The Choreography of Fertilization

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Abstract

At fertilization, a continuum is established between the final phases of oogenesis and the formation of a new individual. In mammals, the two processes are overlapped. The fertilizing spermatozoon represents the paternal contribution to zygote constitution and at the same time the trigger for the completion of meiosis. Oocytes can mimic fertilization, being able to recapitulate autonomously many of the events of early embryonic development. However, without the sperm contribution development to term cannot occur. The sperm, in fact, carries not only the paternal chromosomes, but also cytoskeletal elements and biochemical cues that are essential to complement and regulate the oocyte cellular legacy. Therefore, oocyte-sperm fusion creates a unique cellular machinery whose regulation in time and space influences the long term destiny of the ensuing embryo.

Keywords

Oocytes • Activation • Fertilization • Pronuclei • Development

Introduction

Fertilization is the process by which the male and female gametes recognize each other, interact, and fuse to give rise to a new cell from which a fully formed individual will ultimately develop. It, therefore, represents the bridge between generations, accomplishing the functions of reproduction and transmission of genetic variation.

From a mammalian "oocentric" standpoint, fertilization is also a time of transition, during which oogenesis is finally achieved with the completion of meiosis, while the journey of the newly formed embryo has already started through the fusion of the oocyte with the spermatozoon. The mature oocyte is at the same time the stage and the main actor of the performance of fertilization. It can in fact undergo activation, i.e., recapitulate many of the events of fertilization – including cortical granule release, completion of meiosis, and formation of the female pronucleus – in the absence of paternal contribution. Nevertheless, this choreography would be largely imperfect without the participation of the spermatozoon. This chapter describes the major events occurring

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during fertilization in human and other mammalian species. In particular, sperm-oocyte interaction, gamete fusion, activation events, pronuclear formation and development are illustrated with the intent to give emphasis to both physiological and applicative implications – i.e., in vitro fertilization (IVF) for fertility treatment – of the fertilization process.

Initial Contact: Involvement of Cumulus Cells in Fertilization

Cumulus cells have an important role in fertilization in vivo. It has been shown that the organization of the oocyte, cumulus cells, and extracellular matrix is essential for successful fertilization in the oviduct [1, 2]. In particular, infertility of female mice defective for pentraxin synthesis, a structural constituent of the cumulus mass extracellular matrix, is caused by severe cumulus mass abnormality and inability to support oocyte fertilization in vivo, but not in vitro [3]. The cumulus mass functions physically to entrap spermatozoa and guide them to the oocyte [4, 5], having a chemotactic effect probably mediated by chemokine signaling [6, 7]. Once reached the cumulus cell-oocyte complex (COC), spermatozoa search a passage through the extracellular matrix. The disassembly of the extracellular matrix, observed in vitro in the presence of nonphysiological excess of spermatozoa, may be due to sperm motility and sperm hyaluronidase activity [8] or may be regulated in an autocrine or paracrine manner by cumulus cells. In vivo, only a few spermatozoa may be found around the oocyte at any one time, a condition that probably allows persistence of an intact cumulus mass for a relative long period of time after fertilization.

Sperm–Oocyte Interaction and Fusion

Once the spermatozoon reaches the oocyte surface, the two gametes recognize, bind, and fuse with each other. In the 1970s, studies in invertebrate models suggested the concept of the existence of species-specific sperm receptor(s) on the oocyte vestment, the zona pellucida (ZP) [9]. In mammals, sperm competence to recognize the oocyte is acquired as a consequence of a posttesticular maturation process. This transformation is started in the epididymis where sperm mature, acquiring the ability to swim progressively, advance through the female reproductive tract, undergo capacitation and acrosome reaction, and finally fertilize the oocyte [10].

During capacitation, spermatozoa change their movement to a hyperactivated motility [11, 12]. At the same time, the membrane surface is extensively remodeled. Of particular importance is the activation of the signal transduction pathway of sperm protein kinase A and protein kinase C [13–15], making spermatozoa competent to bind the ZP and respond to this cell recognition event with the initiation of acrosome reaction. In mouse, the ZP is formed from three glycoproteins, ZP1, ZP2, and ZP3 [16]. Even if the molecular mechanism that directs sperm-ZP binding remains controversial, one of the most accepted hypotheses is that spermatozoa interact in a species-specific manner with O-linked carbohydrate ligands of ZP3 [17], provided that this zona protein is structurally associated with ZP2 [18]. In the last several years, a number of candidate ZP3 receptors have been identified. At present, the prevailing opinion is founded on the idea that a sperm multimeric complex is responsible for the interaction with ZP3 and that the assembly of this complex is mediated by chaperons that are themselves activated during capacitation [19–21].

Following sperm-ZP binding, the acrosome reaction is induced. The inner acrosomal membrane is exposed, and enzymes, such as a serine protease and acrosin, are secreted permitting the digestion of the ZP [22]. The hyperactivated sperm cell can then drill through the zona pellucida with sequential local zona digestion and rebinding.

The next steps are adhesion and fusion of the gametes' plasma membranes. After ZP penetration, the spermatozoon accesses the perivitelline space and begins to fuse with the oocyte surface starting from the equatorial segment, located between the inner acrosomal membrane and the plasma membrane overlying the nucleus in the posterior half of the sperm head [23]. Microvilli

present on the oocyte surface envelop the sperm head, preceding sperm–oocyte fusion. On the basis that sperm rarely fuse with mouse oocytes in regions of the oolemma lacking microvilli [24], many studies have been undertaken to find binding and fusogenic molecules present on oocyte microvilli and the sperm equatorial segment [25, 26].

Cell adhesion molecules have been proposed to be involved in sperm–oocyte adhesion, as they mediate somatic cells adhesion. In particular, a role has been suggested for integrins after the identification of an integrin ligand-like disintegrin domain in fertilin α (alpha), fertilin β (beta), and cyritestin sperm ligands [27, 28].

