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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 (P_4) 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 P_4 status on *FGF2* expression ($P<0.05$) and a significant interaction between P_4 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 *FRS3* 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.

Reproduction (2014) **147** 825–834

Introduction

Fibroblast growth factors (FGFs) are members of a large family of growth factors (Itoh & Ornitz 2004, Anteby *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, IIIb and IIIc, 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. FGFR2IIIb, which is highly specific for binding FGF7 and FGF10 (Lu *et al*. 1999, Ornitz 2000, Ornitz & Itoh 2001), and FGFR2IIIc, 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 (E_2) 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,

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-13-0373
Online version via www.reproduction-online.org

Ocon-Grove *et al*. 2008). *FGF2* mRNA has also been detected in the bovine endometrium where it is thought that secretion influences the production of interferon τ (IFNT), the pregnancy recognition signal in ruminants (Michael *et al*. 2006). Addition of FGF2 to culture medium *in vitro* increases the size of trophectoderm outgrowths in mice (Taniguchi *et al*. 1998), promotes gastrulation in rabbit embryos (Hrabe de Angelis *et al*. 1995) and increases blastocyst formation (Fields *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 (P_4) concentrations and which mediates the proliferation of endometrial epithelial cells (Koji *et al*. 1994), where it is thought to function as a progestamedin, i.e. a protein that mediates P_4 effects on cells not expressing a P_4 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 P_4 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 P_4; 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 estrumate, 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 P_4-releasing intravaginal device (1.55 g P_4: CEVA, Animal Health Ltd, Chesham, UK) to elevate circulating concentrations of P_4 (Carter *et al*. 2008). This resulted in four treatment groups: i) pregnant, high P_4 concentration; ii) pregnant, normal P_4 concentration (PN); iii) cyclic, high P_4 concentration and iv) cyclic, normal P_4 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 H_2O. 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 qbase^{plus} 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 reference targets *ACTB*, *RPL19* and *ERK1*. All expression data for genes of interest are presented as the mean $\pm$ S.E.M. of the calibrated normalised expression values (CNRQ) 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 a 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.

Table 1 Accession numbers and symbols of genes used to generate primer pair sequences (5′–3′ direction) for qRT-PCR.

Accession number	Gene symbol	Gene name	Forward primer	Reverse primer	Amplification efficiency (*E*)
NM_174055.2	*FGF1*	*Bos taurus* fibroblast growth factor 1 (acidic) mRNA	CCTGCCTGTGAGTCCCCCGA	AGCTGCCTTCCTCCAACCTAGCC	1.981
NM_174056	*FGF2*	*Bos taurus* fibroblast growth factor 2 (basic) mRNA	GTGCAAACCGTTACCTTGCT	ATTAGACTCCAATCGTTCAAAAA	1.938
NM_001193131.1	*FGF7*	*Bos taurus* fibroblast growth factor 7, mRNA	AGGACAGTGGCTGTTGGAAT	TGCAGTCCTCATTGCATTCT	
XM_001249706.1	*FGF10*	Predicted: *Bos taurus* fibroblast growth factor 10, transcript variant 1, mRNA	GCCGAGTTATGGATGTTGGT	TGGAAGCGTAAGACCTGGTG	1.996
XM_592611.4	*FGF10*	Predicted: *Bos taurus* fibroblast growth factor 10, transcript variant 2, mRNA	CGTCCTCTGCGGGGAGGCAT	TACCGCTGACCTTCCCGTTCTCA	1.981
NM_001110207.1	*FGFR1*	*Bos taurus* fibroblast growth factor receptor 1	CTCTCAGAGACCCACCTTCAA	AAGCTGGGGGAGTATTGGTC	1.943
AJ419173	*FGFR2IIIb*	*Bos taurus* partial mRNA for fibroblast growth factor receptor 2, isoform IIIb	TGGAGTTTGTCTGCAAAGTG	GCTTCAGAACCTTGAGATAGGG	3.525
AJ413268.1	*FGFR2IIIc*	*Bos taurus* partial mRNA for fibroblast growth factor receptor 2, isoform IIIc	CACCACGGACAAAGAAATTG	CAACCATGCAGAGTGAAAGG	1.853
NM_001031751.1	*HGF*	*Bos taurus* hepatocyte growth factor, mRNA	TGCAAGACAGTGTTTCCCTTC	TCTCCTCTCCCATGGACATC	2.034
NM_001012999.2	*MET*	*Bos taurus MET* proto-oncogene (hepatocyte growth factor receptor), mRNA	AGCAGAGGCGGGAGCCAAA	ACCCAGGAACCGCGCGGAAGA	1.882
NM_001040516.1	*RPL19*	*Bos taurus* ribosomal protein L19	GAAAGGCAGGCATATGGGTA	TCATCCTCCTCATCCAGGTT	1.952

