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Uterine MHC Class I Molecules and β_2-Microglobulin Are Regulated by Progesterone and Conceptus Interferons during Pig Pregnancy[1]

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MHC class I molecules and β_2-microglobulin (β_2m) are membrane glycoproteins that present peptide Ags to TCRs, and bind to inhibitory and activating receptors on NK cells and other leukocytes. They are involved in the discrimination of self from non-self. Modification of these molecules in the placenta benefits pregnancy, but little is known about their genes in the uterus. We examined the classical class I swine leukocyte Ags (SLA) genes *SLA-1*, *SLA-2*, and *SLA-3*, the nonclassical *SLA-6*, *SLA-7*, and *SLA-8* genes, and the β_2m gene in pig uterus during pregnancy. Uterine SLA and β_2m increased in luminal epithelium between days 5 and 9, then decreased between days 15 and 20. By day 15 of pregnancy, SLA and β_2m increased in stroma and remained detectable through day 40. To determine effects of estrogens, which are secreted by conceptuses to prevent corpus luteum regression, nonpregnant pigs were treated with estradiol benzoate, which did not affect the SLA or β_2m genes. In contrast, progesterone, which is secreted by corpora lutea, increased SLA and β_2m in luminal epithelium, whereas a progesterone receptor antagonist (ZK137,316) ablated this up-regulation. To determine effects of conceptus secretory proteins (CSP) containing IFN-δ and IFN-γ, nonpregnant pigs were implanted with mini-osmotic pumps that delivered CSP to uterine horns. CSP increased SLA and β_2m in stroma. Cell-type specific regulation of SLA and β_2m genes by progesterone and IFNs suggests that placental secretions control expression of immune regulatory molecules on uterine cells to provide an immunologically favorable environment for survival of the fetal-placental semiallograft. *The Journal of Immunology,* **2008, 181: 2494–2505.**

Implantation is the process by which the blastocyst attaches to the uterus for juxtaposition of embryonic membranes with maternal uterine endometrium to establish histotrophic and hematotrophic exchange of nutrients and gases leading to the establishment of a functional placenta. During placentation, intimate physical contact between uterine and placental cells facilitates bidirectional interactions involving spatiotemporally regulated endocrine, paracrine, and autocrine modulators that mediate cell-cell and cell-matrix interactions essential for successful establishment and maintenance of pregnancy (1–3). These interactions may also, at least in part, prevent rejection of the conceptus (embryo/fetus and associated extraembryonic membranes), which is a semiallograft within the uterine environment.

Medewar recognized that the laws of transplantation biology dictate rejection of the conceptus as a semiallogeneic tissue with paternal as well as maternal histocompatibility Ags (4, 5), however many details of how the conceptus is protected from a potentially hostile immune environment remain unclear. Nevertheless, the conceptus in utero secures its own position, establishes an immunological truce with its mother, and obstructs or directs her immune system to contribute to the immunologic privileged state of the trophoblast (6).

The bulk of immune response to tissue grafts is directed to the MHC Ags. MHC molecules are polymorphic cell surface glycoproteins that present peptide Ags to TCRs, and bind to inhibitory and activating receptors on NK cells and other leukocytes. MHC class I molecules are categorized as either classical or nonclassical and contain a transmembrane α-chain associated noncovalently with an extracellular β-chain called β_2-microglobulin (β_2m).[3] The classical MHC class I molecules are expressed on most somatic cells and present peptides derived from self-proteins or from proteins of intracellular pathogens to CTL; therefore, they are involved in immune recognition of foreign pathogens and transplanted tissues. Additional class I MHC molecules termed nonclassical are less polymorphic and are restricted in cell type-specific expression (7). Protection of the conceptus from the maternal immune system involves down-regulation of MHC class I by the trophoblast (7–11). Additionally, in some species such as humans, nonclassical monomorphic MHC class I molecules are expressed by the trophoblast, which may protect the trophoblast cells from NK cell attack and T lymphocytes (7, 8). However, when placentation is noninvasive, trophectoderm cells do not express any MHC class I during the first trimester of pregnancy.

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[3] Abbreviations used in this paper: β_2m, β_2-microglobulin; SLA, swine leukocyte Ag; ISG, IFN-stimulated gene; CSP, conceptus secretory protein; LE, luminal epithelium; GE, glandular epithelium; USP, ubiquitin-specific protease; IRF, IFN regulatory factor.

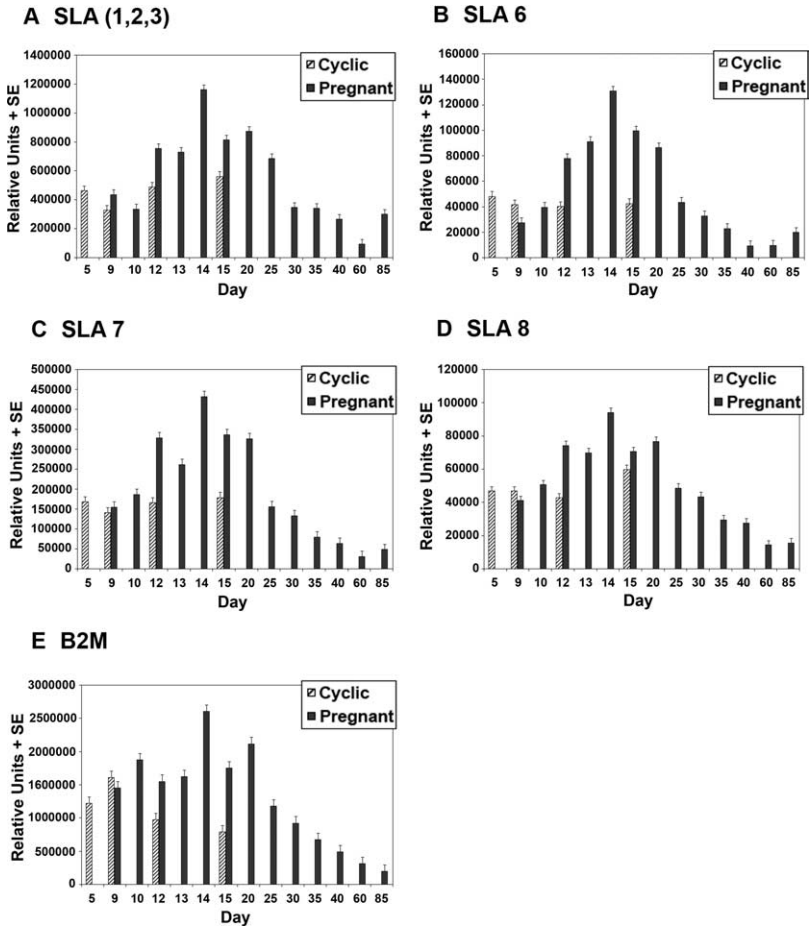

FIGURE 1. Steady-state levels of mRNA for the classical *SLA-1*, *SLA-2*, *SLA-3* genes (*A*), the nonclassical *SLA-6*, *SLA-7*, and *SLA-8* genes (*B–D*), and β_2m gene (*E*) in pig endometria during the estrous cycle and pregnancy were determined by slot blot hybridization. The mRNA levels, expressed as least square mean of relative units of cpm with overall SEM, are normalized for differences in sample loading using 18 S rRNA. The mRNA levels represent 20 μg of total endometrial mRNA per sample. Each of these mRNAs was increased during pregnancy over the estrous cycle.

Notable examples of noninvasive placentation include the entire placenta of pigs, all regions of placentation excluding the invasive chorionic girdle that develops into the endometrial cups of horses, and the interplacentomal uterine-placental interface of ruminants (7, 10, 12).

Cytokines and hormones regulate expression of MHC molecules during conceptus development, as well as the tissue differentiation and remodeling that occurs at the uterine-placental interface (13–21). Mattsson et al. (22) reported in mice increased uterine, but not placental, expression of MHC class I and class II molecules in response to IFN. Choi and coworkers (23) reported complex regulation of MHC class I and β_2m in uterine and placental tissue of sheep. In these studies, MHC class I and β_2m were inhibited in endometrial luminal epithelium (LE), but paradoxically stimulated by IFN-τ, the pregnancy recognition signal in sheep (3), in both endometrial stromal cells, and in glandular epithelium (GE) (23).

In pigs, conceptuses secrete estrogens on days 11 and 12 of pregnancy as the signal for pregnancy recognition (24). In addition, conceptus estrogens modulate uterine gene expression responsible for uterine remodeling for implantation and placentation from days 13 to 25 of gestation (25). The importance of estrogen is underscored by the fact that premature exposure of the pregnant uterus to estrogen on days 9 and 10 results in degeneration of all pig conceptuses by day 15 (26).

