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The contribution of human sperm proteins to the development and epigenome of the preimplantation embryo

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BACKGROUND: Knowledge of the proteomic composition of the gametes is essential to understand reproductive functions. Most of the sperm proteins are related to spermatogenesis and sperm function, but less abundant protein groups with potential post-fertilization roles have also been detected. The current data are challenging our understanding of sperm biology and functionality, demanding an integrated analysis of the proteomic and RNA-seq datasets available for spermatozoa, oocytes and early embryos, in order to unravel the impact of the male gamete on the generation of the new individual.

OBJECTIVE AND RATIONALE: The aim of this review is to compile human sperm proteins and to identify and infer their origin and discuss their relevance during oocyte fecundation, preimplantation embryogenesis and epigenetic inheritance.

SEARCH METHODS: The scientific literature was comprehensively searched for proteomic studies on human sperm, oocytes, embryos, and additional reproductive cells and fluids. Proteins were compiled and functionally classified according to Gene Ontology annotations and the mouse phenotypes integrated into the Mouse Genome Informatics database. High-throughput RNA datasets were used to decipher the origin of embryo proteins. The tissue origin of sperm proteins was inferred on the basis of RNA-seq and protein data available in the Human Protein Atlas database.

OUTCOMES: So far, 6871 proteins have been identified and reported in sperm, 1376 in the oocyte and 1300 in blastocyst. With a deeper analysis of the sperm proteome, 103 proteins with known roles in the processes of fertilization and 93 with roles in early embryo development have been identified. Additionally, 560 sperm proteins have been found to be involved in modulating gene expression by

regulation of transcription, DNA methylation, histone post-translational modifications and non-coding RNA biogenesis. Some of these proteins may be critical for gene expression regulation after embryo genome activation, and therefore, may be potentially involved in epigenetic transmission of altered phenotypes. Furthermore, the integrative analysis of the sperm, oocyte and blastocyst proteomes and transcriptomes revealed a set of embryo proteins with an exclusive paternal origin, some of which are crucial for correct embryogenesis and, possibly, for modulation of the offspring phenotype. The analysis of the expression of sperm proteins, at both RNA and protein levels, in tissues not only from the male reproductive tract but also from peripheral organs, has suggested a putative extra-testicular origin for some sperm proteins. These proteins could be imported into sperm from the accessory sex glands and other tissues, most likely through exosomes.

WIDER IMPLICATIONS: These integrative proteome and transcriptome analyses focused on specific groups of proteins, rather than on enriched pathways, identified important sperm proteins which may be involved in early embryogenesis and provided evidence which could support the hypothesis of paternal epigenetic inheritance. The putative extra-testicular origin of some sperm proteins suggests not only the involvement of accessory sex glands in fertilization and epigenetic information transmission, but also that some proteins from additional organs could possibly contribute information to the offspring phenotype. These findings should stimulate further research in the field.

Key words: sperm / proteomics / embryo development / fertilization / epigenetic inheritance / RNA / extra-testicular

Introduction

Classically, the role and the relative contribution of the sperm cell to the embryo has not been fully appreciated, and minimized only to the contribution of the paternal DNA (Miescher, 1874; Sutton, 1903; Baccetti and Afzelius, 1976; Dahm, 2005; Maruyama and Singson, 2006). However, taking advantage of the current high-throughput 'omic' technologies, growing evidence supports the idea that the male gamete is much more than a vehicle to deliver half of the DNA to the new individual. In fact, the sperm cell provides DNA containing different epigenetic marks, such as DNA methylation (Hammoud *et al.*, 2010; Siklenka *et al.*, 2015), post-translational modifications (PTMs) of histones and protamines (Carrell *et al.*, 2008; Brykczynska *et al.*, 2010; Brunner *et al.*, 2014; Castillo *et al.*, 2015; Siklenka *et al.*, 2015) and a differential distribution of genes within the nucleohistone and the nucleoprotamine chromatin domains (Arpanahi *et al.*, 2009; Hammoud *et al.*, 2009, 2011; Erkek *et al.*, 2013; Castillo *et al.*, 2014a) to the zygote. These epigenetic marks are also combined with a complex population of sperm RNAs and proteins (de Mateo *et al.*, 2011; Jodar *et al.*, 2013, 2015; Amaral *et al.*, 2014a; Castillo *et al.*, 2014a, 2014b), some of which seem to be crucial for early embryogenesis and the future health of the offspring and, possibly, further generations (Krawetz, 2005; Carrell, 2012; Rando, 2012; Castillo *et al.*, 2015; Chen *et al.*, 2016b).

Focusing on the sperm proteomic contribution to the zygote, the application of strategies based on liquid chromatography (LC) coupled to tandem mass spectrometry (MS-MS) has resulted in high-confidence identification of thousands of sperm proteins (Baker *et al.*, 2013; Wang *et al.*, 2013; Amaral *et al.*, 2014a; Vandenbrouck *et al.*, 2016; Jodar *et al.*, 2017). However, in order to fully understand the impact of the sperm proteome to the developing embryo, it is important to also consider the different steps taking place in the biogenesis of both male and female gametes as well as the initial proteomic changes on preimplantation embryo development. The sperm cell is the end cellular product of spermatogenesis, a highly complex process of differentiation that involves very marked genetic, chromatin, biochemical, structural and cellular changes (Fig. 1) (Davies and Mann, 1947; Fawcett and Chemes, 1979; Mezquita, 1985; Poccia, 1986; Oliva and Dixon, 1991; Green *et al.*, 1994; de Kretser *et al.*, 1998; Kimmins and Sassone-Corsi, 2005; Sutovsky and Manandhar, 2006; Oliva, 2006; Carrell *et al.*, 2016). Once released by the testis, the spermatozoa travel through the epididymis, and all along the male reproductive tract, coming into intimate contact with secretions from the different accessory sex glands. This contributes to the acquisition of the potential for sperm motility and the ability to fertilize the oocyte (Saez *et al.*, 2003; Sullivan *et al.*, 2005; Dacheux and Dacheux, 2014; Sullivan and Mieusset, 2016). In contrast, oogenesis results in an oocyte accumulating most of the cytoplasm and small polar bodies (Mattson and Albertini, 1990; Bukovsky *et al.*, 2005; Zuccotti *et al.*, 2011; Coticchio *et al.*, 2015; Conti and Franciosi, 2018) (Fig. 1). Interestingly, and similarly to sperm development, the interactions between the oocyte and its surroundings (granulosa cells and the follicular fluid) are also crucial for correct oocyte maturation (Gilula *et al.*, 1978; Eppig, 1985; Driancourt and Thuel, 1998; Salustri *et al.*, 2003; Chang *et al.*, 2016; Richani and Gilchrist, 2018) (Fig. 1). Although most of the proteins present in the mature sperm and oocyte are synthesized during spermatogenesis and oogenesis, respectively, it is important to note that some proteins may be imported from the supporting reproductive fluids and cells to the male and female gametes (Salustri *et al.*, 2003; Gilchrist *et al.*, 2008; Baker *et al.*, 2012; Intasqui *et al.*, 2013; Ambekar *et al.*, 2015; Zamah *et al.*, 2015; Johnson *et al.*, 2015; Martin-DeLeon, 2015; Saez and Sullivan, 2016; Sullivan and Mieusset, 2016; Chang *et al.*, 2016; Machtinger *et al.*, 2016; Hamad, 2017; Jodar *et al.*, 2017). In fact, emerging evidence shows the existence of an active communication and transference of proteins and other components between gametes and fluids, most probably through the exosome pathway (Johnson *et al.*, 2015; Jodar *et al.*, 2016; Machtinger *et al.*, 2016; Hamad *et al.*, 2017).

The protein profile of the human male gamete has been extensively studied during recent years, and up to 6238 different proteins had been previously compiled (Amaral *et al.*, 2014a; Jodar *et al.*, 2017). However, it remains to be elucidated whether some sperm proteins are required for early embryogenesis, or only represent

