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Characterisation of the cellular and molecular responses of ovine oocytes and their supporting somatic cells to pre-ovulatory levels of LH and FSH during *in vitro* maturation

Matthew Cotterill, Sally L Catt[1] and Helen M Picton

Division of Reproduction and Early Development Research Group, Faculty of Medicine and Health, Leeds Institute for Genetics, Health and Therapeutics, University of Leeds, Clarendon Way, Leeds LS2 9JT, UK and [1]Department of Obstetrics and Gynaecology, Monash Institute of Medical Research, 27-31 Wright Street, Clayton, Victoria 3168, Australia

Correspondence should be addressed to M Cotterill; Email: medmcot@leeds.ac.uk

Abstract

The response of Graafian follicles to pre-ovulatory surge levels of FSH and LH *in vivo* triggers the terminal differentiation of granulosa cells and oocyte maturation. In polyovular species, the LH-driven signalling uses the epidermal growth factor (EGF)-like ligands AREG, EREG and BTC to promote oocyte maturation and cumulus expansion. This experimental series used a physiologically relevant ovine *in vitro* maturation (IVM) system to evaluate the impact of exposure to pre-ovulatory levels (100 ng/ml) of LH and FSH on ovine cumulus cell expression of EGF-like ligands *in vitro*. The serum-free sheep IVM system supported high levels (91.4%) of gonadotrophin-induced maturation of cumulus-enclosed oocytes and embryo development to the blastocyst stage (34.5%). Results were equivalent to a serum-based IVM system (85.1% IVM, 25.8% blastocyst rate; $P > 0.05$) but were significantly different ($P < 0.05$) to serum-free medium without gonadotrophins (69.5% IVM; 8.0% blastocyst rate). Ovine *BTC* was cloned and sequenced. Gonadotrophin-induced *AREG*, *EREG*, *BTC* and *EGFR* expressions were quantified in cumulus and mural granulosa cells during IVM. A rapid induction of *AREG* expression was apparent in both cell types within 30 min of gonadotrophin exposure *in vitro*. *LHCGR* (*LHR*) was detected in mural cells and *FSHR* in both cumulus and mural granulosa cells. The data confirm the involvement of *AREG* and *EGFR* during gonadotrophin-induced cumulus expansion, oocyte maturation and the acquisition of developmental competence by sheep oocytes matured *in vitro*.

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Introduction

It is widely accepted that the mutual cooperation between the cellular compartments of the follicle and the presumptive gamete is critical for oocyte developmental competence (Russell & Robker 2007). The importance of this relationship can be attributed to the need for autocrine and paracrine signalling to allow essential nutrients, proteins and growth factors to promote follicle and oocyte growth (Gilchrist *et al*. 2006, Hussein *et al*. 2006, Cecconi *et al*. 2008) and to regulate oocyte and cumulus metabolism (Sugiura *et al*. 2007). Nuclear and cytoplasmic maturation of mammalian oocytes occurs *in vivo* following a prolonged period of growth and development within ovarian follicles. During this time, oocytes accumulate the full complement of RNA and proteins needed to support their nuclear progression to metaphase II (MII) as well as fertilisation and the early development of the zygote before embryonic genome activation (Picton *et al*. 1998). The resumption of oocyte meiotic maturation is driven by the pre-ovulatory surge of LH and FSH that trigger a cascade of events in the cumulus–oocyte complex (COC) and drive meiotic progression of the oocyte and cumulus mucification and expansion (Su *et al*. 2002, Russell & Robker 2007, Gilchrist *et al*. 2008).

In a direct response to the pre-ovulatory trigger of the LH surge, studies on mice have revealed that three members of the epidermal growth factor (EGF) family, amphiregulin (*Areg*), epiregulin (*Ereg*) and betacellulin (*Btc*), were up-regulated in theca and mural granulosa cells following binding of LH to its cognate receptors (Park *et al*. 2004). Subsequently, limited expression of the EGF-like ligands was also detected in murine cumulus cells (Shimada *et al*. 2006). Furthermore, studies on pig (Prochazka *et al*. 2011) and human (Zamah *et al*. 2010) follicles suggest a link between AREG and oocyte developmental competence. The release of AREG, EREG and BTC is thought to facilitate communication via the cumulus cells through receptor binding with EGF receptors (EGFR, ERBB1, ERBB3 and ERBB4), therefore producing a target cell response via the MAPK signalling

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cascade (Conti *et al*. 2006). Furthermore, it has been shown in mice that prostaglandin endoperoxide synthase 2 (*Ptgs2*), tumour necrosis factor-stimulated gene 6 (*Tnfaip6* (*Tsg6*)) and hyaluronan synthase 2 (*Has2*) are up-regulated through this response (Park *et al*. 2004). To add further evidence to the significance of these findings, murine experiments have shown that the stimulation of oocyte-secreted factors with FSH activates MAPK3/1 and MAPK14 pathways that increase *Has2*, *Tnfaip6*, *Ptgs2* and pentaxin-3 (*Ptx3*) expression allowing cumulus expansion (Diaz *et al*. 2006).

The importance and mechanism of action of the EGF-like ligands during gonadotrophin-induced meiotic maturation of oocytes in monovular species such as the sheep remain to be fully investigated. This question can be most easily addressed using a physiologically relevant *in vitro* maturation (IVM) system that allows ovine oocytes and their companion somatic cells to progress through all the biological checkpoints and signalling events required to facilitate oocyte meiotic progression and cumulus mucification and expansion *in vivo*. Many approaches have been used to support the IVM of oocytes that impact on the mechanism of initiation of oocyte maturation *in vitro* and the length of time in culture (24–48 h) from a range of species including mouse (De la Fuente *et al*. 1999), cow (Brum *et al*. 2005) and sheep (Szollosi *et al*. 1988). Meiotic progression can occur spontaneously (Hartshorne *et al*. 1994), or be induced by the removal of COCs from the follicle (Leonardsen *et al*. 2000, Nogueira *et al*. 2005) or by the disconnection of oocytes from their cumulus cells (Pincus & Enzmann 1935). Furthermore, the constituents of the IVM culture system can have a profound bearing on oocyte developmental competence as assessed by measurement of oocyte fertile capacity, zygote cleavage and embryo progression to the blastocyst stage (Rizos *et al*. 2002). Other factors that are known to affect the developmental competence of IVM oocytes and the embryos they derive include COC group culture, IVM media composition (de Oliveira *et al*. 2005, Ishizaki *et al*. 2009) and the inclusion or exclusion of serum (Gomez *et al*. 2008, Arunakumari *et al*. 2010).

The current experiments aimed to investigate the relevance and role played by the EGF-like ligands in the signalling pathways driving the gonadotrophin-induced pre-ovulatory cascade that results in the meiotic maturation of ovine COCs. Experiments were designed to test the impact of the binding of surge levels of FSH and LH to their cognate receptors in mural and cumulus granulosa cells on the expression of the EGF-like family members *AREG*, *EREG*, *BTC* and *EGFR* during maturation of ovine COCs *in vitro*. To achieve this goal, it was first necessary to develop and validate a physiologically relevant, gonadotrophin-driven, serum-free IVM system for sheep oocytes.