Peri-implantation pig conceptuses also secrete IFNs. The major species is type II IFN-γ and the other is type I IFN-δ (27, 28). In contrast to sheep conceptuses, in which a type I IFN-τ is the signal for maternal recognition of pregnancy (3), the IFNs produced by pig conceptuses do not appear to be antiluteolytic (29). However, both sheep and pig conceptus IFNs increase expression of a number of IFN-stimulated genes (ISGs) in uterine stroma (3, 30–32). Although physiological roles for these IFNs in the pig uterus have not been determined, emerging evidence suggests that induction or increases in uterine ISGs by conceptus IFNs is a phenomenon of early pregnancy in many, if not most, mammals (30–35).

Our working hypothesis is that pig conceptus IFNs increase uterine endometrial expression of the classical and nonclassical MHC class I molecules. In pigs these molecules are known as classical swine leukocyte Ag (SLA) class I genes (*SLA-1*, *SLA-2*, *SLA-3*) and nonclassical SLA class I (*SLA-6*, *SLA-7*, *SLA-8*) genes (36), and β_2m gene during pregnancy. The temporal cell type-specific expression of these genes plays a role in preventing immune disruption of pregnancy. Therefore, the objective of the present study is to determine 1) the temporal and spatial expression of SLA class I and β_2m genes in pig endometrium during pregnancy, and 2) whether their expression is regulated by estrogen, progesterone, or conceptus secretory proteins (CSPs) that contain IFN-γ and IFN-δ. Results provide compelling evidence that pig conceptus trophectoderm cells induce uterine stromal expression of SLA class I and β_2m genes through secretion of IFN-δ or IFN-γ, but expression is silenced in LE as a possible means of preventing immune rejection at the uterine-placental interface.

Materials and Methods

Animals and tissue collection

Experimental and surgical procedures complied with the Guide for Care and Use of Laboratory Animals and were approved by the Texas A&M University Laboratory Animal Care and Use Committee. Pigs were observed daily for estrus (day = 0) and exhibited at least two estrous cycles of normal duration before use in these studies.

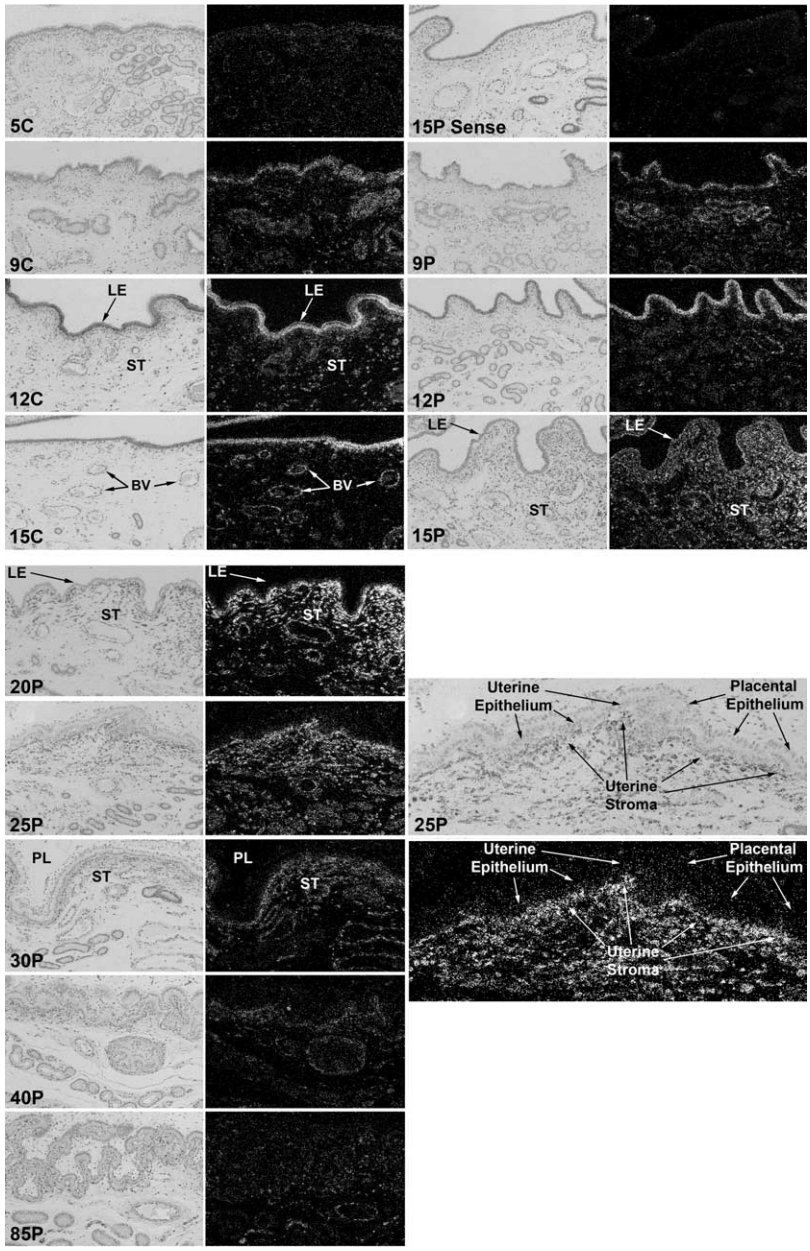

FIGURE 2. In situ hybridization analysis of *SLA-1*, *SLA-2*, and *SLA-3* mRNA in pig endometria. Corresponding brightfield and darkfield images from different days of the estrous cycle (C) and pregnancy (P) are shown. A representative section from day 15 of pregnancy hybridized with radio-labeled sense RNA probe (Sense) serves as a negative control. An expanded view of the photomicrographs of the uterine-conceptus interface on day 25 of pregnancy indicating the lack of hybridization for *SLA-1*, *SLA-2*, and *SLA-3* mRNAs in epithelia at this interface is shown. LE, stratum compactum stroma (ST), placenta (PL), and blood vessels (BV) are indicated. The width of each field is 870 μm.

Study one. To evaluate the effect of pregnancy on endometrial gene expression, pigs were assigned randomly to either cyclic or pregnant status. Those in the pregnant group were bred when detected in estrus and 12 and 24 h thereafter. Pigs were hysterectomized either on day 5, 9, 12, or 15 of the estrous cycle or day 9, 10, 12, 13, 14, 15, 20, 25, 30, 35, 40, 60, or 85 of pregnancy (n = 3 pigs/day/status).

Study two. To evaluate the effect of estrogen on endometrial gene expression, pigs were assigned randomly to receive daily injections (i.m.) of either 5 ml of corn oil vehicle or 5 mg of 17β-estradiol benzoate (5 mg in 5 ml of corn oil; Sigma-Aldrich) on days 11–14 postestrus (n = 5 pigs/treatment). This dose of 17β-estradiol is used to induce pseudopregnancy in pigs (25). All pigs were hysterectomized on day 15 postestrus.

Study three. To evaluate the effect of pig CSPs on endometrial gene expression, pigs (n = 3) were injected (i.m.) with 5 mg of 17β-estradiol benzoate (5 mg in 5 ml of corn oil; Sigma-Aldrich) on days 11–15 postestrus. On day 12 postestrus (coincident with secretion of IFNs by pig conceptuses (27, 37)), each pig was surgically implanted with two indwelling ALZET osmotic pumps (Durect Corporation) with a constant delivery rate of 10 μl/h. Each uterine horn was isolated via midline celiotomy, clamped, and severed from the uterine body at ~5 inches from the uterotubal junction while preserving the mesometrium and vascular supply to the uterine horn. The transected ends of each uterine horn and uterine body were sutured closed, and the serosa of the antimesometrial borders of the horn and body were sutured together to prevent twisting of the uterine horn. For each pump, a catheter was attached and inserted ~2 cm into the lumen of one uterine horn. Before surgery, pumps were filled and equilibrated per the manufacturer's instructions. For each pig, one uterine horn was infused from a pump filled with porcine serum albumin (35 mg; Sigma-Aldrich), whereas the other uterine horn was infused from a pump filled with porcine CSP (35 mg). All pigs were hysterectomized on day 16 postestrus (coincident with maximal antiviral activity in pig uterine flushings (37)).

Preparation of porcine CSP. As previously described (38), conceptuses from day-15 to day-17 pregnant pigs (coincident with maximal production of IFNs by conceptuses (27, 37)) were recovered by flushing uterine horns and cultured for 30 h. After recovery, medium was dialyzed (m.w. cutoff, 3500; Spectrum Laboratories), concentrated (m.w. cutoff, 5000; Millipore), filter sterilized, assayed for protein concentration (Bio-Rad), and stored at 4°C because IFN-γ is unstable to freezing and thawing.

