



# Inactivation of porcine interleukin-1 $\beta$ results in failure of rapid conceptus elongation

Jeffrey J. Whyte<sup>a</sup>, Ashley E. Meyer<sup>a</sup>, Lee D. Spate<sup>a</sup>, Joshua A. Benne<sup>a</sup>, Raissa Cecil<sup>a</sup>, Melissa S. Samuel<sup>a</sup>, Clifton N. Murphy<sup>a</sup>, Randall S. Prather<sup>a</sup>, and Rodney D. Geisert<sup>a,1</sup>

<sup>a</sup>Division of Animal Sciences, University of Missouri, Columbia, MO 65211

Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved December 1, 2017 (received for review October 13, 2017)

**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 1 $\beta$ , 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<sup>+/+</sup>) conceptuses but did not occur in ILB2-null (IL1B2<sup>-/-</sup>) conceptuses. Although the morphological transition of IL1B2<sup>-/-</sup> 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-1 $\beta$  | pregnancy

**E**stablishment 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- $\tau$ , which interferes with endometrial production of prostaglandin F2 $\alpha$  (PGF2 $\alpha$ ), while synthesis and release of estrogens by pig conceptuses stimulate the endometrial production of PGF2 $\alpha$  to be sequestered and metabolized to an inactive metabolite within the uterine lumen (2–4). While conceptus IFN- $\tau$  and estrogens serve as the maternal recognition signals of pregnancy in ruminants and pigs, respectively (4), conceptuses of both species must expand across the endometrial luminal surface of the uterine horns to inhibit PGF2 $\alpha$  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 PGF2 $\alpha$  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 non-invasive, 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 trophoblast 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 trophoblast (13). Mesoderm expression of bone morphogenetic protein 4 (BMP4) stimulates phosphorylated SMAD1/5/8 in the overlying trophoblast, which could stimulate growth and expansion between the trophoblast 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 1 $\beta$  (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.**

Author contributions: J.J.W., R.S.P., and R.D.G. designed research; J.J.W., A.E.M., L.D.S., J.A.B., R.C., M.S.S., and C.N.M. performed research; M.S.S. cared for animals; J.J.W. analyzed data; and R.D.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This open access article is distributed under [Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 \(CC BY-NC-ND\)](https://creativecommons.org/licenses/by-nc-nd/4.0/).

<sup>1</sup>To whom correspondence should be addressed. Email: geisertr@missouri.edu.

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1718004115/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1718004115/-DCSupplemental).

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. S2A) 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. S2B). 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*<sup>-/-</sup> 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*<sup>-/-</sup> and *IL1B2*<sup>+/+</sup> Fibroblasts.** The two colonies of *IL1B2*<sup>-/-</sup> cells were used for SCNT to produce *IL1B2*<sup>-/-</sup> embryos. Concurrently, identical wild-type (*IL1B2*<sup>+/+</sup>)

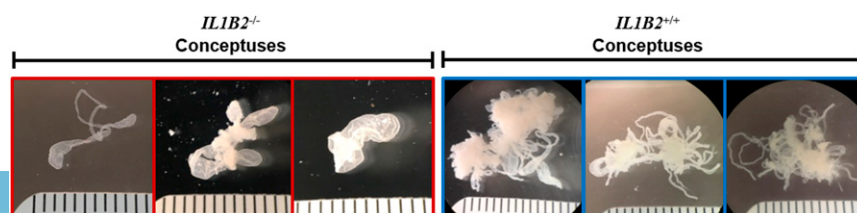
fibroblasts which had undergone electroporation without CRISPR/Cas9 and also had been sorted by FACS selection were used for SCNT to produce *IL1B2*<sup>+/+</sup> embryos. Early in vitro development of *IL1B2*<sup>-/-</sup> embryos to blastocysts was similar to that of *IL1B2*<sup>+/+</sup> nuclear-transferred embryos (range 30–40% across SCNT). Five embryo transfers for both *IL1B2*<sup>-/-</sup> and *IL1B2*<sup>+/+</sup> into recipient gilts were performed. Equivalent numbers of *IL1B2*<sup>-/-</sup> and *IL1B2*<sup>+/+</sup> blastocysts (~43) were transferred to surrogate gilts. The number of embryos reaching day 14 of development was not significantly different in the *IL1B2*<sup>-/-</sup> and *IL1B2*<sup>+/+</sup> recipients (Table S3). In contrast, the number of embryos that were elongated on day 14 was dramatically different in *IL1B2*<sup>-/-</sup> (4/45 = 9%) and *IL1B2*<sup>+/+</sup> (46/58 = 79%) conceptuses, with negligible elongation occurring in the absence of *IL1B2* expression. Morphological abnormalities in *IL1B2*<sup>-/-</sup> recipients include malformed, nonelongated conceptuses with bulbous ends that did not elongate to normal filamentous threads as observed in all *IL1B2*<sup>+/+</sup> 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*<sup>-/-</sup> conceptuses compared with *IL1B2*<sup>+/+</sup> conceptuses (Fig. 2A). Expression of *IL1B2* was ~64-fold greater in *IL1B2*<sup>+/+</sup> than in *IL1B2*<sup>-/-</sup> conceptuses. Suppression of conceptus *IL1B2* gene expression resulted in the inhibition of conceptus IL1B2 secretion during the period of conceptus elongation (Fig. 2B). Total content of IL1B2 protein in uterine luminal flushing (ULF) averaged 6,000 ng in recipients containing *IL1B2*<sup>+/+</sup> conceptuses but was below the sensitivity of the assay in ULFs of recipients containing *IL1B2*<sup>-/-</sup> conceptuses ( $P < 0.008$ ).

