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Could modifications of signalling pathways activated after ICSI induce a potential risk of epigenetic defects?

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ABSTRACT A calcium signal during oocyte or egg activation is a conserved event in virtually all species analyzed so far. This signal, that is in the form of calcium oscillations in mammals, is spatially and temporally controlled and is mainly supported by calcium release from internal calcium stores, but how it is triggered after fertilization is far from understood. The sperm factor hypothesis of egg activation postulates that sperm delivers a calcium-releasing factor into the egg following sperm-egg fusion. Among the many potential sperm factors, PLCzeta is the strongest *bona fide* sperm factor candidate. However, how sperm-oocyte fusion occurs prior to PLCzeta delivery and oocyte activation is not entirely known. We propose in the first part of this review the possibility that other pathways such as those involving G-proteins, tyrosine kinases or integrins could be activated besides sperm factor injection and could be upstream mechanisms involved in later embryonic development. Among different assisted reproductive technologies (ARTs), intracytoplasmic sperm injection (ICSI) is considered as the best and easiest therapeutic technique to circumvent severe male infertility. Although most reports are reassuring, some recent data suggest a greater incidence of abnormalities in children conceived by ART compared with those conceived normally. Spatio-temporal signals may be missing or abnormal during ICSI, perhaps because membrane fusion and signalling events are bypassed. We discuss in the second part of this review the hypothesis that potential perturbations during the ICSI procedure may have repercussions on epigenetic processes, inducing not only alterations of embryonic development, but also diseases in young children and, perhaps, in adults.

KEY WORDS: *fertilization, oocyte, egg, ICSI, calcium, epigenetics*

Activation of the mammalian egg after fertilization

Many reviews have been published to describe how adhesion and fusion of sperm and egg at fertilization induces the formation of a zygote that develops into a new individual. In mammals, the oocyte that can be fertilized is arrested at metaphase of second meiotic division after ovulation. The metabolism of this oocyte is low, and if no fertilization occurs the oocyte will die. This arrest of the cell cycle is due to high activities of MAPK ("M-phase activating protein kinase") and MPF ("mitosis promoting factor"), the latter being a complex between cdc2 kinase and cyclin B. After fertilization, oocyte activation triggers inactivation of these two kinases, exit of meiosis, and start of embryonic development (Ducibella and Fissore, 2008). Normally, egg activation after fertilization starts from the point of sperm-egg interaction and fusion. In all species studied so far, from invertebrate to mammals, the process of egg activation has not yet been clearly identified, but several hypotheses have been proposed in the literature and include: 1) interaction between oocyte receptor(s) and sperm ligand(s); 2) formation of a pore between the two gametes allowing the diffusion into the oocyte of calcium and/or one (or several) spermatic factor(s); 3) insertion in the oocyte plasma membrane of spermatic plasma membrane elements such as calcium channels (Fig. 1). These different hypotheses are not exclusive and several, if not all, could be employed at

Abbreviations used in this paper: ART, assisted reproductive technologies; Ca, calcium; ICSI, intracytoplasmic sperm injection; MAPK, M-phase activating protein kinase; MPF, mitosis promoting factor.

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fertilization.

In developed countries, it is estimated that 1–3% of children are conceived by ARTs (Assisted Reproductive Technologies), in order to treat human infertility. Several factors associated with assisted reproductive methods might increase the malformation rate, which is regularly discussed in the literature. Firstly, chromosomal anomalies may be carried by the gametes used, which could also be at the origin of male or female infertility itself. Secondly, methods used to stimulate fertility and leading for example to superovulation or *in vitro* maturation of gametes have clearly been reported to be able to perturb epigenetics and genomic imprinting, particularly in the extra-embryonic tissues (Laprise, 2009). ART techniques include intra-uterine insemination, *in vitro* fertilization (IVF) and ICSI (*intracytoplasmic sperm injection*). ICSI is considered today as the best and easiest therapeutically technique to circumvent severe male infertility. Injection of a sperm into an egg circumvents natural sperm selection and could indeed transfer any chromosomal anomaly from paternal origin. Finally, ICSI could also introduce foreign material such as culture medium or exogenous DNA and infectious material (Chan *et al.*, 2000). It is therefore difficult to know the origin and the cause of any chromosomal anomaly detected in children conceived by ICSI. Contrary to classical *in vitro* fertilization, interactions between membranes of sperm and oocyte that normally occur after fertilization are bypassed in ICSI. We discuss in this review the possibility that events triggered during or after fertilization and normally induced at the plasma membrane level could be absent or abnormally generated during ICSI and could have an impact on late development and even after birth.

The fertilization calcium signal

In mammals and all other species analyzed so far, fertilization always triggers in the egg a rapid and transient increase in intracellular free calcium (Ca_i), and various recent reviews have been published that describe and discuss the nature and the role of this ionic signal in egg activation and development of the embryo. In mammals, this "calcium signal" is in the form of Ca_i oscillations, the frequency and intensity of which vary with the species (Swann and Yu, 2008; Ducibella and Fissore, 2008). They are generally observed in human, but seem to be altered after conventional ART or after ICSI as discussed below.

