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Disruption of Bidirectional Oocyte-Cumulus Paracrine Signaling During In Vitro Maturation Reduces Subsequent Mouse Oocyte Developmental Competence[1]

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ABSTRACT

Oocyte-cumulus cell bidirectional communication is essential for normal development of the oocyte and cumulus cells (CCs) within the follicle. We showed recently that addition of recombinant growth differentiation factor 9 (GDF9), which signals through the SMAD2/3 pathway, during mouse oocyte in vitro maturation (IVM) increased fetal viability. This study thus aimed to observe the effects of disrupting oocyte-CC bidirectional communication during IVM on oocyte developmental competence and fetal outcomes. Cumulus-oocyte complexes (COCs) from equine chorionic gonadotropin-primed prepubertal (CBA/C57BL6) mice were cultured with or without 50 mIU/ml follicle-stimulating hormone (FSH) and 10 ng/ml epidermal growth factor (EGF) or 4 μM SMAD2/3 inhibitor SB-431542. Cumulus expansion and first polar body extrusion were then assessed, or COCs were fertilized and stained to evaluate sperm entry or cultured to the blastocyst stage. Embryo development and blastocyst quality were assessed, and Day 4.5 blastocysts were transferred to pseudopregnant recipients to analyze fetal outcomes. SMAD2/3 inhibition or FSH/EGF absence during IVM resulted in decreased cumulus expansion. First polar body extrusion and sperm entry were decreased in the absence of FSH/EGF, whereas only sperm entry was affected in SB-431542-matured COCs. Embryo development and blastocyst rates were unaffected; however, blastocyst quality was significantly altered, with reduced inner cell mass cell numbers in embryos derived from COCs matured in both treatments. When COCs were matured with SB-431542 in the absence of FSH/EGF, cumulus expansion was reduced, but fertilization, embryo development, and embryo quality were not. Inhibition of SMAD2/3 signaling in the presence of FSH/EGF significantly reduced fetal survival but had no effect on implantation or fetal and placental dimensions and morphology.

blastocyst, cumulus cells, EGF, embryo, fetal, FSH, gamete biology, in vitro fertilization, in vitro maturation, oocyte-cumulus bidirectional loop, oocyte development, pregnancy, SMAD2/3

INTRODUCTION

In mammalian Graafian follicles, mural granulosa cells line the follicular wall, and a set of highly specialized somatic cells known as cumulus cells surround the oocyte, forming the cumulus-oocyte complex (COC). These layers of granulosa and cumulus cells mediate external endocrine signals to the oocyte, enabling oocyte growth and maturation. Prior to the ovulatory luteinizing hormone (LH) surge, cumulus cells are connected to the oocyte by transzonal cytoplasmic processes that facilitate intercellular gap junction communication [1]. Through these gap junctions, cumulus cells are able to transport amino acids, key metabolites, and signaling molecules that are essential for oocyte growth and development [2].

The dependency of oocytes on their surrounding cumulus cells has long been established. Oocytes do not metabolize glucose well [3, 4], and they are dependent on cumulus cells for pyruvate and other glycolytic products [5, 6] and amino acids [7], which are essential to their growth and development. However, research in the past decade has shown that the oocyte is not a passive recipient but a key regulator of its own development. Through both paracrine signaling, using oocyte-secreted factors (OSFs), such as growth differentiation factor 9 (GDF9), and gap junctional signaling, oocytes are able to influence surrounding cumulus cells and regulate their immediate microenvironment [2, 8]. Oocytes have been shown to be the rate-limiting factor of follicular growth [9] and have been shown to influence cumulus cell glycolytic activity [10] as well as amino acid transport [11]. Bovine cumulus-enclosed oocytes have been shown to have increased developmental competence when cocultured with denuded oocytes [12]. Furthermore, we have shown recently that addition of recombinant GDF9 during mouse oocyte in vitro maturation (IVM) significantly increased blastocyst quality and fetal survival [13]. This bidirectional communication between the oocytes and their cumulus cells thus appears vital for oocyte growth, development, and survival.

Growth differentiation factor 9 is a member of the transforming growth factor-β (TGF-β) superfamily, and hence it signals through the interaction of two membrane-bound serine/threonine kinase receptors. Growth differentiation factor 9 first binds to its bone morphogenetic protein (BMP) type 2 receptor (BMPR2) [14], and the activated BMPR2 then phosphorylates and activates the type 1 receptor, TGF-β 1 receptor (TGFBR1), also known as the activin A receptor type 2-like kinase 5 (ALK5) [15]. Once activated, TGFBR1 phosphorylates transcription factors SMADs 2 and 3 which, together with SMAD4, translocate to the nucleus and induce gene transcription through interaction with SMAD-response elements in gene promoter regions [16, 17].

Paracrine communication by the oocyte through SMAD2/3 signaling in cumulus cells is required for the process of cumulus expansion [18]. Cumulus expansion is the process

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TABLE 1. Effect of FSH/EGF and SMAD2/3 signaling during IVM on meiotic maturation.*

FSH/EGF†	SB-431542†	IVM oocytes (N)	PB1 extrusions/ IVM oocyte (%)
+	−	56	71.4[a]
−	−	50	34.0[b]
+	+	53	62.3[a]
−	+	51	25.5[b]

* Data represents average percentages of at least 50 oocytes from two replicate experiments analyzed using Fisher exact test.
† +, present; −, absent.
[a,b] Values with different superscript letters represent statistically significant differences ($P < 0.05$).

