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—Review—

Porcine oocyte maturation *in vitro*: role of cAMP and oocyte-secreted factors – A practical approach

Ruth APPELTANT[1)], Tamás SOMFAI[2)], Dominiek MAES[3)], Ann VAN SOOM[3)] and Kazuhiro KIKUCHI[1)]

[1)]*Division of Animal Sciences, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Ibaraki 305-8602, Japan*
[2)]*Division of Animal Breeding and Reproduction, Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization, Ibaraki 305-0901, Japan*
[3)]*Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, B-9820 Merelbeke, Belgium*

Abstract. Polyspermy or the penetration of more than one sperm cell remains a problem during porcine *in vitro* fertilization (IVF). After *in vitro* culture of porcine zygotes, only a low percentage of blastocysts develop and their quality is inferior to that of *in vivo* derived blastocysts. It is unknown whether the cytoplasmic maturation of the oocyte is sufficiently sustained in current *in vitro* maturation (IVM) procedures. The complex interplay between oocyte and cumulus cells during IVM is a key factor in this process. By focusing on this bidirectional communication, it is possible to control the coordination of cumulus expansion, and nuclear and cytoplasmic maturation during IVM to some extent. Therefore, this review focuses on the regulatory mechanisms between oocytes and cumulus cells to further the development of new *in vitro* embryo production (IVP) procedures, resulting in less polyspermy and improved oocyte developmental potential. Specifically, we focused on the involvement of cAMP in maturation regulation and function of oocyte-secreted factors (OSFs) in the bidirectional regulatory loop between oocyte and cumulus cells. Our studies suggest that maintaining high cAMP levels in the oocyte during the first half of IVM sustained improved oocyte maturation, resulting in an enhanced response after IVF and cumulus matrix disassembly. Recent research indicated that the addition of OSFs during IVM enhanced the developmental competence of small follicle-derived oocytes, which was stimulated by epidermal growth factor (EGF) *via* developing EGF-receptor signaling.

Key words: cAMP, *In vitro* embryo production, Oocyte-secreted factors, Porcine

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A series of integrated, efficient techniques is required to produce porcine embryos from follicular oocytes *in vitro* [1]. Three major subsequent phases are oocyte collection and *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC). When using the currently applied protocols with immature porcine oocytes, only 20–30% develop to the blastocyst stage [2–4], and a substantial proportion is polyploid [5]. In an *in vitro* production (IVP) system, the success of embryo development to the blastocyst stage depends on oocyte cytoplasm to facilitate normal fertilization and subsequent embryo development *in vitro* [6]. The process by which immature oocytes acquire this ability during IVM is frequently called "cytoplasmic maturation". When considering the two major obstacles in porcine IVP *i.e.*, high polyspermy rates and low blastocyst developmental rates (reviewed by Nagai *et al.* [7]), the question may arise if these problems are related to insufficient cytoplasmic maturation during IVM. However, it is difficult to directly measure the degree of cytoplasmic maturation by a simple assay [8] because it involves many biological processes, such as the re-distribution of certain organelles, accumulation of glutathione, or the adjustment of cell-cycle regulating protein kinases levels (reviewed by Ferreira *et al.* [9]). Therefore, evaluation of pronuclear formation and developmental ability to the blastocyst stage are the most common indirect ways to assess cytoplasmic maturation.

The somatic compartment around the oocyte (*i.e.*, cumulus cells) possibly plays an important role during oocyte maturation by gap junctional communications and soluble factors (reviewed by Tanghe *et al.* [10, 11]). Despite the major progress that has been made in this field of study, there are still gaps in the knowledge. In this respect, we focused on the second messenger cyclic adenosine monophosphate (cAMP) and oocyte-secreted factors (OSFs), which possibly play a key role in oocyte maturation regulation. Cyclic AMP is produced by cumulus cells, and transported through gap junctions into the oocyte (reviewed by Shimada [12]). However, *in vitro*, this coordinated exchange of cAMP is disturbed because of the removal of the oocyte from the follicle. By adjusting cAMP levels during IVM, an improvement of the nuclear and cytoplasmic maturation, the response during fertilization, and the developmental competence of the oocyte can be obtained in *in vitro* systems [13–16].

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Correspondence: R Appeltant (e-mail: ruthappeltant@affrc.go.jp)

In addition, OSFs, specific soluble growth factors produced by oocytes, influence cumulus cell differentiation to enhance oocyte maturation *via* gap junctional communication and paracrine signals (reviewed by Gilchrist [17]). Consequently, these OSFs help the oocyte to create its own microenvironment during porcine IVM since OSFs regulate the surrounding cumulus cells. These cumulus cells in turn affect the oocyte development. This communication can be considered as an auto-regulatory loop between the oocyte and its supporting somatic cells and seems crucial for oocyte developmental competence (reviewed by Gilchrist [17]).

The general aim of this paper was to review the current knowledge on the bidirectional communication between the oocyte and surrounding cumulus cells to establish a porcine IVP system with improved oocyte developmental potential. Therefore, it is important to reveal to what extent the removal of the oocyte from the follicle can affect intracellular cAMP levels and consequently developmental competence, and if oocytes regulate cumulus cell differentiation and their own development *via* the secretion of soluble paracrine growth factors.

