REPRODUCTION

Fibroblast growth factor 10 enhances bovine oocyte maturation and developmental competence *in vitro*

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Abstract

The ability of oocytes to resume meiosis, become fertilized, and generate viable pregnancies is controlled during folliculogenesis by several endocrine and paracrine factors. The aim of this work is to determine whether fibroblast growth factor 10 (FGF10) is an oocyte competent factor. Transcripts for each of the four FGF receptor types (FGFR) were present in cumulus and oocytes after their extraction from the follicles. FGFR1 transcripts predominated in cumulus cells whereas FGFR2 was most abundant in oocytes. Exposing the cumulus–oocyte complexes to FGF10 during *in vitro* maturation did not affect cleavage rates, but increases ($P<0.05$) in the percentage of embryos at the 8–16-cell stage on day 3 and at the blastocyst stage on day 7, which were evident in FGF10-supplemented oocytes. The progression of oocytes through meiosis and cumulus expansion was increased ($P<0.05$) by FGF10. The importance of the endogenous sources of FGFs was examined by adding anti-FGF10 IgG during oocyte maturation. Blocking endogenous FGF10 activity decreased ($P<0.05$) the percentage of oocytes developing into blastocysts and limited ($P<0.05$) cumulus expansion. Expression profiles of putative cumulus and oocyte competency markers were examined for their involvement in FGF10-mediated responses. FGF10 influenced the expression of *CTSB* and *SPRY2* in cumulus cells and *BMP15* in oocytes. In summary, this work provides new insight into the importance of FGFRs and locally derived FGF10 during oocyte maturation in cattle. Its subsequent impact on *in vitro* embryo development implicates it as a noteworthy oocyte competent factor.

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Introduction

Oocyte competency is defined as the intrinsic ability of oocytes to resume meiosis, accept spermatozoa for fertilization, cleave after fertilization, and facilitate proper embryonic development that leads to the production of healthy offspring (Sirard *et al.* 2006, Li *et al.* 2008). Proper follicle development is vital for oocyte competence, and folliculogenesis is controlled by a variety of endocrine and intraovarian factors (Gilchrist *et al.* 2008, Li *et al.* 2008, Binelli & Murphy 2010).

The oocyte plays an active role in regulating folliculogenesis. Specific members of the transforming growth factor-β (TGFB) superfamily of paracrine factors, most notably bone morphogenetic protein-15 (BMP15) and growth differentiation factor-9 (GDF9), are produced within the oocyte and act on cumulus and granulosa cells to regulate folliculogenesis and oogenesis. GDF9 is vital for folliculogenesis. *Gdf9*-null mice are infertile and the follicles fail to develop past the primary follicle stage (Dong *et al.* 1996). Targeted disruption of *Bmp15* yields a subfertile phenotype in mice characterized by poor oocyte competency (Yan *et al.* 2001). BMP15 overexpression promotes follicular development in mice (McMahon *et al.* 2008). Moreover, specific *BMP15* mutations in sheep improve ovulation and lambing rates (Galloway *et al.* 2000).

Another large class of paracrine-acting factors that have received some recent attention for their abilities to regulate follicular development and oocyte maturation are the fibroblast growth factors (FGFs). At least 22 genes encode various FGFs that function as important paracrine regulators of proliferation, morphogenesis, and angiogenesis in various tissues (Ornitz & Itoh 2001, Itoh 2007). Several FGFs are expressed within oocytes and follicular somatic cells. FGF8 is produced by the mouse oocyte and acts cooperatively with BMP15 to promote glycolysis in cumulus cells in antral follicles (Valve *et al.* 1997, Sugiura *et al.* 2007). Granulosa cell production of FGFs, most notably FGF2 (Knee *et al.* 1994, Ben-Haroush *et al.* 2005), is linked to primordial follicle development, granulosa cell proliferation, and LH receptor expression (Gospodarowicz & Bialecki 1979, Oury & Darbon 1988, Nilsson *et al.* 2001, Garor *et al.* 2009).

There is also evidence for FGFs providing a paracrine link between thecal cells and granulosa/cumulus cells.

FGF7 and FGF10 are produced by thecal cells in cattle (Parrott & Skinner 1998, Buratini *et al.* 2007), and their primary receptor partner, FGF receptor 2b (FGFR2b), presides on granulosa cells (Buratini *et al.* 2007). FGF10 is also detected within the oocyte (Buratini *et al.* 2007). Studying the roles of these thecal- and oocyte-derived molecules is of particular interest since *in vitro* oocyte maturation systems lack thecal cells and their products, and the lack of these molecules may contribute to poor oocyte competency outcomes in *in vitro* production systems for cattle, humans, and potentially other mammals (Gilchrist *et al.* 2008, Li *et al.* 2008, Loureiro *et al.* 2009).

The importance of FGF10 as a paracrine-acting mediator of oocyte competency was examined in this work. FGF10 is an important mediator of mesenchymal–epithelial communication in various organs and tissues (Bellusci *et al.* 1997, Sekine *et al.* 1999, Bazer *et al.* 2009). In addition, intraovarian *FGF10* mRNA concentrations are greater in healthy, growing bovine follicles than in follicles undergoing atresia (Buratini *et al.* 2007). Studies presented herein determined that FGFRs utilized by FGF10 are expressed in cumulus cells and oocytes and that providing FGF10 during *in vitro* maturation (IVM) promotes bovine oocyte maturation, cumulus expansion, and the rate of embryo development *in vitro*. Further studies were completed to provide insight into mechanisms controlled by FGF10 during oocyte maturation.

Results

FGFR transcript profiles in cumulus cells and oocytes

Four genes encode FGFRs in mammals (*FGFR1–4*), and alternatively spliced variants for three of these FGFRs provide an extensive diversity of extracellular domains that bind various FGFs (Powers *et al.* 2000, Itoh & Ornitz 2004). Some FGFs bind to multiple FGFRs (e.g. FGF1 and FGF2) whereas others, such as FGF10, associate primarily with only a few receptor subtypes. FGF10 associates primarily with one spliced variant of FGFR2, termed R2b, and one spliced variant of the FGFR1 group, termed R1b (Miralles *et al.* 1999, Powers *et al.* 2000, Itoh & Ornitz 2004). An initial study was completed to describe the relative expression of these and other FGFRs in cumulus cells and oocytes.

