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Comparing Pig and Amphibian Oocytes: Methodologies for Aneuploidy Detection and Complementary Lessons for MAPK Involvement in Meiotic Spindle Morphogenesis

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1. Introduction

Maintenance of ploidy is crucial to the fate of daughter cells in any reproduction process. Improper genetic material segregation leads in general to mitotic catastrophe and subsequent cell death, but may also lead to the formation of aneuploid cells. Chromosomal unbalance, or aneuploidy, has been one of the main concerns in human reproduction for decades. About 20 % of all human oocytes are aneuploid and this percentage is believed to increase with ovarian aging, which is one of the major detrimental factors in pregnancy achievement (Battaglia *et al*., 1996; Younis, 2011). Besides age, oocyte-related aneuploidy is linked to spindle morphology abnormalities, non-disjunction of chromosomes in meiosis I and II, and related to toxic compounds exposure (Hassold and Hunt, 2001; Wang and Sun, 2006). Most embryos, that are formed from aneuploid oocytes, are non-viable and do not result in pregnancy. However, viable aneuploidic embryos can be produced but in many cases carry genetic disorders, such as monosomies for chromosomes X and 21, autosomal trisomies [for instance, Patau syndrome (trisomy 13), Down syndrome (trisomy 21)] and triploidy. The latter is associated with tumor development (Hitzler and Zipursky, 2005).

Imbalance in chromosome number is thought to result in the failure of the organization/assembly or disassembly of the mitotic or meiotic spindle, which is in charge of the correct segregation of the genetic material to the daughter cells (oocytes and polar bodies in the case of female gametogenesis). Indeed, chromosomes that are not organized correctly on the microtubule spindle apparatus may be lost or inappropriately segregated during cell division, which results in aneuploidy. Abnormalities in spindle organization and chromosomal dynamics are predominant in aging oocytes and are regarded as the major factors responsible for infertility, miscarriage and to some extent, birth defects. For these reasons the cell cycle is considered to be tightly controlled through many checkpoints, in a manner to avoid "madness" at the helm, which would drive to genomic instability. Then, any failure in cell cycle regulation is called to promote accumulation alteration in genomic material and in some cases, may lead to aneuploidy.

Studies of human aneuploidy have been hampered by the lack of a suitable animal model. Most studies so far have been performed using the mouse model, which in many aspects does not allow direct comparison with humans (Alvarez Sedo *et al*., 2011; Neuber and Powers, 2000; Schatten and Sun, 2011). It may be underlined that mouse model is a poor one regarding the methods of centrosome inheritance (due to the fact that mouse follows a maternal method for centrosome inheritance, in contrast to human where fertilization restores centrosome). As well, differences may be observed in the failure of fertilization between the two models. In human oocytes, the failure results from cytoplasm inability to support pronuclear formation while in mouse oocytes, the failure is rather due to inability of spermatozoa to penetrate the oocyte (Neuber and Powers, 2000). Other differences may also be reported between mouse and human oocytes, in contrast to non-rodent oocytes, mouse oocytes depend not upon protein synthesis for meiosis resumption and metaphase II spindle organization depends upon the assembly of cytoplasmic asters, including gamma-tubulin (see Table1). Still, the mouse model could promote strategies to sustain oocyte quality in mammals and provide evidences on the effects of environmental factors that may negatively impact oogenesis (Alvarez Sedo *et al*., 2011; Combelles *et al*., 2005; Farin and Yang, 1994; Gordo *et al*., 2001; Hunter and Moor, 1987; Liang *et al*., 2007; Lu *et al*., 2002; Meinecke and Krischek, 2003; Memili and First, 1998; Schatten and Sun, 2011; Sun *et al*., 1999; Sun *et al*., 2002). Nevertheless, further efforts have to be performed to promote other mammalian non-rodent models, whose meiotic regulation, cytoskeletal organization and fertilization are more closely related to human system (Schatten and Sun, 2011).

	Xenopus oocytes	Mouse oocytes	Pig oocytes	Bovine oocytes	Human oocytes
Does maturation depends upon protein synthesis?	Yes	No	Yes	Yes	n.d.
Does initiation of meiosis depends upon transcription?	No	Yes	No (DO) Yes (COC)	No (DO) Yes (COC)	n.d.
Do maturation and spindle morphogenesis rely on MPF activation?	Yes	Yes	Yes	Yes	n.d.*
Does initiation of meiosis depends upon MAPK network activation?	No	No	No	No	n.d.*
Does spindle morphogenesis depends upon MAPK network activation?	Yes	Yes	Yes	Yes	n.d.*
Does metaphase II spindle formation depends upon cytoplasmic asters assembly?	No	Yes	No	No	No

Table 1. Meiosis characteristics in *Xenopus*, mouse, porcine, bovine and human oocytes. (DO: denuded oocyte; COC: Cumulus Oocyte Complexes ; n.d.: not determined; * Dynamical observations have been solely gathered for these kinases) (Alvarez Sedo *et al*., 2011; Combelles *et al*., 2005; Farin and Yang, 1994; Gordo *et al*., 2001; Hunter and Moor, 1987; Liang *et al*., 2007; Lu *et al*., 2002; Meinecke and Krischek, 2003; Memili and First, 1998; Schatten and Sun, 2011; Sun *et al*., 1999; Sun *et al*., 2002).

Organisms enabling studies of spindle morphogenesis and aneuploidy might be divided into two classes: (1) meiotic models from non-mammalian species, which offer opportunities to unravel the fundamental mechanisms of meiotic spindle morphogenesis, and (2) meiotic models that correspond closely to human oocytes, taking into account both morphology and timing of meiotic maturation. Among the non-mammalian models, *Xenopus* has appeared as a model of choice since it shared similarities regarding mechanisms controlling meiotic progression with many mammalian species (see Table 1). In most vertebrates, oocytes are arrested at the first meiotic prophase until meiotic resumption is induced by hormonal stimulation. In both amphibian and pig models, resumption of meiosis and completion of oocytes maturation depend on protein translation, and are independent upon transcription. The factor promoting entry into M-phase, or division, was first reported in amphibians (Masui and Markert, 1971) and isolated almost two decades later in amphibian oocytes, based on genetic analysis performed in yeast (Gautier *et al*., 1988; Lohka *et al*., 1988). Named M-phase or Meiosis Promoting Factor (MPF), this factor is comprised of a catalytic subunit $p34^{Cdc2/Cdk1}$, and a regulatory subunit Cyclin B, whose association is crucial for the kinase activity of the complex. Together with the Mitogen Activated Protein Kinase (MAPK) pathway, MPF rules the coordination of the cellular reorganization during M-phase (Bodart *et al*., 2005; Haccard and Jessus, 2006; Kotani and Yamashita, 2002). The maintenance of low levels of MPF activity is a prerequisite for the maintenance of meiotic arrest at prophase, while high levels of MPF activity are required for arrest at metaphase II (Bodart *et al*., 2002a).

This chapter aims at comparing pig and *Xenopus* oocytes regarding their respective advantages and inconveniences, in comparison to other animal models. Standard methodologies for spindle morphogenesis analysis and aneuploidy detection in both models will be described. The role of the MAPK network in spindle morphogenesis and in ploidy maintenance will also be largely discussed, since female gametes have offered case studies to emphasize the potential role of MAPK's deregulation in aneuploidy.