Role of cAMP and Oocyte-Secreted Factors during Oocyte Maturation

Role of cAMP

In mammalian cells, cAMP is a second messenger in the process of intracellular signaling [18]. Consequently, a wide range of cellular processes is regulated by cAMP responses [18]. Cyclic AMP exerts an important regulating role in the meiotic process of the oocyte. *In vivo*, follicles control meiotic arrest and resumption. High intracellular cAMP levels, produced by the oocyte, that are mediated by signals from the follicle [19] and continuously provided through gap junctional contacts with cumulus and mural granulosa cells [20], maintain meiotic arrest in the oocyte when the oocyte is inside the follicle [21]. In follicular fluid (FF), substances like hypoxanthine, a natural purine, can contribute to an elevated cAMP concentration inside the oocyte and therefore inhibit germinal vesicle breakdown (GVBD) [22]. A recent study showed that the concentration of hypoxanthine in FF was higher in small than large follicles [23]. This information emphasizes that changes within *in vivo* environment allows oocytes from large follicles to resume meiosis more readily in response to a luteinizing hormone (LH) surge. Furthermore, cAMP degradation 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 [17]. After gonadotropin stimulation, a transient increase followed by a drop in the intracellular cAMP concentration induces the oocyte to resume meiosis [24]. Thereafter, the cAMP concentration decreases to basal levels at approximately the time of GVBD [24]. The scenario *in vitro* is considerably different because the cumulus oocyte complex (COC) is removed from the follicle. Since the control of the follicular environment is lost, cAMP levels drop prematurely (mouse, [25]) causing a spontaneous resumption of meiosis, which is characterized by an accelerated progression to metaphase II (nuclear maturation), producing oocytes with low developmental competence (reviewed by Gilchrist and Thompson [26]). A possible cause for this low developmental potential is incomplete cytoplasmic maturation (reviewed by Gilchrist and Thompson [26]). The aspects of cytoplasmic maturation affected by cAMP are detailed below.

Role of oocyte-secreted factors

Oocytes regulate cumulus cell differentiation *via* the secretion of soluble paracrine growth factors, known as the OSF-regulation of folliculogenesis [27], which is a bidirectional regulatory loop between oocyte and cumulus cells since OSFs regulate the surrounding cumulus cells that in turn affect the oocyte development. The importance of the OSFs produced by the oocyte to create a microenvironment determining its further development, has only recently been acknowledged [17]. Two factors, from the transforming growth factor beta (TGF-β) superfamily, growth differentiation factor 9 (GDF9) [28] and bone morphogenetic protein 15 (BMP15) [29], are the most crucial OSFs. Both factors work through the BMP receptor type II (BMPRII) [30], and downstream through activin receptor-like kinases (ALKs) followed by action on the SMA and MAD related intracellular proteins (SMAD) [31]. More specifically, GDF9 acts on ALK5 and SMAD2/3 [31] and BMP15 on ALK6 and SMAD1/5/8 [32].

It is unknown whether the oocyte itself, with the associated OSFs, is necessary for cumulus expansion. Prochazka *et al*. [33] stated that cumulus expansion was not dependent on the oocyte, although Singh *et al*. [34] observed that the oocyte secretes a cumulus expansion-enabling factor(s) based on an interspecies model. In this model, it was shown that mouse cumulus cells, which are dependent on a cumulus-expansion enabling factor produced by the oocyte, could fully expand in the presence of porcine denuded oocytes (DO) and FSH [34]. Funahashi and Day [35] claimed that cumulus expansion did not depend on intercellular communication between cumulus cells and oocytes, although oocytectomy inhibited the expansion of the corona radiata. In these studies, cumulus expansion was scored using an arbitrary scale from 0 to +4 [33–35]. In a recent study of ours [11], cumulus expansion was measured more objectively using an individual tracking system of COCs during IVM, the defined porcine oocyte medium (POM), and digital image analysis. Using this approach, we demonstrated that the cumulus can expand in the absence of the oocyte [11]. However, oocytectomized complexes (OOXs) do not reach the same level of expansion as intact COCs. The addition of DOs to OOXs improved the level of cumulus expansion. Inhibition of gap junctional communication by carbenoxolone in COCs significantly reduced cumulus expansion at 20 h of IVM [11]. These observations support the research by Singh *et al.* [34] that even though expansion does not require the oocyte, the oocyte enhances cumulus expansion. This occurs both through gap junctional communication and paracrine signaling [11]. Our study [11] also detailed the evolution of the cumulus expansion of COCs, OOXs, and cumulus clumps in a defined medium. After a period of an increase in cumulus area, the three groups showed shrinking of the cumulus area between 20 and 48 h of IVM [11]. Previously, cumulus expansion of COCs in POM was described as the occurrence of a continuously increasing cumulus area [36]. A possible explanation for this discrepancy is possibly the use of different hormones between the two studies. Studies have also shown that factors exist in follicular fluid that support continuous and full cumulus expansion [37–39]. *In vivo*, one of the functions of the expanded cumulus is to guide

the oocyte through ovulation by facilitating oocyte extrusion, to help the ciliated epithelial cells of the infundibulum capture and transport the oocyte towards the fertilization site (reviewed by Tanghe *et al*. [10]). Therefore, the shrinking cumulus area observed between 20 and 48 h of IVM [11] could be due to a deficiency of the *in vitro* environment. However, whether cumulus expansion is a pre-requisite for normal fertilization and embryo development is unknown. Abnormal expansion has been associated with low embryo development in many studies [40, 41]. On the contrary, Yoshioka *et al.* [42] reported a similar penetration and development after IVM in POM + polyvinyl alcohol (PVA) and POM + porcine FF despite the more pronounced cumulus expansion in the latter [43, 44]. In another study, Gomez *et al.* [45] reported reduced cumulus expansion associated with improved blastocyst development after IVM in the presence of denuded oocytes. Moreover, the importance of cumulus cells for fertilization in porcine IVF systems is still unknown. Early reports suggested that cumulus cells are essential for sperm penetration and male pronucleus formation [46]. 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.* [47]). The reasons underlying these contradictory findings remain unclear. 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 the cumulus compartment for the regulation of penetration and monospermy rates during IVF is important for future improvements of the porcine IVP system.

