Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes

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Abstract

This study was designed to identify genes that regulate the transition from FSH- to LH-dependent development in the bovine dominant follicle (DF). Serial analysis of gene expression (SAGE) was used to compare the transcriptome of granulosa cells isolated from the most oestrogenic growing cohort follicle (COH), the newly selected DF and its largest subordinate follicle (SF) which is destined for atresia. Follicle diameter, follicular fluid oestradiol (E) and E:progesterone ratio confirmed follicle identity. Results show that there are 93 transcript species differentially expressed in DF granulosa cells, but only 8 of these encode proteins known to be involved in DF development. Most characterised transcripts upregulated in the DF are from tissue development genes that regulate cell differentiation, proliferation, apoptosis, signalling and tissue remodelling. Semiquantitative real-time PCR analysis confirmed seven genes with upregulated ($P \leq 0.05$) mRNA expression in DF compared with both COH and SF granulosa cells. Thus, the new genes identified by SAGE and real-time PCR, which show enhanced mRNA expression in the DF, may regulate proliferation (cyclin D2; *CCND2*), prevention of apoptosis or DNA damage (growth arrest and DNA damage-inducible, β ; *GADD45B*), RNA synthesis (splicing factor, arginine/serine rich 9; *SFRS9*) and unknown processes associated with enhanced steroidogenesis (ovary-specific acidic protein; DQ004742) in granulosa cells of DF at the onset of LH-dependent development. Further studies are required to show whether the expression of identified genes is dysregulated when abnormalities occur during DF selection or subsequent development.

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Introduction

In females from single-ovulating species, such as cattle and humans, the final stages of antral follicle development begin with (1) stimulation of cohort growth through a transient rise in serum follicle-stimulating hormone (FSH) concentrations, (2) subsequent atresia of an increasing number of cohort follicles during declining FSH and (3) identification ('selection') of a single dominant follicle (DF) which alone continues development despite FSH concentrations reaching nadir levels (Adams *et al.* 1993, Sunderland *et al.* 1994, van Santbrink *et al.* 1995, Baerwald *et al.* 2003). Cohort follicles, therefore, require elevated concentrations of FSH (FSH 'dependent'), while the DF, in contrast, develops relative FSH 'independence' demonstrated by its enhanced growth and oestradiol (E) synthesis in a low FSH environment. However, the DF now requires frequent stimulation by luteinizing hormone (LH) to continue differentiation leading up to ovulation (LH-'dependent' stage of development; Mihm & Bleach 2003). Understanding the cellular mechanisms that permit the transit from the FSH- to the LH-dependent developmental stage in the DF is imperative, as abnormalities in follicle selection and/or subsequent

final differentiation result in anovulation, and are thus main causes of ovarian infertility in cattle and women (Lucy 2001, Evers 2002).

Previous studies have used the bovine DF model to examine changes in the expression of known follicular genes (such as for FSH and LH receptors, steroidogenic enzymes, specific growth factors and their binding proteins) when the DF becomes selected from the cohort (Bao & Garverick 1998, Yuan *et al.* 1998, Canty *et al.* 2006). While this candidate gene approach has merit, it is clear that approaches that utilise global gene and protein expression profiling are required to identify all key factors and pathways which regulate cell differentiation during DF development. Genomic approaches so far used (suppression subtractive hybridisation, custom cDNA microarray) have focussed on the comparison between the highly oestrogenic selected DF at the beginning of the rapid growth phase with subordinate follicles undergoing or initiating atresia (Sisco *et al.* 2003, Evans *et al.* 2004, Fayad *et al.* 2004). Such comparisons have identified genes that regulate E synthesis or apoptosis within the growing cohort (Sisco *et al.* 2003) for example. However, a global transcription profiling approach has not been applied to determine changes that occur in granulosa cells when the most successful cohort follicle becomes the DF.

To address this important step of DF differentiation, our aim was to identify the changes in the granulosa cell transcriptome, which occur as the DF transits from the FSH- to the LH-dependent development stage, enhancing its growth and E synthesis and, at the same time, preventing the onset of atresia. As a consequence, we have used serial analysis of gene expression (SAGE), a comprehensive, unbiased and quantitative gene expression profiling technique to generate and then compare the transcriptome of bovine granulosa cells from the DF before its selection (COH), the DF at the onset of dominance and its largest subordinate follicle fated to undergo atresia (SF).

Results

DF model

Frequent ovarian ultrasound scanning was used in dairy cows to monitor development of antral follicles belonging to the first cohort of the cycle until recovery of the largest, most oestrogenic cohort follicle (COH) before the DF can be identified (4 cows), and of the DF and SF at or just after the time when their divergence in growth was determined (4 cows). All follicles were growing at the time of recovery, which was 1.3 days earlier ($P < 0.05$) for the COH than for the DF and SF (Fig. 1; Table 1). Ultrasound analysis showed < 1 mm difference in diameter between the COH and the other growing cohort members (Fig. 1). Following dissection, the COH were the smallest with the lowest intrafollicular E compared with both the DF and SF ($P < 0.05$, Table 1). All follicles were oestrogenic (oestradiol:progesterone (E:P) ratio in follicular fluid > 1 , Ireland & Roche 1983), but the DF showed a sevenfold increase in the E:P ratio from the COH ($P < 0.05$), and was larger and had higher intrafollicular E leading to a threefold higher E:P ratio than the SF ($P < 0.05$; Table 1). Thus, the DF and SF were recovered at the onset of dominance in the DF (three cows) or 12 h after (one cow) based on morphological and hormonal criteria (Fig. 1; Table 1; Mihm *et al.* 1997, Austin *et al.* 2001).

SAGE analysis of differentially expressed transcripts in the DF granulosa cells

Generation of SAGE libraries involved the sequencing of 14-nucleotide SAGE tags, each of which represents one transcript species. All tags that were differentially expressed ($P \leq 0.05$) in the DF compared with both the COH and SF granulosa cell libraries are shown in Table 2. Only 8 out of the 93 identified tags matched genes previously associated with bovine DF development (descriptions below). The overwhelming majority of genes regulated in the DF have not previously been associated with ovarian function. Functional analysis of the transcripts listed in Table 2 is shown in Fig. 2A–D. The largest proportion of characterised transcripts

Figure 1 Growth of the most oestrogenic cohort (COH, black line), dominant (DF, black line) and the largest subordinate follicles (SF, grey line) in individual cows determined using ultrasound scanning from wave emergence to recovery of ovaries (mean $\pm$ S.E.M. COH, DF and SF diameters measured at the last ultrasound examination before recovery were 6.2 ± 0.5 mm, 8.9 ± 0.4 mm and 7.7 ± 0.2 mm respectively); the second and third (stippled lines) cohort follicles are included in the diagrams depicting COH growth to demonstrate that COH were recovered before a dominant follicle is selected (the square symbol is used once to depict a follicle only detected at the last ultrasound examination).

upregulated in the DF compared with both the COH and SF granulosa cell libraries matched tissue development genes that encompass genes regulating cell differentiation, proliferation, apoptosis, signalling and tissue remodelling (Fig. 2A). In contrast, no such transcripts were detected among those expressed at the highest levels in the SF compared with the DF (intermediate levels) and COH (lowest levels) libraries, while over 80% of transcripts matched genes regulating protein synthesis (Fig. 2D; $P < 0.05$).