Study four. To evaluate the effects of progesterone on endometrial gene expression, pigs were ovariectomized on day 4 postestrus and assigned randomly to receive daily injections (i.m.) of either progesterone (200 mg; Sigma-Aldrich) or progesterone plus ZK137,316 (75 mg), a progesterone receptor antagonist generously provided by Dr. K. Chwalisz (Shering AG, Berlin, Germany), on days 4–12 postestrus. All pigs were hysterectomized on day 12 postestrus (n = 5 pigs/treatment).

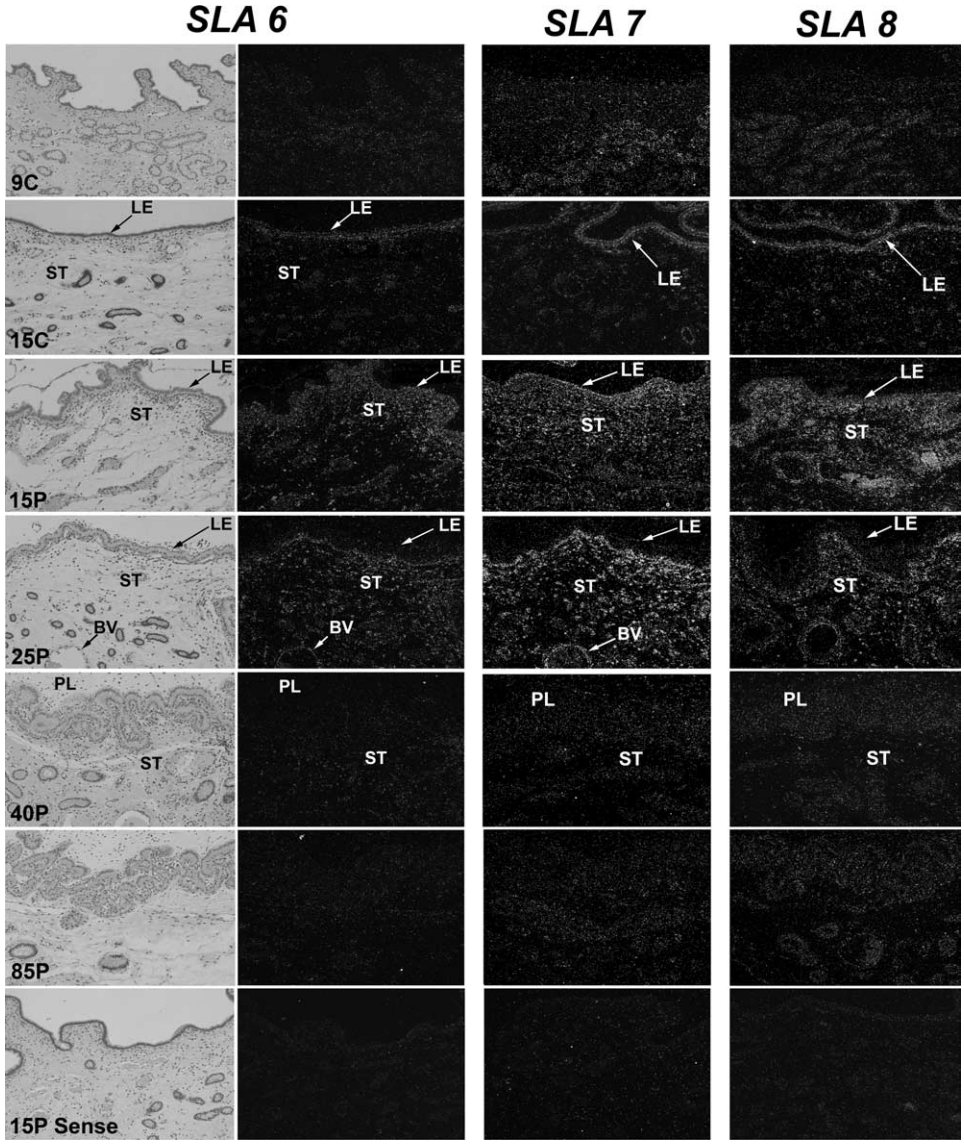

FIGURE 3. In situ hybridization analyses of *SLA-6*, *SLA-7*, and *SLA-8* mRNAs in pig endometria. Corresponding brightfield and darkfield images from different days of the estrous cycle (C) and pregnancy (P) are shown for *SLA-6*, whereas only darkfield images are shown for *SLA-7* and *SLA-8*. Representative sections from day 15 of pregnancy hybridized with radio-labeled sense RNA probes (Sense) served as negative controls. LE, stratum compactum stroma (ST), placenta (PL), and blood vessels (BV) are indicated. The width of each field is 870 μm.

At hysterectomy, several sections (~0.5 cm) from the middle of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Laboratory). Several sections from each uterine horn were also embedded in Tissue-Tek OCT Compound (Miles), snap frozen in liquid nitrogen, and stored at −80°C. The remaining endometrium was physically dissected from the myometrium, frozen in liquid nitrogen, and stored at −80°C for RNA extraction.

RNA isolation and analyses

RNA isolation. Total cellular RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's recommendations.

RT-PCR analyses. A partial cDNA that cross-hybridizes with the classical SLA class I genes (*SLA-1*, *SLA-2*, *SLA-3*), as well as partial cDNAs for *SLA-6*, *SLA-7*, *SLA-8*, β_2m gene, and ubiquitin-specific protease (USP) gene were amplified by RT-PCR as previously described (39). For all genes, porcine uterine endometrial RNA from day 15 of pregnancy was reverse transcribed, then the following gene-specific primers were used to amplify the PCR products: classical SLA class I (forward) 5′-ATACCTGGAGATGGGGAAGG-3′, (reverse) 5′-CCTTGGTAAGGGACACATCG-3′, 500-bp product; *SLA-6* (forward) 5′-TTGGTATCCCGGCCCGGCCACGGTAGTG-3′, (reverse) 5′-TGCCCGATACTGTTCAGCTACTCCC-3′, 443-bp product (40); *SLA-7* (forward) 5′-TATTGCGATCGGAACACACGCATC-3′, (reverse) 5′-GCATGCCACTTCCAGGTAGGCTCTGC-3′, 309-bp product (40); *SLA-8* (forward) 5′-GCCACGGGGAGCCCCGGTACCTTGAG-3′, (reverse) 5′-GAAGCGCTCATGAGCACGGGACTTG-3′, 422-bp product (40); β_2m (forward) 5′-ATGATATCCCACTTTTCACACCGCTCCAGTAGC-3′,(reverse)5′-ATAGATCTGGATTCATCCAACCCAGATGCAGC-3′, 439-bp product (40); and USP (forward) 5′-AGAGGATGACAGTGCCAAGG-3′, (reverse) 5′-CTGCTTCCAACAGGTCTTCC-3′, 473-bp product (GenBank accession no. AF134195). All PCR products were cloned into a pCRII cloning vector using the TA Cloning kit (Invitrogen) and confirmed by sequence analysis. A BLAST search for each was conducted to ensure that only target genes were evaluated.

Slot blot analyses. As previously described (41), duplicate membranes with 20 μg of total RNA per slot were hybridized with radio-labeled antisense porcine classical SLA class I genes *SLA-6*, *SLA-7*, and *SLA-8* and β_2m gene RNA probes generated by in vitro transcription with [α-^{32}P]UTP (PerkinElmer Life Sciences) and a MAXIscript kit (Ambion). To correct for variation in loading, a duplicate membrane was hybridized with a radio-labeled antisense 18 S rRNA (pT718S; Ambion) RNA probe. Hybridization signals were detected by exposure to a PhosphoImager screen and visualized using a Typhoon 8600 variable mode imager (Molecular Dynamics).

In situ hybridization analyses. As previously described (42), deparaffinized, rehydrated, and deproteinated uterine cross-sections (5 μm; 1 cross-section each from three separate blocks from each animal) were hybridized with radio-labeled antisense or sense classical SLA class I genes *SLA-6*, *SLA-7*, and *SLA-8* and β_2m or USP gene RNA probes synthesized by in vitro transcription with [α-^{35}S]UTP (PerkinElmer). After hybridization, washes, and RNase A digestion, autoradiography was performed using NTB liquid photographic emulsion (Eastman Kodak). Slides were exposed at 4°C, developed in Kodak D-19 developer, counterstained with Harris' modified hematoxylin (Fisher Scientific), dehydrated, and protected with coverslips.

