Cumulus expansion and *in vitro* maturation of porcine oocytes

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List of abbreviations

Ab	antibody
ADAMTS-1	a disintegrin and metalloproteinase with thrombospondin-like repeats-1
AI	anaphase I
ALK	activin receptor-like kinase
BME	basal medium Eagle
β-ME	beta-mercaptoethanol
BMP15	bone morphogenetic protein 15
BMPRII	bone morphogenetic protein receptor type II
BSA	bovine serum albumin
CBX	carbenoxolone
Cell-Tak	Cell and Tissue Adhesive
CG	cortical granule
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COC	cumulus-oocyte complex
DABCO	1,4-diazabicyclo[2.2.2]octane
dbcAMP	dibutyryl cAMP sodium salt
DO	denuded oocyte
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor
FITC	fluorescein isothiocyanate
FSH	follicle-stimulating hormone
GDF9	growth differentiation factor 9
GJ	gap junction
GV(BD)	germinal vesicle (breakdown)
HA	hyaluronic acid
hCG	human chorionic gonadotropin
IBMX	3-isobutyl-1-methylxanthine
ICM	inner cell mass

IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* embryo production
LH	luteinizing hormone
M(I)I	metaphase (I)I
MEM	minimum essential medium
(m)NCSU	(modified) North Carolina State University
MPN	male pronucleus
OCM	oocyte conditioned medium
OOX	oocytectomized complex
OPU	ovum pick-up
OSF	oocyte-secreted factor
PBS	phosphate buffered saline
PDE(3)	(type 3) phosphodiesterase
PF	paraformaldehyde
pFF	porcine follicular fluid
PGM	porcine gamete medium
PKA	protein kinase A
POM	porcine oocyte medium
PR	progesterone receptor
proADAMTS-1	proprotein of ADAMTS-1
proMI	prometaphase I
PTH	parathyroid hormone
PVA	polyvinyl alcohol
PZM	porcine zygote medium
SEM	standard error of the mean
SPOM	simulated physiological oocyte maturation
TI	telophase I
TALP	Tyrode's albumin lactate pyruvate
TCM	tissue culture medium
TGF-β	transforming growth factor beta

Chapter 1

General introduction

1.1 Evolution and application of porcine *in vitro* embryo production

In vitro embryo production (IVP) can be classified as an assisted reproductive technology. The development of assisted reproductive technologies has its origin in the continuous search for improvement of reproductive performances (Luvoni 2000). These technologies are important for research purposes as well as for practical applications. One of the most commonly known application in pig production is artificial insemination (Roca *et al.* 2006).

Tremendous progress has already been made in the knowledge of molecular and cellular processes regulating oocyte maturation, fertilization and subsequent developmental competence of the fertilized oocytes. Nowadays, systems for *in vitro* maturation (IVM) of porcine oocytes have been optimized to the extent that over 20 to 30% of blastocysts can be generated starting from immature oocytes (Gil *et al.* 2010). Research in the field of porcine IVP dates from 1965, when Edwards (1965) described for the first time IVM of porcine oocytes. Only a decade later, IVM oocytes were fertilized for the first time *in vivo* (Motlik and Fulka 1974) and *in vitro* (Iritani *et al.* 1978). In 1989, Mattioli *et al*. (1989) reported for the first time blastocyst formation, establishment of pregnancies and birth of live piglets from oocytes matured and fertilized *in vitro*. However, successful piglet production after transfer of IVP blastocysts only took place at the beginning of the 21st century (Marchal *et al.* 2001; Kikuchi *et al.* 2002). The most recent development in this research field is the production of live piglets form cryopreserved embryos (Nagashima *et al.* 2007), zygotes (Somfai *et al.* 2009) and immature oocytes (Somfai *et al.* 2014).

Beside the aim to generate embryos that develop to live piglets, IVP is a crucial tool to study maturation, fertilization and developmental processes of oocytes. The ultimate goal of fundamental research in IVP is to fully understand and subsequently mimic *in vivo* conditions (Luvoni 2000).

Likewise, IVP is indispensable for preservation and distribution of genetic material *via* cryopreservation techniques (Pereira and Marques 2008). The establishment of oocyte and embryo banks could maintain biodiversity by the preservation of endangered species and subspecies from domestic and wild animals and could act as an interface between *in situ* and *ex situ* conservation programs (reviewed by Andrabi and Maxwell (2007)) (Woelders *et al.* 2006). Moreover, genetic resource banking permits oocyte and embryo international

exchange, avoiding animal transportation and its sanitary risks and injuries (Pereira and Marques 2008).

Moreover, IVP constitutes the foundations for further development of advanced biotechniques such as the production of transgenic pigs for biomedical and agriculture applications (Prather *et al.* 2003). Advances in biotechniques have not only been generated by making use of mouse models, but also by using domestic animals, such as cattle and pigs. Porcine models are worldwide recognized as excellent models for human diseases and are considered to be very important to make progress in *in vitro* fertilization (IVF) research. An improved porcine model can provide solutions for human IVF systems to treat infertility and can be an example for other animal models. Moreover, production of normal viable embryos in pigs is important for eventual future applications in the broader field of human medicine (reviewed by Whyte and Prather (2011)). Because of the many physiological similarities to humans, pigs can be used as research models and for creating genetically modified animals as potential donors of tissues and organs for xenotransplantation (Gil *et al.* 2010). Besides biomedical applications, transgenic pigs can be of great value to the agricultural sector (Wheeler and Walters 2001). Changes in the genome could (1) alter the carcass composition such that it is a healthier product, (2) produce pork faster or more efficiently, (3) create animals that are resistant to specific diseases, (4) reduce the major losses normally observed during the first month of porcine embryogenesis, and (5) create animals that are more environmentally friendly (Prather *et al.* 2003).

Despite the aforementioned very promising applications, the IVP system efficacy is still low compared with *in vivo* embryo development due to (1) low competence of IVM oocytes, (2) high incidence of polyspermic fertilization, and (3) reduced blastocyst development because of imperfection of the IVC system (reviewed by Dang-Nguyen *et al.* (2011). To apply the above mentioned biotechniques in pigs at a practical level, improvement of the IVP system efficacy is essential.

1.2 Components of *in vitro* embryo production

1.2.1 Sequential stages of *in vitro* embryo production

A series of integrated, effective techniques is required to produce porcine embryos from follicular oocytes *in vitro* (Funahashi and Day 1997). Three major subsequent phases can be distinguished: collection and *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). A coordinated interplay of these different phases will result in the production of viable embryos, which can be transferred to a recipient animal. Most standard protocols use three different media for the three phases. However, in 2008 Yoshioka *et al.* (2008) designed a basic single medium, based on the composition of porcine oviductal fluid that could be used for the three phases, provided that the necessary adaptations were performed in each phase. Using a basic single medium implied that the embryo did not have to adapt to changes in osmolarity, pH and/or substrate availability (Yoshioka *et al.* 2008). Moreover, advantages were obtained on a practical level by the use of a single basic medium. Because of one basic stock, less time was needed for media preparations, especially because the stock can be stored at 4°C for several months (Yoshioka *et al.* 2008). The use of basic stock solutions also provided benefits for experimental work because it created the possibility of comparing variants of a medium with minimal variation (Gandhi *et al.* 2000).

1.2.2 Collection and *in vitro* maturation

1.2.2.1 General procedure

Retrieval of oocytes can be performed in two ways. First, oocytes can be collected by surgical procedures from donor animals. However, this process is time consuming, expensive and oocyte numbers are limited (Abeydeera 2002). Second, oocytes can be retrieved from ovaries from animals in the slaughterhouse. Ovaries from slaughtered female pigs are inexpensive and

provide an abundant amount of oocytes (Grupen *et al.* 2003). Most commonly, ovaries of prepubertal gilts are used for reproductive studies, although it is known that the developmental potential of prepubertal oocytes is lower than that of oocytes from adult sows (Marchal *et al.* 2001). The fact that pigs are commonly slaughtered at a prepubertal age limits the availability of oocytes from adult sows (Grupen *et al.* 2003). Moreover, more than 50% of adult female pigs are culled because of reproduction failure (de Jong *et al.* 2014), which makes that the quality of such oocytes is questionable. Even if the quality meets the requirements, a more heterogeneous pool of oocytes is obtained when collecting oocytes from adult animals in comparison to prepubertal animals. These practical considerations lead to the use of prepubertal animals instead of adult ones for collection of oocytes.

Transportation of ovaries from the slaughterhouse to the laboratory is conducted in 0.9% NaCl or phosphate buffered saline (PBS) supplemented with antibiotics (Abeydeera 2002). Temperature and duration of transport is a first critical step in the IVP process. Transportation of ovaries at 25°C for up to 5 h seems to be an acceptable way of transportation (Abeydeera 2002). Once arrived in the laboratory, the collection of the oocytes can be started. Several methods such as follicle puncture or follicle dissection can be applied for this purpose. The ovary contains multiple follicle types ranging from small (< 3 mm), over medium (3 to 6 mm) to large (> 6 mm) follicles of which the medium sized follicles are used for oocyte collection (Abeydeera 2002). A wide assortment of different media is generated for IVM of immature oocytes. Mostly, a basic maturation medium is enriched with different kinds of supplements according to the needs of the oocyte in a specific part of the development. In pigs, the IVM period varies between 44 and 48 h and is divided in two parts. In the first 20 to 22 h and the following 22 to 28 h several components such as gonadotropins (equine chorionic gonadotropin and human chorionic gonadotropin), growth factors and serum or porcine follicular fluid (pFF) can be added in different patterns. Removal of hormone supplements from the maturation media after the first IVM period is important to improve cytoplasmic maturation and cumulus expansion (Funahashi and Day 1993). A frequently used growth factor during IVM is epidermal growth factor (EGF), which enhances nuclear maturation by itself and which can stimulate cytoplasmic maturation if used in combination with gonadotropins (Ding and Foxcroft 1994).

During IVM, many research protocols use addition of pFF to a basic maturation medium, mostly North Carolina State University (NCSU) 23 or 37 (Petters and Wells 1993; Funahashi

et al. 1997b). This pFF contains not only several undefined and thus uncontrollable components, but is also a possible source for contamination by viral pathogens (cattle (Galik *et al.* 2002); human (Devaux *et al.* 2003)) or environmental pollutants (pig (Kamarianos *et al.* 2003)). Recently designed media are composed without addition of undefined components such as serum or pFF. The use of undefined components hampers not only the analysis of the specific action of addition of certain substances, but also prevents a good reproducibility of the results because of a lower reliability of the media formulations (Yoshioka *et al.* 2008). By replacing the protein preparations, higher reproducibility of results can be obtained and biosafety of culture media can be ensured (Yoshioka *et al.* 2008). Replacing follicular fluid with polyvinyl alcohol (PVA) in tissue culture medium (TCM) 199 lead to similar blastocyst rates compared to NCSU 23 with pFF addition (Abeydeera *et al.* 1998b). A few years later, pFF was successfully replaced in modified NCSU (mNCSU) 23 by PVA and amino acids (Hong *et al.* 2004). In 2008, a chemically defined, protein-free IVM medium, porcine oocyte medium (POM), was launched (Yoshioka *et al.* 2008). Maturation rates and meiotic progression of oocytes in POM+PVA are similar to POM+pFF or mNCSU 37+pFF, but they decreased in mNCSU 37+PVA (Yoshioka *et al.* 2008). It is clear that POM as a basal medium could sustain maturation in defined circumstances whereas mNCSU 37 could not (Yoshioka *et al.* 2008). An explanation for this phenomenon may be found in the fact that POM contains 22 amino acids (glutamine, hypotaurine, cysteine, 12 basal medium Eagle (BME) essential amino acids and seven minimum essential medium (MEM) non-essential amino acids) in contrast to mNCSU 37 which only contains glutamine and cysteine (Yoshioka *et al.* 2008). Amino acids exerted important specific functions in a protein-free medium such as acting as osmolytes (Yoshioka *et al.* 2008), intracellular buffers (mouse (Edwards *et al.* 1998)), heavy metal chelators (Akaki *et al.* 2009) and energy substrates (mouse (Rose-Hellekant *et al.* 1998)). The fact that essential and/or non-essential amino acids were enhancing the function of a defined medium was also reported in the earlier mentioned study where pFF was replaced by PVA in mNCSU 23 (Hong *et al.* 2004). Not only for nuclear maturation, but also at the level of cytoplasmic maturation, as reflected in cleavage and blastocyst rates after activation, mNCSU 37+PVA seemed to be inferior (Yoshioka *et al.* 2008). Polyspermy and the number of sperm cells that penetrated an oocyte decreased when replacing pFF by PVA in POM as well as in mNCSU 37 (Yoshioka *et al.* 2008). Therefore, it has been suggested that addition of different macromolecules such as PVA or pFF, was influencing the mechanism to block polyspermy in some way (Yoshioka *et al.* 2008). Although the quantity of produced

blastocysts was similar between POM+pFF and POM+PVA, the quality of blastocysts, expressed as total number of cells per blastocyst, was reduced in the PVA supplemented POM (Yoshioka *et al.* 2008). However, this difference could only be observed after IVF and not in artificially activated oocytes, probably referring to inadequacy closely related to fertilization (Yoshioka *et al.* 2008).

The search for additional components depends on which aspect of the maturation process is focused and in which stage of development the oocyte is situated. The media composition tries to reflect the changes, which take place during *in vivo* maturation of porcine oocytes in the best possible way. The key player during IVM is the cumulus-oocyte complex (COC), which refers to the oocyte surrounded by several layers of cumulus cells (Figure 1). Oocyte maturation consists of two processes, nuclear maturation and cytoplasmic maturation. However, also the cumulus cells need to undergo crucial changes during the IVM period, referred to as cumulus expansion.

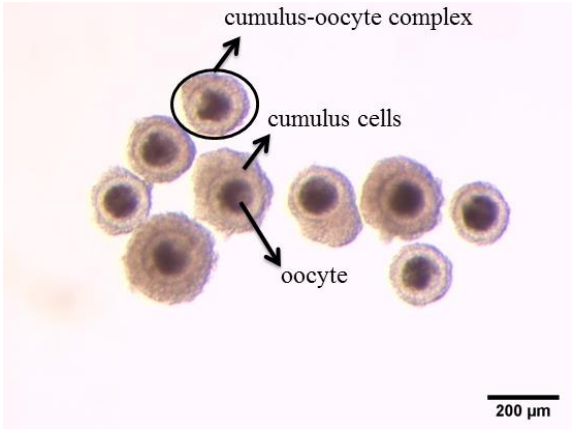


Figure 1: The cumulus-oocyte complex consists of an oocyte that is surrounded by cumulus cells. Magnification x 40. Photograph by R. Appeltant.

The O_2 tension during IVM is a controversial subject. A beneficial effect of 20% O_2 tension during IVM has been reported by Park *et al.* (2005). However, two reports demonstrated beneficial effects of lowering O_2 concentrations to 5% during IVM. Kikuchi *et al.* (2002) reported quality improvement of blastocysts after IVM/IVF and Iwamoto *et al.* (2005) reported a higher activation ability and improved quality of parthenogenetic blastocysts. On

the contrary, Somfai *et al.* (2007) could not report any effect of the oxygen tension during IVM on embryo development.

1.2.2.2 Nuclear maturation

Nuclear maturation is a term that refers to the resumption of meiosis and progression to the metaphase II (MII) stage (Abeydeera 2002) (Figure 2). Metaphase II stage oocytes contain a meiotic spindle with metaphase chromosomes (the metaphase plate) and a polar body that is extruded (Figure 2). At about 35 days after birth of the piglet, all oogonia are in the prophase of the first meiotic division (the germinal vesicle (GV)) (Hunter 2000). Oocytes reach their full size in small antral follicles of 2 to 3 mm in diameter (Motlik and Fulka 1986). The oocyte remains arrested in the prophase of the first meiotic division, until the moment of the gonadotropin surge when the oocyte needs to resume meiosis (Mattioli *et al.* 1994).

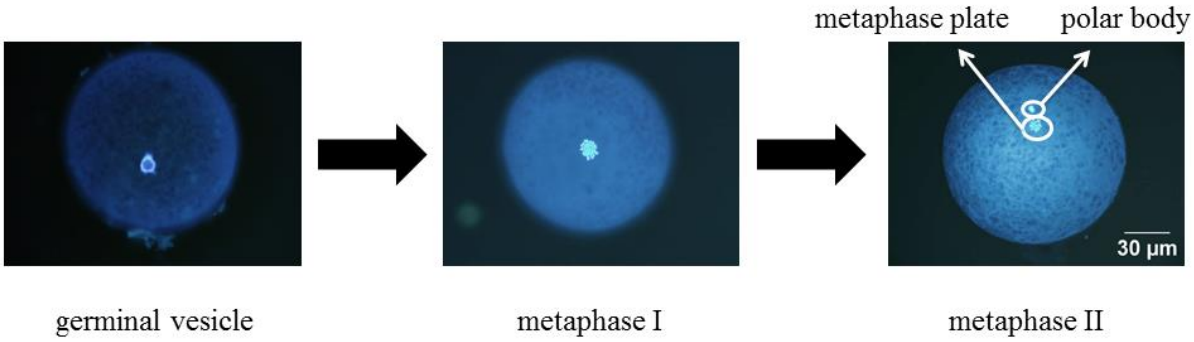


Figure 2: Three major steps in the process of nuclear maturation of (porcine) oocytes: progression from germinal vesicle stage through metaphase I stage to metaphase II stage. Specimen was stained with Hoechst 33342. Magnification x 400. Photographs by R. Appeltant.

The mechanisms of nuclear maturation *in vivo* and *in vitro* differ fundamentally. *In vivo*, the oocyte resumes meiosis after the preovulatory luteinizing hormone (LH) surge (Eppig 1982). On the contrary, *in vitro*, from the moment the oocyte is removed from the follicle during collection, the oocyte resumes meiosis spontaneously (Pincus and Enzmann 1935). This

spontaneous resumption of meiosis during standard IVM techniques does not seem to be a physiological process (reviewed by Gilchrist (2011)). In both cases, meiotic division is resumed starting from GV and progressing until the metaphase of the second meiotic division (MII), which includes germinal vesicle breakdown (GVBD), chromosomes formation and polar body extrusion (Abeydeera 2002). The GV of follicular oocytes maturing *in vivo* or *in vitro*, is divided into four well-defined stages (GV1 to 4) described by Motlik and Fulka (1976). The classification is based on the chromatin changes, and on nucleolus and nuclear membrane disappearance (Motlik and Fulka 1976). In 2004, this classification was further extended with GV0 by Sun *et al.* (2004). Germinal vesicle stage 0 occurs in oocytes derived from small (1 to 2 mm) follicles (Sun *et al.* 2004). As porcine oocytes grow, GV1 configurations increase and GV0 patterns disappear (Sun *et al.* 2004). Germinal vesicle 3 and GV4 are suggested to represent transient events prior to GVBD (Sun *et al.* 2004).

1.2.2.3 Cytoplasmic maturation

Cytoplasmic maturation is a broader term than nuclear maturation and refers to maturational events not directly related to meiotic processes, but to events that prepare the oocyte for fertilization and preimplantation development (Abeydeera 2002). Cytoplasmic maturation involves namely many processes that lead to developmental competence postfertilization (Yamauchi and Nagai 1999) such as the capacity to support male pronucleus (MPN) formation (reviewed by Tanghe *et al.* (2002)) (Funahashi and Day 1993), monospermic fertilization due to a correct cortical granule (CG) exocytosis (Wang *et al.* 1997) and subsequent developmental capability (rat (Vanderhyden and Armstrong 1989); pig (Prather and Day 1998)). Glutathione production is one of the crucial events of cytoplasmic maturation (Eppig 1996). It is the major non-protein sulfydryl compound present in mammalian cells (Abeydeera *et al.* 1998a). Glutathione is involved in many biological functions, such as metabolism, transport and cellular protection (Meister 1983). It protects cells against the effect of reactive oxygen (Meister 1983), is involved in DNA and protein synthesis and amino acid transport (Grupen *et al.* 1995), and supports the MPN formation after fertilization (Yoshida *et al.* 1993).

The composition of the maturation medium is regulating the glutathione content of the oocyte (Yoshida *et al.* 1993) to a large extent. Certain additions to the maturation medium such as EGF (Abeydeera *et al.* 2000), β-mercaptoethanol (Abeydeera *et al.* 1998a), cysteamine (Yamauchi and Nagai 1999) and cysteine (Yoshida *et al.* 1993) promoted glutathione synthesis. Addition of low-molecular-weight thiols, such as β-ME and cysteamine, to the culture medium made more cysteine available for the synthesis of glutathione (Grupen *et al.* 1995). In normal IVP conditions, cysteine was almost completely oxidized to cystine (Mohindru *et al.* 1985), which is poorly utilized for cellular glutathione production (mouse (Ishii *et al.* 1981)). By providing an excess of cysteamine, the complete oxidation to cystine may be prevented (Grupen *et al.* 1995). Not only the addition of specific components to the culture medium is important for the glutathione content, but also the gap junction (GJ) communication between cumulus cells and oocyte. An inflow of glutathione from the cumulus cells to the oocyte will happen through this communication channel (Mori *et al.* 2000). Probably glutathione is one of the most crucial molecules transported through the junction (reviewed by Nagai *et al.* (2006)).

1.2.2.4 The role of cumulus cells and their expansion

From the moment antrum formation takes place, the granulosa cell population starts differentiating in two subpopulations of granulosa cells (mammals (Buccione *et al.* 1990)). The cells immediately surrounding the oocyte differentiate to cumulus granulosa cells and the cells forming the follicular wall are defined as mural granulosa cells (Buccione *et al.* 1990). An intensive metabolic coupling between cumulus cells and oocyte is maintained through cumulus cell process endings, which penetrate through the zona pellucida (cattle (De Loos *et al.* 1991)). Mural granulosa cells, cumulus cells and the oocyte are connected *via* GJ (mammals (Anderson and Albertini 1976)). In response to the preovulatory gonadotropin surge, the cumulus cells start cumulus expansion, which includes production of hyaluronic acid (HA), which is deposited into the intercellular spaces and stabilized by accessory proteins (cattle (Ball *et al.* 1982) and mammals (reviewed by Tanghe *et al.* (2002)). Cumulus cells are important before, during and after ovulation (reviewed by Tanghe *et al.* (2002)). Before ovulation, cumulus cells keep the oocyte under meiotic arrest, participate in the

induction of meiotic resumption and support cytoplasmic maturation of the oocyte (reviewed by Tanghe *et al.* (2002)). It is generally known that under *in vitro* conditions, cumulus cells during IVM support the nuclear maturation of oocytes to the MII stage and are involved in the cytoplasmic maturation (cattle and pig (Nagai 2001)). More specifically, cumulus cells exert a metabolic and protective role of in oocyte cytoplasmic maturation (reviewed by Tanghe *et al.* (2002)). As mentioned in 1.2.2.3, one of the functions of cumulus cells is to transport glutathione to the oocyte through GJs. This way of providing the oocyte with glutathione from the cumulus cells seems to be more important during the early period of oocyte maturation (Ozawa *et al.* 2010). Meanwhile, the oocyte acquires the capacity to synthesize its own glutathione through follicle-stimulating hormone (FSH) stimulation via GJ communication. Thereby the oocyte will be able to synthesize glutathione without GJ communication-mediated support from the cumulus cells in the later half of IVM (Ozawa *et al.* 2010). An adequate synthesis and transportation of glutathione by intact cumulus cells to the oocyte will improve oocyte maturation, fertilization and embryonic development (Maedomari *et al.* 2007). During ovulation, the cumulus cells can conduct the oocyte into the oviduct (hamster (Mahi-Brown and Yanagimachi 1983)). After ovulation, cumulus cells exert some important functions during fertilization (reviewed by Tanghe *et al.* (2002)). Cumulus cells are attracting, trapping and selecting spermatozoa, they facilitate sperm capacitation, acrosome reaction and penetration, and they prevent hardening of the zona pellucida (reviewed by Tanghe *et al.* (2002)).

Despite all the functions mentioned in the previous paragraph, contradictory results have been found concerning the effects of removal of cumulus cells during IVM. In cattle, removal of cumulus cells before 12 h of IVM was detrimental for normal cytoplasmic maturation of the oocyte, but did not influence its nuclear maturation (Chian and Niwa 1994). In pigs, Akaki *et al.* (2009) observed a higher tendency in the percentages of oocytes reaching the MII stage in denuded oocytes compared to COCs. However, other studies in pig demonstrated that removal of cumulus cells had negative effects on meiotic competence of oocytes (Wongsrikeao *et al.* 2005; Maedomari *et al.* 2007). Further research is needed to find a possible elucidation for these contradictory results. However, the results of Akaki *et al.* (2009) can possibly be explained by an inhibitory factor produced by cumulus cells. It is known that porcine cumulus cells can release a factor that can regulate the disruption of GJs, cumulus expansion and quantitatively inhibit GVBD in porcine oocytes (Petr *et al.* 1989;

Isobe and Terada 2001). This factor is probably acting through the mediation of cumulus cells instead of directly on oocytes, which contributes to the lack of inhibitory effect on denuded oocytes (Petr *et al.* 1989). The inhibitory action of this factor can partially be overcome by luteinizing hormone (LH) (Petr *et al.* 1989), but Akaki *et al.* (2009) used gonadotropin-free media, which can be responsible for the maintenance of the inhibitory influence of the cumulus cells on the cumulus-enclosed oocytes, leading to lower nuclear maturation rates in COCs compared to denuded oocytes.

Whether or not cumulus expansion is a pre-requisite for normal fertilization and embryo development remains a matter of debate. Abnormal expansion has been associated with low embryo development in many studies (Qian *et al.* 2003; Bijttebier *et al.* 2008). On the contrary, Yoshioka *et al.* (2008) reported a similar penetration and development after IVM in POM+PVA and POM+pFF despite of the great difference in cumulus expansion in favor of the latter one (Abeydeera *et al.* 1998b; Grupen and Armstrong 2010). In another study, Gomez *et al.* (2012) reported reduced cumulus expansion associated with improved blastocyst development after IVM in the presence of denuded oocytes. In fact the importance of cumulus cells for fertilization in porcine IVF systems is still a debate itself. Early reports suggested that cumulus cells are essential for sperm penetration and MPN formation (Kikuchi *et al.* 1993). More recently, studies have demonstrated that removal of cumulus cells before IVF does not reduce sperm penetration and can even increase penetration with high rates of polyspermy (reviewed by Dang-Nguyen *et al.* (2011)). The reason for these differences among studies is not clear. It is plausible that IVF conditions such as the medium and the origin of sperm used for IVF (such as fresh or frozen, ejaculated or epididymal) contribute to such differences among studies. Clarification of the importance of cumulus compartment for the regulation of penetration and monospermy rates during IVF is important for future improvements of the porcine IVP system.

1.2.2.5 Role of cyclic adenosine monophosphate

1.2.2.5.1 Role of cyclic adenosine monophosphate *in vivo* and *in vitro*

When facing the two main problems in porcine IVP, high polyspermy rates and low blastocyst rates, it could be questioned if an incomplete cytoplasmic maturation during porcine IVP could cause these problems. It is difficult to directly measure the level of cytoplasmic maturation (Day 2000) because it involves many biological processes. Moreover, measuring exactly those two indicators, fertilization parameters and the blastocyst production, are the most commonly used indirect measuring tools for evaluating the cytoplasmic maturation. In this way, changes in the IVM protocol potentially influencing cytoplasmic maturation can be implemented and evaluated by subsequent IVF and IVC.

Cyclic adenosine monophosphate (cAMP) exerts an important regulating role in the meiotic process of the oocyte. *In vivo*, the follicle is in control of meiotic arrest and resumption. High intracellular cAMP levels, produced by the oocyte itself mediated by signals from the follicle (mouse (Mehlmann *et al.* 2002)) and provided continuously through GJ contacts with cumulus and mural granulosa cells (Anderson and Albertini 1976) (Figure 3), maintain meiotic arrest in the oocyte as long as it is inside the follicle (Bornslaeger *et al.* 1986). Furthermore, degradation of cAMP by a type 3 phosphodiesterase (PDE3) of the oocyte is inhibited *via* the supply of cyclic guanosine monophosphate (cGMP) to the oocyte by the cumulus cells (reviewed by Gilchrist (2011)) (Figure 3).

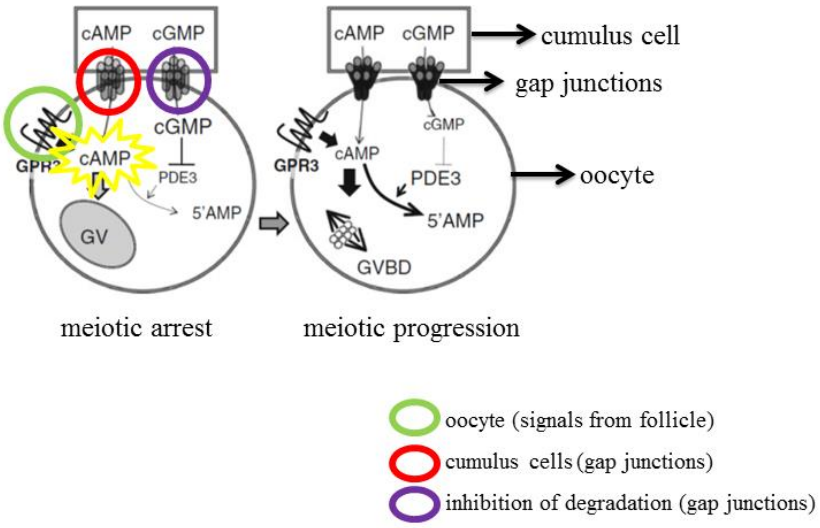


Figure 3: The oocyte-cumulus cell interactions. Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) produced by the cumulus cells are transported through gap junctions towards the oocyte. After the closure of gap junctional communication, the supply of cAMP and cGMP from the cumulus cells to the oocyte is interrupted. In this way, the inhibitory influence of cGMP on a type 3 phosphodiesterase (PDE3) is halted and cAMP is broken down with meiotic resumption as a result. GV: germinal vesicle; GVBD: germinal vesicle breakdown; GPR3: G-protein-coupled receptor 3; 5'AMP: 5'-adenosine monophoshate. Adapted from the review of Shimada (2012).

A transient increase in the intracellular concentration of cAMP after gonadotropin stimulation induced the oocytes to resume meiosis (Mattioli *et al.* 1994). The cAMP value afterwards decreased back to its basal levels at about the time of GVBD (Mattioli *et al.* 1994).

A different mechanism is observed *in vitro*. A problem is that cumulus cells, which are normally connected with the immature oocyte by cellular projections penetrating through the zona pellucida and with the oolemma *via* GJs, are prematurely losing these connections after the COC is being removed from the follicle and that, as a result, cAMP levels drop prematurely. Oocytes then undergo spontaneous maturation, which is characterized by meiotic resumption and progression to the metaphase II stage (nuclear maturation) resulting in oocytes with low developmental competence. One of the possible causes for this is an incomplete cytoplasmic maturation.

1.2.2.5.2 Cyclic adenosine monophosphate-modulating agents

Based on these principles, nuclear maturation, cytoplasmic maturation, the response during fertilization and developmental competence of the oocyte can be improved by adjusting cAMP levels during IVM. Several cAMP-modulating agents such as the PDE3 inhibitor 3-isobutyl-1-methylxanthine (IBMX; rat, (Tsafriri *et al.* 1996)), and the membrane-permeable cAMP analog, dibutyryl cAMP sodium salt (dbcAMP; pig, (Bagg *et al.* 2006)) have been used during IVM to increase cAMP levels, resulting in postponed meiosis, and consequently synchronize nuclear and cytoplasmic maturation of the oocytes (Figure 4).

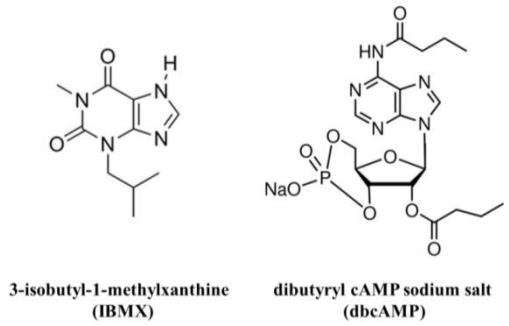


Figure 4: The structural formulae of the cyclic adenosine monophosphate (cAMP)-modulating agents applied in this study: 3-isobutyl-1-methylxanthine (IBMX) (Stemcell-Technologies 29 May 2015) and dibutyryl cAMP sodium salt (dbcAMP) (Sigma-Aldrich 29 May 2015).

Role of 3-isobutyl-1-methylxanthine

IBMX is a non-specific PDE inhibitor, which will prevent the *in vitro* drop in cAMP by inhibiting the increasing action of PDE3. This increased action of PDE3 is caused by the decreased supply of cGMP *via* the GJs because of the premature closure of the GJ communication between oocyte and cumulus cells. When using IBMX, it is important to consider two factors, which strongly determine if IBMX exerts an effect or not. A first factor is the time of IBMX addition, which can either be during oocyte collection, during pre-IVM or during IVM. A second factor is the time of evaluation of the effect of IBMX, which can be

during collection, during pre-IVM, during IVM or during IVC. Several studies investigated the effect of IBMX in combination with other cAMP-modulating agents in various species. Unfortunately, few studies have been conducted for testing the influence of IBMX solely. Bornslaeger *et al.* (1984) demonstrated that IBMX inhibited PDE activity and GVBD in mouse. In the simulated physiological oocyte maturation (SPOM) system (mouse and cattle) IBMX increased cAMP levels during pre-IVM in combination with the adenylate cyclase activator, forskolin, during pre-IVM (Albuz *et al.* 2010). The SPOM system improved COC functions during IVM and developmental programming of the oocyte, although this procedure included the addition of many more other cAMP-modulating agents (Albuz *et al.* 2010). Also in cattle, IBMX addition to the collection medium together with invasive adenylate cyclase was proven to increase blastocyst rate (Luciano *et al.* 1999). On the other hand, in pigs, Somfai *et al.* (2003) could not demonstrate an influence on nuclear progression during IVM and developmental competence using the same combination of IBMX and invasive adenylate cyclase in the collection medium, regardless of the usage of dbcAMP during IVM. On the contrary, addition of IBMX during porcine IVM reversibly blocked meiotic resumption (Laforest *et al.* 2005; Ozawa *et al.* 2008), prolonged the maintenance of GJ communication between the oocyte and the cumulus cells (Ozawa *et al.* 2008), and synchronized oocytes to the GV2 stage when matured with FSH for 20 h (Shimada *et al.* 2003).

Role of dibutyryl cAMP sodium salt

The influence of dibutyryl cAMP sodium salt on nuclear and cytoplasmic maturation

Next to IBMX, another option for modulating the cAMP levels in the oocyte is using the membrane-permeable cAMP analog, dbcAMP. It has been shown that the use of dbcAMP during porcine IVM is crucial to obtain a homogeneous and synchronized pool of oocytes and to improve the rate of early embryonic development to the blastocyst stage after IVF (Funahashi *et al.* 1997a). Furthermore, it is possible that modulation of cAMP levels during IVM could affect sperm penetration into the oocyte during IVF. Until now, it is not clear if the oocyte's ability to block polyspermy is affected by adding dbcAMP to the IVM medium.

Conflicting results are present in literature concerning the influence of dbcAMP on fertilization parameters. Funahashi *et al.* (1997a) could not find any significant difference in sperm penetration rate and monospermy rate between oocytes matured with or without 1.00 mM dbcAMP in the IVM medium. The used dbcAMP concentration was set by Funahashi *et al.* (1997a) according to the findings of Mattioli *et al.* (1994). Somfai *et al.* (2003) confirmed the fact that no significant difference was observed in penetration rate, but revealed that the monospermic fertilization rate significantly increased using 1.00 mM dbcAMP during the first 22 h of IVM. On the contrary, Kim *et al.* (2008) reported a higher penetration rate in the dbcAMP supplemented group but confirmed the lower polyspermy rate. The reason for these divergent results remains unclear.

The influence of dibutyryl cAMP sodium salt on cumulus expansion

Besides the involvement of cAMP levels in nuclear and cytoplasmic maturation of the oocyte, several findings point towards a crucial role in cumulus expansion. During IVM of porcine oocytes, some oocytes tend to adhere to the bottom of the dish. This phenomenon indicates inferior matrix disassembly (Beek *et al.* 2012). To explain this occurrence the composition and degradation of the extracellular matrix of the COC should be examined in more detail. Proteases are one of the products secreted by cumulus cells (Beek *et al.* 2012). Within the metalloproteases, a disintegrin and metalloproteinase with thrombospondin-like repeats-1 (ADAMTS-1), part of the ADAMTS family, plays a crucial role in the ovulation process (mouse (Russell *et al.* 2003)). In pigs, ADAMTS-1 secretion by cumulus cells is a prerequisite for the gonadotropin dependent cumulus expansion (Shimada *et al.* 2004). A disintegrin and metalloproteinase with thrombospondin-like repeats-1 is responsible for the cleavage of versican, a large HA binding proteoglycan, in the expanded COC matrix (mouse (Russell *et al.* 2003)) (Figure 5). As a result of this cleavage, cross-linking properties of versican alter (mouse (Russell *et al.* 2003)) and a stabilization of the expanding matrix is obtained by binding of the cleaved N-terminal domain of versican to HA (Shimada *et al.* 2004).

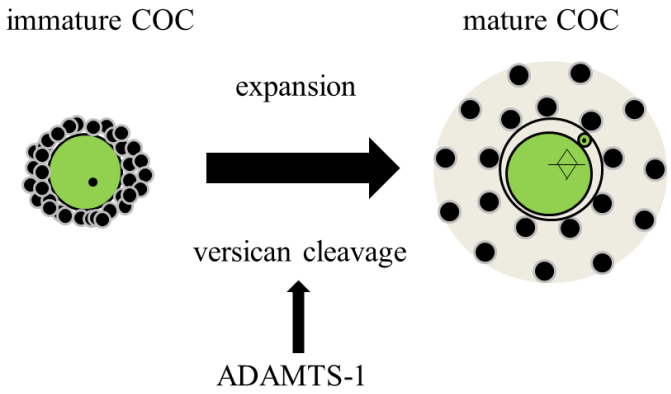


Figure 5: The relation between a disintegrin and metalloproteinase with thrombospondin-like repeats-1 (ADAMTS-1) and versican cleavage during cumulus expansion. Adapted from Curry (2010).

Shimada *et al.* (2004) discovered that inhibiting the function of ADAMTS-1 leads to adherent cumulus cells to the culture dish. Integrins on the surface of cells can interact with the C-terminal of versican and thus promote cell adhesion (Wu *et al.* 2002). *In vitro,* the removal of the C-terminus of versican is crucial in preventing adhesion of the COC to the bottom of the dish. It is known that cumulus expansion is crucial for an optimal nuclear and cytoplasmic maturation and subsequent fertilization of the oocyte (reviewed by Tanghe *et al.* (2002)) (Nagai *et al.* 1993). Hampered cumulus remodeling, followed by adhesion of the COC to the bottom of the dish, could possibly affect maturation, fertilization and developmental competence of porcine oocytes. It has been proven already that the nuclear and cytoplasmic maturation is affected in oocytes attached to the bottom of the culture dish (Somfai *et al.* 2004). Adherent COCs will resume meiosis and reach the MII stage earlier than floating ones surrounded by cumulus matrix, probably caused by the disability of the cumulus cells to maintain the meiotic arrest (Somfai *et al.* 2004). The exact mechanism of the prematurely nuclear maturation remains to be explained, but at the end of the maturation period those oocytes can manifest a certain degree of aging (Somfai *et al.* 2004). Aged oocytes are expected to show heterogeneity concerning cytoplasmic maturation (Somfai *et al.* 2003) and a higher polyspermy rate (Grupen *et al.* 1997).

A possible link between the level of intracellular cAMP and the function of ADAMTS-1 can be found in research about parathyroid hormone (PTH) influence in bone. Miles *et al.* (2000)

showed that in the rat femur metaphysis, ADAMTS-1 mRNA expression is only upregulated by those PTH analogs that are capable of significantly elevating intracellular cAMP levels. Although PTH influences many signal transduction pathways, Miles *et al.* (2000) suggested that the upregulation of ADAMTS-1 expression by PTH, is primarily mediated by the cAMP/protein kinase A (PKA) pathway. Experiments *in vitro* as well as *in vivo*, using forskolin, dbcAMP and agents that activate the cAMP/PKA pathway, revealed this important connection (Miles *et al.* 2000). Apart from the illustration of this connection in bone, little research was done to demonstrate the relationship between the cAMP/PKA signaling pathway and the expression of ADAMTS-1 in COCs. It is widely accepted that pituitary hormones such as LH are acting through the binding of ligand-specific cell surface G protein-coupled receptors, activation of adenylyl cyclase, and the subsequent production of cAMP (reviewed by Richards (2001)). Doyle *et al.* (2004) reported that the molecular mechanism, by which LH is inducing the expression of ADAMTS-1, operates through or independently from the progesterone receptor. Therefore, it is possible that the cAMP pathway affects the expression of ADAMTS-1 in granulosa cells in mice (Doyle *et al.* 2004). Moreover, in other species like human (Törnell and Hillensjö 1993) and rat (Dekel and Phillips 1980), dbcAMP addition inhibited monolayer formation and attachment to the bottom of the culture dish of cumulus cells (Figure 6). No literature reported on the relationship between ADAMTS-1 and cAMP levels in cumulus cells in pigs.

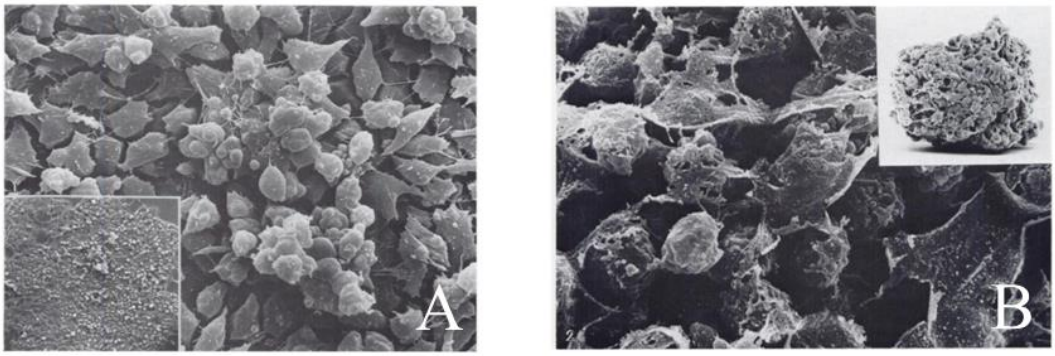


Figure 6: Scanning electron microscope pictures of rat cumulus cells incubated in medium without or with dibutyryl cAMP (dbcAMP) addition. Without dbcAMP addition cumulus cells form a monolayer (A). After dbcAMP addition cumulus cells did not attach to the culture dish and were embedded in matrix (B). Magnification (A) x 2000; overview x 150; (B) x 3000; overview x 400. Adapted from Dekel and Phillips (1980).

1.2.2.6 Role of oocyte-secreted factors

1.2.2.6.1 Nature of oocyte-secreted factors

A great deal of attention has been paid to the influence of the follicle on the oocyte, mostly by using follicular fluid addition for improving oocyte maturation conditions. Nowadays, the focus has shifted more towards the secretion of soluble paracrine growth factors by the oocyte itself for its further development (reviewed by Gilchrist (2011)). These oocyte-secreted factors (OSFs) are helping the oocyte to regulate its own microenvironment during porcine IVM since OSFs are regulating the surrounding cumulus cells, which in their turn will affect the development of the oocyte. This communication is a bidirectional regulatory loop between oocyte and cumulus cells (Figure 7).

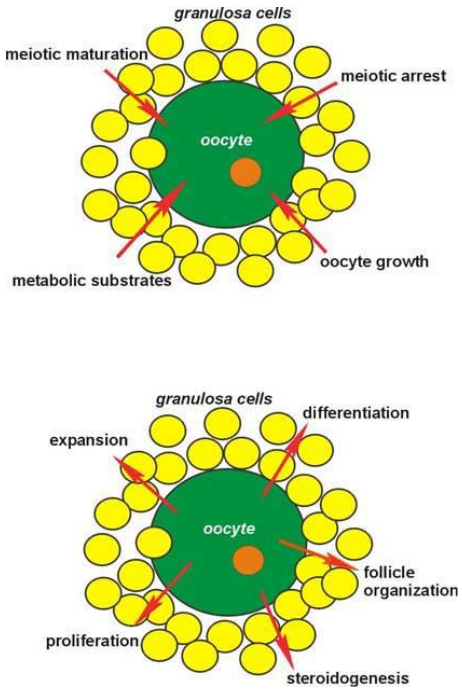


Figure 7: Bidirectional communication between oocyte and cumulus cells. The upper part represents the actions of the cumulus cells on the oocyte and the lower part the action of the oocyte on the cumulus cells. Adapted from Vanderhyden (2002).

Oocytes regulate cumulus cell differentiation *via* the secretion of soluble paracrine growth factors, which is called the OSF-regulation of the folliculogenesis (reviewed by Gilchrist *et al.*

(2008)). Studies in different mammalian species including mice, cattle and pigs, showed that OSFs not only affected cumulus expansion but also several other important parameters of cumulus cells such as cumulus cell apoptosis (cattle (Hussein *et al.* 2005)), luteinization markers (mouse (Eppig *et al.* 1997) and pig (Coskun *et al.* 1995)), cumulus glycolytic enzyme mRNA levels (mouse (Sugiura *et al.* 2005), and steroidogenesis (pig (Coskun *et al.* 1995)) (Figure 8). The importance of the secretion of soluble paracrine growth factors by the oocyte itself to create an own microenvironment determining its further development has only recently been acknowledged (reviewed by Gilchrist (2011)). Two factors of the transforming growth factor beta (TGF-β) superfamily, growth differentiation factor 9 (GDF9) (mouse (Dong *et al.* 1996)) and bone morphogenetic protein 15 (BMP15) (sheep (Galloway *et al.* 2000)) are considered to be the most crucial OSFs. Both factors are working through the BMP receptor type II (BMPRII) (Vitt *et al.* 2002) and downstream through activin receptor-like kinases (ALKs) followed by action on the SMA and MAD related intracellular proteins (SMAD) (Moore *et al.* 2002; Mazerbourg *et al.* 2004). More specifically, GDF9 is acting on ALK5 and SMAD2/3 (Mazerbourg *et al.* 2004) and BMP15 on ALK6 and SMAD1/5/8 (Moore *et al.* 2002) (Figure 8).

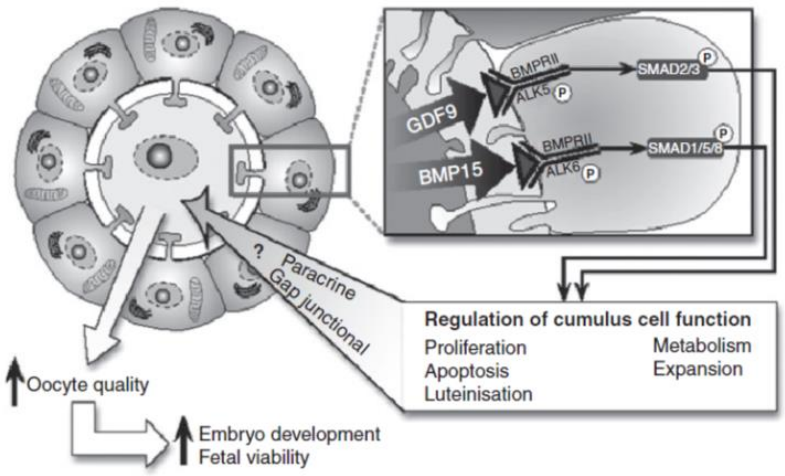


Figure 8: Bidirectional interaction between the oocyte and cumulus cells is crucial for the developmental competence of the oocyte. The oocyte regulates cumulus differentiation via *oocyte-secreted factors (growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15)). Cumulus cells in turn determine the quality of the oocyte* via *gap junctional communication and paracrine signals. BMPRII: BMP receptor type II; ALK: activin receptor-like kinase; SMAD: SMA and MAD related intracellular proteins. Adapted from the review of Gilchrist (2011).*

1.2.2.6.2 Experimental models

The OSF function has been investigated using different kinds of experimental models such as genetic models, immunization models, bioassays of native OSFs or bioassays using candidate recombinant OSFs (reviewed by Gilchrist *et al.* (2008)). All these approaches should be applied complementary to each other to get the full picture.

Studies on the basis of native OSFs can be performed in several ways (Figure 9). The source of OSFs can be the oocyte itself, after removal of the cumulus cells, called denuded oocyte addition, or the culture medium can be conditioned by denuded oocytes, the so called oocyte conditioned medium. Oocyte-secreted factors can be added to monolayers or clumps of granulosa cells or cumulus cells, oocytectomized complexes or intact COCs.

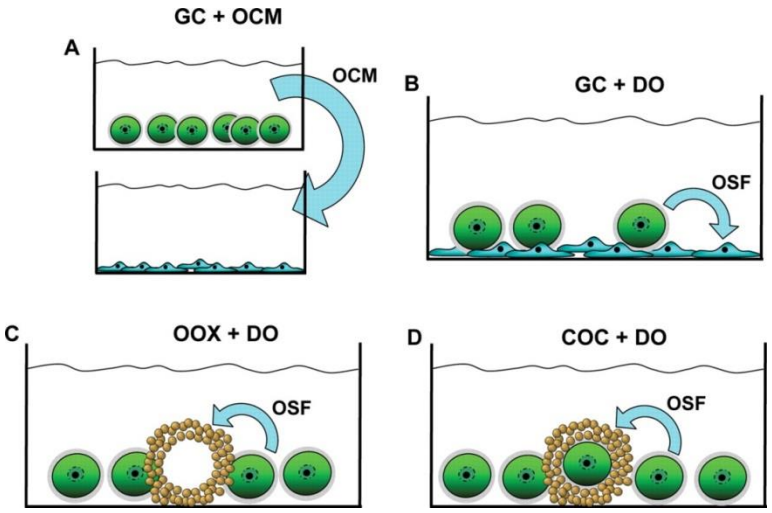


Figure 9: Illustration of the in vitro *models to study the influence of native oocyte-secreted factors (OSF). (A) oocyte conditioned medium (OCM) is used to examine the influence of OSFs on granulosa cells (GC). (B) addition of denuded oocytes (DO) themselves to a culture of GC. (C) denuded oocyte addition to oocytectomized complexes (OOX). (D) denuded oocyte addition to intact cumulus-oocyte complexes (COC). Adapted from the review of Gilchrist* et al. *(2008).*

Oocyte-secreted factors are presumed to be soluble paracrine factors because the denuded oocytes do not physically contact the influenced cell type and oocyte conditioned medium can

exert a biological response (reviewed by Gilchrist *et al.* (2008)). Moreover, OSFs seem to work in a concentration dependent manner: the strongest effect of OSFs on granulosa cells (rat (Lanuza *et al.* 1998)), cumulus cells (cattle (Hussein *et al.* 2005)) or co-cultured oocytes (pig (Gomez *et al.* 2012)) could be observed when more denuded oocytes or/and a lower culture volume were added.

1.2.2.6.3 The use of oocyte-secreted factors in *in vitro* embryo production

Native OSFs influence a numerous amount of cell functions in granulosa cells, cumulus cells and oocytes (reviewed by Gilchrist *et al*. (2008)). When specifically focusing on the improvement of existing IVP systems, the model of COCs with addition of denuded oocytes can give us valuable basic information. Oocyte-secreted factors will be released by denuded oocytes and in this way the addition of OSFs during IVM can be beneficial for oocyte developmental competence. If so, the nature of these OSFs can be verified by the use of the experimental model of recombinant OSFs. Although this very promising potential experimental plan, studies struggled with some practical obstacles. Bioassays with native OSFs were facing technical issues concerning the collection of the necessary amount of material to conduct large-scale co-culture trials, even when using microdrops (reviewed by Gilchrist *et al.* (2008) and reviewed by Gilchrist (2011)). When applying recombinant OSF bioassays, research is obstructed by the fact that commercially available purified GDF9 and BMP15 preparations are lacking their proregions (reviewed by Gilchrist (2011)), although research on and development of highly purified recombinant proteins including proregion-mature region interaction is ongoing (Mottershead *et al.* 2008; Li *et al.* 2009; Li *et al.* 2015).