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 amplicon 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 (Robinson *et al.* 2010) 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, P_4 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,

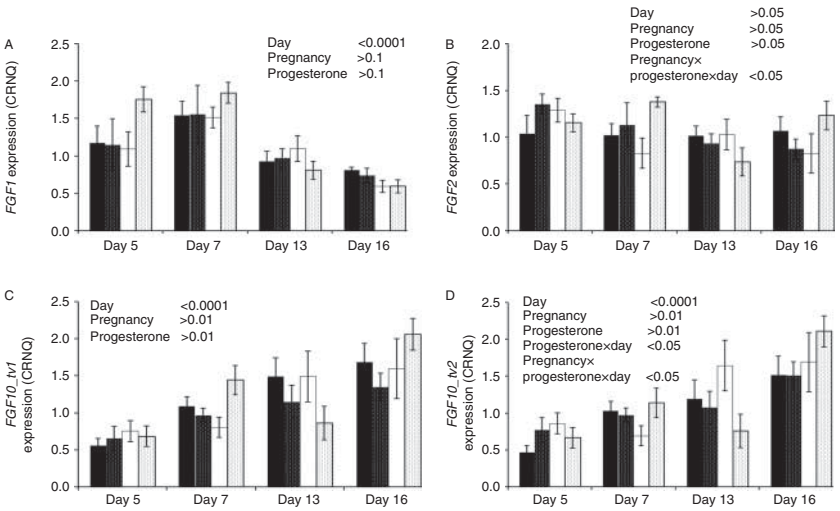

Figure 1 Relative expression values for (A) *FGF1*, (B) *FGF2*, (C) *FGF10* transcript variant 1 and (D) *FGF10* transcript variant 2 in bovine endometrium on days 5, 7, 13 and 16 of the luteal phase of the oestrous cycle/early pregnancy ($n=5$/treatment per time point). Mean (±S.E.M.) expression values are normalised and calibrated relative expression values are given in arbitrary units (AU) for cyclic heifers with normal progesterone concentration (P_4) (solid black bars), cyclic heifers with high P_4 concentration (black bars and white stipple), pregnant heifers with normal P_4 concentration (solid white bars) and pregnant heifers with high P_4 concentration (white bars and black stipple). Overall treatment effects are given in each panel for each gene with significance set at $P<0.05$.