Fertilin α (alpha) (ADAM 1), fertilin β (beta) (ADAM 2), and cyritestin (ADAM 3) are members of the ADAM family (a disintegrin and metalloproteinase domain-containing protein). Fertilin β (beta) was originally identified in the guinea pig as an antigen for an antibody (PH-30) that blocked fertilization [29]. Fertilin α (alpha) was found to form a heterodimer with fertilin β [30]. In turn, cyritestin was originally identified in mouse [31] and monkey [32]. Subsequently, all these molecules have been identified in various other rodents and primates, but functional fertilin α and cyritestin genes have not been identified in human [33]. ADAM proteins contain a metalloproteinase domain, a disintegrin-like domain, a cysteine-rich domain, an EGF-like repeat, and a transmembrane segment with a short cytoplasmic tail. Knockout mice for the genes of three ADAM proteins showed some sperm deficiencies in oolemma-binding activity, but not fusion [34–37].

Integrins are a heterodimeric protein family of a combination formed from 18 α and 8 β subunits [38]. They have been recognized as oolemmal receptors for sperm–oocyte interaction and, in particular, as candidates to recognize sperm ADAMs.

Some integrins are expressed on the surface of mouse oocytes. $\alpha_6\beta_1$-integrin was the first identified candidate for sperm–oocyte binding and fusion [39]. This integrin is also implicated as a receptor for fertilin β and cyritestin [39–41], although studies on oocytes from α_6-knockout mice show that α_6-expression is not required for fertilization [42].

Another family of proteins, tetraspanins, characterized by four transmembrane regions and two extracellular loops, are involved in cell adhesion and other physiological processes. These proteins associate in the plane of the lipid bilayer of the oolemma with other membrane proteins, including integrins, immunoglobulins, proteoglycans, complement regulatory proteins, and growth factor receptors, forming multimolecular complexes, referred to as tetraspanin web [43]. In particular in mouse, CD9, a member of this family, is involved in gamete membrane interaction. In mouse, CD9 is localized over the entire oocyte surface, except the area lacking microvilli where sperm rarely fuse with the oocyte [44]. The same distribution pattern characterizes α_6-integrin. CD9 knockout female mice ovulate normally, and oocytes reach the metaphase II stage but are rarely fertilized. Sperm can bind to oocytes but gametes are generally unable to fuse either in vitro or in vivo [43–45]. In addition, oocytes from CD9 knockout mice can be fertilized by ICSI and give rise to embryos that develop to term, while CD9 knockout females infrequently conceive spontaneously [45]. These observations suggest that CD9 may act in a cooperative fashion with other molecules to facilitate sperm–oocyte fusion. ZP-free oocytes treated with anti-CD9 antibodies showed reduced levels of binding of sperm ligands fertilin α, fertilin β, and cyritestin [40, 41, 46, 47]. These and other data of binding assays suggest a role for CD9 in strengthening the adhesion mediated by ADAMs.

Members of the CRISP family (cysteine-rich secretory protein) are localized on sperm and are thought to be involved in fusion. CRISP1/DE was first identified in rats. It is synthesized by the epididymal epithelium and assembled on rat sperm during epididymal transit. In capacitated rat sperm, CRISP1/DE protein is localized in the equatorial segment [48] and is implicated in gamete membrane interaction. Purified CRISP1/DE protein binds to the entire oocyte surface, except the area lacking microvilli [49]. In addition, anti-CRISP1/DE antibodies prevent sperm fusion with zona-free oocytes [50].

In conclusion, several studies have been carried out to identify molecules involved in sperm–oocyte interaction. Collectively, they suggest the presence of multimeric complexes necessary for interaction on both sperm and oocyte membranes. CD9 appears to be a key component of these complexes in the oocyte. In the case of sperm, multimeric complexes seem to be particularly important, as indicated by the phenotype of fertilin β and cyritestin knockout mice. Furthermore, the fact that antibodies against such molecules cause a reduction in gamete adhesion/fusion is supportive of these hypotheses.

Block to Polyspermic Fertilization

Mammalian oocytes display different strategies to prevent polyspermic fertilization (polyspermy) and avoid the formation of nonviable polyploid embryos.

These blocks occur at the level of the plasma membrane and the zona pellucida. In nonmammals (such as sea urchins and frogs), a rapid (30–60 s) and transient depolarization of the plasma membrane potential is responsible for the membrane block (referred to as "fast block") [51]. On the contrary, in some mammalian species, the membrane block is established approximately 1–2 h after insemination [52–55] and is not generated by a change in oolemma electrical polarization [51]. Rather, inhibition of sperm–oocyte fusion may be dependent on a reduction in sperm adhesion to the oocyte plasma membrane or a detachment after brief or weak adhesion [53, 56–58]. There is evidence that changes in the organization of membrane lipids occur after fertilization [59] and also that fluidity of membrane proteins changes [60], so that molecular differences are present between fertilized and unfertilized oocytes. The use of an intracellular Ca^{2+} chelator has shown that if postpenetration Ca^{2+} signaling (see below) is gradually attenuated, oocytes are increasingly fertilized by more sperm [61], suggesting that the oolemma does not become receptive to sperm according to an all-or-none reaction, but rather that the membrane block is a graded response.

The main system assuring prevention of polyspermy in mammals involves the exocytosis of cortical granules (CGs) from the cortex of the oocyte. CGs fuse with the overlying oolemma and release their contents of enzymes into the perivitelline space (an event referred to as cortical reaction). During the cortical reaction, glycoprotein ZP2 is cleaved by a protease, while β-hexosaminidase B digests the oligosaccharide receptor on glycoprotein ZP3, so that sperm cannot bind to the zona for the absence of a receptor [61]. Furthermore, ZP tyrosine residues are cross-linked preventing proteolytic cleavage. In such a way, sperm penetration is hampered.

The Key Role of Calcium Oscillations in Fertilization

At the intracellular level, Ca^{2+} ions mediate fundamental processes in various cell types. Reports in the sea urchin *Arbacia lixula* consistent with an involvement of Ca^{2+} in fertilization date back as early as the 1930s [62], but the concept that this element was a universal signal for triggering oocyte activation emerged decisively only in the 1970s [63]. Initially in fish [64] and sea urchin [65], a release of Ca^{2+} able to promote activation after sperm-egg fusion was observed as a single and transient increase in cytosolic-free ions, crossing the ooplasm and lasting for several minutes. In mammals, Ca^{2+} changes at fertilization unfold in a different fashion, taking the form of a series of low-frequency, high-amplitude oscillations lasting for hours after sperm penetration [66]. Understanding how and why these oscillations are generated is crucial to appreciate the contribution of the spermatozoon to fertilization and pre- and postimplantation development.

