Activin A and follistatin during the oestrous cycle and early pregnancy in ewes

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Abstract

The activin pathway has been postulated to be involved in regulation of multiple reproductive processes important for survival of the conceptus. These processes include luteinisation of the follicular cells and thus function of the corpus luteum, early embryo development and uterine function including implantation of the conceptus. Therefore, the aim of the current study was to determine whether the concentrations of activin A and follistatin (FST), an activin-binding protein, differed between ewes with a lifetime history of enhanced or reduced embryonic survival (ES). The mRNAs encoding *FST* and activin A (inhibin beta A subunit; INHBA) were present in the uterus and abundant in the uterine luminal or glandular epithelia by day 18 of gestation. A peak of activin A was observed in the systemic circulation around the time of oestrus, and activin A concentrations were elevated in animals with reduced ES during the oestrous cycle and early gestation. Concentrations of activin A in uterine fluid were approximately twofold greater on day 16 of gestation in ewes with reduced ES compared to those with enhanced ES. No consistent differences in FST were observed between these groups. Treatment of luteinising ovine granulosa cells with activin A *in vitro* suppressed progesterone secretion providing evidence of a potential pathway whereby increased concentrations of activin A may decrease ES.

Key Words

- activin
- follistatin
- endometrium
- sheep
- early pregnancy

Journal of Endocrinology (2016) **228**, 193–203

Introduction

In many species, including sheep, >30% of fertilised ova fail to develop to term (Wilmut *et al*. 1985, Diskin & Morris 2008). This reduces production efficiency in domestic animals; thus, reducing this loss is of considerable importance for livestock industries. In sheep that ovulate two or more ova, two types of loss can occur. Firstly, a certain proportion of ewes fail to become pregnant, often ovulating again after ~17 days, which is the normal

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oestrous cycle length for sheep (Quinlivan *et al.* 1966). The failure to become pregnant can be driven by multiple factors including infertility of the male. However, in many sheep, the ewe becomes pregnant, but one or more of the ova released fails to survive, resulting in partial failure of multiple ovulation (PFMO). In cases of PFMO, infertility of the male is excluded because at least one embryo is healthy. It is known that survival of an individual ovum is related to the number of ova ovulated, with ewes ovulating two ova that remain pregnant producing, on average, 1.7 lambs. Pregnant ewes that have ovulated three ova produce, on average, ~2.2 lambs (Hanrahan 1982, Shorten *et al.* 2013). It is also known that individual outlier ewes for PFMO exist, with a lifetime history of having more or fewer lambs born than would be predicted by their observed ovulation rate (OR). This is consistent with these ewes having a natural difference in their ability to support a developing embryo. These differences potentially could be related to differing quality of the ova produced or a differing maternal environment for embryo development. Recently, we reported the identification of a group of genetically similar Coopworth ewes that have a lifetime pattern of high OR with either enhanced or reduced embryonic survival (ES) (O'Connell *et al.* 2013). Therefore, these animals provide a powerful and unique animal model to identify maternal pathways that are potentially important for survival of the embryo.

The activin pathway has been postulated to be involved in regulation of multiple reproductive processes including ovarian follicular development (Knight *et al.* 2012), luteinisation of the follicular cells (Myers *et al.* 2008, Kayani *et al.* 2009), early embryo development (Rajput *et al.* 2013) and uterine function during implantation of the conceptus (Singh *et al.* 2011). As a member of the transforming growth factor beta superfamily of growth factors, activin forms from the dimerisation of two β-subunits of inhibin (INHB), with different combinations forming functionally different isoforms (Knight 1996, Mellor *et al.* 2003). Follistatin (FST), a structurally unrelated protein, is known to bind activin to regulate its activity (Walton *et al.* 2012).

Given the potential of activin to affect ES at multiple levels, we hypothesised that activin, or its binding protein FST, may be differentially expressed between ewes with a lifetime history of reduced or enhanced ES. Therefore, to better understand the potential role of this pathway in regulation of ES, we determined the cellular localisation of *FST* and *INHBA* mRNA expression in the uterus in WT ewes during the late luteal phase of the oestrous cycle and early gestation (experiment 1). For ewes with a lifetime history of enhanced or reduced ES than that predicted by their OR, the concentration of FST and activin A in plasma during the oestrous cycle and early gestation were compared (experiment 2). The expression patterns of mRNAs in uterine tissue as well as the concentrations of FST and activin A in uterine fluid at the beginning of blastocyst implantation were also compared. Additionally, to better understand the mechanisms by which differing concentrations of activin A might lead to differing ES outcomes, we examined the effects of activin A on progesterone production from luteinising ovine granulosa cells, as increased progesterone concentrations during the early luteal phase have been linked to increased ES (Parr 1992, Robinson *et al.* 2008).

Materials and methods

Animals

All animal experiments were conducted following approval by the Animal Ethics Committee of the AgResearch Invermay Agricultural Centre in accordance with the Animals Protection Regulations (Codes of Ethical Conduct) of New Zealand. A schematic of the experiments undertaken is given in Fig. 1. The ewes (*Ovis aries*) were of either Romney or Coopworth (Romney cross) breeds and were sourced from the Invermay research farm. Multiparous ewes were treated with two intramuscular injections of 0.7 ml Estrumate (active ingredient cloprostenol, a prostaglandin analogue, Intervet, Christchurch, New Zealand) 9 days apart to synchronise oestrus. The onset of oestrus (day 0) was detected by mating of the ewe by a vasectomised ram.