**Conceptus *CYP19A1* Gene Expression and ULF Estradiol-17 $\beta$  Analysis.** Conceptus mRNA expression of *CYP19A1* (aromatase) was decreased ( $P < 0.008$ ) in *IL1B2*<sup>-/-</sup> compared with *IL1B2*<sup>+/+</sup> conceptuses (Fig. 3A). Expression of *CYP19A1* was sevenfold greater in *IL1B2*<sup>+/+</sup> than in *IL1B2*<sup>-/-</sup> conceptuses. The content of estradiol-17 $\beta$  (E2) in the ULF was approximately fourfold lower ( $P < 0.05$ ) in recipients containing *IL1B2*<sup>-/-</sup> conceptuses than in those containing *IL1B2*<sup>+/+</sup> conceptuses (Fig. 3B). Although the uterine luminal E2 content of recipients containing *IL1B2*<sup>-/-</sup> conceptuses is significantly less than that in *IL1B2*<sup>+/+</sup> 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 PGF2 $\alpha$  Analysis.** *IL1B2*<sup>-/-</sup> conceptuses had greater mRNA expression for prostaglandin-endoperoxide synthase 2 (*PGTS2*) ( $P < 0.001$ ) than did *IL1B2*<sup>+/+</sup> conceptuses (Table 1). Although *PGTS2* mRNA in *IL1B2*<sup>-/-</sup> conceptuses increased twofold compared with *IL1B2*<sup>+/+</sup> conceptuses, the total uterine luminal content of PGF in the uterine flushings was numerically ( $P < 0.13$ ) greater (243  $\pm$  110 ng) in flushings containing *IL1B2*<sup>+/+</sup> conceptuses than in those containing *IL1B2*<sup>-/-</sup> conceptuses (10  $\pm$  1.7 ng). High uterine PGF content of two flushings from uteri containing *IL1B2*<sup>+/+</sup> 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*<sup>-/-</sup> conceptuses.

**Conceptus Gene Expression.** Gene expression of *IL1B1* and salivary lipocalin (*SAL1*) was very low in both *IL1B2*<sup>-/-</sup> and *IL1B2*<sup>+/+</sup>



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



**Table 1. Conceptus gene expression**

Gene	<i>IL1B2</i> <sup>-/-</sup>		<i>IL1B2</i> <sup>+/+</sup>		Probability
	mean 2 <sup>-ΔCt</sup>	SD	mean 2 <sup>-ΔCt</sup>	SD	
<i>BMP4</i>	0.00089	0.00054	0.00095	0.00023	N.S.
<i>FGF4</i>	0.00030	0.00035	0.00056	0.00023	N.S.
<i>IFND</i>	0.00078	0.00023	0.00037	0.00010	0.02
<i>IFNG</i>	0.59818	0.11588	0.62055	0.19241	N.S.
<i>STAR</i>	0.00027	0.00012	0.00040	0.00019	N.S.
<i>PGTS2</i>	0.00092	0.00007	0.00046	0.00016	0.001

N.S., not significant.

