

The Sphingosine 1-Phosphate (S1P) Signaling Pathway Is Regulated During Pregnancy in Sheep¹

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ABSTRACT

Because sphingosine 1-phosphate (S1P) is a potent stimulator of angiogenesis, we hypothesized that the S1P pathway is activated to stimulate endometrial/placental angiogenesis during pregnancy. We initially localized S1P signaling pathway members in the gravid and nongravid uterine horns of unilaterally pregnant ewes. Sphingosine kinase-1 expression was greater in gravid compared to nongravid horns. In situ hybridization revealed elevated expression of sphingosine 1-phosphate phosphatase (*SGPP1*) in gravid interplacentomal endometrial stroma on Days 20 and 40 compared to the nongravid uterine horn, but expression increased in endometrium of the nongravid uterine horn between Days 40 and 120. *SGPP1* expression increased in placentomes late in gestation. Sphingosine 1-phosphate lyase mRNA was modestly expressed at Day 20 and then decreased. In contrast, sphingosine 1-phosphate receptor 1 (*S1PR1*) mRNA increased in endometrium and caruncular stroma of the gravid uterine horn. Treatment with FTY720 and VPC23019, S1P receptor antagonists, blocked human and ovine endothelial cell invasion using an in vitro model of sprouting angiogenesis. Knockdown of *S1PR1* with siRNA reduced invasion responses as well. We previously reported that delta-like 4 (*DLL4*) and A disintegrin and metalloproteinase with thrombospondin-like repeats 1 (*ADAMTS1*) participate in endothelial cell invasion stimulated by S1P and growth factors in vitro, and thus investigated whether their expression correlated with areas undergoing angiogenesis in vivo. *DLL4* expression was similar to *S1PR1*, while *ADAMTS1* mRNA was expressed by endometria of both nongravid and gravid horns, as well as conceptus and placentomes. These

results establish that S1P signaling pathway members and S1P- and growth factor-regulated genes are prominent in uterine and placental tissue and in some cases are correlated with areas undergoing angiogenesis. Thus, S1P signaling may be crucial for proper fetal-placental development.

ADAMTS1, *DLL4*, endometrium, endothelial, FTY720, S1P, S1P1, siRNA, siRNA gene silencing, sphingosine 1-phosphate, unilateral pregnancy model, uterus

INTRODUCTION

The mammalian uterus undergoes dynamic morphological changes to facilitate pregnancy. Considerable angiogenesis occurs in the uterine endometrium and placenta to ensure the transfer of nutrients from the maternal to the fetal circulation in all mammalian species. Ruminants have a cotyledonary placenta that provides for the conveyance of hematotropic nutrition from the uterus to the fetus primarily through placentomes, which are specialized structures formed through the interdigitation of fetal cotyledons and maternal caruncles [1]. These cotyledons are highly branched, villous, tree-like folds of fetal trophoblast that protrude into maternal endometrial caruncular crypts (aglandular endometrial areas). In sheep, a single layer of epithelial syncytial plaques, which are composed of both trophoblast and endometrium, separate extensive capillary networks within the placenta and uterus. By gestational Day 40 there is maximal juxtaposition of the endometrial and placental microvasculature [2]. Placentomes provide a critical source of hematotropic nutrition for the fetus because the maternal and fetal blood vessels are in very close proximity for exchanging oxygen and micronutrients [1]. Failure of placentome development results in death of the fetus [3]. Additional nutritional support for the fetus is provided by the interplacentomal endometrium, which contains multiple coiled and branched exocrine glands that secrete histotroph, which is a mixture of nutrients, adhesion molecules, enzymes, growth factors, and cytokines taken up by specialized structures of the chorioallantois called areolae [4, 5]. Striking angiogenesis occurs in both placentomes and interplacentomal endometrium. In the ovine interplacentomal endometrium, the capillary density doubles by Day 24 of gestation [6–8]. As gestation progresses, there is increased organization of endothelial cells and formation of new blood vessels throughout the uterus and placenta [9].

Several key signaling pathways mediate angiogenesis, including integrin cell surface receptors, transmembrane metalloproteinases, growth factors, lipids, and extracellular

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matrix proteins. The role of classical angiogenic growth factors, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (FGF2) in uterine angiogenesis during late gestation is well described [1, 9–14]. Additionally, there is much evidence that the lysosphingolipid sphingosine 1-phosphate (S1P) biochemical pathway influences various aspects of angiogenesis [15–22], but little is known about the role of S1P in angiogenesis during pregnancy.

Sphingosine 1-phosphate is generated following the phosphorylation of sphingosine by sphingosine kinase 1 (SPHK1) or sphingosine kinase 2 (SPHK2). Bioactive S1P can be recycled into sphingosine through the actions of sphingosine 1-phosphate phosphatase 1 (SGPP1) and sphingosine 1-phosphate phosphatase 2 (SGPP2) [15, 23]. S1P can also be irreversibly cleaved via sphingosine 1-phosphate lyase (SGPL) to generate ethanolamine phosphate and hexadecenal, which can exit the sphingolipid pathway [15, 23]. Bioactive S1P that has not been recycled or proteolytically cleaved to an inactive form is present in blood and tissues, where it can activate one or more of its five high-affinity G-protein-coupled receptors, S1PR1–S1PR5 [15]. Of the receptors, *S1PR1*, *S1PR2*, and *S1PR3* are expressed in murine endometrium following implantation, and expression correlates with embryonic angiogenesis during development [24, 25]. Further, combining S1P treatment with angiogenic growth factors stimulates endothelial cell invasion of three-dimensional (3-D) collagen matrices approximately 5- to 10-fold compared to either S1P or growth factors alone [22, 26]. These endothelial invasion responses are mediated through the known angiogenic molecules delta-like 4 (DLL4) and A disintegrin and metalloproteinase with thrombospondin-type repeats-1 (ADAMTS1) [22].

This study was designed to test our hypothesis that during pregnancy, S1P is produced locally in the uterus in response to the conceptus and acts via its receptors to coordinate and regulate the expression of critical genes that mediate angiogenesis and facilitate vascular support and maintenance of nutrient exchange at the fetal-maternal interface. The objectives of this study were to 1) investigate the precise temporal and spatial localization of members of the S1P signaling pathway in a unilaterally pregnant model, 2) investigate expression of key genes in response to treatment with a combination of S1P and angiogenic growth factors within placental and interplacental endometrium, and 3) mechanistically determine whether S1P acts via S1P receptors to regulate endothelial cell invasion into 3-D collagen matrices.

MATERIALS AND METHODS

Animals and Experimental Design

Experimental and surgical procedures involving animals in the present studies were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Unilateral Pregnant Ewes

Ewes (Texas A&M, College Station, TX) were checked daily for estrous behavior using vasectomized rams. Following at least one estrous cycle of normal duration, the ovary ipsilateral to the right uterine horn was removed, and a double ligature was placed at the base of the right uterine horn at the bifurcation. At the following estrus (Day 0), ewes were mated to intact rams to generate a nongravid and gravid uterine horn in each bred ewe [27]. Ewes were hysterectomized on either Day 20, 40, 80, or 120 of gestation ($n = 4$ ewes per day). Several sections (thickness ~ 1 – 1.5 cm) of placental (or caruncular) and interplacental (or intercaruncular) tissues from each uterine horn (nongravid and gravid) were placed in fresh 4% paraformaldehyde fixative for 24 h and then embedded in Paraplast Plus (Oxford Labware, St. Louis, MO).

Immunohistochemical Analysis

SPHK1 protein was localized by immunohistochemistry in paraffin-embedded ovine uterine tissues using rabbit anti-human polyclonal SPHK1 IgG (AB46719; Abcam, Cambridge, MA) at 2 μ g/ml and the Rabbit Super ABC kit (Biomedica, Foster City, CA). Antigen retrieval was conducted using boiling citrate. Replacement of primary antibody with nonimmune rabbit IgG (Sigma Chemical Co., St. Louis, MO) at the same final concentration was used as the negative control and diaminobenzidine tetrachloride (Sigma) was used as the chromagen, with all steps conducted according to methods as previously described [28].

In Situ Hybridization Analysis

SGPP1, *SGPL*, *S1PR1*, *DLL4*, and *ADAMTS1* mRNAs were localized in paraffin-embedded uterine tissue as previously described [27, 29]. Briefly, deparaffinized, rehydrated, and deproteinized uterine cross sections (~ 5 μ m; $n = 4$) were hybridized with radiolabeled antisense or sense ovine cRNA probes [27, 29, 30] generated from linearized plasmid DNA templates. Partial cDNAs for *SGPP1*, *SGPL*, *S1PR1*, *DLL4*, and *ADAMTS1* were generated by RT-PCR as described previously [30, 31]. Primers for each target gene were derived from conserved sequences of human and bovine genes using Primer 3 (Version 2.0.0-alpha; <http://primer3.sourceforge.net/>) [32]. Total RNA isolated from endometria and placentomes collected on Days 40, 80, and 120 served as template for S1PR1, and cDNA clones for *SGPP1*, *SGPL*, *DLL4*, and *ADAMTS1* were purchased from Open Biosystems (Huntsville, AL). Amplified PCR products were subcloned into the pCRII cloning vector using a T/A Cloning kit (Invitrogen, Carlsbad, CA) and sequenced in both directions using an ABI PRISM Dye Terminator Cycle Sequencing kit and an ABI PRISM automated DNA sequencer (Applied Biosystems, Foster City, CA) to confirm identity. Probes were synthesized by *in vitro* transcription with [α - 35 S] uridine 5-triphosphate (PerkinElmer Life Sciences, Wellesley, MA). After hybridization, washes, RNase A digestion, and autoradiography were performed using NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY). Slides were exposed at 4°C, developed in Kodak D-19 developer, counterstained with Harris modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated, and protected with cover slips.

