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Optimal doses of EGF and GDNF act as biological response modifiers to improve porcine oocyte maturation and quality

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Summary

It is well documented that both epidermal growth factor (EGF) and glial cell line-derived neurotrophic factor (GDNF) are critical for porcine oocyte maturation, however, little information is known about their mechanism of action *in vitro*. To gain insight into the mechanisms of action of the optimum doses of EGF and GDNF on porcine oocyte maturation, porcine cumulus–oocyte complexes (COCs) were matured in defined porcine oocyte medium supplemented with EGF, GDNF or a combination of both at varying concentrations (0–100 ng/ml) for 44 h. Nuclear and cytoplasmic maturation were determined in terms of nuclear stage after DNA staining with Hoechst and cortical granule distribution after lectin labeling, respectively. Mature oocytes were subsequently collected for gene expression analysis or subjected to *in vitro* fertilization and cultured for 7 days. The results showed that EGF and/or GDNF, when administered in a certain dose (50 ng/μl) to the maturation medium, not only effectively improved the synchronization of nuclear and cytoplasmic maturation processes within the oocyte, but enhanced expression of their corresponding receptors in mature oocytes ($P < 0.05$). Moreover, supplementation with an optimal combination of EGF + GDNF resulted in elevation of TFAM transcripts as well as a decrease of caspase-3 transcripts compared with the other studied groups ($P < 0.05$). Collectively, our results indicate that treatment of porcine oocytes with specific-dose combinations of EGF and GDNF stimulates oocyte quality and competence by transcriptional modulation of genes involved in oocyte survival and competence.

Keywords: Cortical granules, EGF, GDNF, Oocyte maturation, Porcine

Introduction

Regardless of intensive efforts to establish reliable *in vitro* maturation (IVM) and fertilization (IVF) methods in pigs, current IVM and IVF systems still suffer from a low rate and poor quality of *in vitro* produced embryos (Abeydeera, 2002; Dang-Nguyen *et al.*, 2011). Although the precise reason has not been clarified, an emerging body of evidence indicates that this may be attributed, at least in part, to the insufficient cytoplasmic maturation of the oocyte, asynchrony between oocyte nuclear and cytoplasmic maturation and a high rate of polyspermy (Sun *et al.*, 2001; Funahashi, 2003). Consequently, the strategy for increasing the efficiency of IVM needs to be focused on identifying various follicular signalling pathways that are involved in oocyte maturation. Accordingly, in the last few years, various cellular growth factors have received a great deal of attention as regulators of ovarian cell function (Sun & Nagai, 2003). Among the numerous ovarian factors, epidermal growth factor (EGF) and glial cell line-derived neurotrophic factor (GDNF) ligands have received increasing attention in recent years because of their roles in many aspects of oocyte maturation and ovulation in several

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mammals including mouse, pig and human (Linher *et al.*, 2007; Kawamura *et al.*, 2008; Zhao *et al.*, 2011; Toms *et al.*, 2014; Ritter *et al.*, 2015; Sugimura *et al.*, 2015).

Studies in different cell models have demonstrated that EGF and its receptor (EGFR) are involved in the regulation of the activity of a variety of cell signalling pathways, including MAPK and PI3K pathways, which have been shown to stimulate proliferation, growth, and survival of various cell types, including cumulus cells and the oocyte itself (Li *et al.*, 2008; Fujihara *et al.*, 2014; Ritter *et al.*, 2015). Furthermore, the presence of EGF and its receptor at the protein level in the follicle cells, oocyte and the porcine follicular fluid, suggests that it might be an endogenous modulator of ovarian function in this species (Hsu *et al.*, 1987; Singh *et al.*, 1995).

In parallel, *in vitro* studies have revealed that GDNF enhances developmental competence of oocytes from antral follicles in humans (Zhao *et al.*, 2011), porcine (Linher *et al.*, 2007; Valleh *et al.*, 2016) and mouse (Kawamura *et al.*, 2008) species. The biological effects of GDNF were shown mainly to be mediated through its interaction with the RET tyrosine kinase/GFRA1 receptor complex (Sariola & Saarma, 2003; Wang, 2013). Accordingly, GDNF and both its receptors were shown to be expressed in porcine COCs at the mRNA and protein levels and that GDNF protein was also shown to present at similar levels in the follicular fluid derived from both small and large porcine follicles (Linher *et al.*, 2007).

Although the beneficial effects of EGF and/or GDNF on porcine oocyte competence are well established, (Linher *et al.*, 2007; Valleh *et al.*, 2016), many aspects of their mechanism of action still remain unclear. To understand the possible mechanisms of actions of the optimum dose of EGF and/or GDNF on oocyte maturation, we analysed oocyte nuclear and cytoplasmic maturation through fluorescence assessment of oocyte chromatin organization and cortical granules localization, respectively. Moreover, we performed qPCR for various genes including those encoding growth factors receptors (RET, GFRα1, EGFR), telomerase activity (TERT), mitochondrial transcription factor A (TFAM) and genes involved in the regulation of cell survival (BAK, BCL-XL, caspase-3) in matured oocytes.

Material and methods

Culture media

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Selection and culture of cumulus–oocyte complexes

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in 0.9% (w/v) NaCl at 29–32°C. After dissection of the surrounding tissue, the ovaries were rinsed from blood with saline solution or tap water (at 30–32°C) and rapidly kept at 30°C in saline supplemented with penicillin and streptomycin. Cumulus–oocyte cell complexes (COCs) were aspirated from antral follicles (3–8 mm diameter), using an 18-gauge winged infusion set connected to a low pressure vacuum pomp. The follicular contents were allowed to sediment and washed three times with previously equilibrated maturation medium. Only COCs with uniform ooplasm and with at least three layers of compact cumulus cells were selected and used for further experiments. In order to evaluate the possible effects of GDNF and EGF on the proper oocyte nuclear and cytoplasmic maturation, groups of COC were matured randomly in tissue culture medium (TCM)-199 supplemented with 1 mM sodium pyruvate (M-2154), 2 mM L-glutamine (G-6784), 5 µl/ml insulin–transferrin–selenium (I-3146), 10 IU/ml eCG and 5 IU/ml hCG (Suigonan Vet, Intervet Scandinavia, Skovlunde, Denmark), 10,000 U/ml penicillin G (P-3032), 10 µg/ml streptomycin sulfate (G-1264), 4 mg/ml bovine serum albumin (BSA, A-3311), EGF; E-4127)] and/or human recombinant GNDF [10, 50 or 100 ng/ml (GDNF, Prospec, Rehovot, Israel)] at 39°C for 44 h (Table 1).

