

# Basic mechanisms of fertilization and parthenogenesis in pigs

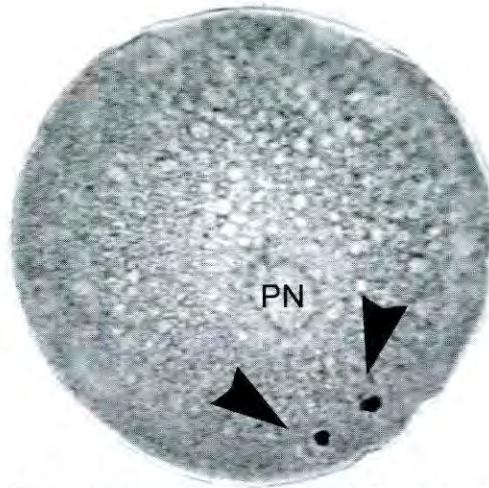
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Fertilization of the egg, or oocyte, initiates the entire developmental process, but while the mechanism by which the spermatozoa triggers the oocyte to resume meiosis has been studied extensively, conclusions about this process are still elusive. Some workers have suggested that a molecule on the surface of the spermatozoon may interact with a receptor on the plasma membrane of the oocyte, thereby triggering the oocyte to resume meiosis. Other workers have focused on a factor or factors located in the cytoplasm of the spermatozoa that is deposited into the cytoplasm of the oocyte. A hallmark response to fertilization in mammals is an increase in the cytoplasmic concentration of free  $\text{Ca}^{2+}$  in the oocyte. Many additional studies have focused on treatments that will induce the oocyte to resume meiosis without being fertilized. The process of resumption of meiosis without a spermatozoon is generally referred to as activation or parthenogenesis. Activation of the oocyte is very important for a number of oocyte- or embryo-related technologies including intracytoplasmic sperm injection (ICSI) and cloning by nuclear transfer. This review will focus on what is known about fertilization and methods to mimic this process, with an emphasis on pigs.

## Introduction

Pig oocytes are arrested at metaphase II of meiosis when ovulated. Fertilization induces the resumption of meiosis, or activates the oocyte, resulting in an oocyte with a single pronucleus and two polar bodies (Fig. 1). Although the downstream events of fertilization have been well characterized, there is still debate as to what triggers activation. The two competing theories are: (i) that sperm binding to the plasma membrane triggers oocyte activation; and (ii) that a factor is deposited into the cytoplasm of the oocyte at sperm–oocyte fusion and that this factor triggers oocyte activation. Proponents of either of these theories admit that complete oocyte activation may be triggered by a combination of these events. A complete understanding of the process of fertilization is needed because failure of adequate fertilization would be disastrous for the reproductive process. In addition, many oocyte- or embryo-related technologies rely on adequate activation of the oocyte. These technologies include *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and nuclear transfer. Many reviews have been written on the mechanisms of oocyte activation in other species such as mice and sea urchins (Swann and Lai, 1997; Macháty and Prather, 1998; Macháty *et al.*, 1999a; Parrington, 2001); thus, the focus of the present review will be on pig oocytes.



**Fig. 1.** Parthenogenetically activated pig oocyte. This oocyte has a single pronucleus (PN) and two polar bodies (arrowheads). Photo courtesy of L. Lai.

### Activation by fertilization

As stated above, one of the hallmarks of fertilization is the release of intracellular  $\text{Ca}^{2+}$ . A series of  $\text{Ca}^{2+}$  bursts or oscillations generally follows this first  $\text{Ca}^{2+}$  transient and continues until the time the pronuclei form. The amplitude and frequency of the  $\text{Ca}^{2+}$  bursts are generally consistent within an oocyte, but do vary between oocytes. Although the  $\text{Ca}^{2+}$  transients are the main feature of fertilization, there are many other cellular and subcellular events that participate in this process. Some of the following features are species-specific; however, for context they will all be presented with a later focus on pigs.