Endothelial Cell Invasion in 3-D Collagen Matrices

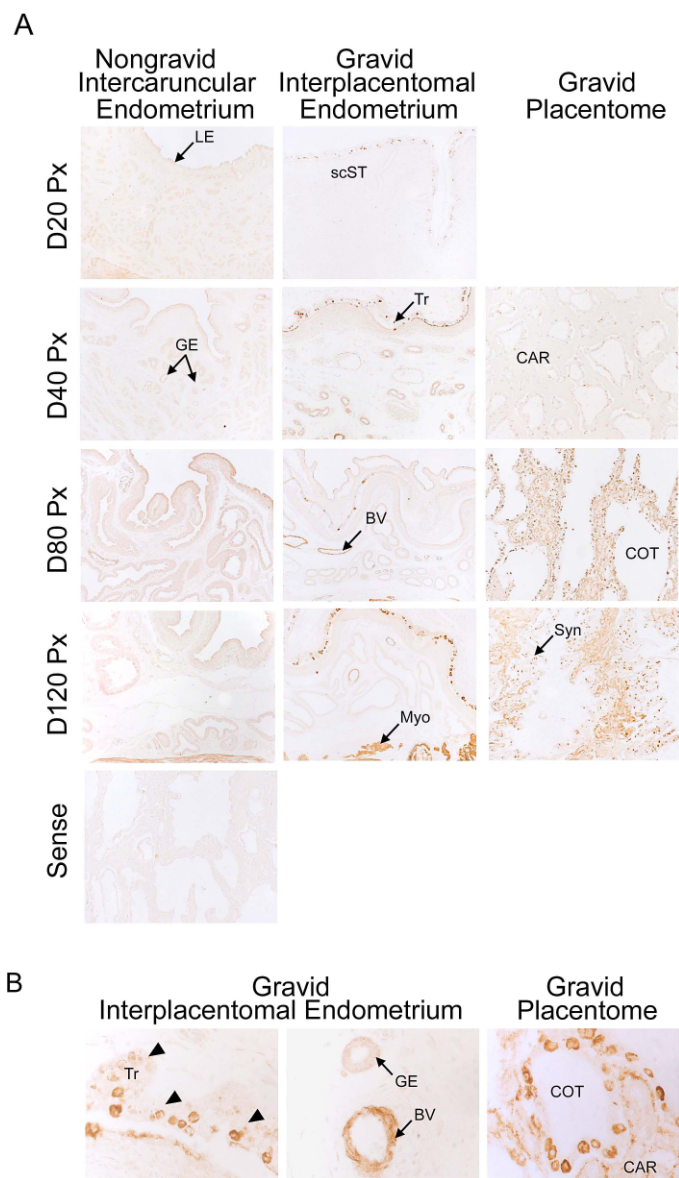
Primary human umbilical vein endothelial cells (HUVECs; Lonza Inc., Allendale, NJ) were grown and maintained as described [33]. Ovine uterine artery endothelial cells (oUAECs) were isolated and propagated as described [26]. Cell passages 3–6 were seeded on the upper surface of 3-D collagen matrices (2.5 mg/ml) containing 1 μ M S1P (Avanti Polar Lipids, Alabaster, AL). Cell monolayers were allowed to attach for 30 min prior to addition of M199 (HUVECs) or MEM (oUAECs) containing a reduced serum II supplement, VEGF (40 ng/ml), FGF2 (40 ng/ml), and ascorbic acid (50 μ g/ml) [22, 33].

Inhibition of Endothelial Invasion Using FTY720

Endothelial cells were seeded with indicated concentrations of FTY720 (0–50 nM; Husker Chemical, Shanghai, China) or vehicle control (dimethyl sulfoxide [DMSO], Sigma), and samples (three wells per treatment) were allowed to invade for 24 h, after which they were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min and stained with 1.09 μ M DAPI (Molecular Probes, Carlsbad, CA). Eyepieces mounted with a reticle (displaying a 10×10 grid that covers an area of 1 mm^2 at $10\times$ magnification) were used for quantifying average numbers of invading cells per standardized field [22, 33]. For each data set, three separate fields for each treatment were recorded and averaged.

siRNA Transfection

siGENOME SMARTpool human *S1PR1* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) control siRNAs were obtained from Dharmcon (Lafayette, CO). Endothelial cells were seeded into 25-cm 2 flasks at 40%–50% confluency. The following day, cells were washed three times with serum- and antibiotic-free Dulbecco modified Eagle medium (DMEM) and transfected with 200 nM siRNA in 2.7 ml antibiotic-free DMEM using 20 μ l siPORT Amine (Ambion, Austin, TX). Flasks were aspirated and supplemented with antibiotic-free growth medium at 8 h posttransfection. This transfection procedure was repeated 2 days after the first transfection. After the second transfection, cells were allowed to recover prior to testing in invasion assays. Lysates were collected by placing collagen matrices in boiling sample buffer for Western blot



(Leeds Instruments Inc., Irving, TX). Photographic plates were assembled using Adobe Photoshop.

RESULTS

Temporal and Spatial Localization of S1P Signaling Pathway Members in the Uterus and Placenta During Pregnancy Supports a Role for S1P in Mediating Angiogenesis

Immunohistochemistry localized SPHK1, an enzyme that phosphorylates sphingosine to SIP, to both uterine and placental tissues (Fig. 1). In interplacental endometrium, basal protein expression was low in nongravid uterine horns on all days of pregnancy. In contrast, SPHK1 was detected in blood vessels within the endometrium of gravid uterine horns by Day 80 and maintained through Day 120 of pregnancy (Fig. 1A). Higher magnification images allow for more detailed evaluation of SPHK1 expression within specific tissues. SPHK1 protein expression was highly upregulated in trophoblasts, uterine vasculature, cells within syncytia, and throughout caruncular tissue of the placentome (Fig. 1B). Additionally, the number of SPHK1-positive cells and the intensity of immunostaining increased from Days 20 through 120 of gestation in gravid tissues (Fig. 1A). In placentomes, SPHK1 was present in the epithelial syncytium from Days 20 through 120 of pregnancy. Interestingly, a dramatic increase of SPHK1 protein expression was observed in caruncular tissue between Days 40 and 80 of pregnancy, and high expression was maintained through Day 120 (Fig. 1A). SPHK1 was also detected in the myometrium of both gravid and nongravid uterine horns, with expression being greater in the gravid uterine horn (Fig. 1A).

In situ hybridization was used to localize mRNAs for both *SGPPI*, the phosphatase that converts S1P to sphingosine, and *SGPL*, which is responsible for the irreversible cleavage of S1P and the exit from the sphingolipid cell signaling pathway. We did not observe widespread and robust hybridization; rather, expression of *SGPPI* mRNA was selectively detectable in unidentified cells scattered within uterine stroma of both nongravid and gravid uterine horns (Fig. 2). In endometria of nongravid uterine horns, *SGPPI*-positive cells were initially detected on Day 40 and remained present through Day 120 of pregnancy (Fig. 2). In contrast, *SGPPI*-positive cells were prominent in the stratum compactum stroma (scST) directly underlying the conceptus on Day 20, became more sparsely distributed throughout the interplacental endometrium on Day 40, and were absent on Days 80 and 120 of pregnancy (Fig. 2). In the placentomes, *SGPPI* was detected in only a few cells within the caruncular stroma on Days 80 and 120 of pregnancy (Fig. 2).

Under the conditions tested, *SGPL* mRNA expression was undetectable in nongravid uterine horns (Fig. 3). However in gravid uterine horns, a pattern of expression similar to *SGPPI*, albeit to a lesser extent, was observed. *SGPL* mRNA localized to a population of cells concentrated in the scST adjacent to attached trophoblasts on Day 20 of pregnancy, then decreased and became more dispersed from Days 40 to 120 of pregnancy (Fig. 3). Interestingly, as pregnancy progressed, cells expressing *SGPL* became less numerous in endometria and more numerous in myometria so that expression levels were high in the myometrium during late pregnancy (Fig. 4, right panel). An increase in *SGPL* mRNA expression levels over basal levels was not detectable in placentomes or trophoblasts on any day of pregnancy studied.