In cattle, Hussein *et al*. (2006) proved that co-culturing of COCs with denuded oocytes or treatment with specific OSFs enhanced oocyte developmental competence. On the contrary, the role of OSFs in porcine cumulus expansion and oocyte developmental potential has not thoroughly been investigated yet. According to Prochazka *et al*. (1991) cumulus expansion was not dependent on the oocyte, although Singh *et al*. (1993) could observe that the oocyte is secreting a cumulus expansion-enabling factor(s) on the basis of an interspecies model. Little information was available about adding denuded oocytes or adding conditioned medium of

denuded oocytes to evaluate the influence of OSFs on intact COCs or oocytectomized complexes during porcine IVM. The only two sources of information on this topic for pigs were reports from Gomez *et al.* (2012) and Romaguera and Grupen (2009). Furthermore, these two studies contradicted each other. It was proven that denuded oocyte addition decreased cumulus expansion of COCs during IVM but improved developmental competence of the oocyte after parthenogenetic activation (Gomez *et al.* 2012). On the other hand, no effect of denuded oocyte addition during IVM on oocyte developmental competence could be observed after activation (Romaguera and Grupen 2009). Moreover, it is important to take into account that most of the previously mentioned studies in pigs are performed in non-defined culture media and cumulus expansion is scored in a subjective manner. To get a clear, unbiased picture of the action of OSFs, it would be recommended to perform these experiments in a defined medium, as done by Romaguera and Grupen (2009). In this way, the culture medium could afterwards be analyzed to identify the active substances. In addition, no information is obtained already about the response after IVF of porcine oocytes treated with OSFs (either through denuded oocyte addition or oocyte conditioned medium).

More knowledge about these oocyte-follicle cell interactions by the action of OSFs could provide opportunities for the development of new approaches to oocyte IVM systems, which can be applied for treatment of human infertility and for optimizing embryo production systems in domestic animals in research as well in practical conditions.

1.2.3 *In vitro* fertilization

In vitro fertilization can be carried out by the use of fresh or frozen-thawed (Figure 10) spermatozoa and of ejaculated or epididymal spermatozoa. When using ejaculates, large variations among boars and among fractions within the same ejaculate are reported (Xu *et al.* 1996a; Xu *et al.* 1996b). Moreover, there can be large differences between different ejaculates from the same boar, even when applying the same IVF protocol (Rath and Niemann 1997). The use of epididymal spermatozoa for IVF is superior over ejaculated semen because of several reasons. First, the motility after thawing is higher with subsequent improved IVF and cleavage rates (Rath and Niemann 1997). Second, an advantage of epididymal spermatozoa is

that they never have been in touch with seminal plasma (Rath and Niemann 1997). Seminal plasma contains decapacitation factors to prevent the acquirement of final fertilizing capacity (Rath and Niemann 1997). The seminal plasma acts in such a way that the sperm cells will only be activated when arriving at the oocyte after migration through the genital tract (Rath and Niemann 1997). During IVF however, the timing of fertilization is managed by the system itself and not by the aid of components such as seminal plasma (Rath and Niemann 1997). Because of the freezing process, the membrane stability of spermatozoa is reduced, which facilitates the acrosome reaction in the presence of COCs without special stimuli (Rath and Niemann 1997). These capacitation-like changes induced by freezing are known as the concept of cryocapacitation (Watson 1995; Peña *et al.* 2004). Certain freezing protocols allow to create 800-1000 spermatozoa doses for IVF from one single boar (Rath and Niemann 1997). In this way, the variable "spermatozoa dose" could be standardized, creating improved *in vitro* procedures to focus on the role of the female component in the IVF process (Rath and Niemann 1997). Freezing one single batch reduces the variability between trials, but the variability among boars is not solved by this technique (Abeydeera 2002). Normally, for each frozen sperm batch of a particular boar, the IVF protocol is adapted only once to determine its optimum IVF parameters (*i.e.* sperm concentration and IVF duration) in order to obtain the best results (Abeydeera 2002). Depending on the source of spermatozoa, the IVF protocol needs to be adapted. Yoshioka *et al.* (2003) described a chemically defined protein-free medium, porcine gamete medium (PGM), and an accompanying procedure to process spermatozoa for IVF of IVM oocytes.

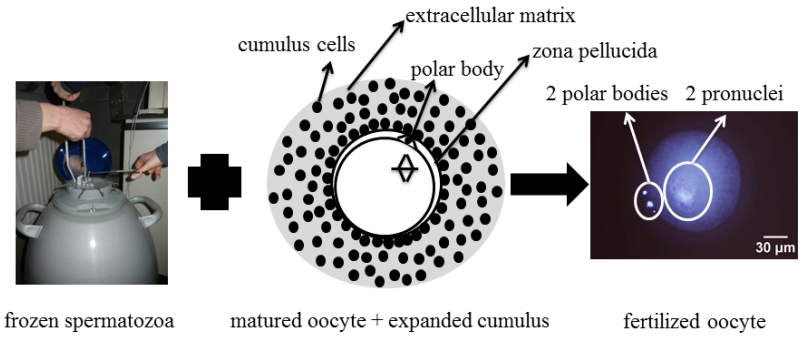


Figure 10: Illustration of in vitro *fertilization with frozen-thawed boar spermatozoa. The fertilized oocyte is stained by Hoechst 33342. Magnification x 400. Photographs and picture by R. Appeltant.*

Once the sperm nucleus has entered the oocyte, several changes should occur. When a matured MII stage oocyte is fertilized, the second polar body will be extruded (Kikuchi *et al.* 1999) and the oocyte will decondense the sperm nuclear chromatin, form a MPN and prevent more than one spermatozoon to enter the oocyte (Figure 10). In order to have the ability of MPN formation after fertilization, it is crucial for the oocyte to produce a sufficient amount of glutathione during oocyte maturation (Yoshida *et al.* 1993). Cysteamine is also reported to play an active role in the reduction of protamine disulfide bonds in the sperm nuclear chromatin which is a requirement for male nuclear formation (Grupen *et al.* 1995). How to prevent entering of more than one spermatozoon will be discussed under 1.3.2.

1.2.4 *In vitro* culture

During IVC, the porcine zygote proceeds through several developmental stages to finally reach the blastocyst stage (Figure 11). In this phase of development, medium composition seems to play a major role (Abeydeera 2002). Throughout history many different media have been designed to achieve the highest possible blastocyst rates. Nevertheless, until the early 1990s, research was confronted with the "four-cell block" when culturing porcine embryos. This block was overcome by the presence of a reduced NaCl concentration or the presence of organic osmolytes in the embryo culture medium (Day 2000). In 2002, Yoshioka *et al*. (2002) developed a chemically defined, protein-free porcine embryo culture medium, porcine zygote medium (PZM), which sustained blastocyst development and showed full term developmental potential after transfer to recipients. Porcine zygote medium was composed on the basis of concentrations of inorganic elements and energy substrates in the porcine oviduct (Yoshioka *et al.* 2002). In the latter study, it was proven that cultured porcine zygotes developed better in PZM than in NCSU 23 (Yoshioka *et al.* 2002). The formulation of NCSU 23 was based on the addition of glucose and a higher NaC1 concentration than in former used media such as Whitten's medium (Petters and Wells 1993).

The currently used IVM systems result in 20 to 30% of blastocysts starting from immature follicular oocytes (Gil *et al.* 2010).

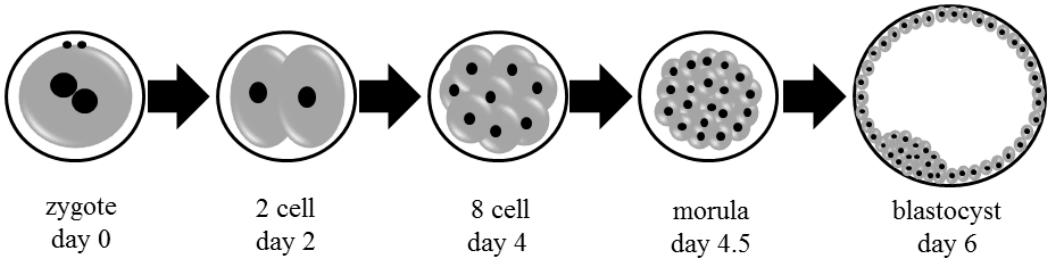


Figure 11: Developmental stages during in vitro *culture of porcine zygotes for 6 days in North Carolina State University 23 medium under 5% O_2 tension. Timing based on Mateusen* et al. *(2005) Pictures by R. Appeltant.*

1.2.5 Embryo transfer

Embryo transfer can happen in two kinds of ways, surgical or non-surgical. The surgical method is the first one used in many research programs (Day 2000). The embryo transfer method of Yoshioka *et al.* (2003) has been proven to be effective. Mainly practical aspects such as the need for surgical facilities and anesthesia are hampering its application into practice (Hazeleger and Kemp 1999). Moreover, the commercial pig sector does not really need extra offspring from a genetic point of view because of the high fecundity of pigs (Hazeleger and Kemp 1999). However, the interest in transferring genetic material of valuable sows and boars is growing worldwide, which necessitates the development of a technique to transfer porcine embryos (Hazeleger and Kemp 1999). Research has been performed on different aspects determining the success of the transfer: (1) the developmental stage of the embryos to be transferred (2) the synchronization of the recipients (3) the resistance during non-surgical insertion of the instrument (4) the hygiene during the transfer procedure, and (5) the location of the transfer (Hazeleger and Kemp 1999). Until recently, commercial utilization of embryo transfer in pigs remained very limited due to the lack of effective non-surgical methods (Martinez *et al.* 2014). However, in 2004, the birth of the first piglets was reported after non-surgical transfer of IVP blastocysts (Suzuki *et al.* 2004). In this study of Suzuki *et al.* (2004), a flexible catheter was used to transcervically transfer the IVP blastocysts into the uterine horn. One of the most recent achievements in the non-surgical transfer is the successful non-surgical deep intrauterine transfer of porcine morulae after 24 h culture in a

chemically defined medium (Martinez *et al.* 2014) resulting in high reproductive performance.

1.3 Current problems in porcine *in vitro* embryo production

1.3.1 Three barriers

Despite the major progress that has been achieved since the start of porcine IVP, the system is still confronted with many obstacles, knowledge gaps and challenges. Three major problems will be discussed: the high polyspermy rates, the low blastocyst developmental rates and low embryo quality, and the culture of low numbers of oocytes/embryos in an individually identifiable manner.

1.3.2 Polyspermy

Polyspermy, or the penetration of more than one spermatozoon (Figure 12), represents a major problem during porcine IVF (reviewed by Nagai *et al.* (2006)). The reason for the high incidence in porcine IVF systems remains to be explained. The porcine oocyte is the ideal model to examine because it is prone to polyspermy. The polyspermy rate in *in vivo* matured porcine oocytes is higher (28%, (Wang *et al.* 1998)) than in other species (11% in cattle, (Leibfried-Rutledge *et al.* 1987)), and after IVM, it even reaches 65% in pigs (Wang *et al.* 1998). This can either be caused by the presence of too many spermatozoa in the immediate vicinity of the oocyte (mouse (Fraser and Maudlin 1978)) and/or by the inability of the porcine COC to prevent penetration of several spermatozoa at the same time (Wang *et al.* 1998).

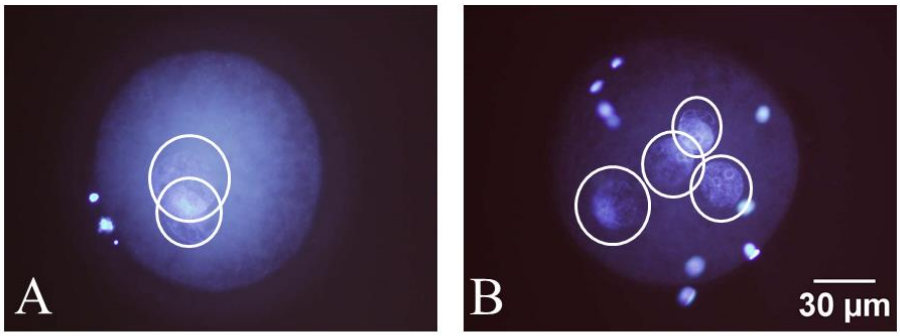


Figure 12: (A) Monospermy or the entry of one spermatozoon (B) polyspermy or the entrance of more than one spermatozoon in the porcine oocyte. A circle indicates a pronucleus. Specimen was stained with Hoechst 33342. Magnification x 400. Photographs by R. Appeltant.

Many processes are involved in blocking polyspermy after fertilization such as oolemma modifications after sperm-oocyte fusion (Sun 2003), cortical granule exocytosis in the perivitelline space (cortical reaction) followed by the zona reaction (Wang *et al.* 1998), and formation of a CG envelope (Sun 2003). Even factors from the oviduct could be necessary to assist in the completion of these cortical and zona reactions (Wang *et al.* 1998). Wang *et al.* (1998) showed that *in vitro* and *in vivo* matured porcine oocytes have the same ability to release CGs as a response on sperm penetration, but major morphologic differences were found in the cortex cytoplasm and the extracellular matrix between *in vitro* and *in vivo* matured oocytes. A possible influence of the oviduct environment *in vivo* on the extracellular matrix can be hypothesized as a defense mechanism against penetration of more than one spermatozoon (Wang *et al.* 1998). Moreover, scanning electron microscopy revealed that the morphology of the zona pellucida and the zona pellucida reaction at the time of sperm penetration greatly differs between ovulated and IVM oocytes (Funahashi *et al.* 2000). Transmission electron microscopy has been used to further elaborate on this aspect and disclosed the changes in the ultrastructure of cross-sectioned zonae pellucidae of IVM and ovulated oocytes before and after sperm penetration (Funahashi *et al.* 2001). It became obvious that the ultrastructure of the outer and inner porcine zonae pellucidae of *in vitro* and *in vivo* matured oocytes have different network organizations before as well as after the zona reaction (Funahashi *et al.* 2001). There is a possibility that the maturation of the zona

pellucida morphology is not completed because the cytoplasmic maturation, defined as developmental competence, is not fully achieved in all MII stage oocytes *in vitro* (Funahashi *et al.* 2000).

Polyspermic embryos can develop until the stage of blastocyst although the ICM cell number is lower compared to normal embryos (Han *et al.* 1999a). Although Han *et al*. (1999a) found that polyspermic embryos can develop to the blastocyst stage at the same rate as normally fertilized zygotes, Somfai *et al.* (2008) showed that the equal ability to develop to blastocyst only occurs when the polyspermic zygotes can achieve the first cleavage. Most polyspermic zygotes are known to form polyploidy embryos (Han *et al.* 1999b). No births of polyploid offspring in pigs have been reported, suggesting that polyploidy may cause embryo/fetal mortality in recipient gilts receiving morphologically normal IVP embryos (Day 2000). However, live piglets have been generated from polyspermic zygotes with diploid or mixoploid caryotypes, suggesting a repair mechanism of embryo ploidy during the development in some polyploid zygotes. (Han *et al.* 1999b).

1.3.3 Embryo production

Porcine embryo production *in vitro* is inferior as regards blastocyst production, both in terms of quantity as well as quality (Figure 13), compared to *in vivo* produced embryos (Nagai 2001). Although mostly the quality of the produced blastocysts is low in comparison to the *in vivo* counterparts, Kikuchi *et al*. (2002) reported high quality porcine blastocysts in a modified *in vitro* system. This success was probably due to the application of low oxygen concentration during IVM and IVF, the use of pyruvate and lactate supplements in the IVC medium during the first two days of IVC, and the use of culture media during the first two days of IVC that were conditioned by oviductal epithelial cells (Kikuchi *et al.* 2002). Quality of embryos is often judged by the count of total number of cells per blastocyst (Kikuchi *et al.* 2002) (Figure 13), but the evaluation of this parameter can be extended by a more advanced differential staining (Yoshioka *et al.* 2002). This differential staining can provide information about the proportion inner cell mass (ICM) cells and the proportion trophectoderm cells within a blastocyst (Yoshioka *et al.* 2002). This information can be useful for embryo transfer

because it is believed that the ratio ICM to total cells in the embryo is more crucial than just the count of the total amount of cells in the embryo (Yoshioka *et al.* 2002).

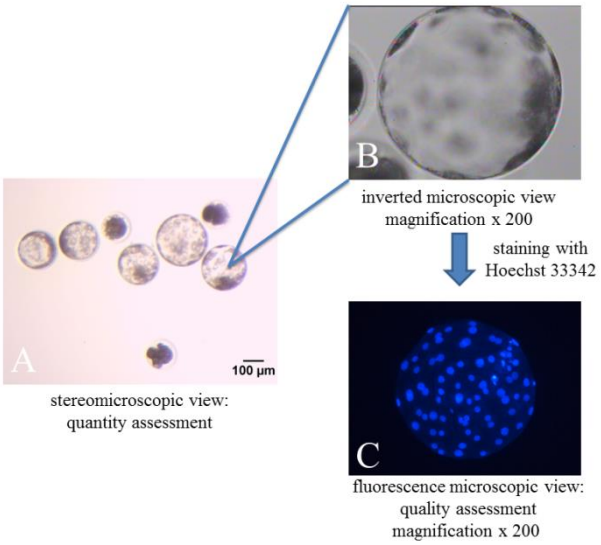


Figure 13: Quantity and quality assessment of blastocysts: (A) evaluation of the ratio of blastocysts under a stereomicroscope; (B) a more detailed view of a blastocyst under an inverted microscope; (C) evaluation of the total number of cells in a blastocyst visualized by a fluorescence microscope after staining with Hoechst 33342. Photographs by R. Appeltant.

The fact that the *in vitro* development of oocytes matured and fertilized *in vitro* was inferior to oocytes matured *in viv*o and fertilized *in vitro* (Yoshida *et al.* 1990) points towards the statement that during the process of IVP the phase of IVM needs improvement.

1.3.4 The culture of low numbers of oocytes in an individually identifiable manner

In general IVP procedures are making use of large group culture systems. However, in research, there is a demand for a system that provides the possibility to follow low numbers of oocytes from follicle to pre-implantation embryo. From this kind of research many relations between follicle parameters and oocyte developmental potential and even early markers for developmental competence (Matoba *et al.* 2010) could be extracted. From the practical point of view, rare pig breeds need adapted systems because of the small number of oocytes per sow obtained after ovum pick-up (OPU) (Brüssow *et al.* 1997) or collection after

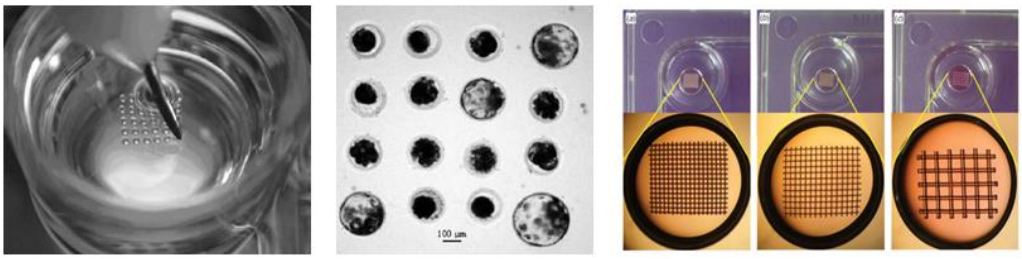
slaughtering. Techniques like cryopreservation and associated IVP systems of small numbers of oocytes can form the base to maintain genetic diversity, to exchange genetic material of endangered or valuable breeds between continents or breeding programs or to provide material for assisted reproduction (Somfai *et al.* 2014). This approach is not only useful for pigs or domestic animals, but is also of great value for comparable research in humans.

Individual embryo culture in microdrops of media has been studied already in species like mice (Paria and Dey 1990), humans (Rijnders and Jansen 1999) and cattle (O'Doherty *et al.* 1997; Goovaerts *et al.* 2012). The focus in these studies was mostly on embryo culture and not on the IVM and IVF phase. Nevertheless, especially in cattle, studies were performed to culture individual immature oocytes to the blastocyst stage (Carolan *et al.* 1996; Hagemann *et al.* 1998; Bunel *et al.* 2015). However, no similar studies on porcine oocytes have been performed to date.

It is important to point out that individual tracking should not be confused with individual culture of oocytes.

The possibility of culturing low numbers of oocytes in groups creates new opportunities such as individual tracking of oocytes and embryos within a small culture group throughout the complete IVP by preventing their movement in the culture dish. This allows the identification of single oocytes while they are exposed to the influence of other accompanying oocytes and therefore they are not considered as systems sustaining individual culture of oocytes. Individual tracking of embryos has already been performed in pigs (Stokes *et al.* 2005; Du *et al.* 2008) and in cattle (Vajta *et al.* 2000; Gopichandran and Leese 2006) and in cattle the all-round tracking of individual oocytes during IVM, IVF and IVC has also been reported (Matoba *et al.* 2010). These techniques open doors towards research on individual characteristics of oocytes while the disadvantages of real individual culture are avoided because the oocyte is still benefitting from the presence of other companion oocytes (so called "apart together" culture systems (Goovaerts *et al.* 2010)). Nevertheless, to date, only a few studies paid attention to the study of the effects of culturing small groups of oocytes as an intermediate situation between large group cultures and individual culture.

Individual tracking of oocytes can be achieved in various ways (Figure 14).



(A) WOW (B) Cell-Tak (C) monofilament meshes

Figure 14: (A) Illustration of the preparation of a Well-of-the-Well (WOW) system. Microwells are made in a four-well dish by the mechanical force of a needle. Adapted from Vajta et al. *(2008). (B) Picture of porcine embryos after 6 days of* in vitro *culture attached to the bottom of the dish by the use of Cell-Tak. Adapted from Stokes* et al. *(2005). (C) Illustration of monofilament meshes small mesh; medium mesh; large mesh. Adapted from Matoba* et al. *(2010).*

One approach is to use the Well-of-the-Well (WOW) system where oocytes or embryos are placed in the bottom of microwells of the plastic culture dish and cultured within a common drop of medium (Vajta *et al.* 2000) (Figure 14 A). Specifically for the WOW system, the time needed to prepare the culture dishes hampers fast working (Matoba *et al.* 2010). A second possible way to identify individual oocytes is to fix them to the bottom of the culture dish by the use of a non-toxic glue such as Cell Tak™, Cell and Tissue Adhesive (Cell-Tak). Cell-Tak is a formulation of polyphenolic proteins extracted from the marine mussel *Mytilus edulis* 1.33 mg/mL in 5% acetic acid. It is known that mussel adhesive proteins can safely be used since they are non-toxic and do not impose immunogenicity (Hwang *et al.* 2004) (BD Cell Tak™, Material Safety Data Sheet). This adhesive coating method for slides or dishes was used already to investigate embryo development in pigs (Stokes *et al.* 2005) (Figure 14 B) and individual oocyte culture until blastocyst stage in cattle (Matoba *et al.* 2010). By the use of Cell-Tak, Stokes *et al.* (2005) determined that the distance between culturing porcine embryos should be between 81 and 160 µm. In this way, embryos could be followed individually. Moreover, if adhered close enough, they could still benefit from diffusible factors released by the neighboring embryos (Stokes *et al.* 2005). Another advantage of this approach is that it does not cause a major change in the optical characteristics of the dish around the embryos or oocytes, which makes it useful when oocytes and embryos are analyzed by photography or

time lapse cinematography. A third option to follow the individual oocytes is to culture them in a monofilament mesh (Booth *et al.* 2007) (Figure 14 C). This technique is superior over the two previous described ones when it comes to preparation time.

The disadvantage of treating the oocytes in such an individual-kind of manner is the significant increase in the time necessary for manipulations of the oocytes during the IVP procedure (Matoba *et al.* 2010) in comparison with manipulations of group culture. This extra time needed can lead to lower output. Despite this disadvantage, however, this way of working is providing double benefit of group culture and of being able to isolate and identify oocytes during the whole IVP period (Matoba *et al.* 2010).

1.4 References

Abeydeera, L.R. (2002) In vitro production of embryos in swine. *Theriogenology* **57**, 257-273

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Prather, R.S., and Day, B.N. (1998a) Presence of β-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization. *Theriogenology* **50**, 747-756

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Rieke, A., and Murphy, C.N. (2000) Development and viability of pig oocytes matured in a protein-free medium containing epidermal growth factor. *Theriogenology* **54**, 787-797

Abeydeera, L.R., Wang, W.H., Prather, R.S., and Day, B.N. (1998b) Maturation in vitro of pig oocytes in protein-free culture media: fertilization and subsequent embryo development in vitro. *Biol. Reprod.* **58**, 1316-1320

Akaki, Y., Yoshioka, K., Noguchi, M., Hoshi, H., and Funahashi, H. (2009) Successful piglet production in a chemically defined system for in-vitro production of porcine embryos: dibutyryl cyclic amp and epidermal growth factor-family peptides support in-vitro maturation of oocytes in the absence of gonadotropins. *J. Reprod. Dev.* **55**(4), 446-453

Albuz, F.K., Sasseville, M., Lane, M., Armstrong, D.T., Thompson, J.G., and Gilchrist, R.B. (2010) Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Hum. Reprod.* **25**(12), 2999-3011

Anderson, E., and Albertini, D.F. (1976) Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *J. Cell Biol.* **71**(2), 680-686

Andrabi, S.M.H., and Maxwell, W.M.C. (2007) A review on reproductive biotechnologies for conservation of endangered mammalian species. *Anim. Reprod. Sci.* **99**(3-4), 223-243

Bagg, M.A., Nottle, M.B., Grupen, C.G., and Armstrong, D.T. (2006) Effect of dibutyryl cAMP on the cAMP content, meiotic progression, and developmental potential of in vitro matured pre-pubertal and adult pig oocytes. *Mol. Reprod. Dev.* **73**(10), 1326-1332

Ball, G.D., Bellin, M.E., Ax, R.L., and First, N.L. (1982) Glycosaminoglycans in bovine cumulus-oocyte complexes: morphology and chemistry. *Mol. Cell. Endocrinol.* **28**, 113-122

Beek, J., Nauwynck, H., Maes, D., and Van Soom, A. (2012) Inhibitors of zinc-dependent metalloproteases hinder sperm passage through the cumulus oophorus during porcine fertilization in vitro. *Reproduction* **144**(6), 687-697

Bijttebier, J., Van Soom, A., Meyer, E., Mateusen, B., and Maes, D. (2008) Preovulatory follicular fluid during in vitro maturation decreases polyspermic fertilization of cumulus-intact porcine oocytes. *Theriogenology* **70**(4), 715-724

Booth, P.J., Watson, T.J., and Leese, H.J. (2007) Prediction of porcine blastocyst formation using morphological, kinetic, and amino acid depletion and appearance criteria determined during the early cleavage of in vitro-produced embryos. *Biol. Reprod.* **77**(5), 765-779

Bornslaeger, E., Mattei, P., and Schultz, R. (1986) Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. *Dev. Biol.* **114**(2), 453-462

Bornslaeger, E., Wilde, M.W., and Schultz, R. (1984) Regulation of mouse oocyte maturation: involvement of cyclic AMP phosphodiesterase and calmodulin. *Dev. Biol.* **105**(2), 488-499

Brüssow, K.P., Torner, H., Rátky, J., Hunter, M., and G, N. (1997) Ovum pick up in swine: the influence of aspiration vacuum pressure on oocyte recovery from preovulatory follicles. *Acta Vet. Hung.* **45**, 189-196

Buccione, R., Schroeder, A., and Eppig, J. (1990) Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol. Reprod.* **41**(1), 176

Bunel, A., Jorssen, E.P., Merckx, E., Leroy, J.L., Bols, P.E., and Sirard, M.A. (2015) Individual bovine in vitro embryo production and cumulus cell transcriptomic analysis to distinguish cumulus-oocyte complexes with high or low developmental potential. *Theriogenology* **83**(2), 228-237

Carolan, C., Lonergan, P., Khatir, H., and Mermillod, P. (1996) In vitro production of bovine embryos using individual oocytes. *Mol. Reprod. Dev.* **45**, 145-150

Chian, R., and Niwa, K. (1994) Effect of cumulus cells present during different periods of culture on maturation in vitro of bovine oocytes. *Theriogenology* **41**(1), 176

Coskun, S., Uzumcu, M., Lin, Y., Friedman, C., and Alak, B. (1995) Regulation of cumulus cell steroidogenesis by the porcine oocyte and preliminary characterization of oocyte-produced factor(s). *Biol. Reprod.* **53**(3), 670-675

Curry, T.E. (2010) ADAMTS1 and versican: partners in ovulation and fertilization. *Biol. Reprod.* **83**(4), 505-506

Dang-Nguyen, T.Q., Somfai, T., Haraguchi, S., Kikuchi, K., Tajima, A., Kanai, Y., and Nagai, T. (2011) In vitro production of porcine embryos: current status, future perspectives and alternative applications. *Animal Science Journal* **82**(3), 374-382

Day, B.N. (2000) Reproductive biotechnologies: current status in porcine reproduction. *Anim. Reprod. Sci.* **60-61**, 161-172

de Jong, E., Appeltant, R., Cools, A., Beek, J., Boyen, F., Chiers, K., and Maes, D. (2014) Slaughterhouse examination of culled sows in commercial pig herds. *Livestock Science* **167**, 362-369

De Loos, F., Kastrop, P., Van Maurik, P., Van Beneden, T.H., and Kruip, T.A. (1991) Heterologous cell contacts and metabolic coupling in bovine cumulus oocyte complexes. *Mol. Reprod. Dev.* **28**(3), 255-259

Dekel, N., and Phillips, D.M. (1980) Cyclic AMP, prostaglandin E2 and steroids: possible mediators in the rat cumulus oophorus mucification. *Biol. Reprod.* **22**, 289-296

Devaux, A., Soula, V., Sifer, C., Branger, M., Naouri, M., Porcher, R., Poncelet, C., Neuraz, A., Alvarez, S., Benifla, J.L., Madelenat, P., Brun-Vezinet, F., and Feldmann, G. (2003) Hepatitis C virus detection in follicular fuid and culture media from HCV+ women, and viral risk during IVF procedures. *Hum. Reprod.* **18**(11), 2342-2349

Ding, J., and Foxcroft, G.R. (1994) Epidermal growth factor enhances oocyte maturation in pigs. *Mol. Reprod. Dev.* **39**, 30-40

Dong, J., Albertini, D.F., Nishimori, K., Kumar, T.R., Lu, N., and Matzuk, M.M. (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383**, 531-535

Doyle, K.M.H., Russell, D.L., Sriraman, V., and Richards, J.S. (2004) Coordinate transcription of the ADAMTS-1 gene by luteinizing hormone and progesterone receptor. *Mol. Endocrinol.* **18**(10), 2463-2478

Du, Y., Lin, L., Schmidt, M., Bøgh, I.B., Kragh, P.M., Sørensen, C.B., Li, J., Purup, S., Pribenszky, C., Molnár, M., Kuwayama, M., Zhang, X., Yang, H., Bolund, L., and Vajta, G. (2008) High hydrostatic pressure treatment of porcine oocytes before handmade cloning

improves developmental competence and cryosurvival. *Cloning and Stem Cells* **10**(3), 325-330

Edwards, L.J., Williams, D.A., and Gardner, D.K. (1998) Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum. Reprod.* **13**(12), 3441-3448

Edwards, R.G. (1965) Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature* **208**(5008), 349-351

Eppig, J.J. (1982) The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. *Dev. Biol.* **89**, 268-272

Eppig, J.J. (1996) Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. *Reprod. Fertil. Dev.* **8**, 485-489

Eppig, J.J., Wigglesworth, K., pendola, F., and Hirao, Y. (1997) Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biol. Reprod.* **56**, 976-984

Fraser, L., and Maudlin, I. (1978) Relationship between sperm concentration and the incidence of polyspermy in mouse embryos fertilized in vitro. *J. Reprod. Fertil.* **52**(1), 103-106

Funahashi, H., Cantley, T., and Day, B. (1997a) Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. *Biol. Reprod.* **57**(1), 49-53

Funahashi, H., Cantley, T.C., and Day, B.N. (1997b) Preincubation of cumulus-oocyte complexes before exposure to gonadotropins improves the developmental competence of porcine embryos matured and fertilized in vitro. *Theriogenology* **47**, 679-686

Funahashi, H., and Day, B.N. (1993) Effects of the duration of exposure to hormone supplements on cytoplasmic maturation of pig oocytes in vitro. *J. Reprod. Fertil.* **98**, 179-185

Funahashi, H., and Day, B.N. (1997) Advances in in vitro production of pig embryos. *J. Reprod. Fertil. Suppl.* **52**, 271-283

Funahashi, H., Ekwall, H., Kikuchi, K., and Rodriguez-Martinez, H. (2001) Transmission electron microscopy studies of the zona reaction in pig oocytes fertilized in vivo and in vitro. *Reproduction* **122**, 443-452

Funahashi, H., Ekwall, H., and Rodriguez-Martinez, H. (2000) Zona reaction in porcine oocytes fertilized in vivo and in vitro as seen with scanning electron microscopy. *Biol. Reprod.* **63**, 1437-1442

Galik, P.K., Givens, M.D., Stringfellow, D.A., Crichton, E.G., Bishop, M.D., and Eilertsen, K.J. (2002) Bovine, viral diarrhea virus (BVDV) and anti-BVDV antibodies in pooled samples of follicular fluid. *Theriogenology* **57**, 1219-1227

Galloway, S.M., McNatty, K.P., Cambridge, L.M., Laitinen, M.P.E., Juengel, J.L., Jokiranta, T.S., McLaren, R.J., Luiro, K., Dodds, K.G., Montgomery, G.W., Beattie, A.E., Davis, G.H., and Ritvos, O. (2000) Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat. Genet.* **25**(3), 279-283

Gandhi, A.P., Lane, M., Gardner, D.K., and Krisher, R.L. (2000) A single medium supports development of bovine embryos throughout maturation, fertilization and culture. *Hum. Reprod.* **15**(2), 395-401

Gil, M.A., Cuello, C., Parrilla, I., Vazquez, J.M., Roca, J., and Martinez, E.A. (2010) Advances in swine in vitro embryo production technologies. *Reprod. Domest. Anim.* **45**, 40-48

Gilchrist, R. (2011) Recent insights into oocyte –follicle interactions provide opportunities for the development of new approaches to in vitro maturation. *Reprod. Fertil. Dev.* **23**(1), 23-31

Gilchrist, R.B., Lane, M., and Thompson, J.G. (2008) Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum. Reprod. Update* **14**(2), 159-177

Gomez, M.N.L., Kang, J.T., Koo, O.J., Kim, S.J., Kwon, D.K., Park, S.J., Atikuzzaman, M., Hong, S.G., Jang, G., and Lee, B.C. (2012) Effect of oocyte-secreted factors on porcine in vitro maturation, cumulus expansion and developmental competence of parthenotes. *Zygote* **20**(02), 135-145

Goovaerts, I.G.F., Leroy, J.L.M.R., Jorssen, E.P.A., and Bols, P.E.J. (2010) Noninvasive bovine oocyte quality assessment: possibilities of a single oocyte culture. *Theriogenology* **74**(9), 1509-1520

Goovaerts, I.G.F., Leroy, J.L.M.R., Langbeen, A., Jorssen, E.P.A., Bosmans, E., and Bols, P.E.J. (2012) Unravelling the needs of singly in vitro-produced bovine embryos: from cumulus cell co-culture to semi-defined, oil-free culture conditions. *Reproduction, Fertility and Development* **24**(8), 1084

Gopichandran, N., and Leese, H.J. (2006) The effect of paracrine/autocrine interactions on the in vitro culture of bovine preimplantation embryos. *Reproduction* **131**(2), 269-277

Grupen, C.G., and Armstrong, D.T. (2010) Relationship between cumulus cell apoptosis, progesterone production and porcine oocyte developmental competence: temporal effects of follicular fluid during IVM. *Reprod. Fertil. Dev.* **22**, 1100-1109

Grupen, C.G., McIlfatrick, S.M., Ashman, R.J., Boquest, A.C., Armstrong, D.T., and Nottle, M.B. (2003) Relationship between donor animal age, follicular fluid steroid content and oocyte developmental competence in the pig. *Reprod. Fertil. Dev.* **15**, 81-87

Grupen, C.G., Nagashima, H., and Nottle, M.B. (1995) Cysteamine enhances in vitro development of porcine oocytes matured and fertilized in vitro. *Biol. Reprod.* **53**, 173-178

Grupen, C.G., Nagashima, H., and Nottle, M.B. (1997) Asynchronous meiotic progression in porcine oocytes matured in vitro: a cause of polyspermic fertilization? *Reprod. Fertil. Dev.* **9**(2), 187-191

Hagemann, L.J., Weilert, L.L., Beaumont, S.E., and Tervit, H.R. (1998) Development of bovine embryos in single in vitro production (sIVP) systems. *Mol. Reprod. Dev.* **51**, 143-147

Han, Y., Abeydeera, L., Kim, J., Moon, H., Cabot, R., Day, B., and Prather, R. (1999a) Growth retardation of inner cell mass cells in polyspermic porcine embryos produced in vitro. *Biol. Reprod.* **60**(5), 1110-1113

Han, Y.M., Wang, W.H., Abeydeera, L.R., Petersen, A., Kim, J.H., Murphy, C., Day, B.N., and Prather, R.S. (1999b) Pronuclear location before the first cell division determines ploidy of polyspermic pig embryos. *Biol. Reprod.* **61**, 1340-1346

Hazeleger, W., and Kemp, B. (1999) State of the art in pig embryo transfer. *Theriogenology* **51**, 81-90

Hong, J.Y., Yong, H.Y., Lee, B.C., Hwang, W.S., Lim, J.M., and Lee, E.S. (2004) Effects of amino acids on maturation, fertilization and embryo development of pig follicular oocytes in two IVM media. *Theriogenology* **62**(8), 1473-1482

Hunter, M.G. (2000) Oocyte maturation and ovum quality in pigs. *Rev. Reprod.* **5**, 122-130

Hussein, T.S., Froiland, D.A., Amato, F., Thompson, J.G., and Gilchrist, R.B. (2005) Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. *J. Cell Sci.* **118**(22), 5257-5268

Hussein, T.S., Thompson, J.G., and Gilchrist, R.B. (2006) Oocyte-secreted factors enhance oocyte developmental competence. *Dev. Biol.* **296**(2), 514-521

Hwang, D.S., Yoo, H.J., Jun, J.H., Moon, W.K., and Cha, H.J. (2004) Expression of functional recombinant mussel adhesive protein Mgfp-5 in Escherichia coli. *Appl. Environ. Microbiol.* **70**(6), 3352-3359

Iritani, A., Niwa, K., and Imai, H. (1978) Sperm penetration in vitro of pig follicular oocytes matured in culture. *J. Reprod. Fertil.* **54**, 379-383

Ishii, T., Hishinuma, I., Bannai, S., and Sugita, Y. (1981) Mechanism of growth promotion of mouse lymphoma L1210 cells in vitro by feeder layer or 2-mercaptoethanol. *J. Cell. Physiol.* **107**(2), 283-293

Isobe, N., and Terada, T. (2001) Effect of the factor inhibiting germinal vesicle breakdown on the disruption of gap junctions and cumulus expansion of pig cumulus-oocyte complexes cultured in vitro. *Reproduction* **121**, 249-257

Iwamoto, M., Onishi, A., Fuchimoto, D.-i., Somfai, T., Takeda, K., Tagami, T., Hanada, H., Noguchi, J., Kaneko, H., Nagai, T., and Kikuchi, K. (2005) Low oxygen tension during in vitro maturation of porcine follicular oocytes improves parthenogenetic activation and subsequent development to the blastocyst stage. *Theriogenology* **63**(5), 1277-1289

Kamarianos, A., Karamanlis, X., Goulas, P., Theodosiadou, E., and Smokovitis, A. (2003) The presence of environmental pollutants in the follicular fluid of farm animals (cattle, sheep, goats, and pigs). *Reprod. Toxicol.* **17**(2), 185-190

Kikuchi, K., Nagai, T., Ding, J., Yamauchi, J., Noguchi, J., and Izaike, Y. (1999) Cytoplasmic maturation for activation of pig follicular oocytes cultured and arrested at metaphase I. *J. Reprod. Fertil.* **116**, 143-156

Kikuchi, K., Nagai, T., Motlik, J., Shioya, Y., and Izaike, Y. (1993) Effect of follicle cells on in vitro fertilization of pig follicular oocytes. *Theriogenology* **39**, 596-599

Kikuchi, K., Onishi, A., Kashiwazaki, N., Iwamoto, M., Noguchi, J., Kaneko, H., Akita, T., and Nagai, T. (2002) Succesful piglet production after transfer of blastocysts produced by a modified in vitro system. *Biol. Reprod.* **66**, 1033-1041

Kim, J.S., Cho, Y.S., Song, B.S., Wee, G., Park, J.S., Choo, Y.K., Yu, K., Lee, K.K., Han, Y.M., and Koo, D.B. (2008) Exogenous dibutyryl cAMP affects meiotic maturation via protein kinase A activation; it stimulates further embryonic development including blastocyst quality in pigs. *Theriogenology* **69**(3), 290-301

Laforest, M.F., Pouliot, E., Guéguen, L., and Richard, F.J. (2005) Fundamental significance of specific phosphodiesterases in the control of spontaneous meiotic resumption in porcine oocytes. *Mol. Reprod. Dev.* **70**(3), 361-372

Lanuza, G.M., Fischman, M.L., and Barañao, J.L. (1998) Growth promoting activity of oocytes on granulosa cells is decreased upon meiotic maturation. *Dev. Biol.* **197**, 129-139

Leibfried-Rutledge, M., Critser, E., Eyestone, W., Northey, D., and First, N. (1987) Development potential of bovine oocytes matured in vitro or in vivo. *Biol. Reprod.* **36**(2), 376-383

Li, J.-J., Sugimura, S., Mueller, T.D., White, M.A., Martin, G.A., Ritter, L.J., Liang, X.-Y., Gilchrist, R.B., and Mottershead, D.G. (2015) Modifications of human growth differentiation factor 9 to improve the generation of embryos from low competence oocytes. *Mol. Endocrinol.* **29**(1), 40-52

Li, Q., Rajanahally, S., Edson, M.A., and Matzuk, M.M. (2009) Stable expression and characterization of N-terminal tagged recombinant human bone morphogenetic protein 15. *Mol. Hum. Reprod.* **15**(12), 779-788

Luciano, A.M., Pocar, P., Milanesi, E., Modina, S., Rieger, D., Lauria, A., and Gandolfi, F. (1999) Effect of different levels of intracellular cAMP on the in vitro maturation of cattle oocytes and their subsequent development following in vitro fertilization. *Mol. Reprod. Dev.* **54**(1), 86-91

Luvoni, G.C. (2000) Current progress on assisted reproduction in dogs and cats: in vitro embryo production. *Reprod. Nutr. Dev.* **40**, 505-512

Maedomari, N., Kikuchi, K., Ozawa, M., Noguchi, J., Kaneko, H., Ohnuma, K., Nakai, M., Shino, M., Nagai, T., and Kashiwazaki, N. (2007) Cytoplasmic glutathione regulated by cumulus cells during porcine oocyte maturation affects fertilization and embryonic development in vitro. *Theriogenology* **67**(5), 983-993

Mahi-Brown, C.A., and Yanagimachi, R. (1983) Parameters influencing ovum pickup by oviductal fimbria in the golden hamster. *Gamete Res.* **8**, 1-10

Marchal, R., Feugang, J.M., Perreau, C., Venturi, E., Terqui, M., and Mermillod, P. (2001) Meiotic and developmental competence of prepubertal and adult swine oocytes. *Theriogenology* **56**, 17-29

Martinez, E.A., Angel, M.A., Cuello, C., Sanchez-Osorio, J., Gomis, J., Parrilla, I., Vila, J., Colina, I., Diaz, M., Reixach, J., Vazquez, J.L., Vazquez, J.M., Roca, J., and Gil, M.A. (2014) Successful non-surgical deep uterine transfer of porcine morulae after 24 hour culture in a chemically defined medium. *PLoS ONE* **9**(8), e104696

Mateusen, B., Van Soom, A., Maes, D.G.D., Donnay, I., Duchateau, L., and Lequarre, A.S. (2005) Porcine embryo development and fragmentation and their relation to apoptotic markers: a cinematographic and confocal laser scanning microscopic study. *Reproduction* **129**(4), 443-452

Matoba, S., Fair, T., and Lonergan, P. (2010) Maturation, fertilisation and culture of bovine oocytes and embryos in an individually identifiable manner: a tool for studying oocyte developmental competence. *Reprod. Fertil. Dev.* **22**(5), 839-851

Mattioli, M., Bacci, M.L., Galeati, G., and Seren, E. (1989) Developmental competence of pig oocytes matured and fertilized in vitro. *Theriogenology* **31**(6), 1201-1207

Mattioli, M., Galeati, G., Barboni, B., and Seren, E. (1994) Concentration of cyclic AMP during the maturation of pig oocytes in vivo and in vitro. *J. Reprod. Fertil.* **100**(2), 403-409

Mazerbourg, S., Klein, C., Roh, J., Kaivo-Oja, N., Mottershead, D.G., Korchynskyi, O., Ritvos, O., and Hsueh, A.J.W. (2004) Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. *Mol. Endocrinol.* **18**(3), 653-665

Mehlmann, L.M., Jones, T.L.Z., and Jaffe, L.A. (2002) Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte. *Science* **297**(5585), 1343-1345

Meister, A. (1983) Selective modification of glutathione metabolism. *Science* **220**(4596), 472-477

Miles, R., Sluka, J., Halladay, D., Santerre, R., Hale, L., Bloem, L., Thirunavukkarasu, K., Galvin, R., Hock, J., and Onyia, J. (2000) ADAMTS-1: a cellular disintegrin and metalloprotease with thrombospondin motifs is a target for parathyroid hormone in bone. *Endocrinology* **141**(12), 4533-4542

Mohindru, A., Fisher, J.M., and Rabinovitz, M. (1985) Endogenous copper is cytotoxic to a lymphoma in primary culture which requires thiols for growth. *Experientia* **41**(8), 1064-1066

Moore, R.K., Otsuka, F., and Shimasaki, S. (2002) Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. *J. Biol. Chem.* **278**(1), 304-310

Mori, T., Amano, T., and Shimizu, H. (2000) Roles of gap junctional communication of cumulus cells in cytoplasmic maturation of porcine oocytes cultured in vitro. *Biol. Reprod.* **62**(4), 913-919

Motlik, J., and Fulka, J. (1974) Fertilization of pig follicular oocytes cultivated in vitro. *J. Reprod. Fertil.* **36**, 235-237

Motlik, J., and Fulka, J. (1976) Breakdown of the germinal vesicle in pig oocytes in vivo and in vitro. *J. Exp. Zool.* **198**(2), 155-162

Motlik, J., and Fulka, J. (1986) Factors affecting meiotic competence in pig oocytes. *Theriogenology* **25**(1), 87-96

Mottershead, D.G., Pulkki, M.M., Muggalla, P., Pasternack, A., Tolonen, M., Myllymaa, S., Korchynskyi, O., Nishi, Y., Yanase, T., and Lun, S. (2008) Characterization of recombinant human growth differentiation factor-9 signaling in ovarian granulosa cells. *Mol. Cell. Endocrinol.* **283**(1-2), 58-67

Nagai, T. (2001) The improvement of in vitro maturation systems for bovine and porcine oocytes. *Theriogenology* **55**, 1291-1301

Nagai, T., Ding, J., and Moor, R. (1993) Effect of follicle cells and steroidogenesis on maturation and fertilization in vitro of pig oocytes. *J. Exp. Zool.* **266**(2), 146-151

Nagai, T., Funahashi, H., Yoshioka, K., and Kikuchi, K. (2006) Up to date of in vitro production of porcine embryos. *Front. Biosci.* **11**, 2565-2573

Nagashima, H., Hiruma, K., Saito, H., Tomii, R., Ueno, S., Nakayama, N., Matsunari, H., and Kurome, M. (2007) Production of live piglets following cryopreservation of embryos derived from in vitro-matured oocytes. *Biol. Reprod.* **76**(5), 900-905

O'Doherty, E., Wade, M., Hill, J., and Boland, M. (1997) Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology* **48**(1), 161-169

Ozawa, M., Nagai, T., Somfai, T., Nakai, M., Maedomari, N., Fahrudin, M., Karja, N.W.K., Kaneko, H., Noguchi, J., Ohnuma, K., Yoshimi, N., Miyazaki, H., and Kikuchi, K. (2008) Comparison between effects of 3-isobutyl-1-methylxanthine and FSH on gap junctional

communication, LH-receptor expression, and meiotic maturation of cumulus–oocyte complexes in pigs. *Mol. Reprod. Dev.* **75**(5), 857-866

Ozawa, M., Nagai, T., Somfai, T.s., Nakai, M., Maedomari, N., Miyazaki, H., Kaneko, H., Noguchi, J., and Kikuchi, K. (2010) Cumulus cell-enclosed oocytes acquire a capacity to synthesize GSH by FSH stimulation during in vitro maturation in pigs. *J. Cell. Physiol.* **222**(2), 294-301

Paria, B.C., and Dey, S.K. (1990) Preimplantation embryo development in vitro: cooperative interactions among embryos and role of growth factors. *Dev. Biol.* **87**, 4756-4760

Park, J.I., Hong, J.Y., Yong, H.Y., Hwang, W.S., Lim, J.M., and Lee, E.S. (2005) High oxygen tension during in vitro oocyte maturation improves in vitro development of porcine oocytes after fertilization. *Anim. Reprod. Sci.* **87**(1-2), 133-141

Peña, F.J., Johannisson, A., Wallgren, M., and Rodriguez-Martinez, H. (2004) Effect of hyaluronan supplementation on boar sperm motility and membrane lipid architecture status after cryopreservation. *Theriogenology* **61**(1), 63-70

Pereira, R.M., and Marques, C.C. (2008) Animal oocyte and embryo cryopreservation. *Cell and Tissue Banking* **9**(4), 267-277

Petr, J., Zetová, L., Fulka, J.J., and Jílek, F. (1989) Quantitative inhibitory influence of porcine cumulus cells upon the maturation of pig and cattle oocytes in vitro. *Reprod. Nutr. Dev.* **29**, 541-550

Petters, R., and Wells, K. (1993) Culture of pig embryos. *J. Reprod. Fertil. Suppl.* **48**, 61-73

Pincus, G., and Enzmann, E.V. (1935) The comparative behavior of mammalian eggs in vivo and in vitro. *J. Exp. Med.* **62**(5), 665-675

Prather, R.S., and Day, B.N. (1998) Practical considerations for the in vitro production of pig embryos. *Theriogenology* **49**, 23-32

Prather, R.S., Hawley, R.J., Carter, D.B., Lai, L., and Greenstein, J.L. (2003) Transgenic swine for biomedicine and agriculture. *Theriogenology* **59**, 115-123

Prochazka, R., Nagyova, E., Rimkevičová, Z., Nagai, T., Kikuchi, K., and Motlik, J. (1991) Lack of effect of oocytectomy on expansion of the porcine cumulus. *J. Reprod. Fertil.* **93**(2), 569-576

Qian, Y., Shi, W.Q., Ding, J.T., Sha, J.H., and Fan, B.Q. (2003) Predictive value of the area of expanded cumulus mass on development of porcine oocytes matured and fertilized in vitro. *J. Reprod. Dev.* **49**, 167-174

Rath, D., and Niemann, H. (1997) In vitro fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen-thawed epididymal semen obtained from identical boars *Theriogenology* **47**, 785-793

Richards, J. (2001) New signaling pathways for hormones and cyclic adenosine 3′, 5′-monophosphate action in endocrine cells. *Mol. Endocrinol.* **15**(2), 209-218