The events of activation have been broken down into two classes: early and late events. Early events include: a sodium influx (Dale *et al.*, 1978), hyperpolarization of the membrane (Miyazaki and Igusa, 1982) and  $\text{Ca}^{2+}$  entry through voltage-gated  $\text{Ca}^{2+}$  channels (Chambers, 1989), followed by an explosive release of  $\text{Ca}^{2+}$  starting at the point of sperm–oocyte fusion. These  $\text{Ca}^{2+}$  releases may be mediated by inositol triphosphate or ryanodine receptors on the endoplasmic reticulum. The first burst of  $\text{Ca}^{2+}$  also causes exocytosis of the cortical granules. It is thought that the contents of the cortical granules modify the zona pellucida such that additional spermatozoa cannot bind to or penetrate the zona pellucida. In mammals, there are a series of intracellular free  $\text{Ca}^{2+}$  oscillations after fertilization. The oscillations are coupled with a  $\text{Ca}^{2+}$  influx and, in pigs, this influx may be mediated by a *trp* gene homologue (Macháty *et al.*, this supplement). Late events include an increase in intracellular pH, polyadenylation and translation of pre-existing mRNAs, resulting in an increase in protein synthesis as well as a change in the quality of the proteins in the oocyte, a decrease in the activity of maturation-promoting factor (MPF), cytostatic factor, mitogen-activated protein kinase (MAPK), myosin light chain kinase (MLCK) and glutathione concentration. If these early and late events occur, then the oocyte will develop pronuclei and begin the developmental programme.

### Parthenogenetic activation

As stated earlier, many manipulations performed on oocytes bypass the normal fertilization process. The two most prominent procedures include ICSI and nuclear transfer. Thus, it is

imperative that we understand the normal responses to fertilization so that we can attempt to mimic them and activate the oocytes parthenogenetically for ICSI and nuclear transfer. The  $\text{Ca}^{2+}$ -dependent and -independent methods of parthenogenetic activation, combination treatments, as well as specific applications to ICSI and nuclear transfer, will be discussed.

### *Calcium-dependent activation*

Early studies in mouse oocytes showed that stimulating protein kinase C with phorbol esters induced a series of  $\text{Ca}^{2+}$  oscillations, and that this was followed by pronuclear formation and, in some cases, limited development. There are numerous ways to induce an intracellular  $\text{Ca}^{2+}$  transient in pig oocytes, including injection of  $\text{Ca}^{2+}$  (Macháty *et al.*, 1996), electrically porating the plasma membrane in the presence of extracellular  $\text{Ca}^{2+}$  (Prather *et al.*, 1989; Sun *et al.*, 1992), treatment with ionophores (Wang *et al.*, 1998a,b, 1999), thimerosal (Macháty *et al.*, 1997a) or ethanol (Didion *et al.*, 1990), stimulation of guanine nucleotide binding proteins (Macháty *et al.*, 1997b) and injection of inositol triphosphate, cyclic-ADPribose (Macháty *et al.*, 1997c) or a crude sperm extract (Macháty *et al.*, 2000). Each of these treatments attacks a different pathway or point on a pathway that results in a  $\text{Ca}^{2+}$  transient.

These observations illustrate why the data on what activates an oocyte at fertilization are so difficult to interpret: many different pathways have the capacity to activate the egg (even nitric oxide has been implicated; Kuo *et al.*, 2000). This is further illustrated by studies in which the mRNA encoding the muscarinic M1 receptor was injected into the cytoplasm of unfertilized oocytes (Macháty *et al.*, 1997b; Kim *et al.*, 1998). After time for translation and insertion into the plasma membrane, the oocytes were treated with acetylcholine. Acetylcholine activated the guanine protein-coupled receptor, which probably activated phospholipase C. Phospholipase C then probably acted on phosphatidyl inositol bisphosphate to yield inositol triphosphate and diacylglycerol. The inositol triphosphate bound to inositol triphosphate receptors located on the endoplasmic reticulum and released  $\text{Ca}^{2+}$  into the cytoplasm. This  $\text{Ca}^{2+}$  release caused release of the cortical granules into the extracellular space, a decrease in histone H1 kinase activity, a shift in protein profiles consistent with fertilization and pronuclear formation (Macháty *et al.*, 1997b; Kim *et al.*, 1998). Furthermore, oocytes activated in this manner could develop to the blastocyst stage and these blastocysts had nucleoli and mitochondria that had morphologies consistent with normal blastocyst stage embryos (Kim *et al.*, 1998).

