Human chorionic gonadotropin increases serum progesterone, number of corpora lutea and angiogenic factors in pregnant sheep

Megan P T Coleson*, Nicole S Sanchez*, Amanda K Ashley, Timothy T Ross and Ryan L Ashley

Department of Animal and Range Sciences, New Mexico State University, PO Box 30003, MSC 31, Las Cruces, New Mexico 88003, USA

Correspondence should be addressed to R L Ashley; Email: ryashley@nmsu.edu

*(M P T Coleson and N S Sanchez contributed equally to this work)

Abstract

Early gestation is a critical period when implantation and placental vascularization are established, processes influenced by progesterone (P_4). Although human chorionic gonadotropin (hCG) is not endogenously synthesized by livestock, it binds the LH receptor, stimulating P_4 synthesis. We hypothesized treating pregnant ewes with hCG would increase serum P_4 , number of corpora lutea (CLs) and concepti, augment steroidogenic enzymes, and increase membrane P_4 receptors (PAQRs) and angiogenic factors in reproductive tissues.

The objective was to determine molecular alterations induced by hCG in pregnant sheep that may promote pregnancy. Ewes received either 600 IU of hCG or saline i.m. on day 4 post mating. Blood samples were collected daily from day 0 until tissue collection for serum P_4 analysis. Reproductive tissues were collected on either day 13 or 25 of gestation and analyzed for PAQRs, CXCR4, proangiogenic factors and steroidogenic enzymes. Ewes receiving hCG had more CL and greater serum P_4 , which remained elevated. On day 25, StAR protein production decreased in CL from hCG-treated ewes while HSD3B1 was unchanged; further, expression of CXCR4 significantly increased and KDR tended to increase. PAQR7 and CXCR4 protein was increased in caruncle tissue from hCG-treated ewes. Maternal hCG exposure influenced fetal extraembryonic tissues, as VEGFA, VEGFB, FLT1, and ANGPT1 expression increased. Our results indicate hCG increases serum P_4 due to augmented CL number per ewe. hCG treatment resulted in greater PAQR7 and CXCR4 in maternal endometrium and promoted expression of proangiogenic factors in fetal extraembryonic membranes. Supplementing livestock with hCG may boost P_4 levels and improve reproductive efficiency.

Reproduction (2015) **150** 43–52

Introduction

Most pregnancy losses in mammalian species occur during early pregnancy (Edey 1969, Reynolds & Redmer 2001, Reynolds *et al.* 2010, 2013). Several factors contribute to embryonic death including insufficient production of progesterone (P_4) by the corpus luteum (CL), as suboptimal P_4 synthesis is indicative of maternal inability to maintain pregnancy (Kittok *et al.* 1983). P_4 is required for establishment and maintenance of pregnancy in all mammals. Growth and development of the conceptus (embryo/fetus and associated extraembryonic membranes) requires P_4 signaling to regulate endometrial functions critical for implantation and placentation. Human chorionic gonadotropin (hCG) is a potent luteotropic hormone (Norman & Litwack 1987) responsible for increased P_4 synthesis by the CL upon binding the luteinizing hormone receptor (LHCGR). Although livestock do not produce hCG, it binds to the LHCGR, which they do

express. Due to LHCGR activation by hCG and the substantially longer half-life than luteinizing hormone (LH) in mammals (Cole 2012), it may be useful for increasing P_4 production in livestock species. In beef heifers, serum P_4 concentrations and conception rates following artificial insemination increased following hCG administration (Funston *et al.* 2005). In ewes, treatment with hCG increased P_4 and total CL weight 12 and 36 h after administration (Nephew *et al.* 1994). Further, hCG increased the number and size of CLs in ewes and these accessory CLs could be responsible for the dramatic increase in serum P_4 levels observed (Farin *et al.* 1988, Shabankareh *et al.* 2012). However, it is unclear if the increase in serum P_4 is exclusively due to increased CL numbers or if hCG is stimulating synthesis of steroidogenic enzymes. Addition of this exogenous hormone may boost serum P_4 levels and decrease pregnancy loss in livestock, thereby increasing animal reproductive efficiency.

P₄ signaling in the uterus primes the endometrium to ensure a receptive environment for the developing conceptus. P₄ elicits its effects through nuclear P₄ receptors (PRs) as well as membrane P₄ receptors (PAQR family). Paradoxically, the elevated P₄ during early pregnancy leads to a downregulation of PR and in sheep the PR protein is undetectable in endometrial luminal epithelium and glandular epithelium after days 11 and 13 of pregnancy respectively (Spencer & Bazer 1995, Spencer *et al.* 1995). While PRs have been researched extensively, not much is known about PAQRs. Interestingly, expression of PAQRs is upregulated by P₄ (Karteris *et al.* 2006) and may play functional roles during early pregnancy. Because hCG has noted effects on increasing P₄ synthesis, one objective of this study was to investigate the effects of exogenous hCG on expression and synthesis of the PAQR family of receptors.

