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Several fibroblast growth factors are expressed during pre-attachment bovine conceptus development and regulate interferon-tau expression from trophectoderm

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Abstract

The trophectoderm-derived factor interferon tau (IFNT) maintains the uterus in a pregnancy-receptive state in cattle and sheep. Fibroblast growth factors (FGFs) are implicated in regulating *IFNT* expression and potentially other critical events associated with early conceptus development in cattle. The overall objectives of this work were to identify the various *FGFs* and FGF receptors (*FGFRs*) expressed in elongating pre-attachment bovine conceptuses and determine if these FGFs regulate conceptus development and/or mediate IFNT production. *In vitro*-derived bovine blastocysts and *in vivo*-derived elongated conceptuses collected at day 17 of pregnancy express at least four *FGFR* subtypes (*R1c, R2b, R3c, R4*). In addition, transcripts for *FGF1, 2,* and *10* but not *FGF7* are present in elongated bovine conceptuses. The expression pattern of *FGF10* most closely resembled that of *IFNT,* with both transcripts remaining low in day 8 and day 11 conceptuses and increasing substantially in day 14 and day 17 conceptuses. Supplementation with recombinant FGF1, 2 or 10 increased *IFNT* mRNA levels in bovine trophectoderm cells and bovine blastocysts and increased IFNT protein concentrations in trophectoderm-conditioned medium. Blastocyst development was not affected by any of the FGFs. In summary, at least four FGFRs reside in pre- and peri-attachment bovine conceptuses. Moreover, conceptuses express at least three candidate FGFs during elongation, the time of peak *IFNT* expression. These findings provide new insight for how conceptus-derived factors such as FGF1, 2, and 10 may control *IFNT* expression during early pregnancy in cattle.

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Introduction

In cattle, sheep, and presumably other ruminants, the trophectoderm-derived factor, interferon-tau (IFNT), is responsible for sustaining a pregnant state by restricting the pulsatile release of prostaglandin F2α from the endometrial epithelium and thereby preventing regression of corpus luteum and return to estrus (Demmers *et al*. 2001, Spencer *et al*. 2004). IFNT also controls the expression of several uterine-derived factors that prepare the uterus for placental attachment, modifies the uterine immune system, and regulates early conceptus development (Austin *et al*. 1996, Teixeira *et al*. 1997, Ott *et al*. 1998, Spencer *et al*. 1998, Pru *et al*. 2001, Wang & Goff 2002, Gifford *et al*. 2008). It is not surprising, therefore, that insufficient production of IFNT or failure of the maternal system to recognize this signal leads to pregnancy failures in cattle (Roberts 1991, Thatcher *et al*. 1994, 2001, Inskeep & Dailey 2005).

Bovine embryo production of IFNT begins at the late morula and early blastocyst stage as the trophoblast cell lineage first develops (day 6–7 of pregnancy; Hernandez-Ledezma *et al*. 1992, Kubisch *et al*. 1998). In cattle, *IFNT* mRNA levels peak around days 14–16 of pregnancy and remain elevated until implantation occurs around days 19–21 of pregnancy (Ealy *et al*. 2001, Mann *et al*. 2006, Robinson *et al*. 2006). A few selective uterine- and conceptus-derived factors are known regulators of *IFNT* expression (Roberts *et al*. 2003, 2004). One of these is fibroblast growth factor 2 (FGF2). FGF2 is expressed by the luminal and glandular epithelium and detected in the uterine lumen of cows and ewes throughout the estrous cycle and early pregnancy (Michael *et al*. 2006, Ocon-Grove *et al*. 2008). Studies using a bovine trophectoderm cell line (CT1) and *in vitro*-produced (IVP) bovine blastocysts reveal that supplementation with FGF2 increases *IFNT* mRNA and protein levels (Michael *et al*. 2006, Rodina *et al*. 2008).

FGF2, also known as basic FGF, was the first identified member of what is now a large family of FGFs. At least 22 genes encode multiple FGFs in the human and mouse, and their protein products contain a variety of biological activities in tissues and organs (Powers *et al*. 2000, Itoh & Ornitz 2004, 2008). These FGFs interact with a group of tyrosine kinase receptors known as FGF receptors, or FGFRs. Four genes encode these receptors

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(*FGFR1-4*), and alternative splicing in extracellular regions generates a variety of receptor isotypes (Powers *et al*. 2000, Kim *et al*. 2001, Sleeman *et al*. 2001, Bottcher & Niehrs 2005). One of these splicing events occurs within the third immunoglobulin (Ig)-like domains of *FGFR1-3*, where splicing one or two exons from the coding transcript generates receptors subtypes termed IgIIIb and IgIIIc. These spliced variants recognize different ligands (Johnson *et al*. 1991, Werner *et al*. 1992, Powers *et al*. 2000). For example, the IgIIIb form of FGFR2 (R2b) interacts primarily with FGF1, 3, 7, 10, and 22 whereas the IgIIIc form (R2c) interacts with FGF1, 2, 4, 6, and 9 (Powers *et al*. 2000, Itoh & Ornitz 2004).

Several FGFs and FGFRs are associated with early conceptus development in mammals. FGF2 increases trophectoderm outgrowth size in mice and stimulates gastrulation in rabbit conceptuses (Haimovici *et al*. 1991, Hrabe *et al*. 1995, Taniguchi *et al*. 1998). In the pig, uterine-derived FGF7 acting through its receptor, R2b, stimulates trophectoderm proliferation (Ka *et al*. 2001). In mice, FGF4-induced activation of its receptor partner, R2c, is critical for maintaining a trophoblast stem cell lineage that supports normal placental development (Feldman *et al*. 1995, Arman *et al*. 1998). FGF4 supplementation is required to prevent mouse trophoblast cell differentiation during culture (Tanaka *et al*. 1998, Hughes *et al*. 2004). This type of supplementation is not needed to maintain bovine trophectoderm in culture. They continue to proliferate without differentiating for extensive periods in culture (Talbot *et al*. 2000, 2007, Kubisch *et al*. 2001, Shimada *et al*. 2001).

Bovine and ovine conceptuses produce multiple FGFs. Transcripts for *FGF2* and several *FGFR* spliced variants are detected in elongated ovine conceptuses (Ocon-Grove *et al*. 2008). Also, transcripts for *FGF1*, *2*, *7*, and *10* and several *FGFRs* exist in post-attachment stage placentae in sheep and cattle (Chen *et al*. 2000, Pfarrer *et al*. 2006). These FGFs likely facilitate placental development in a variety of ways throughout gestation. This laboratory is specifically interested in uncovering the actions of these FGFs as bovine conceptuses develop and begin to attach to the uterine lining early in pregnancy. The overall objectives of this work were to identify the various *FGFs* and *FGFRs* expressed in elongating pre-attachment bovine conceptuses and determine if these FGFs contain the ability to regulate conceptus development and/or mediate IFNT production during pre-attachment development.