Although a simple electrical pulse in the presence of  $\text{Ca}^{2+}$  will generally induce an oocyte to begin the developmental process, the pattern of events associated with normal fertilization is not always followed. Normally, histone H1 kinase activity decreases after fertilization and stays low until the first cleavage; however, with electrical activation H1 kinase activity initially decreases, but increases later in the first cell cycle (Leal and Liu, 1998). Although a single electrical pulse provides a single  $\text{Ca}^{2+}$  transient that is sufficient to induce development to the blastocyst stage, it is thought that the quality (amplitude and frequency) of the  $\text{Ca}^{2+}$  transient or transients is very important and may determine the degree of development after stimulation (Ozil, 1990).

As stated earlier, there is an increase in intracellular pH at fertilization in many lower species such as the sea urchin. However, this has not been demonstrated at fertilization in mammals. Recently, Ruddock *et al.* (2000a) observed an increase in intracellular pH when pig oocytes were treated with 7% (v/v) ethanol or A23187 and there appears to be more than one mechanism regulating the change in pH. Similar observations were made in murine and bovine oocytes (Ruddock *et al.*, 2000b). However, the increase in intracellular pH in pigs is independent of intracellular  $\text{Ca}^{2+}$  concentration (Ruddock *et al.*, 2001).

### Calcium-independent activation

Although intracellular  $\text{Ca}^{2+}$  is released at fertilization, this is not a requisite for activation of the oocyte. Indeed, the early studies on treatment with phorbol esters were not consistent with phosphorylation status; cells in metaphase have proteins that are already phosphorylated and need to be dephosphorylated to push the cell into the next interphase (histones, nuclear lamins). Thus, studies that have evaluated treatments that would decrease the phosphorylation status or inhibit phosphorylation would appear to be more relevant. Inhibition of MAPK, MPF and MLCK (Green *et al.*, 1999) has resulted in pronuclear formation of pig oocytes. In contrast, stimulation of protein kinase C (Sun *et al.*, 1997) resulted in cortical granule release, but not pronuclear formation, whereas stimulation of tyrosine kinase activity resulted in both cortical granule release and pronuclear formation (Kim *et al.*, 1999).

In addition to the treatments that inhibit kinase activity, other treatments can result in oocyte activation. One of the more popular techniques is to inhibit protein synthesis. As cyclin B is being made continually and this manufacture is necessary for maintaining high concentrations of MPF, inhibiting the production of cyclin B will cause MPF activity to decrease. This lower MPF activity results in the resumption of meiosis by the oocyte and formation of a pronucleus. This response may be species-specific as Nussbaum and Prather (1995) found that cycloheximide treatment alone did not result in activation, but when used in combination with an electrical pulse, it enhanced the rates of pig oocyte development; in other species, activation can be induced with the cycloheximide treatment only (Macháty and Prather, 1998).

In relation to altering MPF activity, the cyclin-dependent kinase inhibitor butyrolactone I can result in cleavage rates of 40%. However, when combined with an electrical pulse, butyrolactone I can result in 59% of the treated oocytes progressing to the blastocyst stage (Dinnyes *et al.*, 1999).

### Combination treatments

As stated above, attacking more than one point on the pathway can result in improved rates of pronuclear formation, as well as improved rates of development. A combination of an electrical pulse and cycloheximide is superior to either treatment alone in promoting pronuclear formation (Nussbaum and Prather, 1995). Similarly, Grocholova *et al.* (1997) used a combination of A23187 and cycloheximide to improve development of parthenogenetic oocytes. For the thimerosal treatment to result in pronuclear formation the action on the sulphhydryl groups must be reversed or the spindle will be damaged irreversibly. To facilitate that reversal, thimerosal followed by dithiothreitol treatment results in  $\text{Ca}^{2+}$  release and 42% of oocytes developing to the compact morula or blastocyst stage (Macháty *et al.*, 1997b). Thimerosal causes  $\text{Ca}^{2+}$  oscillations and no development if not reversed with dithiothreitol (Macháty *et al.*, 1999b).