TABLE 2. Effect of FSH/EGF and SMAD2/3 inhibition on sperm entry during IVM.*

FSH/EGF†	SB-431542†	IVM oocytes (N)	Decondensing sperm heads/ IVM oocyte (%)
+	−	42	78.6[a]
−	−	32	56.3[b]
+	+	40	55.0[b]
−	+	43	46.5[b]

* Data represents average percentages of at least 32 oocytes from two replicate experiments analyzed using Fisher exact test.
† +, present; −, absent.
[a,b] Values with different superscript letters represent statistically significant differences ($P < 0.05$).

where cumulus cells form a hyaluronan-rich viscoelastic matrix in response to gonadotrophin signals. Cumulus expansion facilitates ovulation and is necessary for female fertility in vivo, because mice with defective cumulus cell matrix formation are anovulatory and have compromised fertility [19]. In vitro, cumulus expansion can be stimulated through the use of follicle-stimulating hormone (FSH) and epidermal growth factor (EGF) ligands [20, 21]. However, these alone are insufficient to induce cumulus expansion without an oocyte-secreted, cumulus-enabling factor, because mouse COCs with surgically removed oocytes are unable to expand unless cocultured with denuded oocytes or with recombinant GDF9 [22–24]. Similarly, studies in our laboratory have shown that the oocyte-secreted, cumulus expansion-enabling factor is unable to induce cumulus expansion on its own without ligand (e.g., FSH) stimulation [22], reflecting the importance of bidirectional communication between the oocytes and their surrounding cumulus cells. Furthermore, upon gonadotropin stimulation, the oocyte has been suggested to permit mitogen-activated protein kinase (MAPK) activation in cumulus cells, an event which, in turn, is necessary for cumulus expansion and oocyte maturation [25].

Taken together, it appears that FSH/EGF and SMAD2/3 signaling have an interactive effect on cumulus expansion and meiotic maturation, and thus we hypothesized that these signaling pathways during IVM are also likely to have an impact on oocyte developmental competence. This study therefore sought to investigate the importance of oocyte and cumulus bidirectional communication through the SMAD2/3 and FSH/EGF signaling pathways during IVM on oocyte maturation, cumulus expansion, and subsequent embryonic and fetal development.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise specified.

Isolation and Culture of COCs

All animals were purchased from Laboratory Animal Services (Adelaide, 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 14L:10D cycle, and food and water were supplied ad libitum.

Cumulus-oocyte complexes were obtained from 21- to 25-day-old CBA/C57BL6 female mice 46–48 h after administration of an intraperitoneal injection of 5 IU of equine chorionic gonadotropin (Folligon; Intervet, Bendigo, Australia). Germinal vesicle (GV)-stage COCs were aspirated using a 30.5-gauge needle from large antral follicles into Waymouth MB 752/1 medium supplemented with 5% fetal calf serum (Invitrogen, Carlsbad, CA) buffered with HEPES and sodium bicarbonate (handling medium). Cumulus-oocyte complexes were washed once in handling medium then cultured for 17.5–18 h at 37°C in 6% CO_2, 5% O_2, and 89% N_2 in Waymouth MB 752/1 medium supplemented with 5% (v/v) fetal calf serum. In vitro maturation treatments included: 1) the positive control, 50 mIU/ml recombinant human FSH (Puregon; Organon, Oss, the Netherlands) and 10 ng/ml EGF; 2) absence of FSH/EGF; 3) 4 μM SB-431542 (generously donated by GlaxoSmithKline, Stevenage, U.K.); and 4) FSH/EGF plus SB-431542. The carrier control for SB-431542 was COCs treated with 0.04% (v/v) dimethyl sulfoxide (DMSO) plus FSH/EGF. SB-431542 has no toxicity to cells and acts as a competitive ATP-binding site kinase inhibitor with high specificity toward ACVR1B (ALK4), TGFBR1 (ALK5), and ACVR1C (ALK7) when used at <10 μM [26]. SB-431542 was used at 4 μM, because this concentration was shown previously to prevent cumulus expansion [18]. Only COCs with a uniform covering of compacted cumulus cells were used in this study.

Cumulus Expansion Assessment

Cumulus expansion was assessed blinded to treatments according to the 0–4 scale, and the cumulus expansion index (CEI) was calculated as described previously [27, 28]. Using this scale, score 0 indicates no detectable response characterized by the detachment of cumulus cells from the oocyte to assume a flattened monolayer of fibroblastic appearance. A score of +1 indicates the minimum observable response where COCs remain spherical, cumulus cells have a glistering appearance, and most cells remain compacted around the oocyte. For score +2 complexes, only the outermost layers of cumulus cells have expanded; score +3 complexes have all layers except the corona radiata (cells most proximal to the oocyte) prominently expanded; and a score of +4 indicates the maximum degree of expansion including the corona radiata [27].

Assessment of Meiotic Maturation and Sperm Penetration

After 18 h of IVM, oocytes were denuded by repeat gentle pipetting of COCs in MOPS-G1 [29] containing 1 mg/ml hyaluronidase. Oocytes then were washed in MOPS-G1 and then stained with 3 μM 4′-6-diamidino-2-phenylindole (DAPI) for 10 min to facilitate assessment of meiotic maturation status by the presence of nuclei-stained first polar bodies (PB1s). To assess the ability of sperm to bind to and penetrate the in vitro-matured oocytes, the presence of a decondensing sperm head was investigated. Oocytes were cultured with capacitated sperm as described below for 0.5 h, denuded, washed in MOPS-G1, and stained with DAPI for 10 min. The DAPI-stained oocytes/zygotes were mounted in modified MOPS-G1 (formulated without phenol red and albumin) and analyzed under ultraviolet light at 200× magnification for the presence of the PB1 or the sperm head in oocytes. Analysis of sperm penetration included all in vitro-matured oocytes, regardless of meiotic status.