2. Comparing advantages and inconveniences of pig and amphibian oocytes as models for spindle morphogenesis and aneuploidy studies

Xenopus has mainly been used in an aneuploidy context for cancer study purposes and has been less regarded as a valuable source for reproductive studies. Although it may appear as an attractive model, enabling structural approaches of spindle morphogenesis, it is not appropriate for accurate studies of genetic imbalance. To study the unbalance, porcine oocytes have appeared as a valuable and suitable model.

2.1 Strengths and disadvantages of amphibian oocyte model

The current understanding of meiosis regulation in vertebrate oocytes has benefited from studies performed in amphibian models such as *Xenopus laevis*. Fully-grown oocytes, blocked in prophase I resume meiosis upon hormonal stimulation and arrest in metaphase II as mature oocytes, or eggs, in anticipation of fertilization. Neither somatic cells nor *in vitro* systems offer a similar amenability for *in vivo* studies of the mechanisms that control cell cycle and orchestrate the cellular reorganization that occurs during mitosis or meiosis (Liu and Liu, 2006). This model could be advantageous to analyze the role of molecular networks

involved in the morphological events of cell division. Indeed, *Xenopus* oocytes offer impressive opportunities for studies at the biochemical level: oocytes contain high levels of proteins (each oocyte contains for example 50 to 70 ng of the catalytic subunit of Protein Kinase A (PKA), 1 oocyte is equivalent to 50 000 somatic cells) and makes it possible to perform anti-MAPK immunoblotting on one-tenth of a single cell, allowing to study feed-back regulation mechanisms within one cell. Thus, extracts of either oocytes or eggs from amphibian are an abundant source for cytoskeletal and cell cycle proteins. In addition to their physiological synchronization in G2-like state or metaphase block, *Xenopus* oocytes are also known and used for their high capacity of protein synthesis (200-400 ng per day, per oocyte) and their size (1.2 to 1.4 mm), which facilitates micromanipulations, exploration of signaling network or electrophysiological properties. Finally, the use of this model of lower vertebrate fits into the ethical policy of the 3R (Replace, Refine, Reduce) on animal experimentation, which aims at promoting non mammalian alternative models in order to noticeably reduce and refine the use of upper vertebrates in experimental studies.

In addition, for several decades *Xenopus* oocytes have appeared to be one of the best cellular model to study *in vivo* enzymatic activities, assembly of cell division spindle, aneuploidy and characterization of parthenogenetic events. Nevertheless, this model suffers from several disadvantages: (1) amphibians are not oviparous models and oocytes maturation, as well as fertilization, is independent of any cumulus cells; (2) oocytes contain yolk, which does not facilitate structural analysis, and exhibit autofluorescence; (3) *Xenopus laevis* is a allotetraploid species, making it less/not suitable for genetic studies and careful analysis of genetic imbalances.

2.2 Strengths and disadvantages of porcine oocyte model

As mentioned above, mouse models may be optimal for studying molecular mechanisms underlying the maturation of mammalian oocytes, but several differences may been outlined when comparing to the maturation of non-rodent oocytes (Table 1). Porcine oocytes are closely related to human oocytes, in term of morphology and timing of meiotic maturation. Nuclear envelope breakdown, also called germinal vesicle breakdown (GVBD) in oocytes, occurs after 20 hours (2-5 hours in mice). Oocytes are reaching the second meiotic metaphase block approximately 40 hours after the lutheinizing hormone (LH) peak (*in vivo*) or after isolation from follicles (*in vitro* condition) (9-13 hours in mouse). It is known that pig oocytes rely on *de novo* protein synthesis for GVBD, whereas this occurs independently of protein synthesis in murine oocytes.

Several studies have reported that fertilization of pig oocytes resembles more that of lower vertebrates than mice (Long *et al*., 1993; Sun *et al*., 2001a). In sea urchin, starfish, porcine or bovine oocytes, sperm interaction with the oocyte or their penetration is impaired by microfilament inhibitors (Schatten *et al*., 1982). In murine oocytes, however, microfilament inhibitor Latrunculin A did not block sperm penetration (Schatten *et al*., 1986). Similarly, it was reported that the microfilament modulator JAS inhibited sperm incorporation and prevented cortical granules (CG) exocytosis in murine oocytes (Terada *et al*., 2000). In murine oocytes, pronucleus migration is blocked upon inhibition of microfilament assembly (Schatten *et al*., 1989; Terada *et al*., 2000), while in pigs (Sun *et al*., 2001a) or urchins (Schatten *et al*., 1992), microtubule assembly is not required for pronucleus formation. During

fertilization in sea urchins, pigs, cattle and humans, sperm introduces the centrosome into the egg. Microtubules nucleated by centrosomes cause the syngamy between male and female pronuclei (Kim *et al*., 1997; Sun *et al*., 2001a; Sutovsky *et al*., 1996; Van Blerkom *et al*., 1995). In mice, the situation is quite different because centrosomes are maternally inherited. Microtubules are organised by numerous cytoplasmic sites and microtubules and microfilament activity are required for pronuclear migration. For the above-mentioned reasons, porcine egg may be a more appropriate model for studies with the aim to make a comparison with human reproduction.

The access to porcine ovaries is straightforward since they can be obtained as "bioproducts" from local slaughterhouses. Nevertheless, working with pig oocytes may be hampered because of the heterogeneity of the isolated oocytes and the heterogeneity of the donor animals. Two types of ovaries may be obtained from slaughterhouses, depending whether killed animals are monitored or not. When performed, monitoring includes exact reproduction phase, age and farming conditions (including composition of feeding rations). Monitoring is expensive, but often necessary to obtain better standard conditions. For any work using porcine oocytes isolated from ovaries obtained from a slaughterhouse, it is necessary to perform a rigorous and strict selection of the ovaries and oocytes. Apart from ovaries collected at slaughterhouses, oocytes might be obtained by endoscopic ovum pick-up (OPU) from living animals. Antral follicles are punctured and cumulus oocyte complexes (COCs) are aspirated with the follicular fluid into an aspiration cannula using vacuum pressure. In contrast to the previous method, oocytes obtained in this way are homogenous and of high quality. Unfortunately, this method is expensive and labor-intensive and results in a low recovery of oocytes compared to the previous method.

Porcine and murine oocytes are different in their *in vitro* requirements during maturation. In contrast to mouse oocytes, porcine oocytes require cumulus cells for successful maturation, and they are very sensitive to maturation conditions and manipulation. For instance, culture conditions of cumulus cells may alter the meiotic spindle morphology (Ueno *et al*., 2005), may impair successful micro-injection and subsequent scanning by confocal microscopy during maturation. Thus, live imaging experiments in this model are time-consuming and may appear as "complex".

3. Chromatin configuration, spindle morphogenesis and aneuploidy detection

3.1 *Xenopus laevis* oocytes

3.1.1 Morphological signs of maturation by external observation

In *Xenopus* oocytes, spindle formation is associated with migration of nucleoplasm and chromosomes to the apex of the cells. Upon hormonal stimulation, the germinal vesicle breaks down at its basis and migrates towards the apex of the oocyte, creating a large white area without pigment. This area is called white spot or maturation spot (Figure 1). The chromosomes are condensed and the first metaphase spindle is formed at the cortical area, located near the plasma membrane. After extrusion of the first polar body, the metaphase II spindle will be anchored at the plasma membrane. A dot may be detected within the white spot, corresponding to the anchored metaphase II spindle at the plasma membrane.

