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Predictive value of bovine follicular components as markers of oocyte developmental potential

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Abstract. The follicle is a unique micro-environment within which the oocyte can develop and mature to a fertilisable gamete. The aim of this study was to investigate the ability of a panel of follicular parameters, including intrafollicular steroid and metabolomic profiles and theca, granulosa and cumulus cell candidate gene mRNA abundance, to predict the potential of bovine oocytes to develop to the blastocyst stage *in vitro*. Individual follicles were dissected from abattoir ovaries, carefully ruptured under a stereomicroscope and the oocyte was recovered and individually processed through *in vitro* maturation, fertilisation and culture. The mean (± s.e.m.) follicular concentrations of testosterone ($62.8 \pm 4.8\ ng\ mL^{-1}$), progesterone ($616.8 \pm 31.9\ ng\ mL^{-1}$) and oestradiol ($14.4 \pm 2.4\ ng\ mL^{-1}$) were not different ($P > 0.05$) between oocytes that formed (competent) or failed to form (incompetent) blastocysts. Principal-component analysis of the quantified aqueous metabolites in follicular fluid showed differences between oocytes that formed blastocysts and oocytes that degenerated; L-alanine, glycine and L-glutamate were positively correlated and urea was negatively correlated with blastocyst formation. Follicular fluid associated with competent oocytes was significantly lower in palmitic acid ($P = 0.023$) and total fatty acids ($P = 0.031$) and significantly higher in linolenic acid ($P = 0.036$) than follicular fluid from incompetent oocytes. Significantly higher ($P < 0.05$) transcript abundance of *LHCGR* in granulosa cells, *ESR1* and *VCAN* in thecal cells and *TNFAIP6* in cumulus cells was associated with competent compared with incompetent oocytes.

Additional keywords: cattle, follicular fluid, IVF, metabolomics, mRNA expression, oocyte quality.

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Introduction

As the use of *in vitro* maturation (IVM) and single embryo transfer (SET) becomes more routine in assisted reproductive technology (ART), the selection of oocytes or embryos with the highest developmental potential is critical to a successful outcome. New technologies, such as transcriptomics, proteomics and metabolomics, have allowed researchers and clinicians to investigate more sophisticated methods for oocyte and embryo selection, with the emphasis on the predictive value of superfluous fluids and tissues, such as follicular fluid, follicle cells and cumulus cells, for non-invasive assessment of oocyte quality (Revelli *et al.* 2009). Recent studies on bovine oocytes have highlighted the potential of metabolomic technologies in this area (Sinclair *et al.* 2008; Bender *et al.* 2010). Both of these studies suggest that metabolomic analysis of follicular fluid may be a useful tool for characterising oocyte quality. Most recent data from human ART research points at the use of a panel of parameters to predict subsequent oocyte developmental potential (Lédée *et al.* 2007). However, it is often difficult to obtain 'clean' data from human ART research as the numbers of tissues analysed in one clinic are often insufficient to generate statistically robust data and therefore datasets may be compiled from more than one clinic using different protocols. Bovine *in vitro* embryo production provides an excellent resource for the identification of predictive markers of oocyte and embryo potential as large numbers of oocytes are easily retrieved from the ovaries of slaughtered cattle and experimental conditions can be standardised for each trial. From the bovine perspective, markers of oocyte quality have applications in research focussed on mechanisms to improve cow fertility. This study tested the hypothesis that markers in follicular fluid or on cumulus cells are predictive of oocyte development to the blastocyst stage. The specific objective of the present study was to assess the relationship between a panel of non-invasive markers of oocyte

Table 1. List of intrafollicular steroid hormones, mRNAs and metabolomic extraction measured in each follicle that were used to evaluate follicular differentiation, function and oocyte developmental competence
Abbreviations are described in Tables S3 and S4

Tissue type	Follicular parameters
Follicular fluid	testosterone, progesterone, oestradiol, fatty acids, amino acids, urea, glucose
Granulosa and theca cell mRNA	*AMH*, *CYP19A*, *ESR1*, *ESR2*, *FSHR*, *LHCGR*, *HSD3B1*, *VCAN, PTGS2*, *PTX3*
Cumulus cell mRNA	*ESR1*, *VCAN*, *PTGS2*, *PTX3*, *AVEN*, *HSP90B1*, *WASL*, *BAG3*, *BUB3*, *ATRX*, *EGR1*, *TNFAIP6*

competence from follicular fluid and tissues and oocyte development following IVM, *in vitro* fertilisation (IVF) and *in vitro* culture (IVC). To this end, the steroid hormone and metabolomic profiles of follicular fluid and candidate gene mRNA expression profiles of granulosa and theca cells were analysed in relation to oocyte development *in vitro*. In a second experiment, the relationship between the relative abundance of a panel of candidate genes in cumulus-cell biopsies and the developmental competence of the oocyte was assessed.

Materials and methods

All chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise indicated.