In Vitro Fertilization and Embryo Culture

After 17.5–18 h of IVM, COCs were washed twice in fertilization medium (α-minimal essential media [Invitrogen] supplemented with 3 mg/ml bovine serum albumin [BSA], 75 mg/L penicillin G, and 50 mg/L streptomycin sulphate) and coincubated with capacitated sperm from CBA/C57BL6 mice with proven fertility for 4 h at 37°C in 6% CO_2, 5% O_2, and 89% N_2. Presumptive zygotes were washed once in MOPS-G1, then again in G-1 v3 (Vitrolife, Kungsbacka, Sweden) and cultured in groups of approximately 10 in 20-μl G-1 v3 drops overlaid with mineral oil at 37°C in 6% CO_2, 5% O_2, and 89% N_2. Fertilization rates were determined by the number of two-cell embryos, which was assessed 20 h after the 4-h gamete coincubation. Two-cell embryos were then transferred to fresh G-1 v3 drops for another 25–27 h, after

TABLE 3. Effect of FSH/EGF exposure during IVM on subsequent embryo development.*

Treatment	2-Cell embryos/ IVM oocyte†	8-Cell embryos/ 2-cell embryos‡	Blastocysts/ 2-cell embryo§	Hatching blastocysts/ 2-cell embryo‖
With FSH/EGF	83.5 ± 1.9[a]	41.8 ± 4.2	65.3 ± 3.2	39.8 ± 3.2
Without FSH/EGF	75.9 ± 1.8[b]	40.7 ± 4.1	66.2 ± 3.5	43.9 ± 3.4

* Data represents average percentages ± SEM of ~50 replicates representing a total of 515–537 oocytes (~10 oocytes per replicate) in 11 separate experiments.
† Two-cell embryos were counted after 20 h of culture.
‡ Eight-cell (morula) embryos were counted after 45–47 h of culture.
§ Blastocysts were counted after 96–100 h of culture.
‖ Hatching blastocysts were counted after 96–100 h of culture.
[a,b] Values with different superscript letters represent statistically significant differences ($P < 0.05$).

which embryos were assessed for their rate of development as determined by the percentage of eight-cell and compacted embryos. All embryos were then transferred into G-2 v3 (Vitrolife) medium in 20-μl drops overlaid with mineral oil for 47–49 h at 37°C in 6% CO_2, 5% O_2, and 89% N_2. Embryonic morphology was assessed at the end of the culture period (96–100 h of embryo culture) to determine blastocyst development.

Differential Staining

Blastocyst inner cell mass (ICM) and trophectoderm (TE) cell numbers were determined using a differential nuclei staining protocol described by Gardner et al. [30]. Briefly, 0.5% pronase at 37°C was used to dissolve the zona pellucida. Blastocysts were then incubated for 10 min in 10 mM 2,4,6-trinitrobenzenesulfonic acid at 4°C, and then in 0.1 mg/ml anti-dinitrophenyl-BSA for 10 min at 37°C with washes in HEPES-buffered modified G1 medium in between each step. Blastocysts were then placed in guinea pig serum with propidium iodide for 5 min at 37°C and then stained with bisbenzimide in ethanol at 4°C overnight. Prior to mounting in a glycerol drop on a siliconized slide, blastocysts were washed in 100% alcohol. Analysis was performed under a fluorescent microscope at 200× magnification, where ICM and TE cells appeared blue and pink, respectively, under ultraviolet light.

Embryo Transfer

Swiss female recipient mice were mated with vasectomized males and anesthetized on Day 3.5 of pseudopregnancy with 2% Avertin (0.015 ml/g body weight) prior to embryo transfer. Six blastocysts from one treatment were randomly assigned to each uterine horn. A total of 42 embryos were transferred per treatment. On Day 18 of pregnancy, the percentage of implantations, fetal development, fetal and placental weight, and fetal crown-to-rump length were assessed.

Statistical Analysis

All data represent three or more experimental replicates except when stated. Cumulus expansion and embryonic development were analyzed using a 2×2 ANOVA and univariate general linear analysis of variance using least-significant differences when treatments had equal error variance and a Dunnett T3 posthoc when treatments had unequal error variance. Meiotic maturation, sperm entry analysis, and pregnancy outcomes were assessed using a Fisher exact test. Treatments with a P value of ≤ 0.05 were taken to be significantly different. All statistical analyses were performed using SPSS version 13.0 for windows (SPSS, Chicago, IL) or GraphPad online software (GraphPad, La Jolla, CA).

RESULTS

Effect of FSH/EGF and SMAD2/3 Signaling During IVM on Cumulus Expansion

As expected, cumulus expansion did not occur in the absence of FSH/EGF (CEI, 0.3 ± 0.2 vs. 2.6 ± 0.2 with FSH/EGF). Addition of SB-431542 in the presence of FSH/EGF also significantly reduced cumulus expansion (CEI = 0.6 ± 0.2) to levels similar to those when FSH and EGF were absent. Interestingly, SB-431542 and absence of FSH/EGF did have an interactive combined negative impact on the morphology of the COCs observed at the end of the maturation period. Almost all complexes had total detachment of the cumulus cells from the oocytes to assume a flattened monolayer of fibroblastic appearance adhered to the bottom of the culture dish, rendering most oocytes completely denuded. As such, an observation that had not been described previously within the Vanderhyden scoring system [27], this treatment was excluded from cumulus expansion analysis. There was no significant difference in cumulus expansion between FSH/EGF (2.6 ± 0.2) and the carrier control (DMSO and FSH/EGF, 3.1 ± 0.3; $P > 0.05$).

