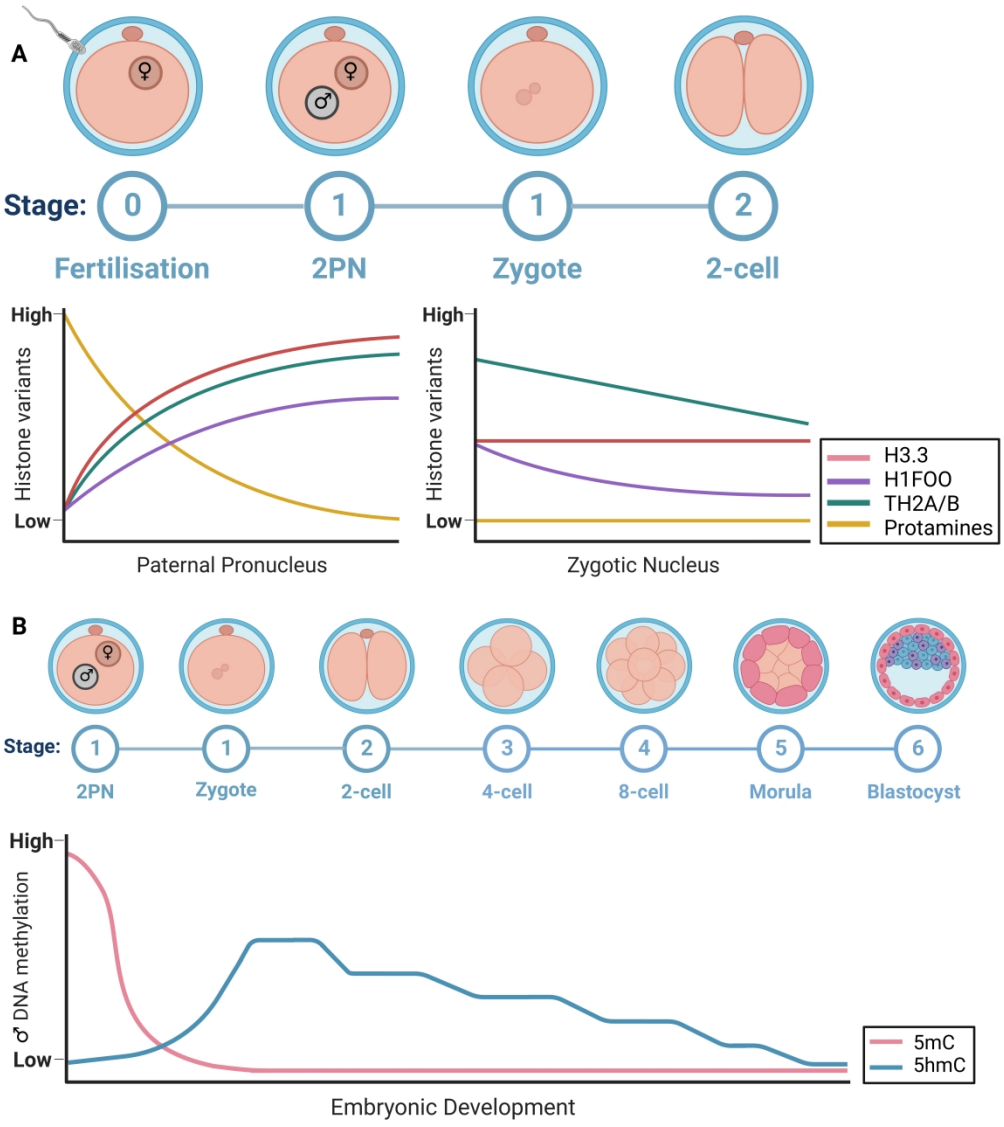

Figure 5

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Table I Studies on the effect of sperm DNA fragmentation on early embryo development.

