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—Mini Review—

Aging of Oocyte-coating Structures and Dicalcin

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Abstract: *Maternal fertility decreases with aging, possibly owing to qualitative changes in the egg itself and the egg-housing condition of the female reproductive tract. Since sperm first interact with the egg-coating structures, age-dependent alterations of egg-coating structures may be related to reduced fertility in aged female mammals. Quantitative genetic and biochemical studies of specimens prepared from aged mammals have revealed altered expression patterns of antioxidant- and apoptosis-related proteins in cumulus cells, and a substantial decrease in the amount of ZP-constituent glycoproteins. Histochemical studies have demonstrated an increase in the number of apoptotic cumulus cells and significant alteration in the appearance of ZP with irregular plaques in aged specimens. Biophysical studies have shown that both susceptibility to digestion with protease and mechanical stiffness are reduced in the ZP aged in vitro. We recently characterized a novel suppressive factor of fertilization, dicalcin, in the cumulus-oocyte complex. The expression level of dicalcin in the cultured normal human fibroblasts increases with the passage of time, which implies an age-dependent increase in its expression in the normal female reproductive tract. The potential increase in dicalcin expression with aging would represent a qualitative change of the egg-coating structure, augment its inhibitory role on fertilization, thereby causing decreased fertility in aged female mammals.*
Key words: *Aging, Fertilization, Zona pellucida, Cumulus cell, Dicalcin*

Introduction

It is well known that the fertilization rate decreases as maternal age increases, and this decrease has often been attributed to the gradual depletion of oocytes as well as to a decrease in oocyte quality with aging [1–4]. However, natural fertilization occurs in the oviductal lumen as a result of sequential, well-coordinated sperm-egg interaction, and therefore the decreased fertility observed naturally may involve gradually-occurring dysfunction of fertilization processes. Since the fertilization process begins with the proper interaction between sperm and egg-coating structures, characterization of age-dependent changes in egg-coating structures will be helpful for understanding the molecular basis of reduced fertilization with aging.

The innate egg-coating structures of eutherian mammals are composed of (i) the zona pellucida (ZP), a relatively thick, elastic acellular coat comprised of several glycoproteins, which lies immediately next to the egg, and (ii) the cumulus oophorus, the outermost layer of the cumulus-oocyte complex (COC), which is constituted by cumulus cells and fibrous extracellular matrix, including hyaluronic acid (HA) and fibronectin [5]. According to the currently prevailing model of fertilization processes, acrosome-intact capacitated sperm recognize oligosaccharides of ZP-constituent glycoproteins, and then sperm initiate acrosomal reaction (AR), penetrates the ZP, and fuses with egg plasma membrane (for reviews see [6–11]). Recent studies using advanced genetic and recording techniques have revealed new and valuable details regarding mechanisms of fertilization. For example, Gahlay *et al.* found that eggs from transgenic mice, mutated to prevent glycosylation on ZP proteins, are still capable of binding to sperm and being fertilized, which suggests that oligosaccharides on ZP proteins may not be necessary for sperm-binding and induction of the AR [12]. Jin *et al.* demonstrated that most fertilizing murine sperm undergo the AR during the passage through the cumulus oophorus, which strongly suggests that the site of initiation of the AR is somewhere within the cumulus

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oophorus, not the ZP [13]. As confirmed by many previous studies, the ZP certainly has the ability to induce the AR, but the ZP may not be the sole site for AR initiation. Instead, the cumulus oophorus is likely to be essential for induction of the AR and subsequent fertilization success, particularly under physiological conditions. Thus, although much attention has been paid to the ZP as a uniquely important extracellular coat of the egg, we consider the cumulus oophorus as an essential part of the egg-coating structure in this review.

We have recently identified a novel suppressive mediator of fertilization, named dicalcin. Dicalcin was originally identified in frog species (*Xenopus laevis* and *Rana catesbeiana*) [14, 15], and the primary structure of dicalcin consists of two S100-protein-like regions connected by a linker region, featuring this single molecule as a "dimer form of S100 calcium-binding protein". S100 proteins constitute a family of small (10-14 kDa) calcium-binding proteins and, to date, more than twenty members have been identified. S100 proteins exist as homo or hetero dimers in solution, regulate various extra- and intracellular events, thereby exerting important functions in a variety of cells (for reviews see [16, 17]). Frog dicalcin is present in the vitelline envelope (VE), a structure equivalent to the mammalian ZP and, through binding to VE-constituent glycoprotein, it remarkably inhibits fertilization *in vitro* [18]. Most recently, our phylogenetic and biochemical analyses revealed that mouse S100A11, one of the S100 protein family, is the mouse counterpart of frog dicalcin. Mouse S100A11, which is thereafter referred to as mouse dicalcin to make this review more readily understandable, is present in the plasma membrane of cumulus cells in the COC in the oviduct, and suppresses *in vitro* fertilization in a dose-dependent manner [19].

