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HIF2 α -induced lysyl oxidase safeguards successful pregnancy by remodelling collagens at the feto-maternal interface

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During the initial stage of gestation, precise constructions of the microenvironment at the feto-maternal interface are critical for successful embryonic development to term. It consists of the fetal placenta and the maternal decidua, contributing to favorable pregnancy outcomes and long-term health in both mother and child. Remarkably, although our previous work demonstrated that physiological uterine hypoxia promotes blastocyst implantation into the decidua basalis and onset of trophoblastic invasion via the hypoxia-inducible factor (Hif) signaling pathway, it remains unclear which key regulatory cascades are triggered underlying. Here, we harnessed recent advances of the spatial transcriptomic technology in combination with genetic mouse models to gain a more comprehensive understanding of physiological uterine hypoxia. We revealed that hypoxia-induced remodeling of the extracellular matrices (ECMs) allows the blastocyst-derived trophoblastic cells to invade into the uterine stroma upon implantation. Mechanistically, Hif2 α drives the expression of lysyl oxidase (Lox) that crosslinks collagen I fibrils and their stabilization in the vicinity of the attached embryos. Uterine-specific knockout (uKO) of *Hif2a* and *Lox* compromised the process of embryo invasion, leading to female infertility. Collectively, our findings provide mechanistic insight into how hypoxia governs early embryonic invasion and contributes to successful pregnancy.

- Hypoxia in the primary decidual zone induces Hif2 α , which regulates lysyl oxidase (Lox) expression to remodel the extracellular matrix at the feto-maternal interface.
- Genetic disruption of *Hif2a* or *Lox* in the uterus impairs trophoblast invasion and compromises fertility, highlighting a critical hypoxia-driven signaling axis.
- Remodeling of the maternal decidual ECM network directly influences embryonic invasive capacity and subsequent placental formation, underscoring the importance of maternal tissue architecture in pregnancy outcomes.

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INTRODUCTION

Embryo implantation into the lining of the uterus is crucial for a successful pregnancy [1–3]. Implantation defects in mice, for example, can cause miscarriage and pre-term births [1, 2], and such defects may contribute to infertility in humans, which affects around 15% of couples globally [1].

The mouse blastocyst comprises an inner cell mass, which will go on to form the embryo proper, in a fluid-filled space surrounded by a layer of trophoblast cells called the trophectoderm, which will eventually give rise to the placenta. Implantation-competent blastocysts enter the uterine cavity on the morning of Day 4 after fertilization (Day 1 = the day of vaginal plug found). They space out, and by midnight of that day, they attach to the luminal epithelium lining the uterus. By the following morning, Day 5, an implantation crypt has formed around each blastocyst,

and the neighboring stroma cells of the uterus begin to differentiate into decidua cells, a process known as decidualization.

The stroma cells nearest the blastocyst differentiate into epithelial-like primary decidual cells, which, by Day 6, form a primary decidual zone (PDZ) around the blastocyst in the implantation crypt. The cells of this layer adhere to each other tightly due to large amounts of the intercellular adhesion proteins E-cadherin and ZO-1 [4], and the PDZ is avascular, so it provides a permeability barrier that protects embryos from immune cells, pathogens and other harmful agents in the maternal circulation [5, 6]. Consequently, this layer is thought also to be hypoxic [7]. Physiological uterine hypoxia has been proposed to promote blastocyst attachment and trophoblast differentiation, in part through the hypoxia-inducible factor (Hif) signaling pathway, yet

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the downstream targets and mechanisms remain only partly understood.

Attachment of the blastocyst facilitates differentiation of PDZ cells in the afternoon of Day 5 [5, 8]. The luminal epithelial cells facing the blastocyst begin to detach from each other, creating gaps in the epithelium [8–10]. The basement membrane underlying the luminal epithelium collapses, and the number of epithelial cells decreases due to engulfment by trophoblasts or cell death [8, 9]. By the evening of Day 5, trophoblasts migrate out of the blastocyst, through the gaps in the epithelium and invade the PDZ. Subsequently, direct contacts with PDZ cells guide the trophoblasts to the secondary decidual zone (SDZ), which comprises polyploid cells differentiated from the outer stroma region, where trophoblasts eventually differentiate into the placenta lineage. Coincident with trophoblastic invasion, the extracellular matrix (ECM) network undergoes spatiotemporal remodeling: notably, the expression and distribution of collagen I (Col I) and collagen IV (Col IV) change upon embryo attachment [11–14]. Col I is a fibrillar collagen that forms dense, mesh-like structures in the tissue stroma [15, 16]. Col IV, by contrast, is a network-forming collagen and a major component of the basement membrane, which separates epithelia from their surrounding tissue compartments [16]. Previous studies have shown that ECM remodeling is tightly regulated during implantation and that aberrant ECM composition is associated with implantation failure and pregnancy disorders [17–19]. A recent *in vitro* study culturing embryos on collagen gels suggested that endometrial ECM facilitates embryo growth [17]. ECM components, including collagens, are degraded by metalloproteases (MMPs), which are inhibited by tissue inhibitors of MMPs (TIMPs) [15, 18]. In the mouse uterus, *Mmp2* is constitutively expressed during pregnancy, and *Mmp9* is strongly expressed in trophoblasts during invasion [19]. *Timp-1* and *-2* are highly induced after embryo attachment, which may inhibit *Mmp2* function [19]. However, despite these advances, it remains unclear how maternal ECM networks are actively remodeled *in vivo* during implantation, and how their regulation by hypoxia contributes to the success or failure of pregnancy.

In this study, we aimed to elucidate how hypoxia at the fetomaternal interface, and specifically in the primary decidual zone (PDZ), contributes to the successful establishment of pregnancy. Building on our previous finding that the hypoxia-inducible transcription factor *Hif2 α* is indispensable for implantation, we sought to identify the downstream pathways by which hypoxia-*Hif2 α* regulates extracellular matrix remodeling around the implantation site. In particular, we focused on whether collagen organization and its enzymatic regulation by lysyl oxidase (*Lox*) play critical roles in enabling trophoblast invasion and placenta-tion. To address this, we combined spatial transcriptomics with ultrastructural analyses and genetic mouse models, allowing us to dissect the mechanisms by which uterine hypoxia orchestrates tissue remodeling required for embryo implantation.

RESULTS

Hif2 α deletion compromises collagen remodeling in the decidua

To investigate the potential mechanisms by which the decidua, especially PDZ, regulates the invasion of the trophoblast cells into the endometria, we performed spatial transcriptome analysis of mouse uterine tissues on Days 4, 5 and 6 of pregnancy. To identify the implantation sites, we intravenously injected Chicago blue dye (Fig. 1A), stained sections of tissue for histology and processed them for spatial transcriptomics (Fig. 1B and Supplementary Fig. 1). Uniform manifold approximation and projection (UMAP) analysis of the spatial transcriptomics data of whole tissues identified stromal cells and myometrial cells as well as luminal and glandular epithelial cells (Fig. 1C and Supplementary Fig. 1A;

Supplementary Table 1). We analyzed further the stromal cells and identified a range of well-documented endometrial cell types, which evolved over the course of these three days of pregnancy: on Day 4, before the embryo attachment occurred, the stromal cell types comprised mainly non-proliferative and epithelial-mesenchymal transition (EMT)-like cell types; on Day 5, after attachment, the number of proliferative and the embryo-attached cells increased, and on Day 6, when the embryos invade the lining of the uterus, PDZ and SDZ cells appeared (Fig. 1D and Supplementary Fig. 1B; Supplementary Table 2). MSigDB Hallmark and GO term analyses of the data for the PDZ and SDZ cells revealed upregulation of hypoxia signaling (Fig. 1E) and collagen-containing extracellular matrix gene transcription (Fig. 1F) in both cell types. These findings are consistent with previous evidence that the PDZ is avascular [6] and therefore hypoxic, and that stromal *Hif2 α* is indispensable for embryo invasion [10].