Another treatment that is often used when development of parthenogenotes is desired is cytochalasin B. It is not known whether cytochalasin B affects any of the  $\text{Ca}^{2+}$  pathways, but it does depolymerize microfilaments and, thus, prevents polar body emission. Retaining the second polar body should result in a doubling of the number of chromosomes in the developing embryo. Cha *et al.* (1997) increased the percentage of 2n chromosome spreads by treating the activated oocytes with cytochalasin B. Jolliff and Prather (1997) used electrical activation followed by cytochalasin B and obtained fairly normal *in vivo* development to day 12. Kure-bayashi *et al.* (2000) found that development could proceed to day 29 *in vivo*. The lack of further development is probably a result of genomic imprinting (Reik and Walter, 2001).

Although some of these treatments result in cortical granule release they do not prevent subsequent fertilization (Wang *et al.*, 1997, 1998a,b). Thus, there is much to learn about the zona pellucida and methods of inducing hardening.

In conclusion, the most widely used method of activation for nuclear transfer is electrical activation (Prather *et al.*, 1989). Variations to a single pulse of electricity have been added to improve the rates of development (Liu and Moor, 1997).

#### *Activation for ICSI or nuclear transfer*

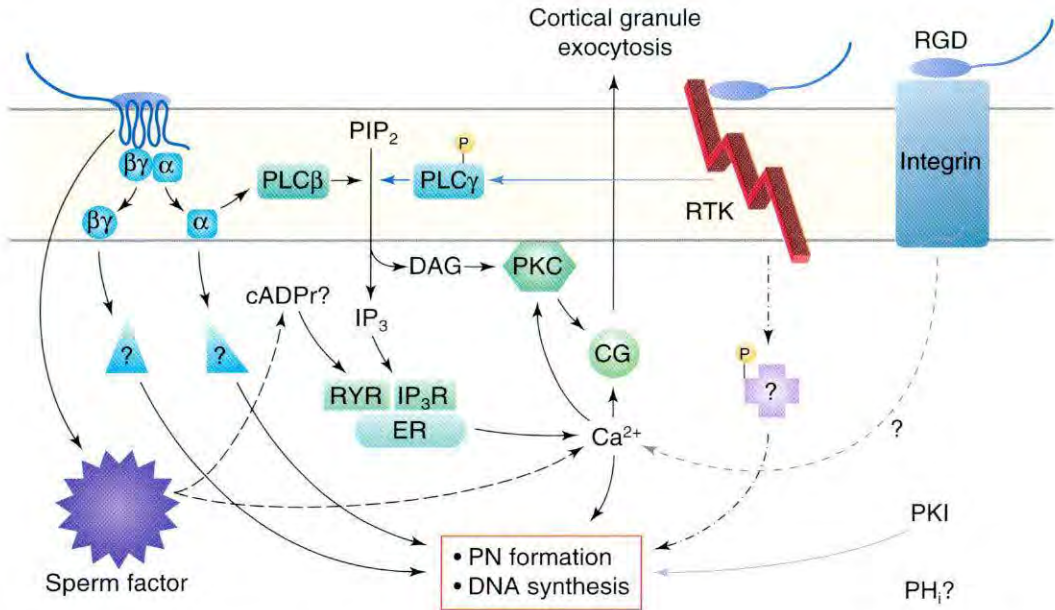
A variety of different activation parameters has been used for ICSI and nuclear transfer. In some cases the injection procedure for ICSI activates the oocyte. Martin (2000) produced an ICSI-derived piglet without any activation stimuli to the *in vivo*-matured oocytes other than injection of a fresh spermatozoon. In contrast, Kolbe and Holtz (2000) used  $\text{Ca}^{2+}$  ionophore to activate *in vivo*-matured ICSI oocytes. Lai *et al.* (in press) obtained a piglet when electrical activation was applied after injection of the spermatozoon; the oocytes did not develop as well without the electrical application.

