Fibroblast growth factor requirements for in vitro development of bovine embryos

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Abstract

The overall goal was to describe the importance of fibroblast growth factors (FGFs) during development of the bovine embryo. An inhibitor of FGF receptor kinase activity (SU5402) was used to examine whether FGF signaling is required for embryo development. Addition of 20 μM SU5402 on Day 0 (Day of IVF) reduced (P < 0.04) the percentage of oocytes becoming blastocysts on Day 7 compared to controls (5.9 ± 2.1 vs 16.9 ± 2.4; average ± SEM). Also, Day-8 blastocysts placed into individual culture drops of medium containing SU5402 tended to have decreased (P = 0.08) blastomere numbers at Day 11 (211.1 ± 27.5 vs 297.8 ± 25.0). A second series of studies determined if supplemental FGF2 enhances development in vitro. There was no effect of FGF2 on cleavage or blastocyst development rates when 5 or 100 ng/mL FGF2 was provided immediately after fertilization. Also, FGF2 supplementation beginning on Day 5 post-fertilization did not significantly affect blastocyst rates or the number of trophoblast and inner cell mass cells. However, addition of 500 ng/mL FGF2 at both Day 0 and Day 4 increased (P = 0.03) the percentage of oocytes that became blastocysts on Day 7 compared with controls (27.4 ± 1.3 vs 19.7 ± 1.3). In a final study, the thermal-protective ability of FGF2 was examined by adding FGF2 1 h before exposing Day 5 embryos to heat shock. Addition of FGF2 did not significantly influence embryo thermal-tolerance. In conclusion, FGF receptor activation was important for optimal blastocyst formation and FGF2 supplementation increased bovine blastocyst formation when provided at high concentrations.

Keywords: Pregnancy; Uterus; Embryo; SU5402; Fibroblast growth factor; Trophoblast

1. Introduction

Bovine embryos derived from in vitro production (IVP) systems differ from embryos produced in vivo in many regards. Embryos derived from IVP have distinct gene expression profiles [1] and contain marked differences in their number and ratio of inner cell mass (ICM) and trophectoderm (TE) cells [2]. Also, IVP-derived embryos are inferior to in vivo-generated embryos at achieving development to term after transfer to recipients, and are associated with increased rates of fetal and placental abnormalities [2–5].

One scheme for improving the competency of IVP embryos for generating live and healthy offspring after transfer was adding specific uterine- or embryo-derived factors. Several paracrine factors produced by the uterus improved in vitro development rates [2,6–8]. Also, pregnancy rates after transfer can be improved by exposure of IVP embryos to specific paracrine factors before transfer. For example, pregnancy loss after transfer of IVP embryos was reduced for embryos...
treated with colony stimulating factor-2 (CSF2) [2]. Exposure of IVP embryos to insulin growth factor-1 (IGF1) before transfer improved pregnancy rates in heat-stressed cows, but not in cows exposed to a thermoneutral environment [3].

Another group of uterine factors that may modify development in ways that improve embryo competence is the fibroblast growth factor (FGF) family of paracrine factors. At least 23 FGFs exist in mammals; these paracrine factors exert mitogenic, morphogenic, and angiogenic activities in various cells and tissues throughout embryonic, fetal and postnatal development [9,10]. Each of the four genes encoding receptors for FGFs (FGFRs) is transcribed in bovine blastocysts [11–13], and several FGFs are produced by ovine and bovine endometria during early pregnancy [11,14,15]. One of central interest is FGF2, which is produced by luminal and glandular epithelium and is detectable in the uterine lumen throughout early pregnancy in cattle and sheep [16,17]. In addition, FGF2 and other FGFs are produced in conceptuses throughout pre-, peri- and post-attachment development [11,12,18].

Actions of FGFs during embryogenesis are beginning to be realized in cattle. Addition of FGF2 alone or in combination with other uterine factors increased development of bovine embryos in vitro [6,7]. Also, specific single nucleotide polymorphisms (SNPs) in bovine FGF2 have been associated with IVF success and blastocyst formation [19,20]. Several FGFs, including FGF2, stimulated TE production of interferon-tau (IFNT), the antiluteolytic factor in ruminants [11,16,21].

