



Kent Academic Repository

Serrano Albal, María, Silvestri, Giuseppe, Kiazim, Lucas G, Vining, Lucy M, Zak, Louisa J, Walling, Grant A, Haigh, Alexandra M, Harvey, Simon C, Harvey, Katie E and Griffin, Darren K. (2022) *Supplementation of porcine in vitro maturation medium with FGF2, LIF, and IGF1 enhances cytoplasmic maturation in prepubertal gilts oocytes and improves embryo quality*. *Zygote*, 30 (6). pp. 801-808. ISSN 1469-8730.

Downloaded from

<https://kar.kent.ac.uk/96935/> The University of Kent's Academic Repository KAR

The version of record is available from

<https://doi.org/10.1017/S0967199422000284>

This document version

Publisher pdf

DOI for this version

Licence for this version

CC BY (Attribution)

Additional information

Versions of research works

Versions of Record

If this version is the version of record, it is the same as the published version available on the publisher's web site. Cite as the published version.

Author Accepted Manuscripts

If this document is identified as the Author Accepted Manuscript it is the version after peer review but before type setting, copy editing or publisher branding. Cite as Surname, Initial. (Year) 'Title of article'. To be published in **Title of Journal**, Volume and issue numbers [peer-reviewed accepted version]. Available at: DOI or URL (Accessed: date).

Enquiries

If you have questions about this document contact ResearchSupport@kent.ac.uk. Please include the URL of the record in KAR. If you believe that your, or a third party's rights have been compromised through this document please see our [Take Down policy](https://www.kent.ac.uk/guides/kar-the-kent-academic-repository#policies) (available from <https://www.kent.ac.uk/guides/kar-the-kent-academic-repository#policies>).

Research Article

Cite this article: Serrano Albal M *et al.* (2022) Supplementation of porcine *in vitro* maturation medium with FGF2, LIF, and IGF1 enhances cytoplasmic maturation in prepubertal gilts oocytes and improves embryo quality. *Zygote*. 30: 801–808. doi: [10.1017/S0967199422000284](https://doi.org/10.1017/S0967199422000284)

Received: 4 February 2022

Revised: 30 May 2022

Accepted: 1 June 2022

First published online: 1 September 2022

Keywords:

Blastocyst; COCs; *In vitro* fertilization; Pig

Author for correspondence:

María Serrano Albal. School of Biosciences, University of Kent, Canterbury, UK.

E-mail: ms2104@kent.ac.uk

Supplementation of porcine *in vitro* maturation medium with FGF2, LIF, and IGF1 enhances cytoplasmic maturation in prepubertal gilts oocytes and improves embryo quality

María Serrano Albal¹ , Giuseppe Silvestri¹ , Lucas G. Kiazim¹, Lucy M. Vining², Louisa J. Zak³, Grant A. Walling³, Alexandra M. Haigh⁴, Simon C. Harvey², Katie E. Harvey² and Darren K. Griffin¹

¹School of Biosciences, University of Kent, Canterbury, UK; ²Biomolecular Research Group, School of Psychology and Life Sciences, Canterbury Christ Church University, Canterbury, UK; ³Topigs Norsvin Research Center, Beuningen, The Netherlands and ⁴JSR Genetics Ltd, Southburn, Driffield, East Yorkshire, UK

Summary

In porcine *in vitro* production (IVP) systems, the use of oocytes derived from prepubertal gilts, whilst being commercially attractive, remains challenging due to their poor developmental competence following *in vitro* maturation (IVM). Follicular fluid contains important growth factors and plays a key role during oocyte maturation; therefore, it is a common supplementation for porcine IVM medium. However, follicular fluid contains many poorly characterized components, is batch variable, and its use raises biosecurity concerns. In an effort to design a defined IVM system, growth factors such as cytokines have been previously tested. These include leukaemia inhibitory factor (LIF), fibroblast growth factor 2 (FGF2), and insulin-like growth factor 1 (IGF1), the combination of which is termed 'FLI'. Here, using abattoir-derived oocytes in a well established porcine IVP system, we compared follicular fluid and FLI supplementation during both IVM and embryo culture to test the hypothesis that FLI can substitute for follicular fluid without compromising oocyte nuclear and cytoplasmic maturation. We demonstrate that in oocytes derived from prepubertal gilts, FLI supplementation enhances oocyte meiotic maturation and has a positive effect on the quality and developmental competence of embryos. Moreover, for the first time, we studied the effects of follicular fluid and FLI combined showing no synergistic effects.

Introduction

The developmental potential of porcine oocytes and embryos is affected by several intrinsic and extrinsic factors that can compromise the efficiency of *in vitro* production (IVP) systems (Hunter, 2000; Weaver *et al.*, 2013; Teplitz *et al.*, 2020). One such factor is the oocyte source; oocytes can be collected from adult donors or prepubertal gilts (Marchal *et al.*, 2001). From a commercial standpoint, the use of ovaries from prepubertal gilts is highly attractive as it gives the opportunity to shorten generation times, thereby facilitating the introduction of new genetics more quickly (Sherrer *et al.*, 2004). This approach nonetheless presents many challenges and limitations that are mainly related to the ability of the oocytes to undergo nuclear and cytoplasmic maturation during *in vitro* maturation (IVM) (Marchal *et al.*, 2001; Pawlak *et al.*, 2012). Indeed, in recent years, research has focussed on the improvement of culture conditions for prepubertal porcine oocytes with the aim of assisting them in completing nuclear and cytoplasmic maturation more efficiently (Uhm *et al.*, 2010; Appeltant *et al.*, 2016; Yuan, *et al.*, 2017; Teplitz *et al.*, 2020). Observing the position of the cortical granules (CGs) allows for the determination of cytoplasmic maturation. CGs are specialized secretory vesicles, present only in female germ cells that are randomly distributed throughout the cytoplasm of immature oocytes and migrate towards the cortical cytoplasm during meiotic maturation. Upon fertilization, CGs undergo exocytosis to release their contents with the aim to harden the zona pellucida (Burkart *et al.*, 2012). Furthermore, some recent studies have reported that CGs function beyond fertilization, having an effect on the regulation of embryonic cleavage and preimplantation development (Liu, 2011; Kulus *et al.*, 2020).

Follicular fluid plays a key role during oocyte maturation in sows (Tatemoto *et al.*, 2004), and, as such, sow follicular fluid (sFF) is a common supplement used in pig IVM medium (Pawlak *et al.*, 2018). It is known to support *in vitro* oocyte development by providing the culture environment with a variety of important growth factors (Lédée *et al.*, 2008). The growth factors found in sFF are naturally produced by ovarian somatic cells during final follicle development,

© The Author(s), 2022. Published by Cambridge University Press. This is an Open Access article, distributed under the terms of the Creative Commons Attribution licence (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted re-use, distribution and reproduction, provided the original article is properly cited.

acting as both autocrine and paracrine mediators of ovarian function and regulating oocyte maturation and oocyte developmental competence (Song *et al.*, 2011). However, the complete constitution of sFF is unknown and its use results in a non-defined culture system and raises potential biosecurity concerns. Batch variation has also been demonstrated in sFF and its composition is known to change with the donor's age and stage of the follicles from which it is recovered (Sun *et al.*, 2011). Furthermore, variation in the concentrations of any of the components of sFF can affect IVM outcomes and therefore oocyte developmental competence (De Oliveira *et al.*, 2006; Pawlak *et al.*, 2018).