Experiment 1: to determine the expression pattern of *FST* and *INHBA* mRNAs in normal sheep, oestrous cycles of Romney ewes were synchronised as described above, and oestrous behaviour was monitored daily. A subset of the ewes detected in oestrus was immediately placed with a fertile ram to initiate pregnancy. The remaining ewes were retained for tissue collection during specified days of the oestrous cycle. Pregnancy was confirmed at the time of collection of the uterine tissue, and those animals that failed to become pregnant after exposure to the fertile ram were excluded from the study. After euthanasia using captive bolt and exsanguination, tissues were collected at day 14 ($n=4$) or day 16 ($n=5$) of the oestrous cycle or day 14 ($n=4$), day 16 ($n=3$) and day 18 ($n=4$) of gestation (day 0=oestrus/mating).

For experiment 2, Coopworth ewes were selected based on their lifetime history of producing more lambs

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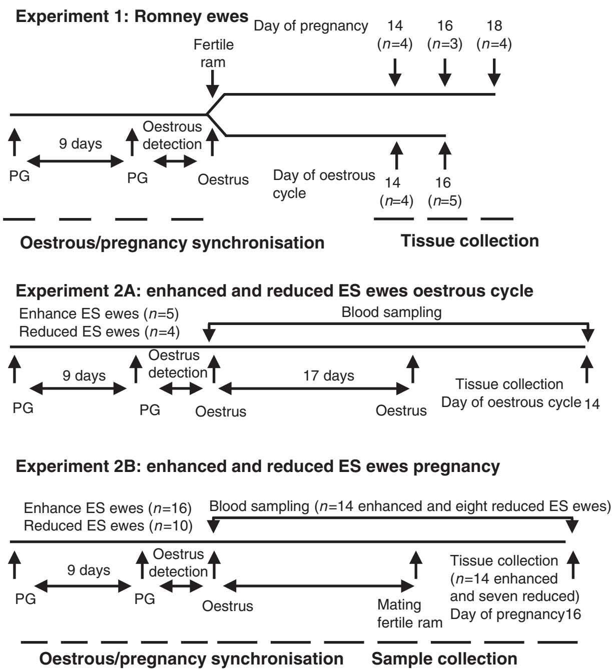

Figure 1
Schematic of timing of blood and tissue collections for experiments 1, 2A and 2B. In experiment 1, oestrous cycles of Romney ewes were synchronised with two injections of a prostaglandin (PG) analogue given 9 days apart. Day of oestrous was detected using vasectomised rams. One group of ewes was immediately placed with a fertile ram when detected in oestrous to initiate pregnancy whereas the remainder of the ewes were left with the vasectomised ram. Tissues were collected on days specified. For experiments 2A and 2B, oestrous cycles of ewes with enhanced or reduced ES were synchronised as for experiment 1. Following oestrous synchronisation, the next natural oestrus was determined using a vasectomised (experiment 2A) or fertile (experiment 2B) rams. Blood samples and tissues were collected as specified.

(enhanced ES, $n=21$) or fewer lambs (reduced ES, $n=14$) than predicted by their OR using the equation developed by Hanrahan (1982):

Predicted litter size

$$= 0.015 + (0.926 \times \mathrm{OR}) - (0.0763 \times \mathrm{OR}^2)$$

Selection of animals was as described by O'Connell *et al.* (2013). An ES score was calculated by subtracting the predicted litter size from the observed litter size using a minimum of three records per ewe. OR of the ewe was measured using laparoscopy (Kelly & Allison 1976), and litter size was determined at lambing each year. The enhanced ES ewes had 0.32 more lambs than predicted whereas the reduced ES ewes had, on average, 0.53 fewer lambs than predicted. For experiment 2A, the ewes were exposed to a vasectomised ram ($n=5$ and $n=4$ for enhanced and reduced ES respectively) on the day of the second detected oestrus following synchronisation. For experiment 2B, ewes were bred by a fertile ram ($n=16$ and $n=10$ for enhanced and reduced ES respectively) on the day of the second detected oestrous following synchronisation. Ewes were then slaughtered on day 14 of the oestrous cycle or day 16 of gestation. Day 14 of the oestrous cycle was chosen, as this represents the start of the rise of prostaglandin secretion and thus the onset of luteal regression in the non-pregnant ewes (Shorten *et al.* 2010). Day 16 of gestation was chosen, as this represents the beginning of implantation of the conceptus (Spencer *et al.* 2004). Two of the 26 ewes mated with the fertile ram failed to become pregnant (from the reduced ES phenotype) and were excluded from the experiment. Collection of the tissues during the oestrous cycle and pregnancy occurred over two successive breeding seasons.

Sampling

For experiment 1, only one uterine horn was flushed on days 16 and 18 of gestation to confirm pregnancy; thus, this uterine flush sample was not used for further analysis. For experiments 1, 2A and 2B, tissue samples from the mid-region of the uterine horn were fixed in 4% v/v paraformaldehyde and embedded in paraffin wax for histological sectioning. For experiments 2A and 2B, daily blood samples were collected from ewes into heparinised tubes via venipuncture from the time of detection of first oestrus until tissue collection. For experiment 2B, following euthanasia of potentially pregnant ewes, uteri were flushed with sterile Tris-buffered saline (10 ml per horn, pH 7.2) with the recovered fluid from both horns pooled and frozen. Due to a calculation error, three ewes (two from the enhanced ES group and one in the reduced ES group) were collected later in pregnancy than intended; thus, the uterine fluid from these ewes was excluded from analysis.