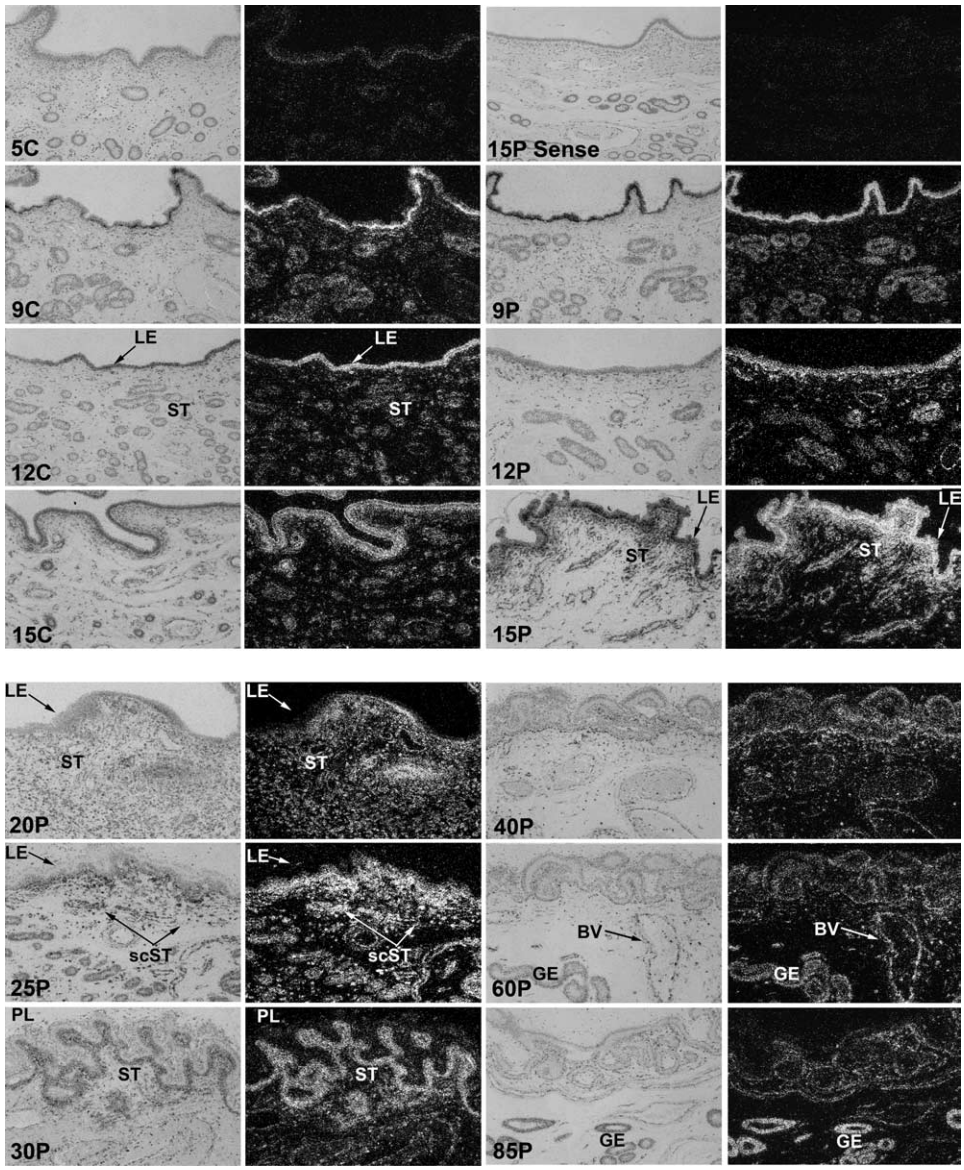

FIGURE 4. In situ hybridization analysis of β_2m mRNA in pig endometria. Corresponding brightfield and darkfield images from different days of the estrous cycle (C) and pregnancy (P) are shown. A representative section from day 15 of pregnancy hybridized with radio-labeled sense RNA probe (Sense) served as a negative control. LE, GE, stratum compactum stroma (ST), placenta (PL), and blood vessels (BV) are indicated. The width of each field is 870 μm.

Immunofluorescence analyses

As previously described (43), frozen uterine cross-sections (~8–10 μm; 1 cross-section each from three separate blocks from each animal) were fixed in −20°C methanol, washed in PBS containing 0.3% v/v Tween 20 (rinse solution), blocked in 10% normal goat serum, incubated overnight at 4°C with 10 μg/ml mouse anti-porcine MHC class I (PT85A; VMRD), or 10 μg/ml mouse IgG (negative control; Sigma-Aldrich) and detected with 2 μg/ml fluorescein-conjugated goat anti-mouse IgG (Chemicon International). Slides were overlaid with Prolong anti-fade mounting reagent (Molecular Probes) and a coverslip.

SLA class I protein was colocalized with either β_2m protein or von Willebrand factor in frozen uterine cross-sections by immunofluorescence staining as previously described (44). Briefly, sections were cut, fixed, washed, and blocked as we described. After dipping in rinse solution at room temperature, sections were incubated overnight at 4°C with the initial primary Ab (10 μg/ml mouse anti-porcine MHC class I or mouse IgG). Following washes, sections were incubated with 2 μg/ml initial secondary Ab (fluorescein-conjugated goat anti-mouse IgG), washed, and incubated overnight at 4°C with the second primary Ab (20 μg/ml rabbit anti-human β_2m, RDI-CBL307; Research Diagnostics, 10 μg/ml rabbit anti-human von Willebrand factor, AB7356; Chemicon International, or 10 μg/ml rabbit IgG, negative control; Sigma-Aldrich). Following washes, sections were incubated with 2 μg/ml secondary Ab (Texas Red-conjugated goat anti-rabbit IgG; Molecular Probes), washed, dipped in distilled-deionized water, and overlaid with anti-fade mounting reagent as described.

Photomicrography

Digital photomicrographs of in situ hybridization (brightfield and darkfield images) and immunofluorescence staining were evaluated using an Axioplan 2 microscope (Carl Zeiss) interfaced with an Axioplan HR digital camera and Axiovision 4.3 software. For immunofluorescent colocalization of proteins, digital camera settings were evaluated to confirm that no "spectral bleed through" FITC signal was detectable in the Texas Red filter set and vice versa. In these studies, once the distribution of individual Ags was established, the codistribution of two Ags was investigated simultaneously in individual sections using compatible primary and FITC or Texas Red secondary Ab combinations with appropriate filter sets. Individual fluorophore images were recorded sequentially with AxioVision 4.3 software and evaluated in multiple fluorophore overlay images recorded in Zeiss Vision Image (.zvi) file format, which were subsequently converted to Tagged Image File (.tif) format. Photographic plates were assembled using Adobe Photoshop (version 6.0; Adobe Systems). All sections from each day per treatment were assessed as a group, and sections exhibiting the most representative hybridization or immunostaining pattern for each day per treatment were selected for inclusion in photographic plates.

Statistical analyses

All slot blot hybridization data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System. Data were analyzed using the 18 S rRNA as a covariate to correct for differences in

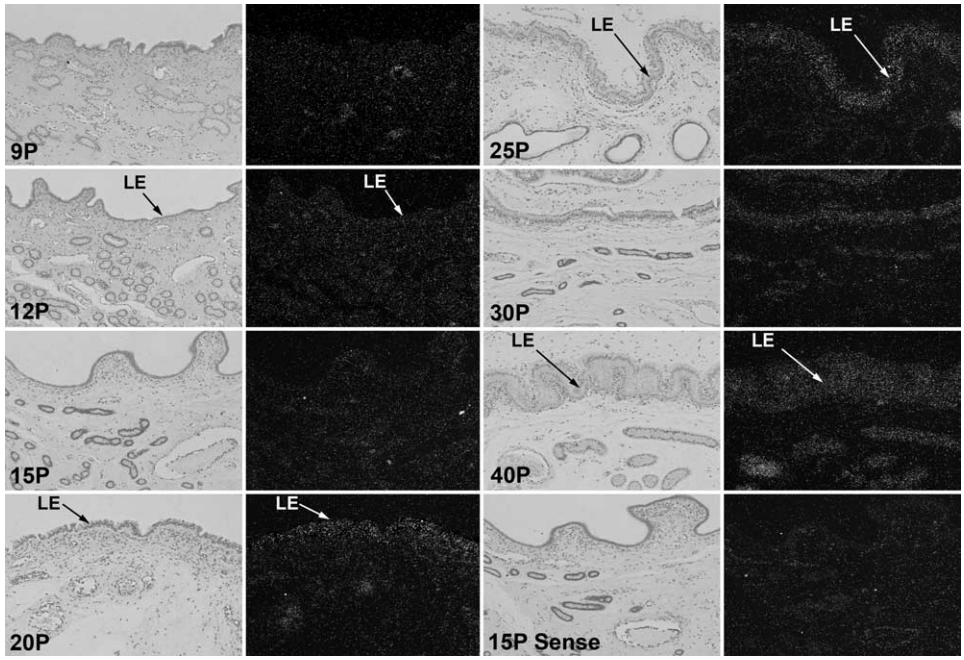

FIGURE 5. In situ hybridization analysis of USP mRNA in pig endometria. Corresponding brightfield and darkfield images in LE from different days of pregnancy (P) are shown. A representative section from day 15 of pregnancy hybridized with radio-labeled sense RNA probe (Sense) served as a negative control. The width of each field is 870 μm.