Oocyte-secreted factors can be added to monolayers or clumps of granulosa cells or cumulus cells, OOXs, or intact COCs. They are presumed to be soluble paracrine factors because the DOs do not come in physical contact with the influenced cell type and oocyte conditioned medium can exert a biological response [27]. Moreover, OSFs apparently work in a concentration-dependent manner: the strongest effect of OSFs on granulosa cells [48], cumulus cells [49] or co-cultured oocytes [45] was observed when more DOs or/and a lower culture volume were added. The addition of DOs to OOXs affects cumulus expansion and several other important parameters, such as cumulus cell apoptosis (cattle [49]), luteinization markers (*e.g.*, the expression of LH receptor mRNA in mice [50] and progesterone production in pigs [51]), cumulus glycolytic enzyme mRNA levels (mouse [52]), and steroidogenesis (pig [51]). It is possible that these effects of DOs are caused by the anti-apoptotic effect of BMP15 on cumulus cells [49]. With fewer apoptotic cumulus cells, the overall functionality of the cumulus cells is improved, resulting in higher mRNA levels and increased progesterone production. Together, these findings suggest that the addition of DOs could be beneficial for an IVM production system for OOXs. Regarding nuclear maturation, no enhancement was observed after DO addition using undefined and defined maturation media [11, 45].

Interplay between cAMP and oocyte-secreted factors

These two previously discussed biosystems should not be considered as totally independent. Recently the beneficial effect of the treatment of developing COCs with dibutyryl cAMP sodium salt (dbcAMP) and OSFs on the acquisition of oocyte developmental competence by enhancing epidermal growth factor (EGF) receptor (EGFR) functionality has been demonstrated in a study by Sugimura *et al.* [53]. *In vivo*, prior to the LH surge, follicle stimulating hormone (FSH) enhanced the expression of LH receptors on mural granulosa cells [54] and the expression of EGFR on cumulus cells [55] (Fig. 1). In a later phase, LH induces the expression of EGF-like peptides (amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC)) that activate EGFR [56, 57] (Fig. 1). These EGF-like peptides stimulate EGFR in the mural and cumulus cell compartment, concomitant with a positive autocrine feedback loop on their own expression [58]. Two main processes regulated by EGFR are the expansion of cumulus cells and meiotic oocyte maturation [55]. This coordinated interplay of events possibly occurs only in pre-ovulatory COCs, after the LH surge. Oocytes from small antral follicles (3 mm diameter) have a lower developmental capacity than oocytes from large antral follicles [16]. However, since these oocytes can grow into large follicles to developmentally competent oocytes, the oocyte is not intrinsically defective, but requires the correct stimulus to gain developmental competence [53]. Cumulus oocyte complexes from small antral follicles contain the same amount of EGFR mRNA as larger antral follicles, but substantially less total-EGFR protein leading to impaired phospho-EGFR and phospho- extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling [59]. In this way, EGF will not stimulate the EGFR and ERK1/2 signaling pathways in those cumulus cells implying a low developmental potential of the oocyte [59] (Fig. 1). However, the combined treatment of dbcAMP and OSFs, more specifically GDF9 and BMP15, promoted EGF receptor functionality, which is, as indicated above, an essential element in the acquisition of developmental potential [53] (Fig. 1). More specifically, OSFs stimulated AREG signaling in COCs derived from small antral follicles (< 4 mm) [59] and dbcAMP positively interacted with COCs from medium-sized follicles (> 4–6 mm) concerning meiotic maturation and developmental potential of the oocyte, but the combination of dbcAMP and BMP15 increased the meiotic maturation and the combination of dbcAMP, BMP15, and GDF9 increased blastocyst rates of COCs from small antral follicles in an AREG-stimulated IVM system [53]. Moreover, only this combination of AREG stimulation together with cAMP modulation, BMP15, and GDF9 positively influenced cumulus expansion and expression of the matrix-related genes [53]. The added products all synergistically stimulated the cumulus cell EGFR-ERK1/2 signaling pathway in AREG stimulated IVM [53] (Fig. 1). Therefore, the improved developmental competence of oocytes derived from small follicles can be attributed to an enhanced EGFR signaling after a combinational treatment of dbcAMP and the OSFs, BMP15, and GDF9 [53] (Fig. 1). After the addition of dbcAMP, BMP15, and GDF9, AREG stimulation appeared to be more beneficial than gonadotropin supplementation [53].

In earlier studies, the establishment of a functional EGFR signaling pathway in developing oocytes depended on the correct stimulation by FSH, followed by cAMP and PKA responses [55, 60, 61]. However, recently it has been demonstrated that this pathway is not the only requirement for functional EGFR signaling, but the combination of cAMP together with OSFs may be necessary [53]. It is assumed that cAMP may stimulate BMP15-GDF9-induced SMAD signaling and therefore increase the developmental potential [53, 62]. Moreover, EGF-peptides/EGFR and cAMP-PKA signaling pathways interact

