The Common and Species-Specific Roles of Oviductal Proteins in Mammalian Fertilization and Embryo Development

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Proteins are biomolecules responsible for a vast range of functions within living cells and tissues. Over 150 different proteins are present in the fluid of the oviduct, where mammalian oocytes are fertilized and zygotes start to develop. Although fertilization can be achieved in vitro without any contributions from the oviduct or oviductal proteins, some of these proteins play crucial or yet unknown roles in fertilization and embryo development. The inclusion of specific oviductal proteins or purified fractions of oviductal fluids with beneficial effects on gametes, zygotes, or embryos might help to reduce the adverse effects of the periconceptional environment in in vitro*–derived embryos and thereby also improve the yield and quality of assisted reproductive technologies (ARTs). The proteins discussed here include osteopontin (SPP1), glycodelin, oviductin (OVGP1), plasmin, heat shock proteins (HSPA8, Grp78, HSP60), lactoferrin, deleted in malignant brain tumors 1 (DMBT1), and fetuin.*

Keywords: oviductal proteins, fertilization, embryo, ARTs, epigenetics

The physiology of the mammalian oviduct has been studied intensively in recent years. All information, new and old, contributes to our better understanding of fertilization and the development of preimplantation embryos and assists with the further improvement of assisted reproductive technologies (ARTs), including *in vitro* fertilization (IVF) or the culture of preimplantation embryo (EC).

In mammals, the oviduct is where oocytes are fertilized and zygotes start to develop. It provides the microenvironment necessary for the encounter between the spermatozoa and oocyte (reviewed by Coy et al. 2012a). The oocytes released from the ovarian follicles are captured quickly by the oviductal infundibulum with a very low margin of error. They are transported to the ampullary region of the oviduct, where they meet the spermatozoa. The latter reach the ampulla after passage through the uterus. Fertilized eggs (zygotes) develop as they pass through the lower (isthmic) segment of the oviduct (reviewed by Yanagimachi 1994). Finally, early stage embryos at 4- to 8-cell, morula, or blastocyst stages, depending on the species, enter the uterus and survive within its secretions until they become implanted and the placenta starts to develop. All these steps take place in the lumen of the oviduct and uterus, which contain oviductal and uterine fluids. Both fluids are dynamic and of complex composition and contain glycosaminoglycans, energy metabolites, amino acids, inorganic salts, and a number of proteins (more than 150 have been identified in the oviduct; Avilés et al. 2010), some of them specifically secreted by the local epithelium and some others coming from the blood serum. Recently, the presence of exosomes in the oviduct have also been described, bringing about the idea that proteins included in these vesicles could address oviduct interactions with gametes and embryos (Al-Dossary et al. 2013).

By contrast, the *in vitro* procedures of some ARTs (maturation, fertilization, embryo culture) occur in a Petri dish containing a liquid medium (culture medium). These culture media used in mammalian *in vitro* fertilization (IVF) and embryo culture have a composition similar to biological fluids with regards to mineral salts, carbohydrates, or amino acids. In this respect, they are chemically semidefined. However, all of them require a protein source for the successful completion of the process. For years, blood serum was used as a source of proteins, but various problems made its use unadvisable. It was shown that the so-called "large offspring syndrome" in cattle and sheep (Young et al. 1998, Fernandez-Gonzalez et al. 2004) and various locomotive abnormalities in mice (Fernandez-Gonzalez et al. 2004)

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Table 1. The differences in IVF procedures and results among different mammals.

Species	Source of oocytes	Source of spermatozoa	Percentage of blastocysts per oocyte collected	Reference
Rabbit	Ovary (preovulatory follicles)	Epididymis	60–65	Zeng et al. 1999
Mouse	Oviduct	Epididymis	42–98	Szczygiel et al. 2002
Cattle	Ovary (IVM)	Ejaculated (frozen–thawed)	30–40	Rizos et al. 2008
Goat	Ovary (IVM)	Ejaculated (frozen–thawed)	45	Souza-Fabjan et al. 2014
Sheep	Ovary (IVM)	Ejaculated (frozen–thawed)	25–40	De Matos et al. 2002
Pig	Ovary (IVM)	Ejaculated or epididymal (Fresh or frozen)	6–38	Coy and Avilés 2010
Horse	Ovary (preovulatory follicles)	Ejaculated (Fresh better) (ICSI)	25–35	Hinrichs 2005
Human	Ovary (preovulatory follicles)	Ejaculated or epididymal (Fresh or frozen)	More than 45	Meintjes et al. 2009

were, in part, due to the presence of serum in the culture media. These reasons meant that most of the research in recent years has focused on the production of serum-free, chemically semidefined media, trying to achieve performance similar to those reached in the presence of serum. However, in this approach, it was forgotten that blood serum and oviductal fluid are of different composition. In this sense, the potentially harmful factors present in blood serum might or might not be present in the oviductal fluid, and their concentrations, if present, could be lower in the oviduct than in blood vessels. In addition, the specific factors secreted by epithelial cells into the oviductal lumen could be of crucial importance for the correct interaction between sperm and oocytes for the initial embryo cleavage in this anatomical region and for the correct establishment of the epigenetic marks in the conceptus.

This article is first focused on the selection of a set of proteins among the 150 identified in the oviduct (Avilés et al. 2010) with already demonstrated benefits on IVF in mammals to investigate whether the roles they play in one species can be assumed for other species and whether these proteins could be thought of as potential additives for IVF culture media. Before describing the actions, roles, and possible uses of various oviductal proteins in ARTs, we considered it necessary to briefly discuss current IVF protocols in different mammals in order to get an idea about the specific steps of the process (*in vitro* maturation, IVM; IVF; or EC) and the specific proteins that could help to increase the efficiency of such protocols.

