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Involvement of TLR7 and TLR8 in conceptus development and establishment of pregnancy in sheep

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Abstract

Toll-like receptors (TLRs) belong to the innate immune system and regulate inflammatory events that affect mammalian reproduction. In Study 1, we demonstrated that abundance of ovine *TLR1–TLR9* mRNAs in the uterus differs due to reproductive status (*TLR2*, *TLR3*, *TLR7*, and *TLR8*) and the day of the estrous cycle and pregnancy (*TLR1–TLR3*, *TLR5–TLR7*, and *TLR9*). Expression of TLR7 and TLR8 proteins was localized primarily to uterine epithelia and stroma and regulated in a temporal manner. In Study 2, we determined that ovine conceptuses express *TLR7* and *TLR8* on all days studied and that expression of the envelope protein of ovine endogenous retrovirus (enJSRV-*Env*) declined in conceptus trophectoderm from Day 13 to Day 16 of pregnancy. In Study 3, loss-of-function experiments were conducted *in vivo* using morpholino antisense oligonucleotides (MAOs) injected into the uterine lumen to block synthesis of TLR7 and TLR8 proteins, individually and jointly. Conceptuses were recovered on Day 16 to assess their morphology. MAO-treated conceptuses were developmentally retarded, produced less interferon tau (IFNT), and had fewer binucleate cells (BNCs) compared with MAO-Controls. Moreover, expression of enJSRV-*Env* mRNA in MAO-*TLR7* conceptuses was greater than that for MAO-Control and MAO-*TLR8* conceptuses, but similar to MAO-*TLR7*/*TLR8* conceptuses. Results of this study indicated differences in *TLR1–TLR9* expression due to reproductive status and the day of the estrous cycle and pregnancy. TLR7 and TLR8 also influence development, enJSRV-*Env* abundance, secretion of IFNT, and formation of BNCs by conceptuses. These findings corroborate our hypothesis that TLR7 and TLR8 mediate pathways whereby enJSRV-*Env* regulates key peri-implantation events in conceptus development and differentiated functions of trophectoderm cells.

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Introduction

The intrauterine immune system in mammals must be finely regulated during pregnancy to allow for development of the semi-allogeneic conceptus (embryo and the associated extra-embryonic membranes) and, at the same time, protect against invading pathogens. Progesterone, the hormone of pregnancy (Spencer *et al*. 2004), has been reported to down-regulate the immune system at the maternal–conceptus interface to establish an anti-inflammatory milieu that favors continuation of pregnancy (Hansen 1998). However, large numbers of immune cells are recruited into the endometrium during the estrous cycle and pregnancy to participate in events of uterine remodeling, maternal tolerance, vascularization, and placentation (Segerson *et al*. 1991, Leonard *et al*. 2006, Laskarin *et al*. 2007, Gomez-Lopez *et al*. 2010, Nagamatsu & Schust 2010, Mansouri-Attia *et al*. 2012). Lacking this influx of immune cells or their aberrant function leads to infertility and pregnancy loss (Greenwood *et al*. 2000, Plaks *et al*. 2008, Guerin *et al*. 2009, Jabbour *et al*. 2009, Erlebacher 2013). Therefore, it is unlikely that the maternal immune system is suppressed during pregnancy. Rather it is appropriately tuned to be permissive to the presence of the conceptus and collaborate with cytokines from the conceptus to ensure a successful outcome of pregnancy.

Pattern recognition receptors (PRRs) are innate immune cell receptors involved in the initiation of immunological responses to highly conserved pathogen-associated molecular patterns (PAMPs) (Janeway & Medzhitov 2002). Among the families within PRRs are the Toll-like receptors (TLRs), which have been most studied and considered to be the first line of defense against pathogens (Akira & Hemmi 2003).

Mammalian TLRs are transmembrane type I proteins with a leucine-rich repeat ectodomain (LRR) for ligand recognition, a single transmembrane domain which differs among TLRs, and a TIR domain for signal transduction (Akira *et al*. 2001). Of the 13 TLRs identified in mammals (Hansen *et al*. 2011, Jungi *et al*. 2011), ten are known to be expressed in domestic ruminants (Menzies & Ingham 2006, Chang *et al*. 2009), which display high homology with their human counterparts (Nalubamba *et al*. 2007, Tirumurugaan *et al*. 2010). Besides TLR10, for which no function has been established, TLR ligand specificity

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depends on cellular location with some being expressed on the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) for recognition of bacterial and fungal compounds, and others being localized to the endosomal compartment (TLR3, TLR7, TLR8, and TLR9), where they mainly bind nucleic acids from viral pathogens (Kawai & Akira 2009, Kumar *et al*. 2009).

The finding of TLRs in non-typical immune cells such as the epithelia (Young *et al*. 2004, Turner *et al*. 2012) has implicated these receptors in many physiological events besides pathogen recognition (Li *et al*. 2010). Available evidence indicates potential involvement of TLRs in mammalian reproduction, which requires an appropriate inflammatory balance among steroid hormones, cytokines, and prostaglandins to assure cyclical uterine remodeling for establishment and maintenance of pregnancy (King & Critchley 2010, Ott & Gifford 2010, Dorniak *et al*. 2011). TLR-mediated cell signaling cascades involve the cytokine inductor nuclear factor kappa β (NFKB) and interferon regulatory factors (IRFs) to induce type I interferons (IFNs) (Kumar *et al*. 2009). Both of these pathways are actively regulated in the female reproductive tract, and their deregulation leads to infertility and disease (Spencer *et al*. 1998, King *et al*. 2001, Fleming *et al*. 2009, Ross *et al*. 2010, Hadfield *et al*. 2011, Maybin *et al*. 2011).

Gestation has been considered a Th2 or anti-inflammatory environment, achieved through suppression of the NFKB pathway and increased abundance of interleukin 10 (IL10), IL4, or IL5 (Piccinni *et al*. 2001, Hadfield *et al*. 2011). However, Th1 (pro-inflammatory) molecules such as tumor necrosis factor alpha (TNFA) and IFNgamma (IFNG) are also necessary for uterine receptivity, implantation, and placental development in some species (Ashkar & Croy 2001, Hess *et al*. 2007, Paulesu *et al*. 2010, Warning *et al*. 2011, Granot *et al*. 2012). Moreover, type I IFNs play an indisputable role during early pregnancy in ruminants, as the conceptus signals its presence to the mother by producing high levels of interferon tau (IFNT). IFNT abrogates development of the luteolytic mechanism and activates IFNT-regulated pathways in the uterus, which influence gene expression within the maternal environment leading to production of histotroph (Spencer & Bazer 2004, Gray *et al*. 2006, Bazer *et al*. 2008).