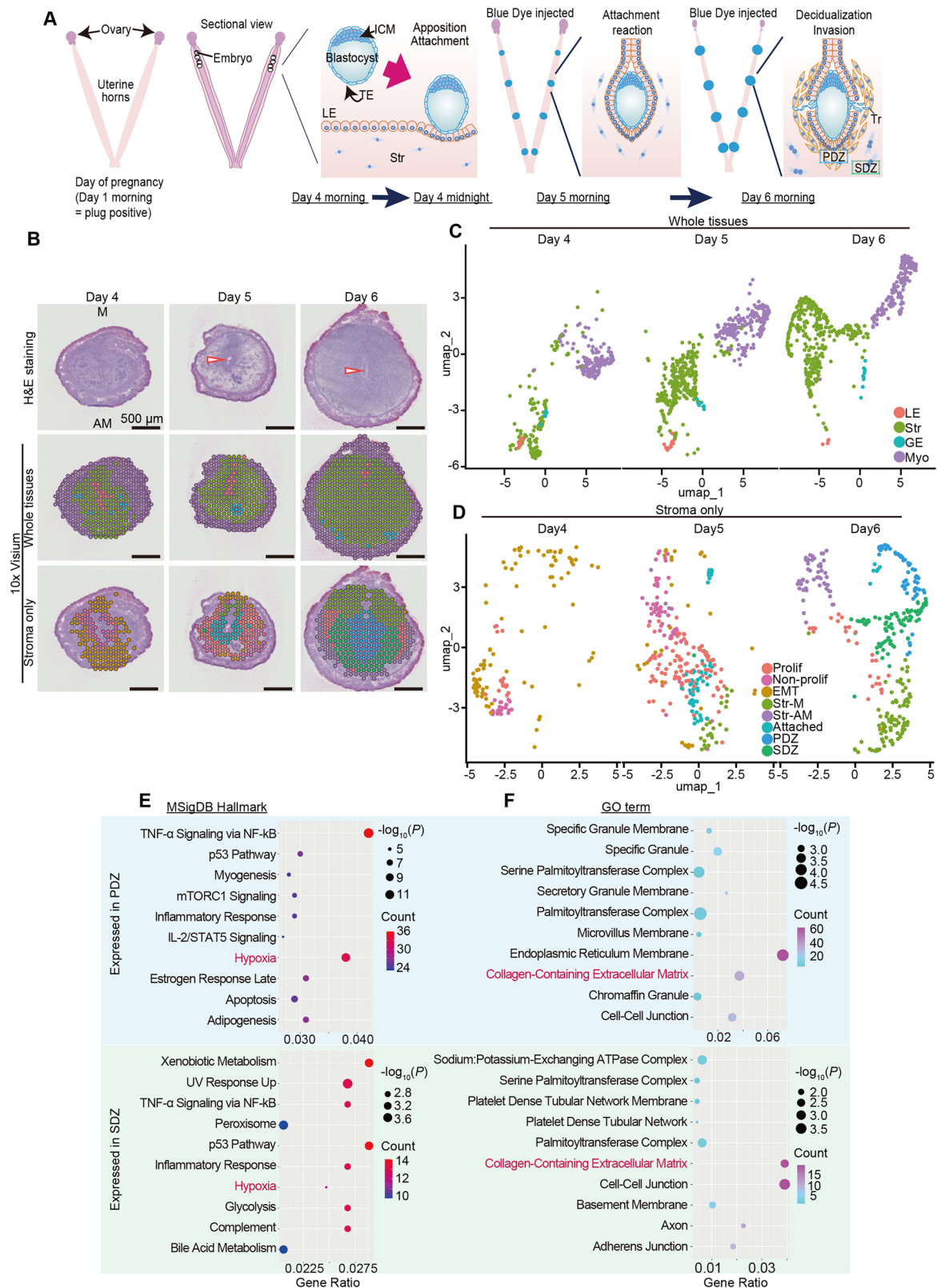
Spatial transcriptome analysis of uterus tissues from the implantation sites of *Hif2 α* uKO mice and control mice on Day 6 of pregnancy identified five cell types in both, including PDZ and SDZ cells (Fig. 2A, B and Supplementary Fig. 2A; Supplementary Table 3). There were no significant alterations in the ratios of each cell cluster per total stromal cells (Supplementary Fig. 2B). We focused on the PDZ cells for further analysis because the PDZ region is avascular and hypoxic as well as these cells express high levels of *Hif2 α* [20, 21] (Supplementary Fig. 3). By comparing gene expression in PDZ cells of *Hif2 α* uKO and control uteri, we found 167 upregulated genes in the *Hif2 α* uKO and 397 upregulated genes in the controls, of which 40 genes were targets of *Hif* proteins (Fig. 2C and Supplementary Table 4). We predicted the molecules upstream of these upregulated genes in the control by using the enrichment analysis tool Enrichr [22] and the RNA-seq data resource ARCHS⁴, which showed that genes upregulated in the control were targets of *HIF1 α* and *HIF2 α* (Fig. 2D). Moreover, reactome analysis indicated that the upregulated genes specifically in either control or *Hif2 α* uKO PDZ cells were involved in collagen assembly, biosynthesis and modification trimerization and fibril crosslinking (Fig. 2E), suggesting that *Hif2 α* deletion in the PDZ compromises collagen remodeling in the extracellular matrix.

To validate our conclusions from spatial transcriptomics, we used transmission electron microscopy (TEM) to analyze embryo implantation sites in *Hif2 α* uKO and control uteri on Day 5 of pregnancy. We found control epithelial cells were detached from the basement and trophoblasts invaded decidua, while there was poor collagen deposition in *Hif2 α* uKO decidua, with a substantial epithelium on the basal membrane, and flawed trophoblast invasion (Fig. 3A). Immunostaining of the implantation sites on Day 6 showed that Col I fibrils were abnormal in the absence of *Hif2 α* : Col I fiber network was evident in the controls while it was fragmented in *Hif2 α* uKO decidua (Fig. 3B).

We hypothesized that the abnormal Col I network in *Hif2 α* uKO decidua might be due to low expression of *Lox* as the *Lox* promoter contains a *Hif*-response element, and expression of this gene is induced by *Hif* proteins under hypoxic conditions [23]. Moreover, spatial transcriptomics showed *Lox* was highly expressed at PDZ implantation sites in the control on Day 6 (Supplementary Fig. 3). To investigate this hypothesis, we used *in situ* hybridization to analyze *Lox* expression at implantation sites in *Hif2 α* uKO and control uteri on Day 6 and found much reduced expression in the absence of *Hif2 α* when compared with the control (Fig. 3C and Supplementary Fig. 4). These data indicate that *Lox*-induced changes in collagen architecture in the decidual stroma may be important for *Hif2 α* -mediated embryo implantation.

Lox deletion in the uterus compromises fertility

To investigate whether *Lox* plays a similar role in *Hif2 α* -mediated trophoblast invasion, we used *in situ* hybridization to examine *Lox*



expression in the stroma surrounding blastocysts during early pregnancy and found high *Lox* mRNA levels on Day 5 and Day 6 (Supplementary Fig. 5A), consistent with a previous report [24]. *Lox* expression was localized to the stroma adjacent to the blastocysts (Supplementary Fig. 5A), similar to *Hif2a* expression

[21]. These findings suggest that *Lox* functions after embryo attachment.

To assess the biological significance of *Lox* in pregnancy, we established mice with a conditional deletion of *Lox* in the uterus (*Lox* uKO mice) by mating *Lox*-floxed mice with *Pgr-Cre* (uterine-

Fig. 1 Spatial transcriptomics of peri-implantation uteri reveals enrichment of hypoxia- and ECM-related gene transcription in the decidua. **A** Schematic diagram of embryo implantation. Four days after fertilization, blastocysts arrive in the uterine horns. By the midnight of Day 4, they attach to the luminal epithelium (LE). From the morning of Day 5 onwards, implantation sites can be visualized by injecting Chicago blue dye intravenously, due to the increased vascular permeability of the stroma (Str) during the attachment reaction. By the morning of Day 6, the primary decidual zone (PDZ) and secondary decidual zone (SDZ) become evident, and trophoblasts (Tr) invade the decidua. ICM, inner cell mass; TE, trophectoderm. **B** Histology (top) and spatial transcriptomics analysis (using 10x Visium) of sections of whole tissue (middle) and stroma only (bottom) from pregnant uteri on Days 4–6. Arrowheads indicate blastocysts. Scale bar, 500 μ m. **C** Uniform manifold approximation and projection (UMAP) analysis of spatial transcriptomics data from whole tissues, as in **(B)** and categorized by cell type, as indicated in the color key. LE, luminal epithelial cells; Str, stromal cells; GE, glandular epithelial cells; Myo, myometrial cells. **D** UMAP analysis of spatial transcriptomics data from stroma as in **(B)** and categorized by stromal cell types, as indicated in the color key. Prolif, proliferating cells; Non-prolif, non-proliferating cells; EMT, epithelial-mesenchymal transitional cells; Str-M, mesometrial stromal cells; Str-AM, anti-mesometrial stromal cells; PDZ, PDZ cells; SDZ, SDZ cells. **E** MSigDB Hallmark prediction of upstream transcription factors and **F** gene ontology (GO) analysis in PDZ (top) and SDZ cells (bottom), determined using Enrichr. In both **E** and **F**, bubble size reflects statistical significance ($-\log_{10}$ adjusted *P*-value), while color scale represents the average expression level of the associated genes. “Gene Ratio” indicates the proportion of genes in each term relative to the total number of genes.

specific Cre) mice (Supplementary Fig. 5B). Exons 3 and 4 of the *Lox* gene were flanked by *loxP* sites to facilitate Cre-mediated deletion. We validated *Pgr-Cre*-mediated genomic deletion of the floxed region in uterine tissues by PCR (Supplementary Fig. 5C) and the absence of *Lox* mRNA from implantation sites by in situ hybridization (Supplementary Fig. 5D).

When *Lox* uKO female mice were mated with fertile male mice, the pregnancy success rate was significantly lower than that when control females were mated (Fig. 4A), and those *Lox* uKO females that successfully delivered had litter sizes only half of the average litter size in controls (Fig. 4B). On Day 12 of pregnancy, we observed embryo resorption and hemorrhaging between the implantation sites in the uteri of *Lox* uKO females, which was not observed in the controls (Fig. 4C), and the lower weights of the uteri indicated impaired embryo growth (Fig. 4D). Indeed, the ratio of abnormal embryos per total litters was significantly increased in uKO uteri (Supplementary Fig. 6).

Embryo resorption can be caused by defects in implantation [1, 2, 25]. To investigate whether implantation defects might explain the resorbed embryos we saw in the uteri of *Lox* uKO females, we examined the uteri of pregnant *Lox* uKO and control females on Days 5 and 6 of pregnancy, when implantation typically occurs, by staining with Chicago blue dye, as before, to visualize the embryo attachment sites. Surprisingly, we saw similar numbers of blue bands in the uteri of *Lox* uKO and control females on Day 6 (Fig. 4E) and even on Day 5 in *Lox*-deficient uteri (Fig. 4F), indicating apparently normal embryo attachment despite the absence of *Lox*. Moreover, the receptivity-related molecules *Ki67* (a marker of proliferation, which should be expressed in the receptive stroma), *Pgr* and *Esr1* (female hormone receptors), and *FoxA2* (a marker of glandular epithelium) were expressed similarly in the uteri of *Lox* uKO and control females on Day 4 of pregnancy (Supplementary Fig. 7), indicating normal uterine receptivity in *Lox* uKO.