Assessment of oocyte nuclear status and distribution of the cortical granules

Oocyte cytoplasmic and nuclear maturation were analysed respectively through fluorescence assessment of oocyte cortical granule (CG) localization and chromatin organization. Assessment of CG distribution in maturing oocytes was done based on previously described procedures (Wang *et al.*, 1997) with some modifications. Briefly, the oocytes were denuded by a short exposure to 0.1% hyaluronidase/PBS solution followed by pipetting. Then, the oocytes were treated with Tyrode's acid to remove the zona pellucida. After washing three times in PBS containing 0.1% PVA (P-8136), oocytes were fixed with 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature and washed three times in PBS for 5 min each time. This was followed by permiabilization with 0.5% (v/v) Triton X-100 (T-6878) in PBS for 5 min and washing twice in PBS supplemented with 0.1% (w/v) polyvinyl alcohol (PVA) for 5 min. For fluorescence staining, oocytes were then incubated in 1 mg/ml FITC–PNA in PBS for 30 min in the dark. Afterward, the oocytes were washed three times in PBS containing 0.1% (w/v) polyvinyl alcohol (PVA). Subsequently nuclear maturation status of oocytes was evaluated

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Table 1 Effect of various levels of growth factor supplements in the maturation media on oocyte maturation, cleavage, blastocyst and hatching rates*

Group	EGF (ng/ml)	GDNF (ng/ml)	No. of oocytes (*N*)**	No. of properly matured oocytes*** (%)	Embryo development (mean ± SEM)		
					Cleavage, (%)	Blastocyst rate (%)	Hatching rate (%)
1	0	0	225 (15)	1 (6.7) ^{a}	55.18 ± 1.78^{a}	5.03 ± 0. 85^{a}	0 ^{a}
2	10	0	232 (15)	2 (13.3) ^{a}	63.17 ± 1.46^{a,b}	8.08 ± 0. 83^{a}	8.49^{a,b}
3	50	0	230 (17)	7 (41.1) ^{b}	62.71 ± 1.93^{a,b}	14.10 ± 0. 29^{b,c}	26.67^{a,b}
4	100	0	235 (18)	4 (23.5)^{a,b}	56.29 ± 2.64^{a}	8.04 ± 1.01^{a}	8.49^{a,b}
5	0	10	221 (15)	2 (13.3) ^{a}	59.55 ± 1.80^{a,b}	7.20 ± 0. 92^{a}	16.99^{a,b}
6	0	50	240 (18)	5 (33.3)^{a,b}	62.14 ± 2.18^{a,b}	12.82 ± 0. 75^{b}	29.91^{a,b}
7	0	100	220 (20)	6 (30.0)^{a,b}	63.19 ± 3.61^{a,b}	7.65 ± 0. 55^{a}	16.99^{a,b}
8	10	10	235 (18)	3 (16.7)^{a,b}	62.28 ± 1.81^{a,b}	8.05 ± 0. 76^{a}	8.49^{a,b}
9	50	50	245 (15)	11 (57.9)^{c}	68.58 ± 1.69^{b}	16.83 ± .057^{c}	35.30^{b}
10	100	100	229 (16)	3 (18.7)^{a,b}	59.34 ± 1.13^{a,b}	7.78 ± 0. 78^{a}	0 ^{a}

*Data marked with different superscripts per column are significantly different between treatments ($P < 0.05$).
**Numbers in parentheses (*N*) represent the numbers of oocytes used per group and simultaneously analysed for meiotic stage and CG localization after IVM.
***Number of oocytes with both nuclear metaphase II plate and clear continuous monolayer of cortical granules (%).

by staining the oocytes with 10 mg/ml bis-benzamide (Hoechst 33342; Calbiochem) in PBS for 5 min. Stained oocytes were mounted on glass slides using Antifade (Slow Fade, Light Antifade Kit, Eugene, OR, USA), were examined under a Leica DMRB fluorescence microscope and classified according to their meiotic maturation stages. Following morphological evaluation, three representative samples of selected groups of treated oocyte were processed for RT-PCR. For this purpose, pools of oocytes (10 oocytes/tube) were collected into 20 µl of PBS supplemented with 0.1% PVA, snap frozen in liquid nitrogen, and stored at −80°C until analysis.

Oocyte RNA extraction and amplification

Total RNA from pooled oocytes was isolated using the NucleoSpin® RNA XS Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. For each sample, 10 ng of total RNA was used for reverse transcription using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo-Scientific) following the manufacturer's protocol. The relative expression of target genes was assessed by real-time PCR performed on a Light Cycler 480 System (Roche Applied Sciences, Indianapolis, IN, USA) using LightCycler FastStart DNA Master SYBR Green I® kit (Roche Diagnostics). Primer sequences for all target genes except for RET, GFRα1 and EGFR were obtained from previously published data in porcine and bovine (Valleh *et al.*, 2014, 2016). Primers for RET, GFRα1 and EGFR genes were designed using Primer Premier 6.0 software (Premier Biosoft International, Palo Alto, CA, USA) on the basis of complementary DNA sequences available in GenBank (Table 2). Real-time PCR amplification was performed using the Corbett Rotor-Gene 3000™ system (Corbett Research, Australia). Thermal cycling parameters consisted of initial denaturation at 95°C for 2 min followed by 40 cycles with 15 s denaturation at 95°C, 15 s annealing at 60°C and 20 s extension at 72°C. Each real-time PCR experiment was performed using three biological replicates. The expression values (C_T values) of each target gene were normalized to the geometric mean of expression levels of three suitable proposed housekeeping genes (*18S rRNA*, *UBB*, and *GAPDH*; Kuijk *et al.*, 2007) using the comparative C_T method (Schmittgen & Livak, 2008). To verify qPCR product identity, melting point curves were analysed following amplification. The PCR product sizes were confirmed by gel electrophoresis on a standard 2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light.

IVF and embryo culture

Porcine *in vitro* matured oocytes were washed three times with TALP medium and groups of 15–25 oocytes were transferred into each well of a four-well multidish (15 × 10 mm, Nunclon, Roskilde, Denmark) containing 250 µl of TALP medium pre-equilibrated at 38.5°C under 5% CO_2. *In vitro* fertilization of the oocytes and embryo culture were accomplished as previously reported (Valleh *et al.*, 2016). Briefly fresh semen from a mature, fertility tested boar was washed in non-capacitating medium (113 mM NaCl, 5 mM KCl, 5.56 mM glucose, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 22 mM Na-lactate, 5 µg/ml phenol red, 50 µg/ml gentamicin), and then resuspended in capacitating IVF medium (90 mM NaCl, 12 mM KCl,

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Table 2 Primer sequences used in real-time PCR of porcine oocyte

Genes	Sequence (5′→3′)	GenBank porcine	Product size (bp)
BCL-XL	GGTACCTCAGTTCAAACTCATC GTTGACTTTCTCTCCTACAAGC	AF216205.1	277
BAK	CTAGAACCTAGCAGCACCAT CGATCTTGGTGAAGTACTC	XM_001928147.3	151
CASP3	GAGGCAGACTTCTTGTATGC CATGGACACAATACATGGAA	NM_214131	236
HSP70	GTTCAAGAG GAAGCACAAGA GTTGATGCTCTTGTTTAGGT	AY466608	361
TERT	GACCTCCAGCCTTACATGAGA TGTTTTCCATGTCCCCGTAG	AY785158	253
TFAM	CCTTTCCACATACAACCATCGA TCCAGAACTCATCTTGGTAAATTCC	NM_001130211.1	80
EGF-R	TGGAGGAGAAGAAAGTTTGC GATGATCTGCAGGTTTTCCA	NM_214007.1	247
RET	CCGGTCAGCTACTCCTCATC CTGTCGCCTTGACCACTTTT	XM_013983253.1	177
GFRα-1	GTGCCCGTGTGCTCCTAC GCTGACAGACCTTGACTCT	XM_013983784.1	137
GAPDH	TCGGCATCGTGGAAGGAC GGCAGCACCAGTAGAAGCAG	AF017079.1	140/300
18S rRNA	GGCTACCACATCCAAGGAAG TCCAATGGATCCTCGCGGAA	NR_002170	130
UBB	GTCTGAGGGGTGGCTGCTAA TGGGGCAAATGGCTAGAGTG	XM_005653993.1	85