In an effort to design efficient, but defined IVM culture medium, growth factors such as certain cytokines have been tested, alone or in combination, for the culture of porcine oocytes. In particular, leukaemia inhibitory factor (LIF) and fibroblast growth factor 2 (FGF2) have been shown to facilitate meiotic progress during maturation, promoting oocyte quality and its subsequent ability to achieve fertilization. It has been shown that LIF phosphorylates MAPK3/1 and STAT3 in the oocytes, which are important pathways during *in vitro* maturation (Dang-Nguyen *et al.*, 2014; Mo *et al.*, 2014). FGF2 acts as a cofactor, promoting cumulus cell survival and extracellular matrix quality during IVM (Barros *et al.*, 2019; Du *et al.*, 2021). Insulin-like growth factor 1 (IGF1) also has been found to improve IVM outcomes and increase cell survival in response to stress (Oberlender *et al.*, 2013). IGF1 enables the expansion of porcine cumulus cells in response to FSH, promoting the synthesis and retention of hyaluronic acid in porcine COCs, also activated by the MAPK3/1 pathway (Singh and Armstrong, 1997; Němcová *et al.*, 2007).

Interestingly, the use of these three cytokines during IVM, known together as FLI (Yuan, *et al.*, 2017), positively affects outcomes by improving oocyte quality, embryonic development, embryo transfer outcomes and, in cattle, embryo cryosurvival (Yuan *et al.*, 2017; Stoecklein *et al.*, 2021).

Here, we performed a direct comparison between sFF and FLI supplementation of IVM medium using oocytes from prepubertal gilts and semen stored in extender. We tested the hypothesis that FLI can efficiently substitute for sFF during IVM without compromising oocyte nuclear and/or cytoplasmic maturation. Moreover, for the first time we studied the effects of combining sFF and FLI in IVM of oocytes to test the hypothesis that there is a synergistic effect between these components that would suggest a role for additional growth factors that may be present in sFF but absent in FLI.

Materials and methods

All chemicals and reagents used were purchased from Sigma-Aldrich (Gillingham, UK) except when specified otherwise.

Oocyte collection and IVM

Prepubertal gilt ovaries were collected and transported to the laboratory within 5 h in a sealed bag floated in water at 30–35°C. The animals had an approximate weight of 160 kg at slaughter, and their prepubertal status was confirmed by the absence of developed ovarian corpora lutea. Prior to aspiration, the ovaries were washed two or three times in 1× phosphate-buffered saline (PBS) and kept in a water bath at 28°C. The cumulus–oocyte complexes (COCs) were collected by manual aspiration from non-atretic follicles (3–6 mm) using a non-pyrogenic/non-toxic syringe (Henke-Sass Wolf GmbH, Tuttlingen, Germany) fitted with an 18-gauge needle.

The follicular contents were washed three times in a modified HEPES-buffered Porcine X Medium [PXM; Yoshioka *et al.*, 2008; 108 mM NaCl, 10 mM KCl, 0.35 mM KH₂PO₄, 0.40 mM MgSO₄, 5.0 mM NaHCO₃, 25 mM HEPES, 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 4 mg/ml bovine serum albumin (BSA)]; warmed at 38°C.

Selected COCs were washed three times in porcine oocyte medium [POM; Yoshioka *et al.*, 2008; 108 mM NaCl, 10 mM KCl, 0.35 mM KH₂PO₄, 0.4 mM MgSO₄, 25 mM NaHCO₃, 5.0 mM glucose, 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 2.0 mM glutamine, 5.0 mM hypotaurine, 0.1 mM cysteine, 20 µl/ml Basal Medium Eagle (BME) amino acids 50×, 10 µl/ml minimum essential medium (MEM) non-essential amino acids 100×, 10 ng/ml EGF, 50 µM β-mercaptoethanol, 10 µg/ml gentamycin, 4 mg/ml BSA] previously equilibrated overnight at 38.5°C in a saturated humidity atmosphere of 5.5% CO₂ in air. COCs were randomly assigned to groups of 50 for treatment with different supplements: FLI, sFF, both FLI and sFF, or left unsupplemented (please refer to 'Experimental design' for details). COCs were cultured for 20 h in POM supplemented with FSH (0.5 IU/ml), LH (0.5 IU/ml) and dbc-AMP (0.1 mM). Subsequently, COCs were cultured in the same medium but without hormones and dbc-AMP for a further 24 h at 38.5°C and 5.5% CO₂ in humidified air.

In vitro fertilization (IVF) and culture

Extended boar semen [for commercial artificial insemination (AI)] was supplied by JSR Genetics Ltd (Southburn, UK). Sperm preparation was performed using a 35%/70% BoviPure discontinuous density gradient system (Nidacon, Gothenborg, Sweden) following the manufacturer's instructions.

Matured oocytes were washed twice in porcine gamete medium (PGM; Yoshioka *et al.*, 2008; 108 mM NaCl, 10 mM KCl, 0.35 mM KH₂PO₄, 0.4 mM MgSO₄, 25 mM NaHCO₃, 5.0 mM glucose, 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 2.5 mM theophylline, 1 µM adenosine, 0.25 µM L-cysteine, 10 µg/ml gentamycin, 4 mg/ml BSA) and were incubated with sperm for 2 h. Following this co-incubation, oocytes were moved to a clean well in PGM for another 2 h to minimize the risk of polyspermy.

After IVF, presumptive zygotes were denuded and washed twice in porcine zygote medium 5 (PZM5; Yoshioka *et al.*, 2008; 108 mM NaCl, 1 mM KCl, 0.35 mM KH₂PO₄, 0.4 mM MgSO₄, 25 mM NaHCO₃, 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 2.0 mM glutamine, 5.0 mM hypotaurine, 20 µl/ml BME amino acids 50×, 10 µl/ml MEM non-essential amino acids 100×, 10 µg/ml gentamycin, 4 mg/ml BSA) before being transferred to a final 500 µl well of PZM5 supplemented with FLI. Individual wells were overlaid with mineral oil and plates were incubated for 6 days at 38.5°C, in 5.5% CO₂ and 6% O₂ in humidified air.