In situ hybridisation and microscopy

cDNA for *FST* and *INHBA* was prepared in our laboratory, and the hybridisation procedure was as described previously (Tisdall *et al.* 1994, Logan *et al.* 2002). Briefly, 4–6 μm tissue sections were rehydrated and treated with proteinase K; slides were then acetylated to reduce non-specific binding and dehydrated. Hybridisation solution, containing ^{33}P-labelled RNA sense or anti-sense probe, was added to the slides which were then incubated at 50 °C overnight. Non-specific hybridisation was removed with RNase A and stringent washes (2× SSC, 50% formamide at

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65 °C and 0.2× SSC at 37 °C). Slides were dehydrated and coated with LM1 emulsion (Amersham). After 3–4 weeks, slides were developed with D19 developer (Eastman Kodak, Rochester, NY, USA). Localisation of gene expression within a transverse section of the uterine tissue was evaluated qualitatively and not quantitatively. Uterine sections from three to five ewes were assessed for each time point and ES phenotype.

Hormone assay

A FST RIA (Phillips *et al*. 1996) and activin A ELISA assay (Knight *et al*. 1996) were performed on all plasma and uterine fluid samples. The samples from the ewes had been used in previous assays (O'Connell *et al*. 2013), and a limited amount of plasma remained for analysis with samples not being available from some ewes on some days. These values were treated as missing samples. Two animals from the enhanced ES had insufficient samples and were excluded from analysis. The minimum number of animals represented for any comparison was four. Assay detection limits and CV were 0.008 ng/ml and <10% for the activin A ELISA and 2 ng/ml and <10% for the FST RIA respectively.

Total protein in the uterine fluid was measured using a BCA Protein Assay Kit (Cat 23225, Pierce, Rockford, IL, USA) as per manufacturer's instructions.

Quantitative RT-PCR

From experiments 2A and 2B, endometrial tissues from the mid-region of the uterus were used for quantitative RT-PCR. Tissue from a total of nine enhanced ES ewes (three collected during the oestrous cycle and six during pregnancy) and eight reduced ES ewes (four each collected during the oestrous cycle and pregnancy) were available for analysis. Total cellular RNA was isolated using Trizol (Life Technologies) according to the manufacture's recommendations. Further purification was undertaken using a QIAGEN RNeasy Mini Kit (Bio-Strategy Ltd) following the manufacture's protocol. Total cellular RNA (1000 ng) was DNase I treated prior to cDNA synthesis using Superscript III (Life Technologies) using the standard method. The sensitivity of the assay was optimised with a primer matrix approach for all genes using SYBR Green (Life Technologies). Absence of non-specific products was assessed by dissociation curve analysis. Serial dilutions of a standard sample were used to calculate efficiency and range of each assay (Table 1). Identity of amplified products was confirmed by sequencing (Waikato DNA Sequencing Facility, Hamilton, New Zealand). Samples outside the upper range were considered below the sensitivity of the assay. The *RPL19* mRNA was used as the reference to calculate ΔCt. Average ($\pm$s.e.m.) *RPL19* Ct for enhanced ES ewes (20.37 ± 0.24) was not different to the average Ct of reduced ES ewes (20.44 ± 0.25; $P=0.84$). All samples were assayed at least in duplicate. A calibrator sample was assayed on every plate and was used to calculate the $\Delta\Delta$Ct for each sample. Samples that omitted the reverse transcriptase for cDNA synthesis and no template controls were included and gave the expected negative results for all assays.

Culture of luteinising ovine granulosa cells

Collection and culture of ovine granulosa cells were as previously described (Juengel *et al*. 2004). Briefly, ovaries were collected from the local abattoir and transported back to the laboratory. Follicles ~1–2 mm in diameter were dissected from the ovaries and granulosa cells collected using a wire loop to scrape the walls of bisected follicles. A glass pipette was used to remove any oocytes or follicular debris. Cells (100 000/well per 250 µl total volume) were added to the culture plates in McCoys media (Sigma) with penicillin (100 µ/ml), streptomycin (100 µg/ml), 2 mM GlutaMAX-1, androstenedione (30 ng/ml), insulin (10 ng/ml), apo-transferrin (5 µg/ml) and selenium (5 ng/ml). Ovine FSH (3 ng/ml, purified in our laboratory, 1.4×USDA-oFSH-19-SIAFP RP2) and IGF1 (1 ng/ml, Long-R3, GroPrep, Adelaide, SA, Australia) were also included.

Table 1 Primers for the quantitative PCR assays and assay characteristics

Gene	Product size	Forward primer: Sequence	Forward primer: Final Conc (nM)	Reverse primer: Sequence	Reverse primer: Final Conc (nM)	Efficiency (%)	R^2	Range
RPL19	116bp	GACGATACCGTGAATCTA	200	CAGCTTGTGGATATGTTC	300	100.4	0.9956	18.94–26.99
FST	105bp	CGTGTAAAGAAACGTGTG	300	GGTGATGTTAGAACAATCTG	400	102.8	0.9977	20.12–31.13
INHBA	110bp	GTACGTGGAGATAGAGGA	300	CAGGAAGAGCCAGATTTC	300	100.7	0.9918	21.35–29.12