Several lines of evidence suggest that Ca^{2+} oscillations are generated by release of stores of this ion stockpiled in the smooth endoplasmic reticulum. In particular, at the membrane level of this organelle, the inositol trisphosphate (InsP3) receptor appears to be centrally involved in such a mechanism, by binding its ligand and acting as a release channel for Ca^{2+} [67]. In fact, blockage of the binding ability of this receptor with a specific antibody [68] or downregulation of its expression [69] prevents the generation of Ca^{2+} oscillations in

hamster and mouse oocytes, respectively, exposed to sperm. The same effect can be obtained in mouse by inhibiting the receptor expression through injection of siRNA [70]. The critical involvement of the InsP3 receptor implies an increased phosphoinositide metabolism and InsP3 generation. This has been shown to occur in sea urchin [71] and frog eggs [72]. In mammals, technical constraints have prevented direct measurement of InsP3, but the fact that fertilization causes downregulation of the InsP3 receptor is consistent with a mechanism in which sperm penetration generates a rise in InsP3 [69]. Although several hypotheses have been formulated on how a Ca^{2+} response is orchestrated by the oocyte at fertilization, it seems now clear that after gamete fusion the initial trigger for Ca^{2+} oscillations is provided by a factor delivered and released by the spermatozoon. The original clue was the finding that the injection of soluble sperm extracts was able to induce Ca^{2+} oscillations in hamster oocytes [73]. Moreover, a factor derived from mouse sperm was shown to cause oscillations in free Ca^{2+} by inducing the InsP3 pathway [74]. The nature of this factor(s) remained elusive for years, but it is currently accepted that it corresponds to a novel form of phospholipase C, described as PLCζ, described for the first time less than a decade ago [75]. Sperm extracts immunodepleted of this activity are unable to induce Ca^{2+} oscillations in oocytes [76]. A physiological role for PLCζ (zeta) is suggested also by the fact that the amount required to stimulate Ca^{2+} oscillations in microinjected mouse oocytes is comparable to the quantity presumably contained in a single sperm [77].

The discovery of PLCζ (zeta) has been a milestone in the understanding of the involvement of Ca^{2+} in fertilization, but other aspects remain only partially explained. The question of how PLCζ can generate an oscillatory phenomenon is critical in this respect. Repetitive release of Ca^{2+} from the smooth endoplasmic reticulum might be associated to the activity of the InsP3 receptor. It has been proposed that, in the course of a single oscillatory event occurring in mammalian oocytes, the Ca^{2+} releasing activity of the receptor is initially stimulated by a stable increase in InsP3 and inhibited in a successive phase by a negative feedback mechanism elicited by the same Ca^{2+}. This model can explain why a single injection of InsP3 can induce Ca^{2+} oscillations in unfertilized mouse oocytes [78]. However, a particular characteristic of PLCζ suggests that, during fertilization, Ca^{2+} dynamics may also depend on another mechanism. Contrary to other PLCs, PLCζ is in fact promoted by very low concentrations of Ca^{2+} [79]. After gamete fusion, resting concentrations of Ca^{2+} in oocytes are probably sufficient to stimulate an increase in PLCζ activity and, as a consequence, cause a rise in InsP3 concentration. The following release of Ca^{2+} is then anticipated to enhance further this mechanism. These two models based on InsP3 and PLCζ are not mutually exclusive and together could explain the observed oscillatory changes in Ca^{2+}. In fact, the positive feedback mechanism based on the sensitivity of PLCζ to Ca^{2+} could account for the initial Ca^{2+} rise, while the negative feedback action of Ca^{2+} on the InsP3 receptor could be responsible for resetting the ion concentration to resting levels.

The diverse events that concur to the fertilization process are triggered by different intensities of the oscillatory stimulus. For example, a single transient may be sufficient to induce meiotic resumption, while repeated oscillations are required to cause a decrease in kinase activities that regulate the cell cycle [80, 81]. This implies that, irrespective of the apparatus that governs Ca^{2+} dynamics, a downstream biochemical interface is needed for translating the digital language of Ca^{2+} oscillation patterns in precise instructions, expressed in an analogue form, to the terminal effectors. Although calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC) have been proposed as possible decoders [82, 83], the way by which Ca^{2+} oscillations are finely interpreted by the fertilization machinery remains unexplained. It is interesting to note that in mouse oocytes, while a single release or short sequences of Ca^{2+} oscillation are insufficient to trigger full activation, more prolonged series of Ca^{2+} transients are much more efficient in eliciting responses associated to fertilization [84]. The question then arises on the significance of mechanisms that generate and decrypt Ca^{2+} oscillations in mammals, unlike other organisms. Studies in rodent parthenogenetic embryos and fetuses offer a possible

answer to this matter. In mouse blastocysts developed from activated eggs, the relative abundance of cells of the inner cell mass and trophectoderm appears to be influenced by the duration of exposure to Sr^{2+}, an agent which causes Ca^{2+} oscillations [85]. Furthermore, the pattern of Ca^{2+} oscillations by which activation is experimentally obtained in rabbit oocytes has been shown to influence the growth and morphology of the ensuing parthenogenetic fetuses [86, 87]. Therefore, although the most obvious reason for the occurrence of Ca^{2+} oscillations at fertilization lies in the necessity to coordinate the activation events, the dynamics of Ca^{2+} may play a role well beyond fertilization, extending over much later stages of development.

Metaphase II Arrest and Meiotic Resumption

Achievement of meiotic maturation requires a modified cell cycle mechanism, by which two consecutive chromosome segregation events occur without an intervening phase of DNA replication. Although detailed description of regulation of meiosis is given elsewhere in this book (see Chap. 13), the present paragraph offers some basic elements on the same subject in the context of the fertilization process.