In addition to stimulating P₄ production, hCG also serves as a proangiogenic factor at the fetal/maternal interface (for a review see Cole (2012)). hCG influences vascularization by regulating expression of proangiogenic factors such as vascular endothelial growth factor (VEGFA) and angiopoietin one (ANGPT1) (Laitinen *et al.* 1997, Sugino *et al.* 2000, Wulff *et al.* 2000). Further, hCG stimulates proliferation of placental microvascular endothelial cells, which are important for capillary sprout formation during angiogenesis (Herr *et al.* 2007). Haploinsufficiency of VEGFA or its receptors, FMS-like tyrosine kinase receptor 1 (FLT1) and kinase insert domain-containing receptor (KDR), results in defective fetal and placental angiogenesis, culminating in embryonic death by mid-gestation (Fong *et al.* 1995, Shalaby *et al.* 1995, Carmeliet *et al.* 1996, Ferrara *et al.* 1996). ANGPT1 also regulates angiogenesis, as several studies have shown vascular defects when these factors are deficient in mice (Carmeliet *et al.* 1996, Ferrara *et al.* 1996, Maisonpierre *et al.* 1997). Similar to hCG induced functions, activation of C-X-C chemokine receptor type 4 (CXCR4) by its ligand, C-X-C chemokine 12 (CXCL12) amplifies angiogenesis by inducing VEGFA release. In turn, VEGFA increases expression of CXCR4, but not other chemokine receptors, thus establishing a positive feedback loop in which VEGFA induces CXCR4 and CXCL12 expression, and conversely CXCL12/CXCR4 signaling enhances VEGFA expression (Salcedo *et al.* 1999, Mirshahi *et al.* 2000, Salcedo & Oppenheim 2003). We previously reported that placental expression of the CXCL12/CXCR4 signaling axis during early pregnancy corresponds with placental vascularization with marked induction of VEGFA, FLT1, KDR and ANGPT1 (Ashley *et al.* 2011, Quinn *et al.* 2014), yet whether hCG regulates the CXCL12/CXCR4 signaling axis is not known.

Based on the variety of functions induced by hCG in humans, specifically stimulating P₄ synthesis and influencing placental vascularization, administration of

exogenous hCG may improve pregnancy success rates and enhance reproductive efficiency and overall production in livestock. We hypothesized that ewes treated with hCG would have higher serum P₄ concentrations, greater number of CL and concepti, augmented steroidogenic enzymes, and increased expression of PAQRs and angiogenic factors in reproductive tissues. This hypothesis was formulated to cover myriad physiological outcomes induced by hCG to provide a more comprehensive perspective on its role in livestock reproduction. The objectives of the study were to determine molecular alterations induced by hCG administration in pregnant sheep that may serve to promote early pregnancy and elucidate additional enzymatic alterations induced by hCG treatment. Specifically, hCG-induced changes in the regulation and synthesis of angiogenic factors and PAQRs were investigated.

Materials and methods

Animal procedures and tissue collection

All procedures involving animals were approved by the New Mexico State University Animal Care and Use Committee (IACUC #2012-018). Unless indicated, all reagents were purchased from Sigma-Aldrich. Nineteen mixed-aged western whiteface ewes (70.5 ± 1.5 kg body weight) received an intravaginal pessary impregnated with 20 mg flurogestone acetate (Searle, Skokie, IL, USA) for 14 days to synchronize estrus. Three vasectomized rams, fitted with marking harnesses, were placed with ewes, for detection of estrus. Ewes were mated with fertile rams upon detection of their second estrus after pessary removal and onset of estrus was determined as the day serum P₄ was at or below 1 ng/ml (day 0). Ewes were randomly assigned to one of two treatments. Ewes received 600 IU (4.8 ml) of hCG (ProSpec-Tany Techno Gene Ltd., Ness Ziona, Israel, Cat #: hor-250) i.m. (n=9) or saline (4.8 ml, n=10) on day 4 post mating. Treatment day and dose was determined by previous studies utilizing pregnant sheep (Nephew *et al.* 1994, Akif Cam & Kuran 2004) and studies conducted in the laboratory of Dr Tim Ross, where varying doses of hCG as well as single vs multiple injections of hCG have been evaluated in pregnant ewes (RA Ashley and TR Ross, personal communication). This treatment regime was highly effective at increasing serum P₄ concentrations and additional injections were not warranted. Within each treatment, ewes were randomly assigned to one of two groups where half the ewes were euthanized 13 days post mating (control n=4; hCG-treated n=5) and the remaining ewes euthanized 25 days post mating (control n=6; hCG-treated n=5). Ewes were anesthetized with 20 mg/kg body weight of sodium pentobarbital (Vortech Pharmacy, Dearborn, MI, USA) via i.v. administration. The surgical area was shorn and reproductive tract removed using a mid-ventral laparotomy prior to euthanasia by exsanguination. One group of ewes was euthanized on day 13, and CLs were counted. In addition, uteri were flushed with ~20 ml of PBS supplemented with 0.25% BSA; pH 7.1) and numbers of concepti were recorded. The second group of ewes was euthanized on day 25 post mating, and CL and concepti