Many studies have demonstrated that significant age-related changes occur in the ZP as well as in the cumulus cell layer. However, the results have not yet been collated, and therefore it seems timely to review the age-related details regarding the egg-coating structures. In this review, after a brief description of the biology of the egg-coating structures and the effects of dicalcin on fertilization, we will introduce the age-related changes in these structures, and discuss possible age-dependent change of dicalcin expression and its involvement with reduced fertility in advanced aged mammals. The term of "aging" sometimes has different meanings in the literatures, depending upon the experimental conditions utilized therein. For example, it means: (i) to grow older, (ii) the passage of time within the oviductal lumen and (iii) the passage of time under some experimental conditions after preparation or dissection. To avoid potential confusion, in this review, we refer particularly to the latter two cases, (ii) and (iii), as "*in vivo* aging" and "*in vitro* aging", respectively.

Cumulus Cells

Composition and metabolism

The cumulus oophorus comprises cumulus cells and fibrous extracellular matrix, including HA and fibronectin [5]. There is a close intercellular communication between the oocyte and cumulus cells via gap junctions and paracrine factors, and therefore the metabolism of oocyte and cumulus cells is mutually dependent (for review see [20]). Oocytes secrete soluble factors, including GDF9 and BMP15, and regulate cumulus cell differentiation and maturation, and conversely cumulus cells provide nutritional support and environmental information mediated by cAMP (for reviews see [21, 22]). Around the ovulatory phase, cumulus cells starts to synthesize and secrete HA, the major structural backbone of the cumulus matrix, and the COC gradually expands in the so- called cumulus expansion. Cumulus expansion is considered to be important for ovulation because inhibition of HA generation *in vivo* decreases the ovulation rate in the mouse [23]. During cumulus expansion, HA molecules are held together by several cumulus cell- and serum-derived proteins, including inter-α-trypsin inhibitor (IαI), tumor necrosis factor α-induced protein 6 (TNFAIP6 or TSG6) and pentraxin 3, and all of them are considered to strengthen and stabilize the HA network. Knockout studies have demonstrated the crucial role of these HA-stabilizing proteins during *in vivo* fertilization [24–27].

Function

Since the cumulus is absent at fertilization in some species such as the goat, and the eggs without the cumulus are able to fertilize *in vitro* [28–30], the cumulus oophorus has sometimes been considered as unnecessary for fertilization success [11]. However, in other species, including mice, hamsters and pigs, the presence of the cumulus is beneficial for *in vitro* fertilization since the rate of fertilization increases compared to that in its absence [31]. Interestingly, recent studies have demonstrated some physiological actions of cumulus cells, which contributes to fertilization success. Tamba *et al.* demonstrated that cumulus cells produce chemokines, such as CCL7, that serve to attract sperm. Subsequently, they activate

prostaglandin E_2/cAMP signaling, and loosen the extracellular fibronectin network among cumulus cells, which ultimately makes better microenvironment for sperm penetration [32]. Hasegawa *et al*. showed that cumulus cells secrete a glycosylphosphatidylinositol-anchored protein to prevent complement-dependent activation of sperm in the oviduct [33]. Most recently, Jin *et al*. combined genetic engineering and real-time video recording to demonstrate that sperm undergo the AR while passing through the cumulus layer and acquire the ability to fertilize the egg [13]. Thus, the cumulus layer is likely to play a crucial role in naturally occurring fertilization, at least in some mammalian species.

Age-dependent Changes of Cumulus Cells

Changes in gene expression levels

A quantitative PCR analysis has shown that creatin kinase B (CKB) and peroxiredoxin 2 (PRDX2) are expressed at higher levels (~6–8 fold increases) in older (≥38 years) women than in younger (≤28 years) women [34]. In addition, an immunohistochemical study found that the percentage of cumulus cells immunopositive for apoptosis-related proteins was altered in aged women [35]. In that study, the mean percentage of Fas-immunopositive cumulus cells was found to be significantly lower in the older (≥38 years) group than that in the younger (<38 years) group (~30% of younger group vs. ~23% of older group), and the percentage of cumulus cells immunopositive for its cognate ligand, Fas-L, was, in reverse, greater in the older group (~9% of the younger group vs. ~14% of the older group) [35]. Altered expression levels of antioxidant- (CKB and PRDX2) and apoptosis-related (Fas and Fas-L) proteins may be related to some mechanisms for defending cumulus cells against oxidative stress, and thereby serve to prevent their apoptosis in older women.