To assess decidualization at the implantation sites on Day 6, we used in situ hybridization to analyze expression of *Bmp2* and *Ptgs2*, which are markers of decidualization following embryo attachment [1]. Consistent with our observation of normal attachment sites and uterine receptivity in *Lox* uKO females, we observed that these markers of decidualization were expressed similarly in both *Lox* uKO and control females (Fig. 4G and Supplementary Fig. 8) *Ptgs2* expression was seen in mesometrial stroma, and *Bmp2* broadly in decidualizing stromal cells.

Another important feature of pregnant uteri on Day 6 is the epithelial crypt that surrounds each attached embryo [26, 27]. After embryo attachment, the surrounding luminal epithelium layer initiates invagination into the anti-mesometrial pole, accompanied by glandular epithelium, and anomalies in this crypt-shaping, as seen by three-dimensional (3D) imaging, lead to defective pregnancy outcomes [21, 26, 27]. We performed 3D imaging of the luminal and glandular epithelia at implantation

sites in *Lox* uKO and control females on Day 6 and found no abnormalities in crypt shapes (Fig. 4H). Likewise, in *Hif2a*-deleted uteri, we found deeply invaginated implantation crypts (Supplementary Fig. 9), indicating that neither loss of *Hif2a* nor loss of *Lox* influences crypt shape. In addition, embryo attachment and crypt formation on the morning of Day 5 (Fig. 4F and Supplementary Fig. 10A), and PDZ formation with epithelial and avascular characters depicted by ZO-1 and Flk1 staining, which starts on the afternoon of Day 5 (Supplementary Fig. 10B and C) [5], were all normal in *Lox* uKO mice. These data indicate that in the uterus, *Lox* contributes to healthy pregnancy outcomes by regulating early events through an unknown molecular mechanism.

Lox remodels the collagen-containing ECM around implantation sites

To gain insights into the possible mechanisms by which the absence of *Lox* might impact pregnancy, we performed spatial transcriptomics on sections of uterus tissue from *Lox* uKO and control female mice on Day 6 of pregnancy and used UMAP analysis to categorize seven cell types (Fig. 5A, B and Supplementary Fig. 11; Supplementary Table 5). Most strikingly, one category of stromal cells was found only in the *Lox* uKO mice (uKO_Unique_Str: Fig. 5A and Supplementary Fig. 11). These cells were seen specifically around the PDZ region in tissue sections (Fig. 5B), suggesting abnormal stromal cell differentiation. Marker gene analysis revealed lower proliferative activity compared with SDZ cells, as indicated by decreased *Mki67* expression (Supplementary Fig. 11). Notably, uKO_Unique_Str cells expressed *Lox* at much lower levels than other stromal cell types (Supplementary Fig. 11). To further characterize these uKO_Unique_Str cells, we analyzed the uniquely downregulated genes in uKO_Unique_Str cells compared to other cell types by using ARCHS⁴ in Enrichr to predict upstream factors (Fig. 5C). These data predicted that gene expression in uKO_Unique_Str cells is regulated by HIF2 α , among other transcription factors, and by fibrillin 1 (*Fbn1*), a component of the ECM (Fig. 5C). Gene ontology (GO) analysis of the uniquely downregulated genes in uKO_Unique_Str cells further revealed that they were related to collagen-containing ECM pathways and cytoskeleton-related pathways (Fig. 5D). These data indicate that deletion of the *Lox* gene in uKO_Unique_Str cells acts downstream of Hif2 α and affects the ECM and cytoskeleton.

In addition to this unique stromal cell population in the *Lox* uKO uteri, we saw differential gene expression in the PDZ and SDZ cell types when compared to the control. In the PDZ cells, we identified 705 differentially expressed genes: 554 of which were upregulated in *Lox* uKO and 151 in control pregnant females (Fig. 5E and Supplementary Table 6). Prediction of upstream factors targeting genes upregulated in the control included HIF2 α (Fig. 5F). GO analysis revealed that genes involved in extracellular structure and ECM organization were both up- and down-regulated in *Lox* uKO PDZ cells (Fig. 5G). Similarly, the SDZ cells

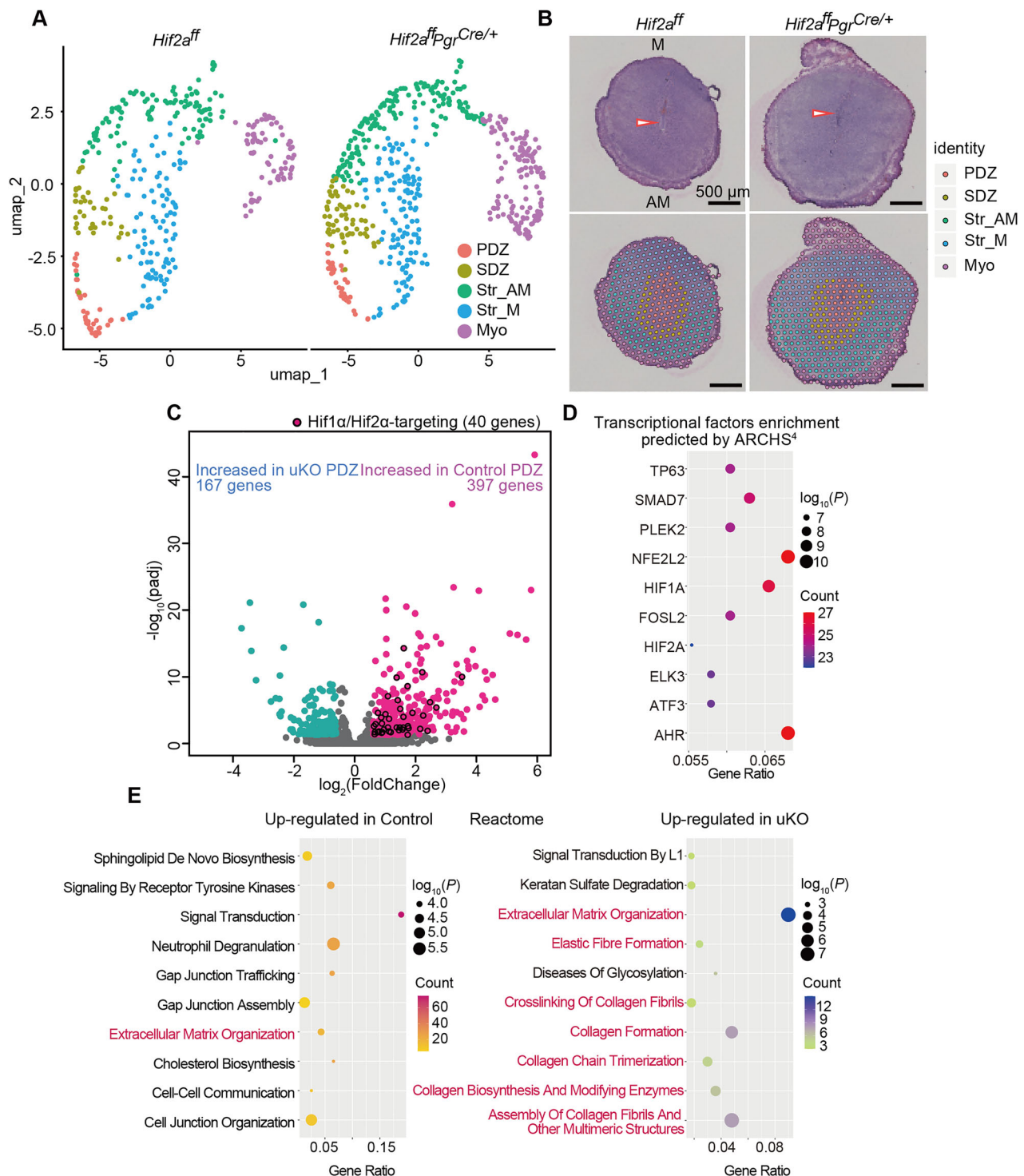
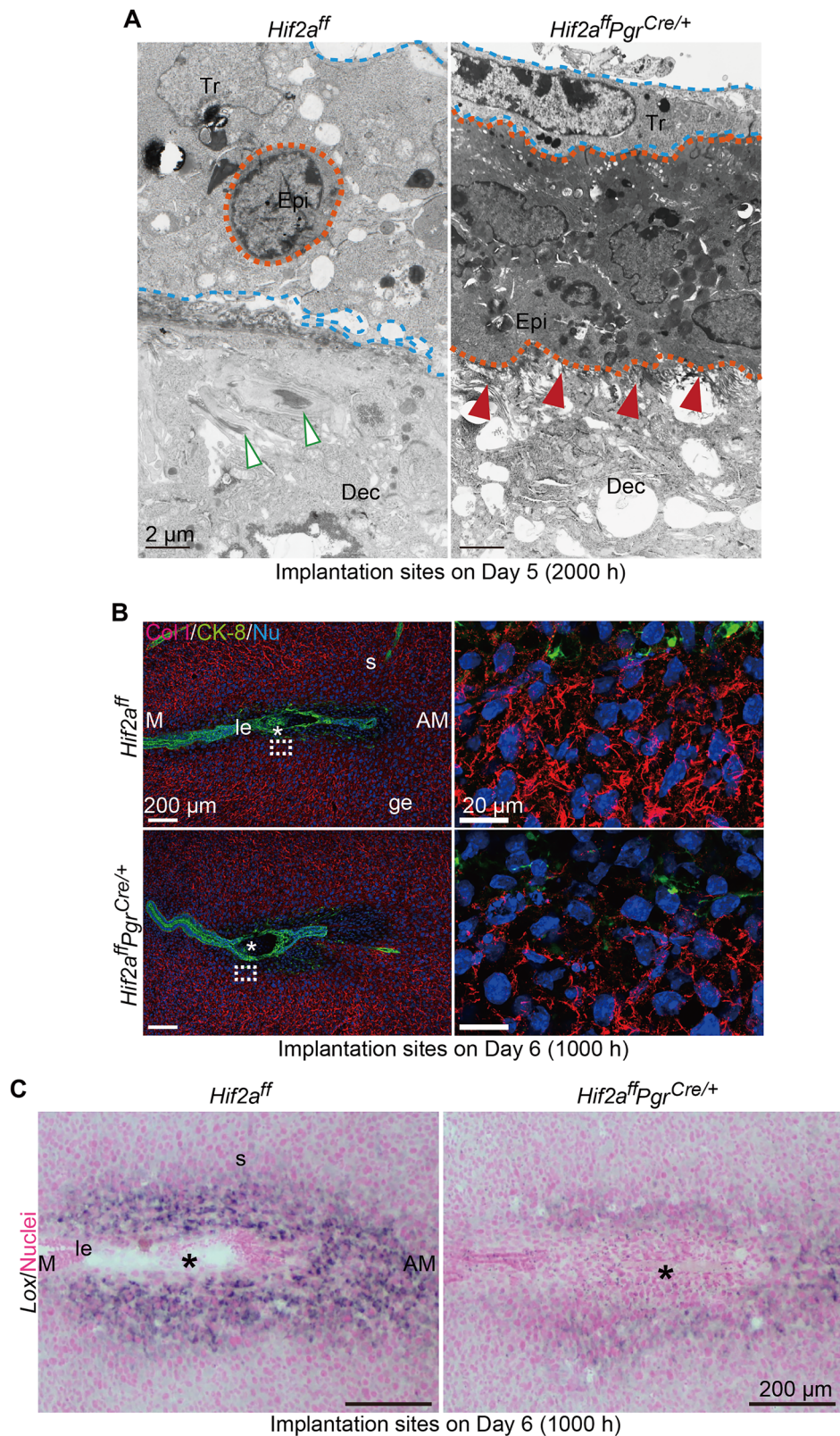