Experiment 1: relationship between follicular parameters and oocyte developmental competence

Ovary collection, follicle dissection and follicle fluid and tissue collection

Pairs of bovine ovaries were collected at a local abattoir, stored on a per animal basis ($n = 49$) and transported to the laboratory in phosphate-buffered saline (PBS) at 35°C within 4 h. Individual follicles ($n = 213$, mean ± s.e.m. diameter 7.92 ± 0.2 mm) were dissected from the ovaries using fine scissors. The diameter of each follicle was measured and the follicles were carefully ruptured using a surgical blade under a stereomicroscope. Once the cumulus–oocyte complex (COC) had been recovered, the follicular fluid was transferred to a 0.6-mL PCR tube and immediately placed on ice until all follicles on a given day were processed ($n = 20$), after which they were centrifuged for 2 min at 2500*g* at 4°C to remove any cells and stored at −80°C until steroid and metabolomic analysis. Morphologically-normal COCs were washed in PBS supplemented with 1 mg mL^{-1} glucose, 36 μg mL^{-1} pyruvate and 0.5 mg mL^{-1} bovine serum albumin (BSA) and processed for *in vitro* embryo production in an individually identifiable manner (Matoba *et al.* 2010). Concurrent with COC processing, granulosa and theca cells were recovered as previously described (Forde *et al.* 2008). Following follicle rupture, granulosa cells were scraped from the theca layer by a glass scraper. The theca layer was then peeled away from the stroma with forceps. Given the nature of the cell types, the granulosa cells are presumed to be very pure while some granulosa cell 'contamination' of thecal cells cannot be ruled out. Therefore, in line with previous publications, these cells are best referred to as 'theca-enriched' cells, although for ease of reading they are referred to as theca cells in the paper. The two cell types were separately transferred to 1.5-mL PCR tubes on ice, centrifuged for 2 min at 21 000*g* at 4°C and each pellet was immersed in 500 μL RNAlater (1 : 5 w/v) and stored at 4°C for 24 h, after which the RNA later was removed by centrifugation for 10 min at 3000*g* at 4°C and removal of supernatant and cell pellets were snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. Follicular parameters that were analysed are listed in Table 1.

In vitro *embryo production*

Dishes for *in vitro* embryo production were prepared with Cell-Tak or a polyester mesh as previously described (Matoba *et al.* 2010). For IVM, COCs were individually placed in groups of 20 on Cell-Tak in 100-μL droplets of IVM medium (TCM 199 with Earle's salts, supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng mL^{-1} epidermal growth factor). Droplets were overlaid with mineral oil and maturation took place at 39°C for ~24 h in a humidified atmosphere of 5% CO_2 in air. Subsequently, IVM medium was replaced with IVF medium (Tyrode's medium supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg mL^{-1} fatty acid-free BSA and 10 μg mL^{-1} heparin–sodium salt (184 U mL^{-1} heparin; Calbiochem, San Diego, CA, USA)). Each droplet was inseminated with frozen–thawed and Percoll-separated (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) spermatozoa from a single bull (Progressive Genetics, Enfield, Ireland) at 1×10^6 spermatozoa mL^{-1}. Gametes were co-incubated for 18–20 h at 39°C in a humidified atmosphere of 5% CO_2 in air. Following IVF, presumptive zygotes were individually denuded by vortexing for 2 min and gentle pipetting with a fine glass pipette and washed in PBS and culture medium. They were then transferred to a new Cell-Tak dish containing 100-μL droplets of synthetic oviduct fluid (SOF) medium (Holm *et al.* 1999) supplemented with 5% (v/v) FCS and cultured at 39°C for 9 days in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 in humidified air. Cleavage and blastocyst rates were assessed on Day 2 (i.e. 48 h after insemination; day of IVF = Day 0) and on Days 7 to 9, respectively. Eleven replicates were carried out (~20 follicles or COCs per replicate); each replicate represents a single day of ovary collection.

Follicular fluid steroid concentrations

Concentrations of testosterone (T4), progesterone (P4) and oestradiol (E2) in all follicles were assayed by radio-immuno assay (RIA) using ^{125}I-total testosterone Coat-a-Count kit (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA), ^{125}I Progesterone Coat-a-Count kit (Siemens Medical Solution Diagnostics) and ^{125}I Oestradiol MAIA kit (Adaltis

Italia S.p.A., Casalecchio di Reno, Italy). Follicular-fluid samples were diluted 1 : 50 with PBS for T4 and 1 : 100 with PBS for P4 and E2; each assay was carried out in duplicate. The inter-assay coefficients of variation (CV) of T4, P4 and E2 were 18.8, 12.5 and 13.7% for low, 6.9, 3.3 and 6.0% for medium and 7.5, 19.5 and 8.0% for high, respectively. The intra-assay CV of T4, P4 and E2 were 7.6, 11.7 and 11.5% (low), 6.5, 6.6 and 8.4% (medium) and 7.6, 4.9 and 12.0% (high), respectively. The sensitivities of the assays were 0.02 ng tube^{-1} for T4, 0.02 ng mL^{-1} for P4 and 0.05 ng mL^{-1} for E2.

Metabolite extraction and analysis

Metabolomic profiling of the aqueous and fatty-acid components of the follicular fluid was carried out as previously described (Bender *et al.* 2010) on a subset of samples from oocytes that cleaved and formed blastocysts or cleaved but subsequently degenerated ($n = 18$, mean ± s.e.m diameter 7.47 ± 0.16 mm). For aqueous metabolite analysis, 40 µL of sample were combined with 160 µL of water and 5 µL of ^{13}C myristic acid (Cambridge Isotopes, Andover, MA, USA) as internal standard before extraction with 800 µL methanol (Jiye *et al.* 2008). Extracts were dried and samples were derivatised and analysed using an Agilent 7890A GC with an Agilent HP-5 ms 30 m × 250 µm × 0.25 µm column coupled with a 5975C MS (Agilent Technologies Ireland Ltd., Cork, Ireland). Compound identification and calibration was achieved by referencing to in-house standards and an amino-acid mix (1 mM amino acid standard solution; Sigma Aldrich, Buchs, Switzerland) using Agilent Chemstation MSD E.02.00.493. (Agilent Technologies Ireland Ltd.) and by comparison of their mass spectra with those in the National Institute of Standards and Technology (NIST) Library 2.0 (2005).