Cumulus–oocyte complexes (COCs) were harvested and cumulus and oocytes were separated after their collection from ovaries. Quantitative (q) RT-PCR was completed to assess the relative expression of the *b* and *c* spliced variant forms of *FGFR1* and *R2* as well as *FGFR3* (primers recognized both *R3b* and *R3c*) and *R4* (no splice variants exist). Transcripts for each *FGFR* were detected in cumulus cells (Fig. 1A). Each of the *FGFR* transcripts was also detected in oocytes, although
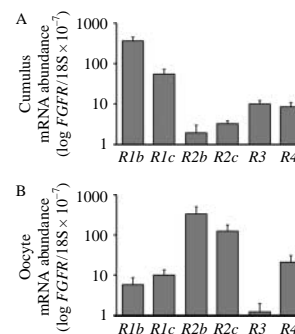

Figure 1 Expression profiles of *FGFRs* in bovine cumulus cells and oocytes prior to maturation. Cumulus and oocytes were separated before maturation, tcRNA was extracted, and qRT-PCR was completed. Log-transformed ratios of the target mRNA to reference RNA (*18S*) were used for statistical analysis. (A) *FGFR* abundance profiles in cumulus cells (each replicate contained cells derived from 20 COCs; 3 replicate studies). (B) *FGFR* profiles in denuded oocytes (20 oocytes/replicate; 3 replicate studies).

the presence of *FGFR3* mRNA was barely detectable ($\sim$37 cycles to reach threshold values; based on 45 cycles of amplification).

FGF10 supplementation during IVM improves embryo development

The role of FGF10 during oocyte maturation was initially examined by collecting the COCs from slaughterhouse-derived ovaries and completing IVM and IVF followed by *in vitro* culture (IVC). Methods described previously were used (Rivera & Hansen 2001, Loureiro *et al.* 2009) with the exception that oocyte maturation medium lacked serum (instead it contained 1 mg/ml polyvinyl alcohol (PVA)). FGF10 supplementation during oocyte maturation did not impact cleavage rates (Fig. 2A), but supplementation with 0.5 ng/ml FGF10 increased ($P<0.05$) the percentage of cleaved embryos at the 8–16-cell stage by day 3 post IVF (Fig. 2B). In addition, 0.5 ng/ml FGF10 increased ($P<0.05$) the percentage of oocytes that developed into blastocysts on day 7 post IVF (Fig. 2C, left side). Similarly, an increase ($P<0.05$) in the percentage of blastocysts on day 7 was evident when data were expressed as the percentage of cleaved embryos that formed blastocysts (Fig. 2C, right panel). The percentage of expanded, hatching, or hatched blastocysts (collectively termed as advanced blastocysts) on day 8 post IVF were also greater ($P<0.05$) in oocyte cultures containing 0.5 ng/ml FGF10 than nontreated controls both when data were expressed as the percentage of oocytes that formed advanced blastocysts (Fig. 2D, left side) or the percentage of cleaved embryos that formed advanced blastocysts (Fig. 2D, right side). A biphasic response to FGF10 supplementation was evident throughout the study. Specifically, effects on the percentage of 8–16-cell embryos, blastocysts on day 7, and advanced blastocysts on day 8
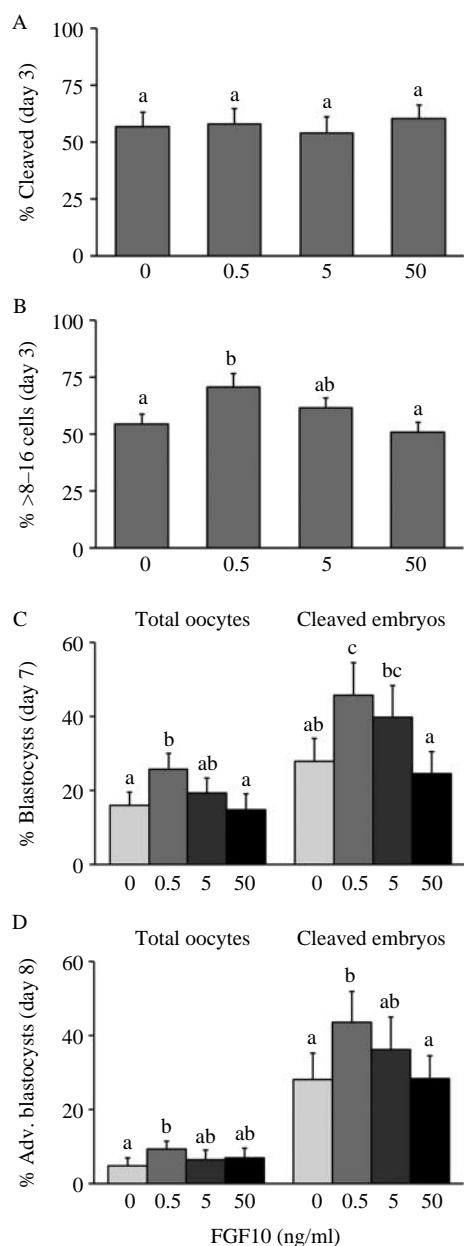
Figure 2 *In vitro* development of bovine embryos is enhanced by adding FGF10 to the medium during oocyte maturation. COCs were matured for 21–22 h in maturation medium containing 1 mg/ml PVA (no serum) and either 0, 0.5, 5, or 50 ng/ml FGF10, then those were fertilized and cultured. Presumptive zygotes were inspected visually using a stereomicroscope to assess embryo development status at specific times. (A) The proportion of zygotes that cleaved at 72 h post IVF. (B) The proportion of cleaved zygotes at 72 h post IVF, which contained between 8 and 16 cells. (C) The proportion of oocytes (left side) or cleaved embryos (right side) that reached the blastocyst stage by day 7 post IVF. (D) The proportion of oocytes (left side) or cleaved embryos (right side) that reached advanced blastocyst stages on day 8 post IVF (expansion, hatching, and hatched). All values represent means and s.e.m.s from eight replicate studies, each containing 20–33 zygotes/treatment group. Different superscripts within each panel represent differences observed due to treatments ($P < 0.05$).

were not observed when 5 or 50 ng/ml FGF10 was provided (Fig. 2B–D).

Differential staining was completed on a subset of blastocysts ($n = 17$–28 blastocysts) to investigate whether FGF10 supplementation during oocyte maturation affects numbers of inner cell mass (ICM) and trophoblast cells. Exposing COCs to 0.5 ng/ml did not affect the numbers of ICM, trophoblast, overall cell numbers, or ICM:trophoblast ratio on day 8 post IVF (37.2 ± 4.1 vs 42.8 ± 2.3 ICM/blastocyst, 134.8 ± 7.8 vs 122 ± 8.7 trophoblast/blastocyst for controls and 0.5 ng/ml treatments respectively).