Evaluation of nuclear stage and cortical granule distribution

Oocytes were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at 4°C, and then washed three times in PBS containing 0.3% BSA and 100 mM glycine for 5 min. After a 5-min treatment with 0.1% Triton X-100 in PBS, oocytes were washed two additional times in PBS (5 min each). To stain the CG, oocytes were cultured in 100 mg/ml fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Invitrogen™, Inchinnan, UK) in PBS for 30 min in a dark box. After staining, oocytes were washed three times in PBS with 0.3% BSA and 0.01% Triton X-100 in PBS. The oocytes were then stained with 10 mg/ml Hoechst H3570 (Invitrogen™) for

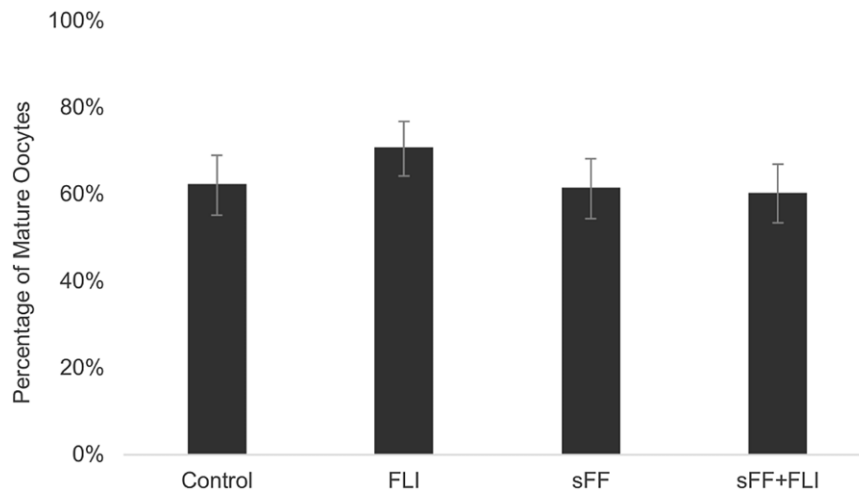


Figure 1. Effect of each treatment on the proportion of mature oocytes. Mean of the effects of the studied supplements on nuclear maturation of prepubertal gilt oocytes after 44 h of culture. Error bars show the 95% confidence interval. Groups did not differ ($P > 0.05$). FLI, combination of the cytokines FGF2, LIF, and IGF1; sFF, sow follicular fluid.

10 min, mounted on non-fluorescent glass slides and visualized using an Olympus BX61 epifluorescence microscope equipped with a cooled charge-coupled device (CCD) camera. Images were taken using 4',6-diamidino-2-phenylindole (DAPI) and FITC filters at $\times 200$ total magnification using SmartCapture3 software (Digital Scientific UK, Cambridge, UK). COCs were classified as mature when they displayed a dye-stained metaphase plate and a polar body; otherwise, they were classified as immature. The distribution of CGs was categorized in three ways as: central, peripheral or complete (Figure 1), where: central distribution indicated a homogenous distribution of CGs throughout the cytoplasm; peripheral distribution, indicated that CGs had begun to localize subjacent to the oolemma, or complete distribution; and where CGs were concentrated subjacent to the oolemma and around the polar body (an indicator of complete cytoplasmic maturation).

Evaluation of blastocysts

Blastocyst morphological appearance was assessed and scored using three grades: (1) excellent: fully expanded blastocyst, spherical, regular border, symmetrical with uniform size cells, obvious inner cell mass (ICM) and densely populated trophectoderm (TE); (2) good, expanded blastocyst with few small blastomeres, fewer cells forming the ICM/TE; (3) poor: expanded or less developed blastocyst with numerous extruded blastomeres, loosely populated TE and possible ICM. After grading, blastocysts from each treatment group were fixed in 4% PFA for 30 min at 4°C and stained with Hoechst H3570 (Invitrogen™) for cell counts. Blastocysts were visualized under an Olympus BX61 epifluorescence microscope as described in the previous section.

Experimental design

Experiment 1: Oocyte nuclear maturation following supplementation of IVM medium FLI or sFF

This experiment was designed to study the effect of FLI supplementation during the IVM culture period on the meiotic competence of COCs. Four groups of ~50 immature COCs were treated during IVM. The first group was supplemented with a combination of three cytokines (Novus Biologicals, Oxon, UK), 'FLI', prepared as described by Yuan *et al.* (2017). The second group was treated with 10% sFF. The third group was treated with the combination of FLI and 10% sFF, to study a possible synergistic

effect. The fourth group of COCs was kept unsupplemented and served as a control. The experiment was repeated four times with 40–50 oocytes per treatment in each replicate ($N = 763$).

Experiment 2: CG distribution (cytoplasmic maturation) following supplementation of IVM medium with FLI or sFF

This experiment aimed to assess CG distribution as an indicator for the level of cytoplasmic maturation of the COCs depending on the treatment during IVM cultured. Treatment groups were as described in Experiment 1. This experiment was replicated three times with 40–50 oocytes per treatment per replicate ($N = 465$). In this case, a greater proportion of oocytes with 'complete' CGs (i.e. neither predominantly peripheral nor central) is the most favourable outcome.

Experiment 3: Post-fertilization outcomes following supplementation of IVM medium with FLI or sFF

The third experiment was designed to test the effects of different supplementations during the IVM process on the oocyte's developmental competence following IVF. This was evaluated using blastocyst rates (blastocyst per inseminated oocyte), blastocyst morphology, and blastocyst cell counts. To estimate the total cell counts, nuclei were stained using Hoechst H3570. The live image of embryo was sequentially divided into smaller sections that could be individually brought into focus to improve the accuracy of the count. The same four IVM groups described above (FLI, sFF, FLI + sFF, and control) were used. The experiment was repeated seven times with 40–50 presumptive zygotes per group per replicate ($N = 1133$). To evaluate the embryo quality of each group, 149 blastocysts were assessed.