The overall goal of the present study was to test the importance of FGF signaling during early embryonic development in cattle. Specific objectives were to: 1) determine if FGFR activation is required for embryo development in vitro; and 2) determine if FGF2 supplementation increases development.

2. Materials and methods

2.1. Materials

Tissue culture medium (TCM)-199 was purchased from Hyclone (Logan, UT, USA). Penicillin and streptomycin were purchased from Millipore Corp. (Billerica, MA, USA). The base Synthetic Oviduct Fluid (SOF) medium was purchased as a custom formulation from Caisson (Sugar City, ID, USA). Bovine FGF2 was purchased from R&D Systems (Minneapolis, MN, USA). The SU5402 was purchased from Symansis (Shanghai, China). A modified SOF referred to as Synthetic Oviduct Fluid-Bovine Embryo 1 (SOF-BE1) was prepared using the formulation listed in Table 1. The PicoPure RNA Isolation Kit was purchased from MDS Analytical Technologies (Sunnyvale, CA, USA). The RNase-free DNase, the High Capacity cDNA Reverse Transcription Kit and TaqMan primers and probe sets were purchased from Applied Biosystems Inc. (Foster City, CA, USA). The RNase A (100 mg/mL) was purchased from Qiagen (Valencia, CA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folltropin-V was purchased from AgTech Inc. (Manhattan, KS, USA). The Tyrode’s albumin lactate pyruvate (TALP) solutions were prepared as described by Parrish et al (1986) [22] using Tyrode’s lactate solutions (TL) prepared as a custom formulation from Caisson (Sugar City, ID, USA). Bovine FGF2 was purchased from R&D Systems (Minneapolis, MN, USA). The SU5402 was purchased from Symansis (Shanghai, China). A modified SOF referred to as Synthetic Oviduct Fluid-Bovine Embryo 1 (SOF-BE1) was prepared using the formulation listed in Table 1. The PicoPure RNA Isolation Kit was purchased from MDS Analytical Technologies (Sunnyvale, CA, USA). The RNase-free DNase, the High Capacity cDNA Reverse Transcription Kit and TaqMan primers and probe sets were purchased from Applied Biosystems Inc. (Foster City, CA, USA). The RNase A (100 mg/mL) was purchased from Qiagen (Valencia, CA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. In vitro production of embryos

The collection of bovine cumulus oocyte complexes and IVM, IVF and IVC procedures were completed as described previously [2,23,24], except that SOF-BE1 was used to culture zygotes after fertilization at 38.5 °C in a humidified atmosphere of 5% (v:v) CO2 and 5% (v:v) O2 with the balance N2. A pool of semen from three bulls (various breeds) was used for each fertilization event, and a different group of bulls were used in each replicate study. Cleavage rate was assessed on
Day 3 (Day 0 = day of IVF). The cleavage rate for all the studies was 71.8 ± 1.0 (average ± SEM). Blastocyst formation and morphology (regular, expanded, hatching, hatched) was assessed on Days 7 and 8 using a stereomicroscope.

2.3. Experimental design

2.3.1. Experiment 1: inhibition of FGF signaling and IFNT mRNA abundance

Blastocysts were harvested at Day 7, washed in SOF-BE1 and randomly allocated to individual 45 μL microdrops of SOF-BE1 containing vehicle [0.2% (v/v) DMSO] or 20 μM SU5402. After 8 h at 38.5 °C in 5% (v/v) CO2, 5% (v/v) O2, and 90% (v/v) N2, 5 μL of 0.5 μg/mL FGF2 was added to half of the drops in each treatment to create a final concentration of 50 ng/mL FGF2. In non-FGF2-treated controls, 5 μL of SOF-BE1 was added to drops. Blastocysts were incubated for an additional 15 h in 5% (v/v) CO2, 5% (v/v) O2, and 90% (v/v) N2 at 38.5 °C before collection of individual blastocysts for qRT-PCR. The experiment was repeated on five separate occasions (n = 11–18 total blastocysts).

The PicoPure RNA Isolation Kit was used to extract total cellular (tc) RNA from individual blastocysts according to the manufacturer’s instructions. For quantitative (q)RT-PCR, tcRNA (20 ng/reaction) was incubated with RNase-free DNase, reverse transcribed and 50 cycles of a two-step PCR was completed using TaqMan primers and probe for IFNT and 18s RNA (internal control), as described previously [11]. Abundance of IFNT mRNA was determined by using the comparative threshold cycle (C_T) method [11].

