Temporal regulation of fibroblast growth factors and their receptors in the endometrium and conceptus during the pre-implantation period of pregnancy in cattle

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Abstract

We hypothesised that the expression pattern of members of the fibroblast growth factor (FGF) family would be altered in the endometrium as the oestrous cycle/early pregnancy progressed associated with changes in the expression pattern of their receptors in the developing embryo/conceptus. Expression of FGF1 and FGF10 transcript variants 1 and 2 increased significantly as the oestrous cycle/early pregnancy progressed. Neither progesterone (P4) supplementation nor pregnancy status significantly affected the expression of any of the FGF ligands studied. However, there was a significant interaction between day, pregnancy and P4 status on FGF2 expression (P < 0.05) and a significant interaction between P4 status and day on FGF10_tv2 expression. FGF10 protein was localised in the luminal and glandular epithelium as well as the stroma but was not detected in the myometrium. By RNA sequencing, the expression of FGF ligands in the developing embryo/conceptus was found to be minimal. The expression of FGF receptor 1 (FGFR1), FGFR2, FGFR3, FGFR4, FGFRL1 and FR33 was significantly affected by the stage of conceptus development. Interestingly, the expression of FGFR1 and FGFR4 was higher during early embryo development (days 7–13, P < 0.05) but decreased on day 16 (P < 0.05) while FGFR2 (P < 0.001) expression was similar from day 7 through to day 13, with a significant increase by day 16 (P < 0.05) that was maintained until day 19 (P > 0.05). In conclusion, these data demonstrate that FGF ligands are primarily expressed by the endometrium and their modulation throughout the luteal phase of the oestrous cycle/early pregnancy are associated with alterations in the expression of their receptors in the embryo/conceptus.

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Introduction

Fibroblast growth factors (FGFs) are members of a large family of growth factors (Itoh & Ornitz 2004, Antebay et al. 2005), which are involved in multiple cellular functions that are essential for embryonic development such as proliferation, migration, angiogenesis, differentiation and cell survival (Gupta et al. 1997, Bottcher & Niehrs 2005). Most FGFs have an N-terminal signal peptide for release but some, such as FGF1 and FGF2, lack the terminal signal peptide and are therefore secreted via non-classical pathways (Jackson et al. 1992, Mignatti et al. 1992). FGFs mediate their biological activity by first associating with heparin/heparan sulphate proteoglycans (Ornitz 2000) before binding to respective FGF receptors (FGFRs), which belong to the tyrosine kinase receptor super family (Bottcher & Niehrs 2005, Ocon-Grove et al. 2008). Four types of FGFRs exist (FGFR1, FGFR2, FGFR3 and FGFR4) and their structure includes a heparin-binding domain and three immunoglobulin-like (Ig) domains (I–III). Alternative splicing in the Ig III domain of FGFR1, FGFR2 and FGFR3 results in two types of isoforms, I1 Ib and I1 Ic, which confers the FGFR with specificity for certain ligands only and is essential for epithelial–mesenchymal signalling (Ornitz 2000). For example, splicing of FGFR2 results in two variants, e.g. FGFR2I1 Ib, which is highly specific for binding FGF7 and FGF10 (Lu et al. 1999, Ornitz 2000, Ornitz & Itoh 2001), and FGFR2I1 Ic, which binds other ligands such as FGF1 and FGF8 (Ornitz 2000, Powers et al. 2000).