Results

FGFR subtype expression in bovine conceptuses

End-point RT-PCR was used to detect the *FGFR* isotypes expressed during early conceptus development in cattle. *FGFR1*, *2*, *3*, and *4* mRNA were detected in bovine conceptuses collected at day 17 post-insemination (Figs 1 and 2) and IVP-blastocysts (Fig. 2). Each *FGFR* was also detected in CT1 cells (Fig. 1), a bovine trophectoderm cell line derived from a bovine blastocyst that produces IFNT (Talbot *et al*. 2000, Michael *et al*. 2006). Amplified products of the correct size were detected in all samples with the exception of one day 17 conceptus sample that consistently lacked an *FGFR1* amplicon (Fig. 1).

Primers used to amplify each *FGFR* spanned the sequence encoding their third Ig-like domains, and the specific spliced variant forms of *FGFR1*, *2*, and *3* expressed by conceptuses and CT1 cells were determined by sequencing amplified products (Ocon-Grove *et al*. 2008). All of the *FGFR1* cDNAs derived from blastocysts and day 17 conceptuses ($n=8$ clones sequenced) had the same sequence and comparative sequence analysis indicated that this sequence encoded *R1c* (Johnson *et al*. 1991, Ocon-Grove *et al*. 2008; GeneID: 281768; GenBank #NP_001103677). All blastocyst- and conceptus-derived *FGFR2* cDNAs ($n=10$ clones sequenced) were identical and their translated product represented *R2b*, which is also known as keratinocyte growth factor (KGF)/FGF7 receptor (Johnson *et al*. 1991, Ocon-Grove *et al*. 2008;

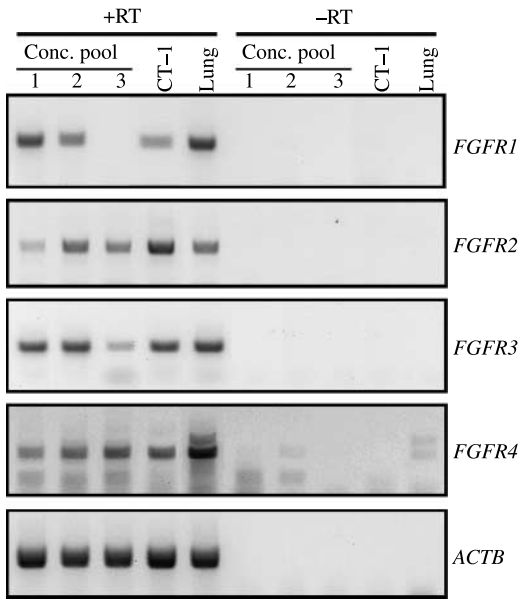

Figure 1 *FGFR* subtypes expressed in day 17 bovine conceptuses and CT1 cells. RT-PCR was completed on tcRNA samples derived from three pools of day 17 bovine conceptuses ($n=3$–5 conceptuses/pool) and one CT1 sample using primers specific for the third Ig-like extracellular domain of bovine *FGFR1*, *2*, *3*, and *4* (Supplementary Table 1). Products were electrophoresed and visualized with ethidium bromide and u.v. light. tcRNA derived from bovine lung was included as a positive tissue control. Primers for β-actin (*ACTB*) were used as a positive RT-PCR control. tcRNA not exposed to reverse transcriptase (−RT samples) was included to verify that amplified products did not result from genomic contamination. Amplified products were sequenced to verify that they represent specific *FGFRs*.

GeneID: 404193; GenBank #XP_001789758). For *FGFR3*, translated products for all cDNAs ($n=10$ clones sequenced) matched the inferred amino acid sequence of *R3c* (GeneID: 281769; GenBank #AAK54132; Ocon-Grove *et al.* 2008). *FGFR4* amplicons of the correct anticipated size ($n=4$ cDNAs sequenced) were identical to *R4* (GeneID: 317696). The less abundant amplicons seen in some of the *FGFR4* reactions were not sequenced (Figs 1 and 2). DNA sequencing also confirmed that CT1 cells express *R1c*, *R2b*, *R3c*, and *R4*.

Expression and abundance of FGF1, 2, 7, and 10 in bovine conceptuses

End-point RT-PCR also was used to determine if *FGF1*, *2*, *7*, and *10* are expressed in day 17 bovine conceptuses (Fig. 3). Transcripts for *FGF1*, *2*, and *10* were detected in all three day 17 bovine conceptus RNA samples were examined. *FGF1* and *2* transcripts but not *FGF10* also were detected in CT1 cell RNA. *FGF7* was not amplified in day 17 conceptus and CT1 samples but was detected in control samples (endometrium and lung). Bovine endometrium was included as a positive control based on previous work describing endometrial *FGF1*, *2*, *7*, and *10* expression in cyclic and pregnant cows and ewes (Chen *et al.* 2000, Pfarrer *et al.* 2006, Michael *et al.* 2006, Ocon-Grove *et al.* 2008). DNA sequencing verified that each PCR product represented the FGFs of interest (data not shown). Smaller, less intense PCR products were observed in some of the *FGF1* reactions (Fig. 3). These products were not sequenced, and this secondary product was not detected when new primers were designed and used for qRT-PCR (see below).

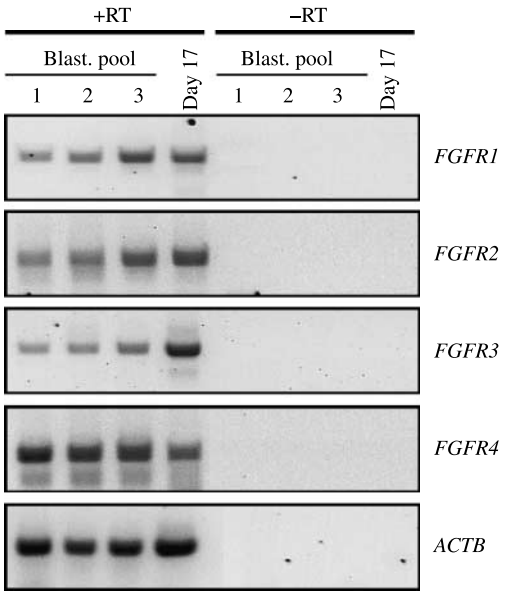

Figure 2 *FGFR* subtypes expressed in bovine IVP-blastocysts. RT-PCR was completed on tcRNA samples derived from three pools of IVP-blastocysts ($n=10$–18 blastocysts/pool) using primers specific for the third Ig-like extracellular domain of bovine *FGFR1*, *2*, *3*, and *4* (Supplementary Table 1). Products were electrophoresed and visualized with ethidium bromide and u.v. light. tcRNA derived from a pooled day 17 bovine conceptus preparation was included as a positive tissue control. Primers for *ACTB* were used as a positive RT-PCR control. tcRNA not exposed to reverse transcriptase (−RT samples) was included to verify that amplified products did not result from genomic contamination.