Results

Validation of a physiological, serum-free IVM media for sheep oocytes

The first experimental series defined the biological parameters of the physiologically relevant, serum-free IVM system that was developed and validated for sheep oocytes. A series of seven repeat cultures were performed on a total of 472 COC of which 164 COCs were cultured in serum-free IVM medium containing concentrations of ovine LH (100 ng/ml) and FSH (100 ng/ml) within the range of physiological values measured during the pre-ovulatory gonadotrophin surge in sheep (Cunningham *et al*. 1975, Narayana & Dobson 1979, Wheaton *et al*. 1982, Atkinson *et al*. 1989). A further 138 COCs were incubated in the serum-free IVM medium without gonadotrophins (−LH/FSH), and 170 COCs were matured in a serum-based IVM medium containing pharmacological levels of ovine LH (5 μg/ml) and FSH (5 μg/ml). Meiotic progression was analysed in a subset of 126 oocytes of which 46 oocytes were denuded and analysed following 24 h of IVM in the serum-based medium. A further 40 oocytes were assessed from each of the serum-free medium −LH/FSH and serum-free IVM medium +LH/FSH groups. There was a high percentage of progression to MII in oocytes cultured using the serum-free medium +LH/FSH and serum-based medium (91.4 and 85.1% respectively). Significantly ($P<0.05$) fewer oocytes progressed to MII (69.5%) in the serum-free medium −LH/FSH in comparison with the other two groups. The numbers and proportions of oocytes from each IVM treatment that were competent to undergo fertilisation and support embryo cleavage and development to the blastocyst stage are shown in Table 1. No significant differences ($P>0.05$) were recorded in the fertilisation rates of oocytes matured in the serum-free medium +LH/FSH (67.7%) and serum-based medium +LH/FSH (75.0%). However, there was a marked reduction ($P<0.05$) in

Table 1 Total numbers of matured oocytes, cleaved embryos and blastocysts generated after IVM in serum-free IVM medium +/− LH/FSH and a serum-based IVM medium +LH/FSH. The values presented are actual numbers cultured with percentages shown in parentheses for each treatment.

Treatment	Inseminated oocytes	Total cleaved (%)	Blastocysts from cleaved embryos (%)	Blastocysts from inseminated oocytes (%)
Serum-free + LH/FSH	124	84 (67.7)	29 (34.5)	29 (23.4)
Serum-free − LH/FSH	98	50 (51.0)*	4 (8.0)*	4 (4.1)*
Serum-based +LH/FSH	124	93 (75.0)	24 (25.8)	24 (19.5)

Statistical evaluations are confined within columns; *statistically significant difference ($P<0.05$) compared with the other treatment groups.

the cleavage rate of embryos derived from oocytes matured in serum-free IVM cultures −LH/FSH (51.0%) compared with the other two groups. There was also a significant decrease ($P<0.05$) in blastocyst production from embryos that cleaved following oocyte IVM in the serum-free medium −LH/FSH (8.0%) to cultures containing pre-ovulatory levels of LH/FSH (34.5%) or serum-based IVM medium (25.8%). When blastocyst rates were compared for all inseminated oocytes, again there was no significant difference ($P>0.05$) between the serum-free IVM medium +LH/FSH and serum-based IVM medium +LH/FSH (23.4 and 19.4%). Blastocyst rates from both these groups were significantly ($P<0.05$) higher than the rates obtained for COCs matured in serum-free medium −LH/FSH (4.1%). Although the highest degree of cumulus expansion (grade 3) was consistently shown to occur in the 124 COCs cultured in serum-based medium, cumulus cells from the same number of COCs ($n=124$) matured in serum-free medium +LH/FSH also exhibited consistently high (grade 2) levels of cumulus expansion. Conversely, maturation in serum-free medium in the absence of gonadotrophins ($n=98$) always resulted in very poor cumulus expansion (grade 1 COCs), as illustrated in Fig. 1.

Impact of pre-ovulatory gonadotrophin exposure on gene expression during serum-free IVM

The second experimental series used the physiologically relevant IVM system detailed above to investigate the putative signalling role(s) played by the EGF-like ligands *AREG*, *EREG*, *BTC* and *EGFR* during the pre-ovulatory cascade in sheep COCs. Validated cDNA libraries that were generated from ovine germinal vesicle (GV) and MII oocytes, cumulus and mural granulosa cells, and a range of somatic tissues were interrogated by PCR to map the expression patterns of key genes relevant to gonadotrophin-stimulated oocyte maturation *in vitro*. Both LH and FSH receptors were absent from GV and MII oocytes (Fig. 2a). In contrast, the expression of *LHCGR* (*LHR*) was detected in mural granulosa cells recovered from antral follicles of 2–5 mm in diameter, whereas *FSHR* expression was detected in both cumulus and mural granulosa cells. The *BTC* gene has not been previously described in sheep cells. A partial nucleotide sequence was therefore obtained for ovine *BTC* as shown in Fig. 3. Comparison of ovine *BTC* to the known sequences for the bovine (NM173896), human (BC011618) and murine (NM007568) gene showed nucleotide similarity of 94, 48 and 23% respectively. Gene expression analysis subsequently revealed that *AREG*, *EREG*, *BTC* and *EGFR* were not detected in GV and MII sheep oocytes (Fig. 2a). In contrast, *EREG*, *BTC* and *EGFR* were detected in both cumulus and mural granulosa cells before LH and FSH exposure *in vitro*. Furthermore, expression of the EGF-like ligands was shown to be widely distributed throughout control ovine tissues (Fig. 2b); *BTC*, *EREG* and *EGFR* expressions were absent from heart tissue.

The impact of *in vitro* treatment with pre-ovulatory surge levels of LH and FSH on the expression of *AREG*, *EREG*, *BTC* and *EGFR* during the IVM of ovine COCs and mural granulosa cells was quantified over a 24 h time course, as shown in Fig. 4. Results were compared with control cells cultured without gonadotrophins. Assessment of cumulus morphology at each time point revealed that the first signs of cumulus expansion were evident 7–8 h after the start of culture in gonadotrophin-supplemented medium. Statistical analyses