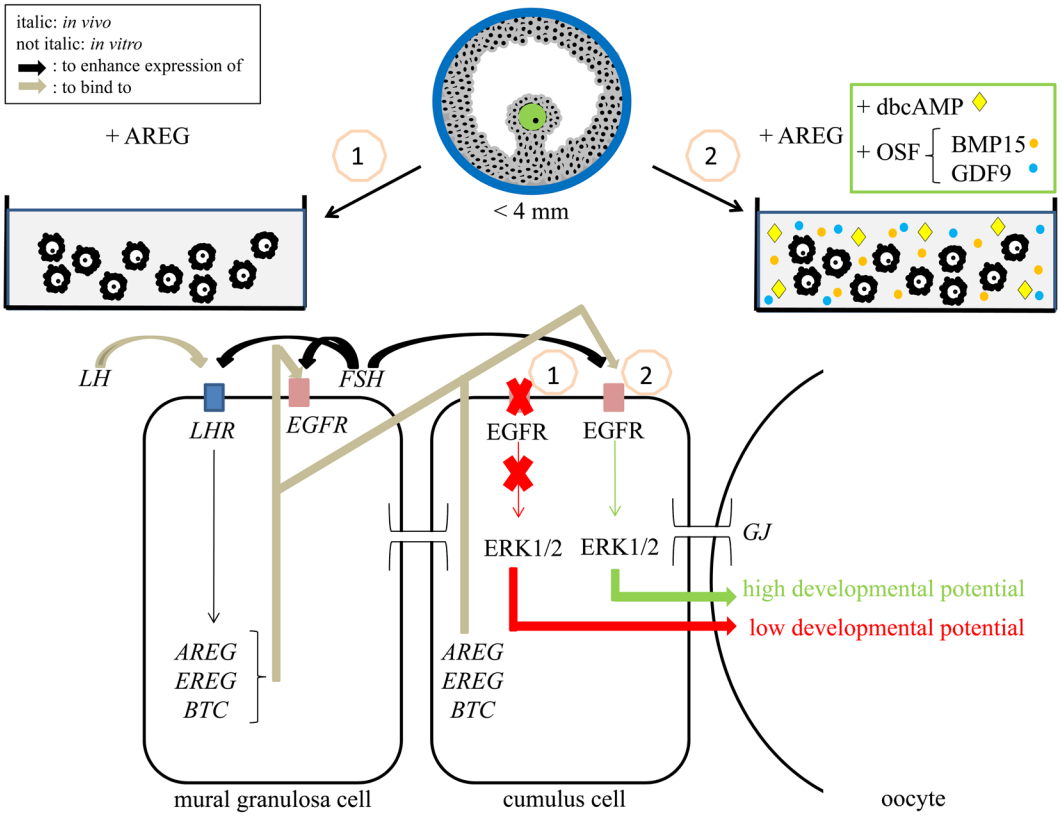

Fig. 1. Hypothetical mechanism for the stimulation of the epidermal growth factor receptor (EGFR)/extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling pathway in cumulus cells of porcine cumulus-oocyte complexes (COCs) derived from small follicles (< 4 mm). Intercellular communication between mural granulosa cells, cumulus cells, and oocytes occurs through paracrine and autocrine signals and gap junctional communication (GJ). *In vivo*, follicle-stimulating hormone (FSH) induces luteinizing hormone (LH) receptors (LHR) and EGFR that can be stimulated by LH and amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC), respectively. *In vitro*, developing oocytes cannot stimulate the EGFR/ERK1/2 pathway without the addition of dibutyryl cAMP sodium salt (dbcAMP) and oocyte-secreted factors (OSFs) leading to a low developmental competence of the oocyte. Addition of dbcAMP and OSFs, such as bone morphogenetic protein (BMP15) and growth differentiation factor 9 (GDF9), in an AREG-stimulated IVM system promotes the EGFR/ERK1/2 signaling pathway, consequently with an improved developmental potential of the oocyte.

and establish a wide intercellular communication all over the COC [53], and the AREG stimulation of COC glycolysis and BMP15 preservation of gap junctional communication can enhance the transfer of necessary metabolites from cumulus cells into the oocyte [63]. Both these events contribute to an enhanced developmental potential of the oocyte [53]. Together, recent research indicated that the co-operative action of cAMP modulation and OSFs resulted in improved EGFR signaling in the somatic compartment, which is necessary for the COC to acquire full oocyte developmental competence [53].

Application of cAMP and Oocyte-Secreted Factors in IVP Systems

Manipulation of intracellular cAMP levels by chemical agents

Nuclear maturation, cytoplasmic maturation, the response during fertilization and developmental competence of the oocyte are possibly improved by increasing cAMP levels during IVM. Intracellular cAMP levels of oocytes can be modulated in several ways by numerous chemicals with different mechanisms of action [26, 64]: (1) membrane permeable cAMP analogs, such as dbcAMP [13–16, 65], (2) adenylate cyclase activators, such as forskolin [22, 66] and invasive adenylate cyclase [13], and (3) phosphodiesterase inhibitors, such as the non-specific inhibitor 3-isobutyl-1-methylxanthine (IBMX) [13, 65, 67], phosphodiesterase type 4-specific inhibitor, rolipram [68], and phosphodiesterase type 3-specific inhibitors, milrinone [69] and cilostamide [67]. However, in pigs, the only cAMP-elevating treatment that has improved porcine oocyte developmental competence is dbcAMP (reviewed by Gilchrist and Thompson [26]). Other cAMP-elevating treatments did not show a clear improvement in porcine oocyte developmental competence.

Three-isobutyl-1-methylxanthine is a non-specific PDE inhibitor [70], which prevents the *in vitro* drop in cAMP by inhibiting the increasing PDE3 activity in the oocyte (cattle [70]). This increased of PDE3 activity is caused by a decreased supply of cGMP *via* the gap junctions because of the premature closure of the gap junctional communication between oocyte and cumulus cells (mouse [71, 72]). When evaluating the IBMX efficacy, two factors could determine the outcome: 1) the time of IBMX addition, which can either be during

oocyte collection or IVM and 2) the time of evaluation of the effect of IBMX, which can be during collection, IVM, or IVC. Several studies investigated the effect of IBMX in combination with other compounds in various species; however, only some studies tested the effect of IBMX alone on oocytes. Bornslaeger *et al.* [73] demonstrated that IBMX inhibited PDE activity and GVBD in mouse oocytes. In the "simulated physiological oocyte maturation (SPOM) system" (in mice and cattle) IBMX increased cAMP levels during pre-IVM in combination with, forskolin [70]. The SPOM system improved the developmental potential of the oocyte, although this procedure included the addition of many more cAMP-modulating agents after the pre-IVM phase [70]. In pigs, the addition of IBMX during IVM reversibly blocked meiotic resumption [67, 74], prolonged the maintenance of gap junctional communication between the oocyte and cumulus cells [74], and synchronized oocytes to the germinal vesicle (GV) 2 stage when matured with follicle-stimulating hormone (FSH) for 20 h [75]. However, when added to the collection medium, the combination of IBMX and invasive adenylate cyclase did not influence the nuclear progression during IVM and subsequent developmental competence of porcine oocytes [13]. Our recent research confirmed that the addition of IBMX during collection does not alter the chromatin configuration of porcine oocytes at the end of collection [65]. However, in earlier research [76], concentrations of > 1 mM IBMX were used to maintain the majority of porcine oocytes in GV arrest, a concentration of 0.5 mM was optimal for synchronizing the GV stage during IVM (> 80% at 20 h of IVM) and allowed the formation of LH receptors and optimal hCG binding of COCs [75]. Higher concentrations of 1 mM suppressed LH receptor formation and hCG binding [75].