For the analysis of organic compounds, 50 µL of follicular fluid were combined with 50 µL of water and 10 µL of 2 mg mL^{-1} pentadecanoic acid (C15 : 0) as internal standard and extracted using a 1 : 2 mixture of chloroform : methanol based on the method of (Bligh and Dyer 1959). Extracts were derivatised by methylation using methanolic BF_3. Derivatives were re-suspended in 200 µL hexane and analysed on an Agilent 7890A GC coupled with a 5975C MS with an Agilent HP-5 ms 30 m × 250 µm × 0.25 µm column. Two microlitres were injected in splitless mode, and the initial oven temperature of 70°C was raised to 220°C at 5°C min^{-1}, held for 20 min, and then raised to 320°C at 20°C min^{-1}. Helium was used as carrier gas with a flow of 1.2 mL min^{-1}. Calibration was achieved by comparison of peak areas for fatty acids with reference to a known standard (Supelco 37 compound mix; Supelco, Poole, UK) using Agilent Chemstation MSD E.02.00.493 and by comparison of their mass spectra with those in the National Institute of Standards and Technology (NIST) Library 2.0 (2005). For quality control purposes, two aliquots from a pool of follicular fluid were extracted and analysed in parallel with each batch of samples.

Automatic peak detection was carried out with Agilent Chemstation MSD. Mass spectra deconvolution was performed with AMDIS version 2.65. Peaks with a signal-to-noise ratio (S/N) lower than 30 were rejected. To obtain accurate peak areas for the internal standard and specific peaks or compounds, one quant mass for each peak was specified as target and three masses were selected as qualifiers. Each data file was manually analysed for false positives and negatives in Agilent Chemstation.

The aqueous metabolite data was divided into compounds identified and quantified using external standards and compounds semiquantified relative to the internal standard only. Concentrations given for fatty acids are µg mL^{-1} + s.e.m.; concentrations for quantified amino acids, glucose and urea are µmol L^{-1}.

RNA isolation and quantitative real-time PCR (Q-PCR)

Excess RNAlater was removed and granulosa and theca cell samples were transferred to RNase/DNase-free tubes for analysis of gene expression by Q-PCR. Total RNA was extracted from granulosa and theca cells originating from individual follicles using the guanidine-based TRI Reagent according to the manufacturer's instructions. Subsequently, mRNA was isolated from the eluted total granulosa and theca cell RNA using the mRNA Direct TM Micro kit (Invitrogen). Isolated mRNA was eluted in 7 µL of RNase-free water and was DNase treated with DNase I (Invitrogen) resulting in a total volume of 10 µL. Messenger RNA was reverse transcribed using Superscript III (Invitrogen) and random primers (Invitrogen) according to the manufacturer's instructions (total volume 20 µL). Subsequently, granulosa and theca cell cDNA was diluted (1 : 20) with RNase-free water to a final volume of 380 µL.

Negative controls were generated by omitting RNA from the cDNA synthesis reaction. Reverse transcription PCR employing intron-spanning primers for *PPIA* was used to confirm successful DNase treatment, generating a 482-bp or 108-bp amplicon in the presence or absence of DNA, respectively. Transcript abundance of candidate genes was analysed in granulosa and theca cell samples from follicles associated with embryos that cleaved and developed to the blastocyst stage (competent, $n = 15$) and those that cleaved but failed to develop (incompetent, $n = 15$). The quantification of all gene transcripts was carried out using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and performed on the ABI 7300 Real-Time PCR System (Applied Biosystems) using the standard-curve method of analysis. Primers were designed using Primer 3 software (http://primer3.sourceforge.net/; see Tables S1 and S2 available as Supplementary Material to this paper for gene list and primer details). Each reaction was carried out in a total volume of 15 µL, consisting of 4 µL cDNA, 7.5 µL Power SYBR Green PCR Master Mix and the optimum concentration of forward and reverse primers (Eurofins MWG Operon, Ebersberg, Germany) and RNase-free water. Reactions were performed in duplicate in 96-well plates, and four non-template control samples were included for each primer set. The thermal cycling conditions consisted of 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and annealing for 1 min at 60°C. The specificity of the amplicon was confirmed by melting-curve analysis, consisting of 40 cycles at 95°C for 15 s and 60°C for 1 min, followed by 95°C for 15 s and 60°C for 15 s. The mRNA abundance for each target gene was normalised against the levels of the constitutive housekeeping gene *PPIA* in all cell types.

Experiment 2: relationship between expression of candidate genes in cumulus cell biopsies and developmental competence of bovine oocytes

Preliminary experiments were carried out to establish the effect of taking a cumulus-cell biopsy from immature and *in vitro*-matured COCs on development to the blastocyst stage. Based on the results of these experiments, a subsequent experiment was carried out where a biopsy was taken from individual *in vitro*-matured bovine COCs and snap frozen in liquid nitrogen. The COCs were then fertilised and cultured *in vitro* in an individually identifiable manner using a polyester mesh system as previously described (Matoba *et al.* 2010). Following 7 days of culture, cumulus cell biopsies were pooled in groups of five according to whether the oocytes from which they were derived cleaved and developed to the blastocyst stage or cleaved but failed to reach the blastocyst stage ($n = 5$ replicate pools each).