In general, calcium acts as a second messenger through multiple pathways and controls a wide range of cellular functions, from exocytosis to gene expression and even cell apoptosis (Scharenberg *et al.*, 2007; Berridge, 2009) Ca_i signals are interpreted by specific proteins that are responsible for the onset of development. Many of these proteins are protein kinases (CaMKII,

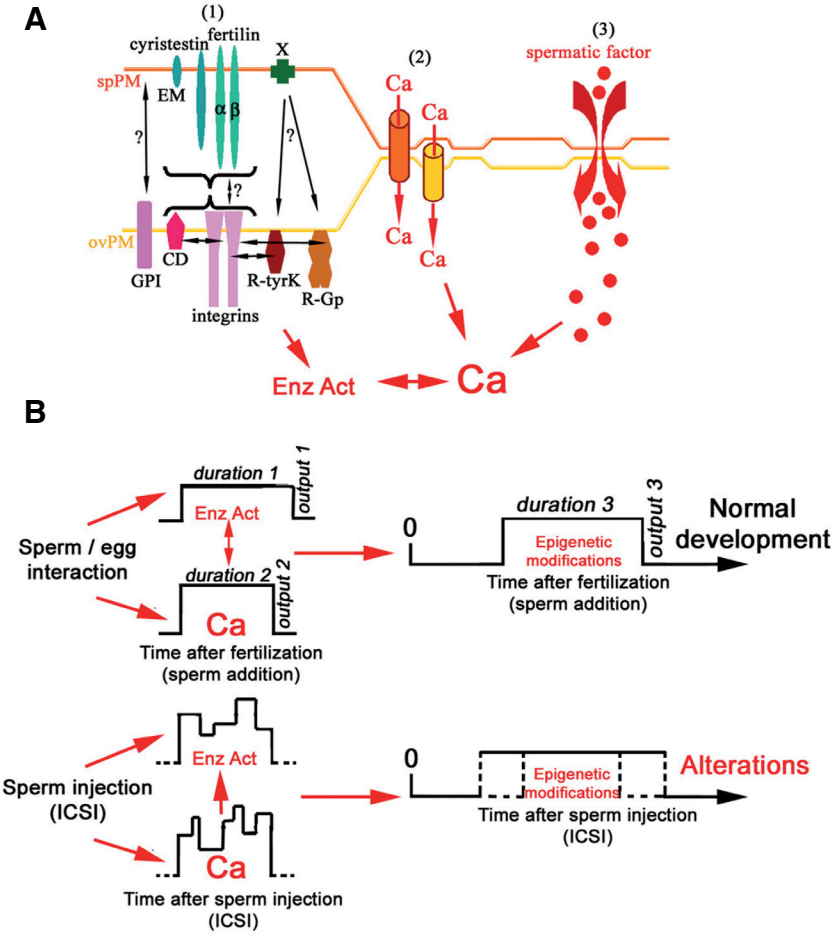

Fig.1. Interaction between sperm and egg after fertilization that is bypassed after ICSI. (A) *Three possibilities that are not exhaustive.* **(1)** *Interaction between sperm ligands and receptor(s) expressed at the surface of the oocyte. Molecules of the sperm plasma membrane (spPM) can bind receptors of the plasma membrane of the oocyte (ovPM): fertilin, cyritestin (ADAM3), elements of the extracellular matrix (EM) such as fibronectin, these elements being ligands of integrins, protein complement regulator the receptor of which are unknown, or diverse other molecules (X) which could bind receptor with tyrosine kinase activity (R-tyrK) or receptors bound to G proteins (R-Gpt). Tetraspanins CD9 and CD91 could interact with integrins, R-tyrK or R-Gpt as it is the case in T lymphocytes. All these molecules can be part of a complex capable of inducing adhesion and fusion between gametes, but also enzymatic activities (Enz Act) such as tyrosine kinase that can be involved or not in triggering a Ca signal.* **(2)** *Role of Ca channels. Calcium channels are already present in the plasma membrane of the oocyte. Moreover, several classes of calcium channels are also present in the sperm plasma membrane and are inserted into the oocyte plasma membrane after sperm fusion. The sperm channels may be activated after their incorporation. Both oocyte and sperm channels would trigger Ca increase in the vicinity of ER large enough to modulate the shape of the Ca_i signal.* **(3)** *Formation of a pore between gametes allowing the diffusion of one (or several) sperm factor(s).* **(B)** *Ca signal and enzymatic activities regulate epigenetic modifications at early times after fertilization. Enzymatic activities and Ca signal normally induced after sperm-egg interaction would constitute two outputs (output 1 and 2 respectively) of well-defined duration and amplitude. This would regulate epigenetic modifications that also need to occur in a precise window of time and amplitude for normal development. Altered outputs 1 and 2 triggered after ICSI would lead to abnormal epigenetic modifications.*

PKC, MPF, MAPK, MLCK) whose activity is directly or indirectly regulated by Ca_i and varies not only during oocyte maturation but also at early times following fertilization (Ducibella and Fissore, 2008). In order to avoid activation of unwanted cellular process, Ca_i increases are spatially and temporally controlled and a wide range of calcium channels are present in the plasma and reticulum membranes to achieve their spatial and temporal control (Boulware and Marchant, 2008; Rizzuto *et al.*, 2009).

It has become a dogma that this event is necessary and sufficient to activate eggs in all species that have been studied so far, since blocking the calcium increase with Ca^{2+} buffers inhibits egg activation and inducing an artificial calcium increase activates the egg. It is important to determine the criteria that are used to judge egg activation. For example, in almost all schema proposed in the literature to explain how a sperm activates an egg, the calcium signal is upstream of all events that lead to development of the zygote. However, only "early events" are chosen as the criteria for judging egg activation, i.e. those normally occurring before blastocyst formation. Whether the Ca_i signalling events have an impact on long-term embryonic development has not been well examined. Furthermore, Ca independent signalling pathways could be activated concomitantly with the Ca_i signal, and be involved in events occurring after blastocyst formation. Several questions are therefore apparent: 1) could abnormal development or diseases be induced after ICSI because of an altered Ca signal transduction? 2) Could signal transduction pathways that are bypassed by ICSI none-the-less have a role to play during development?