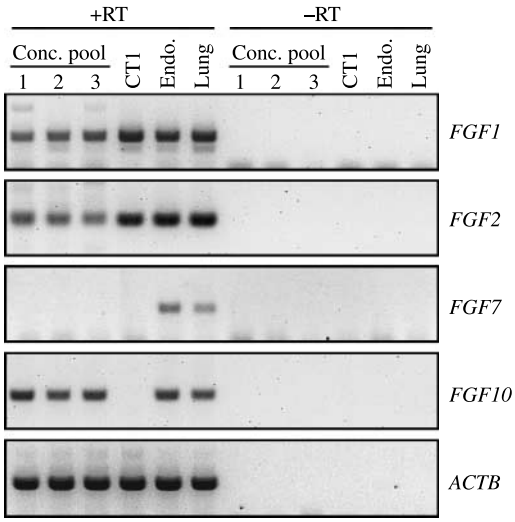

Figure 3 Expression of *FGF1*, *2*, *7*, and *10* mRNA in day 17 bovine conceptuses. RT-PCR was completed on tcRNA from three pools of day 17 bovine conceptuses ($n=3$–5 conceptuses/pool) and one CT1 sample with primers specific for bovine *FGF1*, *2*, *7*, and *10* (Supplementary Table 1). Products were electrophoresed and visualized with ethidium bromide and u.v. light. tcRNA from bovine endometrium and lung were included as positive controls. Primers for *ACTB* were used as a positive RT-PCR control. tcRNA not exposed to reverse transcriptase (−RT samples) was included to verify that amplified products did not result from genomic contamination. Amplified products were sequenced to verify specificity of amplification.

The relative abundance of *FGF1*, *2*, and *10* mRNA populations throughout pre- and peri-attachment conceptus development was determined with qRT-PCR. The internal RNA loading control, *18S* RNA, could not be used to normalize *FGF* mRNA in this study since its concentrations varied depending on the stage of conceptus development (Table 1). More specifically, concentrations of *18S* RNA in tcRNA increased ($P<0.05$) as conceptuses progressed from blastocysts to elongated, filamentous conceptuses. Due to this outcome, the amount of tcRNA in each reaction was used to adjust *FGF* values across these developmental stages (Table 1). *FGF1* mRNA was not detected in IVP-blastocysts. Its relative abundance remained low in conceptuses collected on days 11 and 14 of pregnancy and increased ($P<0.05$) in day 17 conceptuses. *FGF2* mRNA was detected at all stages of conceptus

Table 1 Relative concentrations of *FGF* and *IFNT* mRNA in bovine conceptus at different stages of development.

Conceptus stage	*FGF1*	*FGF2*	*FGF10*	*IFNT*	*18S*
Blastocyst	–	0.037 ± 0.024^{a}	–	0.008 ± 0.009^{a}	0.04 ± 0.02^{a}
d11	0.008 ± 0.004^{a}	0.074 ± 0.024^{ab}	0.122 ± 0.054^{a}	0.010 ± 0.005^{a}	0.33 ± 0.15^{b}
d14	0.003 ± 0.001^{a}	0.158 ± 0.055^{b}	1.02 ± 0.37^{ab}	0.93 ± 0.36^{b}	3.73 ± 1.32^{c}
d17	0.94 ± 0.34^{b}	0.096 ± 0.023^{b}	2.61 ± 0.87^{b}	3.93 ± 1.26^{c}	5.09 ± 1.81^{c}

Different superscripts represent differences in relative abundance of mRNA species within each column ($P<0.05$).

development, and concentrations were greater ($P<0.05$) in day 14 and 17 conceptuses than in day 8 IVP-blastocysts. Day 11 conceptuses contained intermediate amounts of *FGF2* mRNA. *FGF10* mRNA was not detected in IVP-blastocysts and low levels were detected in day 11 conceptuses. The relative abundance of FGF10 mRNA was substantially greater ($P<0.05$) in day 14 and 17 conceptuses. As anticipated, concentrations of *IFNT* mRNA were low in IVP-blastocysts and day 11 conceptuses and then increased ($P<0.05$) in day 14 conceptuses and then again in day 17 conceptuses ($P<0.05$).

A separate analysis was used to describe the relative abundance of *FGF1*, *2*, and *10* mRNA within each stage of bovine conceptus development (Fig. 4). Relative abundance of *18S* RNA was used to normalize *FGF* values within each stage of pregnancy examined. *FGF2* was the only FGF transcript identified in IVP-blastocysts. It also was the most abundant *FGF* transcript in day 11 conceptuses. In day 14 conceptuses, *FGF2* and *10* mRNA were present at similar levels. By comparison, *FGF1* mRNA abundance was low ($P<0.05$) in day 11 and 14 conceptuses. *FGF10* was the most prevalent transcript in day 17 conceptuses, where it represented ~85% of the *FGF* transcripts among the three specific *FGFs* examined in these conceptuses.

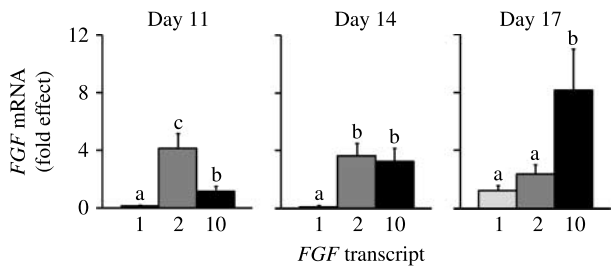

Figure 4 Expression profiles of *FGF1*, *2*, and *10* during bovine conceptus development. TcRNA was extracted from bovine conceptuses collected on days 11, 14, and 17 post-insemination ($n=5$ pools/stage of development; 2–5 conceptuses/pool). TaqMan qRT-PCR was completed using primers/probes specific for *boFGF1*, *2*, and *10* (Supplementary Table 3). Abundance of *18S* RNA was used as an internal control to normalize *FGF* values within each stage of development. ΔC_t values were used to analyze the data within each stage of development and data are presented as mean fold-differences ± S.E.M. from the lowest expression value within each stage. Different superscripts represent treatment differences within each stage of development ($P<0.05$).

