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RESEARCH

Expression of tripartite motif family-like protein 1 and 2 in early conceptus development and placentation in the pig

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Abstract

The eutherian placenta is highly complex, evolving to regulate the inflammatory phase of pregnancy during conceptus attachment and placental tissue development. Tripartite motif family-like (TRIMLs) proteins are implicated in downregulating inflammation. In mammals, TRIML1 and TRIML2 show preferential expression in gonads, preimplantation embryos and placenta. TRIML1 domains differ between eutherians and marsupials, while TRIML2 is absent in marsupials, suggesting it may play a unique role in regulating the inflammatory phase during conceptus attachment, critical for establishing and maintaining pregnancy to term. This study aimed to investigate the expression pattern of *TRIML1* and *TRIML2* in various tissues, as well as during embryo development, conceptus attachment, and placental formation in pigs. Transcripts for *TRIML2* were detected in embryos, conceptuses, extraembryonic membranes, ovary and testis but not in any of the other tissues examined. In contrast, *TRIML1* expression was only observed in testis. *In situ* hybridization of *TRIML1* and *TRIML2* confirmed these results. The specific expression of *TRIML2* in immune privileged sites is consistent with it serving as an anti-inflammatory factor to provide immunological protection of the eutherian placenta. To further investigate the role of TRIML2, CRISPR/Cas9 gene editing was employed to knock out either TRIML1 (control) or TRIML2. *TRIML1*$^{-/-}$ and *TRIML2*$^{-/-}$ porcine fetal fibroblasts were used for somatic cell nuclear transfer, and the resulting embryos were transferred into surrogate gilts. Early conceptus and placental development were not affected by the loss of conceptus TRIML2. Although a tissue-specific expression pattern was found, TRIML1 or TRIML2 are not required for pregnancy establishment in the pig.

Lay summary

This study investigates the expression and possible roles of two specific proteins in pigs. These proteins are implicated in regulating inflammation and are thought to be important for the proper development of the placenta, which is essential for a successful pregnancy. TRIML2 was found in embryos and certain tissues, including ovary, testis and placenta, while TRIML1 appears only in the testis. We further used gene-editing techniques to generate pig embryos lacking these proteins to test whether their absence would affect early pregnancy and placental development. Lack of either TRIML1 or TRIML2 did not disrupt the early stages of pregnancy or placental formation, indicating that these proteins may not be critical for these processes in pigs.

Keywords: conceptus; TRIML2; implantation; pig; pregnancy

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Introduction

With the exception of monotremes, most mammalian species are viviparous. Viviparity is not exclusive to mammals, as there are numerous examples of closely related reptilian species that can be oviparous or viviparous (Amoroso 1952, 1968, Guillette & Hotton 1986). Eutherian (placental) mammals have various lengths of extended pregnancy with birth of young. Although marsupials are viviparous, they have a more simplified intrauterine development and a short gestation (Renfree & Shaw 2021). Marsupials have a choriovitelline placenta, but some also have a short-lived chorioallantois placenta (Renfree *et al.* 2013). Even in the egg-laying monotreme, the embryo is supported by a simple placenta during early development. Both the monotreme hatchling and marsupial newborn rely on a repertoire of uterine milk proteins for further development (Renfree & Shaw 2021).

Viviparity required fundamental changes to adapt the placenta for the prolonged gestation in the maternal uterus. All eutherian mammals feature a version of a placenta, containing a chorion, yolk sac, allantois and amnion. However, the type of placentation in mammals can be quite varied, from the invasive hemochorial placenta of humans and primates to the noninvasive epitheliochorial placenta of the pig (see Geisert & Spencer 2021). Extended lengths of gestation also required establishment of maternal immunotolerance to the semi-allographic conceptus and later development of the placenta. The eutherian placenta is very complex and has evolved by regulating the inflammatory phase of pregnancy during the period of conceptus attachment to the uterine surface before the formation of the placental tissues (Stadtmauer & Wagner 2020*a*,*b*). Following the pre-attachment phase of conceptus development during pregnancy, conceptus attachment to the uterine epithelial surface induces a localized pro-inflammatory response within the endometrium (Griffith *et al.* 2017). In marsupials, shedding of the acellular egg coat and the initiation of attachment of the chorion to the uterine surface immediately stimulates a pro-inflammatory response that induces parturition (Renfree & Shaw 2021). In eutherian mammals, conceptus attachment stimulates a highly regulated localized immune and pro-inflammatory response in the endometrium to increase angiogenesis and blood flow necessary to support the placenta and fetus to term. This pro-inflammatory phase is short-lived, as the development of the placenta in eutherian mammals has evolved to have an anti-inflammatory phase between the inflammatory attachment reaction and the inflammatory response to initiate parturition (Chavan *et al.* 2017). Evolutionary pressures have shaped changes in placental development and fetomaternal contact to support therian reproduction.

Tripartite motif (TRIM) proteins are a family of more than 80 proteins that have undergone extensive replication throughout evolution. In general, TRIM proteins consist of an N-terminal conserved really interesting new gene (RING) domain followed by one or two B-box domains and a coil–coil domain (Ozato *et al.* 2008, Hatakeyama 2017). TRIM proteins act as E3 ubiquitin ligases (Ozato *et al.* 2008) and are involved with multiple cellular functions, with roles in antiviral activity, inflammation and innate immunity (van Gent *et al.* 2018, Giraldo *et al.* 2020). The tripartite motif family-like 2 (TRIML2) is only present in eutherian species and is unique in that it lacks or contains a modified region of both the RING and B-box domains (Zhang *et al.* 2020). Loss of the RING domain removed E3 ubiquitin ligase activity of the protein. Although its specific molecular functions are not well understood, TRIML2 has been shown to be involved with a reduction in proinflammatory cytokine production in human trophoblasts (Zhang *et al.* 2020) and proapoptotic events by interacting with the p53 signaling pathway (Kung *et al.* 2015). Evidence supports that *TRIML2*'s origin is due to a gene duplication event in the lineage of eutherian amniotes, which also gave rise to *TRIML1* (Zhang *et al.* 2020). TRIML1 shows preferential expression in the testis, but is not unique to only the eutherian amniote, as there is a marsupial TRIML1; however, the marsupial TRIML1 contains a B-box domain that the eutherian TRIML1 does not contain (Zhang *et al.* 2020). *TRIML1* and *TRIML2* are expressed in the human and mouse placenta, brain and testis (Zhang *et al.* 2020), with the brain and testis being considered immune privileged sites. Information regarding the expression pattern of *TRIML1* and *TRIML2* in other species is scarce.