Multicapture composite images of Day 80 placentomes, the highly vascularized structures responsible for hematotropic support of the conceptus, demonstrated cell type-specific

FIG. 1. Immunohistochemical localization of SPHK1 in sections of nonpregnant and pregnant uteri collected from unilaterally pregnant ewes on Days 20, 40, 80, and 120 of gestation (A) and Day 80 of gestation (B). Rabbit IgG applied to a Day 80 placentome served as a negative control. GE, uterine glandular epithelium; Tr, placental trophoblast/chorion; BV, blood vessels; Myo, myometrium; CAR, caruncular tissue; COT, cotyledonary tissue; scST, endometrial stratum compactum stroma; Syn, epithelial syncytia of the placentome. Arrowheads indicate placental trophoblasts in (B). Representative images are shown from independent samples (n = 4). Width of each field is 940 μ m (A) and 220 μ m (B).

analysis using anti-human polyclonal antisera directed to S1PR1 (OPA1-15004; Affinity Bioreagents, Golden, CO) and verification of gene knockdown as previously described [22].

Photomicrography

Digital photomicrographs of in situ hybridization (bright field and dark field images) and immunohistochemical staining were evaluated using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axiocam HR digital camera and Axiovision 4.1 software (Carl Zeiss). Multicapture composite images were collected sequentially as individual overlapping frames that were subsequently assembled using Adobe Photoshop (version 6.0; Adobe Systems Inc., San Jose, CA). Digital photographs of HUVEC invasion assays were captured using an Olympus CKX41 microscope

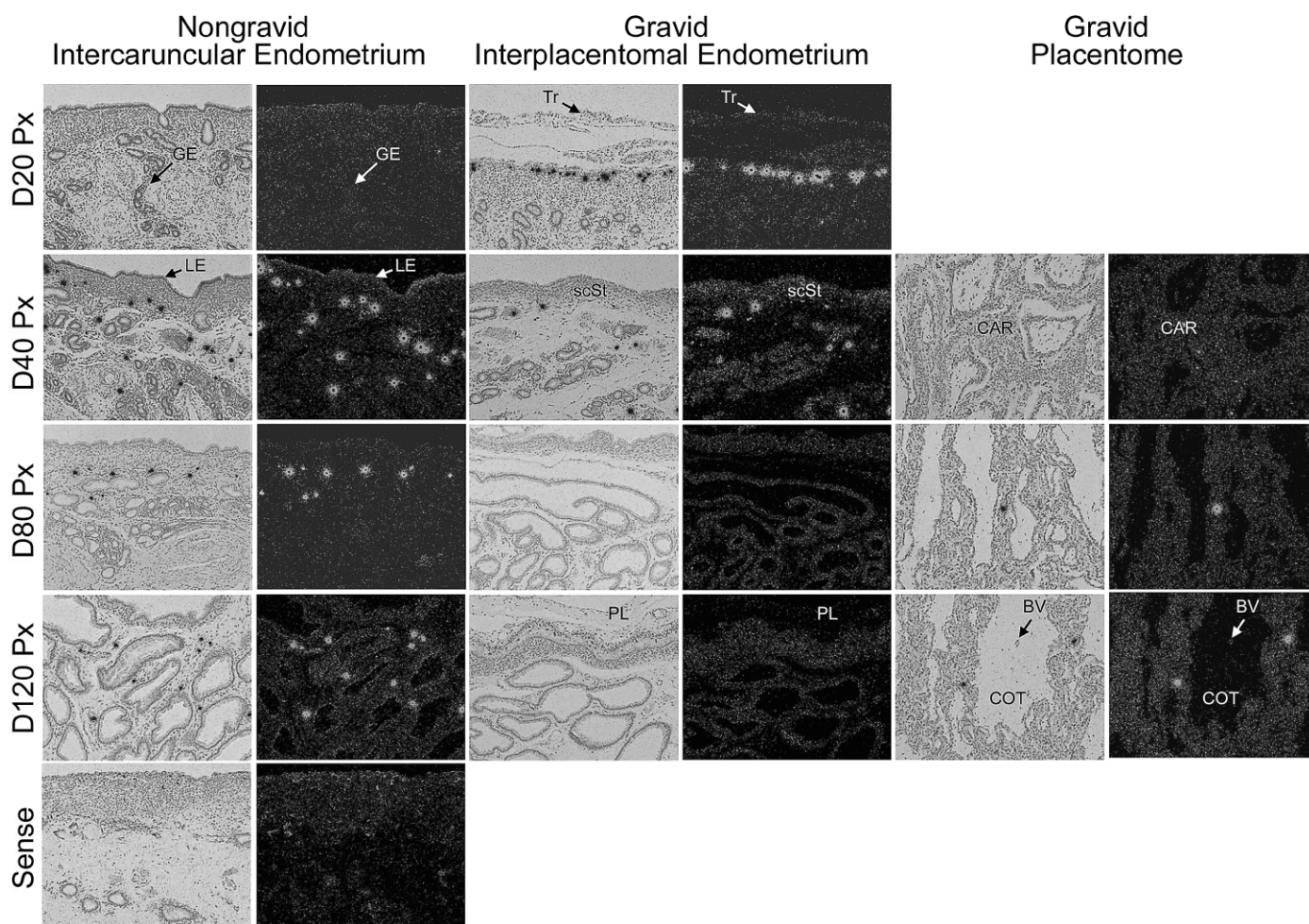


FIG. 2. In situ hybridization analysis of *SGPP1* mRNA in sections of nongravid and gravid uteri collected from unilaterally pregnant ewes on Days 20, 40, 80, and 120 of gestation. Corresponding bright field and dark field images of representative cross sections are shown. A section from Day 80 nongravid intercaruncular endometrium hybridized with radiolabeled sense cRNA probe served as a negative control. LE, endometrial luminal epithelium; GE, endometrial glandular epithelium; scSt, endometrial stratum compactum stroma; Tr, placental trophoderm; PL, placental chorion; CAR, caruncular tissue; COT, cotyledonary tissue. Representative images are shown from independent samples ($n = 4$). Width of each field is 940 μm .

expression patterns for SPHK, *SGPP1*, and *SGPL* (Fig. 4). Immunoreactive SPHK1 was detectable throughout the epithelial syncytia and caruncular stroma, with particularly high expression in blood vessels and trophoderm of the interplacentomal endometria. In situ hybridization revealed mRNA expression of the phosphatase *SGPP1*, which was detected intermittently in caruncular stroma. The lyase, *SGPL*, was abundant in the myometrium, but otherwise not significant in caruncular and cotyledonary tissue (Fig. 4). Collectively, these results suggest that immunoreactive SPHK1 is abundantly expressed in placentomes, where it may serve to phosphorylate sphingosine and form S1P, whereas phosphatase or lyase mRNA expression levels are low to undetectable under the conditions used. These data suggest that increased levels of bioactive S1P may be available to promote angiogenesis within placentomes.

S1PR1 Is Expressed in Vascularized Regions of the Pregnant Uterus and Placenta, and Inhibition of *S1PR1* Blocks Endothelial Cell Sprouting Responses

S1P signaling is mediated through S1PR1, S1PR2, S1PR3, S1PR4, and S1PR5 receptors. Because transgenic mice lacking S1P1 fail to form a functional vasculature [34], we investigated

the pattern of *S1PR1* expression in unilaterally pregnant ewes. In situ hybridization revealed that *S1PR1* mRNA was present in both the nongravid and gravid uterine horns (Fig. 5). In the stroma of the nongravid uterine horn, *S1PR1* levels were highest on Day 40 and then steadily declined throughout pregnancy. In interplacentomal regions of gravid uterine horns, *S1PR1* mRNA was expressed within the scST, glandular epithelium (GE), and conceptus trophoderm. On Day 20, *S1PR1* mRNA expression was detectable in scattered cells within scST. As pregnancy progressed, this expression pattern waned; however, there was a marked increase in *S1PR1* mRNA in all cell types at the fetal-maternal interface (trophoderm and scST) and within the uterine glands at Day 80 (Fig. 5). Within placentomes, *S1PR1* mRNA was localized to cotyledonary blood vessels as well as small terminal vessels of the maternal caruncle underlying the epithelial syncytia at Day 80 in composite images (Fig. 5, composite image). Expression in blood vessels of placentomes was highest at Day 40 and then decreased gradually through Day 120 of gestation (data not shown).