of elongation. Thus, all *IL1B2*<sup>+/+</sup> and *IL1B2*<sup>-/-</sup> 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 non-human 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*<sup>-/-</sup> embryos to blastocysts was similar to that of *IL1B2*<sup>+/+</sup> nuclear-transferred embryos, and in utero blastocyst hatching and growth of spherical conceptuses were not different between *IL1B2*<sup>-/-</sup> and *IL1B2*<sup>+/+</sup> embryos. However, during the period of rapid conceptus elongation, *IL1B2*<sup>-/-</sup> conceptuses developed an abnormal oblong morphology that failed to elongate in comparison with the long, filamentous *IL1B2*<sup>+/+</sup> conceptuses. The synthesis and secretion of IL1B2 was ablated in *IL1B2*<sup>-/-</sup> conceptuses, as IL1B2 was absent in ULFs of recipients containing *IL1B2*<sup>-/-</sup> 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*<sup>-/-</sup> and *IL1B2*<sup>+/+</sup> conceptuses, while the expression of *IFND* and *PGTS2* was slightly greater in *IL1B2*<sup>-/-</sup> conceptuses. These results indicate that, although *IL1B2*<sup>-/-</sup> 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*<sup>-/-</sup> conceptus is not known, as the luminal content of PGF tended to be lower in the uterine flushing of *IL1B2*<sup>-/-</sup> conceptus recipients than in the uterine flushing of *IL1B2*<sup>+/+</sup> 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*<sup>-/-</sup> and *IL1B2*<sup>+/+</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> and *IL1B2*<sup>+/+</sup> conceptuses, and *IFND* expression was even twofold greater in *IL1B2*<sup>-/-</sup> conceptuses. These results again indicate that the failure of *IL1B2*<sup>-/-</sup> 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 trophoblast 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 trophoblast cell 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 trophoblast 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*<sup>-/-</sup> and *IL1B2*<sup>+/+</sup> conceptuses will be necessary to determine the alteration in gene expression during elongation.

**Table 2. Endometrial gene expression**

Gene	<i>IL1B2</i> <sup>-/-</sup>		<i>IL1B2</i> <sup>+/+</sup>		Probability
	mean 2 <sup>-ΔCt</sup>	SD	mean 2 <sup>-ΔCt</sup>	SD	
<i>IL1R1</i>	0.01457	0.00233	0.01549	0.00275	N.S.
<i>IL1RAP</i>	0.01167	0.00243	0.00773	0.00053	0.02
<i>PGFS</i>	0.01396	0.00185	0.00825	0.00194	0.002
<i>PTGES</i>	0.00215	0.00025	0.00210	0.00034	N.S.
<i>SAL1</i>	0.19210	0.03115	0.19027	0.01835	N.S.
<i>SPP1</i>	0.02946	0.00372	0.02138	0.00409	0.01

N.S., not significant.

There is a spatiotemporal increase 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  $\alpha$  (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*<sup>-/-</sup> conceptuses. The decrease in *CYP19A1* expression and E2 synthesis in the *IL1B2*<sup>-/-</sup> 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 NF $\kappa$ B (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*<sup>+/+</sup> or *IL1B2*<sup>-/-</sup> conceptuses. There was actually a slight increase in endometrial expression of *IL1RAP*, *PGFS*, and *SPP1* in *IL1B2*<sup>-/-</sup> recipients. Thus, the failure of *IL1B2*<sup>-/-</sup> 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/](http://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-Offfinder ([www.rgenome.net/cas-offfinder/](http://www.rgenome.net/cas-offfinder/)) (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:NSRRRC\_0005) were thawed, cultured, and transfected with the four ZsGreen1-Cas9-sgRNA vectors targeting *IL1B2* (with sgRNAs 1, 2, 4, and 5;

0.5  $\mu$ g 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*<sup>-/-</sup> 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*<sup>-/-</sup> or *IL1B2*<sup>+/+</sup>) 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  $\times$  g at 4 °C for 10 min and preserved at -80 °C until utilized for *IL1B*, E2, and PGF2 $\alpha$  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*<sup>-/-</sup> conceptuses. Due to the entailment of elongated *IL1B2*<sup>+/+</sup> 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*<sup>+/+</sup> and *IL1B2*<sup>-/-</sup>).

**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 <sup>-2 $\Delta$</sup> CT method. Because RT-PCR analysis showed that porcine  $\beta$ -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. 17 $\beta$ -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).