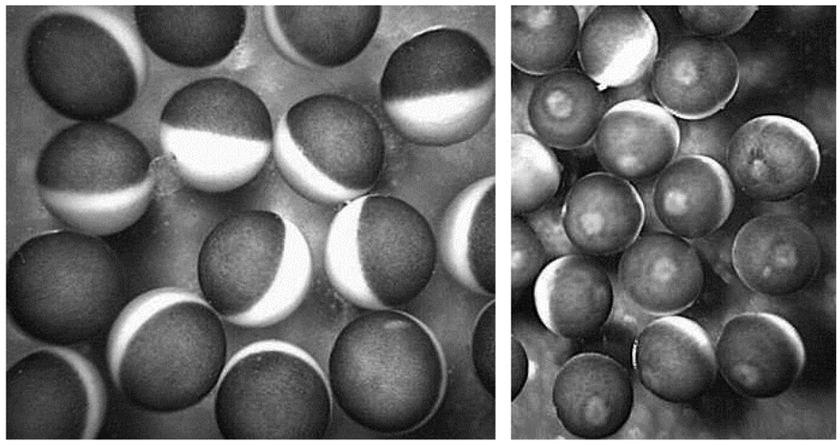

Fig. 1. External morphology of *Xenopus* oocytes. Immature oocytes (left panel) exhibit a dark pigmented hemisphere where the nucleus, or germinal vesicle, is found. Upon stimulation by progesterone, a small white spot appears at the apex of the cell, which is correlated with GV migration and its breakdown (right panel).

3.1.2 Spindle morphogenesis and aneuploidy detection

Classically, oocytes are fixed overnight in Smith's fixative, embedded in paraffin and sectioned (7µm thickness). Sections are then stained with Nuclear Red to detect nuclei and chromosomes, and with picroindigocarmine, which reveals cytoplasmic structures (Bodart *et al*., 1999). This method enables the detection of spindle and condensed chromosomes, even if not located near the plasma membrane. Atypical structures can be detected in depth of the oocytes, such as tripolar spindle or nuclear envelope reformation around chromosomes in deep cytoplasm (Figure 2, 3). These methods can be coupled with electrophysiological procedures or calcium measurements to perform oocyte-by-oocyte analysis (Bodart *et al*., 2001).

To perform immunocytological studies, oocytes are fixed in cold methanol, which is gradually replaced by butanol before embedding in paraffin. 7 µm-thin sections are subsequently incubated with antibodies directed towards spindle structures (e.g. towards alpha-tubulin). Structures are revealed with second antibodies conjugated with a fluorescent marker such as Oregon Green or Fluorescein Isothiocyanate. To detect structures located near the plasma membrane, section and embedding should be avoided. In this case, oocytes are fixed in cold methanol, which is gradually replaced by PBS (phosphate buffered saline). Next the oocytes are incubated in a low percentage of detergent for permeabilization purposes together with diluted antibodies and revealed as previously mentioned. Treated oocytes are bisected and animal halves are placed on standard slides with mounting medium including glycerol and Hoechst 33342, which reveals chromosomes, and observed under an epifluorescent microscope (Figure 4) (Baert *et al*., 2003; Bodart *et al*., 2005; Bodart and Duesbery, 2006).

To overcome autofluorescence and to increase the number of observed structures, many studies have been undertaken using egg extract together with sperm nuclei (Cross and Powers, 2009; Garner and Costanzo, 2009; Maresca and Heald, 2006). Though these methods are of interest, they have to be performed in a cell-membrane-free context and only focus on

mitotic spindle formation. Indeed differences remain, albeit meiosis and mitosis share quite a lot of similarities: (1) centrosomes are absent in oocytes, (2) kinetochores appear later in MI and (3) the Ran GTPase might be dispensable for MI but requested for MII and mitosis (Brunet *et al*., 1999; Dumont *et al*., 2007).

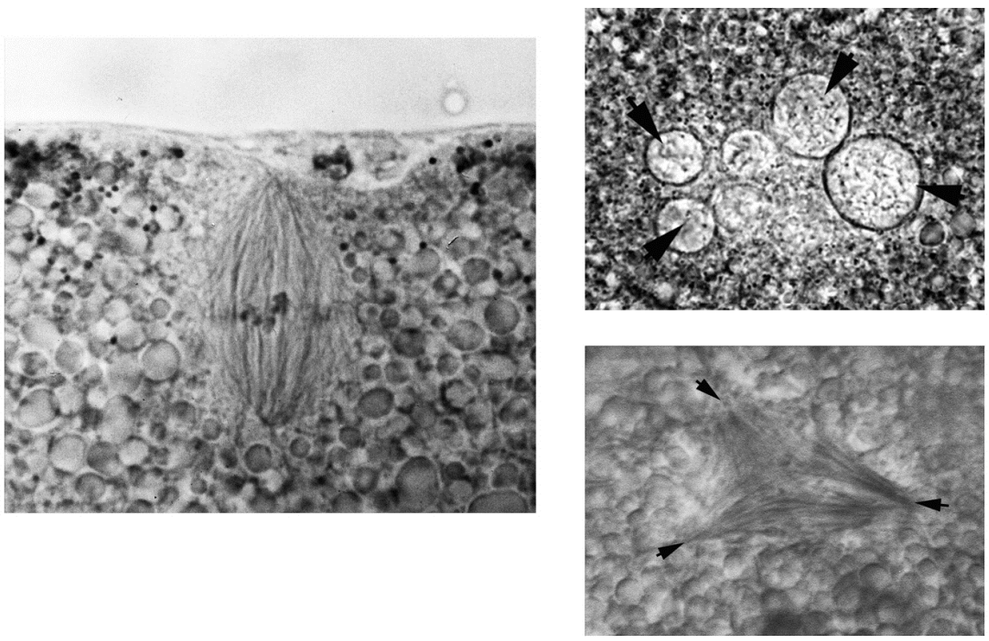

Fig. 2. Bipolar spindle and first polar body (left panel), abnormal tripolar spindle (right panel, bottom), and multiple envelope reformation around chromosomes in deep cytoplasm (right panel, top). Metaphase II spindle has, like seen in most species, a typical barrel shape.

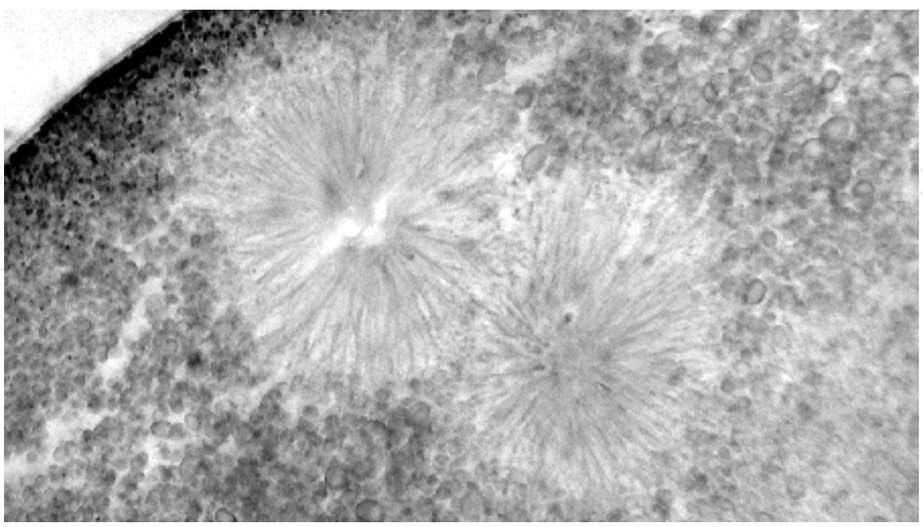

Fig. 3. Double aster formation in *Xenopus* oocyte where MAPK activity has been impaired. Structures are located in the subcortical layer but are not associated with the plasma membrane.