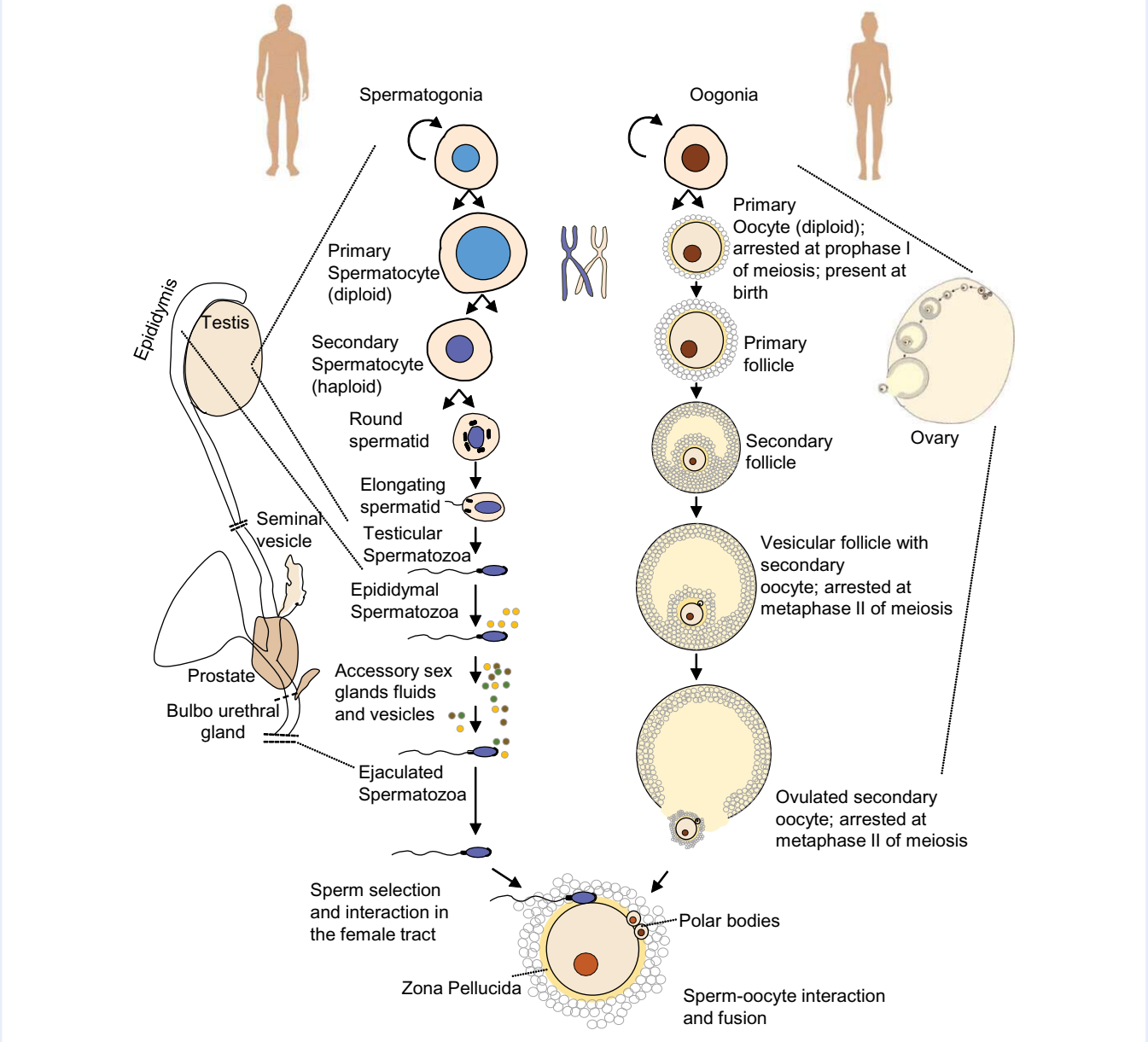

Figure 1 Processes of spermatogenesis, oogenesis and fertilization. The process of spermatogenesis takes place in the testis and is initiated with the replication of spermatogonia (upper left). Spermatogonia differentiate into spermatocytes which undergo genetic recombination at meiosis producing haploid round spermatids. Round spermatids experience marked chromatin and morphological changes, including extreme DNA condensation, the formation of the flagella and the acrosome, the elimination of most of the cytoplasm and the adoption of a hydrodynamic shape, differentiating first into elongating spermatid and finally into the testicular spermatozoon (left). Testicular spermatozoa are released to the epididymis, where they initiate a maturation process that includes the acquisition of motility and other cellular functions and potentials. During the process of ejaculation, the mature sperm cell enters into close contact with the seminal fluid, which is constituted by accessory sex glands fluids and extracellular vesicles (left). The process of oogenesis takes place in the ovary (upper right). Oogonia replicate and differentiate into primary oocytes, which are present at birth and remain arrested at prophase I of meiosis. The developing follicle grows and progresses in order to give rise to the vesicular follicle, which contains the secondary oocyte, arrested at metaphase II of meiosis until ovulation (right). The entry of the sperm cell into the oocyte, through the penetration of the zona pellucida and oocyte plasma membrane, triggers the completion of oocyte meiosis (bottom).

spermatogenic leftovers required for spermatogenesis, sperm maturation or sperm functions such as sperm motility. In the present review, the large amount of information and datasets currently available on the proteomic content of gametes, preimplantation embryos and related reproductive fluids and supporting cells have been compiled, curated, integrated and analyzed. This information has been combined with the corresponding RNA-seq datasets, in order to identify, infer the origin of, discuss and provide further evidence of

the proteomic paternal contribution to the preimplantation embryogenesis, and to offer additional support to the hypothesis of the epigenetic inheritance of alterations to the phenotype through the paternal line.

Because of the large amount of data available, this review is mainly focused on the human model. However, and since very relevant data in different animal model systems are also available, the reader is referred to original articles and excellent reviews which are complementary to the subject (Wasbrough et al., 2010; Skerget et al., 2013, 2015; Kwon et al., 2015; Zhou et al., 2015; Baker, 2016; Holt and Fazeli, 2016; McDonough et al., 2016; Ntostis et al., 2017; Swegen et al., 2017).

Methods

The cellular and the fluid proteomes, corresponding to human sperm, oocytes, blastocysts, cumulus cells, mid-secretory endometrium and follicular fluid, were compiled after an exhaustive literature search using the Web of Science database. Only proteomic articles published in English and available online until the end of September 2017 were included in the search. Subsequently, and in order to reduce as much as possible the potential false-positive identification of proteins, we selected only those proteomic studies whose protein detection relied on mass spectrometry (MS) approaches, and whose protein identification criteria included at least two peptides per protein with a false discovery rate (FDR) ≤5% for each peptide, which corresponds to an estimated FDR ≤0.25% per protein (Table I). Only proteins with a known gene name were included.

For the generation of the human sperm proteome, proteomic profiles were acquired from: (1) original articles (de Mateo et al., 2011; Amaral et al., 2013; Baker et al., 2013; Wang et al., 2013; among others, see the complete list of references in Table I) already compiled in comprehensive reviews previously published by our group (Amaral et al., 2014a; Jodar et al., 2017), and (2) additional recently published sperm proteomic catalogs (Zhu et al., 2013; Intasqui et al., 2013; Azpiazu et al., 2014; Frapsauce et al., 2014; Sun et al., 2014; Wang et al., 2015, 2016; Yu et al., 2015; Jumeau et al., 2015; Liu et al., 2015; Hetherington et al., 2016; Vandenbrouck et al., 2016; Carapito et al., 2017; Saraswat et al., 2017). Only those original articles in which specific methods were referenced to ensure the proper elimination of potentially contaminating cells were considered. In total, 46 published proteomic studies performed using purified ejaculated human sperm were included in the analysis reported herein, and are enlisted in Table I. However, it must be noted that some specific characteristics of the males from which the sperm samples were obtained, such as their fertility, age, body mass index (BMI), life-style or exposure to toxins, were not always specified in the sperm proteomic studies included in this analysis. Thus, it is possible that some of the proteins detailed in the current dataset could reflect an altered phenotypic state. Additionally, in order to further reduce the chance of any false-positive identifications, just those proteins with identified unique peptides were integrated in the final list. This compilation resulted in high-confidence identification of 6871 non-redundant proteins in human spermatozoa (reported in Supplementary Table 1).

Two different strategies were used to infer the functional involvement of the sperm proteins at oocyte fecundation and consecutive processes of human reproduction. The first one was according to their association to the Gene Ontology (GO) Biological Process annotations enclosed in the Gene Ontology Consortium Database (http://www.geneontology.org/; (Ashburner et al., 2000; The Gene Ontology Consortium, 2017)). The second one was according to their association to the Phenotypes and Mutant Alleles data from the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org/phenotypes.shtml). In particular, gene lists associated to GO terms related to fertilization, formation of the zygote and the blastocyst, and implantation were retrieved from de Gene Ontology Consortium database (Release date December, 7th 2016). The GO-derived list of genes was subsequently compared to the human sperm proteome dataset. Similarly, those sperm proteins functionally involved in epigenetic processes were predicted by retrieving gene lists associated to the GO terms related to the processes of epigenetics, transcription, DNA methylation, histone PTMs and small non-coding RNA biogenesis from the Gene Ontology Consortium Database (Release date December, 7th 2016). The complete list of GO terms included in these analyses is described in Supplementary Table 2. However, it cannot be assumed that all the known protein-related functions revealed from the GO data analyses are executed in all the tissues where the proteins are expressed. For that reason, additional tools were used in a complementary manner, such as the analysis of published data obtained after targeted protein depletion in mice. In fact, the animal model data become especially valuable to predict protein functions, due to the ethical limitations associated with conducting of functional studies in humans. Therefore, sperm proteins whose corresponding gene-disrupted mice resulted in 'embryonic lethality before implantation' phenotype, according to the MGI database (Release date December, 7th 2016), were identified and classified according to the developmental stage in which the impairment takes place (first divisions, morula or blastocyst; Fig. 2). Additionally, knock-out data related to the 'male infertility' phenotype from the MGI database, and GO terms associated to different stages of spermatogenesis and sperm maturation were also retrieved, in order to discriminate those proteins that might represent spermatogenesis leftovers with no further function at subsequent stages (Supplementary Table 2).

In order to allow a high-confidence identification of preimplantation embryo proteins with a potential sperm-specific origin, the proteome of the human oocyte and preimplantation embryo were also compiled and compared to the human sperm proteome. The human oocyte proteomic profile was obtained from a single study on mature oocytes without granulosa cells (Virant-Klun et al., 2016), and the human preimplantation blastocyst proteome profile was compiled from proteomic reports using 6-day embryos (blastocyst stage; Table I) (Jensen et al., 2013; Kaihola et al., 2016). Those proteins identified exclusively in treated groups or culture media, and those that did not fit the same MS identification criteria indicated above for the sperm proteome were discarded for the current analysis. In total, 1376 and 1300 non-redundant proteins were compiled for the oocyte and the blastocyst, respectively (Supplementary Table 1).

Since the human oocyte and blastocyst proteomes are less covered than the sperm proteome, and due to the fact that these cells are in close contact with their surroundings, the protein catalogs of additional oocyte and preimplantation embryo interacting cells, tissues and fluids were also compiled (Table I, Supplementary Table 1). Specifically, the literature was searched for proteins identified in human cumulus cells (179 proteins) (McReynolds et al., 2012; Braga et al., 2016), human follicular fluid (1394 proteins) (Lee et al., 2005; Angelucci et al., 2006; Kim et al., 2006; Liu et al., 2007; Hanrieder et al., 2008, 2009; Estes et al., 2009; Lo Turco et al., 2010, 2013; Jarkovska et al., 2010; Kushnir et al., 2012; Twigt et al., 2012; Ambekar et al., 2013, 2015; Severino et al., 2013; Bianchi et al., 2013; Hashemitabar et al., 2014; Regiani et al., 2015; Wu et al., 2015; Zamah et al., 2015; Lewandowska et al., 2017; Lim et al., 2017; Oh et al., 2017) and human endometrium (tissue and fluid) on mid-secretory and decidualization phases (2082 proteins) (Zhang et al., 2006; Fowler et al., 2007; Chen et al., 2009, 2015; Domínguez et al., 2009; Parmar et al., 2009; Scotchie et al., 2009; Hannan et al., 2010; Paule et al., 2010; Stephens et al., 2010; Garrido-Gomez et al., 2011). Although individual characteristics such as BMI, life-style and exposure to toxins were not

Table I Proteomic studies selected for the compilation of the proteomes of human sperm, oocyte, blastocyst, cumulus cells, follicular fluid and mid-secretory endometrium. Only proteomic studies in which the elimination of contamination cells was ensured, protein detection relied on mass spectrometry approaches, and protein identification was based on ≥2 peptides/protein and FDR ≤5% per peptide were included in the compilations. When applicable, only those proteins identified in the control/non-treated group were included in the analyses.

