Molecular determinants of a competent bovine corpus luteum: first- vs final-wave dominant follicles

E Gregson¹, R Webb¹, E L Sheldrick¹, B K Campbell², G E Mann¹, S Liddell¹ and K D Sinclair¹

¹School of Biosciences, University of Nottingham, Loughborough, UK and ²School of Clinical Sciences, University of Nottingham, Nottingham, UK

Correspondence should be addressed to K D Sinclair; Email: kevin.sinclair@nottingham.ac.uk

Abstract

Reproductive management in cattle requires the synchrony of follicle development and oestrus before insemination. However, ovulation of follicles that have not undergone normal physiological maturation can lead to suboptimal luteal function. Here, we investigated the expression of a targeted set of 47 genes in (a) a first-wave vs final-wave dominant follicle (DF; the latter destined to ovulate spontaneously) and (b) 6-day-old corpora lutea (CLs) following either spontaneous ovulation or induced ovulation of a first-wave DF to ascertain their functional significance for competent CL development. Both the mass and progesterone-synthesising capacity of a CL formed following induced ovulation of a first-wave DF were impaired. These impaired CLs had reduced expression of steroidogenic enzymes (e.g. *STAR* and *HSD3B1*), luteotrophic receptors (*LHCGR*) and angiogenic regulators (e.g. *VEGFA*) and increased expression of *BMP2* (linked to luteolysis). Relative to final-wave DFs, characteristic features of first-wave DFs included reduced oestradiol concentrations and a reduced oestradiol:progesterone ratio in the face of increased expression of key steroidogenic enzymes (i.e. *CYP11A1*, *HSD3B1* and *CYP19A1*) in granulosa cells and reduced expression of the HDL receptor *SCARB1* in thecal cells. Transcripts for further components of the TGF and IGF systems (e.g. *INHA*, *INHBA*, *IGF2R* and *IGFBP2*) varied between the first- and final-wave DFs. These results highlight the importance of hormones such as progesterone interacting with local components of both the TGF and IGF systems to affect the maturation of the ovulatory follicle and functional competency of the subsequent CL.

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Introduction

Since the introduction of ovulation synchronisation into mainstream reproductive management of cattle (Pursley *et al.* 1995), a plethora of studies have examined in detail the benefits of follicle synchrony in reproductive management programs (Bisinotto *et al.* 2014, Wiltbank & Pursley, 2014). However, it has been shown that ovulation of follicles that have not undergone normal physiological maturation can lead to suboptimal luteal function compared with spontaneous ovulation. For example, following synchronisation, Perry *et al.* (2005) found that ovulation of smaller follicles (presumed to be short of full maturity) resulted in decreased pregnancy rates. This was associated with lower oestradiol (E2) on the day of insemination together with impaired subsequent luteal function. In contrast, they reported no effect of ovulatory follicle size when ovulation occurred spontaneously. Furthermore, Bisinotto *et al.* (2010) found differences in pregnancy rate following artificial insemination (AI) according to wave of the ovulated follicle, with higher pregnancy rates following ovulation of a second- than a first-wave dominant

follicle (DF). However, less is known about the impact of ovulatory control programs on the detailed molecular control mechanisms underpinning the adequacy of the ovulatory follicle and resulting corpus luteum (CL).

The expression of several genes involved with ovulation, luteinisation and CL function is under endocrine control. Production of the prostaglandin PGF_{2α}, for instance, has been shown to be regulated by progesterone (P4) (Skarzynski & Okuda, 1999, Okuda *et al.* 2004). A first-wave DF undergoes selection during a period of low circulating P4, whereas, during later follicular waves, DF selection occurs during the luteal phase of the oestrous cycle in the presence of higher concentrations of circulating P4 (Savio *et al.* 1988, Ginther *et al.* 1989). There is evidence that P4 supplementation before induced ovulation (around the time of ovulatory DF selection) can increase pregnancy per AI (Wiltbank *et al.* 2011, Colazo *et al.* 2013), which is likely due to the beneficial effects of P4 supplementation on the development of the first-wave DF (Bisinotto *et al.* 2010).

Table 1 Transcripts quantified in bovine dominant follicles and corpora lutea by GeXP.

Gene	Full name	Accession No.	Primers
TGF- β superfamily			
<i>AMH</i>	Anti-Mullerian hormone	NM_173890	F: aggtgacactatagaatacgtgagctgagcgtagacct R: gtacgactcactataggagacaggctgatgaggagctt
<i>BMP2</i>	Bone morphogenetic protein (BMP) 2	NM_001099141	F: aggtgacactatagaataactttggacaccagggttg R: gtacgactcactatagggaactccgcacatgcctctt
<i>BMP4</i>	BMP4	NM_001045877	F: aggtgacactatagaatagctccaccacgaagaacat R: gtacgactcactatagggatagctgtgtgatgagggtgcc
<i>BMP6</i>	BMP6	XM_869844.3	F: aggtgacactatagaatgtcatgtgggcatittgt R: gtacgactcactatagggaaccaacacaggagaagtgcc
<i>BMPRI1A</i>	BMP receptor, type IA	NM_001076800	F: aggtgacactatagaatgtgtgtgtgtcatagctgc R: gtacgactcactatagggaatggctttatgcgattgg
<i>BMPRI1B</i>	BMP receptor, type IB	NM_001105328	F: aggtgacactatagaataatggaacagcagaggaaatgc R: gtacgactcactatagggaaggtccacggagaagaaaa
<i>BMPRI2</i>	BMP receptor, type II	XM_617592	F: aggtgacactatagaatactgtcacacaataggcgctg R: gtacgactcactatagggaactggacatgaatgctcaga
<i>INH1A</i>	Inhibin, alpha	NM_174094	F: aggtgacactatagaatagctcacctcccagtttc R: gtacgactcactatagggaaggtgggaccatctcatc
<i>INH1B1A</i>	Inhibin, beta A	NM_174363	F: aggtgacactatagaataacaaagaaggcagtgactg R: gtacgactcactatagggaagctggagacagggaagatg
<i>INH1B2</i>	Inhibin, beta B	NM_176852	F: aggtgacactatagaataagatcatcagcttcgccc R: gtacgactcactatagggaactcaggtagagccacaggc
Insulin/IGF family			
<i>IGF1</i>	Insulin-like growth factor (IGF) 1	NM_001077828	F: aggtgacactatagaatagaagatgcccatcacatcct R: gtacgactcactatagggaagcctcctcagatcacagctc
<i>IGF1R</i>	IGF1 receptor	XM_606794.3	F: aggtgacactatagaatacaaggcaatctgtctcatca R: gtacgactcactatagggaagttccctctagctgtctcc
<i>IGF2</i>	IGF2	NM_174087	F: aggtgacactatagaataacagcgagacacttgcaaaa R: gtacgactcactatagggaagcgtgtgtgtgtgtgtg
<i>IGF2R</i>	IGF2 receptor	NM_174352	F: aggtgacactatagaataggaccttctactgagcgtg R: gtacgactcactatagggaagttctggagctgaaggctcg
<i>IGFBP2</i>	IGF binding protein 2	NM_174555	F: aggtgacactatagaatacaagggtggcaaacatcac R: gtacgactcactatagggaaggtgttacagccatgct
<i>IGFBP4</i>	IGF binding protein 4	NM_174557	F: aggtgacactatagaatacaggtcccccttactcctc R: gtacgactcactatagggaactttctccatcaggcacat
<i>IGFBP5</i>	IGF binding protein 5	NM_001105327.1	F: aggtgacactatagaatagatcgaagagactcccgtg R: gtacgactcactatagggaagtcagcttcttctgcgtgc
<i>INSR</i>	Insulin receptor	XM_590552	F: aggtgacactatagaataaaggagggccccctaccagaa R: gtacgactcactatagggaatgtacggcggttcacagaaa
Steroidogenic mediators			
<i>CYP11A1</i>	Cytochrome P450, family 11, subfamily A, polypeptide 1	NM_176644.2	F: aggtgacactatagaataaagtttgacccaaccagggtg R: gtacgactcactatagggaagtggtccagtcaccgatatg
<i>CYP17A1</i>	Cytochrome P450, family 17, subfamily A, polypeptide 1	NM_174304	F: aggtgacactatagaataagacaaccaaaagggtcattg R: gtacgactcactatagggaagtcaggatcctcattctga
<i>CYP19A1</i>	Cytochrome P450, family 19, subfamily A, polypeptide 1	NM_174305	F: aggtgacactatagaataaaggcaagagcaacaagcat R: gtacgactcactatagggaattggcgctaattccaaga
<i>ESR1</i>	Oestrogen receptor 1	NM_001001443	F: aggtgacactatagaatagggtgtacatggacagcagca R: gtacgactcactatagggaatcaggtaatagggcacctg
<i>ESR2</i>	Oestrogen receptor 2	NM_174051	F: aggtgacactatagaatagacagaccacaagcccaaat R: gtacgactcactatagggaagtttcacgcaaggactctt
<i>HSD3B1</i>	Hydroxyl-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	NM_174343.2	F: aggtgacactatagaatagcagaaaaccaaggagtgga R: gtacgactcactatagggaatcacctgtgtgtcccctg
<i>PGR</i>	Progesterone receptor	XM_583951.4	F: aggtgacactatagaatgttctcgctctacggggac R: gtacgactcactatagggaatgtgtacaggacgactccag
<i>SCARB1</i>	Scavenger receptor class B, member 1	NM_174597.2	F: aggtgacactatagaataaactgggaacatccagc R: gtacgactcactatagggaatgggatgagcagtagga
<i>LRP8</i>	Low-density lipoprotein receptor-related protein 8	NM_001097565.1	F: aggtgacactatagaataccctgcaagggttcattgtat R: gtacgactcactatagggaagaaatggcctcatttcca
<i>SHBG</i>	Sex hormone-binding globulin	NM_001098858	F: aggtgacactatagaataccagagtcattggaggcta R: gtacgactcactatagggaatcccaagtcggaactca
<i>STAR</i>	Steroidogenic acute regulatory protein	NM_174189.2	F: aggtgacactatagaatactactgcccaggaaagatgc R: gtacgactcactatagggaagaacctaggagagagccgc