Studied parameter	Model	Aim	SDF test	n	Conclusion	Study
Embryo genomic stability	Bovine	Determine effect of SDF on embryonic genome	WGS	N/A	Higher incidence of genetic abnormalities (chromosomal aberrations, aneuploidies, chaotic mosaicism) in embryos originating from sperm with SDF Embryos prone to unequal cleavage division	(Middelkamp *et al.*, 2020)
Embryo genomic stability	Human	Evaluate paternal contribution to embryonic aneuploidy in patients with poor pregnancy prognosis after IVF	N/A	1549	Chaotic mosaicism elevated in embryos originating from men with nonobstructive azoospermia with high SDF	(Magli *et al.*, 2009)
Embryo morphokinetics	Bovine	Correlate oxidative status with SDF and its influence on embryonic development	Alkaline comet assay	30	Lower cleavage rates, higher apoptosis rates, normal blastocyst rates in embryos originating from sperm with SDF SDF does not affect the number of blastomeres that an embryo can produce	(Simões *et al.*, 2013)
Embryo morphokinetics	Human	Investigate if SDF has an impact on embryonic development	TUNEL	220	Kinetics delayed in embryos originating from sperm with SDF. Likely due to additional time required to activate DNA repair mechanisms before cell division. SDF does not determine embryo quality, blastulation rate, or clinical outcome	(Esbert *et al.*, 2018)
Embryo morphokinetics	Human	Investigate effect of SDF on fertilisation, blastulation, and pregnancy rates after ICSI	TUNEL	82	High levels of SDF were associated with low blastulation and pregnancy rates, but fertilisation rate was not affected.	(Sedó *et al.*, 2017c)

					High levels of SDF were associated with embryo arrest and activation of the apoptotic pathway.	
Embryo morphokinetics	Human	Investigate effect of SDF on fertilisation, implantation, and pregnancy rates after ICSI	SCD test (Halosperm)	475 ICSI cycles	High levels of SDF were associated with poor embryo development, lower implantation rate, and higher miscarriage rate.	(Borges *et al.*, 2019)
Embryo morphokinetics	Human	Investigate effect of SDF on embryo quality, progression of embryo development, and implantation and pregnancy rates after IVF and ICSI	Alkaline comet assay	215 infertile men	High levels of SDF were associated with negative effects on embryo quality starting on day 2 of development, which lead to lower implantation and pregnancy rates	(Simon *et al.*, 2014b)
Embryo morphokinetics	Human	Investigate effect of SDF on embryo quality and fertilisation, blastulation, implantation, and pregnancy rates	SCD test	161 IVF cycles	High levels of SDF were associated with negative effects on day 3 embryo quality and blastulation, implantation, and pregnancy rates. Fertilisation rate was not affected.	(Zheng *et al.*, 2018)
Embryo morphokinetics	Human	Investigate effect of single stranded and double stranded SDF on embryo development, and blastulation and implantation rates	Comet assay	195 embryos	Double stranded SDF caused delays in embryo development and impaired implantation. Single stranded SDF did not cause such effects.	(Casanovas *et al.*, 2019)
Embryo morphokinetics	Human	Investigate effects on embryo morphokinetics caused by SDF using time-lapse imaging	N/A	978 embryos	High levels of SDF caused delays n cell cleavage and blastulation.	(Setti *et al.*, 2021)
Embryo morphokinetics	Human	Investigate effects of SDF on embryo morphokinetics parameters, cleavage patterns, and embryo quality	N/A	1152 embryos	High levels of SDF lead to a reduction in fertilisation and blastulation rate after ICSI. High levels of SDF also caused chaotic cleavage patterns.	(Wang *et al.*, 2022)
Embryo morphokinetics	Human	Investigate effects of SDF on embryo formation rate after IVF and ICSI	SCD test (Halosperm)	53 IVF/ICSI cycles	High levels of SDF lead to a reduction of day 3 embryo formation rates.	(Kim *et al.*, 2019b)

n, samples size; WGS, whole genome sequencing; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labelling; SDF, sperm DNA fragmentation; SCD, sperm chromatin dispersion; N/A, not available.