Proteomic study	References
Human sperm cell	
Descriptive whole cell proteomics	Johnston *et al.* (2005), Martinez-Heredia *et al.* (2006), Baker *et al.* (2007), de Mateo *et al.* (2007), Li *et al.* (2007), Wang *et al.* (2013)
Descriptive subcellular proteomics	Kim *et al.* (2007), Naaby-Hansen and Herr (2010), Naaby-Hansen *et al.* (2010), de Mateo *et al.* (2011), Gu *et al.* (2011), Nixon *et al.* (2011), Amaral *et al.* (2013), Baker *et al.* (2013)
Descriptive proteomics targeting post-translational modifications	Lefièvre *et al.* (2007), Vigodner *et al.* (2013), Yu *et al.* (2015)
Comparative proteomics	Pixton *et al.* (2004), Zhao *et al.* (2007); Martínez-Heredia *et al.* (2008), Chan *et al.* (2009), Frapsauce *et al.* (2009), Siva *et al.* (2010), Thacker *et al.* (2011), Paasch *et al.* (2011), Xu *et al.* (2012), Parte *et al.* (2012), Zhu *et al.* (2013), Intasqui *et al.* (2013), Amaral *et al.* (2014b), Azpiazu *et al.* (2014), Liu *et al.* (2015), Hetherington *et al.* (2016), Saraswat *et al.* (2017)
Functional proteomics	Ficarro *et al.* (2003), Secciani *et al.* (2009), Redgrove *et al.* (2011), Frapsauce *et al.* (2014), Sun *et al.* (2014), Wang *et al.* (2015)
Detection of missing proteins	Jumeau *et al.* (2015), Vandenbrouck *et al.* (2016), Carapito *et al.* (2017)
Compilation of published protein catalogs	Amaral *et al.* (2014a), Wang *et al.* (2016), Jodar *et al.* (2017)
Human oocyte	
Comparative proteomics	Virant-Klun *et al.* (2016)
Human blastocyst	
Descriptive whole cell proteomics	Jensen *et al.* (2013)
Comparative proteomics	Kaihola *et al.* (2016)
Human cumulus cells	
Comparative proteomics	McReynolds *et al.* (2012), Braga *et al.* (2016)
Human follicular fluid	
Descriptive proteomics	Lee *et al.* (2005), Angelucci *et al.* (2006); Hanrieder *et al.* (2008, 2009), Twigt *et al.* (2012), Ambekar *et al.* (2013), Bianchi *et al.* (2013), Zamah *et al.* (2015), Lewandowska *et al.* (2017)
Comparative proteomics	Kim *et al.* (2006), Liu *et al.* (2007), Estes *et al.* (2009), Jarkovska *et al.* (2010), Lo Turco *et al.* (2010, 2013), Kushnir *et al.* (2012), Severino *et al.* (2013), Hashemitabar *et al.* (2014), Ambekar *et al.* (2015), Regiani *et al.* (2015), Wu *et al.* (2015), Oh *et al.* (2017), Lim *et al.* (2017)
Human endometrium (tissue and fluid) on mid-secretory and decidualization phases	
Comparative proteomics	Zhang *et al.* (2006), Fowler *et al.* (2007), Chen *et al.* (2009, 2015), Parmar *et al.* (2009); Scotchie *et al.* (2009), Domínguez *et al.* (2009), Stephens *et al.* (2010), Hannan *et al.* (2010), Paule *et al.* (2010), Garrido-Gomez *et al.* (2011)

possible to control, only those proteins identified in healthy, fertile or donor women, younger than 40 years old were included in the respective protein profiles (Supplementary Table 1). Furthermore, published RNA data from human sperm (Johnson *et al.*, 2015), oocyte, and preimplantation embryo (Dang *et al.*, 2016) have been used as an additional tool to predict the gamete-origin of the blastocyst proteins.

Finally, the tissue expression data enclosed in The Human Protein Atlas database (Uhlén *et al.*, 2005; Uhlen *et al.*, 2015) was retrieved (Release date June, 8th 2017) and compared to the comprehensive human sperm proteome, in order to identify the potential tissue origin of each sperm gene product. In particular, sperm proteins were predicted to be acquired in extra-testicular stages of sperm maturation, when fitting the following criteria: RNA levels in testis <25 transcripts per million and no protein expression in testicular seminiferous tubules. Antibody detection on testis sections enclosed in The Human Protein Atlas database were individually checked for all those sperm proteins fitting the criteria mentioned above, in order to ensure the lack of antibody staining and discard false-positive matches. The functional involvement of this subset of sperm proteins was predicted by enrichment analyses on GO annotations related to biological processes, by using the tools from the Gene Ontology Consortium database. The significance of the enrichment analyses was calculated by a Fisher Exact Test. *P*-value <0.05 adjusted for multiple-comparisons with the Bonferroni correction were considered significant.

Involvement of the sperm proteome at oocyte fecundation and beyond

The first generation of a compiled proteome profile of the sperm cell was published in 2014, and it included 6198 non-redundant proteins from 30 different studies (Amaral *et al.*, 2014a). Since then, many

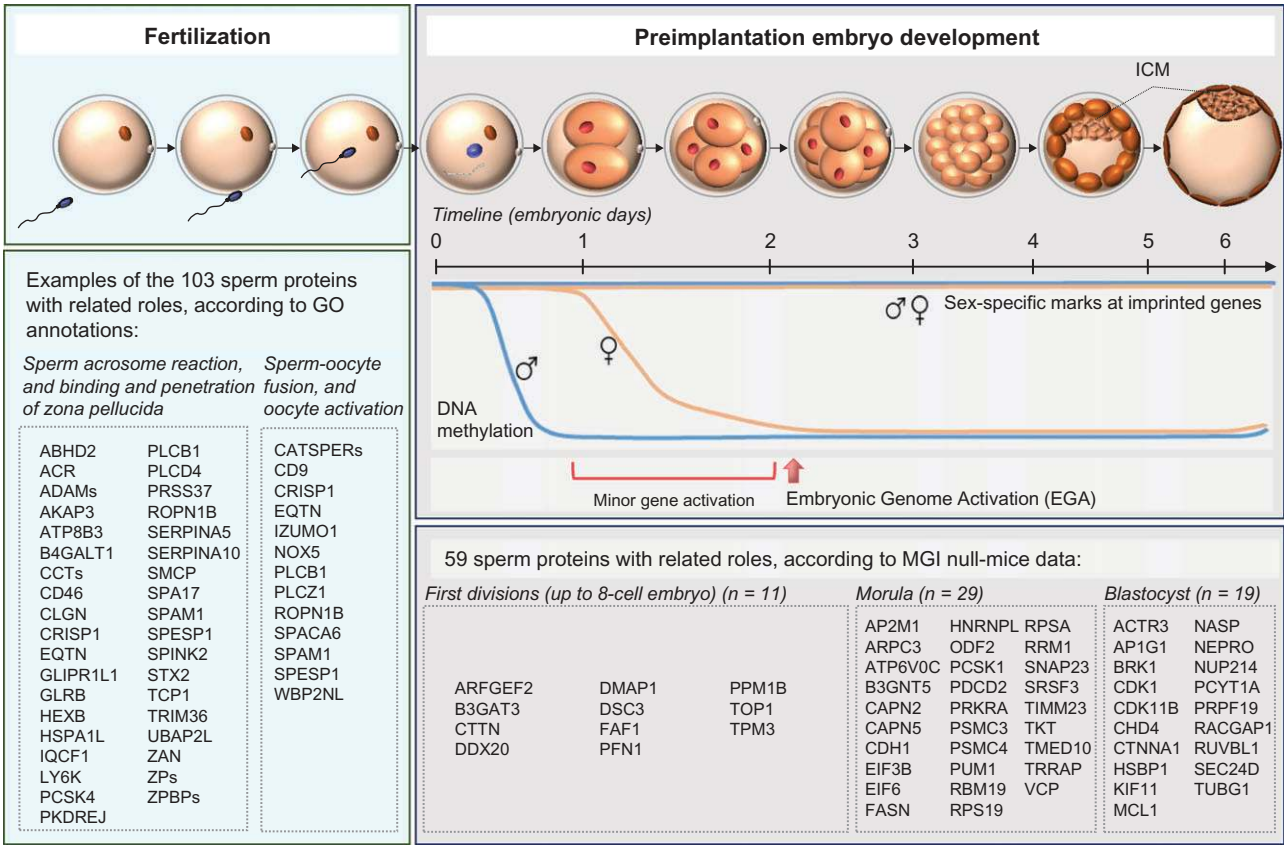

Figure 2 Sperm proteins functionally involved in the processes of fertilization and preimplantation embryo development. Examples of the subset of 103 sperm proteins involved in the process of fertilization, as revealed by the functional analyses based on gene-specific Gene Ontology annotations (left). The proteins indicated here are functionally related to the correct acrosome reaction, binding of sperm to zona pellucida, penetration of the zona pellucida, fusion of sperm to the oocyte plasma membrane and oocyte activation (left). Upon fertilization, the preimplantation embryo development starts with the fusion of male and female pronuclei in the zygote, which develops over approximately six days in order to reach the blastocyst stage (upper right; ICM: inner cell mass). In humans, the embryo genome activation (EGA) takes place between the 4-cell and 8-cell embryo stages, although minor gene activation occurs beforehand, between the 2-cell and 4-cell embryo stages (middle right). In addition, the paternal genome is demethylated within the first 24 h of embryo development, much faster than the maternal genome (middle right). The analysis of the phenotype data from gene-null mice enclosed in the Mouse Genome Informatics database revealed the potential critical role of 59 sperm proteins in different processes occurring during the first divisions of the embryo (up to 8-cell stage; 11 proteins), the formation of the morula (29 proteins) and the development of the blastocyst (19 proteins), as indicated in boxes (bottom right).

additional proteomic reports focused on the male gamete have been published (Table I) (Amaral *et al.*, 2014b; Azpiazu *et al.*, 2014; Jumeau *et al.*, 2015; Wang *et al.*, 2015, 2016; Hetherington *et al.*, 2016; Vandenbrouck *et al.*, 2016; Carapito *et al.*, 2017; Jodar *et al.*, 2017). In this review, we have updated the sperm protein profile while following strict inclusion criteria, in order to minimize as much as possible potential false-positive protein identifications (see Methods). By doing this, the sperm protein list has risen to 6871 proteins, and represents the most complete and reliable catalog of human sperm proteins to date (Supplementary Table I).

