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Vaginal Squamous Cell Carcinoma Develops in Mice with Conditional *Arid1a* Loss and Gain of Oncogenic *Kras* Driven by Progesterone Receptor Cre

Xiyin Wang,* Mariana S.L. Praça,* Jillian R.H. Wendel,* Robert E. Emerson,† Francesco J. DeMayo,‡ John P. Lydon,§ and Shannon M. Hawkins*

From the Department of Obstetrics and Gynecology, Indiana University School of Medicine, Indianapolis, Indiana; the Department of Pathology and Laboratory Medicine,† Indiana University School of Medicine, Indianapolis, Indiana; the National Institute of Environmental Health Sciences,‡ Research Triangle Park, North Carolina; and the Department of Molecular and Cellular Biology,§ Baylor College of Medicine, Houston, Texas*

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Address correspondence to Shannon M. Hawkins, M.D., Ph.D., Indiana University School of Medicine, 550 N. University Blvd, UH2440, Indianapolis, IN 46202. E-mail: shhawkin@iu.edu.

Oncogenic KRAS mutations are a common finding in endometrial cancers. Recent sequencing studies indicate that loss-of-function mutations in the *ARID1A* gene are enriched in gynecologic malignant tumors. However, neither of these genetic insults alone are sufficient to develop gynecologic cancer. To determine the role of the combined effects of deletion of *Arid1a* and oncogenic *Kras*, $Arid1a^{flox/flox}$ mice were crossed with $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$ mice using progesterone receptor Cre ($Pgr^{Cre/+}$). Histologic analysis and immunohistochemistry of survival studies were used to characterize the mutant mouse phenotype. Hormone dependence was evaluated by ovarian hormone depletion and estradiol replacement. $Arid1a^{flox/flox}$; $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$; $Pgr^{Cre/+}$ mice were euthanized early because of invasive vaginal squamous cell carcinoma. Younger mice had precancerous intraepithelial lesions. Immunohistochemistry supported the pathological diagnosis with abnormal expression and localization of cytokeratin 5, tumor protein P63, cyclin-dependent kinase inhibitor 2A, and Ki-67, the marker of proliferation. Ovarian hormone deletion in $Arid1a^{flox/flox}$; $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$; $Pgr^{Cre/+}$ mice resulted in atrophic vaginal epithelium without evidence of vaginal tumors. Estradiol replacement in ovarian hormone—depleted $Arid1a^{flox/flox}$; $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$; $Pgr^{Cre/+}$ mice resulted in lesions that resembled the squamous cell carcinoma in intact mice. Therefore, this mouse can be used to study the transition from benign precursor lesions into invasive vaginal human papillomavirus—independent squamous cell carcinoma, offering insights into progression and pathogenesis of this rare disease. *(Am J Pathol 2021, 191: 1281—1291; https://doi.org/10.1016/j.ajpath.2021.03.013)*

Recent sequencing studies, including The Cancer Genome Atlas (TCGA), PanCancer, and Memorial Sloan-Kettering Integrated Mutation Profiling and Actionable Cancer Targets (MSK-IMPACT), have highlighted potentially impactful mutations across gynecologic cancers.[1,2] Worldwide in 2020, nearly 1.4 million women were diagnosed with gynecologic cancer, and >672,000 women with gynecologic cancer died,[3] up from 528,000 deaths in 2018.[4] In silico analyses of multiple, large, publicly available datasets have found that a small subset of genes are mutated across uterine cervix, uterine corpus, ovary, vulval, or vaginal cancers.[5] However, the functional role of each of these genes in female reproductive tract

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X.W. and M.S.L.P. contributed equally to this work.

Current address of X.W., Graduate School of Biomedical Sciences, Mayo Clinic, Rochester, MN; of M.S.L.P., Department of Obstetrics and Gynecology, Federal University of Minas Gerais and Group of Health, Mater Dei, Belo Horizonte — MG, Brazil.

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malignant tumors remains mostly unknown. Models of these diseases have the potential to improve the understanding of disease pathogenesis, accelerate the discovery of novel therapeutics, and improve the lives of many women worldwide.