The expression of TRIML1 and TRIML2 in the placenta of humans and mice (Zhang *et al.* 2020) may function to regulate the pro-inflammatory response to trophoblast attachment to the endometrial surface and interaction with the chorion following placentation. Knockdown of *Triml1* expression in mice resulted in fewer developed blastocysts that failed at producing neonates after transfer of embryos. This indicated that murine Triml1 was expressed in the embryo before implantation (Tian *et al.* 2009) and is necessary for embryo survival. The epitheliochorial placentation in the pig provides a unique model to evaluate the expression and further requirement of TRIML1 and TRIML2 during early embryo development, conceptus attachment and placentation. The objectives of this study were to i) determine the expression patterns of *TRIML1* and *TRIML2* during early embryo development, conceptus attachment and placental formation during pregnancy in the pig, and ii) evaluate the role of placental TRIML1 or TRIML2 during establishment and maintenance of pregnancy through gene editing of conceptus *TRIML1* and *TRIML2*.

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Materials and methods

Animals

All procedures used in this study were conducted in accordance with the Guide for Care and Use of Agricultural Animals in Research and Teaching and approved by the University of Missouri-Columbia Institutional Animal Care and Use Committee under Protocol 8813. Recipient gilts used for embryo transfer were Large White by Landrace crossbred gilts of similar age (8–10 months) and weight (100–130 kg). Gilts were observed for estrous behavior twice daily, with the onset of estrus designated as day 0 of the estrous cycle.

TRIML1 and TRIML2 tissue expression

Pig tissues (brain, muscle, stomach, pancreas, adipose tissue, colon, esophagus, kidney, heart, lung, duodenum, mammary gland, endometrium, oviduct, ovary, follicle, corpus luteum and testis) were collected from adult wild-type (WT) pigs for RNA extraction and analyzed by end-point PCR for *TRIML1* and *TRIML2* expression. In addition, porcine cumulus–oocyte complexes, oocytes in metaphase II (matured), *in vitro* fertilized 2-cell, 4-cell and 8-cell stage embryos, morulae, blastocysts, day 12 and 15 conceptuses, day 21 placenta and day 30 chorioallantois and amnion were subjected to RNA isolation and analyzed for *TRIML1* and *TRIML2* gene expression.

Total RNA isolation

Total RNA from tissue was obtained by using the Trizol-based RNeasy Mini Kit (Qiagen, USA; Cat. 74104) according to the manufacturer's protocol. Briefly, frozen tissue samples were homogenized in Trizol using a gentleMACS tissue dissociator (Miltenyi Biotec, USA) and the lysates loaded into RNeasy spin columns. Total RNA from pools of porcine cumulus–oocyte complexes, oocytes in metaphase II, 2-cell, 4-cell and 8-cell stage embryos, morulae and blastocysts was isolated using the PicoPure RNA isolation kit (Applied Biosystems, USA; Cat. 12204-01) following the manufacturer's protocol. Membrane-bound samples were subjected to on-column DNase treatment (RNase-Free DNase Set; Qiagen; Cat. 79254) to eliminate genomic DNA contamination. Total RNA yield and purity (260/280 nm ratio) were established by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). RNA samples were stored at −80°C.

cDNA synthesis and PCR analysis

Total RNA (1 μg for tissue samples; 500 ng for oocyte/embryo pools) was reverse transcribed in a 20 μL reaction mix by using the iScript cDNA Synthesis kit (BioRad, USA; Cat. 1708891) according to the manufacturer's protocol. Amplification of cDNA was performed in an Eppendorf Mastercycler Pro and incubated at 25°C for 5 min, reverse transcribed at 42°C for 30 min, and heat-inactivated at 85°C for 5 min. Stocks of cDNA were stored at −20°C until used for PCR. Tissue gene expression was assessed by end-point PCR by using primers specific for *TRIML1* and *TRIML2* mRNA (Supplementary Table 1 (see section on Supplementary materials given at the end of the article)). Exon–exon junction spanning primers were designed based on the *Sus scrofa* GenBank Ref-Seq mRNA (NCBI). Porcine *GAPDH* (Supplementary Table 1) was used as a positive control for the cDNA. PCR reactions were carried out in a final volume of 25 μL using OneTaq Hot Start DNA Polymerase (New England BioLabs, USA; Cat. M0481S). Annealing temperatures were optimized for each primer assay. PCR products were run on a 2% agarose ethidium bromide gel for visualization.

TRIML1 and *TRIML2 in situ* hybridization assays

In situ hybridization was utilized to determine tissue expression and localization of *TRIML1* in testis and *TRIML2* in testis, conceptuses and placentae in the pig by using the RNAscope Multiplex Fluorescent Reagent Kit v2 Assay (ACDbio, USA). The procedures followed manufacturer protocols. In brief, paraffin-embedded sections (5 μm thickness) were baked at 60°C for 1 h, deparaffinized in xylene, and rehydrated in a gradient of ethanol. Sections were then incubated in RNAscope hydrogen peroxidase for 10 min, followed by incubation in RNAscope 1× target retrieval buffer for 15 min at 98°C. Tissue sections were outlined with a hydrophobic barrier pen and incubated with RNAscope protease plus reagent for 15 min at 40°C. The mRNA in the tissue was hybridized by incubating in custom-designed RNAscope probes for *Sus scrofa TRIML1* (Ss-TRIML1-No-XHsMm; 1:50 dilution) or *TRIML2* (Ss-TRIML2-O1; 1:50 dilution) for 2 h at 40°C. Slides were sequentially incubated in RNAscope amplifier components and tagged with TSA vivid fluorescent dyes (1:2,000 dilution). An RNAscope DapB probe was used as a negative control to evaluate nonspecific signals. Nuclei were stained with DAPI and slides were coverslipped with VectaMount mounting medium (VectorLabs, USA). Images were captured with a Leica DM5500 B upright microscope and a Leica DFC450 C camera using Leica Application Suite X (LAS X).

Immunohistochemistry for TRIML1

In addition to *in situ* hybridization, immunohistochemistry was performed on fixed porcine testis tissue to determine tissue localization of TRIML1 protein. Paraffin-embedded (5 μm thickness) sections were deparaffinized and pretreated for epitope retrieval by incubating sections for 30 min in boiling Reveal Decloaker (pH 6.0; Biocare Medical, USA; Cat. RV1000M). After cooling to room temperature,

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sections were blocked with 6% hydrogen peroxide in methanol for 10 min at room temperature. Incubation with 2% normal goat serum for 30 min was used for nonimmune blocking. Sections were incubated with rabbit anti-TRIML1 antibody (Cusabio, USA; Cat. CSB-PA822741LA01HU; 1:500 dilution) overnight at 4°C. Slides were then incubated with a biotinylated anti-rabbit secondary antibody and the Vectastain Elite ABC (VectorLabs) reagents according to the manufacturer's protocol. Staining was developed with 3,3′-diaminobenzidine (DAB) substrate (VectorLabs) for 5 min. Sections that did not receive the primary antibody were used as a negative control. Samples were not counterstained to enhance visualization of DAB labeling. Sections were coverslipped with synthetic mounting medium before imaging. Images were captured in bright-field with a Leica DM5500 B upright microscope and Leica DFC450 C camera using a Leica Application Suite X (LAS X).