Biological activity of FGF1, 2, and 10 on bovine trophectoderm and IVP-blastocysts

The ability of these conceptus-derived FGFs to stimulate IFNT production was first examined using CT1 cells. A dose-responsive increase in both *IFNT* mRNA (Fig. 5A) and protein (Fig. 5B) concentrations were observed after incubating CT1 cells in medium containing commercially available rbFGF1, rbFGF2, and rhFGF10. rbFGF1 and 2 were better than rhFGF10 at increasing ($P<0.05$) *IFNT* mRNA abundance, (5–50 ng/ml versus 500 ng/ml). No such disparities in effective doses among FGF preparations were observed when IFNT protein

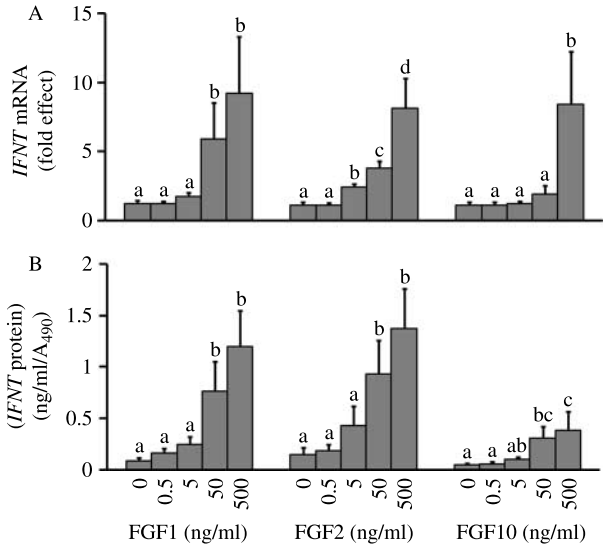

Figure 5 Increase in the relative abundance of *IFNT* mRNA and biologically activity of IFNT in conditioned medium after FGF1, 2, and 10 treatments. CT1 cells were incubated in medium containing 0, 0.5, 5, 50, or 500 ng/ml of rbFGF1, rbFGF2, or rhFGF10. A: After 24 h, tcRNA was extracted and qRT-PCR was performed to determine the relative abundance of *IFNT* mRNA ($n=4$ replicate experiments; 2 wells/treatment/replicate experiment). Abundance of *18S* RNA was used as an internal control to normalize *IFNT* mRNA values. ΔC_t values were used to analyze the data and mean fold-differences ± S.E.M. from the lowest expression value are presented. B: After 48 h, conditioned medium was collected and IFNT concentrations (pg/ml/A_{490}) were determined by using an antiviral assay ($n=5$ replicate experiments; 2 wells/treatment/replicate experiment). Viable CT1 cell number was determined by quantifying the amount of tetrazolium (MTS) oxidation (absorbance at 490 nm). Antiviral results were adjusted accordingly. Different superscripts represent treatment differences within panels ($P<0.05$).

concentrations in conditioned-CT1 medium were examined. Increases ($P<0.05$) in IFNT protein levels were evident for each recombinant preparation when supplemented at a concentration of 50 ng/ml.

A study was completed to determine if rhFGF7 could influence *IFNT* mRNA levels in CT1 cells (data not shown). FGF7 interacts solely with R2b (Lu *et al.* 1999, Powers *et al.* 2000), thereby making it an ideal candidate for examining if this receptor subtype is involved with mediating FGF effects on *IFNT* expression. Provision of 5 ng/ml rhFGF7 was sufficient to increase ($P<0.05$) *IFNT* mRNA abundance in CT1 cells. Exposure to larger doses of rhFGF7 (50 and 500 ng/ml) caused further increases in *IFNT* mRNA concentrations.

A subsequent study was completed to determine if FGF1, 2, and 10 supplementation influences development and *IFNT* expression in bovine blastocysts (Fig. 6).

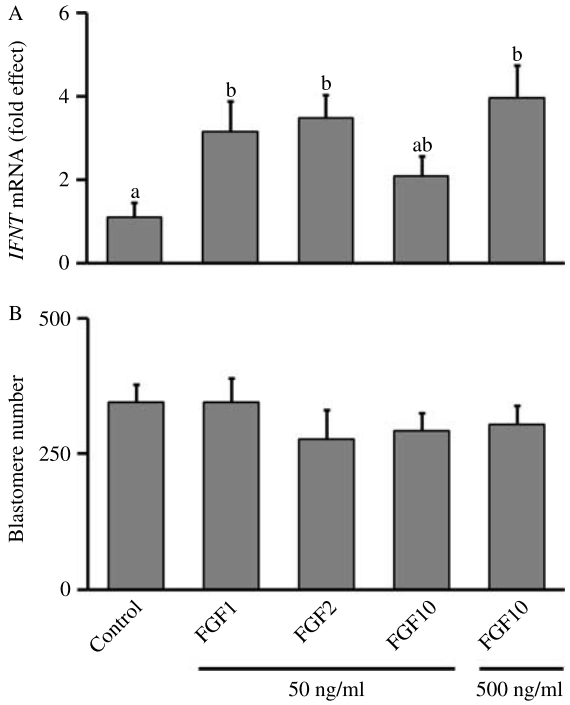

Figure 6 The effect of FGF1, 2, or 10 supplementation on *IFNT* mRNA concentrations and blastomere numbers in cultured bovine blastocysts. Individual IVP-blastocysts were placed in 30 µl drops of medium containing rbFGF1 (50 ng/ml), rbFGF2 (50 ng/ml), rhFGF10 (50 or 500 ng/ml), or vehicle only (50 µg/ml BSA) and incubated at 38.5 °C for 24 or 48 h. A: After 24 h, tcRNA was extracted and qRT-PCR was performed to determine the relative abundance of *IFNT* mRNA ($n=14$–22 blastocysts/treatment spread over four replicate experiments). Abundance of *18S* RNA was used as an internal control to normalize *IFNT* mRNA values. ΔC_t values were used to analyze the data and mean fold-differences ± S.E.M. from the lowest expression value are presented. B: After 48 h, number of nuclei per blastocyst was determined after Hoechst 33342 staining and epifluorescence microscopy ($n=22$–25 blastocysts/treatment spread over four replicate experiments). Different superscripts represent treatment differences within (A) and (B); $P<0.05$).