(Continued)

Table 1 (Continued).

Gene	Full name	Accession No.	Primers
Cytokines			
<i>IL1B</i>	Interleukin (IL) 1, beta	NM_174093.1	F: aggtgacactatagaatatgaacccatcaacgaatga R: gtacgactactatagggatggatgttccatctcccat
<i>IL2</i>	IL2	NM_180997.1	F: aggtgacactatagaatacaaacgggtgcacactctca R: gtacgactactatagggagaatccttgatctctctgggg
<i>IL6</i>	IL6	NM_173923.2	F: aggtgacactatagaataagctctcattaagcgcatgg R: gtacgactactatagggatctgcgatcttttcttcag
<i>IL8</i>	IL8	NM_173925	F: aggtgacactatagaataaccaatggaaacgaggtctg R: gtacgactactatagggacacacagaccacacag
<i>KITLG</i>	KIT ligand	NM_174375	F: aggtgacactatagaataagcattgccagattctttt R: gtacgactactatagggagaactgttaccgccaatgt
<i>MIF</i>	Macrophage migration inhibitory factor	NM_001033608.1	F: aggtgacactatagaatacaactctgcgacatgaacg R: gtacgactactatagggacgtttattgtcctccagg
<i>PTPRC</i>	Protein tyrosine phosphatase receptor type C	BC148881	F: aggtgacactatagaatacggagatgcaggatcaaat R: gtacgactactatagggaccagatcatctccagaaa
Apoptotic regulators			
<i>CCND2</i>	Cyclin D2	NM_001076372.1	F: aggtgacactatagaataagcagtagcgtcaggaccag R: gtacgactactatagggagaagagagagagcggattg
<i>CFLAR</i>	CASP8- and FADD-like apoptosis regulator	NM_001012281.1	F: aggtgacactatagaataactaggctccagaaatggcag R: gtacgactactatagggagctgactcatagccagg
<i>GADD45B</i>	Growth arrest and DNA damage-inducible, beta	NM_001040604.1	F: aggtgacactatagaataacgaacccctcacagac R: gtacgactactatagggagtggtttccgcagcaagttt
Angiogenic regulators			
<i>HIF1A</i>	Hypoxia-inducible factor 1, alpha subunit	NM_174339.3	F: aggtgacactatagaatatgcctctgaaactccaagc R: gtacgactactatagggactggggcatggtaaaagaaa
<i>VEGFA</i>	Vascular endothelial growth factor A	NM_174216.1	F: aggtgacactatagaataagcaaggcaagaaatccct R: gtacgactactatagggatcctggtgagacgtctggtt
Miscellaneous			
<i>FGF1</i>	Fibroblast growth factor 1	NM_174055	F: aggtgacactatagaatgaacgcgttctaataatgcc R: gtacgactactatagggatgagagggaatcatgccag
<i>FSHR</i>	FSH receptor	NM_174061	F: aggtgacactatagaataatgtttccaggagcctct R: gtacgactactatagggatgacccctagcctgagtcac
<i>SRSF9</i>	Splicing factor, arginine/serine-rich 9	NM_001083398	F: aggtgacactatagaataatgccctgcgtaaacctgg R: gtacgactactatagggaaatccaccacgtgtctcag
<i>PTGFR</i>	Prostaglandin F ₂ α receptor	BD187584	F: aggtgacactatagaatatgccactttttctaggcag R: gtacgactactatagggatggcattgcaaacaaatga
House-keeping genes			
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034	F: aggtgacactatagaatacacccctcaagattgtcagca R: gtacgactactatagggagggtcataagtcctccacga
<i>H2AFZ</i>	H2A histone family, member Z	NM_174809.2	F: aggtgacactatagaataatccagtggtgtgattccag R: gtacgactactatagggatttgggtgggaaagctaa
<i>RPLP0</i>	Ribosomal protein, large, P0	NM_001012682.1	F: aggtgacactatagaatacttctgaaaaggtaaggc R: gtacgactactatagggagactcctccgactcctctt

With the foregoing discussion in mind, our hypothesis was that the hormonal milieu within which a DF develops affects its ability to form a viable CL, that this is related to the expression of genes with key roles in regulating DF development, subsequent luteinisation and CL function, and that the expression of these genes differs between the first- and final-wave DFs. It was also hypothesised that the CL formed following induced ovulation of a first-wave DF would be smaller and less capable of P4 production than those formed following spontaneous ovulation and that this would be associated with altered expression of genes involved in cellular differentiation, tissue growth and steroidogenesis.

To test these hypotheses, we conducted an experiment that involved 24 cyclic virgin heifers in which we compared the expression of a targeted set of genes (Table 1), with established physiological effects within the bovine ovary, in follicles and CLs of differing size at contrasting stages of the oestrous cycle. Specifically, we wanted to compare the molecular characteristics of (a) a first-wave DF to that of a final-wave DF destined to ovulate spontaneously and (b) a 6-day-old CL following spontaneous ovulation to a 6-day-old CL following induced ovulation of a first-wave DF. These data were related to quantitative measures of steroidogenesis and local and systemic growth factor and hormone concentrations.

