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Effects of Differing Oocyte-Secreted Factors During Mouse *In Vitro* Maturation on Subsequent Embryo and Fetal Development

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Short title: Effect of OSFs on mouse oocyte developmental competence

Key Words: oocyte *in vitro* maturation (IVM), GDF9, BMP15, oocyte-secreted factor (OSFs).

Abstract

Purpose: We hypothesised that varying native oocyte-secreted factor (OSF) exposure or using different recombinant OSF peptides would have differential effects on post-*in vitro* maturation (IVM) embryo and fetal development.
Methods: Mouse cumulus oocyte complexes (COCs) were treated with the purified mature domain of GDF9 and/or BMP15 or were co-cultured with denuded oocytes (DOs) from 0h or 3h of IVM. DOs were matured for 3h as either intact COCs+/-FSH before denuding, or as DOs+FSH. COCs were fertilised and blastocyst development was assessed on days 5 and 6, and either differentially stained for ICM numbers or vitrified/warmed embryos were transferred to recipients to assess implantation and fetal rates.
Results: No improvement in embryo development was observed with the addition of GDF9 and/or BMP15 to IVM. In contrast, embryos derived from COCs co-cultured with DOs had significantly improved blastocyst rates and ICM numbers compared to controls ($P < 0.05$). The highest response was obtained when DOs were first added to COCs at 3h of IVM, after being pre-treated (0-3h) as COCs+FSH. Compared to control, co-culture with DOs from 3h did not affect implantation rates but more than doubled fetal yield (21% vs 48%; $P < 0.05$). GDF9 Western blot analysis was unable to detect any differences in quantity or form of GDF9 (17 and 65 kDa) in extracts of DO at 0h or 3h.
Conclusions: This study provides new knowledge on means to improve oocyte quality *in vitro* which has the potential to significantly aid human infertility treatment and animal embryo production technologies.

Introduction

In vitro maturation (IVM) of oocytes is an alternative system used in assisted reproductive technology for generating embryos *in vitro,* which reduces or eliminates the need to administer gonadotrophins to patients. In addition, IVM is widely used in the domestic animal sector to further advanced-breeding technologies. However, there is a discrepancy in the success rate of conventional *in vitro* matured oocytes compared to *in vivo* matured oocytes. In women, it is reported that the pregnancy rate post-IVM is less than half of the pregnancy rate post-IVF (*in vitro* fertilisation) [1]. To overcome this challenge, it is important to understand factors that regulate the maturation and development of oocytes which can be implemented in clinical and veterinary scenarios to improve the success rate of IVM [2].

Oocytes acquire developmental competence in the ovarian follicle. There are numerous molecules, proteins and cellular processes involved in the bi-directional communication axis between the oocyte and follicular somatic cells [3]. The oocyte regulates this communication network to a significant degree. The oocyte secretes soluble factors (OSFs) which are transmitted to cumulus cells via paracrine signalling to regulate a multitude of important cumulus cell processes, including; proliferation [4, 5], apoptosis [6], differentiation [7], luteinisation [8], metabolism [9-12] and expansion [13, 14]. Another type of communication between the oocyte and cumulus cells is via gap junctional signalling, mediated by cumulus trans-zonal cytoplasmic projections that abut the oocyte membrane, enabling transport of cAMP, purines/pyrimidines, amino acids and other small regulatory molecules [15-18]. Cumulus cells provide signals and molecules to the oocyte to regulate meiosis and promote developmental competence [19, 20]. Removal of cumulus cells during maturation has a significant detrimental effect on nuclear maturation, normal fertilization and developmental competence of oocytes [21, 22]. Follicle-stimulating hormone (FSH) is typically added to IVM medium to stimulate cumulus expansion [17] and increase normal fertilization rates and fetal development of IVM oocytes [23, 24].

Growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15) are two well-known soluble growth factors derived from oocytes that are important for normal cumulus cell function and hence normal oocyte development [19]. In mouse oocytes, the expression level of GDF9 mRNA is much higher compared to BMP15 [25], reflecting the relative importance of their roles in folliculogenesis. Homozygous GDF9 mutant mice are sterile due to a block in follicular development beyond the primary follicle stage [26], whereas BMP15 null mice demonstrate only a mild reduction in ovulation and fertilization rates [27]. Homozgous GDF9 or BMP15 mutant sheep derived either by natural mutation or active immunization are sterile, however, heterozygous carriers of mutations in either GDF9 or BMP15 have an increased ovulation rate and multiple pregnancies [28-30]. GDF9 and BMP15 also play an important role in human fertility, where several mutations of GDF9 may be potentially associated with polycystic ovary syndrome [31] and rare variants of GDF9 contribute to dizygotic twinning [32]. Moreover, rare mutations of GDF9 and BMP15 are associated with premature ovarian failure [33-35].

GDF9 and BMP15 in the proteolytically processed form consist of two structural parts: the pro-region and the mature region [36]. GDF9 and BMP15 are processed and secreted as non-covalent complexes of the pro- and mature regions, the mature region being the bioactive receptor binding region, whereby the pro-region plays a vital and species-specific role in regulating bioactivity [37-39]. Immunizing mice against the pro-regions of GDF9 or BMP15 caused ovary abnormalities and smaller litter sizes [40].

However, the exact forms of GDF9 and BMP15 secreted by oocytes *in vivo* and *in vitro* are unclear. Mouse *in vitro* matured oocytes secrete GDF9 as a mixture of the unprocessed pro-protein and mature domain [41]. Rat IVM oocytes secrete the mature domains of GDF9 and BMP15 [42]. Sheep follicular fluid contains GDF9 and BMP15 proteins in the unprocessed pro-protein form only [43], whereas sheep oocytes secrete the mature domains of GDF9 and BMP15 during IVM [42]. To our knowledge, the only commercially available forms of GDF9 and BMP15 produced in a recombinant mammalian cell expression system are from R&D Systems and these consist of purified mature domains only.

IVM COCs have abberant gene expression and altered matrix protein profiles in cumulus cells compared to *in vivo* matured oocytes [44], and *in vitro* matured oocytes also have notably decreased expression of BMP15 compared to *in vivo* matured oocytes (Gilchrist *et al*., unpublished). Currently, studies show that addition of exogenous native OSFs during IVM significantly improves subsequent goat [45], cow [20, 46, 47] and pig [48] embryo development, and recombinant GDF9 and BMP15 in their pro-mature form improve *in vitro* matured cattle [12, 46, 47] and mouse oocytes [49]. In addition, differing degrees of improved bovine oocyte competence were observed depending on the temporal pattern of addition of OSFs during IVM [46]. The current study explores in more detail the effects of different forms of OSFs added during IVM on oocyte developmental competence. We examine the effects of commercially available forms of recombinant GDF9 and BMP15 and of native OSFs. We also explore factors that affect the production of native OSFs by mouse oocytes, such as the timing of addition of denuded oocytes (DOs) and the presence of cumulus cells and FSH before denuding COCs and addition to IVM. Outcomes examined were embryo development, cryotolerance of blastocysts and fetal measures following embryo transfer.