The in-depth analyses and data mining of the sperm molecular composition are contributing to an increase in our knowledge about sperm function. Previous enrichment analyses, focused on the identification of those biological pathways likely to be the most active in human sperm, had revealed metabolism (including protein and RNA metabolism), membrane trafficking, apoptosis, cell cycle, hemostasis, and meiosis as the most significant pathways in the whole male gamete (Amaral *et al.*, 2014a). However, there is growing evidence showing the role of the spermatozoon in embryogenesis and transmission of epigenetic marks important for early and future events of the offspring (Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010; Vavouri and Lehner, 2011; Castillo *et al.*, 2014a, 2015; Wei *et al.*, 2014b; Fullston *et al.*, 2015), which are functions that are not revealed when enrichment analyses are performed using the whole set of sperm proteins (Castillo *et al.*, 2014b). Therefore, in the present review we have focused our study of the sperm proteome on specific GO terms, rather than on the basis of enrichment analyses. Interestingly, a remarkable number of sperm proteins functionally related to different stages of fertilization, early embryogenesis, and gene expression modulation have been predicted (Supplementary Tables 3 and 4).

This analysis provides valuable information that may contribute to enhance the current understanding of the role of the male gamete in the generation of the new individual.

Sperm proteins and fertilization

Fertilization is a complex multistep process that relies on tightly regulated events taking place in both gametes, the sperm cell and the oocyte. Of relevance, our analysis of the sperm proteome based on the use of public GO annotations revealed the presence of 103 sperm proteins that might be functionally related to different steps of the fertilization process, such as sperm capacitation, sperm acrosome reaction, sperm penetration and sperm–oocyte fusion (Fig. 2; Supplementary Table 3) (Brucker and Lipford, 1995; Visconti *et al.*, 1998; Bedford, 2004; De Jonge, 2005; Krawetz, 2005; Sutovsky and Manandhar, 2006; Visconti, 2009; Amaral *et al.*, 2014a; Dacheux and Dacheux, 2014; Cuasnicú *et al.*, 2016; Sullivan and Mieusset, 2016; Torabi *et al.*, 2017).

In particular, this subset of sperm proteins includes components with well-known roles in the acquisition of sperm motility and the hyperactivation occurring during sperm capacitation, such as the A-kinase anchor proteins 3 and 4 (AKAP3 and AKAP4) and the sperm-specific cation channels CATSPERs (Ficarro *et al.*, 2003; Singh and Rajender, 2015; Williams *et al.*, 2015) (Supplementary Table 3). Also, the male gamete contains proteins known to be indispensable for fertilization once it reaches the oocyte, such as the Izumo sperm-egg fusion protein 1 (IZUMO1; Fig. 2), which directs sperm–oocyte fusion by binding its complementary oocyte protein Juno (Inoue *et al.*, 2005; Sutovsky, 2009; Bianchi *et al.*, 2014), and the sperm protein PLCzeta (PLCZ1), which induces the Ca^{2+} oscillations in the oocyte that are required for oocyte activation, the formation of the maternal pronucleus and the initiation of embryogenesis (Yoon and Fissore, 2007) (Fig. 2).

Interestingly, published data from targeted gene deletions in mice support the essential role that have been predicted for some of the human sperm proteins associated to fertilization-related GO terms in this study. For instance, it has been shown that the acrosomal sperm protein Proprotein convertase subtilisin/kexin type-4 (PCSK4) null mice produce mutant sperm lacking the ability to proteolytically process the acrosin binding protein (ACRBP), impairing thus the capability of the sperm to bind to the zona pellucida and to fertilize the oocyte (Gyamera-Acheampong *et al.*, 2006; Tardif *et al.*, 2012). Similarly, deficient mice of sperm acrosomal equatorial segment proteins, such as the equatorin (EQTN) and the sperm equatorial segment protein 1 (SPESP1; Fig. 2), also produce sperm with reduced fertilizing capacity (Toshimori *et al.*, 1998; Wolkowicz *et al.*, 2003, 2008; Fujihara *et al.*, 2010; Hao *et al.*, 2014). In fact, the lack of SPESP1 also affects the correct amount and localization of several other sperm proteins involved in gamete fusion, such as the A Disintegrin And Metalloproteinase proteins (ADAMs), the EQTN and the IZUMO1 (Cuasnicú *et al.*, 2016). It is also remarkable the identification of the sperm protein cysteine-rich secretory protein 1 (CRISP1), which is an epididymal androgen-regulated glycoprotein imported to sperm during sperm maturation and involved in sperm motility, sperm penetration and sperm–oocyte fusion (Cameo and Blaquier, 1976; Krätzschmar *et al.*, 1996; Maldera *et al.*, 2014; Ernesto *et al.*, 2015) (Fig. 2). Of relevance, CRISP1 interacts with oocyte complementary sites, such as the glycoprotein ZP3 and, in fact, CRISP1-deficient sperm are not able to penetrate into the oocyte (Da Ros *et al.*, 2015).

The presence of this body of experimental evidence showing the role of this subset of sperm proteins in fertilization is remarkable since it is validating the results from the GO annotations-based data analysis of the present review. Therefore, it supports the application of this strategy in the exploration of other potential functions of the sperm cell.

Sperm proteins and preimplantation embryo development

The analysis of the human sperm proteome profile has also allowed us to identify and highlight a subset of 93 proteins that may be functionally related to the formation of the zygote and the following stages of embryo development prior implantation (Supplementary Table 3), which in humans takes place approximately at Day 7 of development (Niakan *et al.*, 2012). Early embryogenesis is characterized by dramatic changes in chromatin organization, including the replacement of paternal protamines by maternal histones (Oliva and Dixon, 1991; Wright, 1999; Oliva, 2006; Inoue *et al.*, 2011; Kong *et al.*, 2018), and the extensive epigenetic reprogramming of maternal and paternal genomes, which returns the zygote to a genetic state able to generate any cell type in the body (Reik and Walter, 2001; Reik *et al.*, 2001). This is exemplified by the post-fertilization DNA demethylation wave observed first in paternal and subsequently in maternal genome, except for both parental methylation imprinting marks (Mayer *et al.*, 2000; Wei *et al.*, 2014a) (Fig. 2). However, it is not until the third day of development, within the 4- and 8-cell stages (Fig. 2), when the main embryonic genome activation (EGA) takes place (Niakan *et al.*, 2012), although a minor wave of transcription has been observed earlier at the 2-cell stage (Vassena *et al.*, 2011) (Fig. 2). Interestingly, the rapid demethylation occurring in the paternal genome could enable this first minor wave of transcription (Santos and Dean, 2004). Afterwards, the embryo begins to synthesize proteins of its own, moving towards the stages of morula and blastocyst prior to subsequent implantation (Fig. 2).

It is important to note that, due to the fact that the sperm cell is the result of complex series of cellular and molecular modifications, it becomes a challenge to distinguish proteins that are players in the process of early embryo development, from those that, in contrast, simply represent spermatogenesis leftovers with no relevant function in further stages. Although further research and novel approaches are demanded to decipher the functionality of the sperm proteins in humans, the studies already performed with animal models are contributing to shed light into this specific question. For that reason, we have added to our analysis the valuable phenotypic data contained in the MGI database (see Methods) as a tool to predict the potential function of these proteins in human early embryogenesis (Fig. 2; Supplementary Table 3). We found that the human sperm cell contains 59 different proteins whose depletion in mice through knockout studies resulted in very marked impairments at different stages of the preimplantation embryo development (Fig. 2; Supplementary Table 3). Specifically, the male gamete delivers to the zygote 11 proteins related to embryo lethality during the first divisions from zygote to 8-cell stage, such as the transmembrane glycoprotein desmocollin

3 (DSC3) (Fig. 2). Interestingly, the crucial impact of DSC3 on the correct development of the embryo might be suggested by its function in the regulation of cell adhesion, which is necessary for the formation of blastomeres, occurring around Day 1 of development in humans (Den et al., 2006; Garrod and Chidgey, 2008) (Fig. 2). Therefore, this developmental function could be attributed to the sperm-derived DSC3, since it may take place before the activation of the embryonic genome (around Day 2 of development in humans; Fig. 2). Similarly, the human spermatozoon also contains 29 proteins whose deficiencies induce impairments at the morula stage, and 19 proteins at the blastocyst formation (Fig. 2; Supplementary Table 3). This is the case of the proteins lactosylceramide 1,3-*N*-acetyl-beta-D-glucosaminyltransferase (B3GNT5) and choline-phosphate cytidylyltransferase A (PCYT1A), which are both related to the formation of lipid membranes. Specifically, B3GNT5 catalyzes the biosynthesis of the lactoseries of glycosphingolipids (Henion et al., 2001) and its depletion results in embryo lethality at morula stage due to alterations in cell adhesion and signaling processes (Biellmann et al., 2008). PCYT1A, in contrast, is involved in the initiation of the synthesis of phosphatidylcholine, the most abundant phospholipid in mammalian cellular membranes (Vance and Vance, 2004). Remarkably, PCYT1A-null mice embryos were found to fail in the formation of a blastocyst capable of achieving implantation (Wang et al., 2005). This information might be extrapolated to humans, although it is important to take into account that the transcription of embryonic genes is already active at morula and subsequent stages. Therefore, further experimental evidence is necessary to determine whether these proteins and the rest of the sperm proteins belonging to this group are functional during human preimplantation embryogenesis, as well as the potential specific paternal origin of these proteins.