To demonstrate that S1P functionally stimulates angiogenic sprouting through the S1PR1, we utilized a previously described assay [22, 33] in which primary endothelial cells form sprouting structures and invade into 3-D collagen

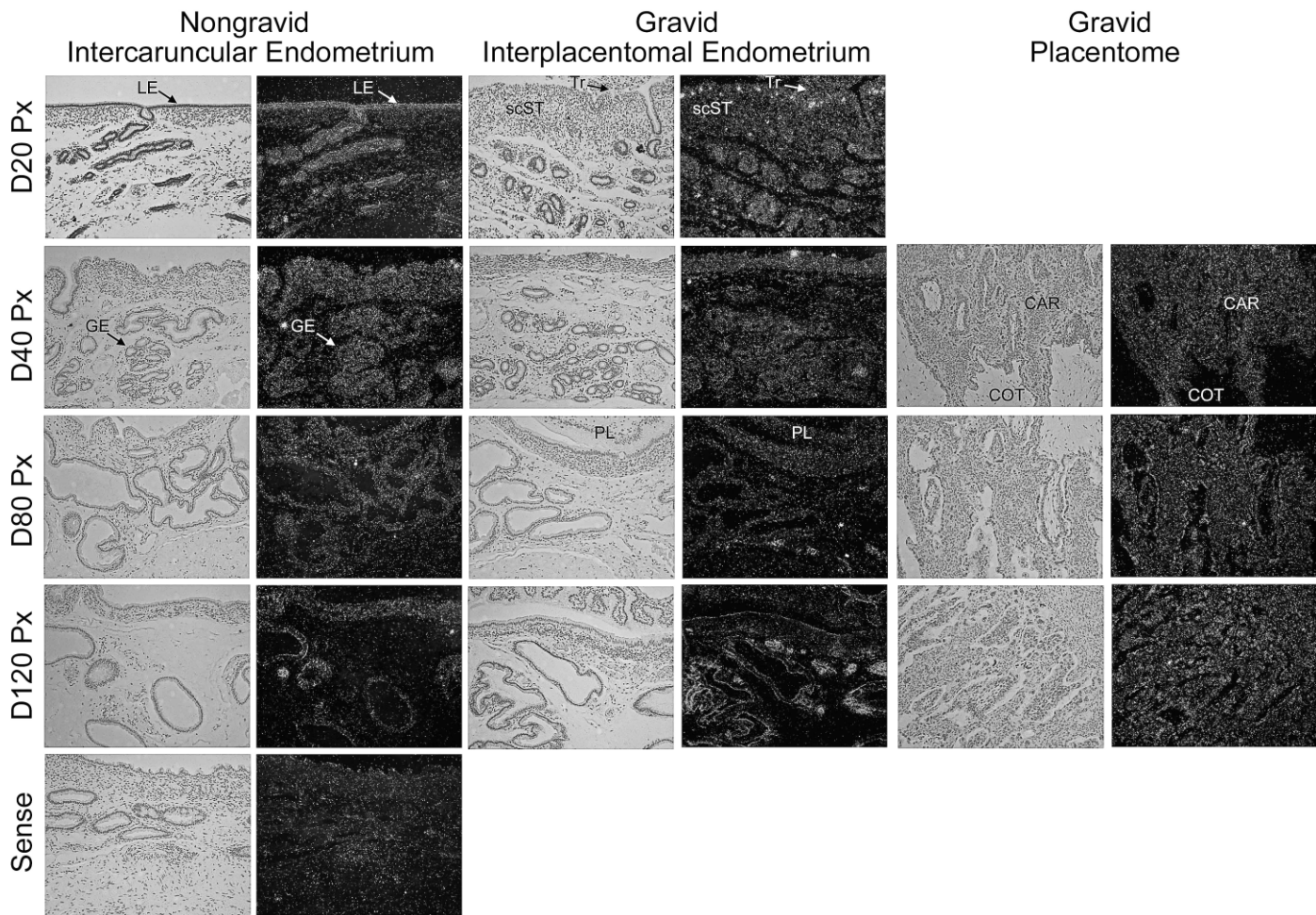


FIG. 3. In situ hybridization analysis of *SCPL* mRNA in sections of nongravid and gravid uteri collected from unilaterally pregnant ewes on Days 20, 40, 80, and 120 of gestation. Corresponding bright field and dark field images of representative cross sections are shown. A section from Day 80 nongravid intercaruncular endometrium hybridized with radiolabeled sense cRNA probe served as a negative control. LE, endometrial luminal epithelium; GE, endometrial glandular epithelium; scST, endometrial stratum compactum stroma; Tr, placental trophoblast; PL, placental chorion; CAR, caruncular tissue; COT, cotyledonary tissue. Representative images are shown from independent samples ($n = 4$). Width of each field is 940 μm .

matrices in response to combined treatment with 1 μM S1P, 40 ng/ml of angiogenic growth factors, VEGF and FGF2, and 50 $\mu\text{g/ml}$ of ascorbic acid. Initial experiments tested the effects of FTY720 and the S1PR1, S1PR2, and S1PR5 antagonist [35]. FTY720 decreased S1P and growth factor-stimulated HUVEC invasion dose-dependently, whereas DMSO, the vehicle control, had no effect on invasion (Fig. 6A). Further, a role for S1PR1 in the 3-D sprouting response was conducted using VPC23019, a functional S1PR1 and S1PR3 antagonist. Like FTY720, VPC23019 similarly interfered with S1P and growth factor-induced invasion of HUVEC in 3-D collagen matrices (Fig. 6B). RT-PCR analyses to determine expression of S1P receptors in HUVECs were carried out and revealed expression of *S1PR1* and *S1PR3* (Fig. 6C). A similar expression pattern was seen in oUAECs (Fig. 6D). These data demonstrate the S1P receptor expression profile is comparable in primary HUVEC and ovine endothelial cell lines. The expression of S1PR1 was confirmed in HUVECs and oUAECs, but we were unable to successfully demonstrate expression of S1PR3 by Western blot analyses (data not shown). Further, invasion of oUAECs stimulated by S1P and growth factors was inhibited by comparable doses of FTY720 and VPC23019 (Fig. 6E), demonstrating that HUVECs and oUAECs 1) invade similarly,

2) exhibit a similar S1P receptor expression profile, and 3) are sensitive to FTY720 and VPC23019.

To demonstrate a functional requirement for S1PR1, HUVECs were treated with small interfering RNA (siRNA) directed to human *S1PR1* and *GAPDH* control. Specificity of the siRNAs for S1PR1 was determined using Western blotting, which revealed a successful, albeit partial, knockdown of S1PR1 in extracts isolated from invading cultures (Fig. 7A). Although S1PR1 protein expression was not completely silenced by siRNA knockdown, invasion responses at 24 h were decreased ($P < 0.05$) compared to GAPDH control (Fig. 7, B and C). Thus, this in vitro model confirms a functional requirement for S1PR1 in endothelial sprouting responses that is consistent with the expression of S1PR1 and SPHK1 at areas of new blood vessel growth in the uterus and placenta during pregnancy.

The S1P- and Growth Factor-Regulated Genes, DLL4 and ADAMTS1, Display Different Patterns of Expression in the Uterus and Placenta, Which Suggests Complementary Roles in Support of Pregnancy

Previously, we demonstrated in vitro that *DLL4* and *ADAMTS1* expression is regulated at the mRNA level by

S1P and angiogenic growth factors. Further, both *DLL4* and *ADAMTS1* were required for HUVEC sprouting responses in 3-D collagen matrices [22]. Based on these results, we used in situ hybridization to investigate whether *DLL4* and *ADAMTS1* mRNA were regulated in ovine uteri and placentae during pregnancy and whether the expression correlated with angiogenesis. *DLL4* is a receptor for the Notch signaling pathway and a known marker of newly sprouting endothelial cells [36, 37]. In interplacentomal endometria, *DLL4* mRNA expression was low to nondetectable in the nongravid uterine horn on all days of pregnancy (Fig. 8, left panels). In contrast, *DLL4* mRNA was specifically expressed by a discrete population of cells in the scST of the gravid interplacentomal endometrium on Days 20 and 40 of gestation. By gestational Day 80, *DLL4* mRNA was detectable throughout the fetal-maternal interface (trophoblast, luminal epithelia [LE], and scST), and there was robust expression by GE on Days 80 and 120 (Fig. 8, center panels). Within the placentome, *DLL4* mRNA was expressed specifically in the small terminal vessels of the maternal caruncular stroma underlying the epithelial syncytia that lines the caruncular crypts of the placentome (Fig. 8, right panels).

In situ hybridization analysis revealed that *ADAMTS1* mRNA is expressed in a number of uterine and placental cell types of both nongravid and gravid uterine horns (Fig. 9). In endometria of nongravid uterine horns, *ADAMTS1* mRNA was expressed by LE, stroma, and superficial GE on Day 20 of gestation. Moderate expression of *ADAMTS1* was maintained in the nongravid LE and stroma, while mRNA in the GE increased to maximum levels by Day 120 of pregnancy (Fig. 9). In the interplacentomal endometria of the gravid uterine horn, *ADAMTS1* mRNA was expressed by conceptus trophoblast/chorion, as well as endometrial LE, scST, and GE on Day 20 of gestation. Expression in all of these cell types increased steadily to very high levels through Day 120 of pregnancy (Fig. 9). There was also abundant *ADAMTS1* mRNA expressed within placentomes in the cotyledonary vasculature, the caruncular stroma, and the epithelial syncytia (Fig. 9).

Multicapture composite images of Day 80 placentomes, the highly vascularized structures responsible for hematotropic support of the conceptus, revealed distinct expression patterns for *DLL4* and *ADAMTS1* (Fig. 10). Significantly, *DLL4* mRNA was expressed throughout the placentome and was localized to cotyledonary blood vessels and small terminal vessels of the maternal caruncle underlying the epithelial syncytia of the placentome. This closely resembled *S1PR1* mRNA expression (Fig. 5). Separate evaluation of an entire Day 80 placentome revealed that *ADAMTS1* mRNA expression was most abundant in the chorioallantois and proximal edges of the cotyledonary villi lining distal portions of the caruncular crypts (Fig. 10). Levels of *ADAMTS1* decreased in the syncytia near the base of the caruncular crypts, and expression was lost upon reaching the placental capsule (Fig. 10). Thus, the expression of these S1P- and growth factor-regulated genes at key sites within the developing placentome overlaps with angiogenesis in some cases and suggests S1P might synergize with growth factors or other stimuli to support growth and development of the conceptus during pregnancy.