RNA loading and for effects of day and status and their interaction where appropriate. All tests of significance were performed using the appropriate error terms according to the expectation of the mean square for error. Slot blot hybridization data are presented as least squares mean with SE.

Results

Effects of pregnancy from study one

Partial cDNAs for classical SLA class I genes *SLA-1*, *SLA-2*, and *SLA-3*, the nonclassical class I genes *SLA-6*, *SLA-7*, and *SLA-8*, as well as β_2m gene were detected in day-15 pregnant pig endometria by RT-PCR analyses. Steady-state mRNAs in endometrium from the estrous cycle and pregnancy were then examined using antisense RNA probes and slot blot hybridization. (Fig. 1). There was no significant change in total steady-state mRNA during the estrous cycle for any of these mRNAs ($p > 0.1$). During the ~115 days of pig pregnancy, steady-state mRNAs increased between day 9 and 14, decreased between day 14 and 60, and remained low through day 85 (classical and nonclassical SLA class I mRNAs, $p < 0.005$, quartic effect of day; β_2m mRNA, $p = 0.06$, quartic effect of day) (Fig. 1).

In situ hybridization was used to localize classical and nonclassical SLA class I mRNAs as well as β_2m mRNAs to specific cell types within the endometria of cyclic and pregnant pigs. Messenger RNA for *SLA-1*, *SLA-2*, and *SLA-3* increased in the LE, GE, and blood vessels between days 5 and 9 of the estrous cycle and remained in these cell types through day 15 (Fig. 2). During pregnancy, the pattern of expression for *SLA-1*, *SLA-2*, and *SLA-3* mRNAs was the same as observed for the estrous cycle through day 12. However by day 15, expression increased in the stratum compactum stroma (note the difference in the spatial distribution of mRNA in endometrium from day 15 of the estrous cycle vs day 15 of pregnancy, Fig. 2), where it remained detectable through day 40 of pregnancy. Significantly, *SLA-1*, *SLA-2*, and *SLA-3* mRNAs were no longer detectable in the LE by day 20 of pregnancy (expanded view of day 25 shown in Fig. 2).

The three nonclassical SLA class I mRNAs exhibited similar patterns of expression in pig endometria by in situ hybridization. *SLA-6*, *SLA-7*, and *SLA-8* mRNAs increased in LE after day 9 of the estrous cycle and early pregnancy (Fig. 3). These mRNAs then decreased in LE after Day 15 of pregnancy and were not detectable in LE on day 25 (Fig. 3). However, similar to the classical SLA mRNAs, *SLA-6*, *SLA-7*, and *SLA-8* mRNAs increased in the stratum compactum stroma by day 15 of pregnancy, where they remained through day 25, and then decreased to undetectable levels by day 40 (Fig. 3). Expression was also detected in endothelial cells in endometrial stroma on all days of the estrous cycle and pregnancy (Fig. 3).

In situ hybridization for β_2m mRNA was generally more intense than was observed for SLA mRNAs; however, the general patterns of expression for these mRNAs were similar. The β_2m mRNA increased in the LE and GE between days 5 and 9 of the estrous cycle and pregnancy, and remained in these cell types through day 15 (Fig. 4). During pregnancy, the pattern of expression for β_2m mRNA was the same as observed for the estrous cycle through day 12. However by day 15, expression increased in the stratum compactum stroma (note the difference in the spatial distribution of mRNA in endometrium from day 15 of the estrous cycle vs day 15 of pregnancy) (Fig. 4). The β_2m remained detectable in the stratum compactum stroma through day 30 and became undetectable in LE by day 20 of pregnancy. Expression of β_2m mRNA by GE was more robust than the SLA mRNAs on all days examined, and diverged from the general pattern of SLA mRNA expression by further increasing in GE between days 60 and 85 of pregnancy (Fig. 4). Additionally, β_2m mRNA was detectable in endothelial cells in the endometrial stroma on all days of the estrous cycle and pregnancy (Fig. 4).

Because mRNAs for *SLA-1*, *SLA-2*, *SLA-3*, *SLA-6*, *SLA-7*, and *SLA-8* and β_2m gene decreased in uterine LE between days 15 and 20 of pregnancy, endometrial expression of the USP gene, a negative regulator of type I IFN signaling (45), was examined. In situ hybridization revealed that USP mRNA increased between days 15 and 20 of pregnancy in the LE, where it continued to be expressed through day 40 (Fig. 5).

Immunofluorescence analysis of pig endometria indicated that immunoreactive classical SLA class I molecules increased in LE between days 9 and 12 of the estrous cycle and pregnancy (Fig. 6). Consistent with in situ hybridization results, SLA class I molecules were maintained in the LE of cyclic endometrium. In pregnant endometrium, SLA class I molecules were not detectable in LE by day 15 and remained absent from LE through day 85 (Fig. 6). Similar to the temporal and spatial expression of SLA mRNAs, immunoreactive SLA class I molecules increased in the stratum

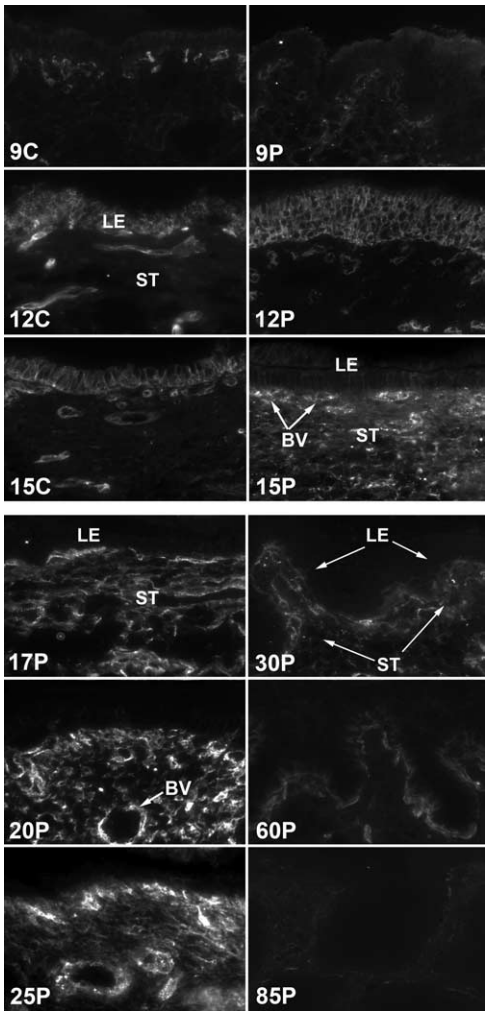

FIGURE 6. Immunofluorescence localization of classical SLA class I molecules in frozen cross-sections of pig endometria during the estrous cycle (C) and pregnancy (P). A nonrelevant mouse Ig (mIgG) served as a negative control as illustrated in Fig. 9. LE, stratum compactum stroma (ST), and blood vessels (BV) are indicated. The width of each field is 540 μm.