were counted and weighed. Caruncles, CLs and fetal extraembryonic membranes were collected, diced into smaller pieces, and immediately immersed in liquid nitrogen, then stored at -80°C .

Blood collection and serum P_4 assessment

Daily blood samples were collected via jugular venipuncture into serum separator tubes (Corvac, Kendall Health Care, St Louis, MO, USA) from all ewes starting on the date of marking by the ram until euthanasia. Tubes were incubated at room temperature for at least 30 min prior to being centrifuged at 4°C for 15 min at 1500 *g*. Serum was harvested and subsequently stored at -20°C until assayed. Serum P_4 concentrations were determined using RIA procedures described by Schneider and Hallford (1996; Coat-A-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) and performed by the New Mexico State University Endocrinology Laboratory. Inter- and intra-assay CV were 4.7 and 6.7% respectively.

RNA isolation and cDNA synthesis

Total cellular RNA was extracted from tissues using 1 ml of Tri Reagent BD (Molecular Research Center, Inc., Cincinnati, OH, USA) per 100 mg of tissue, according to the manufacturer's directions. RNA was eluted in nuclease-free water, and subsequently treated with DNase using the TURBO DNA-free kit (Ambion, Foster City, CA, USA) to ensure samples were not contaminated with genomic DNA. The quantity and purity of RNA was determined using a NanoDrop-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA samples were stored at -80°C until further analysis. Complementary DNA was synthesized from 1 μg RNA using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's recommendations. The products were diluted to a final volume of 100 μl (10 ng/ μl).

Quantitative real-time PCR

The quantitative real-time PCR (qPCR) analysis was performed using a CFX96 Touch Real-Time PCR Detection System and components of the iQ SYBR green supermix (Bio-Rad Laboratories) as previously described (Quinn *et al.* 2014). Forward and reverse primers were used at a final concentration of 0.525 μM and 2 μl of cDNA for each sample was assayed. The specific primers employed are shown in Table 1. The efficiency of all primers was tested using varying levels of cDNA (50–0.05 ng per reaction); all were within acceptable limits (Livak & Schmittgen 2001) and no primer-dimers were formed. The qPCR conditions were 95°C for 3 min followed by 40 cycles of 95°C (30 s), 55°C (30 s) and 72°C (15 s). Then a melt curve was performed to assure no primer-dimers were present. C_q values were attained during logarithmic amplification phase of PCR cycling. The *GAPDH* amplicon did not change across days or pregnancy status and was used to normalize each target via the $2^{-\Delta\Delta C_q}$ method (Livak & Schmittgen 2001).

Table 1 Primer pair sequences for targets listed used in qPCR^a.

Target	Sequence	Accession no.
<i>GAPDH</i>	5'-CGTTCTCTGCCTTGACTGTG-3' 5'-TGACCCCTTCATTGACCTTC-3'	NM_001190390
<i>PAQR7</i>	5'-GCAGGCCAAGTCTGAGTTCT-3' 5'-GCAGGAAGATGGTCTGCACT-3'	DQ318952
<i>PAQR8</i>	5'-CTACCCGGTCATGAGGAAGA-3' 5'-AGATGATCTGGAGGGTGTGG-3'	BC148084
<i>PAQR5</i>	5'-ACACCTTCAGTCCATGTCC-3' 5'-AGTCATGGAAGGTGGTGCTC-3'	XM_605853
<i>CXCR4</i>	5'-GAGTCGATGCTGATCCCAAT-3' 5'-AAGGCTATCAGAAGCGCAAG-3'	NM_174301
<i>VEGFA</i>	5'-AAATGCTTTCTCCGCTCTGA-3' 5'-TCACCAAAGCCAGCACATAG-3'	EU857623.1
<i>VEGFB</i>	5'-CAAGTCCGAATGCAGATCCT-3' 5'-TGTCTGGCTTCACAGCACTC-3'	XM_004019872.1
<i>FLT1</i>	5'-TCCACAAATCTTGGCCTTTC-3' 5'-GTGCAGATGGACGAGGACTT-3'	XM_004012285.1
<i>KDR</i>	5'-GCTCCACCAGCTCTGAAAAC-3' 5'-TGAGAGCCCCTGATTACACC-3'	NM_001278565.1
<i>ANGPT1</i>	5'-AGGAGGCTGGTGCCTATCTC-3' 5'-TCTGGAGCATGTGATGGAAC-3'	XM_004011787.1
<i>STAR</i>	5'-GAGTGGAACCCAGTGTCAC-3' 5'-GGGGCATCTCCTCGTAGAGT-3'	NM_001009243.1
<i>HSD3B1</i>	5'-GTGACCGAGGAGGAGG-3' 5'-AGGAAGCTCACTATTTCAC-3'	NM_001135932.1
<i>ISG15</i>	5'-GGTATCCGAGCTGAAGCAGTT-3' 5'-ACCTCCCTGCTGTCAAGGT-3'	NM_001009735.1