Rijnders, P.M., and Jansen, C.A.M. (1999) Influence of group culture and culture volume on the formation of human blastocysts: a prospective randomized study. *Hum. Reprod.* **14**(9), 2333-2337

Roca, J., Vazquez, J.M., Gil, M.A., Cuello, C., Parrilla, I., and Martinez, E.A. (2006) Challenges in pig artificial insemination. *Reprod. Domest. Anim.* **41 (Suppl. 2)**, 43-53

Romaguera, R., and Grupen, C.G. (2009) Relationship between follicle size and factors secreted by porcine oocytes and cumulus cells during maturation in vitro. *Reprod. Domest. Anim.* **44**, 89

Rose-Hellekant, T.A., Libersky-Williamson, E.A., and Bavister, B. (1998) Energy substrates and amino acids provided during in vitro maturation of bovine oocytes alter acquisition of developmental competence. *Zygote* **6**(04), 285-294

Russell, D.L., Doyle, K.M.H., Ochsner, S.A., Sandy, J.D., and Richards, J.S. (2003) Processing and localization of ADAMTS-1 and proteolytic cleavage of versican during cumulus matrix expansion and ovulation. *J. Biol. Chem.* **278**(43), 42330-42339

Shimada, M. (2012) Regulation of oocyte meiotic maturation by somatic cells. *Reprod. Med. Biol.* **11**(4), 177-184

Shimada, M., Nishibori, M., Isobe, N., Kawano, N., and Terada, T. (2003) Luteinizing hormone receptor formation in cumulus cells surrounding porcine oocytes and its role during meiotic maturation of porcine oocytes. *Biol. Reprod.* **68**(4), 1142-1149

Shimada, M., Nishibori, M., Yamashita, Y., Ito, J., Mori, T., and Richards, J.S. (2004) Down-regulated expression of A disintegrin and metalloproteinase with thrombospondin-like repeats-1 by progesterone receptor antagonist is associated with impaired expansion of porcine cumulus-oocyte complexes. *Endocrinology* **145**(10), 4603-4614

Sigma-Aldrich (29 May 2015) N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt. *http://www.sigmaaldrich.com/catalog/product/sigma/d0627?lang=en&region=BE*

Singh, B., Zhang, X., and Armstrong, D. (1993) Porcine oocytes release cumulus expansion-enabling activity even though porcine cumulus expansion in vitro is independent of the oocyte. *Endocrinology* **132**(4), 1860-1862

Somfai, T., Kikuchi, K., Onishi, A., Iwamoto, M., Fuchimoto, D.-i., Papp, g.B., Sato, E., and Nagai, T. (2004) Relationship between the morphological changes of somatic compartment and the kinetics of nuclear and cytoplasmic maturation of oocytes during in vitro maturation of porcine follicular oocytes. *Mol. Reprod. Dev.* **68**(4), 484-491

Somfai, T., Kikuchi, K., Onishi, A., Iwamoto, M., Fuchimoto, D., Papp, Á.B., Sato, E., and Nagai, T. (2003) Meiotic arrest maintained by cAMP during the initiation of maturation enhances meiotic potential and developmental competence and reduces polyspermy of IVM/IVF porcine oocytes. *Zygote* **11**(3), 199-206

Somfai, T., Ozawa, M., Noguchi, J., Kaneko, H., Karja, N.W.K., Fahrudin, M., Nakai, M., Maedomari, N., Dinnyés, A., Nagai, T., and Kikuchi, K. (2008) In vitro development of polyspermic porcine oocytes: relationship between early fragmentation and excessive number of penetrating spermatozoa. *Anim. Reprod. Sci.* **107**(1-2), 131-147

Somfai, T., Ozawa, M., Noguchi, J., Kaneko, H., Kuriani Karja, N.W., Farhudin, M., Dinnyés, A., Nagai, T., and Kikuchi, K. (2007) Developmental competence of in vitro-fertilized porcine oocytes after in vitro maturation and solid surface vitrification: Effect of cryopreservation on oocyte antioxidative system and cell cycle stage. *Cryobiology* **55**(2), 115-126

Somfai, T., Ozawa, M., Noguchi, J., Kaneko, H., Nakai, M., Maedomari, N., Ito, J., Kashiwazaki, N., Nagai, T., and Kikuchi, K. (2009) Live piglets derived from in vitro-produced zygotes vitrified at the pronuclear stage. *Biol. Reprod.* **80**(1), 42-49

Somfai, T., Yoshioka, K., Tanihara, F., Kaneko, H., Noguchi, J., Kashiwazaki, N., Nagai, T., and Kikuchi, K. (2014) Generation of live piglets from cryopreserved oocytes for the first time using a defined system for in vitro embryo production. *PLoS ONE* **9**(5), e97731

Stemcell-Technologies (29 May 2015) IBMX. *http://www.stemcell.com/en/Products/All-Products/IBMX.aspx*

Stokes, P.J., Abeydeera, L.R., and Leese, H.J. (2005) Development of porcine embryos in vivo and in vitro; evidence for embryo 'cross talk' in vitro. *Dev. Biol.* **284**(1), 62-71

Sugiura, K., Pendola, F.L., and Eppig, J.J. (2005) Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev. Biol.* **279**(1), 20-30

Sun, Q.-Y. (2003) Cellular and molecular mechanisms leading to cortical reaction and polyspermy block in mammalian eggs. *Microsc. Res. Tech.* **61**(4), 342-348

Sun, X.-S., Liu, Y., Yue, K.-Z., Ma, S.-F., and Tan, J.-H. (2004) Changes in germinal vesicle (GV) chromatin configurations during growth and maturation of porcine oocytes. *Mol. Reprod. Dev.* **69**(2), 228-234

Suzuki, C., Iwamura, S., and Yoshioka, K. (2004) Birth of piglets through the non-surgical transfer of blastocysts produced in vitro. *J. Reprod. Dev.* **50**(4), 487-491

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., and de Kruif, A. (2002) Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol. Reprod. Dev.* **61**(3), 414-424

Törnell, J., and Hillensjö, T. (1993) Effect of cyclic AMP on the isolated human oocyte-cumulus complex. *Hum. Reprod.* **8**(5), 737-739

Tsafriri, A., Chun, S., Zhang, R., Hsueh, A., and Conti, M. (1996) Oocyte maturation involves compartmentalization and opposing changes of cAMP levels in follicular somatic and germ cells: studies using selective phosphodiesterase inhibitors. *Dev. Biol.* **178**(2), 393-402

Vajta, G., Korösi, T., Du, Y., Nakata, K., Ieda, S., Kuwayama, M., and Nagy, Z.P. (2008) The Well-of-the-Well system: an efficient approach to improve embryo development. *Reprod. Biomed. Online* **17**(1), 73-81

Vajta, G., Peura, T.T., Holm, P., Páldi, A., Greve, T., Trounson, A.O., and Callesen, H. (2000) New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. *Mol. Reprod. Dev.* **55**, 256-264

Vanderhyden, B. (2002) Molecular basis of ovarian development and function. *Front. Biosci.* **7**, d2006-2022

Vanderhyden, B., and Armstrong, D.T. (1989) Role of cumulus cells and serum on the in vitro maturation, fertilization, and subsequent development of rat oocytes. *Biol. Reprod.* **40**, 720-728

Vitt, U.A., Mazerbourg, S., Klein, C., and Hsueh, A.J.W. (2002) Bone morphogenetic protein receptor type II is a receptor for growth differentiation factor-9. *Biol. Reprod.* **67**, 473-480

Wang, W.H., Abeydeera, L.R., Prather, R.S., and Day, B.N. (1998) Morphologic comparison of ovulated and in vitro–matured porcine oocytes, with particular reference to polyspermy after in vitro fertilization. *Mol. Reprod. Dev.* **49**(3), 308-316

Wang, W.H., Hosoe, M., and Shioya, Y. (1997) Induction of cortical granule exocytosis of pig oocytes by spermatozoa during meiotic maturation. *J. Reprod. Fertil.* **109**, 247-255

Watson, P.F. (1995) Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod. Fertil. Dev.* **7**, 871-891

Wheeler, M.B., and Walters, E.M. (2001) Transgenic technology and applications in swine. *Theriogenology* **56**, 1345-1369

Whyte, J.J., and Prather, R.S. (2011) Genetic modifications of pigs for medicine and agriculture. *Mol. Reprod. Dev.* **78**(10-11), 879-891

Woelders, H., Zuidberg, C.A., and Hiemstra, S.J. (2006) Animal genetic resources conservation in The Netherlands and Europe: poultry perspective. *Poult. Sci.* **85**, 216-222

Wongsrikeao, P., Kaneshige, Y., Ooki, R., Taniguchi, M., Agung, B., Nii, M., and Otoi, T. (2005) Effect of the removal of cumulus cells on the nuclear maturation, fertilization and development of porcine oocytes. *Reprod. Domest. Anim.* **40**, 166-170

Wu, Y., Chen, L., Zheng, P.S., and Yang, B.B. (2002) Beta1-Integrin-mediated glioma cell adhesion and free radical-induced apoptosis are regulated by binding to a C-terminal domain of PG-M/versican. *J. Biol. Chem.* **277**(14), 12294-12301

Xu, X., Ding, J., Seth, P.C., Harbison, D.S., and Foxcroft, G.R. (1996a) In vitro fertilization of in vitro matured pig oocytes: effects of boar and ejaculate fraction. *Theriogenology* **45**, 745-755

Xu, X., Seth, P.C., Harbison, D.S., Cheung, A.P., and Foxcroft, G.R. (1996b) Semen dilution for assesment of boar ejaculate quality in pig IVM and IVF systems. *Theriogenology* **46**, 1325-1337

Yamauchi, N., and Nagai, T. (1999) Male pronuclear formation in denuded porcine oocytes after in vitro maturation in the presence of cysteamine. *Biol. Reprod.* **61**, 828-833

Yoshida, M., Ishigaki, K., Nagai, T., Chikyu, M., and Pursel, V.G. (1993) Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus'. *Biol. Reprod.* **49**, 89-94

Yoshida, M., Ishizaki, Y., and Kawagishi, H. (1990) Blastocyst formation by pig embryos resulting from in-vitro fertilization of oocytes matured in vitro. *J. Reprod. Fertil.* **88**, 1-8

Yoshioka, K., Suzuki, C., Itoh, S., Kikuchi, K., Iwamura, S., and Rodriguez-Martinez, H. (2003) Production of piglets derived from in vitro-produced blastocysts fertilized and cultured in chemically defined media: effects of theophylline, adenosine, and cysteine during in vitro fertilization. *Biol. Reprod.* **69**(6), 2092-2099

Yoshioka, K., Suzuki, C., and Onishi, A. (2008) Defined system for in vitro production of porcine embryos using a single basic medium. *J. Reprod. Dev.* **54**(3), 208-213

Yoshioka, K., Suzuki, C., Tanaka, A., Anas, I.M.K., and Iwamura, S. (2002) Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biol. Reprod.* **66**, 112-119

Chapter 2

Aims of the study

Despite the major progress that has been made since the start of porcine *in vitro* embryo production, the process is still confronted with many obstacles, gaps in the knowledge and challenges. Three major problems are currently most prevalent: high polyspermy rates, low blastocyst rates and inferior blastocyst quality, and difficulties to culture low numbers of oocytes/embryos in an individually identifiable manner, all of which may find their origin in an inadequate oocyte maturation system. The general aim of this dissertation was to get more insight in the bidirectional communication between the oocyte and the surrounding cumulus cells in order to be able to establish a porcine *in vitro* embryo production system with less polyspermy and with an improved oocyte developmental potential. In this respect, we focused our attention on the second messenger cAMP and the oocyte-secreted factors, which have been suggested to play a key role in the regulation of the oocyte maturation.

In order to achieve this general aim, specific objectives were formulated as follows:

1. To investigate if increasing the cAMP concentration during *in vitro* maturation of pig oocytes could improve cumulus maturation and subsequent fertilization *in vitro* (**Chapter 3**).

2. To realize objective 1, there was a need to develop a method for collecting and immobilizing individual cumulus cells enabling quantitative immunofluorescence analysis of proteins (**Chapter 4**).

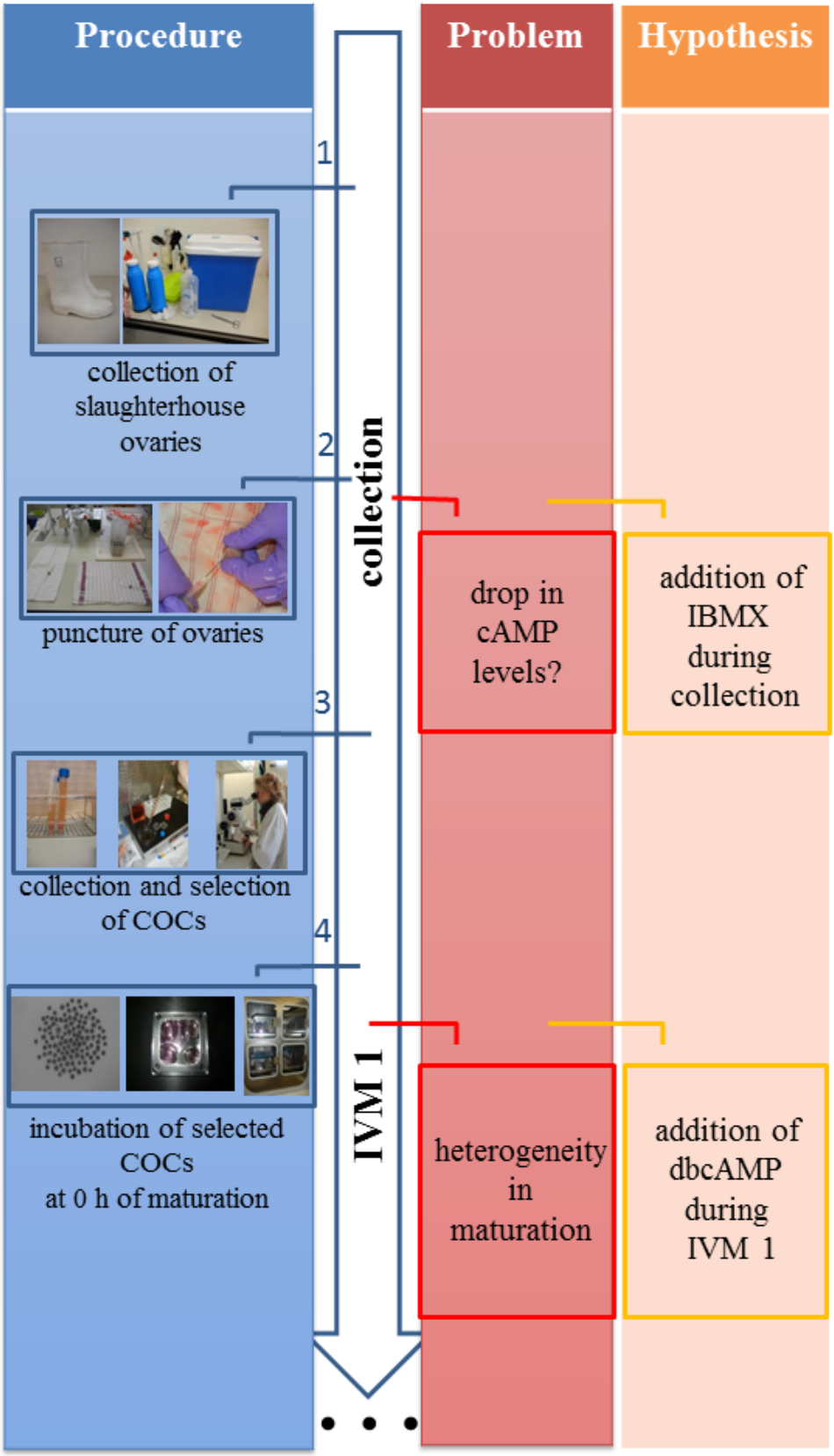
3. To examine the interactions between oocytes and cumulus cells during *in vitro* maturation of porcine cumulus-oocyte complexes in a chemically defined medium *inter alia* by the effect of denuded oocytes on cumulus expansion and oocyte maturation (**Chapter 5**).

4. To create a "living apart together" *in vitro* embryo production system that allows the objective measurement of cumulus expansion in the co-culture experiment of objective 3 (**Chapter 5**).

5. To investigate the practical usefulness of co-culture with denuded oocytes during *in vitro* maturation on fertilization and developmental competence of cumulus-enclosed porcine oocytes in a defined system (**Chapter 6**).

The following process diagrams visualize the procedures, problem areas and hypotheses discussed in this dissertation.

In Figure 1 an overview related to the role of cAMP during porcine *in vitro* embryo production is presented (**Chapter 3**).



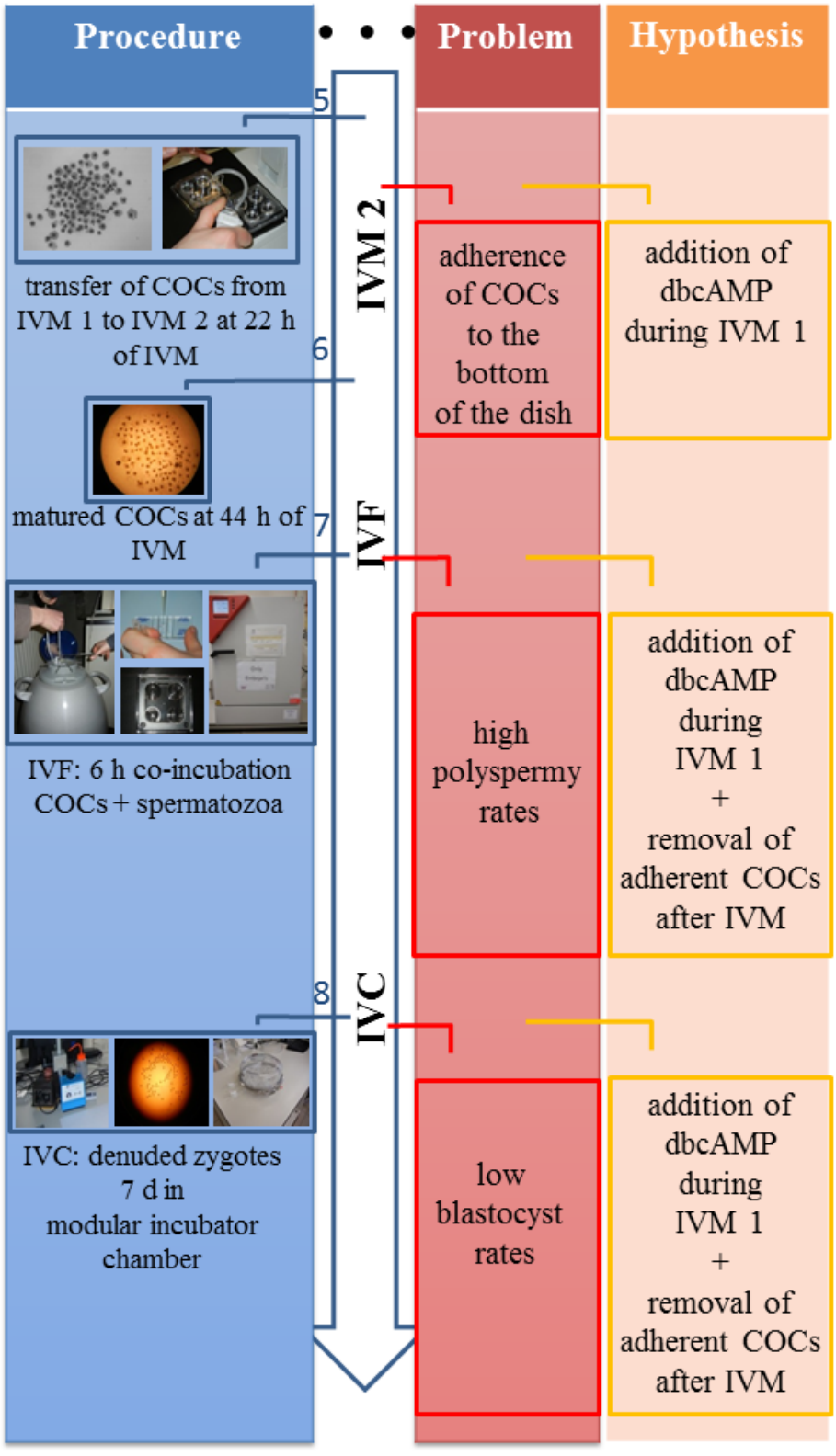
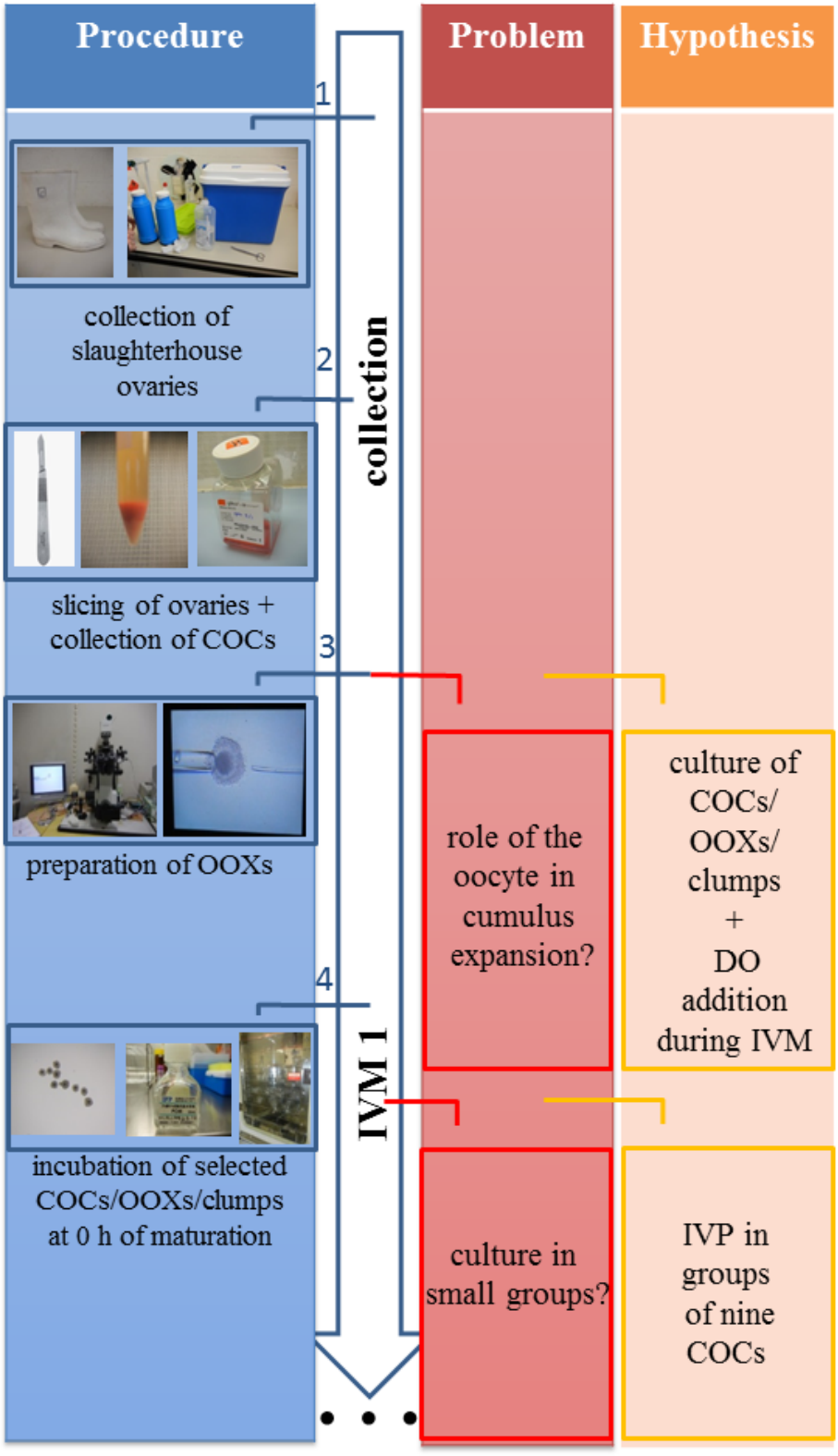


Figure 1: Schematic overview of the role of cAMP during porcine in vitro *embryo production discussed in* ***Chapter 3****. COC: cumulus-oocyte complex; IVM:* in vitro *maturation; IVF:* in vitro *fertilization; IVC:* in vitro *culture. Photographs by R. Appeltant.*

The following process diagram (Figure 2) visualizes the procedures, problem areas and hypotheses about the role of OSFs during porcine *in vitro* embryo production discussed in **Chapters 5** and **6**.



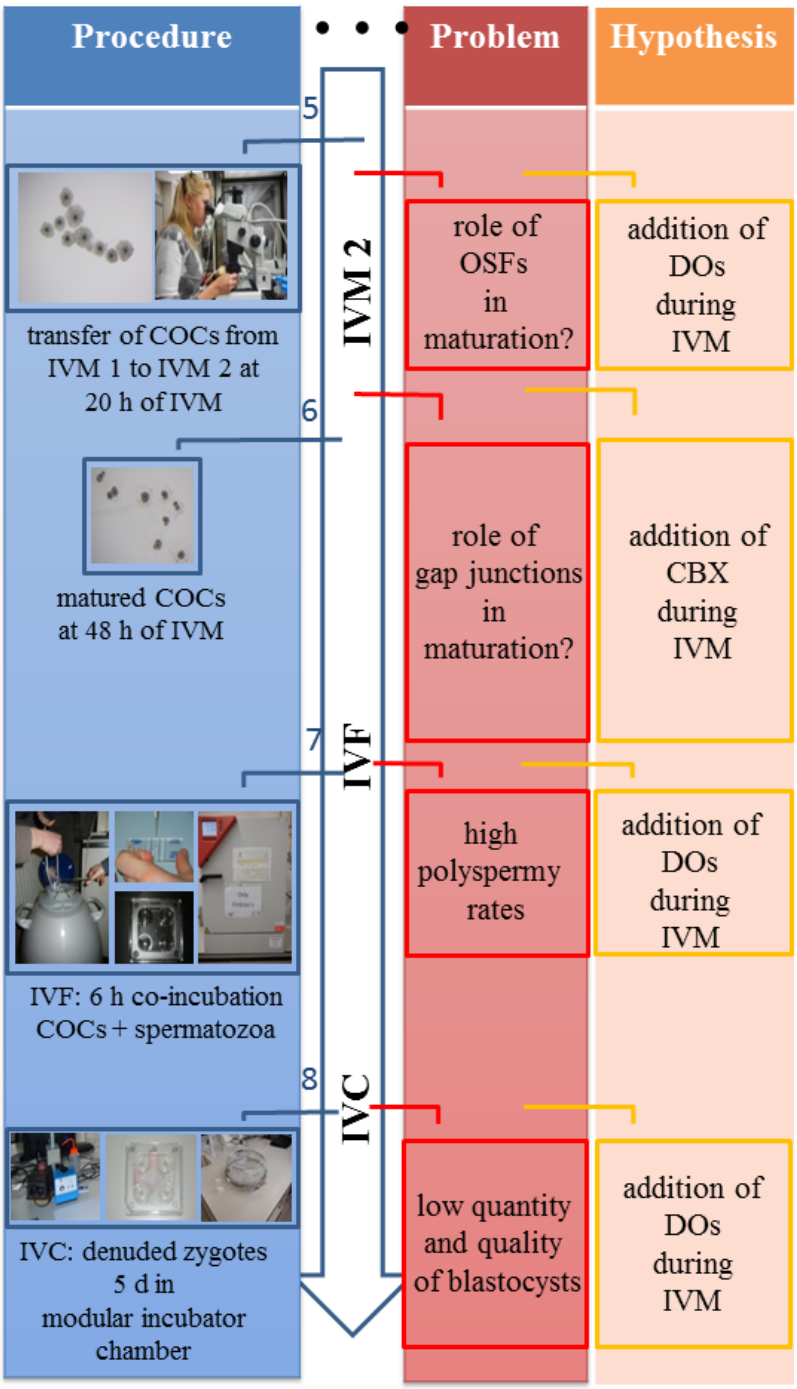


Figure 2: Schematic overview of the role of oocyte-secreted factors (OSFs) on porcine in vitro *embryo production described in* ***Chapters 5*** *and* ***6****. COC: cumulus-oocyte complex; OOX: oocytectomized complex; DO: denuded oocyte; CBX: carbenoxolone; IVM:* in vitro *maturation; IVF:* in vitro *fertilization; IVC:* in vitro *culture. Photographs by R. Appeltant.*

Chapter 3

Increasing the cyclic adenosine monophosphate concentration during *in vitro* maturation of pig oocytes improves subsequent fertilization by affecting cumulus functions

Adapted from:

Appeltant, R., Beek, J., Vandenberghe, L., Maes, D., and Van Soom, A. (2015) Increasing the cAMP concentration during *in vitro* maturation of pig oocytes improves cumulus maturation and subsequent fertilization *in vitro*. *Theriogenology* **83**, 344-352

3.1 Abstract

Porcine IVF faces various problems like incomplete cytoplasmic maturation of the oocyte and polyspermy. Previous studies proved the importance of cAMP in regulating nuclear and cytoplasmic maturation of oocytes. This study investigated the effect of the cAMP modulating agents 3-isobutyl-1-methylxanthine (IBMX) and dibutyryl cAMP sodium salt (dbcAMP) on several parameters during *in vitro* production of porcine embryos. First, we wanted to see if oocyte collection in IBMX could meiotically arrest oocytes and, as such, improve synchronization of nuclear and cytoplasmic maturation. To this end, cumulus–oocyte complexes (COCs) were collected from gilts in HEPES-buffered Tyrode balanced salt solution medium with 0.5 mM IBMX or without IBMX. At the end of oocyte collection, the effect of IBMX on chromatin configuration was evaluated. However, no differences could be observed in nuclear configuration between IBMX− and IBMX+ oocytes ($P \geq 0.05$). Second, we added dbcAMP during IVM to improve cytoplasmic maturation and evaluated cumulus expansion (lack of adhesion), a disintegrin and metalloproteinase with thrombospondin-like repeats-1 (ADAMTS-1) levels in cumulus cells, fertilization, and blastocyst rates. Cumulus–oocyte complexes were matured in modified North Carolina State University medium 37 with or without 1 mM dbcAMP. Frozen-thawed, epididymal, boar spermatozoa were used for IVF. After IVF, presumed zygotes were cultured for 7 days in North Carolina State University medium 23. Penetration rate decreased in dbcAMP+ (57.3%) compared with dbcAMP− (67.8%), but the polyspermy rate also decreased (43.3% vs. 53.4%, respectively) leading to an increased normal fertilization rate (56.7% vs. 46.6%, respectively; $P < 0.05$). Only 7.2% of the COCs showed adhesion in dbcAMP+, which was lower than 15.7% in dbcAMP− ($P < 0.05$) probably because of an upregulation of the ADAMTS-1 protein by dbcAMP. When the adherent oocytes were removed during maturation, no difference could be detected between the blastocyst rate of dbcAMP− and dbcAMP+ (17.1% and 21.0% on Day 7, respectively; $P \geq 0.05$). In conclusion, the use of IBMX during collection did not cause a meiotic arrest. Using dbcAMP during IVM caused a greater normal fertilization rate, a lower rate of adherent COCs during IVM, higher levels of ADAMTS-1 in cumulus cells, and an equal blastocyst rate after screening out adherent COCs. These findings contribute to a better understanding of cAMP involvement in porcine oocyte maturation and provide a basis to develop an improved system with less polyspermy and higher blastocyst rates.

3.2 Introduction

Currently, porcine *in vitro* embryo production (IVP) with immature oocytes results in 20 to 30% of blastocysts (Gil *et al.* 2010). Polyspermy, or the penetration of more than one sperm cell into the oocyte, represents a major problem during porcine *in vitro* fertilization (IVF) (Nagai *et al.* 2006). The polyspermy rate with *in vivo* matured oocytes is higher in pigs (28%, (Wang *et al.* 1998)) than in other species (11% in cattle (Leibfried-Rutledge *et al.* 1987)). With porcine oocytes matured *in vitro*, polyspermy rates reach levels of 65% (Wang *et al.* 1998). Polyspermy can be due to the presence of too many spermatozoa in the immediate vicinity of the oocyte (Fraser and Maudlin 1978) and/or by the inability of the porcine cumulus-oocyte complex (COC) to prevent penetration of several spermatozoa at the same time (Wang *et al.* 1998).

Nuclear and cytoplasmic maturation, the response during fertilization and developmental competence of the oocyte can be improved by adjusting cAMP levels during *in vitro* maturation (IVM). *In vivo*, the follicle is in control of meiotic arrest and resumption. High intracellular cAMP levels, produced by the oocyte itself (Mehlmann *et al.* 2002) and provided continuously through gap junctional contacts with cumulus and mural granulosa cells (Anderson and Albertini 1976), maintain meiotic arrest in the oocyte as long as it is inside the follicle (Bornslaeger *et al.* 1986). Furthermore, degradation of cAMP by a type 3 phosphodiesterase of the oocyte is inhibited *via* the supply of cyclic guanosine monophosphate to the oocyte by the cumulus cells (Gilchrist 2011). During collection, COCs are removed from the follicle and thus the contacts between follicle and oocyte are interrupted. This causes a spontaneous resumption of meiosis, producing oocytes of low developmental competence.

To solve this problem, several cAMP modulating agents such as the non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; rat, (Tsafriri *et al.* 1996)), and the membrane-permeable cAMP analog, dibutyryl cAMP sodium salt **(**dbcAMP; pig, (Bagg *et al.* 2006)) have been used during IVM to increase cAMP levels, postpone meiosis and consequently synchronize nuclear and cytoplasmic maturation of the oocytes. In the simulated physiological oocyte maturation (SPOM) system applied in other species such as mice and cattle, IBMX improves COC functions during IVM and developmental

programming of the oocyte (Albuz *et al.* 2010). On the other hand, in pigs, Somfai *et al.* (2003) could not demonstrate an influence on nuclear progression during IVM and developmental competence using IBMX in the collection medium. Nevertheless, it has been shown that the use of dbcAMP during porcine IVM is crucial to obtain a homogeneous and synchronized pool of oocytes and to improve blastocyst rates (Funahashi *et al.* 1997).

It is not clear if the oocyte's ability to block polyspermy is affected by adding dbcAMP to the IVM medium. Funahashi *et al.* (1997) pointed out that dbcAMP did not influence penetration and polyspermy rate, Somfai *et al.* (2003) found similar penetration rates but lower polyspermy rates, and Kim *et al.* (2008) measured lower polyspermy rates but higher penetration rates. Decreasing polyspermy rates after IVF of cumulus intact oocytes can be due to an improved cumulus expansion (Bijttebier *et al.* 2008). Hence, probably, cAMP levels are not only regulating nuclear and cytoplasmic maturation through acting *via* the oocyte, but also by influencing cumulus expansion.

During IVM, it has been noticed that some COCs stick to the bottom of the dish. Adhesion may be due to inferior cumulus matrix disassembly because of inadequate cleavage of versican in the expanded COC matrix by a disintegrin and metalloproteinase with thrombospondin-like repeats-1 (ADAMTS-1) (Wu *et al.* 2002; Russell *et al.* 2003; Shimada *et al.* 2004). Doyle *et al.* (2004) suggested that LH can possibly influence the expression of ADAMTS-1 through a cAMP dependent, progesterone receptor independent pathway. It is clear that maintaining high cAMP levels in the oocyte can sustain a better maturation of the oocyte and possibly supports cumulus expansion.

From literature it can be stated that (1) IBMX halts meiotic progression in other species, whereas for pigs, no effect could be shown thus far. We hypothesized that IBMX could still exhibit an effect in pigs, but only during collection rather than in further stages. (2) dbcAMP in the IVM medium influences the fertilization outcome, possibly explaining higher blastocyst rates, but conflicting results were obtained concerning penetration and polyspermy rates. (3) cAMP levels could possibly be regulating ADAMTS-1 expression, which is involved in cumulus expansion. We hypothesized that similarly dbcAMP could reduce the occurrence of adherent COCs by upregulating the expression of the ADAMTS-1 protein in the cumulus cells. Removal of adherent low quality COCs during maturation could provide a new selection tool for good quality oocytes resulting in a higher blastocyst rate. We analyzed (1)

the chromatin configuration at the end of collection of porcine oocytes collected in a medium supplemented with IBMX, (2) fertilization parameters after IVM with dbcAMP, and (3) the proportion of adherent COCs after IVM with dbcAMP, ADAMTS-1 protein levels in adherent and floating cumulus cells in both treatment groups, and blastocyst rate after removal of adherent COCs during IVM.

3.3 Materials and methods

3.3.1 Chemicals

Unless otherwise stated, all chemicals used were obtained from Sigma-Aldrich (Bornem, Belgium).

3.3.2 Collection and *in vitro* maturation

Ovaries (50-80) of prepubertal gilts were collected in a local abattoir and transported to the laboratory at 37 °C in a 0.9% NaCl solution supplemented with kanamycin. Ovaries of Piétrain crossbred prepubertal gilts of approximately 6.5 months of age, weighing 105 to 115 kg were used. Randomization was applied at the level of collection of the ovaries and at the level of collection of the COCs. The medium for collection and washing of the oocytes was a modified HEPES-buffered Tyrode balanced salt solution consisting of 3.1 mM KCl, 114 mM NaCl, 2 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 2.1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 10 µg/mL gentamycin sulfate, 10 mM HEPES and 3 mg/mL bovine serum albumin (BSA) (wash).

Antral follicles of 3 to 6 mm were aspirated to collect the COCs. After aspiration, the oocyte selection started with a screening for those oocytes that had a multilayered compact cumulus and a homogeneous ooplasm. The basic maturation medium was BSA-free North Carolina

State University 37 (NCSU 37) (Petters and Wells 1993) supplemented with 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 50 µM β-mercaptoethanol, 10 IU/mL equine chorionic gonadotropin (eCG) (Folligon, Intervet, The Netherlands), 10 IU/mL human chorionic gonadotropin (hCG) (Chorulon, Intervet, The Netherlands), and 10% porcine follicular fluid. Follicular fluid was collected by aspiration of 3 to 6 mm follicles from ovaries of prepubertal gilts, centrifugation (10 min, 100 × g, 22 °C) and snap freezing of the supernatant. Groups of 50 to 55 oocytes were incubated into 500 µL maturation medium, supplemented with eCG and hCG for the first 22 h (38.5 °C, 5% CO_2 in air). For the next 22 h, the oocytes were matured in a 500 µL hormone free maturation medium (38.5 °C, 5% CO_2 in air).

3.3.3 *In vitro* fertilization

The basic medium for IVF was modified Tyrode's albumin lactate pyruvate (TALP) medium (Rath *et al.* 1999) supplemented with 3 mg/mL fatty acid-free BSA (fraction V) and 1.10 mM sodium pyruvate (FERT-TALP) (Romar *et al.* 2003). For the fertilization, frozen-thawed, epididymal boar spermatozoa were used. After thawing, the semen was centrifuged (3 min, 390 × g, 22 °C) in an Androhep extender that consists of 27 mM trisodium citrate, 6.4 mM Titriplex III EDTA, 2.5 mg/mL BSA (fraction V), 16 mM $NaHCO_3$, 38 mM HEPES, 144 mM D(+)-glucose.H_2O, and 50 µg/mL gentamycin sulfate. Subsequently, the centrifuged pellet of semen was resuspended in FERT-TALP. Presumed matured COCs were transferred in groups of 50 to 55 to FERT-TALP drops under mineral oil. The COCs and the spermatozoa were co-incubated for 6 h (38.5 °C, 5% CO_2 in air).

3.3.4 *In vitro* culture

After 6 h, the presumed zygotes were vortexed for 3 min in HEPES buffered (25 mM HEPES) TALP medium (HEPES-TALP). Presumed zygotes were then washed in culture medium that

was NCSU 23 with 4 mg/mL BSA (Petters and Wells 1993) and selected for intact oolemma under a stereomicroscope. Subsequently, the presumed zygotes were cultured in a modular incubator chamber (38.5 °C, 5% CO_2, 5% O_2).

3.3.5 Assessment of the germinal vesicle stage

After collection and selection of the COCs, they were vortexed in HEPES-buffered Tyrode balanced salt solution to remove the cumulus cells, washed in phosphate buffered saline (PBS) supplemented with 1 mg/mL polyvinylpyrrolidone and fixed in 4% of paraformaldehyde (PF). The DNA was stained with Hoechst 33342 (Molecular Probes, Ghent, Belgium). For the evaluation of the nuclear DNA, a DMR fluorescence microscope (Leica microsystems, Brussels, Belgium) was used. The germinal vesicle (GV) stage was defined according to Sun *et al.* (2004) (Figure 1). The oocytes were assigned to one of seven different categories consisting of GV stage 0 (GV0), 1 (GV1), 2 (GV2), 3 (GV3), 4 (GV4), GV breakdown (GVBD), and metaphase I (MI).

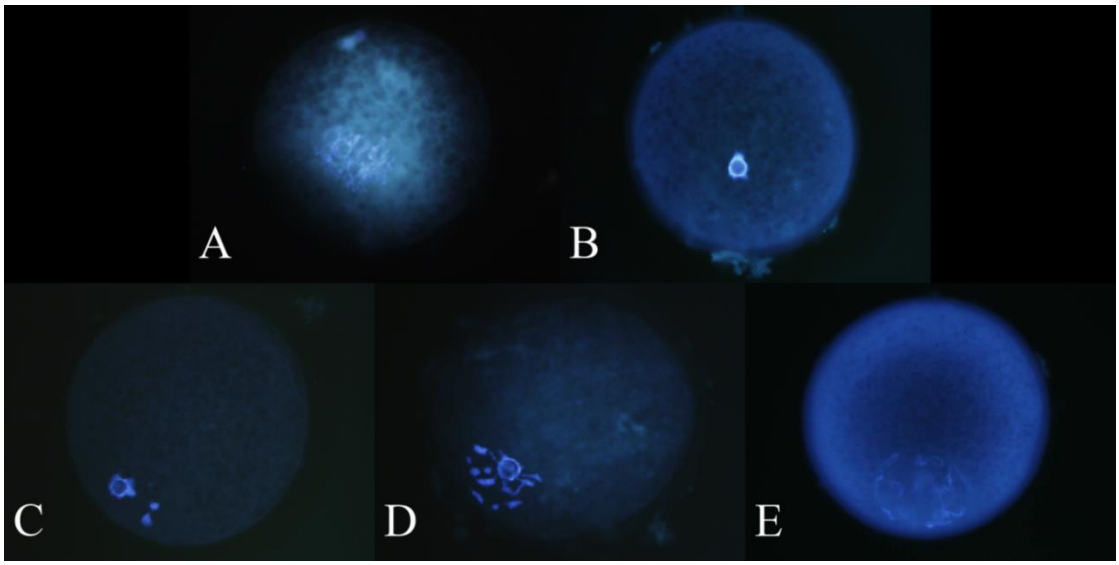


Figure 1: Nuclear chromatin configurations of porcine germinal vesicle (GV) oocytes assessed as described by Sun et al. *(2004) at the end of collection. (A) GV0; (B) GV1; (C) GV2; (D) GV3; (E) GV4. Magnification x 400.*

3.3.6 Assessment of adherent cumulus-oocyte complexes during *in vitro* maturation

At 22 h and 44 h of IVM, COCs that adhered to the bottom of the dish or oocytes that lost their cumulus because of adhesion of cumulus cells to the bottom of the dish were removed from the group. Cumulus-oocyte complexes were considered as adherent when they stuck to the bottom and could not be removed without scratching on the bottom with a pipette. Oocytes that came off, but lost their complete cumulus layer because of adhesion were also considered as adherent COCs. This classification is based on Types 3 and 4 COCs as defined by Somfai *et al.* (2004). The total of removed COCs at both time points was calculated at the end of IVM.

3.3.7 Assessment of ADAMTS-1 protein levels in cumulus cells during *in vitro* maturation

Quantitative immunofluorescence analysis was performed on adherent and floating cumulus cells in both treatment groups, without and with dbcAMP. Because ADAMTS-1 is secreted by cumulus cells into the extracellular matrix and the proprotein is located in secretory vesicles in the cumulus cells (Russell *et al.* 2003), the proprotein was stained. Cumulus cells were collected at 40 h of IVM because it was proven that the proprotein was present at that time of IVM (Shimada *et al.* 2004). Consequently, cumulus cells were immobilized on Cell-Tak coated slides and fixed with 3% PF. Permeabilization was performed by incubating the cumulus cells in 0.5% Triton X-100 and 0.05% Tween 20 for 1 h at 4 °C. Subsequently, the cumulus cells were blocked overnight at 4 °C using 30% goat serum and 0.15% Tween 20. After permeabilization and blocking, cumulus cells were incubated overnight at 4 °C with primary antibodies (H-60; Santa Cruz Biotechnology, Inc.). ProADAMTS-1 was visualized by fluorescein isothiocyanate-labeled goat anti-rabbit antibodies (Molecular Probes, Ghent, Belgium). Nuclei were located by Hoechst 33342. Two kinds of negative controls were included. First, the contribution of non-specific staining of primary antibodies was evaluated by substitution of the primary antibody by rabbit immunoglobulin G whole molecule (Rockland Immunochemicals Inc., Gilbertsville, PA, USA). Second, the intra-cytoplasmic localization of proADAMTS-1 was evaluated by replacing the permeabilization solution by

PBS. The intensity of proADAMTS-1 was measured from digital images using converted grayscale images and the "threshold" and "measure" functions of the NIH ImageJ (version 1.47) software (Abràmoff *et al.* 2004). Digital images were captured by LAS version 4.1 (Leica, Van Hopplynus N.V., Brussel, Belgium) applying the same standardized settings for each picture.

3.3.8 Assessment of fertilization

The same procedure of staining as for the GV stage was followed. After 22 h of *in vitro* culture (IVC), the presumed zygotes were washed and fixed overnight in 4% PF. The day after, the presumed zygotes were stained with Hoechst 33342. The evaluation of the fertilization parameters was performed under a Leica DMR fluorescence microscope (Leica microsystems). Oocytes with more than one pronucleus or a pronucleus and one or more decondensed sperm head(s) were judged as penetrated. The penetration rate is expressed in respect to the total number of non-degenerated presumed zygotes. Zygotes with two pronuclei or one pronucleus and one decondensed sperm head and two polar bodies were classified as normally (monospermic) fertilized. On the contrary, the zygotes that contained at least one pronucleus and two or more other pronuclei or decondensed sperm heads were considered to be polyspermic. Normal fertilization rate and polyspermy rate are expressed in respect to the number of penetrated oocytes.

3.3.9 Assessment of embryo development

After 6 and 7 days of IVC (Day 0 is the day of IVF), the embryo development was evaluated based on the cleavage rate, the morula percentage and the rate of blastocyst formation visualized under a stereomicroscope. The cleavage rate was calculated on the basis of the number of cleaved zygotes at Day 7 of IVC. Non-cleaved zygotes consisted of one blastomere associated with no or a minimum of fragmentation. An embryo was defined as a morula when

it reached the compacted morula stage defined by Mateusen *et al.* (2005). Blastocyst formation was recorded from the moment a blastocoel cavity was formed (Mateusen *et al.* 2005).

3.3.10 Experimental design

3.3.10.1 Experiment 1: effect of IBMX on the nuclear morphology at the end of collection

One group of COCs was collected, selected, and washed in HEPES-TALP medium without IBMX (control group, IBMX−) and another group of COCs was collected, selected, and washed in HEPES-TALP medium supplemented with 0.5 mM IBMX (IBMX+). This concentration was based on the findings of Shimada *et al.* (2003) concerning the influence of IBMX on the formation of LH receptors during maturation. The average collection time in both groups was 140 min. After collection, the oocytes of both groups were stained to evaluate the GV stage. The proportion of oocytes in each category was counted and expressed as a percentage of the non-degenerated oocytes.

3.3.10.2 Experiment 2: effect of dbcAMP on fertilization parameters

One group of COCs was matured without addition of dbcAMP (control group, dbcAMP−), and another group was matured with addition of 1 mM dbcAMP to the maturation medium during the first 22 h (dbcAMP+). After 44 h of IVM, COCs of both groups were co-incubated for 6 h with frozen-thawed epididymal semen. After 22 h of IVC, fertilization parameters were evaluated.

Several fertilization parameters were investigated. First, the percentage of matured oocytes and penetrated oocytes were calculated with respect to the total number of oocytes. Second,

the normal fertilization and polyspermy rates were counted as a percentage of the penetrated oocytes.

Preliminary trials in which IVF was performed without spermatozoa revealed that dbcAMP reduced parthenogenetic activation (data not shown). This can be explained by the synchronizing ability of dbcAMP whereby less oocytes are aged (Funahashi *et al.* 1997) and subsequently less oocytes acquire the ability to be activated already (Kikuchi *et al.* 1995).

3.3.10.3 Experiment 3: effect of dbcAMP on adhesion of the cumulus-oocyte complexes and blastocyst percentage

One group was matured without the use of 1 mM dbcAMP (control group, dbcAMP−), and in the other group, 1 mM dbcAMP was added during the first 22 h of IVM (dbcAMP+). To evaluate the influence of dbcAMP on IVP of porcine embryos, several variables were investigated, more specifically the amount of adherent COCs during IVM and cleavage percentage, morula percentage and blastocyst percentage on Days 6 and 7 of IVC.

Preliminary trials in which IVF was performed without spermatozoa revealed no influence of dbcAMP addition to the IVM medium on cleavage or blastocyst rate (data not shown).

3.3.10.4 Experiment 4: effect of dbcAMP on proADAMTS-1 protein levels in cumulus cells

One group was matured without dbcAMP (−), and in the other group, 1 mM dbcAMP was added during the first 22 h of IVM (+). At 40 h of IVM, cumulus cells of adherent (A) and floating (F) COCs were collected in both treatment groups. Quantitative immunofluorescence was performed to reveal the proADAMTS-1 protein levels of the cumulus cells in the four treatment groups: adherent COCs of dbcAMP− (A−), adherent COCs of dbcAMP + (A+), floating COCs of dbcAMP− (F−), and floating COCs of dbcAMP + (F+). In each group, 20 cells were measured per replicate and three replicates were performed. So, in total, 60 cells

were examined per group. The intensity of proADAMTS-1 in cumulus cells of each group was expressed as a ratio in respect to ratio 1, which was the average intensity of the cumulus cells in A−.

3.3.11 Statistical analysis

Statistical analysis was performed using SPSS Statistics version 20. Three replicates were performed for experiments 1, 3 and 4; four replicates were performed for experiment 2. In every statistical model, $P < 0.05$ was considered as statistically significant (two-sided test).

For experiment 1, the proportions of oocytes at each GV stage were analyzed using the Poisson distribution, with the count of oocytes as dependent variable and group and category as factors.

For experiments 2 and 3, binary logistic regression was applied for the analysis of the parameters penetration rate, polyspermy rate, normal fertilization, adherent COCs, cleavage rate, morula rate and blastocyst rate. The proportion of the parameter to be measured was included as dependent variable, and group (dbcAMP addition or not) as independent variable. Replicate was included as random factor in every model. In Figure 2, Figure 3, Table 1 and Table 2, proportions are presented as percentages. Since the data are approached as proportions instead of continuous data, no error bars are included in the figures.

For experiment 4, Kruskal Wallis one-way ANOVA (k samples) was used because the content of proADAMTS-1 in cumulus cells was normally distributed but the equality of error variances could not be assumed. As *post-hoc*, the Mann-Whitney U (2 samples) test was applied. In Figure 4, results are presented as mean ratios of proADAMTS-1 intensity levels. Error bars in the figure illustrate the standard error of the mean (SEM).

