Inactivation of porcine interleukin-1β results in failure of rapid conceptus elongation

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Conceptus expansion throughout the uterus of mammalian species with a noninvasive epitheliochorial type of placentation is critical establishing an adequate uterine surface area for nutrient support during gestation. Pig conceptuses undergo a unique rapid morphological transformation to elongate into filamentous threads within 1 h, which provides the uterine surface to support development and maintain functional corpora lutea through the production of estrogen. Conceptus production of a unique interleukin 1β, IL1B2, temporally increases during the period of trophoblast remodeling during elongation. CRISPR/Cas9 gene editing was used to knock out pig conceptus *IL1B2* expression and the secretion of IL1B2 during the time of conceptus elongation. Trophoblast elongation occurred on day 14 in wild-type ($IL1B2^{+/+}$) conceptuses but did not occur in ILB2-null ($IL1B2^{-/-}$) conceptuses. Although the morphological transition of $IL1B2^{-/-}$ conceptuses was inhibited, expression of a number of conceptus developmental genes was not altered. However, conceptus aromatase expression and estrogen secretion were decreased, indicating that IL1B2 may be involved in the spatiotemporal increase in conceptus estrogen synthesis needed for the establishment of pregnancy in the pig and may serve to regulate the proinflammatory response of endometrium to IL1B2 during conceptus elongation and attachment to the uterine surface.

porcine | conceptus development | CRISPR/Cas9 | interleukin-1b | pregnancy

Establishment of pregnancy in domestic farm species, such as pigs, sheep, and cattle, requires that the conceptus(es) inhibit luteolysis to extend the secretion of progesterone from the corpus luteum beyond the length of the normal estrous cycle so that continued embryo development will be supported until term (1, 2). Ruminant conceptuses produce IFN-τ, which interferes with endometrial production of prostaglandin F2α (PGF2α), while synthesis and release of estrogens by pig conceptuses stimulate the endometrial production of PGF2α to be sequestered and metabolized to an inactive metabolite within the uterine lumen (2–4). While conceptus IFN-τ and estrogens serve as the maternal recognition signals of pregnancy in ruminates and pigs, respectively (4), conceptuses of both species must expand across the endometrial luminal surface of the uterine horns to inhibit PGF2α production locally or release and establish adequate uterine surface area for nutrient flow for continued embryo and fetal development to term (5, 6). The pig possesses both a local and systemic vascular pathway for PGF2α to reach the ovaries to induce luteolysis during the estrous cycle (7). Therefore, not only do conceptuses of this litter-bearing species migrate throughout both of the long uterine horns to inhibit luteolysis, but the expansion of each conceptus also competes with littermates for uterine space. Expansion of pig conceptuses within the uterine lumen is essential, as the noninvasive, epitheliochorial-type placentation in the pig depends upon adequate uterine surface area to develop and survive.

Early conceptus development in ruminants involves progesterone-stimulated, endometrial-secreted factors that increase conceptus cell proliferation and growth to expand its trophoblast through the ipsilateral uterine horn (2, 8). Although early conceptus development in pigs following blastocyst hatching involves cellular proliferation similar to that of the ruminant conceptus, pig conceptuses undergo a unique rapid transformation in morphology that occurs through cellular remodeling (9, 10). Pig conceptuses proliferate to reach a 2- to 6-mm spherical diameter by day 10 of gestation. Upon reaching a 9- to 10-mm ovoid morphology at day 11–12 of gestation, conceptuses transform to a tubular form that rapidly elongates to a thin filamentous thread greater than 100 mm in length in less than 1–2 h (9, 11). Loosening of the trophectoderm junctional complexes allows cellular fluidity to permit remodeling of the conceptus through movement and migration of the underlying endoderm cells that form a thin filamentous conceptus (12). The timing of the rapid conceptus elongation is associated with the epiblast differentiation and mesodermal outgrowth. Mesoderm cellular differentiation and expansion involve epiblast production of FGF4, which stimulates FGF receptor 2 expressed on the trophectoderm (13). Mesoderm expression of bone morphogenetic protein 4 (BMP4) stimulates phosphorylated SMAD1/5/8 in the overlying trophoectoderm, which could stimulate growth and expansion between the trophectoderm and the underlying extraembryonic endoderm, possibly to release factors to stimulate receptor-driven cellular pathways involved in conceptus remodeling and elongation (14, 15). Although there is a temporal association of peri-implantation epiblast FGF4 production and signaling with conceptus elongation, FGF4 does not appear to stimulate elongation (13), indicating that another factor initiates rapid pig conceptus elongation.

Tuo et al. (16) first identified interleukin 1β (IL1B) as a highly differentially expressed gene during the period of rapid pig conceptus elongation. Conceptus *IL1B* mRNA and protein abundance increase during the tubular-to-filamentous transition, with maximal expression and secretion at the height of rapid

Significance

Establishment of pregnancy in pigs requires that conceptuses produce estrogen to maintain functional corpora lutea throughout gestation and rapidly elongate to provide adequate surface area for nutrient flow through its epitheliochorial placenta. Early conceptus development in the pig is unique in that conceptuses rapidly elongate to long filamentous threads within 1 to 2 h. Conceptus *IL1B2* gene and protein expression increase during the period of conceptus elongation. Using the CRISPR/Cas9 gene-editing system, we successfully knocked out pig conceptus *IL1B2* expression and demonstrate that conceptus elongation was inhibited. This research provides insight into the biological role of IL1B2 in pig conceptus development and demonstrates the use of the CRISPR/Cas9 gene-editing system to evaluate genes during early conceptus development.