Materials and methods

Sample collection

A total of 24 post-pubertal Hereford×Holstein heifers (mean±S.E.M. live weight of 417.5±7.3 kg and a body condition score (BCS) of 2.53±0.05 units; Lowman *et al.* (1976)) were allocated to one of the three treatment groups (A–C) according to live weight and BCS, giving eight animals per treatment group. Animals were group housed on straw bedding and given *ad libitum* access to water and hay. Mineralised concentrates were given twice daily at a rate of 5 kg per animal per day, rising to 6 kg as the animals gained weight in line with their metabolisable energy and protein requirements (AFRC 1993). All procedures were performed under the auspices of the Animal Scientific Procedures Act (1986) and approved by the University of Nottingham ethics review committee.

Oestrous cycles were synchronised initially using two intramuscular prostaglandin (PG) injections (2 mL Estrumate; Intervet UK Ltd., Milton Keynes, UK) given 11 days apart. An intramuscular injection of GNRH (2.5 mL Receptal; Intervet UK Ltd., Milton Keynes, UK) was given 48 h after the initial dose of PG (Fig. 1). Timing of ovulation was confirmed by transrectal real-time B-mode ultrasonography using an Aloka SSD-500v scanner (Aloka Co. Ltd., Tokyo, Japan) equipped with a 5 MHz linear array on nominal day –1 and +1 of the anticipated day of ovulation. We (KD Sinclair and GE Mann, unpublished data) have previously observed that transrectal ovarian ultrasonography on the expected day of ovulation can delay or inhibit this event in some animals. Heifers in group A were killed on day 6 after synchronised ovulation (day 0) to recover a first-wave DF and a 6-day-old, spontaneous CL. Animals in group B were given 5 mL GNRH and 2 mL PG on day 6 to cause ovulation of the first-wave DF and regression of the spontaneous CL, then killed on day 13 to retrieve a 6-day-old, induced CL and a DF. Animals in group C were given 2 mL PG on day 18 and killed on day 19 to retrieve a final-wave DF and a regressing CL. All animals were blood sampled daily by jugular venipuncture and samples were analysed for plasma P4 and insulin-like growth factor 1 (IGF-1). Additional blood samples were taken from group B at 0, 1 and 2 h after GNRH injection on day 6 for plasma LH analysis. To monitor ovarian follicular development and to confirm cyclicity, animals in group C underwent transrectal ultrasonography daily, except on the day of expected ovulation. All other animals underwent transrectal ultrasonography on the days before and following expected ovulations (including that following initial synchronisation; day 0) and on the day before killing.

Animals were blood sampled before transportation to an on-site abattoir for killing. Ovaries from each animal were recovered, transferred to the laboratory within 10 min of killing and processed immediately. The largest follicle (≥11 mm) was dissected from each pair of ovaries. Follicular fluid was aspirated from this large follicle (presumed to be a DF), the largest subordinate follicle (SF) and a selection of smaller subordinate follicles (2–6 mm) from each pair of ovaries and stored at –20°C. Granulosa cells were then scraped from the DF and washed in PBS before storage at –80°C in RLT+ lysis buffer (Qiagen). The thecal sheet was then peeled away from the DF wall using a pair of fine

forceps, washed in PBS and stored at –80°C in RLT+ lysis buffer (Qiagen).

CLs were dissected from ovaries, measured, weighed and then divided into three sections. The first section was minced using a scalpel blade, washed in PBS and then centrifuged at 1500 g for 3 min. RLT+ lysis buffer (Qiagen) was added to the cell pellet, which was stored at –80°C to be homogenised immediately before RNA extraction. The second section was minced and washed in PBS and then divided further to give three 25 mg (±2 mg) samples per animal, which were snap frozen in liquid nitrogen and stored at –80°C before P4 analysis by ELISA. The third section was also minced, washed and divided to give three 25 mg (±2 mg) samples per animal. These samples were re-suspended in 2 mL culture medium (M199 containing 0.068 mM L-glutamine) and incubated at 38°C for 30 min in a shaking water bath at 70 strokes per minute. They were then centrifuged at 1500 g for 5 min and the tissue and spent media were snap frozen separately in liquid nitrogen and stored at –80°C before P4 analysis by ELISA.

Hormone assays

A commercially available ELISA kit (Ridgeway Science, St. Briavels, UK) was used to measure P4 in follicular fluid, blood plasma, spent culture media and CL extracts as described previously (Wonnacott *et al.* 2010). CL tissue samples were ethanol extracted before P4 assay. Double-distilled ethanol (5 mL) was added to each sample, on ice. The samples were homogenised for 30 s (Polytron PT400; Kinematica, Lucerne, Switzerland), evaporated to dryness using a speedvac (Savant DNA 110; Thermo Fisher Scientific) and then re-dissolved in 1 mL PBS. Plasma standards and quality controls (QCs) (Ridgeway Science, St. Briavels, UK) were used when analysing blood plasma samples, and buffer standards and QCs (Ridgeway Science, St. Briavels, UK) were used when analysing all other samples, with intra- and inter-assay coefficients of variation of 6.69 and 5.68% respectively.

Oestradiol was measured by radioimmunoassay, as described previously (Kanakkaparambil *et al.* 2009). The intra- and inter-assay coefficients of variation for this assay were 4.63 and 11.63% respectively. A commercially available bovine ELISA kit (LH Detect; ReproPharm, Nouzilly, France) was used to measure LH in blood plasma. The intra- and inter-assay coefficients of variation for this assay were 5.49 and 15.24% respectively.

A commercially available kit was used to measure IGF1 in blood plasma (DRG Instruments GmbH, Marburg, Germany) from day 0 and the day of killing (refer to Fig. 1). No sample dilution was necessary. The intra- and inter-assay coefficients of variation for this assay were 4.43 and 7.21% respectively.

Transcript expression

RNA extraction was performed using a commercially available kit (RNeasy mini kit; Qiagen) and RNA concentration was determined using a NanoDrop ND-1000 UV-vis spectrophotometer (Thermo Fisher Scientific). Samples were diluted in RNase-free water to a concentration of 20 ng/μL before a further gDNA removal step using another

Figure 1 (A) Oestrous cycle manipulation timeline. Oestrous cycles of 24 Hereford × Holstein heifers were synchronised using prostaglandin (PG) and gonadotrophin-releasing hormone (GNRH). Animals were given further injections according to their treatment group, indicated by letters A–C in brackets. Group A animals were killed at day 6 to recover a first-wave dominant follicle (DF) and 6-day-old corpus luteum (CL); group B animals ovulated on day 7 and were killed on day 13 to recover a 6-day-old induced CL; group C animals were killed on day 19 to recover a final-wave DF. (B) Plasma progesterone was monitored from day 0 to killing for group A (closed circles), group B (open circles) and group C (triangles).

commercially available kit (TURBO DNA-free; Ambion). mRNA was denatured at 70°C for 10 min using a thermal cycler (BioRad) before RT and subsequent transcript expression analysis.