Materials and Methods

All chemicals and media were purchased from Sigma Chemical (St Louis, MO, USA) unless indicated otherwise.

Isolation and maturation of COCs

Mice used in this study were maintained in the Animal House, Medical School, University of Adelaide. This study was approved by the local animal ethics committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Twenty-one to twenty-eight day-old SV129 mice were injected intraperitoneally with 5 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet, Castle Hill, Australia), and ovaries were collected 46-48 hours (h) later. Ovaries were cleaned of any connective tissues and placed in HEPES-buffered αMEM (handling medium, GIBCO, USA) supplemented with 3 mg/ml

fatty-acid free bovine serum albumin (FAF BSA, MP Biomedicals, USA), 1 mg/ml fetuin and 50 µmol 3-isobutyl-1-methylxanthine (IBMX). All antral follicles were punctured with 27-gauge needles and immature COCs collected in handling medium, then washed twice with handling medium minus IBMX. Only COCs with compact cumulus cells (CCs) were taken. The rationale behind the use of IBMX was to maintain all COCs in the same nuclear stage before transfer into maturation medium or being denuded. For the control group, 20 COCs were cultured in 50 µl IVM drops [(αMEM supplemented with 3 mg/ml FAF BSA, 1 mg/ml fetuin and 50 mIU/ml FSH (Puregon, Organon, Oss, Netherlands)] overlaid with mineral oil in 60 mm Petri dishes (Falcon, Becton Dickinson, USA) for 17-18 h at 37^0C in a humidified atmosphere of 5% CO_2 in air. For treatment groups, 20 COCs were cultured with 50 DOs in 50 µl IVM drops (1 DO/µl). In addition, the effect of exogenous recombinant human BMP15 (Catalogue No: 739-G9, R&D Systems, Minnaepolis, MN, USA) and recombinant mouse GDF9 (Catalogue No: 5096-BM, R&D Systems) in mouse IVM was examined. Both of these growth factors are supplied as homodimers of the mature region. COCs were exposed to graded doses (50 ng/ml, 100 ng/ml, and 200 ng/ml) of exogenous BMP15 and GDF9, as well as a combination of both proteins.

Generation of denuded oocytes as a source of native OSFs

DOs were generated by vortexing COCs for ~2 minutes in αMEM handling medium in order to remove the CC.

There were then a number of different processing methods used to produce native OSFs before co-culture with immature COCs (see Table 1 and Fig. 1):

1. COCs were denuded at 0 h before co-culture of 50 DOs with 20 COCs in IVM medium containing FSH. For COCs+DOs, IBMX was not present in the handling medium, and therefore >90% of DOs were at the GVBD stage. This group was utilized to assess the benefits of DOs as a source of native OSFs and will be referred to as COCs+DOs. By contrast, for COCs+DOs (0h [GV]), IBMX was present in the handling medium and therefore these DOs were at the GV stage.
2. COCs were matured first for 3 h in separate IVM medium containing FSH. After 3 h of IVM, COCs were denuded and 50 DOs were co-

cultured with 20 COCs for another 14-15 h in IVM medium containing FSH (Fig. 1a). This group is based on the design by Hussein et al (2011), and was utilized to examine temporal, cumulus cell and FSH effects on the production of native OSFs. This group is referred to as COCs+DOs at 3h+FSH+CC.

3. Intact COCs were matured first for 3 h in separate IVM medium without FSH (Fig. 1b). After 3 h of IVM, COCs were denuded and 50 DOs were co-cultured with 20 COCs for another 14-15 h in IVM medium containing FSH. This group assessed the production of native OSFs in the absence of FSH during the first 3 h of maturation and is referred to as COCs+DOs at 3h-FSH+CC.
4. COCs were denuded at 0 h and then matured for 3 h as DOs in separate IVM medium containing FSH, 50 of these DOs were co-cultured with 20 COCs for another 14-15 h in IVM medium containing FSH (Fig. 1c). This group is referred to as COCs+DOs at 3h+FSH-CC and was designed to assess the effect of CCs on production of native OSFs.

Oocyte nuclear maturation

An experiment was performed to assess whether adding native OSFs affects the timing of oocyte nuclear maturation. At 12 h and 15 h of IVM, COCs were denuded and fixed in paraformaldehyde for 30 minutes, then stained with DAPI. The presence of the first polar body was determined using an upright microscope (Nikon, TE 2000-E) under UV light.

***In vitro* fertilization**

Sperm from CBA F1 male mice aged 6-24 weeks and of proven fertility were incubated for 1 hour in IVF medium (COOK® IVF, Research medium, Catalogue No: K-RVWA- 50, Australia) under 5% CO_2 at 37^0C for capacitation. Post-IVM, COCs were transferred into a 90 µl equilibrated IVF drop overlayed with mineral oil and then 10 µl of capacitated sperm were added into the drops. Three hours post-fertilization under 5% CO_2 at 37^0C, the presumptive zygotes were denuded of sperm and cumulus cells. Insemination day was counted as day 1 (0 h).

In vitro culture of embryos

Five to ten presumptive zygotes were cultured in 20 µl drops of equilibrated culture media (COOK® IVC, Research medium, Catalogue No: K-RVCL-50, Australia) overlayed with mineral oil under 6% CO_2, 5% O_2 and 89% N_2 atmosphere. Embryos were cultured for six days, with cleavage rates assessed on day 2 (24 h post-fertilization) and blastocysts assessed on day 5 (96-100 h post-fertilization) and day 6 (120-24 h post-fertilization).

Differential staining of blastocysts

Differential staining was performed on day 6 blastocysts to assess inner cell mass (ICM) and trophectoderm (TE) cell. Briefly, expanded, hatching and hatched blastocysts were placed into pronase (5 mg/ml) at 37^0C until the zona dissolved. The zona-free blastocysts were incubated in 10 mM trinitrobenzene sulfonic acid (TNBS) in 0.4% polyvinyl alchol (PVA) in phosphate-buffered saline (PBS) at 4^0C for 10 minutes then washed twice before being transferred into 0.1 mg/ml anti dinitrophenol-BSA antibody at 37^0C for 10 minutes. Embryos were then incubated in 10 µg/ml propidium iodide for 5-10 minutes at 37^0C, followed by 4 µg/ml Hoechst 33342 in 96% ethanol at 4^0C overnight. The blastocysts were transferred onto a glass microscope side, covered by a cover slip and assessed immediately under UV fluorescence using an upright microscope (Nikon, TE 2000-E, excitation, 340-380 nm; emission, 440-480 nm), where ICM cells appeared blue and TE cells appeared pink.