Statistical analysis

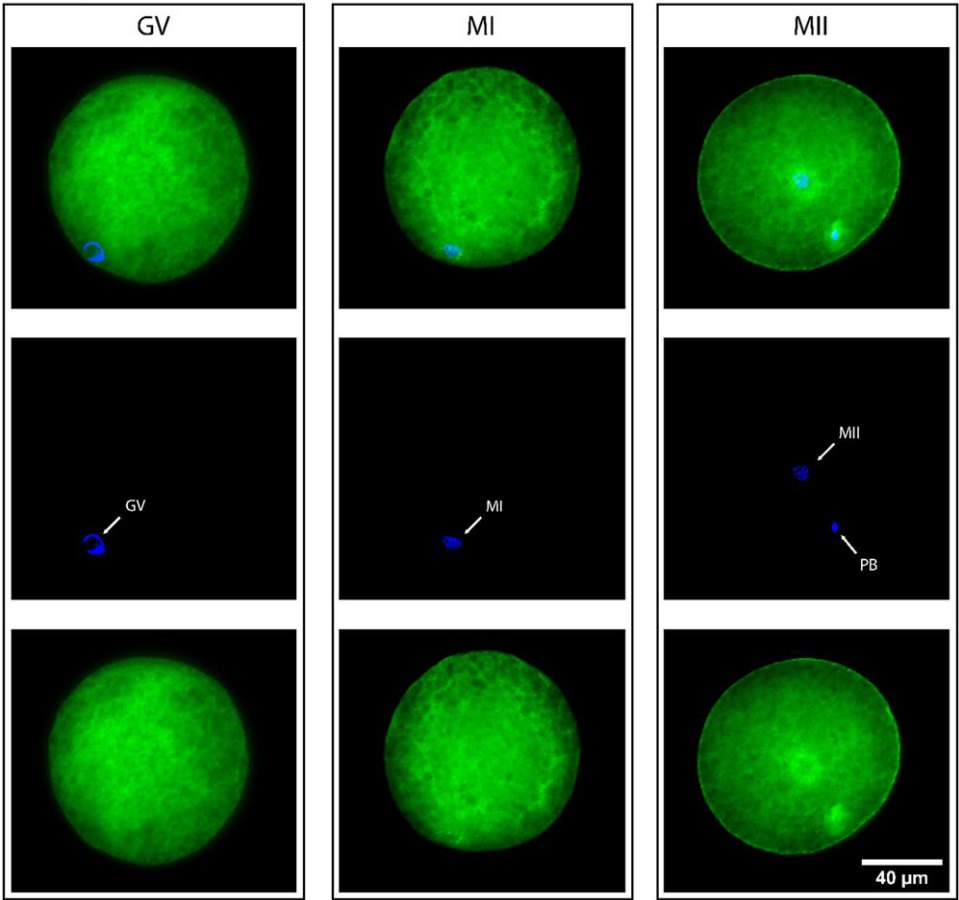
Data were analyzed using SPSS software (Version 26, IBM). The statistical analysis of oocyte nuclear maturation and blastulation rates were completed by fitting a binomial generalized linear model (GLM) with logit link functions. The numbers of blastomeres per blastocyst were log transformed and were assessed using analysis of variance (ANOVA). When analyzing cytoplasmic maturation and the morphology evaluation of the embryos the data were tested using Kruskal–Wallis test. When an interaction was detected amongst the variables, multiple comparisons were completed using the Bonferroni correction. The data in tables are presented as mean $\pm$ standard error of the mean (SEM), whilst in the graphs they

Table 1. Distribution of cortical granules (cytoplasmic maturation) in oocytes after 44 h of *in vitro* maturation

Group	N	CG distribution		
		Central, N (mean % ± SEM)	Peripheral, N (mean % ± SEM)	Complete, N (mean % ± SEM)
Control	113	37 (32.7 ± 6.7) ^a	42 (37.2 ± 9.1) ^a	34 (30.1 ± 3.4) ^a
FLI	123	22 (17.9 ± 1.4) ^b	41 (33.3 ± 10.6) ^b	60 (48.8 ± 11.7) ^b
sFF	115	23 (28.7 ± 2.0) ^a	39 (33.9 ± 3.5) ^a	43 (37.4 ± 4.5) ^a
sFF + FLI	114	30 (26.3 ± 2.4) ^{a,b}	35 (30.7 ± 3.2) ^{a,b}	49 (43.0 ± 3.4) ^{a,b}
P-value	0.012*			

Data are shown as mean ± standard error of the mean (SEM) (N = 3 replicates for each group).
^{a,b}Different superscript letters indicate significant differences amongst the groups.
*Kruskal-Wallis test (H₃ = 10.995).

Figure 2. Cortical granule (CG) distribution in oocytes after IVM. GV: Homogenous distribution of CGs throughout the cytoplasm in a germinal vesicle (GV) stage oocyte denoted with the arrow. MI: Peripheral distribution on metaphase I (MI) stage oocyte, CGs begin to locate subjacent to the oolemma. MII: Complete distribution on a mature oocyte (metaphase II, MII), CGs are concentrated at the periphery of the cytoplasm and around the polar body (PB). CGs stained in green (FITC-PNA), DNA stained in blue (Hoechst), ×200 total magnification. Each column displays an individual oocyte; the top row displays a combined image, with the subsequent images showing individual fluorophores (FITC or Hoechst).



appear as mean with the error bars showing the 95% confidence interval of each variable. Results were considered to be statistically significant when *P*-values were < 0.05.

Results

Effect of FLI and/or sFF supplementation on nuclear maturation

The assessment of the nuclear stage of 763 oocytes after maturation (Figure 1) showed the highest proportion of mature oocytes in the group treated with FLI (70.9%). The use of sFF during IVM gave an outcome slightly lower than the group with no supplementation

(control), 61.5% and 62.3% respectively. The lowest rate was found in the combination group (sFF + FLI) with a 60.3%. However, due to the intergroup variation, these differences were not statistically significant (GLM; $\chi_1 = 1.059$; *P* > 0.05).

Effect of FLI and/or sFF supplementation on oocyte CG distribution (cytoplasmic maturation)

The analysis of the distribution of the CGs after IVM culture (Table 1, Figure 2) showed a higher incidence of ‘complete’ distribution (Figure 2c) on the groups treated with FLI (48.8%), second on the combination of FLI and sFF (43.0%), third in the control group (32.7%) and finally for sFF (28.7%). The incidence of a

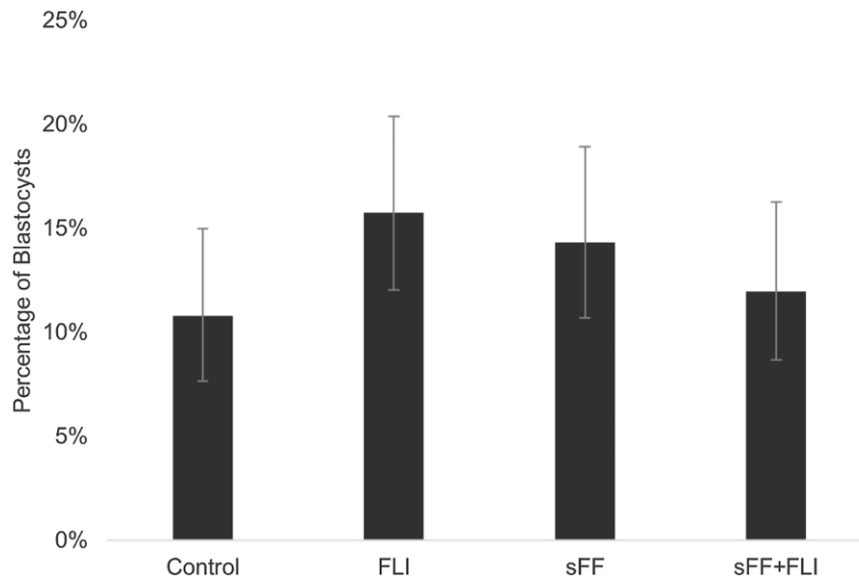


Figure 3. Proportion of blastocyst formation after the different treatments on *in vitro* maturation. Mean of the effects of the studied supplements on blastulation rates after matured prepubertal gilt oocytes were fertilized. Error bars show the 95% confidence interval. Groups did not differ ($P > 0.05$). FLI, combination of the cytokines FGF2, LIF, and IGF1; sFF, sow follicular fluid.