The absence of the effect of IBMX during collection in pigs could possibly suggest that during oocyte collection, intracellular cAMP in oocytes might not drop to a level that could cause spontaneous maturation. This lack of effect of IBMX addition in pigs is in contrast with the results obtained in other species, including cattle and mice [70, 73, 77], where cAMP levels were increased, GVBD was inhibited, and blastocyst rates were improved by adding IBMX to the collection medium. Therefore, these findings strongly suggest that the action of IBMX is species-specific; although, it should be considered that the different findings concerning IBMX effects could also be due to differences in the collection methods used between species and studies.

The generally used dbcAMP concentration of 1.00 mM was set by Funahashi *et al.* [14] according to the findings of Mattioli *et al.* [24]. At this concentration, dbcAMP reversibly blocked GVBD in porcine oocytes without any negative side effects [14]. Exposure of COCs to dbcAMP should occur only during the first 20–22 h of a total of 44–48 h of IVM [14] because a high concentration of cAMP throughout the whole IVM period would maintain meiotic arrest [24]. Keeping cAMP levels high during the early stages of IVM of the porcine oocyte by dbcAMP reversibly inhibited the spontaneous meiotic progression [69], causing a synchronization of meiotic maturation during the second half of the IVM culture, when the oocytes are released from the blocking effect of this agent [13, 14]. Such treatment has been reported to increase normal fertilization rates [13, 15] and subsequent embryo development [13–16]; however, it has not been clarified if this effect is due to the prevention of premature oocyte aging by the synchronization of meiotic maturation or *via* other effects. Furthermore, it is possible that modulation of cAMP levels during IVM could affect sperm penetration into the oocyte during IVF. It is unknown whether the ability of the oocyte to block polyspermy is affected by modulating cAMP levels *via* the IVM medium. Conflicting results have been published concerning the influence of cAMP modulation on fertilization parameters. Funahashi *et al.* [14] did not find any significant difference in sperm penetration and monospermy rates between oocytes matured with or without 1.0 mM dbcAMP in the IVM medium. Somfai *et al.* [13] confirmed 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.* [15] reported a higher penetration rate in the dbcAMP supplemented group, but confirmed the lower polyspermy rate. We recently showed that dbcAMP addition during the first part of IVM decreased the polyspermy rate of porcine oocytes after IVF [65]. This result was in line with the reports of Somfai *et al.* [13] and Kim *et al.* [15], but contradicted the results of Funahashi *et al.* [14]. The reason behind this discrepancy remains unknown. A possible explanation can be that each research group operated with its own materials and methods, which also determined fertilization outcome. Differences in the applied IVF protocols may contribute to a variation in results between research groups. 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 may vary considerably. Moreover, the expression of parameters such as penetration and polyspermy rate is not always clearly defined, making interpretation and comparison difficult. However, we consider the decreased polyspermy rate in our study is a genuine result. First, because of the already shown synchronizing ability of dbcAMP, nuclear maturation will proceed in a more harmonized way [14]. This nuclear synchronization facilitates cytoplasmic maturation, which could better sustain monospermic fertilization. Second, gap junctional communication promotes cytoplasmic maturation by transferring the necessary metabolites to the oocytes [78]. With the addition of dbcAMP, these connections are preserved for a longer period [79] and thus the cytoplasmic maturation and normal fertilization [78] are enhanced. Third, dbcAMP increases the plasminogen activator (PA) [80]. Tissue-type PA could be involved in the block against polyspermy. In rats, tissue-type PA is released during the cortical granule exocytosis and could have a possible role in the zona hardening as part of the defense against polyspermy [81]. Fourth, Bijttebier *et al.* [41] showed that an improved cumulus matrix quality could provide enhanced protection against polyspermy. Mucification is directly correlated with high cAMP levels in rats [82]. Furthermore, it was demonstrated that pre-incubation of COCs with dbcAMP caused an extensive proliferation of porcine cumulus cells [83] and that dbcAMP addition to the IVM medium in pigs resulted in an improved cumulus matrix disassembly [65]. Moreover, in parallel with the reduced polyspermy rate, we also observed decreased penetration [65]. Presumably, cAMP modulation could have been responsible for an improved cumulus matrix and consequently reduced penetration and polyspermy rates. The phenomenon of dbcAMP affecting cumulus cells, emphasizes the importance of the