Statistical analysis

Steroid concentrations (least-square means ± s.e.m.) in follicles that yielded oocytes that developed to the blastocyst stage, that cleaved but failed to develop or that were not cleaved after insemination were compared by analysis of variance (PROC MIXED in SAS, SAS Institute Inc., Cary, NC, USA). Regarding the metabolomic analysis of follicular fluid, principle component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) was performed using SIMCA-P+11 (Umetrics, Umea, Sweden). The quality of the models formed by PCA and PLS-DA were investigated by interrogation of the R^2 and Q^2 values. The R^2 parameter is a representation of how much of the variation within the dataset is explained by the components of the model. The Q^2 parameter gives an indication of how good the model is at class prediction. Correlation analysis, general linear model analysis and Bonferroni posthoc testing was performed using SPSS 14 (IBM, New York, NY, USA). Significance was assumed using $P < 0.05$ as a cut-off.

Gene expression data were analysed using the standard-curve method. Values were normalised to the average values of the reference gene and means were compared by Student's *t*-test. Differences were considered to be significant when $P < 0.05$.

Results

Relationship between follicular parameters and oocyte developmental competence

Oocyte competence

Developmental data from Experiment 1 indicated that follicle dissection and individual culture on Cell-Tak did not affect cleavage or blastocyst formation rate (Table 2; $P > 0.05$). In Experiment 2, taking a cumulus biopsy from immature COCs resulted in a significant reduction in subsequent blastocyst development compared with unmanipulated controls, irrespective of whether culture took place in groups (Table S3) or individually in a mesh system (Table S4). In contrast, biopsy of the expanded cumulus cells associated with *in vitro*-matured oocytes had no detrimental effect on subsequent development (Tables S1 and S2). Thus, subsequent experiments were carried out solely on *in vitro*-matured oocytes.

Table 2. Effect of follicle dissection and individual IVM, IVF and IVC on Cell-Tak on development of IVP bovine embryos (Experiment 1, 11 replicates)

No statistically-significant differences ($P > 0.1$)

Culture method	No. of oocytes	No. of zygotes	Cleaved[A] (%)	Blastocyst yield[A] (%)	
				Day 7	Day 7–9
Cell-Tak	213	197	79.1 ± 3.8	20.8 ± 2.9	28.3 ± 3.4
Group	215	215	81.7 ± 2.4	23.5 ± 5.1	25.8 ± 4.7

[A]Cleaved embryos or blastocysts as a percentage of number of zygotes.

Follicular T4, P4 and E2 concentration

The mean (± s.e.m.) follicular concentrations of T4, P4 or E2 were not different ($P > 0.05$) between oocytes that developed to the blastocyst stage (competent oocytes) and oocytes that cleaved after fertilisation and then degenerated (incompetent oocytes; Fig. 1).

Relationship between follicular granulosa and theca cell candidate gene expression and oocyte developmental competence

The results are presented in Figs 2 and 3. Briefly, *LHCGR* mRNA abundance was significantly higher in granulosa cells associated with competent oocytes compared with incompetent oocytes ($P < 0.05$). The expression of *VCAN* and *ESR1* was higher in thecal cells associated with competent compared with incompetent oocytes ($P < 0.05$ and $P < 0.08$, respectively). The abundance of all other transcripts tested was not different between groups.

Relationship between cumulus cell candidate gene expression and oocyte developmental competence

The results are presented in Fig. 4. Briefly, *TNFAIP6* mRNA abundance was significantly higher in mature cumulus cells from competent oocytes compared with incompetent oocytes ($P < 0.06$). The remaining candidate genes were not significantly differentially expressed.

Metabolite profile of follicular fluid

Fatty-acid profile. A total of 16 fatty acids were identified and quantified and the total fatty acid (SFA), total monounsaturated fatty acid (MUFA), total polyunsaturated fatty acid (PUFA), (n-3) PUFA and (n-6) PUFA contents were determined (Table 3). ANOVA analysis of the fatty-acid data showed that follicular fluid from oocytes that cleaved but subsequently degenerated was significantly higher in palmitic acid ($P = 0.023$) and total SFA ($P = 0.031$) and significantly lower in linolenic acid ($P = 0.036$) compared with follicular fluid from competent oocytes (Table 3). Multiple-regression analysis of aqueous compounds with follicle parameters showed that T4 concentrations were positively correlated with arachidonic acid ($P < 0.001$), pentadecanoic acid ($P < 0.001$) and heptadecanoic acid ($P = 0.045$) but was negatively correlated with oleic acid ($P = 0.005$). P4 concentrations were positively correlated with heptadecanoic acid ($P = 0.002$) and arachidonic acid ($P = 0.045$).