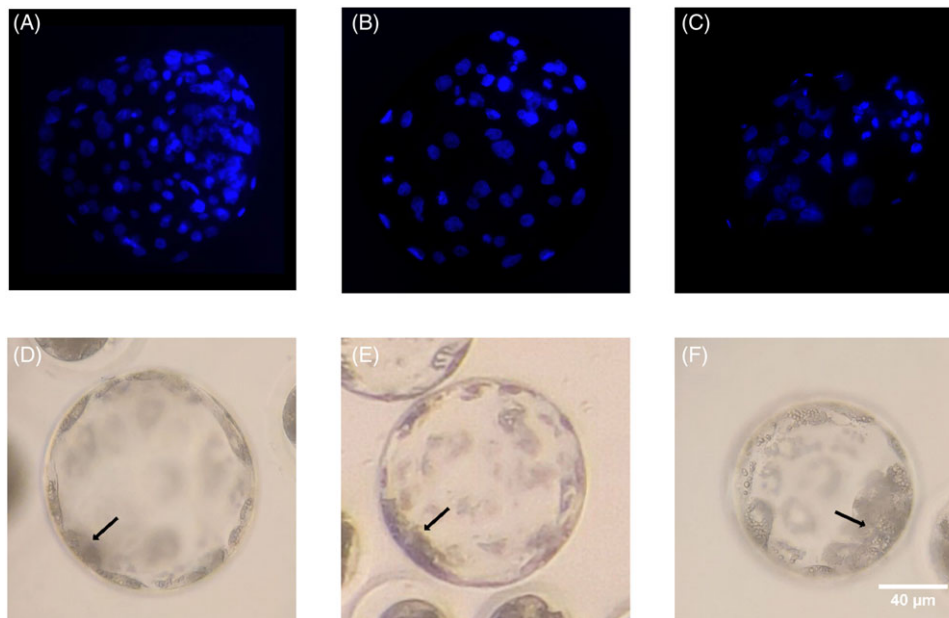


Figure 4. Pig blastocyst morphology. (A–C) Images taken by the use of epifluorescence microscopy at $\times 200$ total magnification. Nuclei in blue (Hoechst staining). (D–F) Phase-contrast optical microscopy images at $\times 200$ total magnification. (A, D) Blastocysts of excellent morphology (grade 1); (B, E) blastocysts of good morphology (grade 2); (C, F) blastocysts of poor morphology (grade 3). Arrows denote the inner cell mass (ICM) of the blastocysts.

peripheral CG distribution (Figure 2b) was similar in the four groups. Intergroup comparison showed that there were significant differences between the groups (Kruskal–Wallis; $H_3 = 10.995$; $P = 0.012$).

Effect of the use of FLI and/or sFF during IVM on embryo developmental competence

After fertilization, the highest rate of blastocyst formation per inseminated oocyte (Figure 3) was found in the group treated with FLI (15.7%), followed by the sFF treatment group (14.3%). The proportion of blastocysts from the combination group (sFF + FLI) was similar to the control group (12.0% and 10.8%, respectively). The differences on the proportion of blastocyst formed between the groups did not show any significant difference after statistical analysis (GLM; $\chi_1 = 0.044$; $P > 0.05$).

The morphological assessment of the blastocyst (Figure 4) generated by each group revealed that the oocytes treated with FLI during IVM developed into better quality embryos (Table 2). The highest proportion of the embryos with excellent morphology (Figure 4a,d) came from the FLI treatment group ($37.0 \pm 12.1\%$) and this was significantly higher than in the other groups (Kruskal–Wallis; $H_3 = 8.162$; $P = 0.043$). This group also had the lower proportion of poor-quality embryos ($19.6 \pm 13.4\%$). In contrast, the group with a higher percentage of poor-quality embryos (Fig. 4c,f) was the control group ($34.5 \pm 13.6\%$). Interestingly, the group cultured in a combination of sFF and FLI had the second highest proportion of excellent quality embryos ($29.4 \pm 4.8\%$), but also the second highest proportion of poor-quality blastocyst ($29.4 \pm 6.6\%$).

To further assess the developmental competence, the number of cells was counted for each blastocyst. The average number of cells

Table 2. Blastocyst quality after the different treatments on *in vitro* maturation

Group	N	Grade		
		Excellent, N (mean % ± SEM)	Good, N (mean % ± SEM)	Poor, N (mean % ± SEM)
Control	29	6 (20.7 ± 6.7) ^a	13 (44.8 ± 9.3) ^a	10 (34.5 ± 13.6) ^a
FLI	46	17 (37.0 ± 12.1) ^b	20 (43.5 ± 10.5) ^b	9 (19.6 ± 13.4) ^b
sFF	40	10 (25.0 ± 9.6) ^a	24 (60.0 ± 13.2) ^a	6 (15.0 ± 4.6) ^a
sFF + FLI	34	10 (29.4 ± 4.8) ^{a,b}	14 (41.2 ± 5.8) ^{a,b}	10 (29.4 ± 6.6) ^{a,b}
P-value	0.043*			

Data are shown as mean ± SEM (N = 7 replicates for each group).
^{a,b}Different superscript letters indicate significant differences amongst the groups.
*Kruskal-Wallis test (H₃ = 8.162).

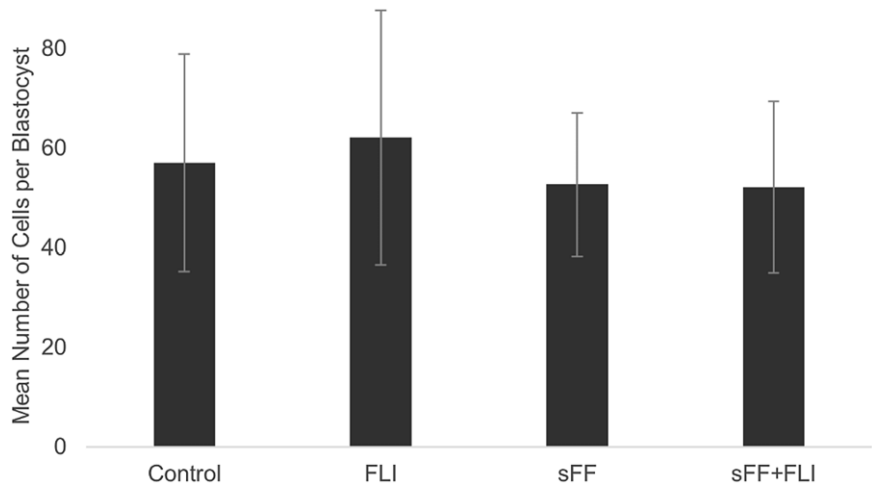


Figure 5. Average number of cells per blastocyst. Mean of the effects of the different combinations on the number of cells per blastocyst after matured prepubertal gilt oocytes were fertilized. Error bars show the 95% confidence interval (N = 73). Groups did not differ (P > 0.05). FLI, combination of the cytokines FGF2, LIF, and IGF1; sFF, sow follicular fluid.