In mammals, meiosis is initiated already in the fetal life but is arrested at the prophase stage shortly afterward. The process is reinitiated only at the time of ovulation, with the consequence that human oocytes can remain in meiotic prophase arrest for up to four decades. Luteinizing hormone (LH) surge at ovulation produces a signaling cascade that ultimately promotes meiotic resumption and progression to metaphase II. This effect is mediated by a characteristic dynamics of a key cell cycle regulator, the kinase activity M-phase-promoting factor (MPF), whose constituents are Cdc2 (referred to also as cyclin-dependent protein kinase 1/CDK1) and cyclin B1. At the GV stage, MPF activity is low. Its increase prompted by the ovulatory stimulus causes meiotic resumption (signified by GV breakdown) and entry into meiosis I (MI). At the transition between MI and meiosis II (MII), cyclin B1 undergoes only limited degradation, which coincides with a partial and brief reduction in MPF activity. This determines that entry into interphase and chromosome duplication are prevented, so that haploid oocytes can be ultimately generated. Exit from MI and extrusion of the first polar body is therefore followed by direct entry into metaphase II, after further synthesis of cyclin B1 and reestablishment of high MPF activity. At the metaphase II stage, the oocyte can pause for several hours waiting for the sperm [88, 89]. Metaphase II arrest is made possible by maintenance of high MPF levels. MPF activity tends to decay rapidly, a reason why in somatic cell it can be detected only transiently (30–40 min) during the M phase [90]. In fact, a soon as metaphase II is attained, cyclin B1 is usually subjected to ubiquitination by the anaphase-promoting complex/cyclosome (APC/C) and proteasome degradation. Instead, in oocytes that have extruded the first polar body, MPF is stabilized for a much longer period of time to prevent exit from metaphase II. The nature of the regulatory element responsible for MPF stabilization, referred to as cytostatic factor (CSF), has remained elusive for decades, but evidence gained in the last few years has shed new light on its molecular identity. Originally in the early 1970s [91], CSF was functionally described as an activity meeting some key criteria including (a) appearance and steady increase peaking at metaphase II during maturation, (b) ability to induce M-phase arrest in blastomeres upon injection, and (c) inactivation following augmented levels of intracellular Ca^{2+} that occur at fertilization. Studies conducted mainly in *Xenopus* over the subsequent 20 years established that CSF could coincide biochemically with a pathway including Mos, MAPK/extracellular signal-regulated kinase (MEK), mitogen-activated protein kinase (MAPK), and ribosomal S6 kinase (Rsk) [88]. However, how the Mos pathway could ultimately inhibit the activity of APC/C and thereby prevent ubiquitination and degradation of cyclin B1 was unclear for another decade. More recent evidence has suggested that endogenous meiotic inhibitor 2 (Emi2) is the missing link between the Mos-MAPK pathway and APC/C [92–94].

Emi2 is targeted by both Cdc2-cyclin B1 and the Mos-MAPK pathway. Emi2 phosphorylation by Cdc2-cyclin B causes the dissociation of the Emi2-APC/C complex [95]. The free form of APC/C is active and could induce exit from metaphase II. This effect is antagonized by the Mos-MAPK pathway, which promotes Emi2 dephosphorylation, reconstitution of the Emi2-APC/C complex, and APC/C inhibition [96]. In this regulatory network, it is important to note how a rise in intracellular Ca^{2+} can influence Emi2 activity and, by this means, promote meiotic resumption. In fact, upon fertilization, activation of CamKII by Ca^{2+} transients causes a regulatory cascade involving a series of Emi2 modifications including phosphorylation, formation of a complex with Plx-1, ubiquitination, and ultimately degradation [97]. In this manner, APC/C can be converted in the active form and drive exit from metaphase II. Future studies will clarify whether this model is also applicable to mammalian species.

Centrosome Origin and Formation in the Fertilized Egg

Following completion of oocyte meiosis with the extrusion of the second polar body (PBII), the haploid maternal and paternal chromosome complements become organized in their respective pronuclear structures. At the beginning of their life cycles, pronuclei are usually distant from each other. Therefore, on a cell scale, they need to sustain a potentially long journey before they can make contact in the innermost part of the oocyte and share their chromosomal contents upon breakdown of pronuclear envelopes. This translocation is carried out and directed by an aster of microtubules originating from a single centrosome that acts as an organizing center for microtubules.

Differences exist among mammals, but generally the centrosome of the fertilized egg includes a centriole of paternal origin and pericentriolar material (PCM) of maternal derivation [98]. Exception to this rule is the mouse in which the spermatozoon does not contribute a centriole and the centrosome is entirely maternally derived [99]. The dual origin of the centrosome observed in the fertilized egg of most species reflects the history of this organelle in oocytes and spermatozoa during gametogenesis. Early during oogenesis, the two centrioles are lost while centrosomal proteins are retained [100]. Acentriolar centrosomes are characteristically found at the poles of the metaphase I (MI) and metaphase II (MII) spindles during oocyte meiosis [101, 102]. These centrosomes include proteins, e.g., γ-tubulin and nuclear mitotic apparatus (NuMA) protein, found in the more canonical centriolar centrosomes of the mitotic apparatus. Centrosomal proteins are also dispersed in the ooplasm. They are not easily detectable, but may be observed as aggregates of increasingly larger size in response to pH and intracellular Ca^{2+} changes or stimuli that cause oocyte activation [103]. A different picture characterizes mature sperm, in which most pericentriolar material has been shed while the two centrioles, termed proximal and distal, have been retained [104]. The proximal centriole, adjacent to the basal plate of the sperm head, is distinguished by nine triplets of microtubules [105] and some pericentriolar proteins, including γ-tubulin [106]. This is the sperm centriole that will exclusively contribute to the constitution of the centrosome of the fertilized egg, upon fusion of the two gametes [98]. The distal centriole, positioned perpendicularly to the proximal centriole and coaxially to the sperm flagellum, is regarded as a degenerated structure because it does not show an intact nine-triplet organization, typical of normal centrioles [105]. It also includes appendages, whose function is still little understood, that are normally found connected to the mother centriole of mitotic centrosomes [107].