**ACKNOWLEDGMENTS.** We thank Dr. Chris Tuggle and Dr. Martine Schroyen for providing IL1B2 sequence data; Dr. Kristin Whitworth and Madison Hennessy for technical assistance; and the National Swine Resource and Research Center (NIH Grant U42OD011140 to R.S.P.) for providing

swine fetal-derived fibroblasts cells. This project was supported by US Department of Agriculture, National Institute of Food and Agriculture, Agriculture and Food Research Initiative Competitive Grant 2013-67015-21023 (to J.J.W., R.S.P., and R.D.G.).

- Geisert RD, et al. (2017) Rapid conceptus elongation in the pig: An interleukin 1 beta 2 and estrogen-regulated phenomenon. *Mol Reprod Dev* 84:760–774.
- Spencer TE, Forde N, Lonergan P (2016) Insights into conceptus elongation and establishment of pregnancy in ruminants. *Reprod Fertil Dev* 29:84–100.
- Bazer FW, Marengo SR, Geisert RD, Thatcher WW (1984) Exocrine versus endocrine secretion of prostaglandin F in the control of pregnancy in swine. *Anim Reprod Sci* 7:115–132.
- Bazer FW, et al. (2010) Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Mol Hum Reprod* 16:135–152.
- Gray CA, et al. (2001) Endometrial glands are required for preimplantation conceptus elongation and survival. *Biol Reprod* 64:1608–1613.
- Dhindsa DS, Dziuk PJ (1968) Influence of varying the proportion of uterus occupied by embryos on maintenance of pregnancy in the pig. *J Anim Sci* 27:668–672.
- Anderson LL, Rathmacher RP, Melampy RM (1966) The uterus and unilateral regression of corpora lutea in the pig. *Am J Physiol* 210:611–614.
- Lonergan P, Forde N (2015) The role of progesterone in maternal recognition of pregnancy in domestic ruminants. *Adv Anat Embryol Cell Biol* 216:87–104.
- Geisert RD, Brookbank JW, Roberts RM, Bazer FW (1982) Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. *Biol Reprod* 27:941–955.
- Matton BA, Overstrom EW, Albertini DF (1990) Transitions in trophoblast cellular shape and cytoskeletal organization in the elongating pig blastocyst. *Biol Reprod* 42:195–205.
- Ross JW, Malayer JR, Ritchey JW, Geisert RD (2003) Characterization of the interleukin-1beta system during porcine trophoblastic elongation and early placental attachment. *Biol Reprod* 69:1251–1259.
- Geisert RD, Johnson GA, Burghardt RC (2015) Implantation and Establishment of Pregnancy in the Pig. *Regulation of Implantation and Establishment of Pregnancy in Mammals: Tribute to 45 Year Anniversary of Roger V. Short's "Maternal Recognition of Pregnancy"*, Advances in Anatomy, Embryology and Cell Biology, eds Geisert RD, Bazer FW (Springer, Cham, Switzerland), pp 137–164.
- Valdez Magaña G, Rodríguez A, Zhang H, Webb R, Alberio R (2014) Paracrine effects of embryo-derived FGF4 and BMP4 during pig trophoblast elongation. *Dev Biol* 387:15–27.
- Blomberg L, Hashizume K, Viebahn C (2008) Blastocyst elongation, trophoblastic differentiation, and embryonic pattern formation. *Reproduction* 135:181–195.
- Jeong W, Lee J, Bazer FW, Song G, Kim J (2016) Fibroblast growth factor 4-induced migration of porcine trophoblast cells is mediated via the AKT cell signaling pathway. *Mol Cell Endocrinol* 419:208–216.
- Tuo W, Harney JP, Bazer FW (1996) Developmentally regulated expression of interleukin-1 beta by peri-implantation conceptuses in swine. *J Reprod Immunol* 31:185–198.
- Mathew DJ, et al. (2015) Activation of the transcription factor nuclear factor-kappa B in the uterine luminal epithelial cells by interleukin 1 Beta 2: A novel interleukin 1 expressed by the elongating pig conceptus. *Biol Reprod* 92:107.
- Degrelle SA, Blomberg LeAnn, Garrett WM, Li RW, Talbot NC (2009) Comparative proteomic and regulatory network analyses of the elongating pig conceptus. *Proteomics* 9:2678–2694.
- Geisert RD, Renegar RH, Thatcher WW, Roberts RM, Bazer FW (1982) Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. *Biol Reprod* 27:925–939.