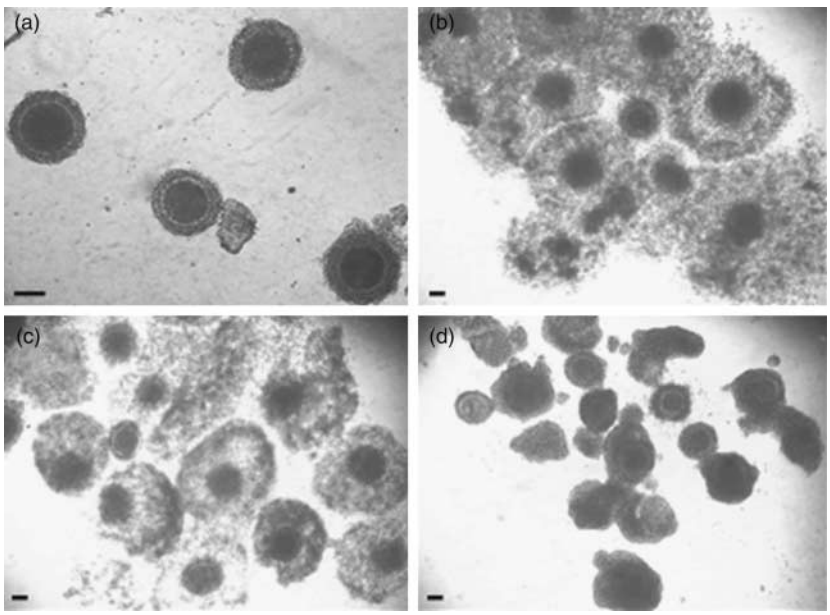

Figure 1 Effect of culture media composition on the morphology of ovine COCs during IVM. (a) Compact COCs before IVM; (b) cumulus expansion in COCs cultured in serum-based IVM medium + LH/FSH after 24 h of culture; (c) cumulus expansion in COCs cultured in serum-free IVM medium supplemented with LH/FSH, after 24 h of culture; (d) reduced cumulus expansion in COCs cultured in serum-free medium without LH/FSH after 24 h of culture. Scale bars represent 100 μm.

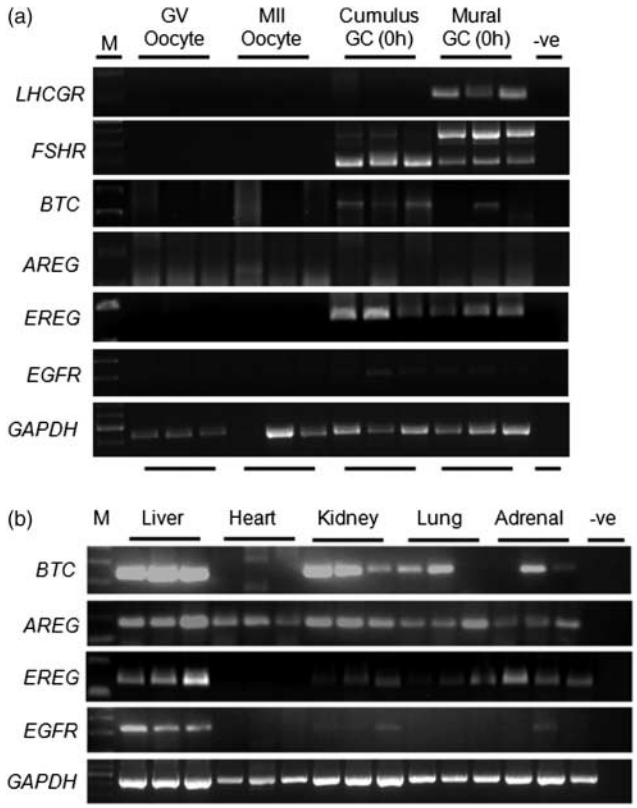

Figure 2 Representative gene expression profiles of *LHCGR* (213 bp), *FSHR* (465 and 300 bp), *BTC* (239 bp), *AREG* (171 bp), *EREG* (142 bp) and *EGFR* (289 bp) in replicate samples of (a) ovine GV and MII oocytes, cumulus and mural granulosa cells harvested before LH and FSH exposure and (b) cDNA libraries from a range of ovine somatic tissues.

assessed the overall impact of time and gonadotrophin treatment on gene expression levels as well as treatment vs time interactions. This analysis revealed measurable levels of *AREG* expression in both cumulus and mural sheep granulosa cells. Furthermore, *AREG* expression in COCs was significantly altered ($P<0.05$) over time such that *AREG* levels increased rapidly to a maximum after 30 min of exposure to pre-ovulatory FSH and LH relative to COCs cultured without gonadotrophins; thereafter, expression declined. In mural granulosa cells, there was a significant ($P<0.01$) overall effect of gonadotrophin treatment on *AREG* expression compared with cells cultured without LH and FSH and treatment vs time interactions tended towards significance ($P=0.065$). In parallel with the cumulus cells, there was a significant ($P<0.001$) and rapid increase in *AREG* expression in mural granulosa cells with peak expression being recorded after 30 min of exposure to LH/FSH *in vitro* compared with time 0. Furthermore, after 30 min of culture with gonadotrophins, *AREG* expression was significantly higher ($P<0.05$) than *AREG* expression at all time points in mural cells cultured without LH/FSH. Thereafter, *AREG* expression returned to basal levels within 2 h of the start of culture ($P>0.05$).

In contrast, although the levels of *BTC* expression in COCs and mural cells appeared to decrease with time in LH/FSH-supplemented cultures, overall neither *BTC* nor *EREG* expression was significantly ($P>0.05$) affected by gonadotrophin treatment or time in either cell type. Furthermore, due to the variability in the data, there were no significant effects of either LH/FSH treatment or time on *EGFR* expression in COCs ($P>0.05$) compared with control COCs cultured without gonadotrophins. There were, however, significant effects of both gonadotrophin treatment ($P<0.01$) and time ($P<0.05$) on *EGFR* expression in mural granulosa cells such that the induction of *EGFR* was maximal ($P<0.01$) after 6 h of LH/FSH exposure *in vitro* compared with the 0 h, 30 min and 2 h time points. The peak level of *EGFR* expression attained after 6 h of gonadotrophin treatment was significantly higher ($P<0.01$) than gene expression levels measured at the 0 h, 30 min, 2, 4, 6 and 8 h time points in control cells cultured without LH/FSH.

Discussion

This represents the first report of the expression profiles of the EGF-like ligands in ovine COCs during gonadotrophin-induced IVM. The data confirm that the distribution of *AREG*, *EREG*, *BTC* and *EGFR* expression was confined to the somatic compartment of ovine follicles and was undetectable in GV or MII oocytes from this species. Furthermore, cumulus and mural granulosa cell *AREG* and mural cell *EGFR* were rapidly induced following exposure to pre-ovulatory surge levels of LH and FSH *in vitro*. The gonadotrophin-induced up-regulation of cumulus *AREG* expression preceded morphological evidence of cumulus expansion.