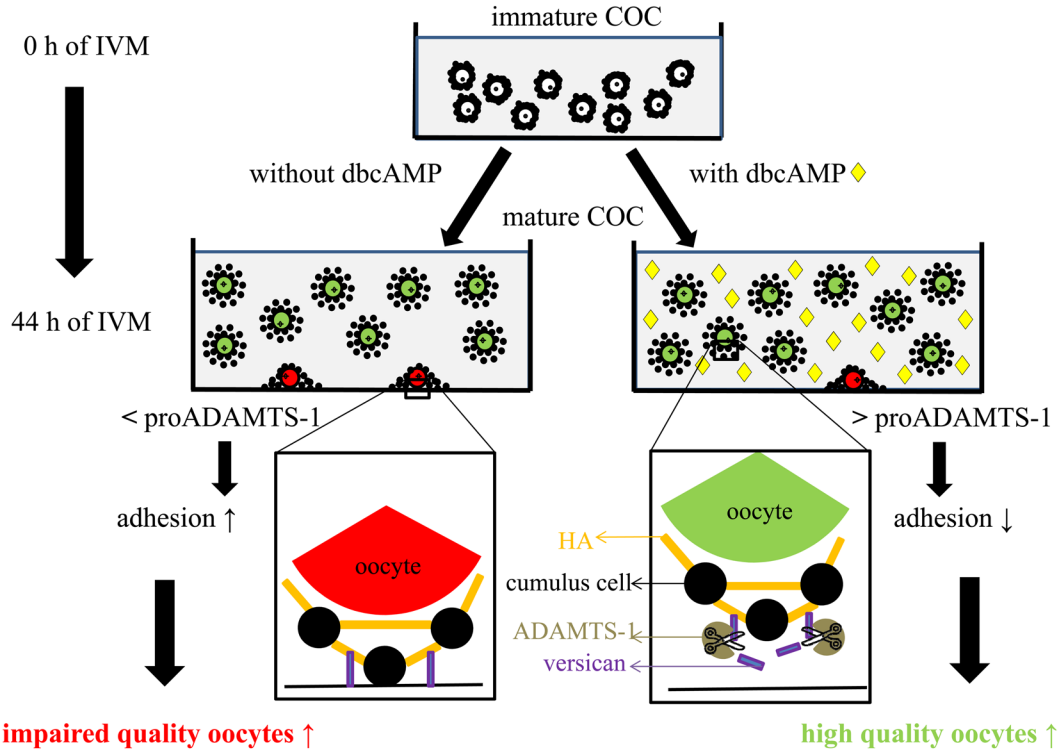

Fig. 2. Relationship between dibutyryl cAMP sodium salt (dbcAMP), the proprotein of a disintegrin and metalloproteinase with thrombospondin-like repeats-1 (proADAMTS-1), versican, and adherent cumulus-oocyte complexes (COCs) during *in vitro* maturation (IVM). Addition of dbcAMP upregulates proADAMTS-1, which promotes the cleavage of versican and thus prevents adherence of the COC to other cells, or specifically for *in vitro* culture to the bottom of the culture dish. Adherent COCs on the bottom of the dish have an impaired quality (red) in comparison to the floating ones (green) [88, 89].

used IVF protocol (*e.g.,* the presence of cumulus cells during IVF) strongly determining the obtained fertilization results and possibly clarifying the divergent results between different research groups.

Besides the involvement of cAMP levels in the nuclear and cytoplasmic maturation of the oocyte, several findings also illustrated a crucial role of cAMP in cumulus expansion. During IVM of porcine oocytes, some oocytes adhered to the bottom of the dish (Fig. 2). This phenomenon indicates inferior matrix disassembly [84]. To explain this occurrence, the composition and degradation of the extracellular matrix of the COC should first be discussed in more detail. Proteases are among the products secreted by cumulus cells [84]. 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 [85]. In pigs, ADAMTS-1 secretion by cumulus cells is a prerequisite for gonadotropin-dependent cumulus expansion [86]. 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 [85] (Fig. 2). As a result of this cleavage, the cross-linking properties of versican are altered [85] and the expanding matrix is stabilized by binding the cleaved N-terminal domain of versican to HA [86]. Shimada *et al.* [86] reported that inhibiting the function of ADAMTS-1 led to cumulus cells adhering to the culture dish. Integrins on the surface of cells can interact with the C-terminal of versican and thus promote cell adhesion [87]. *In vitro*, the removal of the C-terminus of versican is crucial to prevent the adhesion of the COC to the bottom of the dish. 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 shown that nuclear and cytoplasmic maturation is affected in oocytes attached to the bottom of the culture dish [88]. Adherent COCs will resume meiosis and reach the metaphase II stage earlier than floating ones surrounded by cumulus matrix, probably caused by the disability of the cumulus cells to maintain the meiotic arrest [88]. The exact mechanism of premature nuclear maturation is unclear, but at the end of the maturation period these oocytes manifest a certain degree of aging [88]. Aged oocytes are expected to show heterogeneity concerning cytoplasmic maturation [13] and a higher polyspermy rate [89].

A possible link between the level of intracellular cAMP and the function of ADAMTS-1 was found in research regarding the role of parathyroid hormone (PTH) in bones. Miles *et al.* [90] showed that in the rat femur metaphysis, ADAMTS-1 mRNA expression is only upregulated by those PTH analogs that can significantly elevate intracellular cAMP levels. Although PTH influences many signal transduction pathways, Miles *et al.* [90] 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 [90]. Apart from this connection in bone, little is known about the relationship between the cAMP/PKA signaling pathway and expression of ADAMTS-1 in COCs. It is widely accepted that pituitary hormones, such as LH, act through the binding of ligand-specific cell surface G protein-coupled receptors, activation of adenylyl cyclase, and the subsequent production of cAMP [91]. Shimada *et al.* [86] demonstrated 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 [92, 93]. However, Doyle *et al.* [94] reported that the molecular mechanism by which LH induces 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 [94]. Moreover, in other species, including humans [95] and rats [82], the addition of dbcAMP inhibited monolayer formation and attachment onto the bottom of the culture dish of cumulus cells. Only recently, data were obtained about the relationship between adherent complexes, ADAMTS-1, and cAMP levels in cumulus cells in pigs [65]. Appeltant *et al*. [65] were the first to demonstrate the cAMP-dependent regulation of ADAMTS-1 in porcine cumulus cells. First, the latter relationship was shown by a decreased proportion of adherent complexes at 44 h of IVM after treatment with dbcAMP [65] (Fig. 2). Second, upregulation of the proprotein of ADAMTS-1 (proADAMTS-1) levels in cumulus cells was demonstrated when dbcAMP was added to the IVM medium, for floating and attached COCs [65] (Fig. 2). Moreover, cumulus cells of floating complexes contained consistently more proADAMTS-1 than attached complexes [65].