The abundance of TLRs in immune cells depends on the dominant cytokine milieu (O'Mahony *et al*. 2008) and, therefore, it seems reasonable that strong conceptus–maternal signaling during the course of pregnancy influences expression of TLRs in the female reproductive tract and conceptus. Indeed, human endometrial epithelia and stromal cells express TLR1–TLR10, with higher levels during the progesterone-dominated secretory phase of the menstrual cycle (Jorgenson *et al*. 2005, Aflatoonian *et al*. 2007, Hirata *et al*. 2007). Moreover, a microarray analysis found significant up-regulation of genes involved in the TLR pathway in the mouse uterus during the implantation period (Pan *et al*. 2006). In addition, TLR expression in the placenta is regulated in a temporal and spatial manner (Koga & Mor 2010) and the trophoblast modulates the maternal environment through TLR-mediated pathways (Abrahams *et al*. 2004, 2005). Despite evidence for their involvement in uterine biology of humans and mice, there is a lack of information regarding the role of TLR-mediated pathways in ruminants. Reports on expression of TLR1–TLR10 in bovine endometria describe differences in expression between epithelial and stromal cells (Herath *et al*. 2006, Davies *et al*. 2008) and their abundance in uterine endometrium of goats (Tirumurugaan *et al*. 2010).

Therefore, this study examined temporal and cell-specific patterns of expression of TLRs in uteri of cyclic and pregnant ewes during the period of pregnancy recognition signaling by IFNT. In addition, *in vivo* loss of translation of *TLR7* and *TLR8* mRNAs in the trophectoderm was achieved during this same period of pregnancy using morpholino antisense oligonucleotides (MAOs) with the aim of assessing the role of both TLR7 and TLR8 in conceptus development, production of IFNT, and formation of binucleate cells (BNCs). Finally, levels of mRNA coding for the envelope protein of ovine endogenous retrovirus (enJSRV-*Env*) were analyzed in MAO-treated conceptuses, as these viral particles influence implantation in the sheep (Dunlap *et al*. 2005, 2006*a*, Black *et al*. 2010). The overall aim of this study was to advance understanding of the role of TLRs in key events that regulate maternal recognition of pregnancy and implantation in the ewe.

Material and methods

Experimental design

Mature Rambouillet ewes (*Ovis aries*) were observed daily for estrus (Day 0 is the day of onset of estrus) in the presence of vasectomized rams and assigned to experiments after exhibiting at least two estrous cycles of normal duration (16–18 days). All experimental and surgical procedures were performed in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Study 1

At onset of estrus and on Day 1, ewes were mated to either a vasectomized ram or an intact ram of proven fertility. Ewes were then assigned randomly to be ovariectomized–hysterectomized on Day 10, 12, 14, or 16 of the estrous cycle or Day 10, 12, 14, 16, 18, or 20 of pregnancy ($n=4$–5 ewes/day and status) as described previously (Spencer *et al*. 1999). Pregnancy was confirmed by the presence of a morphologically normal conceptus and a functional corpus luteum (CL).

At hysterectomy, sections (~0.5 cm) from the mid-portion of each uterine horn ipsilateral to the CL were fixed with fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol (v/v) for 24 h, dehydrated through a graded series of alcohol to xylene, and then embedded in Paraplast-Plus (Oxford Labware, St Louis, MO, USA). The remaining endometrium of the ipsilateral uterine horn was physically dissected from the myometrium, frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction processes.

Study 2

Ewes were mated at the onset of estrus (Day 0) and on Day 1 to fertile rams. At mating, ewes were assigned randomly in groups for recovery of the conceptus on Day 13, 14, 15, or 16 of pregnancy (n=4/5 conceptuses/day) by flushing the uterus with 10 mM Tris buffer. Conceptuses were snap frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

Study 3

Morpholino oligonucleotides designed and synthesized by Gene Tools (Philomath, OR, USA) were directed against mRNAs coding for *TLR7* (MAO-TLR7; GAAACTGTCTCTT-CAATGTCCACAT) and for *TLR8* (MAO-TLR8; TCAGAAG-CAAAAAGTGAAGGGTCAT). The MAO-Control had the sequence CCTCTTACCTCAGTTACAATTTATA. All morpholinos were synthesized with a 3′-lissamine modification to detect and confirm uptake of each MAO by the trophectoderm, while confirming absence of uptake by uterine epithelia (see Wang *et al.* 2014).

Rambouillet ewes were mated on Days 0 and 1 to rams of proven fertility. On Day 8 post-mating, ewes were subjected to a mid-ventral laparotomy as described previously (Dunlap *et al.* 2006*a*). The base of the uterine horn ipsilateral to the CL was double ligated to prevent migration and growth of the conceptus into the contralateral uterine horn. This procedure does not affect development or implantation of ovine conceptuses (Dunlap *et al.* 2006*a*). MAO-*TLR7*, MAO-*TLR8*, and MAO-Control were complexed with Gene Tools Endo-Porter delivery reagent (50 µl) and diluted to a final volume of 1 ml with OPTI-MEM (Invitrogen). Double knock-down of translation of both MAO-*TLR7* and MAO-*TLR8* (hereafter noted as MAO-*TLR7*/*TLR8*) was prepared by mixing equal volumes of MAO-*TLR7* and MAO-*TLR8*. The respective MAOs were introduced into the uterine lumen (n=6/8 ewes/treatment) via a catheter inserted just below the tubo-uterine junction. After the MAOs were discharged into the uterine lumen, the uterine horn was gently massaged to distribute the morpholinos throughout the uterine lumen. The outside of the uterus was then rinsed with saline containing 10% glycerol to reduce adhesions and then returned to the abdominal cavity.

Morpholino-treated ewes were hysterectomized on Day 16. The uterine horn injected with a morpholino was flushed with 10 ml sterile PBS (pH 7.2). If the conceptus was present, its morphology was recorded (spherical, tubular, and elongated) and the volume of the uterine flushing recovered was recorded. Photomicrographs of the conceptus were obtained using an inverted microscope fitted with a digital camera. Portions of each conceptus were placed in optimal cutting temperature (OCT) compound (Miles, Oneonta, NY, USA), frozen in liquid nitrogen, and stored at −80 °C. Another portion of the conceptus was fixed with freshly prepared 4% (wt/vol) paraformaldehyde in PBS and embedded in paraffin wax. The uterine flush was clarified by centrifugation (5000 ***g*** for 15 min at 4 °C), aliquoted, and stored at −80 °C. The amount of IFNT in the uterine flush was quantified by RIA with a range of detection of 0.1–13 ng/ml (Antoniazzi *et al.* 2013). The intra- and inter-assay coefficient of variation values were 6.2 and 4.0% respectively.