FGFs have wide distribution and several members have been associated with different reproductive tissues. FGF1 has been localised in the primate and ovine uterus (Gupta et al. 1997, Samathanam et al. 1998), where its levels increase in response to oestradiol (E2) treatment and are associated with mitogenic activity (Samathanam et al. 1998), while it has also been detected in bovine placentomes (Pfarrer et al. 2006). FGF2 is expressed by the ovine endometrium and conceptus during early pregnancy, and peri-attachment conceptuses possess several types of FGFRs (Gupta et al. 1997, Antebay et al. 2005).
with progesterone (P₄) concentrations and which expression in the primate uterus is positively associated a stromally derived paracrine growth factor, whose gastrulation in rabbit embryos (Hrabe de Angelis et al. 2011) and primitive endoderm formation in cattle (Yang et al. 2011). FGF7 (keratinocyte growth factor) is a stromally derived paracrine growth factor, whose expression in the primate uterus is positively associated with progesterone (P₄) concentrations and which mediates the proliferation of endometrial epithelial cells (Koji et al. 1994), where it is thought to function as a progastamedin, i.e. a protein that mediates P₄ effects on cells not expressing a P₄ receptor in their own right (Koji et al. 1994). FGF10, a homologue of FGF7, is also a paracrine mediator that stimulates proliferation of epithelial cells (Lu et al. 1999). FGF10 mRNAs have also been detected in ovine endometrial tissue where it has been implicated in endometrial function as well as in growth and development of the conceptus (Satterfield et al. 2008). In cattle, FGF10 is expressed by the conceptus (Cooke et al. 2009) and other reproductive tissues such as follicles, where it is involved in mediating Signals from theca cells and/or oocytes to granulosa cells (Buratini et al. 2007); furthermore, its addition to culture medium enhances bovine oocyte maturation and developmental competence (Zhang et al. 2010).

To date, there has been no comprehensive analysis of the expression of FGF ligands and receptors in both the endometrium and conceptus during the pre-implantation period of pregnancy in cattle. Ozawa et al. (2013) demonstrated the presence of FGFR1, FGFR2, FGFR3 and FGFR4 in bovine embryos up to the blastocyst stage and showed that FGFR activation is needed to maximise IFNT expression and permit outgrowth formation. However, there is a paucity of data relating to post-hatching stages of development, probably a reflection of the difficulty in obtaining such stages in vivo. This period from blastocyst formation to initiation of implantation (around day 19 in cattle) is arguably more important given that it encompasses the period of conceptus elongation, IFNT production, pregnancy recognition and implantation. Recent data from our group have determined that ligands expressed in the endometrium during the time of pregnancy recognition (day 16) have their cognate receptors expressed in the conceptus, indicative of conceptus maternal dialogue prior to implantation in cattle (Mamo et al. 2012). We hypothesised that the expression pattern of members of the FGF family would be altered in the endometrium as the oestrous cycle/early pregnancy progressed and would be modulated by P₄ supplementation. In addition, we hypothesised that the modulation of these genes would be associated with changes in the expression pattern of their receptors in the developing embryo/conceptus.

Materials and methods

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1876) and European Community Directive 86/609/EC, and sanctioned by the Animal Research Ethics Committee of University College Dublin. Unless otherwise stated all reagents were sourced from Sigma.

Animal model and tissue collection

The animal model and tissue collection used in this study were described previously (Forde et al. 2010). The oestrous cycles of 263 cross-bred beef heifers were synchronised using a controlled internal drug-releasing device (CIDR, 1.94 g P₄; InterAg, Hamilton, New Zealand). One day prior to CIDR device removal, all heifers received an i.m. injection of 0.5 mg prostaglandin F₂α analogue (cloprostenol estramate, Schering–Plough Animal Health, Hertfordshire, UK). Of the 210 heifers that displayed standing oestrus within a narrow time window, 140 were artificially inseminated with semen to generate a pregnant group, while the remaining heifers were left as a non-inseminated cyclic control group (n = 70). On day 3 of the oestrous cycle/early pregnancy, half of each group were randomly assigned to receive a P₄-releasing intravaginal device (1.55 g P₄; CEVA, Animal Health Ltd, Chesham, UK) to elevate circulating concentrations of P₄ (Carter et al. 2008). This resulted in four treatment groups: i) pregnant, high P₄ concentration; ii) pregnant, normal P₄ concentration (PN); iii) cyclic, high P₄ concentration and iv) cyclic, normal P₄ concentration (CN). All heifers were randomly assigned for slaughter on either days 5, 7, 13 or 16 of the oestrous cycle/early pregnancy. Within 30 min of slaughter, the reproductive tracts of all heifers were retrieved and flushed with 20 ml PBS containing 10% FCS. For the inseminated heifers, only tissues from those with an appropriately developed embryo/conceptus for the day of pregnancy were further processed. One whole cross section of the uterine horn, with an approximate length of 25-mm, ipsilateral to the corpus luteum (CL), was fixed for 24 h in 10% buffered formalin for immunohistochemical (IHC) analysis. Samples for immunohistochemical (IHC) analysis were then processed by dehydration through a series of ascending concentrations of alcohol, cleared in xylene and finally impregnated with paraffin wax prior to sectioning. For quantitative real-time PCR (qPCR) analysis, strips of endometrial tissue (~300 mg) were removed from the mid-section of the ipsilateral horn, immersed in 1:5 w/v RNAlater and transported back to the laboratory on ice. Samples were stored at 4°C for 24 h, removed from RNAlater, placed into a new tube and stored at −80°C prior to RNA extraction. For both qPCR analysis and IHC analysis, tissues from five animals per treatment per time point were processed (i.e. four treatments × 4 days × five animals = a total of 80 animals).
Analysis of mRNAs for members of the FGF family in the endometrium by qPCR