3.4 Results

3.4.1 Effect of IBMX on nuclear morphology at the end of maturation

There were no differences between both groups in the nuclear morphology of the GV stages ($P \geq 0.05$). The distribution of the categories in both groups was as follows: 0.2% vs. 0.2% (GV0); 39.5% vs. 40.2% (GV1); 19.4%, vs. 18.3% (GV2); 26.4% vs. 29.8% (GV3); 13.6% vs. 10.2% (GV4); 0.2% vs. 0.0% (GVBD); 0.7% vs. 1.3% (MI) (IBMX− and IBMX+, respectively) (Figure 2). Most of the oocytes were still in the GV stage. Almost none were at GV0, GVBD and MI, whereas GV1 and GV3 were most prominent. The rate of degenerated oocytes was similar in both groups.

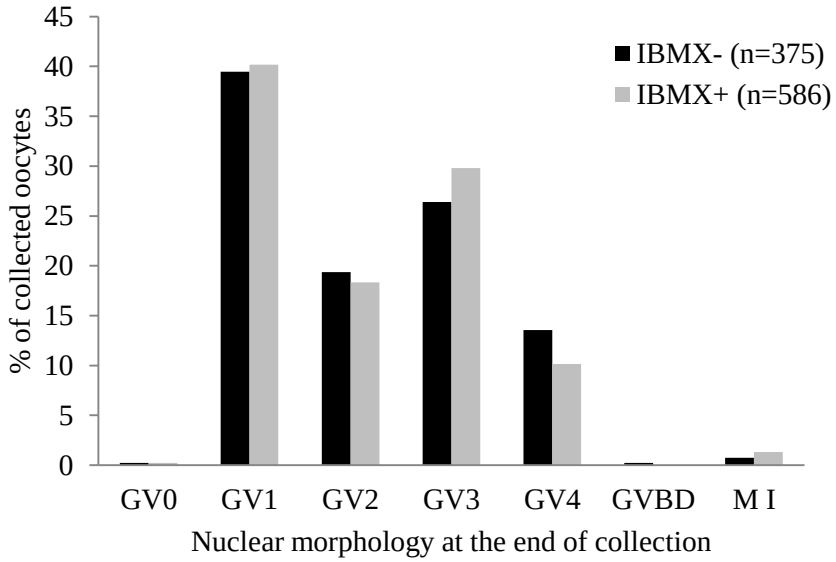


Figure 2: Effect of addition of 0.5 mM IBMX in the collection medium on the nuclear morphology of porcine oocytes at the end of collection. At the end of collection, seven different categories of chromatin configuration were observed: germinal vesicle stage 0 (GV0), 1 (GV1), 2 (GV2), 3 (GV3), 4 (GV4), GV breakdown (GVBD), and metaphase I (MI). No difference was observed in the proportion of collected oocytes in all the categories between the IBMX− (no IBMX addition) and IBMX+ groups (IBMX addition; $P \geq 0.05$). The number of oocytes examined is given in parentheses next to the treatment group. Three replicates were performed. IBMX: 3-isobutyl-1-methylxanthine.

3.4.2 Effect of dbcAMP on fertilization parameters

The dbcAMP+ group showed a lower penetration rate than the dbcAMP− group (57.3% and 67.8%, respectively) but a higher normal fertilization rate (56.7% and 46.6%, respectively) because of a lower polyspermy rate (43.3% and 53.4%, respectively; Table 1; $P < 0.05$). The degeneration rate for the examined oocytes was similar in both experimental groups.

Table 1: Effect of adding 1 mM dbcAMP during the first 22 hours to the in vitro *maturation (IVM) medium on* in vitro *fertilization (IVF) of porcine oocytes.*

IVM treatment	Examined (n)	Penetrated (%)[c]	Polyspermy (%)[d]	Fertilized normally (%)[d]
dbcAMP −	335	67.8[a]	53.4[a]	46.6[a]
dbcAMP +	365	57.3[b]	43.3[b]	56.7[b]

"n" represents the total number of examined presumed zygotes.
[a,b]Within a column, percentages with a different superscript differ significantly ($P < 0.05$). Data were obtained by performing four replicates.
dbcAMP: dibutyryl cAMP sodium salt
[c] Percentage of non-degenerated examined oocytes.
[d] Percentage of penetrated oocytes.

3.4.3 Effect of dbcAMP on adhesion of the cumulus-oocyte complex and embryo development

The percentage of cumulus adhesion was different between both groups: 15.7% vs. 7.2% (without and with dbcAMP addition, respectively; Table 2; $P < 0.05$).

No differences were observed between dbcAMP− and dbcAMP + for cleavage (75.0% and 79.0% on Day 6 and 75.7% and 82.3% on Day 7, respectively), morula percentage (7.1% and 10.3% on Day 6 and 2.5% and 4.0% on Day 7, respectively), and blastocyst percentage (15.0% and 14.3% on Day 6 and 17.1% and 21.0% on Day 7, respectively; Figure 3, Table 2; $P \geq 0.05$).

Table 2: Effect of 1 mM dbcAMP during the first 22 hours of in vitro *maturation (IVM) on adherence of COCs to the dish and blastocyst rate on Day 7.*

IVM treatment	Examined (n)[c]	Adherent COCs (%)	Examined (n)[d]	Blastocyst (%)
dbcAMP−	332	15.7[a]	280	17.1[a]
dbcAMP+	332	7.2[b]	300	21.0[a]

[a,b]Within a column, percentages with a different superscript differ significantly ($P < 0.05$). Data were obtained by performing three replicates.
COCs: cumulus-oocyte complexes; dbcAMP: dibutyryl cAMP sodium salt
[c] Number of oocytes in which maturation was started.
[d] Number of presumed zygotes in which embryo cultivation was started.

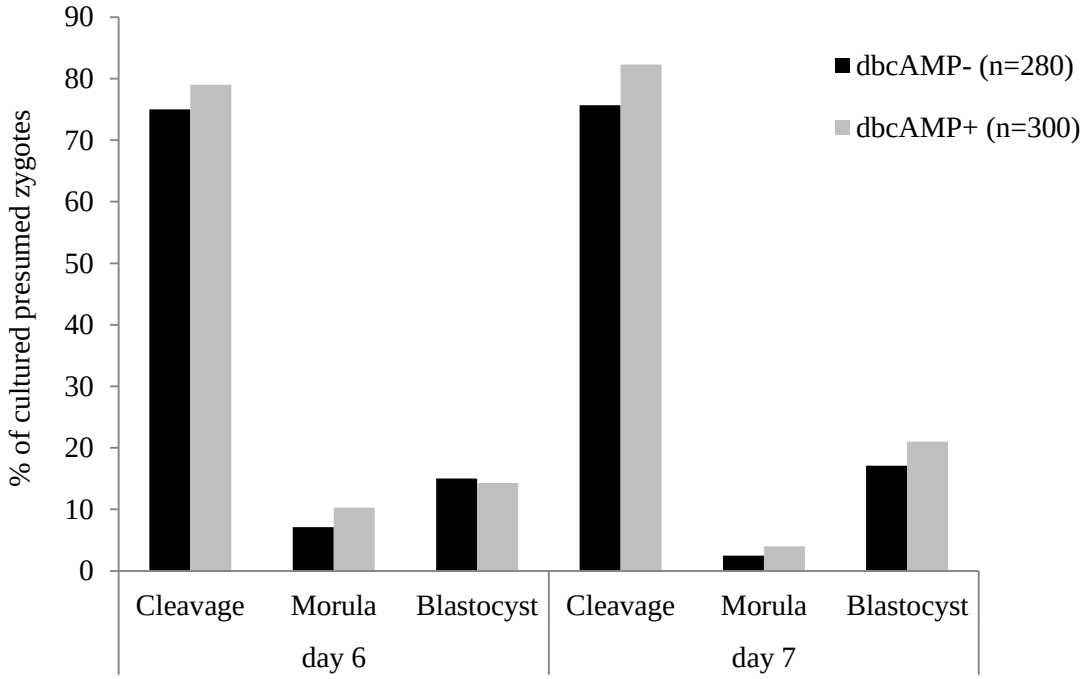


Figure 3: Percentage of cleavage, morula and blastocyst on Days 6 and 7 of porcine in vitro *embryo culture. The number of examined cultured presumed zygotes is mentioned in parentheses next to the definition of the two experimental groups. In one group 1 mM dbcAMP was added to modified North Carolina State University (NCSU) 37* in vitro *maturation (IVM) medium during the first 22 h of IVM (dbcAMP+), and in one group, there was no dbcAMP addition (dbcAMP−). After* in vitro *fertilization (IVF), presumed zygotes were cultured in NCSU 23. No differences were observed between both groups for all of the parameters illustrated on Days 6 and 7 ($P \geq 0.05$), except on Day 7 cleavage was borderline ($P = 0.05$). Three replicates were performed. dbcAMP: dibutyryl cAMP sodium salt.*

3.4.4 Effect of dbcAMP on ADAMTS-1 protein levels in cumulus cells

The ratio of the intensity levels of proADAMTS-1 after quantitative immunofluorescence differed between all four tested groups A−, A+, F− and F+ (1.00, 1.12, 1.20 and 1.34, respectively; $P < 0.05$; Figure 4). Both kinds of negative controls showed no detectable immunofluorescence signal.

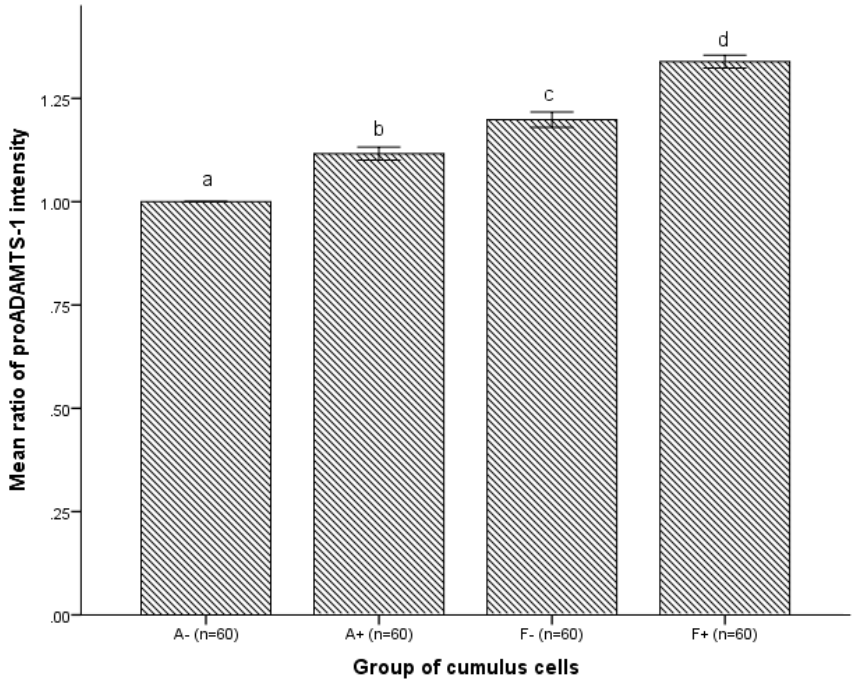


Figure 4: Intensity ratios of proADAMTS-1 protein levels in the different groups of cumulus cells. Quantitative immunofluorescence of proADAMTS-1 protein was performed on cumulus-oocyte complexes (COCs) matured without dibutyryl cAMP sodium salt (dbcAMP) addition during in vitro *maturation (IVM) (−) and with dbcAMP addition (+). In each treatment group, cumulus cells of adherent (A) and floating (F) COCs were investigated. The number of cumulus cells examined is given in parentheses next to the treatment group. Three replicates were performed. Error bars illustrate the standard error of the mean (SEM).* $^{a\text{-}d}$*The mean ratios of proADAMTS-1 intensity with different superscripts differ significantly ($P < 0.05$). ProADAMTS-1: proprotein of a disintegrin and metalloproteinase with thrombospondin-like repeats-1.*

3.5 Discussion

In our study we have shown that the nuclear morphology at the end of collection of immature porcine oocytes is not affected by addition of IBMX to the collection medium, indicating that progression to meiosis is not halted by IBMX addition to the collection medium. Although the collection period (140 min) is short in comparison to the IVM period of 44 h, we hypothesized a possible influence of IBMX during collection based on data from other species. The lack of effect of IBMX addition during collection in this study is in contrast to results obtained in other species like mouse and cattle. In the latter species, IBMX addition during collection increased cAMP levels during collection, inhibited GVBD and improved blastocyst rate (Bornslaeger *et al.* 1984; Luciano *et al.* 1999; Albuz *et al.* 2010). This points to a species-specific working mechanism of IBMX. This species-specific mechanism could mean that in pigs, GVBD possibly proceeds later than in the other discussed species. However, in earlier research (Fan *et al.* 2002), much higher concentrations (>1 mM) of IBMX were used to maintain the majority of porcine oocytes in GV arrest, we based the concentration (0.5 mM) in the present study on a more recent study of Shimada *et al*. (2003). The latter study showed that this concentration was the optimal concentration for synchronizing the GV stage during IVM (>80% at 20 h of IVM) and allowing the formation of LH receptors and the optimal hCG binding of COCs. Higher concentrations as 1 mM caused a suppressed LH receptor formation and a suppressed hCG binding (Shimada *et al.* 2003).

Although the nuclear progression was not affected, we must notice that there could still be the possibility that IBMX elevates intracellular cAMP levels and consequently improves the IVP process. However, Somfai *et al.* (2003) performed a 2 x 1 factorial design of the combination of invasive adenylate cyclase + IBMX and dbcAMP and examined the effect on nuclear progression, oocyte maturation, fertilization parameters and subsequent embryonic development by blastocyst rate and the cell number in each blastocyst. That study could not demonstrate any difference between groups treated with or without IBMX for each parameter. From our results and those of Somfai *et al.* (2003), we can suggest that omitting cAMP modulating agents during collection did not affect maturation and subsequent development after IVF. A possible explanation for the absence of effect of IBMX during collection could

be that possibly decreasing cAMP levels during collection do not harm the further development of the oocyte. Probably spontaneous maturation is not induced during collection, but during IVM.

Because our data are complementary to an earlier report on IBMX and pig oocyte maturation, we can conclude that IBMX addition to the collection medium did not significantly influence, either meiotic progression during maturation (Somfai *et al.* 2003) or nuclear morphology at the end of collection.

Addition of dbcAMP to the IVM medium resulted in a significantly lower polyspermy rate and consequently a higher normal fertilization rate (Table 1). From these results, we can deduce that dbcAMP addition is beneficial to obtain more normally fertilized zygotes within the penetrated oocytes. The detected decrease in polyspermy rate after the use of dbcAMP confirms the findings from Somfai *et al.* (2003) and Kim *et al.* (2008) but contrasts the study of Funahashi *et al.* (1997) who found a similar polyspermy rate. The lower penetration rate in our study conflicts the results of the three studies mentioned before where the same (Funahashi *et al.* 1997; Somfai *et al.* 2003) or a higher penetration rate (Kim *et al.* 2008) was found. The reason for these divergent results remains unclear. However, differences in the applied IVF protocols may contribute to variation in results between research groups. None of the previous mentioned studies applied exactly the same method and from that viewpoint, it is hard to make exact comparisons. Factors such as the IVF medium, sperm concentrations, ejaculated or epididymal, frozen or fresh semen or spermatozoa, IVF co-incubation time and the presence of cumulus cells during IVF vary considerably. Even the expression of the parameters like penetration and polyspermy rate is not always clearly defined what makes interpretation and comparison difficult. Nevertheless we can provide possible explanations for the obtained results in this study. The cause of a decrease in penetration rate in the presence of dbcAMP during preceding IVM of COCs could be due to an improved cumulus matrix constitution induced by dbcAMP. An improved cumulus matrix can act as a barrier for spermatozoa, leading to a lower penetration and polyspermy rate (Bijttebier *et al.* 2008). Many processes are involved in blocking polyspermy by the oocyte after fertilization such as cortical granules (CG) exocytosis (cortical reaction) and the zona reaction (Wang *et al.* 1998). Even factors from the oviduct may assist in the completion of these cortical and zona reactions (Wang *et al.* 1998). The improvement of blocking polyspermy by dbcAMP addition can act through several possible mechanisms (Somfai *et al.* 2003). First, the addition of

dbcAMP will induce a more homogeneous maturation of oocytes (Funahashi *et al.* 1997). Nuclear maturation and cytoplasmic maturation are related in several ways, including cortical granule migration (Sun *et al.* 2001). Thus, a more synchronized nuclear maturation could be the base for a better cytoplasmic maturation and consequently less polyspermy. A second possible mechanism by which polyspermy can be reduced could be based on the fact that the addition of dbcAMP causes a prolongation of the maintenance of the gap junctional communication between oocytes and cumulus cells (Flagg-Newton *et al.* 1981). In this way, cytoplasmic maturation is directly promoted by enhancing metabolism between both cell types. It is widely accepted that gap junctional communication is important for several aspects of cytoplasmic maturation, normal fertilization by transferring factors promoting removal of sperm nuclear envelopes and inflow of GSH from the cumulus cells to the oocytes (Mori *et al.* 2000) and embryonic development. A third possible way by which dbcAMP is helping to prevent polyspermy can be found in its action on plasminogen activator (PA). Dibutyryl cAMP sodium salt can induce an increase in PA concentration (Kim and Menino 1995). Tissue-type PA (tPA) could be involved in the block against polyspermy in parallel with findings in rats in which tPA is released during the cortical granule exocytosis and thus could have a possible role in the zona hardening as part of the defense against polyspermy (Zhang *et al.* 1992). A fourth possible explanation could be based on the same mechanism that is involved in reducing the penetration rate. In parallel with the findings of Bijttebier *et al.* (2008) we could expect a better protection against polyspermy because of the improved cumulus matrix quality by dbcAMP that prevents some spermatozoa to reach the oocyte.

The third part of the study investigated if addition of dbcAMP could improve cumulus expansion by upregulation of ADAMTS-1 and thus reduce the proportion of adherent COCs. A significantly higher percentage of adherent COCs was observed in the dbcAMP− group (Table 2). Moreover, we proved that dbcAMP is increasing the content of proADAMTS-1 in cumulus cells indicating a possible pathway on which dbcAMP is acting concerning cumulus quality (Figure 4). These findings confirm our hypothesis. Revealing this possible connection between dbcAMP addition and improved cumulus expansion is important to create new modulating possibilities of cumulus expansion during IVM. It is known that cumulus expansion is crucial for an optimal nuclear and cytoplasmic maturation and subsequent fertilization of the oocyte (Nagai *et al.* 1993; Tanghe *et al.* 2002). Hampered cumulus remodeling, followed by adhesion of the COC to the bottom of the dish, could possibly affect

maturation, fertilization and developmental competence of porcine oocytes. We presumed a link between cAMP levels and ADAMTS-1 because adding dbcAMP reduced the number of adherent COCs and because adhesion to the bottom of the dish can be explained by an inferior cumulus matrix disassembly by inadequate cleavage of versican by ADAMTS-1 (Wu *et al.* 2002; Russell *et al.* 2003; Shimada *et al.* 2004). This link between the level of intracellular cAMP and the function of ADAMTS-1 can also be found in research about parathyroid hormone (PTH) influence in bone. Miles *et al.* (2000) showed that in rat femur metaphysis, ADAMTS-1 mRNA expression is only upregulated by those PTH analogs that are capable of significantly elevating intracellular cAMP levels. Although PTH influences many signal transduction pathways, Miles *et al.* (2000) suggested that the upregulation of ADAMTS-1 expression by PTH, is primarily mediated by the cAMP/protein kinase A (PKA) pathway. Apart from the illustration of this connection in bone, little research was done to demonstrate the relationship between the cAMP/PKA signaling pathway and the expression of ADAMTS-1 in COCs. It is widely accepted that pituitary hormones such as LH are acting through the binding of ligand-specific cell surface G protein-coupled receptors, activation of adenylyl cyclase, and the subsequent production of cAMP (Richards 2001). Doyle *et al.* (2004) reported that the molecular mechanism, by which LH is inducing the expression of ADAMTS-1, operates through or independently from the progesterone receptor. Therefore, it is possible that the cAMP pathway affects the expression of ADAMTS-1 in granulosa cells in mice (Doyle *et al.* 2004). No literature was found about the relationship between ADAMTS-1 and cAMP levels in granulosa cells in pigs.

Our results showed for the first time that attachment of porcine COCs to the dish can be influenced by adding dbcAMP to the maturation medium and that dbcAMP addition is upregulating the proprotein level of ADAMTS-1 in cumulus cells in pigs (Figure 5).

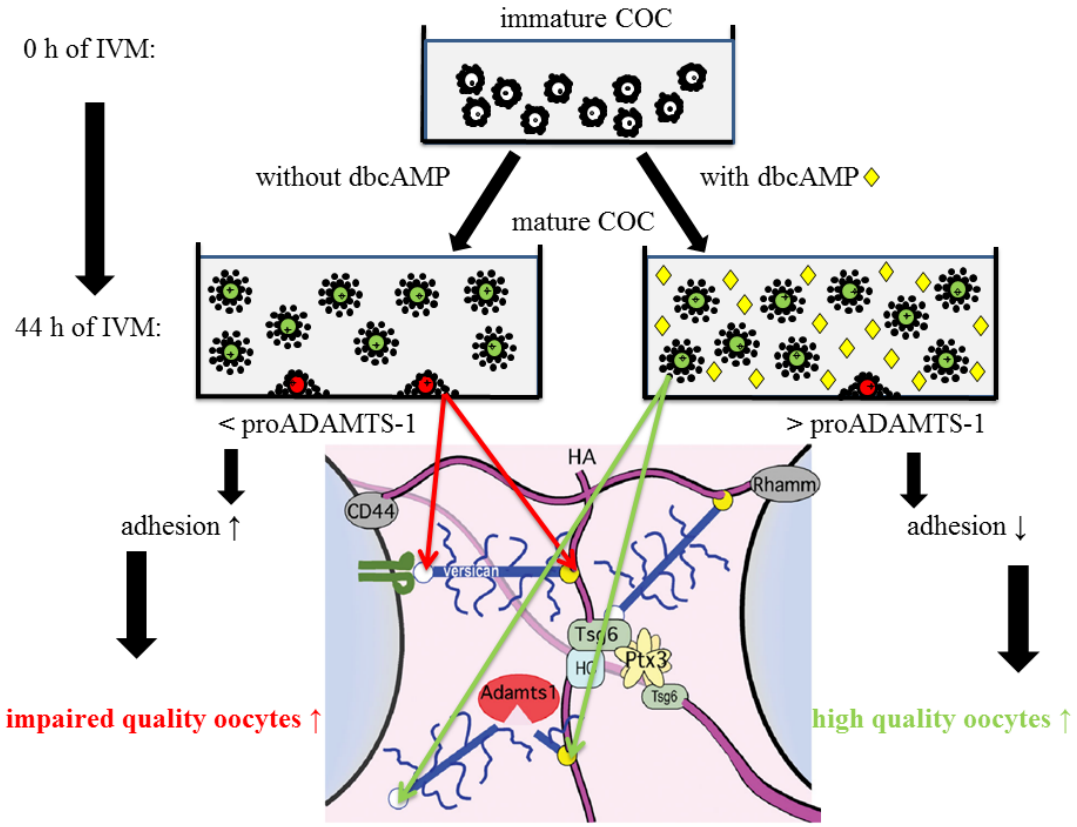


Figure 5: Relation between dibutyryl cAMP sodium salt (dbcAMP), the proprotein of a disintegrin and metalloproteinase with thrombospondin-like repeats-1 (proADAMTS-1), versican and adherent cumulus-oocyte complexes (COCs) during in vitro *maturation (IVM). Addition of dbcAMP upregulates proADAMTS-1, which promotes the cleavage of versican and thus prevents adherence of the COC to other cells or specific for* in vitro *circumstances to the bottom of the culture dish. Adherent COCs to the bottom of the dish have an impaired quality (red) in comparison to the floating ones (green) (Grupen* et al. *1997; Somfai* et al. *2004). The detailed part of the cumulus matrix composition is adapted from Russell and Robker (2007); other pictures by R. Appeltant.*

Revealing this dbcAMP-proADAMTS-1 relationship, it could be expected that higher levels of proADAMTS-1 result in higher levels of ADAMTS-1, an increased cleavage of versican and thus a lower number of adherent COCs due to an improved cumulus matrix disassembly after dbcAMP addition during the first part of IVM. Nevertheless, it must be remarked that adhesion is not totally prevented because several other factors are also determining the adhesion to the dish, e.g., the characteristics of the dish itself (Somfai *et al.* 2004).

Our results also showed that systematic removal of the adherent oocytes during IVM caused no significant difference in blastocyst rate on Day 7 between the dbcAMP− and the dbcAMP+ group (Table 2). Literature data mostly report a significant increase in blastocyst percentage when oocytes are matured in the presence of dbcAMP (Funahashi *et al.* 1997; Somfai *et al.* 2003). The lower blastocyst rate in the dbcAMP− group could be explained by the higher number of adherent oocytes. As adhesion of the cumulus cells to the culture dish has been associated with an impaired cumulus expansion, this inferior cumulus maturation could negatively affect fertilization outcome since the cumulus itself plays an important role during fertilization (Tanghe *et al.* 2002). Moreover, it has already been found that nuclear and cytoplasmic maturation are affected in oocytes attached to the bottom of the culture dish (Somfai *et al.* 2004). Adherent COCs resume meiosis and reach the metaphase II stage earlier than floating ones, probably caused by the disability of the cumulus cells to maintain the meiotic arrest (Somfai *et al.* 2004). As a consequence, those oocytes can manifest a certain degree of aging at the end of the maturation period (Somfai *et al.* 2004), which causes heterogeneity concerning cytoplasmic maturation (Somfai *et al.* 2003), an increased ability of the cytoplasm to initiate oocyte activation (Somfai *et al.* 2004), and a higher polyspermy rate (Grupen *et al.* 1997). From these findings, we can conclude that COCs attached to the bottom of the dish have an impaired oocyte quality. By removal of adherent COCs, the proportion of COCs that have the highest risk for inferior nuclear and cytoplasmic maturation are removed. In the present study, this selection of matured oocytes likely reduced the difference in blastocyst development from oocytes matured in medium with or without dbcAMP. Moreover, when the adherent COCs are removed during IVM, the dbcAMP− group reaches the same level of blastocyst development as the dbcAMP+ group. The difference in blastocyst percentage between dbcAMP-treated and non-treated oocytes in former research is only partially explicable by this factor because a tendency of higher blastocyst rate in the dbcAMP+ group remains even with removal of the adherent COCs. The trend to higher blastocyst rates after adding dbcAMP can partially be explained by the observed increased monospermic fertilization in experiment 2. Although Han *et al.* (1999) found that polyspermic embryos can develop to the blastocyst stage in the same rate as normal fertilized zygotes, Somfai *et al.* (2008) showed that the equal ability to develop to blastocyst only occurs when the polyspermic zygotes can achieve the first cleavage. Moreover, the impaired cytoplasmic maturation in adherent COCs will reduce the blastocyst percentage after IVC.

3.5.1 Conclusion

The use of 0.5 mM IBMX to the collection medium did not influence the state of the germinal vesicle of porcine oocytes at the end of collection. This indicates that IBMX is not capable of causing a meiotic arrest in collected oocytes. Addition of 1 mM dbcAMP in the medium during the first 22 h of IVM induced a significantly lower penetration rate accompanied by a significantly lower polyspermy rate after IVF, resulting in a significantly higher percentage of normally fertilized zygotes in pigs. An *in vitro* embryo production system for pigs using 1 mM dbcAMP during the first 22 h of IVM decreased adhesion of COCs to the bottom of the culture dish because of an upregulation of the proADAMTS-1 content in cumulus cells. No significant difference could be shown in the blastocyst percentage between dbcAMP− and dbcAMP+ groups when the adherent COCs were removed during IVM.

These clarifications in the effect of modulators of cAMP regulated processes during porcine IVP may help to improve the current applied IVP systems in order to obtain less polyspermic fertilization and a higher developmental competence of *in vitro* matured porcine oocytes.

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3.7 References

Abràmoff, M., Magalhães, P., and Ram, S. (2004) Image processing with ImageJ. *Biophotonics International* **11**(7), 36-43

Albuz, F.K., Sasseville, M., Lane, M., Armstrong, D.T., Thompson, J.G., and Gilchrist, R.B. (2010) Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Hum. Reprod.* **25**(12), 2999-3011

Anderson, E., and Albertini, D.F. (1976) Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *J. Cell Biol.* **71**(2), 680-686

Bagg, M.A., Nottle, M.B., Grupen, C.G., and Armstrong, D.T. (2006) Effect of dibutyryl cAMP on the cAMP content, meiotic progression, and developmental potential of in vitro matured pre-pubertal and adult pig oocytes. *Mol. Reprod. Dev.* **73**(10), 1326-1332

Bijttebier, J., Van Soom, A., Meyer, E., Mateusen, B., and Maes, D. (2008) Preovulatory follicular fluid during in vitro maturation decreases polyspermic fertilization of cumulus-intact porcine oocytes. *Theriogenology* **70**(4), 715-724

Bornslaeger, E., Mattei, P., and Schultz, R. (1986) Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. *Dev. Biol.* **114**(2), 453-462

Bornslaeger, E., Wilde, M.W., and Schultz, R. (1984) Regulation of mouse oocyte maturation: involvement of cyclic AMP phosphodiesterase and calmodulin. *Dev. Biol.* **105**(2), 488-499

Doyle, K.M.H., Russell, D.L., Sriraman, V., and Richards, J.S. (2004) Coordinate transcription of the ADAMTS-1 gene by luteinizing hormone and progesterone receptor. *Mol. Endocrinol.* **18**(10), 2463-2478

Fan, H.-Y., Li, M.-Y., Tong, C., Chen, D.-Y., Xia, G.-L., Song, X.-F., Schatten, H., and Sun, Q.-Y. (2002) Inhibitory effects of cAMP and protein kinase C on meiotic maturation and MAP kinase phosphorylation in porcine oocytes. *Mol. Reprod. Dev.* **63**(4), 480-487

Flagg-Newton, J., Dahl, G., and Loewenstein, W. (1981) Cell junction and cyclic AMP. I. Upregulation of junctional membrane permeability and junctional membrane parti¬cles by

administration of cyclic nucleotide or phosphodiesterase inhibitor. *J. Membr. Biol.* **63**(1-2), 105-121

Fraser, L., and Maudlin, I. (1978) Relationship between sperm concentration and the incidence of polyspermy in mouse embryos fertilized in vitro. *J. Reprod. Fertil.* **52**(1), 103-106

Funahashi, H., Cantley, T., and Day, B. (1997) Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. *Biol. Reprod.* **57**(1), 49-53

Gil, M.A., Cuello, C., Parrilla, I., Vazquez, J.M., Roca, J., and Martinez, E.A. (2010) Advances in swine in vitro embryo production technologies. *Reprod. Domest. Anim.* **45**, 40-48

Gilchrist, R. (2011) Recent insights into oocyte –follicle interactions provide opportunities for the development of new approaches to in vitro maturation. *Reprod. Fertil. Dev.* **23**(1), 23-31

Grupen, C.G., Nagashima, H., and Nottle, M.B. (1997) Asynchronous meiotic progression in porcine oocytes matured in vitro: a cause of polyspermic fertilization? *Reprod. Fertil. Dev.* **9**(2), 187-191

Han, Y., Abeydeera, L., Kim, J., Moon, H., Cabot, R., Day, B., and Prather, R. (1999) Growth retardation of inner cell mass cells in polyspermic porcine embryos produced in vitro. *Biol. Reprod.* **60**(5), 1110-1113

Kikuchi, K., Izaike, Y., Noguchi, J., Furukawa, T., Daen, F.P., Naito, K., and Toyoda, Y. (1995) Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged in vitro. *J. Reprod. Fertil.* **105**(2), 325-330

Kim, J.S., Cho, Y.S., Song, B.S., Wee, G., Park, J.S., Choo, Y.K., Yu, K., Lee, K.K., Han, Y.M., and Koo, D.B. (2008) Exogenous dibutyryl cAMP affects meiotic maturation via protein kinase A activation; it stimulates further embryonic development including blastocyst quality in pigs. *Theriogenology* **69**(3), 290-301

Kim, N., and Menino, A. (1995) Effects of stimulators of protein kinase A and C and modulators of phosphorylation on plasminogen activator activity in porcine oocyte-cumulus cell complexes during in vitro maturation. *Mol. Reprod. Dev.* **40**, 364-370

Leibfried-Rutledge, M., Critser, E., Eyestone, W., Northey, D., and First, N. (1987) Development potential of bovine oocytes matured in vitro or in vivo. *Biol. Reprod.* **36**(2), 376-383

Luciano, A.M., Pocar, P., Milanesi, E., Modina, S., Rieger, D., Lauria, A., and Gandolfi, F. (1999) Effect of different levels of intracellular cAMP on the in vitro maturation of cattle oocytes and their subsequent development following in vitro fertilization. *Mol. Reprod. Dev.* **54**(1), 86-91

Mateusen, B., Van Soom, A., Maes, D.G.D., Donnay, I., Duchateau, L., and Lequarre, A.S. (2005) Porcine embryo development and fragmentation and their relation to apoptotic markers: a cinematographic and confocal laser scanning microscopic study. *Reproduction* **129**(4), 443-452

Mehlmann, L.M., Jones, T.L.Z., and Jaffe, L.A. (2002) Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte. *Science* **297**(5585), 1343-1345

Miles, R., Sluka, J., Halladay, D., Santerre, R., Hale, L., Bloem, L., Thirunavukkarasu, K., Galvin, R., Hock, J., and Onyia, J. (2000) ADAMTS-1: a cellular disintegrin and metalloprotease with thrombospondin motifs is a target for parathyroid hormone in bone. *Endocrinology* **141**(12), 4533-4542

Mori, T., Amano, T., and Shimizu, H. (2000) Roles of gap junctional communication of cumulus cells in cytoplasmic maturation of porcine oocytes cultured in vitro. *Biol. Reprod.* **62**(4), 913-919

Nagai, T., Ding, J., and Moor, R. (1993) Effect of follicle cells and steroidogenesis on maturation and fertilization in vitro of pig oocytes. *J. Exp. Zool.* **266**(2), 146-151

Nagai, T., Funahashi, H., Yoshioka, K., and Kikuchi, K. (2006) Up to date of in vitro production of porcine embryos. *Front. Biosci.* **11**, 2565-2573

Petters, R., and Wells, K. (1993) Culture of pig embryos. *J. Reprod. Fertil. Suppl.* **48**, 61-73

Rath, D., Long, C., Dobrinsky, J., Welch, G., Schreier, L., and Johnson, L. (1999) In vitro production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. *J. Anim. Sci.* **77**(12), 3346-3352

Richards, J. (2001) New signaling pathways for hormones and cyclic adenosine 3′, 5′-monophosphate action in endocrine cells. *Mol. Endocrinol.* **15**(2), 209-218

Romar, R., Coy, P., Ruiz, S., Gadea, J., and Rath, D. (2003) Effects of oviductal and cumulus cells on in vitro fertilization and embryo development of porcine oocytes fertilized with epididymal spermatozoa. *Theriogenology* **59**(3), 975-986

Russell, D.L., Doyle, K.M.H., Ochsner, S.A., Sandy, J.D., and Richards, J.S. (2003) Processing and localization of ADAMTS-1 and proteolytic cleavage of versican during cumulus matrix expansion and ovulation. *J. Biol. Chem.* **278**(43), 42330-42339

Russell, D.L., and Robker, R.L. (2007) Molecular mechanisms of ovulation: co-ordination through the cumulus complex. *Hum. Reprod. Update* **13**(3), 289-312

Shimada, M., Nishibori, M., Isobe, N., Kawano, N., and Terada, T. (2003) Luteinizing hormone receptor formation in cumulus cells surrounding porcine oocytes and its role during meiotic maturation of porcine oocytes. *Biol. Reprod.* **68**(4), 1142-1149

Shimada, M., Nishibori, M., Yamashita, Y., Ito, J., Mori, T., and Richards, J.S. (2004) Down-regulated expression of A disintegrin and metalloproteinase with thrombospondin-like repeats-1 by progesterone receptor antagonist is associated with impaired expansion of porcine cumulus-oocyte complexes. *Endocrinology* **145**(10), 4603-4614

Somfai, T., Kikuchi, K., Onishi, A., Iwamoto, M., Fuchimoto, D.-i., Papp, g.B., Sato, E., and Nagai, T. (2004) Relationship between the morphological changes of somatic compartment and the kinetics of nuclear and cytoplasmic maturation of oocytes during in vitro maturation of porcine follicular oocytes. *Mol. Reprod. Dev.* **68**(4), 484-491

Somfai, T., Kikuchi, K., Onishi, A., Iwamoto, M., Fuchimoto, D., Papp, Á.B., Sato, E., and Nagai, T. (2003) Meiotic arrest maintained by cAMP during the initiation of maturation enhances meiotic potential and developmental competence and reduces polyspermy of IVM/IVF porcine oocytes. *Zygote* **11**(3), 199-206

Somfai, T., Ozawa, M., Noguchi, J., Kaneko, H., Karja, N.W.K., Fahrudin, M., Nakai, M., Maedomari, N., Dinnyés, A., Nagai, T., and Kikuchi, K. (2008) In vitro development of polyspermic porcine oocytes: relationship between early fragmentation and excessive number of penetrating spermatozoa. *Anim. Reprod. Sci.* **107**(1-2), 131-147

Sun, Q., Lai, L., Bonk, A., Prather, R., and Schatten, H. (2001) Cytoplasmic changes in relation to nuclear maturation and early embryo developmental potential of porcine oocytes: effects of gonadotropins, cumulus cells, follicular size and protein synthesis inhibition. *Mol. Reprod. Dev.* **59**, 192-198

Sun, X.-S., Liu, Y., Yue, K.-Z., Ma, S.-F., and Tan, J.-H. (2004) Changes in germinal vesicle (GV) chromatin configurations during growth and maturation of porcine oocytes. *Mol. Reprod. Dev.* **69**(2), 228-234

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., and de Kruif, A. (2002) Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol. Reprod. Dev.* **61**(3), 414-424

Tsafriri, A., Chun, S., Zhang, R., Hsueh, A., and Conti, M. (1996) Oocyte maturation involves compartmentalization and opposing changes of cAMP levels in follicular somatic and germ cells: studies using selective phosphodiesterase inhibitors. *Dev. Biol.* **178**(2), 393-402

Wang, W.H., Abeydeera, L.R., Prather, R.S., and Day, B.N. (1998) Morphologic comparison of ovulated and in vitro–matured porcine oocytes, with particular reference to polyspermy after in vitro fertilization. *Mol. Reprod. Dev.* **49**(3), 308-316

Wu, Y., Chen, L., Zheng, P.S., and Yang, B.B. (2002) Beta1-Integrin-mediated glioma cell adhesion and free radical-induced apoptosis are regulated by binding to a C-terminal domain of PG-M/versican. *J. Biol. Chem.* **277**(14), 12294-12301

Zhang, X., Rutledge, J., Khamsi, F., and Armstrong, D. (1992) Release of tissue-type plasminogen activator by activated rat eggs and its possible role in the zona reaction. *Mol. Reprod. Dev.* **32**(1), 28-32

Chapter 4

Method for collecting and immobilizing individual cumulus cells enabling quantitative immunofluorescence analysis of proteins.

Extended version based on:

Appeltant, R., Maes, D., and Van Soom, A. (2015) Method for collecting and immobilizing individual cumulus cells enabling quantitative immunofluorescence analysis of proteins. *Anal. Biochem.* 480, 31-33

4.1 Abstract

Most immunofluorescence methods rely on techniques dealing with a very large number of cells. However, when the number of cells in a sample is low (*e.g.*, when cumulus cells must be analyzed from individual cumulus-oocyte complexes), specific techniques are required to conserve, fix, and analyze cells individually. We established and validated a simple and effective method for collecting and immobilizing low numbers of cumulus cells that enables easy and quick quantitative immunofluorescence analysis of proteins from individual cells. To illustrate this technique, we stained proprotein of a disintegrin and metalloproteinase with thrombospondin-like repeats-1 (proADAMTS-1) and analyzed its levels in individual porcine cumulus cells.

4.2 Aims and results

Much research in life sciences and medicine is depending on protein biosynthesis studies (Hortin *et al.* 2006). Protein biosynthesis includes several regulatory steps like protein gene expression, protein transcription, protein translation and protein post-translational modifications. Each level contains a variety of biotechniques for analysis of the specific component in cells or fluids (Hortin *et al.* 2006). Protein expression can be judged by qualitative or quantitative biological analysis (Hortin *et al.* 2006). Detection of the presence of a specific protein (qualitative analysis) in cells is possible even when sample size is limited. However, the measurement of the level of expression (quantitative analysis) requires much larger cell numbers per sample. Despite major advances in biotechnology, most detection methods still need a considerable amount of biological tissue to be able to perform the analysis. For instance, quantitative analysis based on immunofluorescent flow cytometry is performed with tens of thousands of cells (Enright *et al.* 2003). Analysis by quantitative Western blotting requires less but still requires a few tens of cumulus-oocyte complexes (COCs) (Hussein *et al.* 2005; Dunning *et al.* 2007). In the context of our recent research on the proprotein of a disintegrin and metalloproteinase with thrombospondin-like repeats-1 (proADAMTS-1) protein levels in porcine cumulus cells (Appeltant *et al.* 2015), we were confronted with issues concerning practical feasibility for collecting the necessary amount of

material in each treatment group. In that study, the levels of proADAMTS-1 expression of cumulus cells of porcine COCs attached to the bottom of the dish and of floating complexes were compared. The little amount of material retrieved from the attached cumulus cells could hinder subsequent analysis in the Western blot system. Concerning quantitative immunofluorescence, most cell localization studies apply immunohistochemistry on tissue sections (Russell *et al.* 2003; Richards *et al.* 2005). Nevertheless, in our case the material to be studied was a limited number of free cumulus cells (sometimes only 100 cumulus cells per COC) after *in vitro* culture. In this way, the remaining option was quantitative immunocytochemistry. Most immunocytochemistry protocols use techniques such as centrifugation of cells (Yung *et al.* 2010), preparation of cell smears (Cordell *et al.* 1984), or manipulation of large, easily visible, non-attached entities like embryos in culture wells (Wydooghe *et al.* 2011). These techniques cannot be used efficiently when only a limited number of cells are available. Therefore immobilization of target cells during the fixation and staining procedure is necessary. Immobilization of COCs was already reported by the use of Cell Tak^{TM} Cell and Tissue Adhesive (Cell-Tak) coated coverslips (Hussein *et al.* 2005; Grupen and Armstrong 2010), polylysine-coated coverslips (Hernandez-Gonzalez *et al.* 2006) or laminin coated coverslips (Richards *et al.* 2005) to follow the entity during staining procedures. Since the focus in the latter studies was on the COC as a whole, the COC was attached to the coverslip but not processed further. However, when expression levels of proteins should be measured in individual cumulus cells by fluorescence intensity measurement, overlapping of cumulus cells must be avoided. Therefore, we have developed a method for obtaining a single layer of cumulus cells without overlapping, which allows quantitative immunofluorescent staining procedures and quick evaluation of individual cells. We applied this technique for the first time in porcine cumulus cells to stain the proADAMTS-1 protein content (Appeltant *et al.* 2015). To immobilize cumulus cells, COCs were rolled over a glass slide surface covered with the cell adherent Cell-Tak. Only a small area of the glass slide was coated with Cell-Tak as illustrated in Figure 1. In this area the cumulus cells concentration was approximately 207 cumulus cells/mm^2.

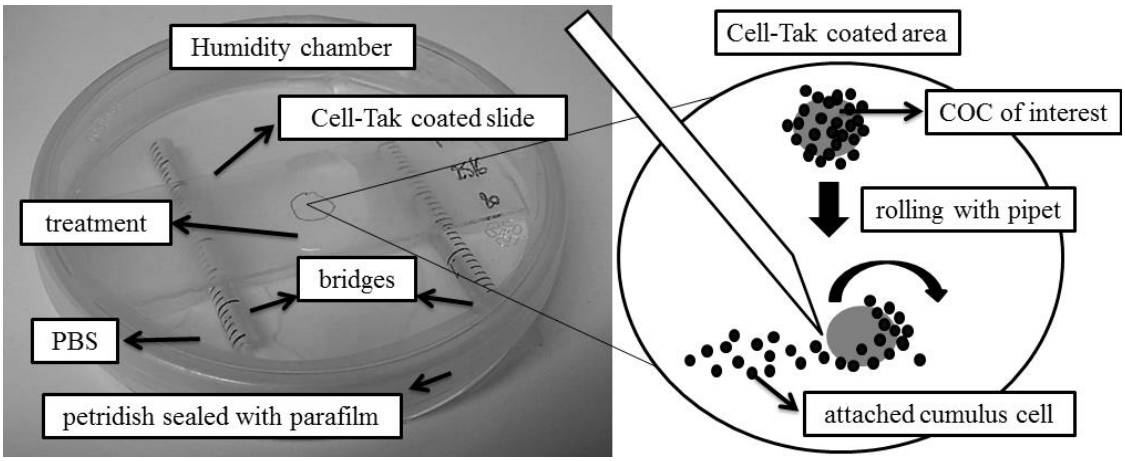


Figure 1: Illustration of the humidity chamber and a schematic overview of the collection method of cumulus cells through the adhesive power of Cell-Tak coated areas.

Before implementation, one of our concerns was if the Cell-Tak coating would be of adequate strength to maintain the attachment of the cumulus cells during the many washing and procedure steps. Since the cumulus cells were attached to the bottom of the slide, the removal of the volume covering the cells and replacing it with other working solutions had to be done by gentle pipetting. We observed that the cells were attached firmly enough to stay adherent during the entire staining procedure, which included fixation, permeabilization, blocking, incubation with primary and secondary antibodies and several washings.

The result of processing cumulus cells throughout the immobilization and staining protocol is illustrated in Figure 2.

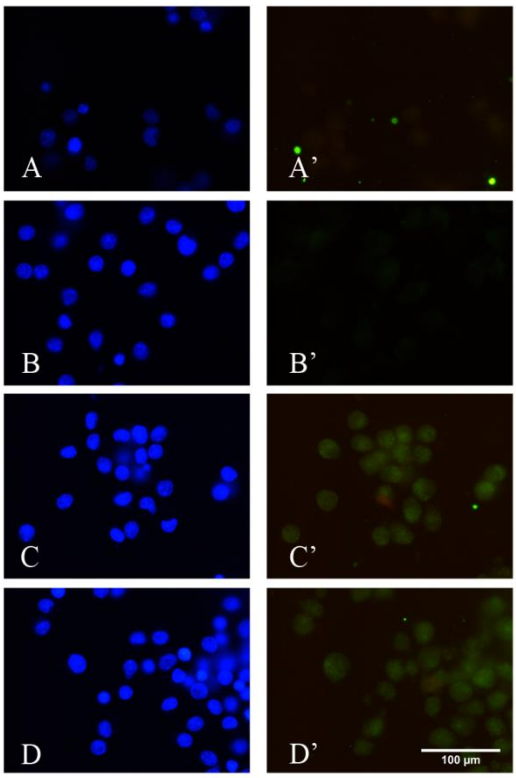


Figure 2: ProADAMTS-1 levels in cumulus cells. The same cumulus cells were observed with Hoechst 33342 (A, B, C and D) and indirect FITC-labeled proADAMTS-1 (A', B', C' and D'). A-A': the negative control using rabbit immunoglobulin G whole molecule. B-B': the negative control using PBS instead of permeabilization. Quantitative immunofluorescence of proADAMTS-1 was performed on cumulus–oocyte complexes matured without dbcAMP addition (C-C') and with dbcAMP addition (D-D') during in vitro *maturation (IVM). The difference in intensity levels between C' and D' was objectively measured by ImageJ software. ProADAMTS-1: proprotein of a disintegrin and metalloproteinase with thrombospondin-like repeats-1; FITC: fluorescein isothiocyanate; PBS: phosphate buffered saline; dbcAMP: dibutyryl cAMP sodium salt.*

Fluorescent signal was detectable in cumulus cells cultured either in the absence (dbcAMP−) or presence (dbcAMP+) of the proADAMTS-1 agonist dibutyryl cAMP sodium salt (dbcAMP) (Doyle *et al.* 2004; Appeltant *et al.* 2015) (Figure 2 C' and D'). Both kinds of negative controls (detailed below) showed no detectable immunofluorescence signal (Figure 2 A' and B'), which verified that the immunostaining was specific to intracellular

proADAMTS-1. Furthermore, Cell-Tak coating did not appear to interfere with measuring intensity levels of the proADAMTS-1 staining since background staining was not observed (Figure 2). In the positive control group (dbcAMP+) the relative intensity levels of proADAMTS-1 after quantitative immunofluorescence were significantly higher than the intensity levels of the cumulus cells of COCs in the dbcAMP− group (1.12 and 1.00, respectively; $P < 0.05$).

In conclusion, we established and validated a simple and effective method for collecting and immobilizing low numbers of cells, which enables easy and quick quantitative immunofluorescence analysis of proteins from individual cells.

4.3 Methods

4.3.1 Chemicals

Unless otherwise stated, all chemicals used were obtained from Sigma–Aldrich (Bornem, Belgium).

4.3.2 Collection and *in vitro* maturation

Porcine COCs were collected and *in vitro* matured as described by our previous study (Appeltant *et al.* 2015). Briefly, ovaries of prepubertal gilts were collected in a local abattoir and transported to the laboratory at 37 °C in a 0.9% NaCl solution supplemented with kanamycin. The medium for collection and washing of the oocytes was a modified HEPES-buffered Tyrode balanced salt solution. Antral follicles of 3 to 6 mm were aspirated to collect the COCs. After aspiration, the oocyte selection started with a screening for those oocytes that had a multilayered compact cumulus and a homogeneous ooplasm. The basic maturation medium was bovine serum albumin (BSA)-free North Carolina State University (NCSU) 37

(Petters and Wells 1993) supplemented with 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 50 µM β-mercaptoethanol, 10 IU/mL equine chorionic gonadotropin (eCG) (Folligon®, Intervet, The Netherlands), 10 IU/mL human chorionic gonadotropin (hCG) (Chorulon®, Intervet, The Netherlands) and 10% porcine follicular fluid. Groups of 50 to 55 oocytes were incubated into 500 µL maturation medium, supplemented with eCG and hCG for the first 22 h (38.5 °C, 5% CO_2 in air). For the next 22 h, the oocytes were matured in 500 µL hormone free maturation medium (38.5 °C, 5% CO_2 in air).

4.3.3 Preparation and use of a humidity chamber

We developed a closed system where the slides could be kept moist and that enabled easy handling during the whole fixation and staining period. The humidity chamber consisted of a 100 mm diameter plastic dish, where two kinds of bridges were set up by the aid of pieces of a 1 mL serological pipette attached by tape (Figure 1). In the beginning of the fixation procedure, 3 mL of phosphate buffered saline (PBS; Gibco, Ghent, Belgium) was added to the bottom of the dish, which provided humidity during the whole staining procedure. In this humidity chamber, the glass slides used for sampling were placed on the bridges above the PBS level. Moreover, the raising of the slide allowed easy manipulation of the slide during staining procedures. When slides are needed to be stored for up to several weeks, the dish can be sealed with parafilm to avoid evaporation.

4.3.4 Preparation and use of slides with Cell-Tak coating

The coating of slides with Cell-Tak was performed based on descriptions in literature (Stokes *et al.* 2005; Matoba *et al.* 2010). In brief, a working solution of Cell-Tak was prepared by adding 0.1 M $NaHCO_3$ (pH 8.0) to an equal volume of Cell-Tak (formulation of polyphenolic proteins extracted from the marine mussel *Mytilus edulis* 1.33 mg/mL in 5% acetic acid; BD Bioscience, Bedford, MA, USA). Five µL Cell-Tak working solution was applied to coat a small surface of the slide that would serve as working area. After drying for 20 min, the

coated surface was washed three times with sterile water (10 µL each) and dried completely. These slides were prepared the night before use and stored at 4 °C in the humidity chamber (Figure 1).