0.5 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM $MgSO_4$, 2 mM Na-pyruvate, 8 mM $CaCl_2$, 1.9 mM caffeine, L-glutamine, penicillin–streptomycin, 5 μg/ml phenol red, 4 mg/ml BSA), and finally preincubated for 10–15 min. After incubation, the supernatant was recovered and 250 μl of the suspension were added to wells with oocytes to a final concentration of 1×10^5 cells/ml. At 24 h post-insemination (hpi), presumptive zygotes were washed twice with fresh TALP to remove both remnant cumulus cells and attached sperm cells. Finally, putative zygotes were transferred to synthetic oviductal fluid supplemented with essential (M-5550) and non-essential (M-7145) amino acids (SOFaa) and were incubated at 38.5°C in a humidified atmosphere consisting of 90% N_2, 5% O_2 and 5% CO_2 for 7 days. Cleavage and blastocyst rate were calculated in relation to the initial number of cultured oocytes on days 2 or 7, respectively. The percentage of hatched blastocysts was also calculated relative to the total number of blastocysts on day 8.

Statistical analysis

An analysis of variance (ANOVA) was carried out after arcsine transformation of cleavage and blastocyst rate data (binomial response). Statistical differences in relative mRNA expression between experimental groups were assessed using repeated measurement ANOVA followed by Tukey–Kramer multiple comparisons. Pearson pairwise correlation analyses were used to determine the strengths of associations between expression patterns of selected genes in mentioned analysed groups. The values are presented as mean ± SEM unless stated otherwise. A P-value ≤ 0.05 was considered to be statistically significant, unless otherwise specified. All data were statistically analysed using JMP software version 9.0 (SAS Institute Inc., Cary, NC, USA) and graphical presentation were performed using the GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, USA).

Results

For evaluation of the results of growth factor treatment on oocyte maturation, at least 15 oocytes per treatment groups were examined for nuclear status and distribution of CG (Fig. 1*A–E*). The nuclear and cytoplasmic maturation status of oocytes after IVM was categorized into the following two patterns. Pattern A: Oocytes with metaphase II plate containing either a polar body or two bright chromatin spots, and with a clearly visible continues CG monolayer just beneath the oolemma (Fig. 1*E*). Pattern B: Oocytes that possessed clumped or strongly condensed chromatin or a metaphase plate without a polar body, and/or with a partial or incomplete CG migration pattern (Fig. 1*D*). Pattern A was predominantly found in

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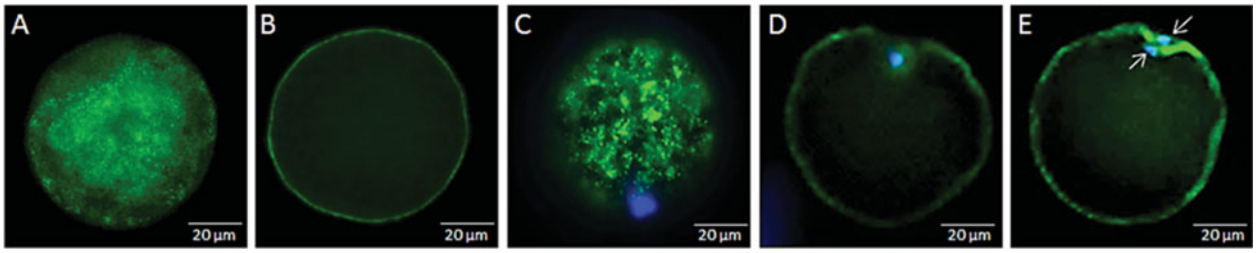

Figure 1 Confocal microscopic images of cumulus-denuded porcine oocytes after staining with fluorescein FITC–PNA and/or propidium iodide showing the pattern of cortical granules distribution (*A–E*; green) and with chromatin (*C–E*; blue). (*A*) Cytoplasmic immature oocytes, where CGs are diffused as large aggregates throughout the ooplasm. (*B*) Cytoplasmic mature oocytes with a clearly visible CG monolayer just beneath the oolema. (*C*) An immature oocyte with an intact germinal vesicle (GV), in which cortical granules distributed in clusters throughout the entire cytoplasm. (*D*) A representative example of incomplete matured oocyte with highly condensed chromatin, without a polar body and with incomplete or partial CG migration. (*E*) Completed matured oocyte in which chromosomes are at metaphase stage (both arrows), first polar body (outside arrowhead) had extruded and a clear continuous CG monolayer is located beneath the oolema.

good-quality matured oocytes examined after maturation in oocyte culture medium supplemented with growth factors at concentrations of 50 ng/ml (Table 1; groups 3, 6 and 9), whereas, pattern B typically was found in poorly matured oocytes at the end of the maturation period.

The number of oocytes presenting synchronous nuclear maturation and cytoplasmic maturation was significantly higher in oocytes treated with an optimum-dose combination of GDNF and EGF when compared with control or single growth factor treatment groups (Table 1 and Fig. 1*E*), ($P < 0.05$).

Furthermore, both EGF and GDNF alone or the combined addition of EGF and GDNF increased the proportion of oocytes that developed to the blastocyst stage by day 8 after IVF in a dose-dependent manner (Table 1); the effect was significant at concentrations 50 ng/ml for EGF and GDNF alone and a combination of the two when compared with the controls (Table 1), ($P < 0.05$).

The expression level of EGF and GDNF receptor subtypes is proposed to contribute to differences in functional responses mediated by these receptors. Therefore, to explore the possible mechanism of dose-dependent effects of these growth factors on oocyte competence, we examined the mRNA expression levels of their corresponding receptors (i.e. RET, GRFα-1, EGFR) in mature oocytes. Using qPCR, we found that mature oocytes expressed EGFR and RET; however GRFα-1 mRNA levels approached the limit of detection in the qPCR assay. Hence, we focus our analysis only on expression patterns of EGFR and RET in our experimental groups (Fig. 2).

The results showed that oocyte treatment with high-dose (100 ng/µl) of EGF and GDNF (alone and in combination) led to a pronounced reduction in the levels of their respective receptor mRNAs compared with those oocytes cultured in medium supplemented with a lower dose (50 ng/µl) of growth factors (Fig. 2) ($P < 0.05$). Moreover, supplementation of the maturation medium with the optimum concentration (50 ng/µl) of growth factors induced the expression of their respective receptors compared with the control group (Fig. 2) ($P < 0.05$).