These observations were recorded using a physiologically relevant IVM system that facilitated gonadotrophin-driven cumulus expansion and oocyte meiotic maturation as well as gonadotrophin-enhanced oocyte developmental competence, characterised by increased rates of embryo development. The current findings also confirm and extend data generated in other studies that COCs matured in serum-free media without gonadotrophins have impaired cumulus expansion (Danfour 2001, Cecconi *et al*. 2008). Indeed in mice, it has been suggested that gonadotrophin-induced cumulus expansion is reliant on specific factors secreted from the oocyte (Buccione *et al*. 1990). An important consequence of pre-ovulatory surge levels of the gonadotrophins includes the secretion of hyaluronic acid from the cumulus cells that leads to their mucification and expansion (Schoenfelder & Einspanier 2003). Both *FSHR* and *LHCGR* must be expressed in the somatic cells of the follicle for each gonadotrophin to work effectively. The current study found the expression of *LHCGR* to be mainly localised to the mural cells

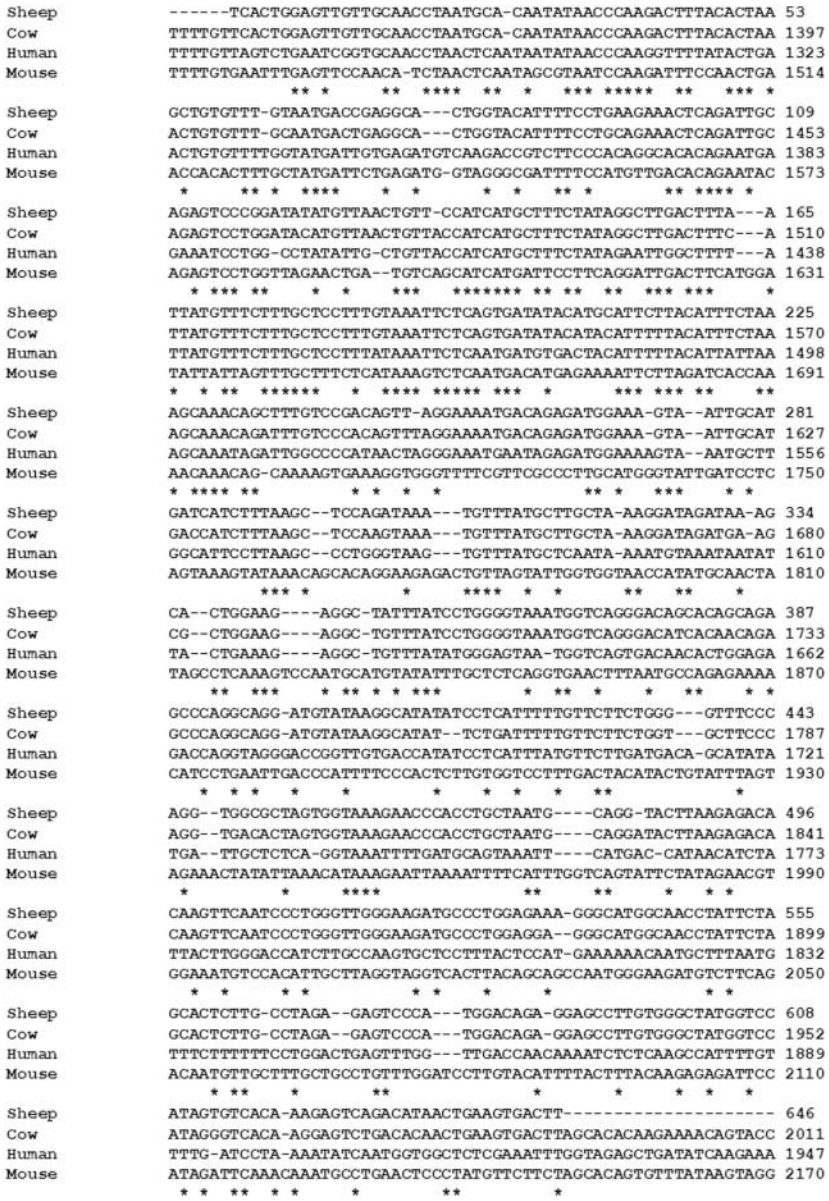

Figure 3 The nucleotide sequence of betacellulin (646 bp) obtained from sheep ovarian somatic cells. The DNA sequence has nucleotide homology with cow (94%), human (48%) and mouse (23%). *Complete sequence homology between the four species.

whereas *FSHR* was present in both cumulus and mural granulosa cells (Fig. 2a). The expression patterns of ovine *LHCGR* and *FSHR*, including their spliced variants, in cumulus and mural granulosa cells reported here are consistent with the previous reports on sheep (Tisdall *et al*. 1995, McNatty *et al*. 2000, Logan *et al*. 2002). Furthermore, our data suggest that expression of *FSHR* splice variants differs between cumulus and mural granulosa cells and that these variants may therefore be functionally relevant.

Accumulating evidence indicates the involvement of the EGF-like ligands in conveying the pre-ovulatory gonadotrophin signal to the oocyte via the follicular somatic cells. Initial studies in polyovulatory species such as mice suggested that the expression of *Areg*, *Ereg* and *Btc* was restricted to the mural granulosa cells (Park *et al*. 2004). However, subsequent experiments have shown a limited expression in cumulus cells 4 h post-exposure to human chorionic gonadotrophin (hCG) *in vitro* (Shimada *et al*. 2006). Murine data indicate that LH binding to *LHCGR* in mural granulosa cells (Peng *et al*. 1991) will up-regulate *Areg*, *Ereg* and *Btc* (Park *et al*. 2004) and that cumulus expansion occurs as a consequence of the EGF family members binding to EGFR to stimulate the expression of genes including *Ptgs2*, *Has2* and *Tnfaip6* (Park *et al*. 2004). The mechanism of *Areg*, *Ereg* and *Btc* up-regulation in murine cumulus is thought to be conveyed by the actions of prostaglandins and progesterone receptors on these cells (Shimada *et al*. 2006). In accordance with the murine data, the current study in sheep has detected *AREG*, *EREG* and *BTC* expression in the granulosa and