RNA isolation and quantitative real-time PCR analysis

Total cellular RNA was isolated from endometrial samples and conceptuses using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The quantity and quality of total RNA were determined by spectrometry and by denaturing agarose gel electrophoresis respectively. Total RNA samples were digested with RQ1 RNase-Free DNase (Promega) and subsequently cleaned-up using an RNeasy Mini Kit (Qiagen). Total RNA (2100 ng) was reverse transcribed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's instructions. Control reactions in the absence of reverse transcriptase were prepared for each sample to detect genomic DNA contamination. The resulting cDNA was stored at −20 °C for further analyses.

Quantitative PCR (qPCR) was performed using the ABI prism 7900HT system (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems) as specified by the manufacturer. Specific oligonucleotide primers were designed and analyzed using Primer Express Software for Real Time PCR v3.0 (Applied Biosystems). The primers were blasted using available databases to ensure specificity of each gene in this study and amplicons were verified by sequencing. Forward and reverse primer sequences for all genes analyzed in endometrial samples and conceptuses are listed in Tables 1 and 2 respectively. Primer specificity and efficiency ($-3.22 > \text{slope} > -3.44$) were confirmed using a test amplification run. Each individual sample was run in triplicate using the following conditions: 50 °C for 2 min, 95 °C for 10 min, and then 95 °C for 15 s and 60 °C for 1 min for 40 cycles. A dissociation curve was generated to determine amplification of a single product. The threshold line was set at the linear region of the plots above the baseline noise, and threshold cycle (C_t) values were determined at the cycle number at which the threshold line crossed the amplification curve. Mean C_t values for each gene were normalized against average C_t values for the reference gene (ovine alpha-tubulin, *TUBA*). The relative quantification (RQ) of each gene was calculated using the $2\Delta\Delta C_t$ method.

Immunohistochemical analyses

Immunoreactive TLR7 and TLR8 proteins were localized in paraffin-embedded samples from uteri of cyclic and pregnant ewes using the rabbit Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions. Briefly, antigen retrieval was performed using boiling

Table 1 Primer sequences used for qPCR in the ovine endometrium.

Genes	Accession numbers	Forward primers	Reverse primers
TLR1	NM_001135060.2	AGATGCTGAGAGCCTTCAAG	TTAGACAGTTCCAGACTCAC
TLR2	NM_001048231.1	CAGGTCAAGGCTTTTCACAC	CCAGGCTTCTTCTCTGTCTT
TLR3	NM_001135928.1	CAAGTGTCACTGAAGTCATTA	CACAGGTACAATCAAATGGAT
TLR4	NM_001135930.1	GTGGAGACAAACCTAGTATC	CAGGTTGGGAAGGTCAGAAA
TLR5	NM_001135926.1	GACTGCCTTGACCTTCTCTTAG	TGCGGTTGTGACTGTCCTGATA
TLR6	NM_001135927.1	GCGTGTGTCTGATATGGTTA	AAACGACATTGAAGGCACTG
TLR7	NM_001135059.1	CTGTGATGTCACTCTGGATG	TGAGGTTGGTGGCATTGGCA
TLR8	NM_001135929.1	TCCACATCCCAGACTTTCTA	GTTCTTGTCCTCACTCTCTT
TLR9	NM_001011555.1	GTTCTCTCGTATCCCTGTCG	TAGTAGCAGTTGCCGTCCAT
TUBA	AF251146.1	GGTCTTCAAGGCTTCTTGGT	CATAATCGACAGAGAGGCGT

citrate and endogenous peroxidase activity was blocked by incubating tissue in methanol with 0.3% hydrogen peroxide for 15 min at room temperature. Slides were incubated overnight at 4 °C with the following primary antibodies: rabbit polyclonal antibodies against TLR7 (ab45371, Abcam, Cambridge, MA, USA) and TLR8 (PA1-12830, Thermo Scientific-Pierce antibodies, Rockford, IL, USA) used at final dilutions of 1:250 and 1:700 respectively.

Fluorescence microscopy and paraffin-immunohistochemistry were utilized to determine knockdown or absence of TLR7 and/or TLR8 proteins in conceptus trophectoderm using the same antibodies described earlier. In addition, cryosections of the uterus were observed for presence of red colored lissamine-tagged MAOs. DAPI-counting medium was used to visualize the nuclei in the cryosections. In addition, immunoreactive placenta-associated glycoprotein (PAG) was detected using the same immunohistochemical procedures described in the previous section for detection of TLR7 and TLR8 except that antigen retrieval was performed with protease treatment at 37 °C. Positive immunostaining for PAG is unique to BNCs. The antibody to ovine PAG (kindly provided by Jonathan A Green, University of Missouri) was incubated overnight at a final dilution of 1:400 for conceptus tissue. PAG-stained sections were counterstained with hematoxylin before affixing coverslips. In all cases, negative controls were prepared with rabbit IgG at the same concentration as the primary antibody and photomicrographs taken using a Zeiss Axioplan2 microscope fitted with an AxioCam HRc camera (Carl Zeiss, Thornwood, NY, USA). Total conceptus area (in mm^2) visible in each slide was measured using Image J1.46r (US National Institutes of Health, Bethesda, MD, USA). Those results were then used along with determinations of PAG-positive BNCs in each conceptus to quantify the number of BNCs per conceptus area.

Statistical analysis

Data were subjected to least-squares ANOVA using Mixed and General Linear Model procedures of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Data obtained from ovine uterine endometria were analyzed for main effects of day and status (cyclic or pregnant), and Day × Status interaction. Data obtained from conceptuses on Days 13, 14, 15, and 16 of pregnancy and from conceptuses following MAO treatments were assessed for effects of treatment. Effects of morpholino treatments on concentrations of IFNT in uterine flushings were analyzed using ANOVA and orthogonal contrasts to determine differences among treatments (MAO-Control vs MAO-*TLR7*, vs MAO-*TLR8*, and vs MAO-*TLR7*/*TLR8*). The number of BNCs was quantified by determining numbers of PAG-positive BNCs per unit (mm^2) of conceptus area in each MAO treatment group. $P \leq 0.05$ was considered statistically significant. Data are expressed as least-squares means with overall S.E.M.