Sperm proteins and the hypothesis of the paternal epigenetic inheritance

Previous studies on the impact of parental health on the offspring have been mainly focused on the female partner, especially during the pregnancy period. This is due to the fact that several poor health outcomes for offspring have been demonstrated from harmful maternal exposures or life-style, either prior to or during pregnancy (reviewed in Brion et al., 2008; Feng et al., 2014). However, recent findings have shown how the paternal life-history experiences have a greater influence on the future health of the offspring than previously thought. Some remarkable evidence supporting this hypothesis have been found in animal models, such as the detection of metabolism alterations on rodent offspring caused by the paternal diet (including caloric restrictions, low protein diets or high fat diets) (Anderson et al., 2006; Carone et al., 2010; Ng et al., 2010; de Castro Barbosa et al., 2016). Other examples are the observation of a decreased fear response and the appearance of depressive symptoms in the offspring from traumatized male mice (Dietz et al., 2011; Rodgers et al., 2013; Gapp et al., 2014), as well as the presence of cognitive impairments and of higher levels of anxiety and aggressiveness in the offspring from males with postnatal exposures to nicotine and heroin, respectively (Farah Naquiah et al., 2016; Renaud and Fountain, 2016). Interestingly, in some cases those harmful phenotypes could be found not only in the offspring (F1), which is due to intergenerational inheritance, but also in further generations, what is known as transgenerational inheritance.

Similar observational data as that indicated above for animal models has been generated in humans, also suggesting the existence of intergenerational and transgenerational effects as a consequence of paternal life-history experiences (reviewed in Pembrey et al., 2014). This is exemplified by the observed correlation between paternal overfeeding during mid-childhood (from 9 to 11 years old) and the lower longevity of sons and grandsons due to an increased risk of diabetes (Bygren et al., 2001; Kaati et al., 2002). Also, an association has been observed between early paternal smoking habits and a higher BMI in the male progeny at 9 years old (Pembrey et al., 2006).

Currently, several groups are working to elucidate the molecular mechanisms that could explain the above observations, supporting the hypothesis of the transmission of some phenotype alterations through the paternal line (Sharma and Rando, 2017). Potential candidates have been proposed to provide the basis for this epigenetic inheritance, such as the sperm DNA methylation (de Castro Barbosa et al., 2016; Donkin et al., 2016; Soubry et al., 2016), sperm histone marks including both the PTMs and the nucleohistone-nucleoprotamine pattern (Zeybel et al., 2012; Vassoler et al., 2013; Castillo et al., 2015; Siklenka et al., 2015) and the set of sperm small non-coding RNAs (sncRNAs) (Rassoulzadegan et al., 2006; Grandjean et al., 2015; Chen et al., 2016a; Sharma et al., 2016). Interestingly, all those epigenetic marks are maintained in the mature spermatozoa and are able to escape from the epigenetic reprogramming occurring in the zygote (Borgel et al., 2010; Chen et al., 2016b), and therefore could act as scaffold for the establishment of new epigenetic signatures in the embryo genome (Brunner et al., 2014; Castillo et al., 2014a, 2014b, 2015). In any case, these sperm epigenetic marks might regulate gene expression at either transcriptional or post-transcriptional levels in the early embryo. For instance, it has been demonstrated that the translational RNA (tRNA) fragments altered in founder males under a low protein diet are able to modulate the abundance of a subset of genes known to be expressed in preimplantation embryos (Sharma et al., 2016). Therefore, the altered phenotype observed in the offspring might be caused by altered gene expression during early embryo development induced by changes in the sperm epigenetic profile.

However, in addition to sperm DNA containing epigenetic marks, and the complex population of sperm sncRNAs (Krawetz et al., 2011; Jodar et al., 2013; Pantano et al., 2015), the sperm cell could also provide the oocyte with a large number of zinc finger- and bromodomain-containing proteins, transcription factors, histone modifiers, and other DNA- and RNA-related proteins that might also be critical for the regulation of gene expression in the early embryo, either at transcriptional or post-transcriptional levels (Castillo et al., 2014a, 2014b, 2015). In fact, the gene-specific GO annotations-based analysis undertaken in this review has revealed a total of 560 sperm proteins with known roles in the regulation of gene expression in other cells or tissues (Table II and Supplementary Table 4). This subset of sperm proteins includes (1) transcription factors and transcription factor-related proteins (381 proteins), (2) chromatin modifiers able to modulate the DNA methylation pattern (25 proteins), (3) chromatin modifiers that might regulate histone PTMs (118 proteins) and (4) proteins that participate in the regulation of the transcription, processing, and function of non-coding RNAs (36 proteins) (Table II; Supplementary Table 4). However, it is not known how many of these proteins could have these roles in the early embryo as

Table II Subgroups and examples of sperm proteins that could modulate gene expression. A total of 560 sperm proteins have been identified as potential modulators of gene expression according to their association with specific GO annotations (see Supplementary Table 4). The 560 proteins have been sub-classified into four subgroups according to their function: (1) Transcription factors or other gene expression-related proteins (381 proteins); (2) Proteins that may regulate the genome methylation and demethelytation patterns (25 proteins); (3) Proteins able to modulate several histone post-translational modifications (PTMs; 118 proteins) and (4) Proteins involved in the biogenesis, processing and functions of the small non-coding RNAs (sncRNAs; 36 proteins).

Subgroups of sperm proteins related to the regulation of gene expression (n = 560) and the corresponding role (GO annotations)	**Examples of specific sperm delivered proteins**
1. Transcription factors or gene expression-related proteins (n = 381)	
Transcription factors	ZHX2, GTF3C5, ATF5, ZNF219, MYEF2, SLTM, CSRNP2, GZF1, STAT1, STAT6
Regulation transcription factor import	IPO11
Termination of transcription	DHX38, CPSF2, CPSF3, CPSF2
2. DNA methylation regulators (n = 25)	
DNA demethylation	APEX1, C2orf61, APOBEC3C
DNA methylation	TRIM28, USP7, MPHOSPH8, DNMT1
Imprinting regions	GNAS
3. Histone PTMs regulators (n = 118)	
Acetylation	ELP3, WDR5, CAMK1
Deacetylation	MTA1, CHD4, HDAC1
Phosphorylation	PRKCD, PRKAA1
Dephosphorylation	PPM1F, PPP5C
Methylation	MEN1, NASP, SUZ12
Demethylation	KDM4B, JMJD1C
Ubiquitination	RNF40, CUL4B
4. sncRNA biogenesis, processing and function regulators (n = 36)	
sncRNA biogenesis	SNIP1, BMPR1A, GTF2B, GTF3C1, NFATC4, CPSF3L
sncRNA processing	PRKRA, HNRNPA2B1, SRRT, DROSHA
sncRNA function	CNOT1, EIF6, TNRC6A, AGO3

they could simply represent spermatogenic leftovers. For instance, 69 of those 560 sperm proteins have a known role during spermatogenesis or sperm maturation, according to either their association to a sperm-related GO annotation or the observation of infertility in their respective null mice (Supplementary Table 4). This is the case of the sperm protein Probable ATP-dependent RNA helicase DDX4 (DDX4), whose corresponding null mice are infertile due to arrest of male meiosis (Kuramochi-Miyagawa *et al.*, 2010). In contrast, we found a total of 28 proteins, out of the 560 sperm proteins related to epigenetics, as being potentially involved in the regulation of gene expression during early embryogenesis. In fact, this subset of sperm proteins either showed associations to GO terms related to early embryogenesis or their targeted deletion in mice resulted in embryonic lethality before implantation (Supplementary Table 4). For instance, no 8-cell embryos were observed after target depletion of the nuclear autoantigenic sperm protein (NASP), which has been suggested to participate in both nucleosome remodeling and maintenance of the methylation pattern during the preimplantation embryo epigenetic reprogramming (Mohan *et al.*, 2011).

To the best of our knowledge, there is a lack of studies regarding the human sperm proteome contributing to explain the mechanisms implicated in paternal epigenetic inheritance, although, as presented above, some sperm proteins might modify gene expression at the transcriptional and post-transcriptional levels in the early embryo (Table II). However, the proteomic data profiles from obese patients may be used to assess this hypothesis, since several authors have suggested that obese males might transmit altered metabolic phenotypes to their offspring (Bygren *et al.*, 2001; Ng *et al.*, 2010; Rando, 2012). Of relevance, three sperm proteins related to the regulation of gene expression, according to the analysis undertaken in this review (Supplementary Table 4), were found to be deregulated in a comparative proteomic analysis of spermatozoa from obese and lean human males: the Eukaryotic Translation Elongation Factor 1 Alpha 1 (EEF1A), the CCR4-NOT Transcription Complex Subunit 1 (CNOT1) and the Elongator Acetyltransferase Complex Subunit 3 (ELP3; Supplementary Table 4) (Liu *et al.*, 2015). Interestingly, CNOT1 is a scaffolding component of the mRNA deadenylase complex CCR4-NOT, which participates in processes such as mRNA degradation, miRNA-mediated repression, translational repression and general regulation of transcription. Moreover, the CCR4-NOT complex represses the expression of early trophectoderm transcription factors in embryonic stem cells, suggestive of a critical role in the

maintenance of the totipotency of blastomeres during early embryogenesis (Zheng et al., 2012). Also, ELP3 has been described as one of the factors responsible for paternal DNA demethylation upon oocyte fertilization (Okada et al., 2010), among other functions such as the modification of tRNAs (Svejstrup, 2007). It is interesting to note that the modifications of tRNA fragments seem to be crucial for the transmission of altered metabolic phenotypes due to paternal high fat diets (Chen et al., 2016b). The deregulation of the proteins CNOT1 and ELP3 in sperm proteomes from obese males and the ability of those proteins to regulate epigenetic marks suggest that they are potential candidates for the transmission of obesity-related paternal environmental information to the new individual. Altogether, our findings reinforce the hypothesis of a potential role of some sperm proteins in the intergenerational epigenetic inheritance of phenotype alterations.