Table II Sperm transcripts potentially involved in oocyte fertilisation and embryo development.

Sperm Transcript	Function	Possible effects on fertilisation and embryo development	Species	Study
c-MYC	A proto-oncogene involved in several carcinogenic processes	Possibly involved in sperm capacitation	Mouse	Ahmad and Naz (1991); Anbara *et al.* (2018)
COX7C	Cytochrome oxidase subunit 7C is part of the mitochondrial electron transport chain, and is thus involved in ATP production.	It is part of the mitochondrial electron transport chain, and is thus involved in ATP production. Relative abundance of *COX7C*-transcript in sperm is negatively correlated with fertility rates, which could be due to an inefficient translation of the protein at the end of spermatogenesis, thereby impairing mitochondrial function.	Cattle	Card *et al.* (2017)
DBY (DDX3Y)	DEAD-box helicase 3 Y-linked is involved in ATP and RNA binding. Mutations in this gene are related to male infertility and the Sertoli-cell only syndrome.	*Ddx3y*-mRNA is retained in the post-acrosomal region of capacitated sperm and is transferred to the oocyte during fertilisation. Injection of antisense *Ddx3y*-mRNA into the male pronucleus of zygotes reduces embryo development, and affects the sex ratio of the resulting developed embryos.	Mouse	Yao *et al.* (2010)
FOXG1	Forkhead box protein G1 is a transcription factor that appears to be involved in brain development in the embryo.	Although sperm-borne *Foxg1*-transcripts are present in zygotes at both the pronuclear stage and the two-cell stage, they do not seem to be translated into protein, at least in early development stages.	Mouse	Fang *et al.* (2014)
FTO	Alpha-ketoglutarate-dependent dioxygenase (also known as fat mass and obesity-associated protein) is involved in the biosynthesis of different molecules (e.g. collagen), post-translational modifications, and metabolism.	Relative levels of FTO-mRNA in sperm are correlated with fertilisation rate, cleavage rate and the proportion of high quality embryos	Human	Pereira *et al.* (2021)
GABRA1	GABA A-α1 receptor is a ligand-gated chloride channel that forms the heteropentameric receptor for GABA, a neurotransmitter.	Transcript levels of the *GABRA1* in sperm are negatively correlated with the proportion of good quality embryos at the cleavage stage.	Human	Kaewman *et al.* (2021)

GPX4	Glutathione peroxidase 4 reduces phospholipid hydroperoxide, protects cells from oxidative damage and prevents them from ferroptosis.	High levels of *GPX4*-transcripts in sperm are likely to produce symmetric embryos, whereas low levels are more likely to produce asymmetric embryos.	Human	Meseguer *et al.* (2006)
HSPA1A (HSP70)	Heat shock protein 70 is a chaperone protein, important for the structural and functional competence of sperm.	Visualised in nervous system of transgenic mice during their embryonic development; however, it is not essential for development.	Murine	Rupik *et al.* (2006); Reddy *et al.* (2018)
HLA class 1	Essential for the immune system (endogenous pathway), as they bind short peptides that derive from proteolysis of intracellular proteins.	Could be involved in protection of the embryo from the mother's immune system.	Human	Chiang *et al.* (1994); Signorelli *et al.*, (2012)
HLA-E	Human leukocyte antigen-E (major histocompatibility complex, class I, E) is involved in both innate and adaptive immune responses.	The content of *HLA-E* mRNA in sperm is higher in fertile individuals than in infertile patients. Interestingly, HLA-E protein is not present in uncapacitated or capacitated sperm. In addition, sperm-borne *HLA-E* transcript in zygotes is detectable after 24 h of sperm injection (but not other transcripts, such as *PRM2)*.	Human	Avendaño *et al.* (2009)
HSPD1 (HSP60)	Heat shock protein 60 (also known as chaperonin) is involved in the transport and folding of mitochondrial proteins encoded by nuclear DNA.	Expression content of HSPD1 is correlated to sperm motility and litter size, the higher the abundance of the transcript the smaller the litter size.	Pig	Pang *et al.* (2022)
INTSI	Involved in the transcription and processing of snRNAs U1 and U2	mRNA levels increase immediately after fertilisation and before embryo genome activation. Knockout of this gene in mouse is lethal for embryos at the blastocyst stage.	Human	Sabath *et al.* (2020)
PAWP (WBP2NL)	WBP2 N-terminal like (also known as postacrosomal sheath WW domain-binding protein) could play a role in meiotic resumption and pronuclear formation.	Patients suffering from globozoospermia have lower levels of *PAWP*-transcript than fertile individuals. These transcript levels, however, do not predict fertilisation rates after ICSI-AOA.	Human	Tavalee and Nasr-Esfahani (2016)
PLCZ1	Phospholipase C zeta (PLCζ) hydrolyses PIP_2 of oocyte vesicles into $InsP_3$ and DAG, and is suggested to be the main sperm-borne oocyte activation factor.	Patients suffering from globozoospermia have lower levels of *PLCZ1*-transcript than fertile individuals. These transcript levels, however, do not predict fertilisation rates after ICSI-AOA.	Human	Tavalee and Nasr-Esfahani (2016)