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Cells were cultured in a 37 °C incubator with 5% CO_2 for 6 days. Media (200 µl) was replaced every 48 h. These culture conditions induce luteinisation of the granulosa cells, as progesterone concentrations in media from the last 48 h of culture were over fivefold greater than concentrations measured from media collected after the first 48 h of culture. A preliminary dose response of 1, 10 and 100 ng/ml of rhActivin A (R&D Systems, Pharmaco, Auckland, New Zealand) was undertaken with maximal inhibition of progesterone observed at 10 ng/ml; therefore, the effects of rhActivin A at 10 ng/ml on progesterone and cell number (as assessed by DNA content) was measured in triplicate in five independent pools of granulosa cells. The media from the last 4800 h of culture was frozen at −20 °C for determination of progesterone concentrations by RIA (Lun *et al*. 1998). The intra- and inter-assay coefficients of variation (CV) for three standard sample assayed at approximately 20, 50 or 80% binding both averaged <20% and no samples were below the sensitivity of the assay. At the end of culture, following collection of the media, unattached cells were removed by washing, and attached cells were lysed using incubation at 37 °C in distilled water for 1–2 h. Cell lysates were frozen at −70 °C until assayed for DNA content using a Hoechst 33258 based dye assay (Juengel *et al*. 2004). Intra- and inter-assay CV were <10%, and no samples were below the sensitivity of the assay.

Statistical analyses

Data for concentrations of activin A and FST in plasma were analysed following natural log transformation to normalise the data. Samples below the sensitivity of the assay were estimated using the fill-in method (Gleit 1985). Transformed values were analysed using REML fitting day of sample, group (enhanced or reduced ES) and pregnancy status, as well as two-way interactions, as main effects with animal fitted as a random variable. An autoregressive error structure was used to allow for heterogeneity between repeated measures. Concentrations of *INHBA* and *FST* mRNA were analysed using REML with pregnancy status and ES group as main effects with ewe as a random effect. No interaction was observed between pregnancy status and ES group; so, the data were combined for final analysis of ES group. Samples below the sensitivity of the assay (INHBA only) were estimated using the fill-in method (Gleit 1985). Concentrations of *INHBA* mRNA were transformed (natural log) to normalise the data before analysis. Concentrations of activin A and FST in uterine fluid were analysed with ANOVA using total protein as a covariate. Following transformation (natural log), concentrations of progesterone and DNA following culture of granulosa cells with activin A or control media were compared using a paired *t*-test.

Results

Expression of FST and INHBA mRNA in uterine tissues

Expression of *FST* mRNA was observed in the luminal epithelium (LE) on days 14 and 16 of the oestrous cycle and during gestation (Fig. 2). On day 18 of gestation, expression was detected in the LE as well as trophectoderm cells of the conceptus (Fig. 2). No obvious differences in patterns of expression were seen between the enhanced and reduced ES groups on either day 14 of the oestrous cycle or day 16 of gestation (data not shown).

In uterine tissue collected from ewes during the oestrous cycle, *INHBA* mRNA was observed in the LE and stratum compactum stromal cells of the endometrium (Fig. 3). In uterine tissue from pregnant ewes, *INHBA* mRNA expression on day 14 was similar to that observed on day 14 of the oestrous cycle but with endometrial glands also containing *INHBA* mRNA. By day 18 of pregnancy, expression appeared to be limited to the uterine glands with no *INHBA* mRNA observed in other uterine cell types or the implanting conceptus. No differences in the patterns of expression were apparent between the ES groups (data not shown).

Concentrations of FST and activin A in plasma

As illustrated in Fig. 3, concentration of FST in plasma was affected by day of the oestrous cycle ($P<0.001$), ES group ($P<0.05$), pregnancy status ($P<0.01$) and pregnancy status by ES group interaction ($P<0.001$). Overall, concentrations of FST decreased ($P<0.05$) ~10% between days −7 and −3 to reach a nadir on day −1 with day 0 being the day of oestrus onset. Following ovulation, increased ($P<0.05$) concentrations of FST were detected by day 2 with peak values ~20% above the nadir established by day 6 of the cycle. Overall, ewes with enhanced ES had 1.5-fold more FST than ewes with reduced ES. Ewes with enhanced ES had higher concentrations of FST during the oestrous cycle, but this was not apparent for pregnant ewes (Fig. 4).

Concentrations of activin A in plasma were affected by day of the oestrous cycle ($P<0.001$), ES group ($P<0.001$) and pregnancy status ($P<0.01$), but no interactions were detected ($P>0.15$). Overall, ewes with