per blastocyst (Figure 5) was highest in the FLI group (62.1 cells per blastocyst), followed by the control (57.1 cells per blastocyst) and the lower number of cells using sFF as treatment (52.7 and 52.2 cells per blastocyst on the sFF and sFF + FLI groups, respectively). Comparison of treatment groups indicated that they did not differ (ANOVA; F₃ = 1.039; P > 0.05).

Discussion

Whilst FLI supplementation during IVM always resulted in improved outcomes compared with control experiments and often to other treatment groups, not all findings were statistically significant. However, the use of FLI consistently yielded better results. By contrast, the use of sFF alone had very little effect on oocyte maturation, yielding similar results to the control group, and when used in combination with FLI outcomes were only marginally improved. The areas in which statistically significant differences were observed include cytoplasmic maturation (distribution of CGs) and blastocyst quality, meaning that the positive effect of FLI supplementation also extended to *in vitro* culture (IVC). However, our results showed that FLI supplementation did not statistically improve nuclear maturation, blastulation rate, or the number of cells per blastocyst (contrary to a previous study by Yuan *et al.*, 2017). The lack of significance could be influenced by statistical power, as the number of blastocysts analyzed differed from the total number of blastocysts obtained; the difference in this

number is a result of fragile blastocysts being destroyed during the fixing process. It is widely accepted that the survival rate of porcine IVP blastocysts is very low because of their poor quality (Zijlstra *et al.*, 2008). Here we show that the use of FLI during IVM improved the synchrony between nuclear and cytoplasmic maturation, improving overall oocyte quality (Marchal *et al.*, 2001; Pawlak *et al.*, 2012). This synchrony could positively influence the zygote’s developmental competence and its ability to undertake normal cell division, resulting in the symmetrical division of the blastomeres and lower fragmentation rates, which is demonstrated in the results. FLI supplementation not only improved the quality of the resulting embryos that developed to blastocyst stage, but has previously been shown to enhance the efficiency of cattle and sheep IVP and embryo quality (Stoecklein *et al.*, 2021; Tian *et al.*, 2021). The viability to implant and birth rates of the embryos have not been tested in this study. However, other publications have shown an increase in the postimplantation viability of embryos treated with growth factors (Zheng *et al.*, 2008; Biswas *et al.*, 2018). It is common to have supplements in the medium that act additively to improve *in vitro* embryo production, with publications showing an increase in the blastulation rates after treatment with individual cytokines, separately as well as in combination (Valleh *et al.*, 2017; Yuan, *et al.*, 2017; Stoecklein *et al.*, 2021). Furthermore, Liu *et al.* (2020) demonstrated the positive effect that cytokines had on promoting porcine oocyte maturation and Redel *et al.* (2021)

showed that the combination of FLI with gonadotropins enhanced oocyte development and improved cumulus cell expansion. Procházka *et al.* (2021) demonstrated a similar activation pattern of MAPK3/1 between medium supplemented with or without FLI, showing only an increase in MAPK3/1 phosphorylation during the first hour of culture in the FLI medium. This suggests that COCs cultured with FLI experience enhanced MAPK3/1 stimulation that resulted in the development of highly competent oocytes. Our data demonstrate that combining FLI and sFF did not have a synergistic effect on IVP outcomes, which could be attributed to factors such as the variation in sFF composition (De Oliveira *et al.*, 2006; Sun *et al.*, 2011). The donor's origin, age, and the stage of the follicles from the ovary may be one of the reasons why the results shown in this publication differed from the information published previously on the effect of FLI on porcine maturation (Yuan, *et al.*, 2017; Redel *et al.*, 2021).

Porcine IVP has, to the disappointment of many in the industry, lagged behind its bovine counterpart. By common consent, porcine IVP is technically more challenging at all stages than bovine (or even human) and incremental improvements, such as those reported in this study, are always welcome. The potential for routine IVP in the pig breeding industry is considerable in terms of biosecurity, cost and environmental benefits for transport of both male and female genetics (nationally and internationally). Moreover, using peri-pubertal gilts as a source of ovaries, as in this study, has great potential for the rapid introduction of new genetics through the shortening of generation intervals. Finally, as a resource for gene editing and biobanking, porcine embryos hold great potential provided that the barriers to their production can be overcome. The results of this study therefore represent a significant advance. Given the added problems of batch variability and biosecurity associated with the use of sFF, our future strategies will rely on the use of FLI alone.

Acknowledgements. The authors wish to thank the staff of Cranswick Country Foods (Hull, UK) for their assistance in sourcing the samples required for this study.

Author contributions. Conceptualization: MSA, GS, DKG, LJZ; formal analysis: MSA, GS; funding acquisition: DKG; investigation: MSA, GS, LGK, LMV; methodology: MSA, GS; resources: AMH, LJZ, GAW; supervision: DKG, LJZ, KEH, SCH; writing – original draft: MSA; writing – review and editing: MSA, GS, LGK, LMV, KEH, SCH, DKG, LJZ. All the authors have read and agreed to publish this version of the manuscript.

Funding. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) LINK awards scheme (BB/R00708X/1).

Institutional review board statement. This project involved the use of abattoir-derived material only and so did not fall within the Animals (Scientific Procedures) Act 1986. It therefore did not require review by an Animal Welfare and Ethical Review Body.

Informed consent statement. Not applicable.

Conflicts of interest. The authors declare no conflict of interest.