(A) Granulosa cell transcripts upregulated in the DF compared with both the COH and SF SAGE libraries (Table 2): SAGE analysis identified 17 characterised transcripts upregulated in the DF, which matched genes that either have not previously been associated with DF development (four tags) or have never been associated with ovarian function (13 tags). The cellular role of 1 (ovary-specific acidic protein; OSAP) out of the 17 genes is currently unknown, while the remaining 16 genes

Table 1 Characteristics of first-wave follicles (means $\pm$ S.E.M.) recovered from four cows before (COH) and four cows after (DF and SF) the onset of dominance.

Follicle parameters	COH	DF	SF
Time of follicle recovery			
Interval from onset of oestrus (h)	63 $\pm$ 0.2 ^a	96.5 $\pm$ 0.6 ^b	
Interval from cohort emergence (days)	1.3 $\pm$ 0.3 ^a	2.6 $\pm$ 0.1 ^b	
Diameter after dissection (mm)*	7.5 $\pm$ 0.6 ^a	11.2 $\pm$ 0.3 ^b	9.9 $\pm$ 0.2 ^c
Follicular fluid hormone concentrations (ng/ml)			
Oestradiol (E)	26.3 $\pm$ 5.0 ^a	319.8 $\pm$ 136.5 ^b	79 $\pm$ 13.3 ^c
Progesterone (P)	12.9 $\pm$ 1.5 ^a	24.4 $\pm$ 4.6 ^b	17.4 $\pm$ 2.6 ^{a,b}
E:P ratio	2.1 $\pm$ 0.5 ^a	15.0 $\pm$ 6.6 ^b	4.9 $\pm$ 1.0 ^c

^{a,b,c}Different superscripts within rows indicate significant differences between follicle groups ($P < 0.05$).

*Measurements were available for only three COH for analysis.

have known functions in intracellular signalling (annexin A2 (*ANXA2*), calmodulin 2 (phosphorylase kinase, δ) (*CALM2*), chloride intracellular channel 1 (*CLIC1*), stimulated by retinoic acid gene 6 homolog (mouse) (*STRA6*)), cell proliferation and apoptosis (cyclin D2 (*CCND2*), growth arrest and DNA damage-inducible, β (*GADD45B*), macrophage migration inhibitory factor (glycosylation-inhibiting factor; *MIF*), replication factor C (activator 1) 4, 37 kDa (*RFC4*)), cell structure (actin, β (*ACTB*), motile sperm domain containing 3 (*MOSPD3*)), protein transport (sorting nexin 9 (*SNX9*)), and RNA (splicing factor, arginine/serine-rich 9 (*SFRS9*), mago-nashi homolog, proliferation-associated (*Drosophila*) (*MAGO*)), protein (DnaJ (Hsp40) homolog, subfamily A, member 1 (*DNAJA1*), solute carrier family 22 (organic cation transporter), member 17 (*SLC22A17*)) and lipid synthesis (stearoyl-CoA desaturase (δ -9-desaturase) (*SCD*)). Five further tags matched uncharacterised genes. From the remaining five unmatched tags, full sequences were determined for two tags using RACE. These two transcripts encoded a novel 3' truncated aromatase variant (deposited under NCBI accession number DQ004742 and recently matched to Bt.4447), and an untranslated mRNA sequence recently matched to the UniGene cluster Bt.33875 (deposited under NCBI accession number AY999166). In addition, SAGE analysis of transcripts upregulated in the DF granulosa cells confirmed four known genes encoding cytochrome P450 aromatase (*CYP19A1*), glutathione S-transferase A2 (*GSTA2*), inhibin α -subunit (*INH*A) and glia-derived nexin (*SERPINE2*), which regulate follicular E synthesis, antioxidant activity and extracellular tissue remodelling in the selected DF (Mihm *et al.* 1997, Sisco *et al.* 2003, Evans *et al.* 2004, Fayad *et al.* 2004).

(B) Granulosa cell transcripts downregulated in the DF compared with both the COH and SF SAGE libraries (Table 2): Out of the 16 tags downregulated in the DF, 14 tags matched characterised genes that encode proteins with cellular functions such as signalling (guanine nucleotide-binding protein (G-protein), β polypeptide 2-like 1 (*GNB2L1*), phospholipase D family, member 3 (*PLD3*)), proliferation (anaphase promoting complex subunit 4 (*ANAPC4*)), structure (actin, γ 1 (*ACTG1*)),

protein synthesis (ribosomal proteins L21 (*RPL21*), L10a (*RPL10A*), L12 (*RPL12*), L3 (*RPL3*)), transport (adaptor-related protein complex 1, μ 2-subunit (*AP1M2*)), secretion (serglycin (*SRGN*)) and metabolism (F-box protein 10 (*FBXO10*)), nitrogen metabolism (glutamate dehydrogenase 1 (*GLUD1*)) and lipid synthesis, transport and metabolism (prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy) (*PSAP*), prostaglandin E synthase (*PTGES*), scavenger receptor class B, member 2 (*SCARB2*)). Only two of the identified genes, *PTGES* and *PSAP*, have previously been associated with ovarian function, specifically luteinisation of granulosa cells (Filion *et al.* 2001, McRae *et al.* 2005).

(C) Granulosa cell transcripts expressed at the intermediate levels in the DF SAGE library (Table 2): nine out of the twelve characterised transcripts downregulated in the DF, but lowest in the SF compared with the COH granulosa cell library, encoded proteins not previously associated with ovarian activity. These proteins have diverse cellular functions in signalling (calreticulin (*CALR*)), metabolism (cytochrome c oxidase subunit VIIb (*COX7B*), glutathione peroxidase 1 (*GPX1*)), DNA binding (high-mobility group nucleosomal binding domain 2 (*HMGN2*), poly(rC) binding protein 1 (*PCBP1*)), RNA synthesis (non-POU domain containing, octamer binding (*NONO*)), protein synthesis (Tu translation elongation factor, mitochondrial (*TUFM*)) and metabolism (heat shock 70 kDa protein 8 (*HSPA8*), heat shock protein 90 kDa β (Grp94), member 1 (*HSP90B1*)) (Table 2). The remaining three transcripts encoded genes (inhibin, β A (*INHBA*), hydroxy- δ -5-steroid dehydrogenase, β - and steroid δ -isomerase 1 (*HSD3B1*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ϵ polypeptide (*YWHA ϵ*)) previously found to show higher mRNA expression in the newly selected DF (Bao & Garverick 1998, Sisco *et al.* 2003, Fayad *et al.* 2004; Table 2).