2.3.2. Experiment 2: inhibition of FGF signaling and embryonic development

The experimental design was a 2 × 2 factorial design that examined the effect of adding SU5402 or its carrier (control) at either Days 0 or 4 post-IVF (Day 0 = day of IVF) on subsequent embryo development. Following fertilization (6–8 h after beginning IVF), presumptive zygotes were placed 45 μL microdrops of SOF-BE1 containing either vehicle [0.2% (v/v) DMSO] or 20 μM SU5402 (n = 20–30 presumptive zygotes/drop). At Day 4, an additional 5 μL of SOF-BE1 containing DMSO or SU5402 was added. Cleavage was recorded at Day 3, and number of blastocysts and stage of development (hatched, hatching, expanded, non-expanded, and early blastocyst) was recorded on Days 7 and 8. The experiment was repeated on six separate occasions (n = 22–86 presumptive zygotes/treatment/replicate).

2.3.3. Experiment 3: effect of blocking FGF signaling on total cell number in blastocysts

Day 8 blastocysts were washed in PBS-PVP and cultured individually in 50 μL microdrops of TCM 199 containing 5% (v/v) fetal bovine serum (Invitrogen Corp.) as described previously [21]. Microdrops contained either vehicle [0.2% (v/v) DMSO] or 20 μM SU5402. On Day 11, blastocysts were processed and incubated to Hoechst 33342 (0.5 mg/mL) as described [21], to determine number of nuclei. The experiment was repeated on five separate occasions (n = 14–18 blastocysts/treatment).

Total, ICM, and TE cell numbers were determined as described [21,25], except that immediately before beginning the fixation and staining steps, embryos were exposed to 1 μg/μL RNase for 1 h at 38.5 °C. After incubation, blastocysts were washed in 0.01 M PBS [pH 7.4] containing 1 mg/mL polyvinylpyrrolidone (PVP), stained with 100 μg/mL propidium iodide in PBS-PVP with 0.2% (v/v) Triton for 30 s, repeatedly washed in PBS-PVP, and then fixed in PBS-PVP containing 4% (wt/vol) paraformaldehyde and 1 μg/mL Hoechst 33342 for 15 min. After repeated washing in PBS-PVP, blastocysts were placed on microscope slides with cover slips containing a small drop of glycerol and examined using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany) with Zeiss filter sets 02 (DAPI filter) and 15 (rhodamine filter) to distinguish nuclei of TE cells (red and blue) and ICM (blue).

2.3.4. Experiment 4: effect of FGF2 supplementation from Days 0–8 on development

Presumptive zygotes were produced by IVM/IVF and then placed in SOF-BE1 containing 0, 5, or 100 ng/mL FGF2 on Day 0. Cleavage rates were determined on Day 3, and number of blastocysts and stage of blastocyst development was determined on Days 7 and 8. The experiment was repeated on seven separate occasions (n = 26–62 oocytes per treatment/replicate).

2.3.5. Experiment 5: effect of FGF2 supplementation from Days 5–8 on development

On Day 5 post-IVF, embryos with ≥ 16 cells were washed in SOF-BE1 and placed in 50 μL microdrops SOF-BE1 containing 0, 5, or 100 ng/mL FGF2 (n = 10–23 embryos/drop). Number of blastocysts and stage of blastocyst development was determined on Days 7 and 8, and numbers of TE and ICM were determined on Day 8. The experiment was replicated on nine separate occasions (n = 10–23 embryos/treatment/replicate).
2.3.6. Experiment 6: effect of FGF2 supplementation from Days 0–8 or 4–8 on development

Presumptive zygotes were produced by IVM/IVF and then cultured in 45 μL microdrops of SOF-BE1 containing 0 or 500 ng/mL FGF2 (n = 20–30 presumptive zygotes/drop). On Day 4, 5 μL of SOF-BE1 was added to half of the microdrops of each treatment, and 5 μL of SOF-BE1 containing 500 ng of FGF2 was added to the other half of the microdrops for each treatment. Thus, embryos received either 0 ng/mL FGF2, 500 ng/mL FGF2 added at Day 0, 500 ng/mL FGF2 added at Day 4, or 500 ng/mL FGF2 added at Day 0 and Day 4. Cleavage rates were determined on Day 3, and number of blastocysts, stage of blastocyst development, and numbers of TE and ICM were determined on Day 7. The experiment was replicated on nine separate occasions (n = 39–75 presumptive zygotes/treatment/replicate).

