Exogenous growth differentiation factor 9 in oocyte maturation media enhances subsequent embryo development and fetal viability in mice

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BACKGROUND: Successful oocyte in vitro maturation (IVM) would eliminate the need for hormonal stimulation used in assisted reproduction techniques. Unfortunately, oocytes matured in vitro have compromised developmental competence possibly due to disrupted oocyte–cumulus communication resulting from inappropriate levels of oocyte-secreted factors such as growth differentiation factor 9 (GDF9). Hence, the aim of this study was to investigate the effects of exogenous GDF9 during IVM of mouse oocytes on development and subsequent fetal viability.

METHODS: Cumulus–oocyte complexes from pregnant mare’s serum gonadotrophin primed mice were cultured with or without 200 ng/ml exogenous recombinant GDF9, 50 mIU/ml FSH and 10 ng/ml epidermal growth factor (EGF). After 18 h, cumulus expansion was scored and oocytes were fertilized in vitro. Cleavage, blastocyst development, blastocyst total, inner cell mass (ICM) and trophectoderm cell numbers were assessed. Viability of embryos was assessed by transfer to recipient females and pregnancy outcome determined at day 15. RESULTS: Oocytes matured with exogenous GDF9 in the presence of FSH and EGF had higher rates of development, percentage of hatching blastocyst and blastocyst total and ICM cell numbers (all P<0.05). Although implantation rate and fetal and placental weights were not affected, the number of viable fetuses at day 15 was increased with exogenous GDF9. CONCLUSIONS: Exogenous GDF9 during IVM improved embryo development and fetal viability and provides a promising approach for human IVM.

Keywords: blastocyst; cumulus cells; IVF; in vitro maturation; oocyte-secreted factors

Introduction

In vitro maturation (IVM) of oocytes poses an attractive alternative to the use of exogenous hormonal stimulation for assisted reproduction techniques which has a number of adverse side effects, the most serious being ovarian hyper-stimulation syndrome. Unfortunately, oocytes matured in vitro result in embryos with compromised development compared with oocytes matured in vivo (Combelles et al., 2002; Dieleman et al., 2002) and result in lower pregnancy rates per embryo transfer (Chian et al., 2004; Le Du et al., 2005; Soderstrom-Anttila et al., 2006). Hence, there is a great need to improve our understanding of what regulates an oocyte’s developmental potential, as well as to improve oocyte IVM efficiency.

In a pre-ovulatory follicle, fully grown meiotically competent oocytes are surrounded by a group of highly specialized somatic cells, called cumulus cells, and are collectively known as cumulus–oocyte complexes (COCs). Oocyte development and viability are highly dependent on these cumulus cells as they provide the oocyte with essential metabolic substrates (Leese and Barton, 1985; Downs and Utecht, 1999) and amino acids (Eppig et al., 2005) and suppress and subsequently promote meiotic progression (Tanghe et al., 2002). Although the oocyte has been previously thought to be a passive recipient of cumulus cell functions, it is now increasingly evident that the oocyte is not inactive but more likely a principal regulator of its own developmental competence. Oocytes control ovarian folliculogenesis (Eppig et al., 2002) and play key regulatory roles on cumulus cell functions such as differentiation, metabolism, expansion and gene expression, which in turn are necessary for oocyte survival and successful ovulation [reviews; (Eppig, 2001; Gilchrist et al., 2004a; Sugiura and Eppig, 2005)]. This bi-directional oocyte–cumulus cell paracrine regulatory loop is likely to play an important role in the acquisition of oocyte developmental competence and subsequent embryogenesis. IVM routinely involves the transfer of COCs from their enclosed follicular environment into culture media, which potentially compromises the complexity.
of somatic–oocyte cellular interactions that occur in vivo. Thus, the reduced developmental competence of IVM oocytes may possibly be attributed to inappropriate levels or composition of oocyte-secreted factors. Our laboratory has recently tested this hypothesis and demonstrated that addition of native or recombinant oocyte-secreted factors during bovine IVM improved post-IVF preimplantation embryo development, although subsequent developmental potential (post-transfer) was not examined (Hussein et al., 2006).