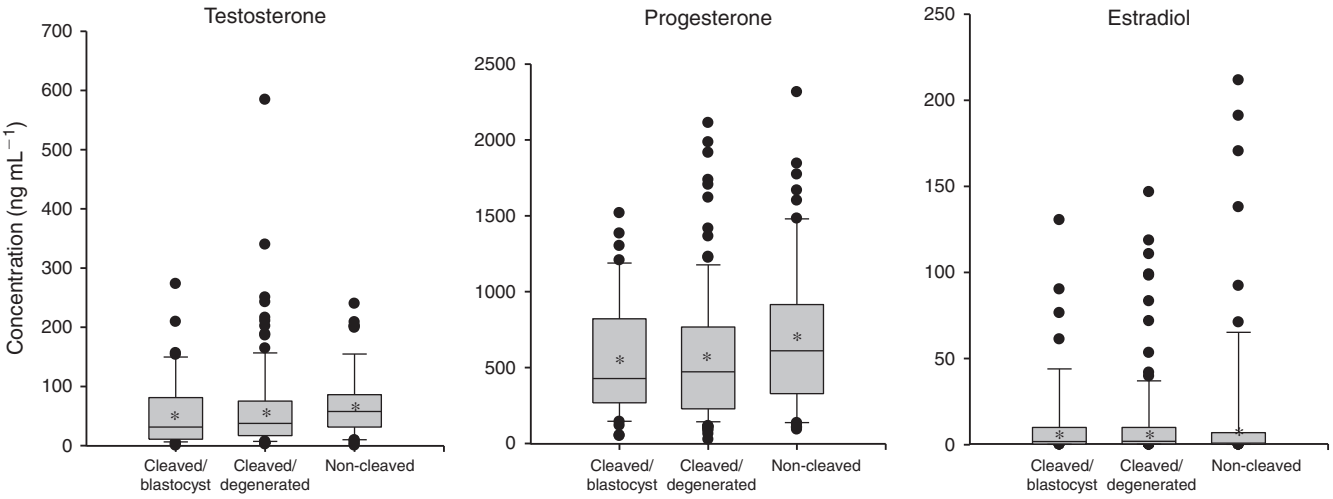

Fig. 1. Steroid hormone concentration (ng mL^{-1}) in bovine follicular fluid yielding oocytes that failed to cleave, cleaved but degenerated or cleaved and developed to the blastocyst stage following maturation, fertilisation and culture *in vitro*. The parameters indicate two quartiles with the 25th and 75th percentiles (rectangle), median (horizontal line), 10th and 90th percentiles (whiskers), the mean (*) and the outliers (individual circles). No significant differences ($P > 0.05$).

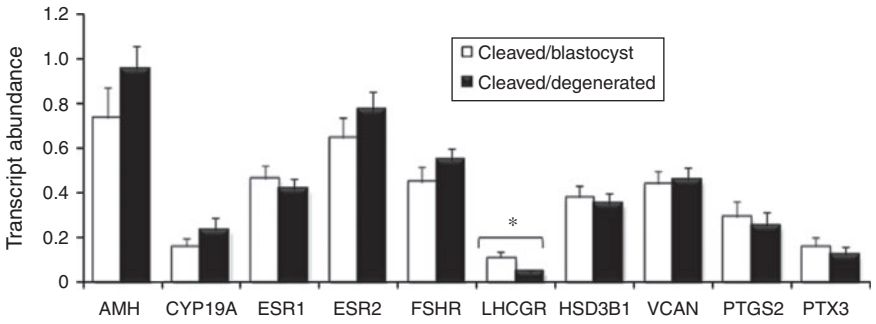

Fig. 2. Relative mRNA abundance (mean ± s.e.m.) of 10 candidate genes in bovine granulosa cells associated with oocytes that reached blastocyst stage or degenerated in culture. *$P < 0.05$.

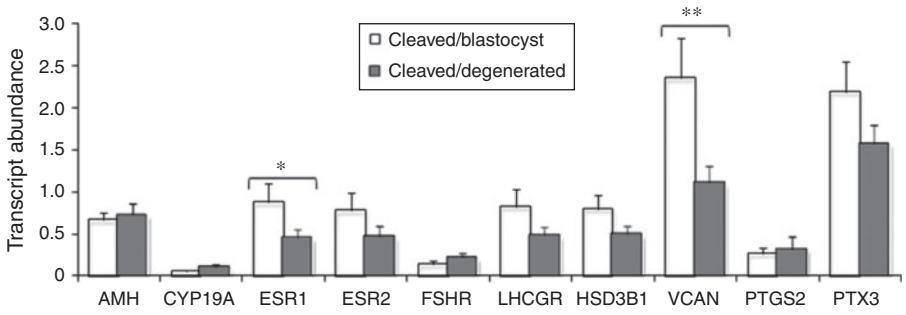

Fig. 3. Relative mRNA abundance (mean ± s.e.m.) of 10 candidate genes in bovine theca cells associated with oocytes that reached blastocyst stage or degenerated in culture. **$P < 0.05$, *$P = 0.08$.

Amino acid profile. A total of 55 aqueous compounds were analysed of which 19 were quantified (Table 4) and 38 semi-quantified. Principal-component analysis of the quantified data showed separation between oocytes that formed blastocysts and oocytes that degenerated ($R^2 = 0.561$). Further analysis using PLS-DA generated a robust model that could predict which oocytes formed blastocysts ($R^2X = 0.552$; $R^2Y = 0.874$; $Q^2 = 0.722$; see Fig. 5). Analysis of the corresponding variable

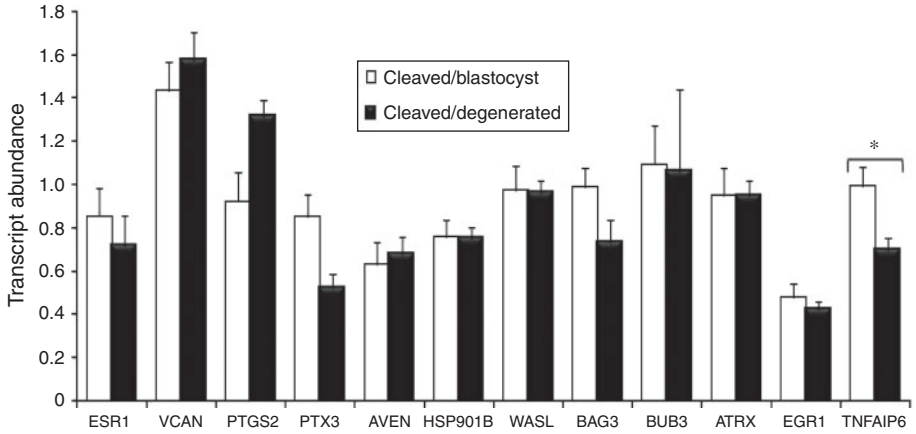

Fig. 4. Relative mRNA abundance (mean ± s.e.m.) of 12 candidate genes in bovine cumulus cells associated with oocytes that reached blastocyst stage or degenerated in culture. *$P = 0.06$.