PRM1 and *PRM2*	Protamines 1 and 2 are the main nucleoproteins of sperm chromatin and allow packaging of DNA, protecting the genetic message.	Possibly preventing abnormally packed sperm from exposure to DNA damage resulting in higher reproductive outcome. Both transcript and protein levels of PRM1 and PRM2 in sperm are correlated with their fertilising ability and the proportion of grade A embryos. Transcript levels of protamine-1 and protamine-2, and protamine-1:protamine-2 mRNA ratio are lower in sperm from patients whose female partners suffer from two or more consecutive miscarriages before the 20th week of gestation compared to those from both healthy controls and couples undergoing IVF/ICSI but not experiencing miscarriage. *PRM1-mRNA:PRM2-mRNA* ratio may have an effect on early embryo development. In addition, *PRM2*-transcript levels are negatively correlated to sperm progressive motility.	Mouse, Human	Depa-Martynów *et al.* (2007; 2012); Valcarce *et al.* (2013); Rogenhofer et al. (2017); Corral-Vazquez et al. (2021)
PSBG1	Pregnancy-specific β 1-glycoprotein (PSBG1, PSG1) is a member of the immunoglobulin superfamily and is a major protein of the syncytiotrophoblast.	The content of *PSG1* mRNA in sperm is higher in fertile individuals than in infertile patients. Interestingly, PSG1 protein is not present in uncapacitated or capacitated sperm.	Human	Avendaño *et al.* (2009)
SESN1	Sestrin-1 (SESN1) negatively regulates the TORC1 signalling pathway, and could prevent the accumulation of ROS through its alkylhydroperoxide reductase activity.	It is involved in embryogenesis and has been detected in the sperm of different species. It is required for left-right symmetry in Zebrafish embryos, and is present in mouse and pig embryos.	Pig, Mouse, Zebrafish	Ko *et al.* (2000); Peeters *et al.* (2006); Yang *et al.* (2009)
ITPRID2 (SSFA2)	ITPR-interacting domain-containing protein 2 is a sperm surface antigen involved in the early cleavage of the fertilised oocyte.	It may provide an extranuclear signal to influence the cleavage program of the fertilised oocyte.	Human	Naz (1992)
TNP1	Transition nuclear protein 1 (TNP1, also known as TP1 and STP1) replaces histones and is subsequently replaced by protamines in mature sperm.	Not essential, yet it can lead to an abnormal pattern of chromatin condensation and reduced fertility when absent.	Mouse	Yu *et al.* (2000)
TR-KIT	Truncated form of c-kit (receptor tyrosine kinase). KIT Proto-Oncogene, Receptor Tyrosine Kinase (CD117) plays an essential role in the regulation of cell survival and proliferation and gametogenesis, among other functions.	Patients suffering from globozoospermia have lower levels of *TR-KIT*-transcript than fertile individuals. These transcript levels, however, do not predict fertilisation rates after ICSI-AOA.	Human	Tavalee and Nasr-Esfahani (2016)

