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Localisation and endocrine control of hyaluronan synthase (HAS) 2, HAS3 and CD44 expression in sheep granulosa cells

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Abstract

The aim of the present study was to investigate the hormonal regulation of hyaluronan (HA) components in sheep granulosa cells. HA components are present in the reproductive tract and have a range of physical and signalling properties related to reproductive function in several species. First, abattoir-derived ovaries of sheep were used to determine the localisation of HA synthase (HAS) 1–3 and CD44 proteins in antral follicles. Staining for HAS1–3 and CD44 proteins was most intense in the granulosa layer. Accordingly, the expression of HAS2, HAS3 and CD44 mRNA was measured in cultured granulosa cells exposed to 0–50 ng mL–1 of 17β-oestradiol and different combinations of oestradiol, gonadotropins, insulin-like growth factor (IGF)-1 and insulin for 48–96 h (1 ng mL–1 FSH, 10 ng mL–1 insulin, 10 ng mL–1 IGF-1, 40 ng mL–1 E2 and 25 ng mL–1 LH.). mRNA expression was quantified by real-time polymerase chain reaction using a fold induction method. The results revealed that the hormones tested generally stimulated mRNA expression of the genes of interest in cultured granulosa cells. Specifically, oestradiol, when combined with IGF-1, insulin and FSH, stimulated HAS2 mRNA expression. Oestradiol and LH had synergistic effects in increasing HAS3 mRNA expression. In conclusion, we suggest that the hormones studied differentially regulate HAS2, HAS3 and CD44 in ovine granulosa cells in vitro. Further work is needed to address the signalling pathways involved.

Introduction

Folliculogenesis proceeds under the coordinated control of systemic gonadotropin hormones and locally produced growth factors. Growing follicles become sensitive to gonadotropins in the preantral stages, whereas antral follicles become increasingly dependent on gonadotropins. Insulin-like growth factor (IGF)-1 mediates the transition from gonadotropin responsiveness to dependency via enhancement of gonadotropin receptor expression and stimulation of steroidogenesis (Silva et al. 2009; Scaramuzzi et al. 2011). Dominant follicles gain ovulatory potential by transition from FSH to LH dependency accompanied by an increase in 17β-oestradiol (E2) in the follicular fluid (Campbell et al. 1999; Fortune et al. 2004; Hunter et al. 2004; Baerwald et al. 2012).

The multiple layers of granulosa cells (GCs) in growing follicles are surrounded by a basal lamina that separates them from the theca. This specialised extracellular matrix (ECM) manages fluid dynamics and provides mechanical support. Hyaluronan (HA) is an important component of the ECM, present in various tissues of many species and contribute to cell signalling pathways as well as to the physical characteristics and stability of different cell types (Itano and Kimata 2002; Adamia et al. 2005). In addition, HA actively participates in both intra- and extracellular pathways, influencing cellular behaviour such as migration, growth, motility, adhesion and differentiation (Toole 2001; Toole et al. 2002; Bodevin-Authelet et al. 2005; Itano 2008).

Three main isoforms of HA synthases (HAS), namely HAS1, HAS2 and HAS3, are responsible for the synthesis of different-sized HA on cell membranes in mammalian tissues and these isoforms have 55%–71% of amino acid sequences in common in their structures (Weigel and DeAngelis 2007; Itano 2008; Erickson and Stern 2012; Bernert 2013). Large-sized HA (>2 × 106 Da) is synthesised by HAS2, whereas smaller-sized HA (between 2 × 105 and 2 × 106 Da) are synthesised by HAS1 and HAS3 (Itano and Kimata 2002). Small-size HA has functional roles that include immune stimulatory, inflammatory and angiogenic effects, as well as induction of CD44 cleavage (Stern 2004). Large-size HA has mainly structural roles, such as water absorbance and space filling. These characteristics of large HA support the role of the ECM in maintaining tissue integrity, together with its functional roles as an anti-angiogenic, immune-suppressive and anti-apoptotic factor (Stern 2004). Indeed, production of large-size HA by HAS2 in ovarian follicles has been linked to lack of vascularisation in the GCs due to blockade of endothelial cell migration and proliferation (Temple et al. 2000).

Various elements of the HA system are present in different parts of the reproductive tract. For example, HAS was detected using immunohistochemistry in pig ovary (Miyake et al. 2009), ovine cervix (Perry et al. 2012) and uterus (Raheem et al. 2013) and human endometrium (Nykopp et al. 2010). In addition, the HA receptor (CD44) was immunolocalised in ovine cervix (Perry et al. 2010) and uterus (Raheem et al. 2013), pig ovary (Miyake et al. 2006) and oviduct (Tienthai et al. 2003), bovine oocytes, embryos and oviduct (Ulbrich et al. 2004; Marei et al. 2012; Marei et al. 2013), cumulus and mural granulosa (Ohta et al. 1999; Marei et al. 2012) and cervical cells (El Maradny et al. 1997).

The main HA-binding proteins, so called hyaladherins, are CD44 and receptor for Hyaluronan-mediated motility (RHAMM) (Laurent et al. 1995; Toole 2004). The CD44 receptor has three domains, of which the second interacts extracellularly with both large and small HA (Marzioni et al. 2001; Bernert 2013). Interaction of HA with its main receptor (CD44) influences cellular activities such as cell migration, proliferation and differentiation (Bernert 2013).

The control of HA synthesis, utilisation and degradation in reproductive tissues has been studied to some extent, but has not been fully characterised. In the present study, we hypothesised that the hormones tested may control HA synthesis via mediation of HAS, particularly HAS2 and HAS3, in sheep GCs.

Materials and methods

Experimental design

Experiment 1 investigated the presence of different isoforms of HAS (HAS1, HAS2, HAS3) and hyaluronan receptor (CD44) by immunohistochemistry in follicles of sheep ovaries collected from a local abattoir at different stages of the oestrous cycle during the breeding season.

Experiment 2 aimed to study the role of E2 in regulating HA synthesis in sheep GCs. Serum-free GC cultures were treated with different concentrations of E2 (0, 3.12, 12.5, 25 and 50 ng mL–1). Expression of HAS2, HAS3 and CD44 mRNA was measured after 24, 48, 72 and 96 h exposure using real-time polymerase chain reaction (PCR).

Experiment 3 aimed to test the effects of different reproductive hormones (gonadotropins and E2), as well as insulin and IGF-1, on the expression of HAS2, HAS3 and CD44 mRNA. Cultured GCs were treated

with different combinations of 1 ng mL–1 FSH, 10 ng mL–1 insulin, 10 ng mL–1 IGF-1, 40 ng mL–1 E2 and 25 ng mL–1 LH. HAS2, HAS3 and CD44 mRNA expression was measured after 48 h culture.

In Experiments 2 and 3, gene expression was compared between cells harvested from basic culture medium only (control) and those cells for which the basic culture medium was supplemented with individual hormones or combinations at the stated concentrations.

Chemicals and reagents

Unless stated otherwise, reagents were purchased from Sigma-Aldrich (Gillingham, UK).

Immunohistochemistry

For detection of HAS1, HAS2, HAS3 and CD44, ovaries collected from the abattoir were fixed immediately in 4% formalin, pH 7.0 (VWR, Leicestershire, UK), for 48 h, then embedded in paraffin wax using a standard automated procedure. Sections (5 µm) were mounted on 'Superfrost' slides (VWR) and then dewaxed in histoclear III (AGTC Bioproducts Ltd, Hessle, UK) for a maximum of 2 h, followed by rehydration in an ethanol gradient series.

Rehydrated sections were washed twice in distilled water for 5 min each time, followed by antigen retrieval in Tris-EDTA buffer containing 10 mM Tris base (Bio-Rad, Hemel Hempstead, UK) and 1 mM EDTA (pH 9). Antigen retrieval involved incubating sections in boiled Tris-EDTA buffer for 25 min. Endogenous peroxidase activity was quenched by applying 3% v/v H2O2 in phosphate-buffered saline (PBS) for 15 min. Slides were then incubated in 0.25% NH4Cl in PBS for 15 min before being washed in distilled water. Non-specific binding was blocked by incubation for 1 h in 5% normal horse serum, obtained from the species in which the secondary antibody was raised. The normal serum was diluted in buffer containing 1× Tris-buffered saline (TBS), 0.1% w/v gelatin, 0.1% v/v Triton X-100 and 0.05% v/v Tween 20. The composition of the 1× TBS was 0.05 M Tris base (Bio-Rad) and 0.9% NaCl (pH 7.6).