Expression of 47 genes (Tables 1 and 2) known to regulate DF and CL function was quantified using the GenomeLab GeXP Genetic Analysis System (Beckman Coulter Inc., High Wycombe, UK). This method utilises gene-specific primers that have a universal sequence tag. Forward universal primers within the PCR buffer are fluorescently labelled, allowing detection and quantification of up to 30 size-separated products within a single PCR (Wu *et al.* 2008, Rai *et al.* 2009). Transcripts were

divided arbitrarily between two multiplex reactions. Due to the size and relative importance of the *LHCGR* transcript in ovulation and luteinisation, a separate multiplex reaction was designed to amplify several regions of the mRNA (Table 2).

RT-PCR were performed using reagents (including an internal standard) and software provided by Beckman Coulter Inc. (High Wycombe, UK). For each of the three different multiplex reactions, reverse primers (Sigma-Genosys Ltd., Poole, UK) were mixed together at concentrations ranging from 60 to 1500 nM in 10 mM Tris–HCl solution, pH 8.0 (Sigma-Aldrich), and forward primers (Sigma-Genosys Ltd., Poole, UK) were mixed together at a concentration of 200 nM each using the same solution. Reverse primer concentrations were adjusted to allow for variation in initial concentrations of mRNA templates and primer efficiencies. Forward primers contained universal sequence tags used for amplification after the first few cycles of PCR. For primer sequences (Tables 1 and 2). RT was carried out including the mixture of reverse primers, and 35 cycles of PCR were carried out including the mixture of forward primers, as per the manufacturer's instructions. The resulting PCR products were diluted 1:30 in water and 2 µL diluted sample were mixed with 0.5 µL DNA size standard-400 and 37.5 µL sample loading solution in an appropriate well of a 96-well electrophoresis plate and covered with mineral oil. The plate was then placed in a GeXP Genetic Analysis System, which separates the PCR products by capillary electrophoresis.

Data were checked using the fragment analysis module of the GenomeLab GeXP system software and any samples lacking a peak from the internal standard, Kan^r, were repeated. The fragment data and peak area were then imported into the eXpress Analysis module of eXpress Profiler software, in which fragments are linked with gene information giving expression, in arbitrary fluorescence units, for each transcript within each well. This was then exported into Microsoft Excel and transcript expression was normalised within each sample by dividing the target expression by the average expression of the three control genes, giving target expression relative to *GAPDH*, *H2AFZ* and *RPLP0* in relative fluorescence units. Although the GeXP multiplex technology is tried and tested, by way of validation in our hands, we compared the expression of a number of genes using quantitative real-time PCR and GeXP (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

Western blotting

In support of the transcript data that emerged from this study, and given that the follicular fluids collected were committed fully to steroid analyses, additional pairs of bovine ovaries were collected from a local abattoir, retaining individual animal

Table 2 Primers designed to amplify regions of the luteinising hormone/chorionic gonadotrophin receptor (*LHCGR*) (NM_174381) by GeXP.

Product name	Product location	Primers
<i>LHCGR</i> ex2	Exons 2–4	F: aggtgacactatagaatacacctatctccctatcaaagtaatcc R: gtacgactcactataggacagaggagattgtaaacgc
<i>LHCGR</i> ex8	Exons 8–11	F: aggtgacactatagaatagactgaaggaaaatgcacg R: gtacgactcactataggagaggtgtcttggtgaagcaga
<i>LHCGR</i> ex11	Within exon 11	F: aggtgacactatagaatattgttaggcacatcaggcaaa R: gtacgactcactataggacatgttcatggattggaag

Table 3 Structures present on the ovaries of heifers at slaughter. Group A were slaughtered at day 6 after synchronised ovulation; Group B were given prostaglandin and GnRH on day 6 to induce CL regression and ovulation on day 7 and were slaughtered on day 13; Group C were slaughtered on day 19. A: Size of the dominant follicle, and size and mass of the corpus luteum (CL), together with progesterone production. B: Oestradiol and progesterone concentrations in follicular fluids from dominant, largest subordinate and a selection of small (2–6 mm) subordinate follicles.

	Treatment group			Probability
	A (n=8)	B (n=7)	C (n=8)	
A				
Size of dominant follicle				
Diameter (mm)	15.12±0.85	16.57±0.61	14.75±0.65	-
Size and mass of corpus luteum (CL)				
Diameter (mm)	23.88±1.89 ^a	18.14±1.03 ^b	21.00±0.53 ^{ab}	0.021
Mass (g)	6.36±1.47 ^a	2.94±0.44 ^b	3.92±0.29 ^{ab}	0.049
Total P4 content (mg)	209±81 ^a	31±8 ^b	49±8 ^b	0.035
P4 production (ng/25 mg tissue)	771±161 ^a	191±54 ^b	102±35 ^b	<0.001
P4 synthetic capacity (mg/CL)	163±36 ^a	23±8 ^b	17±6 ^b	<0.001
B: Oestradiol (E2) and progesterone (P4) concentrations in follicular fluids				
E2 (ng/mL)				
Dominant follicle	181.4±103.5	576.9±109.6	407.2±109.5	0.056
Small subordinate follicle	0.60±0.75	2.56±0.75	1.43±0.76	-
Largest subordinate follicle	7.93±7.42	3.51±9.82	15.12±7.42	-
P4 (ng/mL)				
Dominant follicle	81.5±11.3	67.3±13.0	75.7±11.3	-
Small subordinate follicle	284.3±56.3	197.5±56.5	85.7±56.3	0.058
Largest subordinate follicle	274.9±116.2	234.5±124.2	275.6±116.2	-
E2:P4 ratio				
Dominant follicle	2.55±1.60	8.98±1.65	6.01±1.65	0.054
Small subordinate follicle	0.004±0.003	0.022±0.008	0.020±0.007	-
Largest subordinate follicle	0.25±0.20	0.009±0.17	0.40±0.21	-

Values are given as mean±SE, letters in superscript indicate significant differences ($P<0.05$).

identity, and classified according to the stage of the oestrous cycle by assessing gross morphology of the CL, based on the observations and classification of Ireland *et al.* (1980). Pairs of ovaries presenting healthy, non-atretic follicles were classified as originating from either the early follicular or early luteal phases. The largest follicle (10–14 mm) per pair of ovaries was dissected, aspirated and granulosa cells scraped and washed as described previously.

Follicular fluid samples (5 µL) in Laemmli buffer were subjected to electrophoresis on 10% SDS–polyacrylamide gels. Proteins were blotted onto nitrocellulose membrane (Optitran BA-S 83, Schleicher & Schuell Bioscience GmbH, Dassel, Germany). Membranes were incubated for 60 min at 21°C with blocking solution (PBS, pH 7.4 with 0.05% Tween 20 and 3% non-fat milk powder) and then incubated overnight at 4°C in the same solution containing the specific primary antiserum (rabbit anti-IGFBP-2, Upstate Biotechnology) diluted 1:1500. The membranes were washed three times with PBS–Tween and then incubated with HRP-labelled anti-rabbit IgG (BioRad) diluted 1:25,000 in blocking solution for 60 min at 21°C. Membranes were washed twice for 10 min with PBS–Tween and once with PBS. The bands were visualised using enhanced chemiluminescence (ECL, GE Healthcare) and detected on BioMax Light film (Carestream). Bands were quantified using Image J software.

Statistical analysis

All statistical analysis was performed using Genstat version 11.1.0.1504 (VSN International Ltd., Hemel-Hempstead, UK).