Calcium release from the endoplasmic reticulum

Increase in Ca_i in the egg is mostly due to a release of Ca_i from endoplasmic reticulum (ER). Several types of Ca channels are involved: inositol trisphosphate receptors (IP_3-R) (Berridge, 2009), ryanodine receptors (RyR) whose activation is regulated either by Ca^{2+} influx or by cADPr (cyclic adenosine diphosphate ribose) (Yin *et al.*, 2008), or channels activated by NAADP (nicotinic acid adenine dinucleotide phosphate) (Patel *et al.*, 2010). In hamster, only IP_3-R seems to be involved (Miyazaki *et al.*, 1992). In mouse, RyR, although present and functional in oocytes, would not be solicited during oocyte activation, and only IP_3-R, aggregated in clusters of the RE, would be necessary (Fitz Harris *et al.*, 2003). NAADP could play a key-role in Ca_i signal origin in various species but has not yet been demonstrated to be involved in mammals (Santella *et al.*, 2004). In human oocytes, propagation of the Ca_i signal differs from that observed in other mammals, whatever the protocol of fertilization. This signal is characterized by a rapid increase in Ca_i at the periphery of the oocyte, followed by a slower increase in Ca_i deeper in the cytoplasm. The fact that ryanodine-sensitive calcium stores are located at the periphery of the mature oocyte suggests a role at the origin of the fertilization calcium signal in human (Sousa *et al.*, 1996). In any case, the IP_3-R1 seems to be required to generate Ca_i oscillations and activate mammalian eggs (Ducibella and Fissore; 2008), and how IP_3 production is activated after fertilization is then an important issue that needs to be clarified.

Role of calcium channels located in the plasma membrane

The oocyte is an excitable cell, and hyperpolarisation occurs during fertilization in mammals (Tosti, 2010). Dependence between calcium modification and plasma membrane potential has been argued by Miyazaki (1989) suggesting a role of voltage-dependent channels in the Ca rise. Voltage dependent calcium channels are present in plasma membrane: L type Ca channels have been described in bovine oocytes and T-type channels in early mouse embryos (Tosti, 2010). Ca channels could be activated during interaction with the sperm, as described in sea urchin (David *et al.*, 1988), which would allow a sufficient amount of calcium into the oocyte to stimulate the surrounding IP_3-Rs and generate the Ca signal. These Ca channels could also be directly coupled to IP_3-R or RyR as it is the case in muscle (Berridge, 2009). Insertion of sperm Ca channels into the oocyte plasma membrane during sperm / egg fusion has also been proposed several years ago in sea urchin (McCulloh and Chambers, 1992) but this hypothesis has never been proven, even in this species. These activated channels may behave as described above. Mammalian sperm contain various Ca channels: VOC ("voltage activated channels"), ROC ("receptor-activated channel") and SOC ("store-activated-channel"),that are involved during the acrosome reaction (Tosti, 2010). Finally, a TRP-3 type Ca channel has recently been involved in the fertilization process in C *elegans*. Mutations of this channel lead to sterility of hermaphrodite and male animals, sperm being motile but incapable of fertilizing oocytes (Xu and Sternberg, 2003). It is important to note that Ca channels of this type are expressed in human (Castellano *et al.*, 2003). Whether some types of Ca channels of the oocyte plasma membrane are involved or not in the initiation of the fertilization Ca_i signal, they play an important role in “refilling” intracellular calcium stores to maintain them full during several hours and later in the fertilized mammalian oocyte (Ducibella and Fissore, 2008).

Role of mitochondria

Mitochondria are present in large number in eggs and act as a relay in Ca_i signalling at fertilization. They are necessary to maintain Ca_i oscillations for a long time in mouse oocytes (Dumollard *et al.*, 2009). Studies on ascidians and mouse have shown that sperm-triggered Ca_i oscillations are transduced into mitochondrial Ca_i signals that stimulate mitochondrial respiration. Mitochondrial Ca_i uptake can substantially buffer cytosolic Ca_i concentration and the concerted action of heterogeneously distributed mitochondria in the mature egg may modulate the spatiotemporal pattern of sperm-triggered Ca_i oscillations (Dumollard *et al.*, 2009). Furthermore, inadequate redistribution of mitochondria at MII, unsuccessful mitochondrial differentiation, or decreased mitochondrial transcription induce significantly lower rate of embryo development (Dumollard *et al.*, 2009), even after activation by intracytoplasmic sperm injection (Nagai *et al.*, 2006). Alterations of mitochondrial calcium signalling mechanisms could then not only affect the fertilization Ca_i signal and, consequently, embryonic development, but also lead the oocyte toward apoptosis, a cellular death program that involves mitochondria and is used by oocytes when they are not fertilized (Dumollard *et al.*, 2009).

The sperm factor

The sperm factor hypothesis has strongly been built up by the fact that human oocytes can start embryonic development after ICSI, since during this procedure, sperm / egg interaction is short-

circuited. This hypothesis also comes from the fact that sperm extracts obtained in invertebrate such as ascidia as well as in mammals such as human are capable of generating Ca oscillations in mouse, hamster, human or sea urchin oocytes, and even in somatic cells. These sperm extracts are then non species specific, which suggests that the sperm factor is universal and identical in all species. The sperm factor would be either soluble, or insoluble and associated with the perinuclear material, and is thermo labile since it loses its activating effect after heating (Swann and Yu, 2008). During more than fifteen years, several candidates have been proposed, some have then been clearly forgotten, others remaining to be proved to be "the good one". As an example, a 33 kDa proteic factor was purified, then cloned and called “oscillin”. Oscillin was described as specifically localized in the cytosol, in the sperm equatorial plate, the zone that is involved during gametes interaction. Testicular slices from sterile men correlated with the absence of oscillin protein using anti-oscillin antibodies. It was also shown that oscillin could bind an oocyte component, creating a complex capable of activating both RyR and IP_3-R. In fact, oscillin was a glucosamine-6-phosphate deaminase present in various other tissues and was then clearly ruled as being the "sperm factor" (Ducibella and Fissore, 2008).