Individual IVP-blastocysts were placed in medium drops containing 50 ng/ml rbFGF1 or rbFGF2, 50 or 500 ng/ml rhFGF10, or no FGF treatment (control) and *IFNT* mRNA abundance (Fig. 6A) and blastomere numbers (Fig. 6B) were determined after 24 and 48 h respectively. Incubation with medium containing 50 ng/ml rbFGF1 or 2 increased ($P<0.05$) *IFNT* mRNA abundance compared with the control. Blastocysts required 500 ng/ml rhFGF10 to observe this effect. Blastomere numbers, as determined by quantifying the number of stained nuclei in blastocysts after 48 h exposure to FGF treatment, were not affected by any of the FGF proteins at any of the doses examined.

Discussion

This laboratory previously described a role for FGF2 in stimulating IFNT production in bovine conceptuses (Michael *et al.* 2006, Rodina *et al.* 2008). Initially, the uterine epithelium was proposed as the primary site of FGF2 production in cattle (Michael *et al.* 2006), but work contained herein and other findings (Daniels *et al.* 2000, Lazzari *et al.* 2002, Ocon-Grove *et al.* 2008) establish that bovine conceptuses also produce FGF2. *FGF2* mRNA is detected at the morula/blastocyst stage in this and other studies (Daniels *et al.* 2000, Lazzari *et al.* 2002), and transcript abundance increases in conceptuses undergoing elongation (days 14 & 17). Also, at least two of the primary receptor partners for FGF2, R1c, and R3c, preside in ovine conceptuses (Ornitz 2000, Powers *et al.* 2000, Ocon-Grove *et al.* 2008). An additional receptor subtype, R2b, is also produced by ovine conceptuses (Chen *et al.* 2000, Ocon-Grove *et al.* 2008). FGF2 interacts with R2b with a lower affinity than R1c and R3c (Ornitz 2000, Powers *et al.* 2000), thereby suggesting that additional FGFs may impact conceptus development by acting through R2b.

Bovine conceptuses contain the same *FGFRs* as elongating ovine conceptuses (Ocon-Grove *et al.* 2008). In addition, this work identified the expression of *R4* in bovine IVP-blastocysts and elongated conceptuses. Amplifying the IgIII region for each *FGFR* permitted the identification of specific *FGFR* spliced variants (*R1c*, *R2b*, *R3c*). It remains possible that additional variant forms for *R1*, *2*, and *3* exist in bovine conceptuses and perhaps these subtypes could have been identified if additional cloned products were sequenced or if receptor subtype-specific primer sets were employed. That being said, verifying the presence of *R2b* throughout early bovine conceptus development provided the impetus for focusing on ligands that interact with this receptor subtype. Investigating this receptor isoform was of particular interest because the R2b and R2c subtypes are implicated in regulating trophectoderm development in other species (Feldman *et al.* 1995, Arman *et al.* 1998, Ka *et al.* 2001).

The primary ligands for R2b are FGF1, 3, 7, 10, and 22 (Ornitz 2000, Powers *et al*. 2000). In this work, transcripts for *FGF1* and *10* were detected, whereas *FGF7* transcripts were absent in bovine conceptuses. Tissue-specific expression of *FGF3* and *22* is highly restrictive in other species (e.g., cerebellum, retina, and inner ear for *FGF3*; cerebellum and skin for *FGF22*; Wilkinson *et al*. 1989, Umemori *et al*. 2004, Komi-Kuramochi *et al*. 2005). Therefore, these FGFs were not examined in this work. Transcript levels of *FGF1* were low throughout bovine conceptus development, whereas *FGF10* mRNA concentrations were low or non-existence on or before day 11 but were readily apparent in day 14 and 17 conceptuses. The relative amount of *FGF10* mRNA rivaled that of *FGF2* in day 14 conceptuses and represented to predominant *FGF* transcript in day 17 conceptuses. Chen *et al*. (2000) identified that the extraembryonic mesoderm is the primary site of *FGF10* expression in ovine conceptuses. The extraembryonic mesoderm emerges from the epiblast (i.e., the gastrulating inner cell mass) around days 14–16 of pregnancy in cattle and soon thereafter extends between the trophectoderm and endoderm to establish the inner chorionic layer and the outer yolk sac layer (Betteridge & Flechon 1988, Hue *et al*. 2001, Maddox-Hyttel *et al*. 2003, Degrelle *et al*. 2005, Robinson *et al*. 2006). Although, localization studies were not completed herein to verify the sources of *FGF10* expression during conceptus elongation, our assessment of *FGF10* mRNA abundance is supportive of the concept that the profound rise in *FGF10* expression in day 14 and 17 conceptuses stems from the formation and expansion of extraembryonic mesoderm.

It is interesting that the ontogeny of conceptus-derived *FGF10* expression resembled that of *IFNT*. Relative abundances for both transcripts were low in day 11 conceptuses and increased profoundly at days 14 and 17 of pregnancy. Since mesoderm formation occurs around the same time as conceptus elongation and maximal *IFNT* expression (Ealy *et al*. 2001, Mann *et al*. 2006, Robinson *et al*. 2006), it is possible that a product of this tissue, such as FGF10, could be required for maximal *IFNT* expression as well as other aspects of early conceptus development and elongation. Timely epiblast development certainly is linked to conceptus elongation and survival. Bovine embryos derived from IVP and nuclear transfer (NT) usually have lower survival rates than their *in vivo*-generated counterparts after transfer, and fewer IVP and NT embryos contain epiblasts at day 14 of pregnancy (Bertolini *et al*. 2002, Alexopoulos *et al*. 2008). Also, fewer pregnancies resulted from the transfer of day 14 bovine conceptuses containing either non-intact or undetectable epiblasts as compared with the transfer of conceptuses containing intact epiblasts (Fischer-Brown *et al*. 2004). Further work is needed to determine if paracrine-acting factors produced by the epiblast, and more specifically by the newly formed mesoderm, promote trophectoderm proliferation and gene expression.