Methods

We performed a computerized search in PubMed regarding the protocols and rates of success for IVF in different mammals, looking for similarities and differences among species. To select the candidate proteins to be revisited in light of their role in IVF, a second search using *oviduct*, *oviductal protein*, and *IVF* as main keywords was performed. The criteria for the inclusion of one protein in the list were the following: (a) the protein was highly conserved among species and has shown an effect on IVF in any species; (b) the protein was not highly conserved but has shown an effect on human IVF; (c) the protein had been proposed as essential for fertilization. The identities in the aminoacid sequences of the different proteins were found using the free Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) in the United States. To more reliably check the function of the proteins identified, the phenotypes (related to fertilization) of the knockout mice for each protein were searched.

Differences in IVF in mammalian species

IVF is a powerful tool to understand and assist mammalian fertilization and embryo development. One should be aware that there is no single IVF protocol that works for all species of mammals. A protocol that works very well for one species is not necessarily suitable for another species. Similarly, a protein with a demonstrated role in one species may not always play the same role in a different species, as will be illustrated in the following paragraphs.

Table 1 lists the success rates of IVF in seven arbitrarily selected mammalian species. The success rate of IVF is largely dependent on (a) the source of oocytes (collected from ovary or oviduct, matured *in vivo* or *in vitro*), (b) the source of spermatozoa (collected from epididymis or semen), and (c) the components of culture medium and culture condition.

The rabbit was the first mammalian species with unequivocal evidence of successful IVF. Thibault and colleagues (1954) used *in vivo*–capacitated spermatozoa for insemination, collected from the uterus of mated females, because no one at that time knew how to capacitate rabbit spermatozoa *in vitro*. Today, rabbit epididymal spermatozoa can be capacitated using hypertonic and isotonic solutions with excess sodium bicarbonate (Zeng et al. 1999). According to Lan and colleagues (2008), rabbit oocytes matured *in vivo* are fertilized better than those matured *in vitro*. It should be noted that the rabbit and a few other species are rather "exceptional" among mammals in that the egg's zona pellucida (ZP) does not block polyspermic fertilization; it is the egg's plasma membrane that blocks polyspermy (Yanagimachi 1994).

Mouse IVF is achieved routinely by using epididymal spermatozoa, which are capacitated readily in a simple

media, and oocytes collected from oviducts (Szczygiel et al. 2002). After insemination *in vitro*, a large number of spermatozoa may attach to each oocyte, which does not happen *in vivo* (Stefanini et al. 1969). One should be aware that epididymal spermatozoa, unlike ejaculated spermatozoa, have not been exposed to the seminal plasma, which contains a variety of sperm-coating materials, and this can be a key point in explaining the observed differences in sperm–ZP attaching *in vivo* and *in vitro*. It is also important to be aware that the ZP of a mouse oocyte is modified by a protease of cortical granule origin during oocyte activation by a fertilizing spermatozoon (Burkart et al. 2012). This prevents excess spermatozoa from binding to the ZP (Burkart et al. 2012). It should be noted that in some other species, such as the pig, spermatozoa continue attaching to the ZP, either under *in vivo* (Hunter 1974) or *in vitro* conditions (Coy et al. 2008), even though an effective polyspermy block has been established. Therefore, the role of cortical granule protease in the prevention of polyspermy can be of different importance among different species.

For cattle artificial insemination (AI) or IVF, the spermatozoa in semen are commonly cryopreserved until use. Mature oocytes are collected by transvaginal ovum pickup (OPU) after superovulation (443,533 of them were collected in 2012, according to the International Embryo Transfer Society, IETS, 2013 Statistics and Data Retrieval Committee Report; *www.iets.org*). Alternatively, immature oocytes are collected from the ovaries of slaughtered cows and matured *in vitro* (13,922 according to IETS 2013). Thawed and washed spermatozoa are capacitated by heparin before IVF. Semen cryopreservation, AI, vitrification of preimplantation embryos, and transcervical embryo transfer are now routinely performed in the cattle industry (Rizos et al. 2008, Ha et al. 2014). In 2012, more than 890,875 fresh and frozen cattle embryos were transferred worldwide, of which 384,999 were *in vitro*–produced embryos. Brazil is now the global leader of cattle embryo transfer (IETS 2013).

The situation in small ruminants is quite different. The number of embryos transferred in ewes and goats (mainly in Australia and South Africa) is steadily increasing, but virtually all transferred embryos were produced *in vivo* (IETS 2013). IVF in these species is usually performed using protocols adapted from other animals, such as the cow. A major problem is the difficulty in collecting qualitatively adequate oocytes that can be processed successfully *in vitro*, because most of the slaughtered animals are prepuberal and not sexually mature, resulting in poor developmental rates (Casao et al. 2010). The final yield of transferable preimplantation embryos rarely reaches 30%, although recent investigations have reported significant improvements in goats (de Souza-Fabjan et al. 2014).

The porcine species is probably the one in which IVF is easier than in many other farm animals in terms of successful sperm penetration into oocytes. However, the problem lies in the fact that too many spermatozoa enter each oocyte, resulting in a high incidence of pathological polyspermy (Coy and Avilés 2010). Techniques for *in vitro* maturation of immature oocytes have developed steadily, with a success rate as high as 85% of the oocytes from antral follicles undergoing nuclear maturation (Coy et al. 1999). However, because of the low incidence of monospermic fertilization (no more than 30%), the development of fertilized oocytes to transferable embryos is rather poor (Grupen 2014).