Effect of FSH/EGF and SMAD2/3 Signaling During IVM on Meiotic Maturation

Given the deficient cumulus expansion observed both in the absence of FSH/EGF and/or the presence of SB-431542, the need for SMAD2/3 and FSH/EGF signaling to complete meiosis 1 was investigated at the end of the 18-h maturation period. Lack of FSH and EGF in IVM significantly ($P < 0.001$) reduced PB1 extrusion to 34.0% at 18 h after maturation, compared with 71.4% when matured with FSH and EGF (Table 1). Inhibition of SMAD2/3 had no effect on PB1 extrusion (62.3%) in the presence of FSH/EGF, but only 25.5% of oocytes completed meiosis 1 after 18 h of culture without FSH/EGF in the presence of SB-431542. Two-way ANOVA analysis

TABLE 4. Effect of SMAD2/3 signaling during IVM on subsequent embryo development.*

Treatment	2-Cell embryos/ IVM oocyte†	8-Cell embryos/ 2-cell embryos‡	Blastocysts/ 2-cell embryo§	Hatching blastocysts/ 2-cell embryo‖
DMSO control + FSH/EGF	79.5 ± 1.8	52.3 ± 4.3	70.7 ± 3.5	46.1 ± 2.8
SB-431542 + FSH/EGF	74.2 ± 2.8	46.7 ± 4.5	71.0 ± 3.5	45.3 ± 3.6

* Data represents average percentages ± SEM of ~40 replicates representing a total of 400–429 oocytes (~10 oocytes per replicate) from eight separate experiments.
† Two-cell embryos were counted after 20 h of culture.
‡ Eight-cell (morula) embryos were counted after 45–47 h of culture.
§ Blastocysts were counted after 96–100 h of culture.
‖ Hatching blastocysts were counted after 96–100 h of culture.

TABLE 5. Effect of FSH/EGF and SMAD2/3 signaling during IVM on subsequent embryo development.*

FSH/EGF[†]	SB-431542[†]	2-Cell embryos/ IVM oocyte[‡]	8-Cell embryos/ 2-cell embryos[§]	Blastocysts/ 2-cell embryo[‖]	Hatching blastocysts/ 2-cell embryo[#]
+	−	78.5 ± 3.7	67.7 ± 6.4[a]	73.7 ± 5.9	50.0 ± 10.3
−	−	72.4 ± 3.1	56.6 ± 6.3[a]	76.8 ± 5.6	53.8 ± 6.7
+	+	75.0 ± 6.7	48.8 ± 10.8[a,b]	66.1 ± 10.0	41.2 ± 4.2
−	+	66.1 ± 6.5	25.1 ± 10.0[b]	58.7 ± 12.3	38.1 ± 8.1

* Data represents average percentages ± SEM of at least five replicates representing at least 50 oocytes (~10 oocytes per replicate) from two separate experiments.
[†] +, present; −, absent.
[‡] Two-cell embryos were counted after 20 h of culture.
[§] Eight-cell (morula) embryos were counted after 45–47 h of culture.
[‖] Blastocysts were counted after 96–100 h of culture.
[#] Hatching blastocysts were counted after 96–100 h of culture.
[a,b] Values with different superscript letters represent statistically significant differences ($P < 0.05$).

confirmed no interaction between SB-431542 and FSH/EGF; thus, inhibition of SMAD 2/3 had no additional consequences over the effects of lack of FSH/EGF on completion of meiotic maturation. There was no significant difference ($P = 0.64$) between the DMSO control and IVM with FSH/EGF on polar body extrusion (68% and 72%, respectively).

Effect of FSH/EGF and SMAD2/3 Signaling During IVM on Sperm Penetration

To investigate whether inhibition of oocyte-to-cumulus bidirectional communication and the resultant lack of cumulus expansion during IVM would have an effect on sperm penetration, oocytes were incubated with sperm from male mice with proven fertility for 30 min and stained to assess the presence of a sperm head within the oocytes. Both the absence of FSH/EGF and inhibition of SMAD2/3 significantly ($P < 0.05$) decreased sperm entry relative to the FSH/EGF control. Inhibition of SMAD2/3 in the absence of FSH/EGF did not further decrease sperm entry (Table 2). There was no significant difference ($P = 0.50$) in sperm penetration between the carrier (DMSO) control and positive (FSH/EGF) control (69% and 79%, respectively).

Effect of FSH/EGF and SMAD2/3 Signaling During IVM on Subsequent Embryo Development

The effects of either FSH/EGF and/or SB-431542 during IVM on subsequent embryo development were determined. The absence of FSH and EGF marginally but significantly decreased the percentage of two-cell embryos per IVM oocyte, compared with when the ligands were present (Table 3). Lack of FSH/EGF during IVM had no effect on the rate of development or the ability of resulting embryos to develop into blastocysts. The percentage of hatching blastocysts was also not significantly different from when FSH and EGF were used during IVM.

Inhibition of SMAD2/3 in the presence of FSH and EGF during IVM had no significant effect on two-cell embryo development, rate of embryo development, or blastocyst and hatching blastocyst formation when compared with its DMSO control (Table 4).

