Deletion of Arid1a in Reproductive Tract Mesenchymal Cells Reduces Fertility in Female Mice

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ABSTRACT

Women with endometriosis can suffer from decreased fecundity or complete infertility via abnormal oocyte function or impaired placental-uterine interactions required for normal pregnancy establishment and maintenance. Although AT-rich interactive domain 1A (SWI-like) (ARID1A) is a putative tumor suppressor in human endometrial cancers and endometriosis-associated ovarian cancers, little is known about its role in normal uterine function. To study the potential function of ARID1A in the female reproductive tract, we generated mice with a conditional knockout of Arid1a using anti-Müllerian hormone receptor 2-Cre. Female Arid1a conditional knockout mice exhibited a progressive decrease in number of pups per litter, with a precipitous decline after the second litter. We observed no tumors in virgin mice, although one knockout mouse developed a uterine tumor after pregnancy. Unstimulated virgin female knockout mice showed normal ovarian, ovarian, and uterine histology. Uteri of Arid1a knockout mice showed a normal decidualization response and appropriate responses to estradiol and progesterone stimulation. In vitro studies using primary cultures of human endometrial stromal fibroblasts revealed that small interfering RNA knockdown of ARID1A did not affect decidualization in vitro. Timed pregnancy studies revealed the significant resorption of embryos at Embryonic Day 16.5 in knockout mice in the third pregnancy. In addition to evidence of implantation site hemorrhage, pregnant Arid1a knockout mice showed abnormal placental morphology. These results suggest that Arid1a supports successful pregnancy through its role in placental function.

ARID1A, female reproductive tract, fertility, genetically engineered mouse models, placenta, tumor suppressor, uterus

INTRODUCTION

The AT-rich interactive domain 1A (SWI-like) (Arid1a) gene encodes a large nuclear protein that is a key subunit of the multiprotein SWI/SNF chromatin-remodeling complex that is present in all eukaryotes. The SWI/SNF complex regulates gene expression for a variety of cellular processes, including differentiation, proliferation, DNA repair, and tumor suppression. The complex uses ATP as energy to mobilize nucleosomes, thereby modulating the accessibility of promoters to transcriptional activation or repression [1, 2].

Loss of ARID1A has been discovered in many human cancers, including renal cell carcinoma, gastric carcinoma, bladder tumors, pancreatic cancer, colon cancer, breast cancer, endometriosis-associated ovarian carcinomas, and uterine endometrioid carcinomas [3–20]. Considering gynecological cancer types, ARID1A mutation is a driver mutation for endometrial cancer, giving an advantage to epithelial cell growth in vitro [19]. Evidence from ovarian cancer cell lines supports the idea that ARID1A functions as a tumor suppressor by binding to tumor protein p53 (TP53) and regulating cyclin-dependent kinase inhibitor 1A (CDKN1A) [20].

Although ARID1A-inactivating mutations occur in a wide variety of tumors of the female reproductive tract in humans, inactivation of ARID1A alone is insufficient for tumor initiation in the female reproductive tract of mice [21, 22]. Studies using adenovirus-driven Cre to delete Arid1a in the ovarian surface epithelium showed that deletion of Arid1a alone is not sufficient for ovarian cancer formation. Rather, female reproductive tract tumor formation required additional mutation in phosphatase and tensin homolog (Pten) or phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (Pik3ca) [22, 23]. Studies using progesterone receptor (Pgr)-driven Cre to delete Arid1a in the uterus showed that deletion of Arid1a alone is not sufficient for endometrial cancer formation [21]. Although these studies suggest the potential cooperation between mutation of Arid1a and other tumor suppressors in tumorigenesis, little is known about the role of ARID1A in the normal female reproductive tract.

ARID1A is essential for mammalian development because deletion of Arid1a in traditional knockout mice causes embryonic lethality resulting from defects in mesoderm...
development and embryonic stem cell self-renewal, differentiation, and cell lineage decisions [24]. To overcome this early defect and study the global roles of ARID1A in mammals, others have developed Arid1a floxed alleles [24] for the conditional ablation of Arid1a, which has shed light on the role of Arid1a in regulating key genes in cardiac development [25]. Recently, we showed that deletion of Arid1a from the Pgr-positive cells of the uterus resulted in complete sterility with defective embryo implantation and uterine decidualization. The underlying mechanism of this sterility phenotype was from the effects of ARID1A and PGR on expression of Kruppel-like factor 15 (Klf15) and on uterine epithelial cell proliferation [21]. To study the broader role of Arid1a in the female reproductive tract, we used the anti-Müllerian hormone receptor 2 (Amhr2-Cre) mouse model [26], which has Cre recombinase inserted into the Amhr2 gene. Therefore, Cre is expressed in cells that express Amhr2, which include mesenchymal cells of the uterus such as uterine stromal and myometrial cells, oviductal cells, and somatic cells of the ovary such as surface epithelial and granulosa cells [26–30]. Expression of Amhr2 is detectable as early as the 8-cell stage [31], and its expression in the gonad is present beginning at Embryonic Day 12.5 [27]. Amhr2-Cre mice have been successfully used to study the roles of many genes in female reproduction and cancer, including vascular endothelial growth factor a (Vegfa) [32], miR-34c [33], signal transducer and activator of transcription 3 (Stat3) [34], transforming growth factor beta receptor 3 (Tgfbri) [35], transformation protein related protein protein 2 (Trp2) [36–38], Pten [28, 36, 37, 39–42], Kirsten rat sarcoma viral oncogene homolog (Kras) [28, 36, 37, 43], beta catenin 1 (Ctnnb1) [29, 39, 44–47], tuberous sclerosis 1 (Tsc1) [48, 49], smooth muscle, actin alpha-2 (Smo) [50–53], adrenomedullary polymyositis coli (Apc) [54], wingless-type MMTV integration site family, member 4 (Wnt4) [55], breast cancer 1, early onset (Brca1) [38], Dicer1 [42, 56–58], nuclear receptor subfamily 2, group F, member 2 (Nrf2) [59], splicing factor 1 (Sf1) [60], activins [61], follistatin (Fst) [62], and Smads [63, 64]. Here, using Amhr2-Cre mice, we show that deletion of Arid1a leads to a progressive loss of fertility via placental disruption.