Gene expression analysis was carried out using qPCR analysis and was performed as described previously (Okumu et al. 2011). Briefly, 100 mg samples of endometrium were homogenised and total RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. On column, DNase digestion and RNA cleanup were performed using a Qiagen Mini Kit (Qiagen). Both quality and quantity of the RNA were determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesised from 5 μg total RNA using Superscript III (Invitrogen) and random hexamers according to the manufacturer’s instructions. All primers were designed using Primer Express Software (Applied Biosystems) and synthesised by Eurofins MWG (Ebersberg, Germany). All reactions were performed using 50 ng cDNA, 10 μl SYBER Green Master Mix (Applied Biosystems) and primers at a concentration of 300 nM (Table 1). Final reaction volumes were made up to a total volume of 20 μl with RNase−DNase-free H2O. All qPCRs were carried out in duplicate on the 7500 Fast Real-Time PCR System (Applied Biosystems). The cycling conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Inclusion of a dissociation curve in each qPCR run ensured specificity of the amplicons. Analysis of the most appropriate normaliser gene was carried out using the geNorm application in qbaseplus Software (Biogazelle, Zwijnaarde, Belgium). The optimal number of reference targets in this experimental situation was determined as three (geNorm V <0.15), when comparing a normalisation factor based on the three or four most stable targets. As such, the optimal normalisation factor was calculated as the geometric mean of the calibrated normalised expression values (CNQR) for each gene in arbitrary units.

Localisation of FGF10 protein in the uterus by IHC

The procedure for IHC was carried out as described previously (Okumu et al. 2011). In summary, 4 μm sections of paraffin wax-embedded tissues were de-waxed in xylene and rehydrated through a series of graded alcohol steps. Sections were then blocked for endogenous peroxidase activity using 1% hydrogen peroxide solution in methanol and non-specific binding using 2% normal mouse serum (Dako Diagnostics, Cambridgeshire, UK). Primary antibody (purified rabbit polyclonal anti-human FGF10, Cambridge Bioscience Ltd, Cambridge, UK) was added to the slides at a 1:12.5 dilution in Tris-buffered saline (TBS) and incubated overnight at 4 °C. The secondary antibody (monoclonal anti-rabbit γ-chain-specific IgG) was added at a dilution of 1:500 in TBS and incubated at room temperature for 45 min. The bound antibody was visualised using an Elite Vectastain ABC Kit (Vector Labs, Peterborough, UK) and DAB, which was prepared according to the manufacturer’s instructions, and the colour developed for 10 min. The slides were then washed, dehydrated through ascending concentrations of alcohol and cleared in xylene.

<p>| Table 1 Accession numbers and symbols of genes used to generate primer pair sequences 5′→3′ direction for qRT-PCR. |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplification efficiency</th>
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<td>FGF1 Bos taurus</td>
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The amplification efficiency of each primer pair used for qRT-PCR analysis was determined using a serial dilution series generated by pooling cDNA from each of the experimental samples used in this analysis. The Cq and quantity values of the dilution series were determined using linear regression and the slope and its S.E.M. were calculated to generate the amplification efficiencies that are taken into account when data are processed for normalisation and calibration procedures in the qBase Software.
Slides were mounted using DPX (AGB Scientific Ltd, Dublin, Ireland) and observed under 10× magnification. Using a digital camera, four images were captured per tissue section (two images showing the luminal epithelium (LE), superficial glands (SG) and stroma (STR), and two images showing the deep glands (DG) and myometrium (MYO)). Intensity of staining for all regions was determined using Image-Pro Plus Software (version 6.2, MediaCybernetics, Bethesda, MD, USA) as described previously (Okumu et al. 2011).