A truncated form of c-kit (tr-kit), a tyrosine kinase receptor of the PDFG receptor family, has also been proposed as a potential sperm factor, since it could trigger oocyte activation, with cell cycle resumption, MAPK kinase inhibition, extrusion of the second polar body and cortical granule exocytosis. This 24 kDa protein was proposed to bind another tyrosine kinase fyn, which would lead to PLCγ stimulation (Sette *et al.*, 2002). However, several points must be clarified to verify this hypothesis. One can for example ask why tr-kit is expressed in the mid-piece of the flagella and not in the equatorial zone which fuses first with the oocyte It has also never been shown whether tr-kit injection induces the same Ca_i oscillations as those induced at fertilization. However, it is possible that tr-kit injection induces activation of pathways involving tyrosine kinase of the src or fyn family, which can bind integrins such as $\alpha 6\beta 1$ (Streuli and Akhtar, 2009), molecules that are expressed in the oocyte as described below.

As mentioned above, the IP_3-Rs are necessary to generate Ca_i oscillations. The IP_3 that activates these receptors comes from hydrolysis of phoshatidylinositol bisphosphate (PIP_2) by a phospholipase C (PLC). PLCs form a family of molecules that respond to defined signalling pathways. For example, PLCβ is downstream G-protein receptors while PLCγ belongs to a tyrosine kinase signalling pathway (Berridge, 2009).The identity of the isoform of the particular PLC upstream the IP_3-R could reveal information concerning the cellular pathway that is stimulated at fertilization. Various data led to the hypothesis that sperm would directly inject a PLC into the oocyte following sperm-oocyte fusion which would produce IP_3 in the oocyte. A PLC was purified and cloned in 2002 from a mouse testis EST library: PLCζ. PLCζ has since been cloned in monkey and human suggesting the ubiquity of PLCζ in Mammals. This PLC does not contain any particular domain usually found in other PLCs, PLCγ or PLCβ, and such as SH2, SH3 or PH. PLCζ is the smallest PLC known to date. Expression of mRNA encoding this protein in mouse oocytes induces Ca_i oscillations similar to those observed after fertilization in this species. In human, injection of complementary RNA of human PLCζ in oocytes that were not activated by ART or ICSI induces blastocyst formation. Ca_i oscillations induced by PLCζ exclusively appear in M phase, after its nuclear localization.PLCζ shows such a very high sensitivity to Ca that it should be active even at rest, but the binding of its C2 domain to particular phosphatidylinositides (PI3P et PI5P) would regulate its activity. All these results strongly argue that PLCζ is the long sought after sperm factor (Swann *et al.*, 2006; Swann and Yu, 2008). However, it is intriguing that PLCζ is not expressed in animals where the genome has been sequenced such as sea urchin, ascidian, *Drosophila*, and *C-elegans*. Therefore, PLCζ cannot be the universal sperm factor and perhaps only exits in vertebrates. An unfertile knock out male mouse would be the best way to confirm that PLCζ is the sperm factor but so far this has not been performed successfully.

Calcium signalling after ICSI

ICSI seems to produce a pattern of Ca_i oscillations slightly different from those described after normal fertilization. In human, the first Ca_i oscillations triggered after ICSI are truncated and delayed, in comparison with those observed after SUZI ("sub zonal insemination"), which however does not alter the following Ca_i oscillations (Tesarik and Sousa, 1994). In mouse, the spatial distribution of the Ca_i rise (Nakano *et al.*, 1997) and duration of Ca_i oscillations (Kurosawa and Fissore, 2003) are not equivalent in eggs subjected to ICSI and those subjected to IVF. Therefore, alterations of the calcium signal can be observed at very early times (alterations of first peaks) or in the number and frequency of following oscillations.

Alterations of early Ca peaks

In ICSI, the sperm is immobilized and then taken by aspiration in the injection pipette. The injecting pipette is pushed against the zona, permitting its penetration and thrusting forward to the inner surface of the oolemma. As the point of the pipette reaches the approximate center of the egg, a break occurs in the membrane. This is reflected by a proximal flow of cytoplasmic organelles and the spermatozoon back up into the pipette. The sperm is then slowly ejected back into the cytoplasm, with oocyte cytoplasm. Rupture of the oocyte plasma membrane is a *sine qua non* condition of fertilization success. In mouse, a first Ca_i rise is inevitably produced by calcium entering through the opening of the plasma membrane during the ICSI procedure and affects the duration, intensity and spatial heterogeneity of the subsequent first Ca_i responses, depending on the intensity of this first non physiological peak (Miyazaki, 1999). ICSI then immediately induces a Ca influx, which can also be obtained after injection of medium that does not contain any sperm, but which would not activate the oocyte by itself (Tesarik and Sousa, 1994; Tesarik, 1998). This Ca influx induced after ICSI seems necessary but not sufficient to activate the oocyte. The different methods used to immobilize the sperm during ICSI (pipeting, squeezing, piezo application) seem to influence the beginning of the Ca_i oscillations. All these techniques damage the sperm membrane. The piezo method is the one that induces Ca oscillations more quickly, and induces the highest percentage of fertilization success (Yanagida *et al.*, 2001). Rupture of the sperm membrane is likely required to release one or several factor(s) involved in generating the Ca_i oscillations (Marangos *et al.*, 2003). These results suggest

that the ICSI procedure itself takes the place of the "start signal" that is normally activated after sperm and egg interaction.