In the horse, maturing oocytes can be aspirated from preovulatory follicles through a needle pierced through the flank and also by OPU (Deleuze et al. 2010). High percentages (65%–80%) of cumulus–oocyte complexes can be successfully recovered when they are in large follicles (more than 35 millimeters in diameter). Although approximately 60% of cultured oocytes mature *in vitro* when they are collected from smaller antral follicles (Deleuze et al. 2010), equine IVF is still difficult to achieve. Even after intracytoplasmic sperm injection (ICSI) into oocytes, only 25%–35% develop to blastocysts *in vitro* (Hinrichs 2005).

In humans, the World Health Organization (WHO) estimates that infertility affects about 20% of couples in developed countries, and this figure is increasing every year. In developing countries (except for China), it was estimated that in 2002, more than 186 million women of reproductive age (15 to 49 years old) were infertile. This number represents more than one in four women of reproductive age in these countries. A new WHO study, published at the end of 2012, has shown that the overall burden of infertility in women from 190 countries has remained similar in estimated levels and trends from 1990 to 2010 (Mascarenhas et al. 2012). Since the first baby was born by IVF in 1978 (Steptoe and Edwards 1978), the development of the ART industry has been spectacular, with hundreds of thousands of private and public infertility clinics worldwide. However, in over 60% of cases, the birth of a child by IVF is not possible (Meintjes et al. 2009). This fact has led to the use of different approaches to assist couples to procreate, including the management of reproductive health, infertility treatments (hormonal therapy, AI, IVF, embryo transfer), or gynecological interventions. Transfers of embryos (usually more than one, depending on the countries) at day 3 or 5 usually give percentages of successful pregnancy of more than 45%, and live birth is around 40% (Stephens et al. 2013). Although the techniques for ovarian stimulation, oocytes retrieval, IVF (including ICSI), and embryo culture and transfer have all developed rapidly, it is important to pay more attention to the native intraoviduct environment to design media close to the native tubal fluid in order to further increase the safety and efficiency of IVF. In fact, there is some evidence suggesting that culture media can induce epigenetic changes affecting development and future disease risk (El Hajj and Haaf 2013).

Oviductal proteins potentially affecting fertilization and early embryo cleavage

The candidate proteins matching the criteria for the inclusion in the study were osteopontin (SPP1), glycodelin

Table 2. The percentages of identities relative to the human protein after alignment of sequences (from BLAST at NCBI).

	Species							
Protein	**Human**	**Rabbit**	**Mouse**	**Cow**	**Sheep**	**Goat**	**Pig**	**Horse**
SPP1	X	65	59	58	57	57	65	70
PAEP	X	–	30	43	–	–	–	–
OVGP1	X	81	76	71	71	72	74	(Pseudogene)
Plasminogen	X	82	79	78	84	88	80	79
HSP70 (HSPA8)	X	100	99	99	99	99	99	100
Grp78 (HSPA5)	X	99	99	99	99	99	99	98
Grp94 (HSP90)	X	94	97	98	98	97	98	98
Lactoferrin	X	74	71	70	71	71	71	75
DMBT1	X	74	65	72	64	46	74	61
Fetuin B	X	66	64	66	66	66	60	63

Table 3. Phenotypes of transgenic mice deficient in various oviductal proteins with potential roles in fertilization.

Mouse gene knockout (protein)	Impaired phenotype	Reference
SPP1 (osteopontin)	None	Rittling et al. 1998
PAEP (glycodelin)	?	Not found
OVGP1 (oviductin)	–	Araki et al. 2003
PLAT and *PLAU* (tPA and uPA)	Male/female fertility	Carmeliet et al. 1994
HSPA5 (Grp78)	Embryo development	Luo et al. 2006
HSP70	Male fertility	Dix et al. 1996
HSP90B1 (Grp94)	Embryo development	Wanderling et al. 2007
DMBT1	–	De Lisle et al. 2008
LTF (lactoferrin)	–	Ward et al. 2003
Fetub (Fetuin-B)	Female infertility	Dietzel et al. 2013

(PAEP), oviductin (OVGP1), plasminogen, heat shock proteins (HSPA8, Grp78, HSP60), lactoferrin, deleted in malignant brain tumors 1 (DMBT1), and fetuin b. All of them except glycodelin (PAEP) showed identities with the amino acid sequence of the human protein above 60% (table 2) and have been reported to affect sperm–oocyte interactions, fertilization, or early embryo development (Gabler et al. 2003, Chiu et al. 2007a, Coy et al. 2008, 2012b, Elliott et al. 2009, Teijeiro and Marini 2012, Dietzel et al. 2013, Zumoffen et al. 2013). Consequently, they could be considered as potential additives for IVF and embryo culture media. Moreover, fetuin b has been proposed as essential for fertilization (Dietzel et al. 2013) and, although still not described in the oviduct, is present in the follicular fluid and could potentially reach the ampulla during ovulation. For this reason, it was also included in the study.