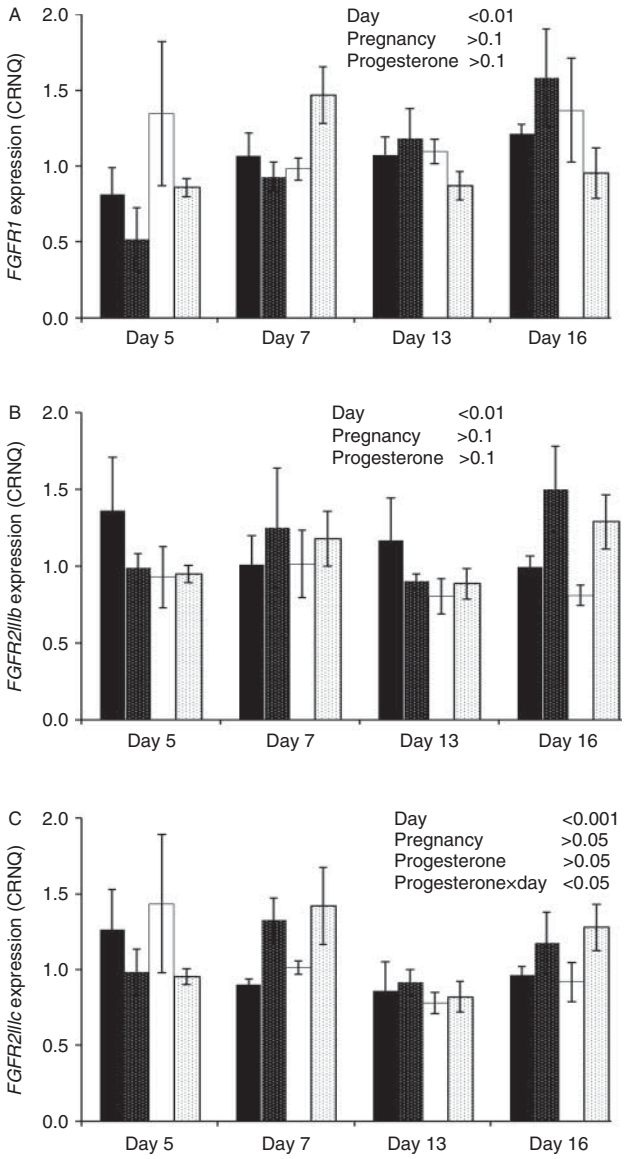

Figure 2 Relative expression values for (A) *FGFR1*, (B) *FGFR2IIIb* and (C) *FGFR2IIIc* in the bovine endometrium on days 5, 7, 13 and 16 of the luteal phase of the oestrous cycle/early pregnancy ($n=5$/treatment per time point). Mean (±S.E.M.) expression values are normalised and calibrated relative expression values are given in arbitrary units (AU) for cyclic heifers normal progesterone (P_4) concentration (solid black bars), cyclic heifers high P_4 concentration (black bars and white stipple), pregnant heifers normal P_4 concentration (solid white bars) and pregnant heifers high P_4 concentration (white bars and black stipple). Overall treatment effects are given in each panel for each gene with significance set at $P<0.05$.

maintained its expression on day 13 and subsequently increased on day 16 ($P<0.05$). Neither P_4 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 P_4 status on *FGF2* expression ($P<0.05$) and a significant interaction between P_4 status 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 *FGFR2IIIb* and *FGFR2IIIc* 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 *FGFR2IIIb* and *FGFR2IIIc* (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 *FGFR2IIIc* ($P=0.017$), 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 progestamedin 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 cytoplasm and the cellular membranes (Fig. 3) with no nuclear staining. 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*, *FGFRL1* 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