Increase in the number of apoptotic cumulus cells

There are some clinical reports suggesting a positive relationship between the age-dependent increase in the percentage of apoptotic cumulus cells and the fertilization rate. Lee *et al.* showed that the incidence of apoptotic cumulus cells is significantly higher in older (≥41 years) women than in younger (≤30 years) women (~1.6% of the older group vs. ~0.6% of the younger group), and the fertilization rate following IVF-ET of identical individuals was significantly reduced in older women compared with younger women (~40% of the older group vs. ~88% of the younger group) [36]. Høst *et al*. also reported that at the ICSI treatment, the percentage of apoptotic cumulus cells in non-fertilized COCs is greater (~8.5%) than that (~5.1%) in fertilized COCs [37]. The results of these studies suggest an age-dependent correlation between the apoptosis of cumulus cells and the fertilization rate; therefore, the apoptotic status of the whole cumulus layer may affect the establishment of the microenvironment for fertilization success.

The Zona Pellucida

Appearance

The thickness of ZP shows considerable variation across species (e.g., 1–2 μm thin in marsupials and 16 μm in the pig) [38]. At the light microscopic level, ZP has an optically homogeneous structure in some species (e.g., mice and rats), whereas it has two or more distinct layers with different optical densities in others (e.g., rabbits and pigs) [38]. Scanning electron microscopical analyses have revealed that the outer surface of the ZP has a spongy appearance with 0.3–0.5 μm pores, whereas the inner one is more densely packed and has a fine granular or microtubular appearance in most mammals [39]. Since the outer ZP with fenestration is more penetrable for sperm in humans [40], the spongy appearance is considered to reflect the maturity of the ZP and favor sperm penetrability.

Composition

ZP is composed of several substances, including protein, sugar and inorganics. For example, porcine ZP contains protein (71%), neutral hexose (19%), sialic acid (2.7%), and sulfate (2.4%) [41]. The major constitutive proteins are represented by a few different ZP proteins. For example, the human ZP contains four ZP proteins: ZP1 (100 kDa), ZP2 (~75 kDa), ZP3 (~55 kDa) and ZP4 (~65kDa), and the murine ZP has three ZP protein: ZP1 (~200kDa), ZP2(~120 kDa) and ZP3(~83kDa) (for a review see [42]). There is a comparable sequence homology among ZP proteins in different species. Sequence homologies of ZP2 and ZP3 among different mammals are relatively high (e.g., ~57% identity between human and mouse ZP2 and ~67% for ZP3), whereas ZP1 shows a lesser homology (~33% between human and mouse ZP1) [43]. ZP proteins are synthesized in growing oocytes, secreted simultaneously and assembled with each other by noncovalent interactions, forming ZP filaments within the ZP. The association of ZP proteins depends upon

the presence of a hallmark ~260-amino-acid-sequence motif, called the ZP domain, that is conserved among ZP proteins in several tissues, including epithelial cells and neural tissues [44]. On the basis of stoichiometry of the interaction between ZP proteins, the schematic arrangement of ZP filaments is assumed as follows: ZP2 and ZP3 constitute a long filament with a width of ~7 nm, and ZP1 crosslinks each filament [45].

Oligosaccharide distribution within the ZP

During the past few decades, oligosaccharides coupled to ZP proteins have been considered to play a key role in the interaction between sperm and the ZP in a variety of species, including mammals and amphibians (for a review see [7]). An immunohistochemical study of ZP proteins of various species (e.g., mouse, rat, hamster, rabbit, cat, dog and pig) demonstrated species-specific variations in lectin-binding patterns, and the extent of the variation was correlated with the evolutionary relationship among the species: greater similarity between closely related species and lower one between distantly related species [46]. For example, *Dolichos biflorus* agglutinin (DBA, a lectin with a preferential binding to α-GalNac terminal) binds only to the mouse ZP proteins, whereas *Griffonia simplicifolia* (GS-I, preferential binding to α-Gal) binds to ZP proteins of mice and rats, but not to ZP proteins of hamsters and rabbits. The same study also showed that lectin-reactive residues were unevenly distributed throughout the depth of the ZP. For example, DBA and GS-I reacted with the inner portion of the ZP, whereas *Ricinus communis* agglutinin I (RCA-I, preferential binding to β-Gal), wheat germ agglutinin (WGA, preferential binding to GlcNAc) and peanut agglutinin (PNA, Gal-β1-3-GalNAc) interacted with the outer portion of the ZP. Thus, specific oligosaccharides such as α-Gal, α-GalNAc are distributed differently throughout the same ZP, and these variations in distribution may underlie molecular mechanisms involved with species-specific interactions between sperm and ZP.

Association of oviductal proteins with the ZP

Several lines of evidence have shown that the ZP interacts with some oviductal proteins that exist extracellularly in the oviductal lumen (for reviews see [47, 48]). They include oviductin and osteopontin, both of which are known to be secreted from the oviductal epithelium into the oviduct and act there on the COC, affecting the efficiency of fertilization. Immunohistochemical studies have shown that exogenously applied oviductin and osteopontin interact with bovine ZP, demonstrating a uniform distribution throughout the ZP [49]. However, their target ZP proteins and the precise mechanism of their actions remain unknown.