The combined effects of SMAD2/3 inhibition and FSH/EGF absence on oocyte maturation in vitro were then investigated and compared to IVM without FSH/EGF only and SMAD2/3 inhibition in the presence of FSH/EGF. In vitro maturation with FSH/EGF only was used as the positive control (Table 5). Oocytes matured under these combined conditions had no significant difference in their ability to form two-cell embryos. However, SB-431542 in the absence of FSH/EGF led to a significant decrease in embryo developmental rate, as shown in the lower percentages of eight-cell/compacted embryos compared with IVM without SB-431542, with or without FSH/EGF ($P < 0.05$). Two-way ANOVA analysis revealed that this decreased developmental rate was not due to an interaction between FSH/EGF and SB-431542. There were no differences between treatments in blastocyst and hatching blastocyst development.

Effect of FSH/EGF and SMAD2/3 Signaling During IVM on Blastocyst Cell Numbers

Blastocyst quality was assessed by the determination of blastocyst cell numbers and cell allocation to the TE and ICM. In vitro maturation without FSH/EGF resulted in a significant decrease ($P < 0.05$) in total blastocyst cell numbers compared with when the ligands were present (48.3 ± 3.3 vs. 63.3 ± 3.5; $P < 0.05$). This could be attributed largely to a significant decrease ($P < 0.001$) in blastocyst ICM cell numbers: IVM without FSH/EGF produced blastocysts with approximately half the ICM size as IVM with FSH/EGF. There was no significant difference in TE cell numbers with the presence or absence of FSH/EGF (Table 6).

Despite no decrease in blastocyst development rates, SMAD2/3 inhibition in the presence of FSH and EGF also had a similar effect, with a tendency for lower total cell numbers (38.1 ± 2.5 vs. 43.3 ± 2.4) and a significant decrease in ICM cell numbers (10.1 ± 1.0 vs. 15.7 ± 1.0; $P < 0.001$), whereas TE cell numbers were unaffected (Table 7).

Absence of FSH/EGF and inhibition of SMAD2/3 during IVM did not have a combined effect on resulting blastocyst

TABLE 6. Effect of FSH/EGF exposure during oocyte IVM on blastocyst quality.*

Treatment	Total cells	ICM cells	TE cells
With FSH/EGF	63.3 ± 3.5[a]	17.2 ± 1.2[b]	46.1 ± 2.8
Without FSH/EGF	48.3 ± 3.3[c]	8.8 ± 0.9[d]	39.5 ± 2.7

* Data represents average cell numbers ± SEM of 27–31 blastocysts from four replicate experiments.
[a–d] Values with different superscript letters represent statistically significant differences: [a,c]$P < 0.05$; [b,d]$P < 0.001$.

TABLE 7. Effect of SMAD2/3 signaling during oocyte IVM on blastocyst quality.*

Treatment	Total cells	ICM cells	TE cells
DMSO control + FSH/EGF	43.3 ± 2.4	15.7 ± 1.0[a]	27.6 ± 1.9
SB-431542 + FSH/EGF	38.1 ± 2.5	10.1 ± 1.0[b]	27.5 ± 1.9

* Data represents average cell numbers ± SEM of 25–38 blastocysts from four replicate experiments.
[a,b] Values with different superscript letters represent statistically significant differences ($P = 0.001$).

TABLE 8. Effect of FSH/EGF and SMAD2/3 signaling during oocyte IVM on blastocyst quality.*

FSH/EGF[†]	SB-431542[†]	Total cells	ICM cells	TE cells
+	−	66.2 ± 4.4	19.5 ± 1.4[a]	46.7 ± 3.2
−	−	58.2 ± 3.9	11.3 ± 1.2[b]	46.9 ± 3.3
+	+	56.4 ± 9.9	11.2 ± 0.9[b]	45.2 ± 9.4
−	+	52.7 ± 3.7	9.8 ± 0.7[b]	42.9 ± 3.1

* Data represents average cell numbers ± SEM of approximately 10 blastocysts from two replicate experiments.
[†] +, present; −, absent.
[a,b] Values with different superscript letters represent statistically significant differences ($P \leq 0.001$).

quality. Aside from reduced blastocyst ICM cell numbers when compared with embryos derived from oocytes matured with FSH/EGF only, blastocysts resulting from oocytes that had been matured with both FSH/EGF absence and SMAD2/3 inhibition did not have total, ICM, or trophectoderm cell numbers significantly different from that of the other treatments, as shown in Table 8.

Effect of SMAD2/3 Inhibition During IVM on Pregnancy Outcomes

Inhibition of SMAD2/3 did not have any effect on the capacity of the blastocyst to implant but significantly reduced fetal survival per embryo transferred (50.0 ± 6.3 control vs. 30.1 ± 4.4; $P < 0.05$; Fig. 1). Fetuses that developed from oocytes matured with SMAD2/3 inhibition were not significantly different in weight, placental:fetal weight ratio, or crown-to-rump length compared with the DMSO control (Fig. 2). There were also no morphological abnormalities in fetuses developed from oocytes matured with SMAD2/3 inhibition.

DISCUSSION

We have shown previously that the addition of recombinant GDF9 during IVM increases blastocyst development [12] and subsequent fetal viability [13]. The purpose of this study was thus to investigate the effects of disrupting this oocyte-cumulus bidirectional communication during IVM on subsequent embryonic and fetal development.