Sections were overlaid with rabbit polyclonal anti-human HAS2 (Santa Cruz, Dallas, TX, USA), HAS3 (Santa Cruz), HAS1 (Abgen, San Diego, CA, USA) or mouse anti-human CD44v6 monoclonal (AbD Serotec, Kidlington, UK) primary antibodies overnight in a humidified chamber at 4°C. The anti-HAS antibodies were used at a dilution of 1 : 50 dilution and the anti-CD44 antibody was diluted 1 : 100 in TBS with Tween 20 (TBST).

The next day, sections were washed twice in TBST for 5 min each time. Horseradish peroxidase-conjugated polyclonal pig anti-rabbit (Dako UK Ltd, Ely, Cambridgeshire, UK) and rabbit anti-mouse (Dako) IgGs were used as secondary antibodies. Secondary antibodies (1 : 200 dilution in PBS) were applied for 1 h at room temperature; slides were then washed in PBS, pH 7.4, for 5 min.

Signals from HAS1, HAS2, HAS3 and CD44 proteins were visualised with 3,3′-diaminobenzidine (liquid DAB+ substrate, chromogen system; Dako). The slides were counterstained in 10% Harris haematoxylin for 20 s and then washed under running water to remove excess stain from the slides. Finally, the slides were dehydrated in a gradient of ethanol before being mounted under a coverslip (VWR) with hard-setting DPX mounting medium (VWR).

Semiquantitative analysis of immunostaining

Immunostaining was assessed semiquantitatively using a histochemical score (H-score) system, ranging between 0 and 300, as described previously (Raheem et al. 2013), where a score of 0 corresponds to no staining and a score of 300 corresponds to maximum staining.

GC isolation for culture

Sheep ovaries were collected during the breeding season (October, November and December) from a local abattoir. Ovaries were transported to the laboratory within 1 h at 37°C in PBS. Intact ovaries were washed three times with PBS before and then again after one rinse with 70% ethanol. Thereafter, the ovaries were maintained in PBS containing 100 U mL–1 penicillin and 100 µg mL–1 streptomycin at 37°C during processing. GCs were collected by aspiration of healthy follicles within the 2–4 mm diameter range. Follicular fluid was aspirated using a 23-gauge needle mounted on a 10-mL syringe. The aspirate was passed through a 100-µm cell strainer to remove debris and oocytes; the resulting cell suspension was then centrifuged at 800g for 10 min. Erythrocyte contamination was removed by resuspending the cell pellet in 1 mL sterile water followed immediately by the addition of 20 mL McCoy's 5A medium to readjust osmolarity. The cell suspension was centrifuged at 800g for 5 min and resuspended in 1 mL culture medium as described below.

GC culture

The yield of viable cells was counted using Trypan blue exclusion on a haemocytometer. Cultures were set up containing 100 000 cells per well in 96-well plates. The culture plates were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Culture media were changed every second day and cultures were maintained for up to 96 h. The basic medium used for GC culture was McCoys 5A medium,

containing 20 mM HEPES, 100 U mL–1 penicillin, 100 μg mL–1 streptomycin, 2 mM l-glutamine, 2.5 μg mL–1 holotransferrin, 4 ng mL–1 sodium selenite and 1 mg mL–1 bovine serum albumin (BSA; Campbell et al. 1996; Allegrucci et al. 2003). This basic medium, including 1 ng mL–1 FSH and 10 ng mL–1 insulin, was used for control cultures. Where required for test samples, the medium was supplemented with individual hormones or combinations, as follows: for Experiment 2, E2 (0, 3.12, 12.5, 25, 50 ng mL–1); for Experiment 3, 1 ng mL–1 FSH, 10 ng mL–1 insulin, 10 ng mL–1 IGF-1, 40 ng mL–1 E2 and 25 ng mL–1 LH. The expression of HAS2, HAS3 and CD44 mRNA was determined after 48h culture.

Assessment of GC viability, proliferation and luteinisation

The viability of freshly isolated GCs was checked by the Trypan blue exclusion test before culture. GC proliferation in vitro was monitored by the cell Titer96 Aqueous one solution cell proliferation assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, in a 96-well plate, 20 μL cell titre solution was mixed with 100 μL fresh culture medium per well and incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO_2. This assay uses formazan to determine the number of viable cells at the time of culture termination. Tetrazolium salt reduction to formazan was detected at a wavelength of 490 nm using a microplate reader (Tecan, Reading, UK).

GC luteinisation was checked using radioimmunoassay (RIA) analysis of spent culture medium at 48 and 96 h to measure E2 and progesterone production, as described by Robinson et al. (2002). For GC cultures in Experiments 2 and 3, the concentrations of FSH and insulin applied were those that, in preliminary experiments, together resulted in an approximate 1 : 1 ratio of secreted E2 : progesterone throughout the culture period.

RNA isolation and reverse transcription

Culture medium was removed and cultured GC were washed using sterile PBS on ice. Total RNA was extracted through RNeasy Mini Kit columns (Qiagen, West Sussex, UK) according to the manufacturer's instructions. The concentration and purity of the isolated RNA samples was determined using an Infinite 200 NanoQuant (Tecan, Reading, UK). All samples had an absorbance at 260 : 280 nm (A260/280) ratio between 1.9 and 2.1. RNA integrity was evaluated using formaldehyde gel to determine the presence of intact 18S and 28S bands. Total RNA (500–1000 ng) was treated for potential genomic DNA carryover in a single reaction in accordance with the guidelines supplied by DNase (Promega, Southampton, UK). DNase-treated RNA was reverse transcribed using random hexamer primers and processed accordingly (Reverse Transcription System Kit; Promega).

Primer design and real-time PCR

Sequences of the genes of interest (HAS2, HAS3, CD44) and the housekeeping gene (β2 microglobulin, B2M) were obtained from GenBank published in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/gene). Primer sequences were designed online using primer3 web software (http://frodo.wi.mit.edu/primer3/input.htm, accessed 25 February 2011). Primer alignment specificity was checked using the BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Sequence information, accession numbers and expected product lengths, together with the running conditions, are given in Table 1. All oligonucleotides were commercially synthesised as highly purified salt-free products (MWG-Biotech, London, UK). The primers of each gene were tested by conventional PCR amplification using a Multiplex PCR kit (Qiagen), 25 ng cDNA and 0.5 μM primers. For each real-time PCR assay, the master mix contained a final concentration of 1× relative qPCR SYBR Green Mix (AB gene, Epsom, UK). The optimal annealing temperature (resulting in minimal threshold cycle (Ct)) was determined using the temperature gradient function of the real-time PCR machine (qPCR; CFX 96 Real-Time PCR Detection System; Bio-Rad) using identical reactions with control cDNA samples. A melting curve analysis was performed for each amplicon between 50°C and 95°C and the reading temperature was set so that any smaller non-specific products, such as dimers, were melted (if present) before fluorescence acquisition. The unknown cDNA samples (equivalent to 25 ng reverse-transcribed RNA) were amplified in duplicate in the same assay using a master mix containing 1× KAPA SYBR green mix (Anachem, Boston, MA, USA), forward and reverse primer mix (0.5 μM) and nuclease-free water in 20 μL reactions. Thermal cycling conditions applied to each assay consisted of an initial taq activation at 95°C for 15 min, followed by 38 cycles of denaturation (95°C for 30 s), annealing (61°C and 59°C for 30 s) for genes of interest and the housekeeping gene and extension (72°C for 20 s). A no-template control (NTC) was included on every plate for each set of primers. To minimise variation, all samples included in each analysis were derived from the same reverse transcription batch, prepared under the same conditions, and were analysed on a single plate in duplicate. The Ct values for unknown samples were obtained using a real-time PCR machine (Bio-Rad). Relative quantified expression of the genes evaluated across treatments was measured by comparing the Ct values of the gene of interest against that of the housekeeping gene using the ΔΔCt method (Hettinger et al. 2001; Luo and Wiltbank 2006).

