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Review

# Mechanisms underlying oocyte activation and postovulatory ageing

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Mammalian oocytes undergo significant growth during oogenesis and experience extensive cytoplasmic and nuclear modifications immediately before ovulation in a process commonly referred to as oocyte maturation. These changes are intended to maximize the developmental success after fertilization. Entry of a spermatozoon into the oocyte, which occurs a few hours after ovulation, initiates long-lasting oscillations in the free intracellular calcium ($[Ca^{2+}]_i$) that are responsible for all events of oocyte activation and the initiation of the developmental programme that often culminates in the birth of young. Nevertheless, the cellular and molecular changes that occur during maturation to optimize development are transient, and exhibit rapid deterioration. Moreover, fertilization of oocytes after an extended residence in the oviduct (or in culture) initiates a different developmental programme, one that is characterized by fragmentation, programmed cell death, and abnormal development. Inasmuch as $[Ca^{2+}]_i$ oscillations can trigger both developmental programmes in mammalian oocytes, this review addresses one of the mechanism(s) possibly used by spermatozoa to initiate these persistent $[Ca^{2+}]_i$ responses, and the cellular and molecular changes that may underlie the postovulatory cellular fragmentation of ageing mammalian oocytes.

Mammalian oocytes acquire the ability to be fertilized and to give rise to viable embryos in waves of growth and maturation. This process commences approximately at the time of puberty and continues for a significant portion of the lifespan of females of most mammalian species. While arrested at the diplotene stage of the first meiosis, also commonly referred to as the germinal vesicle (GV) stage, follicle-enclosed oocytes grow to their maximal size. During this time, oocytes accumulate significant amounts of mRNAs and proteins that will be used later during maturation, fertilization and zygote development. After the completion of oocyte growth, the follicle continues to grow until it reaches ovulatory size. It is at this time that an LH surge induces the release of a metaphase II (MII)-arrested oocyte into the oviduct, concluding the ovarian life of the oocyte. The transition from the GV stage to MII immediately preceding ovulation is referred to as 'oocyte maturation' and entails a complex sequence of nuclear and cytoplasmic events that prepares the oocyte for fertilization and initiation of development.

Although the time required to complete oocyte growth and maturation is measured in weeks or months, the fertilizable lifespan of mammalian oocytes is remarkably short, less than 10 h. Fertilization within this narrow window of developmental opportunity results in oocyte activation and normal embryonic development. Oocyte activation is a process in which the fertilizing spermatozoon initiates repetitive increases in the free intracellular calcium concentration ($[Ca^{2+}]_i$). These $[Ca^{2+}]_i$ oscillations signal all events of activation including release of cortical granules and prevention of polyspermy, resumption and completion of meiosis, and pronuclear formation and subsequent mitotic cleavages (reviewed by Schultz and Kopf, 1995). In contrast, oocytes that are fertilized after this optimal period, when oocyte 'ageing' sets in, exhibit severely compromised developmental success that often culminates in fragmentation of blastomeres and embryonic death (Marston and Chang, 1964 and references therein). $[Ca^{2+}]_i$ oscillations induce both developmental outcomes (Gordo *et al.*, 2000a); therefore, this review will summarize information describing the optimization of $Ca^{2+}$ release mechanism(s) of oocytes during maturation, how the spermatozoa might initiate oscillations, and the cellular and molecular events that may underlie postovulatory oocyte ageing.

## Oocyte maturation: preparation for fertilization

Oocyte maturation in most mammalian species is completed within 12–24 h, and during this brief period significant qualitative changes in protein synthesis take

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place that are intended to initiate and regulate resumption of meiosis, prevent polyspermic fertilization, and promote exit from the MII arrest (reviewed by Wassarman and Albertini, 1994); in other words, these changes make oocytes 'activation competent'. Re-initiation of meiosis is made possible by the activation of maturation-promoting factor (MPF), which comprises the products of the *p34 cdc2* and *cyclin* genes (Draetta and Beach, 1988). The activation of MPF is responsible for inducing germinal vesicle breakdown and promoting progression into meiosis II (Dekel, 1996). In addition to MPF, activation of mitogen-activated protein kinase (MAPK) plays a role in regulating the transition from meiosis I to meiosis II and, more importantly, in the MII stage arrest (reviewed by Ferrel, 1999). These kinases, mostly in conjunction with other kinases or phosphatases, may also regulate the completion of 'cytoplasmic maturation' that occurs before ovulation.

One of the cytoplasmic functions that is modified during maturation is the ability to release $Ca^{2+}$ (reviewed by Carroll *et al.*, 1996). Sperm-induced $[Ca^{2+}]_i$ oscillations are the hallmark of mammalian fertilization and although the mechanism(s) used by the spermatozoon to initiate these oscillations is still under investigation, it has become apparent that the inositol 1,4,5-trisphosphate receptor isoform-1 ($IP_3R$-1) is the channel of choice during fertilization (Miyazaki *et al.*, 1993). In support of this, the sensitivity of $IP_3R$-1, as evidenced by enhanced $Ca^{2+}$ responses to stimulation with $IP_3$, increases gradually during maturation from the GV stage and peaks at MII (Fujiwara *et al.*, 1993; Mehlmann and Kline, 1994; He *et al.*, 1997). Fertilization of oocytes at different stages of maturation induces oscillations only in oocytes that have nearly completed maturation (Fujiwara *et al.*, 1993; Jones *et al.*, 1995), indicating that a maximally sensitized $IP_3R$ system is required for the initiation of oscillations in mammalian oocytes.