^aAll primer pairs were designed to amplify efficiently, produce a single amplicon, not generate primer-dimers and produced BLAST specificity to expressed gene. Accession numbers to corresponding targets are reported.

Protein isolation and immunoblotting

Protein was isolated from ovine tissues by homogenizing 100 mg of tissue in 1 ml of RIPA buffer (50 mM Tris (pH 7.4), 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 1.0% TritonX-100) supplemented with phosphatase and protease inhibitor cocktails (Roche Applied Science). Samples were placed on ice for 15 min, then centrifuged at 12 000 *g* for 10 min at 4°C . The supernatant was collected and stored at -80°C . Concentrations of protein were determined using the BCA protein assay (Pierce, Rockford, IL, USA).

Unless indicated, all reagents used for western blot analysis were purchased from Bio-Rad Laboratories, Inc. Equal protein amounts (ranged from 30 to 50 μg depending on tissue) were combined with 6 $\times$ dye (187 mM Tris (pH 6.8), 6% SDS, 30% glycerol, 440 mM β -mercaptoethanol, 0.2% bromophenol blue, deionized water) and denatured on a heat block at 100°C for 5 min, then cooled and subjected to SDS-PAGE. After electrophoresis, protein was transferred to PVDF membranes for immunoblotting. After blocking in 5% non-fat dry milk or 5% BSA (Sigma-Aldrich) made in Tris-buffered saline plus tween (TBS-T; 68.4 mM Tris base, 10 mM NaCl, 0.10% tween 20, pH 7.6) for 1 h at room temperature, membranes were incubated with primary antibody in blocking solution over night at 4°C . Unless indicated, antibodies were purchased from Santa Cruz Biotechnology. Antibodies specific to FLT1 (sc-316), KDR (sc-315), CXCR4 (sc-9046), VEGFA (sc-152), ANGPT1 (Abcam, ab95230), HSD3B1 (sc-30820), StAR (sc-25806), ISG15 (#2743BC; Cell Signaling Technology, Danvers, MA, USA), PAQR5 (Abcam, ab123979), PAQR7

(Abcam, ab79517) or ACTB (sc-47778) were used to probe membranes. Membranes were washed, and an appropriate secondary antibody (goat anti-rabbit IgG-HRP, sc-2004; donkey anti-goat IgG-HRP, sc-2020; donkey anti-rabbit IgG-HRP, sc-2317) was added in blocking solution for 1 h at ambient temperature. The concentration of antibody varied per tissue, but ranged from 1:500 to 1:2000 for primary antibodies and 1:5000–1:20 000 for secondary antibodies. Proteins were visualized by Clarity Western ECL Substrate peroxide solution and Luminol/enhancer solution for 5 min and immediately detected using the ChemiDoc XRS and Image Lab Software Version 3 (Bio-Rad Laboratories). If antibody removal was required in order to assess loading controls, membranes were stripped in stripping buffer (TBS-T, 143 μ M β -mercaptoethanol, 2% SDS) heated to 65 °C, then washed with TBS-T and blocked in 5% non-fat dry milk for 1 h at room temperature. Following the stripping procedure membranes were assessed to assure lack of chemiluminescence in the absence of antibody before being probed for the loading control.

Statistical analysis

The experimental design was completely randomized. Number of CLs and concepti per ewe were analyzed by χ^2 using the frequency procedure of SAS (SAS Inst., Inc., Cary, NC, USA). P_4 concentrations were analyzed using the mixed procedure of SAS with repeated function. Treatment and ewe were in the whole plot and day and day by treatment interaction were in the subplot. For qPCR the C_q value of the gene of interest was normalized to the C_q value of *GAPDH* for each sample and analysis was run on the $2^{-\Delta\Delta C_q}$ values using GraphPad Prism (version 6 from GraphPad Software, Inc., La Jolla, CA, USA). The chemiluminescent signals for western blots were quantified using the mean value (intensity) using Image Lab Software (version 4.1). Each band of interest was normalized by dividing by mean value (intensity) for the protein of interest divided by mean value (intensity) of ACTB. Significant changes were determined at $P < 0.05$ using an unpaired, two-tailed Student's *t*-test. If the variance significantly differed, Welch's correction was used.