MATERIALS AND METHODS

Generation and Genotyping of Arid1a Conditional Knockout Mice

Arid1a conditional allele (Arid1a<sup>fllox/fllox</sup>) mice have been described previously [24] and were maintained in a C57BL/6J;129S5/Brd mixed hybrid background. Arid1a<sup>fllox/fllox</sup> mice were bred to Amhr2<sup>cre/cre</sup> mice [26] to generate Arid1a<sup>fllox/fllox;Amhr2<sup>cre/cre</sup></sup> mice. Arid1a<sup>fllox/Amhr2<sup>cre/cre</sup></sup> male mice were crossed to Arid1a<sup>fllox/fllox</sup> female mice to generate final breeder pairs of Arid1a<sup>fllox/fllox;Amhr2<sup>cre/cre</sup></sup> male mice and Arid1a<sup>fllox/fllox</sup> female mice. These final breeders were used to generate the experimental female mice: Arid1a<sup>fllox/fllox;Amhr2<sup>cre/cre</sup></sup> mice (termed Arid1a conditional knockout [cKO] mice) and Arid1a<sup>fllox/Amhr2<sup>cre/cre</sup></sup> mice (termed control mice) (Supplemental Fig. S1A; Supplemental Data are available online at www.bioreprod.org). Arid1a<sup>fllox/Amhr2<sup>cre/cre</sup></sup> mice were not generated because previous studies show that Arid1a heterozygosity (Arid1a<sup>+/−</sup>) leads to embryonic lethality in mice, similar to homozygous deletion [24]. All mice were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals under an approved protocol. The Baylor College of Medicine Genetically Engineered Mouse Core performed rederivation and initial breeding. Mice were genotyped at 12–14 days of postnatal life from tail biopsies by PCR analyses using specific primers [22, 65] (Supplemental Table S1 and Supplemental Fig. S1B). For the Arid1a floxed allele, the following conditions were used with Arid1a forward and reverse primers: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C (denaturation), 45 sec at 60°C (annealing), and 45 sec at 72°C (extension). For the Cre allele, the following conditions were used with Amhr2-Cre forward and reverse primers: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C (denaturation), 45 sec at 60°C (annealing), and 45 sec at 72°C (extension).

Fertility Analysis and Studies Involving Parous Uteri

To evaluate reproductive performance, 6-wk-old female control and Arid1a cKO mice were bred to wild-type C57BL/6J;129S5/Brd hybrid male mice of proven fertility. The numbers of litters and pups were recorded over a 6-mo period. After the 6-mo fertility studies were completed, female mice were rested for 2 mo. Mice were then euthanized, and the parous uteri were fixed for histology.

Tissue Collection, Histological Analysis, and Timed Pregnancy

At times listed in experimental design below, mice were euthanized; reproductive organs were excised and fixed for histology or snap frozen for RNA or protein isolation. Uteri, implantation sites, and placenta were fixed in 4% paraformaldehyde (Sigma), and ovaries were fixed in 10% neutral buffered formalin (EMD Millipore). The Baylor College of Medicine Human Tissue Acquisition and Pathology Core performed tissue processing and paraffin embedding. Sections were cut at 5 μm and stained with hematoxylin (VWR) or eosin (VWR) or periodic acid-stain (Sigma) using standard techniques. Tissue and serum samples from timed pregnancies were obtained by mating Arid1a cKO and control female mice with intact wild-type C57BL/6J;129S5/Brd hybrid male mice. The morning that the vaginal plug was observed was designated as 0.5 days postcoitus (dpc). Mice were euthanized on Day 4.5, 8.5, 9.5, or 16.5 of pregnancy, and the number of implantation sites was counted.
PBS and incubated with the appropriate species-specific horseradish peroxidase-conjugated secondary antibody (2 μg/ml; Vector Laboratories) for 1 h at room temperature. Immunoreactivity was detected using the Vectastain Elite DAB kit (Vector Laboratories). Sections were then briefly counterstained with hematoxylin, dehydrated, and mounted. Slides were analyzed by light microscopy.