Table 3. Fatty-acid composition of follicular fluid from single samples: blastocyst-forming versus degenerate follicles (Experiment 1, 11 replicates)

Values are expressed as mean ± s.e.m. (μg mL^{-1}). *P* values are reported for differences between +blastocyst ($n = 8$) and degenerate ($n = 7$) calculated using ANOVA; NS, not significant

Fatty acid	+ Blastocyst	Degenerate	*P* value
Myristic (C14 : 0)	2.879 ± 0.225	4.632 ± 0.919	NS
Pentadecanoic (C15 : 1)	0.332 ± 0.076	0.357 ± 0.093	NS
Palmitoleic (C16 : 1)	4.013 ± 0.621	5.286 ± 0.584	NS
Palmitic (C16 : 0)	18.462 ± 1.329	26.605 ± 3.076	0.023
Heptadecanoic (C17 : 1)	0.434 ± 0.119	0.778 ± 0.200	NS
Heptanoic (C17 : 0)	0.887 ± 0.239	1.615 ± 0.392	NS
γ-Linolenic (C18 : 3n6)	1.386 ± 0.542	0.883 ± 0.145	NS
Linoleic (C18 : 2n6)	33.981 ± 6.942	31.999 ± 5.403	NS
Linolenic (C18 : 3n3)	6.992 ± 1.139	3.941 ± 0.488	0.036
Oleic (C18 : 1n9c)	25.202 ± 2.850	29.382 ± 3.688	NS
Stearic (C18 : 0)	17.250 ± 1.689	23.370 ± 3.986	NS
Arachidonic (C20 : 4n6)	6.250 ± 0.885	5.167 ± 1.933	NS
EPA (C20 : 5n3)	3.090 ± 0.510	2.499 ± 0.931	NS
DGLA (C20 : 3n6)	5.753 ± 2.615	2.499 ± 0.931	NS
Arachidic (C20 : 0)	0.042 ± 0.014	0.067 ± 0.017	NS
DHA (C22 : 6n3)	0.289 ± 0.095	0.294 ± 0.067	NS
Total SFA[A]	39.519 ± 3.123	56.289 ± 6.515	0.031
Total MUFA[B]	29.982 ± 3.411	35.803 ± 4.127	NS
Total PUFA[C]	57.742 ± 8.564	52.629 ± 7.078	NS
Total (n-3)[D]	10.872 ± 1.464	6.734 ± 0.906	NS
Total (n-6)[E]	47.370 ± 8.401	45.896 ± 7.366	NS

[A]Total SFA = $\sum (C14:0, C16:0, C17:0, C18:0, C20:0)$
[B]Total MUFA = $\sum (C15:1, C16:1, C17:1, C18:1n9c)$
[C]Total PUFA = $\sum (C18:3n6, C18:2n6, C18:3n3, C20:4n6, C20:5n3, C20:3n6, C22:6n3)$
[D]Total (n-3) = $\sum (C18:3n3 + C205n3 + C22:6n3)$
[E]Total (n-6) = $\sum (C18:3n6 + C18:2n6 + C20:4n6 + C20:3n6)$

Table 4. Quantified aqueous compounds in follicular fluid from single samples

Amino acid, urea and glucose composition of follicular fluid from single samples: blastocyst-forming versus degenerate follicles (Experiment 1, 11 replicates). Values are expressed as mean ± s.e.m. (μmol L^{-1}). *P* values are reported for differences between +blastocyst ($n = 9$) and degenerate ($n = 9$) calculated using ANOVA; NS, not significant

Metabolite	+ Blastocyst	Degenerate	*P* value
L-alanine	436.75 ± 25.37	282.74 ± 32.54	0.002
L-valine	252.09 ± 56.51	271.85 ± 64.02	NS
L-leucine	239.85 ± 48.83	335.64 ± 50.74	NS
L-proline	82.12 ± 20.72	90.47 ± 18.92	NS
L-isoleucine	62.05 ± 13.06	97.79 ± 20.73	NS
Glycine	660.71 ± 41.32	360.66 ± 11.79	<0.001
L-serine	166.60 ± 34.82	196.93 ± 38.45	NS
L-threonine	110.10 ± 26.11	130.06 ± 24.63	NS
L-glutamine	584.86 ± 31.01	461.06 ± 69.57	NS
L-methionine	14.07 ± 6.03	37.91 ± 15.75	NS
L-aspartate	88.45 ± 4.98	64.49 ± 13.19	NS
L-cysteine	4.43 ± 1.15	6.18 ± 1.41	NS
L-phenylalanine	103.71 ± 29.14	121.23 ± 29.18	NS
L-glutamate	207.56 ± 25.08	120.00 ± 15.84	0.009
L-arginine	185.31 ± 44.91	231.15 ± 56.98	NS
L-lysine	97.19 ± 12.82	127.18 ± 16.77	NS
L-tyrosine	91.44 ± 11.69	113.35 ± 15.56	NS
Urea	1708.05 ± 73.82	2082.53 ± 101.31	0.009
Glucose	1434.07 ± 144.37	1511.77 ± 130.34	NS

importance plots showed that L-alanine, glycine and L-glutamate were positively correlated with blastocyst development. Urea was negatively correlated with blastocyst formation. ANOVA analysis validated these results (Table 4). Multiple-regression analysis of aqueous compounds with follicle parameters showed that L-leucine was positively correlated with T4 ($P = 0.03$) whereas L-glutamine was negatively correlated with P4 ($P = 0.007$). Receiver-operating curve analysis was used to identify parameters that were predictive of oocyte development. Follicular steroid concentrations had no predictive value (area under curve (AUC) = 0.35). However, the aqueous metabolites were predictive of potential to develop to the blastocyst stage and a combination of the fatty acids and aqueous data showed a slight improvement in predictive potential (Fig. 6).