Results

Serum P_4 concentrations and CLs and concepti numbers

A day by treatment interaction ($P < 0.05$) was observed for serum P_4 concentrations for all ewes until day 13 (Fig. 1A). Serum P_4 concentrations were similar among treatments through day 3. Ewes receiving hCG on day 4 had greater ($P < 0.05$) serum P_4 concentrations than control ewes from days 4 to 13. No treatment by day interaction was detected ($P > 0.20$) for serum P_4 concentration between days 14 and 25; therefore, treatment effects were examined across sampling days. During this period, hCG-treated ewes maintained elevated ($P = 0.001$) serum P_4 values (10.9 ± 0.6 ng/ml) compared to controls (6.0 ± 0.6 ng/ml). Treatment with hCG tended ($P = 0.108$) to increase number of concepti (Fig. 1B). On day 25, in the control group, three ewes had singletons, two had twins, and one bore triplets, whereas all hCG treated ewes had twins. No difference was observed between mean weight of concepti for control and hCG-treated ewes (339.7 and 473.6 ± 74.7 mg, respectively, $P = 0.20$). Ewes receiving hCG had greater number of CL ($P = 0.006$) compared to control ewes (Fig. 1C). Control ewes had ≤ 3 CL, whereas 87.5% of ewes that received hCG had ≥ 3 CL (Fig. 1C). Further, CL weight (g) per concepti was significantly greater ($P < 0.05$) in hCG ewes compared to control (Fig. 1D).

Gene expression and protein production

Quantitative PCR was utilized to quantify changes in mRNA and western blot analysis for protein production. No changes were seen in mRNA expression for genes of interest from day 13 CL tissue (data not shown). On day 25, mRNA for *CXCR4* was significantly increased ($P \leq 0.001$) in CL collected from hCG-treated ewes compared to control and gene expression for *KDR*

Figure 1 Administration of hCG to pregnant sheep increases serum progesterone concentration and number of corpora lutea (CLs). (A) Day 0 is the day of mating or day that P_4 concentrations were at or below 1 ng/ml and daily blood collections began. On day 4, ewes received either 600 IU of hCG i.m. in 4.8 ml of saline or saline alone. On days 13 and 25, tissues were collected from hCG-treated ($n = 9$) or control ($n = 10$) ewes. For each tissue collection date, the reproductive tract was removed, CLs were counted and specific maternal tissues were collected. On day 25, fetal extraembryonic membranes were also collected. Concepti and CLs were counted visually during necropsies. (B) Ewes receiving hCG tended ($P = 0.108$) to have an increase in number of concepti at 25 days post mating. (C) The number of CLs per ewe from days 13 and 25 significantly increased (** $P = 0.0056$) following hCG treatment and (D) CL weight per concepti was significantly greater (** $P < 0.001$) in hCG ewes compared to control.

tended to increase ($P=0.06$) with hCG treatment (Fig. 2A). Expression of *PAQR8* and *PAQR5* decreased ($P\leq 0.05$) in CL in hCG-treated ewes compared to control (Fig. 2A). Protein for CXCR4, KDR and *PAQR5* was detected in CL from day 25 but did not differ between treatments (Fig. 2B). *PAQR8* protein was not detected in CL tissue (data not shown).

Because previous research demonstrated hCG would boost P_4 levels in ewes, expression of two steroidogenic enzymes, StAR and HSD3B1, was assessed. The function of a cholesterol transporter, StAR, is the rate-limiting step in steroidogenesis, and the enzyme HSD3B1 converts pregnenolone to P_4 , so increased expression of either might increase serum P_4 . No differences were detected in CL between control and hCG-treated ewes on day 13 or day 25 for production of HSD3B1 protein (data not shown). On day 25 expression of StAR was significantly

Figure 2 Exposure to hCG decreases StAR and ISG15 in the corpus luteum (CL). RNA or protein was isolated from CL on day 25 post-mating ($n=6$ control ewes; $n=4$ hCG treated ewes). (A) Expression of mRNA and (B) protein production of CXCR4, KDR, *PAQR8*, *PAQR5*, StAR, and ISG15 were evaluated. (C) Despite mRNA changes, only StAR and ISG15 protein levels significantly changed. In all graphs, $^{\$}P<0.1$, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

increased ($P\leq 0.05$) in CL from hCG treated ewes compared to controls (Fig. 2A), yet production of StAR protein was lower ($P\leq 0.05$) in CL from hCG-treated ewes (Fig. 2B and C). As serum P_4 was increased with administration of hCG to pregnant ewes, evaluation of the potential hCG-mediated effects on interferon τ production by the conceptus was assessed by changes in the classic interferon stimulated gene ISG15. In caruncular tissue on days 13 and 25, ISG15 protein was similar between control and hCG-treated ewes (data not shown). No difference in production of ISG15 was observed in CL tissue from hCG-treated ewes compared to control on day 13 (data not shown). However, less ($P\leq 0.005$) ISG15 protein was detected in CL from hCG-treated ewes on day 25 compared to control (Fig. 2B and C).