Because ADAMTS-1 is secreted by cumulus cells into the extracellular matrix and the proprotein is located in secretory vesicles in the cumulus cells (Russell *et al.* 2003), the proprotein in the cumulus cells was stained. Cumulus cells were collected at 40 h of IVM because it was proven that the proprotein was present at that time of IVM (Shimada *et al.* 2004). Collection of the cumulus cells from floating COCs started with removing the COCs from the IVM well and washing those complexes in Medium 199 HEPES (M199 HEPES; with Hank's salts; Gibco, Ghent, Belgium). They were treated with a 0.1% hyaluronidase solution during 30 to 60 s to release the cumulus cells from the sticky cumulus matrix cloud and were washed in PBS. To mount the cumulus cells on the slide, the Cell-Tak coated area was covered with 30 µL PBS. To make cumulus cells stick to the Cell-Tak coated area of the slide, a narrow-bore pipette was used to press them gently to the bottom of the drop of PBS. Then, the narrow-bore pipette was used to roll the COC gently over the Cell-Tak coated area without overlapping of cumulus cells with each other or with the oocyte (Figure 1). Consequently, the cumulus cells stayed attached to the bottom. In this way, we could strip the cumulus cells layer by layer from the entire oocyte resulting in a surface covered with a single layer of cumulus cells attached to the Cell-Tak coated glass slide, which could subsequently be processed for immunostaining.

When COCs are firmly attached to the bottom of the culture dish a slightly different method was used for the removal of cells from culture dishes and for the subsequent fixation of the samples. After removal of floating COCs, the maturation medium covering the attached complexes was removed by a narrow-bore pipette and replaced by PBS. Subsequently, PBS was removed in the same way and again replaced by fresh PBS to wash the attached complexes. Then, the attached cumulus cells of COCs were scraped from the bottom of the culture dish by a fine glass pipette with a diameter of approximately 200 µm and with rounded edges in order not to harm the cells. Consequently, the collected cumulus cells were placed in the 30 µL of PBS on the Cell-Tak coated slide. Furthermore, the associated COCs of those attached cumulus cells were also scraped from the bottom of the culture dish and were treated in the same way as those of the floating complexes described above to immobilize them on the Cell-Tak coated slide area.

4.3.5 Assessment of proADAMTS-1 protein levels in cumulus cells

To fix immobilized cells for immunostaining, paraformaldehyde (PF) was applied in two steps. First, 100 µL of 3% PF was gently added to the PBS drop without disturbing the attachment of the cumulus cells. Second, an additional 200 µL of 3% PF was added to the existing drop. Cumulus cells were incubated in 3% PF for 20 to 30 min at room temperature (RT). The concentration of 3% PF was based on the findings of Luque *et al.* (2003) that 3% PF fixation was superior over 2% methanol fixation for ADAMTS-1 immunofluorescence. After the immobilization and fixation of the cumulus cells, the cumulus cells were washed in PBS containing 0.5% BSA (PBS-BSA). Permeabilization was performed by incubating the cumulus cells in 0.5% Triton X-100 and 0.05% Tween 20 in PBS (Wydooghe *et al.* 2011) for 1 h at 4 °C. The cumulus cells were washed in PBS-BSA and blocked overnight at 4 °C using 30% goat serum and 0.15% Tween 20. The next day, cumulus cells were washed in PBS-BSA and incubated overnight at 4 °C with primary antibodies (H-60; Santa Cruz Biotechnology, Inc.) (4 µg/mL in blocking solution). After washing, the cells were incubated with fluorescein isothiocyanate (FITC) labeled goat anti-rabbit antibodies (Molecular Probes, Ghent, Belgium) for 1 h at RT in the dark (10 µg/mL in blocking solution). After another washing step, nuclei were stained with Hoechst 33342 by incubating them for 10 min in the dark at RT.

To verify the specificity of the staining, two kinds of negative controls were included. First, the contribution of non-specific staining of primary antibodies was evaluated by substitution of the primary antibody by rabbit immunoglobulin G whole molecule (Rockland Immunochemicals Inc., Gilbertsville, PA 19525, USA) (Figure 2 A-A'). Second, the intra-cytoplasmic localization of proADAMTS-1 was verified by replacing the permeabilization solution by PBS (Figure 2 B-B'). As dbcAMP can be considered as an activator of proADAMTS-1 levels in cumulus cells (Appeltant *et al.* 2015), in one group dbcAMP was added to the medium during the first 22 h of IVM. This dbcAMP+ group served as a positive control for both the cumulus cells of attached and the cumulus cells of floating COCs.

To evaluate the immunofluorescence signal a DMR microscope (Leica microsystems, Brussels, Belgium) equipped with excitation filters BP 360/40 nm and BP 450-490 nm, suppression filters BP 470/40 nm and LP 515 nm, and a 100 W mercury lamp was used. Since evaluation of differences in proADAMTS-1 intensity levels with the naked eye is too

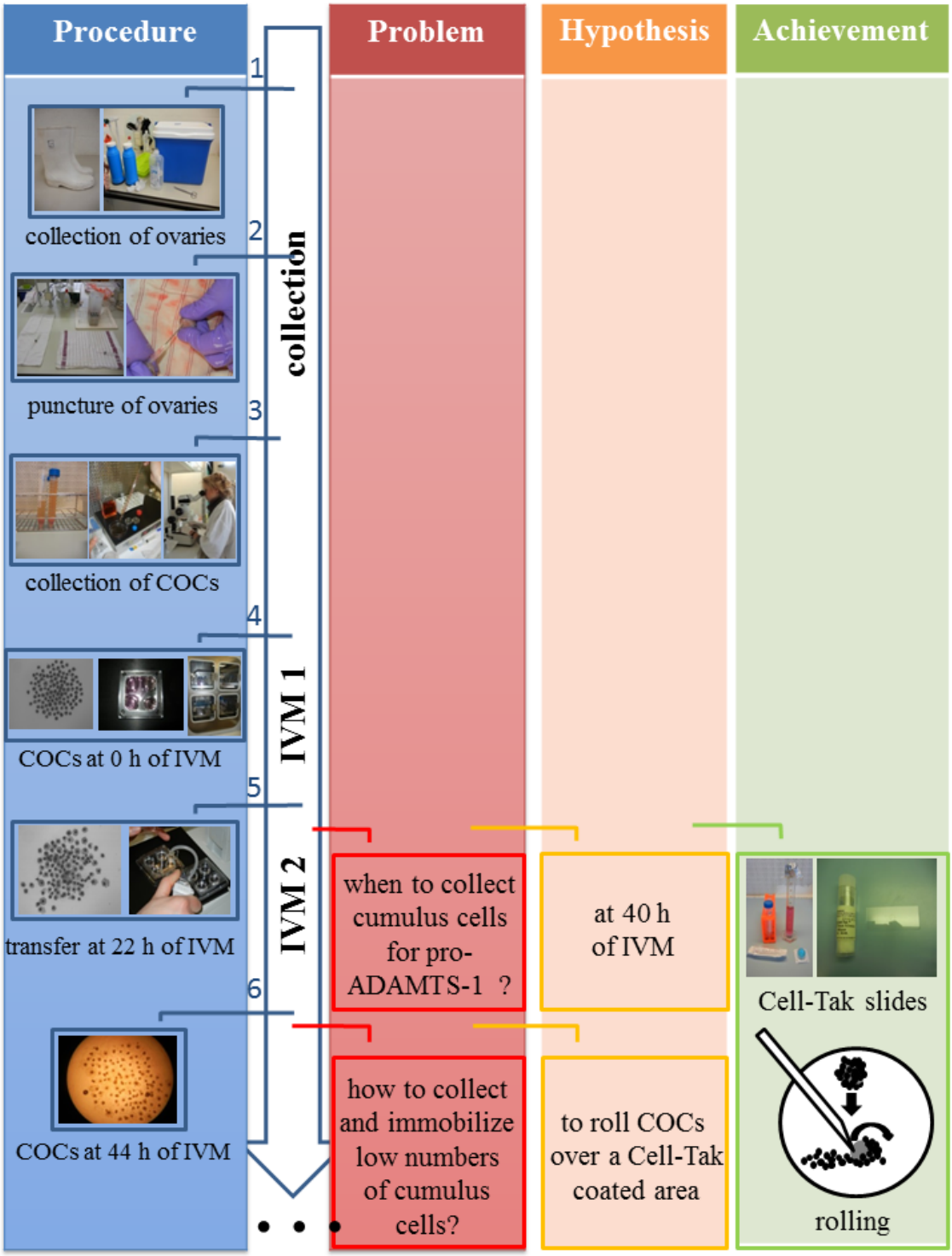
subjective, we processed the samples by digital photography and by National Institutes of Health (NIH) ImageJ (version 1.47) software (Abràmoff *et al.* 2004). In this way an objective assessment of differences in intensity levels between groups could be obtained. Digital images were captured by LAS version 4.1 (Leica, Van Hopplynus N.V., Brussels, Belgium) applying the same settings for each picture (exposure time 1.4 s). To minimize photobleaching, only two pictures per slide were taken. Every picture was captured by a fixed procedure. The cells were searched with the Hoechst filter cube (A4). Because the maximum excitation and emission of FITC (490 nm and 525 nm, respectively) are not overlapping with the maximum excitation and emission (350 nm and 461 nm, respectively) of Hoechst 33342, this cell searching with the Hoechst filter cube is not influencing the intensity level of FITC. Consistently, the shutter was closed before switching to the FITC filter cube (I3). When all the picture settings were ready to use, the shutter was opened, a picture was taken and thereafter the shutter was closed immediately. Subsequently, the same steps were repeated to take a second picture in a different area on the same slide. The expression of proADAMTS-1 was determined by the measurement of mean values of fluorescence intensity (given in pixels) in cells from those digital images using the NIH ImageJ (version 1.47) software. Briefly, color images were converted to 16 bit grayscale images. Measurement area for each cell was marked by the "threshold" function of the software and pixel density in each measurement area was assayed by the "measure" function. Relative expression levels of proADAMTS-1 in each group were defined as a ratio of their mean fluorescent intensity with that of the dbcAMP− group that was considered as value "1". In each group, 20 cells were measured per replicate and three replicates were performed. Therefore, in total, 60 cells were examined per group.

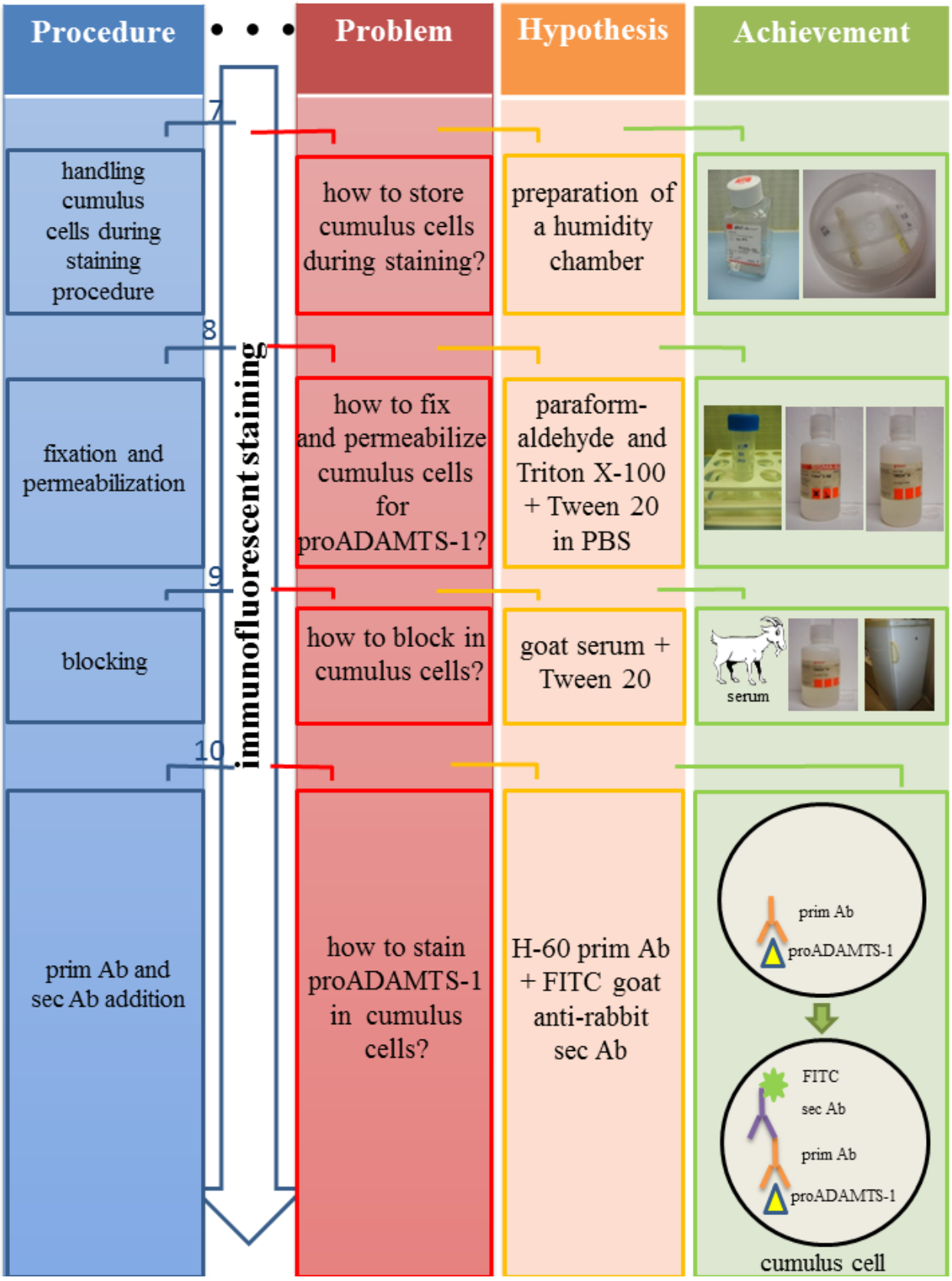
4.3.6 Statistical analysis

Statistical analysis was performed using SPSS Statistics version 20. In the statistical model, $P < 0.05$ was considered as statistically significant (two-sided test). Kruskal Wallis one-way ANOVA (k samples) was used because the content of proADAMTS-1 in cumulus cells was normally distributed, but the equality of error variances could not be assumed. As *post-hoc*, the Mann-Whitney U (2 samples) test was applied. Three replicates were performed.

4.4 Schematic overview

Figure 3 illustrates an overview of the procedures, the stated problems, hypotheses and achievements of the new developed method to collect and immobilize individual cells for immunofluorescent staining. This technique was illustrated by the staining of proADAMTS-1.




Procedure
Problem
Hypothesis
Achievement
7
handling cumulus cells during staining procedure
how to store cumulus cells during staining?
preparation of a humidity chamber
8
fixation and permeabilization
how to fix and permeabilize cumulus cells for proADAMTS-1?
paraform-aldehyde and Triton X-100 + Tween 20 in PBS
9
blocking
how to block in cumulus cells?
goat serum + Tween 20
serum
10
prim Ab and sec Ab addition
how to stain proADAMTS-1 in cumulus cells?
H-60 prim Ab + FITC goat anti-rabbit sec Ab
prim Ab
proADAMTS-1
FITC
sec Ab
prim Ab
proADAMTS-1
cumulus cell
immunofluorescent staining

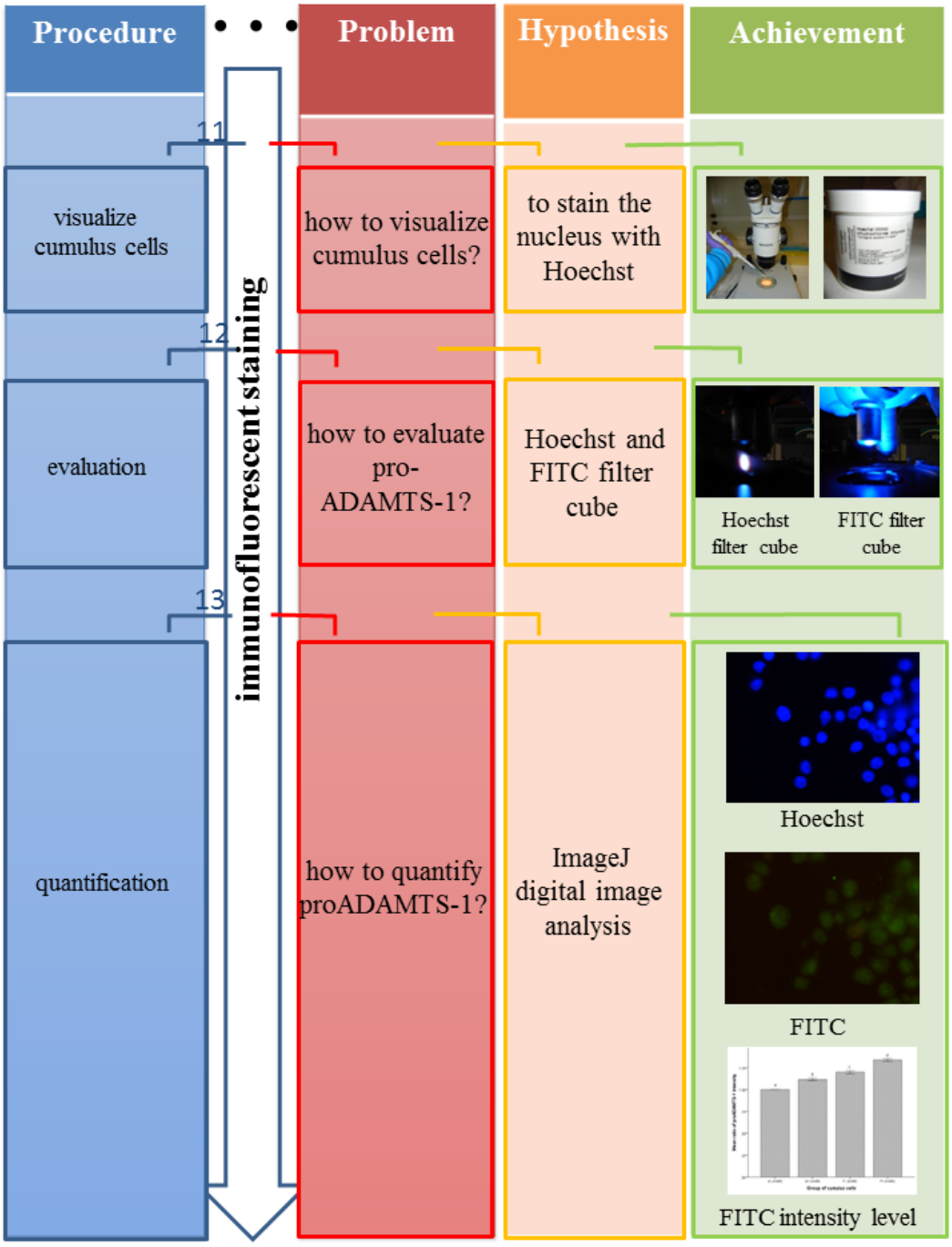


Figure 3: Schematic overview of the developed protocol for collection and immobilization of individual cells for immunofluorescent staining of proADAMTS-1. COC: cumulus-oocyte complex; IVM: in vitro *maturation; proADAMTS-1: proprotein of a disintegrin and metalloproteinase with thrombospondin-like repeats-1; PBS: phosphate buffered saline; Ab: antibody; prim: primary; sec: secondary; FITC: fluorescein isothiocyanate. Photographs and pictures by R. Appeltant.*

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4.6 References

Abràmoff, M., Magalhães, P., and Ram, S. (2004) Image processing with ImageJ. *Biophotonics International* **11**(7), 36-43

Appeltant, R., Beek, J., Vandenberghe, L., Maes, D., and Van Soom, A. (2015) Increasing the cAMP concentration during in vitro maturation of pig oocytes improves cumulus maturation and subsequent fertilization in vitro. *Theriogenology* **83**, 344-352

Cordell, J.L., Falini, B., Erber, W.N., Ghosh, A.K., Abdulaziz, Z., MacDonald, S., Pulford, K.A., Stein, H., and Mason, D.Y. (1984) Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J. Histochem. Cytochem.* **32**(2), 219-229

Doyle, K.M.H., Russell, D.L., Sriraman, V., and Richards, J.S. (2004) Coordinate transcription of the ADAMTS-1 gene by luteinizing hormone and progesterone receptor. *Mol. Endocrinol.* **18**(10), 2463-2478

Dunning, K.R., Lane, M., Brown, H.M., Yeo, C., Robker, R.L., and Russell, D.L. (2007) Altered composition of the cumulus-oocyte complex matrix during in vitro maturation of oocytes. *Hum. Reprod.* **22**(11), 2842-2850

Enright, B.P., Jeong, B.S., Yang, X., and Tian, X.C. (2003) Epigenetic characteristics of bovine donor cells for nuclear transfer: levels of histone acetylation. *Biol. Reprod.* **69**(5), 1525-1530

Grupen, C.G., and Armstrong, D.T. (2010) Relationship between cumulus cell apoptosis, progesterone production and porcine oocyte developmental competence: temporal effects of follicular fluid during IVM. *Reprod. Fertil. Dev.* **22**, 1100-1109

Hernandez-Gonzalez, I., Gonzalez-Robayna, I., Shimada, M., Wayne, C.M., Ochsner, S.A., White, L., and Richards, J.S. (2006) Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: Does this expand their role in the ovulation process? *Mol. Endocrinol.* **20**(6), 1300-1321

Hortin, G.L., Jortani, S.A., Ritchie, J.C., Valdes, R., and Chan, D.W. (2006) Proteomics: a new diagnostic frontier. *Clin. Chem.* **52**(7), 1218-1222

Hussein, T.S., Froiland, D.A., Amato, F., Thompson, J.G., and Gilchrist, R.B. (2005) Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. *J. Cell Sci.* **118**(22), 5257-5268

Luque, A., Carpizo, D.R., and Iruela-Arispe, M.L. (2003) ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and sequestration of VEGF165. *J. Biol. Chem.* **278**(26), 23656-23665

Matoba, S., Fair, T., and Lonergan, P. (2010) Maturation, fertilisation and culture of bovine oocytes and embryos in an individually identifiable manner: a tool for studying oocyte developmental competence. *Reprod. Fertil. Dev.* **22**(5), 839-851

Petters, R., and Wells, K. (1993) Culture of pig embryos. *J. Reprod. Fertil. Suppl.* **48**, 61-73

Richards, J.S., Hernandez-Gonzalez, I., Gonzalez-Robayna, I., Teuling, E., Lo, Y., Boerboom, D., Falender, A.E., Doyle, K.H., LeBaron, R.G., Thompson, V., and Sandy, J.D. (2005) Regulated expression of ADAMTS family members in follicles and cumulus oocyte complexes: evidence for specific and redundant patterns during ovulation. *Biol. Reprod.* **72**(5), 1241-1255

Russell, D.L., Doyle, K.M.H., Ochsner, S.A., Sandy, J.D., and Richards, J.S. (2003) Processing and localization of ADAMTS-1 and proteolytic cleavage of versican during cumulus matrix expansion and ovulation. *J. Biol. Chem.* **278**(43), 42330-42339

Shimada, M., Nishibori, M., Yamashita, Y., Ito, J., Mori, T., and Richards, J.S. (2004) Down-regulated expression of A disintegrin and metalloproteinase with thrombospondin-like repeats-1 by progesterone receptor antagonist is associated with impaired expansion of porcine cumulus-oocyte complexes. *Endocrinology* **145**(10), 4603-4614

Stokes, P.J., Abeydeera, L.R., and Leese, H.J. (2005) Development of porcine embryos in vivo and in vitro; evidence for embryo 'cross talk' in vitro. *Dev. Biol.* **284**(1), 62-71

Wydooghe, E., Vandaele, L., Beek, J., Favoreel, H., Heindryckx, B., De Sutter, P., and Van Soom, A. (2011) Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining. *Anal. Biochem.* **416**(2), 228-230

Yung, Y., Maman, E., Konopnicki, S., Cohen, B., Brengauz, M., Lojkin, I., Dal Canto, M., Fadini, R., Dor, J., and Hourvitz, A. (2010) ADAMTS-1: a new human ovulatory gene and a cumulus marker for fertilization capacity. *Mol. Cell. Endocrinol.* **328**(1-2), 104-108

Chapter 5

Interactions between oocytes and cumulus cells during *in vitro* maturation of porcine cumulus-oocyte complexes in a chemically defined medium: effect of denuded oocytes on cumulus expansion and oocyte maturation

Adapted from:

Appeltant, R., Somfai, T., Nakai, M., Bodó, S., Maes, D., Kikuchi, K., and Van Soom, A. (2015) Interactions between oocytes and cumulus cells during *in vitro* maturation of porcine cumulus-oocyte complexes in a chemically defined medium: effect of denuded oocytes on cumulus expansion and oocyte maturation. *Theriogenology* **83**, 567-576

5.1 Abstract

The aim of the present study was to clarify interactions between oocytes and cumulus cells on the level of cumulus expansion and oocyte maturation during IVM of cumulus-oocyte-complexes (COCs) in a chemically defined medium using a system that allows individual tracking of oocytes. Especially the influence of oocyte-secreted factors was investigated by the aid of addition of denuded oocytes (DOs), as a possible approach to improve the IVM system. The basic maturation medium was porcine oocyte medium (POM) with addition of gonadotropins only during the first 20 h of IVM. During IVM, COCs were kept fixed to the bottom of culture dish by adhesive Cell-Tak coating, which enabled individual tracking of COCs during IVM. Size changes of COCs during IVM were measured by digital image analysis. Cumulus expansion in POM of intact COCs increased in a typical manner until 20 h and decreased in size subsequently until 48 h of IVM ($P < 0.05$). Removal of oocytes from COCs by oocytectomy allowed the expansion of cumulus cells to some extent, although their expansion ability was lower than that of COCs ($P < 0.05$). Addition of DOs (COCs to DOs ratio of 9:16) did not improve cumulus expansion and oocyte maturation rates of intact COCs ($P \geq 0.05$) but did enhance cumulus expansion of oocytectomized complexes ($P < 0.05$). Furthermore, removal of cumulus cells before IVM increased oocyte maturation rates compared with COCs (52.3% and 32.9%, respectively) ($P < 0.05$) and a similar effect was observed in COCs when the gap junction inhibitor carbenoxolone was added to the IVM medium: carbenoxolone repressed the expansion of COCs at 20 h of IVM. In conclusion, the porcine oocyte enhances cumulus expansion both by gap junctional communications and presumably by oocyte-secreted factor production. Nevertheless, the presence of oocytes is not a prerequisite for this process. In return, cumulus cells maintain meiotic arrest in cumulus-enclosed oocytes during the initial culture through gap junctions. On the basis of these findings, future research could investigate if co-culture with DOs during IVM is beneficial for fertilization and embryo development.

5.2 Introduction

During the last decade, major advances have been made in the development of *in vitro* maturation (IVM) systems for porcine oocytes, which allow 20 to 30% of cumulus-enclosed immature oocytes to develop to the blastocyst stage (Gil *et al.* 2010). However, porcine IVM systems still require further improvement because the efficacy of embryo development is lower compared to IVM systems in cattle (30 to 40% (Rizos *et al.* 2002)). Moreover, not only higher quantities but also better quality of the produced embryos is required. Especially the development of IVM systems for oocytes in small groups is needed because reduced numbers of oocytes used for group culture decrease maturation efficiency (O'Doherty *et al.* 1997). Such a system is essential when oocytes from specific donor animals are to be matured in an identifiable manner, because they are allocated in groups according to the donor, leading to variations in oocyte number from one to 50.

A better understanding of the interactions between follicle cells and oocytes is essential for the improvement of IVM systems. One of the possible approaches is the utilization of oocyte-secreted factors (OSFs). This approach is based on the bidirectional communication between the oocyte and the surrounding cells such as granulosa cells and cumulus cells (Buccione *et al.* 1990a). During this process, oocytes regulate cumulus cell differentiation *via* the secretion of soluble paracrine growth factors (aka OSF), which play important roles in regulation of the folliculogenesis (Gilchrist *et al.* 2008). Hussein *et al.* (2006) proved already in cattle that co-culture of cumulus-oocyte-complexes (COCs) with denuded oocytes (DOs) or treatment with specific OSFs enhanced oocyte developmental competence. On the contrary, in pigs, only a few studies have aimed to reveal the effects of oocytes and OSFs on cumulus expansion, nuclear maturation of other oocytes, and their developmental competence with conflicting results. Some authors have demonstrated that the presence of the oocyte enhances cumulus expansion (Singh *et al.* 1993; Vanderhyden 1993), whereas others reported that removal of oocytes from COCs does not affect cumulus expansion during IVM (Prochazka *et al.* 1991). To date, the effects of addition of DOs to the media or use of media conditioned with DOs on nuclear maturation and post-fertilization developmental competence of oocytes in a defined medium remain unknown. On the other hand, using a non-defined North Carolina State University 23 medium supplemented with porcine follicular fluid (pFF) described repressed

cumulus expansion during IVM of COCs in the presence of DOs, which was associated with improved developmental competence (Gomez *et al.* 2012). All the previously mentioned studies were based on non-defined culture media supplemented either with serum or pFF often not specifically designed for the IVM of porcine oocytes. In addition, cumulus expansion was evaluated by subjective observation and grading. These protein supplementations exert adverse effects on cumulus expansion as pFF is known to enhance and serum may suppress cumulus expansion in pigs (Bijttebier *et al.* 2009). Unknown factors in sera and pFF can potentially interact with oocytes and cumulus cells (Pinyopummintr and Bavister 1991; Reed *et al.* 1993; Yoshioka *et al.* 2008), which makes interpretation of results aiming to understand the interaction between these cells difficult. Therefore, the use of defined media is necessary for this purpose. Recently, a chemically defined maturation medium called porcine oocyte medium (POM) has been developed, which enables porcine IVM without serum or pFF supplementation with acceptable nuclear maturation rates (Yoshioka *et al.* 2008).

The aim of the present study was to clarify interactions between oocytes and cumulus cells during IVM using a set-up for small groups of COCs in chemically defined POM medium. Because OSFs play a crucial role in cumulus expansion in mouse and cattle (Dragovic *et al.* 2005; Hussein *et al.* 2006) and, in turn, cumulus plays an important role for nuclear and cytoplasmic maturation of the oocyte (Tanghe *et al.* 2002), we hypothesized a potential positive influence of adding DOs during maturation on cumulus expansion and nuclear maturation of co-cultured COCs. Furthermore, we investigated the importance of the existence of the oocyte and gap junctions (GJs) between cumulus and oocytes on cumulus expansion and oocyte maturation both by oocytectomy and the use of GJs inhibitors.

5.3 Materials and methods

5.3.1 Oocyte collection and *in vitro* maturation

Ovaries (20-30) of prepubertal crossbred gilts (Landrace x Large White) were collected in a local abattoir and transported to the laboratory at 35 °C in a Dulbecco's phosphate buffered saline (PBS) within 2 h. Randomization was applied at the level of collection of the ovaries and at the level of collection of the COCs. Cumulus-oocyte complexes were collected by dissection of 2 to 6 mm follicles in Medium 199 (M199 with Hank's salts; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% (v:v) fetal bovine serum (Gibco, Life technologies, Carlsbad, CA, USA), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), 100 IU/mL penicillin G potassium (Sigma-Aldrich), and 0.1 mg/mL streptomycin sulfate (Sigma-Aldrich). After dissection, the COC selection started with a screening for those oocytes that had a multilayered compact cumulus and a homogeneous ooplasm. The basic medium for IVM was POM (Yoshioka *et al.* 2008) supplemented with 3 mg/mL polyvinyl alcohol. During the first 20 h of IVM, 10 IU/mL of equine chorionic gonadotropin (eCG) (Serotropin; ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/mL of human chorionic gonadotropin (hCG) (Puberogen 500 U; Novartis Animal Health, Tokyo, Japan) were added to the IVM medium. Subsequently, the COCs were matured in the same medium without hormones for an additional 28 h. The oocytes were matured at 39 °C in 5% CO_2, 5% O_2, and 90% N_2. Every group of COCs was matured in a drop of 100 µL maturation medium under paraffin oil (Paraffin Liquid; Nacalai Tesque, Inc., Kyoto, Japan) in a Petri dish (Falcon 351008, Becton Dickinson Labware, Franklin Lakes, NJ, USA). A maximum of two drops per Petri dish was used. In each culture drop either nine COCs or nine oocytectomized COCs (OOXs) with or without 16 DOs (depending on an experimental design described subsequently) were matured in Petri dishes either with or without Cell-Tak (BD Bioscience, Bedford, MA, USA) coating. Ratio of 9:16 of COCs to DOs was chosen based on preliminary trials. These trials showed no significant differences between the ratios 9:9, 9:16, and 9:36 of COCs to DOs concerning nuclear maturation and cumulus expansion. Moreover, to obtain a constant distance of the DO to the COC or OOX a frame of nine COCs or OOXs surrounded by 16 DOs provided the best model to form a frame (Figure 1). In this way, every COC undergoes the influence of the OSF

of the same number of DOs at the same distance spread over the whole grid formation. The use of 16 DOs/100 µL (=0.16 DO/µL) fits within the required range to observe effects of OSF (Hussein *et al.* 2005). The Cell-Tak coating enabled attachment of COCs and DOs to the bottom of the dish (Matoba *et al.* 2010) and allowed accurate measurement of the size of COCs by digital image analysis and their individual follow-up during the whole maturation period. The coating of culture dishes with Cell-Tak was performed based on descriptions in literature (Matoba *et al.* 2010). The approximate distance apart COCs was between 105 and 180 µm. This distance is slightly over the range required to obtain an optimal blastocyst volume and cell number when culturing zygotes (Stokes *et al.* 2005), but it is necessary during IVM to maintain the ability of measuring and following individual COCs in these set-ups (Figure 1) (Matoba *et al.* 2010).

At 20 h of IVM, the 100 µL drop under oil was aspirated by a pipette without disturbing the adherence of the COCs and replaced by 100 µL of IVM medium with 3 mg/mL polyvinyl alcohol without hormones for an additional 28 h of IVM.

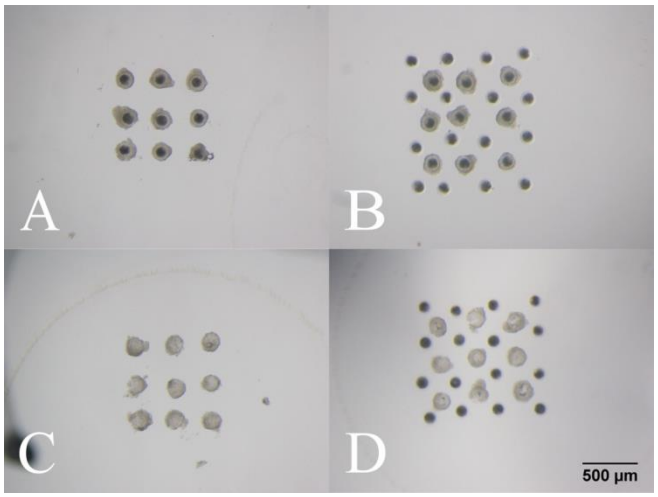

Figure 1: Representative images of COC and OOX grids matured on an adhesive Cell-Tak coated surface with and without DOs. (A) a grid of nine intact COCs; (B) a grid of nine intact COCs and 16 DOs; (C) a grid of nine OOXs; (D) a grid of nine OOXs and 16 DOs. Scale bar represents 500 µm. COCs: cumulus-oocyte complexes; OOXs: oocytectomized COCs; DOs: denuded oocytes.

5.3.2 Oocytectomy

The method used was a modification of the one described by Buccione *et al.* (1990b). After collection, COCs were transferred to a 20 µL drop of M199 covered with paraffin oil (Paraffin Liquid; Nacalai Tesque, Inc.) on the cover of a Petri dish (Falcon 35-1005; Becton, Dickinson and Company, Franklin lakes, NJ, USA). Oocytectomized complexes were obtained by using a pair of micromanipulators (MB-U; Narishige, Tokyo, Japan) on an inverted microscope (IX70; Olympus, Tokyo, Japan) that was equipped with Hoffman modulation contrast. Each individual COC was fixed by creating negative pressure with the holding pipette. The aspiration pipette was prepared from borosilicate glass capillary tubes (Sutter Instrument Co., Novato, CA, USA) with an external diameter of the pipette tip of 4 to 5 µm according to Nakai *et al.* (2005). The content of the oocyte in the COC was aspirated using a Piezo-actuated micromanipulator (PMAS-CT150; Prime Tech Ltd., Tsuchiura, Japan). After aspiration of the cytoplasm, the empty zona of the oocyte was collapsed, but it recovered within a few minutes to the normal spherical shape. By this method, 18 OOXs could be prepared in approximately 30 min. After oocytectomy, the OOXs were placed in maturation medium in groups of nine OOXs.

5.3.3 Preparation of cumulus clumps

To obtain a collection of only cumulus cells without any remnants of cytoplasm, oolemma, or zona pellucida, intact COCs were denuded by mouth pipetting in collection medium. Clumps of cumulus cells of an average size of 10,000 pixels, using the “threshold” and “measure” functions of the National Institutes of Health ImageJ (version 1.47) software (Abràmoff *et al.* 2004), were used for specific experiments described subsequently.

5.3.4 Preparation of denuded oocytes

To obtain the necessary number of DOs for each experimental set-up, COCs were denuded by pipetting through a narrow-bore pipette in collection medium. The COCs used as a source for DOs fulfilled the same requirements as the COCs used for IVM.

5.3.5 Assessment of cumulus expansion

Cumulus expansion was measured at 0, 20, and 48 h of IVM. At each time point, a digital image of the COC grid was captured at the same magnification. A magnification of x 57.6 was chosen to allow taking one picture in which all the oocytes are visible in the grid. The size of each COC was measured individually from digital images by expressing the total two-dimensional area of each COC as total number of pixels using the "threshold" and "measure" functions of the National Institutes of Health ImageJ (version 1.47) software (Abràmoff *et al.* 2004). Using these results, the cumulus expansion levels were calculated for each COC at 0, 20, and 48 h of IVM, in which the value of 0 h was considered as the basis of comparison. Cumulus expansion at 20 and 48 h were expressed as ratios with respect to ratio 1 at 0 h of IVM. Depending on the experiment, statistical analysis was performed in three different ways: the difference between different groups at one time point, the difference within one group between different time points, and the difference of the evolution of the curves of the function of the cumulus expansion of the groups evolving between two time points. The last analysis assesses how the groups are evolving from one time point to another according to a (mathematical) function. In this way, not only the measured starting and end points of the cumulus expansion are taken into account, but also the mathematical model to connect these points.

5.3.6 Assessment of oocyte nuclear maturation

After 48 h of IVM, oocytes were denuded by a brief treatment with 0.1% hyaluronidase in the collection medium followed by pipetting through a narrow-bore glass pipette. Then oocytes were mounted on a glass slide and fixed with acetic alcohol (acetic acid to ethanol, 1:3 v:v) for at least 3 days. Subsequently, the fixed oocytes were stained by 1% (wt/vol) orcein (Sigma-Aldrich) in acetic acid and rinsed in glycerol to acetic acid to water (1:1:3 v:v:v). The stained oocytes were evaluated under a phase-contrast microscope with a x 40 objective. On the basis of their nuclear stage, oocytes were classified according to the previous report (Maedomari *et al.* 2007): germinal vesicle (GV) stage, GV breakdown or prometaphase I stage, metaphase I stage, anaphase I or telophase I stage, metaphase II (MII) stage and degenerated oocytes. Degenerated oocytes were not used for the assessment of nuclear maturation.

5.3.7 Experimental design

5.3.7.1 Experiment 1: effect of oocytectomy on cumulus expansion

We used oocytectomy to clarify the importance of the oocyte for cumulus expansion in the defined POM medium. Three experimental groups were included: (1) intact COCs (COC group), (2) OOXs (OOX group), and (3) cumulus clumps (clump group). The clump group was included as a control group to anticipate the effects of possible remnants of cytoplasm in the OOX in parallel with the study by Singh *et al.* (1993). In each group, nine COCs or nine OOXs or nine clumps, respectively, were matured in each 100 μL drops of defined IVM medium for 48 h as described previously. The dishes were not coated with Cell-Tak. Cumulus expansion was measured at 0, 20 and 48 h of IVM. Three replicates were performed.

5.3.7.2 Experiment 2: effect of co-culture with denuded oocytes on maturation rate and cumulus expansion of cumulus-oocyte complexes

The aim of this experiment was to investigate if DOs placed to the proximity of COCs affected nuclear maturation and cumulus expansion. In each of the 100 µL IVM drops, nine COCs were aligned in a 3 × 3 grid on the adhesive surface of Cell-Tak dishes with or without incorporating 16 DOs in the grid (DO+ and DO− groups, respectively) as shown in Figure 1 A and Figure 1 B, respectively. Cumulus expansion of the COCs at 20 and 48 h of IVM was compared between the two groups. Moreover, the evolution over time between both groups was examined. At 48 h IVM, in both groups (DO− and DO+) the nuclear state of all the oocytes (both DOs and COCs) was evaluated. In this way, the nuclear maturation rates of COCs of DO−, COCs of DO+ and the DOs of DO+ were examined. Five replicates were performed.

5.3.7.3 Experiment 3: effect of co-culture with denuded oocytes on cumulus expansion of oocytectomized complexes

The aim of this experiment was to investigate if DOs placed to the proximity of OOXs affected cumulus expansion. Similarly to the design of *Experiment 2*, in each of the 100 µL IVM drops, nine OOXs were aligned in a 3 × 3 grid on the adhesive surface of Cell-Tak dishes with or without incorporating 16 DOs in the grid (DO+ and DO− groups, respectively) as shown in Figure 1 C and Figure 1 D, respectively. The grids DO− and DO+ were matured for 48 h. As a control, intact COCs were included (COC). The cumulus expansion in both experimental groups was compared at 20 and 48 h of IVM. Three replicates were performed.

5.3.7.4 Experiment 4: effect of the gap junction inhibitor carbenoxolone on cumulus expansion and oocyte maturation of cumulus-oocyte complexes and denuded oocytes during *in vitro* maturation

The aim of this experiment was to investigate if functioning GJs play an important role for cumulus expansion and nuclear progression in the defined IVM medium. Cumulus-oocyte complexes were arranged in grids in IVM droplets as described in *Experiment 2*. The IVM medium was either supplemented with or without 50 µM of the GJ inhibitor 3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate (CBX; Sigma Chemical Co.; St. Louis, MO) throughout the entire 48 h of IVM (CBX+ and CBX– groups, respectively). In both groups, DOs were incorporated in grids to investigate the adverse effects of CBX on oocyte nuclear maturation apart from its actions on GJs. Cumulus expansion of the COCs at 20 and 48 h of IVM was compared between the two groups. At 48 h IVM, in both groups, the nuclear state of oocytes (both DOs and COCs) was evaluated. Three replicates were performed.

5.3.8 Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) Statistics version 20 (SPSS IBM, Brussels, Belgium). Hypothesis testing was performed using a significance level of 5% (two-sided test). Depending on the experiment, three to five replicates were performed.

Statistical analysis of cumulus expansion was performed based on the ratios. Evolution of the curves of the function of the cumulus expansion at 0, 20 and 48 h of IVM was analyzed using linear mixed models with group as factor and time and time^2 as covariates and an unstructured covariance type including the intercept. To evaluate cumulus expansion between groups at one time point and within one group between 0 and 20 h, ANOVA was used because cumulus expansion was normally distributed and showed an equality of error variances. The difference between 20 and 48 h within one group was analyzed by syntax in linear mixed models. In

figures and tables, results are presented as ratios of cumulus expansion. Error bars in the figures illustrate the standard error of the mean (SEM).

Nuclear maturation rate was analyzed using binary logistic regression. The proportion of oocytes in a category was included as a dependent variable and group was included as an independent variable. Replicate was included as a covariate. In the tables, proportions are presented as the mean percentage ± SEM.

5.4 Results

5.4.1 Development of an *in vitro* maturation system

An overview of the systems applied in this study is shown in Figure 2. Depending on the parameters to be measured and the intended research purposes, decisions were made about the number of oocytes to be cultured and the use of Cell-Tak during IVM to fix the complexes to the bottom of the dish. This system provided several advantages in view of the intended research goals. Oocytes could be identified individually, which created the opportunity to follow-up cumulus expansion in an individual manner during the entire IVM period. Moreover, since the COCs and DOs were fixed to the bottom of the dish, COCs experienced the influence of a constant number of DOs on a fixed distance instead of a variable effect of a variable number of DOs floating by. One of the disadvantages of examining the influence of floating DOs on floating COCs is illustrated in Figure 2. In the row of "9 COC+36 DO –" at 48 h of IVM, the COCs and DOs diverged to such an extent that the complexes did not fit in one picture anymore (Figure 2). Moreover, it can be noticed that DOs grouped together, away from the COCs. When interpreting OSF effects in such a system, it can be questioned to what extent the increased distance between DOs and COCs should be taken into account. Also the opposite situation was observed, where DOs made contact with the cultured COCs. Exactly to avoid these kinds of variations in the experimental set-up, our applied Cell-Tak adherence guaranteed a fixed distance between COCs and DOs.

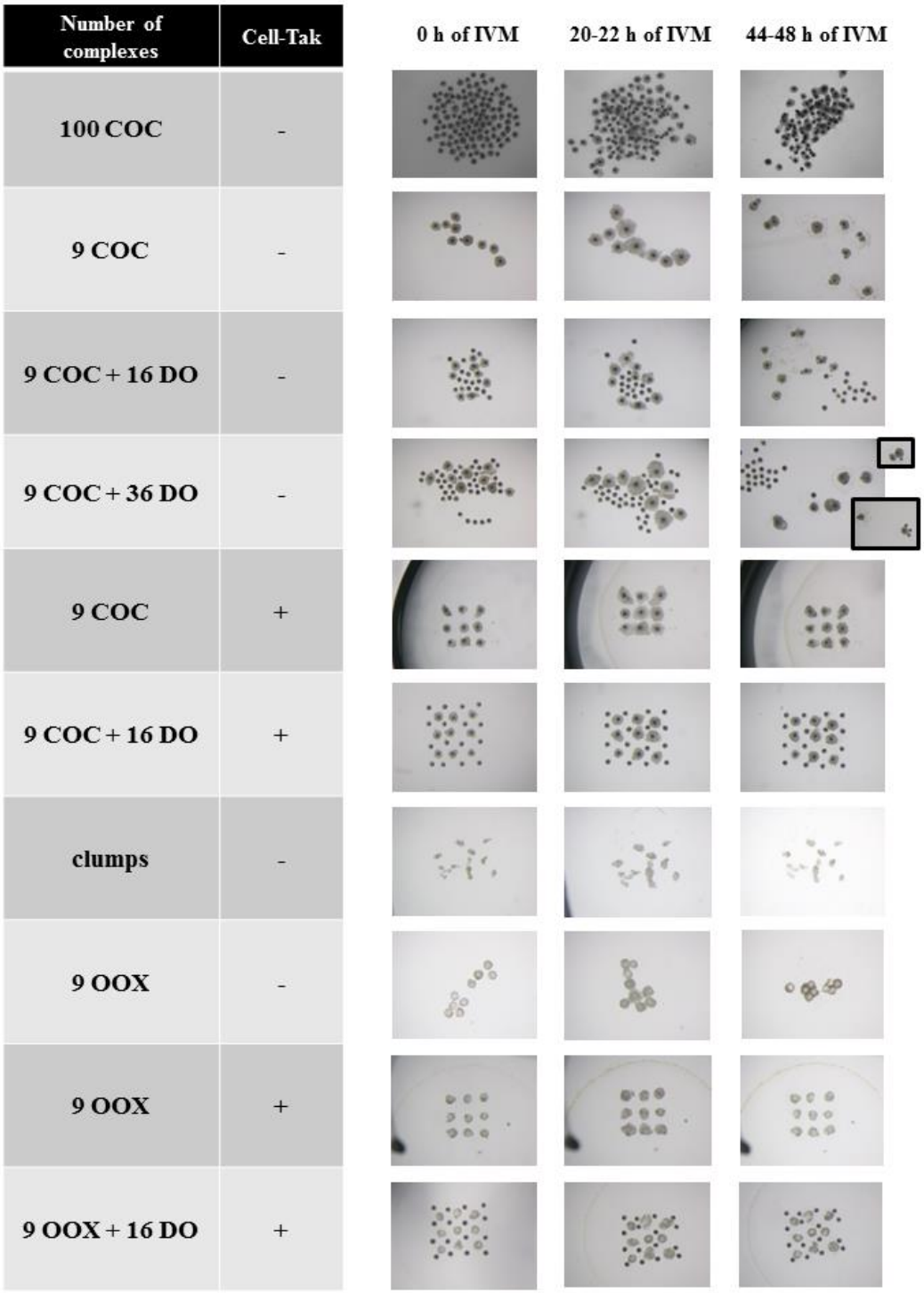

Figure 2: Overview of the in vitro *experimental set-ups used in this study. The variables determining the system were the number of cultured oocytes and the use of Cell-Tak to enhance adherence of the complexes to the bottom of the culture dish. Magnification x 40 from "100 COC-" to "9 COC + 36 DO-" and magnification x 57.6 from "9 COC" to "9 OOX + 16 DO". IVM:* in vitro *maturation; COC: cumulus-oocyte complex; OOX: oocytectomized complex; DO: denuded oocyte. Photographs by R. Appeltant.*

5.4.2 Experiment 1

By means of micromanipulation, oocytectomy was performed by aspirating the oocyte from the COC leaving an empty zona pellucida and surrounding cumulus cells behind (Figure 3).

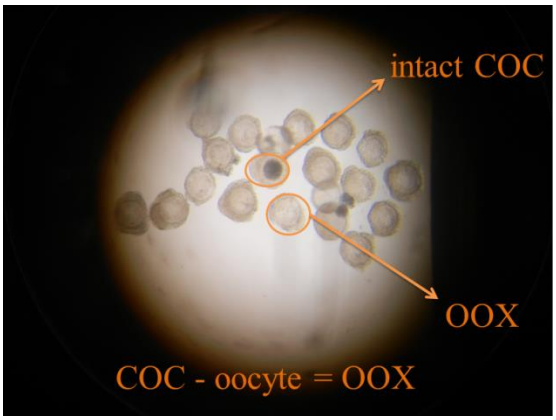

Figure 3: Photograph of a group of oocytectomized complexes (OOX) and one intact cumulus-oocyte complex (COC). Magnification x 57.6. Photograph by R. Appeltant.

The pattern of cumulus expansion during IVM followed a similar trend of increasing and decreasing in the three groups (COC, OOX, and clump group) (Figure 4). Remarkably, in the OOX and clump groups, the relative size of the cumulus increased in the absence of the oocyte during the first 20 h of IVM (from 1.00 to 1.45 and from 1.00 to 1.53, respectively) ($P < 0.05$) (Figure 4). At 20 h of IVM, the cumulus expansion in the COC group was higher compared with those of the OOX and clump groups (1.84 vs. 1.45 and 1.53, respectively) ($P < 0.05$) (Figure 4). At 48 h of IVM, the relative cumulus expansion in the COC group was increased with respect to its status at 0 h of IVM (ratio 1.08 vs. 1.00, respectively) ($P < 0.05$) (Figure 4). At 48 h of IVM, on the contrary, the relative size of OOXs was not different from that at 0 h IVM ($P \geq 0.05$), whereas the size of clumps was decreased with respect to their size at 0 h of IVM ($P < 0.05$) (ratio 0.98 vs. 1.00 and 0.48 vs. 1.00, respectively) (Figure 4). The curves of the evolution over time from 0 to 20 h and from 20 to 48 h were different between the three groups ($P < 0.05$).