2.3.7. Experiment 7: thermoprotective effect of FGF2 in bovine embryos

The experimental design was a 2 × 2 factorial with two treatments (0 vs 500 ng/mL FGF2) incubated at two temperatures (38.5 or 42 °C). On Day 5 post-IVF, embryos with ≥16 cells were washed in SOF-BE1 and placed in 25 μL microdrops SOF-BE1 containing 0 or 500 ng/mL FGF2 (n = 8–15 embryos/microdrop). Cultures were incubated 38.5 °C for 1 h in 5% (v/v) CO2, 5% (v/v) O2, and 90% (v/v) N2 then were incubated either at 38.5 or 42 °C for 15 h in a 5% (v/v) CO2 in humidified air atmosphere. After this incubation, embryos were washed in SOF-BE1, placed into fresh 25 μL microdrops SOF-BE1, and incubated at 38.5 °C in 5% (v/v) CO2, 5% (v/v) O2, and 90% (v/v) N2 until Days 7 and 8, when blastocyst development was determined.

2.4. Statistical analyses

The effects of SU5402 treatment and FGF2 concentrations on percentage of cleaved embryos, percentage of blastocysts, and percentage of advanced blastocysts, were analyzed by least-squares ANOVA, using the GLM procedure of SAS (SAS version 9.2, SAS Institute Inc., Cary, NC, USA). Percentage data were calculated for each replicate and arcsin transformation was completed before analysis. Analysis of differences between individual treatments were further partitioned with pair-wise comparisons (probability of difference [PD/FF] analysis; SAS). Data for qRT-PCR were analyzed by using the ΔC_T values, but data are presented as fold-changes relative to control values. The model included main effects and all interactions. Results are reported as least-squares means ± SEM. When transformation was employed, least-squares means were derived from untransformed data, whereas probability values were derived from arcsin-transformed data.

3. Results

3.1. Experiment 1: inhibition of FGF signaling and IFNT mRNA abundance

An initial study was completed to verify the ability of SU5402 to block FGFR-induced responses in bovine embryos. Treatment with FGF2 induced increases in IFNT mRNA abundance which were used as the indicator of blastocyst responsiveness to treatments [11,16,21]. Addition of 50 ng/mL FGF2 increased IFNT mRNA (3.5 ± 0.68 fold increase in relative mRNA abundance versus non-treated control [mean ± SEM]; P = 0.03). Co-treatment with FGF2 and 20 μM SU5402 prevented this increase (1.1 ± 0.57 fold effect versus non-treated control). Also, providing the inhibitor in the absence of exogenous FGF2 reduced basal concentrations of IFNT mRNA (0.44 ± 0.24 fold effect versus non-treated control; P = 0.005).

3.2. Experiment 2: inhibition of FGF signaling and embryonic development

The importance of embryonic-derived FGFs acting through FGFRs during early development was determined by addition of SU5402 from Days 0 to 8 or Days 4 to 8 post-IVF (Day 0 = Day of IVF; Fig. 1). Addition of SU5402 immediately after fertilization did not affect the percentage of cleaved embryos at Day 3 (data not shown), but decreased (P = 0.04) the percentage of oocytes that were blastocysts at Day 7 (Fig. 1A). There was a numerical reduction in the percentage of blastocysts formed at Day 8, but this effect was not significantly different from its control (Fig. 1A). Providing SU5402 at Day 0 did not affect the percentage of blastocysts that were advanced in development (expanded, hatching, hatched) at Days 7 or 8 (Fig. 1B). Addition of SU5402 on Day 4 did not affect blastocyst formation at Days 7 or 8 (Fig. 1A), or the percentage of advanced blastocysts at Days 7 or 8 (Fig. 1B).