**RNA Isolation and Real-Time Quantitative PCR Analysis**

Total RNA was extracted using the mirVana microRNA isolation kit (Life Technologies, Inc.). After nucleic acid quantification on a NanoDrop ND-1000 (Thermo Scientific), RNA was treated with Turbo DNase (Life Technologies, Inc.) according to the manufacturer’s protocol. DNase-treated RNA (1000 ng) was reverse transcribed in a 50 μl reaction using 250 U Superscript III reverse transcriptase (Life Technologies, Inc.) with random primers (Life Technologies, Inc.). Samples were diluted to 100 μl and 2 μl was used for each quantitative PCR (qPCR) reaction. Real-time qPCR was performed on the ABI StepOnePlus using either predesigned TaqMan Gene Expression Assays (Life Technologies, Inc.) or custom primers designed using Primer Express software (Life Technologies, Inc.) for SYBR green (Supplemental Table S1). Levels of mouse ribosomal protein L13a (Rp13a) or 18s were used as endogenous controls for mouse uterus samples as expression of Rp13a and 18s was shown not to change in response to steroid hormone stimulation [66, 71]. Levels of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were used for placenta as previous studies have shown this is a reasonable endogenous control for mouse placenta [72, 73]. Levels of human ribosomal protein L19 (RPL19) were used as endogenous controls for human samples [66, 74–76]. TaqMan PCR was performed using TaqMan universal PCR master mix (Life Technologies, Inc.), and PCR with custom primers was performed using SYBR Green PCR master mix (Life Technologies, Inc.) in a 10-μl reaction. The reaction conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C (denaturation) and 1 min at 60°C (annealing/extension). Each sample was analyzed in duplicate or triplicate, and a nontemplate control (nuclease-free water) was included on each plate for each primer-probe set. All custom primers had an efficiency of 85%–110%. All SYBR green runs had dissociation curves to predict potential primer-dimers. The relative quantity of transcript was calculated using the 2^(-ΔΔCt) method [77].

**Institutional Review Board Approval, Collection of Human Tissues, Creation of Primary Cultures, and Transfection of Cells**

All human tissues were collected under Baylor College of Medicine Institutional Review Board approval with written informed consent, and cultures were described as created previously [76]. Cells were transfected and endogenously used in vitro decidualization as described previously [78]. Briefly, 2 days before induction of in vitro decidualization, cells in 6-well plates were treated with Dulbecco-modified Eagle medium/F12 with 2% charcoal-stripped fetal bovine serum (Life Technologies, Inc.) containing 5 μM RA/Invitrogen. On the day of decidualization induction, which was

**RESULTS**

**Arid1a cKO Mice Did Not Develop Ovarian Cancer**

To generate female mice lacking expression of Arid1a in the reproductive tract, Arid1a^lox/lox^ mice [24] and Amhr2^cre/+^ mice [26] that express Cre recombinase under the control of the Amhr2 promoter were obtained. Supplemental Figure S1A shows the detailed breeding strategy to generate Arid1a^lox/lox^, Amhr2^cre/+^ mice (termed Arid1a cKO mice) and Arid1a^lox/lox^ (termed control mice). Previous studies show that similar strategies are successful in disrupting ARID1A function [21, 24, 25].

We found that female Arid1a cKO and control mice were born healthy. To confirm tissue-specific recombination and ablation of Arid1a, we performed qPCR and immunohistochemical staining on tissues from virgin 12-wk-old female mice. Quantitative PCR revealed a 1.58-fold decrease (n = 8, Student t-test, P < 0.05) in Arid1a in whole uteri of adult Arid1a cKO mice compared with control mice (Fig. 1A). This sizeable but incomplete ablation of Arid1a in the uterus may be due to the lack of Arid1a deletion in the uterine epithelium. Next, we used immunohistochemistry to examine the cell type-specific expression of ARID1A. In uteri from virgin control female mice, ARID1A was expressed in the nuclei of uterine luminal and glandular epithelial cells, uterine stromal cells, and myometrial cells (Fig. 1, B and C). In uteri from virgin Arid1a cKO female mice, there was a lack of ARID1A staining in the uterine stroma but observable staining in uterine luminal and glandular epithelial cells (Fig. 1, D and E). This cell type-specific depletion of ARID1A in the uterus is consistent with previous work showing AMHR2 expression in uterine stromal cells [30]. Thus, as expected, ablation of Arid1a in the uterus of cKO mice was cell type specific.

Amhr2-Cre should lead to recombination in somatic cells of the ovary, including granulosa cells and ovarian surface epithelial cells [26, 28]. In virgin 12-wk-old female control mice, ARID1A was detected in ovarian theca cells, in somatic cells of ovarian follicles at all stages of follicular development, and in ovarian surface epithelial cells (Supplemental Fig. S2, A and B). In unstimulated virgin female Arid1a cKO mice, there was decreased ARID1A staining in granulosa cells and ovarian surface epithelial cells (Supplemental Fig. S2, C and D). Previous work had shown that deletion of Pten and Dicer using Amhr2-Cre lead to high-grade serous ovarian cancers that arose in the oviduct [42]. ARID1A was detected in nuclei of the columnar epithelium of the oviduct in both virgin control (Supplemental Fig. S2, E and F) and Arid1a cKO mice (Supplemental Fig. S2, G and H).