Vitrification and warming of blastocysts

Hatching and expanded blastocysts on day 6 were vitrified and then within 1-2 weeks those blastocysts were warmed and assessed for cryotolerance, prior to embryo transfer. Vitrification and warming solutions were prepared in HEPES-buffered αMEM. The vitrification solution consisted of 2.3 M dimethylsulfoxide (DMSO) + 3 M ethylene glycol supplemented with 0.75 M sucrose. The corresponding equilibration solution contained half the concentration of cryoprotectants of the vitrification solution and no sucrose. Vitrification steps were performed at 37^0C. Blastocysts were placed in

equilibration solution for 3 minutes then transferred to vitrification solution, with minimal transfer of equilibration solution (less than 3 µl), placed on a fibre-plug™ (CVM kit, Cryologic, Victoria, Australia) and touched on a pre-cooled steel block in liquid nitrogen, and then sealed in a pre-cooled straw (CVM kit, Cryologic) for storage in liquid nitrogen. The time taken from leaving equilibration solution to vitrification on the block was 30 - 40 sec. Vitrified blastocysts were warmed at 37^0C and sequentially passed through 3 solutions of sucrose (0.3 M, 0.25 M and 0.15 M) in HEPES-buffered αMEM for 5 minutes each. Blastocysts were held in culture medium for 2-3 h and then blastocyst morphology was assessed before being transferred into foster mothers. The criteria used for blastocyst cryosurvival were as follows [50]; normal: blastocysts expanded with no signs of lysis, collapse or degeneration; abnormal: blastocysts with signs of lysis, collapse and degeneration.

Embryo transfer and assessment of fetal outcomes

Vitrified/warmed blastocysts assessed as normal were transferred into pseudopregnant recipients (Swiss female mice aged 12-20 weeks, mated with vasectomised F1 CBA males). On day 3.5 of pseudopregnancy, recipients were anaesthetized with 2% Avertin (0.015 ml/g body weight) prior to embryo transfer. For each recipient, six normal expanded or hatching blastocysts post-vitrification/warming were transferred to each uterine horn, with control embryos in one horn and treatment embryos in the other. Eight replicates were performed with a total of 96 blastocysts transferred into 8 recipients. The number of implantation sites, fetuses, fetal and placental weights and fetal crown to rump lengths were assessed on day 17 post-embryo transfer.

Immunodetection of oocyte GDF9

DOs and oocyte-conditioned media were collected for western blot analysis of the form and quantity of GDF9 protein. Briefly, COCs were collected and denuded at 0 h and suspended at a ratio of 8 DOs/µl in IVM medium in Eppendorf tubes. DOs were matured for 17-18 h under 5% CO_2 at 37^0C. Post-IVM, DOs were pelleted by centrifugation and oocyte-conditioned medium was transferred to a separate tube and snap frozen in PBS. Oocytes were washed once in PBS, after which the PBS was removed and the cells snap

frozen. Oocytes and oocyte-conditioned medium were also collected from the 3h+FSH+CC group (Fig. 1a). COCs were matured first for 3 h in IVM medium, then denuded and matured for an extra 14-15 h in fresh IVM medium. Post-IVM, oocytes and oocyte-conditioned medium were processed as above.

Samples (30-60 DOs or media equivalent) were boiled for 3 minutes in 1xLDS reducing buffer [15ul, 1 x Novex NuPAGE LDS sample buffer (NP0009)] with mercaptoethanol (Novex NuPAGE NP0007, Carlsbad, USA) and centrifuged. The supernatant was fractionated by SDS-PAGE for 1 h at RT on 4-12% Bis-Tris pre-cast gel (BioRad, Hercules, Ca, USA, Cat#345-0125) in MES buffer (Novex, NuPAGE, NP0002) followed by electrotransfer at 30V overnight at 4°C onto a nitrocellulose membrane (Hybond-C Extra, GE Healthcare Life Sciences, Bucks, UK). The membrane was incubated with biotinylated GDF9-monoclonal antibody 53 (Oxford Brookes University, Oxford, UK) at 1:2,000 for 1hr at RT then washed, followed by a further incubation with horseradish peroxidase-conjugated anti-mouse IgG (SNN2004, Lot No 892329B, Biosource), at 1:10,000 for 1 h at RT. Immunoreactive proteins were detected using Lumilight chemiluminesence reagents (Roche Diagnostics, GmbH Mannheim, Germany). Membranes were scanned on the BioRad ChemiDoc MP system and the images analysed using Image J software (Biorad). Recombinant mGDF9 (17k) was used as a reference protein. Biotinylated molecular wt protein standards (Cat No. MW001) were obtained from R&D Systems, (Minneapolis, USA). Parallel controls (blanks) were undertaken with oocyte extracts in the absence of biotinylated antibody but in the presence of HRP-IgG antibody.

Statistical analyses

All proportional data for embryo development were arcsine transformed and analysed using either an independent t-test or multivariate ANOVA with least significant difference (LSD) post-hoc tests. Log transformation was performed for blastocyst cell number when data were not normally distributed, and analysed using an independent t-test or one way ANOVA with either LSD or Dunnet's T3 post-hoc tests, depending on whether the data did or did not pass the homogeneity of variance test, respectively. Fetal measurements were analysed with independent t-tests with LSD post-hoc tests. Oocyte

nuclear maturation, survival rate post-warming of blastocysts and post-embryo transfer data for fetal survival and implantation rates were assessed using a Chi-square test. All statistical analyses were performed using SPSS version 13 (SPSS Inc, Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$. Data are expressed as means ± SEM.

Results

Effect of co-culture of intact mouse COCs with native OSFs during IVM

Exposure of intact COCs to exogenous native OSFs from DOs throughout IVM significantly ($P < 0.05$) increased the cleavage rate compared to COCs cultured alone. ICM cell numbers were also significantly increased in blastocysts derived from COCs exposed to DOs, whereas TE and total cell numbers were not affected (Table 2).

Effect of mature form of GDF9 and BMP15 (R&D Systems) on mouse oocyte developmental competence

In contrast to the effect of native OSFs, improvements in embryo development were not observed with the addition of mature domain GDF9 (Table 3) or mature BMP15 (Table 4) to IVM medium at 50 ng/ml, 100 ng/ml or 200 ng/ml, or a combination of both proteins at the same doses (Table 5).

Temporal effect of co-culture of intact mouse COCs with native OSFs during IVM

A previous study by [46] demonstrated that there is a temporal effect of native OSFs on bovine oocyte developmental competence. Based on this result, we assessed mouse oocyte developmental competence following co-culture of COCs with DOs in a temporal manner. COCs were co-cultured with DOs from 0 h (0h [GV] stage) or with DOs precultured as COCs for 3h+FSH+CC (refer to Fig. 1a). Following co-culture of COCs with DOs from 0-17 h (0h [GV] stage), a significant increase was observed in cleavage rate compared to controls, however there were no significant differences in blastocyst rates or ICM number (Table 6). By contrast, treating COCs with DOs from 3h (3h+FSH+CC) significantly ($P < 0.05$) increased blastocyst and hatching

blastocyst rates measured at day 6 and subsequent ICM cell numbers, compared to the control group.