Age-dependent Changes of the ZP

ZP appearance and expression levels of ZP proteins

Scanning electron microscopic observation have revealed that the ZP of oocytes, dissected from the oviduct 37 h after an hCG injection (i.e. *in vivo* aged ZP), had irregular plaques and ZP-constituent filaments were separated from one another by a space of ~0.3 μm in width [50]. A quantitative microarray analysis using young (5- to 6-weeks) and old (42- to 45-weeks) murine oocytes demonstrated that the amounts of ZP1, ZP2 and ZP3 mRNAs in old oocytes declined to 48%, 42% and 37% of those in young oocytes, respectively [51]. Another quantitative PCR analysis using young (170-d-o) and old (4-y-o) gilt oocytes showed that the copy numbers of ZP1, ZP2 and ZP3 in older oocytes were reduced to 87%, 78% and 68% of those in younger oocytes, respectively [52]. Thus, the expression level of every ZP protein decreases with aging, although the precise relationship between the expression level of ZP proteins and the fertilization rate remains unknown.

Oligosaccharide distribution within the ZP

Since oligosaccharides within the ZP are considered to be the essential signal for sperm-ZP interaction, age-dependent alterations in their distribution patterns within the ZP was expected to be observed. However, no differences with aging have yet been demonstrated. An immunohistochemical study by Longo *et al*. reported unaltered staining patterns of ConA and ferritin between “*in vitro*“ aged (12 h after dissection from the oviduct) and unaged ZPs (1 h after dissection): ConA stained the inner layer of the ZP and polycationized ferritin stained the whole ZP uniformly [50].

Susceptibility of ZP to digestion with protease

After fertilization, ZP modification occurs as a consequence of cortical granule exocytosis and chemical reaction by an internal substance, possibly glycosidase, which changes the biochemical properties of the ZP. Due to this modification, the ZP of the fertilized egg becomes more resistant to proteolysis, which is considered to prevent polyspermy (for a review see [53]). An alteration of ZP similar to the above has also been shown to occur spontaneously in the course of oocyte aging in an *in vitro* experiment. During the

treatment of mouse ZP with α-chymotrypsin, the dissolution rate declined to ~50% of the control in *in vitro* aged eggs (12 h after dissection from the oviduct) compared with that of the control (1 h after dissection) [50], which implies that potential reorganization of the ZP occurs in aged oocytes, thereby causing the observed decrease in fertility and penetrability of *in vitro* aged COCs [54–56].

Mechanical stiffness of ZP

Vision-based *in situ* cellular force measurement has enabled the characterization of the mechanical stiffness of ZP-bearing oocytes. It should be noted that this stiffness represents the overall stiffness of oocytes, i.e. oocyte itself and ZP. According to this measurement, old (40~45-weeks) oocytes have significantly lower stiffness compared with young (8~12-weeks) oocytes (~51% of the force necessary for the identical deflection in the young oocytes) [57], which suggests that aged ZP is "softer" in the physical sense.

Dicalcin

Identification as a novel suppressive factor of fertilization in Xenopus laevis

Dicalcin is a recently identified, novel suppressive mediator of fertilization. In the frog *Xenopus laevis*, dicalcin is localized prominently in the frog egg-coating extracellular envelope, called the vitelline envelope (VE), and binds to frog gp41 (the equivalent molecule of mammalian ZP3). Exogenously applied dicalcin inhibits sperm-binding to the VE and sperm-penetration through the VE, resulting in a remarkable dose-dependent reduction in the efficiency of *in vitro* fertilization (~14 % of control in the presence of 4 μM dicalcin). Meanwhile, the inactivation of endogenous dicalcin by the specific antibody dramatically increases the efficiency of fertilization (~208% of control in the presence of 50 mg/L antibody) [18]. Thus, the efficiency of *in vitro* fertilization is dependent upon the amount of dicalcin within the frog VE. We further characterized the molecular action of frog dicalcin in extensive biochemical experiments, and our results strongly suggest that dicalcin induces some conformational changes in gp41, through binding to the protein core portion of gp41, and probably regulates the surface configuration of oligosaccharides on gp41 as well as the three-dimensional structure of the entire filamentous VE, which ultimately leads to alteration of an overall distribution of oligosaccharides within the VE (Fig. 1).