Results

Expression of TLRs in the endometrium during the estrous cycle and pregnancy

Expression of *TLR1*–*TLR9* mRNAs was assessed in uterine endometria from cyclic and pregnant ewes by qPCR, and the relative abundance of each TLR was compared (Fig. 1). The pattern of expression of *TLR2*, *TLR7*, *TLR8*, and *TLR9* mRNAs was affected by the day after the onset of estrus and pregnancy status (Day × Status, $P < 0.01$). The abundance of these *TLR* (*TLR2*, *TLR7*, *TLR8*, and *TLR9*) mRNAs increased from Day 10 to Day 12 of the estrous cycle, and then declined from Day 12 to Day 16; however, expression was maintained between Days 10 and 16 in endometria of pregnant ewes.

With the exception of *TLR4*, the abundance of the other TLRs differed in response to the day of the estrous cycle or the day of gestation (Day; $P < 0.01$). During the estrous cycle, expression increased starting from Day 10 and attained maximum levels on Day 12 for *TLR1*, *TLR3*, and *TLR6*, or Day 14 for *TLR5*. Then, their expression declined on Day 16. In pregnant ewes, relative levels of these TLRs between Days 10 and 14 were similar to that

Table 2 Primer sequences used for qPCR in the conceptus.

Genes	Accession numbers	Forward primers	Reverse primers
TLR7	NM_001135059.1	CTGTGATGTCACTCTGGATG	TGAGGTTGGTGGCATTGGCA
TLR8	NM_001135929.1	TGTGTTTAGAGGAAAGGGATTGG	TCTGCATGAGGTTGTCGATGA
enJSRV-*Env*	AF105220.1	GGATCTGGACCCCTCGACAT	TGTCTATGCCTATGCCAATGCT
TUBA	AF251146.1	GGTCTTCAAGGCTTCTTGGT	CATAATCGACAGAGAGGCGT

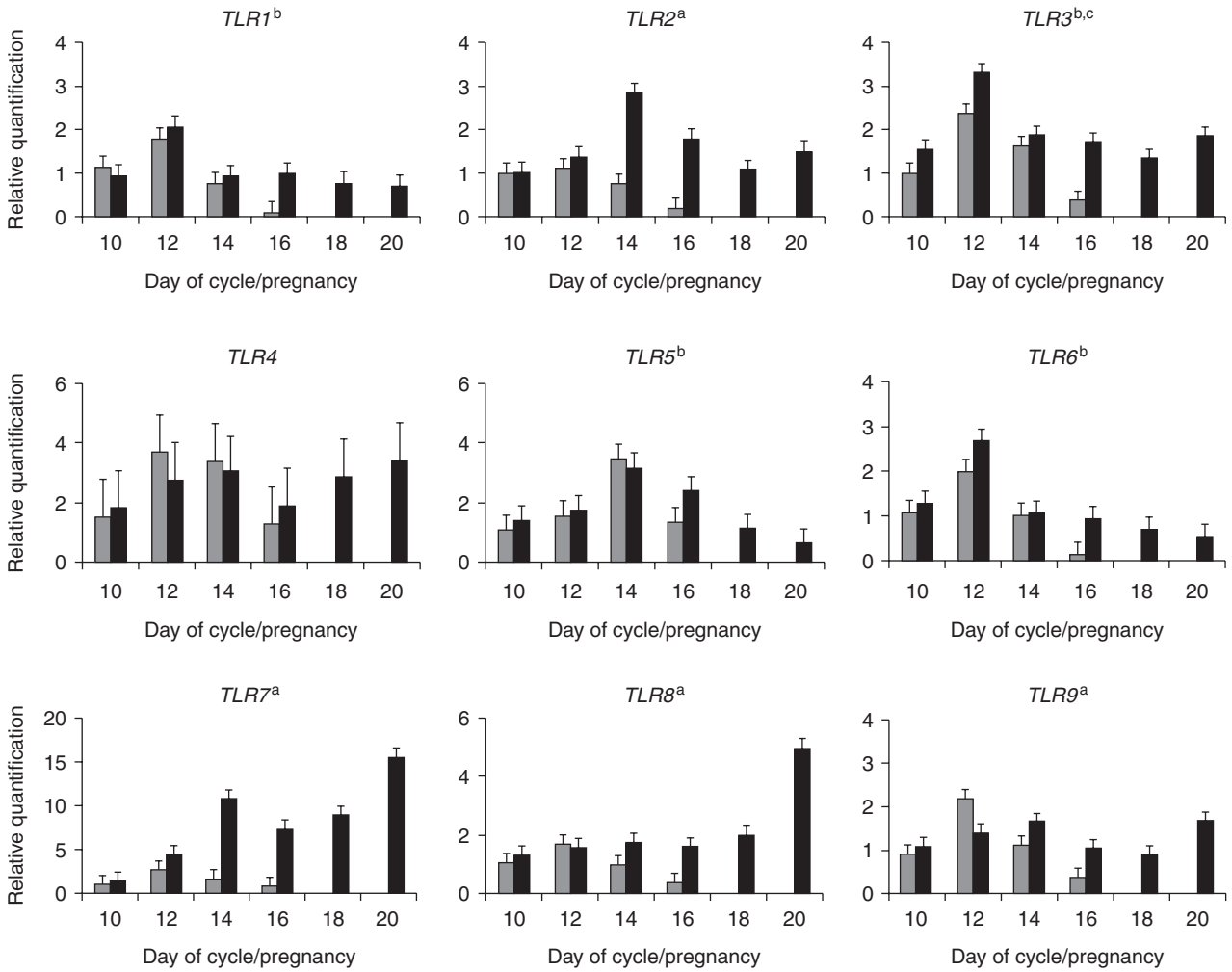

Figure 1 Relative abundance of mRNAs for *TLR1–TLR9* in ovine uterine endometria during the estrous cycle and early pregnancy. Data are expressed as abundance of each *TLR* relative to *TUBA*. Differences ($P \leq 0.05$) are denoted with superscripts as follows: [a]Day × Pregnancy Status interaction, [b]Day effect and [c]Pregnancy Status effect.

for cyclic ewes. Then, between Days 16 and 20, expression did not change for *TLR1*, *TLR2*, *TLR3*, or *TLR6*, but decreased for *TLR5*, and increased for *TLR7* and *TLR9*.

Expression of *TLR2*, *TLR3*, *TLR7*, and *TLR8* was affected by pregnancy status ($P < 0.01$), as their expression was more abundant in endometria of pregnant ewes than in that of cyclic ewes. Although there were significant increases in expression of mRNAs for *TLR7* and *TLR8* in endometria from pregnant ewes, expression of those *TLR* mRNAs in ovine conceptuses was not significantly different between Days 13 and 16 of pregnancy (Fig. 2).