Effect of exposure of COCs to native OSFs on nuclear maturation

Native OSFs regulate CC cGMP levels [51] which affects meiosis and, according to [20], co-culture of COCs with DOs increased nuclear maturation rate in bovine oocytes. Since the highest competence treatment group from the previous experiment was COCs co-cultured with DOs from 3h (3h+FSH+CC), we hypothesised that an effect on oocyte nuclear maturation could account for this enhanced oocyte quality in mice. However, in the present experiment, there was no significant difference in the maturation rates of control oocytes or COCs exposed to DOs (3h+FSH+CC) at 12h or 15h of IVM (Table 7).

Temporal effects on oocyte GDF9 levels

Given the beneficial effects of pre-culture of oocytes with their CCs for 3 h with FSH on OSF-stimulated oocyte developmental competence (Table 6), it was attempted to quantify the amount and form of GDF9 expressed within and secreted by oocytes. The band for the un-processed pro-protein was observed at ~65 kDa and the mature protein at ~17 kDa, with the mature protein less abundant in oocyte extracts (Fig. 2). Three hours of pre-culture (3h+FSH+CC) had no effect on the total quantity or the proportion of pro- versus mature-GDF9 in oocytes. No GDF9 bands were observed in the DO conditioned media.

The role of FSH and cumulus cells in the production of native OSFs

We hypothesized that FSH and cumulus cells are two factors that affect the production of native OSFs. In this experiment, DOs were generated using different methods (see Fig. 1). Following co-culture of COCs with precultured DOs (3h+FSH+CC; see Fig. 1a), a significantly increased blastocyst rate on day 5 was observed compared to control, which contributed to an improvement in ICM number compared to control (Table 8). DOs that were pre-cultured as intact COCs but without FSH (3h-FSH+CC, Fig. 1b) did not stimulate developmental competence in their co-cultured COCs; these

exhibited embryo developmental parameters equivalent to the control and ICM numbers significantly lower than those stimulated with FSH (3h+FSH+CC). Interestingly, there was a significant improvement in blastocyst rate on day 6 from COCs co-cultured with DOs that were without CCs from 0-3h (3h+FSH-CC, see Fig. 1c) compared to the control. Overall, all treatment groups co-cultured with DOs tended to show an improvement in embryo development.

Effect of exposure of COCs to native OSFs during IVM on cryo-survival of blastocysts

With respect to cryopreserved blastocysts, the blastocyst re-expansion rate post-vitrification/warming (normal morphology of blastocysts) did not differ significantly from control for blastocysts derived from COCs exposed to native OSFs (COCs+DOs at 3h+FSH+CC; Table 9). Therefore exposure of COCs to native OSFs did not measurably alter the cryotolerance of blastocysts.

Effect of exposure of COCs to native OSFs on pregnancy rates and fetal outcomes

After 2 h of warming, the morphologically normal blastocysts were transferred into pseudo pregnant recipients. There was no difference in implantation rates between the control and treatment group (COCs co-cultured with DOs [3h+FSH+CC]). However, the proportion of fetuses that developed to day 17 per implantation site, from COCs co-cultured with native OSFs, was more than double that of the control (21% vs 48%, $P < 0.05$, Fig. 3). Moreover, based on gross morphological criteria, those fetuses were normal, with no significant differences between treatments in fetal or placental weight or fetal crown-to-rump length (Table 10).

Discussion

Our results show that treatment of COCs during IVM with native OSFs, enhances oocyte quality, as measured by subsequent embryo cleavage, blastocyst and fetal survival rates. These results are supported by previous studies which have shown that exogenous native OSFs (obtained by co-

culture with DOs), when used as supplements in oocyte IVM, significantly improve embryo development in cattle [20, 46, 47], pigs [48] and goats [45]. In contrast, addition of recombinant mature homodimers of GDF9 and/or BMP15 (R&D Systems) in IVM media did not improve mouse embryo development (current study). However, previous results from our laboratory using a complex of pro- and mature-domains of GDF9 and BMP15 (pro-mature protein) showed a positive effect on bovine as [12, 46, 47] well as mouse [49] oocyte developmental competence.

From this study, we suggest that there are a number of factors which influence the capacity of exogenous OSFs to improve mouse embryo and fetal development post-IVM namely; the form of recombinant OSFs, the capacity of cumulus cells and FSH to regulate native OSF production, and the temporally regulated potency of the native OSF pool. Supplementation of IVM with OSFs has the potential to dramatically improve the efficiency of IVM and therefore its applicability in human and veterinary clinics [2], as we saw more than a doubling in fetal yield using this approach. However, this was only achieved using native and not recombinant OSFs, and while this illustrates the principal clearly, co-culture with denuded oocytes is not practical in a clinical scenario. Even though the exact identity of growth factors secreted by denuded oocytes *in vitro* remains poorly characterised, the principal OSFs are thought to be GDF9, BMP15, BMP6 fibroblast growth factor 8 (FGF8) and FGF10, with widely differing roles for individual growth factors between species [11, 26, 28-30, 41, 42, 52, 53]. Notably, the only commercially available recombinant preparations of GDF9 and BMP15 produced in mammalian cells, had no effect on post-IVM embryo development, despite the extensive dose range tested. By contrast, recombinant FGF10 added during bovine IVM enhances subsequent embryo development [52]. We hypothesize that the failure of these preparations of GDF9 and BMP15 to improve mouse IVM is because these proteins are supplied as isolated homodimers of their mature regions, lacking their prodomains. For the same reason we would predict that the GDF9 and BMP15 preparations produced in *E.coli* would be ineffective in IVM. GDF9 and BMP15 are likely to form a heterodimer, although so far the production of a recombinant preparation has proved controversial [55, 56] and it has not yet been tested in IVM.

When full length recombinant GDF9 and BMP15 are expressed in mammalian cells such as human embryonic kidney 293H cells, the proteins are processed (pro and mature domains are proteolytically cleaved) and secreted into the medium as dimers or monomers of pro- and mature regions, that are non-covalently bound as pro-mature complexes [36]. Our in-house produced GDF9 and BMP15 preparations [41, 54], which contain both the pro- and mature domains, when added to IVM, significantly improve the quality of the blastocysts generated (as measured by ICM cell number) and thus improve fetal survival rates in mouse [49], as well as increase embryo development in cattle [12, 46, 47].