Several molecular mechanisms may account for the heightened sensitivity of the $IP_3R$-1 at MII. First, a noticeable, albeit not overwhelming, increase in the density of these receptors has been observed during maturation in oocytes of mammalian and non-mammalian species (Mehlmann *et al.*, 1996; He *et al.*, 1997; Kume *et al.*, 1997). Second, the phosphorylation status of the receptor may change during maturation, resulting in enhanced conductivity of the receptor. The $IP_3R$-1 has multiple phosphorylation consensus sequences and phosphorylation of selected sites affects the conductivity of the channel (Patel *et al.*, 1999), although the phosphorylation status of the $IP_3R$-1 in mammalian oocytes and zygotes has not been investigated. In addition, $IP_3Rs$ form complexes with other proteins and this, in turn, may also alter the conductivity of the channel (Bultynck *et al*, 1999). Other changes that may augment $IP_3R$-1-mediated $Ca^{2+}$ release during maturation include increased $Ca^{2+}$ store content and redistribution of the endoplasmic reticulum (ER) (Jones *et al.*, 1995; Shiraishi *et al.*, 1995). Therefore, several changes that occur concomitantly during oocyte maturation are intended to maximize $Ca^{2+}$ release at the time of fertilization, underscoring the significance of this signalling event not only for exit of meiosis, but also for increased developmental success of early embryos. On the other hand, prolonged residence in the oviduct without fertilization results in gradual inactivation of the $Ca^{2+}$ release mechanisms, and this may be responsible, at least in part, for the fragmentation and poor developmental success of zygotes that result from delayed fertilization.

## Oocyte activation: signalling mechanism for sperm-induced $[Ca^{2+}]_i$ oscillations

The signalling mechanism used by the spermatozoa to initiate and maintain $[Ca^{2+}]_i$ oscillations has not been elucidated, although several hypotheses have been proposed (Schultz and Kopf, 1995; Stricker, 1999); the evidence for and against each hypothesis has been reviewed recently and therefore will not be discussed here (Swann and Lai, 1997; Swann *et al.*, 2001). Instead, the focus will be on some of the less thoroughly reviewed aspects of the 'Fusion/Sperm Factor (SF)' hypothesis. This hypothesis proposes that a factor from the spermatozoon initiates $[Ca^{2+}]_i$ oscillations when delivered into the ooplasm upon fusion of the gamete membranes. The strongest experimental evidence in support of this hypothesis emanates from the ability of spermatozoa to initiate $[Ca^{2+}]_i$ oscillations, when injected directly into the ooplasm, despite bypassing the presumably required interactions of the gamete membranes (Tesarik *et al.*, 1994; Nakano *et al.*, 1997). Likewise, the demonstrated ability of sperm fraction or factor preparations to initiate $Ca^{2+}$ release when they are injected into oocytes of several mammalian species provides additional support for this hypothesis (Wu *et al.*, 1997; Swann *et al.*, 2001). Nevertheless, the lack of success in the isolation and characterization of the sperm active molecule(s) has delayed progress. The following discussion will focus on some of the outstanding issues such as the number of active SF molecules, the location of SF, and the regulation of its release.

### How many sperm factors?

Injection of SFs into mammalian oocytes can initiate, and in many cases replicate, the $Ca^{2+}$ responses induced by fertilization. In most studies the injected SFs were obtained after sonication of spermatozoa or after cycles of freezing and thawing, and the processed supernatants were denominated soluble or cytosolic SFs, since these lysis procedures are generally expected to release most of the cytosolic components of the cell. However, given the special configuration of sperm cells and their lack of obvious cytoplasm, it is presently unknown which of the sperm domains contribute proteins to these soluble fractions. Moreover, it has become readily apparent that the soluble sperm proteins are not the only fractions able to trigger $Ca^{2+}$ release. Results from experiments using the intracytoplasmic sperm injection (ICSI) technique, which makes

possible the injection of whole spermatozoa into oocytes, showed that injection of demembranated sperm heads, which are consequently devoid of soluble SFs, is still able to induce $Ca^{2+}$ release (Perry *et al.*, 2000). These results were interpreted to mean that the spermatozoa may be equipped with more than one SF, and that the soluble SF may not be involved in the persistence of oscillations during fertilization as these fractions are likely to be lost before gamete fusion. This interpretation may be premature for several reasons. First, the protein compositions of the soluble and the less soluble SFs have not been compared extensively. Second, these experiments were carried out exclusively using mouse spermatozoa that are known to contain lower soluble $Ca^{2+}$ activity than spermatozoa from hamsters or pigs (Swann *et al.*, 2001; M. Kurokawa, H. Wu and R. A. Fissore, unpublished), which have been used mainly for the preparation of soluble SFs. Remarkably, our unpublished chromatographic fractionation studies of soluble and less soluble (Triton X-resistant) pig SFs using Superose 12 and hydroxyapatite columns reveal that the $Ca^{2+}$ active molecules from both SFs exhibit very similar elution profiles in both columns, indicating that the same $Ca^{2+}$ active molecule(s) may be present in both soluble and less soluble fractions. Whether the putative molecule has diverse locations in the spermatozoon, or may be associated with different proteins that in turn regulate or modify its release remains to be elucidated.