Because loss of ARID1A in epithelial cells is a common feature of human endometriosis-associated ovarian cancers [9, 11], we performed long-term survival studies with virgin female control and Arid1a cKO mice (n = 20 per genotype). We observed no differences (P = 0.575) between genotypes in body weight over time (Supplemental Fig. S3). No control or Arid1a cKO mice were euthanized due to disease. Furthermore, at 1 yr of age, both virgin control and virgin Arid1a cKO mice had normal reproductive tract histology (data not shown). These results indicate that virgin female Arid1a cKO mice did not develop ovarian cancer.

**Arid1a cKO Mice Exhibited a Progressive Decline in Fertility**

To evaluate fertility, female control and Arid1a cKO mice (n = 9 per genotype) were housed with wild-type males for 6 mo. Arid1a cKO mice were less fertile than control mice, with

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significantly fewer pups per litter (Student t-test, \( P < 0.05 \); Fig. 2A) and significantly fewer litters per month (cKO: 0.7 ± 0.3; control: 1.1 ± 0.1; Student t-test, \( P < 0.05 \)). Litter size was significantly smaller in Arid1a cKO mice (4.0 ± 1.9 pups per litter) compared with control female mice (7.5 ± 1.5 pups per litter; Student t-test, \( P < 0.001 \)). Furthermore, this subfertility...
Arid1a cKO Mice Had Low Penetration of Uterine Tumors after Pregnancy

After the fertility experiments, parous female mice were dissected at 8 mo of age to evaluate the reproductive tract. Control mice had grossly normal reproductive tracts, with no uterine or ovarian tumors (Supplemental Fig. S4A and data not shown). Parous control mice also had normal uterine histology, with similar ARID1A expression as virgin female mice (Supplemental Fig. S4B). Parous Arid1a cKO mice had normal uterine histology, with similar ARID1A expression as virgin female mice (Supplemental Fig. S4D). However, of the nine parous Arid1a cKO mice, one mouse had a uterine tumor, although both ovaries, both oviducts, and the opposite uterine horn in this mouse were grossly and histologically normal (Supplemental Fig. S4, A and D, and data not shown). Histological analysis showed that the tumor was a benign smooth muscle tumor with complete ablation of ARID1A in both the uterine stroma and epithelium (Supplemental Fig. S4C). In the normal horn, ARID1A expression was detected in the nuclei of glandular and luminal epithelial cells (Supplemental Fig. S4D), similar to its expression in virgin Arid1a cKO uteri. Overall, other than one benign uterine tumor, parous Arid1a cKO and virgin Arid1a cKO female mice had normal histology of female reproductive tract with no difference in ARID1A expression or localization.

Virgin Arid1a cKO Mice Had Normal Reproductive Tracts, Gonadotropin Levels, and Steroid Hormone Responses

Amhr2-Cre deletes the target allele in mesenchymal cells of the uterus, oviduct, and ovary, all of which may contribute to the subfertility phenotype observed in Arid1a cKO mice. Previous in vivo models of subfertility with Amhr2-Cre have shown defects in virgin or primigravid reproductive tract such as Stat3 [34], Kras [37], Pten [41], Wnt4 [55], and Nr2f2 [59]. Therefore, we undertook a systematic approach to examine the contribution of Arid1a deletion in each organ system in virgin female mice.

To characterize the uterus of Arid1a cKO mice, virgin mice were euthanized at 12 wk of age. Gross observation showed that the uterus of Arid1a cKO mice was smaller than that of control mice (Fig. 3A), and measurement of uterine weight revealed significantly lighter uteri in Arid1a cKO mice than in control mice (n = 8). Student t-test, P < 0.05; Fig. 3B). Furthermore, Arid1a cKO mice had significantly more uterine glands per mm² of surface area encapsulated by the myometrium than control mice (n = 4, P < 0.05; Fig. 3C). Therefore, we undertook a systematic approach to examine the contribution of Arid1a deletion in each organ system in virgin female mice.

To examine gonadotropin hormone levels, we measured FSH and LH serum hormone levels of unstimulated, randomly cycling adult Arid1a cKO and control mice. We observed no
significant differences between virgin control and Arid1a cKO mice in FSH levels (control: 5.758 ± 0.625 ng/ml, n = 12; cKO: 7.270 ± 1.789 ng/ml, n = 10; Student t-test, P = 0.402) or LH levels (control: 0.406 ± 0.133 ng/ml, n = 12; cKO: 0.222 ± 0.0633 ng/ml, n = 10; Student t-test, P = 0.253). To evaluate steroid hormone production, we measured serum E2 levels in virgin adult unstimulated, randomly cycling mice. There were no significant differences in E2 levels between control (11.62 ± 7.437 pg/ml, n = 11) and Arid1a cKO (5.200 ± 1.078 pg/ml, n = 10; P = 0.419) mice.