The specific oocyte-secreted factors that are involved in this bi-directional regulatory loop remain currently unclear. A prime candidate is growth differentiation factor 9 (GDF9), a member of the transforming growth factor beta (TGFβ) superfamily. GDF9 is an oocyte-specific paracrine factor which is expressed throughout most stages of folliculogenesis (McGrath et al., 1995; Elvin et al., 1999a) and persists after fertilization through preimplantation embryo development (Pennetier et al., 2004). Previous studies have demonstrated the ability of GDF9 to mimic certain oocyte functions in the absence of the oocyte, including regulating cumulus cell progestosterone synthesis (Elvin et al., 2000), suppressing LH receptor expression (Elvin et al., 1999a), promoting granulosa cell growth (Gilchrist et al., 2006) and regulating cumulus expansion (Elvin et al., 1999a; Dragovic et al., 2005). These known regulatory roles demonstrate that GDF9 is a key regulator of normal cumulus cell function and therefore imply that GDF9 is probably a key element in the oocyte–cumulus regulatory loop.

FSH and epidermal growth factor (EGF) are routine additives during IVM as both have long been shown to improve oocyte developmental competence (Downs et al., 1988; Merriman et al., 1998). Like oocyte-secreted factors, either component is necessary for cumulus expansion in the mouse (Vanderhyden et al., 1990; Park et al., 2004). Both EGF and FSH stimulate the mitogen activated protein (MAP) kinase pathway, subsequently leading to oocyte meiotic maturation (Su et al., 2002; Farin et al., 2007), and similarly recombinant GDF9 has also been shown to activate the MAP kinase pathway independent of FSH yet had no effect on oocyte meiotic maturation (Su et al., 2003).

Hence, the aim of this study was to investigate the effects of exogenous GDF9 during mouse IVM both in the presence and absence of FSH and EGF on subsequent embryonic development and fetal outcomes.

**Materials and Methods**

All chemicals and reagents were purchased from Sigma (St Louis, MO, USA) unless otherwise specified. Recombinant mouse GDF9 was produced and partially purified as previously described (Gilchrist et al., 2004b; Dragovic et al., 2005; Hickey et al., 2005; Hussein et al., 2005). Briefly, human embryonic kidney 293H-cell lines were stably transfected with recombinant mouse GDF9 and the protein was partially purified from the conditioned medium using hydrophobic interaction chromatography (HIC). Conditioned medium from non-transfected 293H cells was also subjected to the same HIC purification process and used as the negative control (designated; 293H).

**Isolation and culture of COCs**

All animals were purchased from Laboratory Animal Services (Adelaide, South Australia) and treated in accordance with the Australian Code of Practice For The Care and Use of Animals for Scientific Purposes. Mice were housed in a 14-h light and 10-h dark cycle and food and water were supplied ad libitum.

COCs were obtained from a cohort of either 5 or 10 female 21–25 day old CBA/C57BL6 mice 46–48 h after administration of an i.p. injection of 5 IU pregnant mare’s serum gonadotrophin (Folligon; Intervet, Bendigo, Victoria, Australia). COCs were released from large antral follicles into Waymouth MB 752/1 medium supplemented with 5% fetal calf serum (FCS) (In Vitrogen; Carlsbad, CA, USA) and buffered with HEPES and sodium bicarbonate (Handling medium). COCs were washed once in handling medium then randomly distributed and cultured for 18 h at 37 °C in 6% CO2, 5% O2, 89% N2 in Waymouth MB 752/1 medium supplemented with 5% v/v FCS with or without 50 mlU/ml recombinant human FSH (Puregon; Organon, Oss, The Netherlands) and 10 ng/ml EGF (IVM controls), recombinant mouse GDF9 (200 ng/ml) or the equivalent 7% v/v control parent cell line 293H conditioned medium. The concentration of recombinant GDF9 was chosen based on a dose response assessment of its effects on cumulus expansion of mouse oocyte cytokeratin–keratinized complexes (Dragovic et al., 2005). Only COCs with a uniform covering of compacted cumulus cells were used in this study. Approximately 30–40 COCs were cultured per treatment in each replicate.