Osteopontin. Osteopontin (SPP1) is a phosphoprotein that was first discovered in bone-forming osteoblasts. It is synthesized in a variety of tissues and secreted in body fluids. It is involved in immune regulation, signal transduction, and cell adhesion (Wang and Denhardt 2008). Among its reproductive functions, it has been shown that its presence or absence in bull seminal plasma is related to male fertility (Cancel et al. 1997). In the oviduct, its secretion by the oviductal mucosa has been detected in bovine species (Gabler et al. 2003). The role of osteopontin in different steps of fertilization has become clear in the pig and cow. Depending on the dose used, osteopontin in IVF medium reduces the sperm acrosome reaction and incidence of polyspermy, resulting in an increase of normal fertilization *in vitro* in the pig (Hao et al. 2006). Similarly, the addition of an osteopontin antibody decreases sperm–oocyte binding and the fertilization rate *in vitro* in the cow (Goncalves et al. 2008). Osteopontin contains an arginine-glycine-aspartic acid (RGD) sequence, and it was proposed that osteopontin might bind primarily to an integrin on oocytes via its GRGDS sequence, thereby decreasing the chances for sperm binding (Goncalves et al. 2008). Because the pig IVF systems usually result in excess spermatozoa bound to the ZP and polyspermy, the addition of osteopontin to the culture medium helps reduce the incidence of this pathology. By contrast, this problem has lower incidence in the bovine IVF, and osteopontin should not be an additive to include in the fertilization medium in this species.

No significant effect of osteoponin is found in horse IVF (Mugnier et al. 2009). Osteoponin-knockout mice are fertile; the litter size is normal, and offspring develop normally (table 3; Rittling et al. 1998), suggesting that this protein can modulate fertilization in the oviduct but that it is not essential, at least for the mouse. From all these data—and as it will be later explained with OVGP1—the role of osteopontin in fertilization seems to be species dependent (table 4).

Table 4. Examples of different effects of oviductal proteins on fertilization events depending on the species.

Fertilization event	Pig	Mouse	Human
Inhibition of sperm–ZP binding after fertilization	No	Yes (ovastacin)	Yes (lactoferrin)
ZP hardening in the oviduct	Yes (OVGP1, SPP1)	No	No
Inhibition of ZP hardening	No	Yes (Fetuin)	?

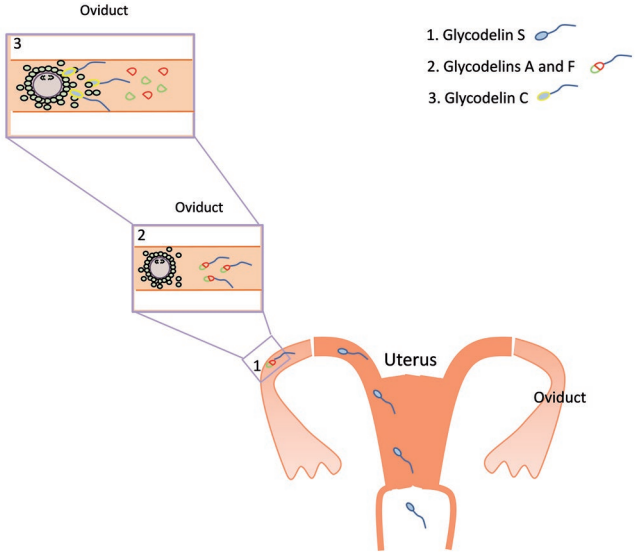

Figure 1. (1) Spermatozoa carry out glycodelin S from a seminal plasma origin during their transit through the uterus. At arrival in the oviduct, glycodelins A and F bind to spermatozoa. (2) Spermatozoa reaching the ampulla and close to the cumulus–oocyte complex still carry the glycodelins A and F, and their ability to bind to the zona pellucida (ZP) is inhibited. (3) However, when the spermatozoa interact with the cumulus oophorus cells, they convert glycodelins A and F to glycodelin C, which displaces the two former isoforms and enhances zona-binding ability.

Glycodelin. Glycodelins (PAEP, placental protein 14, or progestagen-associated endometrial protein) are glycoproteins with at least four glycoforms. They are present in the amniotic fluid (glycodelin A), seminal plasma (glycodelin S), follicular fluid (glycodelin F), and cumulus oophorus (glycodelin C; Yeung et al. 2009). The human oviduct secretes glycodelins, and oviductal cells *in vitro* produce glycodelins A and F (Chiu et al. 2007b). The main role of glycodelins is related to the postimplantation development of embryos as they are secreted by endometrial glands during early pregnancy (Lee et al. 2011). Glycodelins in species other than humans have seldom been studied. According to Yeung and colleagues (2009), the glycodelin glycoforms bind to different receptors on the sperm head. There are two glycodelin-F receptors, one of which is shared with glycodelin A in the plasma membrane overlying the acrosome. Glycodelin S also possesses two receptors distributed over the whole sperm head, whereas glycodelin-C receptors are distributed unevenly over the whole head, with a higher concentration in the equatorial region (reviewed by Yeung et al. 2009). Deglycosylation abolishes the binding and therefore the action of the glycodelins on spermatozoa. Glycodelin S within the seminal plasma suppresses cholesterol efflux from the sperm plasma membrane to prevent precocious sperm capacitation. Whereas glycodelins A and F within the follicular and oviduct fluids inhibit the premature acrosome reaction of spermatozoa and sperm binding to the zona, glycodelin C within the cumulus oophorus replaces these two glycodelins to enhance sperm binding to the ZP and their acrosome reaction (figure 1; Chiu et al. 2007a). This led these researchers to assume that, at least in humans, cumulus cells are important in modulating sperm–ZP interactions, and for that reason, they do not recommend the use of cumulus-free oocytes for human IVF. Because no data are available about the phenotype of glycodelin-null mice (table 3), the level of the importance of this protein for mammalian fertilization in general remains to be clarified.