CRISPR/Cas9 guides design and transfection of porcine fetal-derived fibroblast cells

Guide RNAs (gRNAs) were designed to specifically target the *Sus scrofa TRIML1* and *TRIML2* genes based on the NCBI genome sequence by using the CRISPick design tool (Broad Institute). The pig *TRIML1* and *TRIML2* genes are located on chromosome 17, where *TRIML1* contains six exons while *TRIML2* contains eight. Guide RNAs for *TRIML1* were designed to span the start codon on exon 1. Guide RNAs for *TRIML2* are located on exon 2 and exon 3. Guide sequences are provided in Supplementary Table 2. To minimize off-targeting events, each gRNA was tested by using NCBI Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Cas-OFFinder (https://www.rgenome.net/cas-offinder/; Bae *et al.* 2014) tool. Annealed gRNA oligonucleotides were cloned into the pX330-mCherry vector (Addgene, USA), which contains expression cassettes for human codon-optimized *Cas9* and the chimeric gRNA. The ability of the gRNA pairs to cleave was tested *in vitro*. Two guides for each *TRIML1* and *TRIML2* were transfected together to optimize cutting efficiency. Porcine fetal-derived fibroblasts at a final concentration of 1×10^6 cells/mL were electroporated with three consecutive 250 V, 1 ms square wave pulses in a 2 mm gap cuvette in the presence of 2 μg of the pX330-mCherry vector harboring each of the gRNA (4 μg in total) using methods previously described (Sullivan *et al.* 2024).

Clonal expansion and colony screening

Following transfection, transfected cells were plated using a dilution curve to promote colony growth from a single cell. Fetal fibroblast cells were seeded onto 100 mm cell culture dishes at a low density that allowed single-cell colony formation. The cell culture media consisted of Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 0.5% GlutaMax (Gibco), 12% fetal bovine serum (HyClone, USA), 2 ng/mL fibroblast growth factor (Sigma-Aldrich, USA) and gentamicin (Gibco). After 10 days, cell colonies were individually picked, propagated and screened for biallelic genomic modifications via PCR. Primers for *Sus scrofa TRIML1* and *TRIML2* (Supplementary Table 1) were designed based on the *Sscrofa11.1* GenBank genome sequence (NCBI). PCR reactions were carried out in a final volume of 25 μL using LA *Taq* DNA Polymerase (Takara, USA). The PCR products were run on a 1.5% agarose gel stained with ethidium bromide and bands were screened for biallelic insertions or deletions. The PCR amplicon from individual cell colonies with biallelic edits was cloned into a pCR™ 2.1-TOPO™ TA vector (Invitrogen, USA) and TOPO-cloned colonies were submitted to Sanger sequencing to identify edits on each allele. Colony '95' contained a 368-base pair deletion spanning the start codon for *TRIML1* (Supplementary Fig. 1). No WT alleles were detected. Colony '153' contained a frameshifting 328-base pair deletion flanking exons 2 and 3 for *TRIML2* (Supplementary Fig. 1). No WT alleles were detected. Cells from these two colonies were propagated in 6-well plates, cryopreserved and used to derive *TRIML1*$^{-/-}$ and *TRIML2*$^{-/-}$ embryos.

Somatic cell nuclear transfer (SCNT)

Since *TRIML1* transcripts were not detected in early embryos and conceptuses, SCNT-derived *TRIML1*$^{-/-}$ embryos were used in this study as control counterparts for *TRIML2*$^{-/-}$ pregnancies. *TRIML1*$^{-/-}$ and *TRIML2*$^{-/-}$ fibroblast cells at ~80% confluency were used as donor cells for SCNT as previously described (Whitworth *et al.* 2014) with modifications to the maturation system (Yuan *et al.* 2017). To produce embryos *in vitro*, ovaries from prepubertal gilts were obtained from either a slaughterhouse (Banner Creek, LLC, USA) or purchased from Applied Reproductive Technologies (USA). Oocytes were matured *in vitro* and the cumulus cells were removed with 0.03% hyaluronidase and gentle vortexing. The polar body and adjacent cytoplasm containing the metaphase II plate were removed from the oocyte by micromanipulation. A donor fibroblast cell (*TRIML1*$^{-/-}$ or *TRIML2*$^{-/-}$) was then inserted into the perivitelline space (Lai & Prather 2003) and electrically fused to the ooplasm with two DC pulses at 1.2 kV/cm for 30 μsec using a BTX Electro Cell Manipulator (Harvard Apparatus, USA). After fusion, the cloned zygotes were chemically activated using phenanthroline (Uh *et al.* 2019) and cultured for 14–16 h in histone deacetylation inhibitor scriptaid. Embryos were then removed from scriptaid and cultured in MU4 media for 5–6 days (Spate *et al.* 2015, Chen *et al.* 2018, 2020).

Embryo transfer

Recipient gilts were monitored for signs of estrus and used for embryo transfer. Morula to blastocyst stage embryos (n = 30–50) were surgically transferred into the oviduct of the recipient gilts near the ampullary–isthmic junction on day 4 or 5 after first standing estrus as previously described (Lee *et al.* 2013).

Embryo collection

Recipient gilts were euthanized via jugular injection of pentobarbital sodium and phenytoin sodium (Euthasol; Virbac) on either day 15 (n = 1 *TRIML2*$^{-/-}$), 17 (n = 1 *TRIML2*$^{-/-}$), 21–22 (n = 6 *TRIML1*$^{-/-}$, n = 4 *TRIML2*$^{-/-}$) or 34–36 (n = 4 *TRIML1*$^{-/-}$, n = 3 *TRIML2*$^{-/-}$) of gestation. The day of pregnancy for conceptus/fetus collection is based on the day of recipient estrous cycle. The reproductive tracts were excised from the abdomen, placed on ice and immediately transported to the laboratory for processing. The uterine horns were rinsed and trimmed free of the mesometrium. Day 15 and 17 conceptuses were recovered from the uterus by flushing each uterine horn twice with 30 mL of PBS. Conceptuses retrieved from the uterine luminal flushings were examined and the morphology and viability assessed. Before flushing, a segment of each uterine horn near the uterine body was clamped, cut transversely and the section processed for fixation and paraffin-embedding. Conceptuses and endometrial tissue samples pooled from five random sections along the mesometrial side of each uterine horn were placed into individual 1.5 mL microtubes, flash frozen in liquid nitrogen and stored at −80°C until used for RNA or genomic DNA extraction.

Placental tissue and fetuses were dissected from day 21–22 and day 34–36 pregnancies by cutting down along the anti-mesometrial side of the uterine horn, flash frozen and stored as previously described. Sections of the uterine horn with the attached placenta were removed and fixed in formalin for paraffin-embedding.

