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Gestation-related gene expression and protein localization in endometrial tissue of Suffolk and Cheviot ewes at gestation Day 19, after transfer of Suffolk or Cheviot embryos

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ABSTRACT

The objective of this study was to investigate the gene expression of progesterone and estrogen receptor α (PR, ERα), insulin-like growth factor (IGF) 1, IGF-2, their receptor (IGFR1), IGF-binding proteins (BP) 1 to 6, insulin receptor, adiponectin receptors (AdipoR1/2), cyclooxygenase 2 (PTGS2), mucin 1 and to localize PR, ERα, IGF-1, IGFR1, PTGS2, and proliferating cellular nuclear antigen (PCNA) in the endometrium of pregnant (Day 19) Suffolk and Cheviot ewes carrying Suffolk and Cheviot embryos transferred within and reciprocally between breeds. Gene expression was determined by real-time quantitative polymerase chain reaction (RT-qPCR), and antigen determination was measured by immunohistochemistry in the luminal epithelium (LE), superficial and deep glands (SG, DG, respectively) and superficial and deep stroma. Gene expression of *PR, IGF-1, IGFBP2,* and *IGFBP5* was higher in Suffolk than that in Cheviot ewes ($P < 0.05$). Greater abundance of *IGF-2* and *IGBP3* expression was found in Cheviot ewes carrying Cheviot embryos than Cheviot ewes carrying Suffolk embryos ($P < 0.05$). No staining for PR and ERα was observed in the LE, very scarce staining in SG and DG, whereas positive staining was observed in both superficial and deep stroma. No differences were found for PR staining, but Cheviot ewes had higher ERα staining intensity than Suffolk ewes ($P < 0.05$). Positive staining for IGF-1 was observed in all cell types except DG, and staining of IGFR1 was observed in all cell types. No differences among groups in staining were found for IGF-1 or IGFR1 in any cell type. Positive staining of PTGS2 was observed in LE and SG in all groups. An interaction between ewe and embryo breed affected PTGS2 staining ($P < 0.05$), whereby Cheviot ewes carrying Suffolk embryos had a lower PTGS2 staining than Suffolk ewes carrying Suffolk embryos. Positive staining of PCNA was found in LE and SG. Suffolk ewes carrying Suffolk embryos showed lower PCNA immunostaining than Cheviot ewes carrying Suffolk embryos ($P < 0.05$), whereas no differences were observed in ewes carrying Cheviot embryos. This study showed that gestation-related protein expression in the endometrium of Suffolk and Cheviot ewes is affected by both ewe and embryo breed at Day 19 of pregnancy.

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1. Introduction

Successful embryo growth requires synchronized molecular and cellular signaling that leads to an appropriate conceptus-ewe communication [1]. The maternal uterine

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environment can regulate embryo growth very early in gestation, even when uterine capacity is not a limiting factor [2]. Progesterone (P4), the pregnancy hormone, action on the uterus depends on the presence and quantity of its nuclear receptor (progesterone receptor, PR; [3]). During early pregnancy, the embryo is dependent on the histotroph, which includes IGF-1 and IGF-2 that simulate embryo and endometrial development [4,5]. Growth factors act with high affinity via their specific receptor (insulin-like growth factor receptor; IGFR1) and their bioavailability is regulated by IGF binding proteins (BPs) [4].In addition, prostaglandins derived from the cyclooxygenase-2 enzyme (PTGS2) regulate conceptus elongation and implantation via effects on the endometrium and/or conceptus [6]. Moreover, adiponectin (AdipoQ) from a paracrine and/or an endocrine source may play an important role in embryo development [7] via its receptors, AdipoR1/2, because their expression is increased during the window of implantation [8].In addition, mucin-1 (MUC-1) expression has been reported to determine future embryo implantation, as is an antiadhesive component of the luminal epithelium, limiting trophectoderm accessibility [9]. Finally, proliferating cellular nuclear antigen (PCNA) provides a marker of the balance between cell proliferation and death, which is crucial for embryo implantation [10]. Thus, the aim of this study was to investigate endometrial gene expression of PR, estrogen receptor alpha (ERα), insulin receptor (INSR), IGF-1, IGF-2, IGF1R, IGFBPs, AdipoR1/2, MUC-1, and PTGS2 in pregnant (Day 19) Suffolk and Cheviot ewes carrying Suffolk and Cheviot embryos transferred within and reciprocally between breeds. Protein immunostaining of PR, ERα, IGF-1, IGF1R, PTGS2, and PCNA was also performed to describe protein distribution within the endometrium.