References

- Appeltant, R., Somfai, T., Maes, D., Van Soom, A. and Kikuchi, K. (2016). Porcine oocyte maturation *in vitro*: Role of cAMP and oocyte-secreted factors—A practical approach. *Journal of Reproduction and Development*, 62(5), 439–449. doi: [10.1262/jrd.2016-016](https://doi.org/10.1262/jrd.2016-016)
- Barros, R. G., Lima, P. F., Soares, A. C. S., Sanches, L., Price, C. A. and Buratini, J. (2019). Fibroblast growth factor 2 regulates cumulus

- differentiation under the control of the oocyte. *Journal of Assisted Reproduction and Genetics*, 36(5), 905–913. doi: [10.1007/s10815-019-01436-7](https://doi.org/10.1007/s10815-019-01436-7)
- Biswas, D., So, K. H., Hwang, S. U., Yoon, J. D., Kim, M., Kim, D. Y. and Hyun, S. H. (2018). Embryotropic effects of vascular endothelial growth factor on porcine embryos produced by *in vitro* fertilization. *Theriogenology*, 120, 147–156. doi: [10.1016/j.theriogenology.2018.07.024](https://doi.org/10.1016/j.theriogenology.2018.07.024)
- Burkart, A. D., Xiong, B., Baibakov, B., Jiménez-Movilla, M. and Dean, J. (2012). Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy. *Journal of Cell Biology*, 197(1), 37–44. doi: [10.1083/jcb.201112094](https://doi.org/10.1083/jcb.201112094)
- Dang-Nguyen, T. Q., Haraguchi, S., Kikuchi, K., Somfai, T., Bodó, S. and Nagai, T. (2014). Leukemia inhibitory factor promotes porcine oocyte maturation and is accompanied by activation of signal transducer and activator of transcription 3. *Molecular Reproduction and Development*, 81(3), 230–239. doi: [10.1002/mrd.22289](https://doi.org/10.1002/mrd.22289)
- De Oliveira, A. T., Lopes, R. F. and Rodrigues, J. L. (2006). Gene expression and developmental competence of bovine embryos produced *in vitro* with different serum concentrations. *Reproduction in Domestic Animals*, 41(2), 129–136. doi: [10.1111/j.1439-0531.2006.00653.x](https://doi.org/10.1111/j.1439-0531.2006.00653.x)
- Du, C., Davis, J. S., Chen, C., Li, Z., Cao, Y., Sun, H., Shao, B. S., Lin, Y. X., Wang, Y. S., Yang, L. G. and Hua, G. H. (2021). FGF2/FGFR signaling promotes cumulus–oocyte complex maturation *in vitro*. *Reproduction*, 161(2), 205–214. doi: [10.1530/REP-20-0264](https://doi.org/10.1530/REP-20-0264)
- Hunter, M. G. (2000). Oocyte maturation and ovum quality in pigs. *Reviews of Reproduction*, 5(2), 122–130. doi: [10.1530/ror.0.0050122](https://doi.org/10.1530/ror.0.0050122)
- Kulus, M., Kranc, W., Jeseta, M., Sujka-Kordowska, P., Konwerska, A., Ciesiołka, S., Celichowski, P., Moncrieff, L., Kocherova, I., Józkowiak, M., Kulus, J., Wiczorkiewicz, M., Piotrowska-Kempisty, H., Skowronski, M. T., Bukowska, D., Machatkova, M., Hanulakova, S., Mozdziak, P., Jaśkowski, J. M., *et al.* (2020). Cortical granule distribution and expression pattern of genes regulating cellular component size, morphogenesis, and potential to differentiation are related to oocyte developmental competence and maturational capacity *in vivo* and *in vitro*. *Genes*, 11(7), 815. doi: [10.3390/genes11070815](https://doi.org/10.3390/genes11070815)
- Lédée, N., Lombroso, R., Lombardelli, L., Selva, J., Dubanchet, S., Chaouat, G., Frankenne, F., Foidart, J. M., Maggi, E., Romagnani, S., Ville, Y. and Piccinni, M. P. (2008). Cytokines and chemokines in follicular fluids and potential of the corresponding embryo: The role of granulocyte colony-stimulating factor. *Human Reproduction*, 23(9), 2001–2009. doi: [10.1093/humrep/den192](https://doi.org/10.1093/humrep/den192)
- Liu, M. (2011). The biology and dynamics of mammalian cortical granules. *Reproductive Biology and Endocrinology: RB&E*, 9(1), 149. doi: [10.1186/1477-7827-9-149](https://doi.org/10.1186/1477-7827-9-149)
- Liu, X., Hao, Y., Li, Z., Zhou, J., Zhu, H., Bu, G., Liu, Z., Hou, X., Zhang, X. and Miao, Y. L. (2020). Maternal cytokines CXCL12, VEGFA, and WNT5A promote porcine oocyte maturation via MAPK activation and canonical WNT inhibition. *Frontiers in Cell and Developmental Biology*, 8, 578. doi: [10.3389/fcell.2020.00578](https://doi.org/10.3389/fcell.2020.00578)
- 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(1), 17–29. doi: [10.1016/s0093-691x\(01\)00539-8](https://doi.org/10.1016/s0093-691x(01)00539-8)
- Mo, X., Wu, G., Yuan, D., Jia, B., Liu, C., Zhu, S. and Hou, Y. (2014). Leukemia inhibitory factor enhances bovine oocyte maturation and early embryo development. *Molecular Reproduction and Development*, 81(7), 608–618. doi: [10.1002/mrd.22327](https://doi.org/10.1002/mrd.22327)
- Němcová, L., Nagyová, E., Petlach, M., Tománek, M. and Procházka, R. (2007). Molecular mechanisms of insulin-like growth factor 1 promoted synthesis and retention of hyaluronic acid in porcine oocyte-cumulus complexes. *Biology of Reproduction*, 76(6), 1016–1024. doi: [10.1095/biolreprod.106.057927](https://doi.org/10.1095/biolreprod.106.057927)
- Oberlender, G., Murgas, L. D. S., Zangeronimo, M. G., da Silva, A. C., Menezes, T. de A., Pontelo, T. P. and Vieira, L. A. (2013). Role of insulin-like growth factor-I and follicular fluid from ovarian follicles with different diameters on porcine oocyte maturation and fertilization *in vitro*. *Theriogenology*, 80(4), 319–327. doi: [10.1016/j.theriogenology.2013.04.018](https://doi.org/10.1016/j.theriogenology.2013.04.018)
- Pawlak, P., Cieslak, A., Warzych, E., Zejden, Z., Szumacher-Strabel, M., Molinska-Glura, M. and Lechniak, D. (2012). No single way to explain