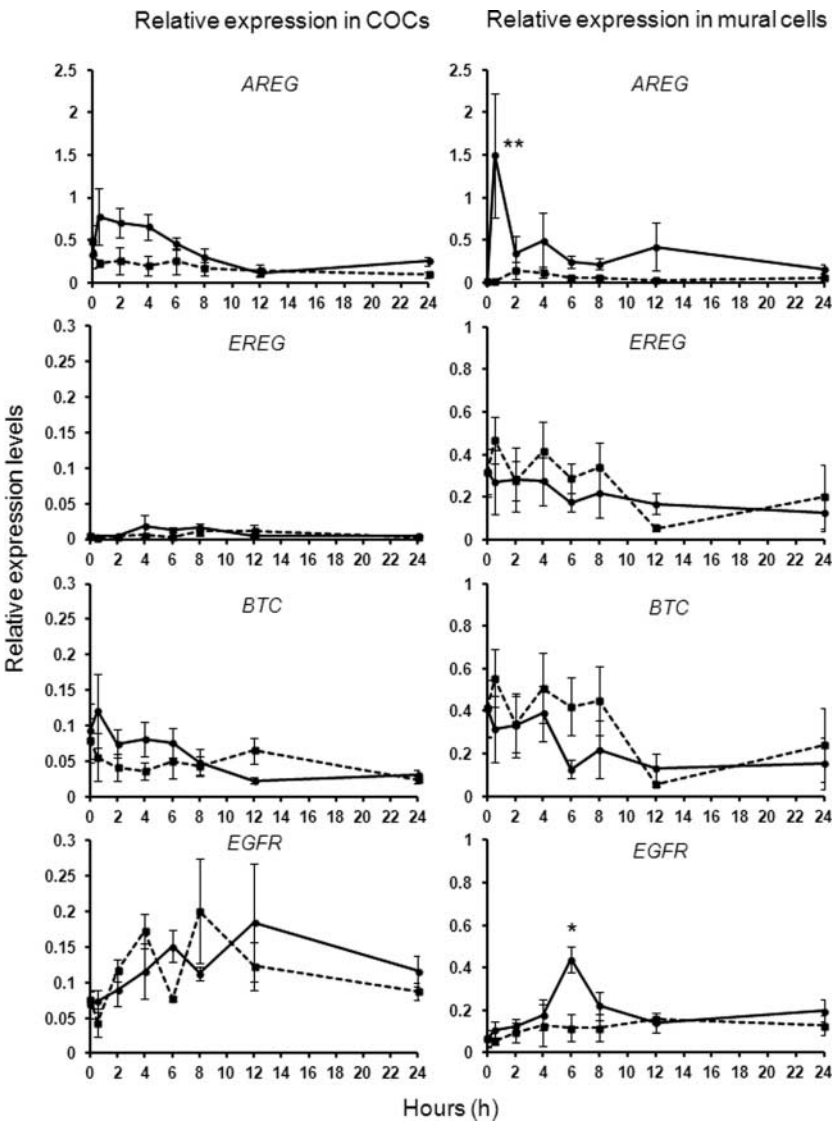

Figure 4 Impact of culture duration and gonadotrophin treatment on gene expression profiles of *AREG*, *EREG*, *BTC* and *EGFR* in ovine COCs and mural granulosa cells over 24 h during oocyte maturation in serum-free IVM media supplemented with LH/FSH (solid line) or without LH/FSH (broken line). The relative expression level of each gene is shown. For AREG, **indicates significant differences ($P<0.001$) to time 0 in cells cultured with LH/FSH and also to all the time points in control cells cultured without gonadotrophins ($P<0.05$). For EGFR, *indicates a significant difference ($P<0.01$) between gene expression levels in mural granulosa cells after 6 h of exposure to LH/FSH *in vitro* relative to 0 h, 30 min and 2 h, within treatment, as well as significant differences to control cells cultured without gonadotrophins and analysed after 0 h, 30 min, 2, 4, 6 and 8 h of culture. Values plotted are mean±S.E.M. for four replicate cultures. Note that the scales are different for *AREG* expression over time in each cell type. A further breakdown of the significant effects of treatment, time and treatment vs time interactions is provided in the text.

cumulus compartments of ovarian follicles; none of these genes were expressed by GV or MII oocytes (Fig. 2a). Furthermore, the results demonstrated a rapid induction of *AREG* expression in both cumulus and mural granulosa cells in sheep in response to exposure to pre-ovulatory levels of LH/FSH. The expression of *AREG* was maximal in both cumulus and mural cells within 30 min of exposure to LH and FSH when compared with controls (Fig. 4). Recent studies on bovine and human have also demonstrated the presence of *AREG*, *EREG* and *BTC* in cumulus cells (Zamah *et al*. 2010, Caixeta *et al*. 2011). Another study has previously detected the presence of *AREG* in the sheep mammary gland (Forsyth *et al*. 1997). The detection of *BTC* in mural granulosa and cumulus cells before gonadotrophin exposure as reported here mirrors previous observations in cattle (Caixeta *et al*. 2011). No significant increases were induced in the expression of *EREG* or *BTC* during 24 h of IVM when COCs and mural granulosa cells were cultured with or without surge levels of LH/FSH, but rather the expression of *BTC* in sheep cumulus and mural cells appeared to decrease over this time frame. Similarly, *BTC* expression profiles have been reported in pigs and humans (Zamah *et al*. 2010, Prochazka *et al*. 2011). The expression of the EGF-like molecules in cumulus and mural granulosa cells has also been demonstrated in rhesus macaque (Nyholt de Prada *et al*. 2009). Further rhesus macaque research has shown an elevated expression of *AREG* and *EREG* 24 h post-hCG treatment (Fru *et al*. 2007). Furthermore, *AREG* and *EREG* are expressed in human mural granulosa cells (Freimann *et al*. 2004), and *AREG* has been detected in human cumulus cells (Feuerstein *et al*. 2007).

All EGF-like ligands including AREG, EREG and BTC use the same EGF receptor (Shimada *et al*. 2006). Studies have shown that exposure to AG1478, a potent and

specific inhibitor of EGFR, significantly compromises cumulus expansion and oocyte maturation in rats (Reizel *et al*. 2010) and primates (Nyholt de Prada *et al*. 2009). In these studies, *EGFR* expression was detected in both mural and cumulus sheep granulosa cells both pre- and post-LH and FSH exposure *in vitro* (Figs 2a and 4), adding weight to the suggestion that the EGF-like ligands influence gene expression in both cell compartments in this species. There was no detectable expression of *EGFR* in either GV or MII ovine oocytes. Furthermore, a significant ($P<0.05$) increase in *EGFR* expression was induced in mural granulosa cells in response to 6 h of exposure to LH/FSH *in vitro* (Fig. 4). However, maximal *EGFR* expression lagged behind the peak of *AREG* expression observed in mural granulosa cells by 5.5 h. The observed discrepancy between the rapidity of induction of the expression of *AREG* and *EGFR* during the gonadotrophin-driven signalling, which leads to the expansion and mucification of cumulus cells against the luteinisation of mural granulosa cells in the sheep, requires further investigation. While the exact function of EGFR in mural granulosa cells is unknown, these receptors would likely contribute to the positive feedback mechanism(s) that amplify the effects of the EGF-like ligands on cumulus cell function. Indeed, the activation of EGFR generates intercellular responses via the MAPK pathway, commonly known to play roles in oocyte maturation and cumulus expansion (Panigone *et al*. 2008). The downstream kinases to this pathway are MAPK1/3 (ERK1/2) and their sustained phosphorylation has been shown to be maintained by active EGFR in rat pre-ovulatory follicles (Reizel *et al*. 2010). The MAPK pathway is known to be functional in sheep COCs during oocyte maturation and cumulus expansion (Cecconi *et al*. 2008).