The current results demonstrated that blastocyst development and fetal survival rates were further improved when COCs were co-cultured with DOs from 3 h (versus control), if those DOs were matured as COCs for the first 3 hours in the presence of FSH, then subsequently denuded and added to IVM. This result supports previous findings in bovine IVM oocytes, which suggested a temporal effect on secretion of OSFs [46]. It has been demonstrated that even a short exposure of COCs to FSH significantly increases subsequent bovine blastocyst development, via protein kinase C activation [23]. Hence, the FSH-treated CCs may in some manner improve the quantity and/or quality of factors which are secreted by the resultant DO. During the 3 h maturation, the oocyte and cumulus cells communicate with each other through gap junctional communication and via paracrine factors, which presumably provides enough time for the oocyte to obtain the beneficial effect of FSH through the receptors in cumulus cells. This effect may be mediated more by paracrine factors rather than gap junctional communication. After meiotic resumption, gap junctional communication between the oocyte and cumulus cells is lost [18]. The action of OSFs can be observed even without direct contact between cumulus cells and oocytes, suggesting that they are acting in a paracrine manner [6]. In the present study, the cleavage rate of mouse DOs post co-culture with COCs was significantly higher compared to DOs cultured alone (result not shown). In cattle, the developmental competence of DOs co-cultured with COCs is significantly improved compared to DOs cultured alone [20, 47].

Based on the notable improvement in oocyte developmental competence from the 3h+FSH+CC group, we anticipated that COCs matured first for 3 h in medium containing FSH would produce different quantities and/or forms of GDF9, compared to oocytes denuded at 0 h. However, unfortunately we were unable to detect any GDF9 in oocyte-conditioned medium, unlike the recent study by Lin et al (2012). This may be due to our oocyte-conditioning medium procedures or the western blot being insufficiently sensitive to detect actual secreted forms. Also, we could not observe any effect of the 3 h pre-culture with FSH treated cumulus cells on the GDF9 produced within the oocyte. It showed that there was no difference in the amount or the proportion of either the pro-protein or the mature form of GDF9 in DOs at 0 h or 3 h. While GDF9 is the principal OSF produced by mouse oocytes [25, 41], other OSFs such as BMP15 [57], BMP6 [53], FGF10 [52] and FGF8 [11]. may be processed and interact differently in DOs at 0 h and 3 h.

Overall, we have shown that there is an obvious advantage to treatment of COCs with exogenous OSFs during IVM. Mouse exogenous OSFs did not affect oocyte nuclear maturation, in contrast with bovine oocytes matured *in vitro*, where co-culture with DOs improves nuclear maturation [20]. Clearly exogenous OSFs have major effects on key aspects governing the developmental program of oocytes, although it is not yet clear how and by which exact mechanisms this is achieved. Other groups have shown that native OSFs play a significant role in; the activity of hyaluronic acid synthase 2 (*HAS2*), which correlates with mucification and expansion of CCs [13], increased *GPX1* gene expression (related to the production of glutathione peroxidise as an antioxidant in oocytes), and enhanced expression of steroidogenic acute regulatory protein (STAR), which contributes to oocyte nuclear and cytoplasmic maturation [20]. Bovine oocytes matured with the mature form of BMP15 showed a significant increase in glucose uptake [9], and those matured with pro-mature BMP15 exhibited increased oxidative phosphorylation activity in the oocyte [12].

The results of this current study confirm that OSFs notably improve embryo development and fetal survival in mice, and extend this knowledge by demonstrating for the first time that the effect of OSFs on mouse oocytes

depends on timing, presence of FSH and cumulus cells. The observation that recombinant GDF9 and BMP15 in their mature form do not improve mouse embryo development leads us to the conclusion that the mature forms of those growth factors is not the functional form that improves the quality of IVM mouse oocytes. The beneficial effect of native OSFs on oocyte quality and subsequent embryo development can be applied in the veterinary clinic; however, application of co-culture of COCs with DOs is not possible in human IVF clinics since it is unethical and not feasible to generate supernumerary DOs simply for OSF supplementation of media. Thus, the next step is to identify and purify the proteins that are secreted by oocytes, such as GDF9 and BMP15, into a functional form that can be added into IVM medium, to improve the quality of IVM oocytes and subsequent embryo development and pregnancy rates.

Conflict of Interest

The University of Adelaide owns a patent family on the applications of GDF9 and BMP15 in oocyte in vitro maturation. RBG and JGT are inventors.

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Figure Legends:

Fig. 1

(**a**) Schematic illustration of methods of producing native OSFs based on pretreatment of oocytes with and without cumulus cells and FSH

Intact COCs cultured for 3 h in separate IVM medium containing FSH. After 3 h, COCs were denuded of CCs and the resultant DOs were then co-cultured with a separate cohort of COCs + FSH for 14-15 h, before the COCs underwent standard IVF and embryo culture (3h+FSH+CC) (**b**) COCs pre-cultured for 3 h in separate IVM medium without FSH, before denuding and co-cultured with a separate cohort of COCs + FSH for 14-15 h (3h-FSH+CC) (**c**) COCs were denuded at 0 h and then pre-cultured for 3 h in separate IVM medium containing FSH, then these DOs were co-cultured with a separate cohort of COCs + FSH for 14-15 h (3h+FSH-CC)

Fig. 2

(**a**) GDF9 Western blot comparison of denuded oocyte (DO) extracts at 0h and 3h (3h+FSH+CC) of pretreatment. mGDF9 refers to recombinant mGDF9 standard. Blank refers to DO sample in the absence of the GDF9 primary antibody. The 65kDa band corresponds to the unprocessed GDF9 pro-protein while the 17kDa band corresponds to the mature domain. The 90 kDa-180 kDa bands seen in all samples are attributed to the non-specific presence of biotinylated proteins in the cell extracts

(**b**) Quantitative analysis of Western blot denuded oocyte extracts at 0 and 3h (3h+FSH+CC) of pretreatment from 5 separate experiments. The Western blot image was quantified using BioRad ChemiDoc MP system and analysed using Image J software (Biorad)

Fig. 3

Effect of treatment with native OSFs during IVM on subsequent pregnancy outcomes. Blastocysts were derived from standard IVM (control) or from COCs exposed to native OSFs during IVM (COC+DO; 3h+FSH+CC pretreatment). Control and treatment blastocysts were vitrified on day 6 and then later warmed and transferred to pseudo pregnant recipients. Six control and six treatment blastocysts were transferred into each uterine horn of a recipient (n=8). Pregnancy outcomes were analysed on day 17. Implantation rate; implantation sites/embryos transferred (n = 48 embryos transferred/treatment). Fetal yield; day 17 fetuses/implantation sites

Table 1 Summary of methods used to generate differing native OSFs

Treatments	Pre-treatment of OSFs	Explanation
Control	not applicable	20 COCs were cultured in 50 µl IVM drops
COC + DO	not applicable	20 COCs were cultured with 50 DOs in 50 µl IVM drops. DOs were added at 0 h at the GVBD stage.
COC + DO	0h [GV]	20 COCs were cultured with 50 DOs in 50 µl IVM drops. DOs were added at 0 h at the GV stage, as IBMX was present in the handling medium and during denuding.
COC + DO	3h+FSH+CCs	COCs were pre-cultured for 3 h in separate IVM medium containing FSH. After 3 h, these COCs were denuded and 50 resultant DOs were then co-cultured with 20 COCs for another 14-15 h (Fig. 1a).
COC + DO	3h-FSH+CCs	COCs were pre-cultured for 3 h in separate IVM medium without FSH, before denuding and co-culture with 20 COCs for another 14-15 h (Fig. 1b).
COC + DO	3h+FSH-CC	COCs were denuded at 0 h and then cultured for 3 h as DOs in separate IVM medium containing FSH, then 50 resultant DOs were co-cultured with 20 COCs for another 14-15 h (Fig. 1c).