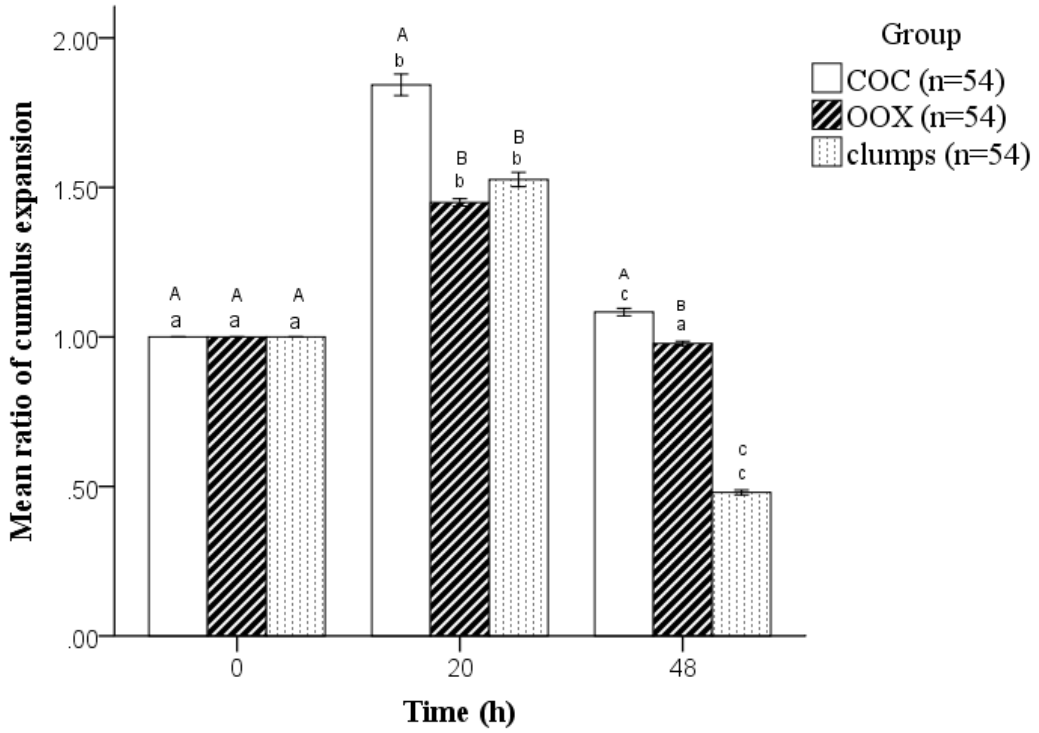

Figure 4: The extent of the relative cumulus expansion at 0, 20 and 48 h of in vitro *maturation (IVM) of COCs, OOXs, and clumps of cumulus cells. Cumulus expansion is presented as ratio of total size measured in pixels at each time point in respect to the original size of the subject at 0 h of IVM. The error bars denote SEM. Three replicates were performed. Within the same group, the mean ratios of cumulus expansion with different superscripts (a-c) differ significantly and at the same time point, the mean ratios of cumulus expansion with different superscripts (A-C) differ significantly (P < 0.05). COCs: cumulus-oocyte complexes; OOXs: oocytectomized COCs; SEM: standard error of the mean.*

5.4.3 Experiment 2

There was no difference in the extent of relative cumulus expansion between the DO− and DO+ groups at 20 h (1.83 and 1.75, respectively) and 48 h of IVM (1.41 and 1.47, respectively) (Figure 5). Nevertheless, the curves of the evolution over time of the cumulus expansion were different between DO− and DO+ groups (P < 0.05) (Figure 5). At 20 h of IVM a trend to lower cumulus expansion could be observed in the DO+ group (Figure 5). On

the other hand, this trend was followed by a trend of a lower cumulus matrix reduction in the next 28 h compared with the decrease in cumulus expansion in the DO− group (Figure 5). Therefore, a trend to a higher cumulus expansion at the end of the 48 h of IVM period was observed in the DO+ group compared with the DO− group (Figure 5).

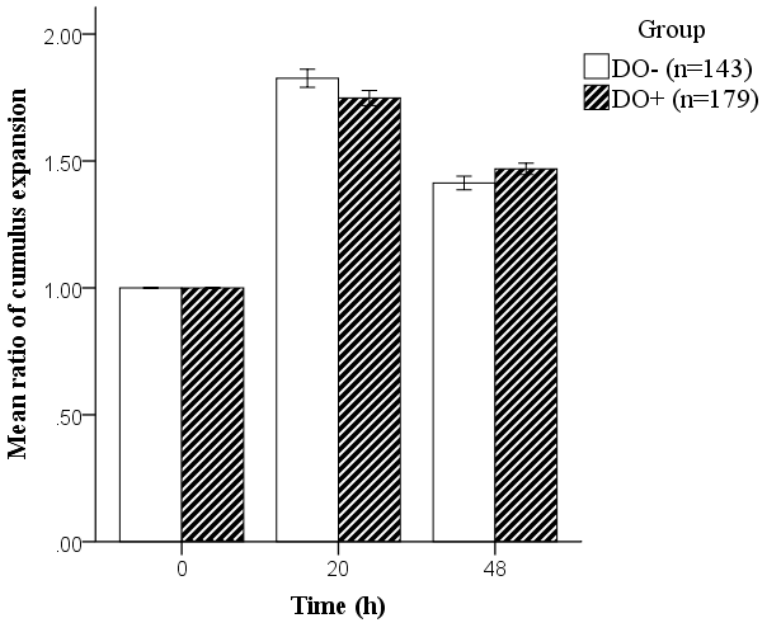

Figure 5: The extent of the relative cumulus expansion at 0, 20 and 48 h of in vitro *maturation (IVM) of COCs aligned in a 3 × 3 grid on the adhesive surface of Cell-Tak dishes with or without incorporating 16 DOs in the grid (DO+ and DO− groups, respectively). Cumulus expansion is presented as ratio of total size measured in pixels at each time point in respect to the original size of the subject at 0 h of IVM. The error bars denote SEM. Three replicates were performed. At the same time point, no significant differences could be observed between the mean ratios of cumulus expansion of both groups (P ≥ 0.05). COCs: cumulus-oocyte complexes; SEM: standard error of the mean; DOs: denuded oocytes.*

The maturation rates are shown in Table 1. The maturation rate of cumulus intact oocytes (*i.e.*, the percentages of those at the MII stage) in the DO− group did not differ from that of the DO+ group (39.0% and 32.9%, respectively) (Table 1). The percentage of DOs at the MII stage (52.3%) was higher than that of the COC-derived oocytes from the DO− and DO+ group (39.0% and 32.9%, respectively) ($P < 0.05$). Denuded oocytes of the DO+ group showed a lower percentage of metaphase I stage oocytes than the COCs of the DO− and DO+

group (13.1%, 28.6%, and 29.9%, respectively) ($P < 0.05$) (Table 1). No differences were observed between COCs of DO−, COCs of DO+, and DOs of DO+ concerning percentage of oocytes at GV, prometaphase I, and anaphase I or telophase I stages.

Table 1: Nuclear status of porcine oocytes in COC grids with and without DOs at 48 h of in vitro *maturation (IVM).*

Group	Oocyte type	Total	GV (%)	proMI (%)	AI+TI (%)	MI (%)	MII (%)
DO−	COC	105	31.4 ± 8.9[a]	0.0 ± 0[a]	1.0 ± 0.6[a]	28.6 ± 10.2[a]	39.0 ± 5.4[a]
DO+	COC	167	35.9 ± 10.1[a]	0.6 ± 0.4[a]	0.6 ± 0.4[a]	29.9 ± 6.1[a]	32.9 ± 8.8[a]
DO+	DO	298	32.9 ± 4.0[a]	0.7 ± 1.3[a]	1.0 ± 0.7[a]	13.1 ± 2.4[b]	52.3 ± 4.9[b]

Percentages are expressed as the mean ± standard error of the mean. Within the same column, the mean percentages with different superscripts (a and b) differ significantly. DO− and DO+ symbolize COC grids without and with addition of DOs, respectively. Five replicates were performed.
AI + TI: anaphase I or telophase I; COC: cumulus-oocyte complex; DO: denuded oocyte; DO+: COC grid with DO addition; DO−: COC grid without DO addition; GV: germinal vesicle; MI: metaphase I; MII: metaphase II; proMI: prometaphase I.

5.4.4 Experiment 3

At 48 h of IVM, a higher relative cumulus expansion level was recorded in the DO+ group than in the DO− group (1.19 and 1.08, respectively) ($P < 0.05$) (Figure 6). Neither the DO− nor the DO+ OOXs reached the level of cumulus expansion recorded in the COC group at 48 h of IVM (1.08 and 1.19 vs. 1.52, respectively) (Figure 6).

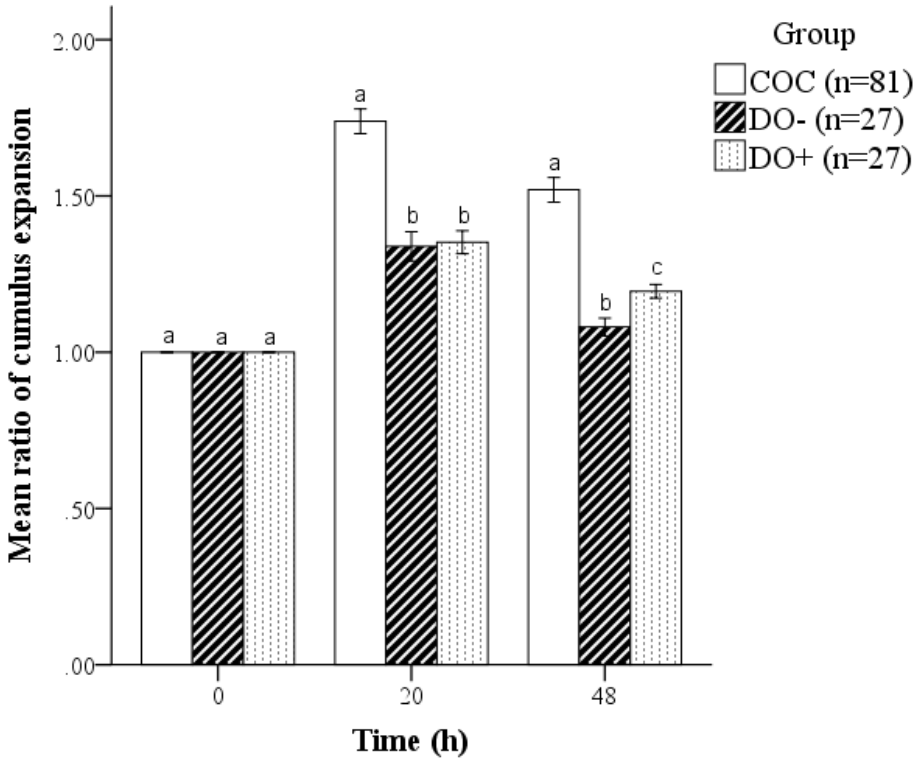

Figure 6: The extent of the relative cumulus expansion at 0, 20, and 48 h of in vitro *maturation (IVM) of OOXs aligned in a 3 × 3 grid on the adhesive surface of Cell-Tak dishes with or without incorporating 16 DOs in the grid (DO+ and DO− groups, respectively). The control group consisted of COCs in a 3 × 3 grid on Cell-Tak dishes (COC). Cumulus expansion is presented as a ratio of total size measured in pixels at each time point in respect to the original size of the subject at 0 h of IVM. The error bars denote SEM. Three replicates were performed. Within the same time point, the mean ratios of cumulus expansion with different superscripts (a-c) differ significantly (P < 0.05). COCs: cumulus-oocyte complexes; OOXs: oocytectomized COCs; SEM: standard error of the mean; DOs: denuded oocytes.*

5.4.5 Experiment 4

At 20 h of IVM, the CBX− group showed a higher relative cumulus expansion than the CBX+ group (2.08 and 1.46, respectively) (P < 0.05) (Figure 7). This difference between CBX− and CBX+ could not be observed at 48 h of IVM anymore (1.35 and 1.29, respectively) (Figure 7).

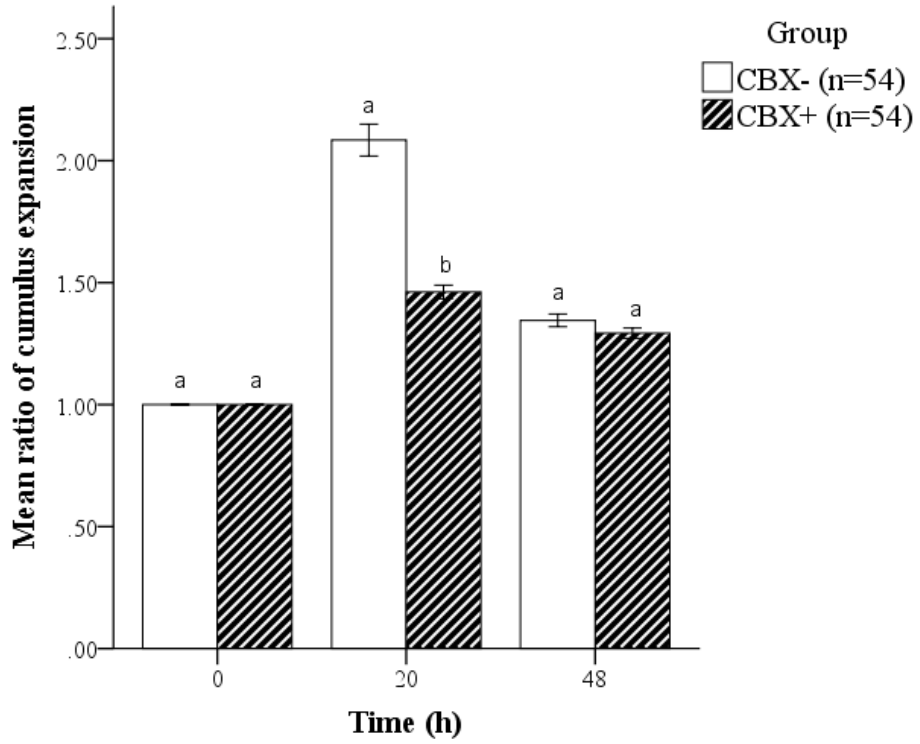

Figure 7: Effect of the gap junction inhibitor carbenoxolone (CBX) on cumulus expansion of COCs at 0, 20, and 48 h of in vitro *maturation (IVM). Cumulus-oocyte complexes were aligned in a 3 × 3 grid on the adhesive surface of Cell-Tak dishes with 16 DOs in the grid without or with CBX (CBX− and CBX+, respectively). Cumulus expansion is presented as a ratio of total size measured in pixels at each time point in respect to the original size of the subject at 0 h of IVM. The error bars denote SEM. Three replicates were performed. Within the same time point, the mean ratios of cumulus expansion with different superscripts (a and b) differ significantly (P < 0.05). COCs: cumulus-oocyte complexes; DOs: denuded oocytes; SEM: standard error of the mean.*

At 48 h of IVM, the percentage of oocytes at the MII stage in COCs of the CBX− group was lower compared with that of the CBX+ group (21.2% and 45.1%, respectively) ($P < 0.05$) (Figure 8). Consequently, at 48 h IVM, in the CBX+ group the oocyte maturation rate of COCs did not differ from that the DOs in the same treatment group (45.1% and 38.9%, respectively) ($P \geq 0.05$), whereas in the CBX− group, the MII rate of DOs was higher than that of the COCs (54.4% and 21.2%, respectively) ($P < 0.05$) corroborating with the results of *Experiment 2* (Figure 8).

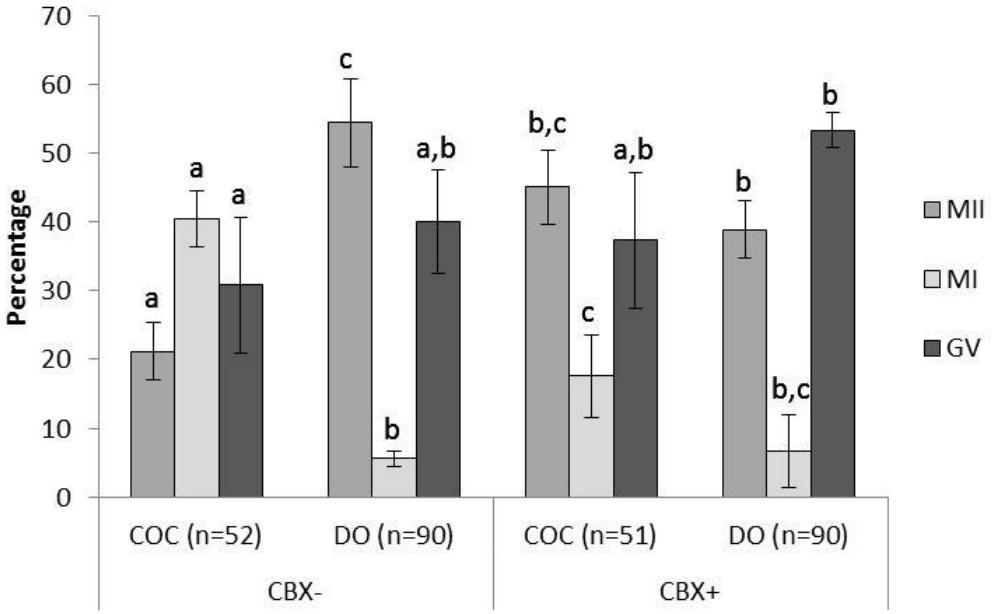

Figure 8: Effect of the gap junction inhibitor carbenoxolone (CBX) on oocyte maturation during in vitro *maturation (IVM). Cumulus-oocyte complexes (COCs) and DOs are matured without and with addition of CBX (CBX− and CBX+, respectively) to the maturation medium. Cumulus-oocyte complexes are aligned in a 3 × 3 grid on the adhesive surface of Cell-Tak dishes with addition of 16 DOs in the grid. Bars represent the percentage (mean ± SEM) of the oocytes that reached metaphase II (MII), metaphase I (MI), or germinal vesicle (GV) stage at 48 hours of IVM. The error bars denote SEM. Three replicates were performed. Within the same maturation category, bars with different superscripts (a-c) differ significantly (P < 0.05). DOs: denuded oocytes; SEM: standard error of the mean.*

5.5 Discussion

5.5.1 Establishment of *in vitro* maturation system

In the present study, we established a system for IVM of porcine oocytes in small groups, which allows the exact measurement and individual tracking of COC size (and therefore cumulus expansion) during IVM. Using this system, we could investigate the effect of oocytes (either as the organic member of the COC or as DOs used for co-culture) on cumulus expansion in a defined medium, and also investigate the effects of the presence of cumulus cells and functional GJs on oocyte nuclear maturation in this system. This culture system used the fixation of COCs and DOs to the bottom of the culture dish by Cell-Tak coating. Cell-Tak has already been shown to be efficient for the individual tracking of porcine and bovine embryos and bovine COCs during culture without adverse effects (Stokes *et al.* 2005; Matoba *et al.* 2010). To our knowledge, the present study is the first attempt for the use of this system for IVM of porcine COCs. Our preliminary experiment did not reveal any effect of Cell-Tak coating on oocyte maturation (data not shown). Cumulus expansion was slightly but significantly increased by the use of Cell-Tak (data not shown). A possible explanation is that by attaching the COCs to the bottom of the dish, the COCs had one dimension less to expand, and consequently, the expansion was increased in other directions. Furthermore, application of digital image analysis for this system enables the precise measurement and tracking of size changes of COCs during culture, which is capable of providing more accurate results on cumulus expansion compared with subjective grading generally applied in previous studies.

5.5.2 Effects of oocytes on cumulus expansion

In *Experiment 1*, when COCs, OOXs, or cumulus clumps were cultured in defined POM, the same evolution of cumulus expansion over time could be seen in all groups. The cumulus compartment expanded during the first 20 h and then their size decreased in the next 28 h of

IVM. This proves that the cumulus cells are not totally dependent on the oocyte to expand. However, a significant difference could be observed in the degree of expansion among intact COCs, OOXs, and clumps, in which the presence of oocytes in COC group resulted in a higher degree of cumulus expansion. These results suggest that the presence of oocytes does enhance cumulus expansion, although it is not a prerequisite for this process. When DOs were added in the COC grids, no significant differences in cumulus expansion were observed at 20 and 48 h of IVM. Nevertheless, the evolution during the entire time period (from 0 to 48 h) of the cumulus expansion was significantly different between DO− and DO+ groups. Consequently, we can presume that the dynamics to reach 20 and 48 h differs between the groups, but they are ending at the same level. To investigate these dynamics between the measured time points, more measuring points should be considered. In this study we did not include additional measuring points in order to disturb the growth of the COCs as little as possible. Further research can reveal the possible mechanisms causing the difference in the evolution of the expanding curve. Addition of DOs to OOXs improved the cumulus expansion at 48 h of IVM, which suggests that DOs could have affected cumulus expansion by altering medium characteristics. This effect of DOs could be overruled in the COC group by the presence of the COC-derived oocytes. Alteration in medium characteristics by oocytes is possible by secreting soluble factors such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) proteins, which in mammalian species affect the development and behavior of follicular cells (Hussein *et al.* 2006). It was already indicated that porcine oocytes secrete cumulus expansion-enabling factors by the use of an interspecies model (Singh *et al.* 1993). Brankin *et al.* (2003) proved that porcine OSFs influence granulosa and theca cell proliferation and steroidogenesis. Although the specific role of GDF9 and BMP15 in pigs remains to be elucidated, the specific expression of BMP15 at the time of cumulus expansion could argue its role in cumulus expansion (Li *et al.* 2008). In mice, Dragovic *et al.* (2005) reported that GDF9 is one of the factors that can enable FSH-induced cumulus expansion. Another possible way for oocytes to alter the medium around them in a way to enhance the function of other cells is the reduction of harmful agents. For instance, an excess amount of oxygen (harmful by causing oxidative stress) may be reduced in the medium around DOs by their oxidative metabolism, and the antioxidant systems of the DOs can detoxify diffusible reactive oxygen species (Guerin *et al.* 2001). The exact mechanism of alteration in medium characteristics remains to be elucidated. On the other hand, in *Experiment 4*, supplementation of the IVM medium with GJ inhibitor CBX reduced the extent

of cumulus expansion during the first 20 h of IVM, which suggests that during this period, gap junctional communications may also play a role in the oocyte-mediated enhancement of cumulus expansion. Therefore, it is suggested that oocytes may affect cumulus expansion both by altering medium characteristics and *via* gap junctional communications.

The pattern of expanding and subsequently shrinking of the cumulus area was observed for the first time in the present study. Mito *et al.* (2013) described cumulus expansion in defined POM as a continuous expanding phenomenon. This different development could be explained by the use of different hormonal supplements used for IVM and the different methods used for the evaluation of cumulus expansion in the two studies. In contrast to earlier reports (Prochazka *et al.* 1991; Singh *et al.* 1993), OOXs and clumps could not reach the same level of expansion as intact COCs (COC group) at 20 h of IVM. This observation supports the determination of Singh *et al.* (1993) that although the expansion is not dependent on the oocyte, the oocyte produces cumulus expansion-enabling factors, in this set-up reflected as a significant difference in degree of expansion. In our literature evaluation, no information was found about the further evolution of the cumulus expansion of OOXs and clumps beyond the 24 h of IVM. We also measured this parameter at the end of the maturation period (48 h) and, remarkably, observed a drastic decrease in expansion in the group of the cumulus clumps, ending significantly lower than the area measured at 0 h of IVM.

5.5.3 Effect of cumulus cells on oocyte maturation

The exact role of cumulus cells during the initiation of meiotic resumption in pigs has been a matter of debate. Early studies suggested the inhibition of a GJ-mediated blocking signal coming from cumulus cells as a key factor for triggering meiosis in oocytes (Gilchrist 2011), whereas others suggested that a cumulus-secreted soluble factor(s) may be responsible for the reinitiation of nuclear progression in oocyte during IVM (Xia *et al.* 2000; Gilchrist 2011). Our results have revealed that, under the present IVM conditions, a significantly higher percentage of DOs reached the MII stage by 48 h of IVM than did the cumulus-enclosed oocytes. This confirms that cumulus cells play a role in the maintenance of meiotic block in porcine oocytes during IVM from which they can be released by the removal of cumulus cells before culture.

In a previous report, using a very basic defined medium for IVM, Akaki *et al.* (2009) observed the same phenomenon as our results: a higher tendency in the percentages of oocytes reaching MII in DOs compared to COCs. Furthermore, our results suggest that a blocking effect of cumulus cells is likely to be mediated through gap junctional communication between the cumulus and the oocytes since the addition of the GJ inhibitor CBX to the IVM medium increased the percentage of MII stage oocytes in COCs in a similar manner to denudation despite of its apparent negative side effects on oocyte maturation observed in DOs. Regarding the use of CBX, it must be noted that although previously this reagent was applied in a 100 µM concentration to block GJs in porcine COCs (Ozawa *et al.* 2010), in our preliminary experiment this concentration was largely detrimental to the nuclear progression of oocytes irrespective of the presence of cumulus cells causing a general meiotic arrest at the GV stage (data not shown). This adverse effect of CBX may be independent of its effect on GJs as it was generally observed in cumulus free oocytes as well.

Although removal of the cumulus cells allows nuclear maturation, it is very detrimental to the cytoplasmic maturation of the oocyte that is necessary to acquire its ability for fertilization and subsequent development (Chian and Niwa 1994; Maedomari *et al.* 2007). On the other hand, several studies have reported that during IVM of porcine COCs, an activating signal from cumulus cells triggers the meiotic resumption of oocytes (Gilchrist 2011). On the basis of the aforementioned observations, removal of cumulus cells is likely to induce a forced maturation that may not resemble the natural process.

5.5.4 Factors affecting oocyte maturation

In COCs (irrespective of the presence of DOs in culture medium), most oocytes that failed to reach the MII stage were arrested at the GV stage (DO− 31.4% and DO+ 35.9%) (Table 1). This suggests that in COCs, the cumulus-mediated meiotic resumption did not occur in most oocytes. The reason for this remains to be elucidated. The low levels of maturation rates observed in our study can be explained by the deliberate use of a very basic defined POM in order to reveal the fundamental interactions between oocyte and cumulus cells without components that might overshadow basic processes between cumulus cells and oocytes. The

only addition to the POM medium was a combination of gonadotropins during the first 20 h of IVM based on the report of Funahashi *et al.* (1996) because they were found to be critical to resume meiosis (Mattioli *et al.* 1994) and induce cumulus expansion (Tanghe *et al.* 2002). When applied for a standard IVM procedure, Yoshioka *et al.* (2008), who developed POM, also recommended using the meiosis synchronizing component dibutyryl cAMP sodium salt (dbcAMP) together with hormones during the first maturation period. Addition of dbcAMP to non-defined media did not alter the maturation rate at the end of IVM according to the study by Funahashi *et al.* (1997). Nevertheless, dbcAMP has the potential to increase the number of oocytes reaching MII stage in a chemically defined gonadotropin-free medium as shown in the study by Akaki *et al.* (2009). Moreover, the stimulation of the cAMP pathway is necessary for successful meiotic resumption (Akaki *et al.* 2009). In fact, meiosis synchronization of porcine COCs during the first half of IVM by dbcAMP has been reported to be beneficial for the meiotic and developmental competence of oocytes (Funahashi *et al.* 1997; Somfai *et al.* 2003; Bagg *et al.* 2006). Previously, Mattioli *et al.* (1994) have revealed that a transient increase in cAMP in porcine oocytes caused by the gonadotropin surge is the signal that triggers the resumption of meiosis *in vivo*. It is possible, that the hormone treatment used in the present study was not efficient enough to reproduce the natural process of meiosis activation. Being a cAMP analog, dbcAMP supplementation during the first part of IVM may resemble the natural process of meiosis *in vitro*, which might be important for the normal nuclear progression. Therefore, we hypothesized that dbcAMP could possibly promote the progression to MII in our study design. To verify our hypothesis, we performed an additional experiment (five replicates) and investigated if supplementation of the IVM medium with 1 mM dbcAMP during the first 20 h of IVM affects the final rate of MII stage oocytes in COCs. Cumulus-oocyte complexes were cultured in 3 x 3 grids together with 16 DOs. Surprisingly, the results indicated that dbcAMP supplementation did not significantly improve MII rates compared with COCs cultured without dbcAMP supplementation. However, there was a trend towards a higher metaphase II rate in the dbcAMP supplemented group (data not shown).

Another important factor that could possibly contribute to the low maturation rates may be the low numbers of COCs (nine) cultures in each IVM drop. Culture of COCs in large numbers in a common drop may be beneficial for the maturation process by the increased concentration of paracrine and autocrine factors secreted by oocytes and cumulus cells. Also, cultured cells consume oxygen; therefore increasing the numbers of cells in the culture drop may reduce

oxidative stress on oocytes that is known to be detrimental to their developmental competence. In this respect, supplementation of the culture medium with antioxidants may be a possible way to improve the defined IVM medium for the culture of low numbers of oocytes. Supporting this theory, the supplementation of POM with β-mercaptoethanol was found to increase glutathione concentration in oocytes, which could potentially neutralize toxic effects of oxidative stress (Akaki *et al.* 2009).

Even if the number of oocytes is sufficient to support maturation, it could still be questioned if the created distance between the mutual COCs or between COCs and DOs could affect the maturation rates. Preliminary trials dealing with these factors were performed and could not reveal any significant differences. The use of Cell-Tak did not affect the maturation rate of COCs (data not shown). Direct contact between DOs and COCs by using floating DOs in a grid formation of attached COCs did not improve maturation rates compared to a grid with both COCs and DOs attached (data not shown).

5.5.5 Is co-culture with denuded oocytes a useful approach to improve *in vitro* maturation/*in vitro* fertilization of cumulus-oocyte complexes?

In a previous study, using a non-defined, pFF supplemented medium, co-culture with DOs during IVM significantly improved embryo developmental competence of cumulus-enclosed oocytes (Gomez *et al.* 2012). However, in this latter study, parthenogenetic activation was used to generate embryos. The efficacy of co-culture with DOs to improve oocyte maturation in defined media for small groups of COCs and also to improve efficacy of IVF systems remained to be elucidated. Our results show that addition of DOs to the IVM droplet did not affect nuclear maturation of COCs, which confirms the findings of Gomez *et al.* (2012). On the other hand, unlike in the report by Gomez *et al.* (2012), DOs evidently enhanced cumulus expansion to some extent as detailed previously. The capability of porcine DOs for improving mouse cumulus cell expansion has already been demonstrated by Singh *et al.* (1993) but no experiments evaluated the influence of porcine DOs on porcine OOXs until now. Buccione *et al.* (1990b) described that the FSH stimulated cumulus expansion of mouse OOXs co-cultured with DOs or denuded-oocyte conditioned media was directly correlated with the number of

oocytes. So this cumulus expansion can be even more improved by adding more DOs. In this line of thought, further research should focus on examining and determining the ideal ratio between DOs and OOXs. In previous studies in different mammalian species including mice, cattle and pigs, DO addition to OOXs not only affected cumulus expansion but also several other important parameters such as cumulus cell apoptosis (Hussein *et al.* 2005), luteinization markers (Gilchrist *et al.* 2008), cumulus glycolytic enzyme mRNA levels (Sugiura *et al.* 2005), and steroidogenesis (Coskun *et al.* 1995). All these findings together point in the same direction that adding DOs could be beneficial for an IVM production system for OOXs and COCs. Despite the observed inhibiting effect of the cumulus cells on the nuclear maturation in *Experiment 4* of this study, the presence of cumulus cells is crucial to sustain the cytoplasmic maturation for the capacity of the oocytes to respond to subsequent IVF and *in vitro* culture (IVC) (Tanghe *et al.* 2002). Cumulus cells play roles in many processes concerning nuclear and cytoplasmic maturation of oocytes, their penetration by sperm and subsequent development (Tanghe *et al.* 2002). Therefore, the level of cumulus expansion may reflect the capability of oocytes to these processes or even directly affect them. Yoshioka *et al.* (2008) have demonstrated that in a protein (pFF) free POM, porcine oocytes are capable of reaching the MII stage and can be fertilized *in vitro* at high rates despite of their minimum degree of cumulus expansion when compared with oocytes matured in a pFF-supplemented POM. On the other hand, the cumulus compartment has been considered as an obstacle for sperm penetration; therefore, its expansion level may make a difference for IVF outcome (Tanghe *et al.* 2002). Further studies need to be performed to clarify the importance of cumulus expansion and the use of DO co-culture during IVM of COCs for their practical use to improve IVF results and subsequent embryo development.

5.5.6 Conclusion

We have established a basic IVM system for small groups of oocytes, which allowed the precise measurement and tracking of cumulus expansion during IVM in an individual manner in a defined medium. We clarified that cumulus cells can expand independently of the oocyte. However, oocytes do enhance cumulus expansion both as organic members of COCs through GJs and also as DOs added to culture drops by modifying medium characteristics. This

indicates the potential enhancing power of DOs. Further research is necessary to improve overall maturation rates and to clarify if DO addition during IVM can lead to better fertilization results and higher embryo developmental competence because of the secretion of OSF.

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5.7 References

Abràmoff, M., Magalhães, P., and Ram, S. (2004) Image processing with ImageJ. *Biophotonics International* **11**(7), 36-43

Akaki, Y., Yoshioka, K., Noguchi, M., Hoshi, H., and Funahashi, H. (2009) Successful piglet production in a chemically defined system for in-vitro production of porcine embryos: dibutyryl cyclic amp and epidermal growth factor-family peptides support in-vitro maturation of oocytes in the absence of gonadotropins. *J. Reprod. Dev.* **55**(4), 446-453

Bagg, M.A., Nottle, M.B., Grupen, C.G., and Armstrong, D.T. (2006) Effect of dibutyryl cAMP on the cAMP content, meiotic progression, and developmental potential of in vitro matured pre-pubertal and adult pig oocytes. *Mol. Reprod. Dev.* **73**(10), 1326-1332

Bijttebier, J., Tilleman, K., Dhaenens, M., Deforce, D., Van Soom, A., and Maes, D. (2009) Comparative proteome analysis of porcine follicular fluid and serum reveals that excessive α2-macroglobulin in serum hampers successful expansion of cumulus-oocyte complexes. *Proteomics* **9**(19), 4554-4565

Brankin, V., Mitchell, M., Webb, B., and Hunter, M. (2003) Paracrine effects of oocyte secreted factors and stem cell factor on porcine granulosa and theca cells in vitro. *Reprod. Biol. Endocrinol.* **1**(1), 55

Buccione, R., Schroeder, A., and Eppig, J. (1990a) Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol. Reprod.* **41**(1), 176

Buccione, R., Vanderhyden, B., Caron, P., and Eppig, J. (1990b) FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev. Biol.* **138**(1), 16-25

Chian, R., and Niwa, K. (1994) Effect of cumulus cells present during different periods of culture on maturation in vitro of bovine oocytes. *Theriogenology* **41**(1), 176

Coskun, S., Uzumcu, M., Lin, Y., Friedman, C., and Alak, B. (1995) Regulation of cumulus cell steroidogenesis by the porcine oocyte and preliminary characterization of oocyte-produced factor(s). *Biol. Reprod.* **53**(3), 670-675

Dragovic, R.A., Ritter, L.J., Schulz, S.J., Amato, F., Armstrong, D.T., and Gilchrist, R.B. (2005) Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. *Endocrinology* **146**(6), 2798-2806

Funahashi, H., Cantley, T., and Day, B. (1997) Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. *Biol. Reprod.* **57**(1), 49-53

Funahashi, H., Kim, N., Stumpf, T., Cantley, T., and Day, B. (1996) Presence of organic osmolytes in maturation medium enhances cytoplasmic maturation of porcine oocytes. *Biol. Reprod.* **54**(6), 1412-1419

Gil, M.A., Cuello, C., Parrilla, I., Vazquez, J.M., Roca, J., and Martinez, E.A. (2010) Advances in swine in vitro embryo production technologies. *Reprod. Domest. Anim.* **45**, 40-48

Gilchrist, R. (2011) Recent insights into oocyte –follicle interactions provide opportunities for the development of new approaches to in vitro maturation. *Reprod. Fertil. Dev.* **23**(1), 23-31

Gilchrist, R.B., Lane, M., and Thompson, J.G. (2008) Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum. Reprod. Update* **14**(2), 159-177

Gomez, M.N.L., Kang, J.T., Koo, O.J., Kim, S.J., Kwon, D.K., Park, S.J., Atikuzzaman, M., Hong, S.G., Jang, G., and Lee, B.C. (2012) Effect of oocyte-secreted factors on porcine in vitro maturation, cumulus expansion and developmental competence of parthenotes. *Zygote* **20**(02), 135-145

Guerin, P., El Mouatassim, S., and Menezo, Y. (2001) Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum. Reprod. Update* **7**(2), 175-189

Hussein, T.S., Froiland, D.A., Amato, F., Thompson, J.G., and Gilchrist, R.B. (2005) Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. *J. Cell Sci.* **118**(22), 5257-5268

Hussein, T.S., Thompson, J.G., and Gilchrist, R.B. (2006) Oocyte-secreted factors enhance oocyte developmental competence. *Dev. Biol.* **296**(2), 514-521

Li, H.-K., Kuo, T.-Y., Yang, H.-S., Chen, L.-R., Li, S.S.-L., and Huang, H.-W. (2008) Differential gene expression of bone morphogenetic protein 15 and growth differentiation factor 9 during in vitro maturation of porcine oocytes and early embryos. *Anim. Reprod. Sci.* **103**(3-4), 312-322

Maedomari, N., Kikuchi, K., Ozawa, M., Noguchi, J., Kaneko, H., Ohnuma, K., Nakai, M., Shino, M., Nagai, T., and Kashiwazaki, N. (2007) Cytoplasmic glutathione regulated by

cumulus cells during porcine oocyte maturation affects fertilization and embryonic development in vitro. *Theriogenology* **67**(5), 983-993

Matoba, S., Fair, T., and Lonergan, P. (2010) Maturation, fertilisation and culture of bovine oocytes and embryos in an individually identifiable manner: a tool for studying oocyte developmental competence. *Reprod. Fertil. Dev.* **22**(5), 839-851

Mattioli, M., Galeati, G., Barboni, B., and Seren, E. (1994) Concentration of cyclic AMP during the maturation of pig oocytes in vivo and in vitro. *J. Reprod. Fertil.* **100**(2), 403-409

Mito, T., Yoshioka, K., Noguchi, M., Yamashita, S., and Hoshi, H. (2013) Recombinant human follicle-stimulating hormone and transforming growth factor-alpha enhance in vitro maturation of porcine oocytes. *Mol. Reprod. Dev.* **80**(7), 549-560

Nakai, M., Saito, E., Takizawa, A., Akamatsu, Y., Koichi, M., Hisamatsu, S., Inomata, T., Shino, M., and Kashiwazaki, N. (2005) Offspring derived from intracytoplasmic injection of sonicated rat sperm heads. *J. Mamm. Ova Res.* **22**(3), 159-162

O'Doherty, E., Wade, M., Hill, J., and Boland, M. (1997) Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology* **48**(1), 161-169

Ozawa, M., Nagai, T., Somfai, T.s., Nakai, M., Maedomari, N., Miyazaki, H., Kaneko, H., Noguchi, J., and Kikuchi, K. (2010) Cumulus cell-enclosed oocytes acquire a capacity to synthesize GSH by FSH stimulation during in vitro maturation in pigs. *J. Cell. Physiol.* **222**(2), 294-301

Pinyopummintr, T., and Bavister, B. (1991) In vitro-matured/in vitro-fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined, protein-free culture media. *Biol. Reprod.* **45**(5), 736-742

Prochazka, R., Nagyova, E., Rimkevičová, Z., Nagai, T., Kikuchi, K., and Motlik, J. (1991) Lack of effect of oocytectomy on expansion of the porcine cumulus. *J. Reprod. Fertil.* **93**(2), 569-576

Reed, M., Estrada, J., Illera, M., and Petters, R. (1993) Effects of epidermal growth factor, insulin-like growth factor-I, and dialyzed porcine follicular fluid on porcine oocyte maturation in vitro. *J. Exp. Zool.* **266**(1), 74-78

Rizos, D., Ward, F., Duffy, P., Boland, M., and Lonergan, P. (2002) Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* **61**(2), 234-248

Singh, B., Zhang, X., and Armstrong, D. (1993) Porcine oocytes release cumulus expansion-enabling activity even though porcine cumulus expansion in vitro is independent of the oocyte. *Endocrinology* **132**(4), 1860-1862

Somfai, T., Kikuchi, K., Onishi, A., Iwamoto, M., Fuchimoto, D., Papp, Á.B., Sato, E., and Nagai, T. (2003) Meiotic arrest maintained by cAMP during the initiation of maturation enhances meiotic potential and developmental competence and reduces polyspermy of IVM/IVF porcine oocytes. *Zygote* **11**(3), 199-206

Stokes, P.J., Abeydeera, L.R., and Leese, H.J. (2005) Development of porcine embryos in vivo and in vitro; evidence for embryo 'cross talk' in vitro. *Dev. Biol.* **284**(1), 62-71

Sugiura, K., Pendola, F.L., and Eppig, J.J. (2005) Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev. Biol.* **279**(1), 20-30

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., and de Kruif, A. (2002) Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol. Reprod. Dev.* **61**(3), 414-424

Vanderhyden, B. (1993) Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s). *J. Reprod. Fertil.* **98**(1), 219-227

Xia, G., Kikuchi, K., Noguchi, J., and Izaike, Y. (2000) Short time priming of pig cumulus-oocyte complexes with FSH and forskolin in the presence of hypoxanthine stimulates cumulus cells to secrete a meiosis-activating substance. *Theriogenology* **53**(9), 1807-1815

Yoshioka, K., Suzuki, C., and Onishi, A. (2008) Defined system for in vitro production of porcine embryos using a single basic medium. *J. Reprod. Dev.* **54**(3), 208-213

Chapter 6

Influence of co-culture with denuded oocytes during *in vitro* maturation on fertilization and developmental competence of cumulus-enclosed porcine oocytes in a defined system

Adapted from:

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6.1 Abstract

Co-culture of cumulus-oocyte complexes (COCs) with denuded oocytes (DOs) during *in vitro* maturation (IVM) was reported to improve the developmental competence of oocytes *via* oocyte-secreted factors in cattle. The aim of the present study was to investigate if addition of DOs during IVM can improve *in vitro* fertilization (IVF) and *in vitro* culture (IVC) results for oocytes in a defined *in vitro* production system in pigs. The maturation medium was porcine oocyte medium supplemented with gonadotropins, dibutyryl cAMP sodium salt (dbcAMP) and β-mercaptoethanol. Cumulus-oocyte complexes were matured without DOs or with DOs in different ratios (9 COCs, 9 COCs+16 DOs and 9 COCs+36 DOs). Consequently; oocytes were subjected to IVF as intact COCs or after denudation to examine if DO addition during IVM would affect cumulus or oocyte properties. After fertilization, penetration and normal fertilization rates of zygotes were not different between all tested groups irrespective of denudation before IVF. When zygotes were cultured for 6 days, no difference could be observed between all treatments groups in cleavage rate, blastocyst rate and cell number per blastocyst. In conclusion, irrespective of the ratio, co-culture with DOs during IVM did not improve fertilization parameters and embryo development of cumulus-enclosed porcine oocytes in a defined system.

6.2 Introduction

In vitro embryo production (IVP) is indispensable for preservation and distribution of genetic material (Pereira and Marques 2008). Besides, IVP constitutes the foundations for further development of advanced biotechniques such as the production of transgenic pigs for biomedical applications (Prather *et al.* 2003). Pigs are routinely used as a model for human biomedical science (reviewed by Whyte and Prather (2011)). Moreover, transgenic pigs can be of great value to the agricultural sector (Wheeler and Walters 2001). Changes in the genome could (1) alter the carcass composition such that it is a healthier product, (2) produce pork faster or more efficiently, (3) create animals that are resistant to specific diseases, (4) reduce the major losses normally observed during the first month of swine embryogenesis,

and (5) create animals that are more environmentally friendly (Prather *et al.* 2003). To allow further improvement in new biotechnologies in pigs, it is crucial to sustain these methods with reliable *in vitro* embryo production (IVP) systems (Prather *et al.* 2003). During the last decade, advances have already been made in the development of *in vitro* maturation (IVM) systems for porcine oocytes, which allow 20 to 30% of cumulus-enclosed immature oocytes to develop to the blastocyst stage *in vitro* (Gil *et al.* 2010). Recently, chemically defined media have been developed for IVP in pigs, which allow omitting unwanted non-defined factors from the system (Yoshioka *et al.* 2008). For IVM in mammals, culture of oocytes in large groups is advantageous since reduced numbers of oocytes used for group culture decrease maturation efficiency (O'Doherty *et al.* 1997). Moreover, future development of the IVM systems for small groups of oocytes will be necessary since this approach allows the culture and tracking of oocytes collected from specific donors in an individually identifiable manner. In general, porcine IVM systems still require further improvement since the efficacy of embryo development is still lower compared to *in vivo* matured oocytes (Yoshida *et al.* 1990).

One of the possible approaches for the improvement of IVM systems is the utilization of oocyte-secreted factors (OSFs). This approach is based on the bidirectional communication between the oocyte and the surrounding cells such as granulosa cells and cumulus cells (Buccione *et al.* 1990a). During this process, oocytes regulate cumulus cell differentiation *via* the secretion of soluble paracrine growth factors, which play important roles in regulation of the folliculogenesis (Gilchrist *et al.* 2008). Many studies proved already that OSFs regulate several cumulus cells functions, which in turn are crucial for the oocyte to acquire developmental competence (reviewed by Li *et al.* (2008b)). In this way, the oocyte is actively regulating its own microenvironment (Gilchrist and Thompson 2007). Alteration of medium characteristics by extra helper-oocytes is possible by secreting soluble proteins such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), which in mammalian species affect the development and behavior of follicular cells (Hussein *et al.* 2005; Hussein *et al.* 2006). Co-culture of cumulus-oocyte-complexes (COCs) with denuded oocytes (DOs) or treatment with specific OSFs enhanced oocyte developmental competence in cattle (Hussein *et al.* 2006), goats (Romaguera *et al.* 2010) and mice (Yeo *et al.* 2007). In pigs, contradictory results have been obtained so far. According to Gomez *et al.* (2012), co-culture with DOs during IVM in a non-defined medium containing porcine follicular fluid

improved developmental competence of COCs after parthenogenetic activation but repressed cumulus expansion. On the contrary, Romaguera and Grupen (2009) did not observe any beneficial effect of OSFs in co-culture conditions on the developmental competence of porcine oocytes. Most of the previously mentioned studies were based on non-defined culture media supplemented either with serum or follicular fluid. Unknown factors in sera and follicular fluid can potentially interact with oocytes and cumulus cells (Pinyopummintr and Bavister 1991; Reed *et al.* 1993; Yoshioka *et al.* 2008), which makes interpretation of results aiming to understand the interaction between these cells difficult. To reveal the possible effect of OSFs on fertilization parameters and oocyte developmental competence, the use of defined media is necessary to avoid interference with undefined components. Undefined components may not only hamper the analysis of the action of certain substances added to the medium, but they may also spoil the reproducibility of the results because of the lower reliability of the media formulations (Yoshioka *et al.* 2008). Besides containing several undefined and thus uncontrollable components, follicular fluid is also a possible source for contamination by viral pathogens (cattle (Galik *et al.* 2002); human (Devaux *et al.* 2003)) or environmental pollutants (pig (Kamarianos *et al.* 2003)). Moreover, once the positive effect of DOs is verified, the defined medium can still potentially be analyzed after culture of DOs for the presence of OSFs. Recently, we have investigated the influence of DO addition during IVM on nuclear maturation and cumulus expansion of COCs in defined media (Appeltant *et al.* 2015). Our results revealed that addition of DOs to the IVM droplet did not affect nuclear maturation of COCs (Appeltant *et al.* 2015), which confirmed the findings of Gomez *et al.* (2012) using a non-defined medium. On the other hand, unlike in the report of Gomez *et al.* (2012), DOs evidently enhanced cumulus expansion to some extent (Appeltant *et al.* 2015). To date, the efficacy of co-culture with DOs to the media to improve efficacy of IVF systems of porcine oocytes using defined media remains unknown.

Consequently, the aim of the present study was to investigate if co-culture with DOs during IVM is beneficial for fertilization and embryo development of COCs cultured in small groups in defined media. To determine if OSFs can potentially influence the IVF process either by affecting the cumulus compartment or directly the oocyte, fertilization rates of matured COCs were assessed both by fertilizing them either as intact COCs or after denudation.

6.3 Materials and Methods

6.3.1 Chemicals

Unless otherwise stated, all chemicals used were obtained from Sigma–Aldrich (Bornem, Belgium).

6.3.2 Oocyte collection and *in vitro* maturation

Ovaries (40-60) of prepubertal gilts were collected in a local abattoir and transported to the laboratory at 37 °C in a 0.9% sodium chloride solution supplemented with kanamycin. Ovaries of Piétrain crossbred prepubertal gilts of approximately 6.5 months of age, weighing 105 to 115 kg were used. Randomization was applied at the level of collection of the ovaries and at the level of collection of the COCs. Cumulus-oocyte complexes were collected by dissection of 2 to 6 mm follicles in Medium 199 HEPES (M199 HEPES; with Hank's salts; Gibco, Ghent, Belgium). After dissection, the COC selection started with a screening for those oocytes that had a multi-layered compact cumulus and a homogeneous ooplasm. The basic medium for IVM was porcine oocyte medium (POM) (Yoshioka *et al.* 2008) supplemented with 3 mg/mL polyvinyl alcohol (PVA) and 50 µM β-mercaptoethanol (β-ME). During the first 20 h of IVM 10 IU/mL equine chorionic gonadotropin (eCG), 10 IU/mL human chorionic gonadotropin (hCG) and 1.0 mM dibutyryl cAMP sodium salt (dbcAMP) were added to the IVM medium (POM 1). At 20 h of IVM, the groups of oocytes were washed three times in the same medium without hormones and dbcAMP (POM 2) and transferred to drops with POM 2 for an additional 28 h of IVM. The oocytes were matured at 39 °C in 5% CO_2 in air. Every group of COCs was matured in a drop of 100 µL IVM medium under mineral oil in a petri dish (Falcon 351008, Becton Dickinson Labware, Franklin Lakes, NJ, USA). A maximum of four drops per petri dish was applied. In each culture drop either 9 COCs with or without 16 DOs or 36 DOs (depending on experimental design as described

below) were matured. Ratio 9 COCs-16 DOs was chosen based on our previous research dealing with cumulus expansion and nuclear maturation (Appeltant *et al.* 2015). Buccione *et al.* (1990b) described that the FSH-stimulated cumulus expansion of mouse oocytectomized complexes co-cultured with DOs or medium conditioned with DOs directly correlated with the number of oocytes. In line with these thoughts, we also included another ratio of 9 COCs-36 DOs based on the results obtained in the study of Gomez *et al.* (2012). The use of 16 DOs/100 µL (=0.16 DO/µL) and 36 DOs/100 µL (=0.36 DO/µL) fits within the required range to observe effects of OSFs (Hussein *et al.* 2005).

6.3.3 Preparation of denuded oocytes

To obtain the necessary number of DOs for each experimental set-up, COCs were denuded by vortexing for 5 min in collection medium. The COCs used as a source for DOs fulfilled the same requirements as the COCs used for maturation and were selected based on the presence of an intact oolemma under a stereomicroscope.

6.3.4 *In vitro* fertilization

The basic medium for IVF was modified Tyrode's albumin lactate pyruvate (TALP) medium (Rath *et al.* 1999) supplemented with 3 mg/mL fatty acid-free bovine serum albumin (BSA fraction V) and 1.10 mM sodium pyruvate (FERT-TALP) (Romar *et al.* 2003). For the fertilization, frozen-thawed epididymal boar spermatozoa were used. After thawing, the semen was centrifuged (3 min, 390 × *g*, 22 °C) in an Androhep extender, which consisted of 27 mM tri-sodium citrate, 6.4 mM Titriplex III EDTA, 2.5 mg/mL BSA (fraction V), 16 mM $NaHCO_3$, 38 mM HEPES, 144 mM D(+)-glucose.H_2O and 50 µg/mL gentamycin sulfate. Subsequently, the centrifuged pellet of semen was resuspended in FERT-TALP. Presumed matured COCs were transferred in groups of 9 COCs to 100 µL FERT-TALP drops under mineral oil. Depending on the experimental design (described below) the presumed matured

COCs were denuded or not, prior to fertilization. Denudation was performed by a brief treatment with 0.1% hyaluronidase in collection medium followed by pipetting through a narrow-bore glass pipette. The oocytes and the spermatozoa were co-incubated for 6 h (38.5 °C, 5% CO_2 in air).