The recognition of the mammalian oocyte's ability to control follicular cell development and function has only emerged recently, in this decade [2, 8]. Oocytes have been shown to orchestrate follicular development [9], secrete mitogens eliciting cumulus and granulosa cell proliferation [31–33], prevent cumulus cell luteinization [34], lower the apoptotic index of cumulus cells [35], and thereby maintain the distinct cumulus cell phenotype [33]. Although it has long been established that preovulatory oocytes can only use oxidative phosphorylation for energy production and are dependent on cumulus cells to metabolize glucose and provide the carboxylic acid substrates [3], it has only been discovered recently that oocytes regulate cumulus cell metabolism, enabling transport of glycolytic products to themselves [10]. Oocytes have also been discovered to be poor synthesizers of cholesterol and manipulate cumulus cell cholesterol synthesis for provision of products of the cholesterol biosynthetic pathway [36].

Oocytes exert these effects through the use of paracrine factors, such as BMP15 and GDF9 [2]. Growth differentiation factor 9 mediates its effects through activation of the SMAD2/3 signaling pathway in cumulus cells. SMAD2/3 signaling has been shown to be the mechanistic pathway that oocytes use to regulate cumulus cell cholesterol synthesis [36], gene transcription, expansion [18], proliferation [31], and differentiation of granulosa cells during follicle antrum formation to the cumulus cell lineage [37]. Given the dependency of cumulus cell functions on oocyte paracrine signaling, we hypothesized that inhibiting SMAD2/3 signaling would be detrimental to oocyte developmental competence. The SMAD2/3 pathway was therefore targeted for disruption of oocyte-to-cumulus communication.

This was achieved through the use of SB-431542, a small molecule that acts as a competitive ATP-binding site kinase inhibitor highly specific for ACVR1B (ALK4), TGFBR1 (ALK5), and ACVR1C (ALK7), the kinases that activate SMAD2/3 signaling [16], When used at concentrations under 10 μM, SB-431542 was found to have no effect on ACVRL1 (ALK1) and ACVR1 (ALK2) , nor did it affect MAPK (ERK), MAPK8 (JNK), or MAPK signaling pathways [26]. The

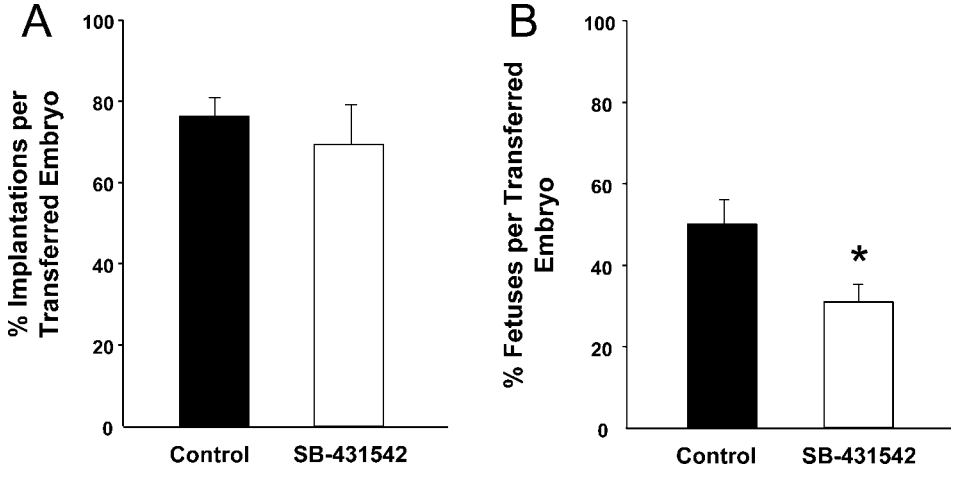

FIG. 1. Effect of SMAD2/3 inhibition in the presence of FSH/EGF during IVM on pregnancy outcomes. Day 4.5 blastocysts developed from COCs matured in the DMSO (0.04% [v/v]) control or with 4 μM SB-431542 in the presence of FSH (50 mIU) and EGF (10 ng/ml) were transferred to pseudopregnant recipients and outcomes were analyzed on Day 18 of pregnancy. Black and white bars represent embryos derived from oocytes matured with the DMSO carrier control and SB-431542 treatment, respectively. Data represent percentage of mean number of implantation sites (**A**) or fetuses (**B**) per embryo transferred ± SEM of seven replicate experiments. *Significant difference with $P < 0.05$. N = 42 embryos transferred per treatment.