In primigravid mice, at 4.5 dpc, P4 levels in Arid1a cKO pregnant mice (7.641 ± 2.296 ng/ml, n = 12) did not differ.
from those in control pregnant mice (14.57 ± 4.571 ng/ml, n = 11; P = 0.426), indicating normal luteal function. When Arid1a was deleted from the uterine epithelium using Pgr-Cre, there was a significant loss of Pgr expression as well as loss of expression of P4 responsive genes [21]. Therefore, we more closely examined steroid hormone receptor and steroid hormone response at the molecular level. Levels of Pgr (n > 4, P = 0.194) and estrogen receptor 1 (Esr1) (n > 4; Student t-test, P = 0.817) were similar between unstimulated virgin adult control and Arid1a cKO mice (Supplemental Fig. S6, A and B). To determine whether the uteri of Arid1a cKO mice show an altered response to steroid hormones, virgin unstimulated mice were ovariectomized and treated with E2 for 3 days or P4 for 6 h [67, 83]. The uteri from virgin Arid1a cKO females underwent a 5.2-fold increase in gross weight in response to E2, similar to the 5.8-fold increase observed for virgin control mice (Supplemental Fig. S7E). Therefore, we more closely examined steroid hormone receptor and steroid expression of P4 responsive genes [21].

Previous work has shown that ARID1A is deficient in hESCs from women with endometriosis [21] and that in vitro decidualization of hESCs from women with endometriosis have a blunted in vitro decidualization response [89]. To investigate the role of ARID1A in decidualization, we performed small interfering RNA knockdown of ARID1A in hESCs from women without endometriosis and then exposed cells to conditions for in vitro decidualization. hESCs transfected with siARID1A showed a 4.7-fold decrease in ARID1A compared with hESCs transfected with siNT (n = 7, Student t-test, P < 0.001; Supplemental Fig. S8A); hESCs transfected with siARID1A and then exposed to in vitro decidualization conditions showed changes in morphology similar to hESCs treated with siNT (data not shown). In addition, hESCs treated with siNT or siARID1A (n = 7) showed similar increases in levels of decidualization markers prolactin (PRL) (Student t-test, P = 0.1441), a ligand of the Wnt family (WNT4) (Student t-test, P = 0.2146), and insulin-like growth factor binding protein 1 (IGFBP1) (Student t-test, P = 0.2591; Supplemental Fig. S8, B–D). Therefore, knockdown of ARID1A in primary cultures of hESCs did not affect in vitro decidualization, similar to how deletion of Arid1a in stromal cells of the mouse uterus in vivo did not affect artificial decidualization (Supplemental Fig. S7).

**Parous Arid1a cKO Mice Exhibited Reduced Fetal Viability**

Our fertility studies showed a progressive decline in the number of pups per litter, although female mice maintained normal decidualization. To investigate the time during pregnancy at which this phenotype becomes apparent, we performed timed pregnancy studies by breeding female control and Arid1a cKO mice at 6 wk with wild-type male mice. During the first pregnancy, for control mice, the number of implantation sites/female was similar to the number of viable embryos/female on Embryonic Days 8.5 and 9.5, with no implantation sites/female on Embryonic Days 8.5 and 9.5, with no

**Table 1. Number of implantation sites and resorbed embryos.**

| Pregnancy number | Genotype | Embryonic day | Implantation sites/female (mean ± SD) | Viable embryos/female (mean ± SD) | % Resorbed embryos/female | P-value |
|------------------|----------|---------------|--------------------------------------|-----------------------------------|--------------------------|---------|
| 1                | Control  | 8.5 (n = 8)   | 9.1 ± 0.5                            | 9.1 ± 0.5                          | 0%                       | 0.9106  |
|                  | cKO      | 8.5 (n = 13)  | 8.4 ± 0.3                            | 8.2 ± 0.3                          | 2.3%                     | 0.7749  |
|                  | Control  | 9.5 (n = 8)   | 8.4 ± 0.7                            | 7.9 ± 0.8                          | 8.3%                     | 0.7401  |
|                  | cKO      | 16.5 (n = 10) | 7.9 ± 0.4                            | 6.5 ± 0.5                          | 17.4%                    |         |
| 3                | Control  | 16.5 (n = 9)* | 11.2 ± 0.6                           | 10.8 ± 0.6                         | 4.1%                     | 0.0177* |
|                  | cKO      | 16.5 (n = 8)* | 10.4 ± 0.5                           | 8.3 ± 0.4                          | 19.4%                    |         |

* Statistical comparisons are between genotypes at similar embryonic day.

**Arid1a DELETION REDUCES FERTILITY**

To further investigate the subfertility phenotype, ovariectomized virgin control and Arid1a cKO mice were treated with hormones, and their uterus was mechanically stimulated to induce decidualization. Gross and histological morphology of the decidual and control horn were similar between virgin control and Arid1a cKO uteri (Supplemental Fig. S7, A–D). In addition, the ratio of decidualized to control horn weight was equivalent in control and Arid1a cKO uteri (n = 7; Student t-test, P = 0.1748; Supplemental Fig. S7E).