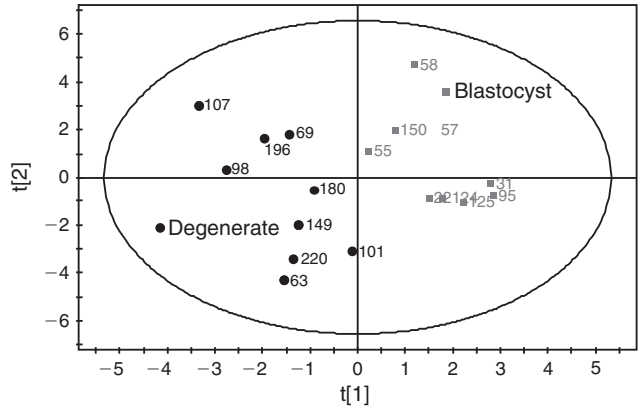

Fig. 5. Principal-component analysis (PCA) scores plot depicting principal component one and principal component two obtained from quantified amino acids in follicular fluid from follicles yielding oocytes that developed to the blastocyst stage and those that cleaved but failed to reach the blastocyst stage (degenerate).

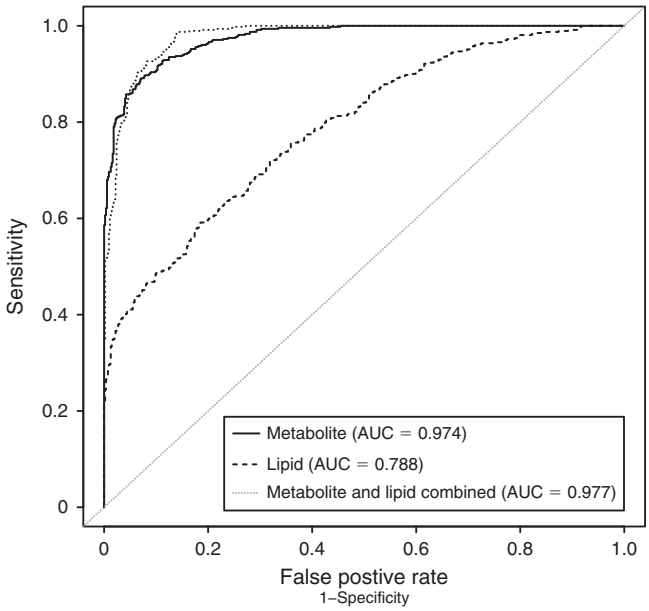

Fig. 6. Receiver Operating Characteristic (ROC) curve produced with the metabolite data, lipid data, metabolite and lipid data combined.

Discussion

The objective of the present study was to test the ability of a panel of non-invasive markers from follicular tissue to predict oocyte developmental competence. Following extensive reviews of the literature a panel of candidate genes was assembled for mRNA expression analysis in follicular granulosa and theca cells. The expression of four of the genes was associated with development to the blastocyst stage; *LHCGR* expression was significantly higher in granulosa cells (GC) associated with competent oocytes compared with incompetent oocytes, *VCAN* and *ESR1* transcript abundance was significantly higher in thecal cells associated with competent compared with incompetent oocytes and *TNFAIP6* was significantly higher in post IVM cumulus cells associated with competent compared with incompetent oocytes. Similar findings have been reported in relation to gene expression in human follicular cells as predictors of pregnancy outcome post IVF, where only two genes from a panel of 10 were significantly different despite controlling for embryo quality at the outset (Hamel *et al.* 2010). The induction of expression of the *LHCGR* mRNA in GC was recently identified as an early event during the follicle selection process (Luo *et al.* 2011) and is essential for follicular maturation in the progression from antral to preovulatory stage (Pakarainen *et al.* 2007). Follicles less than 8 mm in diameter were selected for the present study, excluding the possibility of using selected dominant follicles; therefore, granulosa cell *LHCGR* expression may also be reflective of follicle viability. Similarly, selective deletion of the *Esr1* gene in murine theca cells (thEsr1KO) results in female infertility before the age of 6 months, which is manifested at the level of the ovary by fewer ovulations and therefore fewer corpora lutea, a greater number of early antral follicles and markedly lower or undetectable LH levels in the Esr1KO mice (Lee *et al.* 2009), again suggesting that higher *ESR1* expression is associated with follicle viability. Versican is an abundant extracellular matrix proteoglycan that binds hyaluronic acid and is hormonally regulated in the ovary and is strongly induced at the time of ovulation (Russell *et al.* 2003*b*). Versican is produced by mural granulosa cells in rat and mouse ovaries, but localises selectively to the granulosa–thecal boundary of preovulatory follicles and intensely in expanded COC matrix (Russell *et al.* 2003*b*). Versican is a key substrate for ADAMTS1 proteolysis (Sandy *et al.* 2001), and versican cleavage occurs rapidly in the expanded COC matrix around the time of ovulation (Russell *et al.* 2003*a*, 2003*b*). In *Adamts1*$^{-/-}$ mice, ovulation rate, fertilisation rate and fertility were reduced by 68–75%, versican cleavage in the expanded COC was 75% lower and structural organisation of the COC matrix was strikingly disrupted. Sites of ADAMTS1 activity localised to the granulosa–thecal interface of ovulating follicles, as well as COC and dysmorphogenesis, were evident in these regions of *Adamts1*$^{-/-}$ follicles. Furthermore, the degradation of the COC matrix in oviducts after ovulation was delayed. Our results support the importance of ADAMTS1 in remodelling the extracellular matrix.