Once the fertilizing spermatozoon has been incorporated into the oocyte, maternal and paternal constituents cooperate for the assembly of the zygotic centrosome. Complementary contribution from both parents after selective reduction/modification of centrosomal components during gametogenesis probably responds to the need to avoid the presence of two complete centrosomes at the time of gamete fusion, an event that would lead to tetrapolar spindles during the first mitosis

and disruption of chromosome segregation in the two daughter blastomeres. Following gamete fusion, an early step in centrosome formation is the proteasome-mediated detachment of the sperm proximal centriole from the distal centriole [108]. Proteasome activity at the centrosomal level appears critical because its inhibition has been shown to be associated with abnormal aster formation. Once released, the paternal proximal centriole represents the nucleating force of the rising centrosome. Although some γ-tubulin of paternal origin is associated to the sperm centriole, in the fully formed centrosome, most of this protein is recruited from a diffused ooplasmic reservoir [109]. The amount of maternal γ-tubulin that progressively organizes around the paternal centriole is crucial for centrosomal function, because it determines the size and function of the microtubular aster that drives pronuclear relocation [101, 110]. The aster is formed from microtubuli anchored with their minus ends to the centrosome, while their plus ends progressively grow and radiate to distal ooplasmic locations to make contact with pronuclei. In this fashion, the cytoskeletal network that allows the essential process of pronuclear repositioning is generated. However, centrosomal and aster function embraces other aspects of fertilization. The nucleating capacity of the centrosome and the plasticity of the aster, together with associated motor proteins, represent a logistic infrastructure by which organelles (e.g., mitochondria) and macromolecules are displaced, reassorted, and regulated. An example of that is the centrosomal protein PMC1 and associated factors, such as centrin and pericentrin, which are recruited from distal locations, conveyed along microtubules through a dynein-mediated mechanism, and concentrated in the pericentriolar compartment where they participate in the recruitment of γ-tubulin [111].

Pronuclear Formation, Migration, and Breakdown

Pronuclear formation and development is the crucial process during fertilization by which the parental chromosome complements merge and give rise to the zygotic genome. Pronuclei are formed shortly after gamete fusion. They grow, migrate, and juxtapose over several hours and undergo breakdown immediately before the first cell cleavage. The entire process has been observed in detail in human, initially by conventional microscopy [112] and more recently by time-lapse microscopy [113]. Perturbations in time or space of the unfolding of these highly regulated steps are often diagnostic of developmental failure [114].

Before pronuclear formation, the maternal and paternal chromosome sets follow different paths. In the mature oocyte, the meiotic process is arrested at the metaphase stage of the second division. Activation induced by the spermatozoon prompts resumption of meiosis, separation of bivalents, and elimination of one array of chromatids through the extrusion of the PBII. This sets the stage for the formation of the female pronucleus. Chromatids, while still localized near the point of emission of the PBII, establish contact individually or in groups with membranes probably derived from the Golgi apparatus and become progressively incorporated into vesicles. Immediately after their assembly, these vesicles coalesce to form the female pronucleus. The preliminaries of male pronuclear formation are different. The DNA of the fertilizing sperm is in fact associated with protamines, proteins rich in lysine and arginine required for tight chromatin packaging in the sperm head. Protamines must be removed and replaced by histones before the male pronucleus may be formed [115]. Studies performed with leftover material derived from IVF treatments have described in detail the dynamics of male pronuclear formation in human [112]. Immediately after incorporation into the oocyte cytoplasm, the sperm head undergoes a phase of decondensation occurring progressively according to an anterior-posterior axis and signaled by decrease in opacity and loss of definition of its outline. These two changes in the sperm head are followed by swelling of the chromatin mass whose projected area increases sevenfold in about 60 min. During these steps, it is presumed that disulphide bonds linking sperm proteins are reduced, protamines are shed, and chromatin is decondensed in a more relaxed conformation. Afterward, the mass of the decondensed chromatin

regresses to form a rounded shape, decreasing to a size that however remains larger than one of the original sperm head. This phase of re-condensation is caused by packaging of the sperm DNA with maternal histones and occurs in a time frame of 40–60 min. The re-condensed sperm head then undergoes a second wave of expansion accompanied by appearance of a distinct outline. In the following 30–60 min, numerous small masses of sperm chromatin appear as nucleolar precursors. In such a way, the male pronucleus is formed.

Studies in the rhesus monkey suggest that the application of intracytoplasmic sperm injection (ICSI) to achieve fertilization may affect the process of sperm head decondensation [116, 117]. In fact, when a spermatozoon is microinjected into an oocyte, in some cases the condensation of the apical (acrosomal) part of the sperm head is delayed, perhaps as an effect of partial persistence of the acrosomal cap, which vice versa is completely shed during standard IVF before the spermatozoon penetrates through the zona pellucida. A delay in the disassembly of the acrosomal cap and the underlying perinuclear theca might have significant implications for the remodeling of paternal chromatin. In particular, it might cause a lag in processing of apical chromatin during pronuclear formation, an area where the X chromosome chromatin is preferentially located [118, 119]. Together, these factors might interact and ultimately play a role in the generation of a small increase in sex chromosome aneuploidies detected in children born from ICSI treatments [120]. In microinjected rhesus oocytes, in which delayed chromatin decondensation is observed, formation of the zygotic centrosome and the microtubular aster is unaffected either structurally or temporally [116], a sign that during pronuclear formation the male chromatin does not regulate the egg cytoskeleton. This is consistent with the observation that aster formation is noticed also in oocytes in which the sperm head fails to decondense completely [121].

In human, in general male and female pronuclei are formed almost synchronously. In standard IVF, early observation of pronuclear formation is prevented by presence of cumulus cells surrounding the egg, but after ICSI – case in which cumulus cells are removed – pronuclei become visible as early as 3 h postinsemination (p.i.). More commonly, they may be first observed between 4 and 7 h p.i., while formation delayed to beyond 10 h p.i. is highly likely to be associated with developmental failure. Time-lapse microscopy has had a fundamental role in determining pronuclear dynamics [113]. Usually, the two pronuclei are formed at different locations in the fertilized egg. The female pronucleus emerges invariably next to the site where the PBII is extruded, while formation of the male pronucleus can occur anywhere, depending on the site where gamete fusion has occurred or the spermatozoon has been deposited after microinjection. In mouse, sperm penetration is inhibited in the area surrounding the point of emission of the PBII. Therefore, the male pronucleus is always formed at a significant distance from the female pronucleus. In human, pronuclear formation is accompanied by a still poorly understood cytoplasmic phenomenon described as circular waves of subcortical contractions, occurring 2–10 times with a periodicity of 20–50 min [113]. Another cytoplasmic event, referred to as cytoplasmic flare and concomitant with pronuclear formation, is the propagation across the cell of a single wave of contraction [113]. Considering that this wave usually originates from the site of appearance of the male pronucleus, it is tempting to speculate that it represents a manifestation of the radiation of the sperm aster. Once pronuclei are formed, they are displaced by cytoskeletal forces to establish mutual contact (Fig. 20.1). In particular, the female pronucleus is drawn toward the male counterpart. This phase, occurring in most cases between 6 and 9 h p.i., is crucial. In fact, fertilized eggs that fail to assist pronuclear juxtaposition (Fig. 20.2) are destined to develop into embryos affected by massive blastomere fragmentation and early developmental arrest [122, 123]. Pronuclei may juxtapose while they are still positioned eccentrically and only subsequently be transported to a central or paracentral position. Alternatively, they are moved independently, from separate peripheral positions to the center, where they finally make contact. Peripheral displacement of pronuclei after their initial central localization is also predictive of developmental failure (Fig. 20.3). During the