## Where is the sperm factor(s)?

Although the results obtained using sperm heads demembranated with Triton X-100 underscored the possible functional significance of the detergent-resistant SF fraction, they do not reveal the sperm structure(s) that harbours the $Ca^{2+}$-active molecule. However, these studies indicate that the theca, the sperm perinuclear material, is the structure most likely to carry the $Ca^{2+}$-releasing molecule(s). This interpretation is based on the finding that after Triton X-100 treatment, the theca is the only structure that remains associated with the sperm nucleus (Kimura *et al.*, 1998; Perry *et al.*, 1999). In support of this notion, removal of the sperm theca using SDS and trypsin was shown to eliminate the ability of mouse sperm heads to activate oocytes after ICSI (Kimura *et al.*, 1998). Nonetheless, several questions cannot be resolved by the experimental approach just described. First, a significant amount of soluble SF is released during treatment with Triton X-100, and the location of such SF cannot be determined. Second, whether or not the theca-associated $Ca^{2+}$ activity is removed, or simply inactivated, by the trypsin and SDS treatments was not ascertained because the $Ca^{2+}$ activity was never recovered after the treatments. Finally, it remains to be demonstrated whether the theca may serve as an anchor from which the release of the factor takes place, or whether the active molecule(s) of the SF is an integral component of the theca. These questions are easily testable and the answers should contribute to the elucidation of the location of the SFs in the spermatozoa as well as the mode of release of the factor.

## Mode of release of sperm factor

How the $Ca^{2+}$-active component of the spermatozoon is released and the oocyte conditions that promote or regulate the release are presently not known. For instance, is the factor gradually released or, alternatively, does it move away from the sperm nucleus soon after fusion? Evidence in support of the latter possibility is provided by the finding that soon after entry of the spermatozoon into the oocyte, the increase in $[Ca^{2+}]_i$ appears to originate away from location of entry of the spermatozoon and opposite to the animal pole (Kline *et al.*, 1999; Deguchi *et al.*, 2000). Likewise, the demonstration that fusion of a cytoplast from a telophase-stage fertilized mouse oocyte to an unfertilized oocyte can induce activation indicates that the factor has a cytoplasmic location, although $[Ca^{2+}]_i$ oscillations were not measured in this study (Ogonuki *et al.*, 2001). As suggestive as these studies are of an early cytosolic location of the factor, it is plausible that a fraction of the factor remains associated with the sperm nucleus several hours after sperm entry. What is more, it is possible that the Triton X-100-resistant SF fraction remains associated with the nucleus for several hours, and is responsible for the long duration of the oscillations, especially in large domestic species in which oscillations are known to last in excess of 10 h (Fig. 1). Nevertheless, despite these appealing models of release, basic functional and molecular evidence is needed to elucidate the regulation of release of the factor.

Although the fate of the $Ca^{2+}$-active molecule of the spermatozoon within the first few hours after fusion is presently unknown, it appears that by the time of pronuclear formation, at least a portion of it is associated with the pronuclear structures. Early studies of mouse zygotes by Kono *et al.* (1995) reported that transfer of male or female pronuclei into MII oocytes was able to reinitiate oscillations, whereas fusion of comparably sized cytoplasts was unable to do so. Notably, studies involving injection of physiological amounts of pig SF into mouse oocytes showed that although these fractions can initiate oscillations efficiently, they are not as proficient at associating with the pronuclei. For example, in oocytes fertilized by ICSI, pronuclear envelope breakdown, either spontaneous or induced by exposure to okadaic acid, was accompanied by an increase in $[Ca^{2+}]_i$, whereas SF-generated zygotes failed to show such a response (Gordo *et al.*, 2002a). In addition, oscillations ceased earlier in these oocytes compared with those fertilized normally, even when meiotic exit was inhibited by the addition of colcemid (Gordo *et al.*, 2002a). Thus, the association of the $Ca^{2+}$-active molecule of spermatozoa with pronuclear structures may reflect the presence of an intact SF molecule(s), or SF molecules associated with the correct partners. Such an association of SF with pronuclear binding partners may affect the stability of the factor. Elucidation of the domain(s) or molecules that mediate the association or targetting of SF would be of interest.