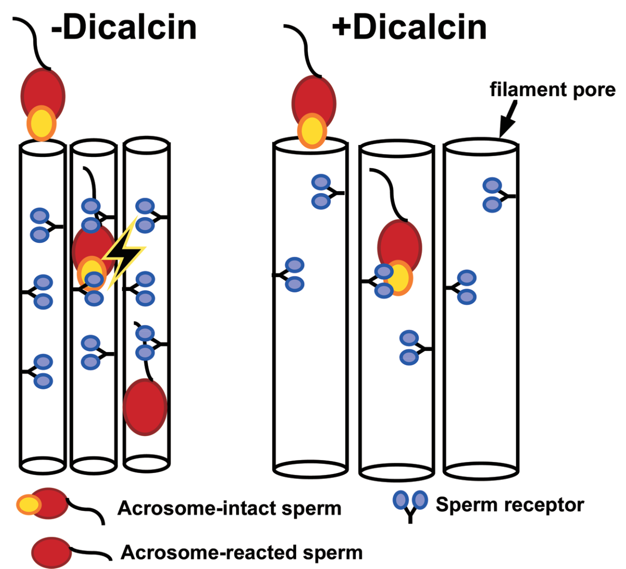

Fig. 1. Schematic model of the suppressive action of frog dicalcin during fertilization.
The frog egg-coating envelope (called the vitelline envelope, VE) contains a filamentous network, pores of which are illustrated in the cylindrical form (filament pore). In the absence of dicalcin (-Dicalcin), a number of sperm-receptors expose to the lumen of the pore and the pore size is proper for the induction of acrosome reaction (AR). In this condition, when sperm enter the VE, they are capable of undergoing the AR and subsequently penetrating the VE. Frog dicalcin binds to gp41, a major VE-constituent glycoprotein, leading to allosteric conformational change of gp41 as well as changes in the three-dimensional structure of VE filaments. These changes induce disorganization of VE filaments: e.g., the sparse distribution of sperm-receptors and the enlargement of the VE pore size (+Dicalcin), which prevents induction of the AR and ultimately causes a remarkable reduction in the fertilization efficiency.

Mouse dicalcin as a potential suppressive factor of fertilization

Most recently, we also identified the mouse counterpart of frog dicalcin, and found that mouse dicalcin also suppresses the efficiency of *in vitro* fertilization in a dose dependent manner [19]. Our molecular phylogenetic and histochemical analyses, revealed that the primary structure and distribution of mouse dicalcin are slightly different from those in the case of frog dicalcin. The molecular weight of frog dicalcin is ~24 kDa, whereas that of the mouse counterpart is ~10 kDa. Frog dicalcin is localized within the VE, whereas mouse dicalcin is not localized significantly in the ZP, and instead, it exists on the

Table 1. Studies on the analyses of age-dependent changes of egg-coating structures

Structure	Source	Method	Part of outcomes	References
Cumulus oophorus	human	Immunostaining	Decreased percentage of Fas-immunopositive cumulus cells	Moffatto *et al*. (2002)
			Increased percentage of Fas-L-immunopositive cumuls cells	
	human	Microarray Quantitative PCR	Increased expression levels of creatine kinase B and peroxiredoxin 2	Lee *et al*. (2010)
	human	Immunostaining	Increased level of percentage of apoptotic cumulus cells	Lee *et al*. (2001) Høst *et al*. (2002)
Zona pellucida	mouse	Scanning electron microscopy	Existence of irregular plaques	Longo (1981)
	mouse	Microarray	Reduced expression levels of ZP proteins	Hamatani *et al*. (2004)
	pig	Quantitative PCR	Reduced expression levels of ZP proteins	Kempisty *et al*. (2009)
	mouse	Biochemical assay	Reduced dissolution rate for the digestion with α-chymotrypsin	Longo (1981)
	mouse	Biophysical assay	Lower stiffness	Liu *et al*. (2010)

plasma membrane of cumulus cells in the COC in the oviduct. These differences between frog and mouse dicalcin may reflect the difference in fertilization competency between them: frog eggs are ready for fertilization when they are oviposited, whereas murine eggs are fully susceptible to fertilization when they are ovulated and deposited in the oviduct as the COC. It appears that dicalcin exists in the egg-coating structures at the time of fertilization-competence, albeit with a slightly different pattern of distribution across species. In the case of mice, the distinct localization on the cumulus cell membrane strongly suggests its action on cumulus cells, and we are currently trying to elucidate the precise mechanism of the action of mouse dicalcin on the COC.