Nineteen out of the twenty characterised transcripts upregulated in the DF, but highest in the SF compared with the COH granulosa cell library, matched genes that encode ribosomal (17 genes) or other proteins (eukaryotic translation elongation factor 1 α 1 (*EEF1A1*), Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)

Table 2 SAGE tags differentially regulated ($P \leq 0.05$) in granulosa cells of dominant follicles (DF) compared with the most oestrogenic cohort follicle (COH) and the largest subordinate follicle (SF).

Tag sequence	COH tags*	DF tags*	SF tags*	Gene symbol	UniGene number†
(A) SAGE tags upregulated in the DF compared with both the COH and the SF					
AGGCCCCCTAC	8890 ^a	35 452 ^b	28 862 ^c	<i>INHA</i>	4897
GACAAGACTA	3383 ^a	17 025 ^b	13 431 ^c	<i>GSTA2</i>	227
TATCCACTAA	7651 ^a	13 366 ^b	8543 ^a	<i>SERPINE2</i>	12 506
CAGTCAATAT	7395 ^a	9517 ^b	5696 ^c	AY999166 now matched to: transcribed locus	33 875
GCTCAACCAG	2045 ^a	2692 ^b	1818 ^a	<i>STRA6</i>	3196
TTGATGTTTG	688 ^a	2408 ^b	747 ^a	<i>CYP19A1</i>	4447
TGTCTACATA	531 ^a	1782 ^b	1272 ^c	Transcribed locus	53 423
AAGAAAATAG	1003*	1555 ^b	606 ^c	<i>SNX9</i>	5507
TTAGAAATGTT	472 ^a	1403 ^b	949 ^c	<i>MAGOH</i>	53 423
AACGCGGCCA	256 ^a	1687 ^b	626 ^c	<i>MIF</i>	15 528
GACCCCTTTT	551 ^a	1138 ^b	606 ^a	<i>ACTB</i>	14 186
TGATTTCACT	197 ^a	1005 ^b	586 ^c	Unmatched	
GTGTTCTCC	118 ^a	720 ^b	384 ^c	<i>MOSPD3</i>	3028
AACTCCAGC	295 ^a	645 ^b	222 ^a	<i>GADD45B</i>	4371
TCTGAATAGT	295 ^a	569 ^b	222 ^a	<i>OSAP</i>	25 070
TTGTTGTTGA	275 ^a	626 ^b	162 ^a	<i>CALM2</i>	12 896
TACCCGCCGT	0 ^a	645 ^b	343 ^c	<i>CCND2</i>	4895
AGGCCCCCTA	39 ^a	512 ^b	61 ^a	Unmatched	
CTCACAGGAT	118 ^a	322 ^b	101 ^a	Transcribed locus	15 298
CTACCCAACG	59 ^a	322 ^b	61 ^a	<i>CLIC1</i>	49 164
AGGGTGAAAC	39 ^a	284 ^b	81 ^a	<i>SFRS9</i>	30 434
GAAAGATACA	39 ^a	246 ^b	0 ^a	DQ004742 now matched to <i>CYP19A1</i>	4447
TGACAGGGTG	39 ^a	190 ^b	20 ^a	Similar to C17orf49	7060
CCAATTTATC	20 ^a	190 ^b	20 ^a	<i>DNAJA1</i>	3274
TAGAATCCAC	20 ^a	209 ^b	0 ^a	Transcribed locus	1411
ATGTGGTGGT	0*	171 ^b	0 ^a	<i>RFC4</i>	13 925
ATGACCCATT	0 ^a	152 ^b	0 ^a	<i>SCD</i>	4798
GCGGCCCGGT	0 ^a	133 ^b	0 ^a	Unmatched	
CTTCTGCCGG	0 ^a	114 ^b	0 ^a	<i>SLC22A17</i>	48 982
TAAATTTCCC	0 ^a	114 ^b	0 ^a	Transcribed locus	36 212
TTGAAAATTA	0 ^a	114 ^b	0 ^a	<i>ANXA2</i>	4314
(B) SAGE tags downregulated in the DF compared with both the COH and the SF					
TTGCATTACC	15 990 ^a	11 451 ^b	13 068 ^c	<i>SRGN</i>	10 209
GCCTGATGGG	5350 ^a	3792 ^b	4706 ^a	<i>RPL21</i>	5211
GCAGAGCTTT	3678 ^a	2199 ^b	3050 ^a	<i>GNB2L1</i>	551
TGAGAACATT	4209 ^a	2029 ^b	2666 ^c	<i>ACTG1</i>	38 667
GGCAAGCCCC	2144 ^a	1479 ^b	2605 ^a	<i>RPL10A</i>	23 381
ACATCATTGA	2203 ^a	1536 ^b	2101 ^a	<i>RPL12</i>	15 530
GGACCACTTA	1495 ^a	891 ^b	1333 ^a	<i>RPL3</i>	3616
TGTATTTCTA	1357 ^a	720 ^b	1151 ^a	<i>SCARB2</i>	11 224
TCCCTATTGC	570 ^a	265 ^b	909 ^a	Unmatched	
CGGTTGCTAT	511 ^a	228 ^b	889 ^c	<i>PSAP</i>	5467
CAACTAGTGA	590 ^a	133 ^b	364 ^a	<i>AP1M2</i>	23 342
CTGGGCCTAG	216 ^a	57 ^b	303 ^a	<i>PLD3</i>	16 204
ATGTCAAAC	275 ^a	38 ^b	202 ^a	cDNA clone IMAGE 8042713	30 784
ACAGTGAGGA	197 ^a	38 ^b	222 ^a	<i>PTGES</i>	49 581
TCAGGAAGGA	118 ^a	0 ^b	121 ^a	<i>ANAPC4</i>	28 388
ACTTAACCTG	118 ^a	0 ^b	121 ^a	<i>GLUD1</i>	55 415
(C) SAGE tags downregulated in the DF compared with the COH, further downregulated in the SF					
TAAATGTGCA	10 483 ^a	8228 ^b	5352 ^c	Transcribed locus	20 295
TTTAAGGTTT	11 604 ^a	4550 ^b	1232 ^c	<i>INHBA</i>	12 760
AGACAAGAGT	7356 ^a	4228 ^b	3191 ^c	Transcribed locus	38 177
AATAAAGTGC	3816 ^a	2995 ^b	2161 ^c	<i>GPX1</i>	4317
AAAAAAAAAA	5153 ^a	2370 ^b	141 ^c	Multiple clusters*	
CTGAGAATGT	2439 ^a	1801 ^b	1131 ^c	<i>CALR</i>	30 105
GAAAAACATT	2203 ^a	1138 ^b	566 ^c	<i>HSPA8</i>	12 309
CAAAAAAAAAA	1868*	1156 ^b	202 ^c	Multiple clusters	
ATTGTTTATG	1888 ^a	815 ^b	343 ^c	<i>HMG2</i>	1758
GAATATTTGT	1554 ^a	815 ^b	404 ^c	<i>TUFM</i>	5344
TAAAAAAAAA	1593 ^a	588 ^b	40 ^c	Multiple clusters	
GAAAAAAAAA	1180 ^a	777 ^b	182 ^c	Multiple clusters	