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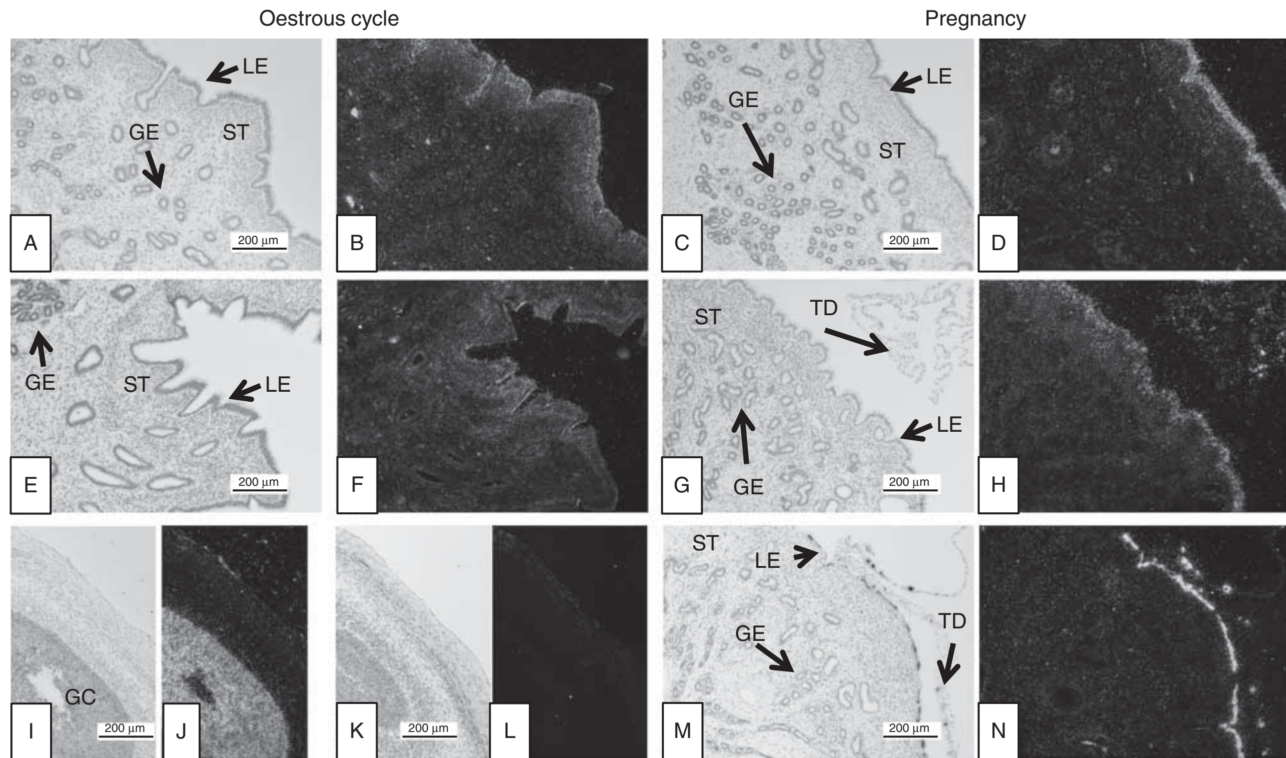

Figure 2
Bright field (A, C, E, G, I, K, and M) or dark field images (B, D, F, H, J, L, and N) of *FST* mRNA expression in uterine tissue collected from control Romney ewes on days 14 (A, B, C, and D), 16 (E, F, G, and H) or 18 (M and N) of the oestrous cycle (A, B, E, and F) or pregnancy (C, D, G, H, M, and N). Control ovarian tissue hybridised with either antisense (I, J; positive control) or sense (K, L; negative control) *FST* RNA probes. Some expression of *FST* mRNA in the LE was observed on days 14 and 16 of the oestrous cycle as well as gestation (A, B, C, D, E, F, G, and H). No expression was observed in the glandular epithelia. On day 18 of gestation, expression in the LE as well as trophectoderm cells was detected. A signal was also detected in the endometrial stroma (ST) for all time periods (A, B, C, D, E, F, G, H, M, and N). Expression was observed in the granulosa cells of the ovarian follicle when hybridised with the anti-sense RNA (I, J; positive control) but not with the sense RNA (K, L; negative control).

enhanced ES had a modest 20% decrease in concentrations of activin A compared to ewes with reduced ES (Fig. 5). Activin A concentrations in plasma were low, being at or just above the detection limits of the assay, on most days of the oestrous cycle or early gestation with the exception of the 2 days around oestrus, where a peak of activin A was detected (Fig. 5).

Concentrations of FST and INHBA mRNA in endometrium of enhanced and reduced ES ewes

Concentration of *FST* mRNA was similar between enhanced and reduced ES ewes (reduced ES ewes 88.2± 24.0% of expression of enhanced ES ewes, $P=0.74$). The concentration (geometric mean 95% confidence range) of *INHBA* mRNA from endometrium of reduced ES ewes was 167.0% (101.9–274.5%) of that observed in enhanced ES, but this was not significantly different ($P=0.13$).

Concentrations of FST and activin A in uterine flushes of enhanced and reduced ES ewes

Concentrations of FST in uterine flushes collected on day 16 of gestation were not different between ewes with enhanced or reduced ES phenotypes (Table 2). In contrast, concentrations of activin A were twofold greater ($P<0.05$) in uterine flushes collected from ewes with a reduced ES compared to those with an enhanced ES phenotype (Table 2).

Effects of activin A on progesterone and DNA content in luteinising ovine granulosa cells

Given that concentrations of activin A in sera, which were increased in reduced ES ewes compared to enhanced ES ewes, peaked during the time that the follicular cells would be undergoing luteinisation, we next examined the