WNT4	Wingless-Type MMTV Integration Site Family, Member 4 plays an important role in the embryonic development of the urogenital tract and the lung, and is required for normal formation of the Müllerian duct in females.	WNT4 plays a critical role in progesterone signalling during embryo implantation and decidualisation, and could be involved in cell division and proliferation. Sperm-borne *Wnt4*-transcripts are present in zygotes at the pronuclear stage, but not at the two-cell stage, and it is translated into protein by the zygote and present in one-cell and two-cell zygotes.	Mouse	Fang *et al.* (2014)

COX7C, cytochrome C oxidase subunit 7C; *DBY/DDX3Y,* human DEAD-box Y RNA helicase; *FOXG1,* forkhead box protein G1; *FTO,* alpha-ketoglutarate-dependent dioxygenase/fat mass and obesity-associated gene; *GABRA1,* GABA A-α1 receptor; *GPX4,* glutathione peroxidase 4; *HSPA1A (HSP70),* heat shock protein 70; *HLA,* human leukocyte antigen; *HLA-E,* human leukocyte antigen-E; *HSPD1 (HSP60),* heat shock protein 60; *INTSI,* major integrase inhibitor; *PAWP (WBP2NL),* postacrosomal sheath WW domain-binding protein/WW domain-binding protein 2 N-terminal like; *PLCZ1,* phospholipase C zeta 1; *PRM1*, protamine 1; *PRM2*, protamine 2; *PSBG1,* pregnancy-specific β 1-glycoprotein; *SESN1,* sestrin-1; *ITPRID2 (SSFA2),* ITPR-interacting domain-containing protein 2/sperm specific antigen 2; *TNP1*, transition nuclear protein 1; *WNT4*, wingless-related integration site family member 4; AOA, artificial oocyte activation; ROS, reactive oxygen species; PIP_2, phosphatidylinositol 4,5-bisphosphate; $InsP_3$, inositol trisphosphate; DAG, diacylglycerol.

Table III Sperm proteins with roles in fertilisation and early embryo development.

Symbol	Protein name	Effects on	Function	Study
ABHD2	Abhydrolase domain-containing protein 2	Acrosome reaction of sperm	It is a serine hydrolase enzyme that controls sperm hyperactivation. Blocking of ABHD2 leads to the inhibition of acrosome reaction	Miller *et al.* (2016); Baggelaar *et al.* (2019)
ACLY	ATP citrate lyase	Recurrent pregnancy loss	Patients whose partners experience recurrent pregnancy loss have higher amounts of this protein in sperm than the control group.	Xue *et al.* (2019)
ACTL7A	Sperm-specific protein actin-like 7A	Fertilisation	It appears to be involved in the formation and fusion of Golgi-derived vesicles during acrosome biogenesis. Relative amounts of ACTL7A in sperm are related to fertilisation rate after IVF/ICSI and embryo development/quality.	Yang *et al.* (2022)
CD151	Tetraspanin CD151	Sperm-oocyte membrane fusion	Located in the equatorial segment of sperm, and suggested to be involved in fertilisation, together with oocyte tetraspanin CD9.	Jankovicova *et al.* (2020)
CRISP proteins	Androgen-dependent epididymal CRISP1 and CRISP4	Sperm-oocyte membrane fusion	They associate with the sperm surface during epididymal maturation. While sperm devoid of CRISP1 are fertile, double KO mice for *Crisp1* and *Crisp4* show immunological deregulation within the reproductive tract and reduced sperm fertilizing ability.	Carvajal *et al.* (2019)
DDX1	DEAD-box helicase 1	Recurrent pregnancy loss	It is involved in the DNA damage repair mechanism. Because the abundance of DDX1 in the extracellular vesicles of seminal plasma is higher in men whose partners experience recurrent pregnancy loss, it is suggested that their sperm could have damaged DNA.	Jena *et al.* (2021)
EQTN	Equatorin	Sperm-oocyte fusion	KO mice models for EQTN show impaired ability of sperm to fuse with the oocyte membrane, and reduced fertility.	Ito *et al.* (2018)