Table 2 Continued.

Tag sequence	COH tags*	DF tags*	SF tags*	Gene symbol	UniGene number [†]
GATTAACATT	1180 ^a	645 ^b	242 ^c	YWHAE	4035
TGTATAAAAA	1141 ^a	588 ^b	81 ^c	HSP90B1	8686
AAAACATATC	944 ^a	455 ^b	61 ^c	Unmatched	
TTTGAAGAAA	728 ^a	379 ^b	81 ^c	COX7B	48 953
AACTTATTAA	629 ^a	228 ^b	61 ^c	NONO	37 860
AATTTTATTT	452 ^a	209 ^b	20 ^c	PCBP1	4620
ACTAAAAAAA	334 ^a	133 ^b	0 ^c	HSD3B1	64 737
(D) SAGE tags upregulated in the DF compared with the COH, further upregulated in the SF					
GCCGGCCCCG	2990 ^a	4152 ^b	5999 ^c	RPS15	4832
AGACAGACAG	1357 ^a	4417 ^b	5938 ^c	EEF1A1	3573
TCCGTGCACC	2399 ^a	3413 ^b	4807 ^c	RPS11	3626
AAGCCCAGCG	2557 ^a	3242 ^b	4302 ^c	RPS16	2686
CTGCCCAGCG	1180 ^a	3394 ^b	5009 ^c	RPL32	21 534
CCCAGCCACT	1298 ^a	3469 ^b	4282 ^c	RPS19	7648
TTGGCTGCCC	531 ^a	2256 ^b	3555 ^c	RPS14	49 145
GCTGCCATCC	983 ^a	1953 ^b	2888 ^c	RPL28	48 857
GTTCCTGGC	551 ^a	1972 ^b	2646 ^c	FAU	4618
CCCACAAGGT	1141 ^a	1668 ^b	2323 ^c	RPL27	4721
GCAGAGTTCG	393 ^a	1630 ^b	2848 ^c	RPS6	7908
GATAAGGCAG	747 ^a	1725 ^b	2282 ^c	RPL4	48 893
CTGCAATACG	846 ^a	1270 ^b	2525 ^c	RPL5	7052
GTGTTGCACA	669 ^a	1384 ^b	2201 ^c	RPS13	2623
TCGGTCTGGG	59 ^a	1611 ^b	2545 ^c	RPS2	48 814
GGCCGCGTTC	354 ^a	1422 ^b	2080 ^c	RPS17	26 469
GGAGGCGCTA	216 ^a	1062 ^b	2141 ^c	RPL24	49 154
ACCGTGCGCA	118 ^a	1138 ^b	2040 ^c	RPS5	1874
CAGTCTCTCA	315 ^a	1232 ^b	1737 ^c	RPS10	49 036
AGCCGCACCA	79 ^a	1175 ^b	1899 ^c	RPL18	4842
TTCAGCTCCA	79 ^a	1138 ^b	1737 ^c	RPS7	7187
CCGGTCGCCC	98 ^a	891 ^b	1313 ^c	RPS9	52 718
CCCATCCGAA	216 ^a	739 ^b	1232 ^c	RPL26	49 129
TCTATATTGC	118 ^a	474 ^b	1151 ^c	Unmatched	
ATCCGGGCGCC	79 ^a	265 ^b	606 ^c	TCEB2	49 100
TCCTATTGCC	39 ^a	228 ^b	545 ^c	Unmatched	
GACTATAGCC	20 ^a	171 ^b	485 ^c	Multiple clusters	
CCTGGGCTCC	0 ^a	133 ^b	364 ^c	Transcribed locus	66 365

a, b, c Different superscripts indicate significant differences ($P \leq 0.05$) between SAGE libraries.

*The number of tags in each library was normalised to a total of 10^6 tags. [†]A minority of tags (18/93) matched with more than one UniGene cluster; in this case the cluster with the complete mRNA sequence and/or the majority of sequences containing the tag is presented. [‡]Five tags could not be matched reliably to one UniGene cluster, because several clusters contained a complete mRNA sequence or similar number of sequences containing the tag sequence.

ubiquitously expressed (FAU)) regulating protein biosynthesis, while the remaining transcript matched the gene transcription elongation factor B (SIII), polypeptide 2 (18 kDa, elongin B; TCEB2), which regulates protein metabolism (Table 2).

Real-time PCR analysis of genes with higher transcript levels in the DF compared with both the COH and SF granulosa cell SAGE libraries

The genes analysed using real-time PCR contained most of the characterised transcripts upregulated in the DF based on the SAGE analysis (Fig. 3). The results confirmed that seven genes (*CYP19A1*, *INHA*, *CCND2*, *GADD45B*, *SFRS9*, *OSAP* and *DQ004742*) show upregulated ($P \leq 0.05$) mRNA expression in the DF compared with both the COH (for *OSAP* $P = 0.06$) and SF granulosa cells. An additional seven transcripts, encoded by *MIF*, *RFC4*, *ANXA2*, *CALM2*, *CLIC1*,

GSTA2 and *SLC22A17*, were upregulated ($P \leq 0.05$) in the DF compared with the SF, and two transcripts (*STRA6*, *AY999166*) tended to be upregulated ($0.05 < P \leq 0.07$) in the DF compared with the COH granulosa cells. Although statistical significance was not always reached, average transcript levels determined using real-time PCR were higher in the DF compared with both the COH and SF granulosa cells for 16 out of the 18 tested genes. mRNA expression levels for *LHCGR*, included as a positive control, were also higher ($P \leq 0.05$) in the DF compared with both the COH and SF granulosa cells.