Could this difference have any incidence on later development? The impact could be direct, by affecting Ca-dependent mechanisms such as those depending on Ca-calmodulin kinases which themselves will transduce an altered signal. ICSI entails breaching the plasma membrane, which results in a Ca_i influx while plasma membrane integrity is being restored. The entering Ca_i can trigger spontaneous Ca^{2+} transients, and such high frequency Ca_i oscillations have been observed after fertilization when membrane integrity is disrupted (Ozil, unpublished observations cited in Ozil *et al.*, 2006), which could well have an impact on late embryonic development, as discussed below. Finally, a few births obtained after ICSI in oocytes that were previously activated by Ca ionophore have recently been reported. This procedure was used when repeated fertilization failed after "classical" ICSI (Eldar-Geva *et al.*, 2003; Chi *et al.*, 2004; Heindryckx *et al.*, 2008; Taylor *et al.*, 2010). It is important to note that this was done directly in human without any previous result obtained in mouse or any other animal species used in laboratories. Ca ionophore triggers a huge increase in Ca_i due to Ca influx from the external medium and leaks from intracellular stores. The pattern of Ca_i oscillations obtained in these conditions is unknown, but is likely abnormal.

Abnormal Ca_i signals could easily be due to the fact that, during ICSI, the sperm factor is being delivered from a different place and at a different rate than during physiological fertilization (as discussed by Nakano *et al.*, 1997). ICSI could modify the early fertilization Ca peaks by altering Ca channels that trigger these peaks. For example, increase in the Ca_i level during ICSI procedure as described previously could modify the activity of IP_3-R which are sensitive to calcium (Berridge, 2009). Finally, Ca channels of the oocyte plasma membrane described above could be bypassed after ICSI.

Modifications in the number and period of Ca_i oscillations

Ca_i oscillations induce MPF inactivation by acting on a Ca/calmodulin upstream MPF and on mechanisms involved in cyclin B destruction. Alterations in the fertilization Ca_i oscillations could then alter mechanisms that depend on MPF activity and that are responsible for early embryonic development (Ducibella and Fissore, 2008). The level of MPF varies with oocyte maturation and can modify the pattern of Ca_i oscillations. However, this impact would be indirect in mammals, as shown in mouse, where MPF determines the timing of formation of the pronucleus which itself regulates Ca_i oscillations after fertilization (Ducibella and Fissore, 2008). The number of Ca_i oscillations also controls early morphological events such as cortical exocytosis and recruitment of maternal mRNA (Ducibella and Fissore, 2008), and acts on cell composition in blastocysts by altering the trophectoderm cell number in mouse (Bos-Mikich *et al.*, 1997). Finally, modifications of Ca_i level can also alter protein synthesis in mouse oocyte (Bos-Mikich *et al.*, 1995).

The pattern of Ca_i oscillations depends on the maturity of each gamete used during ICSI. Several results have shown that the ROSI ("round spermatid injection") or ELSI (elongated spermatid injection) techniques can lead to oocyte activation in mouse, hamster and human that could occur without normal Ca_i oscillations (Yazawa *et al.*, 2000). However, another report shows that the ROSI technique applied to 58 couples did not give any birth (Urman *et al.*, 2002). ICSI applied on oocytes that were matured *in vitro* can also lead to births (Liu *et al.*, 2003), but to our knowledge, no result has been obtained in human concerning the impact of oocyte maturation on the Ca_i signal.

Alterations of late development might well occur when Ca_i oscillations are not accurately generated (Ozil *et al.*, 2006). A precocious interruption of natural regime of Ca_i oscillations alters the incidence of implantation whereas hyper-stimulation of Ca_i signalling events compromises post-implantation development inducing a greater variability in the weight of such induced offspring, which indicates a reduced developmental competence of the blastocysts. More importantly, analysis of global patterns of gene expression by microarray analysis revealed that approximately 20% of the transcripts were mis-regulated when too few oscillations were generated in the embryo. Expression Analysis Systematic Explorer (EASE) analysis indicated that genes preferentially involved in RNA processing and polymerase II transcription were differentially affected, and those involved in cell adhesion mis-expressed which could explain reduced implantation. 3% of the transcripts were mis-regulated following hyper-stimulation, and EASE analysis indicated that genes preferentially involved in metabolism were differentially affected. All these results strongly suggest that altering the fertilization Ca_i signal can have long-term effects on both gene expression and development to term (Ozil *et al.*, 2006).The frequency of the Ca_i oscillations seems to be modulated by the sperm content in a dose-dependent manner, which suggests that the concentration of some sperm factor within the sperm is a crucial element of normal fertilization and oocyte activation (Faure *et al.*, 1999). Sperm used for ICSI and containing low amounts of the sperm factor PLC∂ can trigger enough Ca_i oscillations sufficient to initiate development but insufficient to support development to term (Ozil *et al.*, 2006). Globozoospermia is a severe pathology characterized by the absence of the acrosome and thus sperm are unable to cross the zona pellucida. Although ICSI bypasses zona pellucida crossing, oocyte activation does not occur with these deficient sperm, and this lack of activation seems to be correlated with PLCζ absence. Recent reports suggest that reduced amounts or abnormal forms of PLCζ could be overcome by activating the sperm-injected oocytes with Ca ionophore (Kyono *et al.*, 2008, Tejera *et al.*, 2008, Taylor *et al.,* 2010). Although this treatment could lead to high rates of fertilization and an ongoing pregnancy, we may wonder what the risks are from both a probable abnormal Ca signal as already discussed above and a highly abnormal sperm material since a high percentage of sperm DNA fragmentation is associated with this pathology.