Therefore, during porcine IVM, cAMP modulation caused elevated proADAMTS-1 levels, probably yielding elevated ADAMTS-1 levels, which promoted increased matrix remodeling by the increased cleavage of versican, leading to less adherence to the bottom of the culture dish *in vitro* [65] (Fig. 2). However, it must be noted that the problem of adherent complexes was not totally solved by cAMP regulation, since adherent complexes can occur from several mechanisms, such as the material of the dish or culture system (static or non-static) [88].

Since attached COCs have inferior quality than floating COCs [88], it is important to avoid these complexes during IVP. Besides the addition of dbcAMP, which reduced the number of attached COCs as mentioned above, a practical selection tool during IVM can be applied to select the optimal oocytes to start IVF/IVC. Since adherent complexes display impaired cumulus remodeling [65] and Somfai *et al.* [88] reported inferior nuclear and cytoplasmic maturation in those complexes, fertilization and further developmental competence of such oocytes are possibly affected. The implementation of the systematic removal of adherent (inferior) COCs after IVM revealed that the remaining oocytes developed to the blastocyst stage in the same proportion in the groups treated with or without dbcAMP [65]. Since literature [13, 14] mostly reported an increased blastocyst rate after cAMP modulation, it could be presumed that the reduced blastocyst rate in non-treated COCs without the selection of adherent complexes, was due to the presence of those impaired complexes that were decreasing the blastocyst rates [65].

The use of oocyte-secreted factors in IVP systems in different experimental models

In the past, attention was focused on the influence of the follicle on the oocyte, mostly by the addition of follicular fluid for improving oocyte maturation conditions. Recently, the focus has shifted towards the secretion of soluble paracrine growth factors by the oocyte itself for its further development [17]. In this part of the review, we focused on OSFs to understand the critical changes the COC has to undergo before the oocyte can be fertilized and develop into a viable embryo.

The OSF function has been investigated using different experimental models such as genetic and immunization models, bioassays of native OSFs or bioassays using candidate recombinant OSFs (reviewed by Gilchrist *et al.* [27]). All these approaches should complement each other for a holistic understanding of the processes.

The source of native OSFs can be the oocyte itself after removal of the cumulus cells, called the denuded oocyte (DO) addition, or can be the culture medium conditioned by denuded oocytes, called the oocyte conditioned medium. The addition of 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 was especially promising when culturing low numbers of oocytes in a group because these oocytes suffer from a lack of other surrounding oocytes to sustain their development. By supplying DOs as helper oocytes, a large group culture could possibly be mimicked. In cattle, sheep and mice, these OSFs have been identified as GDF9 and BMP15 [96–99]. Native OSFs influence numerous functions in granulosa cells, cumulus cells, and oocytes (reviewed by Gilchrist *et al*. [27]).

When interpreting results after DO addition on IVM, IVF, and IVC, it is important to notice that the addition of extra oocytes can modify the culture system in several ways. Besides the aimed addition of OSFs, extra cells can exert a non-specific influence during IVM by reducing 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; moreover, the antioxidant systems of the DOs can detoxify diffusible reactive oxygen species [100]. In very basic media such as POM with hormones, this oxygen reducing action may be observed, but in enriched media supplemented with antioxidants, which overrule the oxidative action of extra cells, this effect may be overshadowed and therefore not observed. Consequently, to draw conclusions, every study should reveal if the observed effect is due to specific OSF working mechanisms or non-specific oxygen consumption.

In the study of Gomez *et al.* [45] it was shown that DO addition decreased cumulus expansion of COCs during IVM in pig. It is important to take into account that this study [45] was performed in a non-defined culture medium and cumulus expansion was scored subjectively. However, in recent studies we also assessed the action of OSFs on porcine oocytes using a defined medium [11, 101]. Using a defined medium, differences were not observed after DO addition, neither in cumulus expansion at 20 and 48 h of IVM nor in nuclear maturation at 48 h of IVM [11]. Although no improvement on cumulus expansion of intact COCs could be found, the addition of DOs to OOXs facilitated expansion measured at 48 h of IVM [11]. Consequently, DOs can influence expansion, although, when the oocyte as part of the complex is still present, the DO-effect is

not strong enough to improve expansion.

Furthermore, no differences were observed in penetration, polyspermy, and normal fertilization rates after IVF of porcine IVM oocytes with or without extra helper DOs [101]. Previous research in cattle [96], goats [102], and mice [97] demonstrated an enhanced oocyte developmental potential after DO addition or supplementation of specific OSFs. In pigs, contradictory results are reported. Denuded oocyte addition improved blastocyst rates after parthenogenetic activation in a non-defined medium [45]; however, other studies, using defined medium, could not show any enhancement of the blastocyst proportion and blastocyst quality after activation [103] or IVF [101]. This is clearly in contrast to reports on other species. The possibility that a species-specific mechanism is involved in the OSF action is emphasized by 1), a discrepancy was observed between pigs and other species, and 2), even the same researchers (*i.e.,* Romaguera *et al.*) showed an effect in goats [102], but were not able to show it in pigs [103]. However, even when considering species-specific differences, why the study of Gomez *et al.* [45] contradicts the study of Romaguera and Grupen [103] and our study [101], all performed in pigs, remains unclear. A possible explanation could be the use of defined [101, 103] or undefined [45] maturation media.