Discussion

In this study, we have identified changes in the granulosa cell transcriptome as the DF differentiates from the FSH-dependent cohort and begins its LH-dependent final development. This approach differs from that of the

Figure 2 Ontology analysis of genes encoded by transcripts found to be upregulated (A), downregulated (B) or expressed at the intermediate levels (C and D) in the granulosa cell SAGE library of newly selected dominant follicles (DF) compared with the libraries of the most oestrogenic cohort (COH) and the largest subordinate follicle (SF).

previous studies, which used the bovine DF model to identify genes important for DF survival versus subordinate atresia at the onset of dominance (Sisco *et al.* 2003, Evans *et al.* 2004, Fayad *et al.* 2004). This study has identified more than 80 new genes regulated in the DF, and this knowledge may be very relevant for the study of abnormalities occurring during DF development (such as premature atresia or cystic degeneration) in *post partum* cows, but also in other monovulatory species such as humans. It is also likely that a number of identified genes are involved in the regulation of large follicle development in multiovulatory species. Thus, understanding the roles and regulation of these new genes may become relevant to the development of new approaches to diagnose and treat infertility not only in single-ovulating but also in multi-ovulatory species with agricultural importance such as the pig.

The COH recovered for this study most likely represent the FSH-‘dependent’ development stage of the future DF. This conclusion is based on the previously described timing of growth of the first follicle wave of the oestrous cycle, intrafollicular hormone concentrations and the

fact that, while morphologically unselected and still FSH ‘dependent’, the most oestrogenic cohort follicle 1–2 days after wave emergence will most likely become the DF (Mihm *et al.* 1997, 2000, Austin *et al.* 2001). Growth characteristics and differences in diameter, steroid concentrations and oestrogenic activity established in this study showed the higher differentiation state of the DF recovered at or shortly after the onset of dominance in accordance with previous characterisations of selected DFs (Ginther *et al.* 2003, Mihm & Bleach 2003). As serum concentrations of FSH decline to nadir concentrations at this time, the DF is known to switch from a relative FSH to LH dependence of growth and E production (Mihm & Bleach 2003). This functional switch is clearly paralleled by the acquisition of LH receptors and increased LH binding in the DF granulosa cells (ligand-binding study: Ireland & Roche 1983; *in situ* hybridisation studies: Evans & Fortune 1997, Bao & Garverick 1998; real-time PCR studies: Evans *et al.* 2004; this study). However, it appears that transcript levels for *LHCGR* are relatively low in granulosa cells of the newly selected DF, as mRNA expression was not detected using the SAGE analysis in this study and in another study using *in situ* hybridisation (Evans & Fortune 1997). SAGE tags matched to *LHCGR* were only infrequently found in murine granulosa cells of preovulatory follicles where maximum levels would be expected (McRae *et al.* 2005). Therefore, the SAGE technique may not be sensitive enough to detect very low levels of *LHCGR* transcripts, in contrast to real-time PCR.

This study showed that the majority of characterised transcripts upregulated in the DF granulosa cells are encoded by genes that regulate E synthesis, cell proliferation and survival, signalling, organ development or extracellular tissue remodelling. All these functions are important for tissue development which could directly support the enhanced growth and steroidogenesis characteristic of the DF. The combination of SAGE and real-time PCR clearly identified four genes not previously associated with ovarian function or DF development (*CCND2*, *GADD45B*, *OSAP* and *SFRS9*) and one novel gene (aromatase variant *DQ004742*), which show up-regulated mRNA expression in the DF compared with both the COH and SF granulosa cells. Cyclin D2 regulates the G1 to S transition within the cell cycle promoting proliferation, and its expression is regulated by FSH, E and IGF (Robker & Richards 1998, Kadakia *et al.* 2001). It may not be surprising that the DF shows the highest *CCND2* transcript levels, since it is exposed to the highest free IGF concentrations and the highest intrafollicular E and oestrogen receptor β expression (Mihm *et al.* 2000, Beg *et al.* 2002, Evans *et al.* 2004). Similarly, the gene product GADD45 may act as a potential survival factor in the growing DF, as it is involved in DNA damage repair and control of genomic stability, and has also anti-apoptotic properties (Sheikh *et al.* 2000, De Smaele *et al.* 2001). The splicing factor arginine/serine-rich 9 encoded by *SFRS9*

Figure 3 Semiquantitative real-time PCR analysis of granulosa cell mRNA expression in the most oestrogenic cohort (COH, dark grey bars), dominant (DF, light grey bars) and the largest subordinate follicle (SF, black bars) for genes regulating tissue development (A–C), cellular metabolism (D), RNA and protein synthesis (E) or unknown biological processes (F); three to four individual COH, DF or SF were analysed. *mRNA expression differed ($P \leq 0.05$) or (#)tended to differ ($0.05 < P \leq 0.07$) from DF. #Only a pooled cohort sample was available for analysis.

regulates constitutive splicing of RNA and modulation of the selection of alternative splice sites (Screaton *et al.* 1995). Interestingly, bovine granulosa cells from large follicles express differentially spliced gonadotrophin receptor mRNA variants (*LHCGR*: Kawate & Okuda 1998; *FSHR*: Rajapaksha *et al.* 1996), and such differential expression of splice variants may modify receptor response in growing follicles (Babu *et al.* 2000). In fact, this study has identified a new 3' truncated aromatase mRNA splice variant (DQ004742) expressed in bovine granulosa cells. However, this transcript variant, with orthologues in the sheep, pig and human EST libraries, consists only of exons 1–5 of the aromatase gene, and thus may have no further function following translation (Levallet *et al.* 1998). A new gene with so far unknown functions, *OSAP*, identified in mouse preovulatory follicles, may be a marker of differentiated follicle development, specifically of increased E synthesis, with gene expression responding to gonadotrophic stimulation (Hennebold *et al.* 2000,

Tanaka *et al.* 2003). We propose, therefore, that the new genes identified by the SAGE and real-time PCR analyses, which show enhanced expression in the DF, may regulate proliferation, prevention of apoptosis or DNA damage, RNA synthesis and unknown processes associated with enhanced steroidogenesis in granulosa cells of the DF at the onset of its LH-dependent development. It needs to be determined in the future whether such genes are regulated by LH or, indeed, by E, as mRNA expression is upregulated in the DF at the same time as increased LH receptor transcript levels and enhanced E synthesis are detected.