Oviductin. Oviductin (OVGP1) is an estrogen-dependent oviductal glycoprotein secreted by the oviductal epithelium of most mammals, including humans. It was Oikawa and colleagues (1988) who first found in the hamster that a substance of high molecular weight (200–240 kilomoles) is added to an oocyte's ZP when oocytes are transported from the ovary to the oviduct. This substance was then called *oviductin*. Hamster oviductin binds to both the sperm-head plasma membrane (Kan and Esperanzate 2006) and oocytes' ZP (Oikawa et al. 1988) to promote sperm capacitation and zona penetration by spermatozoa (Kan and Esperanzate 2006). Bovine oviductin also facilitates sperm capacitation and fertilization (King et al. 1994).

In the pig, sheep, goat, cow, hamster, and rabbit, oviductal fluid is responsible for prefertilization ZP hardening (understanding *ZP hardening* as ZP changes that increase the ZP's resistance to proteolysis and reduce sperm binding; Mondéjar et al. 2013). It is the binding of oviductin to ZP that makes it more resistant to sperm attachment and decreases the risk of polyspermy in the pig and the cow (Coy et al. 2008). Interestingly, no ZP hardening in their homologous fluid occurs in the mouse and human (Mondéjar et al. 2013). Mondéjar and colleagues (2013) examined the effects of the oviduct fluids of six different species (cow, pig, goat, sheep, rabbit, and human) on the oocytes of homologous and heterologous species and found that rabbit oviductal fluid had the strongest ZP-hardening effect; it hardened the

ZP of not only rabbit oocytes but also those of mice, rats, hamsters, cows, pigs, ewes, and goats. Even mouse ZP, which did not increase its resistance to proteolysis after exposure to homologous oviductal fluid (table 4), showed a significant change after incubation in rabbit and goat oviductal fluids (Mondéjar et al. 2013). Human ZP did not become resistant to proteolysis after treatment with the fluid of any of five species tested, which included the homologous human fluid (Mondéjar et al. 2013).

It is important to note that ZP hardening by oviductin is transient. It occurs within the ampulla before oocytes are fertilized. By the time zygotes develop to blastocyst, the ZP returns to its initial "soft" stage (Kolbe and Holtz 2005). This could be in part due to the releasing of oviductin from the zona (Coy et al. 2008). Its migration into the perivitelline space and ooplasm of zygotes may facilitate embryo development (Boatman 1997). A soft ZP would certainly make an embryo easier to hatch.

Enhancement of the *in vitro* development of a goat preimplantation embryo by low concentrations of oviductin has been reported (Pradeep et al. 2011). According to Araki and colleagues (2003), oviductin-null female mice are fertile. This does not mean that oviductin is unnecessary in the mouse (table 3). Although it may not be crucially needed for fertilization and embryo development, it may maximize the efficiency of these important biological processes. At least in ungulates, oviductin increases the ZP's resistance to proteolysis, and this ZP hardening might be of crucial importance to warrant monospermic fertilization or sperm selection. The presence of different isoforms of oviductin and different levels of glycosylation of oviductin has been reported (McBride et al. 2004). Studies using recombinant oviductins in different forms would disclose the molecular basis of such physiological differences.

Plasmin. It has been known for many years that cortical granules (CGs) released from the oocytes at fertilization make the oocyte's ZP impenetrable by excess spermatozoa; this process is called the *zona reaction* (Yanagimachi 1994). In the mouse, ovastacin CG protease (Burkart et al. 2012) hydrolyzes a zona glycoprotein ZP2, making acrosome-reacted spermatozoa unable to bind to the zona (table 4). Recently, Gahlay and colleagues (2010) found that the zona reaction can occur in mouse oocytes without ZP2 hydrolysis, making clear the difference between zona reaction (in terms of ZP changes reducing sperm penetration) and ZP hardening (in terms of ZP changes reducing sperm binding). Although it is known that the pig oocyte ZP is hydrolyzed, perhaps by CG protease (Hedrick et al. 1987), pig spermatozoa keep attaching to the ZP after fertilization (Hunter 1974). These excess spermatozoa remain weakly attached to the zona but do not traverse it, and oviductal plasminogen seems to be involved in avoiding the spermatozoal advance. Different components of the plasminogen/plasmin system have been described in the reproductive tract of mammals, from seminal plasma to the uterus, oviduct, or ovary, and their roles are multiple (Ebisch et al. 2008). Coy and colleagues (2012b) proposed that plasminogen in the oviductal fluid of the pig and cow binds to both the ZP and the oocyte's plasma membrane and is activated by plasminogen activators released when the first spermatozoon contacts the oolemma. This activation produces plasmin that prevents the advance of attached spermatozoa by weakening the sperm–zona binding.

According to Mio and colleagues (2012), human spermatozoa passing through the ZP suddenly stop advancing within 10 seconds after the first spermatozoon contacts the oocyte's plasma membrane. It is unknown whether this is due to the action of a few CGs being released during oocyte activation or to the rapid activation of plasmin in the zona, as we mentioned above. The recent discovery of Juno protein as the egg's receptor for sperm Izumo and the description of its release after sperm contact (Bianchi et al. 2014) brings the speculation that oviductal plasminogen, plasmin, Juno, and CG protease are somehow intrincately involved in the polyspermy block on the oocyte's plasma membrane and in the ZP.

Mice lacking two plasminogen activators, tPA and uPA, are significantly less fertile (table 3) than wild-type mice (Carmeliet et al. 1994). Because plasmin and plasmin activators play crucial roles in ovulation and implantation, the exact roles of oviductal plasmin in mouse fertilization, if any, would be difficult to evaluate separately. IVF experiments with male knockout-by-female wild-type gametes and reciprocal incubation could shed light on the function of these factors in sperm and in the oocyte.