Integrated analysis of the preimplantation proteomes: deciphering the potential parental origin of the early embryo proteins

The integrative analysis of the human sperm, oocyte and early embryo proteomes has been used as a complementary approach to determine which human sperm proteins may be involved in early embryo development. This may be inferred by the detection of blastocyst proteins having an unequivocal paternal origin. However, two technical issues should be taken into account in this analysis. The first one is the nearly complete description of the sperm proteome as compared to the limited covered oocyte and embryo proteomes. As observed in Fig. 3A, whereas 6871 proteins have been described in the sperm proteome, only 1376 and 1300 have been indentified in the oocyte and blastocyst proteomes, respectively. These differences are probably due to the relative scarcity of available biological material from human oocytes and early embryos, which becomes technically insufficient for the identification of less abundant proteins (Jensen et al., 2013; Kaihola et al., 2016; Virant-Klun et al., 2016). In contrast, spermatozoa show two main advantages: (1) sperm can be easily purified in large quantities (Martinez-Heredia et al., 2006; Oliva et al., 2008, 2009; Codina et al., 2015), and (2) the male gamete is a well compartmentalized cell that allows the proteomic assessment of different subfractions, such as the head, the chromatin, the tail and the membranes (de Mateo et al., 2011; Amaral et al., 2013; Baker et al., 2013; Castillo et al., 2014a), allowing the identification of less abundant proteins.

The second issue is that, to the best of our knowledge, only the human embryo proteome on the blastocyst stage (5–6 days) is available in the literature (Jensen et al., 2013; Kaihola et al., 2016). As previously mentioned, the activation of the embryo genome occurs mainly between the 4-cell and 8-cell stage (Fig. 2), implying that blastocyst proteins could already have an embryonic origin. However, several proteins have broad half-lives ranging from 30 min to 8 days (Schwanhausser et al., 2011), which suggests that some sperm and oocyte proteins may still be present and functional in the blastocyst stage. Additionally, it is also important to take into account that proteins detected in the blastocyst could derive from the translation of the paternal and maternal mRNAs provided to the zygote, using the maternal translational machinery (Swann et al., 2012). In order to mitigate all these issues, we have also integrated into our proteome analyses the individual RNA-seq data from human sperm (Johnson et al., 2015), oocytes, zygotes and embryos at 2-cell, 4-cell, 8-cell, morula and blastocyst stages (Dang et al., 2016), as a complementary tool to identify potential paternally-derived embryo proteins. Also, in order to minimize the possibility of false-positive identifications, we excluded from the present analysis the blastocyst proteins that have also been identified in the proteomes of additional reproductive cells and fluids highly related to the oocyte and early embryo, such as the follicular fluid, cumulus cells and endometrium, since they may serve as a complementary protein source to the embryo proteome (see Methods and Supplementary Table 1).

Following these strict criteria, we managed to unequivocally classify 173 from the 1300 blastocyst proteins, according to their embryonic (18 proteins), maternal (47 proteins) and paternal (108 proteins) origin (Supplementary Table 5). As observed in Fig. 3B, the transcriptional pattern of some blastocyst proteins classified as Group 1 (18 proteins) clearly reflects an embryonic origin by de-novo transcription after the activation of the EGA. Specifically, those blastocyst proteins have not been detected in any of the cellular and fluid protein profiles of sperm, oocyte, follicular fluid, cumulus cells and mid-secretory endometrium (Supplementary Tables 1 and 5), and their corresponding RNAs were absent in sperm, oocytes and the stages of embryogenesis prior to the EGA (Supplementary Table 5). In contrast, we identified 155 blastocyst proteins with a potential maternal (47 proteins) or paternal (108 proteins) origin, either as proteins already present in the gametes and maintained intact until the blastocyst stage, or as the result of the potential translation of sperm and oocyte RNAs during the first stages of embryogenesis by the maternal translational machinery (Supplementary Table 5). From the subset of blastocyst proteins predicted as maternally derived, 5 of them were exclusively detected in oocytes at the protein level, while their corresponding RNAs were found absent in sperm, oocyte and all the embryo stages assessed. Therefore, the stability of those five embryo proteins during first steps of embryogenesis suggests their slow molecular turnover (Supplementary Table 5). In contrast, the remaining 42 maternal-derived blastocyst proteins identified in this review, follow a specific transcriptional pattern classified as Group 2 in Fig. 3B. In particular, the RNAs encoding this subset of blastocyst proteins are found with high levels in the oocyte, and progressively drop during the different early embryogenesis stages, without any increase upon the EGA. This, together with the fact that those proteins and their corresponding RNAs are absent in the sperm, provides evidence for the maternal origin of these 42 embryo proteins (Fig. 3B).

Unexpectedly, 108 blastocyst proteins were predicted to have a paternal origin, by either (1) their exclusive presence in the sperm protein profile combined with the absence of their corresponding transcripts in sperm, oocyte and all the different early embryogenesis stages (82 proteins), or (2) the solely detection of the corresponding RNA in sperm combined with their absence at protein level in both gametes or reproductive-associated cells or fluids (26 proteins; Group 3, Fig. 3B). Interestingly, this group of paternally-derived blastocyst proteins were found enriched in the GO annotation 'regulation of small GTPase mediated signal transduction' (Bonferroni corrected P-value <0.001). Dynamic analyses of gene expression during mouse preimplantation embryo development have revealed the activation

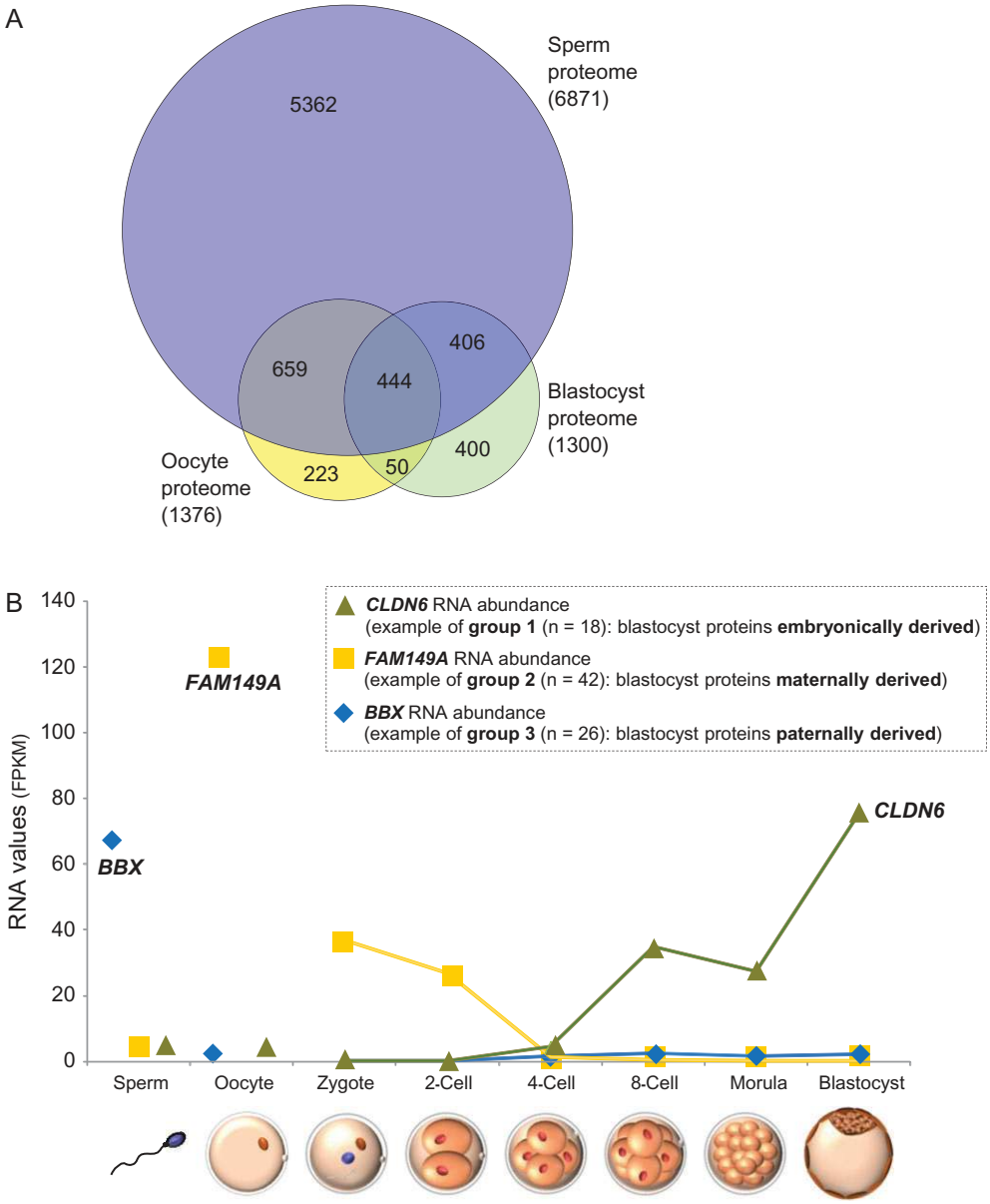

Figure 3 Integrative analyses of proteomic and transcriptomic profiles from gametes and blastocyst. **A.** Venn diagram of the compiled protein profiles of human sperm, oocyte, and blastocyst. The venn diagram analysis shows 5362, 223 and 400 proteins exclusively detected in sperm, oocyte and blastocyst, respectively. A total of 444 proteins are indentified in all three proteomes, 659 are shared between sperm and oocyte, 406 between blastocyst and sperm and 50 between blastocyst and oocyte. **B.** Assessment of the potential origin of blastocyst proteins according to the corresponding transcriptional pattern observed in human gametes and cellular stages of the preimplantation embryo development. The RNA abundance corresponding to blastocyst proteins has allowed classification in three subgroups of a subset of them, according to their potential embryonic, maternal, or paternal origin: (1) Group 1: blastocyst proteins classified as embryonically derived (n = 18), since their corresponding RNAs were absent in both gametes and the levels only increase after embryonic genome activation (EGA). The RNA levels of a component of this group, the claudin-6 (CLDN6) is shown in green. (2) Group 2: blastocyst proteins classified as maternally derived (n = 42). Their corresponding transcripts levels were found with low levels in sperm and high levels in the oocyte. Additionally, the levels of these transcripts decrease during early stages of embryogenesis and they do not increase after EGA. The RNA levels of a component of this group, the protein FAM149A (FAM149A), is shown in yellow. (3) Group 3: blastocyst proteins classified as paternally derived (n = 26). Their corresponding RNAs were found at low levels in oocyte but in high abundance in sperm. Moreover, the levels of these transcripts are low during early stages of embryogenesis and they do not increase after EGA. The RNA levels of a component of this group, the HMG box transcription factor BBX (BBX), is shown in blue.