Oocytes must be enucleated for nuclear transfer. One of the common chemicals used in the enucleation is bisbenzimidazole. Unfortunately, exposure to this compound has deleterious effects on the development of pig oocytes to the blastocyst stage (Tao *et al.*, 2000). Thus, compounds used for enucleation might limit development of nuclear transfer and parthenogenetic embryos.

In many cases there is a benefit to fusing the donor cell to the recipient oocyte or microinjecting the nucleus into the oocyte, before the oocyte is actually activated. Miyoshi *et al.* (2000a) found that a delay of 3 h between fusion and activation improved the rate of blastocyst formation. Tao *et al.* (1999a) used microinjection to transfer the nuclei and attempted to delay the activation up to 4 h after the microinjection while using a  $\text{Ca}^{2+}$ -free medium. The  $\text{Ca}^{2+}$ -free condition was detrimental to development. The rates of development to the blastocyst stage after nuclear transfer have been as low as 3% (Miyoshi *et al.*, 2000b). When presumptive G0 fibroblast cells were transferred to oocytes and electrically activated, only 7% formed blastocysts (Tao *et al.*, 1999b). Betthausen *et al.* (2000) reported 4–8% blastocysts resulting from nuclear transfers that were activated by ionophore followed by 6-dimethylaminopurine. Recently, Kühholzer *et al.* (2001) reported almost 20% blastocysts from electrically activated oocytes. In addition, oocytes from sows resulted in higher rates of development to the blastocyst stage compared with oocytes from gilts. Polejaeva *et al.* (2000) used natural fertilization to achieve normal activation. Nuclear transfer was generally performed as described above with electrical activation. However, the nuclei were transferred to enucleated fertilized oocytes after electrical activation. Thus, the nuclei were transferred to sperm-activated oocytes, which resulted in a number of live born piglets. Onishi *et al.* (2000) produced live pigs by electrical activation and microinjection of oocytes, but did not report on development to the blastocyst stage. Park *et al.* (in press) produced 9–16% blastocysts with two electrical pulses to fuse and activate the oocytes, resulting in five nuclear transfer-derived piglets.

### **Conclusion**

In conclusion, there are a number of methods that can be used to activate pig oocytes artificially. These methods often attack different pathways that lead to parthenogenesis (Fig. 2). These include  $\text{Ca}^{2+}$ -dependent and -independent processes. As there are so many pathways in oocytes that can be stimulated to trigger the resumption of meiosis, it might be concluded that



**Fig. 2.** Summary of pathways that may be activated at fertilization or parthenogenesis. Description of the pathways is included throughout the text. PIP<sub>2</sub>: phosphatidyl inositol bisphosphate; DAG: diacylglycerol; cADPr: cyclic ADP ribose; IP<sub>3</sub>: inositol trisphosphate; RYR: ryanodine receptor; IP<sub>3</sub>R: inositol trisphosphate receptor; PN: pronuclear; PKC: protein kinase C; ER: endoplasmic reticulum; CG: cortical granule; RTK: receptor tyrosine kinase; PLC: phospholipase C; α, β, γ: α, β, γ subunits of a G-protein coupled receptor; p: phosphorylation; PKI: protein kinase inhibitor; RGD: arginine-glycine-aspartic acid peptide.

the oocyte is poised to progress to interphase. Upsetting this apparently delicate balance required for maintenance of the oocyte in metaphase can be accomplished via a variety of methods. Generally, if more than one pathway that results in activation is attacked, greater rates of development to the blastocyst stage can be obtained. Further advancements in the understanding of the basic mechanisms of fertilization will aid in designing strategies to artificially activate pig oocytes for ICSI and nuclear transfer.

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