**Gene expression analysis of FGF family members in the embryo/conceptus during early pregnancy**

Analysis of transcript abundance of members of the FGF family was carried out as described previously (Mamo et al. 2011). Briefly, RNA was extracted from pools of embryos or conceptus tissue from pregnant heifers on days 7, 10, 13, 16 and 19 (n= 5/ day). Days 7 and 10 blastocysts were pooled into groups of five embryos, while individual conceptuses from days 13, 16 and 19 were used for mRNA extraction. These stages represent the blastocyst stage, hatched blastocyst, ovoid conceptus, pregnancy recognition and initiation of implantation stages of development respectively. Extracted RNA was then subjected to library preparation and cluster generation according to the manufacturer’s instructions (www.illumina.com). RNA sequencing (RNAseq) was carried out on the Illumina GA2 sequencer using the standard Illumina protocol for sequencing cDNA samples and the 32 bp reads were processed through the standard software pipeline for the Genome Analyzer and aligned against the BosTau4 genome. A pseudochromosome containing potential splice junction sequences was generated. The ensGene table from the UCSC genome browser (http://hgdownload.cse.ucsc.edu/goldenPath/bosTau4/database/ensGene.txt.gz: Oct 2007 BosTau4) was used to provide exon location information to the CASAVA module. The moderated negative binomial test from the edgeR Bioconductor library was used to generate the lists of differentially expressed transcripts, and transcript abundance took into account the read counts per transcript and generated RPKM (reads per kilobase of exon per million mapped sequence reads) values for all annotated genes, transcripts and exons. A false discovery rate (FDR)-adjusted P value of <0.05 was used as the cut-off for determining significance. The comparative analysis was restricted to 26 957 protein-coding transcripts in version 52 of Ensembl (www.ensembl.org).

**Statistical analyses**

The effect of treatment on both mRNA expression and the intensity of localised protein were determined using SAS (SAS Institute, Inc., Cary, NC, USA). Variables were checked for the assumptions underlying the ANOVA using PROC UNIVARIATE. Variables that violated these assumptions were transformed using the appropriate λ value obtained from PROC TRANSREG. Analysis was done using PROC GLM with day, pregnancy status, P4 status and their two- and three-way interactions included in the model where appropriate. Treatment effects on gene and protein expression were separated by Tukey’s test. The figures show calibrated, normalised and relative expression values (CNRQ) in arbitrary units and the S.E.M.

**Results**

**Temporal changes in endometrial gene expression of members of the FGF family as the oestrous cycle/early pregnancy progresses**

The expression of FGF1 and FGF10 transcript variants 1 and 2 was significantly affected by the day of oestrous cycle/early pregnancy (P<0.0001) with expression of FGF2 approaching significance (P=0.09; Fig. 1). The expression of FGF1 decreased significantly on day 13 when compared with day 7 (P<0.05) and remained low thereafter. The expression of FGF10_tv1 increased from days 5 to 7 (P<0.05) and remained high on days 13 and 16. In contrast, FGF10_tv2 expression increased significantly on day 7 when compared with day 5,
and day of treatment on FGF10\_tv2 expression. Optimisation for FGF7 mRNA revealed a very low expression in our samples and it was consequently omitted from the study.

An overall effect of day on FGFR1 and FGFR2\_IIIb and FGFR2\_IIIc was identified ($P < 0.001$) with FGFR1 mRNA levels increasing significantly from days 5 to 7 ($P < 0.05$) and remaining high until day 16 ($P > 0.05$; Fig. 2A). The temporal pattern of expression of FGFR2\_IIIb and FGFR2\_IIIc (Fig. 2B) was similar; overall expression decreased on day 13 when compared with days 5 and 7 ($P < 0.05$). In addition, a significant interaction between P\_4 concentration and day was identified for the expression of FGFR2\_IIIc ($P = 0.017$), whereby elevated P\_4 concentration increased the expression of this receptor on days 7 and 16.