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elongation (11). Immediately following conceptus elongation, *IL1B* expression rapidly declines, and the uterine luminal content of IL1B protein decreases (11). The pig is unique in that a gene duplication of *IL1B* resulted in the expression of a novel conceptus-specific IL1B isoform, *IL1B2* (17). Both *IL1B1* and *IL1B2* have seven exons, which are positioned in tandem on chromosome 3; however, *IL1B2* contains an alternate exon that may provide differential transcriptional regulation that is specific to the conceptus (17). Conceptus expression of *IL1B2* during elongation is consistent with a pivotal role for IL1B within the primary networks responsible for cellular assembly and organization and for embryonic development, as well as for cell growth and proliferation (18).

Here, we provide evidence that *IL1B2* is involved with the cellular pathway for rapid pig conceptus elongation. We generated a porcine biallelic knockout model using the CRISPR/Cas9 gene-editing system to evaluate the function of *IL1B2* in conceptus development and determined its role in regulating the expression of other genes involved in conceptus development and uterine attachment. Disruption of *IL1B2* expression produced a striking impairment of conceptus elongation during establishment of pregnancy compared with wild-type cloned porcine embryos.

Results

CRISPR Design and Porcine Fibroblast Transfection. Five single-guide RNAs (sgRNAs) were designed (Table S1) to uniquely target the initial coding sequences 1 and 2 of *IL1B2* (located in exons 2 and 3) without modifying the paralogous systemic gene form, *IL1B1* (Fig. S1). Testing of the ability of each sgRNA to cleave its intended target on a 2.4-kb *IL1B2* PCR product was performed in vitro. Comparing the agarose gel band intensities of the unmodified PCR product and the expected cleavage products indicated that sgRNAs 1, 2, 4, and 5 were the most efficient of the five (Fig. S2*A*) and did not target the same location of the systemic *IL1B1* in vitro when each of the IL1B2 sgRNAs was tested on an *IL1B1* PCR product (Fig. S2*B*). These sgRNAs were individually cloned into a plasmid vector that coexpresses the Cas9 nuclease and a GFP under the control of a bidirectional CMV promoter to allow FACS selection. When the four CRISPR/Cas9 vectors were cotransfected into Minnesota minipig fetal fibroblasts suitable for somatic cell nuclear transfer (SCNT), GFP expression was evident by 96 h after electroporation recovery (Fig. S3). Sorting of individual fluorescing cells into 15 96-well plates (n = 1,440 wells) yielded 354 colonies (24.6%), 40 of which had sufficient cells for DNA isolation and PCR genotyping to detect *IL1B2* modification. Two colonies were confirmed by PCR genotyping (Fig. S4) and Sanger DNA sequencing to have biallelic *IL1B2* gene modifications and are referred to as "*IL1B2*$^{-/-}$ cells." These two modified colonies, designated "2-4" and "2-11," represented 5% of the colonies genotyped. The descriptions of each modification are presented in Table S2. The modifications of *IL1B2* in each of the two cell types are biallelic but not homozygous. Since we were interested only in inactivation of the *IL1B2* gene, homozygosity of the alteration was not a requirement. For cell line 2-4, the entire sequences of exons 2 and 3 were excised, corresponding to coding sequences 1 and 2. For cell line 2-11, exon 2 (coding sequence 1) was excised from the first allele, and exons 2 and 3 (coding sequences 1 and 2) were excised from the second allele.

Cloned Embryos from *IL1B2*$^{-/-}$ and IL1B2$^{+/+}$ Fibroblasts. The two colonies of *IL1B2*$^{-/-}$ cells were used for SCNT to produce *IL1B2*$^{-/-}$ embryos. Concurrently, identical wild-type (*IL1B2*$^{+/+}$) fibroblasts which had undergone electroporation without CRISPR/Cas9 and also had been sorted by FACS selection were used for SCNT to produce *IL1B2*$^{+/+}$ embryos. Early in vitro development of *IL1B2*$^{-/-}$ embryos to blastocysts was similar to that of *IL1B2*$^{+/+}$ nuclear-transferred embryos (range 30–40% across SCNT). Five embryo transfers for both *IL1B2*$^{-/-}$ and *IL1B2*$^{+/+}$ into recipient gilts were performed. Equivalent numbers of *IL1B2*$^{-/-}$ and *IL1B2*$^{+/+}$ blastocysts (~43) were transferred to surrogate gilts. The number of embryos reaching day 14 of development was not significantly different in the *IL1B2*$^{-/-}$ and *IL1B2*$^{+/+}$ recipients (Table S3). In contrast, the number of embryos that were elongated on day 14 was dramatically different in *IL1B2*$^{-/-}$ (4/45 = 9%) and *IL1B2*$^{+/+}$ (46/58 = 79%) conceptuses, with negligible elongation occurring in the absence of *IL1B2* expression. Morphological abnormalities in *IL1B2*$^{-/-}$ recipients include malformed, nonelongated conceptuses with bulbous ends that did not elongate to normal filamentous threads as observed in all *IL1B2*$^{+/+}$ conceptuses flushed from the uterine lumen (Fig. 1).