Localization of TLR7 and TLR8 proteins in the uterus and trophectoderm

As shown in Fig. 3, TLR7 and TLR8 proteins were detected in all cells of uteri from cyclic and pregnant ewes, particularly in the uterine luminal (LE) and superficial glandular (sGE) epithelia, and stratum compactum stroma (S).

TLR7 protein was abundant in the uterine LE and stratum compactum stroma on Day 10 of the cycle, but there was stronger expression in the stroma and sGE of uteri from pregnant ewes. Abundance of TLR7 protein increased in uterine LE and sGE as days of the estrous cycle and gestation advanced. In cyclic ewes, TLR7 protein was most abundant in uterine LE and sGE on Day 14, and then decreased to barely detectable amounts on Day 16. In uteri of pregnant ewes, TLR7 expression was similar on Days 12 and 14, before increasing slightly in uterine LE and sGE on Day 16 of gestation. TLR7 protein was equally abundant in uterine sGE on Days 16, 18, and 20 of pregnancy. At this stage, expression of TLR7 decreased in the uterine LE proximal to the conceptus, but it was strong in the trophectoderm of conceptuses on Days 18 and 20 of pregnancy.

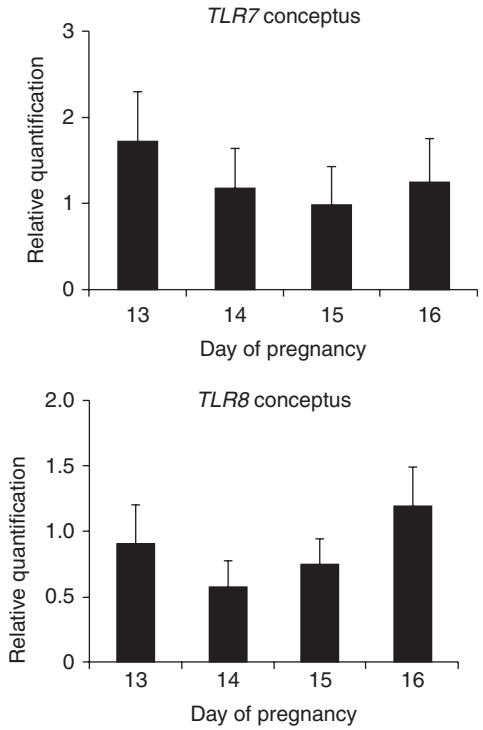

Figure 2 Abundance of mRNAs for *TLR7* and *TLR8* in ovine conceptuses during early pregnancy. Data are expressed as abundance of *TLR7* and *TLR8* relative to *TUBA*.

Expression of TLR8 protein was detected in uterine LE and stromal cells throughout the estrous cycle. For sGE, the abundance was low on Day 10 and increased from Day 12 to Day 16. In endometria from pregnant ewes, TLR8 protein was similar in abundance in uterine LE, sGE, and stroma on Days 10 and 12. Expression of TLR8 decreased slightly on Day 14 before increasing on Day 16, and remaining at that level of abundance on Days 18 and 20. TLR8 protein was also detected in conceptus trophectoderm, with the strongest expression in BNCs. In contrast to TLR7 protein, immunoreactive TLR8 protein was abundant in uterine LE independent of proximity to the conceptus.

Knockdown of TLR7 and TLR8 proteins alters conceptus development from Day 8 to Day 16 of pregnancy

Although treatment with morpholinos did not affect the number of pregnant ewes in each treatment group from which conceptuses were recovered, there were major differences in morphology and degree of development of the conceptuses recovered from ewes that received MAO-*TLR7*, MAO-*TLR8*, and MAO-*TLR7*/*TLR8* compared with MAO-Control ewes (Table 3 and Fig. 4). Although most of the conceptuses elongated, MAO-*TLR7* conceptuses were smaller than the fully elongated and filamentous conceptuses recovered from MAO-Control ewes. Conceptuses from MAO-*TLR8*-treated ewes were more variable in size, but generally smaller when compared with conceptuses from MAO-Control ewes. Conceptuses from ewes treated with MAO-*TLR7*/*TLR8* were much smaller and more fragile, and they failed to elongate to a filamentous form.

To quantify the effect of the morpholino treatment on function of conceptus trophectoderm, concentrations of IFNT in uterine flushings were determined. Consistent with the abnormal morphology and retarded development, concentrations of IFNT in uterine flushings were lower for ewes treated with MAO-*TLR7* (610 ± 185 ng/ml; $P<0.048$), MAO-*TLR8* (577 ± 119 ng/ml; $P<0.049$), and MAO-*TLR7*/*TLR8* (280 ± 133 ng/ml; $P<0.001$) when compared with the MAO-Control (1060 ± 210 ng/ml).

Histological analyses revealed a significant reduction in TLR7 and TLR8 protein expression in conceptuses receiving MAO-*TLR7*, MAO-*TLR8*, and MAO-*TLR7*/*TLR8*, which confirmed the efficiency of morpholino knockdown of translation of the respective mRNAs (Fig. 5A, B, and C). The average number of PAG-positive BNCs (Fig. 6) was less ($P<0.05$) in MAO-*TLR7* (30.0 ± 19.0 BNCs/mm^2) and MAO-*TLR7*/*TLR8*

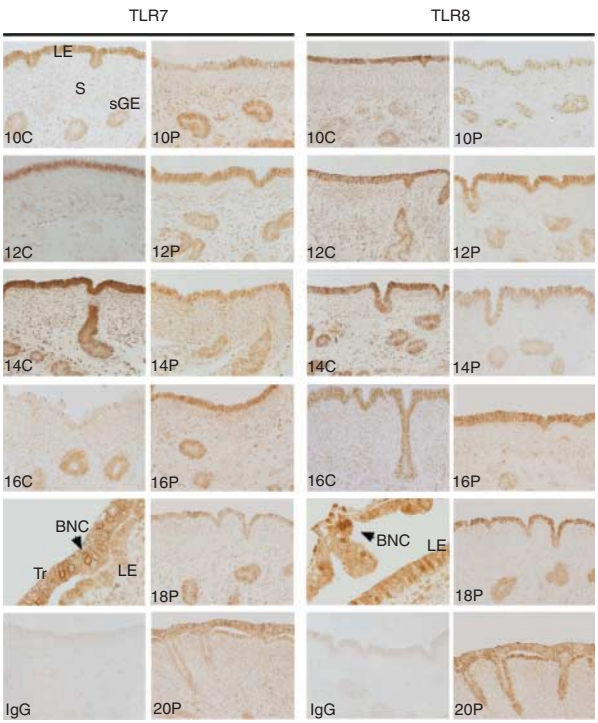

Figure 3 Immunohistochemical localization of TLR7 and TLR8 proteins in uteri of ewes on Days 10, 12, 14, and 16 of the estrous cycle and Days 10, 12, 14, 16, 18, and 20 of pregnancy. Sections were not counterstained. LE, luminal epithelium; sGE, superficial glandular epithelium; S, stroma; Tr, trophectoderm; BNCs, binucleate cells. All photographs were taken from uterine cross-sections at 20× magnification using the same width of field, with the exception of images illustrating TLR7 and TLR8 staining in BNCs, which were taken at 63×magnification.