Table 2 Effect of native OSFs during IVM on subsequent embryo development

Treatments	Number of oocytes	Cleavage[1]	Blastocyst on day 5[2]	Blastocyst on day 6[3]	Hatching blastocyst on day 6[4]	ICM[5]	TE[5]	TCN[5]
Control	224	90.6 ± 1.8[a]	52.8 ± 8.9	77.3 ± 4.6	58.8 ± 8.6	18.5 ± 0.8[a]	55.8 ± 2.1	74.3 ± 2.6
COC + DO[6]	233	96.1 ± 0.8[b]	60.1 ± 4.9	74.3 ± 2.4	68.0 ± 1.9	25.3 ± 1.5[b]	55.5 ± 2.5	80.8 ± 3.5

Values with different superscripts within a column are statistically different ($P < 0.05$)

Data are presented as means ± SEM of 4 replicate experiments. Each replicate experiment consisted of 53-60 oocytes.

[1] Percentage of cleaved embryos per total oocytes

[2] Percentage of blastocysts generated 96-100 h post-fertilization per cleaved embryo

[3] Percentage of blastocysts generated 100-125 h post-fertilization per cleaved embryo

[4] Percentage of hatching blastocysts generated 100-125 h post fertilization per cleaved embryo

[5] Mean blastocyst cell numbers following differential staining of 16 – 18 blastocysts

[6] GVBD-stage denuded oocytes (DO) added from t = 0h of IVM

Table 3 Effect of graded doses of recombinant GDF9 during IVM on subsequent embryo development

Treatment	GDF9 (ng/ml)	Number of oocytes	Cleavage[1]	Blastocyst on day 6[2]	Hatching blastocyst on day 6[3]	ICM[4]	TE[4]	TCN[4]
Control	0	152	82.9 ± 3.3	81.7 ± 4.4	62.6 ± 8.6	16.0 ± 0.8	53.0 ± 2.4	69.0 ± 2.8
GDF9	50	118	74.6 ± 5.2	72.6 ± 8.9	61.8 ± 8.9	16.8 ± 1.1	54.2 ± 3.6	71.0 ± 4.4
GDF9	100	118	74.8 ± 9.6	82.6 ± 7.8	59.1 ± 7.6	16.7 ± 0.9	49.2 ± 2.6	65.9 ± 3.3
GDF9	200	147	86.1 ± 4.7	82.5 ± 8.3	64.3 ± 7.1	17.9 ± 0.7	56.8 ± 3.5	74.8 ± 3.9

Data are presented as means ± SEM of 5 replicate experiments. Each replicate experiment consisted of 10-50 oocytes.

[1] Percentage of cleaved embryos per total oocytes

[2] Percentage of blastocysts generated 100-125 h post-fertilization per cleaved embryo

[3] Percentage of hatching blastocysts generated 100-125 h post fertilization per cleaved embryo

[4] Mean blastocyst cell numbers following differential staining of 28 – 51 blastocysts

Table 4 Effect of graded doses of BMP15 during IVM on subsequent embryo development

Treatment	BMP15 (ng/ml)	Number of oocytes	Cleavage[1]	Blastocyst on day 6[2]	Hatching blastocyst on day 6[3]	ICM[4]	TE[4]	TCN[4]
Control	0	241	66.6 ± 8.6	64.8 ± 8.0	44.3 ± 8.2	16.2 ± 1.0	50.7 ± 2.8	66.9 ± 3.4
BMP15	50	193	77.7 ± 4.7	56.4 ± 8.8	36.5 ± 9.5	16.4 ± 0.7	48.3 ± 2.4	64.8 ± 3.0
BMP15	100	200	75.8 ± 8.5	65.1 ± 7.9	45.0 ± 9.1	17.6 ± 1.1	49.1 ± 2.4	66.7 ± 3.1
BMP15	200	187	76.3 ± 7.1	63.4 ± 8.1	43.1 ± 8.8	18.9 ± 0.8	55.1 ± 2.1	74.0 ± 2.5

Data are presented as means ± SEM of 7 replicate experiments. Each replicate experiment consisted of 14-75 oocytes.
[1]Percentage of cleaved embryos per total oocytes
[2]Percentage of blastocysts generated 100-125 h post-fertilization per cleaved embryo
[3]Percentage of hatching blastocysts generated 100-125 h post fertilization per cleaved embryo
[4]Number of blastocysts staining in each treatment group 38-47

Table 5 Effect of graded doses of GDF9 and BMP15 during IVM on subsequent embryo development

Treatment	GDF9 + BMP15 (ng/ml)	Number of oocytes	Cleavage[1]	Blastocyst on day 6[2]	Hatching blastocyst on day 6[3]	ICM[4]	TE[4]	TCN[4]
Control	0 + 0	78	92.0 ± 1.3	85.4 ± 5.7	84.2 ± 6.9	12.9 ± 0.8	42.8 ± 1.7	55.7 ± 1.9
BMP15 + GDF9	50 + 50	88	91.5 ± 2.7	82.6 ± 9.5	80.5 ± 11.5	12.7 ± 0.7	43.5 ± 2.6	56.2 ± 2.9
BMP15 + GDF9	100 + 100	86	93.2 ± 0.4	78.6 ± 10.8	75.2 ± 11.3	12.9 ± 1.0	37.9 ± 2.1	50.8 ± 2.1
BMP15 + GDF9	200 + 200	85	89.7 ± 5.2	72.3 ± 7.1	67.0 ± 7.6	11.8 ± 1.0	45.5 ± 2.1	57.3 ± 2.7

Data are presented as means ± SEM of 3 replicate experiments. Each replicate experiment consisted of 12-40 oocytes.