Based on the results from the SAGE and real-time PCR analyses, an additional four new tissue development genes (*ANXA2*, *CALM2*, *CLIC1* and *MIF*) and one known gene, *GSTA2*, are very good candidates for further experiments to determine their positive roles in DF granulosa cell proliferation, survival and steroidogenesis. High levels of annexin A2, a member of the family of calcium-binding proteins with multiple roles in

membrane function, phospholipid transport and cell signalling (Camors *et al.* 2005) have been detected in cells during the G1 to S transition phase of mitosis (Chiang *et al.* 1993). Calmodulin can affect gonadotrophin-regulated granulosa cell steroidogenesis through its calcium-binding properties in the laboratory rodents (Carnegie & Tsang 1984, Stocco *et al.* 2002), and macrophage migration inhibitory factor has antioxidant and anti-apoptotic activities (Mitchell & Bucala 2000, Nguyen *et al.* 2003) and may regulate murine follicular development and ovulation (Matsuura *et al.* 2002). In contrast to the previous three genes, expression of the chloride intracellular channel protein 1, which localises to internal organelles and is involved in ion transport (Landry *et al.* 1993), has never been described in ovarian cells. For these genes, both SAGE and real-time PCR analyses showed significantly higher mRNA expression in the DF compared with the SF granulosa cells, and absolutely higher mRNA expression levels in the DF compared with the COH granulosa cells. Overall, the quantitative nature of the data derived from the SAGE libraries appears to be reliable. This is seen most clearly in the accord between results from SAGE and real-time PCR with data from 16 out of the 18 tested genes showing absolutely higher mRNA expression levels in the DF. Also, findings from this study confirmed data for five genes (*CYP19A1*, *GSTA2*, *HSD3B1*, *INHBA* and *SERPINE2*) previously generated using very different genomic and molecular techniques, such as suppressive subtraction hybridisation, cDNA microarray or *in situ* hybridisation (Bao & Garverick 1998, Sisco *et al.* 2003, Evans *et al.* 2004, Fayad *et al.* 2004). Complete confirmation of SAGE results by real-time PCR may not be expected, since the SAGE technique does not account for biological variation due to pooling of RNA samples. Thus, a markedly greater sample size may be required when using real-time PCR to demonstrate significant differences in gene expression for the group of genes, in which mean profiles show absolute, but not statistical, increases in the DF. In addition, the two molecular techniques may target different transcript regions, 3' transcript variants may exist and unequal amplification efficiencies may prevent the detection of low transcript numbers, preventing complete concordance of results.

The inclusion of the FSH-dependent COH in this study of DF development extends previously published data for three genes, *HSD3B1*, *INHBA* and *YWHA*E (Bao & Garverick 1998, Sisco *et al.* 2003, Fayad *et al.* 2004). While the higher transcript levels in the DF versus SF granulosa cells were confirmed for all three genes, SAGE analysis showed the highest expression in the COH and declining expression in the DF granulosa cell SAGE library. This occurs in parallel with declining FSH serum concentrations (Mihm & Bleach 2003). The granulosa cell mRNA expression for *INHBA* is FSH responsive in the laboratory rodent (Aloi *et al.* 1995), but whether expression of all

three genes is directly regulated by FSH during bovine cohort growth needs to be determined in the future.

Finally, a large number of transcripts encoding genes that regulate protein synthesis are abundantly expressed and regulated in the DF granulosa cells at the onset of dominance based on the SAGE analysis. Our findings of high expression (the top 10 abundant transcripts encoding ribosomal proteins represent between 3.3 and 4.5% of the granulosa cell transcriptome) concur with other SAGE studies (Angelastro *et al.* 2002) and other studies profiling gene expression in ovarian tissue (Stanton *et al.* 2002). However, almost all transcripts upregulated in the DF compared with the COH granulosa cells, but expressed at the highest levels in the SF granulosa cells, encode ribosomal proteins (Table 2). The finding of such differential expression in granulosa cells from individual follicles of different developmental stages (FSH or LH dependence) and fates (dominance or atresia) is a completely new result, and may indicate that the regulation of cellular protein synthesis is an important feature of antral follicle growth, signalling the onset of a new intracellular program (Ludwig & Tenhaken 2001). Ribosomal proteins may be involved in differentiation and apoptosis, two cellular programmes clearly important for the DF and SF respectively. For example, the DF shows reduced *RPS15* transcript levels similar to differentiating rat neuronal cells (Angelastro *et al.* 2002). Conversely, several members of the large and small ribosomal subunit protein families have been shown to promote apoptosis onset in the mammalian cell model systems (Khanna *et al.* 2000, Coutts & La Thangue 2006), and upregulated mRNA expression in the SF may, therefore, be part of the mechanism for the initiation of granulosa cell apoptosis.

In conclusion, the combination of genomic profiling with SAGE followed by real-time PCR identified numerous new genes that are candidates for specific cellular and molecular hypotheses with regard to their direct roles in DF differentiation. Thus, enhanced expression of the proliferative gene *CCND2* and the anti-apoptotic gene *GADD45B* in granulosa cells may support further growth of the DF, while enhanced expression of *CYP19A1*, *DQ004742*, *INHA* or *OSAP* may support the increased E synthesising capacity characteristic of DF. In addition, this study has highlighted nuclear events such as RNA splicing which are regulated in the DF at the onset of dominance. We propose that the causative roles of any of these candidate genes in DF differentiation will have to be established in *in vivo* models of DF growth, using intrafollicular gene silencing or enhancing techniques yet to be validated by the research groups working with this model. Elucidating the key cellular pathways for physiological DF differentiation will allow hypothesis-driven investigations of aberrations of DF development, and thus the bovine model may also become invaluable for the testing of candidate genes and proteins influencing DF selection and development in the woman.

Materials and Methods

Animal model and follicle recovery

Eight multiparous (2–11 lactations), non-lactating Holstein–Friesian dairy cows in the luteal phase of the oestrous cycle were treated with a short-term (3–6 days) intravaginal progesterone device (Eazi-Breed CIDR; Animal Reproductive Technologies Ltd, Leominster, UK) and injected with the prostaglandin F_{2α} analogue luproliol (PGF; Prosolvin; Intervet Milton Keynes, UK) before device withdrawal to induce precisely timed luteolysis, oestrus and ovulation. From the time of PGF treatment, ovarian follicular development was monitored daily by transrectal ultrasound scanning using a 7.5 MHz transducer to determine the time of ovulation. ‘Emergence’ (sudden appearance) and subsequent growth of follicles from 3 mm in diameter belonging to the first cohort of the next oestrous cycle was monitored using ultrasound every 12 h until recovery of (1) cohort follicles before the DF can be identified (four cows) and (2) the DF and SF at or just after the time when their divergence in growth was determined (four cows). This time point, which defines the onset of dominance, was reached when the DF had a minimum size of 8 mm at the last ultrasound examination, was larger and grew faster over the last 24-h period than the next largest follicle (Ginther *et al.* 1997, Mihm & Bleach 2003). The DF selection process and the subsequent 3- to 4-day growth phase (Mihm *et al.* 2006) are similar for non-ovulatory and ovulatory DF (Roche *et al.* 1998).