Fig. 2 Spatial transcriptomics of *Hif2^α* uKO uteri reveals upregulation of ECM-related genes in the PDZ. **A** Uniform manifold approximation and projection (UMAP) analysis of spatial transcriptomics data categorized by cell type, as indicated in the color key, in control (*Hif2^{αff}*) and *Hif2^α* uKO (*Hif2^{αff}Pgr^{Cre/+}*) uteri on Day 6 of pregnancy. PDZ, PDZ cells; SDZ, SDZ cells; Str-AM, anti-mesometrial stromal cells; Str-M, mesometrial stromal cells; Myo, myometrial cells. **B** Histology (top) and spatial transcriptomics analysis (bottom) of implantation sites in the control and *Hif2^α* uKO uteri on Day 6. Arrowheads indicate blastocysts. M, mesometrial pole; AM, anti-mesometrial pole. Scale bar, 500 μm. **C** Volcano plot depicting differentially expressed genes (DEGs) in control and *Hif2^α* uKO PDZ cells. Upregulated genes in control cells are shown in magenta and those in *Hif2^α* uKO PDZ cells are shown in blue. DEGs targeted by Hif proteins are indicated by open black circles. **D** Upstream transcription factors of the upregulated genes in control cells predicted using ARCHS4 at Enrichr. **E** Analysis with Reactome at Enrichr of genes upregulated in PDZ cells from controls (left) and in those from *Hif2^α* uKO cells. In **(D)** and **(E)** “Gene Ratio” indicates the ratio number of GO term per the total number of genes.



in *Lox* uKO females showed differential gene expressions compared with controls (Fig. 5H and Supplementary Table 7). HIF2α was predicted to be a highly enriched upstream factor in the controls (Fig. 5I), and, as in PDZ cells, genes involved in extracellular structure and ECM organization were highly

expressed in *Lox* uKO cells and genes involved in collagen-containing ECM processes were highly expressed in control cells (Fig. 5J).

Taken together with our evidence implicate signaling through HIF2α and *Lox* in PDZ cells plays a crucial role in remodeling the

Fig. 3 **Abnormal collagen deposition in the decidua of *Hif2a* uKO uteri during trophoblast invasion.** **A** Representative TEM images of embryo implantation sites in sections of control (*Hif2a*^{fl}) and *Hif2a* uKO (*Hif2a*^{fl} *Pgr*^{Cre/+}) uteri on the evening of Day 5. Trophoblasts and luminal epithelial cells are demarcated by dashed lines in blue or orange, respectively. Red arrowheads, remaining basement membrane; open, green arrowheads, collagen fibers; Tr, trophoblasts; Epi, luminal epithelial cell; Dec, decidual cell. Scale bar, 2 μ m. *n* = 3 for each genotype. **B** Co-immunostaining of collagen I (Col I) and cytokeratin 8 (CK-8), and DAPI staining of DNA in nuclei (Nu) in sections of control and *Hif2a* uKO uteri on Day 6. CK-8 staining indicates luminal epithelia and trophoblasts. The regions indicated by dashed lines are enlarged in the middle and right panels. M, mesometrial pole; AM, anti-mesometrial pole; le, luminal epithelium; ge, glandular epithelium; s, stroma. Asterisks (*) indicate blastocysts. Scale bars, 200 μ m (left), 50 μ m (middle), and 20 μ m (right). *n* = 3 for each genotype. **C** In situ hybridization of *Lox* mRNA (black) in sections of the PDZs of control and *Hif2a* uKO uteri on Day 6. Nuclei were counter stained by Nuclear Fast Red (Pink). Scale bar, 200 μ m. *n* = 3 for each genotype.

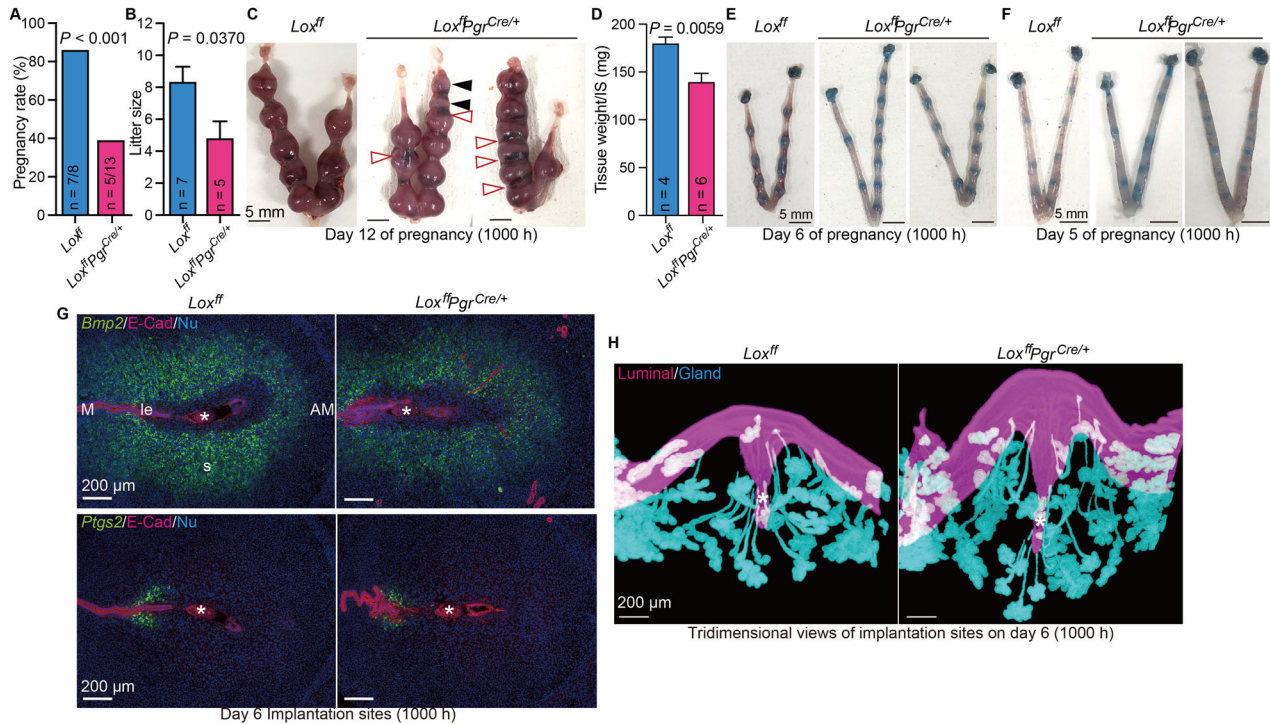


Fig. 4 ***Lox* deletion in the uterus compromises fertility.** Pregnancy rates (**A**) and litter sizes (**B**) of control (*Lox*^{fl}) and *Lox* uKO (*Lox*^{fl}*Pgr*^{Cre/+}) mice are shown. *n* = 8 and 13 in (**A**), *n* = 7 and *n* = 5 in (**B**) respectively. The numbers on the bars in (**A**) indicate dams with successful term delivery per total plug-positive ones. **C** Representative images of pregnant uteri from control and *Lox* uKO mice on Day 12. Open-red arrowheads indicate bleeding, and black arrowheads indicate embryo resorption. Scale bar, 5 mm. **D** Average weight per implantation site in **C**; *n* = 4 and 6, respectively. Representative images of pregnant uteri from control and *Lox* uKO mice on Day 6 (**E**) and Day 5 (**F**). *n* = 12 and 7 in (**E**) *n* = 4 and *n* = 3 in (**F**) respectively. Scale bar, 5 mm. **G** Representative images showing in situ hybridization of mRNAs encoding the decidua markers *Bmp2* (top) and *Ptgs2* (bottom) at implantation sites in control and *Lox* uKO mice on Day 6. E-cadherin (E-Cad; a marker of epithelial cells) was co-immunostained and DNA in nuclei (Nu) was stained with DAPI. M, mesometrial pole; AM, anti-mesometrial pole; le, luminal epithelium; s, stroma. Asterisks (*) indicate blastocysts. Scale bar, 200 μ m. *n* = 3 for each genotype. **H** Three-dimensional imaging of luminal and glandular epithelia at implantation sites in control and *Lox* uKO mice on Day 6. Asterisks (*) indicate blastocysts. Scale bar, 200 μ m. Data are means $\pm$ SEM and statistical significance was determined by using Student's *t* test. *n* = 3 for each genotype.