FASN	Fatty acid synthase	Recurrent pregnancy loss	Patients with sperm leading to partner recurrent pregnancy loss have higher amounts of this protein than the control group.	Xue *et al.* (2019)
FIMP	Fertilization influencing membrane protein	Sperm-oocyte fusion	It is located in the equatorial segment of acrosome-intact sperm, and a weak staining remains after the acrosome reaction in some sperm cells. FIMP is a transmembrane protein involved in sperm-oocyte fusion via a process that is independent of IZUMO1, and is necessary for sperm fertilizing ability.	Fujihara *et al.* (2020)
GPX1	Glutathione Peroxidase 1	Early embryo development	Lower enzyme activity of GPX1 in sperm leads to impaired embryo development at 5 days post-fertilisation	Meseguer *et al.* (2006)
GSTP1	Glutathione S-transferase P1	Recurrent pregnancy loss	It regulates the response to oxidative stress and deactivates the JNK pathway, which is essential to maintain sperm function. The relative amount of GSTP1 in seminal plasma vesicles is lower in men, leading to recurrent pregnancy loss.	Jena *et al.* (2021); Llavanera *et al.* (2021)
HE4	Epididymis protein 4	Fertilisation	Sperm devoid of HE4 are infertile/subfertile	Kant *et al.* (2019)
HISTH1C1	Histone 1.2	Recurrent pregnancy loss	It binds linker DNA between nucleosomes forming chromatin in somatic cells. It is also present in sperm, and extracellular vesicles of seminal plasma from men with partner recurrent pregnancy loss show higher levels of this protein.	Jena *et al.* (2021)
HK1	Hexokinase 1 (HK1)	Recurrent pregnancy loss	Patients whose partner experiences recurrent pregnancy loss have lower amounts of this protein in sperm than the control group.	Xue *et al.* (2019)
HPR	Histone-Protamine Ratio / Histones linked to sperm chromatin	Fertilisation/embryo development	Regardless of whether IVF or ICSI is conducted, HPR > 26% or lower than 6% is associated with lower blastocyst formation.	Fournier *et al.* (2018)
ITGA5 (ITGB1)	Integrin α5β1	Early development	Integrin α5β1 is localized in the acrosomal region or the equatorial segment. Early embryo development is correlated with the localisation of α5β1 in the acrosomal region but not in the equatorial segment.	Vernaz *et al.* (2022)