Conceptus *IL1B2* Gene Expression and Uterine Luminal Flushing Protein Analysis. Gene editing of pig conceptus *IL1B2* inhibited ($P < 0.005$) *IL1B2* mRNA expression in *IL1B2*$^{-/-}$ conceptuses compared with *IL1B2*$^{+/+}$ conceptuses (Fig. 2*A*). Expression of *IL1B2* was ~64-fold greater in *IL1B2*$^{+/+}$ than in *IL1B2*$^{-/-}$ conceptuses. Suppression of conceptus *IL1B2* gene expression resulted in the inhibition of conceptus IL1B2 secretion during the period of conceptus elongation (Fig. 2*B*). Total content of IL1B2 protein in uterine luminal flushing (ULF) averaged 6,000 ng in recipients containing *IL1B2*$^{+/+}$ conceptuses but was below the sensitivity of the assay in ULFs of recipients containing *IL1B2*$^{-/-}$ conceptuses ($P < 0.008$).

Conceptus *CYP19A1* Gene Expression and ULF Estradiol-17β Analysis. Conceptus mRNA expression of *CYP19A1* (aromatase) was decreased ($P < 0.008$) in *IL1B2*$^{-/-}$ compared with *IL1B2*$^{+/+}$ conceptuses (Fig. 3*A*). Expression of *CYP19A1* was sevenfold greater in *IL1B2*$^{+/+}$ than in *IL1B2*$^{-/-}$ conceptuses. The content of estradiol-17β (E2) in the ULF was approximately fourfold lower ($P < 0.05$) in recipients containing *IL1B2*$^{-/-}$ conceptuses than in those containing *IL1B2*$^{+/+}$ conceptuses (Fig. 3*B*). Although the uterine luminal E2 content of recipients containing *IL1B2*$^{-/-}$ conceptuses is significantly less than that in *IL1B2*$^{+/+}$ recipients, the conceptuses were capable of synthesizing and releasing E2 into the uterine lumen, which normally occurs during the period of conceptus rapid elongation (19).

Conceptus Prostaglandin-Endoperoxide Synthase 2 Gene Expression and ULF PGF2α Analysis. *IL1B2*$^{-/-}$ conceptuses had greater mRNA expression for prostaglandin-endoperoxide synthase 2 (*PGTS2*) ($P < 0.001$) than did *IL1B2*$^{+/+}$ conceptuses (Table 1). Although *PGTS2* mRNA in *IL1B2*$^{-/-}$ conceptuses increased twofold compared with *IL1B2*$^{+/+}$ conceptuses, the total uterine luminal content of PGF in the uterine flushings was numerically ($P < 0.13$) greater (243 ± 110 ng) in flushings containing *IL1B2*$^{+/+}$ conceptuses than in those containing *IL1B2*$^{-/-}$ conceptuses (10 ± 1.7 ng). High uterine PGF content of two flushings from uteri containing *IL1B2*$^{+/+}$ conceptuses resulted in a large SEM, as the amounts of PGF in the other three flushings from wild-type conceptuses were similar to those from *IL1B2*$^{-/-}$ conceptuses.

Conceptus Gene Expression. Gene expression of *IL1B1* and salivary lipocalin (*SAL1*) was very low in both *IL1B2*$^{-/-}$ and *IL1B2*$^{+/+}$

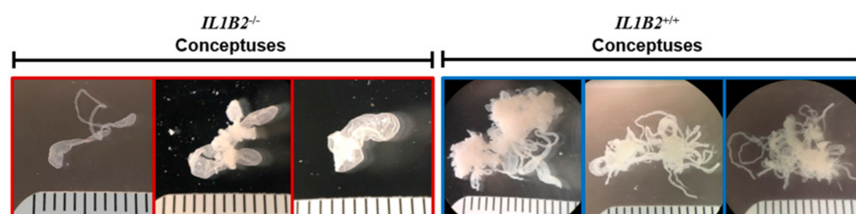

Fig. 1. Morphology of *IL1B2*$^{-/-}$ and *IL1B2*$^{+/+}$ porcine conceptuses at the day 12 stage of development. *IL1B2*$^{-/-}$ conceptuses collected from three surrogate gilts are smaller and do not show the same degree of elongation as *IL1B2*$^{+/+}$ conceptuses. (Scales: each mark represents 1 mm.)