We report here the development and validation of a physiologically relevant, gonadotrophin-driven, serum-free IVM system for sheep oocytes. This method was developed on the basis of the optimised serum-free culture conditions used to induce and maintain oestradiol production by granulosa cells in sheep (Campbell *et al*. 1996), cow (Gutierrez *et al*. 1997) and pig (Picton *et al*. 1999). The IVM medium used in these studies was further supplemented with sodium pyruvate (Wynn *et al*. 1998) as unlike granulosa cells, oocytes preferentially use pyruvate as their energy source (Tsutsumi *et al*. 1990, Downs 1995, Steeves & Gardner 1999). Furthermore, gonadotrophin concentrations within the physiological range of levels measured for the pre-ovulatory surge in sheep were used to trigger oocyte maturation and cumulus mucification and expansion *in vitro*. The peak concentrations of LH and FSH measured during the pre-ovulatory surge in sheep have been shown to range from 40 to 240 ng/ml for LH, and 5 to 350 ng/ml for FSH (Cunningham *et al*. 1975, Narayana & Dobson 1979, Wheaton *et al*. 1982, Atkinson *et al*. 1989). Comparable rates of IVM and fertilisation were achieved using the physiological serum-free IVM system when compared with an established serum-based IVM system containing 10% FCS (Obrien *et al*. 1997). Importantly, the data presented clearly show gonadotrophin-induced meiotic maturation and gonadotrophin-enhanced oocyte developmental competence. Two lines of evidence support this claim: i) oocytes that were exposed to serum-free medium without LH and FSH showed reduced maturation potential and compromised embryo development as evidenced by both nuclear and cytoplasmic maturation and subsequent blastocyst development *in vitro* and ii) oocytes matured in the serum-based IVM medium produced cleavage rates of 75.0% and blastocyst formation of 25.8% from cleaved embryos. All oocytes and embryos detailed here were cultured in groups, and IVM and blastocyst rates generated by the serum-free conditions conform to the rates obtained in other published ruminant IVM/IVF systems. Two studies using the same percentage of serum (10%), as the present work, yielded cleavage and blastocyst rates of 82 and 34% (Obrien *et al*. 1997) and 79 and 58.3% respectively (Zhu *et al*. 2007). Previously, the use of 2% heat-inactivated sheep serum in IVM medium has produced embryo cleavage rates of 88% and blastocyst rates of 31% (Brown & Radziewic 1998). The serum-free IVM system reported here demonstrated cleavage rates of 67.7% and blastocyst formation of 34.5% (Table 1). Previous reports have shown that sheep blastocyst production rates can range from 15 to 70% following the IVM of COCs with and without the addition of FCS or oestrus sheep serum (Thompson *et al*. 1989, 1992, 1995, Gardner *et al*. 1994, Watson *et al*. 1994, Obrien *et al*. 1996, Walker *et al*. 1996, Wang *et al*. 1998, Grazul-Bilska *et al*. 2003). These serum-based culture systems are commonly supplemented with high pharmacological concentrations of 0.5–10 μg/ml LH and FSH. Variability in the blastocyst production rates between this study and the published reports can be attributed to differences in IVM and embryo media culture and conditions, sheep breed and hormonal treatments, as well as differences between the IVF protocols used for handling spermatozoa, oocyte and sperm co-incubation and group culture. Specific information about the expected cleavage and blastocyst rates for sheep oocytes matured in serum-free IVM medium is scarce; however, comparisons with bovine data show cleavage rates of 65.6% and blastocyst rates of 19.8% (Cho *et al*. 2002). Similarly, publications on research with pigs have reported cleavage rates of 62% and blastocyst rates of 16% after serum-free IVM (Wang & Day 2002).

It has been indicated that maturation of the oocyte cytoplasm during the GV to MII transition is vital to support early embryo development (Cha & Chian 1998). The present data demonstrate that IVM culture systems lacking the appropriate gonadotrophin trigger may permit oocyte nuclear meiotic progression but fail to advance

cytoplasmic maturation or support subsequent embryo development (Moor *et al*. 1998, Trounson *et al*. 2001). In the current study, the supplementation of serum-free IVM medium with LH and FSH concentrations that lie within the physiological range measured for the pre-ovulatory surge in sheep (Picton 2002) enhanced the developmental potential of oocytes, indicating that both nuclear and cytoplasmic maturation were synchronised by gonadotrophin exposure. This is in contrast to serum-free IVM systems in species such as mice in which oocyte maturation frequently occurs spontaneously after the removal of oocytes from their follicular environment (Leonardsen *et al*. 2000).

In conclusion, the data reported here used a serum-free IVM system containing the physiological triggers for ovulation to promote gonadotrophin-driven IVM of ovine oocytes. The studies have provided the first demonstration of the expression of *AREG*, *EREG*, *BTC* and *EGFR* in both cumulus and mural granulosa cells during COC maturation *in vitro*, as well as the rapid induction of *AREG* and *EGFR* expression following gonadotrophin exposure. These findings suggest that AREG via binding to EGFR contributes to the signalling mechanism of gonadotrophin-driven cumulus expansion in ovine COCs, which promotes oocyte maturation in response to the pre-ovulatory gonadotrophin surge in this species. The physiologically relevant, serum-free ovine IVM system reported here supports oocyte developmental competence in the presence of pre-ovulatory levels of LH and FSH and produces comparable rates of oocyte maturation, cleavage and blastocyst development to serum-based IVM systems containing pharmacological levels of the gonadotrophins.

Materials and Methods

All reagents used in this series of experiments were purchased from Sigma, unless otherwise stated. Sheep reproductive tracts were collected from a local abattoir and transported to the laboratory at room temperature for the isolation of COCs. Ovaries were cut from the reproductive tracts and subjected to a minimum of three washes in PBS supplemented with penicillin G (100 IU/ml), streptomycin sulphate (100 μg/ml) and amphotericin B (250 ng/ml).

Validation of the serum-free IVM system for sheep oocytes

The first experimental series validated a physiologically relevant, serum-free IVM system for sheep oocytes. Ovine COCs were aspirated from antral follicles of 2–5 mm diameter using a sterile 20 ml syringe attached to a 19-gauge needle pre-loaded with 1 ml pre-warmed HEPES buffered medium 199 ($H199^+$) supplemented with BSA (0.1% w/v). Throughout oocyte handling, COC temperature was maintained at 39 °C using a hotplate (MTG, Medical Technology Vertriebs GmbH, Altdorf, Germany) attached to a stereomicroscope fitted with a heated stage (Tokai Hit Co. Ltd, Shizuoka-ken, Japan). Oocytes with a homogenous cytoplasm surrounded by at least three to four layers of cumulus cells were transferred onto specific IVM medium and an average of 22 oocytes (472 in total) was cultured per group for each IVM medium in four-well Nunc culture plates (Nunclon, Roskilde, Denmark); seven replicate cultures were conducted. Each culture well maintained a ratio of 4 μl medium per COC under washed mineral oil. Each dish contained one of three different treatments: i) serum-based IVM medium (serum-based +LH/FSH), containing ovine LH (5 μg/ml) and ovine FSH (5 μg/ml), glutamax (2 mM) and 10% (v/v) FCS all in medium 199 (Obrien *et al*. 1997); ii) serum-free IVM medium (serum-free +LH/FSH) that contained pre-ovulatory surge levels of ovine LH (100 ng/ml) and ovine FSH (100 ng/ml), bovine transferrin (5 μg/ml), sodium pyruvate (0.47 mM) (Wynn *et al*. 1998), sodium selenite (5 ng/ml), L-glutamax (3 mM), 0.1% (w/v) BSA and the optimised concentrations of insulin (10 ng/ml) and the synthetic analogue of insulin-like growth factor 1 -long R3 IGF1 (10 ng/ml) for use with sheep somatic cells as previously detailed (Campbell *et al*. 1996), all in Alpha Minimal Essential Medium (Danfour 2001); and iii) serum-free IVM media −LH/FSH. The bio-potencies of the ovine LH (NIH-LH-525) and ovine FSH (NIH-FSH-S1) preparations used were 0.56 and 0.9 U/ml respectively. All COCs were matured over 24 h at 39 °C in a 6% CO_2, 5% O_2 and 89% N_2 in a humidified atmosphere.