To understand the molecular mechanisms of the beneficial action of GDNF and /or EGF, the relative transcript levels of HSP70, TERT, TFAM, Caspase-3, BAK and BCL-XL genes were analysed in mature oocytes. We chose to focus on the concentrations 50 ng/ml EGF and 50 ng/ml GDNF and a combination of the two, as these concentrations produced maximum effects on oocyte maturation and embryo development when compared with controls (Table 1). Supplementation of the maturation medium with an optimal dose combination of GDNF and EGF was shown to significantly affect the expression pattern of all investigated genes compared with the controls ($P < 0.05$; Fig. 3). Accordingly, treatment of oocytes with a combination of EGF and GDNF increased the relative abundance of BCL-XL, TFAM and TERT, while decreasing the levels HSP70, Caspase-3 and BAK transcript compared with the control group ($P < 0.05$; Fig. 3). In addition, significant differences in the relative levels of BCL-XL and HSP70 transcript were observed between oocyte treated with EGF alone and those treated with GDNF alone ($P < 0.05$; Fig. 3). However, no significant differences in relative abundance of TERT and BAK were observed among growth factor-treated oocytes ($P > 0.05$; Fig. 3). In addition, treatment of oocyte with a combination of EGF and GDNF increased the relative abundance of TFAM while decreasing the levels of caspase-3 transcripts compared with all other groups ($P < 0.05$; Fig. 3).

Regardless of treatment group, the results of Pearson correlation analysis indicated that the RET levels correlated positively with the EGFR levels ($r = 0.46$;

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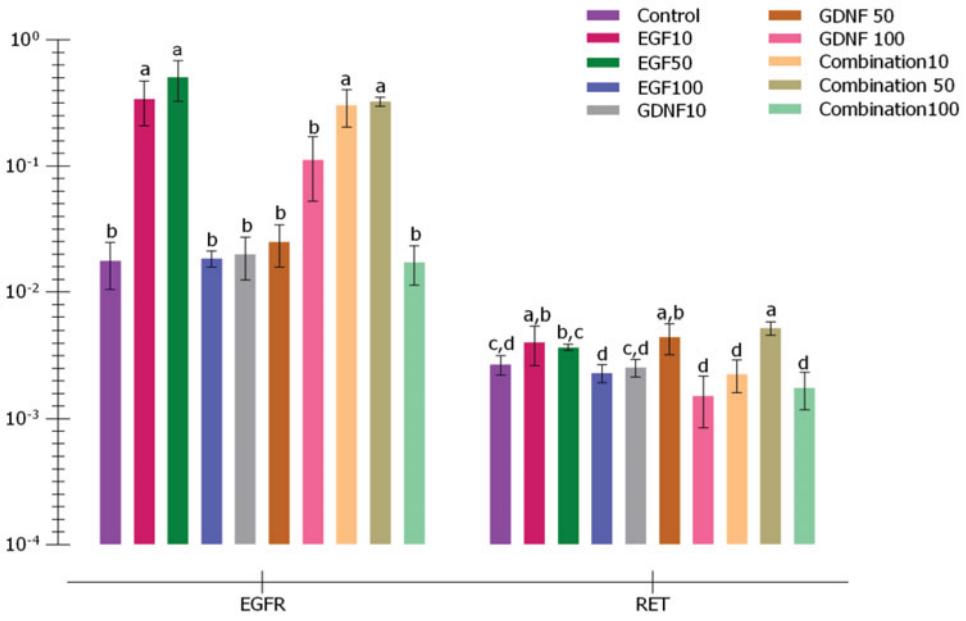

Figure 2 Relative abundance of *EGFR* and *RET* mRNA expression in porcine oocyte matured in control or growth factors supplemented media. Bars with different letters differ statistically within a group ($P < 0.05$).

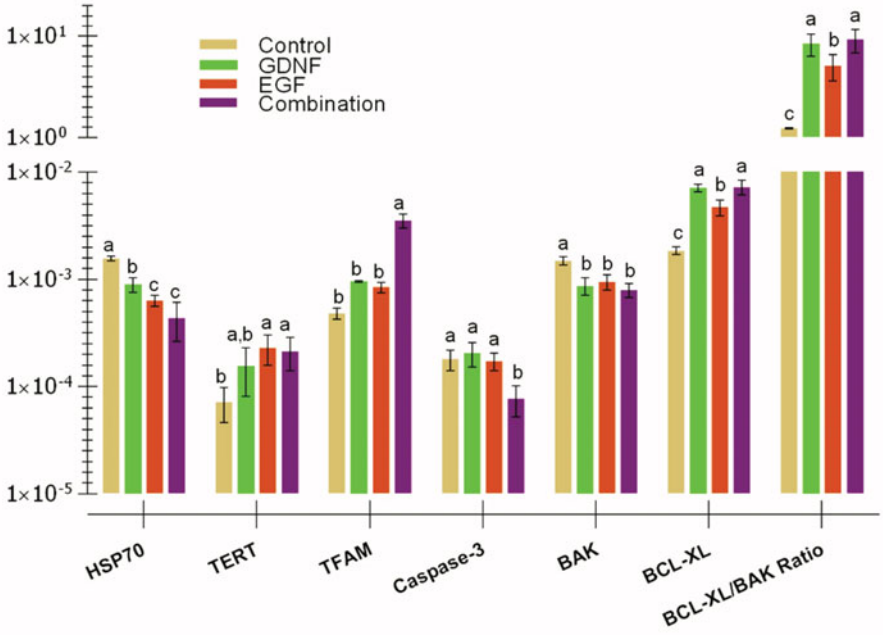

Figure 3 Relative abundance of *HSP70*, *TERT*, *TFAM*, *Caspase-3*, *BAK*, *BCL-XL* and *BCL-XL* to *BAK* ratio (G) mRNA expression in porcine oocyte matured in control or growth factors supplemented media. Bars with different letters differ statistically within a group ($P < 0.05$).

$P < 0.05$), BCL-XL ($r = 0.81$; $P < 0.01$) and TFAM ($r = 0.72$; $P < 0.01$), but negatively correlated with levels of HSP70 ($r = –0.62$; $P < 0.05$), caspase-3 ($r = –0.64$; $P < 0.05$), BAK ($r = –0.67$; $P < 0.05$). Our results also indicated that the levels of EGFR transcript correlated positively with TERT levels ($r = 0.65$; $P < 0.05$) but also correlated negatively with HSP70 levels ($r = –0.76$; $P < 0.05$) in matured oocytes.

Discussion

In this study, the exposure of COCs to specific doses of EGF and/or GDNF during IVM significantly improved the synchronization of oocyte nuclear maturation with cytoplasmic maturation compared other groups. Our results are in line with previously published reports in pig, which implied a stimulating

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effect of EGF on oocyte maturation (Prochazka *et al.*, 2000; Mao *et al.*, 2012). These results are also in agreement with previous reports in pig, indicating that supplementation of maturation medium with GDNF enhances oocyte maturation (Linher *et al.*, 2007; Toms *et al.*, 2014).