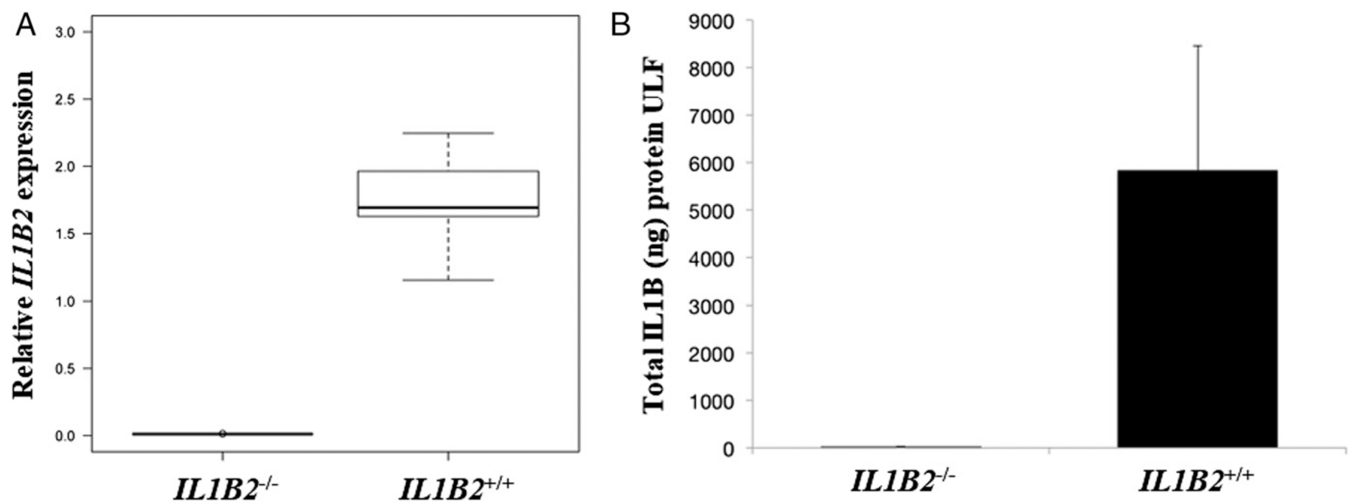

Fig. 2. (*A*) *IL1B2* gene expression in *IL1B2*$^{-/-}$ and *IL1B2*$^{+/+}$ pig conceptuses. Data were normalized to β-actin and are expressed as box plots (minimum, first quartile, median, third quartile, and maximum) of ΔCT relative to *IL1B2*$^{+/+}$ conceptuses (n = 5 gilts and n = 6 conceptuses per gilt; $P < 0.05$). (*B*) Total IL1B protein content ± SEM detected by ELISA in uterine flushings from pregnant gilts carrying *IL1B2*$^{-/-}$ and *IL1B2*$^{+/+}$ conceptuses (n = 5 gilts and n = 3 pooled flushings per gilt; $P < 0.05$).

conceptuses (not detectable by RT-PCR). Conceptus gene expression of *BMP4*, *FGF4*, steroidogenic acute regulatory protein (*STAR*), and IFN-γ (*IFNG*) was not significantly different between *IL1B2*$^{-/-}$ and *IL1B2*$^{+/+}$ conceptuses (Table 1). *IL1B2*$^{-/-}$ conceptuses had a twofold greater mRNA expression for *IFND* ($P < 0.02$) compared with *IL1B2*$^{+/+}$ conceptuses.

Endometrial Gene Expression of Recipients Containing *IL1B2*$^{-/-}$ or *IL1B2*$^{+/+}$ Conceptuses. Gene expression for *IL1B2* in endometrium was lowly abundant (not detectable by RT-PCR). Endometrium gene expression for IL1 receptor 1 (*IL1R1*), PGE synthase-1 (*PTGES*), and *SAL1* was not significantly different in the uteri of recipients containing *IL1B2*$^{-/-}$ or *IL1B2*$^{+/+}$ conceptuses (Table 2). However, endometrial interleukin 1 receptor accessory protein (*IL1RAP*), secreted phosphoprotein 1 (*SPP1*), and PGF synthase (*PGFS*) mRNA expression was significantly (1.5- to 1.7-fold) greater in recipients containing *IL1B2*$^{-/-}$ conceptuses than in recipients with *IL1B2*$^{+/+}$ conceptuses.

Discussion

Establishment of pregnancy in pigs involves conceptus production of estrogens to maintain functional corpora lutea throughout pregnancy (1). Pig conceptuses rapidly elongate throughout the uterine lumen to alter PGF2α movement to prevent luteolysis and to provide an adequate placental surface area for maternal nutrient transfer for development and survival to term. Rapid conceptus elongation normally occurs between day 11 and 12 of gestation (9). However, our preliminary data indicated that in vitro-cultured nuclear-transferred pig blastocysts have delayed development, with only 3- to 4-mm spherical conceptuses present on day 12 of pregnancy and with rapid conceptus elongation occurring on day 14 in recipient females containing cloned embryos. Spherical conceptuses were flushed from the uterine horns of the recipients of *IL1B2*$^{+/+}$ and *IL1B2*$^{-/-}$ conceptuses on day 12 of pregnancy, which demonstrated that *IL1B2*$^{-/-}$ embryos were normal in development until the period