of the genes involved in small GTPases-mediated signal transduction between the 8-cell and the morula stages (Hamatani et al., 2004). Therefore, our results suggest that the male gamete might provide the preimplantation embryo with proteins that ensure the appropriate gene expression pattern of GTPases involved in signal transduction processes. In addition, some of the blastocyst proteins inferred as paternally-derived were found related to already known sperm components required for preimplantation embryogenesis, such as the centrosomal protein 135 (CEP135), which acts as a scaffolding protein during early centriole biogenesis (Ohta et al., 2002). The roles of other potentially paternal-derived proteins are less well known in mammalian early embryogenesis. It is the case of the Rho GTPase Activating Protein 21 (ARHGAP21), whose homolog protein in *Caenorhabditis elegans* is crucial for the establishment of the radial polarity in the early embryo, a process known in mammals as compactation (Anderson et al., 2008; Nance, 2014). Furthermore, some blastocyst proteins potentially derived from the sperm seem to be related to the regulation of epigenetic marks, such as the (A-T) mutated (ATM) kinase, which is involved in the DNA damage response by phosphorylation of numerous substrates including histones (Yamamoto et al., 2012), and the DGCR8 Microprocessor Complex Subunit (DGCR8), which is crucial for primicroRNA processing to mature miRNA (Wang et al., 2007).

This integrative analysis of 'omic' data from reproductive cells and fluids provides the first step for the identification of embryo proteins with a potential paternal origin. The future validation of the impact of these 108 sperm proteins in the embryo, as well as the assessment of their potential roles in preimplantation embryogenesis and epigenetic inheritance may shed new light onto the real contribution of the father to the generation of healthy offspring.

The putative extra-testicular origin of a subset of human sperm proteins and the soma-to-embryo transmission hypothesis

All human sperm proteomic studies conducted to date have been performed in purified ejaculated spermatozoa, and thus, in cells that have been in contact with the seminal fluid. The seminal fluid constitutes at least the 90% of the ejaculated volume and is composed of a mixture of secretions from the accessory sex glands (epididymis, prostate and seminal vesicles), which are rich in lipids, glycans, oligosaccharides, inorganic ions, immune components, DNA, RNAs, miRNAs, proteins and peptides, either free or encapsulated in extracellular vesicles (Saez et al., 2003; Jones et al., 2010; Ronquist et al., 2011; Aalberts et al., 2014; Drabovich et al., 2014; Vojtech et al., 2014; Chiasserini et al., 2015; Jodar et al., 2016, 2017).

During the past years, the existence of communication between the sperm cells and the seminal fluid has been proposed, most probably through extracellular vesicles (Sullivan and Saez, 2013; Jodar et al., 2017). In fact, growing evidence has recently contributed to this hypothesis, such as the enrichment of RNAs from the seminal fluid extracellular vesicles in the peripheral membrane of mouse spermatozoa (Johnson et al., 2015). On the one hand, from a functional point of view, the seminal fluid seems to be not only a medium to carry spermatozoa through male and female tracts, but also a source of nutrition and components that modulate sperm function, motility and fertilizing capacity (Saez et al., 2003; De Jonge, 2005; Aalberts et al., 2014). On the other hand, from the proteomic perspective, the high impact of the seminal fluid on the protein composition of the male gamete has also been shown. Specifically, both ejaculated sperm and seminal fluid were found to contain a remarkable number of common proteins in their respective proteome profiles (Jodar et al., 2017). Interestingly, a previous integrative analysis of proteins and transcripts present in human sperm, extracellular vesicles and testes provided a list of sperm proteins with a potential extra-testicular origin (Jodar et al., 2016).

In this review, we have improved the prediction of the putative tissue origin of the human sperm proteins by integrating the RNA and protein data available in the Human Protein Atlas database. This analysis was based on the premise that the process of translation in the mature ejaculated sperm is blocked and, therefore, those sperm proteins not expressed in any stage of the seminiferous tubules, neither at RNA nor at protein level, may be acquired from extra-testicular tissues. By doing this, we were able to identify with high confidence 165 different sperm proteins potentially provided by the fluids or extracellular vesicles from epididymis, prostate or seminal vesicles (Supplementary Table 6).

From a general perspective, the enrichment analysis focused on GO annotations related to biological processes showed that these potential accessory sex glands-derived sperm proteins were mainly involved in immune response, cell junction organization, response to stimulus, extracellular matrix disassembly, gene expression and keratinization (Bonferroni corrected *P*-value <0.05; Table III). Interestingly, seven of these potential extra-testicular sperm proteins have been related to the processes of fertilization (four proteins) or preimplantation embryo development (three proteins; Supplementary Table 6). For instance, the beta-defensin 126 (DEFB126) is an epididymal protein known to be recruited to the sperm surface during the pass through the distal corpus and proximal cauda parts of the epididymis (Perry et al., 1999) (Fig. 4). Of note, a DEFB126 role in the efficient protection of the sperm from the female immune response has been reported (Yudin et al., 2005), which is in agreement with the GO enrichment analysis in this review with the accessory sex glands-derived sperm proteins (Table III). Additional epididymal proteins recruited by sperm are the epididymal sperm-binding protein 1 (ELSPBP1), which is involved in the correct acquisition of sperm motility (Parte et al., 2012), the binder of sperm protein homolog 1 (BSPH1), which has a role in capacitation (Plante et al., 2014), and the cysteine-rich secretory protein 1 (CRISP1), which has been already highlighted because of its crucial role in sperm penetration and sperm–oocyte fusion (see above; Fig. 4; Supplementary Table 6). Also, remarkable is the fact that sperm seem to carry prostatic proteins that might be relevant for the success of ART. This is the case of the prostatic acid phosphatase (ACPP) which is found deregulated in idiopathic infertile couples who did not achieve pregnancy after artificial insemination (Xu et al., 2012), suggesting that ACPP is crucial for sperm to reach and fertilize the oocyte. Similarly, a total of nine sperm proteins functionally associated to the modulation of gene expression are suggested to originate in the accessory sex glands (Fig. 4; Supplementary Table 6), which is in agreement with recent evidence showing that sperm gain specific non-coding RNAs involved in the epigenetic inheritance of the paternal low protein diet phenotype

Table III Biological processes significantly enriched in the subset of sperm proteins found to have a potential extra-testicular origin. Enrichment analyses performed with tools from the Gene Ontology Consortium database ($P < 0.05$ after Bonferroni correction).

GO biological process annotation	Total number of genes in GO annotation	Number of genes found	Fold enrichment	Bonferroni corrected *P*-value
Sperm proteins potentially derived from accessory sex glands				
Immune system process (GO:0 002 376)	2566	48	2.28	2.50×10^{-04}
Multicellular organismal process (GO:0 032 501)	6703	90	1.63	2.72×10^{-04}
Cell junction organization (GO:0 034 330)	200	12	7.30	1.16×10^{-03}
Response to stimulus (GO:0 050 896)	8033	100	1.51	1.17×10^{-03}
Extracellular matrix disassembly (GO:0 022 617)	79	8	12.32	3.33×10^{-03}
Gene expression (GO:0 010 467)	3763	10	0.32	2.27×10^{-02}
Keratinization (GO:0 031 424)	227	11	5.89	3.03×10^{-02}
Sperm proteins potentially derived from other extra-testicular tissues				
Keratinization (GO:0 031 424)	227	35	10.92	3.30×10^{-21}
Multicellular organismal process (GO:0 032 501)	6703	163	1.72	1.99×10^{-12}
Cell adhesion (GO:0 007 155)	870	43	3.50	1.17×10^{-08}
Immune system process (GO:0 002 376)	2566	77	2.13	7.90×10^{-07}
Programmed cell death (GO:0 012 501)	1040	42	2.86	1.03×10^{-05}
Movement of cell or subcellular component (GO:0 006 928)	1471	48	2.31	4.96×10^{-04}
Cellular macromolecule metabolic process (GO:0 044 260)	7017	59	0.60	1.66×10^{-03}
Extracellular matrix organization (GO:0 030 198)	309	18	4.13	5.35×10^{-03}
Regulation of gene expression (GO:0 010 468)	4618	33	0.51	7.94×10^{-03}
Anion transport (GO:0 006 820)	541	23	3.01	3.19×10^{-02}

through epididymosomes (Sharma *et al.*, 2016). In fact, the importation of sperm proteins and RNAs from accessory sex glands able to regulate gene expression in the embryo, once its genome is activated, might be a good strategy to provide environmental epigenetic information without the need to overcome the hemato-testicular barrier.