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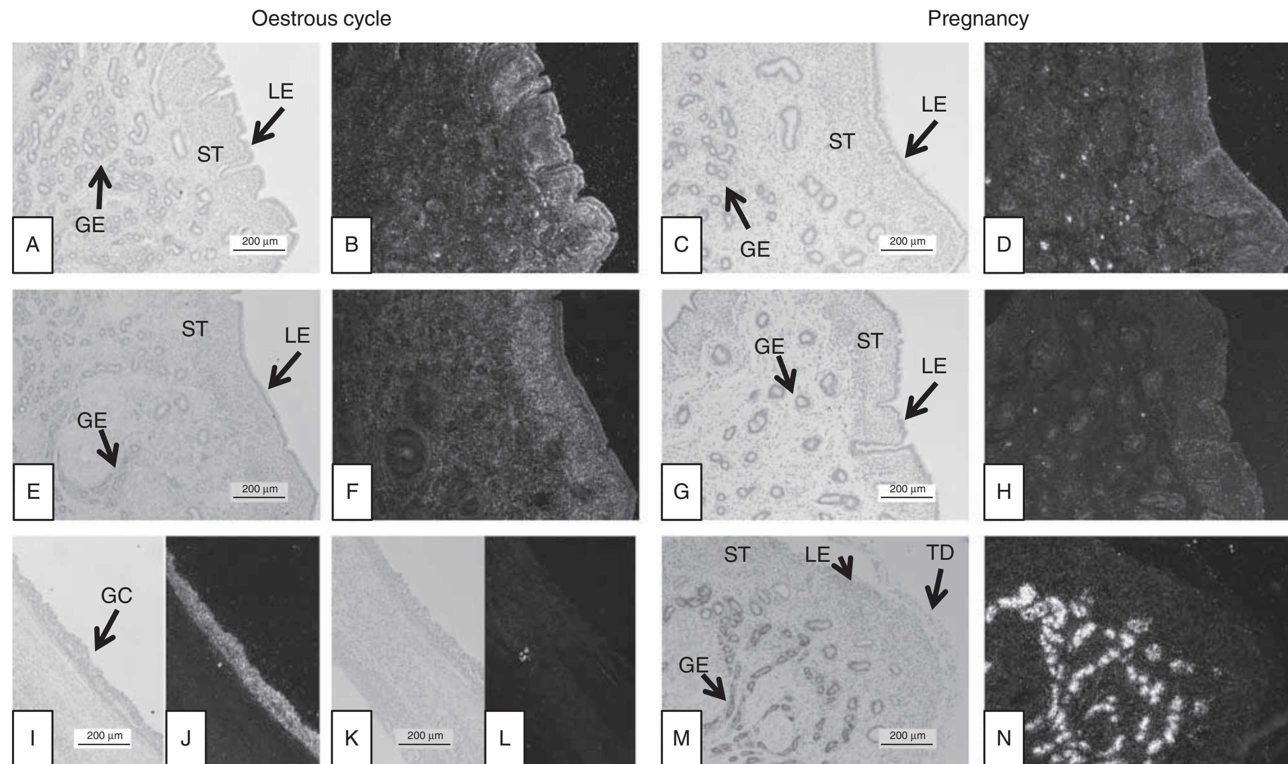

Figure 3
Bright field (A, C, E, G, I, K, and M) or dark field images (B, D, F, H, J, L, and N) of *INHBA* mRNA expression in uterine tissue collected from control Romney ewes on days 14 (A, B, C, and D), 16 (E, F, G, and H) or 18 (M and N) of the oestrous cycle (A, B, E, and F) or pregnancy (C, D, G, H, M, and N). Control ovarian tissue was hybridised with either antisense (I, J; positive control) or sense (K, L; negative control) *INHBA* RNA probes. Expression of *INHBA* mRNA was found to be expressed by the LE and stromal cells of the endometrium compactum in uterine tissue collected during the oestrous cycle. In pregnant uterine tissue, *INHBA* expression was also observed in the glandular epithelia on day 14 of gestation with distinct expression at day 18 of gestation. Expression was observed in the granulosa cells of the ovarian follicle when hybridised with the anti-sense RNA (I, J; positive control) but not with the sense RNA (K, L; negative control).

effects of activin A on progesterone production from luteinising granulosa cells. Concentrations of progesterone in media collected from days 4–6 of culture from ovine granulosa cells treated with activin A were decreased ($P<0.001$) to 7.4±1.5% of that observed in control cells. Treatment with activin A did not affect ($P<0.15$) the amount of DNA measured at the end of culture (activin A treated cells had 70.1±17.3% of the DNA measured in control cells).

Discussion

In the present study, a modest increase of activin A was observed in plasma with a larger increase in uterine fluid in ewes with reduced ES compared to those with enhanced ES. No such variations in concentrations of FST were detected indicative of an increased activin tone. The mechanisms by which increases in activin might cause reproductive failure are unclear. A delicate balance between the pro- and anti-inflammatory environment is mediated through cytokine and chemokine signalling with significant variation among species (van Mourik *et al.* 2009). It is possible that overexpression of activin leads to disruption of this delicate balance resulting in rejection of the developing conceptus. Activin A has also been shown to stimulate proliferation of bovine endometrial cells (Sugawara *et al.* 2010). Alternatively, if activin concentrations are altered in the preovulatory follicle, this could potentially affect oocyte quality or subsequent luteal function. Increased expression of *FST* by the oocyte has also been linked to enhanced oocyte quality as assessed by improved early embryo development (Rajput *et al.* 2013). However, it should be noted that it is unclear if the positive effects of FST on oocyte quality and embryo development are due to neutralisation of activin as treatment with activin A mimicked FST actions, albeit with less potency (Rajput *et al.* 2013).