Cumulus expansion was recorded using a three-level scoring system similar to previously described methods (Buccione *et al*. 1990, Vanderhyden *et al*. 1990, Danfour 2001, Cecconi *et al*. 2008). The expansion of cumulus for each treatment was assessed visually per group after 24 h of IVM. The degree of cumulus expansion was scored as follows:

i) Exhibited very poor or no cumulus expansion.
ii) Demonstrated limited cumulus expansion.
iii) Showed full cumulus expansion.

After 24 h of IVM, an average of six oocytes per IVM treatment group per culture replicate, totalling 126 oocytes overall, was denuded and incubated in a 1 mg/ml solution of bisbenzimide for 5 min before being mounted onto glass slides and evaluated by fluorescent microscopy at a wavelength of 488/533 nm to confirm oocyte nuclear progression. Maturation stages recorded included GV, GV breakdown (GVBD), metaphase I and MII. Samples of denuded GV and MII oocytes, unexpanded cumulus and mural granulosa cells were snap frozen in lysis buffer (100 mM Tris–HCl, 500 mM LiCl, 10 mM EDTA, 1% (v/v) lithium dodecyl sulphate and 5 mM dithiothreitol (DTT)) and stored at −80 °C for molecular analysis.

Following 24 h of IVM, the remaining COCs from each treatment were transferred to four-well Nunc dishes containing 800 μl $H199^+$ medium per well and each COC was gently washed by repeated pipetting to remove excess cumulus cells. The oocytes were twice washed in 800 μl $H199^+$ and 800 μl bicarbonate-buffered synthetic oviduct fluid (Tervit *et al*. 1972), and oocytes from each IVM treatment were transferred to a new four-well dish containing

450 μl IVF media (5% v/v FCS, 0.06 mg/ml penicillin, 0.05 mg/ml streptomycin, 100 μg/ml kanamycin, 0.3 mM pyruvate, 0.1 mM glutamine, 3 mM calcium, 25 mM sodium bicarbonate, 92 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 0.74 mM $MgSO_4 \cdot 7H_2O$ and 7 mM Na lactate) overlaid with pre-equilibrated and washed mineral oil. Oocytes from each treatment were inseminated using frozen–thawed ram sperm (Britbreeds Ltd, Edinburgh, Scotland), at a concentration of 1×10^6 spermatozoa per millilitre. Insemination was conducted in 5% CO_2, 6% O_2 and 89% N_2 in a humidified atmosphere over 24 h. Zygotes were visually assessed for fertilisation and cleavage 24 h after insemination. Fertilised embryos were cultured in 10 μl bovine cleavage media (Cook Medical Australia Pty Ltd, Brisbane, QLD, Australia) for 4 days followed by transfer into 10 μl bovine blastocyst media (Cook), for a further 3 days of culture as used previously (Hollinshead *et al.* 2004, Harvey *et al.* 2007). All embryo cultures were conducted in a 5% CO_2, 6% O_2 and 89% N_2 in a humidified atmosphere at 39 °C. The number of embryos developing to the blastocyst stage was assessed for each IVM treatment on days 6, 7 and 8 post-insemination. The criteria used to assess blastocyst formation were the presence of a partially or fully formed blastocoele cavity and a distinguishable inner cell mass and trophectoderm (Obrien *et al.* 1997).

Impact of pre-ovulatory gonadotrophin exposure on gene expression during serum-free IVM

The second experimental series used the validated serum-free IVM system detailed earlier to evaluate the impact of *in vitro* exposure to pre-ovulatory levels of LH and FSH on the expression of *AREG*, *BTC*, *EREG* and *EGFR* in ovine cumulus and mural granulosa cells. Antral follicles of 3–4 mm diameter were dissected from ovine ovaries, COCs were removed and the mural granulosa cells were scraped from the inside of each follicle into $H199^+$ medium. The COCs were recovered as previously detailed and duplicate drops of serum-free IVM media containing surge levels of FSH and LH were set up to include two COCs per 8 μl micro drop under oil. Viable granulosa cell number was assessed using trypan blue dye exclusion (Picton *et al.* 1999) and cells were seeded at a concentration of 3×10^5 viable cells per well in 96-well dishes and cultured in 200 μl IVM media with and without FSH (100 ng/ml) and LH (100 ng/ml). Both COCs and granulosa cell cultures were incubated at 39 °C in 6% CO_2, 5% O_2 and 89% N_2 in a humidified atmosphere. The COCs and mural granulosa cell samples were removed after 30 min and after 2, 4, 6, 8, 12 and 24 h of culture and were snap frozen in lysis buffer for gene expression analysis. The experiment was repeated four times. The relative expression of *AREG*, *EREG*, *BTC* and *EGFR* was performed using real-time PCR; gene expression at each culture time point was normalised against the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and histone (*HIST2H2AC*). As there was no sequence data for *BTC* in sheep, a partial mRNA sequence was isolated, sequenced and submitted for use at Genbank (accession number: HM229291).

Isolation of mRNA and cDNA synthesis

Each sample was processed to create cDNA libraries to assess the expression of key markers including *FSHR*, *LHCGR*, *AREG*, *EREG*, *BTC* and *EGFR*. The procedure for creating a cDNA library was modified from the previously published protocols (Huntriss *et al.* 2002). Briefly, IVM-derived samples including denuded GV and MII oocytes, cumulus and mural granulosa cells as well as a range of sheep somatic tissues were snap frozen and stored at −80 °C in 10 μl Dynal lysis buffer. Upon use, each sample was heated at 80 °C for 10 min to ensure complete cellular lysis. Using a Dynabead mRNA extraction kit (Life Technologies Ltd, Paisley, UK), 25 μl were added to each sample and agitated for 30 min at room temperature to bind the mRNA. The mRNA was separated from the cellular components and washed in progressive buffer steps supplied with the Dynabead mRNA extraction kit. The samples were re-suspended in 3 μl sterile distilled water and frozen at −80 °C until further use.

First-strand cDNA synthesis (SMART)

The primers used for the cDNA synthesis from the total mRNA within each sample have been previously published (Eberwine *et al.* 1992, Wang *et al.* 2000) and include an $Oligo(dT)_{24}$ primer (5′-aaacgacggccagtgaattgtaatacgactcactatagggcgct$_{24}$-3′) and a template switching primer (5′-aagcagtggtatcaacgcagagtacgcggg-3′). Each primer was diluted to a concentration of 25 μg/μl. A mastermix was assembled by the addition of 1 μl of both the $Oligo(dT)_{24}$ primer and template switching (TS) primer, 2 μl first-strand reaction buffer (5×), 1 μl dNTP mix (10 mM), 1 μl DTT (0.1 M) and 7 μl SuperScript reverse transcriptase RNase-H (200 U/μl) (all supplied by Invitrogen Ltd). A 7 μl aliquot of the mastermix was added to each 3 μl sample from the mRNA isolation step. The reaction was heated for 2 h at 42 °C followed by air cooling for a further 2 min. Samples that were used for real-time PCR were retained at this stage of the experiment. The only exception to the earlier protocol was the substitution of random hexamers (50 μM) for the oligo-dT and -TS primers.