Tissue fixation and paraffin-embedding

Freshly collected tissue samples were fixed in 10% normal buffered formalin for 24–48 h and stored in 70% ethanol until further processing. Samples were paraffin-embedded, sectioned and stained with hematoxylin and eosin following standard procedures by the Veterinary Medical Diagnostic Laboratory core at the University of Missouri.

Results

Tissue-specific expression of *TRIML1* and *TRIML2*

Transcripts for *TRIML1* were present in the testis while not detected in any of the other selected panel of porcine tissues or developing early embryos and placenta (Fig. 1A). Of note, *TRIML1* mRNA was only detected in testis from a pubertal boar, while absent from neonatal testis (Fig. 2D), suggesting that *TRIML1* expression is associated with the onset of spermatogenesis. Transcripts for *TRIML2* were detected in the porcine female and male gonads (i.e. testis and ovary, including follicle and corpus luteum; Fig. 1B), in oocytes, early developing embryos, morulae, blastocysts

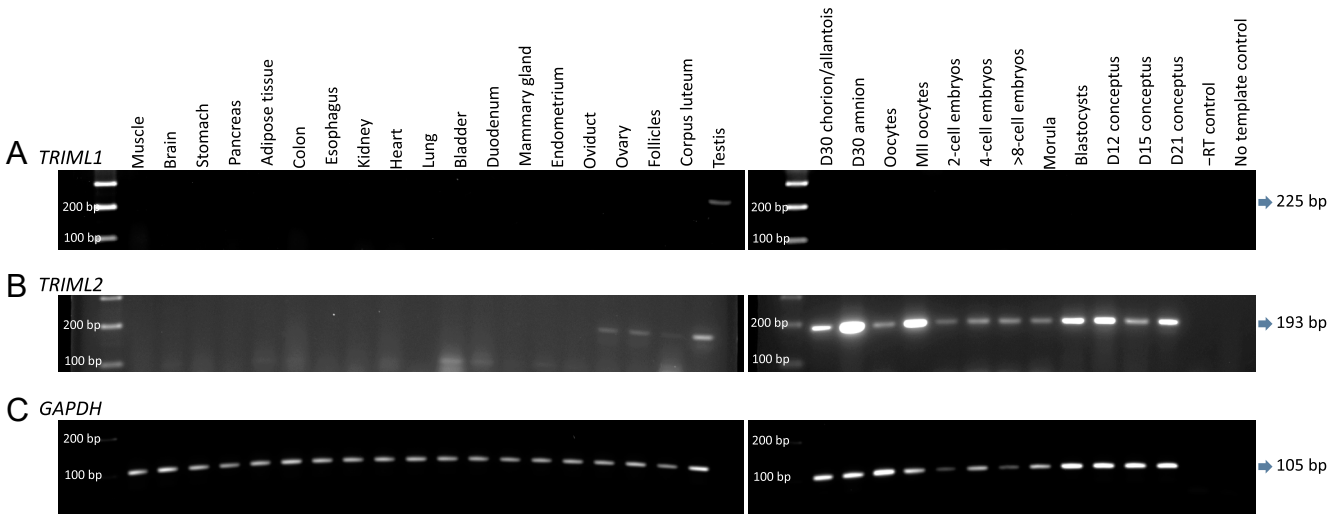

Figure 1

Tissue-specific expression of *TRIML1* and *TRIML2*. End-point PCR for (A) *TRIML1* and (B) *TRIML2* mRNA across different porcine tissues, oocytes, embryos and placenta. Note the specific expression of *TRIML1* in the testis from the selected porcine tissue panel. Transcripts for *TRIML2* were detected in gonads, oocytes, embryos and placenta. (C) *GAPDH* was used as housekeeping for cDNA quality control. Thirty-three cycles were used to amplify *TRIML1*, and 32 cycles were used to amplify *TRIML2* and *GAPDH*. A minus reverse transcriptase control (–RT) of pooled RNA samples and a no-template reaction were included as negative controls.

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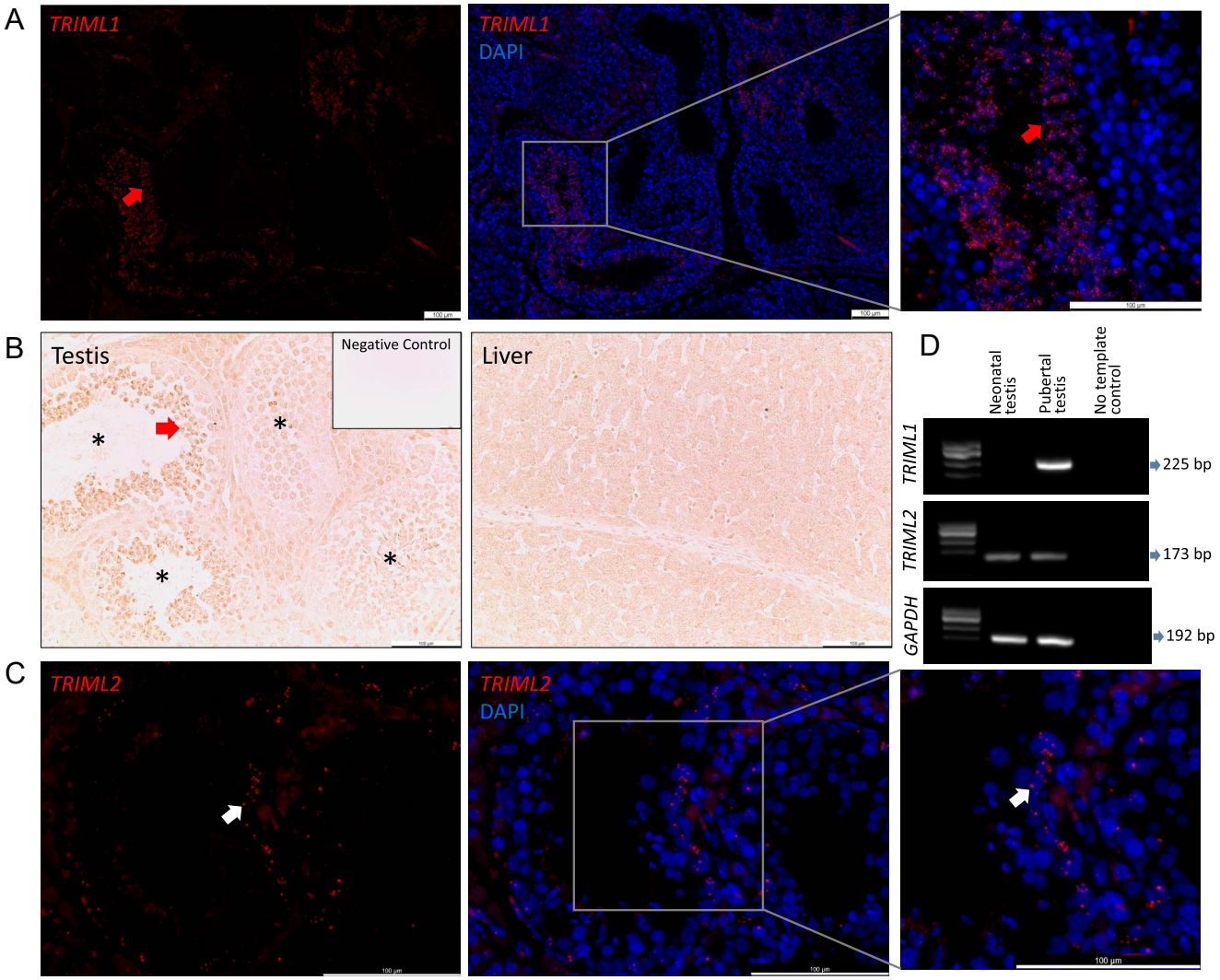