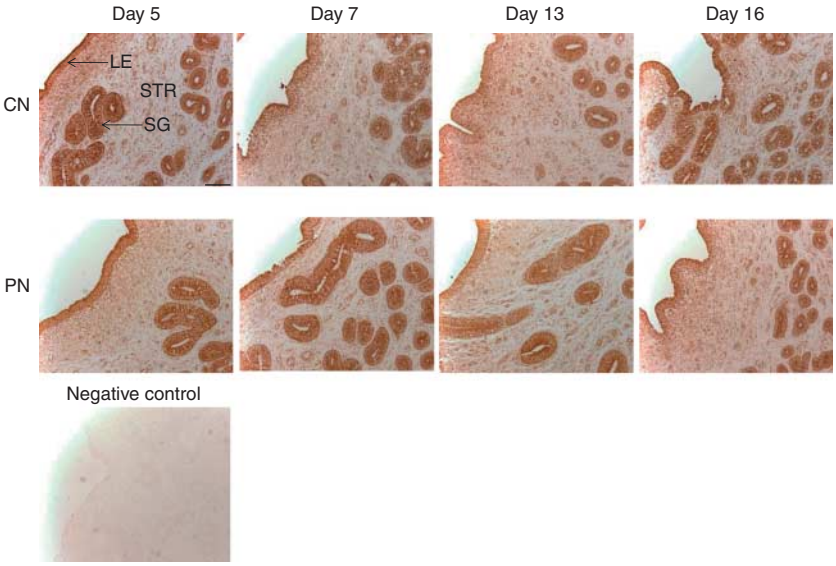

Figure 3 (A) Representative images depicting the localisation of FGF10 protein in the luminal epithelium (LE), superficial glandular epithelia (SG) and stroma (STR) of bovine uterine cross sections in cyclic and pregnant heifers with normal P_4 concentration (CN and PN respectively) on days 5, 7, 13 and 16 of the oestrous cycle/early pregnancy ($n=5$ heifers/treatment per time point). Significant effect of pregnancy, progesterone or day status were identified when $P<0.05$.

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 *FGFRL1* 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 P_4 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 trophectoderm 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 E_2 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 E_2 and rising P_4 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 P_4 concentrations on *FGF2* mRNA expression on days 5 and 7. Previous results from our group have shown that P_4 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), ovoid conceptus (day 13), elongated conceptus (day 16) and beginning of implantation (day 19).

				Average RPKM (±S.E.M.)				
Gene name	**Ensembl transcript ID**	**Ensembl gene ID**	**Gene description**	Day 7	Day 10	Day 13	Day 16	Day 19
FGF1	ENSBTAT00000006851	ENSBTAG00000005198	Acidic fibroblast growth factor	1.00±0.62	0.33±0.18	0.16±0.10	1.34±0.59	1.17±0.72
	ENSBTAT00000043056	ENSBTAG00000005198	Acidic fibroblast growth factor	0.00±0.00	1.43±1.33	0.07±0.07	0.82±0.35	1.14±0.65
FGF10	ENSBTAT00000037849	ENSBTAG00000024820	Fibroblast growth factor 10	1.54±0.94	0.97±0.97	0.41±0.41	3.47±1.15	5.25±1.74
FGF12	ENSBTAT00000016471	ENSBTAG00000012413	Fibroblast growth factor 12	1.67±1.25	3.31±2.02	2.58±0.61	0.80±0.34	0.31±0.11
	ENSBTAT00000046189	ENSBTAG00000012413	Fibroblast growth factor 12	2.67±1.99	1.72±1.06	1.83±0.42	1.13±0.53	0.19±0.07
FGF13	ENSBTAT00000026897	ENSBTAG00000020194	Fibroblast growth factor 13	0.34±0.26	2.02±1.60	0.92±0.42	0.05±0.05	0.05±0.05
	ENSBTAT00000030390	ENSBTAG00000020194	Fibroblast growth factor 13	1.22±0.41	1.34±0.55	0.71±0.26	0.77±0.70	0.03±0.01
	ENSBTAT00000048333	ENSBTAG00000020194	Fibroblast growth factor 13	3.90±1.54	1.59±0.47	1.03±0.30	1.38±0.46	0.06±0.02
FGF16	ENSBTAT00000008839	ENSBTAG00000006722	Fibroblast growth factor 16	2.65±1.80	2.11±0.86	0.32±0.16	2.96±0.81	0.05±0.05
FGF18	ENSBTAT00000000139	ENSBTAG00000000128	Fibroblast growth factor 18	14.44±10.61	6.75±5.95	1.66±1.02	10.00±3.79	0.07±0.02
FGF19	ENSBTAT00000022973	ENSBTAG00000017285	Fibroblast growth factor 19	20.04±8.57	7.72±3.69	7.72±2.82	8.69±1.69	6.76±3.21
FGF2	ENSBTAT00000007477	ENSBTAG00000005691	Basic fibroblast growth factor	1.04±0.53	1.62±0.67	0.93±0.24	1.01±0.22	0.22±0.03
FGF21	ENSBTAT00000015438	ENSBTAG00000011624	Fibroblast growth factor 21	0.00±0.00	1.41±1.19	0.50±0.34	2.87±1.33	0.12±0.12
FGF8	ENSBTAT00000002001	ENSBTAG00000001530	Fibroblast growth factor 8	7.23±1.67	10.45±6.38	2.35±0.97	1.31±0.77	0.23±0.16