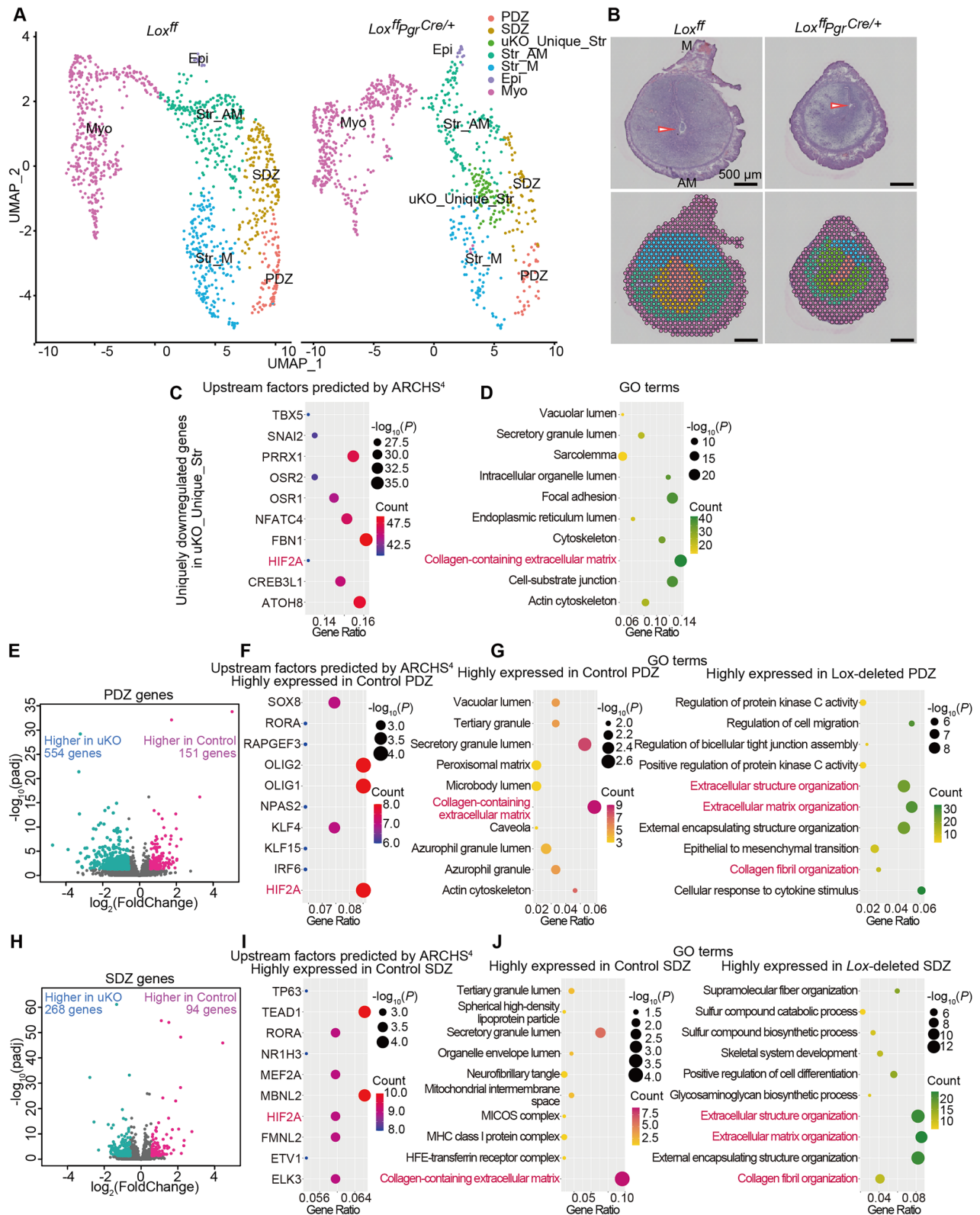
collagen-containing ECM around implantation sites in early pregnancy.

Lox is required for breakdown of the basement membrane and invasion of trophoblasts

To examine whether the altered ECM-related gene expression we saw in the PDZ and SDZ cell types of *Lox* uKO pregnant females when compared to the controls was reflected in the morphology of the tissue around implantation sites, we used TEM to image these sites on Day 5 and Day 6 of pregnancy. By the evening of Day 5, the luminal epithelium of the uterine cavity adjacent to the pre-implantation embryo had been removed in the controls (Fig. 6A). However, in the *Lox* uKO females, the trophoblasts remained near the epithelial layer and within the basement membrane underlying the luminal epithelial cells, whereas in the controls,

they were in direct contact with decidual cells (Fig. 6A). This indicates that in the absence of *Lox*, the basement membrane of the epithelium fails to break down, so the trophoblasts cannot invade the decidual layer.

Anomalies in trophoblast invasion were also evident on the morning of Day 6. Although epithelial cells engulfed by trophoblasts were seen in both *Lox* uKO and control uteri (Fig. 6B, middle panels), in *Lox* uKO uteri the trophoblasts remained within the epithelial layer (Fig. 6B, right bottom panel), whereas in the controls they infiltrated further into the decidual regions, making contact with ECM fibers (Fig. 6B, right top panel). Moreover, ECM fibers were sparse in the stroma of the following the *Lox* uKO uteri (Fig. 6B, right bottom panel), indicating that the anomalies due to loss of *Lox* affect the stroma rather than the epithelia.



We confirmed the anomalous trophoblast invasion on Day 6 by immunostaining sections of tissue at the implantation sites for CK-8, a marker of trophoblast and epithelial cells and for E-Cad, a marker of epithelial cells. In *Lox* uKO uteri, we saw poor trophoblast invasion of the decidua whereas the control uteri exhibited prominent

trophoblast invasion into the primary decidua at the lateral sides (Fig. 6C). Together, these data indicate that *Lox* is required for breakdown of the basement membrane, invasion of trophoblasts from the blastocyst into the decidual layer of the uterus at embryo attachment sites, and for maintenance of ECM fibers in the stroma.

Fig. 5 **Lox remodels the collagen-containing ECM around implantation sites.** **A** Uniform manifold approximation and projection (UMAP) analysis of spatial transcriptomics data categorized by cell type, as indicated in the color key, from control (Lox^{ff}) and Lox uKO ($Lox^{ff}Pgr^{Cre/+}$) uteri on Day 6 of pregnancy. uKO_Unique_Str, stromal cells found only in the Lox uKO. **B** Histology (top) and spatial transcriptomics (bottom) of implantation sites in control and Lox uKO uteri on Day 6. M, mesometrial pole; AM, anti-mesometrial pole. Arrowheads indicate blastocysts. Scale bar, 500 μ m. **C** Upstream transcription factors of uniquely downregulated genes in uKO_Unique_Str cells were predicted by using ARCHS⁴ in Enrichr. **D** Gene ontology analysis of uniquely downregulated genes in uKO_Unique_Str cells performed using Enrichr. **E** Volcano plot depicting differentially expressed genes (DEGs) in control and Lox uKO PDZ cells. Genes upregulated in the control cells are shown in magenta and those in Lox uKO cells in blue. **F** Upstream transcription factors of highly upregulated genes in control PDZ cells predicted using ARCHS⁴ in Enrichr. **G** GO analysis of highly upregulated genes in control PDZ cells performed using Enrichr. **H–J** as for **E–G** for genes upregulated in SDZ cells. In **(C)**, **(D)**, **(F)**, **(G)**, **(I)**, and **(J)**, “Gene Ratio” indicates the ratio number of GO term per the total number of genes.