3.3. Experiment 3: effect of blocking FGF signaling on total cell number in blastocysts

To test whether FGF signaling influences blastocyst development, blastocysts were treated with inhibitor beginning at Day 8 post-IVF, and cell numbers were determined at Day 11. Blastocysts cultured with inhib-
itor tended (P = 0.08) to have fewer cells (211.13 ± 27.49) than untreated controls at Day 11 (297.81 ± 25.14). Based on TUNEL labeling, SU5402 did not induce apoptosis (data not shown).

3.4. Experiment 4: effect of FGF2 supplementation from day 0–8 on development

A series of studies were completed to determine if exogenous FGF2 improved competence of embryos to develop to the blastocyst stage. In the first study, 0, 5, or 100 ng/mL FGF2 was added from Day 0 to Day 8 post-IVF (Fig. 2). Addition of FGF2 did not affect the percentage of cleaved oocytes at Day 3 (not shown) or development to the blastocyst stage at Days 7 and 8 when compared with controls (Fig. 2A). Direct comparison between 5 and 100 ng/mL FGF2 treatment groups identified that the percentage of oocytes that became blastocysts at Day 7 tended to be lower (P = 0.06) for embryos incubated in medium containing 5 ng/mL than 100 ng/mL FGF2 (Fig. 2A). No differences were detected in the percentage of blastocysts at advanced stages at Day 7 due to FGF2 treatment, but the percentage of blastocysts that were advanced at Day 8 was reduced in embryos receiving 5 ng/mL FGF2 when compared with either controls (P = 0.02) or embryos receiving 100 ng/mL FGF2 (P = 0.01; Fig. 2B).

3.5. Experiment 5: effect of FGF2 supplementation from day 5–8 on development

In a follow-up study, the effect of providing exogenous FGF2 between Days 5 and 8 post-fertilization on blastocyst formation and cell number was examined (Fig. 3). Neither concentration of FGF2 affected the percentage of blastocysts at Days 7 or 8 when compared with controls (Fig. 3A). The percentage of advanced blastocysts at Day 8 was lower (P = 0.04) for embryos receiving 5 ng/mL FGF2 than controls (Fig. 3B). There was no effect of either concentration of FGF2 on blastocyst numbers of TE, ICM, or total cells (Fig. 3C), or on the ratio of TE to ICM (data not shown).

3.6. Experiment 6: effect of FGF2 supplementation from day 0–8 or 4–8 on development

Another experiment tested whether increasing the concentration of supplemental FGF2 to 500 ng/mL benefited embryo development (Fig. 4). The FGF2 was added to medium on Day 0, Day 4, and Days 0 and 4.

Fig. 1. SU5402 limited blastocyst formation. Bovine embryos were cultured in medium containing vehicle [0.2% (v:v) DMSO] or 20 μM SU5402 added at Day 0 or Day 4. Embryos were incubated until Day 8. Panel A: Percentage of oocytes that were blastocysts at Days 7 and 8 post-fertilization. Panel B: Percentage of blastocysts that were at advanced stages at Days 7 and 8. Data in each panel are least-squares means ± SEM. Different superscripts in each panel represent means that differed (P < 0.05).

Fig. 2. The effect of supplementing FGF2 in culture medium from Days 0 to 8 post-fertilization on blastocyst formation. Panel A: Percentage of oocytes that were blastocysts at Days 7 and 8 post-fertilization. Panel B: Percentage of blastocysts that were at advanced stages at Days 7 and 8. Data in each panel are least-squares means ± SEM. Different superscripts in each panel represent differences between treatments (P < 0.05).
The percentage of oocytes that cleaved by Day 3 was not affected by treatment (results not shown). The percentage of oocytes that formed blastocysts at Day 7 was not affected by addition of 500 ng/mL FGF2 on Day 0 or Day 4, but addition of FGF2 on both days increased \((P < 0.03)\) the percentage of blastocysts (Fig. 4A). None of the treatments affected the incidence of advanced blastocyst formation at Day 7 (Fig. 4B).

Blastocyst formation and staging was not completed at Day 8. Rather, blastocysts identified at Day 7 were collected and processed to determine ICM, TE and total cell numbers (Fig. 4C). There was no effect of treatment on total cell number, number of ICM, or the TE:ICM ratio, but there was a strong tendency for embryos treated with FGF2 at both Days 0 and 4 to have greater numbers of TE than non-treated controls \((P = 0.06)\).