Blocking endogenous FGF10 action during oocyte maturation reduces subsequent embryo development

Thecal cells are the predominant source for FGF10 in bovine follicles but the oocyte also produces FGF10 in antral follicles (Buratini *et al.* 2007). Anti-FGF10 IgG was used to determine the importance of oocyte-derived FGF10 during IVM. In the first study, the effectiveness of anti-FGF10 IgG at blocking FGF10 actions was examined. Providing an excess of anti-FGF (0.1 μg/ml; 25-fold excess compared with 0.5 ng/ml FGF10) did not affect cleavage rates or rates of early embryonic development (data not shown) but it effectively blocked FGF10 (0.5 ng/ml) from increasing ($P < 0.05$) the percentage of oocytes that formed blastocysts on day 7 post IVF (Fig. 3A). Anti-FGF10 also reduced ($P < 0.05$) the percentage of oocytes that formed blastocysts when comparing this group with nontreated controls (Fig. 3A). Similar effects were observed when analyzing the outcomes based on the percentage of cleaved embryos that formed blastocysts on day 7. Addition of 0.5 ng/ml FGF10 increased ($P < 0.05$) the percentage of cleaved embryos that formed blastocysts on day 7 (41.1 ± 3.9 vs $27.1 \pm 5.1\%$ for controls) whereas addition of anti-FGF10 decreased ($P < 0.05$) the percentage of blastocysts on day 7 ($18.2 \pm 45\%$). Providing control IgG did not affect the percentage of cleaved embryos that formed blastoycsts on day 7 ($35.6 \pm 3.8\%$).

A second study was completed to further define the developmental events affected by anti-FGF10 treatment in the absence of supplemental FGF10 (Fig. 3B). Anti-FGF10 treatment did not affect the cleavage rates or the percentage of oocytes reaching the 8–16-cell stage by day 3 post IVF (data not shown). On day 7, post IVF, fewer ($P < 0.05$) blastocysts resulted from the oocytes exposed to anti-FGF10 (0.1 μg/ml). In addition, providing a 3.9-fold molar excess of FGF10 (50 ng/ml) reversed the negative effect of anti-FGF10 on the percentage of oocytes forming blastocysts on day 7 (Fig. 2B). The same effects of treatments were observed when examining the percentage of cleaved embryos that formed blastocysts on day 7 ($26.0 \pm 4.5\%$ for controls; $14.6 \pm 2.6\%$ for anti-FGF10 treatment; $25.4 \pm 2.9\%$ for IgG control; and $27.8 \pm 3.4\%$ for anti-FGF10 plus 50 ng/ml FGF10).
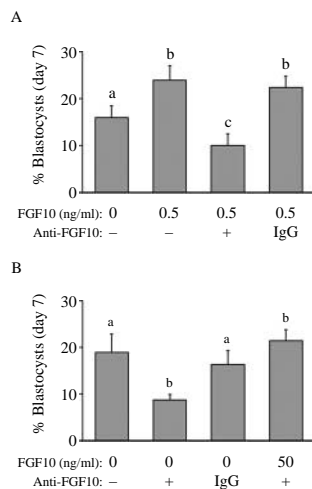
A

B

Figure 3 Evidence for an endogenous source of FGF10 during oocyte maturation *in vitro*. COCs were cultured in maturation medium in the presence or absence of anti-FGF10. FGF10 was included in some samples to verify the effectiveness of the anti-FGF10 treatment. Control IgG (labeled as IgG) was also included. (A) The effects of providing anti-FGF10 (0.1 µg/ml) in the presence or absence of 0.5 ng/ml FGF10 during COC maturation on blastocyst formation on day 7 post IVF (*n*=7 replicate studies). (B) Limiting the effects of anti-FGF10 by providing excess FGF10 (50 ng/ml; *n*=4 replicate studies). Different superscripts within each panel represent differences observed due to treatments (*P*<0.05). Data are presented as the percentage of oocytes that developed into blastocysts on day 7 post IVF.

Effects of FGF10 on oocyte maturation

The physiological basis for the newly described FGF10 effects was explored by determining whether FGF10 supplementation affects oocyte maturation. The percentage of oocytes reaching telophase I or metaphase II (TI/MII) after 21–22 h of maturation were influenced by FGF10 supplementation (Fig. 4A). Supplementation with 0.5 or 5 ng/ml FGF10 did not affect the percentage of TI/MII oocytes but exposure to 50 ng/ml FGF10 increased (*P*<0.05) the percentage of TI/MII oocytes compared with controls. The percentage of oocytes extruding their first polar body were influenced by FGF10 supplementation (Fig. 4A). Supplementation with 0.5 and 50 ng/ml FGF10, but not 5 ng/ml FGF10, increased (*P*<0.05) the first polar body extrusion rates as compared with the control. A related study was completed to determine whether endogenous sources of FGF10 impact bovine oocyte maturation (Fig. 4B). Supplementing anti-FGF10 (0.3 µg/ml) during IVM did not affect the percentage of TI/MII oocytes and the first polar body extrusion rates at 21 h post IVM.

In a third study, the effect of FGF on the early progression of meiosis was examined by describing how FGF10 affects chromatin condensation during IVM (Fig. 4C). Adding 50 ng/ml FGF10 increased (*P*<0.05) the proportion of oocytes containing condensed chromatin after 6 h. Supplementation with 0.5 ng/ml FGF10

or anti-FGF10 did not affect the percentage of oocytes with condensed chromatin.

In a final study, oocytes devoid of cumulus cells were matured *in vitro* in the presence or absence of FGF10 or anti-FGF10 to examine whether FGF10 acted directly on the oocyte to exert its beneficial effects on oocyte maturation (Fig. 5). Bovine oocyte maturation can proceed in the absence of cumulus cells, albeit to a lesser extent than intact COCs (Homa 1988, Lonergan *et al.* 1996). Providing 50 ng/ml FGF10 decreased (*P*<0.05) the percentage of TI/MII oocytes at 21 h post IVM.
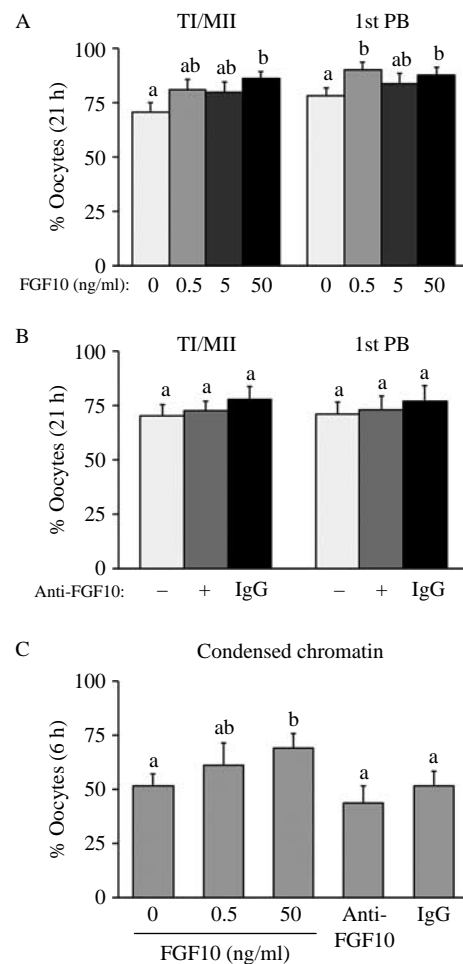
A