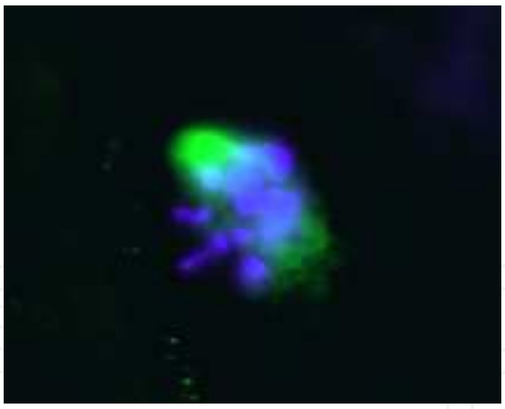

Fig. 4. Bipolar spindle in metaphase-II arrested *Xenopus* oocyte; DNA (blue), beta-Tubulin (green).

3.2 Porcine oocytes

3.2.1 Germinal vesicle stages

Full meiotic competence of pig oocytes is reached in ovarian follicles of 2 mm or more in diameter. Germinal vesicle breakdown is initiated *in vivo* by either preovulatory surge of gonadotropins or atretic degeneration of the follicle. Spontaneous (gonadotropin-independent) maturation occurs upon removal of the oocyte, or the oocyte-cumulus cell complex, from antral follicles when cultured in an appropriate supportive medium. Several protein kinases have been shown to regulate meiotic resumption. MPF is a key regulator of the meiotic resumption. Its inhibition totally blocks GVBD in pig oocytes. Nevertheless, MAPK, Protein Kinase C and Calmodulin-dependent Kinase (CAMKII) also play crucial roles during meiotic resumption in pig oocytes. In pigs, like in other domestic species and amphibians, protein synthesis is a prerequisite for oocyte meiotic resumption. In porcine oocytes, four GV chromatin configurations (GV1-GV4) were described, based on chromatin stages, nucleolus and nuclear chromatin disappearance (Motlik and Fulka, 1976). Stages GV1 (Figure 5), GV2 and GV3 exhibit a typical perinucleolar ring. At stage GV4 chromatin makes clumps and strands, nuclear membrane is less distinct, and nucleolus disappears completely. In oocytes undergoing GVBD, GV membrane disappears completely and chromatin condenses into clumps. Growing oocytes (diameter ≤90 μm) are unable to resume meiosis *in vitro*. Acquisition of meiotic competence in growing pig oocyte rather correlates with its ability to activate both MPF and MAPK pathways (Kanayama *et al*., 2002).

3.2.2 Spindle morphogenesis

Metaphase II spindle is a crucial structure for genetic material segregation within oocytes and eggs; this structure is maintained in a highly dynamic status from ovulation to fertilization. The spindle morphogenesis during maturation begins after GVBD, when microtubule-organizing centres (MTOCs) are recruited in the vicinity of chromosomes: small microtubule asters are observed near the condensed chromatin. During the prometaphase stage, microtubule asters are found in association with each chromatin mass. Randomly growing microtubules are then stabilized and organized into a bipolar spindle. Next, asters elongate and encompass the chromatin at the metaphase-l stage. At this step,

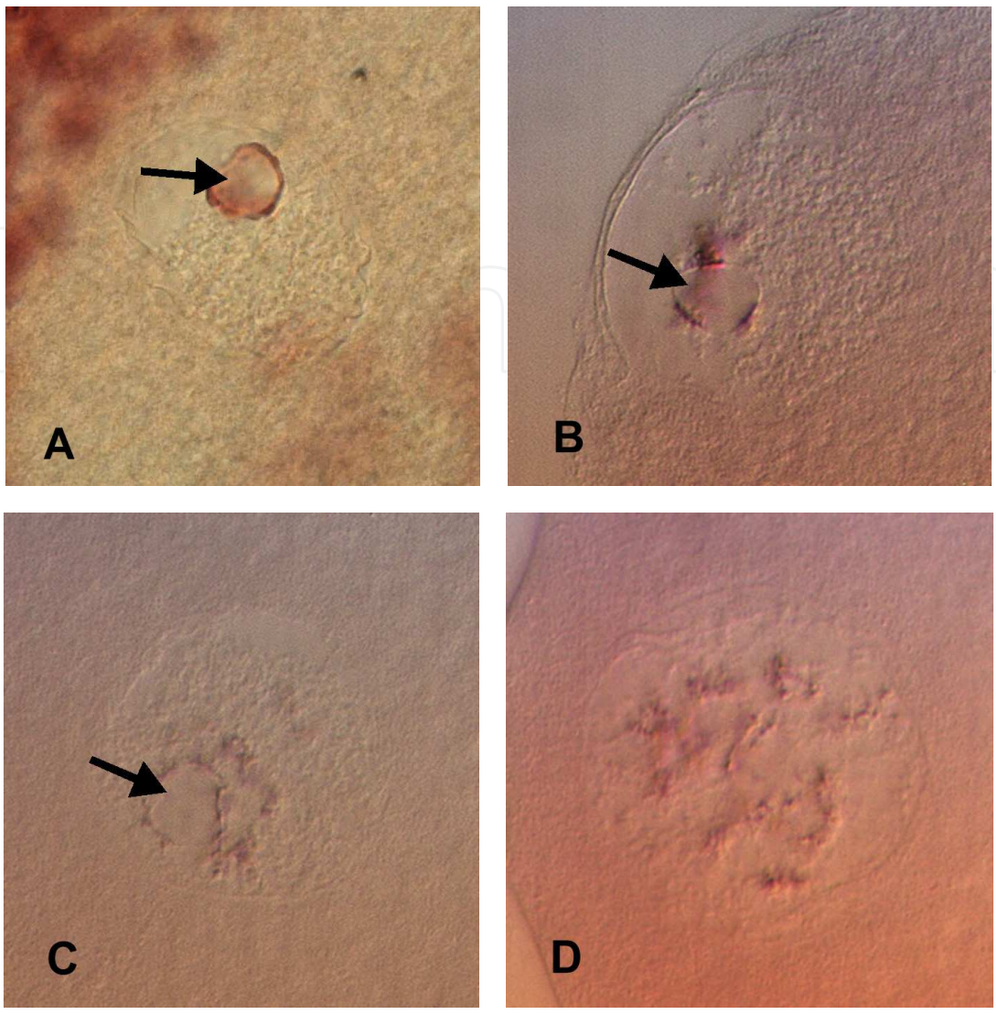

Fig. 5. Morphology of porcine oocyte nucleus exhibiting different GV configurations. (A) GV1: nucleolus surrounded by condensed chromatin, (B) GV2: condensed chromatin are not only around the nucleolus, (C) GV3: chromatin is condensed in many clumps or strands, (D) GV4: nucleolus disappears completely and condensed chromatin is in clumps (black arrows indicate nucleolus).

microtubules are seen only in the spindle (Figure 6, left). During anaphase-I and telophase-I, microtubules are detected around the chromatin (Figure 6, right). At the metaphase-II stage, microtubules are only observed in the second meiotic spindle. The meiotic spindle has a symmetric barrel-shaped structure containing anastral broad poles peripherally located and radially oriented (Kim *et al.*, 1996).