Signalling cascades upstream or independent of the fertilization calcium signal

If the fertilization Ca signal is necessary and sufficient to trigger egg activation, it is therefore essential to know how it is triggered in the egg from the starting point of sperm/egg interaction, and we have mentioned above the different hypotheses that have been proposed in the literature. The first point to consider is that any single cascade could not only lead to a calcium signal but could also contain elements capable of activating Ca-independent pathways. The second point is that sperm could indeed straightfor-

wardly induce a calcium signal, for example through the injection of a sperm factor, but this does not exclude necessarily the stimulation in parallel of other pathways, independent of the Ca signal. The possibility that events may be generated at the plasma membrane level besides this Ca signal after sperm - egg interaction and may have an impact on late development has never been envisaged. If that were the case, it would question the role of various signalling elements such as G proteins that have generally been tested by looking only at their ability to induce a Ca signal, and not on their capability to act on other events that do not necessarily depend on calcium. Finally, since the interaction between membranes of sperm and oocyte that normally occurs after fertilization is bypassed after ICSI, putative signalling pathways generated from the plasma membrane would also be bypassed, or abnormally stimulated. Since ICSI leads to apparently normal development, one must admit that all events necessary to trigger development have been stimulated by ICSI. Organization into nanodomains and clusters of different signal transduction circuits, for example in lipid rafts that have been suggested to play a crucial role in egg activation (Sato *et al.*, 2006), allows fine regulation of duration and intensity of various *in vivo* responses, including Ca or MAP kinase signals (Harding and Hancock, 2008). Therefore, all putative signalling pathways proposed below generated by integrins, tetraspanins, GPI or R-G proteins may have been stimulated after ICSI by crosstalks and/or feedback loops.

Role of tyrosine kinase or G-protein receptors

This hypothesis comes from the fact that several molecules expressed at the sperm surface could bind to the oocyte plasma membrane and behave as ligands of oocyte receptors. Various G proteins (Gαs, Gαi 1-3, Gαo) are expressed in the mouse oocyte. Moreover, microinjection of GTPγS, a GTP analogue, leads to oocyte activation while microinjection of GDPβS, which inhibits G-proteins, cancels the Ca_i signal in hamster. These results are in favor of a G-protein receptor in these species (Runft *et al.*, 2002), but neither receptor of this type nor putative ligand has ever been identified. However, G-proteins could be indirectly activated through other pathways implying tyrosine kinase activity. Similarly, the presence and role of a tyrosine-kinase receptor has never been demonstrated (Runft *et al.*, 2002).

Role of the Gq family G proteins in mouse eggs activation has been investigated by looking at their ability to initiate calcium release, cortical granule exocytosis, recruitment of maternal mRNAs, and cell cycle resumption, but whether they can affect later development is not known. Similarly, injection of SH2 domains or addition of tyrosine kinase inhibitors to block the normal stimulation of PLCγ, or src like kinases also fails to stop sperm induced Ca_i oscillations in mouse eggs (Mehlmann and Jaffe, 2005). These results only strongly suggest that neither PLCγ not src like kinase are sufficient to transduce a Ca_i signal, but cannot exclude the idea that they may be involved in later development, or need to be associated with another event to generate the fertilization Ca signal. As a matter of fact, although Src-family PTKs are not required for fertilization-induced calcium oscillations in mouse, activation of these kinases in distinct regions and at specific times does play a critical role in development of the zygote (Luo *et al.*, 2009). PTKs and G-protein receptors are linked to MAPK activation (Shaul and Seger, 2007), which itself may act on Ca_i oscillations (Matson and Ducibella 2007).

Integrins, tetraspanins and glycosylphosphatidylinositol-proteins

In mammals, several sperm proteins that could behave as specific ligands of oocyte receptors have been identified. Ligands of integrins, that are elements of the extracellular matrix such as fibronectin or fertilin, have largely been described in the literature to be expressed at the sperm surface.Fertilin is composed of two transmembranal subunits, α and β. The β subunit contains a disintegrin domain that could bind an integrin of the egg surface, while the α subunit presents in its intracellular domain a helical motif that is also found in viral fusion proteins, which would allow the membranes of the two gametes to fuse. Fertilin has been cloned in various mammals including human. This molecule belongs to a family of 39 membrane proteins that have been named ADAMs ("A Disintegrin And Metalloprotease domain"). Some of them are expressed in the sperm, and the best studied are fertiline β (ADAM2) and cyritestine (ADAM3). Mutagenesis of these proteins leads to infertile male mice, which suggests a role of these proteins in the process of sperm-egg interaction. However, other ADAMs could also be involved. A new sperm protein, named Izumo, has been identified as required for sperm fusion with oocyte. Although its oocyte receptor is unknown, this receptor could play a role in egg activation (Rubinstein *et al.*, 2006). The search of the putative receptor of fertilin has led to many articles and controversies over the last years. It has been known for many years that various integrins, glycoproteins that are are constituted of two subunits bound non-covalently, α and β, are expressed at the oocyte surface and that inhibition of these molecules with antibodies or specific ligands can alter fertilization. However, knockout experiments have shown that all female mice that do not express β1 integrins, including α2, α3, α5, α6, α9 and αv subunits, or that do not express the αvβ3 or αvβ5 integrins, are fertile. Moreover, combination of these KO with inhibition of the function of other integrins does not alter fusion between the two gametes. All these results argue against a role of integrins in egg activation (Rubinstein *et al.*, 2006). Recently, the integrin α9 subunit has been suggested to play a role in sperm-egg binding and fusion (Vjugina *et al.*, 2009). Finally, it has been reported that the tripeptide RGD, that binds some integrins, can trigger an increase in Ca_i and even Ca_i oscillations in the bovine oocyte (Campbell *et al.*, 2000).

CD molecules are cell surface molecules that are associated with the immune system and could also be involved in the fertilization process Expression of complement regulatory proteins such as DAF ("Decay activating factor") or CD55 have been described on oocytes, but no role in activation has ever been attributed to these molecules. The tetraspanin CD9 plays a role at fertilization since *Cd9*$^{-/-}$ female have a severe reduced fertility. Another tetraspanin, CD81, is also weakly expressed in the oocyte, but its role in adhesion and/or fusion has not yet been clarified, although fertility of female mice lacking CD81 is reduced (Fabryova and Simon, 2009; Rubinstein *et al.*, 2006).