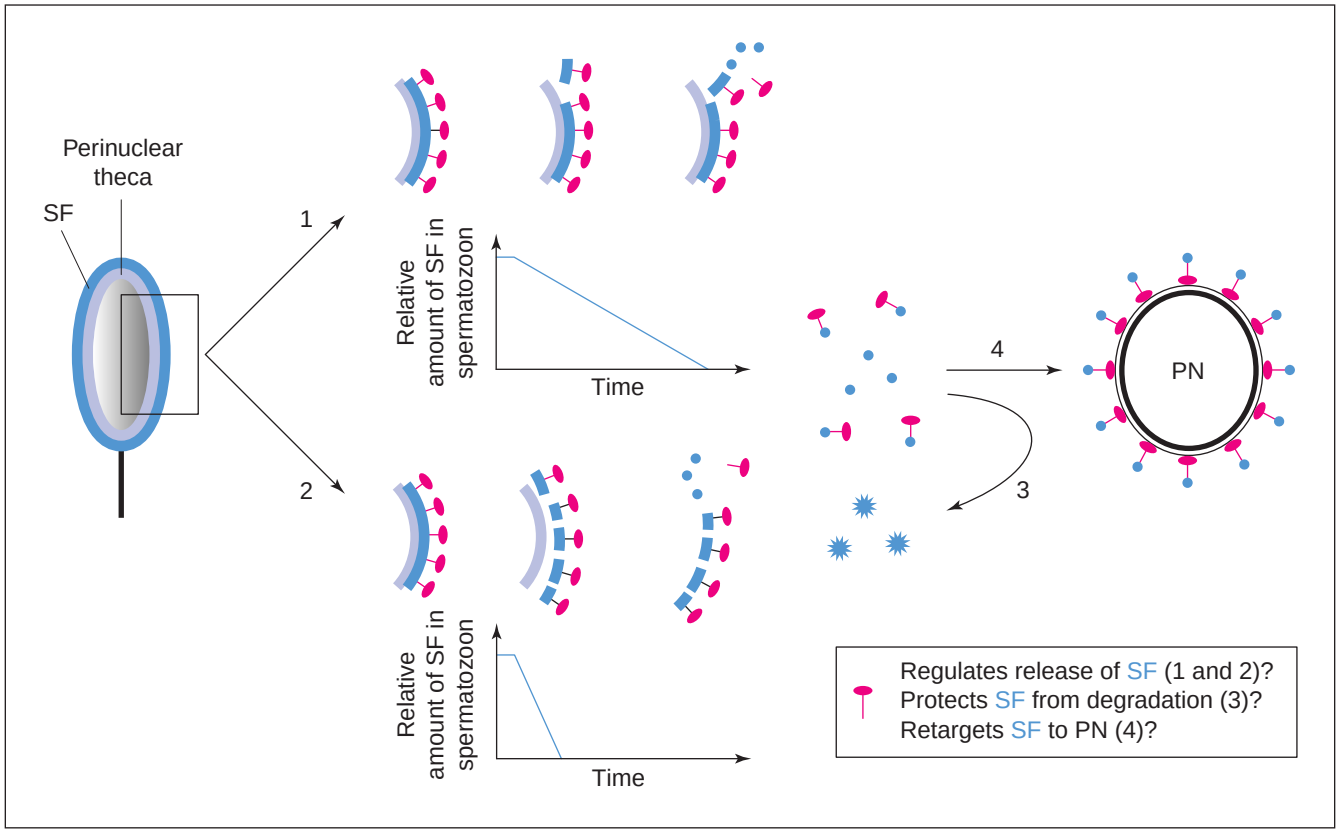


**Fig. 1.** Models for release of the $Ca^{2+}$ active factor from the spermatozoon during fertilization in mammalian oocytes. Two models are shown: (1) the release of the factor from the sperm head is gradual; (2) the factor becomes dissociated from the sperm head within the first hour after fertilization. The symbols in blue denote sperm factor (SF), the $Ca^{2+}$-active molecule(s) of spermatozoa. The symbols in red denote a domain or accessory protein, which may be important in regulating the release of the factor from the spermatozoon, or its stability after release, or its targeting to the pronuclear (PN) envelope area. The supposition for the need of a correctly exposed domain or accessory protein to control some of the functions of SF arises from the findings that the oscillations induced by injections of SF into mammalian oocytes seem to exhibit shorter duration than those initiated by the spermatozoa.

It is important to note that notwithstanding the success of ICSI in several mammalian species, its effectiveness is far from acceptable in large domestic species (Horiuchi and Numabe, 1999; Suttner *et al.*, 2000). The most important limitation appears to be an inability of injected spermatozoa to deliver an appropriate activation stimulus in the oocytes of these species. Therefore, although the Fusion/SF hypothesis may represent the mechanism responsible for oscillations in mammalian species, we are far from understanding fundamental aspects of its mode of release, location and activation, and must contemplate the possibility of significant species-specific variability in mammals.

## Postovulatory oocyte ageing: a prelude to fragmentation and cell death

Oocyte maturation is timed in such a manner that sperm entry, activation and developmental competence are maximal within a few hours after ovulation; in mice nearly 100% of oocytes are expected to be fertilized within 6 h of ovulation (Braden and Austin, 1954). Nonetheless, interference with this timing is now occurring with increased frequency, especially in species in which humans intervene to control the number of ovulations or the genetic makeup of the progeny. The end-result is fertilization of 'aged oocytes'. Zygotes from aged oocytes reportedly show limited developmental success and undergo programmed cell death or apoptosis. Apoptosis, a process of cell selection present in all organisms, mediates the elimination of damaged cells and the strict selection, and consequent attrition, of germ cells that takes place in the gonads before and after birth (reviewed by Dunkel *et al.*, 1997; Morita and Tilly, 1999). The molecules involved in programmed cell death have been extensively reviewed elsewhere (Adams and Cory, 1998; Thornberry and Lazebnik, 1998; Heiden and Thompson, 1999); therefore, we will focus on new evidence involving mechanisms that may underlie apoptosis or fragmentation that ensues after fertilization or activation of ovulated, aged mammalian oocytes.