Figure 2

Cell-specific expression of TRIML1 and TRIML2 in the porcine testis. Representative images of (A) *in situ* hybridization for *TRIML1* mRNA (red punctate signals). Nuclei are in blue. (B) Immunohistochemistry for TRIML1 showing protein expression in spermatids (red arrow) within selected seminiferous tubules (asterisks are positioned in the lumen). For immunohistochemistry, replacement of the primary antibody with normal IgG eliminated specific labeling (negative control; inset). As expected, no labeling was detected in the liver. (C) *In situ* hybridization for *TRIML2* mRNA (red punctate signals; white arrows) within seminiferous tubules. Scale bars equal 100 µm. (D) Transcripts for *TRIML1* were detected by PCR in the pubertal boar testis while absent in the neonatal testis. *TRIML2* mRNA is present in the porcine testis regardless of stage. Thirty-three cycles were used to amplify *TRIML1* and *TRIML2*, and 32 cycles were used to amplify *GAPDH*.

and conceptus tissues collected on days 12, 15 and 21, as well as in the chorioallantois on day 30 of gestation (Fig. 1B). The specific and unique expression of *TRIML2* in the placenta is consistent with it serving as a putative anti-inflammatory factor to provide immunological protection of the eutherian placenta.

Cell-specific expression of *TRIML1* and *TRIML2*

In situ hybridization was performed to validate the PCR findings and assess tissue localization of *TRIML1* and *TRIML2* in porcine tissues.

Testis

Transcripts for *TRIML1* were detected in the seminiferous tubules of the testis (Fig. 2A). Interestingly, TRIML1 mRNA and protein were localized to secondary spermatocytes transforming into round spermatids within the seminiferous tubules (Fig. 2A and B). Transcripts for *TRIML2* were localized in cells near the basal compartment of the lamina propria of the seminiferous tubule (Fig. 2C), suggesting that expression occurs only in the prespermatogonia (stem cells). *TRIML1* expression during the late stages of spermatogenesis is consistent with the absence of transcripts in the neonatal testis,

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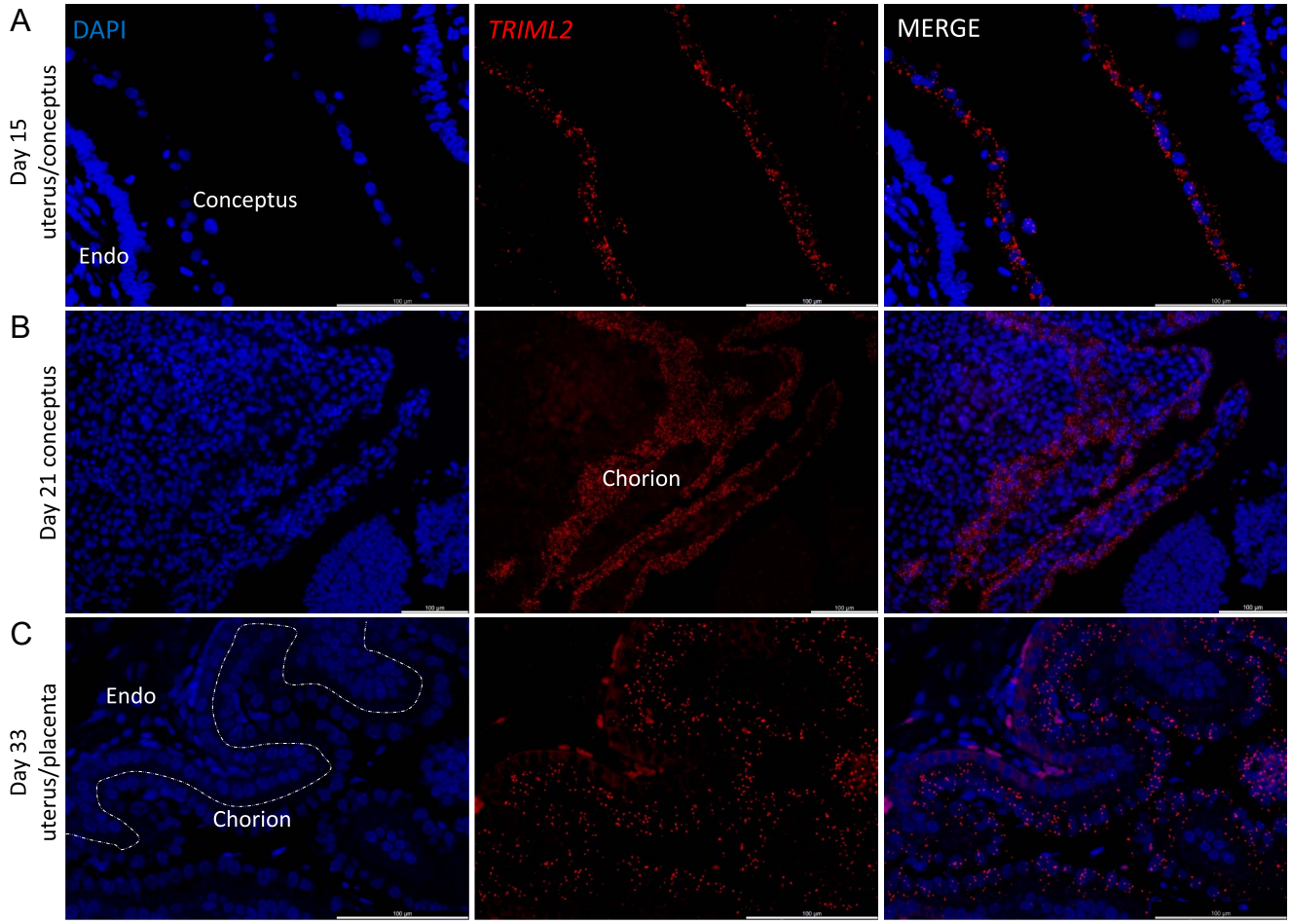

Figure 3

Cell-specific expression of *TRIML2* in the porcine trophoblast and placenta. Representative images of *in situ* hybridization for *TRIML2* mRNA (red punctate signals) in (A) conceptus trophoblast on day 15 of gestation, (B) chorioallantois on day 21 and (C) the placenta–maternal interface on day 33. Nuclei are in blue. Endo, endometrium. Scale bars equal 100 μm.

while transcripts for *TRIML2* are present in the primordial germ cells from both neonatal and pubertal testis (Fig. 2D).