**Cumulus expansion assessment**

Cumulus expansion was scored blinded according to the 0–4 scale and the cumulus expansion index (CEI) was calculated as described (Faghihoun and Downs, 1990; Vanderhyden et al., 1990). Using this scale, score 0 indicates no expansion, characterized by the detachment of cumulus cells from the oocyte to assume a flattened monolayer of fibroblastic appearance leaving a partially or fully denuded oocyte. A score of 1 indicates no expansion but cumulus cells are spherical, and remain compacted around the oocyte. For score 2 complexes, only the outer most layers of cumulus cells have expanded, score 3 complexes have all layers except the corona radiata prominently expanded and a score of 4 indicates the maximum degree of expansion including the corona radiata (cells most proximal to the oocyte) (Vanderhyden et al., 1990).

**IVF and embryo culture**

After 18 h of IVM and following assessment of cumulus expansion, COCs were washed twice in fertilization media [α-Minimal Essential Media (In Vitrogen, Vic, Australia) supplemented with 75 mg/l penicillin G, 50 mg/l streptomycin sulphate and 3 mg/ml bovine serum albumin (BSA)] and co-incubated with capacitated sperm from mice with proven fertility for 4 hrs at 37 °C in 6% CO2, 5% O2, 89% N2. Presumptive zygotes were washed once in MOPS-G1, then again in G-1TH v3 drops for another 25–27 h after which embryos were assessed for their rate of development, to distinguish percentage of faster developing embryos and finally moved into G-2TH v3 (vitrolife) drops over-layered with mineral oil. Thirteen replicates were performed for each experiment. Fertilization rates were scored 18 h after IVF and 2-cell embryos transferred to fresh G-1TH v3 drops for another 25–27 h after which embryos were assessed for their rate of development, to distinguish percentage of faster developing embryos and finally moved into G-2TH v3 (vitrolife) drops over-layered with mineral oil for 47–49 h. Embryonic morphology was assessed at the end of the culture period at 96–100 h post-fertilization to determine blastocyst development.
Differential staining
Blastocyst inner cell mass (ICM) and trophoderm (TE) cell numbers were determined using a differential nuclei staining protocol described by Gardner et al. (2000). Briefly, blastocysts were subjected to 0.5% pronase at 37°C to dissolve the zona pellucida and then incubated in 10 mM 2,4,6-trinitrobenzenesulfonic acid at 4°C for 10 min. Blastocysts were then transferred to 0.1 mg/ml anti-dinitrophenyl-BSA for 10 min at 37°C then placed in guinea pig serum with propidium iodide for 5 mins at 37°C. Blastocysts were then stained with bisbenzimide in ethanol at 4°C overnight. Embryos were washed in 100% alcohol and mounted in a glycerol drop on a siliconized slide. Analysis was performed under a fluorescent microscope at ×400 magnification, where ICM and TE cells appeared blue and pink, respectively, under UV light.

Statistical analysis
All data represent 13 or more experimental replicates. Cumulus expansion and embryonic development were analysed using a three by two-way and univariate general linear analysis of variance. Fetal expansion and embryonic development were analysed using a three-way analysis of variance. All statistical analyses were performed using the Statistical Package for the Social Sciences version 13.0 for windows. Data is expressed as mean ± SEM.

Results

Effect of exogenous GDF9 during IVM on cumulus expansion
Exogenous GDF9 during IVM significantly increased (P < 0.05) cumulus expansion in the presence of FSH and EGF. The CEI was 3.1 ± 0.2 with exogenous GDF9 compared with a CEI of 2.4 ± 0.1 for the 293H control. Although almost no cumulus matrix was observed in the absence of FSH and EGF, exogenous GDF9 resulted in a higher number of COCs staying intact, with a CEI of 2.4 ± 0.1 for the 293H control. Although almost no cumulus matrix was observed in the absence of FSH and EGF, exogenous GDF9 resulted in a higher number of COCs staying intact, with a CEI of 2.4 ± 0.1 for the 293H control. Although almost no cumulus matrix was observed in the absence of FSH and EGF, exogenous GDF9 resulted in a higher number of COCs staying intact, with a CEI of 2.4 ± 0.1 for the 293H control. (P < 0.05). No significant differences were observed between the IVM and 293H controls.

