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Supplementary Information for

Sexually dimorphic effects of forkhead box A2 (FOXA2) and uterine glands on decidualization and fetoplacental development

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Supporting Materials and Methods

Evaluation of the Uterus on GD 8.5.
Bred Control and LIF-replaced FOXA2 cKO dams (n=5 per genotype) were euthanized, and the uterus placed in ice cold Hank's balanced salt solution (HBSS). Each uterine horn was opened longitudinally along the AM side. Using blunt curved forceps, the decidua from each implantation site was detached from the myometrium. With a pair of fine forceps, an opening was made from the M towards the AM pole, and the intact placenta containing fetal membranes and the fetus along with the ectoplacental cone was carefully separated from the M uterus decidua, which was placed in chilled HBSS. Using a stereomicroscope, the M and AM regions of the uterus were separated using a curved iris scissor. Of note, the M decidua is larger in diameter than the AM decidua. Samples were placed in RNAlater (Sigma-Aldrich), flash-frozen in liquid nitrogen, and stored at -80°C. The corresponding fetus was placed in a 1.5-ml tube and stored at -80°C for fetal sex determination by PCR (1). Total RNA was extracted from the M and AM decidua for transcriptome analysis (n = 5 per fetal sex and dam genotype for each region).

Evaluation of Fetal and Placental Development on GDs 12.5 and 17.5.
Bred Control and FOXA2 cKO dams were euthanized on either GDs 12.5 or 17.5. The total number of viable and non-viable fetuses in each gravid horn was determined by visual inspection of fetal heartbeat. The left horn was frozen in chilled heptane over dry ice and stored at -80°C for histological analysis. The right horn was slit open and each placentation site with fetus and its membrane was carefully removed with curved forceps and placed individually into chilled saline using a 12-well culture plate on ice. For GD 12.5, the metrial gland from each placentation site was micro-dissected and snap-frozen for RNA extraction. Next, each fetus and placenta were removed using dissecting microscope, scissors, and curved forceps and weighed. The placenta was placed into a 1.5-ml tube and snap-frozen in liquid nitrogen for RNA extraction. The tail of each fetus was removed, placed into a 1.5-ml tube, and frozen at -20°C for sex determination by PCR (1).

Immunofluorescence Analyses.
Each placentation site of the frozen gravid horn was separated and embedded into OCT media in a cryomold (Fisher, 22363554). Superficial sectioning was done on each placentation site inside a cryostat to expose the fetal tissue and a pinch of it was carefully taken with a clean blade and forceps into a 1.5-ml tube and stored at -20°C to determine the fetal sex using an established PCR method (1). After fetal sex determination, male and female placentation sites from each pregnancy were cryosectioned at 6 µm thickness. The sections were mounted onto Superfrost Plus microscope slides (Fisher, 22037246) and stored at -20°C. To ensure a horizontal cross-section, only the central portions of the placenta section, marked by the central uterine blood vessel, were used for further analyses. Slides with tissue sections were dried at room temperature for 10 min and fixed in ice cold acetone (Fisher, A929) for 10 min. Excess acetone on slides was evaporated in a fume hood. After complete drying, slides were washed thrice (10 min each) with PBS and blocked with 10% normal goat serum (Life Technologies, 50062Z) in PBS for 1 h. Sections were then incubated with a cocktail of primary antibodies to cytokeratin 8 (TROMA-1; 1:50 dilution of rat monoclonal, University of Iowa Developmental Studies Hybridoma Bank) and vimentin (VIM; 1:500 dilution of chicken polyclonal antibody, Novus Biologicals, NB300-223) in 10% normal goat serum overnight at 4°C. After thoroughly washing thrice (PBS for 10 min each), a cocktail of Alexa Fluor conjugated secondary goat anti-chicken and anti-rat antibodies (1:400 dilution of Invitrogen A-21337 and A-11006) in PBS containing 1% BSA was applied to each section for 1 h at room temperature. After washing thrice (10 min each) with PBS, nuclei were stained for 5 min with Hoechst 33342 (2 µg/ml; Life Technologies H3570). A final wash was performed with water twice (5 min each). Fluoromount-G (ThermoFisher, 495802) mounting media was placed on each section before affixing a coverslip that was sealed with nail polish. Processed tissue sections were inspected for fluorescence and images recorded with a Leica DM5500 B upright microscope using the Leica Application Suite X (LAS X).

Morphometric Measurements.
Morphological measurements of the depth of invasion and sizes of placental compartments were performed with NIH Image J software using described methods (2, 3). The depth of intrauterine trophoblast cell invasion was used to determine the trophoblast invasion index (distance of endovascular CK8 positive cell location relative to the trophoblast giant cell layer of the chorioallantoic placenta/total distance from the trophoblast giant cell layer to the outer mesometrial surface of the uterus). The thickness of the junctional zone was estimated from cross-sectional area measurements of VIM immunostained placentation sites. Measurements were expressed as the ratio of the junctional zone to labyrinth zone cross-sectional areas. Depth of intrauterine trophoblast cell invasion and chorioallantoic zone measurements were all made from a histological plane at the center of each placentation site perpendicular to the flat fetal surface of the placenta. The sample sizes for the analyses were at least six placentation sites from at least six different animals per treatment group.