Conceptus and placentae

Consistent with the PCR results, *TRIML1* mRNA was not detected in conceptus or placental tissues of the pig (data not shown). By using RNAscope, transcripts for *TRIML2* were detected in the trophoblast of the developing day 15 pig conceptus (Fig. 3A), as well as in day 21 conceptus (Fig. 3B) and day 30 chorioallantois (Fig. 3C).

TRIML1 and *TRIML2* loss-of-function on conceptus and placental development

Ablation of *TRIML1* and *TRIML2* was confirmed at the gDNA (Fig. 4) and mRNA levels (Fig. 5). Although *TRIML1* expression was not expected in fetuses and placenta, there was a possibility that by knocking out *TRIML2*, *TRIML1* expression would be turned on. Therefore, we assayed both *TRIML1*$^{-/-}$ and *TRIML2*$^{-/-}$ fetuses and placentae to confirm the absence of *TRIML1* mRNA (Fig. 5). Regarding *TRIML2* expression, *TRIML2* mRNA was detected in *TRIML1*$^{-/-}$ day 21–22 fetuses and placentae, as well as in *TRIML1*$^{-/-}$ day 34–36 placentae, as expected. Since the primers used to amplify *TRIML2* mRNA were specifically designed to span the deleted sequence of DNA, PCR products for *TRIML2* were not detected in *TRIML2*$^{-/-}$ fetuses/placentae (Fig. 5).

Ablation of either *TRIML1* or *TRIML2* did not prevent embryo development up to the blastocyst stage *in vitro*, nor the establishment of pregnancy after embryo transfer. Recipient gilts containing *TRIML2*$^{-/-}$ embryos contained developmentally and morphologically normal elongated conceptuses (Fig. 6A), which were attaching to the uterine epithelium surface on days 15 and 17. The endometrium at the site of conceptus attachment was

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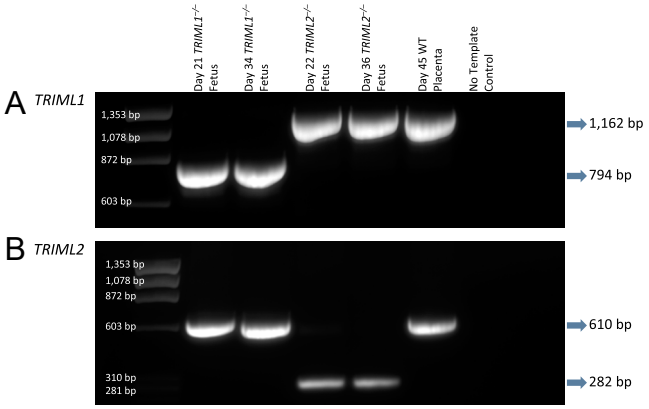

Figure 4

End-point PCR confirmation of genome editing in representative *TRIML1*$^{-/-}$ and *TRIML2*$^{-/-}$ fetuses collected on days 21–22 or 34–36. (A) A 368 bp deletion for *TRIML1* in *TRIML1*$^{-/-}$ fetuses was expected. (B) A 328 bp deletion for *TRIML2* in *TRIML2*$^{-/-}$ fetuses was expected. WT gDNA was included as control. A no-template reaction was included as negative control. Thirty-five and 33 cycles were used to amplify *TRIML1* and *TRIML2*, respectively.

normal and was not inflamed, as would be expected if the *TRIML2*$^{-/-}$ conceptuses induced a strong pro-inflammatory response and rejection of the conceptuses by the endometrium. Recipient gilts containing either *TRIML1*$^{-/-}$ and *TRIML2*$^{-/-}$ embryos established and maintained pregnancy on days 21–22 (3/6 *TRIML1*$^{-/-}$ and 2/4 *TRIML2*$^{-/-}$) and 34–36 (2/4 *TRIML1*$^{-/-}$ and 3/3 *TRIML2*$^{-/-}$). Pregnancy rates were similar to what has been observed in previous

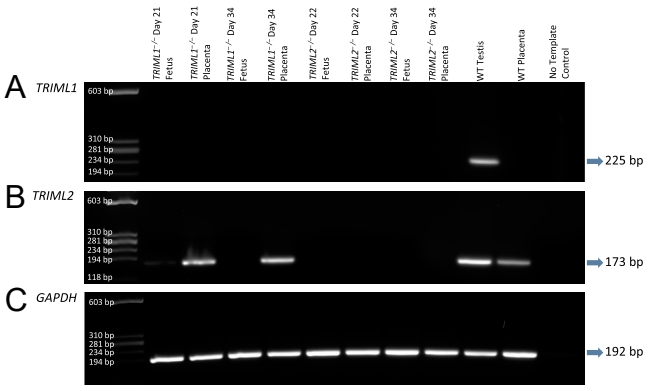

Figure 5

End-point PCR for *TRIML1* and *TRIML2* mRNA in *TRIML1*$^{-/-}$ and *TRIML2*$^{-/-}$ fetuses collected on days 21–22 or 34–36. (A) Transcripts for *TRIML1* were not detected in either genotype. WT testis cDNA was used as a positive control. (B) Transcripts for *TRIML2* were detected only in *TRIML1*$^{-/-}$ fetuses, as expected. WT testis and placenta were used as positive controls. A no-template reaction was included as negative control. Thirty-three cycles were used to amplify *TRIML1* and *TRIML2*, and 32 cycles were used to amplify *GAPDH*.

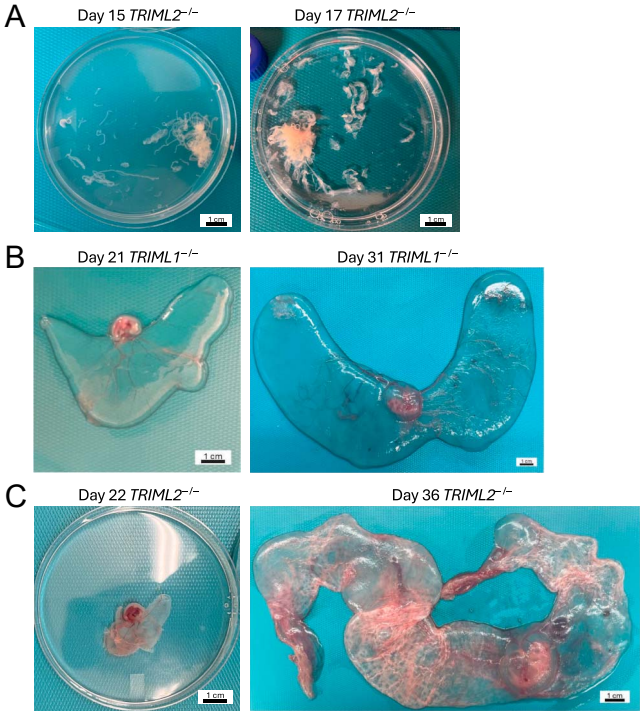

Figure 6

Ablation of conceptus *TRIML1* or *TRIML2* is not associated with fetal or placental gross morphological abnormalities. Representative images of (A) *TRIML2*$^{-/-}$ conceptuses on days 15 and 17, (B) *TRIML1*$^{-/-}$ and (C) *TRIML2*$^{-/-}$ fetuses and extraembryonic membranes on days 21–22 and 34–36 of gestation. Scale bars equal 1 cm.