Table 3 Effect of morpholino antisense oligonucleotide knockdown of translation of *TLR7, TLR8* and *TLR7/TLR8* mRNAs on pregnancy and conceptus development.

Morpholino	Pregnancy rate	Conceptus development	**IFNT** (ng/ml uterine flush)	**BNCs**/mm^2
MAO-Control	85.7% (6/7)	Robust, elongated	1060±210	90.143±33.16
MAO-*TLR7*	75% (6/8)	Thin, fragile, small, some elongated	610±185*	30.018±19.03*
MAO-*TLR8*	83.3% (5/6)	Small, shredded, fragile. Some elongated	577±119*	79.486±60.01
MAO-*TLR7/TLR8*	71.4% (5/7)	Small, fragile, fragmented, shredded	280±133*	12.225±5.46**

*$P<0.05$; **$P<0.01$.

conceptuses (12.2±5.5 BNCs/mm^2; $P<0.01$) when compared with MAO-Control (90.1±33.2 BNCs/mm^2) conceptuses. PAG-positive cells were scarce or absent in MAO-*TLR7/TLR8* conceptuses. Owing to a high degree of variability in the number of BNCs among MAO-*TLR8* conceptuses (79.5±60.0 BNCs/mm^2), numbers of BNCs were not significantly different from MAO-Control conceptuses.

TLR7 and TLR8 regulate abundance of enJSRV-Env in the conceptus

Expression of mRNA coding for the envelope protein of the ovine endogenous beta retroviruses (enJSRV-*Env*) was assessed in conceptuses recovered on different days of pregnancy and from morpholino-treated ewes (Fig. 7). During pregnancy, expression of enJSRV-*Env* was highest on Day 13 and declined progressively to Day 16 ($P=0.01$). Abundance of enJSRV-*Env* in MAO-conceptuses was different among treatment groups. Expression of enJSRV-*Env* in MAO-*TLR7* conceptuses was greater when compared with MAO-*TLR8* ($P=0.05$) and MAO-Control conceptuses ($P=0.01$), but was similar ($P=0.09$) to the expression of enJSRV-*Env* in MAO-*TLR7/TLR8* conceptuses.

Discussion

Results of this study provide initial insight into the expression and potential functions of TLRs in the ovine uterus and conceptus, as well as evidence for involvement of TLR7 and TLR8 in development and differentiated functions of the trophectoderm during the peri-implantation period of pregnancy in ewes.

The functional transcripts for *TLR1–TLR9* previously identified in ruminants (Menzies & Ingham 2006, Chang *et al*. 2009) are expressed by cells of the ovine endometrium in a temporal and cell-specific manner during the estrous cycle and early pregnancy. The abundance of TLRs was maximum in the endometrium between Days 12 and 14 of the estrous cycle and pregnancy, but then decreased on Day 16 in cyclic ewes while remaining similar in abundance in pregnant ewes. This suggests that progesterone influences expression of TLRs as proposed for other species (Jorgenson *et al*. 2005, Aflatoonian *et al*. 2007). Production of progesterone by ovine CL is maximal between Days 9 and 10 of the estrous cycle. After that time, and in the absence of a developing conceptus, luteolysis will occur on Days 15 and 16 to decrease progesterone and allow for an increase in estradiol that leads to estrus and an ovulatory surge of luteinizing hormone for ovulation that marks the beginning of the next estrous cycle (Spencer *et al*. 2004).

The increase in expression of *TLR2*, *TLR7*, *TLR8*, and *TLR9* in the endometrium during early pregnancy probably results from both progesterone-dependent recruitment of immune cells enriched in these particular TLRs (Segerson *et al*. 1991, Plaks *et al*. 2008, Gomez-Lopez *et al*. 2010, Mansouri-Attia *et al*. 2012) and changes in uterine gene expression required for pregnancy (Young *et al*. 2004, Jorgenson *et al*. 2005, Aflatoonian *et al*. 2007, Hirata *et al*. 2007, Turner *et al*. 2012). In view of the significant increase in steady-state levels of both *TLR7* and *TLR8* mRNAs in uteri of early pregnant ewes, we determined cell-specific expression of TLR7 and TLR8 proteins to be particularly abundant in uterine LE and sGE and expressed in a temporal and cell-specific manner that differed due to reproductive

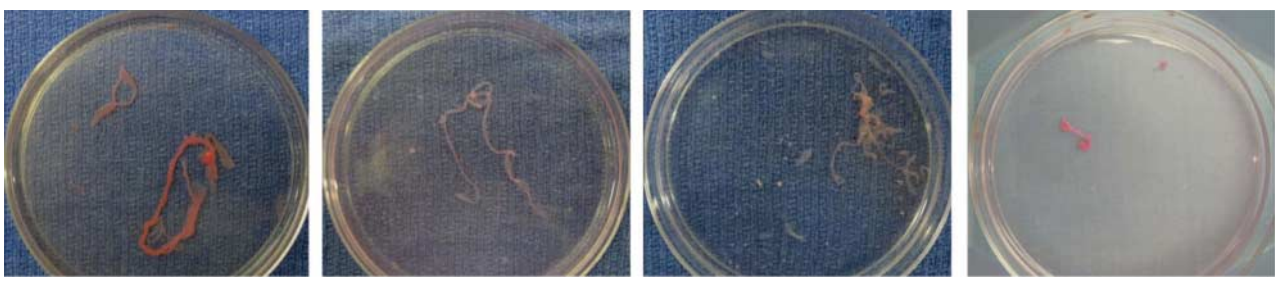

Figure 4 Morphological comparison of MAO-treated conceptuses. Representative images demonstrating differences in gross morphology of MAO-Control, MAO-*TLR7*, MAO-*TLR8*, and MAO-*TLR7/TLR8* conceptuses upon recovery on Day 16 of pregnancy.