Primary cultures of human endometrial stromal fibroblasts (hESCs) have the ability to undergo in vitro decidualization, and this culture system has been used to demonstrate the importance of several factors in uterine decidualization, such as LIF [84], transient receptor potential cation channel, subfamily C, member 1 (TRPC1) [85], transcription factor 23 (TCF23) [86], uterine activating like kinase 2 (ALK2) [78], bone morphogenetic protein 2 (BMP2) [87], and WNT4 [88].
examined uterine differences at the molecular level for the Arid1a subfertility and sterility [90, 91]. Given the changes in placental function when deleted in the uterus, leading to receptors, uterine activin like kinase 4 (ALK4) and uterine labyrinthine zone; Statistical significance (*P < 0.05) was calculated by two-way ANOVA. Bars = 500 μm (C, D, H, I).

FIG. 4. Arid1a cKO mice showed increased embryo resorption during the third pregnancy. Gross morphology of primigravid control (A) and cKO (B) uteri on Embryonic Day 8.5. Conditional knockout implantation sites (D) on Embryonic Day 8.5 showed evidence of hemorrhage, which was not observed at control implantation sites (C). Conditional knockout antimesometrial decidua was thicker than those in control mice (E). Conditional knockout mice (G) showed more resorbed embryos (arrows) than control mice (F) on Embryonic Day 16.5 of the third pregnancy. Midsagittal sections of placental tissue obtained on Embryonic Day 16.5 from control (H) and cKO (I) mice of third pregnancy. Conditional knockout mice had a thinner junctional zone and decidua compared with control mice (I). AMD, antimesometrial decidua; MD, mesometrial decidua; De, decidua; Jz, junctional zone; Lz, labyrinthine zone; Statistical significance (*P < 0.05) was calculated by two-way ANOVA. Bars = 500 μm (C, D, H, I).

(Molecular Differences Existed Between Parous and Virgin Arid1a cKO Mice)

To understand what factors contribute to the significant subfertility phenotype beginning with the third litter in Arid1a cKO mice, we studied multiparous females and their placentas. During the third pregnancy, P4 levels in multiparous Arid1a cKO pregnant mice at Embryonic Day 16.5 (23.34 ± 5.030 ng/ml, n = 7) did not differ from those in control pregnant mice (24.14 ± 5.886 ng/ml, n = 6; Student t-test, P = 0.9195), indicating normal luteal function in parous Arid1a cKO mice during pregnancy. Gross morphology and histology of parous ovaries was similar to virgin ovaries for both control and Arid1a cKO mice showing normal corpora lutea and follicular development (data not shown) further supporting normal luteal function in the first pregnancy and subsequent pregnancies. Finally, AMH levels were similar between virgin and multiparous Arid1a cKO mice (data not shown).

Recently, two different transforming growth factor beta receptors, uterine activin kinase 4 (ALK4) and uterine activin kinase 5 (ALK5), were shown to significantly affect placental function when deleted in the uterus, leading to subfertility and sterility [90, 91]. Given the changes in placental morphology in parous Arid1a cKO females, we examined uterine differences at the molecular level for the underpinnings of this progressive subfertility phenotype. In uteri from 6-mo fertility studies, examination of ARID1A expression in the multiparous uterus showed a slight decrease in ARID1A expression. Expression of ARID1A was present in 50%–80% of epithelial cells in the uteri of Arid1a cKO female mice while expression of ARID1A was present in 80%–95% of epithelial cells in the uteri of control female mice (Fig. 5, A and B). Higher power magnification examination confirmed nuclear ARID1A staining in both Arid1a cKO uteri and control uteri with decreased expression in Arid1a cKO uteri (Supplemental Fig. S9). Previous work had shown that deletion of Arid1a with Pgr-Cre led to proliferation of uterine epithelial cells during early pregnancy, but not uterine stromal cells [21]. In parous uterus, we found that Arid1a cKO uterine epithelial and stromal cells showed increased proliferation compared to multiparous control (Fig. 5, C and D). However, there was no difference in PGR or ESR1 expression (Fig. 5, E–H). These data suggest that ARID1A plays a role in both epithelial and stromal cell proliferation in the uterus. Given the subtle changes in thickness of antimesometrium at Embryonic Day 8.5 in the first pregnancy and the more significant changes in placental morphology at Embryonic Day 16.5 in the third pregnancy, we focused our molecular studies on the plenta.

We discovered a 33% decrease in Arid1a expression in the placentas from Arid1a cKO mothers during the third pregnancy (Fig. 6A), likely an indirect effect and not an effect of knockout on the plenta. Similarly, there was a significant decrease in SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4 (Smarca4), an ARID1A binding partner, expression (Fig. 6B). To gain insight into the molecular underpinnings of the placental phenotype, we examined the levels of molecules involved in placental structure and function. We discovered decreased expression of nodal growth differentiation factor (Nodal), a transforming growth factor beta-receptor ligand (Fig. 6C) in placentas from Arid1a cKO female mice. Additionally, placentas from Arid1a cKO mice exhibited decreased proteocadherin 12 (Pcdh12), a marker of glycogen trophoblast cells, and trophoblast specific protein alpha (Tphpa), a marker of spongiosotrophoblast cells compared to placentas from control female mice (Fig. 6, D and
Genetic aberrations in cyclin-dependent kinases [96]. For colon proliferation of several types of cancers and is associated with pregnancy.