- Strakova Z, et al. (2005) In vivo infusion of interleukin-1beta and chorionic gonadotropin induces endometrial changes that mimic early pregnancy events in the baboon. *Endocrinology* 146:4097–4104.
- De los Santos MJ, et al. (1996) Role of endometrial factors in regulating secretion of components of the immunoreactive human embryonic interleukin-1 system during embryonic development. *Biol Reprod* 54:563–574.
- Bourdieu A, Martel V, Akoum A (2014) Synchronous regulation of the determinants of endometrial receptivity to interleukin 1 at key stages of early embryo implantation in vivo. *Fertil Steril* 101:1183–1193.
- Correia-Álvarez E, et al. (2015) Expression and localization of interleukin 1 beta and interleukin 1 receptor (type I) in the bovine endometrium and embryo. *J Reprod Immunol* 110:1–13.
- Paulesu L, Jantra S, Ietta F, Brizzi R, Bigliardi E (2008) Interleukin-1 in reproductive strategies. *Evol Dev* 10:778–788.
- Smith TP, et al. (2001) Mapping of expressed sequence tags from a porcine early embryonic cDNA library. *Anim Genet* 32:66–72.
- Blomberg LA, et al. (2005) Serial analysis of gene expression during elongation of the peri-implantation porcine trophoblast (conceptus). *Physiol Genomics* 20:188–194.
- Blomberg LA, et al. (2006) Transcriptome profiling of the tubular porcine conceptus identifies the differential regulation of growth and developmentally associated genes. *Mol Reprod Dev* 73:1491–1502.
- Lee SH, et al. (2005) Transcriptional profiling using a novel cDNA array identifies differential gene expression during porcine embryo elongation. *Mol Reprod Dev* 71:129–139.
- Ali S, Mann DA (2004) Signal transduction via the NF-kappaB pathway: A targeted treatment modality for infection, inflammation and repair. *Cell Biochem Funct* 22:67–79.
- Davis DL, Pakrasi PL, Dey SK (1983) Prostaglandins in swine blastocysts. *Biol Reprod* 28:1114–1118.
- Franczak A, Zmijewska A, Kurowicka B, Wojciechowicz B, Kotwica G (2010) Interleukin 1β-induced synthesis and secretion of prostaglandin E<sub>2</sub> in the porcine uterus during various periods of pregnancy and the estrous cycle. *J Physiol Pharmacol* 61:733–742.
- Geisert RD, Rasby RJ, Minton JE, Wettemann RP (1986) Role of prostaglandins in preimplantation development of porcine blastocysts. *Prostaglandins* 31:191–204.
- La Bonnardière C, et al. (1991) Production of two species of interferon by Large White and Meishan pig conceptuses during the peri-attachment period. *J Reprod Fertil* 91:469–478.
- Prutsch N, et al. (2012) The role of interleukin-1β in human trophoblast motility. *Placenta* 33:696–703.
- Librach CL, et al. (1994) Interleukin-1 beta regulates human cytotrophoblast metalloproteinase activity and invasion in vitro. *J Biol Chem* 269:17125–17131.
- Fazleabas AT, Geisert RD, Bazer FW, Roberts RM (1983) Relationship between release of plasminogen activator and estrogen by blastocysts and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig. *Biol Reprod* 29:225–238.
- Roberts RM, Bazer FW (1988) The functions of uterine secretions. *J Reprod Fertil* 82:875–892.
- Windoffer R, Kölsch A, Wöll S, Leube RE (2006) Focal adhesions are hotspots for keratin filament precursor formation. *J Cell Biol* 173:341–348.
- Matsumoto H, Daikoku T, Wang H, Sato E, Dey SK (2004) Differential expression of ezrin/radixin/moesin (ERM) and ERM-associated adhesion molecules in the blastocyst and uterus suggests their functions during implantation. *Biol Reprod* 70:729–736.
- Karmakar S, Das C (2004) Modulation of ezrin and E-cadherin expression by IL-1beta and TGF-beta1 in human trophoblasts. *J Reprod Immunol* 64:9–29.
- Nestler JE (1993) Interleukin-1 stimulates the aromatase activity of human placental cytotrophoblasts. *Endocrinology* 132:566–570.