Unexpectedly, the results of our integrative analysis also suggest that not only the tissues from accessory sex glands are contributing to the sperm protein content. In fact, we found a subset of 286 sperm proteins with no RNA and protein detection either in the testis or in the accessory sex glands (Supplementary Table 7). Therefore, it may be suggested that these proteins could derive from the peripheral tissues outside the male reproductive tract and be acquired by the sperm after testicular maturation. These findings are consistent with the results obtained by Cossetti and colleagues, who showed the presence of a specific RNA from human melanoma in the sperm from mice that were previously subcutaneously inoculated with human melanoma cells (Cossetti *et al.*, 2014). Interestingly, the specific human transcript measured in Cossetti's study was also detected in the extracellular fraction of blood, indicating a potential active transport from mice dermis to the sperm cells through the extracellular vesicles pathway (Cossetti *et al.*, 2014). Our analysis revealed that some sperm proteins are specifically expressed at the protein level in a high variety of tissues, such as brain, lung, pancreas and bone marrow and in the immune system, among others tissues, but absent in testes and accessory sex glands at both transcript and protein levels (Fig. 4). From the functional perspective, proteins potentially derived from tissues other than testis and accessory sex glands were found mainly involved in keratinization, cell adhesion, immune response, programmed cell death, extracellular matrix organization, regulation of gene expression and anion transport, among others (Bonferroni corrected *P*-value <0.05; Table III).

Some of the proteins identified here as potentially originating in extra-testicular tissues were specifically found to be potentially related to processes of fertilization (2 proteins), early embryo development (2 proteins) and modulation of gene expression (12 proteins) (Supplementary Table 7). Interestingly, a remarkable number of sperm proteins possibly originating in the immune system seem to be able to modulate gene expression by the regulation of transcription and the histone modification pattern (Fig. 4). This is the case of the protein–arginine deiminase type-4 (PADI4) which is involved in the modification of histone 1 (H1) by the conversion of arginine residues to the non-coded aminoacid citrulline. This modification induces H1 disassembly from the DNA, leading to a global chromatin decondensation. Although this process was described in neutrophils during the innate immune response to infection (Neeli *et al.*, 2008), it has been recently shown to have a role in the mice early embryo, for reprogramming and for promotion of pluripontency and stem cell maintenance (Christophorou *et al.*, 2014).

Additional experiments are now required in order to validate the potential origin of these groups of sperm proteins that appear to be incorporated into the sperm cell during post-testicular processes. Although the success rate of pregnancies obtained after testicular sperm extraction (TESE) combined with intracytoplasmic sperm injection (ICSI) suggest that extra-testicular sperm proteins might not be crucial for early embryo development, the functionality of these extra-testicular proteins in the fertilization processes that are

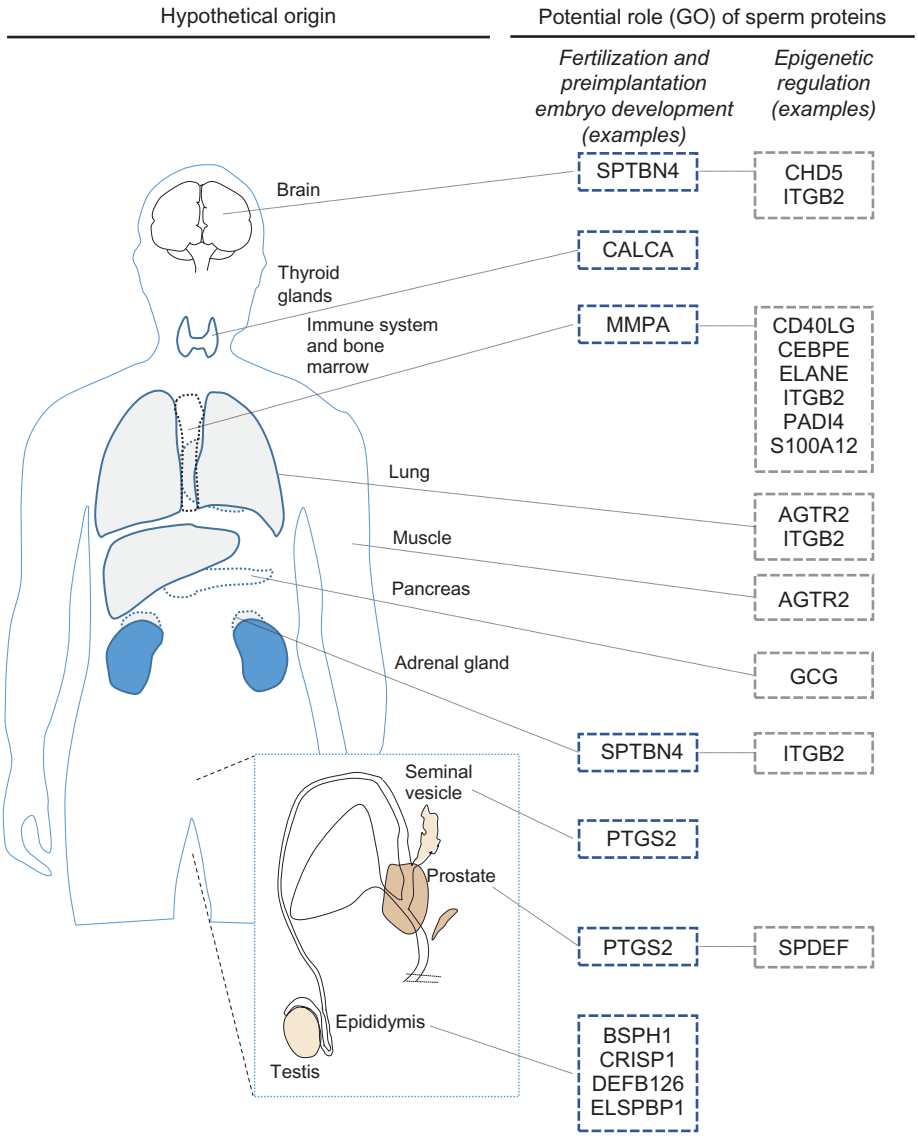

Figure 4 Human sperm proteins with potential extra-testicular origin. Examples of the subset of sperm proteins potentially derived from extra-testicular tissues (165 proteins from accessory sex glands and 286 from other extra-testicular tissues; see Supplementary Tables 6 and 7), according to RNA-seq and protein data enclosed in the Human Protein Atlas database (right). Sperm proteins are classified based on their involvement in processes related to fertilization and preimplantation embryo development (blue squares) and epigenetic regulation (gray squares), according to the Gene Ontology (GO) annotations data enclosed in the Gene Ontology Consortium database.

overcome by ICSI, and as potential modulators of the offspring phenotype should be elucidated. This will contribute to unravel the mechanisms of the transmission of information within organs and generations, such as the transmission of parental olfactory experiences to the offspring (Dias and Ressler, 2014), which could be explained by the putative transport of epigenetic information from peripheral tissues outside male reproductive tract to the sperm.

Discussion and future directions

The characterization of the sperm cell through MS-proteomic strategies combined with the integrative analysis of the data is providing a powerful approach to decipher the molecular aspects of sperm function (Amaral *et al.*, 2014a; Carrell *et al.*, 2016). In fact, the analysis reported in the current review has increased our knowledge into the potential contribution of the father to the generation of healthy offspring. This has been possible thanks to the current availability of sperm protein catalogs, allowing us to generate a nearly complete protein profile of the human male gamete. In addition, since the functionality of a cell is also influenced by its surroundings, impeding the analysis of cellular proteomic data in isolation, we have also proceeded to an exhaustive examination in combination with additional 'omic' data and evidence from related tissues, cells and fluids. Due to the limitations associated with research in human samples, data from studies of animal models

have been also included as a tool to predict the potential functions of the human sperm proteins.

In terms of sperm function, the role of the male gamete during fertilization has been widely studied. In fact, our GO annotations-based analysis of the sperm proteome was in agreement with the many sperm proteins already known and proposed by others to be crucial for the different processes occurring through the sperm journey from the testis to the oocyte (Fig. 2) (Toshimori *et al.*, 1998; Ficarro *et al.*, 2003; Inoue *et al.*, 2005; Fujihara *et al.*, 2010; Maldera *et al.*, 2014; Singh and Rajender, 2015; Cuasnicú *et al.*, 2016). However, less information is available so far about the involvement of the sperm proteins in the correct initiation and early progression of embryogenesis. The analysis shown in the present review has revealed remarkable groups of proteins that may be key players in post-fertilization processes, including not only those taking place during the first stages of the preimplantation embryo development, but also those which could modulate gene expression once the embryo is activated (Supplementary Tables 3 and 4). This raises the possibility that a subset of the sperm proteome could contribute to explain the hypothesis of epigenetic inheritance of phenotype alterations. Reinforcing this idea, we have also observed that some blastocyst proteins revealed as required for correct early embryogenesis appear to be paternally-derived (Supplementary Table 5). Altogether, these findings suggest that the sperm proteome is not only limited to the proteins necessary for oocyte fertilization and spermatogenic leftovers, but also a cargo of additional groups of proteins which are delivered to the embryo and which may have critical immediate and future impacts.

Interestingly, our analyses also add further support to the idea that the protein composition of the mature spermatozoon is not concluded at testicular level, but it is only completed through the potential molecular communication between the sperm cell and the environment (Fig. 4; Supplementary Tables 6 and 7), as has been already proposed (Sullivan and Saez, 2013; Jodar *et al.*, 2017). In fact, it can be hypothesized that since sperm are not able to produce proteins de-novo due to the blockage of their transcriptional and translational machinery, the importation of proteins from secretions of accessory sex glands and possibly other peripheral tissues could be an efficient strategy to maintain the sperm proteomic profile in optimal conditions for its function at fertilization and in potential future events in early embryogenesis. Moreover, these imported proteins during sperm maturation might provide environmental epigenetic information without the need to overcome the hemato-testicular barrier.

Overall the current data suggest that a subset of sperm proteins is crucial for fertilization and beyond. These results should stimulate further experimental studies aimed to elucidate the roles of groups of specific sperm proteins in the processes of fertilization, embryo development and paternal epigenetic inheritance, both in normal and altered conditions.

Supplementary data

Supplementary data are available at *Human Reproduction Update* online.

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Authors' roles

J.C. and M.J.: participation in study design, execution, analysis, manuscript drafting and critical discussion. R.O.: participation in study design, execution, manuscript drafting and critical discussion.

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Conflicts of interest

None of the authors has any conflict of interest.

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