**Localisation of FGF10 in the bovine uterus by immunohistochemistry (IHC)**

We sought to localise FGF10 protein by IHC given that its expression increased as the oestrous cycle/early pregnancy progressed and as it is a known progestagen in sheep (Chen et al. 2000), and we hypothesised that pregnancy and/or P\_4 would affect its localisation. FGF10 protein was localised in the LE, SG epithelium, DG epithelium and STR but was not detected in the MYO. Expression in the epithelial cells was limited to the apical portion of luminal epithelia had a stronger expression of the protein than the basal part with the intensity of the localised protein varying from moderate to high. When the intensity of the localised protein within each cell type of the endometrium (LE, SG, DG and STR) was analysed, pregnancy status and P\_4 concentrations did not affect localisation ($P > 0.05$).

**Changes in the gene expression profile of members of the FGF family during post-hatching embryo development**

The expression of FGF ligands in the developing embryo/conceptus was minimal and no effect of stage of embryo development was observed for any of the ligands detected (Table 2). In contrast, the expression of FGFR1, FGFR2, FGFR3, FGFR4, FGFR1L1 and FRS3 was significantly affected by the stage of conceptus development. These data are summarised in Table 3. Expression of FGFR1 increased from days 7 to 13 ($P < 0.05$) and decreased on day 16 ($P < 0.05$) after which expression remained stable until day 19 (Fig. 4). A significant effect of day was also observed for FGFR2 ($P < 0.001$), where expression was similar from day 7 through to day 13, with a significant increase by day 16 ($P < 0.05$) that was maintained until day 19 ($P > 0.05$). Expression of FGF3 changed only on day 13 with an increased expression.
when compared with day 16 ($P<0.05$). In contrast, FGFR4 expression was high on days 7–13 but decreased significantly on day 16 that was maintained until day 19 ($P<0.05$). The expression of FGFR1 decreased on day 16 when compared with previous time points ($P<0.05$), while a similar expression pattern was observed for FRS3 in the embryo (Fig. 4).

Discussion

This study is the first to perform a comprehensive analysis of the FGF family in the endometrium and conceptus during the pre-implantation period of pregnancy in cattle. The results indicate that FGF1, FGF2 and FGF10 are expressed abundantly in the bovine endometrium and that the expression of FGF10 shows a distinct temporal regulation with the stage of the cycle/early pregnancy. We have also shown that P4 supplementation affects endometrial expression of FGF2 and that there is minimal expression of these ligands in the conceptus, but their receptors are modulated during early embryo development.

Studies using in vivo- and in vitro-produced embryos showed that the environment under which the embryos develop affects the expression of FGFs. Only FGF2 could be detected in culture, while FGF10 was undetectable in in vitro-produced embryos. On the other hand, in in vivo produced embryos, FGF1 and FGF10 were detectable in addition to FGF2 (Cooke et al. 2009). This indicates a possible crucial role of the endometrium and/or its secretions in the subsequent expression of the FGFs by the embryo; for example, in sheep, FGF10 is expressed in the endometrium but its receptors are found in the foetal trophoderm cells, where they mediate mesenchymal–epithelial interactions (Chen et al. 2000).

In the bovine endometrium, FGF1 levels in the current study were similar on days 5 and 7 and then decreased through to day 16. This is in contrast to expression in the bovine conceptus where levels were low on days 11 and 14 and increased on day 17 (Cooke et al. 2009). We therefore propose that endometrial-derived FGF1 may be required as the embryo develops to the blastocyst stage but subsequently other FGF family members, such as FGF10, play a role in conceptus development in cattle.