Necessary transformations of P4 and E2 data were determined by Box–Cox analysis. Analysis of variance (ANOVA) was used to compare E2 concentrations, P4 concentrations and gene expression between stages of the oestrous cycle. DF size, CL weight and CL size were also compared by ANOVA. For transcript analyses, a common approach in simultaneous testing is the Benjamini and Hochberg linear step-up false discovery rate (FDR) controlling procedure (Reiner *et al.* 2003). For such data, an FDR of 0.25 (q) is typically applied to avoid a high proportion of false negatives. P values ($P_{(1)} \leq \dots \leq P_{(m)}$) were ordered along with their respective null hypotheses ($H_{(1)}, \dots, H_{(m)}$), and ranked P_i were compared with the critical value $q \cdot i/m$. In this analysis, $k = \max i$ for which $P_i \leq q \cdot i/m$. We then rejected $H_{(1)}, \dots, H_{(k)}$. Treatment comparisons were then made using the least significant difference test.

Results

Ovarian follicle and CL development

Shortly after the onset of oestrous synchrony (i.e. day –13; Fig. 1A), ultrasound scanning confirmed that a CL was present in all eight heifers allocated to group A, 6/8 heifers allocated to group B and 6/8 heifers allocated to group C. Ultrasound scanning on day –13 (1 day before GNRH) further confirmed the presence of follicles ≥ 8 mm in diameter in all the 24 heifers (size range 8–18 mm). Of the two heifers in group B, one that did not have a CL present at the onset of synchrony subsequently failed to ovulate at day 0 (Fig. 1A), so this

Table 4 Transcript expression in first wave (A), first wave in the presence of a sub-functional corpus luteum (B) and final wave (C) bovine dominant follicles. In general, only transcripts that differed significantly between treatment groups are shown.

Transcript	Treatment group			Probability
	A (n=8)	B (n=7)	C (n=8)	
Granulosa cells				
TGF-β superfamily				
<i>INHA</i>	0.281 ± 0.026 ^a	0.272 ± 0.046 ^a	0.132 ± 0.043 ^b	0.020
<i>INHBA</i>	0.948 ± 0.060 ^a	0.769 ± 0.093 ^{ab}	0.500 ± 0.132 ^b	0.015
Insulin/IGF family				
<i>IGF2R</i>	0.100 ± 0.012	0.092 ± 0.009	0.159 ± 0.037	0.122
<i>IGFBP2</i>	0.200 ± 0.029	0.166 ± 0.014	0.353 ± 0.086	0.059
Steroidogenic mediators				
<i>CYP11A1</i>	0.545 ± 0.048 ^a	0.462 ± 0.050 ^a	0.273 ± 0.079 ^b	0.015
<i>CYP19A1</i>	2.955 ± 0.145 ^a	2.411 ± 0.121 ^a	1.195 ± 0.315 ^b	< 0.001
<i>ESR2</i>	0.231 ± 0.029 ^a	0.199 ± 0.025 ^{ab}	0.123 ± 0.024 ^b	0.024
<i>HSD3B1</i>	0.229 ± 0.028 ^a	0.205 ± 0.025 ^{ab}	0.112 ± 0.038 ^b	0.036
<i>SCARB1</i>	0.433 ± 0.062	0.492 ± 0.062	0.2839 ± 0.066	0.083
Angiogenic regulators				
<i>HIF1A</i>	0.984 ± 0.053 ^a	0.951 ± 0.064 ^a	0.668 ± 0.091 ^b	0.009
Miscellaneous				
<i>PTGFR</i>	0.034 ± 0.004 ^a	0.025 ± 0.007 ^{ab}	0.013 ± 0.005 ^b	0.038
<i>SRSF9</i>	0.601 ± 0.037	0.569 ± 0.031	0.493 ± 0.032	0.079
Thecal cells				
Insulin/IGF family				
<i>IGF2R</i>	0.080 ± 0.009 ^a	0.102 ± 0.014 ^{ab}	0.136 ± 0.012 ^b	0.011
<i>IGFBP2</i>	0.211 ± 0.028 ^a	0.216 ± 0.045 ^a	0.366 ± 0.053 ^b	0.030
Steroidogenic mediators				
<i>SCARB1</i>	0.272 ± 0.048 ^a	0.535 ± 0.073 ^b	0.505 ± 0.099 ^b	0.047
Cytokines				
<i>PTPRC</i>	0.044 ± 0.006 ^a	0.048 ± 0.007 ^a	0.094 ± 0.017 ^b	0.011

Values are mean $\pm$ SE in arbitrary fluorescence units relative to the control genes *GAPDH*, *H2AFZ* and *RPLP0*. Letters in superscript indicate significant differences ($P < 0.05$). Reported transcripts (other than *IGF2R* in granulosa cells) lie within the FDR threshold of 0.25.

animal was removed from any further analysis. All other animals ovulated between 11 am on day -1 and 11 am on day +1 as expected, and diameter of the ovulatory follicle (i.e. its last recorded diameter as determined by ultrasound scanning) did not differ between groups (mean $\pm$ S.E.M. of 13.4 $\pm$ 2.62 mm). Similarly, DF and CL diameter on day 5 did not differ between groups (mean values of 11.6 $\pm$ 2.02 mm and 18.7 $\pm$ 4.32 mm respectively). Plasma P4 concentrations were also found not to differ between groups before day 6 (Fig. 1B). Of the seven animals remaining in group B, all underwent luteal regression, resulting in a decrease in plasma P4 concentrations (Fig. 1B) and ovulated between 11 am on day 6 and 11 am on day 8. Response to GNRH was supported by an immediate increase ($P < 0.001$) in plasma LH (from 1.0 $\pm$ 0.64 pg/mL at the time of GNRH administration to 8.7 $\pm$ 0.83 ng/mL 2 h later), followed by disappearance of the DF within 48 h. Ovulatory follicle diameter was compared between the initial, synchronised ovulation (groups A, B and C; day 0) and induced ovulation (group B; day 7); however, no significant difference was detected (13.4 vs 12.0 mm ($P = 0.09$) measured on days -1 and 6 respectively; Supplementary Fig. 3A). Furthermore, when it came to killing, there was no difference in DF diameter between groups (Table 3A). However, CLs collected from group B at killing were smaller ($P = 0.021$) and weighed less ($P = 0.049$) than those collected from group A (Table 3A). Of the eight heifers in group C, three had a two-wave

cycle, four had a three-wave cycle and one had a four-wave cycle. For DFs at killing in these animals, the time interval from initial visualisation (≤ 2 mm) to killing was 8.25 $\pm$ 0.48 vs 6.0 $\pm$ 0.57 days ($P = 0.024$) for two- and three-wave cycles respectively. There was no significant difference in diameter of the DF between two- vs three-wave cycles (15.5 $\pm$ 1.29 vs 14.0 $\pm$ 2.16 mm). Similarly, there was no difference in FF steroid concentrations between these two groups (E2: 402 $\pm$ 172 vs 412 $\pm$ 156 ng/mL; P4: 69 $\pm$ 13 vs 83 $\pm$ 46 ng/mL).

CL progesterone-producing capacity

Total P4 content (amount of P4 per CL), P4 production (amount of P4 produced per unit of tissue cultured = $P4_{\text{tissue}} + P4_{\text{media}} - P4_{\text{initial tissue}}$) and P4 synthetic capacity (P4 production corrected for total CL weight) were greater ($P = 0.035$, < 0.001 and < 0.001 respectively) for group A than for either group B or C (Table 3A). Furthermore, analyses indicated that diameter of the follicle destined to ovulate was positively ($P = 0.001$) correlated with diameter of the resulting CL 6 days after ovulation for DFs scanned on day -1, but not for DFs scanned on day 6 (i.e. group B) (Supplementary Fig. 3A). However, diameter of the resulting CL was not correlated with its P4 synthetic capacity for either group A or B treatments (Supplementary Fig. 3B), indicating that size of these structures alone does not explain CL functionality.