Effect of exogenous GDF9 during IVM on embryonic development
Fertilization rates, as assessed by cleavage after 20 h of culture, were not different with the addition of GDF9 during maturation compared with controls in the presence of FSH and EGF (Table I). In the absence of FSH and EGF, a significant difference (P < 0.05) was observed between GDF9 and the 293H control. Compared with the 293H controls, addition of GDF9 to the maturation medium in the presence of FSH and EGF significantly (P < 0.05) increased the rate of embryo development to the 8-cell or morula stage but had no effect on rate of embryo development after 49–51 h of culture in the absence of FSH and EGF (Table I). There was no effect of exogenous GDF9 in the oocyte maturation medium on blastocyst development in the presence or absence of FSH and EGF (Table I). However, the percentage of hatching blastocyst per cleaved embryo was significantly increased (P < 0.05) with exogenous GDF9 both in the presence and in the absence of FSH and EGF (Table I).

Effect of exogenous GDF9 during IVM on blastocyst cell numbers
In the presence of FSH and EGF, GDF9 addition during IVM significantly increased (P < 0.05) blastocyst total cell numbers over the 293H control (Table II). ICM cell numbers were significantly increased (P < 0.05) over both controls, whereas exogenous GDF9 had no effect on TE cell numbers (Table II). In the absence of FSH and EGF, no differences in total cell numbers or the numbers of ICM or TE cells were observed with exogenous GDF9, however, both the 293H and GDF9 treatments had fewer TE cells than the IVM only control (Table II).

Discussion
GDF9 is a member of the TGFβ family and is one of just two known oocyte-specific paracrine growth factors. Female mice and sheep that are genetically deficient in GDF9 are infertile with ovarian follicles that do not develop past the primary stage (Elvin et al., 1999b; Hanrahan et al., 2004). GDF9 has also been shown to mimic oocyte functions in oocytecomized cumulus complexes (COCs with removed oocytes), such as suppression of LH receptor expression (Elvin et al., 1999a), stimulation of cumulus cell proliferation (Gilchrist et al., 2006) and regulation of cumulus matrix gene expression such as cyclooxygenase 2 (COX2) and hyaluronan synthase 2 (HAS2) leading to cumulus expansion (Elvin et al., 1999a;...
Dragovic et al., 2005, 2006). Given its fundamental role in female fertility and in the function of cumulus cells in particular, we therefore hypothesized that addition of GDF9 to the developing oocyte during IVM would increase cumulus expansion in intact COCs and improve subsequent embryonic quality and viability.

Indeed, in the presence of FSH and EGF, compared with its 293H control, exogenous GDF9 increased the CEI, the proportion of time developed embryos and hatching blastocysts, despite having no effect on the percentage of embryos reaching the blastocyst stage. Total blastocyst cell numbers have been shown to reflect embryo viability, as demonstrated by increased capacity to develop into a viable fetus after embryo transfer (Lane and Gardner, 1997), and hence, blastocyst cell numbers were quantified. Exogenous GDF9 during IVM resulted in increased blastocyst total cell numbers, with the most significant observations found in increased ICM cells, the portion of the blastocyst that derives the fetus. Concordantly, fetal number was almost doubled after embryonic transfer but no differences were found in implantation rates. Hence, although GDF9 addition during IVM did not affect the ability of embryos to develop into blastocysts, it did significantly enhance blastocyst quality as established by increased fetal survival. Thus, GDF9 addition during the short window of oocyte maturation appears to have a profound effect on developmental programming of the oocyte, a legacy that persists through late preimplantation development and into fetal development.