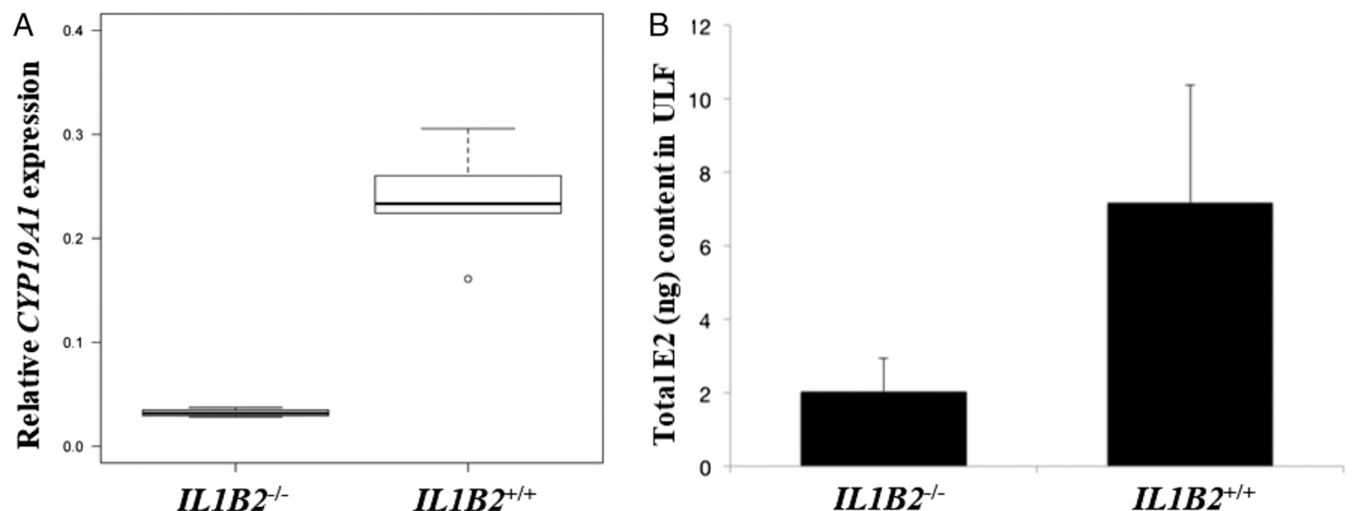

Fig. 3. (*A*) *CYP19A1* gene expression in *IL1B2*$^{-/-}$ and *IL1B2*$^{+/+}$ pig conceptuses. Data were normalized to β-actin and are expressed as box plots (minimum, first quartile, median, third quartile, and maximum) of ΔCT relative to *IL1B2*$^{+/+}$ conceptuses (n = 5 gilts and n = 6 conceptuses per gilt; $P < 0.05$). (*B*) Total E2 protein content ± SEM detected by ELISA in uterine flushings from pregnant gilts carrying *IL1B2*$^{-/-}$ and *IL1B2*$^{+/+}$ conceptuses (n = 5 gilts and n = 3 pooled flushings per gilt; $P < 0.05$).

Table 1. Conceptus gene expression

Gene	$IL1B2^{-/-}$ mean $2^{-\Delta Ct}$	SD	$IL1B2^{+/+}$ mean $2^{-\Delta Ct}$	SD	Probability
BMP4	0.00089	0.00054	0.00095	0.00023	N.S.
FGF4	0.00030	0.00035	0.00056	0.00023	N.S.
IFND	0.00078	0.00023	0.00037	0.00010	0.02
IFNG	0.59818	0.11588	0.62055	0.19241	N.S.
STAR	0.00027	0.00012	0.00040	0.00019	N.S.
PGTS2	0.00092	0.00007	0.00046	0.00016	0.001

N.S., not significant.

of elongation. Thus, all $IL1B2^{+/+}$ and $IL1B2^{-/-}$ conceptuses were harvested on day 14 in the present study.

The proinflammatory cytokine IL1B system is proposed to be involved in implantation and establishment of pregnancy in nonhuman primates (20), women (21), mice (22), and cattle (23). Expression of IL1B is apparent across many species, suggesting that this cytokine is a conserved mediator of vertebrate reproduction and placental viviparity (24). The gene duplication of *IL1B* resulted in the novel pig conceptus-specific alternate exon *IL1B2* which may provide differential transcriptional regulation that is specific to pig conceptus development (17). The nonconserved amino acid substitutions in IL1B2 affect its interaction with IL1R1, which result in different physiological responses to IL1B1 and IL1B2 (17).

Global gene analysis of pig conceptus development during the time of rapid elongation by several laboratories have indicated that *IL1B* is one of the most abundant differentially expressed genes, along with cytokeratin (*KRT*) 8 and 18, stratifin (*SFN*), and (although in lower abundance) *trans*-retinoic acid (11, 14, 25–28), which are genes involved with cell survival, growth, migration, and morphogenesis. *IL1B* and *E2* are two of the top five differently regulated networks during the morphological change in conceptus development (14). Blomberg et al. (26, 27) indicated that a large number of genes (~300) were differentially expressed during the morphological transition in development of the pig conceptuses, many of which are associated with cellular morphology and movement. Since both expression and secretion of ILB2 is greatly increased during the period of rapid pig conceptus elongation, and because of its association with networks involved cellular remodeling, *ILB2* is an obvious candidate gene that may regulate the rapid morphological change that occurs in pig conceptuses.