Table 5 Transcript expression in 6-day-old spontaneous (A), 6-day-old induced (B) and 19-day-old regressing (C) bovine corpora lutea. Only transcripts that differed between treatment groups are shown. Abundance of three regions (*LHCGRex11*, *LHCGRex2* and *LHCGRex8*) with two splice variants lacking exon 3 (*LHCGRex2(-3)*) and exon 9 (*LHCGRex8(-9)*) of the *LHCCR* transcript is given.

Transcript	Treatment group			Probability
	A (n=8)	B (n=7)	C (n=8)	
TGF- β superfamily				
<i>BMP2</i>	0.003 $\pm$ 0.002 ^a	0.021 $\pm$ 0.006 ^b	0.020 $\pm$ 0.006 ^b	0.037
<i>INHBA</i>	0.017 $\pm$ 0.006 ^a	0.039 $\pm$ 0.013 ^{a,b}	0.063 $\pm$ 0.009 ^b	0.009
<i>INHBB</i>	0.016 $\pm$ 0.004 ^a	0.038 $\pm$ 0.016 ^{a,b}	0.063 $\pm$ 0.012 ^b	0.027
Insulin/IGF family				
<i>IGFBP4</i>	0.228 $\pm$ 0.037 ^a	0.116 $\pm$ 0.016 ^b	0.134 $\pm$ 0.031 ^b	0.038
<i>IGFBP5</i>	0.266 $\pm$ 0.022 ^a	0.550 $\pm$ 0.086 ^b	0.635 $\pm$ 0.121 ^b	0.016
Steroidogenic mediators				
<i>CYP11A1</i>	0.933 $\pm$ 0.065 ^a	0.720 $\pm$ 0.126 ^a	0.466 $\pm$ 0.036 ^b	0.002
<i>HSD3B1</i>	0.637 $\pm$ 0.032 ^a	0.415 $\pm$ 0.126 ^b	0.078 $\pm$ 0.024 ^c	<0.001
<i>SCARB1</i>	1.722 $\pm$ 0.092 ^a	1.493 $\pm$ 0.210 ^a	1.038 $\pm$ 0.085 ^b	0.005
<i>STAR</i>	1.350 $\pm$ 0.061 ^a	0.837 $\pm$ 0.185 ^b	0.266 $\pm$ 0.053 ^c	<0.001
Cytokines				
<i>IL1B</i>	0.307 $\pm$ 0.027 ^a	0.246 $\pm$ 0.026 ^{a,b}	0.182 $\pm$ 0.025 ^b	0.011
<i>IL8</i>	0.039 $\pm$ 0.010 ^a	0.062 $\pm$ 0.013 ^a	0.166 $\pm$ 0.038 ^b	0.004
<i>KITLG</i>	0.046 $\pm$ 0.008 ^a	0.024 $\pm$ 0.004 ^b	0.002 $\pm$ 0.004 ^c	<0.001
<i>MIF</i>	0.037 $\pm$ 0.003 ^a	0.034 $\pm$ 0.006 ^a	0.012 $\pm$ 0.004 ^b	<0.001
Apoptotic regulators				
<i>CADD45B</i>	0.444 $\pm$ 0.028 ^a	0.295 $\pm$ 0.040 ^b	0.231 $\pm$ 0.034 ^b	<0.001
Angiogenic regulators				
<i>HIF1A</i>	0.674 $\pm$ 0.034 ^a	0.612 $\pm$ 0.039 ^{a,b}	0.441 $\pm$ 0.047 ^b	0.002
<i>VEGFA</i>	0.037 $\pm$ 0.009 ^a	0.009 $\pm$ 0.005 ^b	ND	0.002
Miscellaneous				
<i>PTGFR</i>	0.500 $\pm$ 0.045 ^a	0.304 $\pm$ 0.056 ^b	0.144 $\pm$ 0.036 ^c	<0.001
<i>SRSF9</i>	0.579 $\pm$ 0.046	0.621 $\pm$ 0.027	0.479 $\pm$ 0.042	0.075
Luteinizing hormone receptor variants				
<i>LHCGRex2</i>	0.392 $\pm$ 0.054 ^a	0.218 $\pm$ 0.085 ^b	0.003 $\pm$ 0.002 ^c	<0.001
<i>LHCGRex2(-3)</i>	0.059 $\pm$ 0.012 ^a	0.017 $\pm$ 0.010 ^b	ND	<0.001
<i>LHCGRex8</i>	0.012 $\pm$ 0.007	ND	ND	–
<i>LHCGRex8(-9)</i>	0.019 $\pm$ 0.009	ND	ND	–
<i>LHCGRex11</i>	0.428 $\pm$ 0.048 ^a	0.281 $\pm$ 0.137 ^a	0.001 $\pm$ 0.001 ^b	0.003

Values are mean $\pm$ S.E.M. in arbitrary fluorescence units relative to the control genes *GAPDH*, *H2AFZ* and *RPLP0*. Letters in superscript indicate significant differences ($P < 0.05$), ND means none detected. Reported transcripts lie within the FDR threshold of 0.25.

Follicular fluid hormone concentrations

As one might expect, follicular fluid P4 concentration was greater ($P < 0.05$) in small follicles than in DFs, and E2 concentration was greater ($P < 0.001$) in DFs than in small follicles (Table 3B). There was a strong indication ($P = 0.058$) that P4 concentrations were greater in small follicles from group A than in small follicles from groups B and C. There was also a strong indication ($P = 0.056$) that E2 concentrations were lower in follicular fluids from DFs in group A than in follicular fluids from either groups B or C. This observation was supported by a lower ($P = 0.054$) E2:P4 ratio in DF fluids from group A compared with groups B and C.

Transcript expression

Transcripts for *AMH*, *BMP2*, *BMP6*, *ESR1*, *FGF1*, *IGF2*, *CYP17A1*, *IL2*, *IL6*, *MIF* and *PCR* were not detected in granulosa cells. Similarly, transcripts for *AMH*, *BMP2*, *BMP6*, *FGF1*, *CYP19A1*, *IL2*, *IL6*, *MIF* and *VEGFA* mRNA were not detected in thecal cells, and transcripts

for *AMH*, *BMP6*, *FGF1*, *IL2*, *IL6*, *INHA*, *LRP8* and *PCR* mRNA were not detected within the CL. Although expressed in our mixed population of ovarian cells during GeXP platform development, the following genes were not expressed in any of our experimental cell types: *AMH*, *BMP6*, *FGF1*, *IL2* and *IL6* (Supplementary Materials and results).

In granulosa cells, expression of *INHA*, *INHBA*, *CYP11A1*, *CYP19A1*, *ESR2*, *HSD3B1*, *HIF1A* and *PTGFR* was greater ($P < 0.05$) in group A (first-wave DF) than in group C (final-wave DF) (Table 4). In thecal cells, expression of *IGF2R*, *IGFBP2*, *SCARB1* and *PTPRC* was lower ($P < 0.05$) in group A than in group C (Table 4). Interestingly, thecal cell *SCARB1* expression was lower ($P < 0.05$) in group A than in group B, and expression of *PTGFR* was only detectable in thecal cells from group B (data not shown). *LHCCR* splice variant expression within granulosa and also thecal cells of the DF did not differ with the stage of the oestrous cycle.