Statistical analysis

In all experiments, data points were determined from at least three independent experiments. Gene expression data were analysed using SPSS version 19 (IBM United Kingdom Limited, Portsmouth, UK)

using a linear mixed model. If the treatment effect was significant, least significance difference (l.s.d.) tests were performed. Data are plotted as the mean ± s.e.m. Two-tailed $P \leq 0.05$ was considered significant. P-values between >0.05 and 0.1 were considered to indicate a non-significant tendency. Effects of treatment were compared with control at each time point individually, based on the mean fold change in mRNA level.

Results

Immunohistochemistry of the HA system in sheep ovarian follicles

As shown in Fig. 1a, HAS1, HAS2, HAS3 and CD44 proteins were present in the antral follicles. In addition, the intensity of HAS and CD44 immunostaining in the theca cell layer appeared to be less than the staining of the GC layer (Fig. 1a). The H-Score values for the corresponding proteins are shown in Fig. 2. The H-Score results are approximately double for HAS2 and HAS3 and nearly fourfold higher for CD44 presence in the GC layer compared with the theca cell layer. The presence of these proteins in stromal cells was negligible compared with follicular cells. There was a tendency for slightly more abundant HAS2 protein in the GC layer than HAS1 or HAS3 protein. There were no obvious differences in levels of HAS isoform proteins in the theca cell layer of antral follicles. The presence of CD44 in the theca cell layer was negligible.

Fig. 1b shows results obtained in the negative controls using rabbit and mouse IgG. The negative control for rabbit IgG was free from non-specific binding, whereas mouse IgG showed some non-specific staining in the follicular fluid and at the endothelial cells of blood vessels in particular. Therefore, CD44 staining in follicular fluid and endothelial cells was considered to be an artefact; however, CD44 staining observed in GC and theca cell layers was considered reliable because the negative control slides were always negative in these areas.

Effects of different concentrations of E2 on HAS2 mRNA expression in cultured GC

Treatment of GC cultures with E2 resulted in increased HAS2 expression. However, the timing of this effect varied according to the concentration of E2 used. At the lowest concentration (3.12 ng mL^{-1}) the increase in HAS2 expression was only evident after 96 h, whereas HAS2 expression in GC treated with higher concentrations of E2 (12.5–50 ng mL^{-1}) increased at 48 h culture, then decreased subsequently at 72 and 96 h ($P < 0.05$; Fig. 3). When the effect of E2 (regardless of concentration) on HAS2 was analysed statistically, a significant ($P < 0.05$) increase in HAS2 expression was evident at 48 h compared with 24 h (Fig. 4).

Effects of different concentrations of E2 on HAS3 mRNA expression in cultured GC

The effect of E2 on HAS3 expression was highly variable among different experimental replicates. HAS3 expression increased gradually with time and was significantly higher at 96 h than at 24 h after treatment with 12.5 and 50 ng mL–1 E2 ($P < 0.05$; Fig. 3). When the effect of E2 (regardless of concentration) on HAS3 was analysed statistically, no significant effect was detected at 48 h compared with 24 h (Fig. 4).

Effects of different concentrations of E2 on CD44 mRNA expression in cultured GC

The effect of E2 on CD44 was less marked and was not consistent among different concentrations of E2. An approximate 0.4-fold increase in CD44 mRNA expression was observed after 96 h compared with expression at 24 h in cells treated with 3.1 and 25 ng mL–1 E2, as well as at 48 h in cells treated with 12.5 ng mL–1 E2 ($P < 0.05$; Fig. 3). When the effect of E2 on CD44 was analysed regardless of the E2 concentration, a significant ($P < 0.05$) increase in CD44 expression was evident at 48 h compared with 24 h (Fig. 4).

Effects of FSH, LH, IGF-1, insulin and E2 on HAS2, HAS3 and CD44 expression HAS2

As shown in Fig. 5, compared with control, IGF-1 tended ($P = 0.058$) to increase HAS2 expression at 48 h in culture, with insulin showing a similar tendency. FSH or LH alone did not significantly change HAS2 expression; however, the combination of FSH and insulin tended ($P = 0.057$) to increase HAS2 expression. The combination of FSH + IGF-1 significantly increased HAS2 expression compared with control.

In the presence or absence of FSH or LH, E2 did not significantly alter HAS2 expression, whereas the combination of E2 and FSH together with insulin, in the presence or absence of IGF-1, significantly increased HAS2 expression compared with control and the FSH + E2-treated group. However, the addition of IGF-1 to this combination (i.e. insulin + FSH + E2) triggered a significant increase in HAS2 mRNA expression compared with control, FSH + E2 and FSH + insulin treatment.

HAS3

As shown in Fig. 5, LH + E2 treatment significantly increased HAS3 expression compared with all other treatments (including control) but excluding the combination of FSH + insulin + E2. All treatments except for the combination of LH + E2 had an effect similar to control and did not significantly change HAS3 expression. Further, the FSH + insulin + E2 treatment had a tendency to increase HAS3 expression compared with FSH + IGF-1 ($P = 0.055$) and insulin ($P = 0.071$) treatments. Although neither

LH nor E2 changed HAS3 expression independently, their combination (i.e. LH + E2) increased HAS3 expression compared with the effect either treatment alone.

CD44

As shown in Fig. 5, E2 and insulin significantly increased, whereas IGF-1 ($P = 0.071$) and FSH ($P = 0.060$) had a tendency to increase, CD44 expression compared with control. Interestingly, compared with control, E2 in the presence of FSH or insulin significantly increased CD44 expression, whereas the combination of E2 + FSH + insulin tended ($P = 0.065$) to increase CD44 expression to a similar extent. In addition, FSH + IGF-1 tended ($P = 0.054$) to increase CD44 expression compared with control. Further, LH in the presence or absence of E2 significantly increased CD44 expression compared with control. The combination of E2 with other factors, excluding IGF-1, generally had an effect on CD44 expression that was similar to that of E2 alone. Compared with FSH alone, the combination of either IGF-1 or insulin together with FSH did not change CD44 mRNA expression. The combination of FSH + IGF-1 + insulin + E2 significantly decreased CD44 expression compared with E2 in the presence or absence of LH.

Discussion

The present study has shown, for the first time, the presence of HAS isoforms and CD44 protein in ovine ovaries and has further demonstrated, in a GC culture model system, that expression of HAS2, HAS3 and CD44 is significantly stimulated by certain reproductive hormones acting alone or in combination.

HA is recognised as a cell signalling molecule that has effects on cell cascades, such as migration and proliferation, through interactions with cell surface receptors (Kosaki et al. 1999; Camenisch et al. 2000; Lee and Spicer 2000; Necas et al. 2008). Synthesis of HAS3 restricts the availability of HAS substrate (uridine diphosphate (UDP)) for the other HAS enzymes (Kultti et al. 2009). Of the three HAS isoforms (i.e. HAS1, HAS2, HAS3), HAS3 is the most resilient to UDP constraint (Rilla et al. 2013), whereas HAS1 is more sensitive to sugar shortage than HAS2 and HAS3. Both the precursor of E2 (cholesterol) and HAS substrate (UDP) originate from the phospholipid compartment of the cell membrane.

The H-Score data (Fig. 2) in the present study revealed that the intensity of HAS and CD44 staining was greater in GCs than in theca cells in antral follicles. HA, as a component of the follicular fluid (Saito et al. 2000; Ohta et al. 2001), is known for its role in cumulus cell expansion. Therefore, the presence of

HAS isoforms in GCs is not surprising. However, the parallel presence of the CD44 receptor was not previously appreciated. This may underlie a range of effects of HA in the reproductive system, possibly facilitating the close control of synthesis and degradation of different lengths of HA molecules in follicles at different stages of development and in different phases of the reproductive cycle. Further investigation of HAS and CD44 intensity in follicular cells of oestrus synchronised ewes may shed further light on the factors influencing the protein content of antral follicles.

The HAS isoforms are responsible for the production of different lengths of HA (Itano et al. 1999; Itano and Kimata 2002). Therefore, we consider that coordination between the ratio of HAS enzymes and their products to CD44 in sheep follicles is important in order to accumulate HA in an appropriate and timely manner as the antral follicle approaches ovulation. Positive staining was observed for HAS2 and HAS3 in the blood vessels and stromal layer of ovarian sections in the present study. HAS isoforms and CD44 expression in human and rat vascular smooth muscle, respectively, have been reported previously (Jain et al. 1996; Sainio et al. 2010; Freudenberger et al. 2011).

Having demonstrated the presence of HA synthesis and signalling components, we wanted to understand the hormonal regulation of HAS and CD44 in the ovary. Therefore, we studied the expression of these components in response to several endocrine signals in a model system, namely GC culture.