- Johnson GA, Burghardt RC, Bazer FW (2014) Osteopontin: A leading candidate adhesion molecule for implantation in pigs and sheep. *J Anim Sci Biotechnol* 5:56.
- Laughlin TD, et al. (2017) Development of pre-implantation porcine blastocysts cultured within alginate hydrogel systems either supplemented with secreted phosphoprotein 1 or conjugated with Arg-Gly-Asp peptide. *Reprod Fertil Dev* 29:2345–2356.
- Jeong W, Kim J, Bazer FW, Song G, Kim J (2016) Stimulatory effects of interleukin-1 beta on development of porcine uterine epithelial cell are mediated by activation of the ERK1/2 MAPK cell signaling cascade. *Mol Cell Endocrinol* 419:225–234.
- Seo H, Kim M, Choi Y, Ka H (2011) Salivary lipocalin is uniquely expressed in the uterine endometrial glands at the time of conceptus implantation and induced by interleukin 1beta in pigs. *Biol Reprod* 84:279–287.
- Kim M, et al. (2015) Analysis of stage-specific gene expression profiles in the uterine endometrium during pregnancy in pigs. *PLoS One* 10:e0143436.
- Garlow JE, et al. (2002) Analysis of osteopontin at the maternal-placental interface in pigs. *Biol Reprod* 66:718–725.
- Bae S, Kweon J, Kim HS, Kim JS (2014) Microhomology-based choice of Cas9 nuclease target sites. *Nat Methods* 11:705–706.
- Bae S, Park J, Kim JS (2014) Cas-OFFinder: A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* 30:1473–1475.
- Whyte JJ, et al. (2011) Gene targeting with zinc finger nucleases to produce cloned eGFP knockout pigs. *Mol Reprod Dev* 78:2.
- Whitworth KM, et al. (2014) Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. *Biol Reprod* 91:78.
- Macháty Z, Wang WH, Day BN, Prather RS (1997) Complete activation of porcine oocytes induced by the sulfhydryl reagent, thimerosal. *Biol Reprod* 57:1123–1127.
- Zhao J, et al. (2009) Significant improvement in cloning efficiency of an inbred miniatur pig by histone deacetylase inhibitor treatment after somatic cell nuclear transfer. *Biol Reprod* 81:525–530.
- Spate LD, Brown A, Redel BK, Whitworth KM, Prather RS (2015) P548 can replace bovine serum albumin in pig embryo culture medium, and improve in vitro embryo development by phosphorylating AKT. *Mol Reprod Dev* 82:315–320.
- Lee K, et al. (2013) Piglets produced from cloned blastocysts cultured in vitro with GM-CSF. *Mol Reprod Dev* 80:145–154.
- Mathew DJ, et al. (2011) Uterine progesterone receptor expression, conceptus development, and ovarian function in pigs treated with RU 486 during early pregnancy. *Biol Reprod* 84:130–139.
- Hall VJ, Christensen J, Gao Y, Schmidt MH, Hyttel P (2009) Porcine pluripotency cell signaling develops from the inner cell mass to the epiblast during early development. *Dev Dyn* 238:2014–2024.
- Joyce MM, et al. (2007) Pig conceptuses secrete estrogen and interferons to differentially regulate uterine STAT1 in a temporal and cell type-specific manner. *Endocrinology* 148:4420–4431.
- Ebeling S, Töpfer D, Weitzel JM, Meinecke B (2011) Bone morphogenetic protein-6 (BMP-6): mRNA expression and effect on steroidogenesis during in vitro maturation of porcine cumulus oocyte complexes. *Reprod Fertil Dev* 23:1034–1042.
- Bolzan E, et al. (2013) The novel effect of hCG administration on luteal function maintenance during the estrous cycle/pregnancy and early embryo development in the pig. *Pol J Vet Sci* 16:323–332.
- Jeong W, et al. (2016) Lysophosphatidic acid (LPA) receptor 3-mediated LPA signal transduction pathways: A possible relationship with early development of peri-implantation porcine conceptus. *Biol Reprod* 94:104.
- Blitek A, Wacławik A, Kaczmarek MM, Kiewisz J, Ziecik AJ (2010) Effect of estrus induction on prostaglandin content and prostaglandin synthase enzyme expression in the uterus of early pregnant pigs. *Theriogenology* 73:1244–1256.
- R Core Team (2016) R: A Language and Environment for Statistical Computing (R Project for Statistical Computing, Vienna), Version 3.4.3.