3.7. Experiment 7: thermoprotective effect of FGF2 in bovine embryos

Since a related growth factor, FGF4, protected cells from heat shock [26], the thermoprotective effects of FGF2 on embryos at Day 5 post-fertilization was determined (Fig. 5). Culture of embryos at 42 °C for 15 h reduced \((P < 0.05)\) the percentage developing to the blastocyst stage at Day 7 and 8 (Fig. 5A). Treatment with 500 ng/mL FGF2 tended to increase \((P = 0.08)\) the percentage of embryos that formed blastocysts at Day 7, but not at Day 8. There was a tendency \((P = 0.06)\) for heat shock exposure to limit the percentage of advanced blastocysts at Day 8 (Fig. 5B). Also, exposure to 500 ng/mL FGF2 and heat shock reduced the percentage of advanced blastocysts at Day 7 when compared with non-heat shocked treatments \((P < 0.07)\), but this effect was not evident at Day 8 (Fig. 5B). No FGF2 treatment by heat shock interaction was observed in any analyses, indicating that FGF2 did not have a thermoprotective effect on blastocyst and advanced blastocyst formation.

4. Discussion

As described previously, the overall goal of this work was to describe the importance of FGFs during development of the bovine embryo. Two approaches were taken to achieve this goal; 1) a chemical inhibitor of FGFR kinase activation was used to examine the impact of endogenous FGFs, and 2) various concentrations of FGF2, a molecule that interacts with several FGFR complexes [10,27], were examined to determine
if supplementing FGF2 affected embryo development, gene expression, and survival following heat shock. Several end points were examined in an attempt to provide a comprehensive examination of the various developmental and physiological impacts of FGF signaling during early embryo development in cattle.

Bovine embryos produce several FGFs prior to implantation [11,12], and the importance of these endogenously-produced FGFs during early bovine embryo development was examined by blocking FGFR activation with SU5402. Blocking the ability of these molecules to activate their receptors indicated that one or more of these molecules regulated embryonic development to the blastocyst stage. This inhibitor is best known for its ability to interfere with kinase activation in FGFR1; however, it also inhibits FGFR2 and FGFR3 at concentrations used in this work [28–33]. An initial study was completed to establish that SU5402 interfered with FGF-dependent actions in bovine embryos. Several FGFs, including FGF2, stimulated IFNT mRNA and protein concentrations in bovine blastocysts and trophoblast cell lines [11,16,21]. Consistent with previous findings, providing FGF2 in culture medium increased the relative abundance of IFNT mRNA in bovine blastocysts. Co-treatment with SU5402 prevented this effect. Also, the addition of SU5402 in the absence of exogenous FGF2 reduced basal IFNT mRNA levels. There are two likely explanations for the reduction in basal IFNT mRNA levels. Blocking
FGFRs could have prevented endogenously produced FGFs from regulating IFNT production. There were FGF2 transcripts at the blastocyst stage in cattle [11,12]. Also, FGF4 mRNA was detected in bovine blastocysts [12]. Although FGF4 contained only marginal sequence similarities with FGF2, both FGFs utilized several of the same FGFR subtypes to elicit cellular responses (e.g., FGFR1, R2c, R3c) [10,34]. Therefore, one or both of these FGFs, and potentially others, may act as endogenous regulators of IFNT expression. Alternatively, perhaps the reduction in basal IFNT mRNA abundance was caused by non-specific actions of SU5402. This inhibitor did not impact apoptosis when provided to blastocysts between Day 8 and 11 post-IVF, but this inhibitor can react with other tyrosine-receptors to several growth factors (e.g., VEGF, PDGF, EGF) [30,35]. Therefore it is possible that SU5402 was acting on other receptors to exert this effect.

One interesting finding of the SU5402 work was that the addition of SU5402 appeared to delay the timing of blastocyst formation. It reduced the percentage of blastocysts at Day 7, but not at Day 8. Transcripts for each of the FGFRs (R1–4) was detected in bovine embryos between the 2-cell and blastocyst stages [12,36,37]. Also, the relative abundance for FGFR1 and R2 increased after the 8-cell stage, coincident with activation of the embryonic genome, and at the blastocyst stage ample amounts of FGFR mRNA and immunoreactive protein preside in bovine embryos [36]. It is unclear whether FGFR protein exists throughout early embryo development, and also it is not clear whether the necessary signaling systems exist to transduce FGF signals during early stages of development.