Heat shock proteins. Heat shock proteins (HSPs) are usually considered intracellular proteins. However, they have been detected in human serum and plasma, along with the finding that HSP70 can be secreted through an unconventional lysosomal pathway (Mambula et al. 2007). Therefore, it is not surprising that different members of the HSP family are present in the oviductal epithelium or fluid in various species, such as the pig (Georgiou et al. 2005), cow (Boilard et al. 2004), ram (Lloyd et al. 2012), mouse (Lin et al. 2012), and human (Lachance et al. 2007). All studies have suggested that HSPs bind to spermatozoa during their transit from the uterus to the oviductal isthmus and ampulla. The suggested roles of HSPs include the enhancement of sperm survival within the female tract (Elliott et al. 2009) and the prevention of premature sperm capacitation, thereby increasing the chance of successful fertilization. It is interesting that the HSPA8 level within a sow oviduct is more than five times higher in the presence of spermatozoa in the oviduct than in their absence (Georgiou et al. 2005). The importance of this family of proteins in fertilization can be evaluated by the phenotypes of transgenic mice for different HSPs (table 3). It is obvious that HSPA5 (Grp78) is essential for embryonic cell growth. HSPA5-null mouse embryos are unable to hatch out of the ZP, with a massive apoptosis in the inner cell mass. HSP70-null male mice have neither postmeiotic spermatids nor mature spermatozoa and are infertile (Dix et al. 1996).

Other members of the family, such as HSP90 (Grp94), are of fundamental importance for embryo development, because disruption of these genes causes the death of mouse embryos on day 7 of gestation (Wanderling et al. 2007). These data corroborate the importance of oviductal HSPs for the success of the reproductive process, especially by their role in sperm function and embryo development. It remains to study their beneficial role as additives in IVF media.

Deleted in malignant brain tumours 1. Deleted in malignant brain tumors 1 (DMBT1) is an estrogen-dependent multifunctional glycoprotein detected first in the endometrial epithelium of the monkey and rat (Tynan et al. 2005). The presence of mRNA for DMBT1 has been detected in bovine, porcine, and human oviducts (reviewed by Mondéjar et al. 2012), and the proteins have been identified in porcine and equine oviductal epithelia and fluid (Mondéjar et al. 2012, Ambruosi et al. 2013). In the sow, DMBT1 is on the apical surface of the oviductal epithelium and is assumed to be a molecule binding to capacitating spermatozoa with a proposed role in a mechanism of negative sperm selection (Teijeiro and Marini 2012). Ambruosi and colleagues (2013) found that the preincubation of porcine and equine oocytes in media containing oviductal fluid or recombinant DMBT1 before IVF increases the incidence of monospermic fertilization. However, nothing else is known about the mechanism by which this oviductal protein acts on the gametes and the deficient knockout mice are fertile (table 3; De Lisle et al. 2008).

Lactoferrin. Lactoferrin (LTF) is a multifunctional glycoprotein related to immune and inflammatory response, the regulation of cellular growth and differentiation, and the protection against cancer development and metastasis (Ward et al. 2005). It has been recently observed in the secretions and epithelium of women's oviducts (Zumoffen et al. 2013). It is abundant in the oviductal fluid during the periovulatory phase of the menstrual cycle, and it binds to capacitated spermatozoa, as well as to the oocyte's ZP (Zumoffen et al. 2013). It inhibits sperm–zona binding, reducing the chance of polyspermic fertilization. Lactoferrin has also been detected in the endometrium of the hamster, rat, and mouse (Teng et al. 2002). However, its expression in the oviducts of mice and rats is much lower than in women: Because the human uterus and utero–tubal junction represent a unique morphofunctional entity and the mixing of uterine and oviduct fluids may be possible (Hunter 2002), lactoferrin from oviductal and uterine origin could converge in the human fluids. This anatomical difference might explain the difference between humans and other mammals regarding lactoferrin concentrations in the oviductal fluid. Lactoferrin-null mice are fertile (table 3), and their offspring grow normally (Ward et al. 2003). Like mouse oviductin, the function of lactoferrin may be maximizing the incidence of normal fertilization *in vivo*.

Fetuin-B. Fetuin, like plasminogen, is a liver-derived plasma protein, but it belongs to a family of protease inhibitors. Although still not identified in the oviduct, fetuin inhibits the hardening of the ZP during *in vitro* maturation of ocytes in the mouse and rat (Schroeder et al. 1990), and it was thought that its presence in follicular fluid contributed to inhibiting CG proteases in immature oocytes before ovulation (Schroeder et al. 1990, Dell'Aquila et al. 1999). From these observations, an attempt was made using fetuin as an agent to prevent the hardening of the ZP during horse oocyte maturation *in vitro*, but the results did not improve the success of IVF (Dell'Aquila et al. 1999). The presence of fetuin in the oviductal fluid remains to be confirmed.

Recently, fetuin has been reported as a protein essential for fertilization (Dietzel et al. 2013). Fetuin-null female mice are infertile because of excessive hardening of the ZP; their infertility is restored only by perforating the ZP using laser beams (Dietzel et al. 2013). By contrast, in cattle, pigs, goats, and sheep, zona hardening occurs normally before fertilization (Coy et al. 2008, Mondéjar et al. 2013), and it has been considered a mechanism contributing to regulate polyspermy (pig), although other roles related to sperm selection cannot be discarded (Coy and Avilés 2010). From these data, it is important to reflect on the usefulness of the transgenic knockout mice models to infer the roles of different genes in species other than mice. As is the case with fetuin or ovastacin, it seems clear that some mechanisms occurring in the follicle or in the oviduct and affecting fertilization are not equally important in the different species (table 4). Other animal models, such as the pig or cow, might be helpful in deciphering the oviductal mechanisms affecting gamete interactions and initial embryo cleavage.