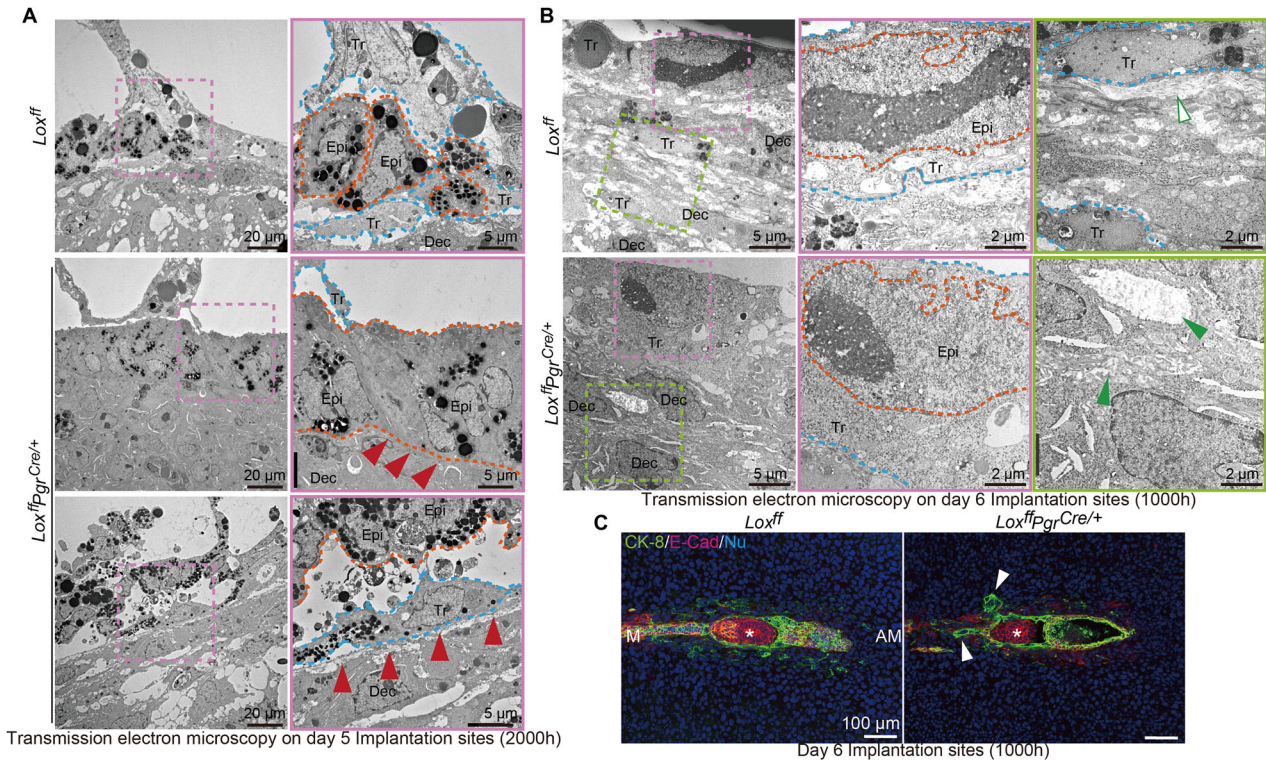
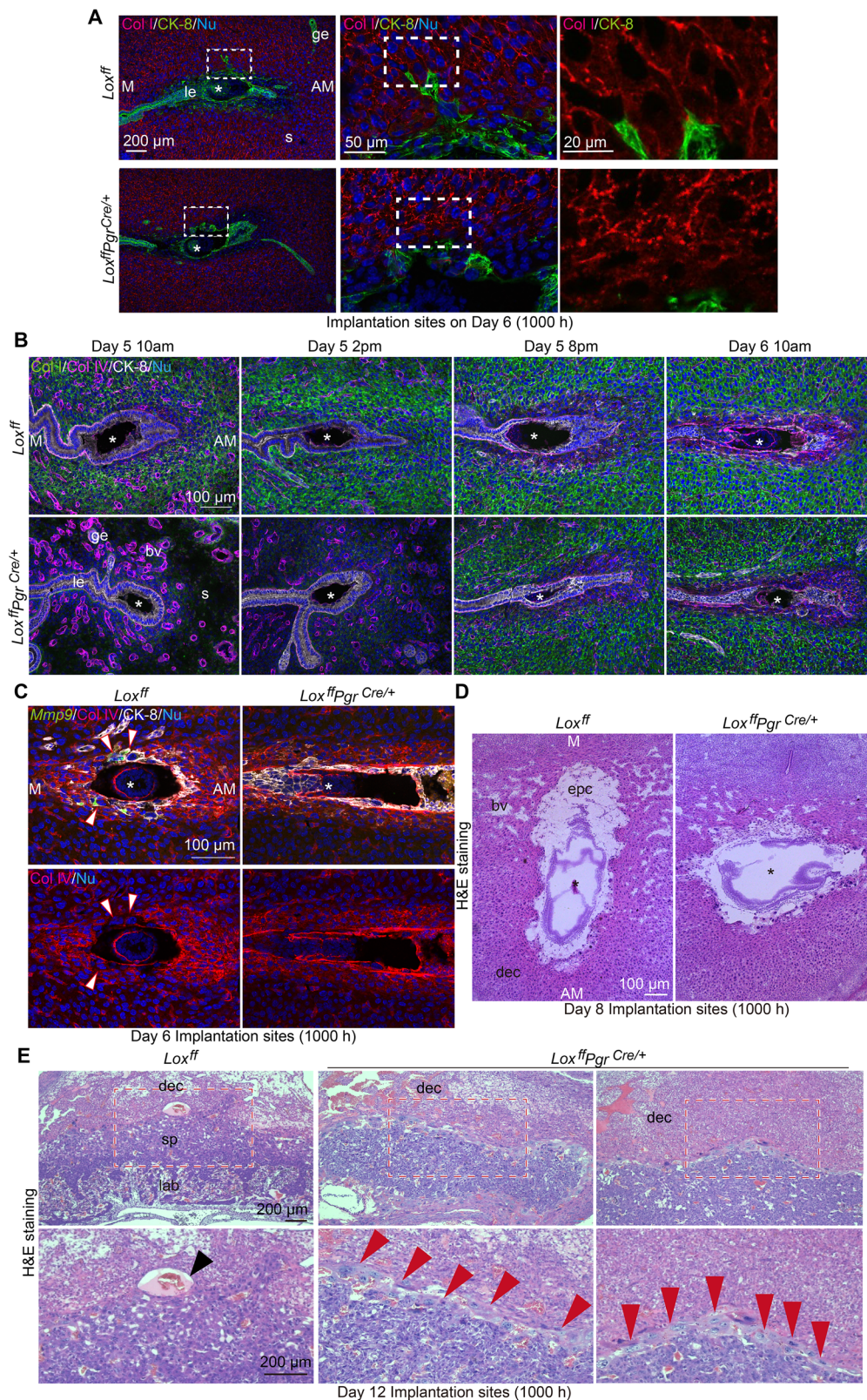


Fig. 6 **Lox is required for breakdown of the basement membrane and invasion of trophoblasts.** **A** Representative TEM images of embryo implantation sites in control (Lox^{ff}) and Lox uKO ($Lox^{ff}Pgr^{Cre/+}$) uteri on the evening of Day 5. The regions indicated by dashed lines are enlarged in the right-hand panels. Scale bars, 20 μ m (left-hand images), 5 μ m (right-hand images). Trophoblasts and luminal epithelial cells are demarcated by dashed lines in blue or orange, respectively. Red arrowheads, remaining basement membrane. Tr trophoblasts, Epi luminal epithelial cells, Dec decidua cells. $n = 3$ for each genotype. **B** As in **A**, but the regions indicated by pink dashed lines are enlarged in the middle panels, and those indicated by green dashed lines are enlarged in the right panels. Scale bars, 5 μ m (left-hand images), 2 μ m (middle and right-hand images). Trophoblasts and luminal epithelia are demarcated by dashed lines in blue or orange, respectively. Open, green arrowheads, collagen fibers; green arrowheads, spotty collagen; blue dashed lines, trophoblasts. $n = 3$ for each genotype. **C** Representative images showing the immunostaining of tissue sections at implantation sites for cytokeratin 8 (CK-8; a marker of trophoblasts and epithelial cells) and E-cadherin (E-Cad; a marker of epithelial cells), and DAPI staining of DNA in nuclei (Nu) in control and Lox uKO uteri on Day 6. White arrowheads, abnormally invading trophoblasts from the ecto-placental cone. M mesometrial pole, AM anti-mesometrial pole. Asterisks (*) indicate blastocysts. Scale bar, 100 μ m. $n = 6$ for each genotype.

Collagens I and IV are remodeled by Lox during embryo invasion

Consistent with our evidence that Lox is required for breakdown of the basement membrane, and for maintenance of ECM fibers in the stroma, the composition of the ECM is known to undergo significant changes during embryo implantation in rodents [11]. To investigate whether the requirement for Lox for breakdown of the basement membrane and invasion of trophoblasts during embryo implantation might be due to effects on these collagen structures in the wall of the uterus, we immunostained sections of Lox uKO and control uteri from mice on Day 6 of pregnancy. In control uteri, we observed prominent Col I fibers surrounding decidual cells and invading trophoblasts, whereas in Lox uKO uteri Col I signals appeared fragmented and trophoblast invasion was impaired (Fig. 7A), similar to those seen in $Hif2a$ -deficient uteri

(Fig. 3). These data suggest that Lox supports trophoblast invasion by crosslinking Col I fibers. We also detected abnormal localization of the Col IV basement membrane in Lox uKO uteri (Fig. 7B). In control decidua, the basement membrane was degraded around invading cells with enlarged nuclei—a characteristic of trophoblasts—whereas in uKO samples the membrane remained intact (Fig. 7B). To further investigate the relationship between these two types of collagens, we performed double immunostaining for Col I and Col IV in uterine sections collected at multiple time points from 10 am on Day 5 to 10 am on Day 6. In control uteri, Col I fibers progressively assembled in the stroma after embryo attachment, while the Col IV-containing basement membrane near the blastocyst degraded (Fig. 7C). In Lox uKO uteri, by contrast, Col I staining was weak at early time points and later appeared stronger; however, as shown in Fig. 7A, Col I remained



fragmented and failed to form normal fibrillar structures. At the same time, Col IV-containing basement membranes persisted adjacent to the blastocysts in uKO samples (Fig. 7C), as further confirmed by higher-magnification images (Fig. 7B). This abnormal network of Col I and IV seen in *Lox* uKO mice may explain the

failure of the basement membrane to break down and the failure of trophoblasts to invade the decidua in the absence of *Lox*, as shown above (Fig. 6).