IZUMO1	Izumo sperm-egg fusion protein 1	Fertilisation	IZUMO1 interacts with its receptor in the oocyte, JUNO; the gametes' membranes then fuse and the spermatozoon is engulfed by the oocyte cytoplasm	Bianchi *et al.* (2014); Jean *et al.* (2019)
LR67 (RPSA)	Laminin receptor (also known as ribosomal protein p40)	Fertilisation	Sperm from infertile men show increased levels of LR67, and also exhibit a reduced ability to interact with the oocyte. In fact, this protein is involved in cell adhesion and is a laminin receptor with high affinity.	Frapsauce *et al.* (2014)
LYPD4	Ly6/PLAUR domain-containing protein 4	Fertilisation	Men with sperm devoid of LYPD4 are infertile/subfertile	Wang *et al.* (2020)
ODF2	Outer Dense Fiber 2	Motility/Fertilisation	Mutations in *ODF2* underlie defective outer dense fibres and morphological abnormalities in the sperm flagellum, thus leading to severe asthenozoospermia and male infertility. In bulls, high relative content of ODF2 in sperm is related to low fertility after artificial insemination.	Kaya *et al.* (2022); Zhu *et al.* (2022)
OPRM1	Mu opioid receptor	Fertilisation	In mouse, morphine, an agonist of OPRM1, reduced fertilisation rates and the number of blastocysts after IVF.	Olabarrieta *et al.* (2020)
P34H	L-xylulose reductase	Sperm binding to zona pellucida of oocyte	L-xylulose reductase participates in the binding of sperm to the zona pellucida. It is also a marker of epididymal maturation in sperm. Low levels of P34H are associated with male infertility.	Frapsauce *et al.* (2014)
PAWP (WBP2NL)	Postacrosomal sheath WW domain-binding protein	Oocyte activation	It is located in the post-acrosomal sheath of sperm and some studies reported that it triggers calcium oscillations in pig, cattle, macaque and human oocytes. In cattle, high relative content of PAWP in sperm is related to low fertility after artificial insemination.	Wu *et al.* (2007); Aarabi *et al.* (2010); Aarabi *et al.* (2014); Kennedy *et al.* (2014); Kaya *et al.* (2022)
PLCβ1	Phospholipase beta 1	Fertilisation / Embryo development	Potentially involved in sperm fertilizing ability, as a male mouse model with a mutation in *Plcb1* gene was reported to exhibit reduced fertilisation rates and embryo development after IVF.	Choi *et al.* (2001)
PLCζ	Phopholipase C Zeta 1	Oocyte activation	Evidence supports the role of this protein as a sperm-borne oocyte activation factor (SOAF). KO male mice are unable to trigger calcium oscillations in oocytes and are subfertile. In humans, the reduction or	Kashir *et al.* (2011); Kashir *et al.* (2013); Yelumalai *et al.* (2015);

			absence of PLCζ leads to impaired oocyte activation and thus male-related infertility.	Hachem *et al.* (2017); Kashir *et al.* (2018)
PRSS37	Probable inactive serine protease 37	Sperm-oocyte fusion and oocyte activation	It is a putative trypsin-like serine protease expressed in the testis. Sperm from *Prss37*$^{-/-}$ male mice are unable to recognize ZP-intact oocytes but they can fertilise COCs, which makes it difficult to determine if this protein plays a relevant role in sperm-oocyte binding. It has been suggested that deficiency of PRSS37 causes mature sperm to be devoid of ADAM3, and reduces their migration ability. In humans, PRSS37 is found in the acrosome and its content decreases after the acrosome reaction. In addition, sperm of men suffering from unexplained infertility have a reduced content of PRSS37 and exhibit premature proteolysis of ADAM2. Yet, fertilisation rates after IVF are not reduced, and the particular function of this protein regarding sperm-oocyte binding remains unclear.	Shen *et al.* (2013); Liu *et al.* (2016); Xiong *et al.* (2021)
PRSS55	Probable inactive serine protease 55	Sperm-oocyte fusion	It is a chymotrypsin-like serine protease, anchored to GPI, and is found in the acrosome. Similar to PRSS37, sperm from *Prss55*$^{-/-}$ male mice show less ability to bind ZP-intact and zona-free mouse oocytes, but does not seem to have fertilising ability. The actual function is not clear, but again seems to be related to the maturation of ADAM3.	Shang *et al.* (2018)
RUVBL1	RuvB-like helicase	Recurrent pregnancy loss	Found in sperm and extracellular vesicles of seminal plasma, it is involved in chromatin decondensation. The relative content of RUVBL1 in extracellular vesicles of seminal plasma is reduced in men associated with recurrent pregnancy loss.	Jena *et al.* (2021)
SOF1	Sperm-oocyte fusion required 1	Sperm-oocyte fusion	*Sof1* KO mice are sterile, and their sperm show an impaired ability to fuse with oocyte plasma membrane.	Noda *et al.* (2020)
SPACA6	Sperm acrosome associated 6	Sperm-oocyte fusion	*Spaca6* KO mice are sterile, and their sperm show an impaired ability to fuse with oocyte plasma membrane. SPACA6 is located in the acrosome and relocates to the equatorial segment after the acrosome reaction.	Noda *et al.* (2020)