Possible involvement of dicalcin in age-related changes of oocyte-coating structures

The expression level of mouse dicalcin has been examined in the aging process in culture. This *in vitro* aging ultimately results in irreversible cessation of cell growth, which is referred to as cellular senescence [58], and may mimic some cellular mechanisms underlying aging process *in vivo*. During culture for 12 days, the expression of mouse dicalcin increases by 5-fold, and is accompanied by accumulation in the nuclei of normal human fibroblasts [59]. Our recent study demonstrated that mouse dicalcin is localized in the oviductal epithelial cells and the membrane of cumulus cells of COCs within the oviduct [19]. It is of interest to examine whether the expression level of mouse dicalcin on the cumulus cell membrane increases with aging. If so, its inhibitory action on fertilization would be augmented, which may cause decreased fertility in females with advanced age. It is also interesting to investigate its expression level on the cumulus membrane with the passage of time in the oviduct (i.e. *in vivo* aged COCs). If ovulated COCs spend longer within the oviductal lumen, they would likely express a greater amounts of dicalcin, which may account for the reduced fertility of *in vivo* aged COCs.

Conclusion

This review focused on age-dependent alterations of egg-coating structures, including the cumulus oophorus and the zona pellucida, and described the biological properties of dicalcin, a suppressive mediator of fertilization, that regulates the overall distribution pattern of oligosaccharides within the frog egg-coating structure. Some biochemical and biophysical properties of egg-coating structures (summarized in Table 1) and possible alteration of dicalcin expression in the female reproductive tract may be causative mechanisms in reduced fertility in aged females. Future studies for understanding the molecular basis for these changes may provide possible treatments for infertility in aged women.

References

1) Armstrong, D.T. (2001): Effects of maternal age on oocyte developmental competence, Theriogenology, 55, 1303–

1322.
2) Klein, J. and Sauer, M.V. (2001): Assessing fertility in women of advanced reproductive age. Am. J. Obstet. Gynecol., 185, 758–770.
3) Navot, D., Bergh, P.A., Williams, M.A., Garrisi, G.J., Guzman, I., Sandler, B. and Grunfeld, L. (1991): Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility. Lancet, 337, 1375–1377.
4) Piette, C., de Mouzon, J., Bachelot, A. and Spira, A. (1990): In-vitro fertilization: influence of women's age on pregnancy rates. Hum. Reprod., 5, 56–59.
5) Yudin, A.I., Cherr, G.N. and Katz, D.F. (1988): Structure of the cumulus matrix and zona pellucida in the golden hamster: a new view of sperm interaction with oocyte-associated extracellular matrices. Cell Tissue Res., 251, 555–564.
6) Tanphaichitr, N., Carmona, E., Bou Khalil, M., Xu, H., Berger, T. and Gerton, GL. (2007): New insights into sperm-zona pellucida interaction: Involvement of sperm lipid rafts. Front. Biosci., 12, 1748–1766.
7) Clark, G.F. and Dell, A. (2006): Molecular models for murine sperm-egg binding. J. Biol. Chem., 281, 13853–13856.
8) Hoodbhoy, T. and Dean, J. (2004): Insights into the molecular basis of sperm-egg recognition in mammals. Reproduction, 127, 417–422.
9) Evans, J.P. (2002): The molecular basis of sperm-oocyte membrane interactions during mammalian fertilization. Hum. Reprod. Update., 8, 297–311.
10) Primakoff, P. and Myles, D.G. (2002): Penetration, adhesion, and fusion in mammalian sperm-egg interaction. Science, 296, 2183–2185.
11) Yanagimachi, R. (1994): Mammalian fertilization. In: The physiology of reproduction (Knobil, E. Neill, JD., eds.) pp.189–317, Raven Press, New York.