Tissue localization studies were not completed in this work, but the trophectoderm is likely one site of FGF1 and 2 production in elongating conceptuses. *FGF1* and *2* mRNA are detected in CT1 cells and localized to the trophectoderm of mid-gestation bovine placentae (Pfarrer *et al*. 2006). Likewise, each of the four *FGFR* subtypes identified in bovine blastocysts and elongating conceptuses probably are expressed in trophectoderm since they were detected in CT1 cells. This certainly is the case for the *R2b* subtype. Its transcripts localize to ovine and bovine trophectoderm during peri- and post-attachment development (Chen *et al*. 2000, Pfarrer *et al*. 2006). *R2b* is also expressed by the uterine epithelium in ewes where it appears to play a central role in regulating uterine activity during pregnancy by interacting with stromal-derived FGF10 (Chen *et al*. 2000). It is possible, but unlikely that this uterine source for FGF10 is released into the uterine lumen. Most FGFs, including FGF10, contain high affinities for heparan sulfate proteoglycans and remain sequestered within the extracellular matrix of tissues rather than being released into the blood or luminal cavities (Ornitz 2000, Powers *et al*. 2000). Very little to no FGF10 is produced by luminal and glandular epithelium. Cell lines developed from ovine endometrial epithelium contain *FGF10* mRNA (Chen *et al*. 2007), but *in situ* studies did not detect *FGF10* transcripts in luminal or glandular epithelium during diestrus and early pregnancy (Chen *et al*. 2000). By contrast, FGF2 is produced by the endometrial epithelium and can be found in the uterine lumen during early pregnancy (Michael *et al*. 2006, Ocon-Grove *et al*. 2008). It remains unknown, if uterine-derived FGF1 is released into the uterine lumen. FGF1 is produced by bovine luminal epithelium during mid-gestation (Pfarrer *et al*. 2006) and, therefore, may have a similar uterine expression pattern as FGF2 prior to placental attachment.

All of the recombinant FGF preparations were able to stimulate *IFNT* mRNA and/or protein levels in bovine trophectoderm and IVP-blastocysts. FGF1 and 2 proteins were similar in their ability to stimulate *IFNT* mRNA, whereas as much as tenfold more FGF10 was required in some cases to elicit biological responses on trophectoderm and blastocysts. It is unclear why FGF10 contained slightly lower biological activity than other FGFs. Perhaps using a human recombinant FGF10 protein that contains a good but not great sequence identity to its bovine homolog caused this effect (91.6% identical amino acid sequence identity to bovine FGF10). Alternatively, issues with freeze/thawing the recombinant preparation, post-thawing handling, and/or protein degradation rate during culture could have contributed to this outcome. Regardless of the reason, rhFGF10 did contain biological activity on both CT1 cells and IVP blastocysts. Therefore, its bovine homolog is probably

also capable of stimulating *IFNT* expression in bovine trophectoderm.

None of the FGF proteins examined affected trophectoderm and blastomere cell numbers. Previous work consistently observed little to no mitogenic effect of boFGF2 (Michael *et al*. 2006, Rodina *et al*. 2008). One interpretation of these findings is that the FGFs under investigation are likely not serving an active role in regulating cell proliferation rate early in conceptus development. However, the ability of selective FGFs to influence early bovine embryo development (i.e., blastocyst formation, blastocyst quality, and ratio of trophoblast/inner cell mass cells) have yet to be fully explored.

It still remains unclear whether one or multiple FGFR subtypes dictate FGF responses in bovine trophectoderm. FGF1 associates equally well with each of the FGFR variants expressed in bovine conceptuses, and FGF2 has a high affinity for R1c and R3c and a moderate affinity for R2b (Ornitz 2000, Powers *et al*. 2000, Itoh & Ornitz 2004). FGF10 is more selective in its receptor partner binding interactions and generally acts through either R2b or R1b (Lu *et al*. 1999, Powers *et al*. 2000). The latter receptor subtype was not detected in conceptus and CT1 screens. Recombinant FGF7was included in one CT1 study to determine if its interaction with R2b was sufficient to induce an *IFNT* mRNA response. *FGF7* is not expressed in pre- and peri-attachment bovine conceptuses and endometrial sources of FGF7 are localized too deep within the stromal region to permit FGF7 from being secreted into the uterine lumen (Chen *et al*. 2000). However, FGF7 acts exclusively through R2b (Lu *et al*. 1999, Powers *et al*. 2000), and its ability to increase *IFNT* mRNA levels in CT1 cells implicates R2b as one of the receptor subtypes responsible for FGF actions on trophectoderm. This observation does not exclude the possibility that other FGFRs also may participate in directing the biological activities of other FGFs on bovine trophectoderm, but the relative importance of other FGFRs in regulating *IFNT* expression and other critical actions during conceptus development remains to be fully explored.

In conclusion, current findings provide new insight for how conceptus-derived factors may control *IFNT* expression during early pregnancy in cattle. Multiple *FGFRs* are expressed by elongating bovine conceptuses, and at least one of these receptor subtypes, R2b, is involved with mediating FGF-induced increases in *IFNT* mRNA and protein production in bovine trophectoderm. Also, several FGFs are expressed by bovine conceptuses. Of particular note is FGF10, a ligand for R2b whose expression increases in day 14 and 17 conceptuses coincident with peak *IFNT* expression. This and potentially other conceptus-derived FGFs as well as uterine-derived FGFs likely are acting in concert to impact IFNT production from bovine trophectoderm. This could well be an essential component for the establishment and maintenance of pregnancy in cattle and sheep.

Materials and Methods

Animal use and tissue collection

All animal experimentation was completed in accordance with Institutional Animal Care and Use Guidelines and with the approval of the Institutional Animal Care and Use Committee at the University of Florida. Healthy, non-lactating Holstein cows ($n=21$) were housed at the University of Florida Dairy Unit (Hague, FL, USA) and fed a maintenance diet. Conceptuses were harvested on days 11, 14, and 17 post-insemination after superovulation. In brief, growth of a new wave of follicles was induced by ablating large follicles (>10 mm diameter) on ovaries with an ultrasound-guided follicle aspiration device (Bilby *et al*. 2006). A controlled drug intravaginal device containing progesterone (1.38 g; Eazi-Breed CIDR; Pfizer Corp., New York, NY, USA) was inserted after follicle ablation. Cows were provided a 4 day regiment of FSH treatment (Folltropin-V; AgTech, Manhattan, KS, USA; 400 mg total) beginning 2 days after follicle ablation, CIDRs were removed on the third day of FSH treatment and cows were injected with Lutalyse (25 mg each time; Pfizer Corp.) twice on the third day of FSH treatment (Sartori *et al*. 2004). Superovulated cows were inseminated with Holstein semen (Genex Cooperative Inc., Shawano, WI, USA) at 12 and 24 h after first detection of standing estrus.