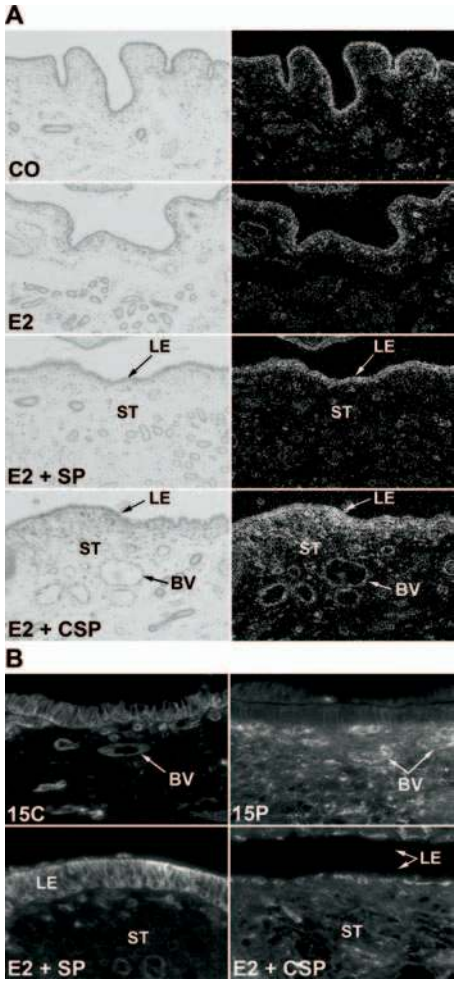

FIGURE 7. Treatment with CSP increased expression of classical SLA class I mRNAs. *A*, In situ hybridization analysis of *SLA-1*, *SLA-2*, and *SLA-3* mRNA. *B*, Immunofluorescence localization of classical SLA molecules. Cross-sections of pig endometria from day-15 cyclic pigs injected i.m. with either corn oil control (CO) or estradiol benzoate (E2), or from estrogen-treated day-16 cyclic pigs in which either control serum proteins (E2 + SP) or CSPs (E2 + CSP) were infused into the uterine lumen are shown. Corresponding brightfield and darkfield images of in situ hybridization from different treatments are shown. A representative section from day 15 of pregnancy hybridized with radio-labeled sense RNA probe (15P Sense) served as a negative control for in situ hybridization as illustrated in Fig. 2. A nonrelevant mouse Ig (mIgG) served as a negative control as illustrated in Fig. 9. LE, luminal epithelium, stratum compactum stroma (ST), and blood vessels (BV) are indicated. The width of each in situ hybridization field is 870 μm and the width of each immunofluorescence localization field is 540 μm.

compactum stroma by day 15 of pregnancy, remained abundant through day 25, and then progressively decreased through day 85 (Fig. 6). An increase of SLA class I molecules in endometrial stroma was not observed during the estrous cycle (Fig. 6). In addition, SLA class I protein was present in endothelial cells of the endometrium throughout the estrous cycle and pregnancy (Fig. 6).

Collectively, results from study one indicate that classical and nonclassical SLA, as well as β_2m, 1) increases in uterine LE before implantation; 2) increases in uterine stratum compactum stroma during the peri-implantation period (days 10–25); and 3) decreases in uterine LE immediately after initial attachment of trophectoderm to uterine LE during early placentation. Therefore, although expression prominently increases within the uterine-placental environment, classical SLA class I mRNA and molecules and nonclassical SLA class I and β_2m mRNAs are conspicuously absent at the immediate interface between uterine and placental epithelia of pigs after day 15, perhaps due to expression of USP in uterine LE.

Effects of exogenous estrogen and CSPs for study two and study three

The i.m. injections of estradiol benzoate did not alter expression of classical SLA class I mRNA in endometrial LE of day-15 cyclic-treated pigs compared with corn oil vehicle treatment (Fig. 7*A*). In contrast, intrauterine infusion of CSPs into the uterine horns of day-16 cyclic pigs treated with exogenous estrogen increased expression of classical SLA class I mRNAs in the stratum compactum stroma compared with intrauterine infusion of control serum proteins (Fig. 7*A*). In agreement with mRNA results, immunofluorescence analysis indicated that intrauterine infusion of CSP increased SLA class I molecules in the stratum compactum stroma (Fig. 7*B*).

Similar to SLA class I mRNA, in situ hybridization for *SLA-6* (Fig. 8*A*), *SLA-7* (Fig. 8*B*), *SLA-8* (Fig. 8*C*), and β_2m gene (Fig. 8*D*) indicated no effect of exogenous estrogen on expression of

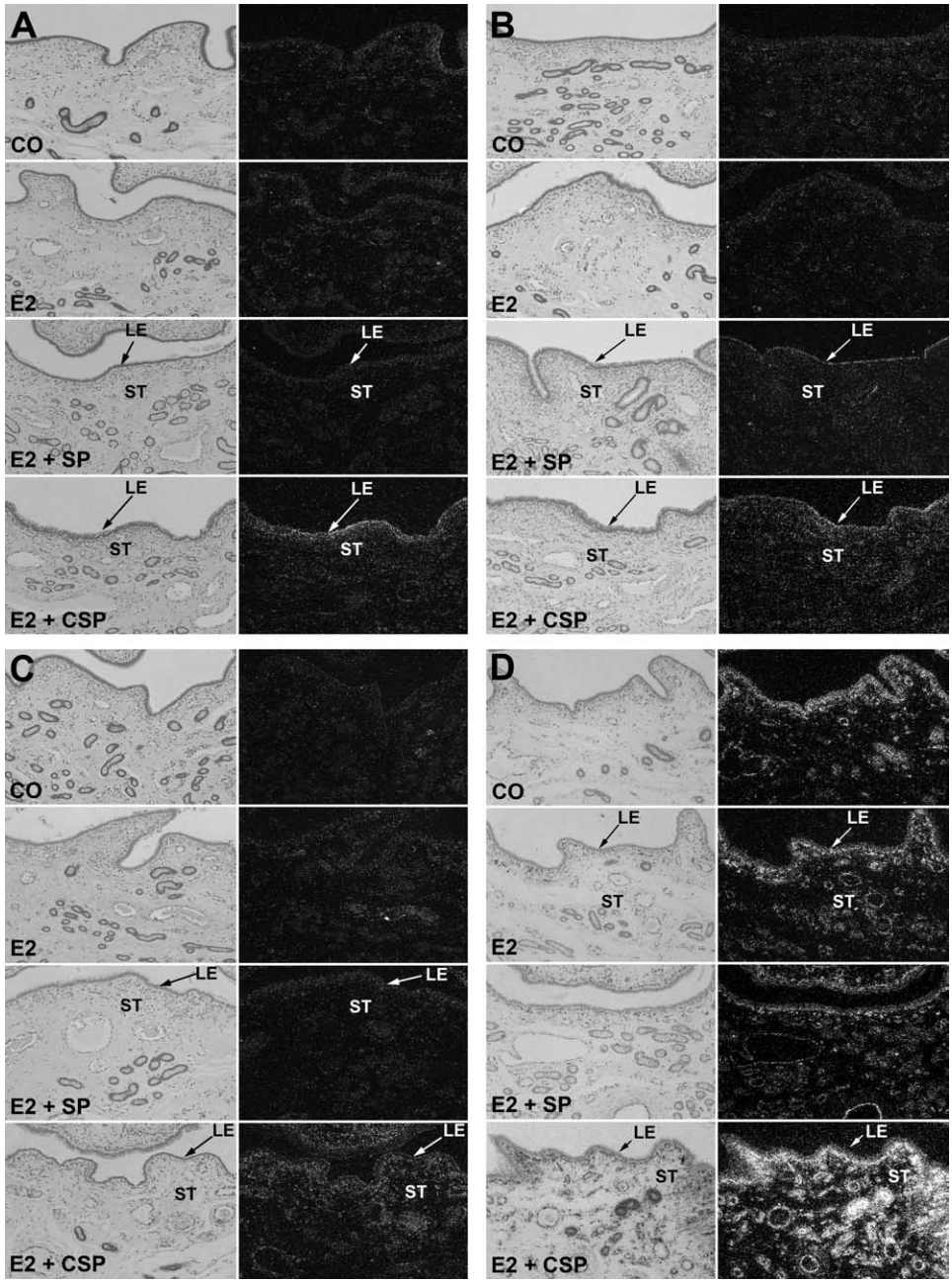

FIGURE 8. In situ hybridization analyses on expression of mRNAs. *SLA-6* (*A*), *SLA-7* (*B*), *SLA-8* (*C*) and β_2m (*D*) mRNAs in cross-sections of pig endometria from day-15 cyclic pigs injected i.m. with either corn oil control (CO) or estradiol benzoate (E2), or from estrogen-treated day-16 cyclic pigs, which received intrauterine infusions of either control serum proteins (E2 + SP) or CSPs (E2 + CSP). Corresponding brightfield and darkfield images from different treatments are shown. Representative sections from day 15 of pregnancy hybridized with radio-labeled sense RNA probes (15P Sense) served as negative controls as illustrated for *SLA-6*, *SLA-7*, and *SLA-8* in Fig. 3 and for β_2m in Fig. 4. LE and stratum compactum stroma (ST) are indicated. The width of each field is 870 μm.

these mRNAs in day 15 cyclic pig endometria. However, intrauterine infusion of CSP into estradiol benzoate-treated day-16 cyclic pigs increased mRNA levels for *SLA-6*, *SLA-7*, *SLA-8*, and β_2m gene in the stratum compactum stroma compared with infusion of serum proteins (Fig. 8, *A–D*).