12) Gahlay, G., Gauthier, L., Baibakov, B., Epifano, O. and Dean, J. (2010): Gamate Recognition in Mice Depends on the Cleavage Status of an Egg's Zona Pellucida Protein. Science, 329, 216–219.
13) Jin, M., Fujiwara, E., Kakiuchi, Y., Okabe, M., Satouh, Y., Baba, S., Chiba, K. and Hirohashi, N. (2010): Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. Proc. Natl. Acad. Sci. USA., 22, 4892–4896.
14) Miwa, N., Shinmyo, Y. and Kawamura, S. (2007): Cloning and characterization of *Xenopus* dicalcin, a novel S100-like calcium-binding protein in *Xenopus* eggs. DNA Seq., 18, 400–404.
15) Miwa, N., Kobayashi, M., Takamatsu, K. and Kawamura, S. (1998): Purification and molecular cloning of a novel calcium-binding protein, p26olf, in the frog olfactory epithelium. Biochem. Biophys. Res. Commun., 251, 860–867.
16) Donato, R. (2003): Intracellular and extracellular roles of S100 proteins. Microsc. Res. Tech., 60, 540–551.
17) Heizmann, C.W., Fritz, G., Schafer, B.W. (2002): S100 proteins: Structure, functions and pathology. Front. Biosci., 7, 1356–1368.
18) Miwa, N., Ogawa, M., Shinmyo, Y., Takamatsu, K. and Kawamura, S. (2010): Dicalcin inhibits fertilization through its binding to a glycoprotein in the egg envelope in *Xenopus laevis*. J. Biol. Chem., 285, 15627–15636.
19) Hanaue, M., Miwa, N., Uebi, T., Fukuda, Y., Katagiri, Y. and Takamatsu, K. (2011): Characterization of S100A11, a suppressive factor of fertilization, in the mouse female reproductive tract. Mol. Reprod. Dev., 78, 91–103.
20) Huang, Z. and Wells, D. (2010): The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome. Mol. Hum. Reprod., 16, 715–725.
21) Sutton-McDowall, M.L., Gilchrist, R.B. and Thompson, J.G. (2010): The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction, 139, 685–695.
22) Schuetz, A.W. and Dubin, N.H. (1981): Progesterone and prostaglandin secretion by ovulated rat cumulus cell-oocyte complexes. Endocrinology, 108, 457–463.
23) Chen, L., Russell, P.T. and Larsen, W.J. (1993): Functional significance of cumulus expansion in the mouse: role for the preovulatory synthesis of hyaluronic acid within the cumulus mass. Mol. Reprod. Dev., 34, 87–93.
24) Salustri, A., Garlanda, C., Hirsch, E., De Acetis, M., Maccagno, A., Bottazzi, B., Doni, A., Bastone, A., Mantovani, G, Beck Peccoz, P, Salvatori, G., Mahoney, D.J. Day, A.J., Siracusa, G., Romani, L and Mantovani, A. (2004): PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization. Development, 131, 1577–1586.
25) Varani, S, Elvin, J.A., Yan, C., DeMayo, J., DeMayo, F.J., Horton, H.F., Byrne, M.C. and Matzuk, M.M. (2002): Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. Mol Endocrinol., 16, 1154–1167.
26) Fulop, C., Szanto, S., Mukhopadhyay, D., Bardos, T., Kamath, R.V., Rugg, M.S., Day, A.J., Salustri, A.J., Hascall, V.C., Glant, T.T. and Mikecz, K. (2003): Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. Development, 130, 2253–2261.
27) Zhuo, L, Yoneda, M., Zhao, M., Yingsung, W., Yoshida, N., Kitagawa, Y., Kawamura, K, Suzuki, T. and Kimata K. (2001): Defect in SHAP-Hyaluronan complex causes severe female infertility. J. Biol. Chem., 276, 7693–7696.
28) Cox J.F (1991): Effect of the cumulus on *in vitro* fertilization of *in vitro* matured cow and sheep oocytes. Theriogenology, 35, 191.
29) Mahadevan, M.M. and Trounson, A.O. (1985): Removal of the cumulus oophorus from the human oocyte for in vitro fertilization. Fertil. Steril., 43, 263–267.
30) Amoroso, E.C., Griffiths, W.F., Hamilton, W.J. (1942): The early development of the goat (*Capra hircus*). J. Anat., 76, 377–406.
31) Kikuchi, K., Nagai, T., Motlik, J., Shioya, Y. and Izaike, Y.