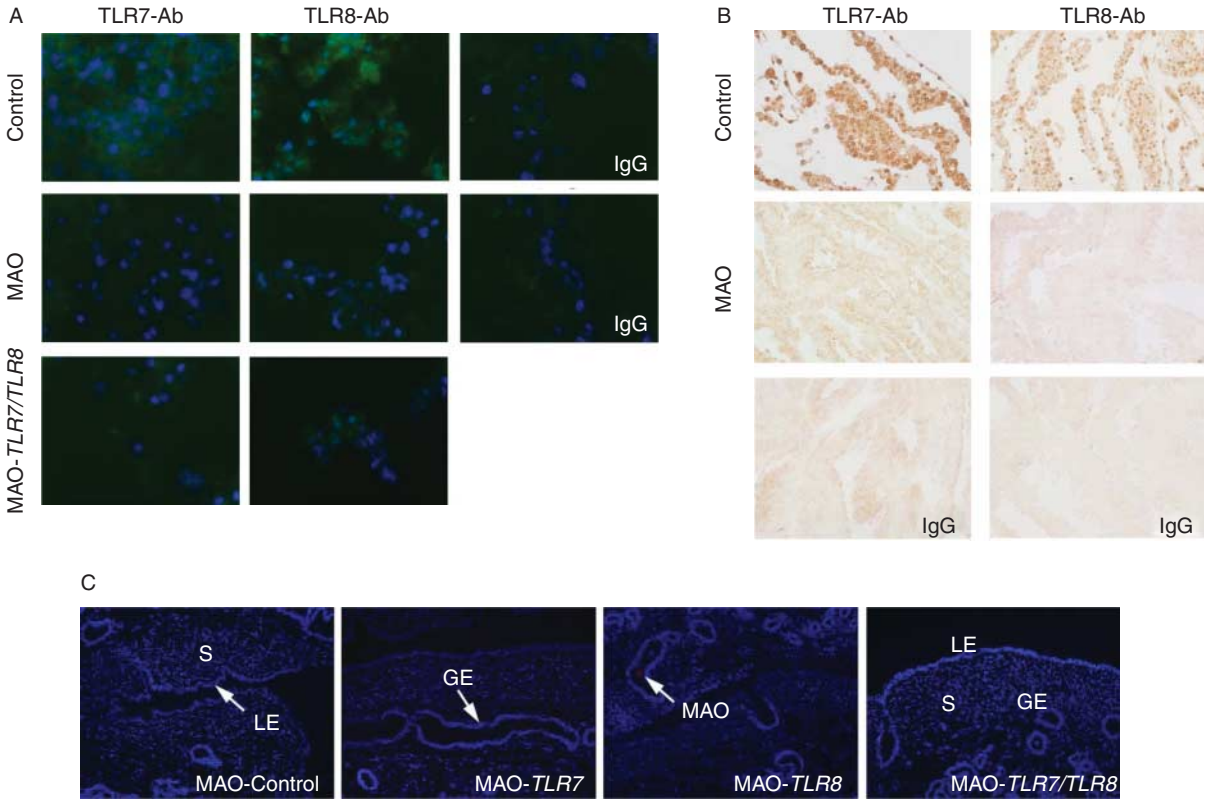

Figure 5 (A, B, and C) Efficiency of morpholino delivery to block translation of TLR7 and TLR8 proteins in the conceptus trophectoderm. (A and B) Immunohistochemical localization of TLR7 and TLR8 in conceptuses recovered from morpholino-treated ewes by (A) fluorescence microscopy and (B) standard paraffin immunohistochemistry. Images demonstrate an efficient morpholino delivery that blocked translation of TLR7 and TLR8 proteins in MAO-*TLR7,* MAO-*TLR8,* and MAO-*TLR7*/*TLR8* conceptuses compared with MAO-Controls. Sections with standard paraffin immunohistochemistry were not counterstained. Representative pictures of immunofluorescence and standard immunohistochemical analysis were taken at magnifications of 40× and 10×, respectively, using the same width of field. Ab, antibody used for the analysis. (C) Immunofluorescence analysis demonstrated absence of lissamine-tagged MAO uptake by the uterine epithelial cells. Image width of field=900 μm. LE, luminal epithelium; sGE, superficial glandular epithelium; S, stroma.

status, which supports results obtained from previous studies (Jorgenson *et al*. 2005, Aflatoonian *et al*. 2007, Hirata *et al*. 2007).

The uterine epithelia constitute a primary barrier against pathogens, while also ensuring tolerance to the implanting conceptus and producing nutrients and adhesion molecules indispensable for pregnancy (Burghardt *et al*. 2002, Spencer & Bazer 2004, Gray *et al*. 2006). Progesterone is claimed to suppress or modify the immune system during pregnancy to avoid rejection of the semi-allogeneic conceptus (Hansen 1998); however, expression of the progesterone receptor declines after Day 12 in all uterine epithelia of ewes. Thus, direct actions of progesterone are limited after Day 12 of pregnancy to stromal cells and myometrium as they continue to express the progesterone receptor throughout gestation (Spencer *et al*. 2004, Bazer *et al*. 2008). In this study, expression of both TLR7 and TLR8 increased in uterine LE and sGE following down-regulation of the progesterone receptor (Spencer & Bazer 1995). The selective loss of a direct influence via progesterone may allow expression of TLRs to be maintained in the epithelia to ensure protection against pathogens and to influence conceptus development.

In ewes, maternal recognition of pregnancy occurs between Days 12 and 14 of gestation when an appropriately elongated and filamentous conceptus signals its presence by secreting IFNT. This cytokine acts in a paracrine manner on uterine epithelia to abrogate development of the luteolytic mechanism (Spencer & Bazer 2004) and to induce expression of genes critical to establishment and maintenance of pregnancy (Bazer *et al*. 2012). The morpholino-treated conceptuses in this study did elongate; however, they secreted less IFNT due to being developmentally retarded and morphologically disorganized compared to control conceptuses. Those results indicate essential roles played by TLR7 and TLR8 in the trophectoderm that influence conceptus development directly or indirectly, and therefore, IFNT production.