AT-rich interactive domain 1A (ARID1A) is one of eight genes whose mutation frequency is significantly higher in gynecologic malignant tumors over other cancers.[5] Loss-of-function mutations in *ARID1A* are frequent in endometriosis-associated ovarian cancers, endometrial cancers, and cervical cancers.[1,2,5–12] More than 40% of endometrial cancers have a mutation in *ARID1A*.[8–10] Of human papillomavirus (HPV)–negative cervical squamous cell carcinomas, 33% contained mutations in *ARID1A*.[12] *ARID1A* encodes a protein in the switch/sucrose nonfermentable chromatin remodeling complex, playing a role in transcriptional regulation and reprogramming.[10,13,14] The ARID1A protein plays an essential role in female reproduction and is ubiquitously expressed in the female reproductive tract.[15,16] Conditional deletion of *Arid1a* with the antimüllerian hormone receptor 2 *Cre* resulted in subfertility because of abnormal placentation.[15] Conditional deletion of *Arid1a* with the progesterone receptor Cre ($Pgr^{Cre/+}$) or the lactotransferrin Cre resulted in infertility because of endometrial dysfunction.[16,17] Deletion of *Arid1a* alone in the mouse female reproductive tract was not sufficient to drive cancer.[13–18] Additional gene deletions were required for the development of gynecologic malignant tumors.[13,14,18–22]

Mutations in *KRAS* are frequent in gynecologic malignant tumors. *KRAS* is mutated in >10% of samples across multiple large datasets.[5] Oncogenic *KRAS* has been detected in up to 30% of endometrial cancers.[23–25] Increased expression of *KRAS* is associated with low overall survival across gynecologic cancers.[5] Conditional expression of $Kras^{G12D}$ alone was insufficient to develop cancer in mice.[26–29] However, *Pten* conditional deletion in $Kras^{G12D}$-expressing mice was sufficient for gynecologic malignant tumors.[27–31] Similar to deletion of *Arid1a* alone, the gain of oncogenic $Kras^{G12D}$ alone in the female reproductive tract was not sufficient to drive cancer,[26,28,29,32] and additional gene deletions were required in genetically engineered mouse models.[19,26–29,31,33–35]

In humans, concurrent mutations in *ARID1A* and *KRAS* are frequent in mesonephric-like müllerian adenocarcinomas of the female reproductive tract.[36] Similarly, simultaneous mutations are common in endometrial and cervical cancer and are rare, yet present, in ovarian cancer.[1,2,5,8,12,37,38] These previous results in humans led to the hypothesis that conditional *Arid1a* deletion in $Kras^{G12D}$-expressing mice using Pgr^{Cre} would generate female reproductive tract cancer.

Materials and Methods

Animal Husbandry and Genotyping

Animal experiments were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (Indianapolis, IN). Animals were handled according to the NIH's *Guide for the Care and Use of Laboratory Animals*.[39] All mice were bred and kept under standard conditions. $Arid1a^{flox/flox}$; $Pgr^{Cre/+}$ mice[16] and $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$ mice[28] or $Pgr^{Cre/+}$ mice[40] and $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$ mice[28] were crossbred and maintained on a C57BL/6J; 129S5/Brd mixed hybrid background to generate $Arid1a^{flox/flox}$; $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$; $Pgr^{Cre/+}$ (AKP), $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$; $Pgr^{Cre/+}$ (KP), and $Pgr^{+/+}$ (control) mice (Supplemental Figure S1). Mice were genotyped at 12 to 14 days of postnatal life from tail biopsy specimens by PCR as previously described.[15,41,42] *Cre*-mediated recombination in Arid1aflox/flox mice removes exon 8, leading to loss of protein.[43] *Cre*-mediated recombination of $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$ mice removes the stop codon, resulting in the expression of oncogenic $Kras^{G12D}$.[44] PgrCre/+ drives *Cre*-mediated recombination where progesterone receptor is expressed.[40] For survival studies, mice were caged, examined twice weekly, and euthanized at humane endpoints.[42] Postmortem tail clips were used to confirm genotyping,[15,41,42] and mice with inconsistent genotypes were reassigned or removed.

Histologic Analyses

Tissues were fixed, stained, and quantified as previously described.[42] The Lower Anogenital Squamous Terminology project nomenclature was used to describe histologic findings.[45] All histologic findings were interpreted by a surgical pathologist (R.E.E.). Antibodies for immunohistochemistry are listed in Table 1. Immunohistochemistry and immunofluorescence were performed as previously described,[42] confirming deletion of ARID1A and expression of KRAS proto-oncogene, GTPase (KRAS)^{G12D} in AKP uterine and vaginal tissues (Supplemental Figure S2).

Serum Analysis

Mice were anesthetized by isoflurane (Abbott Laboratories, North Chicago, IL), and blood was collected in microtainer tubes (Becton Dickinson, Franklin Lakes, NJ) by closed cardiac puncture. The serum was separated by centrifugation and stored at −20°C until use. The University of Virginia Ligand Assay and Analysis Core performed measurements of follicle-stimulating hormone and luteinizing hormone by radioimmunoassay.