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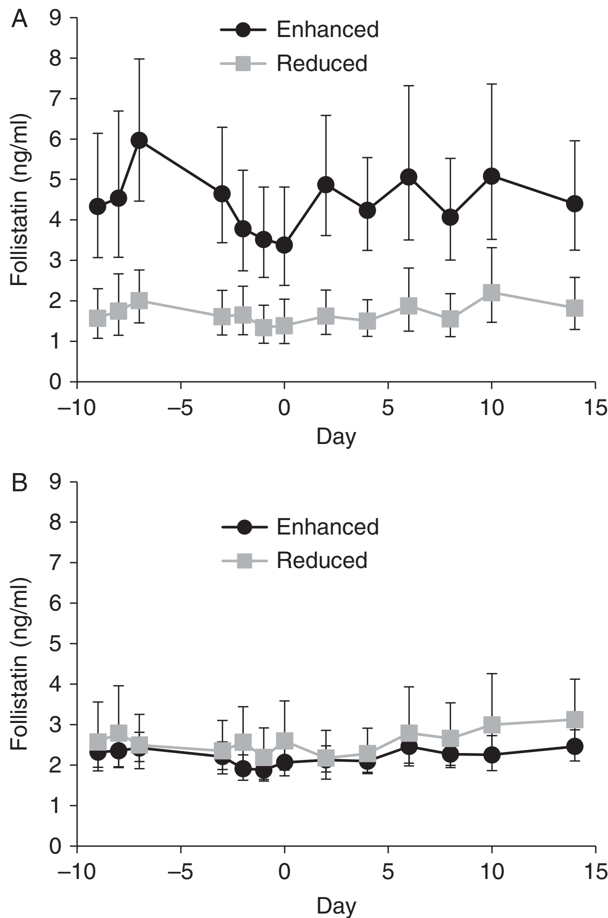

Figure 4
Concentrations (geometric means and 95% confidence limits) of FST in plasma from ewes with enhanced or reduced ES across the cycle. Data collected from non-pregnant ewes are shown in panel A ($n=5$ and $n=4$ for enhanced and reduced ES respectively) whereas data collected from pregnant ewes are shown in panel B ($n=14$ and $n=8$ for enhanced and reduced ES respectively). Day 0 equals the day of oestrus/breeding.

A key pathway that may be affected by increased activin secretion is luteinisation of the granulosa cells and subsequent luteal function. Concentrations of activin A in the peripheral circulation were low during most of the cycle, but a peak of activin A occurred around oestrus. The preovulatory follicle strongly expresses *INHBA* mRNA (Engelhardt *et al.* 1993, Tisdall *et al.* 1994), and this peak could be driven by growth of the preovulatory follicle. However, concentrations of activin A were not higher in follicular fluid of oestrogen-active follicles than oestrogen-inactive follicles (Young *et al.* 2012); so, the source of the peak of activin A observed around oestrous needs to be confirmed.

We have previously shown that these ewes with enhanced ES have increased concentrations of progesterone both in the systemic as well as the uterine circulations, potentially linked to improved function of the corpus luteum (O'Connell *et al.* 2013). In the current study, we have shown that treatment of luteinising ovine granulosa cells with activin A reduced progesterone secretion; thus, the increased activin A concentrations observed around the time of oestrous in the reduced ES ewes may have resulted in poor luteal function in these ewes. Similar to what was observed in the current study, activin A has been shown to reduce luteinisation of granulosa cells *in vitro* in both humans and cattle (Myers *et al.* 2008, Kayani *et al.* 2009). Activin A also suppressed progesterone production *in vitro* from granulosa cells collected from preovulatory follicles in rats (Miro *et al.* 1991).

An increase in activin concentrations has been found to be associated with reproductive failure in women (Rombauts *et al.* 2006). Elevated activin concentrations have been linked to reduced adhesion of the blastocyst to the LE (Stoikos *et al.* 2008). However, others have found higher concentrations of activin A in uterine fluid collected during the periovulatory period in women who became pregnant after intrauterine insemination compared to those that failed to establish a pregnancy (Florio *et al.* 2010). Moreover, using semi-quantitative immunohistochemical techniques, Prakash *et al.* (2006) observed reduced staining for both activin A and FST in women with recurring miscarriages. The differences between these studies could be related to differing days

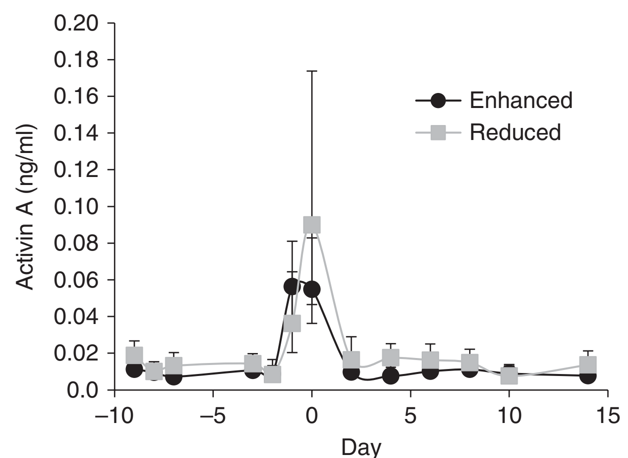

Figure 5
Concentrations (geometric means and 95% confidence limits) of activin A in plasma from ewes with enhanced ($n=19$) or reduced ($n=12$) ES across the cycle. Data collected during the oestrous cycle and early gestation were combined, as there were no group by pregnancy status interactions. Day 0 equals the day of oestrus.

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Table 2 Relative expression of *FST* and activin A expression in uterine flush media collected on day 16 of gestation from ewes with enhanced or reduced ES

Protein (ng/ml)	**Enhanced ES** (n=14)	**Reduced ES** (n=7)	**SED**	***P* value**
activin A	0.45	1.05	0.24	0.022
FST	105.2	121.8	19.8	0.412

of sample collection or methods of measuring activin and FST, but may also signify the requirement for a balance of activin activity, with under or overexpression being detrimental to pregnancy outcome.