The present study has shown that E2 significantly stimulates the expression of HAS2 and CD44 over a 48 h culture period (Fig. 4). The concentrations of E2 selected for experimentation fall within the physiological concentrations in follicular fluid (Tsonis et al. 1984). Oestradiol receptor (ER) β is present in GCs (Hall et al. 2001; Jacob et al. 2006; Juengel et al. 2006; Deroo et al. 2009). Not only does ERβ interact with ERα, but it also regulates cell signalling cascades associated with E2 (Pettersson et al. 1997; Koehler et al. 2005; Galluzzo et al. 2007; Zhao et al. 2008).

There is a complex feedback system associated with the synthesis and signalling of HA and E2, which may explain why the effects observed in our cultures were quite variable. For example, E2 stimulates many intracellular cascades involving phosphatidylinositol 3-kinase and mitogen-activated protein kinase (MAPK). Conversely, MAPKs affect steroidogenesis by regulating steroidogenic acute regulatory protein (StAR) expression (Moriarty et al. 2006; Watson et al. 2012; Manna and Stocco 2011). Similarly, the presence of gonadotropins (FSH or LH) plus E2 enhanced both HAS2 and HAS3 expression in our cultures. Gonadotropins have been reported to enhance HAS2 mRNA expression in bovine cumulus–

oocyte complexes (Schoenfelder and Einspanier 2003). This suggests that the pathway controlling HAS2 and HAS3 may have a link to E2 or gonadotropin pathways.

There was no substantial effect of the treatments evaluated on CD44 expression in our culture system. It seems that CD44 expression may be associated with the level of HA production rather than HAS expression (Nykopp et al. 2009).

The results of the present study indicate that insulin and IGF-1 are more effective than FSH and E2 at enhancing HAS2 expression. The effects of LH on HAS3 expression surpassed those of insulin and FSH, and LH enhanced the effects of E2 in combination with insulin and FSH on HAS3 expression. Therefore, LH has the predominant effect on HAS3. In addition, the results of the present study indicate that E2 is an important hormone regulating CD44 expression in sheep GC because E2 enhanced the effect of FSH on CD44 whereas insulin did not. We postulate that IGF-1, LH and E2 are the decisive factors in HAS2, HAS3 and CD44 expression, respectively. In addition, insulin seems to be a codeterminant of HAS2 and CD44 expression.

Together, the results of the present study indicate that endocrine control of some HA system components (HAS2, HAS3 and CD44) in sheep GCs occurs in a discrete and potentially complementary manner. This work promotes the idea that HA is not solely a physical component of the follicle, but that its signalling role is affected by factors that control female reproduction. The details of HA’s signalling capacity remain to be elucidated.

Table 1. Oligonucleotide primer sequences and amplicon sizes, and their corresponding annealing and plate reading temperatures, used for quantitative polymerase chain reaction assays
HAS, hyaluronan synthase; B2M, beta 2 microglobulin

Gene	Primer sequence (5'-3')	Size (bp)	Accession no.	Temp (°C) Annealing	Temp (°C) Reading
HAS2	Forward: GGGGGAGATGTCCAGATTTT Reverse: ATGCACTGGACACATCCGA	125	NM174079	61	74
HAS3	Forward: ACAGGTTTCTTCCCCTTCTTCC Reverse: GCGACATGAAGATCATCTCTGC	166	NM001192867.1	61	78
CD44	Forward: TATAACCTGCCGATATGCAGG Reverse: CAGCACAGATGGAATTGGG	221	NM174013.3	61	80
B2M	Forward: CGCCAGAAGATGGAAAGC Reverse: GAACTCAGCGTGGGACAGA	167	NM001009284.1	59	76.5

Fig. 1. Immunohistochemical localisation of hyaluronan synthase (HAS) isoforms (HAS 1–3) and the HAS receptor (CD44) in ovine antral follicles. (a) Localisation of HAS1, HAS2, HAS3 and CD44. The intensity of staining is greatest for HAS2, followed by HAS3 and then HAS1. Staining is more intense in the granulosa cell (GC) layer than the theca cell layer. Some staining is apparent in the endothelial layer of blood vessels and the follicular fluid. TCE, theca cell externa; TCI. theca cell interna. (b) Negative controls using rabbit and mouse IgG. Note some staining of endothelial cells of blood vessels and the follicular fluid in the mouse IgG control. Theca cells and GCs were not stained in the negative controls.

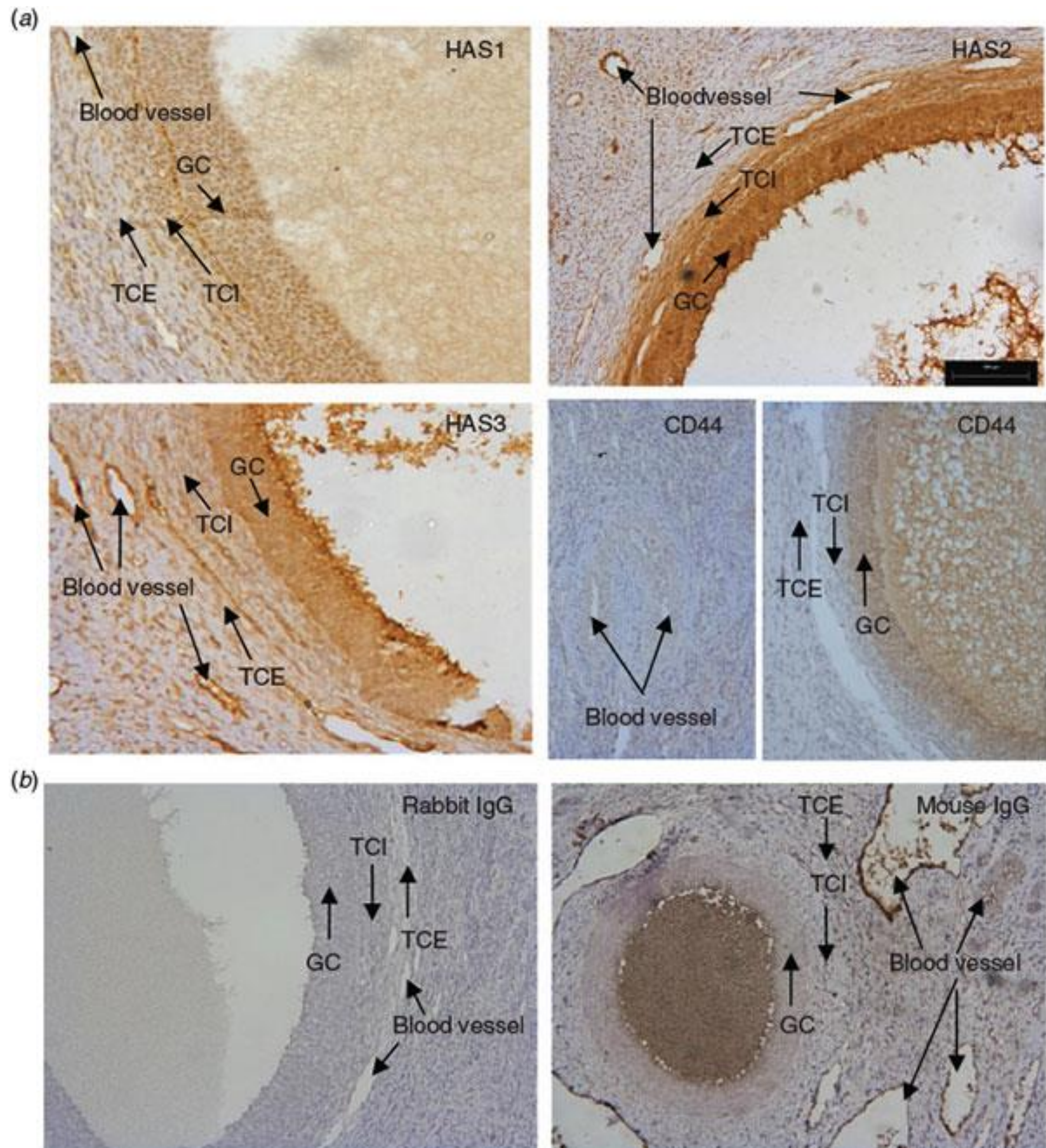


Fig. 2. Semiquantitative measurement of hyaluronan synthase (HAS) isoforms (HAS 1–3) and CD44 proteins immunolocalised in ovine antral follicles using the histochemical score (H-Score) system. Data are given as the mean ± s.e.m. The significance of differences in mean values was evaluated using t-tests (independent sample). Columns with different letters differ significantly (P < 0.05).