In this study, we observed that although supplementation of maturation medium with certain combination doses of EGF (50 ng/µl) and GDNF (50 ng/µl) enhanced oocyte competence, both of these growth factors retarded oocyte maturation and even embryo development at higher concentrations. Based on our data, we cannot specify the principles of the observed dose-dependent beneficial or detrimental effects of EGF and GDNF on proper oocyte maturation, but their beneficial effects could be attributed to their roles in regulation of different aspects of oocyte maturation. EGF was shown to accelerate the timing of polar body extrusion in bovine oocytes through the induction in activities of both MPF and MAPK kinases (Sakaguchi *et al.*, 2002; Li *et al.*, 2008). Accordingly, the EGF-mediated MAPK3/1 pathway was shown to be critical for the normal distribution of chromosomes during oocyte maturation (Ni *et al.*, 2015). Notably, activated EGFR signalling was shown to induce meiotic resumption by downregulating NPPC/NPR2 signalling (Tsuji *et al.*, 2012). Moreover, EGF-like growth factors have also been reported to be involved in the regulation of maternal mRNA translation in mouse oocytes by activation of the PI(3)K–AKT–mTOR pathway (Chen *et al.*, 2013). GDNF has also been shown not only to induce the level of cyclin B1 (CCNB1) protein expression, a proxy for oocyte cytoplasmic maturation (Linher *et al.*, 2007), but also to decrease the meiosis inhibitor WEE1 homolog 2 (WEE1B/WEE2) transcript levels in pig oocytes (Toms *et al.*, 2014). Furthermore, treatment of granulosa cells with GDNF was shown to induce increased expression of both miR-378-3p and miR-378-5p; which are proposed to be actively involved in the regulation of porcine follicle maturation (Toms, 2014). Importantly, both EGF and GDNF have been shown to stimulate synergistically DAZL expression in oocytes derived from antral follicles during IVM, possibly through the promotion of Sp1 binding to the DAZL promoter (Liu *et al.*, 2009). DAZL up-regulation not only has been shown to play an important role in the translational regulation of key proteins associated with oocyte maturation (Liu *et al.*, 2009) but also has been demonstrated to play a critical role in oocyte-to-zygote transition (Chen *et al.*, 2011b). Oocyte-toxic effects of high doses of EGF and/or GDNF is not surprising, as previous studies have shown that, in variety of cell types, prolonged or high-dose exposure to either EGF or GDNF may induce down-regulation of their respective receptors (Mulligan, 2014; Barbieri *et al.*, 2016) through activation either the clathrin or the non-clathrin dependent pathway of receptor internalization (Scott *et al.*, 2005; Richardson *et al.*, 2006; Esseghir *et al.*, 2007; Sigismund *et al.*, 2013).

In this study, oocyte gene expression patterns were somewhat influenced by the growth factors that were supplemented in the maturation medium. These results are in agreement with a series of experiments showing that quantity and quality of the stockpiled mRNA in the matured oocyte is critical for preimplantation embryo development (Sirard *et al.*, 2006; Sirard, 2012). Accordingly, differences in the mature oocyte stockpiled mRNA have been partially attributed to either new transcription or polyadenylation of existing dormant transcripts (Jones *et al.*, 2008). In this study, relative to the control group, supplementation of maturation medium with an optimum combination dose of growth factors was found to not only induce the expression of TERT, TFAM and BCL-XL but concurrently decrease expression levels of HSP70, caspase-3 and BAK in *in vitro* maturated oocytes.

In view of the recent developments in the field of reproduction, oocyte apoptosis has been postulated to be one of the main factors that determines oocyte competence (Tiwari *et al.*, 2015). Programmed cell death (apoptosis) has been shown to be coupled, at least in part, with the activation of the downstream executioner caspase-3 (Lakhani *et al.*, 2006). Caspase-3 activity has also been reported to be determined by the ratio of anti-apoptotic and pro-apoptotic Bcl-2 family genes, such as the *BCL-XL/Bak* ratio, rather than the absolute expression level of each single member of the BCL-2 gene family (Czabotar *et al.*, 2014). Our correlation analysis result is also in agreement with the notion that caspase-3 activity, at least in part, is regulated by the relative activity of the members of the BCL-2 family (Salakou *et al.*, 2007). More interestingly, in our study, marked down-regulation of oocyte caspase-3 expression was found to be associated with the higher blastocyst hatching rate (Fig. 2 and Table 1). This result may in part support the fact that the presence of molecules that regulate the apoptotic mechanism in oocytes may also affect embryo quality (Metcalfe *et al.*, 2004). The mechanisms by which EGF or GDNF inhibit apoptosis signalling in a variety of cell types are not well understood, but recent studies revealed that binding of EGF to its cell surface receptor (EGFR) stimulates a complex array of pro-survival signals including Ras/MAPK, PI3K/Akt, PLCc/PKC, ERK1/2 and Stat pathways (Henson & Gibson, 2006; Krall *et al.*, 2011) that are critical for promotion of survival of various cell types, including the primordial follicle (Henson & Gibson, 2006; Fujihara *et al.*, 2014; Ritter *et al.*, 2015). In a similar manner, GDNF binding to its receptors, GFRα-1 and

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c-Ret, induces initiation of a number of downstream signalling cascades including PI3K/AKT, MEK/ERK, SRC and STAT3 pathways that are known to be critical for cell survival (Sariola & Saarma, 2003; Boku *et al.*, 2013).

In our experiment, the addition of the combination of EGF and GDNF into the maturation medium enhanced the transcription levels of TFAM in mature oocytes, indicating that a correlative link existed between treatment with the combination of tested growth factors and oocyte mitochondrial activity. Mitochondrial metabolic activity has been proposed to be critical in the regulation of distinct signalling pathways during oocyte maturation (Spikings *et al.*, 2007; Ge *et al.*, 2012). Oocyte mitochondrial dysfunction and associated deficiencies in ATP content have been proposed to negatively affect oocyte quality, embryonic development, and even the implantation process (Ge *et al.*, 2012; Chappel, 2013; Fragouli & Wells, 2015). Functionally significant mtDNA replication was reported to proceed during oocyte maturation at the transition from the germinal vesicle (GV) to the MII-arrested oocyte (Mao *et al.*, 2012). Recently it has been suggested that low mtDNA content may be associated with a deficiency in the oocyte maturation process (Keefe *et al.*, 2015). In this regard, mitochondrial transcription factor A (TFAM) activity was not only reported to be essential for maintenance, replication and transcription of mtDNA but its transcriptional level was recently shown to positively correlated with mtDNA copy number in various cell types (Ekstrand *et al.*, 2004), including pig oocyte (Mao *et al.*, 2012). It has also been reported that enhancement of the GDNF/RET/PI3K/NF-κB/mitochondrial pathway is critical for maintenance of proper mitochondrial integrity and function (Meka *et al.*, 2015). EGF-like growth factor, neuregulin, has also been shown to stimulate oocyte mitochondrial biogenesis during *in vitro* maturation after binding to its receptor EGFR (Mao *et al.*, 2012). Moreover, the presence of a statistically significant correlation between the levels of TFAM and transcription of those apoptosis regulatory factor including caspase-3, HSP70 or BCL-XL/BAK ratio index also supports the model that there is functional cross-talk between these signalling molecules in the cell (Jin *et al.*, 2007; Valleh *et al.*, 2014). Similarly, the presence of a positive correlation between oocyte TFAM levels and blastocyst hatching rate, is in agreement with those groups that reported that quality of preimplantation embryos was highly correlated with oocyte ATP content (Stojkovic *et al.*, 2001).