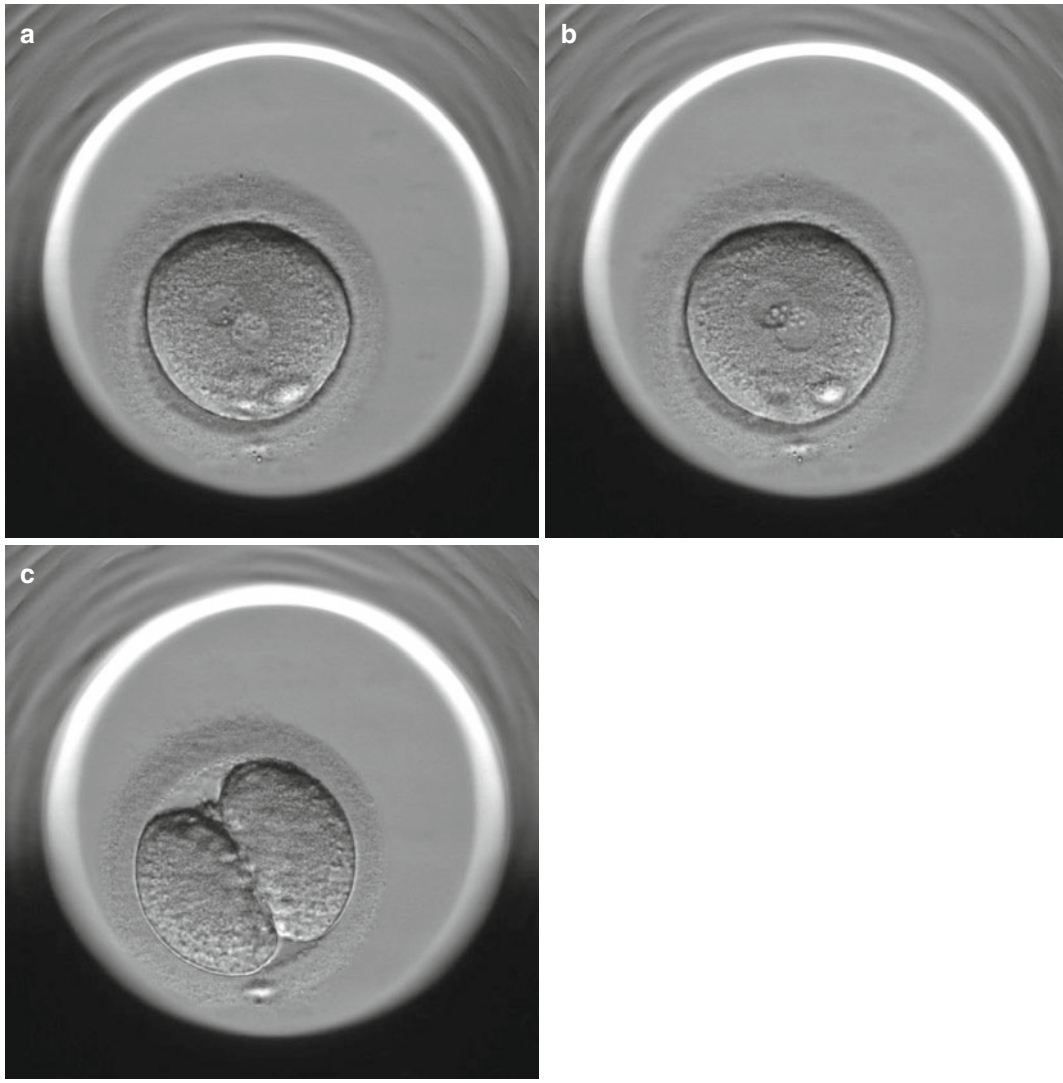

Fig. 20.1 Human fertilized egg showing juxtaposed pronuclei in which chromatin is organized in four to seven nucleoli aligned along the pronuclear contact area (**a**). This organization, which is normally maintained during the later stage during fertilization (**b**), is suggestive of higher implantation ability of the ensuing embryo (**c**)

phases of transportation and juxtaposition, pronuclei increase in size. The diameter of the male pronucleus changes from approximately 16 μ to over 24 μ(mu)m, while the female pronucleus reaches a size of about 22 μm at the end of the growth phase.

Chromatin also rearranges during pronuclear development. In both pronuclei, initially it becomes visible as small masses that subsequently aggregate in nucleoli. Nucleoli are continuously motile while undergoing partial coalescence and alignment along the edge of the nuclear envelope where pronuclei juxtapose. It has been suggested that chromatin organization at such an early stage of development may be predictive of embryo viability. In particular, at the time of fertilization check during a human IVF procedure (16–18 h p.i.), it appears that embryos with higher chances of establishing a pregnancy are characterized by nucleolar alignment along the juxtaposition area