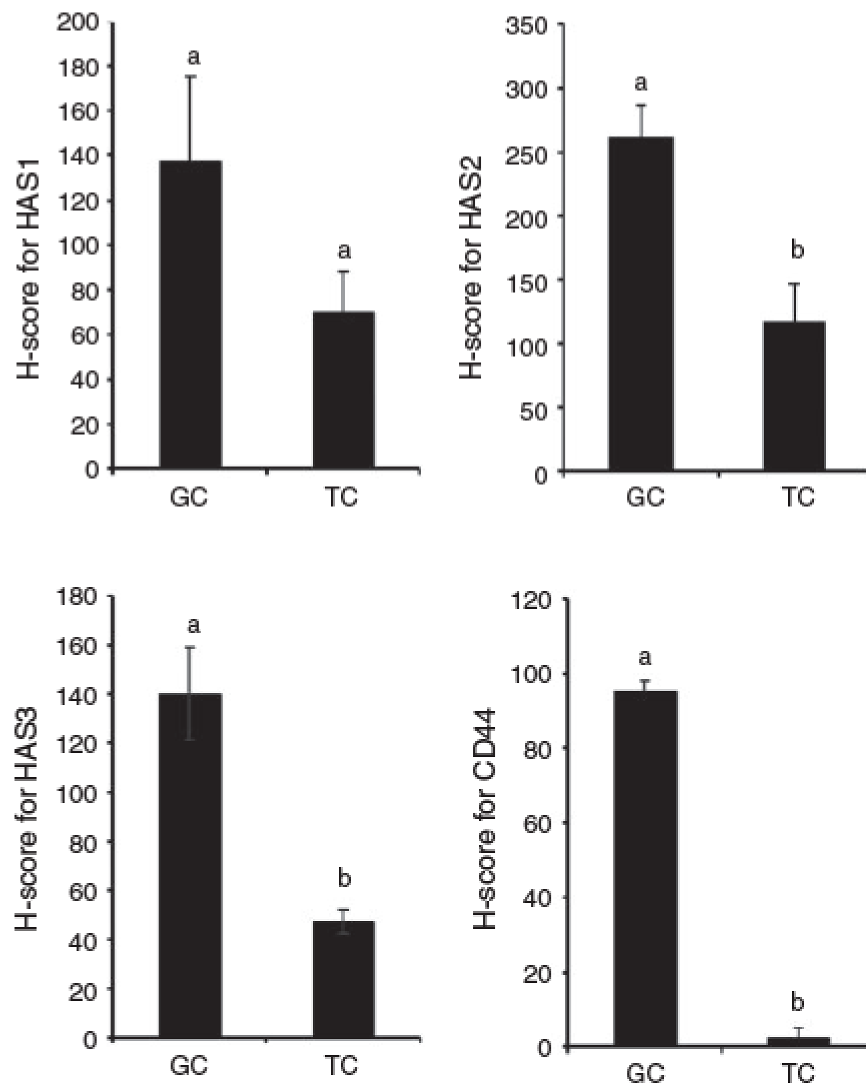


Fig. 3. Hyaluronan synthase (HAS) 2, HAS3 and CD44 expression in granulosa cells in the presence of 3.1, 12.5, 25 and 50 ng mL–1 17β-oestradiol (E2). Data are the mean ± s.e.m. The significance of differences in mean values was evaluated using a linear mixed model followed by l.s.d. pairwise comparison. For each concentration of E2, values with different letters differ significantly ($P < 0.05$).

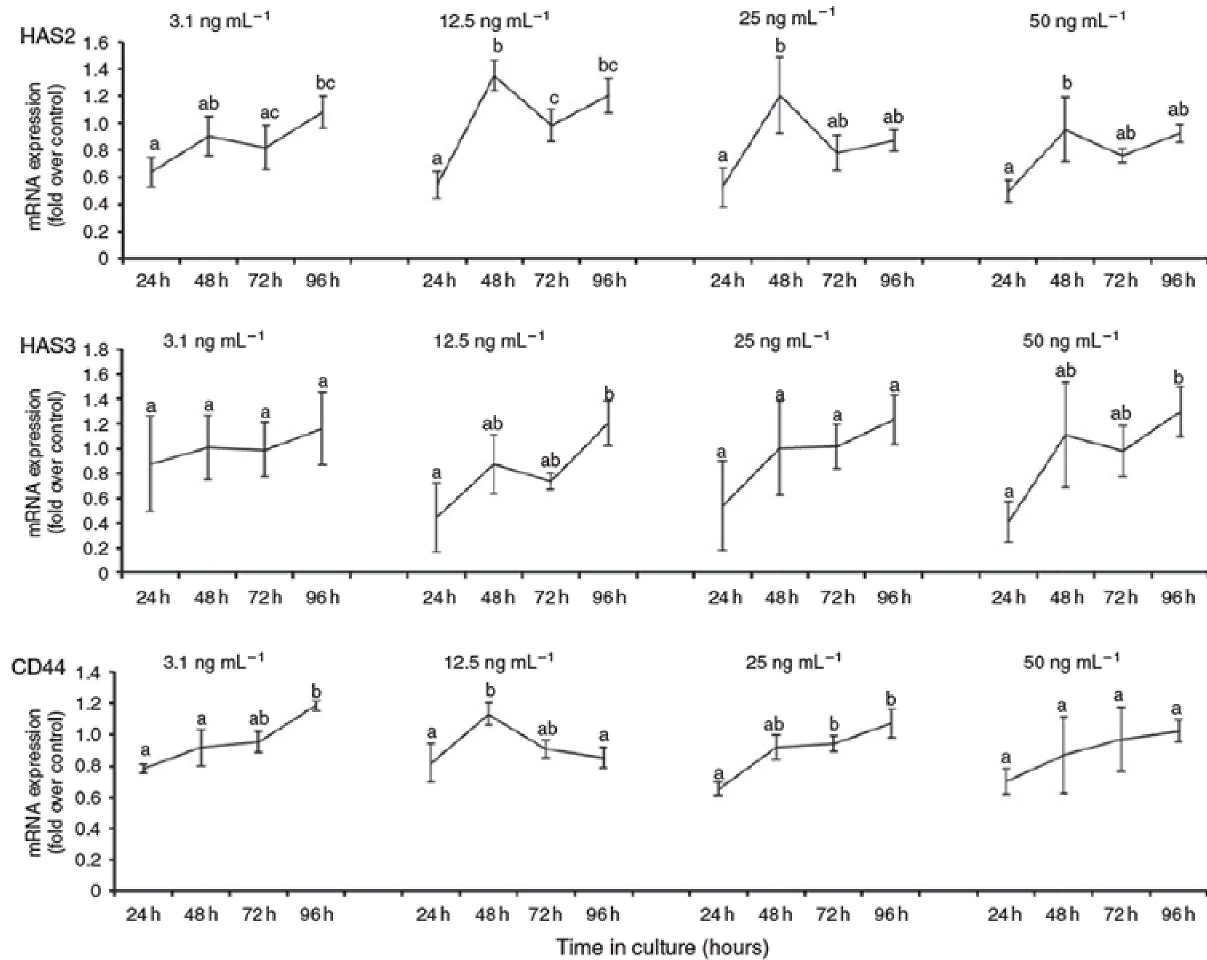


Fig. 4. Hyaluronan synthase (HAS) 2, HAS3 and CD44 expression in granulosa cells in response to 17β-oestradiol (E2) supplementation. Data are the mean ± s.e.m. The significance of differences in mean values was evaluated using a linear mixed model. Columns with different letters differ significantly ($P < 0.05$).