B

C

Figure 4 FGF10 impacts oocyte progression through meiosis and first polar body extrusion. (A and B) After 21–22 h, cumulus was removed and denuded oocytes were processed to determine the proportion of oocytes reaching telophase I (TI), meiosis II (MII; B only) and extruding their first polar body (1st PB). (A) The effect of FGF10 supplementation during IVM on oocyte maturation (*n*=6 replicate studies; 8–10 oocytes/treatment per study). (B) The effect of adding anti-FGF10 (or control IgG; each at 0.3 µg/ml) during IVM on oocyte maturation (*n*=6 replicate studies; 8–10 oocytes/treatment per study). (C) After 6 h of maturation, cumulus was removed and denuded oocytes were processed to determine the proportion of oocytes containing condensed chromatin (*n*=5 replicate studies; 15–20 oocytes/treatment per study). Different superscripts represent differences observed due to treatments (*P*<0.05).
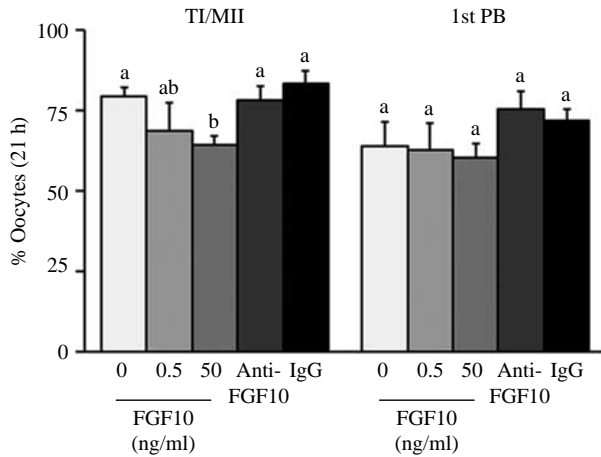
Figure 5 FGF10 does not stimulate maturation in denuded oocytes. Cumulus was removed from oocytes before maturation, and denuded oocytes were incubated in maturation medium containing FGF2 (0, 0.5, or 50 ng/ml) or IgG (0.3 μg/ml anti-FGF2 or control IgG; $n=6$ replicate studies; 15–20 oocytes/treatment per study). After 21–22 h, the proportion of oocytes reaching TI or MII and undergoing 1st PB extrusion were determined. Different superscripts represent differences observed due to treatments ($P<0.05$).

Adding 0.5 ng/ml FGF10 or anti-FGF10 did not affect the percentage of TI/MII oocytes. None of the treatments affected the first polar body extrusion rates.

Effects of FGF10 on cumulus expansion

FGF10 also affected the cumulus expansion rates during IVM. The first study examined the dose–response effect of FGF10 supplementation on the degree of cumulus expansion (rank scoring from 1 to 3; $n=9$ replicate studies; 20–30 COCs/treatment per study). A biphasic response to increasing FGF10 concentrations was observed. Cumulus expansion was increased ($P<0.05$) in oocytes matured in medium containing 0.5 ng/ml when compared with controls (2.43 ± 0.07 for 0.5 ng/ml FGF10 vs 2.20 ± 0.05 for control) but not when greater concentrations of FGF10 were provided (2.24 ± 0.06 and 2.31 ± 0.06 for 5 and 50 ng/ml FGF10 respectively).

Data were also analyzed to determine the proportion of COCs in each rank category. The proportion of oocytes with fully or near fully expanded cumulus (index score=3) were greater ($P<0.05$) in oocytes supplemented with 0.5 ng/ml FGF10 than controls ($51.0\pm5.6\%$ for 0.5 ng/ml FGF10 vs $35.3\pm3.8\%$ for controls) but not when greater amounts of FGF10 were supplemented (34.1 ± 4.5 and $43.8\pm4.3\%$ for 5 and 50 ng/ml FGF10 respectively). TUNEL analysis was completed on a subset of the COCs used in these experiments, and the supplementation with FGF10 did not affect the percentage of apoptotic cumulus cells at 21 h post maturation (data not shown).

The effect of endogenous FGF10 on cumulus expansion rates was examined by anti-FGF10 supplementation

($n=5$ replicate studies; 20–30 COCs/treatment per study). Adding 0.5 ng/ml FGF10 increased ($P<0.05$) the cumulus expansion score and co-supplementation with anti-FGF10 (0.1 μg/ml) blocked ($P<0.05$) this effect (2.06 ± 0.07 for controls; 2.26 ± 0.05 for 0.5 ng/ml FGF10 treatment; 2.05 ± 0.04 for 0.5 ng/ml FGF10 plus anti-FGF10; and 2.21 ± 0.04 for 0.5 ng/ml FGF10 plus control IgG). In a final study, providing anti-FGF10 in the absence of exogenous FGF10 reduced ($P<0.05$) the mean COC expansion score (1.87 ± 0.05 vs 1.7 ± 0.06 for control versus anti-FGF10 exposure; $n=3$ replicate studies; 20–30 COCs/treatment per study).

Effects of FGF10 on cumulus and oocyte gene expression

To examine the molecular basis of FGF10 actions during oocyte maturation, expression profiles for candidate genes were completed on cumulus cells and oocytes. FGF10 modified the relative abundance of only a few transcripts in cumulus cells and oocytes after 21 h of maturation (Fig. 6). Adding 0.5 ng/ml FGF10 decreased ($P<0.05$) the concentrations of *CTSB* and *SPRY2* mRNA in cumulus cells (Fig. 6A) and increased ($P<0.05$) the *BMP15* mRNA abundance in oocytes (Fig. 6B). *BMP15* mRNA abundance was also increased ($P<0.05$) in
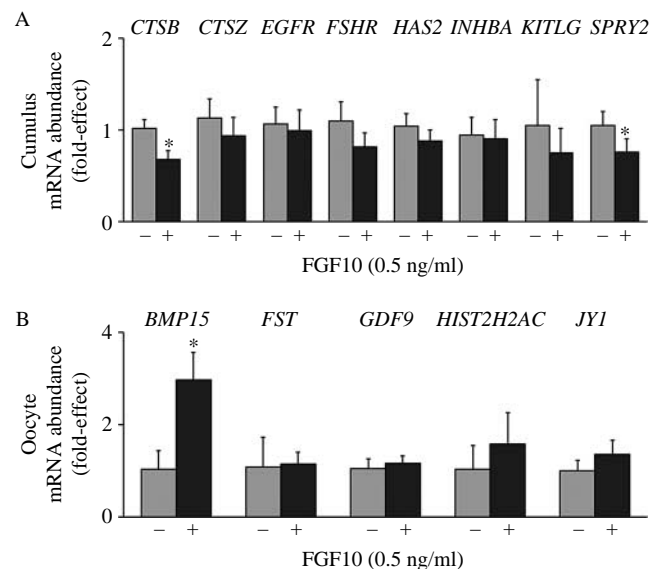