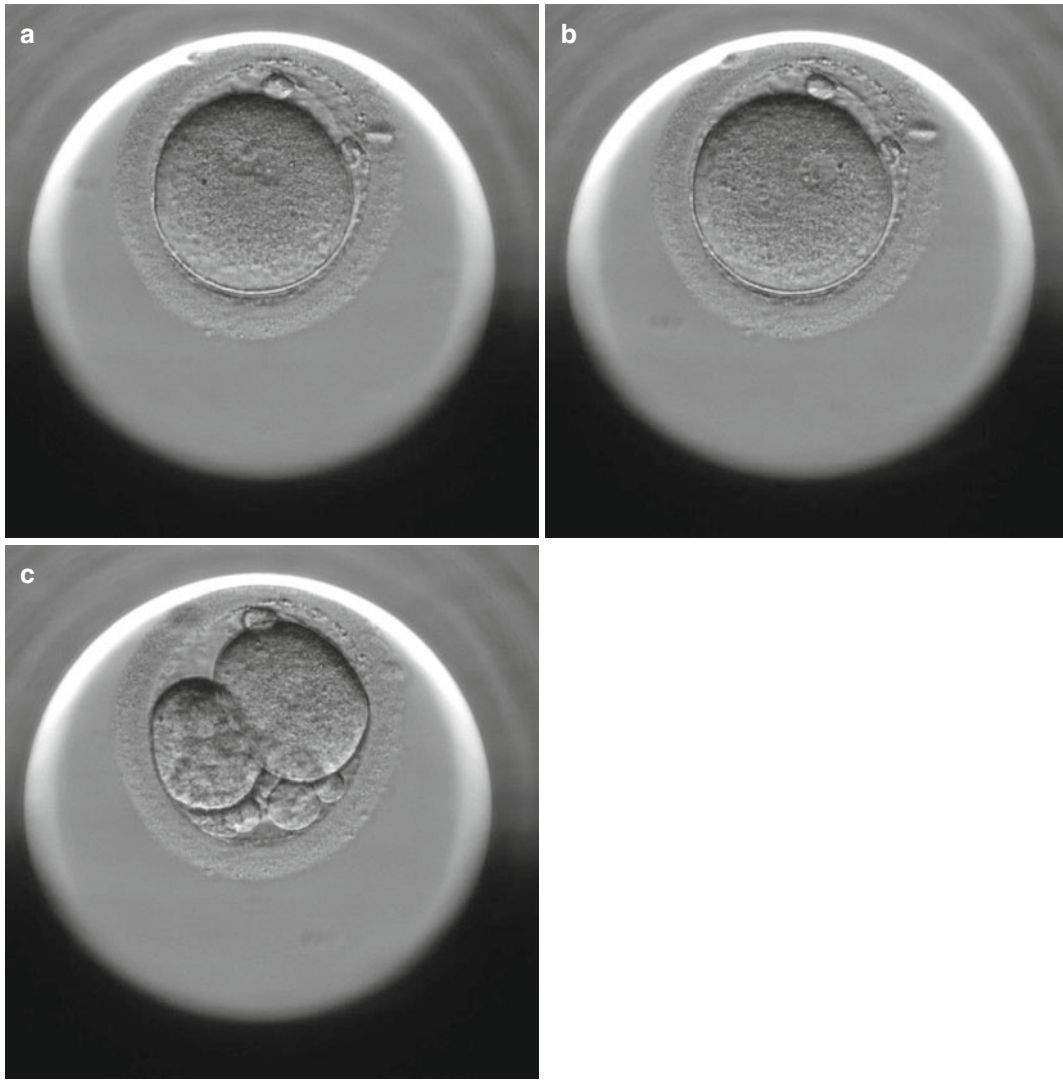

Fig. 20.2 Human fertilized egg in which the process of pronuclear juxtaposition failed. Pronuclei initially juxtaposed (**a**), but separated afterward (**b**). The first cleavage generated two blastomeres of different sizes (**c**), a phenomenon associated with poor developmental competence

of the two pronuclei and a number of nucleoli comprised between four and seven in both pronuclei (Fig. 20.1) [114]. Several studies seem to confirm the developmental significance of nucleolar organization [122, 124], although the underlying biology remains elusive.

Pronuclear and nucleolar development are extensive and active processes requiring extraordinary mechanical and energetic support from the egg. This justifies the massive organelle rearrangement that accompanies pronuclear migration from the periphery to the center. In particular, the entire population of mitochondria is reasserted, driven by movements that are believed to be under the control of dynein molecules shifting on tracks of microtubules [125]. These organelles, which provide the major ATP energy source for mechanical and biochemical processes, may be found aggregated in clusters of various sizes throughout the cytoplasm in mature unfertilized