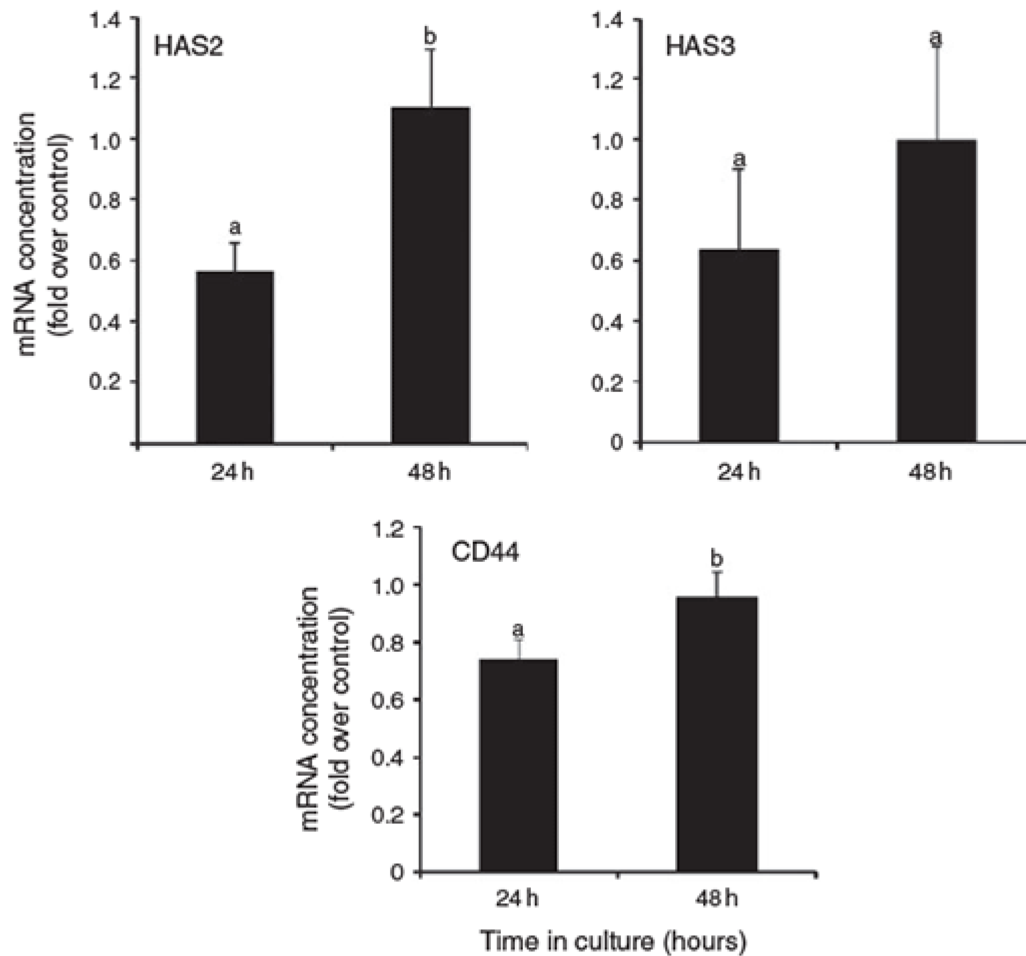
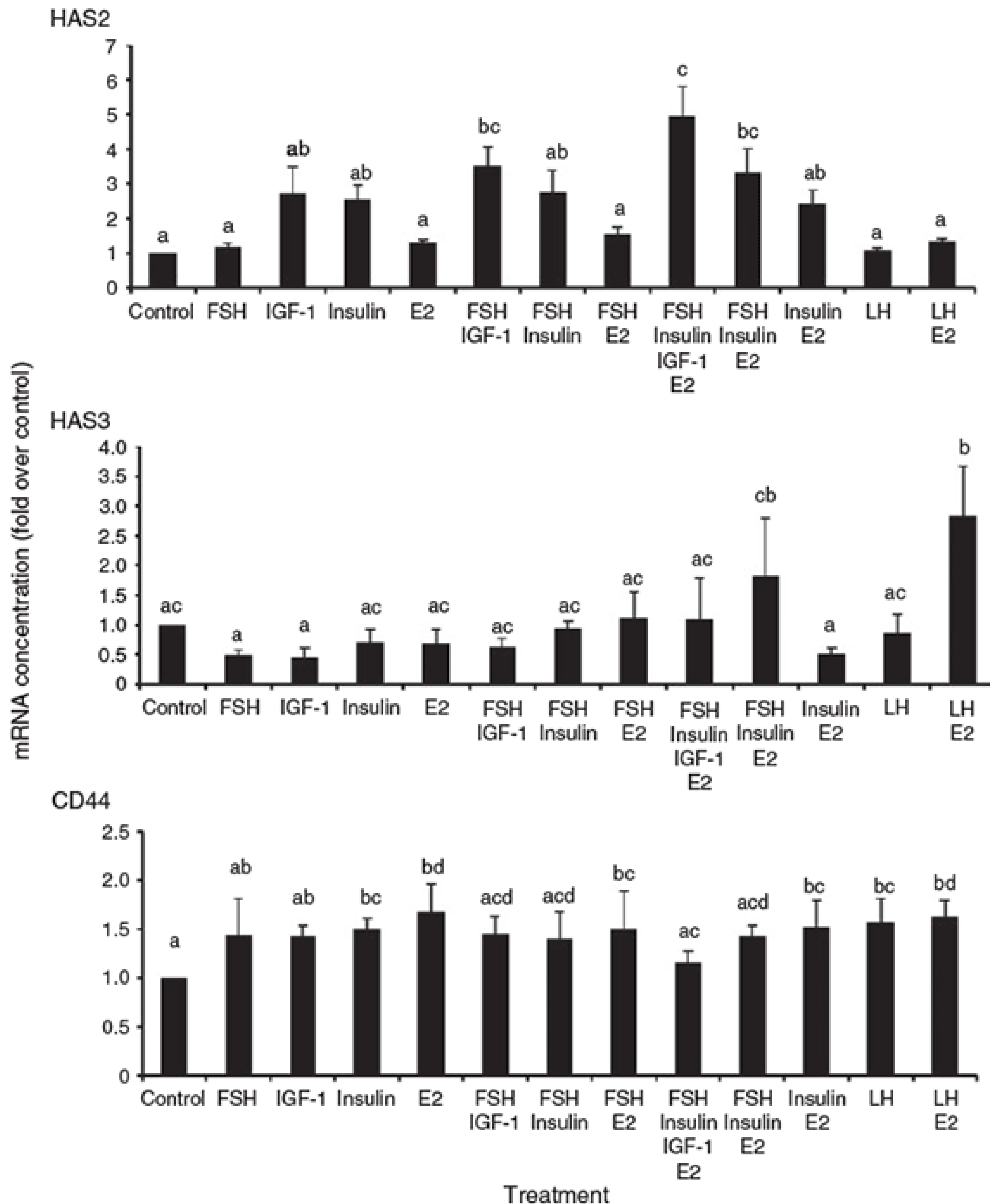


Fig. 5. Effects of FSH, insulin, insulin-like growth factor (IGF)-1, 17β-oestradiol (E2) (1 ng mL–1 FSH, 10 ng mL–1 insulin, 10 ng mL–1 IGF-1, 40 ng mL–1 E2 and 25 ng mL–1 LH) and their combination on hyaluronan synthase (HAS) 2, HAS3 and CD44 expression in granulosa cells cultured for 48 h. Data are the mean ± s.e.m. The significance of differences in mean values was evaluated using a linear mixed model. Columns with different letters differ significantly ($P < 0.05$).