- cytoplasmic maturation of oocytes from prepubertal and cyclic gilts. *Theriogenology*, **78**(9), 2020–2030. doi: [10.1016/j.theriogenology.2012.07.023](https://doi.org/10.1016/j.theriogenology.2012.07.023)
- Pawlak, P., Warzych, E., Cieslak, A., Malyszka, N., Maciejewska, E., Madeja, Z. E. and Lechniak, D. (2018). The consequences of porcine IVM medium supplementation with follicular fluid become reflected in embryo quality, yield and gene expression patterns. *Scientific Reports*, **8**(1), 15306. doi: [10.1038/s41598-018-33550-4](https://doi.org/10.1038/s41598-018-33550-4)
- Procházka, R., Bartková, A., Němcová, L., Murin, M., Gad, A., Marcollová, K., Kinterová, V., Lucas-Hahn, A. and Laurinčík, J. (2021). The role of MAPK3/1 and AKT in the acquisition of high meiotic and developmental competence of porcine oocytes cultured in vitro in FLI medium. *International Journal of Molecular Sciences*, **22**(20), 11148. doi: [10.3390/ijms222011148](https://doi.org/10.3390/ijms222011148)
- Redel, B. K., Spate, L. D., Yuan, Y., Murphy, C. N., Roberts, R. M. and Prather, R. S. (2021). Neither gonadotropin nor cumulus cell expansion is needed for the maturation of competent porcine oocytes *in vitro*. *Biology of Reproduction*, **105**(2), 533–542. doi: [10.1093/biolre/iaab090](https://doi.org/10.1093/biolre/iaab090)
- Sherrer, E. S., Rathbun, T. J. and Davis, D. L. (2004). Fertilization and blastocyst development in oocytes obtained from prepubertal and adult pigs. *Journal of Animal Science*, **82**(1), 102–108. doi: [10.2527/2004.821102x](https://doi.org/10.2527/2004.821102x)
- Singh, B. and Armstrong, D. T. (1997). Insulin-like growth factor-1, a component of serum that enables porcine cumulus cells to expand in response to follicle-stimulating hormone *in vitro*. *Biology of Reproduction*, **56**(6), 1370–1375. doi: [10.1095/biolreprod56.6.1370](https://doi.org/10.1095/biolreprod56.6.1370)
- Song, H. J., Kang, E. J., Maeng, G. H., Ock, S. A., Lee, S. L., Yoo, J. G., Jeon, B. G. and Rho, G. J. (2011). Influence of epidermal growth factor supplementation during *in vitro* maturation on nuclear status and gene expression of canine oocytes. *Research in Veterinary Science*, **91**(3), 439–445. doi: [10.1016/j.rvsc.2010.09.003](https://doi.org/10.1016/j.rvsc.2010.09.003)
- Stoecklein, K. S., Ortega, M. S., Spate, L. D., Murphy, C. N. and Prather, R. S. (2021). Improved cryopreservation of *in vitro* produced bovine embryos using FGF2, LIF, and IGF1. *PLOS ONE*, **16**(2), e0243727. doi: [10.1371/journal.pone.0243727](https://doi.org/10.1371/journal.pone.0243727)
- Sun, Y. L., Ping, Z. G., Li, C. J., Sun, Y. F., Yi, K. L., Chen, L., Li, X. Y., Wang, X. L. and Zhou, X. (2011). Comparative proteomic analysis of follicular fluids from normal and cystic follicles in sows. *Reproduction in Domestic Animals*, **46**(5), 889–895. doi: [10.1111/j.1439-0531.2011.01760.x](https://doi.org/10.1111/j.1439-0531.2011.01760.x)
- Tatemoto, H., Muto, N., Sunagawa, I., Shinjo, A. and Nakada, T. (2004). Protection of porcine oocytes against cell damage caused by oxidative stress during *in vitro* maturation: Role of superoxide dismutase activity in porcine follicular fluid. *Biology of Reproduction*, **71**(4), 1150–1157. doi: [10.1095/biolreprod.104.029264](https://doi.org/10.1095/biolreprod.104.029264)
- Teplitz, G. M., Lorenzo, M. S., Maruri, A., Cruzans, P. R., Carou, M. C. and Lombardo, D. M. (2020). Coculture of porcine cumulus–oocyte complexes with porcine luteal cells during IVM: Effect on oocyte maturation and embryo development. *Reproduction, Fertility, and Development*, **32**(16), 1250–1259. doi: [10.1071/RD20117](https://doi.org/10.1071/RD20117)
- Tian, H., Qi, Q., Yan, F. and Hou, J. (2021). Enhancing the developmental competence of prepubertal lamb oocytes by supplementing the *in vitro* maturation medium with sericin and the fibroblast growth factor 2-leukemia inhibitory factor-insulin-like growth factor 1 combination. *Theriogenology*, **159**(1), 13–19
- Uhm, S. J., Gupta, M. K., Yang, J. H., Chung, H. J., Min, T. S. and Lee, H. T. (2010). Epidermal growth factor can be used in lieu of follicle-stimulating hormone for nuclear maturation of porcine oocytes *in vitro*. *Theriogenology*, **73**(8), 1024–1036. doi: [10.1016/j.theriogenology.2009.11.029](https://doi.org/10.1016/j.theriogenology.2009.11.029)
- Valleh, M. V., Zandi, N. K., Rasmussen, M. A. and Hyttel, P. (2017). Optimal doses of EGF and GDNF act as biological response modifiers to improve porcine oocyte maturation and quality. *Zygote*, **25**(4), 423–433. doi: [10.1017/S0967199417000181](https://doi.org/10.1017/S0967199417000181)
- Weaver, A. C., Kelly, J. M., Kind, K. L., Gatford, K. L., Kennaway, D. J., Herde, P. J. and van Wettene, W. H. (2013). Oocyte maturation and embryo survival in nulliparous female pigs (gilts) is improved by feeding a lupin-based high-fibre diet. *Reproduction, Fertility, and Development*, **25**(8), 1216–1223. doi: [10.1071/RD12329](https://doi.org/10.1071/RD12329)
- Yoshioka, K., Suzuki, C. and Onishi, A. (2008). Defined system for *in vitro* production of porcine embryos using a single basic medium. *Journal of Reproduction and Development*, **54**(3), 208–213. doi: [10.1262/jrd.20001](https://doi.org/10.1262/jrd.20001)
- Yuan, Y., Spate, L. D., Redel, B. K., Tian, Y., Zhou, J., Prather, R. S. and Roberts, R. M. (2017). Quadrupling efficiency in production of genetically modified pigs through improved oocyte maturation. *Proceedings of the National Academy of Sciences of the United States of America*, **114**(29), E5796–E5804. doi: [10.1073/pnas.1703998114](https://doi.org/10.1073/pnas.1703998114)
- Zheng, J., Wen, Y., Song, Y., Wang, K., Chen, D. B. and Magness, R. R. (2008). Activation of multiple signaling pathways is critical for fibroblast growth factor 2- and vascular endothelial growth factor-stimulated ovine fetoplacental endothelial cell proliferation. *Biology of Reproduction*, **78**(1), 143–150. doi: [10.1095/biolreprod.107.064477](https://doi.org/10.1095/biolreprod.107.064477)
- Zijlstra, C., Kidson, A., Schoevers, E. J., Daemen, A. J., Tharasanit, T., Kuijk, E. W., Hazeleger, W., Ducro-Steeverink, D. W., Colenbrander, B. and Roelen, B. A. (2008). Blastocyst morphology, actin cytoskeleton quality and chromosome content are correlated with embryo quality in the pig. *Theriogenology*, **70**(6), 923–935. doi: [10.1016/j.theriogenology.2008.05.055](https://doi.org/10.1016/j.theriogenology.2008.05.055)