6.3.5 *In vitro* culture

After 6 h, the presumed zygotes, of which the cumulus was still present, were vortexed for 3 min and the presumed zygotes, of which the cumulus was already removed, were vortexed for 40 s in HEPES buffered (25 mM HEPES) TALP medium (HEPES-TALP). Presumed zygotes were selected based on the presence of an intact oolemma under a stereomicroscope, washed in HEPES-TALP and culture medium, which was porcine zygote medium (PZM-5) (Yoshioka *et al.* 2002). Subsequently, the presumed zygotes were cultured for 6 days in a modular incubator chamber (38.5 °C, 5% CO_2, 5% O_2).

6.3.6 Assessment of fertilization

The presumed zygotes were washed, fixed in 4% paraformaldehyde (PF) for 1 h at room temperature at 18 h after IVF and stained with Hoechst 33342. The evaluation of the fertilization parameters was performed under a DMR fluorescence microscope (Leica microsystems, Brussels, Belgium). Oocytes with more than one pronucleus or a pronucleus and one or more decondensed sperm heads were judged as penetrated. The penetration rate was expressed in respect to the total number of non-degenerated presumed zygotes. Zygotes with two pronuclei or one pronucleus and one decondensed sperm head and two polar bodies were classified as normally (monospermic) fertilized. On the contrary, the zygotes that contained at least one pronucleus and two or more other pronuclei or decondensed sperm heads were considered to be polyspermic. Normal fertilization rates as well as polyspermy rates were expressed in respect to the number of penetrated oocytes. Maturation rates for the

presumed zygotes in the fertilization trials were also calculated. Oocytes were considered matured if an unpenetrated metaphase II or a penetrated oocyte with two polar bodies (Kikuchi *et al.* 1999) was observed. The maturation rate was expressed in respect to the total number of non-degenerated presumed zygotes.

6.3.7 Assessment of embryo development

The embryo development was evaluated based on the cleavage rate and the rate of blastocyst formation visualized under a stereomicroscope. The cleavage rate was calculated based on the number of cleaved zygotes at 48 h after IVF. Non-cleaved zygotes were defined as presumed zygotes consisting of one blastomere associated with no or a minimum of fragmentation. Blastocyst formation was recorded from the moment a blastocoel cavity was formed (Mateusen *et al.* 2005) and calculated after 6 days of *in vitro* embryo culture (d 0 is the day of IVF).

6.3.8 Assessment of the number of cells in blastocysts

On day 6, blastocysts were washed and fixed overnight in 2% PF. Subsequently, the blastocysts were stained with Hoechst 33342 and mounted on a glass slide in a drop of 1,4-diazabicyclo[2.2.2]octane (DABCO). The number of cells per blastocyst was counted under a DMR fluorescence microscope (Leica microsystems, Brussels, Belgium) (magnification 400 X).

6.3.9 Experimental design

6.3.9.1 Experiment 1: Effect of co-culture with denuded oocytes on fertilization parameters of cumulus-oocyte complexes

Different ratios of DOs were added to COCs during IVM to investigate if DO addition during IVM could improve fertilization parameters. The applied ratios consisted of an increasing number of DOs co-cultured with COCs: 9 COCs (= treatment 1), 9 COCs+16 DOs (= treatment 2) and 9 COCs+36 DOs (= treatment 3) (Figure 1).

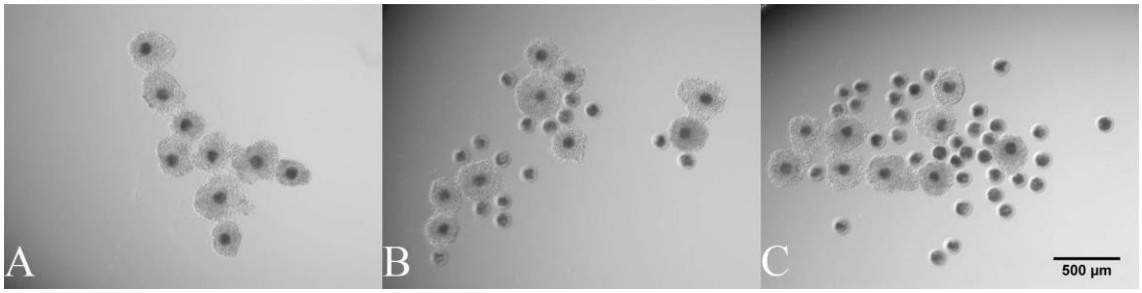

Figure 1: Representative images of the three different applied ratio compositions used in the experimental design at 20 h of in vitro *maturation (A) 9 intact cumulus-oocyte-complexes (COCs); (B) 9 COCs+16 denuded oocytes (DOs); (C) 9 COCs+36 DOs. Photographs by R. Appeltant.*

After 48 h of IVM, COCs of the three treatment groups were subjected to IVF. Presumed matured COCs were either fertilized as intact COCs (= COC-fertilized group) or after denudation (= denuded-fertilized group) to examine if DO addition during IVM would affect the cumulus or the oocyte properties of the COC (Figure 2). Fertilization parameters were evaluated at 18 h after IVF. Several fertilization parameters were investigated. First, the percentage of matured oocytes and penetrated oocytes were calculated in respect to the total number of non-degenerated presumed zygotes. Second, the normal fertilization and polyspermy rates were counted as a percentage of the penetrated oocytes. Six replicates were performed.

Preliminary trials were conducted to set the spermatozoa concentration in order to be able to observe both an increase as well as a possible decrease of penetration and polyspermy rates in comparison to the rates of treatment 1 (data not shown).

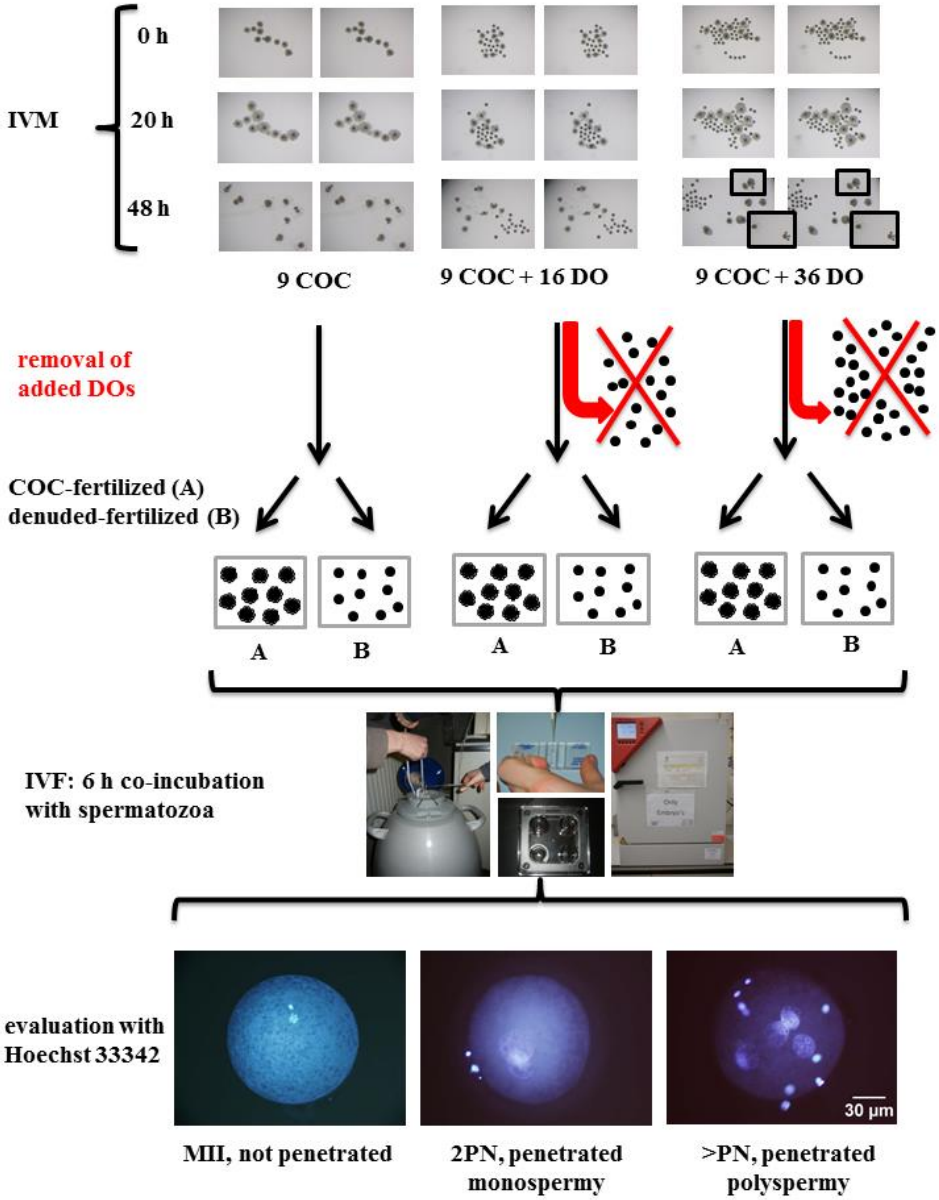

Figure 2: Process diagram of the experimental set-up to investigate the effect of denuded oocyte (DO) addition during in vitro *maturation (IVM) on fertilization parameters (penetration and polyspermy rates) after* in vitro *fertilization (IVF). The matured cumulus-oocyte complexes (COCs) were fertilized as intact COCs (COC-fertilized) or after denudation (denuded-fertilized). Magnification x 40 for IVM photos. Photographs and pictures by R. Appeltant.*

6.3.9.2 Experiment 2: Effect of co-culture with denuded oocytes on developmental competence of cumulus-oocyte complexes

The same treatment groups as in *Experiment 1* were used to examine if DO addition during IVM could improve developmental competence of COCs. After 48 h of IVM, COCs of the three treatment groups were subjected to IVF and cultured for 6 days as described above. Presumed matured COCs were all fertilized as intact COCs. To evaluate the influence of DO addition during IVM on IVP of porcine embryos, several variables were investigated, more specifically the cleavage percentage at 48 h after IVF, blastocyst percentage on day 6 of IVC and the total cell number per blastocyst on day 6. Three replicates were performed.

6.3.10 Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) Statistics version 20. Hypothesis testing was performed using a significance level of 5% (two-sided test). A P-value between 0.05 and 0.1 was considered as a trend towards significance. Depending on the experiment, three to six replicates were performed. Due to the experimental set-up of experiment 1, six replicates were performed to obtain the necessary statistical power.

Fertilization parameters, cleavage and blastocyst percentage were analyzed using binary logistic regression. The proportion of oocytes in a category was included as dependent variable and group was included as independent variable. Replicate was included as a covariate. The number of cells per blastocyst was analyzed by ANOVA because this parameter was normally distributed and showed an equality of error variances. In tables, proportions are presented as percentage ± standard error of the mean (SEM).

6.4 Results

6.4.1 Effect of co-culture with denuded oocytes on fertilization parameters of cumulus-oocyte complexes

No significant differences could be observed among groups 1, 2 and 3 when COCs were fertilized as intact COCs for maturation rates (92.5%, 97.8%, and 94.8%, respectively), penetration rates (62.7%, 58.4%, and 48.5%, respectively), and polyspermy rates (54.8%, 53.8%, and 44.7%, respectively) (Table 1). Nevertheless, a trend towards a lower penetration between treatment 1 and 3 could be noticed (P = 0.079).

Table 1: Effect of DO addition during in vitro *maturation (IVM) on* in vitro *fertilization (IVF) of porcine cumulus-intact oocytes. Proportions are presented as percentage ± SEM.*

IVM treatment	Examined (n)	Matured (%)†	Penetrated (%)†	Polyspermy (%)‡	Fertilized normally (%)‡
1	67	92.5 ± 3.2	62.7 ± 8.0*	54.8 ± 10.7	45.2 ± 10.7
2	90	97.8 ± 1.0	58.4 ± 8.2	53.8 ± 7.1	46.2 ± 7.1
3	97	94.8 ± 2.2	48.5 ± 7.9*	44.7 ± 4.6	55.3 ± 4.6

"n" represents the total number of examined presumed zygotes.
Within a column, percentages did not differ significantly ($P \geq 0.05$). The percentages with * superscripts show a trend of difference (P = 0.079). Data were obtained by performing six replicates.
DO: denuded oocyte; COC: cumulus-oocyte complex; SEM: standard error of the mean.
Treatment 1 = 9 COCs without DOs; treatment 2 = 9 COCs+16 DOs; treatment 3 = 9 COCs+36 DOs.
† Percentage of non-degenerated examined oocytes.
‡ Percentage of penetrated oocytes.

When COC-derived oocytes were fertilized after denudation, there was no significant difference among the three treatment groups in any of the fertilization parameters (Table 2). In groups 1, 2 and 3, maturation rates were 95.3%, 93.2%, and 97.7%, respectively, penetration rates were 88.4%, 90.9%, and 88.4%, respectively, and polyspermy rates were 73.7%, 77.5%, and 71.1%, respectively (Table 2). In addition, the penetration trend was not present in the denuded-fertilized group (Table 2). The degeneration rate for the examined oocytes was similar in all experimental groups (data not shown).

Table 2: Effect of DO addition during in vitro *maturation (IVM) on* in vitro *fertilization (IVF) of porcine denuded oocytes. Proportions are presented as percentage ± SEM.*

IVM treatment	Examined (n)	Matured (%)†	Penetrated (%)†	Polyspermy (%)‡	Fertilized normally (%)‡
1	43	95.3 ± 2.7	88.4 ± 7.4	73.7 ± 9.8	26.3 ± 9.8
2	44	93.2 ± 3.2	90.9 ± 4.7	77.5 ± 5.1	22.5 ± 5.1
3	43	97.7 ± 2.8	88.4 ± 5.8	71.1 ± 6.3	28.9 ± 6.3

"n" represents the total number of examined presumed zygotes.
Within a column, percentages did not differ significantly ($P \geq 0.05$). Data were obtained by performing six replicates.
DO: denuded oocyte; COC: cumulus-oocyte complex; SEM: standard error of the mean.
Treatment 1 = 9 COCs without DOs; treatment 2 = 9 COCs+16 DOs; treatment 3 = 9 COCs+36 DOs.
† Percentage of non-degenerated examined oocytes.
‡ Percentage of penetrated oocytes.

6.4.2 Effect of co-culture with denuded oocytes on developmental competence of cumulus-oocyte complexes

No significant differences in developmental competence could be observed among treatments 1, 2 and 3 for cleavage rates (62.3%, 52.1%, and 50.7%, respectively), blastocyst rates (27.9%, 26.8%, and 31.0%, respectively), and the mean numbers of cells per blastocyst (52.0,

51.3, and 47.0, respectively) (Table 3). Nevertheless, a trend towards a lower cleavage rate among treatment 1 and 3 could be noticed (P = 0.072).

Table 3: Effect of DO addition during in vitro *maturation (IVM) on* in vitro *culture (IVC) parameters of cultured oocytes. Proportions are presented as percentage ± SEM. No. of cells per blastocyst are presented as number ± SEM.*

IVM treatment	Examined (n)	Cleaved (%)†	Blastocyst (%)†	No. of cells/blastocyst
1	61	62.3 ± 6.2*	27.9 ± 9.3	52.0 ± 4.5
2	71	52.1 ± 10.7	26.8 ± 2.2	51.3 ± 5.1
3	71	50.7 ± 11.0*	31.0 ± 4.3	47.0 ± 4.5

"n" represents the number of oocytes in which IVC was started.
Within a column, percentages did not differ significantly (P ≥ 0.05). The percentages with * superscript show a trend of difference (P = 0.072). Data were obtained by performing three replicates.
DO: denuded oocyte; COC: cumulus-oocyte complex; SEM: standard error of the mean.
Treatment 1 = 9 COCs without DOs; treatment 2 = 9 COCs+16 DOs; treatment 3 = 9 COCs+36 DOs.
† Percentage of examined oocytes.

6.5 Discussion

Since OSFs played a crucial role in developmental competence of oocytes in various animal species (Hussein *et al.* 2006; Yeo *et al.* 2007; Romaguera *et al.* 2010) and improved developmental competence of porcine oocytes after parthenogenetic activation (Gomez *et al.* 2012), we hypothesized a potential positive influence of adding DOs during IVM on fertilization results and embryo developmental competence of co-cultured porcine COCs. However, our results have revealed that in a defined system, the fertilization parameters of porcine oocytes after IVF were not significantly affected by DO addition during IVM irrespective of the COC-DO ratio. Nevertheless we could observe a trend towards a lower

penetration for treatment 3 compared to treatment 1 when the oocytes were fertilized as intact COCs (Table 1). However, such a trend was not observed when COC-derived oocytes were fertilized after denuding (Table 2). This suggests that co-culture with DOs during IVM may be acting on the cumulus compartment of the COCs, slightly influencing fertilization parameters. The cause of a decrease in penetration rate in the presence of additional DOs during preceding IVM of COCs could be due to an improved cumulus matrix constitution induced by the added DOs. Our previous results have demonstrated that although DO addition did not improve cumulus expansion of COCs, co-culture with DOs significantly improved the expansion of cumulus cells of oocytectomized complexes (OOXs) in a defined medium (Appeltant *et al.* 2015). This suggests that even though co-culture with DOs has no evident effect on COC expansion, this treatment might still induce quality changes in the cumulus matrix of COCs. In parallel with the findings of Bijttebier *et al.* (2008) an evolution towards a reduction of polyspermy was associated with a trend towards reduction in penetration rate (Table 1), suggesting that an altered cumulus matrix by DO addition may prevent some spermatozoa to reach the oocyte. Although mainly oocyte-related processes are involved in blocking polyspermy during fertilization such as cortical granule (CG) exocytosis (cortical reaction) and the zona reaction (Wang *et al.* 1998), in this study polyspermy was not reduced when COCs were fertilized after removal of the cumulus (Table 2). Therefore, the evolution towards a reduced polyspermy rate as a result for co-culture with DOs during IVM may not have been caused by mechanisms of the oocyte itself but rather by reducing penetration by the cumulus composition. Previous studies using an interspecies model (Singh *et al.* 1993) and oocytectomized porcine COCs (Appeltant *et al.* 2015) have suggested that porcine oocytes secrete a cumulus expansion-enabling factor(s). Nevertheless, this factor is yet to be determined. In previous studies in different mammalian species including mice, cattle and pigs, DO addition to OOXs not only affected cumulus expansion but also several other important parameters such as cumulus cell apoptosis (Hussein *et al.* 2005), luteinization markers (Gilchrist *et al.* 2008), cumulus glycolytic enzyme mRNA levels (Sugiura *et al.* 2005) and steroidogenesis (Coskun *et al.* 1995). Brankin *et al.* (2003) proved that porcine OSFs influence granulosa and theca cell proliferation and steroidogenesis. Although the specific role of GDF9 and BMP15 in pigs remains to be elucidated, the specific expression of BMP15 at the time of cumulus expansion suggests a possible role in cumulus expansion (Li *et al.* 2008a). In mice, Dragovic *et al.* (2005) showed that GDF9 is one of the factors that can enable FSH-induced cumulus expansion. Further research will be needed to clarify the

involvement of these factors in the regulation of penetration through the cumulus compartment in porcine IVP systems.

Following the trend towards a lower penetration rate of the COC-fertilized group in treatment 3 compared to treatment 1 (Table 1), the same trend could be observed for the cleavage rates between those treatment groups (Table 3). Despite these latter trends and the decreasing evolution of polyspermy rates of the COC-fertilized group (Table 1), no differences in blastocyst formation and cell numbers among the groups were observed in Experiment 2 (Table 3). This fact illustrates that the non-significant reduction of polyspermy and the observed trends in penetration and cleavage rates are not associated with any effects on blastocyst formation level. Literature data in cattle and goat IVF oocytes and porcine parthenotes have reported a significant increase in blastocyst percentage when COCs were co-cultured with DOs during IVM (Hussein *et al.* 2006; Romaguera *et al.* 2010; Gomez *et al.* 2012). On the contrary, similarly to the present study, Romaguera and Grupen (2009) could not observe any positive effect on the developmental competence of porcine oocytes after co-culture. Since Hussein *et al.* (2006) and Romaguera *et al.* (2010) were working with different species, it is plausible that the action of DO-derived OSFs on COCs varies among species, which could explain the discrepancy in results between the studies. The fact that the same researchers (*i.e.* Romaguera *et al.*) reported beneficial effects of OSFs in goats, but not in pigs, highlights the possibility that species-dependent mechanisms could be involved. Our results partially contradict the findings of Gomez *et al.* (2012) but they confirm the findings of Romaguera and Grupen (2009), where no beneficial effect on the developmental competence of porcine oocytes could be observed. The reasons for the discrepancy between the results of Gomez *et al.* (2012) and those of Romaguera and Grupen (2009) and our results remain to be elucidated. In fact, there were substantial differences in the set-up of the experiments between our study and that of Gomez *et al.* (2012). In their study, Gomez *et al.* (2012) used a maturation medium supplemented with non-defined porcine follicular fluid, whereas in our study a defined POM medium was used for IVM. Nevertheless, it remains unclear how the differences in medium composition could have contributed to the differences in blastocyst development results between the two studies. Furthermore, our results have shown that the quality of blastocysts, expressed by the total cell number per blastocyst, was not affected by co-culture of COCs with DOs during IVM (Table 3), confirming the previous findings from Gomez *et al.* (2012) for porcine parthenotes, but conflicting the results obtained in cattle by Hussein *et al.* (2006).

Addition of DOs to IVM medium is believed to affect COCs by other means than OSF production. Cultured cells consume oxygen. Therefore increasing the numbers of cells in the culture drop may reduce oxidative stress on oocytes, which is known to be detrimental to their developmental competence. Nevertheless this might not have happened in our system since embryo development was not affected in this study. Regarding this point, it must be noted that in the present study, the POM medium was supplemented with the antioxidant β-ME in contrast to our previous study (Appeltant *et al.* 2015) where a very basic POM was applied to reveal the effects of DO addition during IVM on cumulus expansion and nuclear maturation of cumulus-enclosed porcine oocytes. The lack of effect of DO addition on oxidative stress can be explained by the supplementation of POM medium with β-ME. Because β-ME is known to increase glutathione concentration in oocytes, it could potentially neutralize toxic effects of oxidative stress (Akaki *et al.* 2009).

The results reveal that the extra OSFs supplementation *via* DO addition did not improve fertilization parameters and oocyte developmental competence. In every trial of the present study, a ratio of 0.16 DO/µL and 0.36 DO/µL was applied. These ratios are higher than 0.128 DO/µL, which was described in two previous studies (Gilchrist *et al.* 2001; Gilchrist *et al.* 2006), and are within the range of 0.1 DO/µL to 1 DO/µL as described by Hussein *et al.* (2005). Based on these findings in literature, the applied number of oocytes/µL in the present is believed to be sufficient to reveal any possible effects of OSFs.

The fact that an abundant number of functions of OSFs are described in various species, pointed us towards our hypothesis. Since the purpose of this study was to find a practical tool to increase fertilization and blastocyst rates and no positive effects of DO addition could be observed, no further studies have been performed to elucidate the underlying working mechanism of OSFs. Nevertheless, possible effects of DO-derived OSFs like GDF-9 and BMP-15 on *e.g.* the expression of ovulation related genes and estrogen production in the cumulus cannot be ruled out and may be a subject of future research. Considering the results and also the issue of a very labor intensive protocol of DO production, the use of recombinant OSFs may be a more expedient approach for fundamental research of OSFs in pigs in the future.

In conclusion, our results demonstrate that addition of DOs to IVM medium at a concentration up to 0.36 DO/µL does not significantly improve fertilization and developmental competence

in the present defined system. It has become evident that the defined medium used in this study supplies the necessary components for IVM and that it sufficiently supports the maturation of COCs, even in small groups of oocytes without the need of extra helper-oocytes.

6.6 Acknowledgements

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6.7 References

Akaki, Y., Yoshioka, K., Noguchi, M., Hoshi, H., and Funahashi, H. (2009) Successful piglet production in a chemically defined system for in-vitro production of porcine embryos: dibutyryl cyclic amp and epidermal growth factor-family peptides support in-vitro maturation of oocytes in the absence of gonadotropins. *J. Reprod. Dev.* **55**(4), 446-453

Appeltant, R., Somfai, T., Nakai, M., Bodó, S., Maes, D., Kikuchi, K., and Van Soom, A. (2015) Interactions between oocytes and cumulus cells during in vitro maturation of porcine cumulus-oocyte complexes in a chemically defined medium: effect of denuded oocytes on cumulus expansion and oocyte maturation. *Theriogenology* **83**, 567-576

Bijttebier, J., Van Soom, A., Meyer, E., Mateusen, B., and Maes, D. (2008) Preovulatory follicular fluid during in vitro maturation decreases polyspermic fertilization of cumulus-intact porcine oocytes. *Theriogenology* **70**(4), 715-724

Brankin, V., Mitchell, M., Webb, B., and Hunter, M. (2003) Paracrine effects of oocyte secreted factors and stem cell factor on porcine granulosa and theca cells in vitro. *Reprod. Biol. Endocrinol.* **1**(1), 55

Buccione, R., Schroeder, A., and Eppig, J. (1990a) Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol. Reprod.* **41**(1), 176

Buccione, R., Vanderhyden, B., Caron, P., and Eppig, J. (1990b) FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev. Biol.* **138**(1), 16-25

Coskun, S., Uzumcu, M., Lin, Y., Friedman, C., and Alak, B. (1995) Regulation of cumulus cell steroidogenesis by the porcine oocyte and preliminary characterization of oocyte-produced factor(s). *Biol. Reprod.* **53**(3), 670-675

Devaux, A., Soula, V., Sifer, C., Branger, M., Naouri, M., Porcher, R., Poncelet, C., Neuraz, A., Alvarez, S., Benifla, J.L., Madelenat, P., Brun-Vezinet, F., and Feldmann, G. (2003) Hepatitis C virus detection in follicular fuid and culture media from HCV+ women, and viral risk during IVF procedures. *Hum. Reprod.* **18**(11), 2342-2349

Dragovic, R.A., Ritter, L.J., Schulz, S.J., Amato, F., Armstrong, D.T., and Gilchrist, R.B. (2005) Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. *Endocrinology* **146**(6), 2798-2806

Galik, P.K., Givens, M.D., Stringfellow, D.A., Crichton, E.G., Bishop, M.D., and Eilertsen, K.J. (2002) Bovine, viral diarrhea virus (BVDV) and anti-BVDV antibodies in pooled samples of follicular fluid. *Theriogenology* **57**, 1219-1227

Gil, M.A., Cuello, C., Parrilla, I., Vazquez, J.M., Roca, J., and Martinez, E.A. (2010) Advances in swine in vitro embryo production technologies. *Reprod. Domest. Anim.* **45**, 40-48

Gilchrist, R.B., Lane, M., and Thompson, J.G. (2008) Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum. Reprod. Update* **14**(2), 159-177

Gilchrist, R.B., Ritter, L.J., and Armstrong, D.T. (2001) Mouse oocyte mitogenic activity is developmentally coordinated throughout folliculogenesis and meiotic maturation. *Dev. Biol.* **240**(1), 289-298

Gilchrist, R.B., Ritter, L.J., Myllymaa, S., Kaivo-Oja, N., Dragovic, R.A., Hickey, T.E., Ritvos, O., and Mottershead, D.G. (2006) Molecular basis of oocyte-paracrine signalling that promotes granulosa cell proliferation. *J. Cell Sci.* **119**(18), 3811-3821

Gilchrist, R.B., and Thompson, J.G. (2007) Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro. *Theriogenology* **67**(1), 6-15

Gomez, M.N.L., Kang, J.T., Koo, O.J., Kim, S.J., Kwon, D.K., Park, S.J., Atikuzzaman, M., Hong, S.G., Jang, G., and Lee, B.C. (2012) Effect of oocyte-secreted factors on porcine in vitro maturation, cumulus expansion and developmental competence of parthenotes. *Zygote* **20**(02), 135-145

Hussein, T.S., Froiland, D.A., Amato, F., Thompson, J.G., and Gilchrist, R.B. (2005) Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. *J. Cell Sci.* **118**(22), 5257-5268

Hussein, T.S., Thompson, J.G., and Gilchrist, R.B. (2006) Oocyte-secreted factors enhance oocyte developmental competence. *Dev. Biol.* **296**(2), 514-521

Kamarianos, A., Karamanlis, X., Goulas, P., Theodosiadou, E., and Smokovitis, A. (2003) The presence of environmental pollutants in the follicular fluid of farm animals (cattle, sheep, goats, and pigs). *Reprod. Toxicol.* **17**(2), 185-190

Kikuchi, K., Nagai, T., Ding, J., Yamauchi, J., Noguchi, J., and Izaike, Y. (1999) Cytoplasmic maturation for activation of pig follicular oocytes cultured and arrested at metaphase I. *J. Reprod. Fertil.* **116**, 143-156

Li, H.-K., Kuo, T.-Y., Yang, H.-S., Chen, L.-R., Li, S.S.-L., and Huang, H.-W. (2008a) Differential gene expression of bone morphogenetic protein 15 and growth differentiation factor 9 during in vitro maturation of porcine oocytes and early embryos. *Anim. Reprod. Sci.* **103**(3-4), 312-322

Li, Q., McKenzie, L.J., and Matzuk, M.M. (2008b) Revisiting oocyte-somatic cell interactions: in search of novel intrafollicular predictors and regulators of oocyte developmental competence. *Mol. Hum. Reprod.* **14**(12), 673-678

Mateusen, B., Van Soom, A., Maes, D.G.D., Donnay, I., Duchateau, L., and Lequarre, A.S. (2005) Porcine embryo development and fragmentation and their relation to apoptotic markers: a cinematographic and confocal laser scanning microscopic study. *Reproduction* **129**(4), 443-452

O'Doherty, E., Wade, M., Hill, J., and Boland, M. (1997) Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology* **48**(1), 161-169

Pereira, R.M., and Marques, C.C. (2008) Animal oocyte and embryo cryopreservation. *Cell and Tissue Banking* **9**(4), 267-277

Pinyopummintr, T., and Bavister, B. (1991) In vitro-matured/in vitro-fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined, protein-free culture media. *Biol. Reprod.* **45**(5), 736-742

Prather, R.S., Hawley, R.J., Carter, D.B., Lai, L., and Greenstein, J.L. (2003) Transgenic swine for biomedicine and agriculture. *Theriogenology* **59**, 115-123

Rath, D., Long, C., Dobrinsky, J., Welch, G., Schreier, L., and Johnson, L. (1999) In vitro production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. *J. Anim. Sci.* **77**(12), 3346-3352

Reed, M., Estrada, J., Illera, M., and Petters, R. (1993) Effects of epidermal growth factor, insulin-like growth factor-I, and dialyzed porcine follicular fluid on porcine oocyte maturation in vitro. *J. Exp. Zool.* **266**(1), 74-78

Romaguera, R., and Grupen, C.G. (2009) Relationship between follicle size and factors secreted by porcine oocytes and cumulus cells during maturation in vitro. *Reprod. Domest. Anim.* **44**, 89

Romaguera, R., Morató, R., Jiménez-Macedo, A.R., Catalá, M., Roura, M., Paramio, M.T., Palomo, M.J., Mogas, T., and Izquierdo, D. (2010) Oocyte secreted factors improve embryo

developmental competence of COCs from small follicles in prepubertal goats. *Theriogenology* **74**(6), 1050-1059

Romar, R., Coy, P., Ruiz, S., Gadea, J., and Rath, D. (2003) Effects of oviductal and cumulus cells on in vitro fertilization and embryo development of porcine oocytes fertilized with epididymal spermatozoa. *Theriogenology* **59**(3), 975-986

Singh, B., Zhang, X., and Armstrong, D. (1993) Porcine oocytes release cumulus expansion-enabling activity even though porcine cumulus expansion in vitro is independent of the oocyte. *Endocrinology* **132**(4), 1860-1862

Sugiura, K., Pendola, F.L., and Eppig, J.J. (2005) Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev. Biol.* **279**(1), 20-30

Wang, W.H., Abeydeera, L.R., Prather, R.S., and Day, B.N. (1998) Morphologic comparison of ovulated and in vitro–matured porcine oocytes, with particular reference to polyspermy after in vitro fertilization. *Mol. Reprod. Dev.* **49**(3), 308-316

Wheeler, M.B., and Walters, E.M. (2001) Transgenic technology and applications in swine. *Theriogenology* **56**, 1345-1369

Whyte, J.J., and Prather, R.S. (2011) Genetic modifications of pigs for medicine and agriculture. *Mol. Reprod. Dev.* **78**(10-11), 879-891

Yeo, C.X., Gilchrist, R.B., Thompson, J.G., and Lane, M. (2007) Exogenous growth differentiation factor 9 in oocyte maturation media enhances subsequent embryo development and fetal viability in mice. *Hum. Reprod.* **23**(1), 67-73

Yoshida, M., Ishizaki, Y., and Kawagishi, H. (1990) Blastocyst formation by pig embryos resulting from in-vitro fertilization of oocytes matured in vitro. *J. Reprod. Fertil.* **88**, 1-8

Yoshioka, K., Suzuki, C., and Onishi, A. (2008) Defined system for in vitro production of porcine embryos using a single basic medium. *J. Reprod. Dev.* **54**(3), 208-213

Yoshioka, K., Suzuki, C., Tanaka, A., Anas, I.M.K., and Iwamura, S. (2002) Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biol. Reprod.* **66**, 112-119

Chapter 7

General discussion

7.1 Introduction

In vitro embryo production (IVP) consists of three major subsequent phases: collection and maturation (IVM), fertilization (IVF) and culture (IVC). When using the currently applied protocols and starting from immature porcine oocytes, only 20 to 30% of blastocysts can be obtained, a substantial proportion being polyploid. When considering the two major obstacles in porcine IVP, high polyspermy rates and low blastocyst rates, the question can be raised if these may be related to the fact that cytoplasmic maturation during IVM does not occur properly. This research focused on the cumulus-oocyte complex (COC) during IVM, which refers to the oocyte surrounded by several layers of cumulus cells.

The general aim of this dissertation was to obtain detailed insights in the bidirectional communication between the oocyte and the surrounding cumulus cells in order to establish a porcine IVP system with less polyspermy and with an improved oocyte developmental potential. Moreover, this system should allow the culture of small numbers of oocytes. To acquire a better understanding of the communication between oocytes and their surrounding cumulus cells, we aimed to investigate whether the removal of the oocyte from the follicle can affect its developmental competence by a premature drop in cAMP levels and if oocytes regulate cumulus cell differentiation and their own development *via* the secretion of soluble paracrine growth factors (reviewed by Gilchrist (2011)).

7.2 Role of cyclic adenosine monophosphate-modulating agents

7.2.1 Problem statement

What is exactly going wrong during IVM of porcine oocytes? During collection, COCs are removed from the follicle and thus the contacts between follicle and oocyte are interrupted. Because the control of the follicular environment is lost, cAMP levels are dropping prematurely. This causes a spontaneous resumption of meiosis, producing oocytes of low

developmental competence. We hypothesized that keeping cAMP levels high during early stages of IVM of the porcine oocyte would increase normal fertilization rates and subsequent embryo development because of a synchronization of meiotic maturation. To investigate the influence of cAMP-modulating agents like IBMX and dbcAMP on the IVP in pigs, several specific hypotheses were formulated based on literature findings. In the following sections, we discuss each of those two cAMP-modulating agents separately.

7.2.2 Role of 3-isobutyl-1-methylxanthine

It is known that IBMX halts meiotic progression in oocytes from other species, whereas for pigs, no effect could be shown thus far. We hypothesized that there was still a chance that IBMX could influence meiotic progression in pigs, but only during oocyte collection rather than in further stages. Therefore, the study in **Chapter 3** examined the influence of IBMX addition during oocyte collection, with a special emphasis on the nuclear morphology of the oocytes at the end of collection. In parallel with the findings on IBMX addition during IVM (Shimada *et al.* 2003), we hypothesized that the presence of IBMX during collection would induce an arrest at the germinal vesicle 2 (GV2) stage. Moreover, dbcAMP is also synchronizing to the GV2 stage during IVM of porcine oocytes (Funahashi *et al.* 1997). Funahashi *et al.* (1997) reported that high intracellular cAMP levels caused meiotic progression until GV2 or an arrest at GV2 and once the cAMP levels decreased, meiotic progression would resume. However, we revealed that IBMX addition during collection did not alter the chromatin configuration of porcine oocytes at the end of collection (**Chapter 3**). The lack of effect of IBMX addition during collection apparently contradicts the results obtained in other species like cattle and mice (Bornslaeger *et al.* 1984; Luciano *et al.* 1999; Albuz *et al.* 2010) where cAMP levels were increased, germinal vesicle breakdown (GVBD) was inhibited and blastocyst rates were improved. In human, it was concluded that IBMX was beneficial for developmental competence based on mouse models (Zeng *et al.* 2013; Spits *et al.* 2014). All these results strongly point towards a species-specific mechanism of IBMX. The absence of effect of IBMX during collection in pigs could possibly be explained by the fact that decreasing cAMP levels during collection do not impair the developmental competence of the porcine oocyte and that spontaneous maturation of porcine oocytes is

occurring during IVM, rather than during collection. Hereby, the findings of this study confirm the fact that spontaneous meiotic resumption is substantially slower in pigs than in other species like mouse, rat and bovine (Santiquet *et al.* 2014).

7.2.3 Role of dibutyryl cAMP sodium salt

7.2.3.1 Fertilization parameters

Several research groups reported an effect of addition of dbcAMP to the IVM medium of porcine oocytes, with altered fertilization outcome and blastocyst rates, but conflicting results were obtained concerning penetration and polyspermy rates. Therefore we aimed to elucidate the effect of dbcAMP addition during IVM on fertilization parameters. We showed that dbcAMP addition during the first part of IVM decreased the polyspermy rate of porcine oocytes after IVF (**Chapter 3**). This result corroborated the reports of Somfai *et al.* (2003) and Kim *et al.* (2008), but did not corroborate the earlier results of Funahashi *et al.* (1997), who reported equal polyspermy rates regardless of dbcAMP addition. Possibly the discrepancy can be explained by the fact that each research group operated with its own materials and methods, which are also determining fertilization outcome. We argue that the decreased polyspermy rate is a genuine result. First, because of the already proven synchronizing ability of dbcAMP, nuclear maturation will proceed in a more harmonized way (Funahashi *et al.* 1997). This nuclear synchronization can provide the required circumstances to allow also a decent cytoplasmic maturation, which could therefore better sustain monospermic fertilization. Second, it is known that gap junction (GJ) communication promotes cytoplasmic maturation by transferring the necessary metabolites to the oocytes. By the addition of dbcAMP these connections are preserved for a longer period (Flagg-Newton *et al.* 1981) and thus the cytoplasmic maturation and normal fertilization (Mori *et al.* 2000) will be enhanced. Third, it is reported that dbcAMP increases tissue-type plasminogen activator (Kim and Menino 1995), which could be important for zona hardening (rat (Zhang *et al.* 1992)). Fourth, Bijttebier *et al.* (2008) proved that an improved cumulus matrix quality could provide a better protection against polyspermy. Beside the fact that mucification is directly

correlated with high levels of cAMP in rat (Dekel and Phillips 1980), it was demonstrated that pre-incubation of COCs with dbcAMP caused an extensive proliferation of porcine cumulus cells (Procházka *et al.* 2009) and we now also observed an improved cumulus matrix disassembly after dbcAMP addition to the IVM medium in pigs (see 7.2.3.2.). Moreover, in parallel with the reduced polyspermy rate, also a decrease in penetration was observed. In this way, we can expect that dbcAMP could have been responsible for an improved cumulus matrix and consequently reduced penetration and polyspermy rates.

7.2.3.2 Cumulus expansion and developmental competence

Special attention was given to the fact that cAMP levels were suggested to be regulating a disintegrin and metalloproteinase with thrombospondin-like repeats-1 (ADAMTS-1) expression, which is involved in cumulus expansion by the cleavage of versican. Addition of dbcAMP was used as a tool to manipulate the specific cAMP/ADAMTS-1 pathway to investigate the relation between dbcAMP and adherent complexes. From a more practical point of view, we also verified if systematic removal of adherent COCs during IVM could serve as a selection tool during IVP since it is known that adherent COCs show signs of aging (Somfai *et al.* 2004). Our hypothesis was confirmed on several levels (**Chapter 3**). A first step in the analysis of the relation between dbcAMP and adherent COCs was proven by the decreased proportion of adherent complexes at 44 h of IVM after treatment with dbcAMP. Second, we developed and validated a method in which individual cumulus cells could be collected, immobilized and stained for quantitative immunofluorescence of proteins (**Chapter 4**). We could demonstrate an upregulation of proADAMTS-1 levels in cumulus cells when dbcAMP was added to the IVM medium, for floating as well as for attached COCs. Moreover, cumulus cells of floating complexes contained consistently more proADAMTS-1 than attached complexes, in the group of COCs cultured with as well as without dbcAMP. Regarding the fact that less adherent complexes were present in the dbcAMP-treated group than in the non-treated group, it can be presumed that the elevated levels of proADAMTS-1 caused elevated levels of ADAMTS-1. Consequently, the elevated ADAMTS-1 levels could have been promoting increased matrix remodeling by the increased cleavage of versican, leading to less adherence to the bottom of the culture dish *in vitro*. However, we should note

that still some COCs that were adherent to the bottom of the dish, could be observed since adherent complexes can occur due to several reasons. Other mechanisms such as the material of the dish can contribute to the level of adherence to the bottom of the dish *in vitro* (Somfai *et al.* 2004).

Next, logical deduction let us to conclude that, since dbcAMP-treated cumulus cells contained more proADAMTS-1 than non-treated ones, and since attached cumulus cells contained less proADAMTS-1 than floating ones, and since less complexes adhered when dbcAMP was added to the medium, a link between dbcAMP, proADAMTS-1 and the adherence of COCs has been demonstrated.

Because it is already known that attached COCs have an inferior quality compared to the floating ones (Somfai *et al.* 2004), it is important to avoid these complexes as much as possible. In order to address this problem, this study provided solutions at two levels. First, addition of dbcAMP is reducing the number of attached COCs as mentioned above. Second, a practical selection tool during IVP was applied concerning the remaining attached COCs. Since we showed that adherent complexes had an impaired cumulus remodeling and since Somfai *et al.* (2004) reported an inferior nuclear and cytoplasmic maturation in those complexes, fertilization and further developmental competence of that kind of oocytes were suspected to be affected. The implementation of a systematic removal of adherent (inferior) COCs after IVM revealed that the remaining oocytes developed to the blastocyst stage in the same proportion in groups treated with or without dbcAMP. Since literature (Funahashi *et al.* 1997; Somfai *et al.* 2003) is mostly reporting an increased blastocyst rate after dbcAMP treatment, we hypothesize that the reduced blastocyst rate in non-treated COCs without selection of the adherent complexes, is due to the presence of exactly those impaired complexes that were decreasing the blastocyst rates.

7.3 Role of oocyte-secreted factors

7.3.1 Problem statement

In **Chapters 5** and **6** we focused on these OSFs to understand the critical changes the COC has to undergo before the oocyte can be fertilized and develop into a viable embryo. The role of OSFs can be examined at two levels. First, it can be questioned if the oocyte is necessary for cumulus expansion. Researchers did not fully elucidate the role of the porcine oocyte in cumulus expansion (Prochazka *et al.* 1991; Singh *et al.* 1993). Therefore, we chose to perform more precise experiments using novel protein-free maturation media and micromanipulation to perform oocytectomy of the COC. Second, the role of OSFs can be investigated by the addition of helper denuded oocytes (DOs). Helper DOs can possibly exhibit beneficial effects on cultured COCs of interest by secreting extra OSFs and therefore providing a higher concentration of OSFs in the applied IVP system. This approach is especially promising when culturing low numbers of oocytes because these oocytes are suffering from the lack of other surrounding oocytes to sustain their development. By supplying helper oocytes, large group culture could possibly be mimicked. In cattle, sheep and mice, these OSFs have been identified as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) (Hanrahan *et al.* 2003; Su *et al.* 2004; Hussein *et al.* 2006; Yeo *et al.* 2007). We hypothesized that addition of extra OSFs could enhance cumulus expansion and nuclear maturation (**Chapter 5**). Moreover, since the cumulus matrix is a key player in prevention of polyspermy (Bijttebier *et al.* 2008) and the cumulus cells are influencing the quality of the oocyte by GJ communications and paracrine signals (reviewed by Gilchrist (2011)), we predicted that OSFs would also play a key role in preventing polyspermy and in acquiring oocyte developmental competence (**Chapter 6**).

7.3.2 Role of oocyte-secreted factors during *in vitro* maturation

In analogy with a system in cattle (Matoba *et al.* 2010), we established and validated a new technique for IVM of porcine oocytes, based on the use of Cell-Tak, in which COCs can be tracked during the entire IVM period (**Chapter 5**). To avoid any bias of the use of Cell-Tak, preliminary trials tested its effect on nuclear maturation and cumulus expansion. No differences could be observed in nuclear maturation with or without the use of Cell-Tak (**Chapter 5**). Cumulus expansion was enhanced by Cell-Tak (**Chapter 5**). Probably this increase could be explained by the attachment of the COCs to the bottom of the dish, which eliminated one dimension and caused the cumulus cells to expand more in other directions.

Recent developments in the design of new media are focused on omitting undefined components such as serum or follicular fluid. By replacing the protein preparations by polyvinyl alcohol (PVA), higher reproducibility of results can be obtained and biosafety of culture media can be ensured (Yoshioka *et al.* 2008). Moreover, a defined basic medium supporting IVM could reveal the purest effect of changing conditions such as addition of DOs. Therefore, all the experiments are performed using the defined porcine oocyte medium (POM) (**Chapters 5** and **6**). By using POM, supplemented with the macromolecular component PVA, the negative consequences of an undefined medium were avoided. It is known that POM+PVA induced the same ability to complete nuclear and cytoplasmic maturation as obtained with North Carolina State University (NCSU) 37 containing follicular fluid (Yoshioka *et al.* 2008).

First, we showed whether the oocyte was necessary for cumulus expansion. In earlier research about oocytectomy, cumulus expansion was scored using an arbitrary scale from 0 to +4 (Prochazka *et al.* 1991; Singh *et al.* 1993). In the study of **Chapter 5**, cumulus expansion was measured in a much more reproducible and objective way using an individual tracking system of COCs during IVM and digital photography processed by National Institutes of Health (NIH) ImageJ (version 1.47) software. A group of clumps was included to exclude the influence of remnants of cytoplasm, the oolemma and the zona pellucida in the OOX. We demonstrated that the cumulus can still expand in the absence of the oocyte (**Chapter 5**). Nevertheless, concluding that the oocyte would therefore not affect the cumulus compartment would be an over-hasty conclusion. Despite the presence of expansion, oocytectomized

complexes (OOXs) never reached the same level of expansion as intact COCs, addition of DOs to OOXs improved the level of cumulus expansion and inhibition of GJ communication in COCs significantly reduced cumulus expansion at 20 h of IVM (**Chapter 5**). These observations supported the statement by Singh *et al.* (1993) that even though the expansion was not dependent on the oocyte, the oocyte produced cumulus expansion-enabling factors. We now proved that this expansion-enabling action occurred both through GJ communication and through paracrine signaling. In addition, FSH-stimulated cumulus expansion of mouse OOXs co-cultured with DOs was directly correlated with the number of added oocytes (Buccione *et al.* 1990). Therefore, future research can verify if addition of more DOs would be able to increase cumulus expansion of OOXs even until the COC level.

This study also provided for the first time information about cumulus expansion of OOXs and clumps beyond 24 h of IVM. We demonstrated that also at 48 h of IVM a difference between COCs, OOXs and clumps could be observed (**Chapter 5**). Between 20 h of IVM and 48 h of IVM, the three groups showed a shrinking of the cumulus area (**Chapter 5**). Remarkably the clumps decreased so drastically in cumulus area that the measurement at 48 h of IVM revealed an even lower area than the area measured at 0 h of IVM. The evolution of expanding and subsequently shrinking of the cumulus area was observed for the first time. Previously, cumulus expansion in POM was described as the occurrence of a continuous increasing cumulus area (Mito *et al.* 2013). A possible explanation for this discrepancy could be the use of different hormones in both studies.

Second, we evaluated the influence of DOs, as a source of extra OSFs, on IVM of COCs, using a set-up with a fixed ratio and distance between DOs and COCs. Only the ratio 9 COCs+16 DOs was included in the experiments because of several reasons. First, preliminary trials revealed no difference for these parameters between the ratios 9 COCs+9 DOs, 9 COCs+16 DOs, and 9 COCs+36 DOs. Second, the ratio 9 COCs+16 DOs provided the best base to create a Cell-Tak frame where each of the COCs or OOXs were subjected to the same impact of DOs at the level of number of added DOs as well as at the level of the distance to the DOs. We could not observe any difference in cumulus expansion at 20 h and 48 h of IVM nor in nuclear maturation at 48 h of IVM after DO addition (**Chapter 5**). Nevertheless, a difference in evolution of the mathematical curve from 0 h to 20 h and from 20 h to 48 h of IVM after DO addition to COCs could be observed (**Chapter 5**). This finding indicated that the COCs with and without DO addition were evolving differently over time although the

groups with and without DO addition ended on an equal level at 20 h of IVM and on an equal level at 48 h of IVM. Future research could elucidate the consequences of such an effect. Since the cumulus development through the IVM period is managing so many functions, we expected that this difference in evolution could exert certain effects on the development of the COC.

Although no improvement could be found at 48 h of IVM of DO addition on cumulus expansion of COCs, addition of DOs to OOXs grids did facilitate expansion at 48 h of IVM as mentioned above. Consequently, DOs could influence expansion although, when the oocyte as part of the complex was still present, the DO-effect was not strong enough to improve expansion significantly. It is reported that addition of DOs to OOXs is not only affecting cumulus expansion but also several other important parameters such as cumulus cell apoptosis (cattle (Hussein *et al.* 2005)), luteinization markers (mouse (Eppig *et al.* 1997) and pig (Coskun *et al.* 1995)), cumulus glycolytic enzyme mRNA levels (mouse (Sugiura *et al.* 2005)) and steroidogenesis (pig (Coskun *et al.* 1995)). All these findings together point to the same direction that adding DOs could be beneficial for an IVM production system for OOXs. Moreover, since also other cumulus cell functions besides cumulus expansion are affected, DO addition could be beneficial for COC cultivation although no increase in expansion could be observed.

Concerning the nuclear maturation, no enhancement could be observed after DO addition (**Chapter 5**). Surprisingly, a larger proportion of DOs reached the metaphase II stage than COCs (**Chapter 5**). To investigate this phenomenon, the GJ inhibitor carbenoxolone (CBX) was added during IVM. In this way, we mimicked the situation of the DOs in the COC group and wanted to elucidate the mechanism behind this observation. After CBX addition the percentage of MII stage oocytes in COCs was the same as in DOs, indicating that the cumulus cells were inhibiting meiotic progression in the oocyte through the GJ communication (**Chapter 5**). We suspected the very basic maturation medium (POM with gonadotropins) to inhibit meiotic progression. It was already demonstrated that addition of dbcAMP did enhance nuclear maturation in COCs, but did not in DOs (Akaki *et al.* 2009). Therefore, we tested if the addition of the synchronizing agent dbcAMP could increase nuclear maturation of COCs, but no improvement could be determined (**Chapter 5**). Consequently, the reason for not re-initiating meiosis in our set-up remains to be elucidated. Somehow the currently applied combination of culturing of low numbers of oocytes and the basic IVM medium is not

providing any efficient signal to resume meiosis. When culturing smaller groups of cells, the oxidative stress, which is known to be detrimental to their developmental competence, may be increased because of the lower consumption of oxygen in the presence of fewer cells. To anticipate on this problem, when culturing low numbers of oocytes, supplementation of antioxidants like β-mercaptoethanol could provide better results.