Steroid Hormone Treatment

Mice underwent ovariectomy at 6 weeks. Mice were randomly divided into treatment groups: estradiol pellet (0.25 mg of 17-β-estradiol per 60-day release pellet; Innovative Research of America, Sarasota, FL) or no pellet (sham) and tissues collected after 60 days.

Table 1 Antibodies Used for Immunostaining

Antibody name	Company	Catalog number	Dilution
ARID1A	Santa Cruz Biotechnology (Dallas, TX)	sc-98441	1:100
$KRAS^{G12D}$	Abcam (Cambridge, UK)	Ab221163	1:20
CK5	Cell Signaling Technology (Danvers, MA)	71536S	1:50
p63	Genetex (Irvine, CA)	GTX102425	1:100
p16	Abcam	Ab108349	1:100
Anti–Ki-67	MilliporeSigma (Burlington, MA)	AB9260	1:300
Goat anti-rabbit IgG	Vector Laboratories (Burlington, MA)	BA-1000	1:200

ARID1A, AT-rich interactive domain 1A; CK5, cytokeratin 5; $KRAS^{G12D}$, KRAS proto-oncogene, GTPase; p16, cyclin-dependent kinase inhibitor 2A; p63, tumor protein P63.

Statistical Analysis

Log-rank (Mantel-Cox) test, Fisher's exact test, *t*-test, and multiple *t*-tests were performed using the InStat package for GraphPad Prism version 8 (GraphPad, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Large Vaginal Tumors

As per the criteria for humane endpoints,[42] AKP mice had decreased survival compared with $Pgr^{+/+}$ mice (Figure 1A and Supplemental Figure S3). The median survival of AKP mice was 106 days. AKP mice (10 of 10) exhibited gross, solid lesions protruding from the vagina, resulting in early euthanasia. These lesions were pink-red, with oblong to round growth on the outer part of the vagina (Figure 1B). On dissection, the gross lesions appeared to be confined to the vagina, with grossly normal ovaries, oviducts, and uterus (Figure 1C).

$Pgr^{+/+}$ female mice had normal-appearing vaginal histologic findings with well-defined epithelial layers, including a keratinized outer layer of cells without nuclei, stratified squamous epithelium, and lamina propria (Figure 1D). AKP mice had squamous cell carcinoma with central keratinization surrounded by highly dysplastic squamous epithelium as evidenced by the large, dark nuclei and disorganized cellular pattern, along with nests of cells with abundant eosinophilic cytoplasm (Figure 1E).