[1]Percentage of cleaved embryos per total oocytes

[2]Percentage of blastocysts generated 100-125 h post-fertilization per cleaved embryo

[3]Percentage of hatching blastocysts generated 100-125 h post fertilization per cleaved embryo

[4]Number of blastocysts staining in each treatment group 14-19

Table 6 Effects of exogenous native OSFs during IVM on subsequent embryo development

Treatments	Pre-treatment of OSFs[1]	Number of oocytes	Cleavage[2]	Blastocyst on day 5[3]	Blastocyst on day 6[4]	Hatching blastocyst on day 6[5]	ICM[6]	TE[6]	TCN[6]
Control	N/A	152	87.5 ± 1.6[a]	52.2 ± 7.2	67.4 ± 6.0[a]	53.3 ± 5.6[a]	18.7 ± 1.9[a]	51.1 ± 3.4	69.8 ± 4.3
COC + DO	0h [GV]	155	95.5 ± 2.2[b]	57.5 ± 2.6	77.2 ± 4.4[a,b]	66.9 ± 2.8[a,b]	22.0 ± 1.7[a,b]	52.4 ± 2.4	74.4 ± 3.5
COC + DO	3h+FSH+CCs	153	94.8 ± 1.1[a,b]	65.5 ± 1.7	83.4 ± 2.6[b]	74.5 ± 2.8[b]	24.8 ± 1.3[b]	55.7 ± 2.7	80.5 ± 3.4

Values with different superscripts within a column are statistically different ($P < 0.05$)

Data are presented as means ± SEM of 4 replicate experiments. Each replicate experiment consisted of 37-40 oocytes.

[1]See Figure 1 and Table 1 for explanation of experimental design

[2]Percentage of cleaved embryos per total oocytes

[3]Percentage of blastocysts generated 96-100 h post-fertilization per cleaved embryo

[4] Percentage of blastocysts generated 100-125 h post-fertilization per cleaved embryo

[5] Percentage of hatching blastocysts generated 100-125 h post fertilization per cleaved embryo

[6] Mean blastocyst cell numbers following differential staining of 13-22 blastocysts

Table 7 Effect of native OSFs added at 3 hours on oocyte nuclear maturation

Treatments	Pre-treatment of OSFs[1]	Maturation time (h)	n	MII oocytes (%)
Control	N/A	12	111	92.8
		15	74	91.9
COC + DO	3h + FSH + CCs	12	113	93.8
		15	78	93.6

Data are presented as means of 3 replicate experiments. Each replicate experiment consisted of 33-39 oocytes.
[1]See Figure 1 and Table 1 for explanation of experimental design

Table 8 Effects of cumulus cells and FSH on native OSF efficacy at enhancing post-IVM embryo development

Treatments	Pre-treatment OSFs[1]	Number of oocytes	Cleavage[2]	Blastocyst on day 5[3]	Blastocyst on day 6[4]	Hatching Blastocyst on d6[5]	ICM[6]	TE[6]	TCN[6]
Control	N/A	192	92.7 ± 2.9	52.5 ± 3.4[a]	76.6 ± 3.0[a]	60.8 ± 3.3	11.9 ± 0.6[a]	43.1 ± 2.2	55.0 ± 2.4
COC + DO	3h+FSH+CCs	191	93.6 ± 1.9	65.2 ± 5.6[b]	83.2 ± 2.0[a,b]	69.0 ± 3.4	13.8 ± 0.5[b]	43.6 ± 1.5	57.4 ± 1.6
COC + DO	3h-FSH+CCs	190	94.7 ± 1.9	61.0 ± 3.6[a,b]	79.5 ± 2.5[a,b]	66.7 ± 4.0	11.7 ± 0.5[a]	46.4 ± 1.9	58.2 ± 2.0
COC + DO	3h+FSH-CCs	191	93.7 ± 2.5	63.8 ± 3.5[a,b]	86.2 ± 2.9[b]	70.4 ± 2.7	12.2 ± 0.7[a,b]	44.1 ± 2.0	56.3 ± 2.4

Values with different superscripts within a column are statistically different ($P < 0.05$)

Data are presented as means ± SEM of 5 replicate experiments. Each replicate experiment consisted of 36-39 oocytes.

[1]See Figure 1 and Table 1 for explanation of experimental design

[2]Percentage of cleaved embryos per total oocytes

[3]Percentage of blastocysts generated 96-100 h post-fertilization per cleaved embryo

[4] Percentage of blastocysts generated 100-125 h post-fertilization per cleaved embryo

[5] Percentage of hatching blastocysts generated 100-125 h post fertilization per cleaved embryo

[6] Mean blastocyst cell numbers following differential staining of 33-42 blastocysts

Table 9 Effect of native OSFs during IVM on survival rate post-warming of blastocysts

Treatments	Pre-treatment OSFs[1]	Numbers of blastocysts	Normal (%)	Abnormal (%)
Control	N/A	90	73 (81.1)	17 (18.9)
COC + DO	3h+FSH+CC	92	74 (80.4)	18 (19.6)

Data are presented as means of 6 replicate experiments. Each replicate experiment consisted of 10-19 blastocysts.
[1]See Figure 1 and Table 1 for explanation of experimental design

Table 10 Effect of native OSFs during IVM on subsequent fetal and placental weight and crown-rump length

Treatments	Pre-treatment OSFs[1]	Placental weight (g)	Fetal weight (g)	Crown to rump length (mm)
Control	N/A	0.2 ± 0.01	1.8 ± 0.1	24.5 ± 1.9
COC + DO	3h+FSH+CC	0.2 ± 0.01	1.7 ± 0.1	25.6 ± 0.8

Data are presented as means ± SEM of 6 and 13 fetuses (control and treatment, respectively) from 8 recipients.
[1]See Figure 1 and Table 1 for explanation of experimental design