Collectively, results from study two and study three strongly suggest that classical and nonclassical SLA, as well as β_2m increase in endometrial stroma in response to IFN-γ or IFN-δ present in CSP of the peri-implantation period, but expression of these mRNAs and molecules in the endometrial LE is not induced by conceptus estrogens alone, although these estrogens are accepted to be the pregnancy recognition signal.

Effects of exogenous progesterone for study four

Because *SLA-1*, *2*, *3*, *6*, *7*, and *8* and β_2m mRNAs increased in endometrial LE between days 5 and 15 of pregnancy, which is a period of increased progesterone secretion from the corpora lutea, the effects of exogenous progesterone on endometrial expression of SLA class I and β_2m mRNAs and proteins was examined. Classical SLA class I and β_2m mRNAs were detectable in endometrial LE and blood vessels of the stratum compactum stroma of ovariectomized pigs treated with exogenous progesterone. These effects of progesterone were completely ablated by treatment with the progesterone receptor antagonist, ZK137,316, in both uterine LE and vasculature (Fig. 9*A*).

Results shown in Fig. 9*B* confirm that SLA class I and β_2m proteins colocalize in the endometrial LE and endothelial cells of ovariectomized pigs treated with exogenous progesterone. Immunoreactive SLA class I molecules also colocalized with von Willebrand factor, an endothelial cell marker, in endometrial endothelial cells of the stratum compactum stroma on day 12 in ovariectomized pigs treated with exogenous progesterone (Fig. 9*B*).

Collectively, results from study four strongly suggest that expression of intact SLA class I molecules including classical SLA

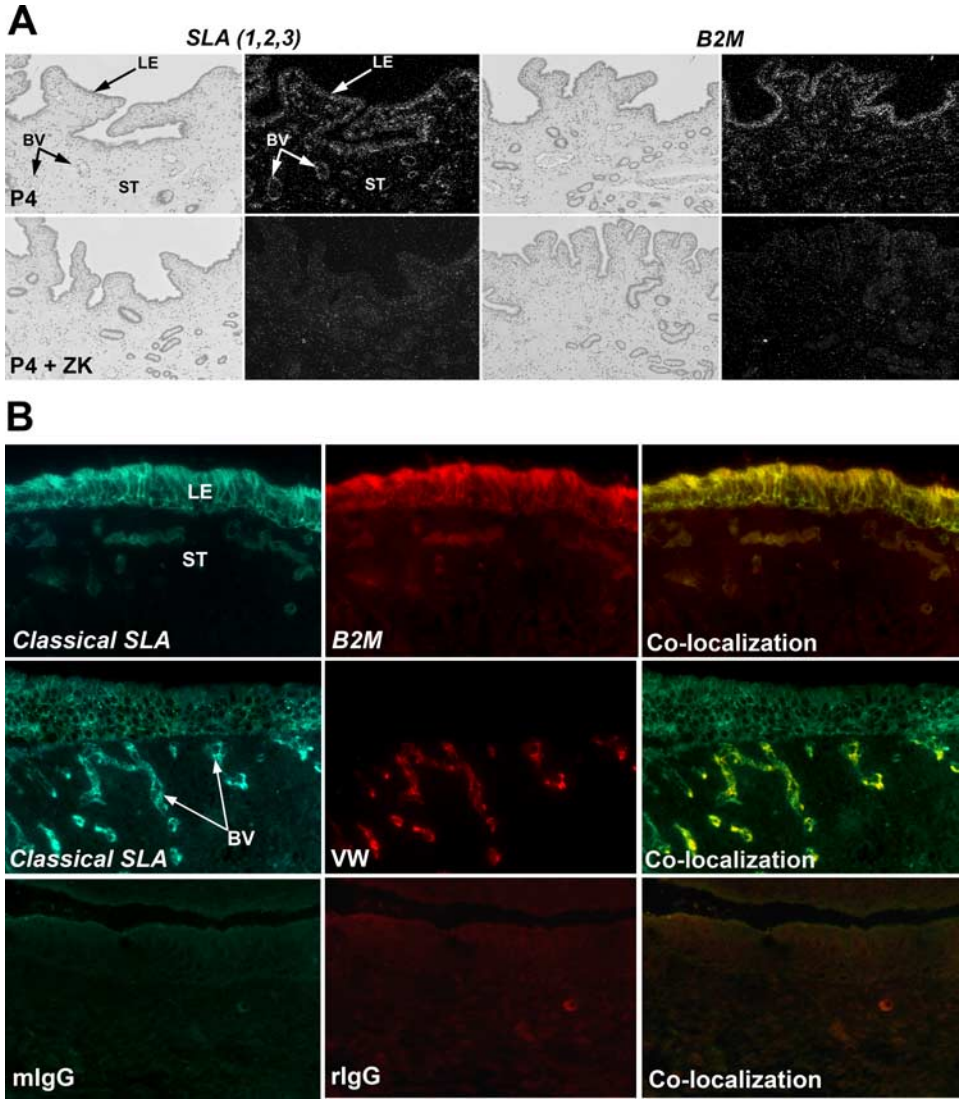

FIGURE 9. The effects of exogenous progesterone on endometrial expression of SLA class I and β_2m mRNAs and proteins. *A*, In situ hybridization analyses for *SLA-1*, *SLA-2*, *SLA-3*, and β_2m mRNAs in cross-sections of endometria from day-12 ovariectomized pigs treated with progesterone (P4) or progesterone and the progesterone receptor antagonist ZK137,316 (P4 + ZK). Corresponding brightfield and darkfield images from different treatments are shown. LE, stratum compactum stroma (ST), and blood vessels (BV) are indicated. The width of each field is 870 μm. *B*, Immunofluorescence colocalization of classical SLA with β_2m and classical SLA with von Willebrand factor (VW) in endometrial cross-sections from day 12 ovariectomized pigs treated with progesterone. SLA immunoreactivity was detected using fluorescein-conjugated anti-mouse IgG (*left*, green fluorescence), whereas the β_2m and von Willebrand factor immunoreactivity were detected using Texas Red-conjugated anti-rabbit IgG (*middle*, red fluorescence). SLA and β_2m proteins were colocalized (*right*, yellow fluorescence) to LE and blood vessels (BV) within the stratum compactum stroma (ST). Nonrelevant mouse Ig (mIgG) was detected using fluorescein-conjugated anti-mouse IgG, nonrelevant rabbit Ig (rIgG) was detected using Texas Red-conjugated anti-rabbit IgG and their colocalization served as negative controls as illustrated (*lower*). The width of each field is 540 μm.

class I and β_2m heterodimers is increased by progesterone produced by corpora lutea in endometrial LE and endothelial cells during the peri-implantation period in pigs.

Discussion

The MHC class I molecules and accompanying β_2m function in immune responses and are involved in discrimination of self from non-self. It is generally accepted that modification or down-regulation of these molecules in placental tissues is beneficial to pregnancy across mammalian species (7–12). The present studies focus on the uterine tissues that represent the maternal component of the fetal-maternal interface of pregnancy. Results illustrate dynamic temporal and cell type-specific regulation of MHC class I molecules and β_2m by progesterone and CSPs in the peri-implantation pig uterine endometrium. Similar to previous results in sheep, MHC class I and β_2m up-regulate in endometrial stratum compactum stroma, but are conspicuously undetectable in LE, suggesting that lack of expression in LE is beneficial to pregnancy in species in which the LE maintains direct contact with the placenta (epitheliochorial placentation). However, clear differences between sheep and pigs in regulation and pattern of expression of these genes were observed. Although pigs and sheep share stromal MHC class I and β_2m expression, pigs lack the IFN-τ responsible for this expression in sheep (23). Therefore it is likely that in pigs, conceptus-derived IFN-δ and IFN-γ increase MHC class I and β_2m in endometrial stroma (27–29). Unlike sheep, which never express MHC class I and β_2m in LE, ovarian progesterone increased expression of SLA class I and β_2m in the uterine LE during the estrous cycle and early pregnancy of pigs. Finally, the present data suggest a mechanism for down-regulation of SLA class I and β_2m in LE that has not been proposed for sheep. Although estrogens secreted by the conceptus or administered exogenously did not directly effect expression of SLA class I and β_2m genes, estradiol does increase expression of IFN regulatory factor (IRF)-2 in LE (30), which together with USP may down-regulate SLA class I and β_2m gene expression in uterine LE between days 15 and 25 of pregnancy. Collectively, for the majority of the first half of gestation, there is abundant uterine expression of SLA and β_2m in the stromal compartment of pig endometrium, but not in LE that is directly apposed to trophectoderm or chorion.