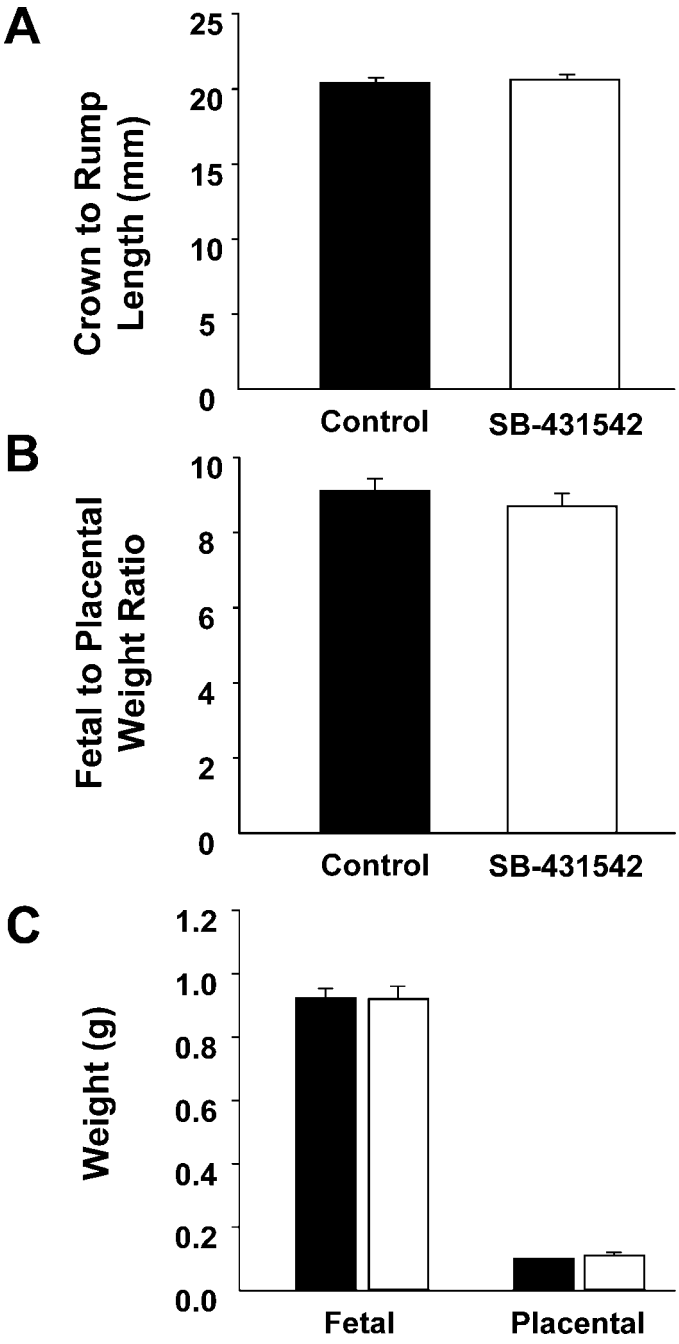

FIG. 2. Effect of SMAD2/3 inhibition in the presence of FSH/EGF during IVM on fetal and placental outcomes. Day 4.5 blastocysts developed from COCs matured in the DMSO (0.04% [v/v]) control or with 4 μM SB-431542 in the presence of FSH (50 mIU) and EGF (10 ng/ml) were transferred to pseudopregnant recipients, and outcomes were analyzed on Day 18 of pregnancy. Black and white bars represent embryos derived from oocytes matured with the DMSO carrier control and SB-431542 treatment, respectively. Data represent percentage of mean fetal crown-to-rump length (**A**), fetal:placental weight ratio (**B**), and fetal and placental weight (**C**) ± SEM of 12–21 fetuses/placentas in seven replicate experiments. N = 42 embryos transferred per treatment.

concentration used in this study was 4 μM, because SB-431542 was found to be nontoxic and have the greatest, but specific, inhibitory effect on expression of cumulus expansion gene transcripts at 4 μM [18].

As summarized in Table 9, SMAD2/3 inhibition during IVM had no effect on meiotic maturation or two-cell embryo formation, but it significantly decreased the ability of sperm to penetrate these oocytes. This is most likely attributed to the lack of an expanded cumulus matrix, resulting in a temporary delay in sperm penetration, which would have been neutralized to that of the controls by the end of the 4-h gamete coincubation period, given that there were no significant differences in two-cell embryo formation. These results are consistent with previous findings, where SMAD2/3 inhibition during bovine IVM had no effect on two-cell embryo development [12], and where GDF9 had no effect on meiotic resumption, despite inducing MAPK activity [25]. Although blastocyst formation was unaffected, blastocyst quality was severely reduced, with significant reductions in ICM cell numbers in blastocysts derived from oocytes matured with SMAD2/3 inhibition compared with controls. Fetal survival rates were also subsequently reduced following embryo transfer of these blastocysts. These effects of SMAD2/3 inhibition are directly opposite those observed when recombinant GDF9, an activator of the SMAD2/3 signaling pathway [31], was added during oocyte IVM, which resulted in increased fertilization rates, blastocyst ICM cell numbers, and fetal survival rates [13]. As such, oocyte-to-cumulus signaling via the SMAD2/3 pathway and the consequential effects on cumulus cell functions have a direct and significant impact on oocyte developmental competence, illustrating the importance of the oocyte-cumulus cell regulatory loop to oocyte development.

TABLE 9. Summary of results of treatments with respect to IVM.*

Parameter	Treatment 1†	Treatment 2‡	Treatment 3§
PB1	↓	–	↓
Sperm penetration	↓	↓	↓
2-Cell formation	↓	–	–
Development rate	–	–	↓
Blastocysts/hatching blastocysts	–	–	–
Blastocyst total cell no.	↓	–	–
ICM cell no.	↓↓	↓↓	↓↓
TE cell no.	–	–	–
Fetal and placental morphology		–	
Fetal survival		↓	

* –, no difference; ↓, significant decrease ($P < 0.05$); ↓↓, very significant decrease ($P \leq 0.001$).
† Without FSH/EGF or SB-431542; FSH/EGF was the control.
‡ SB-431542 with FSH/EGF; IVM with the DMSO carrier control (FSH/EGF + 0.04% v/v DMSO) was the control.
§ SB-431542 without FSH/EGF; FSH/EGF was the control.

It is well established that FSH and EGF have positive effects on meiotic maturation [38], cumulus expansion, oocyte fertilization, and subsequent embryo development and fetal outcomes when used during oocyte IVM [39–43]. These effects are mediated through the cumulus cells to the oocyte, because oocytes do not have FSH receptors [44], and these ligands only improve oocyte developmental competence in the presence of cumulus cells [21, 45–47]. Hence, the FSH and EGF signaling pathways were targeted as the mode for cumulus-to-oocyte communication in this study.