In the present study, the CRISPR/Cas9 gene-editing system successfully knocked out *IL1B2* expression in the pig conceptus. Early in vitro development of $IL1B2^{-/-}$ embryos to blastocysts was similar to that of $IL1B2^{+/+}$ nuclear-transferred embryos, and in utero blastocyst hatching and growth of spherical conceptuses were not different between $IL1B2^{-/-}$ and $IL1B2^{+/+}$ embryos. However, during the period of rapid conceptus elongation, $IL1B2^{-/-}$ conceptuses developed an abnormal oblong morphology that failed to elongate in comparison with the long, filamentous $IL1B2^{+/+}$ conceptuses. The synthesis and secretion of IL1B2 was ablated in $IL1B2^{-/-}$ conceptuses, as IL1B2 was absent in ULFs of recipients containing $IL1B2^{-/-}$ conceptuses.

We decided to evaluate conceptus gene expression of *BMP4*, *FGF4*, *STAR*, and *PGTS2*, as these genes have previously been detected during the period of conceptus elongation. The expression of *BMP4*, *FGF4*, *STAR*, and *IFNG* was similar in $IL1B2^{-/-}$ and $IL1B2^{+/+}$ conceptuses, while the expression of *IFND* and *PGTS2* was slightly greater in $IL1B2^{-/-}$ conceptuses. These results indicate that, although $IL1B2^{-/-}$ conceptuses did not elongate and had an abnormal morphology, the conceptuses were transcriptionally active and viable. The biological relevance of a twofold increase in *PGTS2* gene expression in the $IL1B2^{-/-}$ conceptus is not known, as the luminal content of PGF tended to be lower in the uterine flushing of $IL1B2^{-/-}$ conceptus recipients than in the uterine flushing of $IL1B2^{+/+}$ recipients. Both the conceptuses and endometrium synthesize and secrete PGF into the uterine lumen, confounding any direct evaluation of conceptus prostaglandin synthesis. Expression of *PGTS2* is enhanced by the NFκB system through stimulation of the IL1B receptor (29), which, with the loss of conceptus IL1B2 expression, could reflect the lower content of PGF in uterine flushings from both $IL1B2^{-/-}$ and $IL1B2^{+/+}$ conceptuses (30) and endometrium (31). However, conceptus prostaglandin production is not essential for rapid elongation, as inhibiting PGTS1 and PGTS2 with indomethacin and Banamine (nonspecific PGTS inhibitors) did not block conceptus elongation (32).

The expression of porcine IFN increases immediately following conceptus elongation (33), which allowed us to demonstrate the ability of $IL1B2^{-/-}$ conceptuses to transition to the attachment stage of development although they failed to undergo elongation. In the present study, IFNG was not different in $IL1B2^{-/-}$ and $IL1B2^{+/+}$ conceptuses, and *IFND* expression was even twofold greater in $IL1B2^{-/-}$ conceptuses. These results again indicate that the failure of $IL1B2^{-/-}$ conceptuses to elongate was not caused by a loss of cellular viability.

The rapid transition from an ovoid to a filamentous conceptus does not involve cellular hyperplasia, which is greatly reduced during elongation (9), and apoptosis does not increase during the transition of the conceptuses (18). Alteration of the trophectoderm cell-junctional complexes along with migration of underlying endodermal cells (9) creates the intracellular mechanical tension that alters cellular shape and rapidly remodels the conceptus trophoblast during elongation. In the human placenta, IL1B has been demonstrated to promote cellular motility during human cytotrophoblast formation and the secretion of urokinase plasminogen activator (34) and metalloproteinases (35) during endometrial invasion. During elongation, pig conceptuses produce plasminogen activator (36) and are highly invasive, but endometrial secretion of plasmin/trypsin inhibitor and other protease inhibitors blocks endometrial invasion in the pig (37). The linkage of IL1B2 to primary networks responsible for cellular assembly and organization and for cell growth and proliferation (14) suggests that knockout of IL1B2 would interfere with a number of pathways necessary for conceptus elongation. During the morphological transition of the conceptus during elongation, there is an increase in cytokeratins (KRT7, KRT8, and KRT9), ezrin (EZR), annexin 5 (ANXA), and SFN (18), which are involved with the cellular cytoskeletal architecture, morphogenesis, and migration. Pig conceptus elongation involves an alteration in trophectoderm cellular structure, the development of filopodia, and movement of the underlying endoderm (9). KRN and EZR are involved with the formation of lamellipodia and filopodia (38), which would be essential for the establishment of focal adhesions and cellular migration observed during conceptus elongation (9). EZR is expressed by the endoderm and trophoectoderm of the mouse implanting embryo (39). Karmakar and Das (40) suggested that cytotrophoblast invasiveness of the human placenta could be regulated by IL1B stimulation of urokinase plasminogen activator and metalloproteinases secretion but also by modifying the cytoskeleton architecture by downregulating EZR, E-cadherin, and β-catenin. These pathways would increase cell–matrix and reduce cell–cell interaction, providing an environment appropriate for cell motility. Future global gene analyses comparing $IL1B2^{-/-}$ and $IL1B2^{+/+}$ conceptuses will be necessary to determine the alternation in gene expression during elongation.