Pups in the postimplantation phase beginning with the third progressive decline in fertility with higher parity, with a loss of Arid1a proliferation [82, 97, 98]. Likewise, we found that loss of ARID1A is important for normal tumor formation in the mouse female reproductive tract. Here, by conditionally deleting Arid1a in mesenchymal-derived cells of the female reproductive tract, we provide evidence that loss of Arid1a is not sufficient for tumor formation, suggesting that additional mutations are required for tumor formation in the mouse female reproductive tract. However, we show that ARID1A is important for normal placental morphology and fertility in parous female mice. Female mice with conditional deletion of Arid1a show a progressive decline in fertility with higher parity, with a loss of pups in the postimplantation phase beginning with the third pregnancy.

Studies suggest that ARID1A plays a key role in the proliferation of several types of cancers and is associated with genetic aberrations in cyclin-dependent kinases [96]. For colon cancer, non-small cell lung cancer, and gastric cancer, knockdown of ARID1A enhances cellular proliferation in vitro, and restoration of ARID1A expression suppresses cellular proliferation [82, 97, 98]. Likewise, we found that loss of Arid1a up-regulated Ccnd1 in the virgin uterus in vivo.

**DISCUSSION**

Many previous studies have focused on the role of ARID1A in cancer, particularly as a tumor suppressor or driver mutation in tumors of the female reproductive tract [9, 11, 13, 15, 20, 23]. Thus, our original hypothesis was that deletion of Arid1a with Amhr2-Cre would give rise to ovarian tumors in mice. Here, by conditionally deleting Arid1a in mesenchymal-derived cells of the female reproductive tract, we provide evidence that loss of Arid1a is not sufficient for tumor formation, suggesting that additional mutations are required for tumor formation in the mouse female reproductive tract. However, we show that ARID1A is important for normal placental morphology and fertility in parous female mice. Female mice with conditional deletion of Arid1a show a progressive decline in fertility with higher parity, with a loss of pups in the postimplantation phase beginning with the third pregnancy.

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Additionally, we also observed up-regulation of the pro-apoptotic gene Bcl2 in the Arid1a cKO uterus. Therefore, we believe that the balance of proliferation and apoptosis may preclude the development of cancer in virgin female Arid1a cKO mice. Other studies using adenosivirus-driven Cre to delete Arid1a from the ovarian surface epithelium showed that Arid1a depletion is not sufficient to induce ovarian tumors in mice [22]. Additionally, our work deleting Arid1a in the uterus using Pgr-Cre did not develop endometrial tumors [21]. Consistently, we found that Arid1a deletion from the ovarian surface epithelium using Amhr2-Cre did not induce ovarian tumors in virgin female mice. Thus, we speculate that female reproductive tract cancers in mice require additional genetic hits along with loss of Arid1a to give rise to malignant tumors.

Interestingly, Arid1a cKO female mice had significant subfertility that became apparent after the second pregnancy. Transitionally, this may be comparable to secondary infertility although most of our Arid1a cKO mice were not completely infertile. Male factors did not contribute because we were using fertility-proven intact wild-type male mice for our experiments. Given the deletion of Arid1a in the somatic cells of the ovary, ovarian dysfunction was critically evaluated. In primigravid and multigravida female Arid1a cKO mice, we found similar P4 levels as control as well as similar numbers of corpora lutea. AMH levels, a measure of ovarian reserve, were also similar between Arid1a cKO and control for both virgin and parous female mice. Gonadotropin levels were also similar between virgin Arid1a cKO and control female mice. Thus, we do not see an obvious luteal phase defect or other ovulatory dysfunction in either virgin or parous Arid1a cKO female mice. However, it remains possible that a more subtle luteal defect may contribute to the reduced pups per litter. Anatomically, Arid1a cKO had normal oviductal, uterine, and cervical histology for both virgin and parous female mice, and we found no gross or histological evidence of endometriosis. Thus, we do not believe that anatomical factors lead to secondary subfertility. In women undergoing an evaluation for secondary infertility, this would fall under the category of unexplained infertility and is typically thought to be caused by endometrial dysfunction. Only recently have genomewide

**FIG. 5.** After 6-mo fertility studies, parous uteri from Arid1a cKO mice showed increased proliferation. ARID1A expression was present in >95% of epithelial cells from control (A) but only 50%–80% of epithelial cells from cKO (B) uteri. However, increased proliferation in both epithelium and stromal compartments was apparent in cKO (D) compared to control (C) parous uteri by Ki67 staining. Expression of ESR1 was similar between control (E) and cKO (F) parous uteri. Similarly, expression of PGR was similar between control (G) and cKO parous uteri (H). GE, glandular epithelium; L, lumen; LE, luminal epithelium; M, myometrium; S, uterine stroma. Bars = 25 μm (A, B) and 50 μm (C–H).
profiling techniques been used to identify these subtle molecular changes [99–101].

Our virgin Arid1a cKO female mice had normal implantation, normal decidualization, and normal pregnancy outcomes in the first pregnancy. However, examination of parous uteri showed an increase in both epithelial and stromal proliferation (Fig. 5) with deletion of ARID1A that was not present in virgin uteri even at 1 yr of age (data not shown). Little has been published about the molecular differences between virgin and parous uteri. We can only speculate that the deletion of Arid1a led to indirect changes in gene expression that affected uterine remodeling after pregnancy. We speculate that subtle changes in the uterine stroma after remodeling postpartum lead to changes in placentation with subsequent pregnancies. However, significant work needs to be done to understand the subtle molecular changes between the parous and the virgin uterus before we can tease out the specific changes associated with loss of Arid1a.