This study also revealed that MAO-*TLR7* and MAO-*TLR7*/*TLR8* conceptuses had significantly fewer BNCs in the trophectoderm, which impairs their ability to fuse with the uterine LE and undergo implantation. Moreover, BNCs

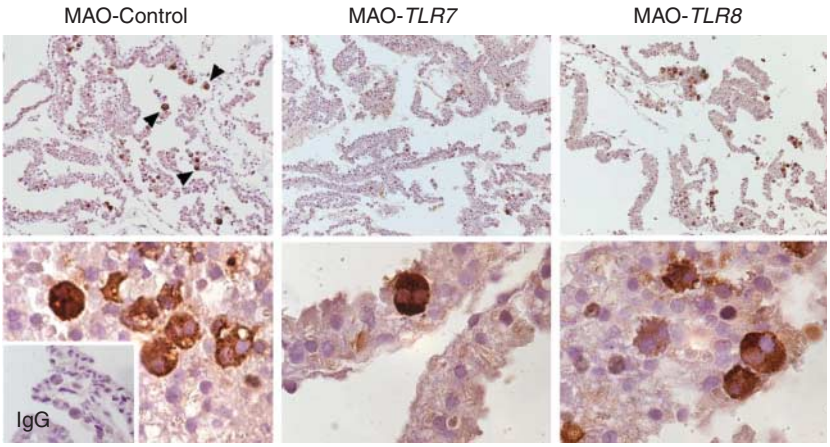

Figure 6 Immunohistochemical localization of PAG proteins in morpholino-treated conceptuses. MAO-*TLR7* and MAO-*TLR7*/*TLR8* conceptuses had significantly fewer PAG-positive BNCs than MAO-Control conceptuses. This effect was especially evident when blocking translation of *TLR7* mRNA. Variability was very high among conceptuses from the MAO-*TLR8* treatment group. Images illustrating specific staining in BNCs were taken at a magnification of 63×.

secrete placental lactogen (CSH1) and progesterone, which stimulate endometrial gland morphogenesis and differentiated functions during pregnancy in support of conceptus development. Interestingly, the ovine endogenous beta retroviruses (enJSRVs) included in sheep genome (Palmarini *et al.* 2001) are responsible for formation of BNCs (Dunlap *et al.* 2005, 2006*a*,*b*). During pregnancy, viral particles shed into the uterine lumen transfect conceptus trophectoderm (Black *et al.* 2010) as early as Day 12 (Dunlap *et al.* 2005) and influence development, IFNT production, and BNC formation (Dunlap *et al.* 2006*a*). Ovine enJSRV Envelope protein is a member of the syncytin family of retroviral proteins with high fusogenic activity responsible for inducing formation of the syncytiotrophoblasts in placenta of various species (Mi *et al.* 2000, Cornelis *et al.* 2013). In the ovine conceptus, mononuclear trophectoderm cells express abundant enJSRV-Env protein by Day 16 of pregnancy (Dunlap *et al.* 2006*a*). By Day 20, its expression appears to be limited to BNCs and syncytia (Dunlap *et al.* 2005), suggesting that this protein induces cell fusion that leads to BNC formation. Blockage of enJSRV-*Env* mRNA translation results in retarded development, decreased secretion of IFNT, and few or no BNCs in ovine conceptuses (Dunlap *et al.* 2006*a*). Thus, enJSRV-Env could interact with TLR7 to influence conceptus development, IFNT production, and BNC formation by ovine trophectoderm cells.

This study revealed that expression of enJSRV-*Env* mRNA by conceptus trophectoderm decreases between Days 13 and 16 of a normal pregnancy. This decline of enJSRV mRNA could result from active processing through recruitment and activation of antiviral ISGs such as Mx or 2′,5′-oligoadenylate synthetase (OAS), which are known to be induced by IFNT (Johnson *et al.* 2001, 2002). On the other hand, enJSRV-*Env* mRNA may accumulate under the influence of a regulatory feedback controlled by secretions from BNCs, which would account for the decline in enJSRV-*Env* mRNA around Day 16 as numbers of BNC increase. Regardless, treatment with MAO-*TLR7* affects enJSRV mRNA expression, which opens the possibility for TLR7 involvement in recognition of maternal viral particles (Black *et al.* 2010) or regulation of enJSRV abundance during the period when expression of IFNT increases rapidly. Till date, this role had been assigned to the proposed cellular receptor, hyaluronidase 2 (HYAL2),

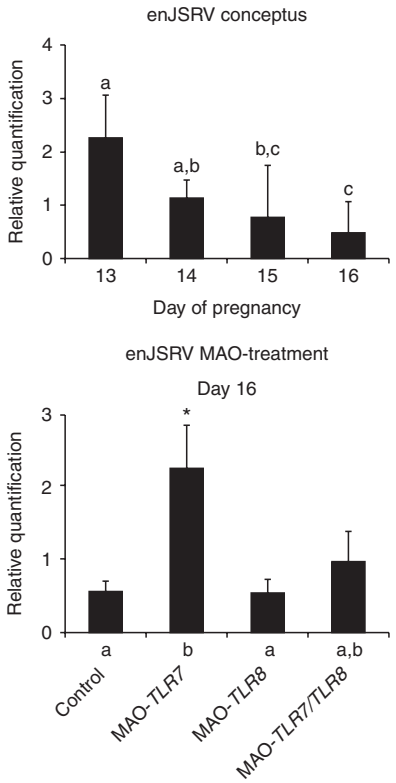

Figure 7 Abundance of mRNA coding for the envelope protein of enJSRV (enJSRV-*Env*) in ovine conceptuses during early pregnancy (top panel) and in MAO-treated conceptuses recovered on Day 16 (bottom panel). Data are expressed as levels of enJSRV-*Env* relative to *TUBA*. Different superscripts (^{a,b,c}) denote significant effects of days. The asterisk (*) indicates a significant treatment effect of MAO-*TLR7* ($P \leq 0.05$).

although it is not expressed by the conceptus until Day 16 when secretion of IFNT is actually decreasing (Dunlap *et al*. 2005). Thus, future experiments are necessary to clarify the mechanism(s) by which TLRs, particularly TLR7, are involved in key events during the peri-implantation period of pregnancy.

In summary, results of this study document patterns of expression of TLR1–TLR9 in ovine uterine endometria and conceptuses during the estrous cycle and the peri-implantation period of pregnancy, with significant differences in temporal and cell-specific expression of endometrial TLR7 and TLR8. Mechanistically, our *in vivo* loss-of-function experiments provide evidence for essential roles of TLR7 and TLR8 in conceptus development, pregnancy recognition signaling by IFNT, and formation of BNCs. These results provide strong evidence in support of our hypothesis that members of the TLR family are critical to the establishment and maintenance of pregnancy in ewes.

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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