DISCUSSION

Angiogenesis within the pregnant uterus occurs rapidly and is critical to provide hematotropic support for fetal-placental development [6, 7, 9]. Insufficient endometrial/placental vascularization can compromise pregnancy in pathological

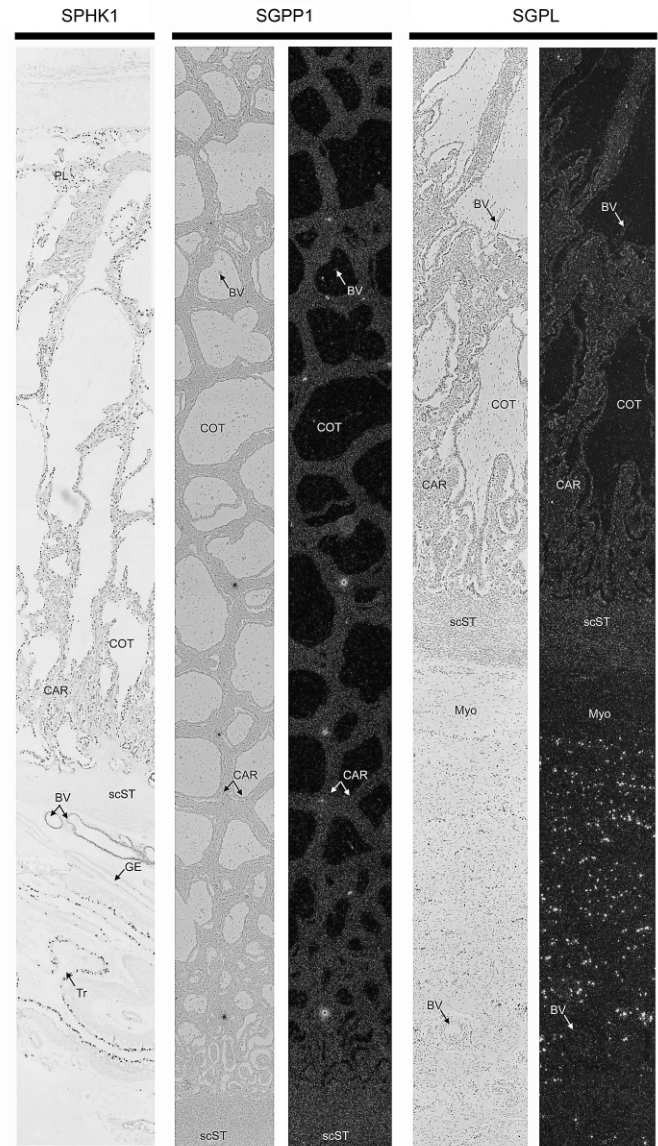


FIG. 4. Composite images of immunohistochemical localization of SPHK1 protein and in situ hybridization analyses of *SGPP1* as well as *SGPL* mRNAs within placentomes from Day 80 of pregnancy. GE, endometrial glandular epithelium; scST, endometrial stratum compactum stroma; BV, blood vessel; CAR, caruncular tissue; COT, cotyledonary tissue; Myo, myometrium. Representative images are shown from independent samples ($n = 4$). Width of each field is 940 μm .

situations such as preeclampsia. Considerable attention has been paid to regulation of uterine/placental angiogenesis by classical angiogenic factors such as VEGF and FGF. In addition, members of the S1P signaling pathway are expressed within mammalian uteri [24, 25], and we recently reported that S1P is a potent angiogenic factor that stimulates endothelial cell invasion and sprouting within 3-D collagen matrices [22]. Results of the present study include a comprehensive examination of S1P signaling pathway members in the ovine uterus and placenta throughout gestation in a unilaterally pregnant model and establish the ability to determine the molecular mechanism(s) by which S1P affects fetal-placental development. Our results clearly indicate that 1) robust expression of SPHK and moderate levels of *SGPP1* and *SGPL*

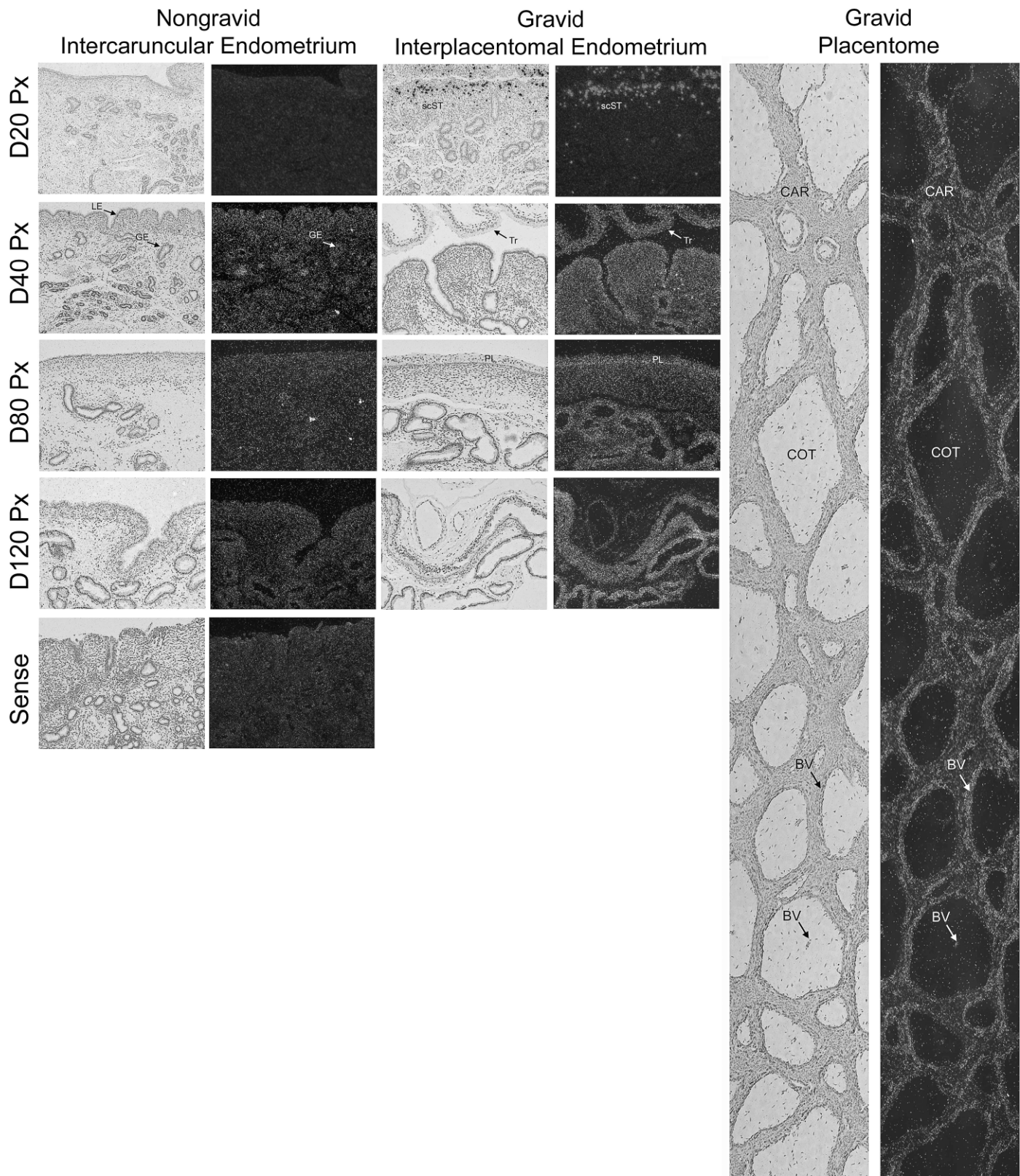


FIG. 5. In situ hybridization analysis of *STPR1* mRNA in sections of nongravid and gravid uteri collected from unilaterally pregnant ewes on Days 20, 40, 80, and 120 of gestation. A composite image of a Day 80 placentome is shown (right panel). Corresponding bright field and dark field images of representative endometrial cross sections are shown. A section from Day 80 nongravid interarcular endometrium hybridized with radiolabeled sense cRNA probe served as a negative control. LE, endometrial luminal epithelium; GE, endometrial glandular epithelium; scST, endometrial stratum compactum stroma; Tr, placental trophoderm; PL, placental chorion; CAR, caruncular tissue; COT, cotyledonary tissue; BV, blood vessel. Representative images are shown from independent samples ($n = 4$). Width of each field is $940 \mu\text{m}$.