We hypothesized that *Mmp9* is a key factor in degrading the Col IV-containing basement membrane during implantation, as it is

Fig. 7 **Collagens I and IV are remodeled by *Lox* during embryo invasion.** **A** Co-immunostaining of collagen I (Col I) and cytokeratin (CK-8) and DAPI staining of DNA in nuclei (Nu) in sections of control (*Lox^{fl}*) and *Lox* uKO (*Lox^{fl}Pgr^{Cre/+}*) uteri on Day 6. The regions indicated by dashed lines are enlarged in right panels. M, mesometrial pole; AM, anti-mesometrial pole; le, luminal epithelium; ge, glandular epithelium; s, stroma. Asterisks (*) indicate blastocysts. Scale bars, 100 μ m (left), and 20 μ m (right). *n* = 6 for each genotype. **B** Immunostaining of collagen IV (Col IV) in sections of control (*Lox^{fl}*) and *Lox* uKO (*Lox^{fl}Pgr^{Cre/+}*) uteri on Day 6. DNA in nuclei (Nu) was stained with DAPI. Labels as in (A). *n* = 6 for each genotype. **C** Co-immunostaining of Col I, Col IV and CK-8 around attached embryos in control (*Lox^{fl}*) and *Lox* uKO tissue sections during embryo attachment and invasion (Days 5–6). DNA in nuclei (Nu) was stained with DAPI. Labels as in (A); bv, blood vessel. Scale bar, 100 μ m. *n* = 3 for each genotype and time point. **D** In situ hybridization of *Mmp9* mRNA in trophoblasts (green, arrowheads) in control and *Lox* uKO uterus tissue on Day 6. Sections were co-immunostained for Col IV and CK-8 and DNA in nuclei (Nu) was stained with DAPI. Top panels show Col IV, CK-8, *Mmp9* staining with DAPI, while bottom ones show only Col IV and DAPI to clarify the area with breakdown of basement membrane. Labels as in (A). Scale bar, 100 μ m. *n* = 6 for each genotype. **E** Representative images of hematoxylin and eosin (H&E)-stained tissue sections at implantation sites on Day 8 in control and *Lox* uKO uteri. Labels as in (C) except asterisks (*) indicate embryos; epc, ectoplacental cone; dec, decidua. Scale bar, 100 μ m. *n* = 3 for each genotype. **F** Representative images of H&E-stained tissue sections at implantation sites on Day 12. The regions indicated by dashed lines are enlarged in the bottom panels. Red arrowheads, trophoblast giant cells. Black arrowhead, spiral artery. dec decidua, sp spongio layer, lab labyrinthine layer. Scale bar, 200 μ m. *n* = 3 for each genotype.

highly expressed by invading trophoblasts [19, 28] and the embryonic deletion of this gene affects trophoblast differentiation and invasion [29]. To test this hypothesis, we used fluorescent in situ hybridization to look for *Mmp9* expression in trophoblasts at implantation sites in *Lox* uKO and control uteri on Day 6 of pregnancy; we found no *Mmp9* expression in *Lox* uKO, whereas there was significant expression in the control (Fig. 7D). Histological analysis of implantation sites on Day 8 of pregnancy showed that the embryos in *Lox* uKO uteri lacked ectoplacental cones, resulting in abnormal embryo shapes (Fig. 7E; similar effects were observed in *Mmp9*-deleted embryos [29]). Moreover, similar analyses on Day 12 revealed abnormal placentation in *Lox* uKO uteri (Fig. 7F; like that observed in *Mmp9*-deleted embryos [29]). The spongio- and labyrinth layers observed in placentas from control uteri were absent from *Lox* uKO, but trophoblast giant cells were seen in their place (Fig. 7F). This abnormal placentation explains the lesser weight of the uteri from *Lox* uKO pregnant females on Day 12 when compared to the controls, as well as the lower pregnancy rates and smaller litter sizes in *Lox* uKO (Fig. 4).

These data indicate that *Lox* facilitates trophoblast invasion during placentation by remodeling Col I in the stroma and Col IV in the basement membrane of the decidua surrounding the blastocyst.

DISCUSSION

In this study, we aimed to understand the mechanism whereby Hif2 α expressed in the hypoxic fetomaternal interface ensures successful pregnancy. Spatial transcriptomics of uterine tissue around implantation sites in Hif2 α uKO mice pointed to the collagen-containing extracellular matrix as a potential factor, and we subsequently demonstrated defective Col I fibers in tissue at the implantation sites. We hypothesized the involvement of *Lox*, which oxidizes and cross-links Col I fibers and has an HIF-response element in its gene promoter. Indeed, *Lox* expression in the uterus tissue lacking Hif2 α was much reduced. Moreover, *Lox* was highly expressed at implantation sites. Subsequently, we created a uterus-specific knockout of *Lox* in female mice and found they had fewer pregnancies and smaller litter sizes than normal. We characterized the implantation stages of pregnancy in these animals and discovered that *Lox* safeguards fertility by maintaining Col I fibrillar structure around implantation sites. It also provokes degradation of Col IV in the basement membrane of the luminal epithelium by inducing the metalloprotease *Mmp9* in trophoblasts, promoting invasion of trophoblasts into the decidual zone, where they will eventually differentiate into a placental lineage. Our findings thus explain how hypoxia in the PDZ, by inducing *Lox* expression, promotes a uterine environment conducive to implantation and successful establishment of pregnancy.

We previously showed that pregnancy defects in Hif2 α uKO could not be mitigated by administering either the P₄ or leukemia inhibitory factor [10], two key molecules involved in early pregnancy [1], suggesting a previously unexplored mechanism, which we have uncovered in this study. The *Lox* uKO did not affect receptivity, attachment, or crypt formation—essential for early pregnancy [1, 2, 26]—but resulted in abnormal embryo invasion by failing to maintain Col I fibers in the stroma and to provoke Col IV degradation in the basement membrane. This aligns with previous reports showing that proper ECM remodeling is indispensable for blastocyst invasion and placental development [28, 29], and highlights *Lox* as a critical downstream effector of Hif2 α in orchestrating this process.

In cancer tissues, extensive cell proliferation causes hypoxia, triggering the transcriptional activities of Hif proteins [30]. *Lox*, a transcriptional target of Hif proteins, is required to crosslink Col I fibers that aid tumor migration [16]. Similar parallels have been noted in invasive placentation, where mechanisms of ECM remodeling share features with tumor invasion [31]. Recent in vitro research demonstrated that human placental trophoblast cells express LOX under low-oxygen conditions to promote extravillous trophoblast lineage commitment and column formation [31]. Considering this evidence together with our observations from this study, *Lox* appears to be crucial for trophoblast invasion at the fetomaternal interface, and this function is potentially conserved across species. LOX is expressed in trophoblasts in humans [32], however, it is unclear whether LOX is found in the decidua as in mice. This might explain the differences in trophoblast invasiveness in the two species. Trophoblast invasion is more profound in primates than in rodents, as they penetrate deeply into maternal tissues to modify maternal spiral arteries in the decidua and myometria [33]. Clinically, shallow embryo invasion is a cause of preeclampsia and intrauterine growth restriction [33]. Thus, our findings provide a mechanistic link between hypoxia-driven LOX activity and ECM remodeling, which could inform translational approaches to diagnose or treat human pregnancy disorders characterized by impaired trophoblast invasion. Assessing LOX expression or activity may serve as a biomarker for implantation competence, while therapeutic modulation of LOX could represent a novel strategy to improve reproductive outcomes.

Evidence about the oxygen requirements for trophoblast growth is inconsistent. Hypoxia enhances trophoblast invasion and proliferation in vitro [31, 34], whereas in vivo, trophoblasts migrate toward higher oxygen levels [35]. In this study, we confirmed that Hif2 α -induced *Lox* expression promotes trophoblast invasion into SDZs, which have more developed blood vessels than PDZs [5, 36]. This observation reconciles previous contradictory findings by suggesting that hypoxia-induced *Lox* provides an initial scaffold for trophoblast entry, after which trophoblasts migrate toward oxygen-rich environments to support

growth and differentiation. We observed persistent hypoxia in *Lox*-deficient deciduae around embryos on day 8 (Supplementary Fig. 12). As embryo growth was similarly impaired, failure to invade high-oxygen environments may hinder proliferation and differentiation of embryonic cells.

Given the omnidirectional expression of *Hif2α* and *Lox* around the embryo, it may be surprising that mouse embryos generally implant toward the lateral side of the implantation crypt. As embryogenesis is reported to require intrauterine forces [37, 38], we hypothesize that embryos sense mechanical forces generated by uterus tissues. The nuclear concentration of Yap, a mechanosensing protein that translocates to the nucleus in response to mechanical stress [39], was lower in the cells of *Lox* uKO deciduae when compared with that in controls (Supplementary Fig. 13). Consistent with our hypothesis, spatial transcriptome analysis predicted high expression of *Tead1* in normal PDZ cells, suggesting that Yap–Tead signaling may contribute to mechanosensing in this context. We propose that the *Hif2α*–*Lox*–ECM axis could influence intrauterine mechanical forces that facilitate embryo development. Future studies employing genetic models, such as uterine- or trophoblast-specific knockouts of Yap or Tead, will be required to test this possibility and to integrate mechanical signaling into the broader framework of implantation biology.

Despite the notable effect of *Lox* deletion on fertility (Supplementary Fig. 14), some females could still carry pregnancies to term, which is in contrast to the fact that *Hif2α* uKO females are completely infertile [10]. We speculate that this may reflect differential expression patterns of *Hif2α* and *Lox* during pregnancy: *Hif2α* is robustly expressed throughout the uterine lining by Day 8, whereas *Lox* expression becomes restricted to certain decidual regions. Hypoxia and *Hif1α* expression, in contrast, are not evident at this stage. Consistent with this, uterine-specific deletion of *Hif1α* decreases fertility but does not abolish it, suggesting that *Hif2α* may contribute to later stages of embryo invasion in a manner partially independent of hypoxia. However, our current Cre-driver system ablates genes before or during implantation, making it difficult to directly assess roles of *Hif2α* in decidua after trophoblast invasion. The development of stage- and tissue-specific, inducible Cre lines will be essential to address these questions and to explore how *Hif2α* may regulate uterine remodeling and pregnancy maintenance beyond the implantation phase.