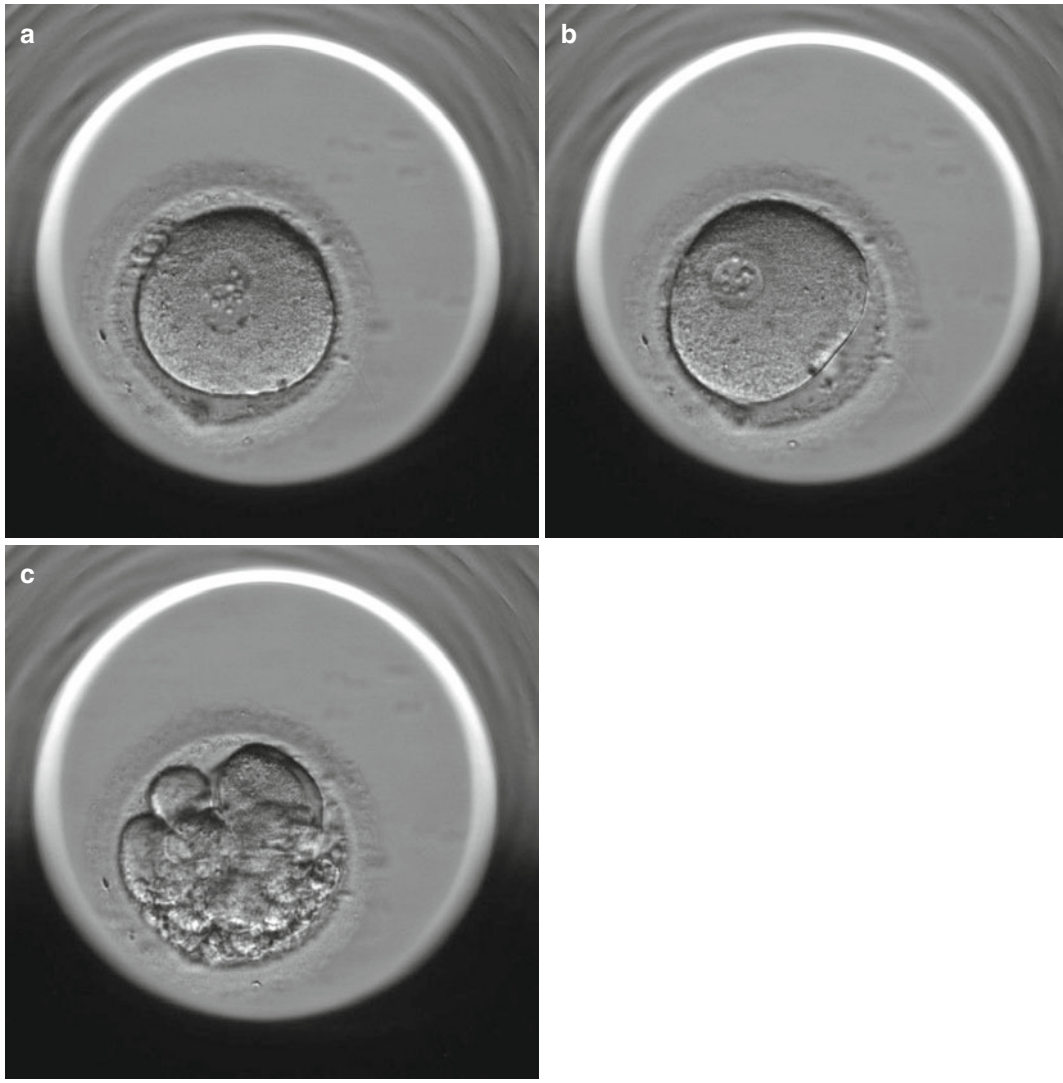

Fig. 20.3 Human fertilized egg. Pronuclei were initially localized in a central position (**a**), but were subsequently displaced to the cortex (**b**), The resulting two-cell embryo was affected by very severe fragmentation (**c**)

oocytes of the rhesus monkey [126]. With the onset of fertilization, they are progressively relocated toward more central positions forming two distinct masses of accumulation along either side of the surface of pronuclear juxtaposition. This arrangement seems to have a developmental significance because in its absence extensive blastomere fragmentation has been observed after the 8-cell stage of the ensuing embryo. Mitochondrial accumulation around the pronuclei has been observed also in the human fertilized egg [127]. Other organelles, such as tubuli and cisternae of the endoplasmic reticulum, are rearranged in a similar fashion, with the effect that starting from 8 to 10 h p.i., the cortical domain, left relatively deprived of subcellular structures, appears as a thinner cytoplasmic halo under conventional microscopic observation [124]. Likewise pronuclear position and nucleolar arrangement, formation of a cytoplasmic halo has been proposed to be predictive of higher implantation potential. As fertilization progresses, the egg also undergoes a significant overall contraction corresponding to a decrease

in diameter of 5–10 μm [113]. This massive phenomenon is not well understood but might be generated by the same mechanisms that control centripetal organelle displacement.

Pronuclear juxtaposition/relocation in central position, formation of the cortical halo, and contraction of the cortex are followed by an apparently uneventful phase lasting several hours in which movement of nucleoli is the only obvious manifestation of cellular activity under light microscopy observation. In reality, as early as 8–10 h p.i., chromosomal DNA starts to duplicate. The S phase of the fertilized eggs extends over 4–7 h and is usually completed by 14–17 h p.i. [128]. At the end of this phase, the 2*n*/4C DNA content is established in preparation for the first cleavage. The timing of DNA synthesis has practical implications with respect to the practice of zygote cryopreservation in human IVF. Irrespective of whether controlled rate freezing of vitrification is applied, during cryopreservation the fertilized egg is subjected to massive physical and biochemical stress that could affect DNA integrity in a phase of synthesis in which this macromolecule is particularly vulnerable. Therefore, cryopreservation should be postponed at around 20 h p.i., at a time when DNA duplication has been completed.

Once DNA duplication has been achieved, the fertilized egg is equipped to deliver two equivalent sets of chromosomes to each of the blastomere of the 2-cell embryo. This requires simultaneous breakdown of pronuclei in order for chromosomes to be released in the cytoplasmic compartment and pooled together in a single set. Pronuclear breakdown usually occurs at around 23–25 h p.i., but its time range is included between 19 and 35 h p.i. [129]. Two to three hours before the breakdown of pronuclei, the cortical cytoplasm undergoes a short event of contraction that accompanies the disappearance of the cortical halo. This might signify a redistribution of mitochondria, endoplasmic reticulum, and other organelles. Once in direct contact with the cytoplasmic environment, chromosomes are progressively recruited onto the mitotic spindle that meanwhile is assembled in a central position.

The Final Act of Fertilization

Fertilization is concluded by the first mitotic division. Geometrically directed by the central position of the mitotic spindle, the cleavage furrow divides the zygote cytoplasm into two blastomeres of equal size. Observations in human suggest that timing, geometry, and regularity of the first cleavage are indicative of the destiny of the ensuing embryo. In human IVF, early timing of the first cleavage appears to be positively associated with implantation ability. In 1997 for the first time, in a retrospective study, a higher pregnancy rate was reported in transfers whose embryos underwent the first cleavage by 25 h postinsemination [130]. The existence of a positive association between early cleavage and implantation ability was subsequently confirmed by a number of studies [131–133]. The recent introduction in routine clinical embryology of time-lapse microscopy (TLM) has opened entirely new opportunities to interpret the significance of embryo developmental morphokinetics, allowing monitoring at short intervals (2–20 min) rather than very few observations at fixed time points. It has been reported that, together with other parameters, the duration of the first cytokinesis is predictive of embryo developmental ability [134]. In particular, embryos that develop to blastocyst perform the cleavage process within a precise time interval (14.3 ± 6.0 min). In addition, embryos able to reach the blastocyst stage start and finish the first cytokinesis in a smooth, controlled fashion. On the contrary, in embryos destined to cleavage arrest, the first cytokinesis occurs over a long period of time, and the cleavage furrow is accompanied by extensive membrane instability. Meseguer et al. [129] have also adopted TLM to predict embryo implantation ability, concluding that in embryos with higher implantation ability, the first cleavage occurs between 24.3 and 27.9 h p.i. and daughter blastomeres are comparable in size. Therefore, it appears that the first cleavage, i.e., the final act of fertilization, is already to some extent informative of embryo developmental ability.

Conclusions

Fertilization joins the final phases of the oocyte life with the first steps of the journey of a newly formed individual. The oocyte is therefore central to the process of reproduction. However, the role of the male gamete is crucial, not only to restore biparental diploidy. In fact, the paternal-derived PLCζ and centriole provide biochemical and cytoskeletal cues that orchestrate distinct fertilization events, such as meiotic resumption and pronuclear formation and migration. In such a way, the legacies of the female and male gametes lay the foundations for successful development.

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