SPAM1	Hyaluronidase PH-20	Penetration of cumulus-oocyte complexes	It has hyalurodinase activity. Double KO mice for *Spam1* and *Hyal5* have a decreased ability to penetrate the COC, and produce less offspring.	Park *et al.* (2019)
SPESP1	Sperm equatorial segment protein 1	Sperm-oocyte fusion and oocyte activation	A sperm protein located in the equatorial region. A *Spesp1* KO model showed reduced offspring, their sperm had reduced ability to fuse with the oocyte, and the localisation of other sperm proteins was altered.	Fujihara *et al.* (2010)
SPTRX3 (TXNDC8)	Spermatid specific thioredoxin-3	Fertilisation	It is a member of thioredoxin family that accumulates in the superfluous cytoplasm of defective human spermatozoa. High relative levels of SPTRX3 in sperm are related to lower success of ART (IVF/ICSI) and a lower pregnancy rate. High SPTRX3 levels are found in sperm of infertile men.	Buckman *et al.* (2013)
SQSTM1	Sequestosome-1 (also known as ubiquitin-binding protein p62)	Fertilisation	Involved in the degradation of sperm mitochondria after fertilisation, as it has been associated with these organelles in pig zygotes.	Song *et al.* (2016); Song *et al.* (2021)
TMEM95	Sperm-egg fusion protein TMEM95	Sperm-oocyte fusion	A sperm membrane protein that participates in sperm-oocyte fusion. *Tmem95* KO mice are infertile.	Lamas-Toranzo *et al.* (2020); Noda *et al.* (2020)
VCP	Valosin-containing protein (also known as transitional endoplasmic reticulum ATPase, TER ATPase, and p97)	Fertilisation	Involved in the degradation of sperm mitochondria after fertilisation, as it has been associated with these organelles in pig zygotes.	Song *et al.* (2016); Song *et al.* (2021)

ABHD2, abhydrolase domain-containing protein 2; ACLY, adenosine 5’ triphosphate citrate lyase; ACTL7A, sperm-specific protein actin-like 7A; CD151, tetraspanin CD151; COC, cumulus oocyte complex; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; CD9, tetraspanin CD9; CRISP, cysteine-rich secretory proteins; CRISP1, cysteine-rich secretory protein 1; CRISP4, cysteine-rich secretory protein 4; KO, knock-out; DDX1, DEAD-box helicase 1; EQTN, equatorin; FASN, fatty acid synthase; FIMP, fertilisation influencing membrane protein; IZUMO1, izumo sperm-egg fusion protein 1; GPX1, glutathione peroxidase 1; GSTP1, glutathione S-transferase P1; HE4, epididymis protein 4;

HISTH1C1, histone 1.2; HK1, hexokinase 1; HPR, Histone-Protamine Ratio; ITGA5 (ITGB1), integrin α5β1; LR67 (RPSA), laminin receptor/ribosomal protein p40; LYPD4, Ly6/PLAUR domain-containing protein 4; ODF2, outer Dense Fiber 2; OPRM1, mu opioid receptor; P34H, L-xylulose reductase; PAWP(WBP2NL), postacrosomal sheath WW domain-binding protein; PLCβ1, phospholipase C beta 1; PLCζ, phospholipase C zeta 1; PRSS37, probable inactive serine protease 37; PRSS55, probable inactive serine protease 55; RUVBL1, ruvB-like helicase; SOF1, sperm-oocyte fusion required 1; SPACA6, sperm acrosome associated 6; SPAM1, hyaluronidase PH-20; SPESP1, sperm equatorial segment protein 1; SPTRX3 (TXNDC8), spermatid specific thioredoxin-3; SQSTM1, sequestosome-1; TMEM95, sperm-egg fusion protein TMEM95; VCP, valosin-containing protein ; ZP, zona pellucida.