The concept of addition of oocyte-secreted factors during IVM is new and has only very recently been proposed. A very recent study from our laboratory demonstrated increased blastocyst formation after exposure to native oocyte-secreted factors or treatment with recombinant GDF9 or bone morphogenetic protein-15 (BMP 15), another oocyte-specific factor, during bovine IVM (Hussein et al., 2006). The results of the current study confirm these findings in another species and extend them by demonstrating for the first time improved fetal development and survival post-transfer. Furthermore, we have also provided evidence that the fetuses and their placentas derived from GDF9 treatment during IVM have normal weight, size and gross morphology, as determined by limb and structural development and overall appearance when compared with published information of in vivo derived mouse fetuses of the same age (Nagy et al., 2003), suggesting no obvious adverse effect on development.

It is important to note at this point that the recombinant GDF9 is only partially purified by chromatography (Hickey et al., 2005) and that contaminating factors originating from the human embryonic kidney 293H parent cell line, present in both the GDF9 preparation and the 293H control, have adverse effects on embryo development (Hussein et al., 2006). Hence, positive effects of our exogenous GDF9 on some parameters of embryo development over that of conventional IVM conditions may be masked by the 293H contaminants in the preparation. It is all possible that there may be interactions between growth factors found in serum and the beneficial actions of GDF9. In spite of this, exogenous GDF9 during IVM with FSH and EGF still resulted in a significant increase of ICM cell numbers over the IVM experimental control.

### Table 2.

<table>
<thead>
<tr>
<th>Culture media</th>
<th>FSH/EGF</th>
<th>Total cell numbers</th>
<th>ICM cell numbers</th>
<th>TE cell numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVM</td>
<td>+</td>
<td>40.2 ± 3.0 b</td>
<td>11.9 ± 1.4 a</td>
<td>28.3 ± 2.5</td>
</tr>
<tr>
<td>293H</td>
<td>+</td>
<td>37.3 ± 2.8 a</td>
<td>7.1 ± 0.8 a</td>
<td>26.4 ± 2.2</td>
</tr>
<tr>
<td>GDP9</td>
<td>-</td>
<td>46.8 ± 1.8 b</td>
<td>15.0 ± 0.7 b</td>
<td>31.8 ± 1.6</td>
</tr>
<tr>
<td>IVM</td>
<td>-</td>
<td>30.3 ± 1.7 e</td>
<td>5.4 ± 0.6</td>
<td>24.9 ± 1.9 a</td>
</tr>
<tr>
<td>293H</td>
<td>-</td>
<td>24.9 ± 1.2 b</td>
<td>7.1 ± 0.5</td>
<td>17.8 ± 1.0 b</td>
</tr>
<tr>
<td>GDP9</td>
<td>-</td>
<td>26.1 ± 1.9 e</td>
<td>7.4 ± 0.8</td>
<td>18.7 ± 1.4 a</td>
</tr>
</tbody>
</table>

Data represents the mean ± SEM of at least 13 experimental replicates. n = 50 oocytes per treatment. Values with different superscripts within the same column and FSH/EGF treatment group are significantly different (P < 0.05).
It would be interesting to study the effects of other oocyte-specific factors on embryo viability as it is likely that the oocyte utilizes a variety or combination of factors to regulate its environment for optimal developmental competence. Our lab has previously shown that recombinant ovine BMP15 improves bovine IVM oocyte developmental competence (Hussein et al., 2006). It is relevant to note, however, the varied degree of importance of BMP15 in different species (McNatty et al., 2005; Yoshino et al., 2006). Unlike GDF9 null mice (Dong et al., 1996) and GDF9 and BMP15 deficient sheep (Galloway et al., 2000; Hanrahan et al., 2004) which are all sterile, BMP15 null mice undergo folliculogenesis but exhibit mild defects in cumulus expansion and preimplantation embryo development (Su et al., 2004) leading to subfertility (Yan et al., 2001). Recent studies have shown that BMP15 expression is up-regulated in peri-ovulatory mouse follicles with oocytes producing active BMP15 only after an ovulatory signal (Gueripel et al., 2006; Yoshino et al., 2006). Thus, it appears that GDF9 is most crucial for follicular function and oocyte–somatic cell communication throughout folliculogenesis across species, whereas BMP15 has a more subtle role in the mouse later in folliculogenesis. Unfortunately to date, recombinant murine BMP15 cannot be produced (Hashimoto et al., 2005), hence the effects of homologous exogenous BMP15 on mouse IVM cannot be elucidated.