In this study, the relative expression of TERT was found to be higher in oocytes matured in medium supplemented with EGF or those derived from medium supplemented with both EGF and GDNF, when compared with the control untreated oocytes. Importantly, telomere length has been demonstrated to be implicated in oocyte competence and participate in reproductive aging (Keefe and Liu, 2009; Kalmbach *et al.*, 2013). Critically short telomeres were shown to trigger the cellular senescence process, which ultimately resulted in p53-dependent apoptosis (Cosme-Blanco *et al.*, 2007). The proportions of fragmented or aneuploid embryos that failed to implant was shown to be higher in those embryos derived from oocytes with shorter telomeres (Keefe & Liu, 2009; Ozturk *et al.*, 2014). In contrast, high telomerase activity in human luteinised granulosa cells was shown to be positively correlated with a greater likelihood of pregnancy (Chen *et al.*, 2011a). Despite the importance of the role of telomere length in oocyte quality, there is a growing body of evidence showing that the catalytic subunit of telomerase (TERT) is not only positively correlated with telomerase enzymatic activity, but also with telomere length (Iqbal *et al.*, 2011). Notably, it has been shown that EGF could promote telomerase activity by direct activation of TERT transcription through stimulating the activation of the Ras/MEK/ERK pathway (Maida *et al.*, 2002). Similarly, GDNF-mediated signalling was also shown to induce expression of the *TERT* gene in mouse male germline stem cells (mGSCs) cell (Sun *et al.*, 2013). Significant positive correlation between TERT and EGFR transcription that was found in this study also gave support to other studies and implies the role of the EGF receptor in the regulation of cell survival (Wang *et al.*, 2000; Lo & Hung, 2006).

Expression levels of Hsp70 were found to be higher in oocytes matured in control medium compared with those oocytes cultured in medium supplemented with EGF and/or GDNF. HSP families have clearly been shown to be essential for normal cell function, but also shown to participate in mechanism(s) underlying the adaptation to various conditions of environmental stress (Mayer & Bukau, 2005). HSP70, which is strongly induced by various stresses, is known to be essential for protection of cellular processes against environmental stress by regulating the levels of important members of the apoptosis network such as BCL-2, p53 and caspase-3 (Li *et al.*, 2015). Accordingly, its variation was proposed as a reliable parameter for measuring the levels of stress in various cell culture models including *in vitro* maturation (IVM) of oocytes (Warzych *et al.*, 2007). The observed significant negative correlation between the levels of HSP70 and expression of both of EGFR and RET genes may also provide further support for the implication of these receptor components in the cell stress response (Myers & Mulligan, 2004; Cao *et al.*, 2011).

In conclusion, the results of this study provide evidence for dose-dependent positive or negative

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effects of growth factors on porcine oocyte maturation and quality. Specifically, we found that appropriate concentrations (50 ng/ml) of the combination of EGF and GDNF enhanced both coordination of oocyte nuclear and cytoplasmic maturation, and embryo development following *in vitro* fertilization of oocytes. Accordingly, these stimulatory effects of exogenous EGF and GDNF on oocyte quality seem to be mediated, in part, by regulating the expression levels of their corresponding receptors as well as the genes involved in the stress response, cell survival/death and mitochondrial functions. Overall, these findings support the arguments of others that oocyte developmental competence depends to a great extent on IVM components and conditions (Sirard *et al.*, 2006).

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Declaration of interest

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References

Abeydeera, L.R. (2002). *In vitro* production of embryos in swine. *Theriogenology* **57**, 256–73.

Barbieri, E., Di Fiore, P.P. & Sigismund, S. (2016). Endocytic control of signaling at the plasma membrane. *Curr. Opin. Cell Biol.* **39**, 21–7.

Boku, S., Nakagawa, S., Takamura, N., Kato, A., Takebayashi, M., Hisaoka-Nakashima, K., Omiya, Y., Inoue, T. & Kusumi, I. (2013). GDNF facilitates differentiation of the adult dentate gyrus-derived neural precursor cells into astrocytes via STAT3. *Biochem. Biophys. Res. Commun.* **434**, 779–84.

Cao, X., Zhu, H., Ali-Osman, F. & Lo, H.W. (2011). EGFR and EGFRvIII undergo stress- and EGFR kinase inhibitor-induced mitochondrial translocalization: a potential mechanism of EGFR-driven antagonism of apoptosis. *Mol. Cancer* **10**, 26.

Chappel, S. (2013). The role of mitochondria from mature oocyte to viable blastocyst. *Obstet. Gynecol. Int.* **2013**, 183024.

Chen, H., Wang, W., Mo, Y., Ma, Y., Ouyang, N., Li, R., Mai, M., He, Y., Bodombossou-Djobo, M.M. & Yang, D. (2011a). Women with high telomerase activity in luteinised granulosa cells have a higher pregnancy rate during *in vitro* fertilisation treatment. *J. Assist. Reprod. Genet.* **28**, 797–807.

Chen, J., Melton, C., Suh, N., Oh, J.S., Horner, K., Xie, F., Sette, C., Blelloch, R. & Conti, M. (2011b). Genome-wide analysis of translation reveals a critical role for deleted in azoospermia-like (Dazl) at the oocyte-to-zygote transition. *Genes Dev.* **25**, 755–66.

Chen, J., Torcia, S., Xie, F., Lin, C.J., Cakmak, H., Franciosi, F., Horner, K., Onodera, C., Song, J.S., Cedars, M.I., Ramalho-Santos, M. & Conti, M. (2013). Somatic cells regulate maternal mRNA translation and developmental competence of mouse oocytes. *Nature Cell Biol.* **15**, 1415–23.

Cosme-Blanco, W., Shen, M.F., Lazar, A.J., Pathak, S., Lozano, G., Multani, A.S. & Chang, S. (2007). Telomere dysfunction suppresses spontaneous tumorigenesis *in vivo* by initiating p53-dependent cellular senescence. *EMBO Rep.* **8**, 497–503.

Czabotar, P.E., Lessene, G., Strasser, A. & Adams, J.M. (2014). Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat. Rev. Mol. Cell. Biol.* **15**, 49–63.

Dang-Nguyen, T.Q., Somfai, T., Haraguchi, S., Kikuchi, K., Tajima, A., Kanai, Y. & Nagai, T. (2011). *In vitro* production of porcine embryos: current status, future perspectives and alternative applications. *Anim. Sci. J. [Nihon chikusan Gakkaiho]*, **82**, 374–82.

Ekstrand, M.I., Falkenberg, M., Rantanen, A., Park, C.B., Gaspari, M., Hultenby, K., Rustin, P., Gustafsson, C.M. & Larsson, N.G. (2004). Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum. Mol. Genet.* **13**, 935–44.

Esseghir, S., Todd, S.K., Hunt, T., Poulsom, R., Plaza-Menacho, I., Reis-Filho, J.S. & Isacke, C.M. (2007). A role for glial cell derived neurotrophic factor induced expression by inflammatory cytokines and RET/GFR alpha 1 receptor up-regulation in breast cancer. *Cancer Res.* **67**, 11732–41.

Fragouli, E. & Wells, D. (2015). Mitochondrial DNA assessment to determine oocyte and embryo viability. *Semin. Reprod. Med.* **33**, 401–9.

Fujihara, M., Comizzoli, P., Keefer, C.L., Wildt, D.E. & Songsasen, N. (2014). Epidermal growth factor (EGF). sustains *in vitro* primordial follicle viability by enhancing stromal cell proliferation via MAPK and PI3K pathways in the prepubertal, but not adult, cat ovary. *Biol. Reprod.* **90**, 86.

Funahashi, H. (2003). Polyspermic penetration in porcine IVM-IVF systems. *Reprod. Fertil. Dev.* **15**, 167–77.