The currently applied distance between COCs was between 105 µm and 180 µm. This distance was larger than the distance defined to culture attached zygotes in order to obtain blastocysts (+/- 160 µm), but the distance was increased to allow the expansion of the cumulus matrix. This adaptation was made in parallel with the research of Matoba *et al.* (2010), but we even applied a lower limit of 180 µm instead of 200 µm applied in their study. Since this distance would not hamper development during IVM, we could use this distance between COCs allowing individual measuring and analysing the cumulus expansion by digital image analysis. In this way, an objective and individual manner of interpreting cumulus expansion was provided without compromising the maturation of the oocytes.

To verify if the applied distance between mutual COCs or between COCs or DOs could have caused the low maturation rates, several preliminary trials were performed. The effect of the use of Cell-Tak and consequently adhering COCs on a certain distance from each other was examined by comparing 9 COCs placed in a grid formation by Cell-Tak and 9 COCs floating in the drop. No difference in nuclear maturation rate could be observed between floating COCs and attached COCs. To test the distance between COCs and DOs, grid formations of COCs were made but in one group DOs were also glued and in the other group the DOs could float between the attached COCs. Again, no significant differences could be observed between the nuclear maturation of COCs with attached DOs and COCs with floating DOs. These results confirmed that the distance between the COCs or the distance between the COCs and DOs applied in this study is not too large to allow the diffusion of the OSFs. In addition, these results correspond to the fact that Brankin *et al.* (2003) proved how extremely potent OSFs are by observing significant effects on granulosa cells by using only 5 oocytes in 200 µL of medium. Even the comparison with the *in vivo* situation emphasized the high potency of the OSFs because a single oocyte is surrounded by 10 to 250 µL of follicular fluid (Brankin *et al.* 2003). Even though the situations described in the latter study, it was worth testing the distance in our specific experimental set-up, but clearly no negative effect of "distance" could be observed.

7.3.3 Role of oocyte-secreted factors during *in vitro* fertilization and *in vitro* culture

To investigate if OSFs, by the aid of DO addition, could enhance the response after IVF and the developmental competence of the oocyte after IVC, different ratios of DOs were added to nine COCs during IVM. To verify if DO addition would be acting on the cumulus compartment or the oocyte itself, the cultured COCs were either fertilized as intact COCs or fertilized after denudation. No differences were observed in penetration, polyspermy and normal fertilization rates between the different ratios in the COC-fertilized as well as in the denuded-fertilized groups (**Chapter 6**). Although no significant differences were observed in the obtained results, some patterns could be determined. These patterns can provide more insight in biologically relevant processes about the working mechanism of OSFs. There was a trend of decreasing penetration between 9 COCs and 9 COCs+36 DOs in the intact fertilized COCs and this trend was not observed in the groups fertilized after denudation (**Chapter 6**). The fact that a discrepancy existed between the COC-fertilized group and the denuded-fertilized group pointed already towards a role of a changing cumulus. Moreover, the results of the native OSF bioassays on IVM proved that DO addition improved cumulus expansion of OOXs. These new findings together with the already known changes of the cumulus function after DO addition to OOX (cumulus cell apoptosis (cattle (Hussein *et al.* 2005)), luteinisation markers (mouse (Eppig *et al.* 1997) and pig (Coskun *et al.* 1995)), cumulus glycolytic enzyme mRNA levels (mouse (Sugiura *et al.* 2005)) and steroidogenesis (pig (Coskun *et al.* 1995))), indicated that OSFs may be involved in the development of the cumulus department during IVM. Consequently, a changing cumulus could influence fertilization parameters like penetration rate because it is known that the cumulus can act as a barrier for spermatozoa (Bijttebier *et al.* 2008).

In contrast to reports on other species, our study could not reveal any differences in blastocyst proportion and blastocyst quality after DO addition during IVM (**Chapter 6**). Research in cattle (Hussein *et al.* 2006), goat (Romaguera *et al.* 2010) and mouse (Yeo *et al.* 2007) demonstrated an enhanced oocyte developmental potential after DO addition or supplementation of specific OSFs. On the contrary, research in pigs reported contradictory results. Denuded oocyte addition improved blastocyst rates after parthenogenetic activation in a non-defined medium (Gomez *et al.* 2012), but another study, using defined medium, could

not prove any enhancement of the blastocyst rate (Romaguera and Grupen 2009). The possibility that a species-specific mechanism could be involved in the OSF action was emphasized by the fact that a discrepancy was observed between pig and other species and that even the same researchers (*i.e.* Romaguera *et al.*) proved effect in goats but were not able to prove it in pigs. However, why the study of Gomez *et al.* (2012) contradicts our study and the one of Romaguera and Grupen (2009) remains unclear. A possible explanation could be found in the use of different maturation media. Gomez *et al.* (2012) used a non-defined medium supplemented with porcine follicular fluid in contrast to our study and the one of Romaguera and Grupen (2009), which used both defined POM medium for IVM.

A trend of decreasing cleavage was observed between 9 COCs and 9 COCs+36 DOs. This was the same trend that was reported on penetration rates between those groups. Nevertheless, it was clear that although these trends occurred, no impact was detected at the level of embryo development such as blastocyst formation.

When interpreting the results after DO addition on IVM, IVF and IVC, it is important to notice that addition of extra oocytes can modify the culture system in several ways. Next to the aimed addition of OSFs, extra cells will consume oxygen and therefore exert a non-specific influence during IVM. In very basic media, this oxygen reducing action may be observed, but when enriched media are used often antioxidants are added, which may overrule the effect of the addition of the extra cells. In our study, the two situations were applied. The effect of OSFs on IVM was examined using a basic medium without addition of extra antioxidants. We observed an improvement of cumulus expansion of OOX when DOs were added. More research could reveal if this effect is due to specific OSF working mechanisms or to non-specific oxygen consumption. In the second part of the study, no effects of addition of extra DOs could be observed after IVF and IVC. However, both research parts should be evaluated in a different way, because in the fertilization and culture experiments the medium was enriched by antioxidants like β-mercaptoethanol. Therefore, the same oxygen reducing effect could have occurred, but because of the enriched medium this effect could have been overshadowed.

The predominant occurrence of the lack of effect of addition of DOs could raise questions about the sufficient administration of extra helper oocytes. However, we did extensive preliminary literature research to set well-thought-out ratios. We expressed the ratio of

oocytes per µL medium to investigate the influence of DO addition to an IVM system. Our studies applied ratios of 0.16 DO/µL or 0.36 DO/µL. These ratios were higher than 0.128 DO/µL, which was described in two previous murine studies (Gilchrist *et al.* 2001; Gilchrist *et al.* 2006), and they were within the range of 0.1 DO/µL to 1 DO/µL as described by Hussein *et al.* (2005) in cattle. Based on these findings in literature, the applied number of oocytes/µL in the present studies was believed to be sufficient to reveal any possible effects of OSFs.

7.4 Future perspectives

7.4.1 Role of cyclic adenosine monophosphate-modulating agents

It can be concluded that maintaining high cAMP levels in the oocyte sustained a better maturation of the oocyte, reflected in not only an improved response after IVF but also in an improved cumulus matrix disassembly.

From a practical point of view, IBMX is not recommended to be used during collection of porcine COCs, but addition of dbcAMP during the first period of IVM is advised strongly. In order to select only the high quality oocytes for further processing in IVP, COCs that show adherence to the bottom of the culture dish after IVM, should be removed.

From a more fundamental point of view, more research should be performed to reveal the exact mechanism by which cAMP levels are involved in the regulation and the production of the ADAMTS-1 protein. In **Chapter 3** the basic dependence was demonstrated, but more insight in the cAMP/ADAMTS-1 pathway could possibly reveal more modulating opportunities. In parallel with the performed research on parathyroid in bones (Miles *et al.* 2000), other cAMP modulating agents like forskolin, an activator of adenylate cyclase that causes cAMP accumulation, could be tested for their influence on ADAMTS-1 levels in porcine cumulus cells. Shimada *et al.* (2004) revealed that porcine ADAMTS-1 expression is regulated by the functionality of the progesterone receptor (PR), which is a member of the nuclear receptor transcription factor superfamily (Schrader and O'Malley 1972; Robker *et al.*

2000). However, Doyle *et al.* (2004) showed in mice that ADAMTS-1 can be induced by luteinizing hormone (LH) using divergent signal transduction pathways, including a PR-independent pathway based on cAMP signaling cascades. The present study is the first to demonstrate this cAMP dependent regulation of ADAMTS-1 in porcine cumulus cells. Further research should be performed in pigs to sustain our findings more profoundly. A detailed mapping of the different pathways by which ADAMTS-1 production can be controlled in porcine cumulus cells will provide a better understanding of the fundamental upstream regulating mechanism of ADAMTS-1 and therefore will create new chances to intervene in the process.

7.4.2 Role of oocyte-secreted factors

The reports of an abundant number of functions of OSFs in various species pointed us towards our hypothesis that DO addition could possibly enhance IVM, IVF and IVC of porcine oocytes. If a positive effect would have been observed, further studies could have been performed to investigate the nature of these OSFs and to elucidate the underlying enhancing mechanism of OSFs. Unfortunately, in general no significant effects could be observed throughout the study. More fundamental research about the basic action of OSFs will not contribute to a technique to improve the IVP system for porcine embryos and will therefore not formulate any answers to our specific hypothesis. In this way *e.g.* gene expression studies will, to our opinion, not reveal solutions for our problem statement. Nevertheless, possible effects of DO-derived OSFs like GDF9 and BMP15 on gene expression of *e.g.* ovulation related genes in the cumulus cannot be ruled out and may be a subject of future research.

When planning to perform future research, one important limiting factor will have to be taken into account: the technical feasibilities hampering large-scale experiments when performing trials in the field of native OSF bioassays. Also other studies reported the technical limitations of bioassays with native OSFs like the collection of the necessary amount of material to conduct large co-culture trials even when using micro drops (reviewed by Gilchrist *et al.*

(2008) and Gilchrist (2011)). To explain this problem more in detail, the power calculation of the performed experiments on DO addition effects after IVF and IVC is exemplified.

Because of the complicated set-up of the experiments, including the abundant number of DOs, which were not contributing to the calculated parameters, some replicates provided only information about nine COCs of a specific treatment group. In fertilization trials, parameters were calculated on the non-degenerated examined (for penetration rate) or even only the penetrated oocytes (for polyspermy and normal fertilization rates), which made each replicate providing even less than nine COCs to calculate the desired parameters. Based on the currently obtained results we performed a power calculation with Win Episcope 2.0. Each calculation included a 95% level of confidence and a power of 80%. This power calculation revealed that up to 190 COCs per treatment group should be included to be able to significantly express the expected difference in penetration between the 9 COCs group and the 9 COCs+36 DOs group (the groups in which now a trend is observed). Since six replicates produced only 67 COCs, more than 17 replicates should have been performed before this observed difference could be stated as significant. Concerning the other parameters, even much more replicates should have been performed because of two main reasons. First, there were always less penetrated COCs than non-degenerated COCs in each replicate, resulting in even less COCs to be examined per replicate. Second, since the expected difference between *e.g.* polyspermy rate between the 9 COCs group and the 9 COCs+36 DOs group was much smaller, the power calculation reported even a much higher number of COCs to be examined up to *e.g.* 382 COCs for polyspermy rate between the 9 COCs group and the 9 COCs+36 DOs group. The arguments mentioned above brought us to the conclusion that simply performing more replicates would not have changed the non-significant observations or trends to significant differences. In this research, we mainly aimed to reveal the real biological advantage of addition of DOs during IVM on IVF and IVC, and not only to obtain statistical significant results. If trials required such a very large number of replicates, the risk of overpowering experiments would have occurred. Therefore in this study, we strongly believe the performed number of replicates was sufficient to demonstrate the practical value of the native OSF bioassay in porcine IVP.

To avoid problems in future research the obtained results on native OSF bioassays from this study and the issue of a very labor intensive protocol of DO production, illustrated by the

power calculation, should be taken into account. Consequently, the use of recombinant OSFs may be a more expedient approach for fundamental research on OSFs in pigs in the future.

7.5 General conclusion

In general, we can conclude that this research provided more insight in the bidirectional communication between oocyte and cumulus cells in the pig, at the level of cAMP dependent pathways and at the level of oocyte-secreted factors.

The take home messages drawn from this research are:

1. Addition of IBMX did not synchronize the germinal vesicle stages of porcine oocytes during collection. However, the use of dbcAMP during IVM did lead to less polyspermy and resulted in a lower rate of adherent COCs due to an elevation of proADAMTS-1 levels in cumulus cells.

2. To sustain the trials about the role of cAMP during IVP with the necessary technical support, a new and simple technique was validated to process individual cells in order to perform an immunofluorescent staining of proteins.

3. A defined IVM system that allowed culturing small numbers of COCs, objective measurements of cumulus expansion and individual tracking, was established. By using this system, it was proven that cumulus cells could expand independently from the oocyte. However, oocytes enhanced cumulus expansion through GJs and through DO addition.

4. No effect of DO addition during IVM could be observed on fertilization parameters and the developmental competence of porcine oocytes.

The key findings of this dissertation are presented in a schematic overview (Table 1).

Table 1: A schematic overview of the timeline of porcine in vitro *embryo production, the research questions, and the key findings of this dissertation.*

Timeline of porcine IVP	**Research question**	**Key finding**
Collection	Can addition of IBMX overcome the hypothesized precocious nuclear progression?	IBMX during collection does not affect nuclear status of oocytes
	What is the role of the oocyte in cumulus expansion?	Expansion to some extent happens independently of the oocyte. However, DO addition improves cumulus expansion in OOX
IVM 1	Can addition of dbcAMP improve cytoplasmic maturation?	dbcAMP addition improves the response after IVF by affecting cumulus matrix disassembly
	Is oocyte culture in small groups possible?	We established and validated a new IVP method for culture of low numbers of oocytes
IVM 2	Can addition of dbcAMP during IVM 1 reduce adherence of COCs to the bottom of the dish during IVM 2?	dbcAMP decreases the adherence of COCs
	What is the effect of OSFs by co-culture with DOs on COC maturation?	DO addition does not improve maturation of COCs
	What is the role of gap junctions in oocyte maturation?	Gap junctional communication is involved in cumulus expansion and the initial transient meiotic arrest of oocytes at the GV stage.

Timeline of porcine IVP	Research question	Key finding
IVF	Can addition of dbcAMP during IVM 1 in combination with removal of adherent COCs after IVM reduce polyspermy rates?	dbcAMP addition and removal decrease polyspermy rates
	Can addition of DOs during IVM reduce polyspermy rates?	DO addition does not reduce polyspermy rates
IVC	Can addition of dbcAMP during IVM 1 in combination with removal of adherent COCs after IVM increase blastocyst rates?	Removal of adherent oocytes causes equal blastocyst rates with and without dbcAMP addition
	Can addition of DOs during IVM improve quantity and quality of blastocysts?	DO addition does not improve quantity and quality of blastocysts

cAMP: cyclic adenosine monophosphate; IBMX: 3-isobutyl-1-methylxanthine; dbcAMP: dibutyryl cAMP sodium salt; COC: cumulus-oocyte complex; IVP: *in vitro* embryo production; IVM: *in vitro* maturation; IVF: *in vitro* fertilization; IVC: *in vitro* culture; OOX: oocytectomized complex; DO: denuded oocyte; OSF: oocyte-secreted factor

7.6 References

Akaki, Y., Yoshioka, K., Noguchi, M., Hoshi, H., and Funahashi, H. (2009) Successful piglet production in a chemically defined system for in-vitro production of porcine embryos: dibutyryl cyclic amp and epidermal growth factor-family peptides support in-vitro maturation of oocytes in the absence of gonadotropins. *J. Reprod. Dev.* **55**(4), 446-453

Albuz, F.K., Sasseville, M., Lane, M., Armstrong, D.T., Thompson, J.G., and Gilchrist, R.B. (2010) Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Hum. Reprod.* **25**(12), 2999-3011

Bijttebier, J., Van Soom, A., Meyer, E., Mateusen, B., and Maes, D. (2008) Preovulatory follicular fluid during in vitro maturation decreases polyspermic fertilization of cumulus-intact porcine oocytes. *Theriogenology* **70**(4), 715-724

Bornslaeger, E., Wilde, M.W., and Schultz, R. (1984) Regulation of mouse oocyte maturation: involvement of cyclic AMP phosphodiesterase and calmodulin. *Dev. Biol.* **105**(2), 488-499

Brankin, V., Mitchell, M., Webb, B., and Hunter, M. (2003) Paracrine effects of oocyte secreted factors and stem cell factor on porcine granulosa and theca cells in vitro. *Reprod. Biol. Endocrinol.* **1**(1), 55

Buccione, R., Vanderhyden, B., Caron, P., and Eppig, J. (1990) FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev. Biol.* **138**(1), 16-25

Coskun, S., Uzumcu, M., Lin, Y., Friedman, C., and Alak, B. (1995) Regulation of cumulus cell steroidogenesis by the porcine oocyte and preliminary characterization of oocyte-produced factor(s). *Biol. Reprod.* **53**(3), 670-675

Dekel, N., and Phillips, D.M. (1980) Cyclic AMP, prostaglandin E2 and steroids: possible mediators in the rat cumulus oophorus mucification. *Biol. Reprod.* **22**, 289-296

Doyle, K.M.H., Russell, D.L., Sriraman, V., and Richards, J.S. (2004) Coordinate transcription of the ADAMTS-1 gene by luteinizing hormone and progesterone receptor. *Mol. Endocrinol.* **18**(10), 2463-2478

Eppig, J.J., Wigglesworth, K., pendola, F., and Hirao, Y. (1997) Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biol. Reprod.* **56**, 976-984

Flagg-Newton, J., Dahl, G., and Loewenstein, W. (1981) Cell junction and cyclic AMP. I. Upregulation of junctional membrane permeability and junctional membrane parti¬cles by administration of cyclic nucleotide or phosphodiesterase inhibitor. *J. Membr. Biol.* **63**(1-2), 105-121

Funahashi, H., Cantley, T., and Day, B. (1997) Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. *Biol. Reprod.* **57**(1), 49-53

Gilchrist, R. (2011) Recent insights into oocyte –follicle interactions provide opportunities for the development of new approaches to in vitro maturation. *Reprod. Fertil. Dev.* **23**(1), 23-31

Gilchrist, R.B., Lane, M., and Thompson, J.G. (2008) Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum. Reprod. Update* **14**(2), 159-177

Gilchrist, R.B., Ritter, L.J., and Armstrong, D.T. (2001) Mouse oocyte mitogenic activity is developmentally coordinated throughout folliculogenesis and meiotic maturation. *Dev. Biol.* **240**(1), 289-298

Gilchrist, R.B., Ritter, L.J., Myllymaa, S., Kaivo-Oja, N., Dragovic, R.A., Hickey, T.E., Ritvos, O., and Mottershead, D.G. (2006) Molecular basis of oocyte-paracrine signalling that promotes granulosa cell proliferation. *J. Cell Sci.* **119**(18), 3811-3821

Gomez, M.N.L., Kang, J.T., Koo, O.J., Kim, S.J., Kwon, D.K., Park, S.J., Atikuzzaman, M., Hong, S.G., Jang, G., and Lee, B.C. (2012) Effect of oocyte-secreted factors on porcine in vitro maturation, cumulus expansion and developmental competence of parthenotes. *Zygote* **20**(02), 135-145

Hanrahan, J.P., Gregan, S.M., Mulsant, P., Mullen, M., Davis, G.H., Powell, R., and Galloway, S.M. (2003) Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (ovis aries). *Biol. Reprod.* **70**(4), 900-909

Hussein, T.S., Froiland, D.A., Amato, F., Thompson, J.G., and Gilchrist, R.B. (2005) Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. *J. Cell Sci.* **118**(22), 5257-5268

Hussein, T.S., Thompson, J.G., and Gilchrist, R.B. (2006) Oocyte-secreted factors enhance oocyte developmental competence. *Dev. Biol.* **296**(2), 514-521

Kim, J.S., Cho, Y.S., Song, B.S., Wee, G., Park, J.S., Choo, Y.K., Yu, K., Lee, K.K., Han, Y.M., and Koo, D.B. (2008) Exogenous dibutyryl cAMP affects meiotic maturation via protein kinase A activation; it stimulates further embryonic development including blastocyst quality in pigs. *Theriogenology* **69**(3), 290-301

Kim, N., and Menino, A. (1995) Effects of stimulators of protein kinase A and C and modulators of phosphorylation on plasminogen activator activity in porcine oocyte-cumulus cell complexes during in vitro maturation. *Mol. Reprod. Dev.* **40**, 364-370

Luciano, A.M., Pocar, P., Milanesi, E., Modina, S., Rieger, D., Lauria, A., and Gandolfi, F. (1999) Effect of different levels of intracellular cAMP on the in vitro maturation of cattle oocytes and their subsequent development following in vitro fertilization. *Mol. Reprod. Dev.* **54**(1), 86-91

Matoba, S., Fair, T., and Lonergan, P. (2010) Maturation, fertilisation and culture of bovine oocytes and embryos in an individually identifiable manner: a tool for studying oocyte developmental competence. *Reprod. Fertil. Dev.* **22**(5), 839-851

Miles, R., Sluka, J., Halladay, D., Santerre, R., Hale, L., Bloem, L., Thirunavukkarasu, K., Galvin, R., Hock, J., and Onyia, J. (2000) ADAMTS-1: a cellular disintegrin and metalloprotease with thrombospondin motifs is a target for parathyroid hormone in bone. *Endocrinology* **141**(12), 4533-4542

Mito, T., Yoshioka, K., Noguchi, M., Yamashita, S., and Hoshi, H. (2013) Recombinant human follicle-stimulating hormone and transforming growth factor-alpha enhance in vitro maturation of porcine oocytes. *Mol. Reprod. Dev.* **80**(7), 549-560

Mori, T., Amano, T., and Shimizu, H. (2000) Roles of gap junctional communication of cumulus cells in cytoplasmic maturation of porcine oocytes cultured in vitro. *Biol. Reprod.* **62**(4), 913-919

Prochazka, R., Nagyova, E., Rimkevičová, Z., Nagai, T., Kikuchi, K., and Motlik, J. (1991) Lack of effect of oocytectomy on expansion of the porcine cumulus. *J. Reprod. Fertil.* **93**(2), 569-576

Procházka, R., Němcová, L., Nagyová, E., Scsuková, S.a., and Mlynarčíková, A.b. (2009) Development of functional LH receptors on pig cumulus-oocyte complexes cultured in vitro by a novel two-step culture system. *Mol. Reprod. Dev.* **76**(8), 751-761

Robker, R.L., Russell, D.L., Espey, L.L., Lydon, J.P., O'Malley, B.W., and Richards, J.S. (2000) Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. *Proceedings of the National Academy of Sciences* **97**(9), 4689-4694

Romaguera, R., and Grupen, C.G. (2009) Relationship between follicle size and factors secreted by porcine oocytes and cumulus cells during maturation in vitro. *Reprod. Domest. Anim.* **44**, 89

Romaguera, R., Morató, R., Jiménez-Macedo, A.R., Catalá, M., Roura, M., Paramio, M.T., Palomo, M.J., Mogas, T., and Izquierdo, D. (2010) Oocyte secreted factors improve embryo developmental competence of COCs from small follicles in prepubertal goats. *Theriogenology* **74**(6), 1050-1059

Santiquet, N., Sasseville, M., Laforest, M., Guillemette, C., Gilchrist, R.B., and Richard, F.J. (2014) Activation of 5' adenosine monophosphate-activated protein kinase blocks cumulus cell expansion through inhibition of protein synthesis during in vitro maturation in swine. *Biol. Reprod.* **91**(2), 51-51

Schrader, W.T., and O'Malley, B.W. (1972) Progesterone-binding components of chick oviduct. *J. Biol. Chem.* **247**(1), 51-59

Shimada, M., Nishibori, M., Isobe, N., Kawano, N., and Terada, T. (2003) Luteinizing hormone receptor formation in cumulus cells surrounding porcine oocytes and its role during meiotic maturation of porcine oocytes. *Biol. Reprod.* **68**(4), 1142-1149

Shimada, M., Nishibori, M., Yamashita, Y., Ito, J., Mori, T., and Richards, J.S. (2004) Down-regulated expression of A disintegrin and metalloproteinase with thrombospondin-like repeats-1 by progesterone receptor antagonist is associated with impaired expansion of porcine cumulus-oocyte complexes. *Endocrinology* **145**(10), 4603-4614

Singh, B., Zhang, X., and Armstrong, D. (1993) Porcine oocytes release cumulus expansion-enabling activity even though porcine cumulus expansion in vitro is independent of the oocyte. *Endocrinology* **132**(4), 1860-1862

Somfai, T., Kikuchi, K., Onishi, A., Iwamoto, M., Fuchimoto, D.-i., Papp, g.B., Sato, E., and Nagai, T. (2004) Relationship between the morphological changes of somatic compartment and the kinetics of nuclear and cytoplasmic maturation of oocytes during in vitro maturation of porcine follicular oocytes. *Mol. Reprod. Dev.* **68**(4), 484-491

Somfai, T., Kikuchi, K., Onishi, A., Iwamoto, M., Fuchimoto, D., Papp, Á.B., Sato, E., and Nagai, T. (2003) Meiotic arrest maintained by cAMP during the initiation of maturation enhances meiotic potential and developmental competence and reduces polyspermy of IVM/IVF porcine oocytes. *Zygote* **11**(3), 199-206

Spits, C., Guzman, L., Mertzanidou, A., Jacobs, K., Ortega-Hrepich, C., Gilchrist, R.B., Thompson, J.G., De Vos, M., Smitz, J., and Sermon, K. (2014) Chromosome constitution of human embryos generated after in vitro maturation including 3-isobutyl-1-methylxanthine in the oocyte collection medium. *Hum. Reprod.* **30**(3), 653-663

Su, Y.-Q., Wu, X., O'Brien, M.J., Pendola, F.L., Denegre, J.N., Matzuk, M.M., and Eppig, J.J. (2004) Synergistic roles of BMP15 and GDF9 in the development and function of the oocyte–cumulus cell complex in mice: genetic evidence for an oocyte–granulosa cell regulatory loop. *Dev. Biol.* **276**(1), 64-73

Sugiura, K., Pendola, F.L., and Eppig, J.J. (2005) Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev. Biol.* **279**(1), 20-30

Yeo, C.X., Gilchrist, R.B., Thompson, J.G., and Lane, M. (2007) Exogenous growth differentiation factor 9 in oocyte maturation media enhances subsequent embryo development and fetal viability in mice. *Hum. Reprod.* **23**(1), 67-73

Yoshioka, K., Suzuki, C., and Onishi, A. (2008) Defined system for in vitro production of porcine embryos using a single basic medium. *J. Reprod. Dev.* **54**(3), 208-213

Zeng, H.t., Ren, Z., Guzman, L., Wang, X., Sutton-McDowall, M.L., Ritter, L.J., De Vos, M., Smitz, J., Thompson, J.G., and Gilchrist, R.B. (2013) Heparin and cAMP modulators interact during pre-in vitro maturation to affect mouse and human oocyte meiosis and developmental competence. *Hum. Reprod.* **28**(6), 1536-1545

Zhang, X., Rutledge, J., Khamsi, F., and Armstrong, D. (1992) Release of tissue-type plasminogen activator by activated rat eggs and its possible role in the zona reaction. *Mol. Reprod. Dev.* **32**(1), 28-32

Summary

In vitro embryo production (IVP) in pigs is an important assisted reproductive technology, which can be used to study maturation, fertilization and developmental processes of oocytes, to preserve genetic material *via* gene banking and to constitute the foundations for further development of advanced biotechniques (**Chapter 1.1**). *In vitro* embryo production consists of several sequential stages with each their specific needs and demands (**Chapter 1.2**). Even though major advances in the knowledge of molecular and cellular processes regulating IVP have been made over the years, research is still confronted with some obstacles that prevent further progress (**Chapter 1.3**). Polyspermy or the penetration of more than one sperm cell keeps on interfering with normal *in vitro* fertilization (IVF) in the pig. Moreover, after *in vitro* culture of porcine presumptive zygotes, only a low percentage of blastocysts will develop and the quality of the resulting blastocysts is inferior compared to that of *in vivo* derived blastocysts. Another issue is that small group culture systems are known to decrease maturation efficiency, although it are exactly these systems that are required when oocytes from specific donor animals have to be matured in an identifiable manner. Hence, there is a need for the development of new IVP approaches solving these issues. When analyzing these problems, the question arises if the cytoplasmic maturation is sufficiently sustained in the currently applied IVP methods. Consequently, it is crucial to understand the complex interplay between oocyte and cumulus cells during *in vitro* maturation (IVM). By focusing on this bidirectional communication, it is possible to better control the coordination and harmonization of cumulus expansion, nuclear maturation, and cytoplasmic maturation during IVM.

Therefore this research focused on the regulatory mechanisms between oocytes and cumulus cells, in order to be able to set up a new IVP mechanism resulting in less polyspermy and an enhanced oocyte developmental potential (**Chapter 2**). The research was subdivided in several specific objectives.

In **Chapter 3**, specific attention was given to cAMP. It is already known that cAMP plays a crucial role in the regulation of nuclear and cytoplasmic maturation. During IVM, the regulatory mechanism of cAMP is disturbed by the removal of the cumulus-oocyte complex

(COC) from the follicle. This removal causes a premature drop in cAMP leading to a non-physiological spontaneous resumption of meiosis because of the disruption of the contact between the inhibitory follicle environment and the COC. To prevent this premature drop in cAMP, the first aim of this study was to test the influence of cAMP-modulating agents like the non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), and the membrane-permeable cAMP analog, dibutyryl cAMP sodium salt (dbcAMP). Our results showed that IBMX addition to the collection medium did not synchronize the germinal vesicle stage of porcine oocytes evaluated at the end of collection. However, addition of dbcAMP to the IVM medium during the first 22 h of IVM had a positive influence, causing a decreased polyspermy rate and a higher normal fertilization rate. Moreover this research revealed for the first time a connection between dbcAMP levels and the number of adherent COCs to the bottom of the culture dish. Addition of dbcAMP reduced the adherent COCs due to an upregulation of the proprotein of a disintegrin and metalloproteinase with thrombospondin-like repeats-1 (proADAMTS-1) in the cumulus cells.

Since it may be interesting to examine single or few COCs, we developed a method that allows collecting and immobilizing individual cumulus cells in order to subject them to a quantitative immunofluorescence analysis of proteins (**Chapter 4**). Most of the currently applied techniques are based on the availability of high numbers of cumulus cells. However, sometimes less biologic material is available and therefore other specific techniques to process a low number of cells are required. We now established and validated a simple and effective method for the collection and immobilization of cells on a slide, by the aid of Cell-Tak, which enables immunostaining of proteins allowing their quantitative analysis in individual cells.

In **Chapters 5** and **6** another important element of the bidirectional communication between oocyte and cumulus cells was highlighted. The oocyte is capable of regulating her own microenvironment by secreting oocyte-secreted factors (OSFs) that influence the differentiation of cumulus cells, which in their turn are enhancing oocyte development. This phenomenon can be considered as a kind of auto-regulatory loop of the oocyte through the cumulus cells. The use of OSFs to improve existing IVP systems in this study was investigated in a specific set-up designed for the culture of low numbers of oocytes using a defined medium.

First (**Chapter 5**), a basic IVM system was developed by the aid of Cell-Tak that allowed individual tracking of oocytes during IVM and provided a system to measure cumulus expansion in a precise and objective way. Moreover, this system was suitable for inclusion of denuded oocytes (DOs) (as a source of OSFs) on a fixed and constant distance of the COC to be evaluated. We proved by the aid of the technique of oocytectomy that cumulus cells could expand independently of the oocyte. However, oocytes were promoting cumulus expansion both as organic members of COCs through gap junctions and also as DOs added to culture drops by modifying medium characteristics.

Since we clearly demonstrated that OSFs could influence cumulus expansion to some extent, in **Chapter 6** we verified if OSFs, by the aid of DO addition during IVM, could improve fertilization parameters and oocyte developmental potential. Our results showed that addition of DOs to an IVM medium at a concentration up to 0.36 DO/µL did not significantly improve fertilization and developmental competence in the applied defined system. Because other research groups could point towards an enhanced oocyte developmental competence after DO addition in other species, a possible species-specific mechanism could be involved.

In **Chapter 7** the results of this dissertation are recapitulated and discussed per topic.

The take home messages drawn from this research are summarized below.

1. Addition of IBMX did not synchronize the germinal vesicle stages of porcine oocytes during collection. However, the use of dbcAMP during IVM did lead to less polyspermy and resulted in a lower rate of adherent COCs due to an elevation of proADAMTS-1 levels in cumulus cells.

2. To sustain the trials about the role of cAMP during IVP with the necessary technical support, a new and simple technique was validated to process individual cells in order to perform an immunofluorescent staining of proteins.

3. A defined IVM system that allowed culturing small numbers of COCs, objective measurements of cumulus expansion and individual tracking was established. By using this system, it was proven that cumulus cells could expand independently from the oocyte. However, oocytes enhanced cumulus expansion through gap junctions and through DO addition.

4. No effect of DO addition during IVM could be observed on fertilization parameters and the developmental competence of porcine oocytes.

In general, we can conclude that this research provided more insight in the bidirectional communication between oocyte and cumulus cells in the pig, at the level of cAMP-dependent pathways and at the level of oocyte-secreted factors.

Samenvatting

In vitro embryo productie (IVP) bij varkens is een belangrijke kunstmatige voortplantingstechniek die kan gebruikt worden om het maturatie-, fertilisatie- en ontwikkelingsproces van eicellen te bestuderen, om genetisch materiaal te bewaren *via* een genenbank en om de funderingen te bouwen voor de ontwikkeling van geavanceerde biotechnieken (**Hoofdstuk 1.1**). De *in vitro* embryo productie bestaat uit verschillende opeenvolgende fases met elk hun specifieke noden en eisen (**Hoofdstuk 1.2**). Ondanks het feit dat er grote vooruitgang is gemaakt op het vlak van kennis van moleculaire en cellulaire processen die IVP reguleren, wordt het onderzoek nog geconfronteerd met obstakels die verdere vooruitgang verhinderen (**Hoofdstuk 1.3**). Bij het varken blijft polyspermie, of de penetratie van meer dan één spermacel, interfereren met een normale *in vitro* fertilisatie (IVF). Bovendien zal, na *in vitro* cultuur van porciene zygoten, slechts een laag percentage zich ontwikkelen tot blastocyst en de kwaliteit van de verkregen blastocysten zal minderwaardig zijn aan blastocysten die *in vivo* verkregen worden. Een ander probleem is dat bij systemen om kleine groepen te cultiveren de maturatie-efficiëntie vermindert, hoewel net deze systemen vereist zijn wanneer eicellen van specifieke donordieren gematureerd moeten worden op een identificeerbare manier. Bijgevolg is er nood aan de ontwikkeling van een nieuwe IVP-aanpak waarbij deze problemen opgelost worden. Bij het analyseren van deze problemen rijst de vraag of de huidig toegepaste IVP methodes de cytoplasmatische maturatie wel voldoende ondersteunen. Bijgevolg, is inzicht in de complexe wisselwerking tussen eicel en cumuluscellen tijdens *in vitro* maturatie (IVM) cruciaal. Door te focussen op deze bidirectionele communicatie kan de coördinatie en de harmonisatie van de cumulusexpansie, de nucleaire en de cytoplasmatische maturatie gedurende IVM beter gecontroleerd worden.

Met het oog op het uitwerken van een nieuw protocol voor IVP dat zou resulteren in minder polyspermie en een beter ontwikkelingspotentieel van de eicel, focuste dit onderzoek op de regulerende mechanismen tussen eicel en cumuluscellen (**Hoofdstuk 2**). Gezien het feit dat er een zeer breed en algemeen doel vooropgesteld werd, werd het onderzoek opgedeeld in meerdere specifieke doelen.

In **Hoofdstuk 3** werd bijzondere aandacht verleend aan cAMP. Het staat reeds geruime tijd vast dat cAMP een cruciale rol speelt in de regulatie van nucleaire en cytoplasmatische maturatie. Tijdens IVM wordt het regulerende mechanisme van cAMP verstoord door de verwijdering van het cumulus-eicel complex (COC) uit de follikel. Deze verwijdering veroorzaakt een voortijdige daling van cAMP wat leidt tot een niet-fysiologische spontane hervatting van de meiose, daar het contact tussen de inhiberende follikelomgeving en het COC verbroken wordt. Om de vroegtijdige daling van cAMP te voorkomen was het eerste doel van deze studie om de invloed van cAMP-modulerende stoffen zoals de aspecifieke phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) en het membraan-permeabele cAMP analoog, dibutyryl cAMP (dbcAMP), te testen. Onze resultaten toonden aan dat IBMX toevoeging aan het collectiemedium het kiemblaasstadium van porciene eicellen, dat geëvalueerd werd in de eindfase van de collectie, niet synchroniseerde. De toevoeging van dbcAMP bij het IVM medium gedurende de eerste 22 uur van IVM had daarentegen wel een positieve invloed en veroorzaakte een verlaagde polyspermie ratio en een hoger normaal fertilisatiepercentage. Bovendien toonde dit onderzoek voor het eerst aan dat er een relatie bestaat tussen het dbcAMP niveau en het aantal COCs die zich aan de bodem van het cultuurplaatje hecht. De toevoeging van dbcAMP reduceerde de aangehechte COCs ten gevolge van een opregulering van proADAMTS-1 in de cumuluscellen.

Gezien het interessant is om individuele of kleine aantallen COCs te onderzoeken, ontwikkelden we een methode die toelaat individuele cumuluscellen te collecteren en te immobiliseren (**Hoofdstuk 4**). Deze aanpak had als doel de proteïnen in de cumuluscellen te onderwerpen aan een kwantitatieve immunofluorescentie analyse. De meest toegepaste hedendaagse technieken zijn gebaseerd op het voor handen zijn van grote aantallen van cellen. Soms is er echter minder biologisch materiaal ter beschikking en dan is er nood aan specifieke technieken om lage celaantallen te verwerken. Wij hebben nu een eenvoudige en effectieve methode voor collectie en immobilisatie van cellen met behulp van Cell-Tak op punt gezet en gevalideerd. Dit liet een immunokleuring van proteïnen toe zodat kwantitatieve analyse van individuele cellen kon uitgevoerd worden.

In **Hoofdstukken 5** en **6** werd nog een ander belangrijk element van de bidirectionele communicatie tussen eicel en cumuluscellen belicht. De eicel is in staat om haar eigen micro-omgeving te reguleren met behulp van de afscheiding van eicel-gesecreteerde factoren. Deze factoren beïnvloeden de differentiatie van de cumuluscellen, die op hun beurt de ontwikkeling

van de eicel bevorderen. Dit fenomeen kan beschouwd worden als een soort auto-regulerende lus van de eicel langs de cumuluscellen om. Het gebruik van eicel-gesecreteerde factoren om bestaande IVP systemen te verbeteren werd in deze studie onderzocht met behulp van een specifieke proefopstelling die ontwikkeld werd om lage aantallen eicellen te cultiveren in een gedefinieerd medium.

Vooreerst (**Hoofdstuk 5**) werd een basis IVM systeem ontwikkeld met behulp van Cell-Tak, wat toeliet eicellen individueel op te volgen gedurende IVM en wat ons toeliet om cumulusexpansie op een precieze en objectieve manier te meten. Bovendien was dit systeem geschikt voor de inclusie van naakte eicellen (als bron van eicel-gesecreteerde factoren) die op een vaste afstand van het te onderzoeken COC bevestigd werden. Met behulp van oocytectomie toonden we aan dat cumuluscellen onafhankelijk van de eicel kunnen uitzetten. De eicellen waren echter wel in staat de cumulusexpansie te bevorderen zowel als organische delen van de COCs *via* gap junctions, als door naakte eicellen, toegevoegd aan cultuurdruppels, die de eigenschappen van het medium veranderden.

Gezien we duidelijk aantoonden dat eicel-gesecreteerde factoren de cumulusexpansie kunnen beïnvloeden tot op een bepaald niveau, verifieerden we in **Hoofdstuk 6** in welke mate eicel-gesecreteerde factoren met behulp van toevoeging van naakte eicellen tijdens IVM, de fertilisatieparameters en het ontwikkelingspotentieel van de eicel konden verbeteren. Onze resultaten toonden aan dat de toevoeging van naakte eicellen bij een IVM medium in een concentratie tot 0.36 naakte eicellen/µL de fertilisatie en het vermogen tot ontwikkelen in het toegepaste gedefinieerde systeem niet op een significante manier kon verbeteren. Gezien andere onderzoeksgroepen bij andere diersoorten na toevoeging van naakte eicellen wel een verbeterd ontwikkelingspotentieel van de eicel konden vaststellen, zou hier mogelijk een diersoort-specifiek mechanisme een rol kunnen spelen.

In **Hoofdstuk 7** worden de resultaten van deze thesis hernomen en per thema besproken.

De specifieke conclusies die we kunnen formuleren zijn:

1. De toevoeging van IBMX gedurende collectie synchroniseerde de kiemblaastadia van de porciene eicellen niet. Daarentegen leidde de toevoeging van dbcAMP gedurende IVM tot lagere polyspermie percentages en resulteerde het in een lager aantal plakkende COCs ten gevolge van het verhoogde proADAMTS-1 niveau in cumuluscellen.

2. Om de experimenten omtrent de rol van cAMP technisch te ondersteunen ontwikkelden en valideerden we een nieuwe en eenvoudige techniek om individuele cellen te verwerken met als doel een immunofluorescente kleuring van proteïnen uit te voeren.

3. Er werd een gedefinieerd IVM systeem voor cultuur van lage aantallen COCs, voor objectieve meting van cumulus expansie en individuele opvolging ontwikkeld. Door dit systeem te hanteren werd aangetoond dat cumuluscellen onafhankelijk van de eicel kunnen expanderen, maar dat eicellen *via* gap junctions en *via* toevoeging van naakte eicellen cumulusexpansie wel bevorderen.

4. Het toevoegen van naakte eicellen gedurende IVM had geen effect op de fertilisatieparameters en het ontwikkelingspotentieel van porciene eicellen.

In het algemeen kan gesteld worden dat dit onderzoek ons meer inzicht bezorgde in de bidirectionele communicatie tussen eicel en cumuluscellen, zowel op het niveau van de cAMP afhankelijke pathways als op het niveau van eicel-gesecreteerde factoren.

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Indeed, lost in the public transport system of Warsaw, my scientific poster holder betrayed me and so I got to chat with two other conference participants from Japan. A few hours later, only at the desk of the hotel, I recognized their names… I realized that those two men were the authors of the papers I had been reading over and over again for one year. From that moment on, I hoped I would have the opportunity to learn from them about the newest approach to the problems in the porcine IVF world. My astonishment was even bigger when they seemed interested in my research. Hesitantly, I talked about the percentages I obtained and about the difficulties I was faced with. They overwhelmed me with advice. When they proposed me to do some research under their supervision in their labs in Japan, I could hardly believe that a small fish as me got this opportunity. Doctor Kikuchi, I am forever indebted to you. Thanks for the confidence, for the time you spent on managing all the administrative burdens for my projects, thanks for the fact that you forgave me so many times not knowing nor understanding the Japanese social rules… But especially thanks for being here. Getting attention from a scientific authority like you makes my research more valuable. Moreover, you want to allow me in your extremely competent team. I cannot find words to express my gratitude.

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Curriculum Vitae

Ruth Appeltant werd geboren op 6 augustus 1987 te Vilvoorde. Toen ze het diploma hoger secundair onderwijs aan het Koninklijk Lyceum te Mechelen behaald had, begon ze in 2005 aan de studie diergeneeskunde te Antwerpen. Na het behalen van dit bachelordiploma, startte ze in 2008 aan de masteropleiding in de diergeneeskunde aan de Universiteit Gent. In 2011 behaalde ze het diploma van dierenarts met grootste onderscheiding in de optie varken, pluimvee en konijn. Ze ontving de IPVS Belgian branch prijs voor haar masterthesis betreffende het onderzoek van reforme zeugen in het slachthuis.

Eind 2011 trad ze als wetenschappelijk bursaal van het Fonds Wetenschappelijk Onderzoek – Vlaanderen in dienst van de vakgroep Verloskunde, Voortplanting en Bedrijfsdiergeneeskunde om zich te specialiseren in de *in vitro* embryo productie bij het varken. Binnen de Reproductive Biology Unit focuste ze gedurende vier jaar op de *in vitro* maturatie van varkenseicellen. In het kader van haar doctoraatsonderzoek heeft ze in 2013 gedurende twee maanden laboratoriumwerk in het NIAS-instituut in Tsukuba (Japan) verricht. Naast haar doctoraatsonderzoek was ze ook werkzaam in de Eenheid Gezondheidszorg Varken binnen de vakgroep. In 2015 voltooide ze de doctoraatsopleiding aan de Doctoral Schools of Life Sciences and Medicine.

Ruth Appeltant is auteur en co-auteur van verschillende wetenschappelijke publicaties in nationale en internationale tijdschriften en ze nam actief deel aan diverse nationale en internationale congressen.

Bibliography

International journals with peer-review

2015

Appeltant, R., Beek, J., Vandenberghe, L., Maes, D., and Van Soom, A. (2015) Increasing the cAMP concentration during *in vitro* maturation of pig oocytes improves cumulus maturation and subsequent fertilization *in vitro*. *Theriogenology* **83**, 344-352

Appeltant, R., Maes, D., and Van Soom, A. (2015) Method for collecting and immobilizing individual cumulus cells enabling quantitative immunofluorescence analysis of proteins. *Anal. Biochem.* 480, 31-33

Appeltant, R., Somfai, T., Kikuchi, K., Maes, D., and Van Soom, A. (2015) Influence of co-culture with denuded oocytes during *in vitro* maturation on fertilization and developmental competence of cumulus-enclosed porcine oocytes in a defined system. *Anim. Sci. J.* In press

Appeltant, R., Somfai, T., Nakai, M., Bodó, S., Maes, D., Kikuchi, K., and Van Soom, A. (2015) Interactions between oocytes and cumulus cells during *in vitro* maturation of porcine cumulus-oocyte complexes in a chemically defined medium: effect of denuded oocytes on cumulus expansion and oocyte maturation. *Theriogenology* **83**, 567-576

Arsenakis, I., Appeltant, R., Rijsselaere, T., and Maes, D. Is there a relationship between semen parameters and meat quality characteristics in Pietrain boars? In preparation

Beek, J., Maes, D., Appeltant, R., Bijttebier, J., Van Steendam, K., Nauwynck, H., and Van Soom, A. Hampered cumulus expansion of porcine cumulus-oocyte complexes by excessive alpha2-macroglobulin is likely mediated via inhibition of zinc-dependent metalloproteases. Second round revision

Beek, J., Nauwynck, H., Appeltant, R., Maes, D., and Van Soom, A. Inhibitors of serine proteases decrease sperm penetration during porcine fertilization *in vitro* by inhibiting sperm binding to the zona pellucida and acrosome reaction. *Theriogenology* Second round revision

Maes, D., Van Soom, A., Appeltant, R., Arsenakis, I., and Nauwynck, H. (2015) Porcine semen as a vector for transmission of viral pathogens. *Theriogenology* Submitted

2014

de Jong, E., Appeltant, R., Cools, A., Beek, J., Boyen, F., Chiers, K., and Maes, D. (2014) Slaughterhouse examination of culled sows in commercial pig herds. *Livestock Science* **167**, 362-369

National popular journals

2012

Appeltant, R. (2012) Onderzoek van reforme zeugen in het slachthuis. *Varkensbedrijf*, 23ste jaargang, nummer 11

Oral presentations

2015

Appeltant, R., Somfai, T., Nakai, M., Bodo, S., Maes, D., Kikuchi, K., and Van Soom, A. (2015) Impact of co-culturing cumulus-enclosed porcine oocytes with denuded oocytes during *in vitro* maturation in a defined medium on cumulus expansion and oocyte maturation. In "Proceedings of the 7th European Symposium of Porcine Health Management (ESPHM), Nantes, France". p. 114

Appeltant, R., Somfai, T., Nakai, M., Bodo, S., Maes, D., Kikuchi, K., and Van Soom, A. (2015) Impact of co-culturing cumulus-enclosed porcine oocytes with denuded oocytes during *in vitro* maturation in a defined medium on cumulus expansion and oocyte maturation. In "Proceedings of the International Pig Veterinary Society Belgian branch (IPVSBb) meeting April, Ghent, Belgium". p. 43-46

2014

Appeltant, R., Beek, J., Maes, D., and Van Soom, A. (2014) Effect van 3-isobutyl-1-methylxanthine op de nucleaire configuratie van varkenseicellen tijdens *in vitro* collectie. Vereniging voor Fertiliteitsstudie (VFS) voorjaarsvergadering, Leuven, Belgium

2013

de Jong, E., Appeltant, R., Beek, J., Boyen, F., Chiers, K., Van Soom, A., and Maes, D. (2013) Slaughterhouse examination of culled sows of commercial pig herds. In: "Proceedings of the 5th European Symposium of Porcine Health Management (ESPHM), Edinburgh, Scotland". Abstract O17

de Jong, E., Appeltant, R., and Maes, D. (2013) Using slaughterhouse data to improve management of reproduction in sows. In "Proceedings of the Satellite Symposium of 9th International Conference on Pig Reproduction (ICPR), Pig Reproduction for practitioners, Olsztyn, Poland". p. 44-51

2011

Appeltant, R. (2011) Reasons for culling of sows and evaluation of the reproductive tract at slaughter. In "Proceedings of the International Pig Veterinary Society Belgian branch (IPVSBb), Ghent, Belgium". p. 42-52

Poster presentations

2015

Appeltant, R., Somfai, T., Nakai, M., Bodo, S., Maes, D., Kikuchi, K., and Van Soom, A. (2015) Impact of co-culturing cumulus-enclosed porcine oocytes with denuded oocytes during *in vitro* maturation in a defined medium on cumulus expansion and oocyte maturation. *Reprod. Fertil. Dev.* **27** (1), 237

Arsenakis, I., Appeltant, R., Sarrazin, S., Rijsselaere, T., Van Soom, A., and Maes, D. (2015) Relationship between carcass quality characteristics and semen quality in Piétrain boars. Accepted for poster presentation at the VIIIth International Conference on Boar Semen Preservation (IBSC), Champaign Urbana Illinois, USA

2014

Appeltant, R., Beek, J., Maes, D., and Van Soom, A. (2014) Effect of 3-isobutyl-1-methylxanthine (IBMX) on the germinal vesicle (GV) stage of porcine oocytes during oocyte collection *in vitro*. In "Proceedings of the International Pig Veterinary Society Belgian branch (IPVSBb), Drongen, Belgium". Abstract P-1

Appeltant R., Beek J., Maes D., and Van Soom A. (2014) Effect of 3-isobutyl-1-methylxanthine (IBMX) in the germinal vesicle stage of porcine oocytes during oocyte collection *in vitro*. *Reprod. Fertil. Dev.* **26** (1), 193

Bodo, S., Somfai, T., Gocza, E., Pribenszky, C., Appeltant, R., Haraguchi, S., Nagai, T., and Kikuchi, K. (2014) Analysis of the timing and pattern of first embryonic cleavage in mono and polyspermic porcine oocytes by time lapse cinematography. *J. Reprod. Dev. Suppl.* **107**, Abstract P-58

2013

de Jong, E., Appeltant, R., Beek, J., Boyen, F., Chiers, K., Van Soom, A., and Maes, D. (2013) Slaughterhouse examination of culled sows in commercial pig herds reveals few macroscopic lesions. In "Proceedings of the 9th International Conference on Pig Reproduction (ICPR), Olsztyn, Poland". Abstract P-74