Progesterone, the hormone of pregnancy, plays a critical role in control of temporal and spatial (cell-specific) changes in gene expression within the uterus (3). Indeed, treatment with progesterone significantly alters the expression of a number of genes in the uteri of rodents, primates, and sheep as determined using microarray analyses (46–48). In the present pig study, progesterone increased expression of SLA class I and β_2m genes in endometrial LE and

stromal endothelial cells during the estrous cycle and early pregnancy. This expression was blocked by ZK137,316, a progesterone receptor antagonist, indicating that induction by progesterone is mediated via progesterone receptors. Because progesterone receptors in pigs are down-regulated in endometrial LE and GE by day 10 of the estrous cycle and pregnancy, but maintained in stromal cells and myometrium (49), the endocrine effects of ovarian progesterone on endometrial LE expression of SLA class I and β_2m genes may be mediated indirectly by either progesterone-induced paracrine-acting factors (progestamedins) produced by the progesterone receptor-positive stromal cells, or by induction of factors in LE that down-regulate progesterone receptors to either allow or stimulate expression of endometrial genes (50, 51).

Given that SLA class I molecules and β_2m are important for host defense, their expression in the LE during the estrous cycle and early pregnancy may be important for preventing uterine infections. At estrus, mucin 1 (MUC1), which forms an apical LE glycocalyx barrier to provide innate immune protection for the uterus from bacterial infections (52), is localized to the endometrial LE, but decreases by day 10 of the estrous cycle and pregnancy (53). This down-regulation of MUC1 is hypothesized to be necessary for conceptus attachment to the LE, but leaves the uterus susceptible to bacterial invasion (52–54). Increased expression of SLA class I and β_2m genes before day 9 of the estrous cycle and pregnancy by ovarian progesterone may compensate for the progesterone-induced loss of MUC1 and provide continued immune protection of the uterus from pathogens.

Results of the present study strongly suggest that classical SLA genes *SLA-1*, *SLA-2*, and *SLA-3*, nonclassical *SLA-6*, *SLA-7*, and *SLA-8*, as well as β_2m mRNAs are induced in the stratum compactum stroma of pigs in response to IFNs secreted by the conceptus. Pig conceptuses secrete both IFN-δ and IFN-γ; ~75% of antiviral activity in pig CSPs is attributed to IFN-γ and the other 25% to IFN-δ (27, 28). Pig conceptus trophectoderm cells express high amounts of IFN-γ mRNA from day 13 through day 20 of pregnancy, and immunoreactive IFN-γ localizes to perinuclear membranes typically occupied by endoplasmic reticulum and Golgi apparatus as well as cytoplasmic vesicles within trophectoderm cells, suggesting trafficking and secretion of IFN-γ into the uterine lumen (28, 31). Interestingly, *SLA-1*, *SLA-2*, and *SLA-3*, but not *SLA-6* or *SLA-7*, were reported to respond to both IFN-γ and IFN-α in a pig kidney cell line (55). Although results of the present study are consistent with those of Tennant et al. (55) for *SLA-1*, *SLA-2*, and *SLA-3*, there are differences between in vivo and in vitro results for *SLA-6* and *SLA-7*. It is perhaps not surprising that stromal cells in vivo, which have distinct spatial relationships to other cell types and the extracellular matrix, respond differently to IFNs than do isolated and cultured kidney cells. Certainly macrophages are highly individualized in tissues, where their functions are a reflection of the systemic and local environment (56). Indeed, it is well established that uterine gene regulation is altered by differences in cell type or epithelial-stromal interactions (57).

Pig conceptuses are unique among mammalian species in that they secrete two IFNs during the peri-implantation period, providing the opportunity for IFN-γ and IFN-δ to work together to regulate endometrial gene expression. In general, both type I and type II IFNs can induce expression of SLA class I through the classical JAK-STAT cell signaling pathway leading to gamma-activation factor binding of gamma activation sequence elements and induction of gene transcription (58). In addition, IFN-δ signals through a similar, yet distinct, pathway leading to ISG factor 3 complex binding of IFN-stimulated response elements in the promoters of several ISGs to initiate transcription (59). Interactions between type I and type II IFNs have been demonstrated (60). Although the type I IFN-α and type II IFN-γ each induce expression of largely nonoverlapping sets of genes, they can also act in concert to produce synergistic interactions leading to mutual reinforcement of physiological responses (61). With cells that are normally relatively unresponsive to IFN-γ, sequential treatment with IFN-γ followed by IFN-α results in greater induction of ISGs (61). In addition, cotreatment with IFN-γ and IFN-α extends the period of ISG expression over IFN-α alone (62). For typical ISGs, type I IFNs induce rapid expression followed by a protein synthesis-dependent suppression of transcription within 6 h. IFN-γ overrides this suppression, allowing continuous expression of ISGs for over 24 h (62). Clearly, IFN-δ and IFN-γ may profoundly influence endometrial physiology through cooperative induction of cytokine-specific transcription factors that allow reinforcement of effects of distinct cell surface ligands, including SLA class I and β_2m genes while maintaining the specificities of the individual inducing IFNs to influence cell function.

Although it is well established that conceptus IFN-τ induces expression of numerous ISGs in the stroma and GE of ruminants (3), and that ISGs increase in the decidualizing stroma of humans and rodents (34, 35), the pregnancy-specific roles of uterine ISGs remain conjectural. Because IFN-γ secreted by pig conceptuses can initiate uterine vascular development (63), it is reasonable to hypothesize that conceptus-derived IFNs up-regulate ISGs such as SLA class I and β_2m genes to facilitate vascular changes necessary to support the developing conceptus. Recently, placental human HLA-G was implicated in regulation of angiogenesis during placental invasion and replacement of endothelial cells at the ends of uterine spiral arteries (64). Indeed, there is evidence that vascular development at the maternal-fetal interface is regulated by a balance of proangiogenic and antiangiogenic factors, and MHC and β_2m molecules may be significant players in this balance (64). Whether IFN-induced SLA class I and β_2m gene products in endometrial stratum compactum stroma of pigs are involved in angiogenesis during pregnancy remains to be determined; however, it is clear that expression of ISGs within the uterine stroma of mammals is a universal response to pregnancy.

Intriguingly, SLA class I and β_2m gene products decrease in endometrial LE as pregnancy progresses so that they are no longer detectable by day 20 of pregnancy. Although the mechanism involved in this down-regulation is unknown, IRF-2, a potent repressor and attenuator of ISG expression (65, 66), is induced in endometrial LE of pigs by estrogens (30). IRF-2 can also bind to the promoter of Ubp43, *Usp18*, a USP in mice used to mediate basal levels of expression. Ubp43 (*Usp18*), a ubiquitin deconjugating enzyme specific for the ubiquitin-like protein ISG15 (67), is up-regulated by type I IFNs (68) and inhibits type I IFN signaling by decreasing JAK1 phosphorylation (67). Although *Usp18* is present in both endometrial LE and the deep GE of cows (69), results of the present study localized USP gene only to LE between days 15 and 20 of pregnancy in pigs, which is coincident with temporal loss of SLA class I and β_2m mRNAs from LE. Given that IRF-2 and USP genes are expressed in the LE during pregnancy, both may play a role in down-regulating SLA class I and β_2m genes. It is generally accepted that SLA class I and β_2m molecules are decreased in the placenta to ensure that the conceptus semiallograft avoids host-vs-graft immune rejection (7–12).

In conclusion, results of the present study suggest that down-regulation of SLA class I and β_2m genes in uterine LE, in coordination with a lack of expression of these genes in placenta (12), may be important for preventing fetal allograft rejection in species

exhibiting epitheliochorial placentation. The temporal cell type-specific regulation of pig endomtrial SLA class I and β_2m by progesterone, IFN-γ, or IFN-δ and perhaps through permissive effects of conceptus estrogens, provides insight into how immune tolerance of the conceptus allograft is achieved. The traditional view, first proposed by Medawar (4, 5), was that the immune system must be circumvented to permit pregnancy. The results presented in this study provide additional evidence that supports reports from multiple investigators implicating the immune system as a dynamic and active player in the complex bidirectional endocrine, paracrine, autocrine, and juxtacrine interactions between uterus and placenta that uniquely define establishment and maintenance of a successful pregnancy.

Disclosures

The authors have no financial conflict of interest.

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