studies from our group using SCNT-derived embryos (Meyer *et al.* 2019, Johns *et al.* 2021, Sullivan *et al.* 2024). The litter size of pregnant *TRIML1*$^{-/-}$ recipients was 2, 8 and 9 on days 21–22 of gestation, and 8 and 9 on days 34–36, while pregnant *TRIML2*$^{-/-}$ recipient gilts contained 5 and 8 embryos on days 21–22, and 3, 8 and 13 embryos on days 34–36. Fetuses and extraembryonic membranes seemed developmentally normal (i.e., presence of well-vascularized membranes filled with clear abundant fluid, localized necrotic tips and developmentally appropriate fetuses) by gross morphological evaluation (Fig. 6B and C). Finally, histological assessment of the maternal–fetal interface in *TRIML1*$^{-/-}$ and *TRIML2*$^{-/-}$ recipients/fetuses revealed abundant vascularization on the endometrium underneath the placenta and the presence of a delimited attachment site between the chorioallantois and endometrial luminal epithelium (Fig. 7). On days 34–36 of gestation, when placentation is completed, placental folds were observed and the fetal chorion laid in apposition to the maternal endometrial luminal epithelium with no signs of tissue rejection (i.e., inflammation accompanied by damage to blood vessels and structural compromise of the tissue's normal histology) in either genotype group.

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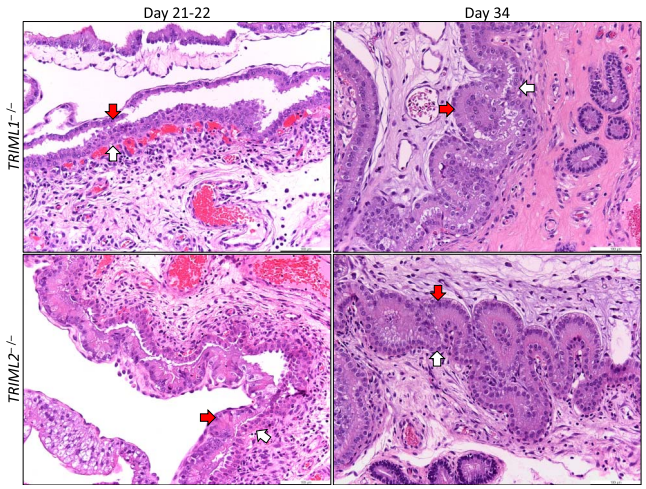

Figure 7

Ablation of conceptus *TRIML1* or *TRIML2* is not associated with apparent histological abnormalities at the maternal–fetal interface. Representative images of hematoxylin and eosin staining of the endometrial (white arrows) and chorioallantois (red arrows) interface in uterine/placental sections obtained from *TRIML1*$^{-/-}$ and *TRIML2*$^{-/-}$ recipients on days 21–22 and 34–36 of gestation. Scale bars equal 100 μm.

Discussion

The evolutionary transition from oviparity to viviparity in eutherian mammals necessitated the long-term adaptation of placenta attachment to the uterine surface, stimulation of uterine growth, increased maternal endometrial vascular expansion for nutrient transport to the developing embryo and coexistence with the maternal immune system to prevent rejection (Renfree *et al.* 2013). Marsupial short gestation lengths are caused by the activation of a pro-inflammatory response to placental attachment following the loss of the protective acellular shell, leading to the induction of parturition during gestation (Renfree & Shaw 2021). Eutherian mammals developed the capability to regulate the short pro-inflammatory period to allow attachment and later formation of the placental membranes to term (Chavan *et al.* 2017, Stadtmauer & Wagner 2020*a*,*b*). Although the endometrium elicits a pro-inflammatory response to trophoblast attachment to the surface epithelium such as marsupials, the eutherian conceptus regulates the response to establish increased angiogenesis and blood flow for the support of pregnancy to term (Chaouat 2013) while escaping maternal rejection.

Although types of placentation and placental invasiveness in mammals are quite varied, attachment of the conceptus to the uterine epithelium invokes a pro-inflammatory response and alteration of immune cells infiltrating the endometrium at the attachment site. The difference between stimulating a pro-inflammatory response for the induction of parturition in marsupials and trophoblast attachment and placental development in eutherian mammals most likely evolved from the adaptation and expression of several unique genes in the placenta, such as estrogen receptor, proteases, cadherins and Cdx2 (Warren & Grutzner 2021). The regulation of the endometrial pro-inflammatory response (Chavan *et al.* 2017) may have resulted from the placenta-specific expression of *TRIML2* during eutherian pregnancy, which is absent in marsupials (Zhang *et al.* 2020). TRIMs have a broad range of biological functions in gene regulation, interference with retrovirus infection, antiviral activity, innate immunity, cellular development and proliferation and oncogenesis (Sardiello *et al.* 2008, Vunjak & Versteeg 2019). TRIML2 is unique compared to other TRIM family proteins as it lacks both the B-box and RING domain and thus has no ubiquitin ligase activity, while TRIML1 has been modified in mammals (Zhang *et al.* 2020).

Within the tissues probed in the present study, expression of *TRIML1* was restricted to the adult testis in the pig. According to tissue-specific RNA-Seq data from the Human Protein Atlas (Djureinovic *et al.* 2014), *TRIML1* is also only expressed in the testis in humans (Robertson *et al.* 2020), although *TRIML1* mRNA has been found in extraembryonic tissues in another study (Zhang *et al.* 2020). This diverges from mice, however, where Triml1 has been detected in the testis (Robertson *et al.* 2020), as well as in the ovary, early embryos and extraembryonic membranes (Zhang *et al.* 2020). The absence of *TRIML1* expression in the pig trophoblast and placenta may be related to the epitheliochorial type of placentation, where the trophoblast does not breach the barrier of the uterine surface epithelium. Testis TRIML1 mRNA and protein were detected in the seminiferous tubules and, interestingly, specifically localized to developing round spermatids. This is consistent with the detection of TRIML1 in early and late spermatids within the seminiferous tubules of the human testis (The Human Protein Atlas; www.proteinatlas.org/search/TRIML1 and Hou *et al.* 2012). Approximately 29 E3 ubiquitin ligase proteins have been reported in the mouse testis and function during various stages of spermatogenesis, with the majority occurring during or following meiosis (Hou *et al.* 2012). The E3 ubiquitin ligases may function in histone modifications, inducing protein modification and degradation essential to spermiogenesis following the completion of meiosis. It appears that *TRIML1* expression is activated as secondary spermatocytes migrate through tight junctions of the Sertoli cells, which form the blood-testis barrier during spermatogenesis (Hou *et al.* 2012). The role that TRIML1 protein plays in the development of secondary spermatocytes and round spermatids has not been explored and warrants further investigation. It is possible that TRIML1 may serve to protect developing and transforming haploid spermatids from immune