The role of integrins at fertilization is not easy to decipher, considering the redundancy of these proteins that often function inside multi-protein complexes and in association with other types of receptors including tyrosine kinase receptors and G-protein receptors (Streuli and Akhtar, 2009). Integrins can mediate, inside micro-domains, a few interactions with other proteins including CD9 and CD81. As an example, CD81 is associated in T lympho-

cytes with $\alpha4\beta1$ in the mobility functions of these cells (Kolesnikova *et al.*, 2004). Palmitoylation of CD9 and CD81, as well as that $\alpha3$, $\alpha6$, and $\beta4$ integrin subunits favors their association inside protein complexes (Yang *et al.*, 2004). As a matter of fact, the tetraspanin CD9 would be involved in human and mouse gamete fusion by helping the formation of clusters containing the integrin $\alpha6\beta1$ (Fabryova and Simon, 2009). Tetraspanins can interact with G-protein receptors (Little *et al.*, 2004). Integrins can also alter cellular behaviour through the recruitment and activation of signalling proteins such as non-receptor tyrosine kinases including focal adhesion kinase (FAK) and c-Src that form a dual kinases complex. The FAK-Src complex can phosphorylate various adaptor proteins such as p130Cas and paxillin. Multiple integrin-regulated linkages exist to activate FAK or Src and to interact with other adaptor molecules such as ILK (integrin-linked kinase), PINCH (particularly interesting new cysteine-histidine rich protein) and Nck2 (a tyrosine kinase adaptor protein). Integrin signalling tightly and cooperatively interacts with receptor tyrosine kinase signalling pathways *via* all these molecules (Streuli and Akhtar, 2009).

Finally, female mice that do not express Pig-A, an enzyme necessary to the binding of glycosylphosphatidylinositol- proteins (GPI), are infertile and produce oocytes that cannot fuse with sperm, which suggests a role of GPI during gamete fusion (Alfieri *et al.*, 2003).

In conclusion, all these proteins that can act as part of multicomponent complexes would be capable not only of inducing adhesion and fusion of gametes, but also to start various types of signalling pathways including a Ca_i signal, tyrosine kinase or MAP kinase activities. It is clear that abolishing one of them only, for example after KO experiment, would not necessarily lead to inhibition of egg activation, if another protein inside this complex has a similar role, as it is the case for integrins, or if one missing leg would not refrain the centipede to walk.

Fertilization and epigenetic modifications

Nature and function of epigenetic mechanisms

Gene expression is not only regulated by genetic mechanisms but also by epigenetic modifications. The epigenetic information is not encoded by the DNA sequence itself but by reversible modifications of DNA and/or associated histones, and can be transmitted from cell to daughter cell and even from one generation to the next generation. DNA methylation and histone modifications interplay to control gene expression (Vaissière *et al.*, 2008). Enzymes that induce histone modifications include acetyltransferases (HATs), arginine and lysine methyltransferases (HMTs), ubiquitin ligases and peptidyl arginine deaminase (PAD) (Nottke *et al.*, 2009). DNA methylation is a process that allows the transfer of a methyl group from S-adenosylmethionine to cytosine residues in the CpG dinucleotides by different methyltransferases (Cheng *et al.*, 2008). The entire genome is subjected to epigenetic modifications during gametogenesis and embryogenesis. Particularly, some of these processes are activated very rapidly after sperm-egg fusion and could well depend on the signalling pathways described above.

DNA demethylation after fertilization

DNA methylation is totally erased in germinal cells and then re-established during gametogenesis (Feil, 2009). Each cell has two copies of each gene, one of maternal origin and the other of paternal origin. However, there are genes for which one copy only is expressed, the other being repressed. This genomic repression depends on the parental origin, and is called the genomic imprinting. Before fertilization, one of the alleles, maternal or paternal, is silenced, and then transmitted to the lineage in this conformation. In human, this process is essential for placenta and embryo development, but mechanisms at the origin of this process are still not well understood (Koerner and Barlow, 2010). In mammals, fertilization triggers an active and rapid DNA demethylation of the paternal genome, followed by a passive DNA demethylation of the maternal genome. The entire genome is then methylated again during embryonic development (Feil, 2009). The occurrence and the extent of DNA demethylation of the paternal genome is however controversial among different mammalian species (Abdalah *et al.*, 2009). The mechanisms at the origin of the active demethylation of the paternal genome, and those protecting the maternal genome at the very beginning of embryogenesis, are just beginning to be deciphered, but still poorly understood.

PGC7/Stella, that was initially identified as a gene predominantly expressed in primordial germ cells (PGCs) has been shown to protect the maternal genome from demethylation (Nakamura *et al.*, 2007). Very recently, the component of the elongator complex5 Elp3 (also called KAT9) has been reported to be involved in this process although these authors do not have evidence indicating that Elp3 directly acts upon DNA as a DNA demethylase. Similar results were obtained with the elongator components Elp1 and Elp4. Interestingly, the role of the SAM radical domain in Elp3 seems to be crucial (Okada *et al.*, 2010). This domain is present in the radical SAM superfamily proteins that use S-adenosylmethionine (SAM) to catalyse a variety of radical reactions. SAM is substrate for an activating enzyme, the pyruvate formate-lyase (PFL) activase, PFL leading to the production of acetyl-CoA + formate from pyruvate + CoA (Wang and Frey, 2007). Interestingly, selection between glucose and lactate by a regulated compartmentation of pyruvate in neurons and astrocytes depends on cytosolic NAD^+/NADH redox conditions (Cerdan *et al.*, 2006), which are themselves crucial for mouse early development (Dumollard *et al.*, 2009). Mitochondria are deeply involved in the regulation of the redox state of cells and of eggs in particular and as mentioned above, also modulate Ca_i oscillations in fertilized eggs (Dumollard *et al.*, 2009). This may look like a "drawer hypothesis", but the fertilization Ca_i signal, mitochondria and redox potential might act together to modify epigenetic information during early development since DNA paternal demethylation occurs at early times after fertilization (Santos *et al.*, 2005).