2. Materials and methods

This experiment was approved by the Massey University Animal Ethics Committee. The animals used in this study were maintained under commercial pastoral farming conditions at Massey University Keeble Farm, Palmerston North, New Zealand.

2.1. Experimental design and animals

Cheviot (C) and Suffolk (S) sheep breeds were used to provide different genotypes according to their mature body size, consistent with previously established protocols for modifying the uterine environment [2,11]. All donors ewes were 4 years of age (nine Suffolk and 13 Cheviot ewes were used), the recipient ewes (58 Suffolk and 52 Cheviot) were of mixed ages (3- to 6-year old) and parities (all multiparous). Pure breed embryos were transferred using standard commercial embryo transfer procedures within and reciprocally between breeds of sheep to create four treatment groups: SinS (Suffolk embryo in Suffolk ewe–large genotype control), SinC (Suffolk embryo in Cheviot ewe–large genotype embryo in small ewe genotype), CinS (Cheviot embryo in Suffolk ewe–small genotype embryo in large ewe genotype), and CinC (Cheviot embryo in Cheviot ewe–small genotype control). On Day 19 of gestation, recipient ewes (n = 9 per treatment group) were sacrificed, and endometrial samples from the middle third of the uterine horn ipsilateral to CL were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical investigation. Other sections were snap frozen in liquid N2 and stored at −80 °C for polymerase chain reaction (PCR) analysis. Day 19 of gestation was chosen because it allowed us to select ewes that maintained pregnancy after embryo transfer and did not return to estrous, and it was also the desired stage for research on uterine functionality (e.g., after maternal recognition of pregnancy but before complete implantation).

2.2. RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA from endometrium tissue collected from Suffolk and Cheviot ewes was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) followed by precipitation with lithium chloride and DNase-treatment with DNA-FreeTM 180 Kit (Ambion, Austin, TX, USA). Concentration of RNA was determined by measuring absorbance at 260 nm, the purity of all RNA isolates was assessed from 260 to 280 absorbance ratio and the integrity by electrophoresis (1% agarose gel). For each sample, complementary DNA (cDNA) was synthesized by reverse transcription using the SuperScript III transcriptase (Invitrogen) with oligo-dT primers, and 1 μg total RNA added as a template.

Sequences and the expected product lengths of primers to amplify cDNA of the target genes *PR, ERα, IGF-1, IGF-2, IGFBP* 1 to 6, *IGFR1, Adipor1/R2, INSR, PTGS2, MUC-1* and of the endogenous controls, hypoxanthine guanine phosphoribosyl-transferase (HPRT), ribosomal protein L19 (RPL19), and β-actin are presented in Table 1. Real-time PCR reactions were performed using 7.5 μL SYBER Green master-mix (Quantimix EASY SYG kit, Biotools B&M Labs, Madrid, Spain), equimolar amounts of forward and reverse primers (200 nM, Operon Biotechnologies GmbH, Cologne, Germany), and 3 μL diluted cDNA (1:7.5 in RNase/DNase free water) in a final volume of 15 μL. Samples were analyzed in duplicate in a 72-disk Rotor-GeneTM 197 6000 (Corbett Life Sciences, Sydney, Australia). Standard amplification conditions were 3 minutes at 95 °C and 40 cycles of 15 seconds at 95 °C, 40 seconds at 60 °C, and 10 seconds at 72 °C. At the end of each run, dissociation curves were analyzed to ensure that the desired amplicon was being detected and to discard contaminating DNA or primer dimers. Samples of cDNA from eight ewes (two from each group) were pooled to provide an exogenous control, and five dilutions (from 100 to 6.25 ng/tube) of this pool were used to perform linear regressions for each gene. The efficiency (E) of the assays was calculated according to the formula $E = (10^{-1}/\text{slope } 206^{-1})$; Table 1) [19]. Gene expression was measured by relative quantification [20] to the exogenous control and normalized to the geometric mean expression of the endogenous control genes (HPRT and RPL19), taking into account the respective efficiencies [20].

2.3. Immunohistochemistry

Immunoreactivity of the proteins of interest was visualized in transverse 5-μm sections from uterine tissue

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