Of the recovered cohort follicles, the follicle with the highest absolute concentrations of E in the follicular fluid, which was also the largest in three out of four cows, was subsequently selected for gene expression profiling. This follicle became the COH representing the FSH-‘dependent’ stage of the future DF, because the most oestrogenic follicle 1–2 days before the onset of dominance will become the DF (Mihm *et al.* 2000). Diameter changes of the COH, DF and SF until recovery determined using ultrasonography are shown in Fig. 1. Ovaries were removed from cows within 2.5 h of the last ultrasound examination following slaughter in the post-mortem facility of the Faculty of Veterinary Medicine, University of Glasgow. Transrectal ultrasound scanning was carried out under the license awarded by the Home Office, UK, according to the ‘Animals and Procedures Act 1986’.

Follicle dissection and recovery of follicular fluid and granulosa cells

Following slaughter, ovaries were removed and placed in 0.05 M ice-cold PBS for the short transport to the laboratory. DFs and SF were identified by matching ultrasound records with ovarian diagrams, follicle surface measurements and calliper measurements after dissecting follicles free from stroma. In cows where ovaries were recovered before the DF could be identified morphologically, five to eight follicles belonging to the new cohort were dissected. The follicular fluid was aspirated gently from all the dissected follicles using a 20 or 25 gauge needle attached to a 1 ml syringe to minimise aspiration of granulosa cells. The follicular fluid was then stored on ice until dissections were completed, and frozen at –20 °C until assayed for E and progesterone concentrations.

Steroid concentrations were subsequently used to identify COH (see above) and confirm DF and SF identities.

Granulosa cells were collected by extensive flushing of the cavity of excised follicles with PBS, centrifuged for 5 min at 700 g and washed twice with PBS. The granulosa cell pellet was re-suspended in 400 µl TRIzol Reagent (Invitrogen Ltd) for the extraction of total RNA. Total time from ovary recovery to granulosa cell RNA extraction was 30–45 min, similar to other successful studies of granulosa cell gene expression in bovine follicles (Evans *et al.* 2004, Mihm *et al.* 2006).

Follicular fluid steroid analysis

E concentrations in the unextracted follicular fluid samples were determined using a validated RIA (Mihm *et al.* 1997). Sensitivity of the assay was 0.04 pg/tube, and the mean intra-assay ($n=4-6$) and inter-assay ($n=2$) coefficients of variation (CV) for a 45 pg/tube quality control sample were 9.1 and 12.2% respectively. P concentrations in the unextracted follicular fluid samples were determined using a validated RIA (Ireland & Ireland 1994, Mihm *et al.* 2006). Sensitivity of the assay was 0.01 ng/tube, and the mean intra-assay CV ($n=5$) for a 1.4 ng/tube quality control sample was 10.5%.

Construction of SAGE libraries

Total RNA was extracted from granulosa cells using TRIzol reagent, according to the manufacturer’s instructions. Poly (A)⁺ RNA was isolated from equal amounts of RNA pooled from four individual follicles at the COH, SF and DF stages using oligo dT cellulose (Invitrogen) or the Oligotex mRNA Mini Kit (Qiagen Ltd). The SAGE libraries were constructed from poly (A)⁺ RNA as described previously (Velculescu *et al.* 1995, O’Shaughnessy *et al.* 2003, McRae *et al.* 2005). The ditags were amplified in two different stages of 23 cycles and 10 or 11 cycles using nested biotinylated primers. Concatemers were cloned into the SphI site of the pZER0-1 vector (Invitrogen) and transfected into DH10B cells (Invitrogen). Selected clones were amplified using PCR and sequenced directly as described previously (Shires *et al.* 2001, O’Shaughnessy *et al.* 2003) using the BigDye terminator v1.1 and the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Warrington, UK). Fourteen-nucleotide SAGE tags were extracted from sequence data, and the frequency of tags was analysed using SAGE 2000 software (<http://www.sagenet.org/protocol/index.htm>). A total of 50 844, 52 747 and 49 512 14-nucleotide SAGE tags were sequenced in the COH, DF and SF libraries respectively. The original SAGE data from the COH, DF and SF libraries can be accessed at <http://www.ncbi.nlm.nih.gov/SAGE/> in the Gene Expression Omnibus repository under GSM48351, GSM48352 and GSM48353.

Generation of full sequence from SAGE tags

For two unmatched SAGE tags (GAAAGATACA and CAGTCAATAT), the full sequence was generated 3’ and 5’ from the tag sequence using pooled bovine granulosa cell RNA and the rapid amplification of complementary ends (RACE) technique (SMART RACE cDNA amplification kit; BD Biosciences, Cowley, UK; Chen *et al.* 2000, O’Shaughnessy *et al.* 2003).

For initial 3' RACE reactions, the SAGE tag plus six degenerate nucleotides at the 5' end acted as the gene-specific primer for PCR. The sequences obtained were subsequently analysed using real-time PCR (see below).

Semiquantitative real-time PCR (real-time PCR)

Relative abundance of 19 selected transcripts was measured in RNA extracted from granulosa cells of individual COH, DF and

SF by real-time PCR. Transcripts were chosen on the basis of SAGE analysis, except for *LHCGR* (encoding the LH receptor) which is a marker gene and protein for DF differentiation (Ireland & Roche 1983, Bao *et al.* 1997, Evans *et al.* 2004) and was chosen to physiologically validate real-time PCR results. The samples of granulosa cell RNA used for real-time PCR were taken from the same RNA used for the construction of the SAGE libraries, following removal of residual genomic DNA by DNase treatment (DNA-free DNase Treatment and Removal

Table 3 Sequences of primers and probes used for quantitative real-time PCR.