MATERIALS AND METHODS

Mice

Hif2α (*Epas1*) uKO mice were established, as previously reported by our group [10]. *Lox*-floxed mice were prepared using the CRISPR–Cas9 system as previously reported [40]. The gRNAs to insert *loxP* sites into introns 2 and 4 (Supplementary Fig. 5B) were injected into fertilized eggs. After germline transmission, *Lox-loxP/+* mice were intercrossed to generate *Lox-loxP/loxP* mice containing homozygous recombinant alleles. *Lox-loxP/loxP* females were then crossed with *Pgr-Cre* [41] males to generate mice with *Lox* deletions throughout the uterus (*Lox* uKO mice).

Evaluation of pregnancy outcomes

To examine pregnancy outcomes, *Lox* uKO or *Lox^{fl/fl}* (control) female mice were mated with C57BL/6N fertile male mice as previously reported [42, 43]. The day when the vaginal plug was detected was considered Day 1 of pregnancy. These female mice were euthanized on the designated days of pregnancy to evaluate pregnancy phenotypes and collect samples. On Day 4, a uterine horn was flushed with saline to confirm the presence of a blastocyst. The Chicago blue dye solution was injected intravenously on Days 5 and 6 [44], enabling visualization of embryo attachment sites as blue bands. When no attachment sites were visible, the uterine horns were dissected and flushed to collect embryos. Uterine tissues were weighed on Days 8 and 12 to evaluate embryo growth. All mice were housed in the University of Tokyo Animal Care Facility according to the institutional

guidelines for the use of laboratory animals. All animal experiments were approved by the Institutional Animal Experiment Committee of the University of Tokyo Graduate School of Medicine (Approval numbers P16-066, P20-076, and A2023M165).

PCR to check the genomic deletion of *Lox*

DNA collected from the uterine tissues was used as a template. One hundred nanograms of DNA was amplified using the primers 5'-AATTACTGCTGAAGCCACC-3' and 5'-GAATCTCTAGAACTGACTGG-3' and rTaq (Takara), with 35 cycles of 98 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The PCR products were electrophoresed on a 2% agarose gel to detect a band shift.

In situ hybridization

We performed fluorescence- or colorimetric ISH as previously reported [5, 45], with some modifications. Frozen sections (12 μm thick) were attached to Superfrost™ Plus Microscope Slides (Fisher Scientific). After fixation and acetylation, the sections were hybridized overnight at 55 °C with a DIG-labeled *Lox*, *Bmp2*, or *Ptgs2* antisense probe. Following probe washing, the sections were treated with an anti-DIG antibody-POD (11207733910, Roche, 1:200) followed by Alexa 488-conjugated tyramide (B40953, Invitrogen) to detect fluorescence signals. For colorimetric ISH, anti-DIG-AP (11093274910, Roche, 1:2000) and NBT/BCIP (S3771, Promega) were used. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI; Dojindo, 1:500) or Nuclear fast red (40871, Muto Kagaku). Fluorescence or colorimetric images were acquired using an AXR confocal microscope (Nikon) or DM2500 (Leica). Quantification of in situ hybridization was performed using ImageJ (NIH).

Spatial transcriptome analysis

Spatial transcriptome analysis using 10× Visium (10× Genomics) was performed according to the manufacturer's protocol. Uterine tissues collected on day 6 of pregnancy from *Hif2α* uKO, *Lox* uKO, and control mice were sectioned into 10 μm slices, mounted on gene expression slides, and sent to KOTAI Bio. Inc (Osaka, Japan). Following a 30 min Proteinase K reaction, the sections were hybridized with spatial tags on the slides and reverse-transcribed in situ. cDNAs were analyzed via RNA sequencing using DNBseq (MGI) with 300 million reads per sample.

Raw FASTQ files and microscope slide images for each sample were processed using the Space Ranger software (version 1.1, 10× Genomics) with the "spaceranger count" pipeline, which used STAR with default parameters for aligning reads against the mouse reference genome mm10 "refdata-gex-mm10-2020-A." This pipeline used the Visium spatial barcodes to generate a feature-spot matrix of unique molecular identifier (UMI) counts. Clustering analysis was performed via Seurat (version 4.0.3), and the clusters were visualized using UMAP. DEGs between genotypes were identified with an adjusted *P*-value < 0.05 and a fold change > 1.5. Enrichr was used to analyze gene ontologies and upstream transcription factors within each cluster, for the data from Days 4–6 of pregnant uteri were previously deposited to the NCBI Gene Expression Omnibus (GEO accession no. GSE253520).

Hematoxylin and eosin (H&E) staining and immunohistochemistry

H&E staining was performed as previously described [5]. Frozen sections (12 μm) or 10% formalin-fixed paraffin-embedded sections (6 μm) were used for day 8 and 12 tissues, respectively. Images were acquired using a DM2500 (Leica) or an Eclipse Si (Nikon).

Immunofluorescence

Frozen sections (12 μm thick) were prepared for immunofluorescence. After fixing in 4% PFA-PBS, the sections were washed with 0.1% (v/v) Tween20-PBS (PBST), and blocked by 5% (w/v) BSA-PBST for 1 h. Sections were then incubated with primary antibodies at 4 °C, O/N. Following primary antibodies were used: Ki67 (Thermo Fisher Scientific, SP6, 1:300), Pgr (Cell Signaling Technology, 8757, 1:300), Esr1 (Abcam, ab32063, 1:300), E-Cadherin (Cell Signaling Technology, 3195, 1:300), CK8 (DSHB, TROMA-1, 1:300), Collagen I (Cell Signaling Technology, 72026, 1:300), Collagen IV (Merck, AB769, 1:300), Flk1 (BD Pharmingen, 550549, 1:300), ZO-1 (Santa Cruz, sc-8147, 1:300), ZO-1 (eBioscience, 14-9776-82, 1:300) and Yap (Cell Signaling Technology, 14074, 1:300). Signals were detected using secondary antibodies conjugated with Alexa Fluor 594 (anti-rabbit

immunoglobulin G; Thermo Fisher Scientific, A21428, 1:500), Alexa Fluor 488 (anti-rat immunoglobulin G; Thermo Fisher Scientific, A11006, 1:500), Alexa Fluor 594 (AffiniPure Donkey Anti-Rabbit IgG; Jackson, 711-585-152, 1:500), Alexa Fluor 488 (AffiniPure Donkey Anti-Goat IgG; Jackson, 705-545-003, 1:500), and Alexa Fluor 647 (AffiniPure Donkey Anti-Rat IgG; Jackson, 712-605-153, 1:500). Sections were incubated with secondary antibodies and DAPI (Dojindo, 2 µg/mL final) for 1 hr, RT. Hypoxic regions were identified by treating mice with Hypoxyprobe-1TM (Hypoxyprobe, Inc.) following slight modifications to a previously described protocol [7]. Briefly, pregnant females were intraperitoneally administered 60 mg/kg of Hypoxyprobe-1 in saline on day 6 or 8 and euthanized 45 min later. Snap-frozen uteri were sectioned and incubated with an FITC-conjugated anti-hypoxyprobe antibody. Images were captured using an AXR microscope (Nikon).

Three-dimensional visualization of the implantation sites

Three-dimensional visualization of Day 5 and 6 implantation sites was performed according to a previously reported protocol [26]. On Days 5 and 6, tissues were incubated with an anti-E-cadherin antibody (Cell Signaling Technology, 24E10, 1:500) to label luminal and glandular epithelial cells and subsequently with an Alexa 555-conjugated anti-rabbit antibody (Thermo Fisher Scientific, A21428, 1:500). Images were captured using an AXR microscope (Nikon). The 3D structures were constructed using the surface tool in Imaris (v9.8, Oxford Instruments). Luminal and glandular epithelial structures were segmented from E-cadherin-stained 3D images using Imaris, according to previous studies [26, 27].

Transmission electron microscopy

TEM was performed as described previously [10]. Briefly, uterine tissues were collected after sequential perfusion with saline and TEM fixative (2% glutaraldehyde/2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Tissues were then sliced into individual implantation sites and fixed with TEM fixative. The fixed tissues were sent to the Hanaichi UltraStructure Research Institute (Okazaki, Japan) for embedding in resin, ultrathin sectioning, and TEM analysis.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10. Data normality was assessed using the Shapiro–Wilk test in GraphPad Prism. The equality of variances was tested using F-test before performing Student's *t* tests. A two-tailed Student's *t*-test or Fisher's exact test was used to determine significance at *P* < 0.05.

Ethics approval and consent to participate

All methods were performed in accordance with the relevant guidelines and regulations. All animal experiments were approved by the Institutional Animal Experiment Committee of the University of Tokyo Graduate School of Medicine (Approval numbers P16-066, P20-076, and A2023M165).

DATA AVAILABILITY

RNA-seq data will be publicly available upon publication (GSE253520 and GSE274059). This study did not involve the development of custom code or algorithms. All software used in this study is publicly available and is cited in the main text and Methods section.

CODE AVAILABILITY

This study did not make any custom code or algorithm. All software used in this study is publicly available and cited in the main text and/or Methods section.

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AUTHOR CONTRIBUTIONS

Conceptualization: SA, RSH, and YH; Funding acquisition: SA, RSH, AS, YH, and YO; Investigation: SA, RSH, AS, XH, DH, CI, RI, TI, MM, and MI; Data analysis: SA and AS; Data interpretation: SA, RSH, AS, NT, YO, and YH; Project administration and supervision: YH; Writing: SA.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All methods were performed in accordance with the relevant guidelines and regulations. All animal experiments were approved by the Institutional Animal Experiment Committee of the University of Tokyo Graduate School of Medicine (Approval numbers P16-066, P20-076, and A2023M165).

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