Table 2. Endometrial gene expression

Gene	$IL1B2^{-/-}$ mean $2^{-\Delta Ct}$	SD	$IL1B2^{+/+}$ mean $2^{-\Delta Ct}$	SD	Probability
IL1R1	0.01457	0.00233	0.01549	0.00275	N.S.
IL1RAP	0.01167	0.00243	0.00773	0.00053	0.02
PGFS	0.01396	0.00185	0.00825	0.00194	0.002
PTGES	0.00215	0.00025	0.00210	0.00034	N.S.
SAL1	0.19210	0.03115	0.19027	0.01835	N.S.
SPP1	0.02946	0.00372	0.02138	0.00409	0.01

N.S., not significant.

There is a spatiotemporal increase rise in estrogen synthesis in the pig conceptus that parallels the increase and decrease of uterine luminal IL1B2 content between days 12 and 15 of pregnancy (1, 11). Conceptus estrogens act through estrogen receptor α (ERA) in the endometrium to signal pregnancy recognition, stimulate uterine secretions, and regulate trophoblast attachment and elongation (1). Expression of *CYP19A1* and E2 synthesis were significantly decreased in $IL1B2^{-/-}$ conceptuses. The decrease in *CYP19A1* expression and E2 synthesis in the $IL1B2^{-/-}$ conceptus would be consistent with IL1B's reported ability to stimulate aromatase expression in human placental cytotrophoblast cells (41).

Elongation not only involves morphological changes in the spherical conceptus; the rapid remodeling also requires the appropriate uterine luminal epithelial expression of integrins and cell-surface receptors such as SPP1, which contain an Arg-Gly-Asp (RGD) peptide sequence that binds to cell-surface integrins (42). Endometrial expression of SPP1, which is stimulated by E2 in the pig, has been proposed to be involved with conceptus attachment through integrin binding (42). Spherical pig conceptuses will not elongate in vitro unless they are supported by an alginate hydrogel either supplemented with SPP1 or conjugated with an RGD sequence that promotes cell adhesion and migration (43). Expression of IL1B2 increased in conceptuses that expanded in the alginate hydrogel supplemented with either SPP1 or RGD. IL1B2 stimulates endometrial IL1R1, which can activate a number of cellular signaling pathways at the conceptus–maternal interface of the endometrium via ERK1/2, MAPK (44), and NFκB (17). Although IL1B has been reported to increase the expression of a number of genes expressed by the endometrium during pregnancy (11, 44–46), expression of *IL1R1*, *PTGES*, and *SAL1* were not different in the endometrium of recipients carrying either $IL1B2^{+/+}$ or $IL1B2^{-/-}$ conceptuses. There was actually a slight increase in endometrial expression of *IL1RAP*, *PGFS*, and *SPP1* in $IL1B2^{-/-}$ recipients. Thus, the failure of $IL1B2^{-/-}$ conceptuses to elongate could be caused not only by a failure of trophoectoderm and endoderm cellular remodeling and migration but possibly also by the inability to express the proper integrins or receptors to interact with SPP1 present on the uterine surface epithelium (47).

The CRISPR/Cas9 gene-editing system successfully knocked out pig conceptus *IL1B2* expression and demonstrated that rapid conceptus elongation does not occur in the absence of conceptus IL1B2 secretion. Conceptus production of IL1B2 appears to regulate conceptus estrogen synthesis, which may serve to regulate the proinflammatory response of endometrium to IL1B2 during conceptus elongation and attachment to the uterine surface. The CRISPR/Cas9 gene-editing system provides a method to determine the role of conceptus genes in development, implantation, placentation, and survival.

Materials and Methods

All procedures used in this study were approved by the University of Missouri-Columbia Institutional Animal Care and Use Committee under Protocol 7868. Additional descriptions of procedures are provided in *Supporting Information*.

CRISPR Design. A set of five sgRNAs was designed by using CRISPR RNA-guided endonuclease (RGEN) tools (www.rgenome.net/mich-calculator/) (48) to uniquely target *IL1B2* (Fig. S1) in a 1,500-bp genomic region without compromising the function of *IL1B1*. To limit possible off-target cleavage events, the selected sgRNAs were scanned with Cas-OFFinder (www.rgenome.net/cas-offinder/) (49). The efficiency and accuracy of each sgRNA was tested in vitro against a PCR-amplified fragment of both *IL1B2* and *IL1B1* and to verify that only the conceptus form of *IL1B* was targeted and the systemic form was not targeted (Fig. S2). After this screening, four of the sgRNAs (1, 2, 4, and 5) were selected (Table S1) to target *IL1B2* in SCNT donor cells. Annealed sgRNA oligos were individually cloned into the pGuide-it-ZsGreen1 vector (Clontech) which simultaneously expresses Cas9, GFP, and the *IL1B2*-specific sgRNA (ZsGreen1-Cas9-sgRNA vector) to yield four CRISPR/Cas9 vectors.