Besides the addition of native DOs to the IVM system, OSFs can be investigated by direct supplementation of media with recombinant OSFs. When focusing on the improvement of existing IVP systems, the model of COCs with the addition of DOs can provide valuable basic information. Oocyte-secreted factors will be released by DOs and the addition of OSFs during IVM can be beneficial for oocyte developmental competence. The nature of these OSFs can be verified by using the experimental model of recombinant OSFs. Although this is a promising potential experimental plan, studies were faced with some practical obstacles. Bioassays with native OSFs faced technical issues concerning the collection of the necessary amount of material to conduct large-scale co-culture trials, even when using microdrops [17, 27, 101]. When applying recombinant OSF bioassays, research was obstructed because commercially available purified GDF9 and BMP15 preparations lacked their proregions [17], although research on and development of highly purified recombinant proteins including proregion-mature region interaction is ongoing [104–106]. Meanwhile, methods are being developed by which recombinant BMP15 is produced in 293T cells, and the majority of these recombinants consist of a non-covalent complex of the processed mature region bound to the corresponding proregion [107–110]. It is important to use these full-length pro-mature protein forms to obtain an increase in developmental potential [53, 63, 111], since isolated mature domains of recombinant BMP15 and GDF9 seemed to be ineffective [112]. This is in line with the findings that pro-domains of TGF-β superfamily growth factors are necessary for correct protein conformation and the regulation of bioactivity [113]. To obtain an enhanced developmental competence of the oocyte, recombinant human BMP15 and GDF 9 should be added to the IVM medium in a concentration of 100 ng/ml [53, 63, 111]. Moreover, recent research focused on the interaction between GDF9 and BMP15 leading to the formation of a heterodimer called cumulin, which could explain the potent synergistic action of these two oocyte-specific growth factors on granulosa cells [114]. Recombinant heterodimeric complexes of pro/mature domains (pro-cumulin) and complexes of covalent mature domains (cumulin) are generated [114]. Both kinds of complexes activated SMAD2/3 and SMAD1/5/8 signaling pathways in granulosa cells, together with the proliferation and upregulation of genes associated with oocyte-regulated differentiation [114]. Although cumulin was more effective on granulosa cells, pro-cumulin stimulated better oocyte developmental competence in COCs [114]. Therefore, pro-cumulin can be considered as a potential additive during IVM to improve oocyte developmental competence [114].

Future Perspectives

The general use of cAMP-modulating agents and oocyte-secreted factors

It can be concluded that maintaining high cAMP levels in porcine oocytes during the first 20–24 h of IVM enhances maturation of the oocyte, reflected in improved response after IVF and improved cumulus matrix disassembly. Practically, the use of cAMP modulating agents is not necessary (recommended) during the collection of porcine COCs. However, the addition of dbcAMP during the first period of IVM is strongly advised. To select only the high quality oocytes for further processing in IVP, COCs that adhered to the bottom of the culture dish after IVM, should be removed. Fundamentally, more research is necessary to reveal the exact mechanism by which cAMP levels are involved in the regulation and production of the ADAMTS-1 protein. More insight into the cAMP/ADAMTS-1 pathway could possibly reveal more modulating opportunities. In parallel with the performed research on parathyroid in bones [90], other cAMP-modulating agents, such as forskolin could be tested for their influence on ADAMTS-1 levels in porcine cumulus cells. 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 may create opportunities to intervene in the process.

Regarding the practical use of OSFs in porcine IVP systems, most studies conclude that DO addition cannot be used as a practical tool to increase cumulus expansion, nuclear maturation, fertilization, and blastocyst formation. In the future, further fundamental research should be performed to investigate the nature of OSFs and elucidate the basic action of OSFs in pigs. Possible effects of DO-derived OSFs on gene expression (*e.g.,* ovulation-related genes) in the cumulus cannot be eliminated and may be revealed in the future. The technical feasibilities hampering large-scale experiments when performing trials in the field of native OSF bioassays should also be considered. Several studies reported the technical limitations of bioassays with native OSFs, such as the collection of the necessary amount of material to conduct large co-culture trials, even when using micro drops [17, 27, 101]. To avoid problems in research, the obtained results on native OSF bioassays of this review and the issue of a very labor intensive protocol of DO production should be taken into account. Consequently, the use of recombinant OSFs may be a more expedient approach to study OSFs in pigs.

The combinational treatment with cAMP modulating agents and oocyte-secreted factors

More insight was gained the interaction between cAMP and OSFs regarding the modulation of maturation of small follicle-derived

oocytes. Research on this topic emphasized the important role of a functioning EGFR signaling pathway in the acquisition of oocyte developmental competence [53, 59, 115, 116]. This combinational treatment suggests the possibility of modifying and improving the developmental competence of developing oocytes. Moreover, EGF responsiveness of the cumulus cell compartment can possibly be considered as an indicator of the cumulus cell differentiation and possibly plays a central role in the acquisition of developmental potential [59]. Fundamentally, it would be interesting to investigate the possible interaction of cAMP with non-SMAD pathways, activated by BMP15 and GDF9 [117], to explain the influence on oocyte developmental potential [53].

Conclusion

This review discussed the bidirectional communication between oocytes and cumulus cells in the pig, at the level of cAMP dependent pathways and oocyte-secreted factors. The use of cAMP modulating agents during collection does not synchronize the germinal vesicle stages of porcine oocytes. However, the use of the cAMP-analog, dbcAMP, during the first 20–24 h of IVM leads to less polyspermy and results in a lower rate of adherent COCs due to elevated proADAMTS-1 levels in cumulus cells. Although cumulus cells can expand independently from the oocyte, oocytes enhance cumulus expansion through gap junctions and by affecting the medium. Nevertheless, co-culture with DOs during IVM does not affect fertilization parameters and the developmental competence of porcine COCs. Regarding the practical use of OSFs, direct supplementation of the IVM medium is advised especially in combination with the use of cAMP modulating agents (dbcAMP) during the first half of IVM to increase oocyte developmental competence after fertilization.

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