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recognition. Testis *TRIML2* expression was localized to what appears to be the prospermatogonia along the basal border of the seminiferous tubule. The detection of *TRIML2* mRNA expression in the porcine neonatal testis suggests that TRIML2 may be involved in the maintenance of basal stem cells for continued spermatogenesis, as TRIML2 was not detected in developing spermatogonia. The putative roles of TRIML2 in stem cell maintenance need to be investigated.

Comparable to mice and humans, *TRIML2* expression is detectable in the pig gonads (i.e., testis and ovary). In addition, *TRIML2* mRNA was detected in follicles, corpora lutea, oocytes, early embryos, trophoblast and placental membranes of the pig. Transcripts for *TRIML2* are localized in the trophoblast of the developing conceptus (day 12–15) and the chorion of the placenta (day 21–35). The tissue-specific localization of TRIML2 suggests it may help regulate inflammation at the maternal–placental interface to establish and maintain pregnancy. It is possible that TRIML2 plays a role in suppressing the pro-inflammatory response and immune cell function following trophoblast attachment to the endometrial surface. Zhang *et al.* (2020) indicated that TRIML2 is involved in suppressing the pro-inflammatory response of the trophoblast, decreasing trophoblast apoptosis and increasing embryo survival in mice. The epitheliochorial type placentation of the pig provides a model for noninvasive placental attachment compared to invasive implantation in mice and humans.

Gene editing was utilized to determine the role of pig conceptus *TRIML2* expression during early conceptus development, placentation and pregnancy establishment. Since *TRIML1* expression is absent in pig conceptuses and placentae, *TRIML1*$^{-/-}$ embryos served as controls for the gene editing, cloning and embryo transfer procedures. Embryo development was not precluded by the ablation of TRIML2, nor was early conceptus development and survival on days 15 and 17 of pregnancy in recipients containing *TRIML2*$^{-/-}$ embryos. Thus, under the conditions utilized in the present study, trophoblast attachment of the *TRIML2*$^{-/-}$ conceptuses did not stimulate an uncontrolled acute inflammatory response that would have disrupted conceptus survival and maintenance of pregnancy. Following the pro-inflammatory period of conceptus attachment to the uterine epithelial surface (days 12–20) in the pig, the maternal–fetal interface becomes anti-inflammatory with the formation of the chorion and continued expansion of the allantois during placental development. Fetal/placental development and survival were not altered in *TRIML2*$^{-/-}$ compared to recipients carrying *TRIML1*$^{-/-}$ embryos on either days 21–22 or 34–36 of gestation. Our results indicate that trophoblast and later placental *TRIML2* expression is not essential for development and maintenance of pregnancy in the pig. These results are consistent with the knockout of either *Triml1* or *Triml2* in the mouse (Telugu & Geisert, unpublished data).

Although TRIML2 has several biological functions that suggest an important role in regulating the interactions necessary for the maintenance of pregnancy at the endometrial/placental interface, early pregnancy does not seem to be affected by the absence of its expression. The period of conceptus attachment to the uterine surface is a key event for the development of viviparity in eutherian mammals. This period is marked by a highly regulated endometrial pro-inflammatory response to increase vascularity and blood flow. Pig conceptuses produce cytokines such as interferon gamma (IFNG), which stimulate the migration and multiplication of maternal immune cells beneath the site of trophoblast attachment to the uterine surface (McLendon *et al.* 2020, Johns *et al.* 2021). Transfer of *IFNG*$^{-/-}$ embryos into recipient gilts revealed that conceptus IFNG production is essential to resolve inflammation, perhaps through unidentified effects on the required recruitment of immune cells to achieve a state of tolerance required for the uterus to be receptive to implantation and placentation for a successful pregnancy outcome (Johns *et al.* 2021). The presence of maternal immune cells, specifically uterine monocytes and macrophages, is essential for increasing angiogenesis and immune tolerance to establish early pregnancy in the pig (Geisert *et al.* 2024). Although conceptus and placental TRIML2 expression does not appear to be essential for the maintenance of pregnancy in the pig, TRIM proteins are well known for their antiviral activity through induction in virus-induced autophagy and adaptive innate immune response (van Gent *et al.* 2018, Giraldo *et al.* 2020). TRIM proteins can also function to regulate the ability of IFNG to induce viral autophagy (Kimura *et al.* 2015). In addition, several TRIM proteins are activated by type I and type II interferons or pathogens (Rajsbaum *et al.* 2008, Carthagena *et al.* 2009, Uchil *et al.* 2013). Therefore, it is possible that other TRIM proteins can substitute to regulate retroviral and innate immune (Grütter & Luban 2012) responses of the endometrium in face of TRIML2 protein absence. For instance, *TRIML2* has been demonstrated to be significantly upregulated in human choriocarcinoma cells when transfected with poly (I:C), while *TRIML1* and *TRIML2* knockdown in response to siRNA significantly increased interleukin 6 and interferon B1 expression when cells were transfected with poly (I:C) (Zhang *et al.* 2020). TRIML2 may function to interfere with retroviral infection at the placental/endometrial interface, which would be interesting to determine the response of TRIML2$^{-/-}$ embryos to viral or pathogen challenge during pregnancy.

In conclusion, our findings reveal that TRIML1 and TRIML2 are uniquely expressed in certain cells from immune-privileged sites, including the testis, ovary, trophoblast and placenta in the pig. While TRIML2 is found in all aforementioned tissues, TRIML1 is expressed only in the porcine testis. Knockout of either conceptus *TRIML1* or *TRIML2* expression does not alter

the early establishment and maintenance of pregnancy in the pig. The function of TRIML1 and TRIML2 in spermatogenesis and male fertility warrants further investigation.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/RAF-24-0107.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

EKE, MS, RDG, KDW, WCW and RSP contributed to the conception and design of the study. EKE and MS developed the knockout models. EKE and RMS performed SCNTs and embryo production. EKE, MS and RDG performed the embryo transfers. EKE, MS, CGL, RDG, RMS, BKR and PRC assisted with animal uterine collection, sampling and analysis of the samples. EKE, MS and RDG analyzed the data. EKE and MS wrote the manuscript. All authors read and approved the final manuscript.

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