## Fertilization or activation induces a different developmental outcome in aged oocytes

The negative impact of delayed fertilization on reproductive success has been known for several decades, but the stage(s) at which embryonic development is compromised, and the mechanism(s) responsible for the developmental failure, were not apparent from earlier studies. Marston and Chang (1964) noted that postovulatory ageing, in mice, hindered embryo development soon after fertilization as they observed that within 24 h after fertilization, grossly mis-shapen and fragmented zygotes developed when insemination was delayed for more than 12 h after ovulation. Subsequent studies in mice confirmed (Juetten and Bavister, 1983) and extended these findings by showing that aged oocytes exhibit abnormal morphological characteristics before fertilization, such as the disappearance of the microfilament-rich area over the meiotic spindle, disruption of and abnormal spindle location, and chromatin disorganization (Webb *et al.*, 1986). These changes appear to be the consequence of decreasing MPF and MAPK activities, and seem to develop faster in the oviduct than *in vitro* (Xu *et al.*, 1997; Abbott *et al.*, 1998). This progressive deterioration leads to oocyte fragmentation even in the absence of fertilization (Takase *et al.*, 1995; Fujino *et al.*, 1996; Tarin *et al.*, 2001). Both spontaneous and fertilization-induced fragmentation may have a genetic component since they appear, at least in mice, to be influenced by the strain(s) under investigation (Warner, *et al.*, 1998; Hawes *et al.*, 2001).

As our understanding of oocyte ageing and its developmental consequences have gradually progressed, insights into the mechanism and role of apoptosis have been revealed in several cellular paradigms. Of particular relevance was the demonstration that the postnatal decline in the female germ cell population is mediated by apoptosis (De Pol *et al.*, 1997; Tilly, 2001). Nevertheless, the molecular mechanism responsible for postovulatory fragmentation of oocytes was not ascertained until Takase *et al.* (1995) showed that ovulated unfertilized mouse oocytes cultured for extended periods undergo cytoplasmic fragmentation accompanied by DNA fragmentation, as determined by terminal deoxynucleotidyl transferase (TdT) nick-end labelling (TUNEL), both of which are classical signs of programmed cell death. Since then, several studies have extended those results by showing that apoptosis is not only observed after fertilization of aged oocytes, or of oocytes from ageing females, but also after exposure of newly ovulated mouse oocytes to chemical or chemotherapeutic compounds (Weil *et al.*, 1996; Perez *et al.*, 1997, 1999a).

Apoptosis requires the expression and activation of specific genes involved in the execution of the cell suicide programme. For the purpose of this review, suffice it to say that mouse oocytes express most of the genes known to participate in this programme (Jurisicova *et al.* 1998; Exley *et al.*, 1999; Rucker *et al.*, 2000). Mouse oocytes express several caspases, which are proteases responsible for the dismantling of cells, and several of the anti- and pro-apoptotic members of the *Bcl-2* gene family. Pro-apoptotic family members promote the release of cytochrome *c*, a component of the mitochondrial respiratory chain that induces the activation of caspases when released into the cytosol, whereas anti-apoptotic members block the release of this factor (Adams and Cory, 1998). Results from gene disruption and overexpression studies in the ovary underscore the importance of these molecules in oocyte cell death. Oocytes from mice lacking caspase 2 or Bax, or overexpressing Bcl-2, are resistant to apoptosis induced by exposure to chemotherapeutic agents or that occurs spontaneously after ovulation (Bergeron *et al.*, 1998; Morita *et al.*, 1999; Perez *et al.*, 1999b). Hence, as proposed for somatic cells (Oltvai *et al.*, 1993), alterations in the amounts or ratios of the protein products of these genes may underlie fragmentation and cell death in aged oocytes too.

## Mitochondrial dysfunction in aged oocytes

Mitochondrial dysfunction is likely to play an important role in the predisposition of aged oocytes to spontaneous and fertilization- or activation-induced fragmentation. Mitochondria harbour important anti-apoptotic as well as apoptogenic compounds, and serve as the bioenergetic centre of the cell by generating ATP (Green and Reed, 1998; Wang, 2001). Therefore, disruption of any of the mitochondrial membranes, or of its ability to generate ATP, is likely to have negative consequences on cell proliferation and survival. With respect to the latter, the ATP content of mouse oocytes appears to decrease steadily with time of culture (Chi *et al.*, 1988), and in human oocytes, there may be a close association between developmental competence and intra-oocyte concentrations of ATP (Van Blerkom *et al.*, 1995). Extended culture times also decrease the mitochondrial membrane potential in human oocytes (Wilding *et al.*, 2001), possibly resulting in swelling of the mitochondrial matrix. In somatic cells, mitochondrial swelling precedes the rupture of the outer mitochondrial membrane, making possible the release of cytochrome *c* and other apoptogenic products (Wang, 2001). Advanced stages of mitochondrial dysfunction are also associated with accumulation of reactive oxygen species, which further compromise cell survival. Importantly, exposure of oocytes to different concentrations of hydrogen peroxide was shown to induce release of cytochrome *c* and to lower the mitochondrial membrane potential (Liu *et al.*, 2000). Moreover, addition of dithiothreitol, a reducing agent, to the culture medium prevented some of the detrimental effects of oocyte ageing on fertilization and development (Tarin *et al.*, 1998).