In caruncle tissue, gene expression of *CXCR4*, *PAQR5* and *PAQR7* tended to increase ($P\leq 0.07$) in hCG-treated ewes on day 13 while *CXCR4* and *PAQR7* transcripts increased ($P\leq 0.05$) in hCG-treated ewes on day 25 compared to control (Fig. 3A). *CXCR4* protein was detected on day 13 and day 25 in caruncle tissue, and hCG treatment increased ($P\leq 0.05$) *CXCR4* on day 25 (Fig. 3B and C). Similarly, production of *PAQR7* was greater in caruncle tissue from hCG-treated ewes on day 25 compared to control (Fig. 3B and C).

Effects of maternal hCG exposure were also noted in fetal extraembryonic tissues. *VEGFA*, *VEGFB*, *FLT1* and *ANGPT1* expression increased in fetal membranes (Fig. 4A). Protein for VEGFA, *FLT1*, KDR and *ANGPT1* was detected in fetal tissue, but did not differ between hCG or control ewes (Fig. 4B).

Discussion

Early pregnancy loss is a serious problem to livestock producers and humans alike. Suboptimal synthesis of P_4 during gestation can contribute to early pregnancy loss underscoring the importance of luteal function. P_4 is required for maintenance of pregnancy and P_4 signaling regulates endometrial functions critical to implantation and placentation. Because hCG is a powerful luteotropic hormone, we sought to determine the molecular alterations induced by hCG administration in pregnant sheep that may serve to foster early pregnancy. In the current study, a single dose (600 IU, i.m.) of hCG on day 4 of gestation resulted in increased serum P_4 concentrations from day 4 through completion of the study on day 25 (Fig. 1A). Our results agree with other studies in sheep (Nephew *et al.* 1994, Ishida *et al.* 1999, Fukui *et al.* 2001) and cattle (Santos *et al.* 2001, Stevenson *et al.* 2007) demonstrating hCG treatment results in greater serum P_4 concentrations. It has been postulated that the increased serum concentrations of P_4 may be due to increased accessory CL formation or increased number of luteal cells secreting P_4 (Fricke *et al.* 1993, Schmitt *et al.* 1996, Santos *et al.* 2001,

Figure 3 CXCR4 and PAQR7 increase in caruncle following hCG treatment. (A) On day 13 of pregnancy, gene expression of CXCR4 ($^{\$}P=0.06$), PAQR7 and PAQR5 ($^{\$}P=0.07$) tended to increase in hCG exposed ewes. By day 25 of pregnancy, CXCR4 and PAQR7 expression in ewes treated with hCG differed. (B) Protein production of PAQR7 and CXCR4 significantly increased (C) in ewes receiving hCG. In all graphs, $^*P<0.05$, $^{**}P<0.005$.

Stevenson *et al.* 2007). Treatment with hCG increased the number of CL compared to control ewes (Fig. 1C), which may account for the sustained increase of P_4 noted in the hCG-treated ewes throughout the study. Our study does not delineate whether the increased number of CL observed in hCG-treated ewes was due to formation of accessory CL, resulting from luteinized follicles, or if treatment with hCG on day 4 of pregnancy induced additional ovulations.

Because of the sustained increase in serum P_4 levels in hCG-treated ewes, we wanted to determine if the rise was due to increased steroidogenesis in the CL. To address this, we analyzed levels of StAR and HSD3B1 in CL from hCG and control ewes. As StAR functions during the rate limiting step in steroidogenesis, in that it facilitates cholesterol transport to the inner mitochondrial membrane, we evaluated StAR levels in CL from hCG-treated and control ewes. StAR mRNA increased in the CL from hCG-treated ewes on day 25 compared to control (Fig. 2A), whereas protein levels decreased (Fig. 2B and C). We anticipated mRNA

expression and protein synthesis to display similar patterns, but transcription and translation are not equivalent, and post-transcriptional regulation of mRNA can govern protein synthesis (Anderson & Kedersha 2009). Why differences exist for StAR mRNA and protein production is unclear, but it is possible that following transcription, the mRNA itself is regulated. For example, mRNA can be restrained in a translationally-silent state following expression, or transported to the cytoskeleton rather than immediately associating with ribosomes (Moore 2005). Additionally, the elevated P_4 in hCG exposed ewes may stimulate a negative feedback loop, governing the amount of StAR in the CL. The enzyme HSD3B1 converts pregnenolone to P_4 and with the increased serum P_4 concentrations observed in our study we anticipated increased expression of HSD3B1.