Studies on FGF2 in both the primate (Samathanam et al. 1998) and mouse uterus (Wordinger et al. 1992) reported no response of FGF2 to altered E2 concentrations. This is in contrast to previous studies on FGF2 in the bovine endometrium whereby FGF2 expression was highest at oestrus and decreased on subsequent days of the luteal phase of the cycle (Michael et al. 2006). Moreover, there was no effect of pregnancy on the abundance of FGF2 mRNA in the endometrium or protein in the endometrium or uterine lumen (Michael et al. 2006). These results are consistent with the present study where no effect of pregnancy was observed and stage of the oestrous cycle/early pregnancy did not alter FGF2 expression in the endometrium. In the context of the results obtained by Michael et al. (2006), these results are not surprising given that the period in which the current study was undertaken represented the luteal phase of the cycle, characterised by low E2 and rising P4 concentrations. The novel aspect of this study was the observed interaction between the stage of the luteal phase of the oestrous cycle/early pregnancy and P4 concentrations on FGF2 mRNA expression on days 5 and 7. Previous results from our group have shown that P4 supplementation advances the downregulation of PGR (Okumu et al. 2010) and modifies the expression of a large number of genes in the endometrium (Forde et al. 2009, 2011) which contribute to a uterine
Table 2  Mean expression values (± S.E.M.) determined by RNA sequencing for all FGF ligands detected in the bovine embryo/conceptus during early pregnancy. Expression values are given for each gene in the blastocyst (day 7), hatched blastocyst (day 10), ovulated conceptus (day 13), elongated conceptus (day 16) and beginning of implantation (day 19).

<table>
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<th>Gene name</th>
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environment that promotes advanced conceptus elongation (Carter et al. 2008, Clemente et al. 2009). FGF2 increases the secretion of IFNT in vitro CT1 cells as well as increasing the output of IFNT from blastocysts cultured in vitro (Michael et al. 2006). There was no overall effect of day, pregnancy status or P4 supplementation on the expression of FGF2 in the endometrium. However, a significant three-way interaction existed reflecting the complex changes in pattern of response to P4 supplementation in the pregnant group across days (no effect on days 5 and 13 and increased expression on days 7 and 19). Therefore, a higher expression of FGF2 mRNA by the high P4 groups during the early luteal phase could be one of the mechanisms by which IFNT secretion is increased by the conceptus derived from P4-supplemented heifers.

FGF10 has been described as a factor that mediates epithelial–mesenchymal interactions (Chen et al. 2000). To the best of our knowledge, this is the first comprehensive description of the expression of both FGF10 transcripts in the bovine uterus. In the current study, regardless of pregnancy status or P4 concentration, FGF10 mRNA levels increased steadily as the P4-dominanted luteal phase progressed. Expression of FGF10 protein was detected in the LE and GE as well as stromal cells. From this, we propose that FGF10 in the bovine endometrium may play a dual role. One of the ways in which the biological activity of FGF10 protein in the bovine uterus could be controlled is by the availability of its main receptor FGFR2IIIb as was previously suggested for FGF10 in the bovine CL (Castilho et al. 2008). In the current study, FGF10 increased in the endometrium but expression of its receptor decreased as the oestrous cycle/early pregnancy progressed. However, FGFR2 in the developing conceptus increased significantly between days 13 and 16, which was maintained throughout conceptus elongation. This is associated with increased IFNT production by the conceptus trophectoderm. In fact, studies involving supplementation of blastocyst culture media with FGF10 showed increased IFNT mRNA expression (Cooke et al. 2009). Given the localisation of FGF10 protein in the LE and GE, we propose that it is secreted in to the uterine lumen, where it is available for binding to its receptor (FGFR2) on the conceptus and contributes to conceptus elongation and possibly contributing to IFNT production during the peri-implantation period of pregnancy. In this study, FGF10 was also localised in the STR of the endometrium throughout the oestrous cycle and early pregnancy. In other species, FGF10 is a known stromal-derived factor that acts on the LE and sGE to facilitate the actions of P4 in cells that lack the PGR, i.e. it is a known progestagen in sheep (Chen et al. 2000). The receptor for FGF10 was detected in the endometrium throughout the oestrous cycle and early pregnancy indicating that
FGF10 may be involved in paracrine signalling in the endometrium.

In the current study, FGF7 mRNA levels were very low (data not shown). A previous study detected FGF7 in bovine endometrial (Cooke et al. 2009) and placental tissues (Pfarrer et al. 2006). In sheep, P4 treatment, pregnancy status and day of the oestrous cycle/early pregnancy did not have a significant effect on expression of FGF7 (Chen et al. 2000, Satterfield et al. 2008). In gilts, FGF7 has specific expression in the LE during conceptus elongation, which is coincident with the downregulation of PGR. In addition, its expression is dependent on an interplay between P4, E2 and oestrogen receptor α (Ka et al. 2007). However, results from this study as well as data in the literature indicate that FGF7 is unlikely to play a significant role in early pregnancy in ruminants.