Results demonstrating that injection of purified mitochondria from somatic cells inhibits spontaneous postovulatory oocyte fragmentation in certain strains of mice (Perez *et al.*, 2000) are consistent with the involvement of mitochondrial dysfunction in oocyte cell death. Likewise,

the beneficial effects of 'cytoplasmic transfer', a process during which cytoplasm from fertile donor oocytes is injected into oocytes from females that have failed to conceive, reportedly to overcome some cases of human infertility, may by explained by the addition of fresh mitochondria during this procedure (Cohen *et al.*, 1998). Nevertheless, the clinical application of these techniques should proceed with caution until studies are carried out to compare the developmental competence to term of these oocytes, and assess potential long-term detrimental effects of these procedures on the offspring.

## $[Ca^{2+}]_i$ oscillations and abnormal development in aged oocytes

As previously mentioned, mammalian oocytes remain fully developmentally competent for a few hours after ovulation. In some cases, unfertilized aged oocytes undergo spontaneous fragmentation. However, in others, late fertilization results in the initiation of development, but high rates of fragmentation and apoptotic death are observed, and some of the surviving embryos appear to exhibit birth defects (Tarin *et al.*, 1999). We have demonstrated that initiation of the $[Ca^{2+}]_i$ signal due to fertilization is capable of inducing fragmentation and activation of caspases in aged oocytes (Gordo *et al.*, 2000), implying that this $[Ca^{2+}]_i$ signal may act as the instigator of apoptosis in aged oocytes. In addition, in human oocytes, caspase activity was detected only after fertilization, in fragments, and in embryos of poor morphology (Martinez *et al.*, 2002).

A question that arises from these results is how can the same signal trigger such divergent developmental paths? Changes in $Ca^{2+}$ homeostasis have been associated with cellular necrosis and programmed cell death, although the mechanism(s) by which $[Ca^{2+}]_i$ changes induce apoptosis is not entirely clear (Trump and Berezesky, 1995; Berridge *et al.*, 1998). Interestingly, $Ca^{2+}$ responses in aged mouse oocytes are abnormal in several respects (Jones and Whittingham, 1996; Igarashi *et al.*, 1997). For instance, the rate of increase of $[Ca^{2+}]_i$ during fertilization or after stimulation by agonists is slower in aged oocytes. Likewise, the amplitude of the responses and the duration of the oscillations are decreased in these oocytes. Collectively, these results indicate altered $Ca^{2+}$ homeostasis in aged oocytes (Takahashi *et al.*, 2000). The molecular basis for this defective $Ca^{2+}$ regulation has not been elucidated, but may involve the aforementioned decrease in ATP in conjunction with reduced Bcl-2 expression in aged oocytes (Gordo *et al.*, 2002b). Low availability of ATP may impair the function of the SERCA pumps, the $Ca^{2+}$-ATPase pumps responsible for the refilling of the endoplasmic reticulum (ER). Reduced amounts of Bcl-2 may also negatively impact the function of the $Ca^{2+}$ pump, although the role of Bcl-2 in $Ca^{2+}$ homeostasis remains controversial (Diestelhorst and Dubyak, 1998; Pinton *et al.*, 2000). Nonetheless, Kuo *et al.* (1998) suggest that Bcl-2 may upregulate the expression of SERCA mRNA and may interact directly with the pump, possibly modulating its function. Therefore, malfunction of the SERCA pump may be responsible for the abnormal $Ca^{2+}$ homeostasis in aged oocytes.

Fertilization-associated $[Ca^{2+}]_i$ oscillations may signal apoptosis in aged oocytes by inducing several cellular responses that simultaneously, and possibly synergistically, promote programmed cell death. First, the initiation of $[Ca^{2+}]_i$ oscillations may result in depletion of $Ca^{2+}$ from the ER, as evidenced by the premature cessation of oscillations in aged oocytes. Low $Ca^{2+}$ in the ER is an effective trigger of cell death in somatic cells (Jiang *et al.*, 1994; Bian *et al.*, 1997) and also suppresses protein synthesis (Soboloff and Berger, 2002), effects which are likely severely to limit embryo development. Second, there appears to be privileged communication between $IP_3R$-mediated $Ca^{2+}$ release and mitochondrial function (Csordás *et al.*, 1999; Szalai *et al.*, 1999). The mitochondria serve as $Ca^{2+}$ stores in the cell and have been shown to participate in the generation of oscillations in several types of cell, including mouse oocytes (Liu *et al.*, 2001) and human embryos (Souza *et al.*, 1997). Under optimal conditions, each of the $IP_3R$-mediated increases in $[Ca^{2+}]_i$ stimulates mitochondrial function and ATP production. However, in the presence of a pro-apoptotic stimulus, $IP_3$-mediated $[Ca^{2+}]_i$ oscillations signal the release of cytochrome *c*, a decrease in the mitochondrial membrane potential, and activation of caspases (Szalai *et al.*, 1999). Therefore, we propose a model whereby postovulatory ageing of oocytes, which results in less ATP and Bcl-2 and an increase in hydrogen peroxide, act as a pro-apoptotic stimulus and, under these circumstances, fertilization-like $[Ca^{2+}]_i$ oscillations trigger cell death (Fig. 2).