Other proteins. Haptoglobin has been identified in cow, pig, and rabbit oviductal tissues and fluids (Lavery et al. 2003, Herrler et al. 2004, Georgiou et al. 2005), but its specific effect on sperm–oocyte interaction has not been elucidated. Similarly, more studies are needed for Spam1 (PH-20), which was detected in the oviductal luminal epithelium and mesothelium of the mouse (Zhang and Martin-DeLeon 2003). It has been proposed that this protein with hyaluronidase activity binds to spermatozoa within the oviduct to assist sperm passage through cumulus oophorus before fertilization (Griffiths et al. 2008).

A major issue to be considered for any of the proteins revisited in the present study is the fact that results from the laboratory, even if they were promising, cannot be assumed as useful at the clinical level until the corresponding trials are performed. A clear example is the case of the granulocite–macrophage colony stimulation factor (GMC-SF). It was shown that this cytokine, added into two different commercial IVF media, increased the proportion of human embryos developing from 2- to 4-cell stage to blastocysts, as well as their inner cell mass size and their development competence assessed by hatching (Sjöblom et al. 1999). However, when these results were translated into the clinical assays some years later, it was observed that the effect of GMC-SF was depending on the concentration of human serum albumin

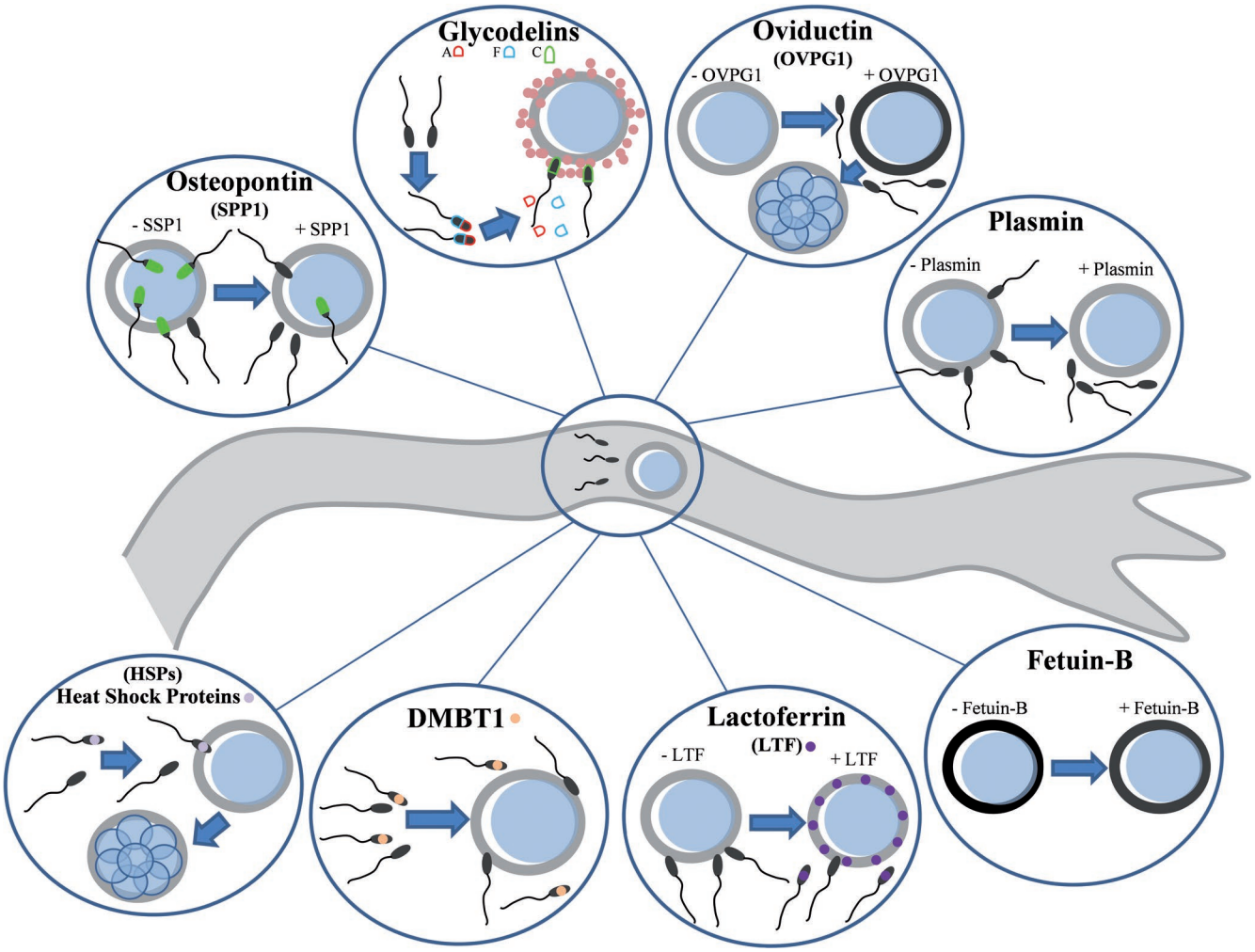

Figure 2. A summary of the proposed roles for some oviductal proteins in mammalian fertilization and embryo development. Osteopontin reduces the sperm acrosome reaction and contributes to the regulation of polyspermy. Glycodelins A and F within follicular and oviductal fluids inhibit the premature acrosome reaction of spermatozoa and sperm binding to the zona, whereas glycodelin C within the cumulus oophorus displaces A and F, enhancing sperm binding to the zona pellucida and acrosome reaction. Oviductin produces zona hardening and decreases sperm–zona binding, helping to avoid polyspermy and to enhance embryo development. Plasmin breaks sperm–zona attachments, avoiding the oocyte's penetration by supernumerary spermatozoa. Different heat shock proteins have different roles in sperm function and embryo development. Deleted in malignant brain tumors 1 binds to capacitating spermatozoa, contributing to negative sperm selection. Lactoferrin binds to capacitated spermatozoa, as well as to the oocyte's zona pellucida, and inhibits sperm–zona binding, reducing the chance of polyspermic fertilization. Fetuin prevents premature zona hardening.

(HSA) in the culture medium and that the benefit was only apparent when GMC-SF was added with the lower HSA concentration, particularly in women with a previous miscarriage (Ziebe et al. 2013). Therefore, protein interactions must be always taken into account when one is trying to extrapolate the laboratory data into real physiological environments in living organisms.

Conclusions

The oviductal fluid contains a large number of proteins with different effects on the fertilization process and the development of preimplantation embryos (figure 2). Some have been studied intensely, but many others have not. Moreover, proteins rarely act alone, and the interactomic system of a living tissue and the corresponding fluid (such as the oviduct) forms the basis of its normal functioning. In this sense, interactions among oviductal proteins might be crucial for their effects on embryo development and, perhaps, on epigenetic reprogramming.

Today, we use chemically defined (without proteins) or semidefined media (with bovine serum albumin) for ARTs. However, these media have important limitations, the most apparent being the lack of a variety of individual proteins and their possible interactions. It is proposed that a great

percentage of failures in the success of the *in vitro* production of embryos in the different species is due to the lack of specific proteins in the culture media. In fact, it was shown in a recent clinical trial that the use of serum globulins as protein additives in the culture media may increase human births by more than 8% (Meintjes et al. 2009).