Figure 6 The effect of FGF10 supplementation on expression profiles of selective transcripts in cumulus cells and oocytes. COCs were cultured for 21 h in maturation medium containing or lacking 0.5 ng/ml FGF10. Cumulus cells and oocytes were separated, tcRNA was extracted from each, and qRT-PCR was completed. (A) Expression profiles for cumulus cells ($n=5$ samples/treatment; each sample contained cumulus cells from 20 to 25 COCs). (B) Expression profiles for oocytes ($n=6$ pools/treatment; each pool contained 20–25 oocytes). ΔC_t values were used for statistical analysis, and data are reported based on fold change ($\pm$ s.e.m.) relative to the control (no FGF10 treatment). Asterisks indicate differences observed between treatment groups for a given transcript ($P<0.05$).

oocytes obtained from FGF10-treated COCs when data were normalized based on oocyte numbers used for qRT-PCR analysis (data not shown).

A second study was completed to describe how FGF10 impacts candidate gene expression after 6 h of maturation. No changes in transcript abundance were detected in the subset of the cumulus-specific transcripts (*CTSB*, *EGFR*, *FSHR*, *HAS2*, and *SPRY2*) and all of the oocyte-specific transcripts (data not shown).

Discussion

Embryos generated from IVM/IVF procedures usually are less able to produce viable offspring than *in vivo*-derived embryos in cattle and humans (Fitzgerald *et al*. 1998, Loureiro *et al*. 2009, Binelli & Murphy 2010). The absence of specific thecal-derived products during IVM may be one reason for these reductions in oocyte and embryo competency. This work determined that at least one thecal- and oocyte-derived product, FGF10, improves embryogenesis when provided during IVM.

The first study described the types of *FGFRs* expressed in bovine cumulus cells and oocytes as they begin IVM. Transcripts for each of the four genes were readily evident in cumulus cells. Oocytes also contained transcripts for all four receptor classes, although very little *FGFR3* mRNA was detected. As described previously, FGF10 reacts primarily with R1b and R2b with high affinity and with several other FGFRs with much lower affinities (Miralles *et al*. 1999, Powers *et al*. 2000, Itoh & Ornitz 2004). By using primer sets that specifically amplify single *FGFR1* and *R2* spliced variants (Naimi *et al*. 2002, Berisha *et al*. 2006, Guerra *et al*. 2008), transcripts for *R1b* and *R2b* were detected in both tissues and it appeared that *R1b* mRNA was the predominant FGF10 receptor partner in cumulus cells whereas *R2b* was more prevalent in oocytes. During the initial validation of primer efficiencies, all primer pairs effectively and specifically reacted with their target sequences (87–104% efficiency). Standard curves (either absolute or relative curves) were not included during the examination of the test samples, and therefore statistical comparisons between amplicons could not be made. To the best of the authors' knowledge, this is the first report of *FGFR1* and *R2* mRNA subtype profiling in bovine COCs. A previous report has described *R3c* and *R4* expression in preantral bovine follicles (Buratini *et al*. 2005). Interestingly, that report indicated that *R3c* was more prevalent than *R4* during preantral development. This was not observed in the present work; rather, *R3* mRNA (both *b* and *c* isotypes) was barely detectable in oocytes. This suggests that *FGFR* transcript profiles are distinct in preantral and postantral stages bovine follicle development.

Cleavage rates were unaffected in oocytes supplemented with FGF10 during IVM, but subsequent embryo development rates were improved with this treatment.

The most obvious postfertilization effects included 1) the proportion of embryos reaching the 8–16-cell stage on day 3, and 2) the proportion of embryos reaching the blastocyst stage on day 7 and advanced blastocysts on day 8 post IVF. It remains unclear exactly how FGF10 treatment promotes embryo development, but exposure to FGF10 did not affect blastomere numbers on day 8 suggesting that the embryotrophic effects of FGF10 do not require improvements in blastomere numbers on days 7 and 8. Similar outcomes were observed by others (Rizos *et al*. 2002, 2003). In those studies, bovine oocyte quality affected subsequent blastocyst yields more so than blastocyst quality. Perhaps FGF10 enhances the ooplasm microenvironment by altering the concentrations of specific molecules that promote embryo development during the first few cleavage events. Embryonic genome activation occurs at the 8–16-cell stage in bovine embryos (Betteridge & Flechon 1988). Improvements in embryo development were detectable this time, and it is quite possible that enhancements in the early developing embryo created a greater proportion of competent 8–16-cell embryos that continued to develop into blastocysts.

Biphasic oocyte responses were evident when FGF10 was added to the maturation medium. Observing maximal responses with 0.5 ng/ml indicates that FGF10 likely interacts with high-affinity FGFRs (i.e. R1b and/or R2b) to elicit its response ($ED_{50}=0.1$–1 ng/ml; Ornitz 2000, Powers *et al*. 2000). Similar biphasic dose responses to FGF supplementation, and other paracrine-acting factors, for that matter, are evident in various cell types (Butterwith *et al*. 1993, Murono *et al*. 1993, Halevy *et al*. 1994, Garcia-Maya *et al*. 2006, Li *et al*. 2009a). This phenomenon could have been caused by receptor downregulation events associated with ligand overloading (Murono *et al*. 1993). Alternatively, this effect could reflect differential receptor usage that could have prompted secondary signaling systems that interfered with the primary signaling response (Wang *et al*. 2004, Garcia-Maya *et al*. 2006, Hayashi *et al*. 2008).

Subsequent investigation into how FGF10 improves oocyte competency determined that FGF10 improves several aspects of oocyte maturation. One feature of maturation that was improved by FGF10 was cumulus expansion. The magnitude of FGF10 effects on cumulus expansion scores and the percentage of COCs that underwent full expansion by 21 h were not great, likely because expansion rates already were fairly great to begin with, but they did occur when using the same concentration of FGF10 required to improve subsequent embryo development (0.5 ng/ml). An endogenous source of FGF10 also appears important for cumulus expansion in cattle. *In situ* hybridization work in bovine follicles found copious amounts of *FGF10* mRNA in thecal cells and immunoreactive FGF10 protein throughout the thecal and granulosa layers (Buratini *et al*. 2007). Oocytes also contain *FGF10* transcripts (Buratini *et al*. 2007).