The identity of the embryo-derived FGFs that optimize the speed of blastocyst formation has not been determined. Others have associated exogenous FGF2 with blastocoel formation in bovine embryos [7,8]. Based on the current studies, we inferred that endogenous FGFs influenced blastocyst development after Day 8, perhaps due to FGF4-dependent actions on bovine TE. It is noteworthy that FGF4 was crucial in the mouse for maintenance of a proliferative TE population [38–40]. In cattle, FGF4 expression was first detected at the morula/blastocyst stage coincident with TE formation and proliferation [12]. A precise action for FGF4 on bovine embryos has not been delineated.

Adding large quantities of FGF2 (1 μg/mL total; 500 ng/mL on Days 0 and 4) increased the percentage of embryos becoming blastocysts and increased the number of TE cells in blastocysts. This finding was consistent with the concept that FGFs, and FGF2 in particular, can regulate the speed of blastocyst formation during culture. However, lower concentrations of FGF2 (5 or 100 ng/mL) were unable to stimulate blastocyst development in this work.

There are at least two potential explanations. Perhaps FGF2 must act through a non-conventional FGFR to control embryo development. There are high-affinity interactions between FGF2 and several FGFR subtypes (R1c, R2c, R3c, R4; ED50 range from 0.05 to 5 ng/mL), but FGF2 can interact with other FGFRs with lower affinities [10,34]. Alternatively, perhaps endogenous sources of FGF2 and/or other FGFs are already being produced in sufficient quantities to provide a near-maximal effect on the developmental potential of IVP embryos. This would explain why such large amounts of FGF2 were needed to detect beneficial effects above that of exogenously produced FGF2.

That 5 or 100 ng/mL FGF2 had no positive effect on embryonic development was inconsistent with a recent report that 50 ng/mL FGF2 improved in vitro blastocyst development [6]. Several procedural distinctions in some aspect of the IVP systems could explain the alternate outcomes observed between that study and ours. However, the cause for this discrepancy in outcomes was unclear. A modified SOF medium containing many of the same supplements was used in their study and ours. Both groups also completed studies under a physiological oxygen tension environment. Interestingly, a consistent theme in this previous report and other reports was that FGF2 acted cooperatively with other growth factors to improve bovine embryo development during culture (e.g., TGFβ, PDGF, IGF1, CSF2) [6–8,41,42]. Perhaps such co-supplementation schemes are needed to fully realize the beneficial effects of FGF2 and other FGFs in our IVP system.

In one of the present studies, addition of 5 ng/mL of FGF2 produced a slight reduction in blastocyst development. This effect was not observed in a subsequent study (Fig. 3). The reason for these disparate responses was not pursued, since it was clear in both studies that this concentration of FGF2 was not beneficial for embryo development.

A final study was completed to determine if FGF2 contained thermoprotective activities on bovine embryos. Mouse male germ cells could be protected from heat shock-induced apoptosis by providing FGF4 [26]. Also, a recent study described a linkage between an FGF4 SNP and heat-stress associated alterations in milk production in cattle [43]. Recombinant bovine FGF4 is not available commercially, and it is not
known whether murine and human FGF4 proteins adequately substitute for their bovine homolog. Since FGF2 reacts with several of the same FGFR subtypes as FGF4 (e.g., FGFR1c, R2c, R3c) [10,34], FGF2 was used as the reactive ligand for this work. No thermal-protective effects of FGF2 were observed. Therefore, either the cellular processes conferring thermoprotection in other cells (e.g., male germ cells) were not in place in early stage bovine embryos, or FGF2 did not adequately substitute for FGF4.

To conclude, observations made using the FGFR inhibitor provided evidence that embryo-derived FGFs were important for development to the blastocyst stage, post-blastocyst development, and IFNT expression, but there was no evidence that FGF2 was important for embryo thermal-tolerance. The identification of the specific FGF responsible for these actions remains unresolved.

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