Discrepancies among species regarding basic biological mechanisms, such as the regulation of polyspermy, are evident with the data provided by *in vitro* experiments. However, it is possible that such differences might be more minor than one might expect if studies could be done *in vivo*. So far, the only species in which the genetic studies have been possible, because of the complexity of producing animals knocked out for specific genes, has been the mouse. However, the recent developing of the CRISPR/Cas9 system for knocking in and out target genes might offer the same opportunity in other species, and some *in vivo* studies could show unexpected similarities in some of the mechanisms discussed here.

We have described the specific role of some oviductal-secreted proteins in fertilization and embryo development and how the same protein may have a different importance in different species. However, there is a long way to go before identifying the function, concentration, and optimum time for adding into the culture medium each protein included in the reproductive fluids. In addition, a theoretical culture medium that could include as additives all the oviductal or uterine proteins, produced in laboratories as recombinant proteins, would cost an unaffordable amount of money. Blood serum cannot be the right source of protein for the culture media, but oviductal and uterine fluids could be. If blood serum or mother's milk are directly collected from living animals or human donors because they are better than their artificial counterparts, why not collect reproductive fluids and include them in biobanks to validate their use in ARTs? Data showing the improvement of IVF results by adding oviductal fluid in the culture media are already available, as well as protocols to collect and store pig and cow undiluted oviductal fluids (Carrasco et al. 2008a, 2008b; patent P201400811). Similar procedures to collect human fluids could be used from volunteer donors attending hospitals or infertility clinics. We propose that studies addressing the development of technologies allowing the collection of oviductal and uterine samples under controlled sanitary conditions in different mammalian species, including women, are necessary for the future improvement of yield and quality of *in vitro* produced embryos.

A number of experiments in the mouse and large animal models as well as epidemiologic studies in humans support our proposal. It has been shown that the use of ARTs can perturb epigenetic gene regulation, leading to abnormal phenotypes (El Hajj and Haaf 2013). Children born after human ART suffer from an increased rate of birth defects and a two- to threefold increased rate of low birth weight (Schieve et al. 2002). There is cumulative evidence in mice and humans that the embryo is sensitive to its very early environment and that the culture media used in ARTs may have long-lasting consequences (Kleijkers et al. 2014). The importance of the stresses to which the embryos have to adapt in artificial environments provided by chemically defined media is today widely recognized. Obesity, increased anxiety, and deficiencies in memory are examples observed in mice due to alterations in the genomic imprinting during embryo culture (Fernandez-Gonzalez et al. 2004). In humans, an adverse periconceptional and/or prenatal environment has been associated with increased rates for many metabolic and cardiovascular diseases (NCDs) in adulthood, and low birth rate is associated with type-2 diabetes, obesity, and hypertension (Gluckman et al. 2010). Despite the vast majority of ART children appearing to be healthy, there is still a lack of large longitudinal studies on the health of adolescents or adults, and the subtle epigenetic effects of ART modulating the susceptibilities to various complex diseases cannot be excluded (El Hajj and Haaf 2013).

Therefore, it is possible that including reproductive fluids in the human and animal ARTs may reduce the adverse periconceptional environment in *in vitro* derived embryos, and lower NCDs rates in the future could be reached. In addition, the efficiency of the current systems for *in vitro* embryo production in the livestock market and, consequently, the trade of embryos all over the world would be also increased.

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