3.2.3 Aneuploidy detection

To detect aneuploidy there are applicable methods such as fluorescence *in situ* hybridization (FISH) or karyotyping from chromosomal spreads. These methods require spreading the metaphase II oocyte on a slide, risking the loss of chromosomal material. Modern cytogenetic method of comparative genomic hybridization is also suitable for aneuploidy

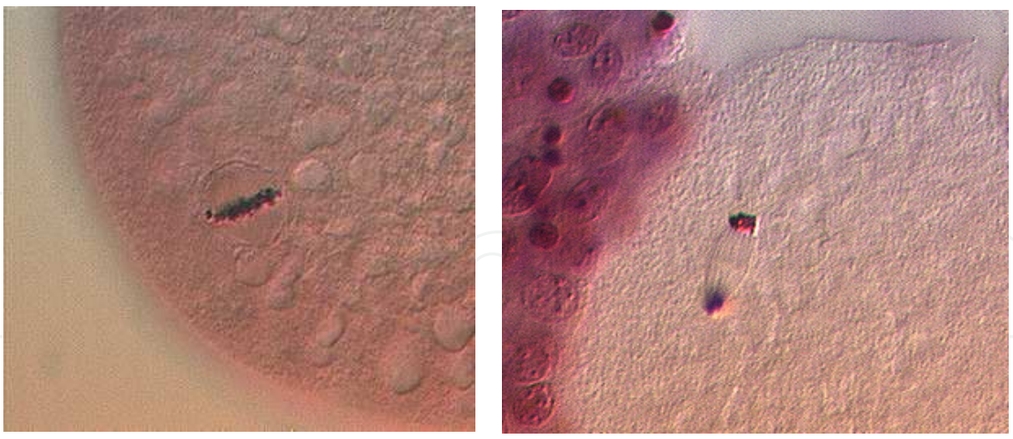

Fig. 6. Spindle detection in pig oocyte; (left) spindle at metaphase-I stage; (right) spindle at telophase-I stage.

detection in oocyte or polar body. The comparative genomic hybridization (CGH) method relies on whole oocyte DNA amplification in a single reaction, preventing artificial changes of chromosome content. These methods are suitable for detection of numeric aberrations, chromosomal non-disjunction and frequency of premature segregation of sister chromatids (an extra or a missing copy of a single chromatid).

3.2.3.1 Karyotyping from spreads

Giemsa staining is suitable only for hyperhaploid spreads. Hypohaploidy is rather considered as an artifact, because air-drying step used in slide preparation can account for chromosome loss during rapid evaporation of alcohol containing fixative. For karyotype analysis, only cells in metaphase can be analyzed because they present identifiable individual chromosomes (Figure 7). Furthermore, poor chromosome morphology and artefactual loss of chromosomes can compromise cytogenetic results.

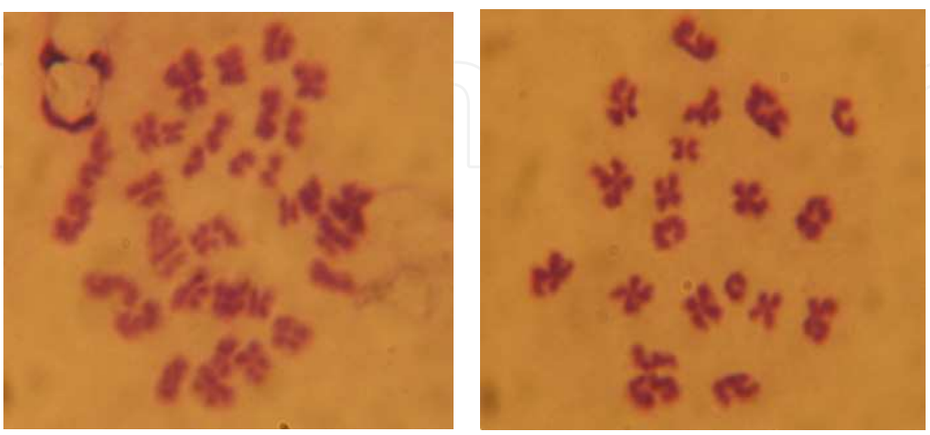

Fig. 7. Chromosomal analysis of porcine oocytes (Giemsa staining); (right) haploid chromosome set (n=19), (left) diploid chromosome set (2n=38) (Lechniak *et al*., 2007)

3.2.3.2 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH, Figure 8) is a well-established technique for chromosome analysis and aneuploidy screening (Foster *et al*., 2010; Lechniak *et al*., 2007). In contrast to previously described methods, FISH data can be obtained from interphase nuclei or at least in cases where not all chromosomes are separated during spreading. Concerning oocyte, analysis of the corresponding polar bodies can be performed in parallel to strength the observations. Unfortunately, the diagnostics of targeted aneuploidies in pig with fluorescence *in-situ* hybridization is limited to chromosome-specific DNA probes. Current protocols have used probes for up to 13 chromosomes in human samples but in pigs, they are suitable for usually two probes (specific for centromeric region of Chromosomes 1 and 10) (Lechniak *et al*., 2007). Results are thus extrapolated for all chromosomes solely based on these 2 probes. Furthermore, premature segregation of sister chromatids is not detectable *via* this method.

3.2.3.3 Comparative genomic hybridization (CGH)

Comparative genomic hybridization (CGH, Figure 9) is a more comprehensive method than karyotyping or FISH techniques. This cytogenetic technique allows the analysis of the full set of chromosomes and it has been applied to detect aneuploidy at the single cell level in interphase or M-phase cells. The CGH protocol requires only 0,5-1 μg of genomic DNA (from oocyte or polar body). By comparison with other farm animals, pigs have a small number of chromosomes (2n=38), which ease up chromosome identification and analysis. This method also enables analysis of the whole chromosomal set and relative contribution of individual chromosomes to resulting aneuploidy. This approach was recently applied in a study looking to detect chromosome abnormalities in first polar bodies and metaphase II-arrested oocytes in pig. CGH method is also suitable for detection of chromosome non-disjunction or premature segregation of sister chromatids (an extra or a missing copy of a single chromatid).

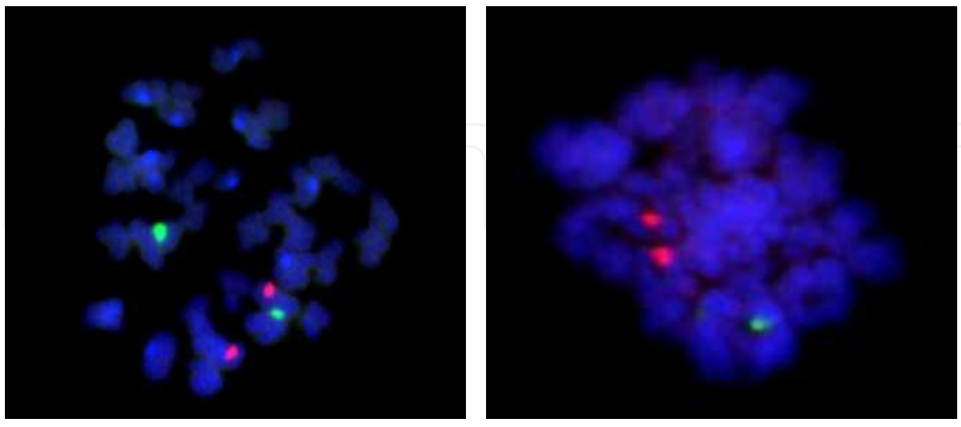

Fig. 8. Chromosomal analysis of porcine oocytes (FISH); (left) diploid oocytes (green signal – chromosome 1, red signal - chromosome 10), (right) aneuploid oocyte (disomy of chromosome 10) (Lechniak *et al*., 2007)