References

Adamia, S., Maxwell, C. A., and Pilarski, L. M. (2005). Hyaluronan and hyaluronan synthases: potential therapeutic targets in cancer. Curr. Drug Targets Cardiovasc. Haematol. Disord. 5, 3–14. doi:10.2174/ 1568006053005056

Allegrucci, C., Hunter, M. G., Webb, R., and Luck, M. R. (2003). Interaction of bovine granulosa and theca cells in a novel serum-free co-culture system. Reproduction 126, 527–538. doi:10.1530/REP.0.1260527

Baerwald, A. R., Adams, G. P., and Pierson, R. A. (2012). Ovarian antral folliculogenesis during the human menstrual cycle: a review. Hum. Reprod. Update 18, 73–91. doi:10.1093/HUMUPD/DMR039

Bernert, B. (2013). Importance of hyaluronan metabolism and signalling in tumour progression. Doctorate thesis, Uppsala University. Bodevin-Authelet, S., Kusche-Gullberg, M., Pummill, P. E., DeAngelis, P. L., and Lindahl, U. (2005). Biosynthesis of hyaluronan: direction of chain elongation. J. Biol. Chem. 280, 8813–8818. doi:10.1074/JBC. M412803200

Camenisch, T. D., Spicer, A. P., Brehm-Gibson, T., Biesterfeldt, J., Augustine, M. L., Calabro, A., Kubalak, S., Klewer, S. E., and McDonald, J. A. (2000). Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. J. Clin. Invest. 106, 349–360. doi:10.1172/JCI10272

Campbell, B. K., Scaramuzzi, R. J., and Webb, R. (1996). Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media. J. Reprod. Fertil. 106, 7–16. doi:10.1530/JRF.0.1060007

Campbell, B. K., Dobson, H., Baird, D. T., and Scaramuzzi, R. J. (1999). Examination of the relative role of FSH and LH in the mechanism of ovulatory follicle selection in sheep. J. Reprod. Fertil. 117, 355–367. doi:10.1530/JRF.0.1170355

Deroo, B. J., Rodriguez, K. F., Couse, J. F., Hamilton, K. J., Collins, J. B., Grissom, S. F., and Korach, K. S. (2009). Estrogen receptor b is required for optimal cAMP production in mouse granulosa cells. Mol. Endocrinol. 23, 955–965. doi:10.1210/ME.2008-0213

El Maradny, E., Kanayama, N., Kobayashi, H., Hossain, B., Khatun, S., Liping, S., Kobayashi, T., and Terao, T. (1997). The role of hyaluronic acid as a mediator and regulator of cervical ripening. Hum. Reprod. 12, 1080–1088. doi:10.1093/HUMREP/12.5.1080

Erickson, M., and Stern, R. (2012). Chain gangs: new aspects of hyaluronan metabolism. Biochem. Res. Int. 2012, Article ID 893947. doi:10.1155/ 2012/893947

Fortune, J. E., Rivera, G. M., and Yang, M. Y. (2004). Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. Anim. Reprod. Sci. 82–83, 109–126. doi:10.1016/ J.ANIREPROSCI.2004.04.031

Freudenberger, T., Rack, K., Dai, G., Dorn, S., Mayer, P., Heim, H.-K., and Fischer, J. W. (2011). Estradiol inhibits hyaluronic acid synthase 1 expression in human vascular smooth muscle cells. Basic Res. Cardiol. 106, 1099–1109. doi:10.1007/S00395-011-0217-5

Galluzzo, P., Caiazza, F., Moreno, S., and Marino, M. (2007). Role of ERb palmitoylation in the inhibition of human colon cancer cell proliferation. Endocr. Relat. Cancer 14, 153–167. doi:10.1677/ERC-06-0020
Hall, J. M., Couse, J. F., and Korach, K. S. (2001). The multifaceted mechanisms of estradiol and estrogen receptor signaling. J. Biol. Chem. 276, 36 869–36 872. doi:10.1074/JBC.R100029200

Hettinger, A. M., Allen, M. R., Zhang, B. R., Goad, D. W., Malayer, J. R., and Geisert, R. D. (2001). Presence of the acute phase protein, bikunin, in the endometrium of gilts during estrous cycle and early pregnancy. Biol. Reprod. 65, 507–513. doi:10.1095/BIOLREPROD65.2.507

Hunter, M. G., Robinson, R. S., Mann, G. E., and Webb, R. (2004). Endocrine and paracrine control of follicular development and ovulation rate in farm species. Anim. Reprod. Sci. 82–83, 461–477. doi:10.1016/ J.ANIREPROSCI.2004.05.013

Itano, N. (2008). Simple primary structure, complex turnover regulation and multiple roles of hyaluronan. J. Biochem. 144, 131–137. doi:10.1093/JB/ MVN046

Itano, N., and Kimata, K. (2002). Mammalian hyaluronan synthases.IUBMB Life 54, 195–199. doi:10.1080/15216540214929

Itano, N., Sawai, T., Yoshida, M., Lenas, P., Yamada, Y., Imagawa, M., Shinomura, T., Hamaguchi, M., Yoshida, Y., Ohnuki, Y., Miyauchi, S., Spicer, A. P., McDonald, J. A., and Kimata, K. (1999). Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. J. Biol. Chem. 274, 25 085–25 092. doi:10.1074/JBC.274.35.25085

Jacob, J., Sebastian, K. S., Devassy, S., Priyadarsini, L., Farook, M. F., Shameem, A., Mathew, D., Sreeja, S., and Thampan, R. V. (2006). Membrane estrogen receptors: genomic actions and post transcriptional regulation. Mol. Cell. Endocrinol. 246, 34–41. doi:10.1016/J.MCE. 2005.11.015

Jain, M., He, Q., Lee, W.-S., Kashiki, S., Foster, L. C., Tsai, J.-C., Lee, M.-E., and Haber, E. (1996). Role of CD44 in the reaction of vascular smooth muscle cells to arterial wall injury. J. Clin. Invest. 97, 596–603. doi:10.1172/JCI118455

Juengel, J. L., Heath, D. A., Quirke, L. D., and McNatty, K. P. (2006). Oestrogen receptor a and b, androgen receptor and progesterone receptor mRNA and protein localisation within the developing ovary and in small growing follicles of sheep. Reproduction 131, 81–92. doi:10.1530/ REP.1.00704

Koehler, K. F., Helguero, L. A., Haldose´n, L.-A., Warner, M., and Gustafsson, J.-A. (2005). Reflections on the discovery and significance of estrogen receptor b. Endocr. Rev. 26, 465–478. doi:10.1210/ER.2004- 0027

Kosaki, R., Watanabe, K., and Yamaguchi, Y. (1999). Overproduction of hyaluronan by expression of the hyaluronan synthase Has2 enhances anchorage-independent growth and tumorigenicity. Cancer Res. 59, 1141–1145.

Kultti, A., Pasonen-Seppanen, S., Jauhiainen, M., Rilla, K. J., Karna, R., Pyoria, E., Tammi, R. H., and Tammi, M. I. (2009). 4-Methylumbelliferone inhibits hyaluronan synthesis by depletion of cellular UDP– glucuronic acid and downregulation of hyaluronan synthase 2 and 3. Exp. Cell Res. 315, 1914–1923. doi:10.1016/J.YEXCR.2009.03.002

Laurent, T. C., Laurent, U. B., and Fraser, J. R. (1995). Functions of hyaluronan. Ann. Rheum. Dis. 54, 429. doi:10.1136/ARD.54.5.429

Lee, J. Y., and Spicer, A. P. (2000). Hyaluronan: a multifunctional, megaDalton, stealth molecule. Curr. Opin. Cell Biol. 12, 581–586. doi:10.1016/S0955-0674(00)00135-6

Luo, W., and Wiltbank, M. C. (2006). Distinct regulation by steroids of messenger RNAs for FSHR and CYP19A1 in bovine granulosa cells. Biol. Reprod. 75, 217. doi:10.1095/BIOLREPROD.105.047407

Manna, P. R., and Stocco, D. M. (2011). The role of specific mitogenactivated protein kinase signaling cascades in the regulation of steroidogenesis. J. Signal Transduct. 2011, Article ID 821615. doi:10.1155/2011/821615

Marei, W. F., Ghafari, F., and Fouladi-Nashta, A. A. (2012). Role of hyaluronic acid in maturation and further early embryo development of bovine oocytes. Theriogenology 78, 670–677. doi:10.1016/J.THERIOGENOLOGY.2012.03.013

Marei, W. F. A., Salavati, M., and Fouladi-Nashta, A. A. (2013). Critical role of hyaluronidase-2 during preimplantation embryo development. Mol. Hum. Reprod. 19, 590–599. doi:10.1093/MOLEHR/GAT032

Marzioni, D., Crescimanno, C., Zaccheo, D., Coppari, R., Underhill, C. B., and Castellucci, M. (2001). Hyaluronate and CD44 expression patterns in the human placenta throughout pregnancy. Eur. J. Histochem. 45, 131–140.