Ge, H., Tollner, T.L., Hu, Z., Dai, M., Li, X., Guan, H., Shan, D., Zhang, X., Lv, J., Huang, C. & Dong, Q. (2012). The importance of mitochondrial metabolic activity and mitochondrial DNA replication during oocyte maturation *in vitro* on oocyte quality and subsequent embryo developmental competence. *Mol. Reprod. Dev.* **79**, 392–401.

Henson, E.S. & Gibson, S.B. (2006). Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. *Cell. Signal.* **18**, 2089–97.

Hsu, C.J., Holmes, S.D. & Hammond, J.M. (1987). Ovarian epidermal growth factor-like activity. Concentrations in porcine follicular fluid during follicular enlargement. *Biochem. Biophys. Res. Commun.* **147**, 242–7.

Iqbal, K., Kues, WA., Baulain, U., Garrels, W., Herrmann, D. & Niemann, H. (2011). Species-specific telomere length differences between blastocyst cell compartments and ectopic telomere extension in early bovine embryos by human telomerase reverse transcriptase. *Biol. Reprod.* **84**, 723–33.

Jin, Y. X., Lee, J.Y., Choi, S.H., Kim, T., Cui, X.S. & Kim, N.H. (2007). Heat shock induces apoptosis related gene expression and apoptosis in porcine parthenotes developing *in vitro*. *Anim. Reprod. Sci.* **100**, 118–27.

Jones, G.M., Cram, D.S., Song, B., Magli, M.C., Gianaroli, L., Lacham-Kaplan, O., Findlay, J.K., Jenkin, G. & Trounson, A.O. (2008). Gene expression profiling of human oocytes following *in vivo* or *in vitro* maturation. *Hum. Reprod*. **23**, 1138–44.

Kalmbach, K.H., Fontes Antunes, D.M., Dracxler, R.C., Knier, T.W., Seth-Smith, M.L., Wang, F., Liu, L. & Keefe, D.L. (2013). Telomeres and human reproduction. *Fertil. Steril.* **99**, 23–9.

Kawamura, K., Ye, Y., Kawamura, N., Jing, L., Groenen, P., Gelpke, M.S., Rauch, R., Hsueh, A.J. & Tanaka, T. (2008). Completion of Meiosis I of preovulatory oocytes and facilitation of preimplantation embryo development by glial cell line-derived neurotrophic factor. *Dev. Biol.* **315**, 189–202.

Keefe, D., Kumar, M. & Kalmbach, K. (2015). Oocyte competency is the key to embryo potential. *Fertil. Steril.* **103**, 317–22.

Keefe, D.L. & Liu, L. (2009). Telomeres and reproductive aging. *Reprod. Fertil. Dev.* **21**, 10–4.

Krall, J.A., Beyer, E.M. & MacBeath, G. (2011). High- and low-affinity epidermal growth factor receptor–ligand interactions activate distinct signaling pathways. *PLoS One* **6**, e15945.

Kuijk, E.W., du Puy, L., van Tol, H.T., Haagsman, H.P., Colenbrander, B. & Roelen, B.A. (2007). Validation of reference genes for quantitative RT-PCR studies in porcine oocytes and preimplantation embryos. *BMC Dev. Biol.* **7**, 58.

Lakhani, S.A., Masud, A., Kuida, K., Porter, G.A., Booth, C.J., Mehal, W.Z., Inayat, I. & Flavell, R.A. (2006). Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* **311**, 847–51.

Li, M., Liang, C.G., Xiong, B., Xu, B.Z., Lin, S.L., Hou, Y., Chen, D.Y., Schatten, H. & Sun, Q.Y. (2008). PI3-kinase and mitogen-activated protein kinase in cumulus cells mediate EGF-induced meiotic resumption of porcine oocyte. *Domest. Anim. Endocrinol.* **34**, 360–71.

Li, Y., Zhang, Z., He, C., Zhu, K., Xu, Z., Ma, T., Tao, J. & Liu, G. (2015). Melatonin protects porcine oocyte *in vitro* maturation from heat stress. *J. Pineal Res.* **59**, 365–75.

Linher, K., Wu, D. & Li, J. (2007). Glial cell line-derived neurotrophic factor: an intraovarian factor that enhances oocyte developmental competence *in vitro*. *Endocrinology* **148**, 4292–301.

Liu, J., Linher, K. & Li, J. (2009). Porcine DAZL messenger RNA: its expression and regulation during oocyte maturation. *Mol. Cell. Endocrinol.* **311**, 101–8.

Lo, H.W. & Hung, M.C. (2006). Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. *Br. J. Cancer* **94**, 184–8.

Maida, Y., Kyo, S., Kanaya, T., Wang, Z., Yatabe, N., Tanaka, M., Nakamura, M., Ohmichi, M., Gotoh, N., Murakami, S. & Inoue, M. (2002). Direct activation of telomerase by EGF through Ets-mediated transactivation of TERT via MAP kinase signaling pathway. *Oncogene* **21**, 4071–9.

Mao, J., Whitworth, K.M., Spate, L.D., Walters, E.M., Zhao, J. & Prather, R.S. (2012). Regulation of oocyte mitochondrial DNA copy number by follicular fluid, EGF, and neuregulin 1 during *in vitro* maturation affects embryo development in pigs. *Theriogenology* **78**, 887–97.

Mayer, M.P. & Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. Cellular and molecular life sciences: *CMLS* **62**, 670–84.

Meka, D.P., Muller-Rischart, A.K., Nidadavolu, P., Mohammadi, B., Motori, E., Ponna, S.K., Aboutalebi, H., Bassal, M., Annamneedi, A., Finckh, B., Miesbauer, M., Rotermund, N., Lohr, C., Tatzelt, J., Winklhofer, K.F. & Kramer, E.R. (2015). Parkin cooperates with GDNF/RET signaling to prevent dopaminergic neuron degeneration. *J. Clin. Invest.* **125**, 1873–85.

Metcalfe, A.D., Hunter, H.R., Bloor, D.J., Lieberman, B.A., Picton, H.M., Leese, H.J., Kimber, S.J. & Brison, D.R. (2004). Expression of 11 members of the BCL-2 family of apoptosis regulatory molecules during human preimplantation embryo development and fragmentation. *Mol. Reprod. Dev.* **68**, 35–50.

Mulligan, L.M. (2014). RET revisited: expanding the oncogenic portfolio. *Nat. Rev. Cancer* **14**, 173–86.

Myers, S.M. & Mulligan, L.M. (2004). The RET receptor is linked to stress response pathways. *Cancer Res.* **64**, 4453–63.

Ni, H., Sheng, X., Cui, X., Gu, M., Liu, Y., Qi, X., Xing, S. & Guo, Y. (2015). Epidermal growth factor-mediated mitogen-activated protein kinase3/1 pathway is conducive to *in vitro* maturation of sheep oocytes. *PLoS One* **10**, e0120418.

Ozturk, S., Sozen, B. & Demir, N. (2014). Telomere length and telomerase activity during oocyte maturation and early embryo development in mammalian species. *Mol. Hum. Reprod.* **20**, 15–30.

Prochazka, R., Srsen, V., Nagyova, E., Miyano, T. & Flechon, J.E. (2000). Developmental regulation of effect of epidermal growth factor on porcine oocyte-cumulus cell complexes: nuclear maturation, expansion, and F-actin remodeling. *Mol. Reprod. Dev.* **56**, 63–73.