(1993): Effect of follicle cells on in vitro fertilization of pig follicular oocytes. Theriogenology, 39, 593–599.
32) Tamba, S., Yodoi, R., Segi-Nishida, E., Ichikawa, A., Narumiya, S. and Sugimoto, Y. (2008): Timely interaction between prostaglandin and chemokine signaling in a prerequisite for successful fertilization. Proc. Natl. Acad. Sci. USA., 105, 14539–14544.
33) Hasegawa, A., Takenobu, T., Kasumi, H., Komori, S. and Koyama, K. (2008): CD52 is synthesized in cumulus cells and secreted into the cumulus matrix during ovulation. Am. J. Reprod. Immunol., 60, 187–191.
34) Lee, M.S., Liu, C.H., Lee, T.H., Wu, H.M., Huang, L.S., Chen, C.M. and Cheng, E.H. (2010): Association of creatin kinase B and peroxiredoxin 2 expression with age and embryo quality in cumulus cells. J. Assist. Reprod. Genet., 27, 629–639.
35) Moffatt, O., Drury, S., Tomlinson, M., Afnan, M. and Sakkas, D. (2002): The apoptotic profile of human cumulus cells changes with patient age and after exposure to sperm but not in relation to oocyte maturity. Fertil. Steril., 77, 1006–1011.
36) Lee, K.S., Joo, B.S., NA, Y.J., Yoon, M.S., Choi, O.H. and Kim, W.W. (2001): Cumulus cells apoptosis as an indicator to predict the quality of oocytes and the outcome of IVF-ET. J. Assist. Reprod. Genet., 18, 490–498.
37) Høst, E., Gabrielsen, A., Lindenbeerg, S. and Smidt-Jensen, S. (2002): Apoptosis in human cumulus cells in relation to zona pellucida thickness variation, maturation stage, and cleavage of the corresponding oocyte after intracytoplasmic sperm injection. Fertil. Steril., 77, 511–515.
38) Dunbar, B.S. (1983): Morphological, biochemical, and immunological characterization of the mammalian zona pellucida. In: Mechanism and Control of Animal Fertilization (Hartmann, J., eds.), pp. 139–157, Academic Press, New York.
39) Phillips, D.M. and Shalgi, R.M. (1980): Surface properties of the zona pellucida. J. Exp. Zool., 213, 1–8.
40) Familiari, G., Nottola, SA., Micara, G., Aragona, C. and Motta, P.M. (1988): Is the sperm-binding capability of the zona pellucida linked to its surface structure? A scanning electron microscopic study of human in vitro fertilization. J. In Vitro Fert. Embryo Trans., 5, 134–143.
41) Dunbar, B.S., Wardrip, N.J. and Hedrick, J.K. (1980): Isolation, physicochemical properties and macromolecular composition of the zona pellucida from porcine oocytes. Biochemistry, 19, 356–365.
42) Wassarman, P.M. (2008): Zona pellucid glycoproteins. J. Biol. Chem., 283, 24285–24289.
43) Hughes, D.C. and Barratt, C.L. (1999): Identification of the true human orthologue of the mouse Zp1 gene: evidence for greater complexity in the mammalian zona pellucida? Biochim. Biophys. Acta., 28, 303–306.
44) Plaza, S., Chanut-Delalande, H., Fernandes, I., Wassarman, P.M. and Payre, F. (2010): From A to Z: apical structures and zona pellucida-domain proteins. Trends. Cell. Biol., 20, 524–532.
45) Wassarman, P.M., Jovine, L., Qi, H., Williams, Z., Darie, C. and Litscher, E.S. (2005): Recent aspects of mammalian fertilization research. Mol. Cell. Endocrinol., 234, 95–103.
46) Skutelsky, E., Ranen, E., Shalgi, R. (1994): Variations in the distribution of sugar residues in the zona pellucida as possible species-specific determinants of mammalian oocytes. J. Reprod. Fertil., 100, 35–41.
47) Goncalves, R.F., Staros, A.L. and Kilian, G.J. (2008): Oviductal fluid proteins associated with the bovine zona pellucid and the effect on *in vitro* sperm-egg binding, fertilization and embryo development. Reprod. Domest. Anim., 43, 720–729.
48) Wagh, P.V. and Lippes, J. (1989): Human oviductal fluid proteins. III. identification and partial purification. Fertil. Steril., 51, 81–88.
49) Wegner, C.C. and Killian, G.J. (1991): In vitro and in vivo association of an oviduct estrus-associated protein with bovine zona pellucid. Mol. Reprod. Dev., 29, 77–84.
50) Longo, F.J. (1981): Changes in the zones pellucidae and plasmalemma of aging mouse eggs. Biol. Reprod., 25, 399–411.
51) Hamatani, T., Falco, G., Carter, M.G., Akutsu, H., Stagg, C.A., Sharov, A.A., Dudekula, D.B., VanBuren, V. and Ko, M.S. (2004): Age-associated alteration of gene expression patterns in mouse oocytes. Hum. Mol. Genet., 13, 2263–2278.
52) Kempisty, B., Antosik, P., Bukowska, M., Lianeri, M., Jaśkowski, J.M. and Jagodziński, P.P. (2009): Assessment of zona pellucid glycoprotein and integrin transcript contents in porcine oocytes. Reprod. Biol., 9, 71–78.
53) Tsaadon, A., Eliyahu, E., Shtraizent, N. and Shalgi, R. (2006): When a sperm meets an egg: block to polyspermy. Mol. Cell. Endocrinol., 252, 107–114.
54) Meyer, N.L. and Longo, F.J. (1979): Cytological events associated with in vitro aged and fertilized rabbit eggs. Anat. Rec., 195, 357–374.
55) Wolf, D.P. and Hamada, M. (1976): Age-dependent losses in the penetrability of mouse eggs. J. Reprod. Fertil., 48, 213–214.
56) Yanagimachi, R. and Chang, M.C. (1961): Fertilizable life of golden hamster ova and their morphological changes at the time of losing fertilizability. J. Exp. Zool., 148, 185–203.
57) Liu, X., Fernandes, R., Jurisicova, A., Casper, R.F. and Sun, Y. (2010): In situ mechanical characterization of mouse oocytes using a cell holding device. Lab. Chip.,10, 2154–2161.
58) Hayflick, L. (1965): The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res., 37, 614–636.
59) Sakaguchi, M., Miyazaki, M., Kondo, T. and Namba, M. (2001): Up-regulation of S100C in normal human fibroblasts in the process of aging in vitro. Exp. Gerontol., 36, 1317–1325.