Our data show minimal expression of ligand members of the FGF family during the pre-implantation period of embryo development (Table 2). RNAseq analysis revealed significant modifications in the expression patterns of the FGFRs as the embryo transitions from a blastocyst though to elongation. This is consistent with the data obtained by Cooke et al. (2009), whereby they reported detection of transcripts for FGFR1, FGFR2, FGFR3 and FGFR4 in in vitro-produced embryos as well as day 17 conceptus and CT1 cells. Cooke et al. (2009) also characterised the ligands and found FGF10 detectable in the bovine conceptus during elongation and its profile was similar to that of IFNT, i.e. FGF10 mRNA expression in the conceptus increased substantially during the mid- and late-luteal stages. This is in contrast to the results of this study which indicate minimal FGF10 expression in the conceptus, i.e. <5 RPKM; however, significant increases in the mRNA for FGF10 are detectable in the endometrium. The discrepancy between this study and data obtained by Cooke et al. may simply reflect the different methods of analysis (qPCR V RNAseq). In addition, qPCR analysis detected FGF10 transcript in day 17 conceptuses and the RNAseq data do demonstrate an average RPKM value of 3.5 and 5.2 on days 16 and 19 respectively with an increase in values of <1 RPKM during the blastocyst stage of development. While FGF10 supplementation of CT1 cells in vitro increased IFNT production, we propose that in vivo endometrial-derived FGF10, rather than conceptus-produced FGF10, is responsible for this effect on IFNT production.

In conclusion, this study provides a comprehensive analysis of members of the FGF family of ligands and receptors in the bovine endometrium and the embryo as it transitions from a blastocyst enclosed in a zona pellucida (day 7) through to a fully elongated conceptus at the initiation of implantation (day 19). These data clearly demonstrate that FGF ligands are primarily expressed by the endometrium and their modulation throughout the luteal phase of the oestrous cycle/early pregnancy is associated with alterations in the expression of their receptors in the embryo.

Table 3 Summary of the expression values of members of the FGF family of ligands and their receptors in the bovine endometrium (qPCR analysis) and conceptus (RNA sequencing analysis). Data are grouped according to ligands and their respective receptors.

<table>
<thead>
<tr>
<th>Ligand (s)</th>
<th>Tissue</th>
<th>Day</th>
<th>P4</th>
<th>P4×day</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF7</td>
<td>Endometrium</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FGF10</td>
<td>Endometrium</td>
<td>↑</td>
<td>NS</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Receptor(s)</td>
<td>Endometrium</td>
<td>↓</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>FGFR2IIb</td>
<td>Conceptus</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ligan(s)</td>
<td>Endometrium</td>
<td>↓</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>FGF1</td>
<td>Endometrium</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FGF8</td>
<td>Endometrium</td>
<td>↓</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Receptor(s)</td>
<td>Endometrium</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FGFR2IIIc</td>
<td>Conceptus</td>
<td>↑</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Receptor(s)</td>
<td>Endometrium</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FGFR1</td>
<td>Conceptus</td>
<td>↑</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>FGFR4</td>
<td>Conceptus</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

An arrow indicates the direction of change in mRNA expression levels due to the variable measured, e.g. day, when P<0.05. ND, not detected in that tissue type; NS, no significant effect; and –, not examined. FGF family ligands were not detected in the conceptus tissue at any of the time points examined. No pregnancy effects were detected.

Figure 4 Average gene expression values in the embryo/conceptus (RPKM±S.E.M.) for receptors in the FGF family. Bars represent a distinct morphological events in embryo development corresponding to blastocyst stage (day 7, solid bars), hatched blastocyst (day 10, open bars), ovoid conceptus (day 13, black bars and white stipple), filamentous conceptus (day 16, white bars and black stipple) and the initiation of implantation (day 19, grey bars). Significant differences in expression of a specific gene across time are denoted by different superscripts (a, b, c and d) when P<0.05.
Temporal regulation of FGF and FGFRs

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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