In the current study, the combination of FSH and EGF during IVM significantly improved oocyte maturation and developmental competence, as evidenced by increased cumulus expansion, on-time embryo development, development to blastocysts, blastocyst hatching rates and increased blastocyst cell numbers. This is not surprising as FSH and EGF have long been known to be beneficial to oocyte meiotic and cytoplasmic maturation and hence developmental competence (Downs et al., 1988; Das et al., 1991; Merriman et al., 1998), and as such, these hormones are common IVM additives. Through different signalling receptors, FSH and EGF both stimulate the MAP kinase pathways in cumulus cells, activating a cascade of cellular consequences. For example, FSH positively influences aspects of cumulus metabolism such as glycolysis (Downs and Utecht, 1999; Roberts et al., 2004), whereas EGF has also been shown to up-regulate glycolysis in human ovarian follicles (Roy and Terada, 1999) and other cell types (Hamer and Dickson, 1990; Quintana et al., 1995). FSH and EGF also induce cumulus expansion by up-regulating...
transcripts necessary for matrix formation, such as HAS2, COX2 and tumour necrosis factor induced protein 6 (Diaz et al., 2006), however importantly, these FSH and EGF mediated functions are critically dependent on the presence of oocyte-secreted factor signalling in cumulus cells, as cumulus expansion cannot occur in the absence of an oocyte (Buccione et al., 1990; Vanderhyden et al., 1990). The effects of GDF9 are mediated through activation of the SMAD two-third pathway (Mazerbourg et al., 2004). Recent evidence from our laboratory demonstrates that oocyte-secreted factor activation of SMAD two-third signalling in cumulus cells is essential to enable FSH or EGF to induce cumulus expansion (Dragovic et al., 2007), suggesting critical interactions between the SMAD two-third and MAP kinase signalling pathways. It is unclear from the results of the present study if such an interaction also regulates oocyte developmental competence. The most notable effects of GDF9 on oocyte competence were observed in the presence of EGF and FSH, yet GDF9 also improved oocyte development to some extent in their absence, as seen by an increase in hatched blastocyst, a response which differs from the regulation of cumulus expansion.

Although live human births have resulted from oocytes matured in vitro, (Barnes et al., 1995; Cha and Chian, 1998; Chian et al., 2000; Mikkelsen et al., 2000) current IVM success rates are significantly reduced compared with periovulatory oocytes collected after superovulation stimulation with gonadotrophins (Mikkelsen et al., 2000; Trounson et al., 2001). Current IVM procedures also result in reduced embryo quality with embryos displaying frequent cleavage retardation and blockage in development (Trounson et al., 1998; Nogueira et al., 2000). Given the current limitations of IVM, it is thus essential that systems be devised to improve the viability of individual COCs in order to advance IVM proficiency in the human. Therefore, the observation that addition of the oocyte-secreted factor GDF9 to the medium increased embryo development and fetal viability is an important novel finding that may provide insight into the formulation of maturation media for the human COC.

Unfortunately, the role of GDF9 and other identified oocyte-secreted factors in human oocyte biology is still unclear. The only implication of their importance is suggested by the observation in a small study involving only eight patients that some of the cumulus cell genes up-regulated by GDF9 are reflective of oocyte developmental competence (McKenzie et al., 2004). Hence, human studies are necessary to identify oocyte-specific paracrine factors actively involved in the human oocyte-to-cumulus bi-directional regulatory loop. This potentially has great implications for our fundamental understanding of human oocyte biology as well as for the development of IVM media and the improvement of clinical IVM success rates.

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