The IgG treatments used for this work likely targeted the oocyte-derived FGF10 and any residual thecal-derived FGF10 that remained bound to extracellular matrix within COCs. The IgG neutralization appeared specific for FGF10. This IgG was used previously to neutralize FGF10 actions on mouse incisor growth (Harada *et al.* 2002). In the present work, providing IgG molecules prevented supplemental FGF10 from stimulating cumulus expansion and subsequent embryo development rates, and providing molar excesses of FGF10 partially overcame the neutralization effects of FGF10 IgG on embryonic development into the blastocyst stage. In addition, providing FGF10 IgG in the absence of supplemental FGF10 decreased cumulus expansion. These observations implicate modifications in cumulus cell activity as a potential source for FGF10-induced increases in oocyte competency.

FGF10 also affects other aspects of oocyte maturation. FGF10 improved the percentage of oocytes containing condensed chromatin after 6 h of maturation, oocytes progressing to TI/MII, and oocytes extruding their first polar body after 21 h. However, the concentrations of FGF10 needed to observe many of these responses were greater than those needed to improve subsequent embryo development. With the exception of the polar body extrusion rates, other benefits to oocyte meiotic maturation required 50 ng/ml FGF10. Providing anti-FGF10 did not affect meiotic maturation rates. These observations indicate that FGF10-dependent improvements in bovine oocyte competency are not dependent on improvements in oocyte maturation; rather, modifications in cumulus function likely are the primary target for FGF10 actions. Cumulus cells are needed for FGF10 to positively impact oocyte maturation. FGF10 supplementation to oocytes devoid of cumulus cells did not improve their ability to mature *in vitro*. Polar body extrusion rates were unaffected by FGF10 supplementation when cumulus cells were absent and meiotic maturation rates decreased when 50 ng/ml FGF10 was added. Moreover, adding FGF10 IgG to block endogenous FGF10 activity did not affect maturation rates in these oocytes.

To further understand how FGF10 improves oocyte competency, a series of transcripts identified by others as putative competency markers in cumulus and oocytes were examined. Several cumulus-specific transcripts are linked to oocyte competency. The gene encoding cathepsin B (*CTSB*) contained an interesting FGF10-dependent expression pattern. Several cathepsin transcripts, including *CTSB* and *CTSZ*, are inversely related to oocyte quality. In one study, cumulus derived from COCs of prepubertal heifers, a model of poor oocyte competency, contained more *CTSB* mRNA than COCs from mature cows (Bettegowda *et al.* 2008). Oocyte quality and postfertilization development could be improved by exposing COCs to a membrane-permeable cathepsin inhibitor (Bettegowda *et al.* 2008). In another

study, cows with a low rate of antral follicle development contained greater amounts of *CTSB* mRNA than cows with higher numbers of antral follicles (Ireland *et al.* 2009). In the present work, *CTSB* mRNA abundance decreased in cumulus from COCs exposed to FGF10. *CTSZ*, by contrast, exhibited no FGF10-dependent changes in transcript abundance. FGF10 supplementation also reduced the cumulus concentrations *SPRY2* mRNA. This is not surprising given that SPRY2 is a modulator of FGF signaling (Sugiura *et al.* 2009). The remaining cumulus competency markers were not impacted by FGF10. Included in this work were the following transcripts: *EGFR* and *FSHR*, receptors essential for normal oocyte maturation and cumulus expansion (Assidi *et al.* 2008, Caixeta *et al.* 2009); *HAS2*, a key player in cumulus expansion (Dragovic *et al.* 2005, Assidi *et al.* 2008); *KITLG*, a component of stem cell survival and oocyte growth (Cho *et al.* 2008); and *INHBA*, an inhibin subunit (Assidi *et al.* 2008).

Several putative oocyte competency factors were also examined for whether they associate with FGF10-mediated increases in oocyte competency. Included among these were *FST* (Hussein *et al.* 2006, Lee *et al.* 2009), *GDF9* (Dragovic *et al.* 2005), *JY1* (Bettegowda *et al.* 2007) and *HIST2H2AC* (Dode *et al.* 2006, Caixeta *et al.* 2009), and *BMP15* (Hussein *et al.* 2006). Changes in overall RNA abundances are evident throughout oocyte maturation and early embryonic development in cattle and other species (Robert *et al.* 2002), and identifying a suitable internal control (or set of controls) is imperative for proper interpretation of outcomes at these stages. This work did not compare transcript profiles over time because of these complications with identifying suitable reference transcripts; rather, control and FGF10-treated oocytes were examined within specific time-points. The abundance of the internal control (*18S*) did not differ among treatments within each time-point. Moreover, correcting the raw C_t values for numbers of oocytes in each RNA sample yielded the same outcomes as the ΔC_t method. These arguments support the use of *18S* as the reference transcript for this work. However, the accuracy of these findings may have been improved if more extensive methods that utilize multiple reference genes (e.g. Relative Expression Software Tool or REST) or include external cRNA to control for variations in RT and/or PCR efficiencies between samples were utilized in this work.

BMP15 was the only oocyte transcript investigated which was affected by FGF10 treatment. It is one of the several members of the TGFB superfamily expressed in oocytes during folliculogenesis and ovulation (Juengel & McNatty 2005, Gilchrist *et al.* 2008). *BMP15* transcripts are first evident at the primary follicle stage of most mammals (Juengel & McNatty 2005, Gilchrist *et al.* 2008). Deficiencies in *BMP15* expression cause primary follicle arrest in ewes (Galloway *et al.* 2000) and are linked with infertility in women (Di Pasquale *et al.* 2004,

2006). Several recent findings implicate BMP15 as a vital mediator of oocyte competency. Recombinant BMP15 induces cumulus expansion in mice (Li *et al.* 2009*b*, Mottershead & Watson 2009) and limits cumulus cell apoptosis in bovine COCs (Hussein *et al.* 2005). In addition, culturing bovine COCs with BMP15 did not affect fertilization rates *in vitro* but increased subsequent blastocyst formation rates (Hussein *et al.* 2006). Perhaps some or all of the FGF10-mediated effects observed in this work resulted from an increase in BMP15 production. The extent of this functional linkage must be described more thoroughly, but it is tempting to speculate that that one of the major actions of FGF10 is to serve as an upstream regulator of *BMP15* expression during the final stages of oocyte maturation.