Conceptuses at day 11 and 14 post-insemination were collected non-surgically after providing an epidural injection of 2% (wt/vol) lidocaine (Sparhawk Laboratories Inc., Shawnee, Mission, KS, USA) by inserting a latex Foley catheter (Agtech Inc.) through the cervix and flushing each horn with 250–500 ml flush solution (Dulbecco's PBS (DPBS; Invitrogen Corp., Carlsbad, CA, USA) with 0.04% BSA (BSA)). Conceptuses at day 17 post-insemination were collected during slaughter at the UF Meats Laboratory by excising reproductive tracts, injecting flush solution into the anterior tip of one uterine horn and collecting fluid and conceptuses from an excised anterior portion of the other uterine horn. Flush solutions were examined macroscopically and microscopically, if needed (day 11) to collect conceptuses. All day 11 conceptuses were ovoid in shape and most could be detected in the petri dish with the naked eye. Approximately, half of the day 14 conceptuses collected were ovoid but readily visible in the petri dish with the naked eye. The remaining day 14 conceptuses were in the initial stages of elongation and ranged from 0.5 to 3 cm in length. All of the day 17 conceptuses were elongated and filamentous, ranging from 5 to 45 cm in length. Conceptuses were pooled together in small groups ($n=3$–5/pool) and snap-frozen in liquid nitrogen and stored at −80 °C. Each pool of the day 14 samples contained a mixture of ovoid and elongating conceptuses. Additional tissues, including endometrium, lung, and brain (hippocampus and cerebrum), were collected from cows during slaughter, snap-frozen in liquid nitrogen and stored at −80 °C until use as positive control samples.

Total cellular (tc) RNA was extracted from day 11 and day 14 conceptus pools using the RNeasy Micro kit (Qiagen Inc, Valencia, CA, USA). The RNAqueous-Midi RNA Isolation Kit (Applied Biosystems/Ambion, Austin, TX, USA) was used to extract tcRNA from d17 conceptuses. The PureLink Micro-to-Midi Total RNA Purification System with Trizol (Invitrogen Corp.) was used to extract tcRNA from other tissues.

Concentration and quality of isolated tcRNA was assessed by using a NanoDro8000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA, USA).

Bovine in vitro *embryo production*

In vitro maturation, fertilization, and cultures procedures were used to generate bovine blastocysts (Rivera & Hansen 2001, Moreira *et al*. 2002, Paula-Lopes & Hansen 2002). Briefly, oocytes from slaughterhouse derived ovaries were matured and fertilized *in vitro* with Holstein semen (Genex Cooperative Inc). After fertilization, presumptive zygotes were placed in groups of 25–30 and incubated in 5% CO_2/5%O_2/90%N_2 at 38.5 °C in 50 µl drops of either synthetic oviduct fluid (SOF; Chemicon International/Millipore, Billerica, MA, USA) or modified potassium simplex optimized medium (mKSOM; Caisson Laboratories, North Logan, UT, USA), depending on the study. Both media were supplemented with 0.3% (w/v) essentially fatty acid free BSA (Sigma Aldrich, St Louis, MO, USA). In one study, expanded and hatched blastocysts formed on day 8 post-fertilization after culture in mKSOM were placed in groups (n=10–18), snap-frozen in liquid nitrogen and stored at −80 °C. TcRNA was extracted using the RNeasy Micro kit (Qiagen Inc.) and quantified by using a NanoDro8000 spectrophotometer (Thermo Fisher Scientific).

In a second set of studies, blastocysts identified on day 8 post-fertilization were collected and washed in DPBS containing 1 mg/ml polyvinyl pyrolidone (PVP; Thermo Fisher Scientific). Individual blastocysts were placed in 30 µl drops Medium 199 (M199) containing 2.5% (v/v) fetal bovine serum (FBS; Invitrogen Corp.) and 10 µM Gentamycin (Sigma Chemical Co.; Rodina *et al*. 2008). The stage of blastocyst development (non-expanded or expanded) was used to balance treatment combinations. Medium was supplemented with 50 ng/ml recombinant (r) bovine (b) FGF1 (R&D Systems), 50 ng/ml rbFGF2 (R&D Systems Minneapolis, MN, USA), 50 or 500 ng/ml r human (h) FGF10 (Invitrogen Corp.) or carrier only (M199 containing 1% (w/v) BSA). In one study, blastocysts were incubated at 38.5 °C in 5% CO_2/5%O_2/90%N_2 for 24 h, then washed once in PBS/PVP, snap-frozen in liquid nitrogen and stored at −80 °C. In a companion study, blastocysts were incubated at the aforementioned conditioned for 48 h then were washed once in PBS/PVP and fixed by incubation in 4%(w/v) paraformaldehyde (Polysciences Inc., Warrington, PA, USA) for 10 min and processed for determining blastomere numbers with Hoechst 33342 staining and epifluorescence microscopy (Rodina *et al*. 2008). Number of Hoechst-positive nuclei was quantified with NIS-Elements software (Nikon Instruments Inc, Melville, NY, USA).

Bovine trophectoderm cell (CT1) culture

Cells were propagated as described previously (Talbot *et al*. 2000, Michael *et al*. 2006) in DMEM containing 10% FBS and other supplements (4.5 g/l D-glucose, non-essential amino acids, 2 mm glutamine, 2 mm sodium pyruvate, 55 µm β-mercaptoethanol, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 250 ng/ml amphoterin B) on Matrigel Basement Membrane Matrix (BD Biosciences, Bedford, MA, USA) at 38.5 °C in 5% CO_2 in air. CT-1 cells were seeded onto 12-well plates. Upon reaching ∼50% confluence, fresh DMEM lacking FBS but containing all other supplements and a serum substitute (insulin/transferrin/selenium; ITS; Invitrogen Corp.) were added to cultures. After 24 h, fresh serum-free DMEM containing 0, 0.5, 5, 50, or 500 ng/ml of either rbFGF1, rbFGF2, or rhFGF10 were provided to cultures (n=2 wells/FGF treatment/replicate experiment). Each treatment, including controls contained 50 µg/ml BSA as a carrier protein. For some studies, tcRNA was extracted 24 h after FGF supplementation using the PureLink Micro-to-Midi Total RNA Purification System with Trizol (Invitrogen Corp.) following manufacturer's parameters (n=4 replicate experiments). TcRNA was stored at −80 °C until further use.

In another study, conditioned medium was collected and stored at −20 °C after 48 h exposure to FGF treatments (n=5 replicate experiments). Viable cell number was determined by using the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Absorbance was measured at 490 nm. A cytopathic antiviral assay was used to determine concentrations of biologically active IFNT in conditioned medium (Roberts *et al*. 1989, Michael *et al*. 2006, Rodina *et al*. 2008). Unconditioned medium and FGF preparations did not contain antiviral activity. Data are expressed as ng/ml of biologically activity boIFNT based on the specific activity of the rbIFNT preparation used as the standard (8.03×10^8 IU/mg) and normalized by the cell proliferation assay absorbance reading to account for well to well variations in CT1 cell density.