Culture and Transfection of Cells. Low-passage-number (2 or 3) cryopreserved male porcine fetal fibroblasts originating from the Minnesota minipig strain (RRID:NSRRC_0005) were thawed, cultured, and transfected with the four ZsGreen1-Cas9-sgRNA vectors targeting *IL1B2* (with sgRNAs 1, 2, 4, and 5; 0.5 μg each) and were used for electroporations performed in duplicate. Wild-type control cells were electroporated without CRISPR/Cas9 vectors. After electroporation, fibroblasts were cultured for 96 h.

FACS Isolation and Genotyping of $IL1B2^{-/-}$ Cells. GFP expression in CRISPR/Cas9-treated cells was confirmed by fluorescence microscopy (Fig. S3), and then cells were sorted individually by FACS using a Beckman Coulter MoFlo XDP cell sorter into 96-well culture plates (50). Wild-type control cells were sorted by FACS into 96-well plates without fluorescence selection. Over the next 14 d colonies were identified and split for genotyping and propagation.

SCNT. Fibroblast colonies (wild type or determined to have the *IL1B2* modification) were used as donor cells for SCNT as previously described (51). Briefly, sow-derived oocytes (Desoto Biosciences) were matured in vitro, cumulus cells were removed from the oocytes, and the polar body and the metaphase II plate were removed. A donor cell ($IL1B2^{-/-}$ or $IL1B2^{+/+}$) was placed in the perivitelline space and electrically fused to the oocyte. Reconstructed zygotes were chemically activated (52) and cultured using our standard protocols (53, 54). After 6 d blastocyst-stage embryos were surgically transferred into the ampullary–isthmic junction of the oviduct of the surrogate gilts at day 4, 5, or 6 after first standing estrus (55).

Embryo Collection. Embryos were collected by uterine flushing as previously described (11, 56). Gilts were killed on day 14 of pregnancy (corresponding to day 12 of embryo development in SCNT embryos, based on previous observations) and were hysterectomized. The exterior of the uterus was washed with PBS, and conceptuses were recovered from the uterine horns by flushing twice with 40 mL of PBS. The collected conceptuses were examined under a stereomicroscope, and the morphology (i.e., spherical, ovoid, tubular, or elongated) was assessed. Endometrial tissue (~5 g) was removed from the mesometrial side of the uterine horn, immediately snap-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation. A 5-mL ULF sample of the first uterine flushing was centrifuged at 3,000 × *g* at 4 °C for 10 min and preserved at −80 °C until utilized for IL1B, E2, and PGF2α analysis.

Total RNA and Genomic DNA Isolation. Total RNA and genomic DNA were extracted from conceptuses and endometrial tissue. For conceptuses, RNA and DNA were analyzed from six samples from each surrogate gilt ($n = 5$ per treatment). RNA and DNA were extracted from individual $IL1B2^{-/-}$ conceptuses. Due to the entailment of elongated $IL1B2^{+/+}$ conceptuses after the flushing of the uterine horns of surrogate gilts, individual conceptus analysis not possible there; pooled samples containing multiple conceptuses were used. For endometrium, samples of ∼5 mg of tissue were harvested from the mesometrial side of the uterine horn at three different locations in each surrogate gilt ($n = 5$ for $IL1B2^{+/+}$ and $IL1B2^{-/-}$).

Real-Time RT-PCR Gene-Expression Analysis. First-strand cDNA for RT-PCR was synthesized from 500 ng of total RNA, and real-time RT-PCR was performed and quantified on a CFX Connect Real-Time PCR Detection System (Bio-Rad). Expression was measured for conceptus genes using primers specific for *IL1B2* (17), *IL1B1* (17), *FGF4* (44), *BMP4* (57), *IFND* (58), *IFNG* (58), *CYP19A1* (59), *STAR* (60), *PTGS1* (61), *PTGS2* (61), and *SAL1* (61). For endometrial genes, *PGFS* (62), *PTGES* (62), *IL1B2*, *IL1B1*, *SPP1* (46), *IL1R1* (11), *IL1RAP* (11), and *SAL1* expression was measured (primer design is listed in Table S4). Relative expression of sequence-specific products was quantified by the $^{-2\Delta}$CT method. Because RT-PCR analysis showed that porcine β-actin (*ACTB*) gene expression was not statistically different ($P > 0.05$) in the conceptus and endometrial RNA, porcine *ACTB* was used as the endogenous control to standardize the amount of RNA in each reaction.

Measurement of IL1B, E2, and PGF in ULF by ELISA. A pig IL1B ELISA Kit (Abcam) was used to detect porcine IL1B in the uterine flushing. 17β-estradiol in the uterine flushings was measured with an E2 radioimmunoassay as previously described for our laboratory (6). PGF in the collected uterine flushing was measured with a PGF ELISA kit (Cayman Chemical Company). Samples and standards were added in duplicate to the 96-well ELISA plate and were processed according to the manufacturer's directions; then absorbance was measured at 412 nm. Concentrations for IL1B, PGF, and E2 were expressed as nanograms per flush volume (40 mL).

Statistical Analysis. All statistical analyses of quantitative data were performed using R statistical software, version 3.3.2 (63).

AGRICULTURAL SCIENCES

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