It remains unclear to what extent using a heterologous recombinant protein had on the outcomes. The human FGF10 protein chosen for this work is 94% identical in amino acid sequence to bovine FGF10. It reacts with approximately the same potency as other FGFs on various tissues, although a subtle reduction in activity was observed when comparing the potency of this protein with other FGFs (FGF1, 2, and 7) for their ability to stimulate interferon-τ production in bovine trophectoderm (TE; Cooke *et al.* 2009). It is presumed that human FGF10 reacts with the same FGFR subtypes as bovine FGF10, although that has not been verified. It remains possible, therefore, that certain aspects of FGF10 activity during oogenesis were not identified by using human FGF10 in bovine COCs.

In summary, work presented herein provides evidence that thecal- and oocyte-derived FGF10 improves oocyte competency. Providing FGF10 to bovine COCs during IVM improved oocyte maturation, cumulus expansion, and subsequent embryo development. The mechanisms controlled by FGF10 have not been elucidated. A closer examination of metabolic, transcriptomic, and proteomic changes regulated by FGF10 is warranted. However, based on the present findings, FGF10 is likely to act on cumulus cells in ways that improve their ability to regulate meiosis and provide ooplasm with components of importance for early embryonic survival.

Materials and Methods

Unless stated otherwise, reagents were purchased from Sigma–Aldrich Co. All studies were completed in accordance and with the approval of the Institutional Animal Care and Use Committee at the University of Florida.

Bovine IVM, IVF, and IVC

Bovine oocyte IVM, IVF, and IVC were completed as described previously (Rivera & Hansen 2001, Loureiro *et al.* 2009). In brief, ovaries from beef and dairy cattle were obtained from Central Beef Packing Co. (Center Hill, FL, USA) and washed several times with 0.9% (w/v) sodium chloride supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin. COCs were collected, and groups of 10–12 COCs were cultured in 50 µl drops of oocyte maturation medium (TCM199 containing Earle's salts (Invitrogen Corp.) supplemented with 25 µg/ml bovine FSH (Bioniche Life Sciences, Belleville, ON, Canada), 2 µg/ml estradiol 17-β, 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, 1 mM glutamine, and 1 mg/ml PVA). Maturation medium was supplemented with varying concentrations of recombinant human FGF10 (Invitrogen Corp.; 0.5–50 ng/ml prepared in TCM199 containing Earle's salts) or immunoglobulin (IgG; anti-FGF10 polyclonal, IgG-purified (SC-7375) or control IgG (SC-2028), Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). In one study, IVM was completed using denuded oocytes. For this, COCs were vortexed for 4 min to remove cumulus before maturation.

After 21–22 h at 38.5 °C in 5% CO_2 in humidified air, COCs were transferred to fertilization medium and exposed to Percoll-gradient-purified bovine spermatozoa (Rivera & Hansen 2001, Loureiro *et al.* 2009). The same pool of semen from three bulls was used throughout the studies. After 8–10 h at 38.5 °C (5% CO_2 in humidified air), cumulus cells were removed by vortexing in 1000 U/ml hyaluronidase and denuded putative zygotes were placed in groups of 25–30 in 50 µl drops of synthetic oviduct fluid (Millipore, Billerica, MA, USA) containing 25 µg/ml gentamicin sulfate, 0.4 mM sodium pyruvate, 2.77 mM myo-inositol, 0.5 mM sodium citrate, 1 mM alanyl glutamine, 5.3 mM sodium lactate syrup, 10 µl/ml nonessential amino acids, 20 µl/ml essential amino acids, and 4 mg/ml fatty acid-free BSA. Drops were covered with mineral oil and maintained at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2 for 8 days. The proportion of cleaved zygotes and the proportion of embryos containing 8–16 blastomeres were recorded on day 3 post IVF. The proportion and stage of blastocysts (early, regular, expanded, hatching, and hatched) were recorded at days 7 and 8 post IVF.

Nuclear maturation, cumulus expansion, and apoptosis following oocyte maturation

After maturation, the degree of cumulus expansion in COCs was scored visually by phase-contrast microscopy on a 1–3 scale (1, poor expansion characterized by few morphological changes compared with before maturation; 2, partial expansion characterized by fair expansion but notable clusters lacking expansion; 3, complete or nearly complete expansion) as described previously (Kobayashi *et al.* 1994).

To assess progression through meiosis after IVM, oocytes were denuded by vortexing for 4 min in saline after 6 or 21 h of maturation. Chromatin condensation status was determined at 6 h and meiotic staging and first polar body extrusion was completed at 21 h. At both time-points, oocytes were fixed with 4% (w/v) paraformaldehyde (Polysciences, Inc., Warrington, PA, USA), permeabilized with 0.1% Triton X-100, and stained for 15 min with 1 µg/ml Hoechst 33342 (Invitrogen Corp.). Chromatin status and meiotic staging were determined with epifluorescence microscopy as described previously (Roth & Hansen 2005, Marei *et al.* 2009). The presence of first polar body extrusion was determined under stereomicroscopy.

In some studies, COCs were processed after IVM to determine the percentage of TUNEL-positive cumulus cells. For this, COCs were washed in 0.01 M PBS (pH 7.2) containing 1 mg/ml polyvinyl pyrrolidone (PBS–PVP) and fixed in 4% (w/v) paraformaldehyde. COCs were permeabilized in 0.5% (v/v) Triton X-100, and 0.1% (w/v) sodium citrate for 30 min at RT in a humidified box. COCs were incubated in 25 μl drops of the TUNEL reaction mixture containing FITC-conjugated dUTP and terminal deoxynucleotidyl transferase (Roche Applied Sciences) for 1 h at 37 °C in the dark. COCs were counterstained with 1 μg/ml Hoescht 33342, mounted on slides, and analyzed with epifluorescence microscopy. The proportion of TUNEL-positive cumulus cells were calculated by dividing the number of TUNEL-positive nuclei with total nuclei numbers in each of the four fields under 200-fold magnification.

Differential staining in blastocysts

Differential staining for TE versus ICM cells was completed as described previously (Block *et al.* 2008). In brief, blastocysts obtained on day 8 post IVF were incubated with 100 μg/ml RNase A (Qiagen) for 1 h and were transferred to as little solution as possible into 100 μg/ml propidium iodide (Invitrogen Corp.), 0.2% Triton X-100, and 1 mg/ml PVP in PBS for 30 s. After three washes in PBS–PVP, embryos were placed into a solution containing 10 μg/ml Hoechst 33342, 4% paraformaldehyde, and 1 mg/ml PVP in 0.01 M PBS (pH 7.2) for 15 min. After a final series of washes, blastocysts were placed into glycerol drops on microscope slides and TE and ICM cells were counted by using epifluorescence microscopy.

Real-time qRT-PCR

Cumulus cells and oocytes ($n=25$–30/group) were separated from each other by vortexing either immediately after isolating COCs from follicles (0 h; no maturation) or after 6 or 21 h after beginning IVM. Denuded oocytes were removed and washed thrice in PBS–PVP. Cumulus cells were transferred to microcentrifuge tubes and centrifuged at 700 ***g*** for 2 min at room temperature to remove residual solution. Both oocytes and cumulus cells were snap-frozen in liquid nitrogen and stored at −80 °C.