Many more of our selected transcripts were differentially expressed in the CL (Table 5) than in either granulosa or thecal cells (Table 4). For the CL,

Figure 2 Plasma IGF1 concentrations at the point of killing for 24 Hereford × Holstein heifers. Animals were synchronised (Fig. 1) and then group A animals were killed at day 6 (after initial synchronised ovulation); group B animals were induced to ovulate on day 7 and killed on day 13; group C animals were killed on day 19. Plasma IGF1 concentrations were lower ($P=0.001$) in group A than in group B and C animals.

the greatest differences in transcript expression were between groups A and C; transcript expression for group B often was intermediate to these contrasting levels.

Figure 3 Follicular fluid IGF2R protein concentration (from cycle stage-determined abattoir-derived ovaries) was greater ($P<0.001$) in early follicular than early luteal phase dominant follicles (A). IGF2R was quantified by western blotting. (B) A typical gel for follicular fluid from two early luteal (EL1 and EL2) and two early follicular (EF1 and EF2) phase dominant follicles, with alternative lanes left blank (–).

Given that the comparison of particular interest lies between groups A and B, it is noteworthy that *BMP2* and *IGFBP5* expression was lower in CLs from group A than from group B. In contrast, expression of *IGFBP4*, *HSD3B1*, *STAR*, *KITLG*, *GADD45B*, *VEGFA*, *PTGFR*, *LHCGR*ex2, -ex2(-3), -ex8 and -ex8(-9) was greater for group A than for group B.

Plasma IGF1 concentration

At day 0 (Fig. 1), plasma IGF1 concentration was 148 ± 47 ng/mL and did not differ between treatment groups. At the point of killing, however, plasma IGF1 was significantly lower ($P=0.001$) in heifers from group A than from groups B and C (Fig. 2).

IGF2R and IGFBP2 expression in supplementary abattoir ovaries

In granulosa cells harvested from cycle stage-determined abattoir-derived ovaries, relative expression of *IGF2R* and *IGFBP2* was greater ($P=0.004$) in cells from early follicular phase (similar to group C) than early luteal phase (similar to group A) dominant follicles (0.267 ± 0.022 vs 0.188 ± 0.018 for *IGF2R*; 0.631 ± 0.060 vs 0.353 ± 0.050 for *IGFBP2*). In agreement with transcript abundance, the concentration of IGFBP2 protein in follicular fluid was greater ($P<0.001$) from early follicular than early luteal phase DFs (Fig. 3).

Discussion

This study reports a number of key findings. Both the mass and P4-synthesising capacity of a CL formed following induced ovulation of a first-wave DF (i.e. CLs from group B in this study) were reduced relative to a CL formed following spontaneous ovulation (i.e. CLs from group A). Indeed, the P4 synthetic capacity of these induced (i.e. group B) CLs was similar to that of a regressing CL (i.e. group C) during the pro-oestrous phase of the cycle and, at a molecular level, they were characterised as having reduced expression of steroidogenic enzymes (i.e. *STAR* and *HSD3B1*) involved in cholesterol transfer into mitochondria and conversion of pregnenolone to progesterone. These induced CLs were further characterised as having reduced expression of *LHCGR* (required for luteal support; Niswender *et al.* 2007) and *VEGFA* (a key angiogenic regulator; Robinson *et al.* 2007), together with increased expression of *BMP2* (linked to luteolysis in regressing CLs; Nio-Kobayashi *et al.* 2015).

Regarding follicular development, relative to final-wave DFs (i.e. those from group C at day 19), key functional features of first-wave DFs (i.e. those from group A at day 6, coinciding with GNRH treatment in group B)

included reduced E2 concentrations and a reduced E2:P4 ratio. These differences occurred in the face of increased transcript expression of key steroidogenic enzymes (i.e. *CYP11A1* (encoding cholesterol side-chain cleavage), *HSD3B1* and *CYP19A1* (encoding aromatase)) in granulosa cells and reduced expression of *SCARB1* (which facilitates cellular cholesterol uptake from high-density lipoproteins; Azhar *et al.* 1998) in thecal cells. Also difference between these two DF groups were transcripts for two inhibin/activin subunits (i.e. *INH A* and *INHBA*), which were both increased in first-wave DFs relative to final-wave DFs. Importantly, given that background plasma and follicular fluid P4 levels were similar between groups A and B (Fig. 1B and Table 3B), it is noteworthy that transcript expression for a range of genes in granulosa and thecal cells from both groups A and B were also similar, highlighting the importance of P4 as a regulator of follicular maturation. Finally, it is also worth noting the differences in transcript expression of *IGF2R* and *IGFBP2* in both granulosa and thecal cells, and protein expression of IGFBP2 in follicular fluid, between first- and final-wave DFs (i.e. group A vs group C). These were consistently lower in first-wave relative to final-wave DFs, when circulating levels of IGF1 were also at their lowest (Fig. 3). These differences seem to be of key significance given that these IGF family members each serve to regulate the bioavailability of both IGF-1 and -2 within the ovarian follicle (Webb and Campbell 2007). However, the issue of proximity to PG administration cannot be discounted. Indeed, *PTGFR* expression was lower in group C ovarian cells (i.e. granulosa and luteal) than in group A, with group B in between. This could be due to direct or indirect actions of PG.

Collectively, these results indicate an important role of P4 during terminal follicle maturation that determines subsequent luteal competence, although the effects of endogenous LH, which are well established (e.g. Quintal-Franco *et al.* 1999) but not determined in this study, and differences in the nature and timing of pharmacological intervention (i.e. PG relative to endogenous or administered GNRH) between groups cannot be discounted. Indeed, in sheep, Murdoch and Van Kirk (1998) found that premature induction of ovulation (i.e. 12 h vs 36 h after PG-induced luteolysis) compromised the formation of a functionally competent CL. In this study, follicles that gave rise to less competent CLs were less oestrogenic than those that gave rise to more competent CLs and the data point to underlying contributions by components of both the TGF and IGF systems.

The 'final-wave' dominant follicle

In this study, one could consider the DF that ovulated around day 0 to be representative of a 'final-wave' DF, although it is recognised that this follicle did not occur

in a natural, uncontrolled oestrous cycle, but rather in one in which both follicle and CL developments were regulated and synchronised (Fig. 1). This was necessary for experimental purposes as it standardised follicle development to a more precisely timed ovulation. From the perspective of assisted reproduction, it is also representative of protocols routinely used for oestrous synchronisation. Furthermore, ovulation of the resultant DF was induced by the endogenous surge of LH that followed the second prostaglandin treatment, and thus more closely resembles the natural ovulatory process than that represented by the GNRH-induced ovulation of a day 6 DF. However, this 'final-wave' DF (i.e. destined to ovulate around day 0) probably developed under a low P4 environment (not determined), given that PG administration preceded GNRH treatment during the initial synchrony programme (Fig. 1A). In contrast, the DFs harvested from group C heifers on day 19 (24 h after PG) better represent the normal final-wave, pre-ovulatory follicle.