Figure 4 Expression of proangiogenic factors in fetal extraembryonic membranes increases following maternal hCG treatment. (A) In fetal membrane tissues collected on day 25, a significant increase was observed in gene expression of VEGFA, VEGFB, FLT1 and ANGPT1 in ewes treated with hCG compared to control ewes. (B) Protein was isolated from fetal extraembryonic membranes on day 25 post-mating and probed for VEGFA, FLT1 or ANGPT1 production. (C) No differences in protein production were observed. In all graphs, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

However, neither mRNA nor protein for HSD3B1 was affected in CL from ewes treated with hCG suggesting the increase in P_4 synthesis is not a result of increased HSD3B1. Taken together, from our study, it appears the increase in serum P_4 concentrations from ewes treated with hCG are due to increased number of CL and not increased steroidogenesis.

In women, hCG stimulates P_4 production for 3–4 weeks following implantation, yet this specific function is only critical for a short time, as reviewed by Cole (2012). Serum levels of hCG actually reach a peak concentration at 10 weeks of gestation, well past implantation, and continues to be produced throughout gestation. Therefore, hCG likely has roles aside from simply stimulating P_4 synthesis. Hyperglycosylated hCG drives invasion and implantation by trophoblast cells and stimulates trophoblast growth (Cole *et al.* 2006, 2008, Sasaki *et al.* 2008, Guibourdenche *et al.* 2010). Additionally, hCG functions as an angiogenic factor and promotes development and growth of uterine spiral arteries (Toth *et al.* 1994, 2001, Zygmunt *et al.* 2002, Herr *et al.* 2007, Berndt *et al.* 2009). Our laboratory focuses on the role of CXCL12/CXCR4 in regulating trophoblast growth, attachment and placentation during early pregnancy. Because hCG influences development of the conceptus and placenta, we examined the results of hCG treatment on the CXCL12/CXCR4 axis. PAQR proteins are present in reproductive tissues in humans (Fernandes *et al.* 2005, Karteris *et al.* 2006), pigs (Qiu *et al.* 2008), mice (Nutu *et al.* 2009), rats (Cai & Stocco 2005) and sheep (Ashley *et al.* 2006, 2009) and are induced by P_4 , yet their regulation in early pregnancy is undefined. In view of the increased serum P_4 in hCG-treated ewes in our study, we assessed the possible impact of hCG mediated changes in the expression of PAQR proteins.

Caruncle tissue from day 13 pregnant ewes treated with hCG had increased gene expression for CXCR4, PAQR7 and PAQR5 compared to control but did not reach statistical significance (Fig. 3; $P=0.06$, 0.07 and 0.07 respectively). By day 25, mRNA and protein for CXCR4 and PAQR7 was significantly elevated in caruncle tissue from hCG-treated ewes compared to controls (Fig. 3). We speculate the increase in CXCR4 and PAQR7 in caruncle tissue is either due to increased circulating P_4 or hCG treatment. Although several groups (Dominguez *et al.* 2003, Kumar *et al.* 2004, Sherwin *et al.* 2007, Ashley *et al.* 2011, Li *et al.* 2011, Barrientos *et al.* 2013) have reported increased CXCR4 in the endometrium from different species during early gestation, to our knowledge reports of P_4 directly increasing CXCR4 do not exist. However, hCG can stimulate CXCR4 synthesis in endometrium; treatment of human first trimester decidua tissue with hCG results in elevated CXCR4 (Sales *et al.* 2011) and similar regulation has been observed in baboons (Sherwin *et al.* 2007). Of note, women that exhibit recurrent implantation failure

display lower levels of endometrial CXCR4 compared to fertile women, despite having normal levels of estradiol and P_4 (Tapia *et al.* 2008). As such, hormonal regulation alone may not be sufficient to control CXCR4 production in the endometrium. Intriguingly, the increase in CXCR4 on day 25 is observed 21 days after hCG administration. Whether hCG influenced CXCR4 production directly or indirectly via altering the uterine environment requires further investigation. Evidence exists that hCG stimulates CXCR4 production in other reproductive tissues, including equine and bovine preovulatory follicles (Sayasith & Sirois 2014) and human granulosa cells (Kryczek *et al.* 2005). Overall, the increase we observed in caruncle tissue on day 25 may be directly due to hCG. Taken together, the increased CXCR4 in endometrium during early pregnancy (Ashley *et al.* 2011, Quinn *et al.* 2014) coupled with reports indicating blastocyst apposition polarizes CXCR4 expression in cultured endometrial epithelial cells (Dominguez *et al.* 2003) and co-culture studies showing elevated CXCR4 in decidualized stromal cells in response to conditioned medium from trophoblasts (Hess *et al.* 2007) suggest embryonic signals regulate CXCR4 to ensure successful pregnancy.