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 P_4 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 P_4 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 P_4 groups during the early luteal phase could be one of the mechanisms by which IFNT secretion is increased by the conceptus derived from P_4-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 P_4 concentration, *FGF10* mRNA levels increased steadily as the P_4-dominated 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 P_4 in cells that lack the PGR, i.e. it is a known progestamedin in sheep (Chen *et al.* 2000). The receptor for FGF10 was detected in the endometrium throughout the oestrous cycle and early pregnancy indicating that

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.

	Tissue	Day	P_4	$P_4 \times$ day
Ligand (s)				
FGF7	Endometrium	ND	ND	ND
FGF10	Endometrium	↑	NS	$P<0.05$
Receptor (s)				
FGFR2IIIb	Endometrium	↓	NS	NS
	Conceptus	↑	–	–
Ligand (s)				
FGF1	Endometrium	↓	NS	NS
FGF8	Endometrium	–	–	–
Receptor (s)				
FGFR2IIIc	Endometrium	↓	NS	$P<0.05$
	Conceptus	↑	–	–
Receptor (s)				
FGFR1	Endometrium	↑	NS	NS
	Conceptus	↑↓	–	–
Receptor (s)				
FGFRL1	Conceptus	↓	–	–
FGFR3	Conceptus	↑↓	–	–
FGFR4	Conceptus	↓	–	–

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.

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, P_4 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 P_4, E_2 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.

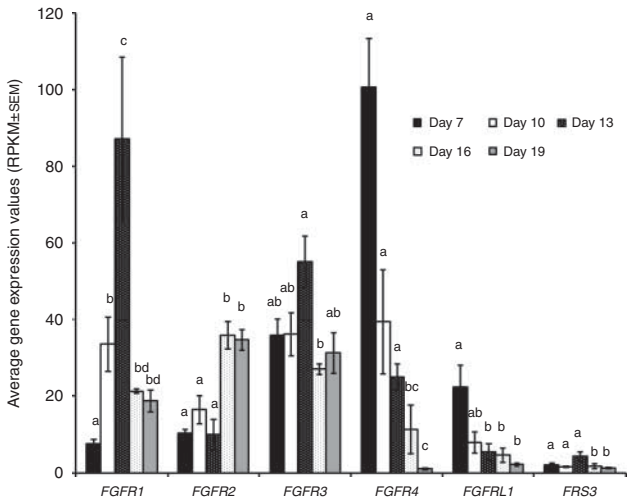

Figure 4 Average gene expression values in the embryo/conceptus (RPKM±S.E.M.) for receptors in the FGF family. Bars represent a distinct morphological events in embryo development corresponding to blastocyst stage (day 7, solid bars), hatched blastocyst (day 10, open bars), ovoid conceptus (day 13, black 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 $P<0.05$.

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.

Funding

This work was funded by the Science Foundation Ireland under grant numbers 06/INI/B62 and 07/SRC/B1156.

Acknowledgements

The authors express gratitude to all the principal investigators, postdoctoral scientists, technical staff and all graduate students involved in sample collection and assays and also thank them for their advice on various aspects of the study.

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Received 13 August 2013
First decision 9 September 2013
Revised manuscript received 17 February 2014
Accepted 19 February 2014