Polar body one extrusion was significantly reduced in oocytes matured in the absence of FSH/EGF. Follicle-stimulating hormone and EGF are both known to have meiotic-inducing effects and override the inhibitory effects of hypoxanthine to induce meiotic maturation in cumulus-enclosed oocytes [38]. Sperm penetration was also lower in oocytes matured in the absence of FSH/EGF compared with the FSH/EGF IVM control. A decrease in two-cell embryo

formation was also observed and was most likely due to the decreased meiotic maturation rates.

Contrary to another study [40], we did not observe any effects of FSH and EGF during oocyte IVM on the ability of oocytes to form blastocysts, but we did observe an effect on blastocyst quality, as depicted by the substantial decrease in total and ICM numbers when FSH and EGF were not present. This is most likely due to differing culture conditions and mouse strains. C57BL6/CBA hybrid mice have been shown to produce blastocysts with a low apoptotic index [48]; IVM in Waymouth media produces maximal rates of blastocyst formation [49]; and IVM with serum has been shown to increase blastocyst formation [50] and blastocyst cell numbers [51]. Similarly, SMAD2/3 inhibition in the presence of FSH/EGF only affected blastocyst quality, with a significant reduction in ICM cell numbers compared with the control.

Stimulation of the MAPK pathway through increased cAMP levels in cumulus cells leads to cumulus expansion, and both GDF9 and FSH/EGF have been shown to activate MAPK through independent pathways [25, 52, 53], increasing cumulus expansion gene transcripts, such as prostaglandin-endoperoxide synthase 2 (*Ptgs2*) and hyaluronan synthase 2 (*Has2*) [18, 19]. However, FSH and/or EGF, together with an oocyte-secreted cumulus expansion-enabling factor, such as GDF9, must be present for mouse cumulus expansion [22, 24], and it has been suggested that oocyte paracrine factors "license" MAPK activation by cAMP in cumulus cells [25]. Although the importance of MAPK to oocyte developmental competence remains to be elucidated, the interactive effect of FSH/EGF and SMAD2/3 signaling could explain why the absence of FSH/EGF and SMAD2/3 signaling produced similar adverse outcomes on oocyte developmental competence.

Aside from maintaining the cumulus cell phenotype for the provision of substrates and metabolites crucial for oocyte development, there are other probable factors and mechanisms for how and why oocyte-cumulus bidirectional communication prior to and during oocyte maturation regulates cumulus cell function to affect oocyte developmental competence. Oocytes denuded from their cumulus cells prior to IVM were found to undergo GVBD at a higher rate than cumulus-enclosed oocytes, but had a lower incidence of PB1 extrusion [54] and could not develop into blastocysts [46]. Ge et al. [54] also showed that removal of cumulus cells prior to IVM altered cortical granule redistribution, spindle assembly, mitochondria distribution and function, and meiosis-promoting factor activity, all of which were significantly rescued to levels comparable to cumulus-enclosed oocytes, when the denuded oocytes were matured on a cumulus cell monolayer derived from GV-stage COCs. Meiotic spindle morphology was also more reflective of in vivo-ovulated oocytes in COCs matured with EGF [55]. Although the presence of cumulus cells is important to the acquisition of oocyte developmental competence, recent studies suggest that the act of expansion itself or the number of cumulus cells left attached to the oocyte at the end of the maturation period is not [46, 47, 56]. Our findings further support this, given that PB1 extrusion in oocytes matured without FSH/EGF but with the SMAD2/3 inhibitor was not significantly different from without FSH/EGF only (25.5% vs. 34.0%; Table 1), nor was there any significant difference in sperm penetration or two-cell embryo formation in all the treatments—IVM without FSH/EGF, SB-431542 with FSH/EGF, and SB-431542 without FSH/EGF—where cumulus expansion was negatively affected, despite the differences in morphology and the number of actual cumulus cells left attached to the oocyte at the end of the maturation period. It is also important to highlight that all subsequent developmental data were expressed as a percentage of two-cell embryos, not the number of IVM oocytes; hence, the lack of differences in blastocyst and hatching blastocyst formation was independent of any events perturbing meiotic maturation and fertilization in COCs matured with both SMAD2/3 inhibition and without FSH/EGF in comparison with when only one inhibitory mechanism was present. This implies that expansion itself is not reflective of oocyte developmental competence in vitro but is an indication of the signaling capability of the oocyte and the cumulus cells.

Increased oocyte maturation rates and developmental competence have been linked to prolonged persistence of oocyte-cumulus cell gap junctional communication associated with high levels of cAMP but independent of cumulus expansion [57, 58]. This is supported by the findings that cumulus cells translate ligand stimulatory signals in a paracrine manner [38, 59], maintaining dictyate arrest and inducing GVBD in a timely, coordinated fashion [60]. Because FSH increases cAMP levels [45], it is possible that bidirectional communication between the oocyte and cumulus via SMAD2/3 and FSH and EGF signaling prolongs gap junction communication, allowing the exchange of factors necessary for optimal oocyte developmental competence. However, this remains for further investigation.

Oocyte-cumulus bidirectional communication is thus an intricate relationship and essential for the development and function of both cell types. The findings of this study show for the first time the interdependency of both directions of oocyte and cumulus communication and demonstrate the importance of their persistence during oocyte maturation to the acquisition of oocyte developmental competence and subsequent embryonic and fetal development.

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