Whether hCG induces synthesis of PAQR7 is not currently known. The increase in PAQR7 in caruncle tissue isolated on day 25 from hCG-treated ewes may be due to increased P_4 production, as P_4 increases synthesis of PAQR7 in human myometrial cells (Karteris *et al.* 2006). The downstream functions once PAQR7 is activated by P_4 and the subsequent biological effects are under investigation by our laboratory. The possibility exists that P_4 may upregulate PAQR7 in caruncular tissue and activation of PAQR7 results in increased CXCR4 protein in the endometrium. The CXCR4 promoter does not contain a P_4 response element, and as noted a direct action of P_4 inducing CXCR4 has not been observed. Yet, CXCR4 expression is upregulated in several different species during the period of implantation and placentation when P_4 levels are high (Kumar *et al.* 2004, Ashley *et al.* 2011, Li *et al.* 2011, Sales *et al.* 2011, Barrientos *et al.* 2013, Quinn *et al.* 2014), and the nuclear PR is undetectable (Spencer & Bazer 1995, Spencer *et al.* 1995).

Establishment of functional fetal and placental blood circulation is one of the earliest events during embryonic development, requiring coordinated activation and regulation of various angiogenic factors (Charnock-Jones *et al.* 2004). Expression of VEGFA, ANG1 and associated receptors are elevated in fetal and maternal placenta during early pregnancy in sheep (Grazul-Bilska *et al.* 2010, 2011, Quinn *et al.* 2014). The increased expression of angiogenic factors, specifically in fetal extraembryonic membranes indicates that the fetus may actively regulate its own vascularization in an autocrine/paracrine fashion. In the current study, angiogenic factors were expressed in maternal caruncular tissue, but did not differ between treatment groups (data not shown). The only significant changes observed in gene

expression for angiogenic factors and respective receptors assayed occurred in the fetal extraembryonic membranes from ewes treated with hCG (Fig. 4). The increased mRNA for the angiogenic factors was striking in the fetal extraembryonic membranes, especially considering that the ewes only received one injection of hCG. We did not see concomitant protein changes, however, two reasons may explain this: i) VEGFA especially is secreted, so we may not be able to observe increases in the fetal tissue itself and ii) our collection date may be too early to detect protein changes. We previously observed increased expression of pro-angiogenic factors during early pregnancy in fetal placenta (Quinn *et al.* 2014), but the noted increase with hCG treatment is intriguing, especially as the hCG-treated ewes tended to have greater number of concepti ($P=0.108$), a phenomenon observed by others as well (Akif Cam & Kuran 2004, Khan *et al.* 2007). The increase in expression of angiogenic factors in fetal extraembryonic membranes may be due to increased histotroph resulting from the increased P_4 synthesized in the hCG-treated ewes. Thus, the elevated levels of P_4 may modify the uterine environment by enhancing the composition of histotroph thereby stimulating expression of angiogenic factors in fetal extraembryonic membranes.

Early pregnancy is a critical period when the fetal-maternal interface is established to facilitate not only embryonic survival, but also subsequent fetal growth and development. Overall, our results indicate that administration of hCG increases serum P_4 due to increased number of CL per ewe. Additionally, hCG stimulates production of PAQR7 and CXCR4 in the maternal endometrium and promotes expression of proangiogenic factors in fetal extraembryonic membranes. The functions of hCG, previously thought to primarily affect maternal tissue, may also stimulate the fetus to promote its own survival. To address this, future directions include *in vitro* studies to elucidate potential mechanisms of hCG-induced signaling which may alter trophoblast survival and/or placental vascularization. In summary, supplementing livestock with hCG may boost P_4 levels and improve reproductive efficiency.

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

Funding was supported by the Howard Hughes Medical Institute Science Education Award #52006932 to NMSU, the Agriculture and Food Research Initiative Competitive Grants #2013-38422-20957 from the USDA National Institute of Food and Agriculture, and the New Mexico Agriculture Experiment Station.

Acknowledgements

The authors would like to express their gratefulness to Kelsey Quinn and Gail Silver for laboratory assistance and to Consuelo Gurule, Raed Halalsheh and Colleen Richardson for their help with sheep husbandry and tissue collections. The authors would also like to express their gratitude to Dr Dennis Hallford for assistance with the serum progesterone analysis.

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Received 4 December 2014

First decision 23 December 2014

Revised manuscript received 6 April 2015

Accepted 9 April 2015