Total cellular (tc) RNA was extracted from cumulus cells using the RNeasy Micro kit (Qiagen) and from oocytes using the PicoPure RNA Isolation kit (MDS Analytical Technologies, Sunnyvale, CA, USA). RNA concentrations were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The $A_{260/280}$ ratios were <1.8 in all cumulus samples. Limited amounts of RNA prevented determination of RNA quality in the oocyte samples. RNA (10 ng/reaction for cumulus samples; entire RNA sample for oocyte samples) was incubated in RNase-free DNase (New England Biolabs Inc., Ipswich, MA, USA) and reverse transcribed using the High Capacity cDNA RT kit (Applied Biosystems, Inc., Foster City, CA, USA). Primer sets (Table 1) were used in combination with a SYBR Green detection system and a 7300 Real-Time PCR System (Applied Biosystems, Inc.) to provide relative quantities of specific transcripts in cumulus and oocyte transcripts. Primers were used at a concentration

Table 1 Primers used for quantitative RT-PCR.

Transcript	Primer sequence (5′–3′)
Oocyte	
BMP15	For: GTCAGCAGCCAAGAGGTAGTG
	Rev: CCCGAGGACATACTCCCTTAC
FST	For: CAGAGCTGCAAGTCCAGTACCA
	Rev: CATGTAGAGCTGCCTGGACAGA
GDF9	For: GGGAAATGTGTTCCTTGCTAATTC
	Rev: AGCAGCAAAACCAAAGGAAGAA
HIST2H2AC	For: GTCGTGGCAAGCAAGGAG
	Rev: GATCTCGGCCGTTAGGTACTC
JY1	For: TTGGAACTTCCATGGACGACC
	Rev: ATTTGCTGGTGATCCCAAGAG
Cumulus	
CTSB	For: CGATGCCCGGGAACAGT
	Rev: GAGCACAGGATCCCTGATC
CTSZ	For: GGGAGAAGATGATGGCAGAAAT
	Rev: TCTTTTCGGTTGCCATTATGC
EGFR	For: ACCCTGATCTGGAAGTTTGCA
	Rev: TCGAGACCTGGCCCTTCAC
FSHR	For: TGGTCCTGTTCTACCCCATCA
	Rev: GAAGAAATCCCTGCGGAAGTT
HAS2	For: TAAATGTGGCAGGCGGAAGAAGG
	Rev: GTCTTTGTTCAAGTCCCAGCAGCA
INHBA	For: GCTACCACGCCAACTACTGTGA
	Rev: AGAGGGATGAGCCCGATGT
KITLG	For: AATGGGCAGCCGTAGCATT
	Rev: TCCAGTAAAAGGCCCCAAAA
SPRY2	For: CACGTGCTGTCTCTGGATCAG
	Rev: GGCCCCTCCGTGTACTCA
FGFRs	
R1b	For: ACGTCCTGGTGACGGAGG
	Rev: CCGGTGCCATCCATTTGA
R1c	For: ACTGCTGGAGTTAATACCACCG
	Rev: GCAGAGTGATGGGAGAGTCC
R2b	For: GTGGAAAAGAACGGCAGTAAATA
	Rev: GAACTATTTATCCCCGAGTGCTTG
R2c	For: CACCACGGACAAAGAAATTG
	Rev: ATGCAGAGTGAAAGGATATCCC
R3	For: GCAGCGGCTACAGGTGCTCA
	Rev: CAGGCCGCGTCCAGTAAGGG
R4	For: GCAGACGCTCCTCACCCGAC
	Rev: CGAGACTCACGAGGCCAGCG

The relative standard curve approach (five serial dilutions of a follicular/oocyte RNA pool) was used to verify primer efficiencies. The following primers sets were designed previously: *CTSB* and *CTSZ* (Bettegowda *et al.* 2008); *BMP15* (Marina *et al.* 2006); *JY1* and *FSHR* (Ireland *et al.* 2009); *HIST2H2AC* (Goossens *et al.* 2005); *HAS2* (Assidi *et al.* 2008); *FGFR1b* (Guerra *et al.* 2008); *FGFR1c* and *R2c* (Berisha *et al.* 2006); *FGFR2b* (Naimi *et al.* 2002). Remaining primers were designed using Primer Express Software (version 3.0; Applied Biosystems, Inc.).

of 200 nM and were mixed with RT products and SybrGreen PCR Master Mix (Applied Biosystems, Inc.). After an initial activation/denaturation step (50 °C for 2 min, 95 °C for 10 min), 40 cycles of a two-step amplification protocol (60 °C for 1 min, 95 °C for 15 s) were completed. A dissociation curve analysis (60–95 °C) was used to verify the amplification of a single product. Amplicons derived from newly synthesized primer pairs were sequenced (UF DNA Sequencing Facility) to ensure the correctness of amplification. Each sample was run in triplicate and a fourth reaction lacking exposure to reverse transcriptase was included to verify the absence of genomic contamination. Relative amount of *18S* RNA was used as an internal control for quantifying relative gene expression. In one

study, the ratio of target to reference RNA was used to determine relative expression ($2^{-C_t(\text{target})}/2^{-C_t(\text{reference})}$). The remaining studies used the comparative threshold cycle (C_t) approach to determine relative abundance (Michael *et al.* 2006). The average ΔC_t value for each transcript was calculated (target $C_t - 18S\ C_t$) and used to calculate the fold-change ($2^{-\Delta\Delta C_t}$).

Statistical analysis

All analyses were completed with least-squares ANOVA using the general linear model of the Statistical Analysis System (SAS for Windows, version 9.0; SAS Institute, Inc., Cary, NC, USA). Statistical analyses used arcsin-transformed percentage data generated from each replicate (experimental unit = average percentage within each replicate). Differences in the individual means were separated further by completing pair-wise comparisons (probability of difference analysis; SAS Institute, Inc.). Percentage data were graphed using nontransformed values and S.E.M.s. Differential staining data were analyzed using embryo as the experimental unit, and data were analyzed to describe the effect of FGF10 treatment on TE and ICM cell numbers and proportion of cells that were ICM (ICM/Total). COC expansion was analyzed in two ways: 1) the mean COC expansion index was calculated for each treatment within each replicate and 2) the proportion of COCs observed within each of the nonparametric rankings (1, 2, and 3). When analyzing qRT-PCR data, either log-transformed ratios or ΔC_t values were used for the statistical analyses. Data were presented either as the ratio of target to reference RNA or as fold differences from control values.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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