Corpus luteum

In bovine-assisted reproduction, either follicle ablation or aspiration (to recover ova) close to the anticipated time of ovulation leads to the formation of small CLs, with reduced capacity to produce and secrete P4 (O'Hara *et al.* 2012). This reduction in P4 secretion is, in turn, associated with reduced expression of *LHCGR* in luteal tissue. These authors commented that this may be due in part to removal of a variable number of granulosa cells that would otherwise have contributed to luteal formation; although given the preferential localisation of *LHCGR* to small (i.e. theca derived) luteal cells (Yuan & Lucy 1996, Mamluk *et al.* 1998), it is uncertain whether this alone could account for reduced *LHCGR* expression. The study of Hayashi *et al.* (2006), however, highlighted the importance of appropriate LH priming before GNRH-induced ovulation for the formation of functionally competent CLs. In this study, DFs from group C best represent 'final-wave' (pre-ovulatory) DFs; however, the timing of their collection (i.e. 24-h post PG administration; Fig. 1) probably precluded exposure to surge levels of LH. In contrast, the 'final-wave' DF that gave rise to a day 6 CL (group A) will probably have been 'older' and larger at the point of ovulation, and almost certainly would have been exposed to higher levels of LH; although these parameters were not determined. Therefore, while molecular features of group C relative to group A DFs (discussed later) provide important information on factors regulating subsequent CL function, they probably do not represent the complete picture.

Molecular basis of luteal support and steroidogenesis

The reduced capacity of induced (group B) CLs to produce P4 is consistent with the reduced expression of *STAR*

and *HSD3B1* observed (Table 5). Reduced expression of transcripts for *IGFBP4* and increased expression of transcripts for *IGFBP5* in group C (regressing), relative to group A (developing), CLs is consistent with earlier reports of CL demise following $\text{PGF}_{2\alpha}$ -induced luteolysis in cattle (Neuvians *et al.* 2003) and sheep (Hastie & Haresign 2006). Although *IGFBP4* generally inhibits IGF action, *IGFBP5* is known to have both IGF-dependent and -independent effects but is generally associated with growth arrest and apoptosis (Kelley *et al.* 1996, Monget *et al.* 1998). What is interesting in this study is that transcript expression for these two binding proteins in group B CLs more closely matches that of group C than group A CLs which, when considered with the P4 data in Table 3, lends further support to the functional inadequacy of these induced CLs. Closer inspection of Table 5 data, however, indicates that there are numerous molecular differences between group B and C CLs, not least of which is transcript expression for steroidogenic enzymes and key cytokines, indicating that while these CLs may be developmentally compromised, they nevertheless retained some residual function.

VEGFA is a potent mitogen that promotes the growth, migration and permeability of vascular endothelial cells in CLs throughout the luteal phase (Robinson *et al.* 2007). Levels of this protein within the CL peak at around day 15 of the oestrous cycle but decline on luteolysis as witnessed in this study (Table 5) and by Guzmán *et al.* (2015). This latter study also demonstrated that there are both pro- and anti-angiogenic isoforms of VEGFA in the bovine CL, and that immediately before luteolysis, there is an increase in anti-angiogenic isoforms. With respect to the various isoforms identified by Guzmán *et al.* (2015), we can deduce from the primers designed for this study that we amplified the single isoform 205, which has only been described as pro-angiogenic. Increased expression of this isoform in group A CLs, relative to group B and C CLs, further serves to confirm their viability.

Several alternatively spliced variants of the *LHCGR* gene have also been reported in the bovine ovary, but only a couple of these variants with open reading frames over the entire sequence are capable of producing a fully functional receptor (Robert *et al.* 2003). The variants reported include a complete deletion of exon 10 and/or partial deletion of exon 11, and there is also a loss of exon 3 in bovine granulosa cells (Nogueira *et al.* 2007). In humans, a splice variant lacking exon 10 produces a protein capable of binding hCG, but not LH (Müller *et al.* 2003) and, in keeping with a further human splice variant lacking exon 9, can form complexes with other *LHCGR* isoforms to reduce overall receptor expression and cAMP accumulation (Nakamura *et al.* 2004, Ndiaye *et al.* 2005, Minegishi *et al.* 2007). Primer design in this study (Table 1) allowed us to confirm the expression of *LHCGR* transcripts lacking exons 3 and 9; however, we were unable to detect transcripts lacking exon 10

in any of the somatic (i.e. granulosa, thecal and luteal) cells studied in the ovary. Relative to CLs from group A (formed from 'final-wave' DFs), expression of all *LHCGR* variants was reduced in CLs from group B (derived from first-wave DFs) and was barely detectable in regressing CLs (group C) (Table 5). Based on quantitative measurements of *LHCGR* expression within the bovine CL during a regular oestrous cycle (Yoshioka *et al.* 2013), we surmise that group B CLs in this study were more similar to regular day 2–3 CLs than day 5–7 CLs. This point is consistent with reduced levels of P4 production by group B relative to group A CLs (Table 3A).

Molecular features of DFs that give rise to CLs

In this study, group C DFs were more oestrogenic than group A DFs (Table 3B); however, transcript expression for *ESR2*, three steroidogenic enzymes (*CYP11A1*, *HSD3B1* and *CYP19A1*) and subunits for inhibin-A and activin-A were decreased (Table 4). These features are consistent with a number of previous observations. For example, increasing levels of LH (Byers *et al.* 1997) and ovarian oestrogens (Sharma *et al.* 1999) are each known to down-regulate *ESR2* expression in granulosa cells and, in cattle, levels of inhibin-A and activin-A in follicular fluid are reduced in large (13–20 mm) follicles with high (>5) compared with low (<5) E2:P4 ratios (Glister *et al.* 2006). Peripheral (Armstrong *et al.* 2001) and follicular (Echternkamp *et al.* 1994) concentrations of IGF1 also increase under these oestrogen-dominated conditions (Fig. 3) as animals enter the follicular phase. Expression of uterine *IGFBP2* mRNA and protein increases towards the late luteal phase and is thought to be under the regulation of P4 (McCarthy *et al.* 2012, Costello *et al.* 2014). Elevated expression of *IGFBP2* transcripts in group C DFs (Table 4) and *IGFBP2* protein in follicular phase fluids (Fig. 3) is consistent with these observations but, on first inspection, is somewhat at odds with earlier studies that indicate that *IGFBP2* levels in follicular fluids decrease in large oestrogen-active and pre-ovulatory follicles (Echternkamp *et al.* 1994, Funston *et al.* 1996). However, in contrast to previous work, this study compared DFs of equivalent size but at different stages of the oestrous cycle. Furthermore, it is noteworthy that (i) follicles were harvested in both luteal and early follicular phases, and (ii) E2:P4 ratios were only slightly greater for group C than for group A DFs (Table 3B) and were similar for abattoir-derived early luteal and early follicular phase DFs (1.27 ± 0.96 vs 2.2 ± 1.50 respectively). Western blot analyses also revealed proteolytic fragments of *IGFBP2* in early follicular phase DFs (data not shown), suggesting initial stages of degradation at the onset of this oestrogen-dominated period. Collectively, these data suggest the presence of an active IGF regulatory system in final-wave DFs to tightly control cellular responses to increased circulating IGF1.

Conclusions

The foregoing discussion focused on differences in functional competency and transcript expression of CLs derived following induced and spontaneous ovulations, together with differences in transcript expression of DFs that give rise to these structures. This study confirms that induced ovulation of a first-wave DF results in the formation of a smaller CL with functionally lower P4 production than one formed following spontaneous ovulation. Furthermore, these smaller induced CLs were characterised as having reduced expression of transcripts required for luteal support, angiogenesis and steroidogenesis, together with increased expression of transcripts associated with luteolysis. Importantly, these differences in CL function were not related to size of the ovulated DF but were associated with their steroidogenic activity. Transcript expression differed between first- and final-wave DFs and was associated with peripheral and local levels of P4 and components of the IGF system. These data indicate that these separate follicular systems interact to affect maturation of the ovulatory follicle transiting from di-oestrus to pro-oestrus in a manner that subsequently alters the functional competency of the CL.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0415>.

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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