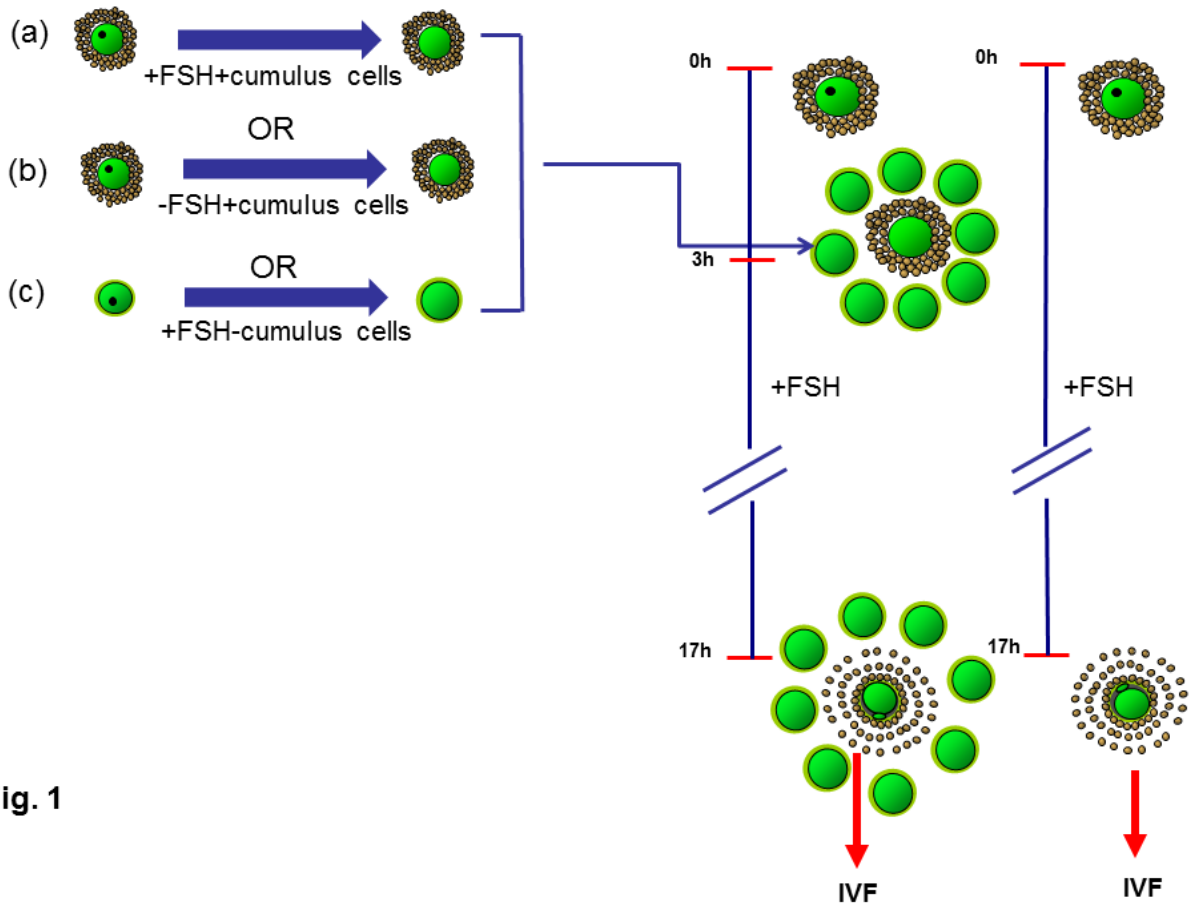

(a)
+FSH+cumulus cells
OR
(b)
-FSH+cumulus cells
OR
(c)
+FSH-cumulus cells
0h
3h
+FSH
17h
IVF
0h
+FSH
17h
IVF

Fig. 1

Fig. 2 (a)

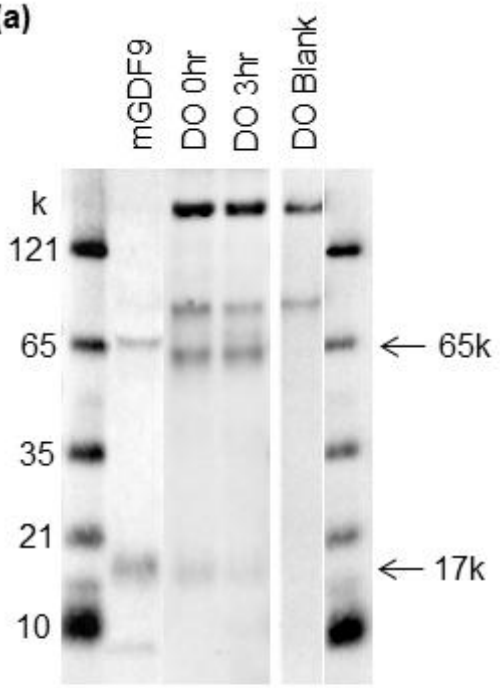

mGDF9
DO 0hr
DO 3hr
DO Blank
k
121
65
35
21
10
65k
17k

Fig. 2 (b)

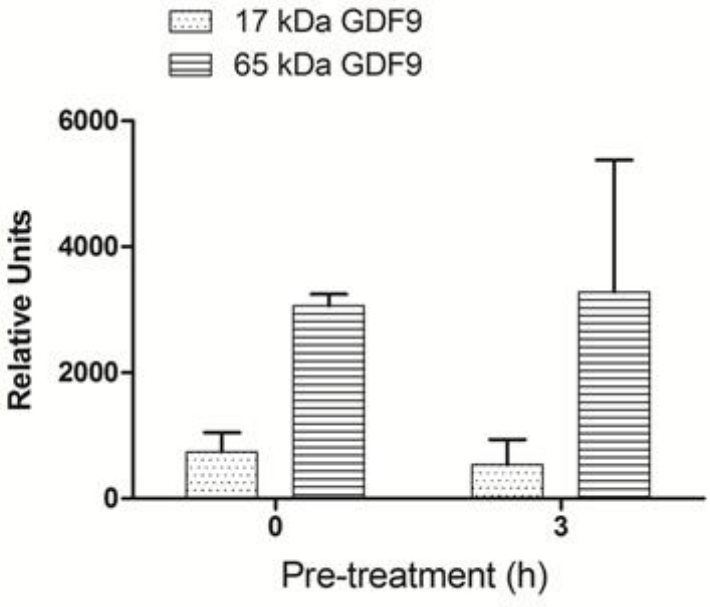
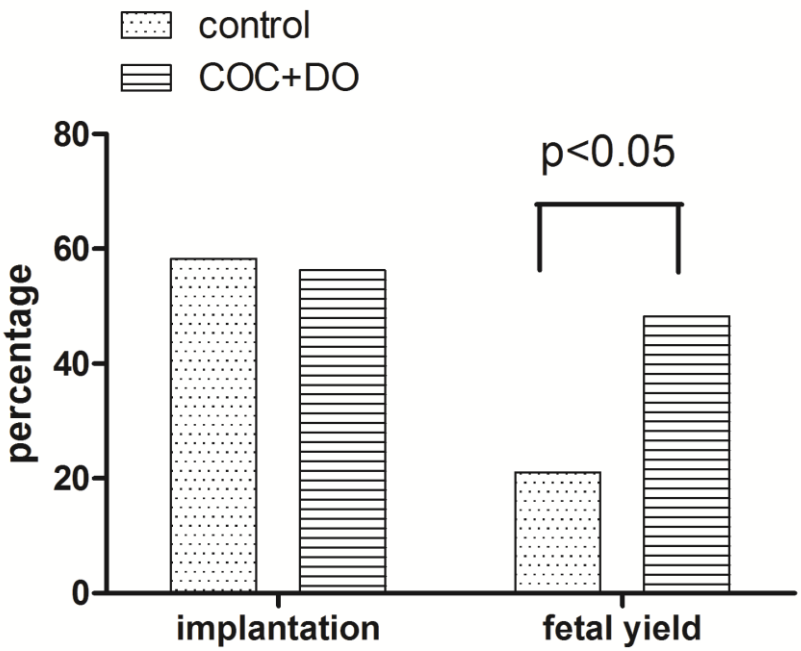

17 kDa GDF9
65 kDa GDF9
6000
4000
2000
0
Relative Units
0
3
Pre-treatment (h)

Fig. 3


control
COC+DO
p<0.05
80
60
40
20
0
percentage
implantation
fetal yield