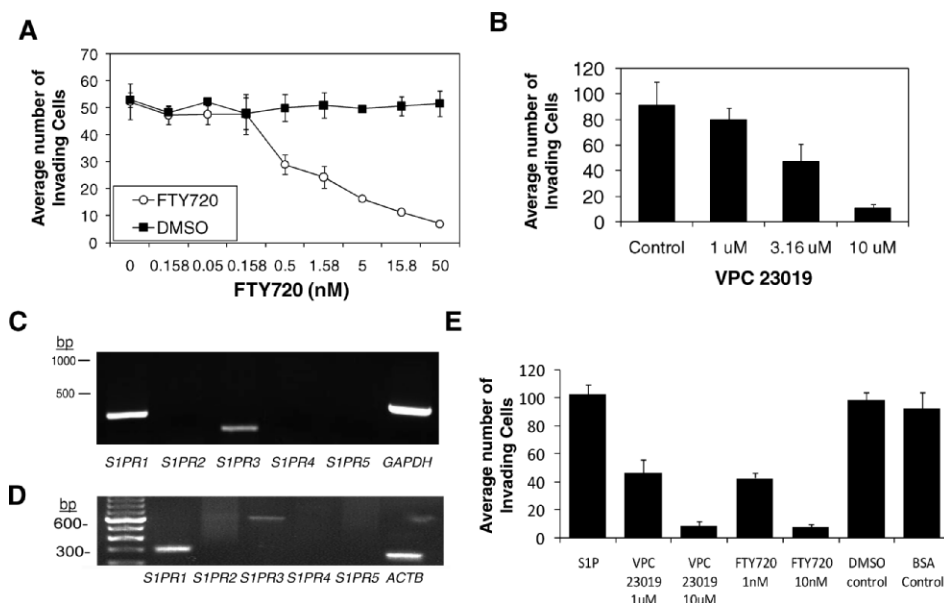


FIG. 6. S1P1 receptor signaling is required for human and ovine endothelial cell sprouting responses in 3-D collagen matrices. In all experiments, endothelial invasion was stimulated with 1 μ M S1P, 40 ng/ml VEGF, and 40 ng/ml FGF2. **A**) FTY720, an S1P receptor antagonist, blocked HUVEC invasion in a dose-dependent manner. **B**) HUVEC invasion was conducted with increasing doses of the S1PR1 and S1PR3 antagonist VPC23019. RT-PCR reactions using primer sets designed for human (**C**) and ovine (**D**) S1PR1–S1PR5 sequences using HUVEC and oUAEc cDNA as a template, respectively. Also pictured are GAPDH and beta actin (ACTB) positive controls. Primer sequences are available upon request. **E**) oUAEc invasion cultures were established in the presence of S1P in combination, with doses of FTY720 and VPC23019 indicated. DMSO and bovine serum albumin served as vehicle controls for FTY720 and VPC23019, respectively. Dose-responses were conducted four times, and duplicate experiments were performed on endothelial cell lines isolated from separate individuals.

are associated with uterine and placental angiogenesis; 2) S1PR1 is present in endometrial and placental tissues undergoing angiogenesis, and antagonism of S1PR1 blocks S1P and growth factor-induced invasion responses in human and ovine endothelial cells; and 3) S1P- and angiogenic growth factor-regulated genes, *DLL4* and *ADAMTS1*, are expressed in a cell-specific manner to optimally influence angiogenic events at the uterine-placental interface.

The present study utilized the unilaterally pregnant ewe to assess effects of the conceptus on regulation of S1P signaling pathway to better understand the role of S1P in mediating angiogenesis during pregnancy. The unilaterally pregnant model provides two distinct environments for assessment of angiogenic changes. The nonpregnant uterine horn does not form placentomes and lacks a placenta and fetus. Because the placenta exerts profound physical and paracrine/endocrine effects at the uterine-placental interface, including effects on angiogenesis, the nonpregnant uterine horn exhibits only moderate angiogenesis in response to maternal factors during

pregnancy. In contrast, the gravid uterine horn exhibits the full angiogenic response to paracrine and endocrine factors of a normal pregnancy. This study is the first to use a unilaterally pregnant animal model for investigation of pregnancy-associated regulation of S1P signaling pathway members. It is important to note that the majority of genes investigated in this study, including *SPHK*, *S1PR1*, *DLL4*, and *ADAMTS1*, were expressed at higher levels in the gravid compared to nonpregnant uterine horn of unilaterally pregnant ewes. Our data are consistent with Skaznik-Wikiel et al. [25], who demonstrated specific upregulation of S1PR1 by the embryo using an oil-induced decidualoma model.

The enzymes SPHK1 and SPHK2 are necessary for the generation of S1P. Results of the present study strongly support a role for SPHK1 in uterine/placental angiogenesis. SPHK1 expression was detected in the trophoblast, raising the possibility that S1P could be produced locally and secreted towards the interplacental endometrium to support a doubling of the vasculature during pregnancy. More important-

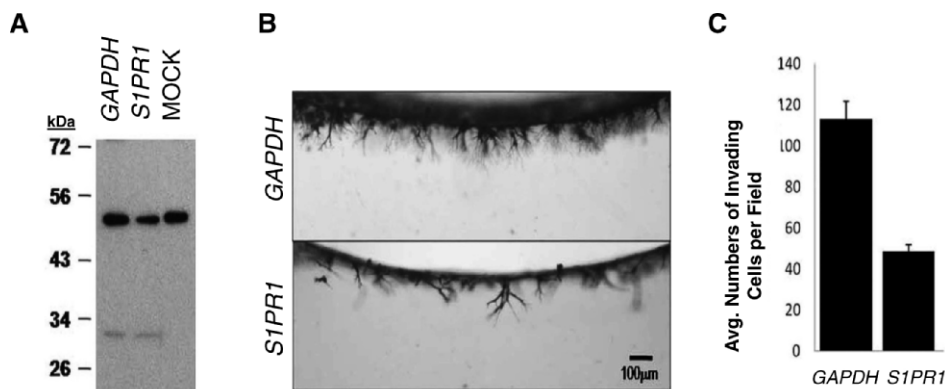


FIG. 7. Silencing of *S1PR1* using custom-pooled siRNA significantly reduced HUVEC invasion. **A**) Extracts taken from 3-D invading cultures (16 h) were probed with antisera directed to S1P1 in Western Blot analysis. **B**) Invading cultures were fixed, stained, and photographed (side-view) at 24 h. **C**) Invasion density was quantified by counting the average numbers of invading cells per standardized field ($n = 3$ fields; mean \pm SEM). $P < 0.05$. Results are representative of duplicate experiments.