Histone modifications after fertilization

Although the female haploid genome is associated with histones in a somatic-like chromatin structure in the fertilizable oocyte, the spermatic chromatin is highly condensed and transcriptionally inert before fertilization but becomes transcriptionally competent in the male pronucleus (Miller *et al.*, 2010). Modifications of DNA-binding histones that form the nucleosome, H2A, H2B, H3 and H4, occur after fertilization and seem to differ between maternal and paternal DNA (Wu *et al.*, 2008). More importantly, these modifications could occur very rapidly after fertilization. For example, histone H4 is highly acetylated in the paternal genome at an early stage and remains acetylated at a low

level in the maternal pronucleus in early one-cell embryos (Wu *et al.*, 2008). A very quick loss of histone H2A variants H2AL1 and H2Al2 from the paternal pericentric heterochromatin regions occurs after sperm-egg fusion (Wu *et al.*, 2008).

Could an abnormal signal generated after fusion of sperm and egg have an impact on early epigenetic modifications? In mammals, the formation of the pronucleus (PN) is under the control of MAPK activity and perhaps depends on the frequency of Ca_i oscillations (discussed in Dulcibella and Fissore, 2008). It is not known why this event occurs 3-4 hours after expulsion of the second polar body in mammalian zygotes, but this delay could be the time required to reconfigure the sperm chromatin (Morgan *et al.*, 2005). Therefore, an altered timing in the formation of PN may result in altered reprogramming of the male genome. Modifications in Ca_i signalling might also alter activities of enzymes involved in histone epigenetic modifications since they are turned on rapidly after fertilization (Santos *et al.*, 2005) and as described above. The activity of the PAD enzymes is calcium dependent (Holbert and Marmorstein, 2005). For example, PADI4 is a Ca_i -dependent peptidylarginine deiminase enzyme that can function to antagonize arginine methylation levels by demethylimination (Hagiwara *et al.*, 2005). Histone deacetylase also interacts with Ca_i /calcineurin -mediated signalling pathways during T cell activation and proliferation (Im and Rao, 2004) and cardiac gene expression (Sanna *et al.*, 2005). Moreover, stimulation of the integrin pathway could lead to histone epigenetic modifications after fertilization. For example, integrin engagement increases histone H3 acetylation and reduces histone H1 association with DNA in murine lung endothelial cells, thus inducing global sensitivity of DNA to nuclease digestion, which reflects alterations in chromatin structure (Rose *et al.*, 2005).

ICSI and epigenetic defects

Can the risk of epigenetic defects increase after ICSI? Most of the studies give reassuring data meanwhile recommending a more thorough investigation because the level of defects is nevertheless statically higher in babies born through ART and among the different reasons, imprinting defects may be preponderant (Palermo *et al.*, 2009). Heterogeneity of the cohorts used in all these studies renders difficult quantification of such risks linked to ART and particularly to ICSI, and it seems difficult to appreciate whether risks are due to the ART procedures, infertility and/or age of parents. More particularly, ART has been associated with an increase in the frequency of apparition of the Prader-Willi or d'Angelman syndrome, that are due to defects of the genetic imprinting of one region of the chromosome 15, and of the Beckwith Wiedemann syndrome, that is due to epigenetic abnormalities in a cluster of genes that are submitted to imprinting in one region of the chromosome 11 and triggers a macrosomy and an increased risk of neoplasy (Manipalviram *et al.*, 2009). Percentages reported in these studies remain however very low and the number of reported cases insufficient for deducing any conclusion.

Defects in methylation reprogramming of PG3 and APC genes (Zechner *et al.*, 2009) and of the methylation status of H19 Imprinting Control region (ICR) and H19 gene expression (Fauque *et al.*, 2007) have been reported to occur during ART in human. Very few data have been reported where epigenetic modifications after ICSI *vs* normal or ART fertilization have been studied. The pattern of histone methylation seems to differ between ART and ICSI in mouse (Van der Heijden *et al.*, 2009) and even in human (Qiao *et al.*, 2010). In rats, the demethylation dynamics of the paternal genome at pronuclear-stage is impaired when ART are used (Yoshiwara *et al.*, 2010). Sperm chromatin remodeling after ICSI is more asynchronous than in ART in mouse embryos (Ajduk *et al.*, 2006).

Conclusion

Signalling pathways involved during ICSI may be different in duration and intensity from those stimulated after normal fertilization. Could this have a repercussion on epigenetic processes? Are epigenetic modifications of the genome linked to Ca_i signalling and/or to pathways independent of Ca generated at fertilization and could they be altered after ICSI because they did not occur in the natural frame of time or at a right intensity? Could modifications of the initial Ca_i signal, for example after activation of the oocyte with Ca ionophore, have any incidence on epigenetic mechanisms? And finally, is it possible that epigenetic defects induced at the time of fertilization lead to diseases and cancers that would occur only at late adult age?

Epigenetic defects can lead to major pathologies including various syndromes linked to chromosome instabilities or mental retardation (Zhao *et al.*, 2007). More specifically, alterations of parental imprinting have been associated with cancer (Esteller, 2007). Moreover, it is accepted that epigenetic alterations that lead to some forms of cancers are probably linked to environmental factors and ageing (Feinberg, 2007). ICSI has been performed since 1992. Children born that way at that time have then just reached the age of young adults. A large number of cancers and of neuronal and muscular diseases are revealed only in adults. Is it possible that children born after ICSI or ART have epigenetic alterations that are undetectable during young life but develop some kind of cancer by ageing or after exposure to some environmental factors?

All reports on the frequency of abnormalities in children conceived by ART look currently reassuring. However, we believe that more studies should be performed to study molecular mechanisms regulating epigenetic, and more particularly focusing on potential relationships with calcium signalling. Studies on epigenetic modifications after fertilization and putative alterations induced by ART should be encouraged. Finally, this review points out the fact that mechanisms of egg activation still remain unclear and need further investigation.

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