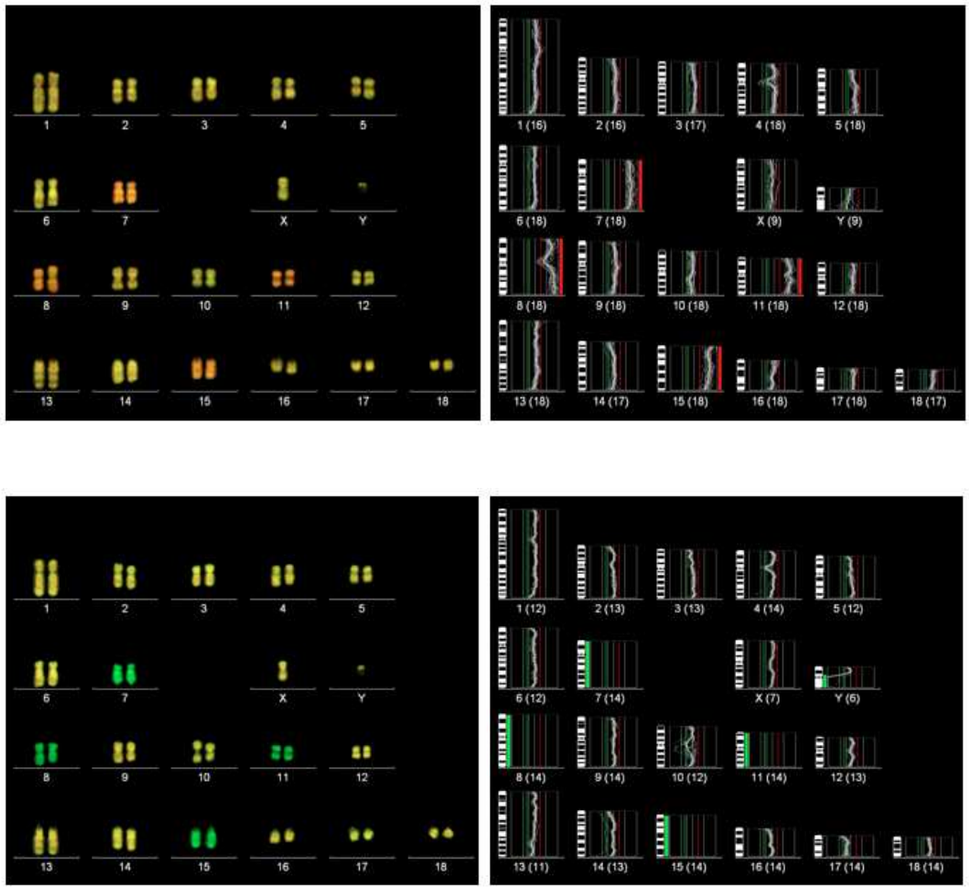

Fig. 9. Comparative genomic hybridization (CGH) in MII porcine oocytes; (upper panel) aneuploid oocyte with trisomy of chromosomes 7, 8, 11 and 15; (lower panel) corresponding polar body with nulisomy of chromosomes 7, 8, 11 and 15 (red signal – DNA from oocyte or polar body, green signal – reference DNA) (Hornak *et al*., 2011).

3.2.3.4 Abnormalities during meiotic spindle morphogenesis in pig oocytes

Abnormalities during meiotic spindle morphogenesis are a hallmark of maternal aging. Spindle aberrations are not found so often in oocytes of animals with shorter reproductive periods like mice. In porcine oocytes, abnormal spindle morphologies include poorly shaped spindle morphologies, grossly disorganized microtubules, sometimes with thick bundles of cytoplasmic microtubules (Figure 10). These morphologies may result from the failure to organize two opposite metaphase spindle poles, either in metaphase I or metaphase II, or from the loss of attachment between microtubules and chromosomes. In oocytes coming from old pigs, one might observe various forms of abnormal spindles like multipolar spindle, large rounded spindle with microtubules emanating from most of its surface, elongated spindle, highly disorganized spindle with scattered microtubules, tripolar spindle or large irregular spindle (Miao *et al*., 2009). In contrast to amphibians, aster formations are rare in mammals. In pigs, they were solely observed after taxol (Sun *et al*., 2001b).

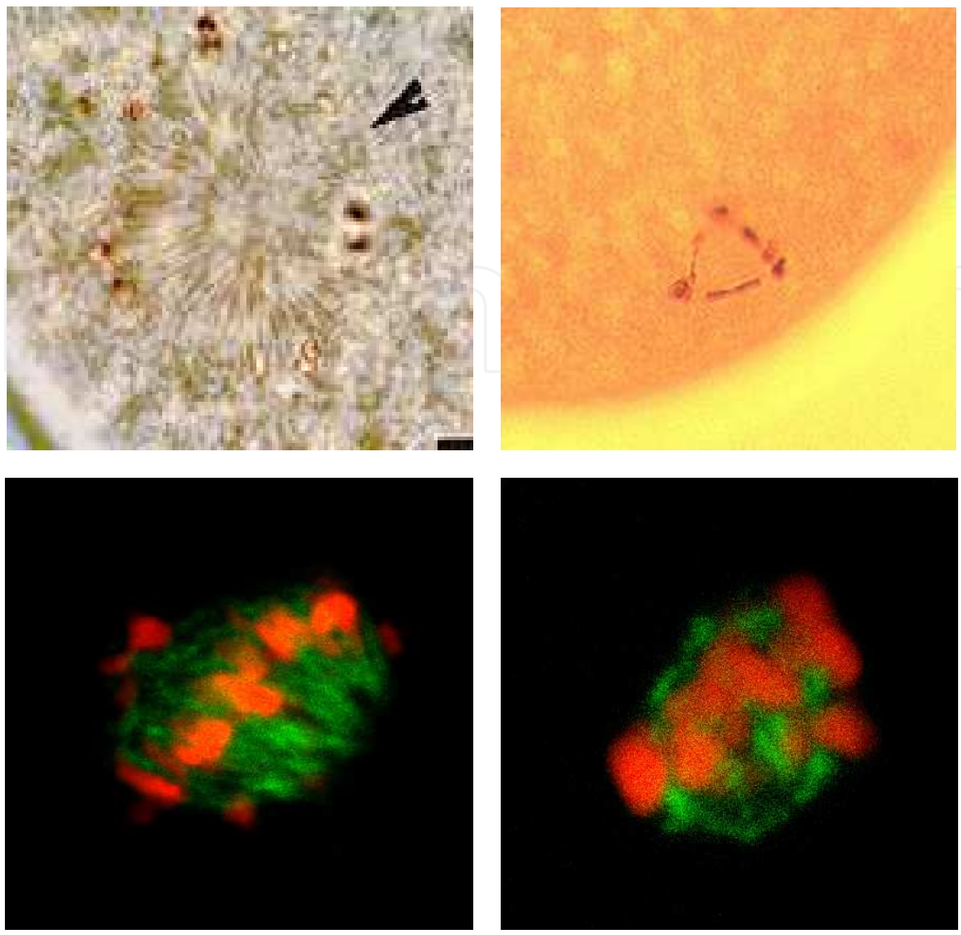

Fig. 10. Typical abnormal spindle morphologies observed in porcine oocytes: aster formation (upper panel, left) (Sun *et al*., 2001b), tripolar spindles (upper panel, right), disorganized spindle with scattered chromosomes (lower panel, left) and irregular spindle (lower panel, right).