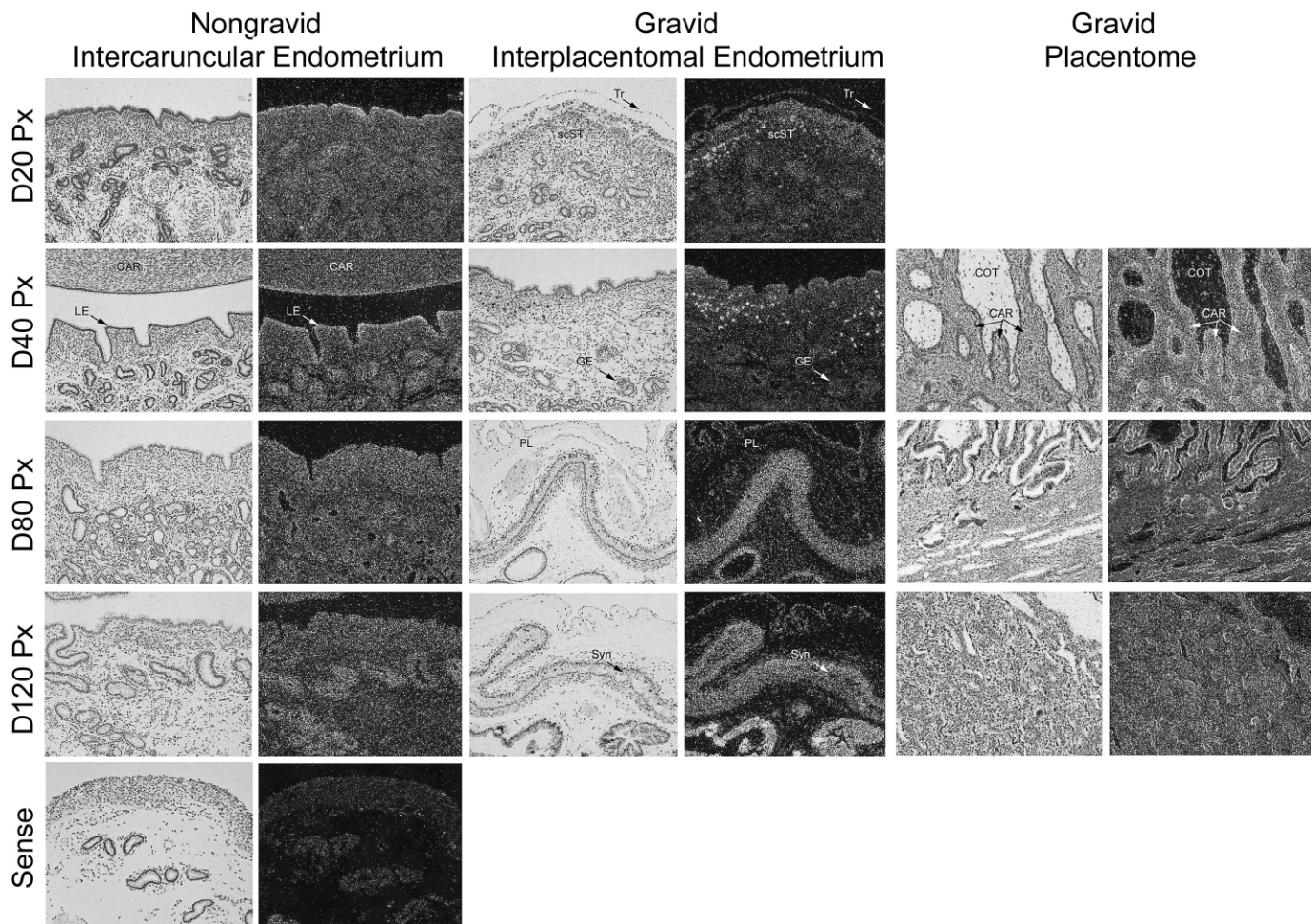


FIG. 8. In situ hybridization analysis of *DLL4* mRNA in sections of nonpregnant and pregnant uteri collected from unilaterally pregnant ewes on Days 20, 40, 80, and 120 of gestation. Corresponding bright field and dark field images of representative endometrial cross sections are shown. A section from Day 80 nonpregnant intercaruncular endometrium hybridized with radiolabeled sense cRNA probe served as a negative control. LE, endometrial luminal epithelium; GE, endometrial glandular epithelium; scST, endometrial stratum compactum stroma; Tr, placental trophoblast; PL, placental chorion; CAR, caruncular tissue; COT, cotyledonary tissue; Syn, epithelial syncytia of the placentome. Representative images are shown from independent samples ($n = 4$). Width of each field is 940 μm .

ly, SPHK was highly expressed in the placentomes that provide the major vascular network for high throughput transfer of molecules from the mother to fetus. SPHK was present in the epithelial syncytium that directly separates the terminal tips of uterine and placental capillaries. In addition, SPHK increased dramatically in the stroma of caruncular (endometrial) tissue, where a logarithmic increase in angiogenesis occurs during pregnancy. Our results further support that SPHK is important for fetal development. In the rat uterus, *Sphk1* is localized to the GE, vasculature, and myometrium [38] and, in a pattern similar to that for sheep, steadily increases throughout gestation. In the mouse, *Sphk1* mRNA is elevated in the decidua (an structure analogous to the sheep placentome) compared to the interimplantation sites [24]. Decidua of pregnant *Sphk1* knockout mice exhibit an abnormal accumulation of dihydrosphingosine and sphingosine, and decidual blood vessels degenerate in proximity to the embryo [24]. Interestingly, inside-out signaling of S1P can be induced by receptor tyrosine kinase activation by various growth factors (e.g., VEGF, transforming growth factor- β , and insulin-like growth factor). This leads to SPHK activation and translocation to the membrane, which induces local production of S1P to stimulate G protein-coupled receptors [39]. Collectively, our work and that of others suggest that SPHK1 is critical in maintaining the uterine environment that

may signal in concert with various growth factor receptor pathways during pregnancy.

Although expression is comparable in the nonpregnant and gravid uterine horns of ewes, both *SGPPI* and *SGPL* were abundantly expressed by cells located intermittently within the stratum compactum stroma, just beneath the basal lamina of the endometrial LE on Day 20 of pregnancy, which overlaps with the attachment phase of implantation in sheep. *SGPPI* recycles S1P into sphingosine; conversely, *SGPL* irreversibly cleaves S1P to exit the lysosphingolipid pathway. One interpretation of this finding is that removal of bioactive S1P is necessary for conceptus implantation. Consistent with this possibility, localization of *SGPPI* within the scST beneath the LE at the site of conceptus attachment would allow S1P produced in response to elevated SPHK1 secreted by the trophoblast to be efficiently recycled. Further, relatively lower *SGPPI* expression levels in gravid versus nonpregnant tissue suggest increased availability of S1P occurs in gravid tissue as pregnancy proceeds. Similar to *SGPPI*, *SGPL* mRNA levels declined in the gravid endometrium and became restricted to the myometrium and supporting serosa. Placentomes exhibited high levels of SPHK1, which phosphorylates sphingosine to form S1P, whereas low-level increases of *SGPPI* and *SGPL* were detectable within placentomes as pregnancy progressed.

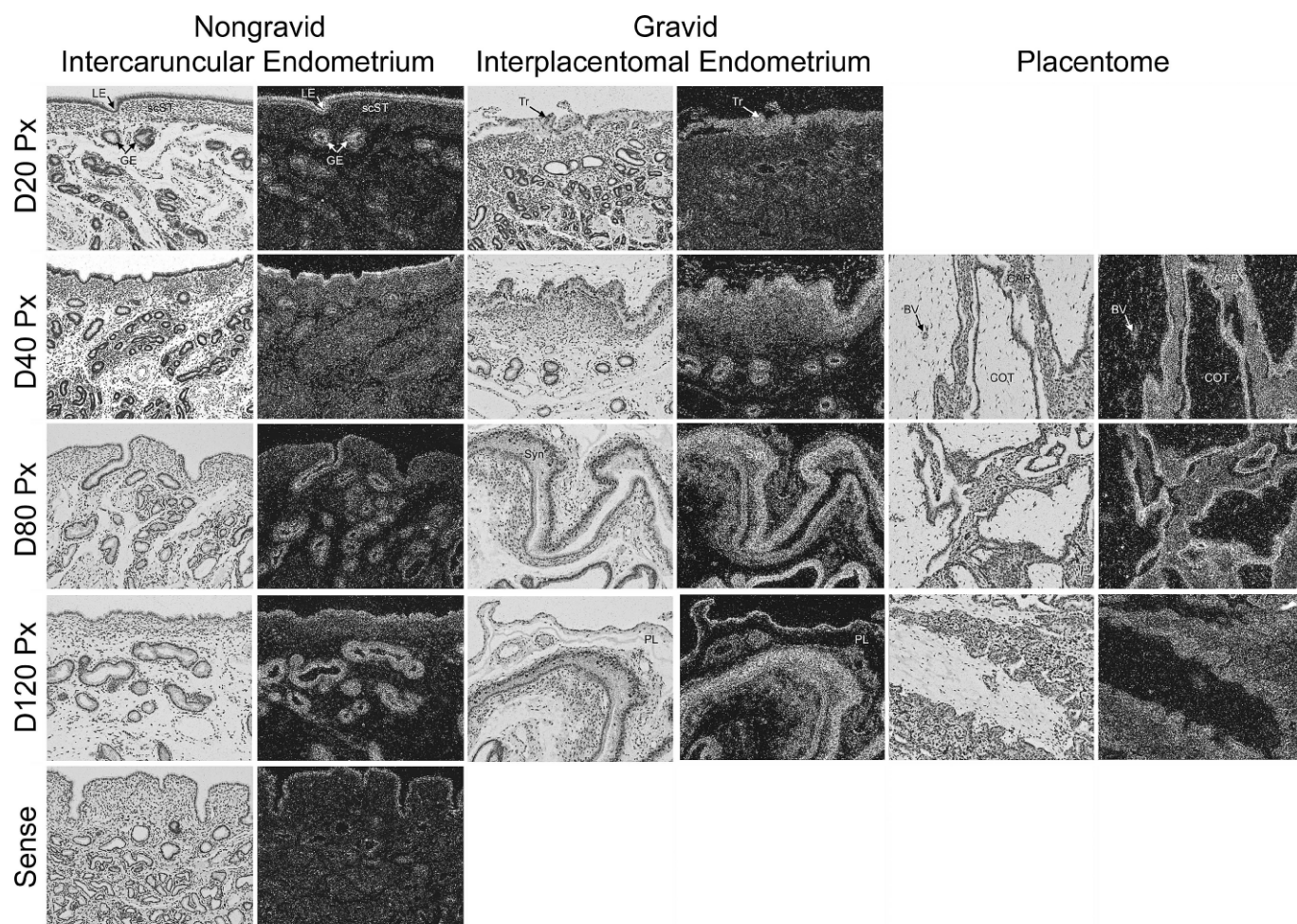


FIG. 9. In situ hybridization analysis of *ADAMTS1* mRNA in sections of nonpregnant and pregnant uteri collected from unilaterally pregnant ewes on Days 20, 40, 80, and 120 of gestation. Corresponding bright field and dark field images of representative endometrial cross sections are shown. A section from Day 80 nonpregnant intercaruncular endometrium hybridized with radiolabeled sense cRNA probe served as a negative control. LE, endometrial luminal epithelium; GE, endometrial glandular epithelium; scST, endometrial stratum compactum stroma; Tr, placental trophoblast; BV, blood vessel; CAR, caruncular tissue; COT, cotyledonary tissue; PL, placental chorion; Syn, epithelial syncytia of the placentome. Representative images are shown from independent samples ($n = 4$). Width of each field is 940 μm .