$[Ca^{2+}]_i$ oscillations are responsible for promoting progression of the cell cycle in mammalian oocytes and the apoptotic effects of $Ca^{2+}$ release on aged oocytes may also rely, at least in part, on this transition. As mentioned, mammalian oocytes are arrested at MII, a stage that appears to confer resistance to apoptosis. For instance, mouse MII oocytes are susceptible to staurosporin-induced apoptosis, but at concentrations significantly higher than those required in somatic cells (Weil *et al.*, 1996). Similarly, oocytes from Mos-null mice, which lack MAPK activity and fail to arrest at MII, progress into the cell cycle and undergo high rates of spontaneous fragmentation (Colledge *et al.*, 1994). Aged oocytes also appear to be resistant to fragmentation by $[Ca^{2+}]_i$ oscillations when arrested at MII. For example, the initiation of oscillations in aged oocytes treated with colcemid, which induces an arrest of the cell cycle at a mitosis-like stage without interfering with $Ca^{2+}$ responses, failed to promote fragmentation, whereas similarly injected oocytes that were transferred into medium devoid of the drug fragmented shortly thereafter (Table 1). Similar fragmentation was observed in aged oocytes when progression of the cell cycle was promoted by exposure to ionomycin and 6-(dimethylamino)purine (DMAP), a common parthenogenetic activation combination (M. Zhang and R. A. Fissore, unpublished). Interestingly, *Xenopus* egg

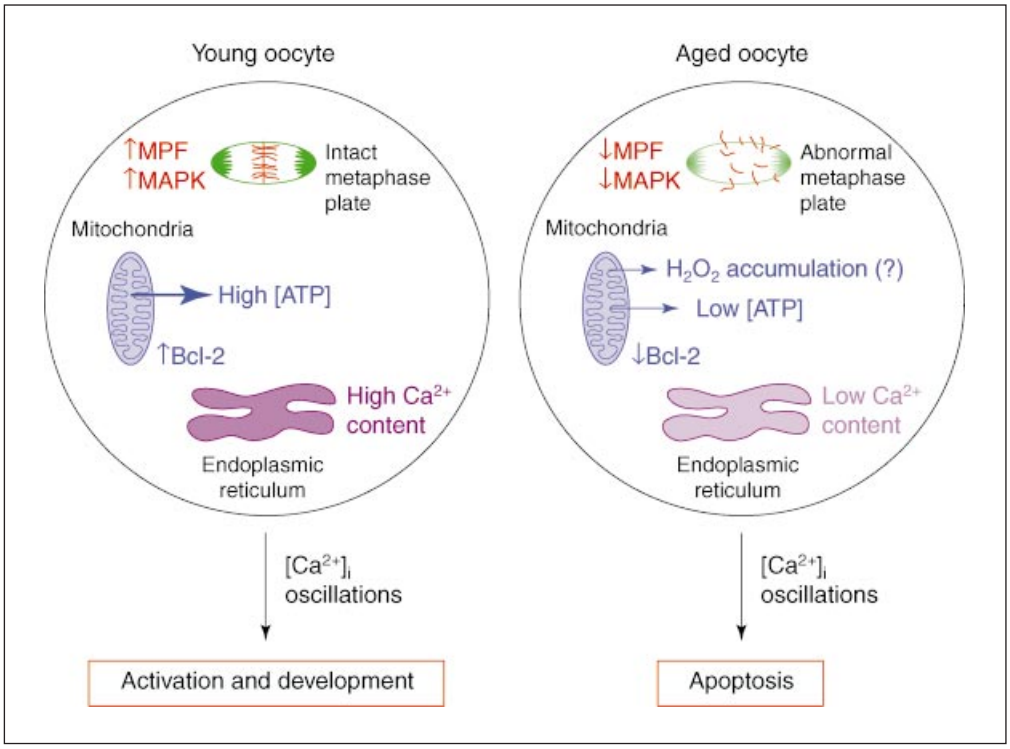


**Fig. 2.** Cellular and molecular changes in mammalian oocytes during ageing that may underlie fragmentation or developmental arrest after activation or fertilization. As oocytes age, a host of cellular functions may be compromised, which in turn compromise the ability of oocytes to initiate normal development. Aged eggs exhibit a steady decline of meiotic kinases, maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK), and show decreased anti-apoptotic proteins and $Ca^{2+}$ contents in the stores. Aged oocytes also appear to have a depressed mitochondrial function, which may result in the accumulation of reactive oxygen species as well as in the release of pro-apoptotic products after $Ca^{2+}$ stimulation. Therefore, activation of aged oocytes may result in fragmentation or cell cycle arrest rather than in the customary normal initiation of development.