4. Aneuploidy case study: Unraveling the role of MAPK pathway in the cellular reorganization during meiosis

In many species, MAPK from the Extracellular regulated kinase (Erk) family has appeared as major player in the network regulating meiosis. The completion of the two meiotic metaphase offer windows of vulnerability towards aneuploidy, where MAPK are playing a crucial role. Nevertheless, the involvement of this pathway has been underestimated in its role for genome integrity. At least, the involvement of MAPK deregulation in aneuploidy remains difficult to untangle and female gametes have offered case studies to unravel MAPK's role in spindle morphogenesis. Here is outlined the involvement of the MAPK network in aneuploidy throughout different models.

4.1 Physical properties of MAPK pathway in amphibian

In many species, MAPK from the Extracellular regulated kinase (Erk) family is a crucial component in the network regulating meiosis. In vertebrate oocytes, the oncoprotein Mos

acts as the upstream activator of mitogen-activated protein kinase kinase (MEK) and MAPK/Erk levels. This oocyte-expressed kinase appeared early during animal evolution and was in charge of regulating female meiosis specializations (Amiel *et al*., 2009). Once accumulated, Mos phosphorylates MEK, which in turn activates MAPK/Erk by dual phosphorylation of a TEY motif (Ferrell and Bhatt, 1997). In amphibian oocytes, Mos-activated cascade prevents DNA synthesis during meiosis and promotes spindle morphogenesis as well as cytostatic activity present in metaphase-II arrested oocytes (Baert *et al*., 2003; Bodart *et al*., 2005; Dupre *et al*., 2002; Sagata, 1997). In this model, physiological role of MAPKKK Raf, has been minored and Mos is thought to literally hijack the control of the MAPK cascade. A specific all-or-none response for MAPK/Erk activation is characteristic in these oocytes, in contrast to the gradual response of MAPK/Erk to external stimuli observed in mammalian somatic cells. The cascade arrangement of the signaling network generates the steepness of MAPK/Erk response in *Xenopus* oocytes. The physical properties of the cascade, which includes ultrasensitivity, bistability and irreversibility (Angeli *et al*., 2004; Ferrell and Machleder, 1998; Huang and Ferrell, 1996; Russo *et al*., 2009), are thought to mainly arise for bistability and ultrasensitivity from the existence of a feed-back loop motif. Indeed, the Mos-MEK-MAPK/Erk network has been found to be embedded in a positive feed-back loop, driven by MAPK/Erk itself (Ferrell and Machleder, 1998; Howard *et al*., 1999; Matten *et al*., 1996) and / or through MPF action (Castro *et al*., 2001; Nebreda *et al*., 1995; Paris *et al*., 1991), which promotes Mos accumulation.

4.2 Role of MAPK/Erk network in spindle morphogenesis

4.2.1 Amphibian oocytes

MAPK/Erk activity was suggested to be required for functional spindle assembly checkpoint in amphibian oocyte extracts (Chung and Chen, 2003; Minshull *et al*., 1994; Takenaka *et al*., 1997). Although presence of MAPK/Erk on kinetochores has been suspected in somatic cells (Willard and Crouch, 2001), proteomic studies failed to find MAPK/Erk associated to isolated human metaphase chromosomes (Uchiyama *et al*., 2005). Thus, whether MAPK/Erk in its active form is a component of kinetochore, and whether it is required for spindle assembly checkpoint function, remains somehow controversial.

From observations made in many species like starfish, jellyfish, urochordates, amphibians and mice, it is thought that control of meiotic spindle morphogenesis and positioning and chromatin organization are conserved functions for Mos and MAPK/Erk network. First observations were made in nullizygous mice for Mos, where MEK activation is impaired and interphase-like structure of microtubules and chromosomes are found between meiotic division, as well as formation of monopolar half-spindle (Araki *et al*., 1996; Tong *et al*., 2003; Verlhac *et al*., 1996). Similar observations were made in other biological systems. MAPK/Erk cascade regulates spindle bipolarity through its direct or indirect effects on microtubule dynamics in amphibian oocytes (Bodart *et al*., 2005; Gotoh *et al*., 1995). No bipolar spindle anchored at the plasma membrane is observed when MAPK/Erk activity is inhibited by chemical inhibitors of MEK such as U0126 (Bodart *et al*., 2002b; Gross *et al*., 2000; Horne and Guadagno, 2003). *Rana japonica* oocytes treated with U0126 also fail to organize a Microtubule Organizing Center (MTOC) at the bottom of the germinal vesicle and chromosomes are partially condensed (Kotani and Yamashita, 2002). Both *in vitro* (Horne and Guadagno, 2003) and *in vivo* (Bodart *et al*., 2005), MAPK activity inhibition leads to the

formation of monopolar spindle or aster-like structures, attesting the failure to establish a bipolar organization (Figure 3). Similarly, when Mos accumulation is prevented *in vivo, Xenopus* oocytes exhibit aster-like structures. Such structures remain to be fully characterized but do not enable oocytes to properly segregate their genomic content (Bodart *et al*., 2005). Finally, by inhibiting the network at different levels, it has been shown that Mos and MAPK/Erk play distinct but complementary roles in spindle morphogenesis (Bodart *et al*., 2005). The latter observations suggested that MAPK/Erk is composed of functional modules, which may exert distinct actions at different levels of spindle organization. Then deregulation in any of this model may drive different type of aneuploidy, depending on the deregulated module.

4.2.2 Porcine oocytes

MAPK is found under an inactive form in pig oocytes at the GV stage, while its level of activity is significantly increased at GVBD time. Microinjection of c-mos RNA into porcine oocytes induces GVBD to occur earlier. But, antisense RNA towards pig c-mos protein does not affect GVBD in denuded oocytes, although MAPK phosphorylation and activation were completely inhibited (Ohashi *et al*., 2003). Other reports show that the presence of MEK inhibitors, PD98059 or U0126 in the maturation medium blocks MAPK activation in both cumulus-enclosed and denuded oocytes, but prevents GVBD only in cumulus-enclosed oocytes (CEOs) (Meinecke and Krischek, 2003). Similarly to other models, MAPK activation is dispensable for meiotic resumption *per se*, but activation of this cascade in cumulus cells is indispensable for the gonadotropin-induced meiotic resumption of porcine cumulus-enclosed oocytes. These observations suggest that MAPK activation is not required for GVBD induction in denuded oocytes but is necessary for GVBD induction in CEOs. MPF activation correlates with GVBD occurrence, even though the activation of MAPK has been completely prevented, indicating that MPF is sufficient to induce GVBD.

Nevertheless, the activity of MAPK in porcine oocytes may be involved in the organization of chromosomes at the metaphase spindle plate (Ye *et al*., 2003). Phosphorylated MAPK is detected at the spindle during the post-GVBD maturation period (Sugiura *et al*., 2001). After GVBD, phosphorylated MAPK and its downstream effector p90rsk distribute to the area around the condensed chromosomes, in the meiotic spindle at the MI stage, in the midzone of the elongated spindle at anaphase I to telophase I transition, and in the spindle at MII stage (Goto *et al*., 2002; Lee *et al*., 2000). MAPK is kept highly phosphorylated from the MI to MII stages, when microtubules are assembled in the spindle (Sun et al., 2001a). Thus, it can be suggested that the MAPK cascade is required not to initiate resumption of maturation but for microtubule dynamics in the meiotic spindle in pig oocytes (Meinecke and Krischek, 2003). Inhibition of MAPK activation during MI-to-MII transition results in the failure of first polar body emission and MII spindle formation (Lee *et al*., 2000). After fertilization, MAPK is kept highly active while the second meiosis resumes and the second polar body extrudes. Finally, MAPK is dephosphorylated during pronucleus formation (Sun *et al*., 2001a). High level of MAPK is important for the oocyte to remain arrested at MII stage and is also necessary for the chromosomes to orderly align at the spindle equator. Low level of MAPK activity may cause instability of chromosomes in the spindles and may alter the precious relationship between microtubules and chromosomes (Ma *et al*., 2005).