Transcriptome Analysis.
Total RNA was isolated from frozen uteri using a standard TRIzol-based protocol (Catalog 15596026, Thermo Fisher). To eliminate genomic DNA contamination, extracted RNA was treated with DNase I and purified using a RNeasy MinElute Cleanup Kit (Qiagen). The quality and concentration of RNA were determined using a Fragment Analyzer (Advanced Analytical Technologies). Libraries were prepared by Novogene using an Illumina TruSeq mRNA kit (Illumina Inc.) and sequenced (paired-end 150; 30 million read pairs) using an Illumina NovaSeq 6000. Adapters were trimmed from reads using cutadapt (version 1.11) and quality trimmed to a sliding window quality score of 30 and minimum length of 20 bp with fqtrim software. Reads were mapped to the *Mus musculus* genome assembly (GRCm38.p5) using HISAT2 (version 2.0.3) (4). Reads overlapping Ensembl annotations were quantified with featureCounts (version 1.5.0) (5). Genes with evidence of expression (counts per million; CPM rowSum > 0) were used for model-based differential expression (DE) analysis using the edgeR-robust method (6). Differentially expressed gene list (logFC $\geq$ 2, FDR $\leq$ 0.05) enrichment analysis was conducted using ShinyGo v0.61 with default settings (7).

Statistical Analyses.
A Shapiro-Wilk normality test was used to assess normality. Depending on this outcome, either an unpaired t-test or Mann-Whitney test was done to compare two data sets. Comparisons for significance was done with a P-value of less than 0.05.

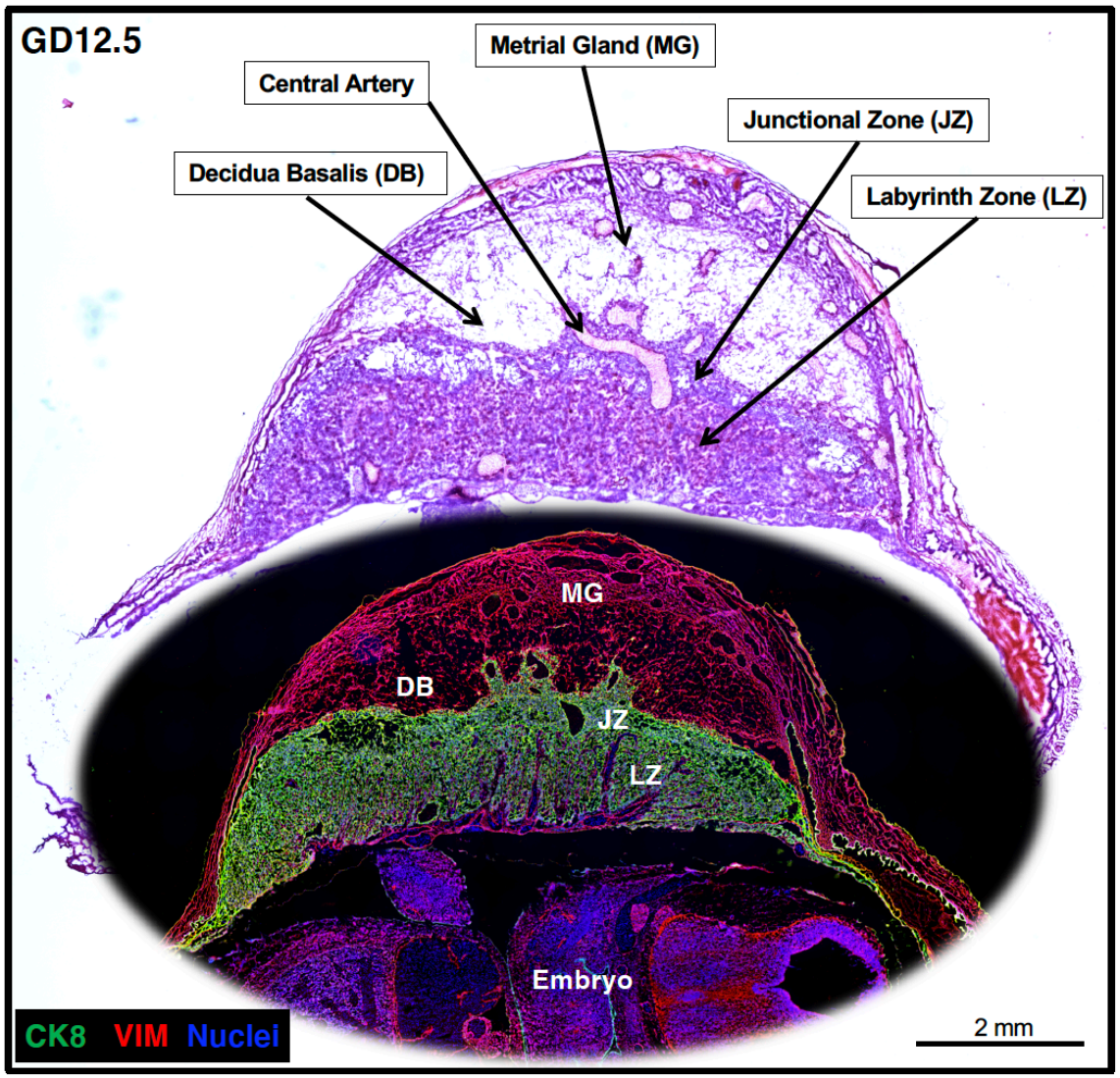

Fig. S1. Placental development in a control dam on GD 12.5. (Upper section) Cross-section of the uterus stained with hematoxylin and eosin. (Lower section) Immunolocalization of the pan trophoblast marker cytokeratin 8 (CK8), vimentin (VIM) with nuclei visualized with Hoechst dye.

Dataset S1 (separate file). RNA-seq analysis of the antimesometrial and mesometrial decidua from control and Foxa2 conditional knockout mice on GD8.5

Dataset S2 (separate file). RNA-seq analysis of the male and female placenta from control and Foxa2 conditional knockout mice on GD12.5

Dataset S3 (separate file). RNA-seq analysis of the metrial gland from control and Foxa2 conditional knockout mice on GD12.5

Dataset S4 (separate file). RNA-seq analysis of the male and female placenta from control and Foxa2 conditional knockout mice on GD17.5

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