The net result may be high levels of S1P that stimulate extensive angiogenesis within the placentome. However, we must cautiously interpret these findings because protein expression is not a reliable indicator of enzymatic activity, and mRNA may not accurately reflect protein expression levels of *SGPP1* and *SGPL*. The expression patterns for both *SGPP1* and *SGPL* within uteri of sheep differ from that in mice, in which both *Sgpp1* and *Sgpl1* increase in decidua versus interimplantation sites of the uterus [24]. The scattered expression patterns of *SGPP1* and *SGPL* in multiple species warrants further investigation because S1P is a known mediator of lymphocyte trafficking, which is increased in the uterus during implantation and placentation [40]. Likewise, we have not investigated lipid phosphate phosphatases, which may also serve to modulate S1P bioavailability [41, 42].

Our study delved into S1P1 receptor expression patterns in a unilaterally pregnant model. Of the five known G protein-coupled receptors (S1P1-5), *S1PR1* and *S1PR3* are expressed in both embryonic and uterine tissues [25, 38], and ablation of *S1PR1* is lethal to the embryo due to hemorrhaging [34]. Further, expression of *S1PR1*, *S1PR3*, and *S1PR5* has been detected within human chorionic plate arteries [43]; however, we were unable to detect significant levels of *S1PR5* mRNA or

protein (data not shown), nor could we detect *S1PR3* protein despite positive expression at the mRNA level (Fig. 6, C and D). Thus, although we cannot rule out a role for *S1PR3* in the present study, we elected to focus on *S1PR1*. Similar to other species, *S1PR1* was expressed by the trophoblast and endometrium at the maternal-fetal interface of interplacentomal regions on all days of pregnancy. However, a major finding of this study was localization of *S1PR1* to the capillary tips of the caruncular vasculature of placentomes, which are areas of active angiogenesis. These vessels supply the epithelial syncytium that separates maternal from fetal tissues and are directly responsible for hematotropic support of fetal development. These results support data from mice, where *S1PR1* is upregulated in the subluminal stromal compartment of the antimesometrial pole of the decidua by Day 5, and in the microvasculature of the mesometrial pole by Day 7 of pregnancy [25]. *S1PR1* is also present at the maternal/fetal interface, as it is expressed by placental vascular stem cells and fetal connective tissues [25]. In humans it is hypothesized that sources of S1P affecting the fetal vasculature likely include the syncytiotrophoblast or cytotrophoblast cells, placental stromal cells, and fetal circulatory cells [43]. Collectively, results from mice, humans, and now sheep indicate that *S1PR1* is an excellent candidate

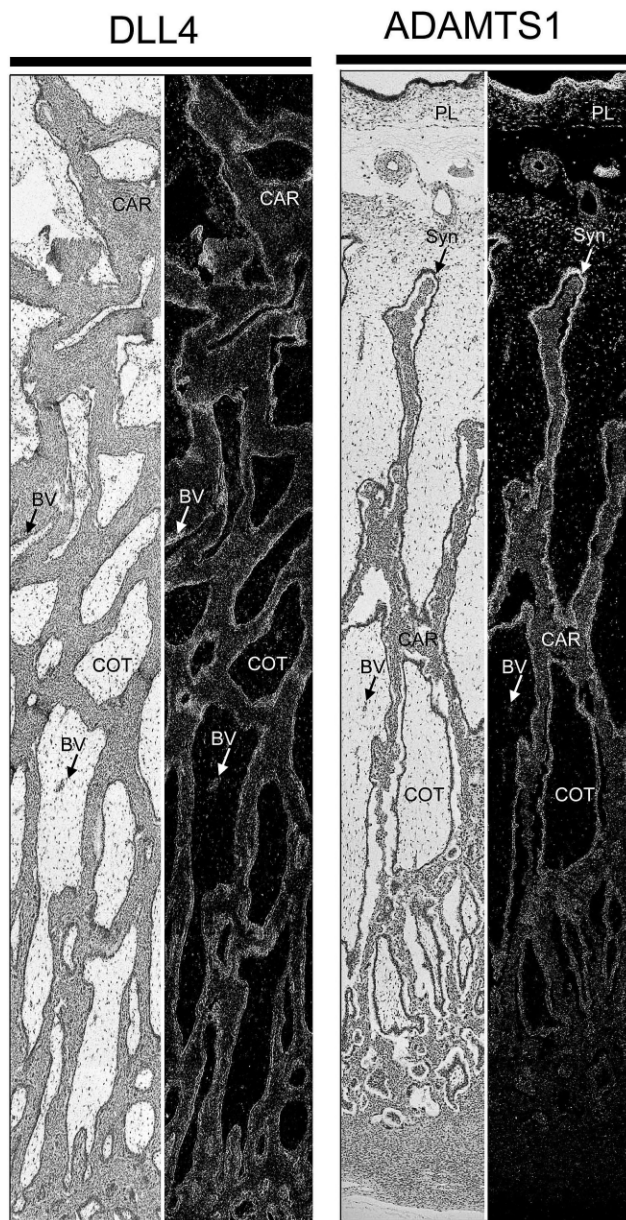


FIG. 10. Composite images of in situ hybridization analyses for *DLL4* and *ADAMTS1* mRNA within placentomes collected from unilaterally pregnant ewes on Day 80 of pregnancy. Corresponding bright field and dark field images of representative placentomes are shown. BV, blood vessel; CAR, caruncular tissue; COT, cotyledonary tissue; PL, chorion; Syn, epithelial syncytia of the placentome. Representative images are shown from independent samples ($n = 4$). Width of each field is 940 μm .

molecule for stimulating angiogenesis. To confirm a functional requirement for S1PR1 in mediating angiogenic sprouting events, an established in vitro 3-D endothelial cell invasion assay was used to demonstrate that FTY720, an S1P receptor antagonist, as well as siRNA specific for S1PR1, significantly decreased endothelial cell invasion and sprouting responses in vitro. These results clearly indicate that *S1PR1* is required for S1P and growth factor-induced endothelial sprouting responses, and these results are also consistent with a role for S1PR1 in regulating angiogenic responses during pregnancy.

Appropriate regulation of gene expression is necessary for maintenance of pregnancy. We previously demonstrated that *DLL4* and *ADAMTS1* were differentially regulated by S1P, VEGF, and FGF2 [22], motivating in situ hybridization studies

conducted here. In the pregnant ewe, *DLL4* displayed a complex pattern of expression that appeared to be driven by the presence of placental/fetal tissues, because *DLL4* expression is relatively low in the nonpregnant uterine horn of unilaterally pregnant ewes. In gravid uterine horns, *DLL4* mRNA was detected throughout the maternal-fetal interface and uterine glands during later stages of gestation. Relevant to angiogenesis, *DLL4* and *S1PR1* mRNA expression patterns were similar. *DLL4*, like *S1PR1*, was observed in the maternal vasculature in placentomes. Interestingly, *DLL4* also increased in the endometrial GE of gravid ewes during the later stages of pregnancy. *DLL4* protein is expressed in human endometrium [50], where both cytoplasmic and apical membrane staining were observed in LE and GE with moderate diffuse staining in the cytoplasm of stromal cells [44]. Similarly, *ADAMTS1* expression at the uterine-placental interface of sheep agreed with results from other species. In the pregnant human uterus, *ADAMTS1* is associated with decidualization of endometrial stroma and localized specifically in the stroma surrounding the spiral arterioles of the secretory endometrium that are the primary source of hematotropic support for fetal development [45]. Interestingly, similar to sheep, the endometrial GE of women also expresses high levels of *ADAMTS1*. In mice, *Adams1* was not present in the stroma, but rather detectable in uterine LE and GE [46]. Coordinate and complementary expression of *DLL4* and *ADAMTS1* suggest necessary roles for each in regulating the morphological changes within interplacentomal and placental endometrium to support pregnancy. Thus, S1P may be acting through S1PR1 receptors to promote angiogenesis within placentomes, but whether *DLL4* and *ADAMTS1* expression patterns are directly regulated by S1P remains to be demonstrated.

Results of the present study demonstrate coordinated temporal regulation of *SPHK*, *SGPPI*, and *SGPL*, which support increased availability of S1P to stimulate placental angiogenesis and provide hematotropic support for pregnancy in sheep. Antagonism of S1PR1 with pharmacological compounds and siRNA blocked S1P- and growth factor-stimulated human and sheep endothelial cell invasion, which was further supported by detection and localization of *S1PR1* to the caruncular capillary tips of placentomes. Thus, S1P may be acting through S1PR1 to promote angiogenic responses. However, effects on other cell types and tissues remain to be defined. Collectively, the spatial expression of these members of the S1P signaling pathway at key sites of angiogenesis within the uterus suggests an important role in supporting growth and development of the conceptus during pregnancy. Future studies antagonizing the S1P pathway in vivo will determine key signals elicited by S1P and determine whether S1P is required for angiogenic responses, vascular and glandular development, and coordinate expression of *ADAMTS1* and *DLL4*.

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