Miyake, Y., Matsumoto, H., Yokoo, M., Miyazawa, K., Kimura, N., Tunjung, W. A. S., Shimizu, T., Sasada, H., Aso, H., and Yamaguchi, T. (2006). Expression and glycosylation with polylactosamine of CD44 antigen on macrophages during follicular atresia in pig ovaries. Biol. Reprod. 74, 501–510. doi:10.1095/BIOLREPROD.105.045641

Miyake, Y., Sakurai, M., Tanaka, S., Tunjung, W. A. S., Yokoo, M., Matsumoto, H., Aso, H., Yamaguchi, T., and Sato, E. (2009). Expression of hyaluronan synthase 1 and distribution of hyaluronan during follicular atresia in pig ovaries. Biol. Reprod. 80, 249–257. doi:10.1095/BIOLREPROD.108.067694

Moriarty, K., Kim, K. H., and Bender, J. R. (2006). Estrogen receptormediated rapid signaling. Endocrinology 147, 5557–5563. doi:10.1210/ EN.2006-0729

Necas, J., Bartosikova, L., Brauner, P., and Kolar, J. (2008). Hyaluronic acid (hyaluronan): a review. Vet. Med. (Praha) 53, 397–411.

Nykopp, T. K., Rilla, K., Sironen, R., Tammi, M. I., Tammi, R. H., Hamalainen, K., Heikkinen, A.-M., Komulainen, M., Kosma, V.-M., and Anttila, M. (2009). Expression of hyaluronan synthases (HAS1–3) and hyaluronidases (HYAL1–2) in serous ovarian carcinomas: inverse correlation between HYAL1 and hyaluronan content. BMC Cancer 9, 143. doi:10.1186/1471-2407-9-143

Nykopp, T. K., Rilla, K., Tammi, M. I., Tammi, R. H., Sironen, R., Hamalainen, K., Kosma, V.-M., Heinonen, S., and Anttila, M. (2010). Hyaluronan synthases (HAS1–3) and hyaluronidases (HYAL1–2) in the accumulation of hyaluronan in endometrioid endometrial carcinoma. BMC Cancer 10, 512. doi:10.1186/1471-2407-10-512

Ohta, N., Saito, H., Kuzumaki, T., Takahashi, T., Ito, M. M., Saito, T., Nakahara, K., and Hiroi, M. (1999). Expression of CD44 in human cumulus and mural granulosa cells of individual patients in in-vitro fertilization programmes. Mol. Hum. Reprod. 5, 22–28. doi:10.1093/ MOLEHR/5.1.22

Ohta, N., Saito, H., Kaneko, T., Yoshida, M., Takahashi, T., Saito, T., Nakahara, K., and Hiroi, M. (2001). Soluble CD44 in human ovarian follicular fluid. J. Assist. Reprod. Genet. 18, 21–25. doi:10.1023/A:1026494528415

Perry, K., Haresign, W., Wathes, D. C., and Khalid, M. (2010). Hyaluronan (HA) content, the ratio of HA fragments and the expression of CD44 in the ovine cervix vary with the stage of the oestrous cycle. Reproduction 140, 133–141. doi:10.1530/REP-09-0424

Perry, K., Haresign, W., Wathes, D. C., Pitsillides, A. A., and Khalid, M. (2012). Cervical expression of hyaluronan synthases varies with the stage of the estrous cycle in the ewe. Theriogenology 77, 1100–1110. doi:10.1016/J.THERIOGENOLOGY.2011.10.012

Pettersson, K., Grandien, K., Kuiper, G. G. J. M., and Gustafsson, J.-A. k. (1997). Mouse estrogen receptor b forms estrogen response elementbinding heterodimers with estrogen receptor a. Mol. Endocrinol. 11, 1486–1496.

Raheem, K. A., Marei, W. F., Mifsud, K., Khalid, M., Wathes, D. C., and Fouladi-Nashta, A. A. (2013). Regulation of the hyaluronan system in ovine endometrium by ovarian steroids. Reproduction 145, 491–504. doi:10.1530/REP-13-0001

Rilla, K., Oikari, S., Jokela, T. A., Hyttinen, J. M. T., Karna, R., Tammi, R. H., and Tammi, M. I. (2013). Hyaluronan synthase 1 (HAS1) requires higher cellular UDP-GlcNAc concentration than HAS2 and HAS3. J. Biol. Chem. 288, 5973–5983. doi:10.1074/JBC.M112.443879

Robinson, R. S., Pushpakumara, P. G., Cheng, Z., Peters, A. R., Abayasekara, D. R., and Wathes, D. C. (2002). Effects of dietary polyunsaturated fatty acids on ovarian and uterine function in lactating dairy cows. Reproduction 124, 119–131. doi:10.1530/REP.0.1240119

Sainio, A., Jokela, T., Tammi, M. I., and Ja¨rvela¨inen, H. (2010). Hyperglycemic conditions modulate connective tissue reorganization by human vascular smooth muscle cells through stimulation of hyaluronan synthesis. Glycobiology 20, 1117–1126. doi:10.1093/GLYCOB/CWQ076

Saito, H., Kaneko, T., Takahashi, T., Kawachiya, S., Saito, T., and Hiroi, M. (2000). Hyaluronan in follicular fluids and fertilization of oocytes. Fertil. Steril. 74, 1148–1152. doi:10.1016/S0015-0282(00)01586-7

Scaramuzzi, R. J., Baird, D. T., Campbell, B. K., Driancourt, M. A., Dupont, J., Fortune, J. E., Gilchrist, R. B., Martin, G. B., McNatty, K. P., McNeilly, A. S., Monget, P., Monniaux, D., Vinoles, C., and Webb, R. (2011). Regulation of folliculogenesis and the determination of ovulation rate in ruminants. Reprod. Fertil. Dev. 23, 444–467. doi:10.1071/RD09161

Schoenfelder, M., and Einspanier, R. (2003). Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle. Biol. Reprod. 69, 269–277. doi:10.1095/BIOLREPROD.102.011577

Silva, J. R. V., Figueiredo, J. R., and van den Hurk, R. (2009). Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. Theriogenology 71, 1193–1208. doi:10.1016/J.THERIOGENOLOGY.2008.12.015

Stern, R. (2004). Hyaluronan catabolism: a new metabolic pathway. Eur. J. Cell Biol. 83, 317–325. doi:10.1078/0171-9335-00392

Tempel, C., Gilead, A., and Neeman, M. (2000). Hyaluronic acid as an antiangiogenic shield in the preovulatory rat follicle. Biol. Reprod. 63, 134–140. doi:10.1095/BIOLREPROD63.1.134

Tienthai, P., Yokoo, M., Kimura, N., Heldin, P., Sato, E., and RodriguezMartinez, H. (2003). Immunohistochemical localization and expression of the hyaluronan receptor CD44 in the epithelium of the pig oviduct during oestrus. Reproduction 125, 119–132. doi:10.1530/REP.0. 1250119

Toole, B. P. (2001). Hyaluronan in morphogenesis. Semin. Cell Dev. Biol. 12, 79–87. doi:10.1006/SCDB.2000.0244

Toole, B. P. (2004). Hyaluronan: from extracellular glue to pericellular cue. Nat. Rev. Cancer 4, 528–539. doi:10.1038/NRC1391

Toole, B. P., Wight, T. N., and Tammi, M. I. (2002). Hyaluronan–cell interactions in cancer and vascular disease. J. Biol. Chem. 277, 4593–4596. doi:10.1074/JBC.R100039200

Tsonis, C. G., Carson, R. S., and Findlay, J. K. (1984). Relationships between aromatase activity, follicular fluid oestradiol-17 beta and testosterone concentrations, and diameter and atresia of individual ovine follicles. J. Reprod. Fertil. 72, 153–163. doi:10.1530/JRF.0.0720153

Ulbrich, S. E., Schoenfelder, M., Thoene, S., and Einspanier, R. (2004). Hyaluronan in the bovine oviduct: modulation of synthases and receptors during the estrous cycle. Mol. Cell. Endocrinol. 214, 9–18. doi:10.1016/J.MCE.2003.12.002

Watson, C. S., Jeng, Y.-J., Hu, G.,Wozniak, A., Bulayeva, N., and Guptarak, J. (2012). Estrogen- and xenoestrogen-induced ERK signaling in pituitary tumor cells involves estrogen receptor-a interactions with G protein-ai and caveolin I. Steroids 77, 424–432. doi:10.1016/J.STEROIDS.2011.12.025

Weigel, P. H., and DeAngelis, P. L. (2007). Hyaluronan synthases: a decadeplus of novel glycosyltransferases. J. Biol. Chem. 282, 36 777–36 781. doi:10.1074/JBC.R700036200

Zhao, C., Dahlman-Wright, K., and Gustafsson, J.-A. (2008). Estrogen receptor b: an overview and update. Nucl. Recept. Signal. 6, e003.