4.3 Mos-MAPK in human oocytes

p42Erk2 is the main form of MAPK in human oocytes (Sun *et al*., 1999). Its pattern of activation by phosphorylation is reminiscent of other mammalian species including mice and pigs. MAPKKK Mos has also been detected in human oocytes and its expression was like in other models restricted to oocytes (Heikinheimo *et al*., 1995; Heikinheimo *et al*., 1996; Pal *et al*., 1994). MAPK is inactive in immature oocyte while its activity increases during maturation and drops after fertilization. Thus, the functional role of MAPK in human oocytes remains an opened question since dynamical observations have been solely gathered (Combelles *et al*., 2005; Sun *et al*., 1999; Trounson *et al*., 2001). MAPK and Mos have been then assumed to exert similar functions to those of other species.

4.4 A role for Mos in the limitation of M-phase rounds?

Mitotic exit is irreversible and irremediably followed by interphase, based on the degradation mechanisms of Cyclin B (Potapova *et al*., 2006). This is not the case during meiosis, where exit from the first meiotic division is not followed by interphase and replication but is immediately followed by the onset of the second division. This lack of irreversibility has raised the following question: how oocytes limit the number of M-phases to just two during maternal meiosis? Switching off the activity of Mos – MAPK/Erk network appeared as an attractive hypothesis for ruling the number of M-phases, though it was clear from studies in jellyfish, starfish and amphibians that this mechanism could not be a universal one. First evidence supporting this hypothesis was recorded in mice oocytes where maintaining MAPK activity inhibits pronucleus formation (Moos *et al*., 1995; Moos *et al*., 1996) and where entry to meiosis III is observed in the presence of high level of MAPK/Erk activity (Verlhac *et al*., 1996). The hypothesis that Mos-MAPK/Erk network could be the determining factor limiting the number of meiosis to two was recently formally tested in urochordates, which are at the crossroad between invertebrates and vertebrates (Dumollard *et al*., 2011). In ascidian eggs, prolonging MAPK activity by expressing murine Mos leads to entry into supernumerary rounds of M-phases, which was attested by the increased number of polar bodies (Dumollard *et al*., 2011). Then, urochordates offer an attractive model to unravel new observations on a conserved role of MAPK/Erk in spindle morphogenesis and to decipher the mechanisms leading to uncontrolled division and polyploidy since the successive rounds of M-phases observed in these cases occur without intervening replication.

5. Concluding remarks

Intensive fundamental research has generated a large amount of experimental data. However, it is crucial to validate this knowledge on animal models closer to humans. For analysis of aneuploidy degree and mechanism of occurrence in mammalian oocytes, pig has appeared as an attractive model. Comparing to non-human primates, pigs are cheaper and easier to maintain in controlled conditions. Porcine physiology assures a high relevance of the data obtained in this species for human-related research. Effective application of special breeds like minipigs, together with new methods for aneuploidy detection like CGH, brings new possibilities for aneuploidy research in mammalians. Usage of porcine oocytes for research on regulation pathways is still limited by the volume of oocytes collected and their sensitivity to manipulation in *in vitro* conditions. Development of *in vitro* cultivation

methods and more effective live-cell imaging systems open new perspectives for aneuploidy research on porcine oocytes. To this extent, the molecular toolkits developed for kinase activity reporter will offer advantages to understand how kinases integrate and achieve cellular functions (Riquet *et al*., 2011). Also, these methods, which aim at developing new sensors, will gain in sensitivity, kinetic properties and in spatial resolution, providing new tools for precise subcellular localization of dynamic structures such as meiotic spindles.

Nowadays pigs are used in several fields of biomedical research and its importance, as biomedical model, will increase in the near future. Last reports detecting aneuploidies in porcine oocytes bring interesting results and indicate significance of the porcine model for the study of aneuploidy in human. Nevertheless, taking advantages of the above-mentioned advantages of *Xenopus* oocytes, this amphibian might be considered as an "old dog", from which one might learn new tricks. It might also provide new insights to the scientific community interested in the field of reproduction. Proteomic approaches of the meiotic microtubule proteome are promising, since they will enable us to build a network of a microtubule-associated interactome: such approach was successfully adopted and began to validate novel spindle components like the human orthologue Mgc81475 (Smu1), which depletion drives mitotic arrest (Gache *et al*., 2010). Further works are requested to validate this observation in other meiotic or mitotic models and to fully elucidate the role of the member of the MAPK/Erk network in spindle morphogenesis and aneuploidy. Nevertheless, approaches driven either in oocytes, eggs or extracts have generated an abundant literature, which has been attractive for modeling purposes. Computational models have been proposed to understand the self-organization of meiotic spindle (Loughlin *et al*., 2010; Schaffner and Jose, 2006; Schaffner and Jose, 2008). These models offer a coherent picture of how microtubule dynamic instability, flux, and nucleation contribute to self-organization of a structure in a steady state.

Understanding of aneuploidy occurrence during meiosis in humans will benefit from experiments performed in various models, such as pigs and amphibians, and from the development of new tools, like sensors, and new approaches, like modeling. Further efforts will be necessary to collect and compare data obtained from these models.

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7. List of abbrevations

Calmodulin-dependent kinase (CAMKII); cumulus-enclosed oocytes (CEOs); cumulus oocytes complexes (COCs); cortical granule (CG); comparative genomic hybridization (CGH); denuded oocyte (DO) ; extracellular regulated kinase (Erk); fluorescence *in situ* hybridization (FISH); Germinal Vesicle (GV); germinal vesicle breakdown (GVBD); lutheinizing hormone (LH); metaphase I (MI); metaphase II (MII); mitogen activated protein kinase (MAPK); mitogen-activated protein kinase kinase (MEK); M-phase or meiosis promoting factor (MPF); microtubule organizing center (MTOC); ovum pick-up (OPU); polar body (PB); phosphate buffered saline (PBS); protein kinase A (PKA);

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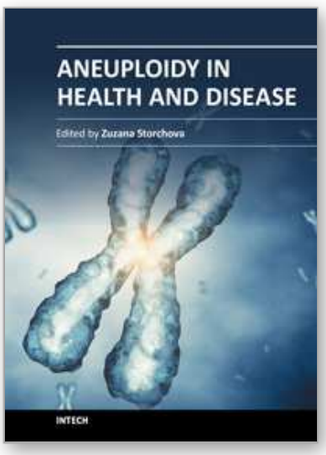

Aneuploidy in Health and Disease

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Aneuploidy means any karyotype that is not euploid, anything that stands outside the norm. Two particular characteristics make the research of aneuploidy challenging. First, it is often hard to distinguish what is a cause and what is a consequence. Secondly, aneuploidy is often associated with a persistent defect in maintenance of genome stability. Thus, working with aneuploid, unstable cells means analyzing an ever changing creature and capturing the features that persist. In the book Aneuploidy in Health and Disease we summarize the recent advances in understanding the causes and consequences of aneuploidy and its link to human pathologies.

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