Only after pregnancy, one female Arid1a cKO mouse was found to have a benign uterine tumor that exhibited a lack of ARID1A in epithelial cells. Given the low penetrance (one in nine) of tumors in parous uteri, a significantly larger number of

FIG. 6. Placentas from Arid1a cKO mice showed molecular dysregulation. Placental mRNA levels of Arid1a (A), Smarca4 (B), Nodal (C), Pcdh12 (D), Tpbpa (E), Ptgs2 (F), Vegfa (G), Angpt1 (H), and Corin (I) were decreased in cKO mice on Embryonic Day 16.5 of the third pregnancy. There was no difference in levels of Gcm1 (J) or Vcam1 (K). Statistical significance (*P < 0.05) was calculated by Student t-test. Gene expression was measured relative to Gapdh.
female mice would need to be examined to determine the statistical significance of this result. Previous studies suggest that glandular and luminal epithelial cells of the uterus are regenerated from uterine stromal cells after pregnancy in Amhr2-Cre mice [102]. After one pregnancy, at most 50% of epithelial cells were regenerated from stroma in a process of mesenchymal epithelial transition (MET) [103]. Thus, deletion of ARID1A in the epithelium could result from Arid1a deletion in uterine stromal cells. This could account for the loss of ARID1A in the one female mouse with a tumor after pregnancy. Interestingly, recurrent copy number variants of Arid1a have recently been discovered in uterine leiomyomata tumors [104], which are the most translationally equivalent human disease to the one tumor. However, other parous Arid1a cKO female mice examined had expression of ARID1A in the epithelium and no tumors. Deletion of Arid1a with Pgr-Cre deleted Arid1a in the epithelium and did not result in similar tumors. However, these female mice were completely sterile and thus could not be studied in the multiparous state [21].

Indeed, we did see a decrease in ARID1A staining in the epithelium after repetitive pregnancy (Fig. 5 and Supplemental Fig. 9) in Arid1a cKO uteri, suggesting that MET is occurring in this model. Similar to published work, we saw at most 50% loss of ARID1A staining in epithelial cells after repetitive pregnancy. While previous work had only examined MET after 1 pregnancy, we have shown that MET remains after repetitive pregnancies and may be long lasting. The MET effect does not seem to be exponential with each pregnancy (i.e., loss of 50% with first pregnancy, loss of another 50% with second pregnancy). However, we cannot speculate as to whether repetitive pregnancy allows for regeneration of ARID1A-positive epithelial cells from ARID1A-positive epithelial cells based on our experimental time point. While a partial deletion of ARID1A does occur in the epithelial cells of the uterus in the Arid1a cKO mice, the phenotype is different than the complete deletion of ARID1A in the epithelial cells of the uterus of the Pgr-Cre female mice. However, it remains possible that the epithelial cell phenotype in the Amhr2-Cre female mice contributes to the reduced pups per litter.

Finally, multiparous Arid1a cKO female mice developed significantly abnormal placentas. The placenta is critical for proper fetal development. Translationally, placental abnormalities can lead to significant maternal morbidities such as pre-eclampsia and fetal complications such as fetal growth restriction or fetal demise. While we did not find gross differences in size of live born pups (data not shown), we did discover significant resorption of embryos at Embryonic Day 16.5 in the third litter of Arid1a cKO female mice (Table 1 and Fig. 4). The placental morphology was abnormal (Fig. 4), and molecular markers of abnormal placental morphology confirmed these differences (Fig. 6). ARID1A is a critical player in chromatin remodeling, cellular proliferation, and gene regulation [2, 104–108]. Although multiple studies have focused on the role of epigenetic markers in chromatin remodeling in the placenta, little is known about the actual gene products involved in placental chromatin remodeling. One study shows that in vitro depletion of SMARCA4 (also known as BRG1), which is a critical member of the SWI/SNF chromatin-remodeling complex and a known protein-binding partner for ARID1A [109] that plays a key role in trophoblast stem cell self-renewal [110], has a similar effect on cellular proliferation as the depletion of ARID1A [111]. We found a decrease in both Arid1a and Smarca4 expression in placentas from multiparous Arid1a cKO females (Fig. 6). In the present study, the molecular markers examined in placentas from Arid1a cKO mice are at best suggestive of abnormal placental morphology.

Although they do not provide a complete understanding of the molecular mechanism of the effect of Arid1a deletion, they suggest that loss of Arid1a in the uterus impairs placental function, evidenced by a subfertility phenotype, increased resorbed embryos, and a thinner junctional zone at implantation sites.

In conclusion, we found that Arid1a deletion alone is insufficient to induce tumorigenesis in the female reproductive tract of mice. ARID1A regulates embryo implantation and development of a functional placenta. Future studies of our Arid1a cKO mouse model should provide molecular insight into the delicate function of SWI/SNF-mediated chromatin remodeling in placental function.

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