**Table 1.** Cell cycle arrest prevents $[Ca^{2+}]_i$ oscillation-induced fragmentation of aged mouse oocytes

| Treatment[a] | Number of oocytes | Number of fragmented oocytes | Per cent age |
|---|---|---|---|
| Colcemid (0.1 μg $ml^{-1}$)–culture | 15 | 1 | 6.6 |
| SF (1mg $ml^{-1}$)–culture | 16 | 10 | 62.5[b]* |
| SF–colcemid–culture | 21 | 2 | 9.5 |
| Colcemid–wash–SF–culture | 13 | 11 | 84.6* |
| Colcemid–SF–colcemid–culture | 18 | 0 | 0 |

[a]Oocytes were aged in potassium simplex optimized medium (KSOM) supplemented with colcemid for 24 h. Colcemid-treated aged oocytes were injected with pig sperm factor (SF) to initiate $Ca^{2+}$ responses. In one group (SF + colcemid), injections and injection after culture were conducted in the presence of colcemid. In the other two groups, injections were done in the absence of colcemid (wash), after which a group was cultured in colcemid-free conditions, whereas the other group of oocytes was returned to colcemid-containing culture conditions.
[b]Experiments were repeated twice.
*Significantly different from those within the column (chi-squared; $P < 0.05$).

extracts are also significantly more resistant to apoptotic stimuli when prepared from MII-arrested eggs compared with those prepared from interphase stage eggs (Faure *et al.*, 1997). Remarkably, when these extracts are maintained in the laboratory for a few hours, they appear spontaneously to initiate changes consistent with apoptosis, raising the possibility that 'extract ageing' also takes place (Smith *et al.*, 2000).

It is possible that critical anti-apoptotic molecules are inactivated or degraded during ageing, and that progression of the cell cycle under these conditions promotes cell death. Toward this end, the decreasing concentrations of MPF and

MAPK that occur during prolonged postovulatory ageing may favour the degradation of anti-apoptotic molecules. Interestingly, in somatic cells, reduced concentrations of MAPK and MPF favour degradation of Bcl-2 and other anti-apoptotic proteins (Boucher *et al.*, 2000; Breitschopf *et al.*, 2000), whereas they do not seem to affect pro-apoptotic proteins (Wada *et al.*, 1998). Remarkably, a similar regulation of anti-apoptotic proteins may occur in effect in oocytes, because reduced amounts of Bcl-2 mRNA and protein, but unchanged amounts of Bax mRNA, have been observed in aged mouse oocytes (Gordo *et al.*, 2002b). In addition, in pig oocytes, fragmentation rates after activation seem to be directly associated with the amount of MPF (Kikuchi *et al.*, 2000).

Progression of the cell cycle in aged oocytes may also promote fragmentation and death by activating apoptotic proteins the functions of which are regulated by phosphorylation. For instance, Bad, one of the pro-apoptotic members of the Bcl-2 family of proteins, is primarily regulated by phosphorylation and its translocation to the ER occurs only in its dephosphorylated state. Calcineurin, a $Ca^{2+}$-dependent phosphatase, dephosphorylates Bad (Wang *et al.*, 1999), and translocated Bad promotes cell death by binding to and inactivating the anti-apoptotic members of the Bcl-2 family (Zha *et al.*, 1996). Therefore, progression of the cell cycle and the consequent changes in the phosphorylation status of the oocyte may activate a molecule(s) such as Bad and, in aged mouse oocytes, which exhibit reduced amounts of anti-apoptotic proteins, fragmentation and cell death may ensue.

It is worth noting that molecular defects other than those outlined here may account for the fragmentation and developmental incompetence of fertilized aged oocytes. Nonetheless, abnormal $Ca^{2+}$ homeostasis, decreased amounts of anti-apoptotic proteins, and exit of meiosis are likely to play an important role in limiting the developmental potential of aged oocytes.

## Conclusions

The initiation of development at fertilization is a tightly controlled process that relies on the structural and molecular integrity of the oocyte and the fertilizing spermatozoon. Under optimal conditions, fertilization induces a series of $[Ca^{2+}]_i$ oscillations that initiate radical alterations in the cellular and molecular organization of the oocyte that are critical for the progression of the embryonic programme. It follows that any changes in this structure that may compromise development, such as alterations induced by ageing, should signal an arrest of development. Oocytes have evolved an elaborate developmental checkpoint whereby the same $Ca^{2+}$ signal is capable of promoting activation or apoptosis, depending on the cellular and molecular integrity of the oocyte. It is critical, therefore, that we further advance our understanding of the pathways that regulate $Ca^{2+}$ release and the mechanisms by which it functions in divergent developmental pathways. Clarification of these pathways may have significant implications in improving the developmental outcome of embryos generated *in vitro* and *in vivo* in humans and animals of agricultural significance.

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