The observed differences in concentrations of activin A were found locally in uterine fluid. The uterus could be synthesising activin A, as mRNA encoding *INHBA*, which dimerises to form activin A, is expressed by the uterus. Average concentrations of *INHBA* mRNA in endometrium of reduced ES ewes were 167% of that observed in enhanced ES ewes, which is consistent with a twofold increase observed in concentrations of activin A in uterine fluid. However, this difference did not reach significance, potentially because concentrations of *INHBA* mRNA were quite variable between animals. This variability could be due to heterogeneity of cell types within the endometrial sample collected (Bauersachs 2014). Expression of *INHBA* mRNA was detected in uterine LE of both day 14 non-pregnant and pregnant ewes, but expression was limited to the glandular epithelium in day 18 pregnant ewes. Expression of *INHBA* has previously been observed in the developing ovine uterus (prior to puberty) with strongest expression in the luminal and glandular epithelia (Hayashi *et al.* 2003). Altered uterine gland development was induced by ovariectomy and linked to reduced expression of *INHBA* mRNA and protein (Carpenter *et al.* 2003). Taken together, these studies support a role for activin A in regulation of uterine function in sheep throughout the lifetime of a ewe.

There was some evidence that plasma concentrations of FST were greater in ewes with enhanced ES, as combined analysis of samples collected in pregnant and non-pregnant sheep revealed increased plasma FST concentrations in ewes with enhanced ES. However, this increased plasma FST was driven by differences observed in the first year, when the ewes were not exposed to a fertile ram, with no differences observed in the second year, when the ewes were exposed to a fertile ram and thus became pregnant during the blood collection period. Given that some of the samples collected from the pregnant sheep were taken before exposure to the fertile ram, and thus these samples would be physiologically equivalent to those samples collected from the non-pregnant sheep, the failure to observe differences in both groups of sheep indicates that the increased concentration of plasma FST is not consistently observed but may represent a differing response of enhanced and reduced ES ewes to environmental conditions. These environmental conditions are unknown at this time, and a better understanding of how environmental factors may regulate FST concentrations may provide new insights into variability in ES. No differences in FST concentrations were observed in uterine fluid nor were any differences observed in expression of *FST* mRNA in the endometrium. However, it is clear that increased concentrations of activin in the low ES ewes were not accompanied by an increase in neutralising FST.

The mRNA encoding *FST* was expressed primarily in the LE with expression also observed in the trophectoderm of the developing conceptus. The expression of *FST* mRNA has also been observed in developing ovine uterine tissue, although expression in the epithelial cells was observed shortly after birth, and expression was not detected in developing glandular epithelium between days 21 and 56 after birth (Hayashi *et al.* 2003). Thus, expression of *FST* mRNA appears to be regulated in the ovine uterus under differing reproductive states. In cattle, the non-gravid and gravid uterine horns, as well as foetal membranes, from day 35 of pregnancy, express *FST* mRNA (Sugawara *et al.* 2010). *FST* mRNA is also expressed in ovarian tissues, particularly the granulosa cells of ovarian follicles as well as other tissues (Tisdall *et al.* 1994). Circulating concentrations of FST were reduced around oestrus compared to the peak levels of activin A at that time being indicative of a higher activin tone around oestrus and ovulation. However, it should be noted that the decrease in FST observed in this study was relatively minor, with only a 20% difference between the nadir and peak values. Previous studies have shown that *FST* expression is unvarying throughout the oestrous cycle (McFarlane *et al.* 2002, Xia *et al.* 2010).

In summary, differences in activin A concentrations, both in the systemic circulation and in uterine fluid, were observed in ewes with differing lifetime ES phenotypes. Ewes with reduced ES had elevated concentrations of activin A, without concomitant changes in FST concentrations, indicative of an increase in activin tone. A peak of activin A was observed around oestrus, and activin A was shown to supress progesterone production by luteinising ovine granulosa cells *in vitro*; thus, the increased activin A

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concentration may underlie the supressed progesterone concentrations observed during the early luteal phase in these animals (O'Connell *et al*. 2013). The expression of *INHBA* and *FST* mRNA in the uterus/conceptus, as well as increased activin A concentrations in uterine fluid of ewes with poor ES, is also consistent with a role for activin A in regulating local interactions between the conceptus and uterus during implantation.

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.

Funding
The research was supported by the New Zealand Ministry of Business, Innovation & Employment (formerly Foundation for Research, Science & Technology), New Zealand (grant number: C10X1001), and AgResearch Core Funding.

Acknowledgements
We thank Ms Susan Hayward and A/Prof. Mark Hedger at the Monash Institute of Medical Research, Monash University for performing the activin A and FST assays. Provision of reagents for the activin A ELISA by Oxford Brookes University is gratefully acknowledged. We would also like to thank Michelle French for performing the quantitative PCR assays. We would also like to thank Laurel Quirke for her help with the *in situ* hybridisation technique and Dr Carey Satterfield and Dr David Phillips for insightful discussion on embryo survival and activin and FST. The help of members of the Animal Reproduction team and the Invermay farm staff for sample collection and animal care and management is also greatly appreciated.

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Received in final form 13 December 2015
Accepted 24 December 2015
Accepted Preprint published online 5 January 2016

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