Richardson, D.S., Lai, A.Z. & Mulligan, L.M. (2006). RET ligand-induced internalization and its consequences for downstream signaling. *Oncogene* **25**, 3206–11.

Ritter, L.J., Sugimura, S. & Gilchrist, R.B. (2015). Oocyte induction of EGF responsiveness in somatic cells is associated with the acquisition of porcine oocyte developmental competence. *Endocrinology* **156**, 2299–312.

Sakaguchi, M., Dominko, T., Yamauchi, N., Leibfried-Rutledge, M.L., Nagai, T. & First, N.L. (2002). Possible mechanism for acceleration of meiotic progression of bovine follicular oocytes by growth factors *in vitro*. *Reproduction* **123**, 135–42.

Salakou, S., Kardamakis, D., Tsamandas, A.C., Zolota, V., Apostolakis, E., Tzelepi, V., Papathanasopoulos, P., Bonikos, D.S., Papapetropoulos, T., Petsas, T. & Dougenis, D. (2007). Increased Bax/Bcl-2 ratio up-regulates caspase-3 and increases apoptosis in the thymus of patients with myasthenia gravis. *In Vivo* **21**, 123–32.

Sariola, H. & Saarma, M. (2003). Novel functions and signalling pathways for GDNF. *J. Cell Sci.* **116**, 3855–62.

Schmittgen, T.D. & Livak, K.J. (2008). Analysing real-time PCR data by the comparative C_T method. *Nat. Protoc.* **3**, 1101–8.

Scott, R.P., Eketjall, S., Aineskog, H. & Ibanez, C.F. (2005). Distinct turnover of alternatively spliced isoforms of the RET kinase receptor mediated by differential recruitment of the Cbl ubiquitin ligase. *J. Biol. Chem.* **280**, 13442–9.

Sigismund, S., Algisi, V., Nappo, G., Conte, A., Pascolutti, R., Cuomo, A., Bonaldi, T., Argenzio, E., Verhoef, L.G., Maspero, E., Bianchi, F., Capuani, F., Ciliberto, A., Polo, S. & Di Fiore, P.P. (2013). Threshold-controlled ubiquitination of the EGFR directs receptor fate. *EMBO J.* **32**, 2140–57.

Singh, B., Rutledge, J.M. & Armstrong, D.T. (1995). Epidermal growth factor and its receptor gene expression and peptide localization in porcine ovarian follicles. *Mol. Reprod. Dev.* **40**, 391–9.

Sirard, M.A. (2012). Factors affecting oocyte and embryo transcriptomes. *Reprod. Domest. Anim.* [*Zuchthygiene*] **47**(Suppl 4), 148–55.

Sirard, M.A., Richard, F., Blondin, P. & Robert, C. (2006). Contribution of the oocyte to embryo quality. *Theriogenology* **65**, 126–36.

Spikings, E.C., Alderson, J. & St John, J.C. (2007). Regulated mitochondrial DNA replication during oocyte maturation is essential for successful porcine embryonic development. *Biol. Reprod.* **76**, 327–35.

Stojkovic, M., Machado, S.A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Goncalves, P.B. & Wolf, E. (2001). Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after *in vitro* maturation: correlation with morphological criteria and developmental capacity after *in vitro* fertilization and culture. *Biol. Reprod.* **64**, 904–9.

Sugimura, S., Ritter, L.J., Rose, R.D., Thompson, J.G., Smitz, J., Mottershead, D.G. & Gilchrist, R.B. (2015). Promotion of EGF receptor signaling improves the quality of low developmental competence oocytes. *Dev. Biol.* **403**, 139–49.

Sun, J.-w., Zhu, H.-j., Chao, L., LI, M.-z. & Hua, J.-l. (2013). GDNF up-regulates c-Myc transcription via the PI3K/Akt pathway to promote dairy goat male germline stem cells (mGSC) proliferation. *J. Integ. Agric.* **12**, 1054–65.

Sun, Q.Y., Lai, L. X., Park, K.W., Kuhholzer, B., Prather, R.S. & Schatten, H. (2001). Dynamic events are differently mediated by microfilaments, microtubules, and mitogen-activated protein kinase during porcine oocyte maturation and fertilization *in vitro*. *Biol. Reprod.* **64**, 879–89.

Sun, Q.Y. & Nagai, T. (2003). Molecular mechanisms underlying pig oocyte maturation and fertilization. *J. Reprod. Dev.* **49**, 347–59.

Tiwari, M., Prasad, S., Tripathi, A., Pandey, A.N., Ali, I., Singh, A.K., Shrivastav, T.G. & Chaube, S.K. (2015). Apoptosis in mammalian oocytes: a review. *Apoptosis* **20**, 1019–25.

Toms, D., Tsoi, S., Dobrinsky, J., Dyck, M.K. & Li, J. (2014). The effects of glial cell line-derived neurotrophic factor on the *in vitro* matured porcine oocyte transcriptome. *Mol. Reprod. Dev.* **81**, 217–29.

Toms, D.D. (2014). Involvement of GDNF and microRNA-378 in the regulation of porcine follicle maturation (Doctoral dissertation).

Tsuji, T., Kiyosu, C., Akiyama, K. & Kunieda, T. (2012). CNP/NPR2 signaling maintains oocyte meiotic arrest in early antral follicles and is suppressed by EGFR-mediated signaling in preovulatory follicles. *Mol. Reprod. Dev.* **79**, 795–802.

Valleh, M.V., Hyttel, P., Rasmussen, M.A. & Strøbech, L. (2014). Insulin-like growth factor 2: a modulator of anti-apoptosis related genes (HSP**70**, BCL2-L1). in bovine preimplantation embryos. *Theriogenology* **82**, 942–50.

Valleh, M.V., Rasmussen, M.A. & Hyttel, P. (2016). Combination effects of epidermal growth factor and glial cell line-derived neurotrophic factor on the *in vitro* developmental potential of porcine oocytes. *Zygote* **24**, 465–76.

Wang, W.H., Sun, Q.Y., Hosoe, M., Shioya, Y. & Day, B.N. (1997). Quantified analysis of cortical granule distribution and exocytosis of porcine oocytes during meiotic maturation and activation. *Biol. Reprod.* **56**, 1376–82.

Wang, X. (2013). Structural studies of GDNF family ligands with their receptors-Insights into ligand recognition and activation of receptor tyrosine kinase RET. *Biochim. Biophys. Acta* **1834**, 2205–12.

Wang, X., McCullough, K.D., Franke, T.F. & Holbrook, N.J. (2000). Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. *J. Biol. Chem.* **275**, 14624–31.

Warzych, E., Wrenzycki, C., Peippo, J. & Lechniak, D. (2007). Maturation medium supplements affect transcript level of apoptosis and cell survival related genes in bovine blastocysts produced *in vitro*. *Mol. Reprod. Dev.* **74**, 280–9.

Zhao, P., Qiao, J., Huang, S., Zhang, Y., Liu, S., Yan, L.Y., Hsueh, A.J. & Duan, E.K. (2011). Gonadotrophin-induced paracrine regulation of human oocyte maturation by BDNF and GDNF secreted by granulosa cells. *Hum. Reprod.* **26**, 695–702.