End-point RT-PCR

All samples were incubated with RNase-free DNase (Applied Biasystems Ambion Inc.) for 30 min at 37 °C then heat-denatured at 75 °C for 10 min immediately before RT. The SuperScript III First,-Strand Synthesis System Kit (Invitrogen Corp.) and random hexamers were used for RT of tcRNA. Non-reverse transcribed DNase-treated RNA was included as a negative control for each sample. Gene-specific primer sets were used to amplify products for *FGFR1*, *2*, *3*, or *4* and *FGF1*, *2*, *7*, or *10* (see Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/). A primer pair specific for β-actin (*ACTB*) cDNA was included as a positive control in these reactions (Supplementary Table 1). PCR amplification was performed using either PfuUltr High-Fidelity DNA Polymerase (Stratagene) or ThermalAc DNA Polymerase (Invitrogen Corp). From 30 to 45 cycles of denaturation (95 °C for 1 min), annealing (55–62 °C for 1 min; depending on the primer set) and DNA synthesis (72 or 74 °C for 1 min; depending on the polymerase) followed by a DNA polishing stage (72–74 °C for 10 min) were completed. The presence and approximate size of amplified products were determined by electrophoresis in an agarose gel (1.2% (w/v)) containing ethidium bromide (100ng/mL) and visualized on an u.v. light box.

Amplicons of the desired size were excised from gels and ligated into the pCR4-Blunt TOPO vector (Invitrogen Corp). Ligation reactions were used to transform chemically competent TOP10 *E. coli* (Invitrogen Corp). Selected colonies were propagated and purified clones were sequenced in both

directions using vector primer sets at the University of Florida DNA Core Facility. Multiple clones from at least three different conceptus or CT1 samples were sequenced to verify that amplified products represented the transcript of interest. Also, DNA sequencing was used to identify specific splice variant forms of *FGFR1-3* in bovine conceptuses and CT1 cells.

Quantitative (q), real-time RT-PCR

The abundance of *FGF* and *IFNT* transcripts in bovine conceptus and CT1 cells was determined by qRT-PCR. All samples were incubated with RNase-free DNase (Applied Biasystems Ambion Inc., Foster sites CA, USA) as described previously before RT with the High Capacity cDNA Archive Kit and random hexamers (Applied Biosystems Inc).

For one study with IVP-blastocysts, primers specific for *boIFNT* and *18S* (internal control; Supplementary Table 2, which can be viewed online at www.reproduction-online.org/supplemental/) were used in combination with a SybrGreen detector system (Applied Biosystems Inc.) and a 7300 Real-Time PCR System (Applied Biosystems Inc.) to quantify *IFNT* mRNA concentrations. 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. Each blastocyst sample was run in duplicate reactions and a third reaction lacking exposure to reverse transcriptase was included for each sample to verify if they were free of genomic contamination. The comparative threshold cycle (C_t) method was used to quantify the abundance of *IFNT* mRNA relative to that of *18S* (Michael *et al.* 2006). In brief, the average ΔC_t value for each sample was calculated (*IFNT*C_t – *18S*C_t) and used to calculate the fold-change in the relative abundance of *IFNT* mRNA.

A TaqMan-based qRT-PCR approach was used to quantify the abundance of *FGF1*, *FGF2*, *FGF10*, *IFNT*, and *18S* RNA (internal RNA loading control) in other studies. Primers and probes specific for each *FGF* transcript and *IFNT* mRNA were synthesized (Applied Biosystems Inc.; Supplementary Table 3, which can be viewed online at www.reproduction-online.org/supplemental/) and labeled with a fluorescent 5′ 6-FAM reporter dye and 3′ TAMRA quencher. The *IFNT* Primer/probe set was developed to recognize every known bovine and ovine *IFNT* isoform (Michael *et al.* 2006). After an initial activation/denaturation step (50 °C for 2 min; 95 °C for 10 min), 50 cycles of a two-step amplification protocol (60 °C for 1 min; 95 °C for 15 s) was used with TaqMan reagent (Applied Biosystems Inc.) and a 7300 Real-Time PCR System to quantify transcript levels. Abundance of *18S* RNA was determined using the *18S* RNA Control Reagent Kit (Applied Biosystems Inc.) containing a 5′-VIC-labelled probe with a 3′-6-carboxy-tetramethylrhodamine quencher. Each RNA sample was analyzed in duplicate (conceptus study) or triplicate (CT1 study) reactions (10–50 ng tcRNA for blastocysts and day 11 conceptuses; 50 ng tcRNA for day 14 and 17 conceptuses and CT1 cells). An additional reaction lacking exposure to the reverse transcriptase was included for each sample to verify if they were free of genomic DNA contamination.

The ΔC_t method was used to contrast abundance of transcripts for *FGFs* and *IFNT* relative to *18S* RNA in all but one study. In one study, relative amounts of *18S* RNA changed across stages of conceptus development, so a relative standard curve approach was used in combination with the amount of tcRNA used in qRT-PCR reactions to describe relative abundances for individual *FGFs* and *IFNT* during conceptus development (Larionov *et al.* 2005, Cikos *et al.* 2007; ABI Prism Sequence Detection System User Bulletin No. 2; Applied Biosystems Inc). One of the day 17 conceptus RNA samples was used a standard sample, and four doses of this preparation (2, 10, 50, 250 ng tcRNA/reaction) was included in each real-time run. The slope and intercept for each *FGF*, *IFNT*, and *18S* curve was used to convert raw C_t values into values that represented the ng of control tcRNA required to equal that detected in each sample by solving the formula: $(10^{((C_t \text{ value} - y \text{ intercept})/\text{slope})}/\text{sample tcRNA})$.

Statistical analyses

All analyses were completed by ANOVA (ANOVA) using the general linear model of the Statistical Analysis System (SAS Institute Inc, Cary, NC, USA). Differences in individual means were partitioned further by completing pair-wise comparisons (probably of difference analysis). When analyzing qRT-PCR data transformed using the ΔC_t method, the ΔC_t values were used for the statistical analyses but data are presented as fold differences from control values. Antiviral activity of CT1 conditioned medium was log-transformed and normalized based on predicted cell number before analysis. Results are presented as arithmetic means ± S.E.M.S.

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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