Although the present study confirmed the feasibility of individual oocyte tracking during IVC as a model for identifying follicle tissue-derived transcripts as potential biomarkers of oocyte competence, the use of gene-expression analysis of mature cumulus cells as a predictor of oocyte competence was only verified for one previously identified candidate gene (*TNFAIP6*). *TNFAIP6* is a secretory protein of the hyaluronan-binding protein family, which is involved in extracellular matrix formation, playing a role in cumulus cell stabilisation and expansion (Fulop *et al.* 2003). Our findings cement earlier recommendations of *TNFAIP6* as a potential cumulus cell indicator of oocyte competence in cattle (Assidi *et al.* 2008; Tesfaye *et al.* 2009). The panel of candidate genes was assembled following a thorough cross-species review of mammalian oocyte and cumulus cell transcriptomic data (O'Shea *et al.* 2012). The poor correlation between our findings and that of

previously published findings is possibly due to the variation in oocyte maturation and sample processing regimes associated with different species. This emphasises the challenge associated with identifying universal somatic cell or follicle-derived predictors of oocyte competence and IVF outcome and highlights the importance of customising biomarker panels according to the oocyte maturation regime within a clinic or laboratory (Grøndahl *et al.* 2009; Adriaenssens *et al.* 2010).

Metabolomic analysis of bovine follicular fluid from single-embryo culture

Our recent comparison of the metabolomic profile of preovulatory follicular fluid from nulliparous heifers and postpartum dairy cows has indicated the potential predictive value of metabolomic analysis of follicular fluid for the assessment of oocyte quality (Bender *et al.* 2010). Consistent with those observations, in the present model both amino acids and fatty acids were found to be predictive of the developmental competence of the oocyte. In contrast to other studies, our results show significant differences in the fatty-acid fraction of competent and degenerate oocytes in terms of their C16 : 0, C18 : 3n3 and saturated fatty-acid concentrations (Sinclair *et al.* 2008). Follicular fluid from degenerate oocytes was significantly higher in palmitic acid as well as in total saturated fatty acids. The detrimental effects of increased palmitic acid in follicular fluid are well documented in the literature. *In vitro* studies have shown that increased palmitic acid concentrations resulted in a decreased rate of blastocyst formation (Leroy *et al.* 2005). Increased concentrations of both palmitic acid and stearic acid impair meiosis, cleavage rate and blastocyst formation (Leroy *et al.* 2005) as well as exerting adverse effects on both bovine (Vanholder *et al.* 2005) and human (Mu *et al.* 2001) granulosa cell growth and function. High concentrations of fatty acids are associated with impaired embryo quality through lipid accumulation (Reis *et al.* 2003), and developmentally competent human oocytes exhibit low levels of saturated fatty acids (Haggarty *et al.* 2006). In the present study, the follicular fluid associated with oocytes that formed blastocysts had higher concentrations of the n-3 PUFA linolenic acid. Our previous results demonstrated that follicular-fluid n-3 PUFA levels were higher in a high-fertility bovine model (Bender *et al.* 2010). Supporting the important role of n-3 PUFA, a recent study demonstrated that a concentration of 50 μM linolenic acid in the culture medium enhanced the number of metaphase II (i.e. mature) stage oocytes resulting from bovine COCs (Marei *et al.* 2009). Overall, the present results demonstrate the potential role of fatty-acid concentrations in predicting oocyte developmental competence.

The amino acids L-alanine, glycine and glutamate were predictive of the developmental ability of the oocyte. Several studies have described the beneficial effects of alanine (Cetica *et al.* 2003) and L-alanine and glycine (Lee and Fukui 1996) on embryonic development. Furthermore, L-alanine, glycine, proline, valine and glutamate were proposed to be predictive for oocyte development (Sinclair *et al.* 2008). Most recently, amino-acid profiling of spent IVM medium revealed higher consumption of glutamine and higher production of alanine during IVM by MII oocytes that failed to cleave by 72 h (Hemmings *et al.* 2012) Taken together, these findings may be used to optimise *in vitro*-maturation conditions through supplementation or oocyte profiling.

Linking in with the altered amino-acid levels, it was also found that urea levels were negatively correlated with blastocyst formation. This is consistent with the findings of De Wit *et al.* (2001) who reported negative effects of urea during IVM on the meiotic progression of bovine oocytes and subsequent cleavage and blastocyst development. An earlier study also reported reduced *in vitro* blastocyst development rates from oocytes recovered from heifers with increased concentrations of plasma urea (Santos *et al.* 2009). As urea concentrations have been shown to be similar in plasma and preovulatory follicular fluid in women, the blood plasma data supports our findings (Jozwik *et al.* 2006).

In conclusion, oocyte developmental potential is reflected in the gene expression signature and metabolomic profile of follicular tissue. Of all parameters measured, receiver-operating characteristic analysis indicated that the best predictor of oocyte developmental competence was the follicular-fluid metabolite profile. Furthermore, when combined, lipid and metabolite profiling improved the predictive capacity.

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