Gene	Accession no.	Primer/probe*	Sequences 5' to 3'
Housekeeping gene <i>ACTB</i>	AY141970	F R P	GTCATCACCATCGGCAATGA CGTGAATGCCGAGGATT CGCTGCCCTGAGGCTCTCTTCCA
Cell differentiation and tissue remodeling <i>CYP19A1</i>	NM174305	F R P	CATAGATTTGCCACTGAGTTGAT GCATTCCAATATGCACTGGTTT TTGCTGAGAAACGTGGTGAACCTACAAGAGA- GAA
<i>INHA</i>	NM174094	F R P	CTCGGATGGAGGTTACTCTTTAAGT GATTCCCTTAGATGCAAGCACAGT TGAGATGGTGCCCAACCTTCTCACC
<i>LHCGR</i>	U20504	F R	TGACCATGGCCCGTCTAAAA TACTACCCAAAGCAATTTATAGATTCAATG
<i>SERPINE2</i>	NM174669	F R P	TTCTCCGCATGGGATTGC GAGACGATGGCCTTGTGATCT CGCGTGAAACGGAGTCGGTAAGA
Cell proliferation and apoptosis <i>CCND2</i>	CB455384	F R	TGCCCCAGTGCTCCTACTTC CGGGTACATGGCAAACCTGA
<i>GADD45B</i>	AV616939	F R	TACGAGTCGGCCAGCTGAT GTCCTCCTCTTCTCGTCGAT
<i>MIF</i>	CO890360	F R	GCATTAGCCCGGACAGGAT GGCCCTCAGGCCGAAGGT
<i>RFC4</i>	CK972781	F R	GGCTCTTTTCGACAACTAGAAGCT CATGGAGTTGATTACAAGTTGAGTTG
Intracellular signalling <i>ANXA2</i>	NM174716	F R	AGCTCAGTTTGGAGGGTGATCA CCGCTCAGCATCAAAGTTAGTGT
<i>CALM2</i>	CK979290	F R	AGCAGAGCTTCGCCATGTG TCTGCTTCCTGATCATTTCATC
<i>CLIC1</i>	BT021003	F R	CACCTTCAATGTCAACCTGTTG TCAGTGCCATACAGCAGGAAAG
<i>STRA6</i>	CR456296	F R	GCCTGCTGCCGCTTAGAG TGACTGGCTGGCCTCCAT
Cell metabolism <i>GSTA2</i>	U49179	F R	GTGCCACCTGCTGAAAAA TTTTCAAATGCAGGGAGATAACG
RNA and protein synthesis <i>SFRS9</i>	CR456210	F R	GTGCCCTTCGCTTCGT GGCCATAATCATAACCGTTCCTT
<i>DNAJA1</i>	CB441342	F R	ATGGAGATATCAAGTGTGTGCTAAATG CTCAGGAAAGTTTACCTTAAATTCAATG
<i>SLC22A17</i>	BI534865	F R	CGATTTCTTCAGCGAATGATCAC TCCTCAATCTGTCGCTCACTATC
Unknown biological process New aromatase sequence matched to UniGene Bt.4447	DQ004742	F	TGGATGGTACTGAAATTTTCATTCTC
<i>OSAP</i>	CB467434	R F R	ACAAAAAAGTGATCTTTCCATGAGTTC ATAGTGCTGAAGGTACAACGGAGAA CCTCCCCGGCAGAGGAT
New sequence matched to UniGene Bt.33 875	AY999166	F R	TCTATGGCATTCCAGCGGATAT GTCTTTTATTTAACATAAGGCCAAACAAG

*F, forward primer; R, reverse primer; P, probe.

Reagent; Ambion Europe Ltd, Huntingdon, UK). The TaqMan method (O'Shaughnessy *et al.* 2002) was used for three transcripts encoding aromatase (*CYP19A1*), inhibin α -subunit (*INHA*) and glia-derived nexin (*SERPINE2*), while SYBR Green (SYBR Green JumpStart Taq ReadyMix; Sigma–Aldrich Company Ltd) was used for the remaining transcripts (Evans *et al.* 2004, Mihm *et al.* 2006). All primers and probes (only for the TaqMan PCR protocol) were designed using Primer Express Software v2.0.0 (Applied Biosystems), synthesised by MWG Biotech AG (Ebersberg, Germany) and used in concentrations of 200 μ M. Reactions were carried out in the Stratagene Thermocycler Mx3000P (Stratagene Europe, Amsterdam, The Netherlands), and the Stratagene algorithm was used to estimate threshold cycle numbers (C_t). Primers and probes are listed in Table 3.

Four transcripts encoding ribosomal protein L2 (*RPL2*), ribosomal protein L36 (*RPL36*), acidic ribosomal phosphoprotein P0 (*RPLP0*) and actin, β (*ACTB*) were tested to determine their suitability as endogenous reference genes in the real-time PCR study. Three of these transcripts (*RPL2*, *RPL36* and *RPLP0*) proved to be unsuitable as reference genes, because the mRNA expression was too low and/or the target gene expression calculated relative to the reference was too variable between follicles from the same stages of development for statistical analysis. However, real-time PCR results using *ACTB* as the endogenous reference gene were consistent between follicles from the same stages of development and with other molecular studies of DF development (Evans *et al.* 2004, Fayad *et al.* 2004, Mihm *et al.* 2006). Thus, *ACTB* was used as the endogenous reference in the real-time PCR study, although it may underestimate transcripts upregulated in the DF (Neuvians *et al.* 2005). Expression levels relative to *ACTB* were calculated using $2^{-\Delta C_t}$.

Analysis of SAGE data and statistical analyses

Differences in tag frequency between the SAGE libraries were analysed using the χ^2 test (Man *et al.* 2000). This study aimed to identify transcripts differentially regulated in the DF as it transits from the FSH- to the LH-dependent development stage. Consequently, an initial comparison was made between the DF and COH libraries that identified 597 differentially expressed tags ($P \leq 0.05$). Expression of these tags was then compared between the DF and SF SAGE libraries, and 93 tags were identified, which were significantly different ($P \leq 0.05$) between the DF and both the COH and SF libraries (Table 2). The SAGEmap facility was then used to match differentially expressed SAGE tags to bovine UniGene clusters (<http://www.ncbi.nlm.nih.gov/SAGE/>) up to and through January 2007. When the tags are matched to more than one UniGene cluster, the cluster with the complete mRNA sequence and/or the majority of sequences containing the tag is presented. The SAGE technique also generates a slightly longer sequence for many tags (15 nucleotides), and this longer sequence was used to map the tag TTGATGTTTG to one UniGene cluster. Bovine (Bt.) UniGene cluster numbers can be accessed at <http://www.ncbi.nlm.nih.gov/SAGE/>, and full gene descriptions can be accessed at http://www.genenames.org/cgi-bin/hgnc_search.pl.

Differentially expressed SAGE tags matched to UniGene clusters also underwent gene ontology analysis based on the GO biological process (www.geneontology.org) regulated by the encoded gene. χ^2 analysis was again used to determine whether the number of encoded proteins regulating tissue development, DNA/RNA synthesis, protein synthesis or unknown biological processes differed ($P \leq 0.05$) between the transcripts expressed at the highest levels in the DF and the transcripts expressed at lower levels in the DF compared with the COH and/or SF libraries. Other differences ($P \leq 0.05$) between the COH, DF and SF not related to the SAGE libraries were analysed using one-way ANOVA or a paired *t*-test (only for DF versus SF comparisons). Steroid concentrations and relative gene expression values were log transformed to pass normality tests if indicated, but untransformed data are presented (mean $\pm$ S.E.M.).

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