PCR amplification of total cDNA and confirmation

A reaction was made up to amplify the cDNA and contained 1 μl of both the $Oligo(dT)_{24}$ primer and template switching primer (1 μg/ml), 5 μl Advantage 2 PCR buffer (10×) (Takara Bio Europe, Paris, France; Clontech, Mountain View, CA, USA), 1 μl Advantage 2 Taq polymerase (50×) (Takara Bio), 1 μl dNTP mix (50×) (10 mM) and 31 μl H_2O. Amplification was performed for 35 cycles at 95 °C for 30 s followed by 65 °C for 6 min, after an initial one-cycle step at 95 °C for 1 min on a PerkinElmer GeneAmp 480 thermal cycler (PerkinElmer, Waltham, CT, USA). To assess expression of *LHCGR*, *FSHR*, *AREG*, *EREG*, *BTC* and *EGFR*, a standard reaction mixture was made up as follows: 1.25 μl PCR buffer, 0.75 μl Mg^{2+}(50 mM), 0.1 μl Taq Polymerase (5 U/μl) (all supplied by BIOTAQ Polymerase Kit, Bioline Ltd, London, UK), 1 μl specific housekeeping PCR primers (25 μM), 2 μl dNTP (1.25 mM), 6.1 μl H_2O and 0.3 μl cDNA sample. The PCR conditions for

Table 2 Primer sequences used to amplify each gene from ovine cDNA reference samples.

Primer name	Sequence	Annealing temperature (°C)	Product size (bp)	Accession number
AREG	5′-CCGGATCCAGTCAGAGTTGAACAGG-3′	50	260	Forsyth *et al.* (1997)
Outside	5′-CCCGAATTCGCTGTGAGTCTTCAT-3′			
AREG	5′-AAAAGGGAGGCAAAAATGGA-3′	60	171	Forsyth *et al.* (1997)
Inside	5′-CTTTTCCCCACATCGTTCAC-3′			
EREG	5′-AGTCCACAGCTGGCTAGGAA-3′	60	142	XM002688367
	5′-CGGGTTTTGTGGAAGACAAT-3′			
BTC	5′-GCAGAAAACTGCCCAGCTAC-3′	60	425	NM173896
Outside	5′-AACTTGCCATCAACCTGGAG-3′			
BTC	5′-CCCCAAGCAGTACAAGCATT-3′	60	239	NM173896
Inside	5′-GACGTTTCCGAAGAGGATGA-3′			
EGFR	5′-TGCAAGGTGCCGTGAGATTCAGC-3′	60	289	HM749883
	5′-CAGCCAGCGGCACACTGGTT-3′			
GAPDH	5′-GAACCACGAGAAGTATAAACA-3′	60	533	AF030943
	5′-AGGAAATGAGCTTGACAAAG-3′			
LHCGR	5′-TGCACACCTGAAGAAGATGC-3′	60	213	L36329
	5′-TGGCTGGGGTAAGTCAATGT-3′			
FSHR	5′-AACAGTAATTTGGAAGAACTGCC-3′	55	523	NM001009289
Outside	5′-ATGGCCAGGATGCTAATA-3′			
FSHR	5′-TGATGTTTTCCAGGGAGCCTC-3′	55	465	NM001009289
Inside	5′-TCTGAGAATATCATACCCCATG-3′			

this reaction consisted of a denaturation step at 95 °C for 5 min for 1 cycle, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. An elongation step at 72 °C for 5 min was followed by a holding step at 4 °C to complete the cycle. The specific primers that were used are shown in Table 2. The primers used to obtain the 646 bp sequence of *BTC* included the forward primer 5′-ggatttgatccctgggtctt-3′ and reverse primer 5′-ggcaagatggtcccaagtaa-3′. The presence of gel bands was confirmed by sequencing all samples at the Biomolecular Analysis Facility, University of Leeds, and sequence data confirmed using BLAST searching.

Real-time PCR

Quantification of *AREG*, *EREG*, *BTC* and *EGFR* expression levels in both cumulus and mural granulosa cells was conducted using an ABI7900HT PCR analyser, using SYBR green technology (Life Technologies). The mRNA was extracted and reverse transcribed as previously stated, and quantification of the total amount of mRNA in each sample was tested to allow statistical comparison of each sample per treatment for each gene. Primers to assess the relative expression of each gene were designed using the web-based programme primer 3 (Rozen & Skaletsky 2000) and are listed in Table 3. The reaction for the real-time PCR was assembled by the addition of 12.5 μl SYBR green master mix (Applied Biosystems), 1.25 μl specific primer (500 pM), 10.5 μl H_2O and 0.5 μl of each sample. All analysis was duplicated to ensure consistency and included negative controls and dissociation curves. Expression of *GAPDH* and *HIST2H2AC* mRNA was used to normalise gene expression levels in each experimental sample.

Statistical analysis

Statistics were performed using the Minitab 15 statistical programme (Minitab Ltd, Coventry, UK). All data sets were checked for normality using the Anderson–Darling test. Oocyte meiotic progression, fertilisation and embryo production data were interrogated using χ^2 analysis. Gene expression data generated by real-time PCR were transformed before factorial ANOVA to determine the effect of treatment, time and treatment vs time interactions. For the ease of interpretation, the gene expression data are presented as mean ± S.E.M. for the number of replicate analyses shown. In all analyses, P values <0.05 were considered to be statistically significant.

Table 3 Primer sequences used for real-time PCR.

Primer name	Sequence	Annealing temperature (°C)	Product size (bp)	Accession number
AREG	5′-AAAAGGGAGGCAAAAATGGA 3′	60	171	Forsyth *et al.* (1997)
	5′-CTTTTCCCCACATCGTTCAC-3′			
EREG	5′-AGTCCACAGCTGGCTAGGAA-3′	60	142	XM002688367
	5′-CGGGTTTTGTGGAAGACAAT-3′			
BTC	5′-GACCGAGGCACTGGTACATT-3′	60	178	HM229291
	5′-GTCGGACAAAGCTGTTTGCT-3′			
EGFR	5′-GAGGTGGTCCTTGGGAATTT-3′	60	114	DQ152947
	5′-CACTGTGTTGAGGGCAATGA-3′			
HIST2H2AC	5′-GAGTAGGCGGCTGGTTCTC-3′	60	109	NM001009270
	5′-GGAGTCCTTCCCAGCCTTAC-3′			
GAPDH	5′-TGATTCCACCCATGGCAAGT-3′	60	93	AF030943
	5′-CGCTCCTGGAAGATGGTGAT-3′			

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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