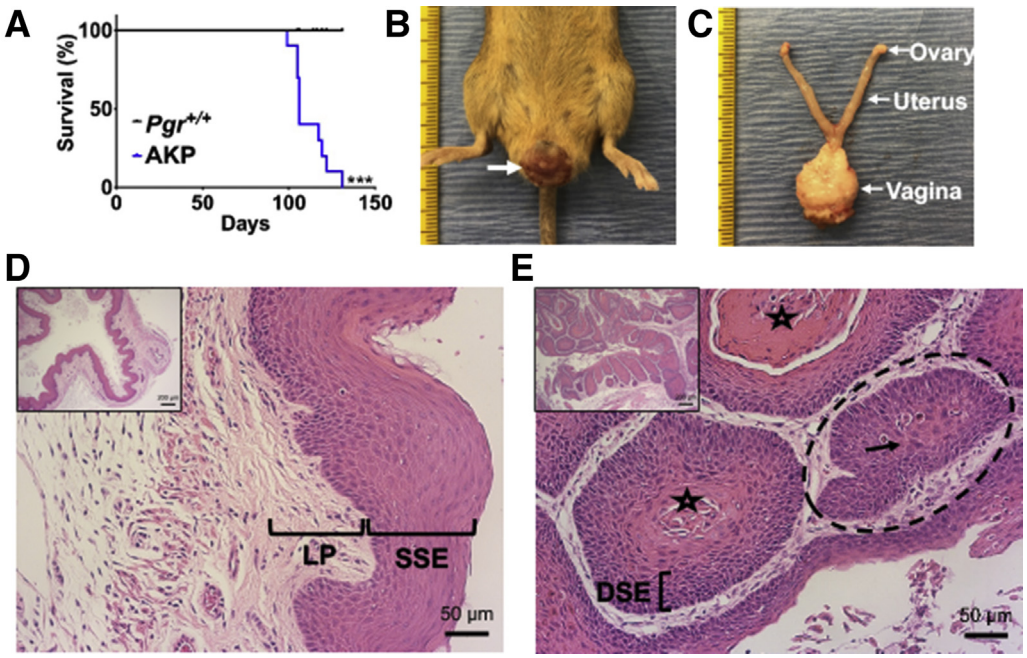

Figure 1 Poor survival and squamous cell carcinoma in *Arid1a*$^{flox/flox}$; *Kras*$^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$; *Pgr*$^{Cre/+}$ (AKP) female mice. **A:** Kaplan-Meier survival curves were analyzed by the log-rank (Mantel-Cox) test. AKP mice had decreased survival compared with control mice. **B:** Gross vaginal tumor burden (**white arrow**) in AKP mice. **C:** Gross lesions were localized to the vagina with grossly normal ovaries, oviducts, uterus, and cervix. Tick marks on ruler indicate 1 mm. **D:** Control vagina had normal keratinized stratified squamous epithelium (SSE) and lamina propria (LP). **E:** AKP vagina exhibited squamous cell carcinoma lined by highly dysplastic squamous epithelium (DSE) with central keratinization (**star**) and nests of cells (**dashed circle**) with abundant eosinophilic cytoplasm (**black arrow**). Hematoxylin and eosin staining. $n = 10$ per group (**A**). ***$P < 0.001$. Scale bars: 50 μm (**D** and **E, main images**); 200 μm (**D** and **E, insets**).

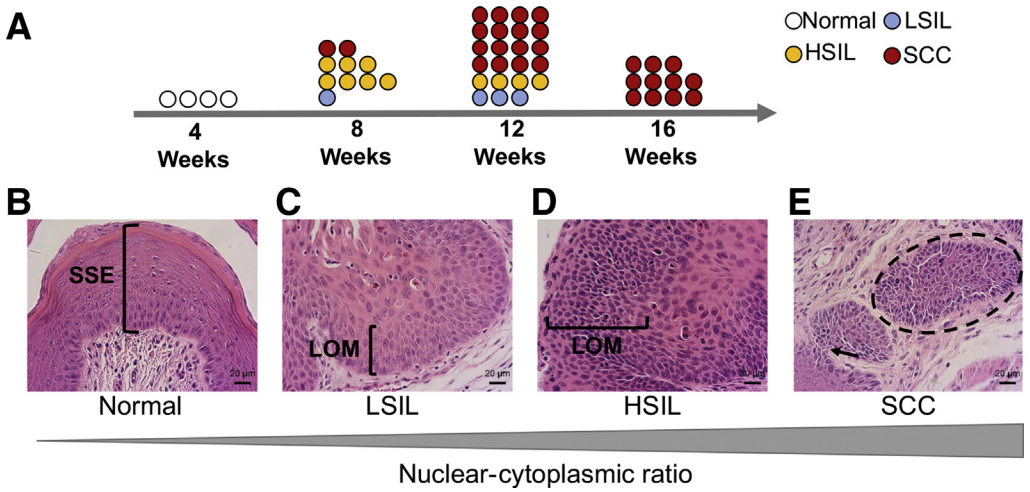

Figure 2 Squamous intraepithelial lesions and squamous cell carcinoma in *Arid1a*$^{flox/flox}$; *Kras*$^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$; *Pgr*$^{Cre/+}$ (AKP) mice. **A:** Schematic timeline of the progression of squamous intraepithelial lesions to squamous cell carcinoma in AKP mice. **B:** At 8 weeks, *Pgr*$^{+/+}$ vagina had normal stratified squamous epithelium (SSE). **C:** Ten percent of AKP vaginas contained low-grade squamous intraepithelial lesions (LSILs) with increased nuclear/cytoplasmic ratios and loss of maturation (LOM) in the lower epithelium. **D:** Seventy percent of AKP vaginas exhibited high-grade squamous intraepithelial lesions (HSILs) with cell crowding, high nuclear/cytoplasmic ratio, and LOM in at least two-thirds of the epithelium. **E:** Twenty percent of AKP vaginas had squamous cell carcinoma with nests of cells (**dashed circle**) and highly dysplastic squamous epithelium invading through the stroma (**black arrow**). Hematoxylin and eosin staining. Scale bar = 20 μm.

Additional examples of squamous cell carcinoma are in Supplemental Figure S4, and individual mouse female reproductive tract histologic findings are listed in Supplemental Table S1.

Early Precancerous Lesions Leading to Cancer

In women, invasive squamous cell carcinoma develops from precancerous lesions, including low-grade squamous intraepithelial lesions (LSILs, also known as vaginal intraepithelial neoplasia 1) or high-grade squamous intraepithelial lesions (HSILs, vaginal intraepithelial neoplasia 2 and 3).[46] As AKP mice aged, the penetrance of squamous cell carcinoma increased (Figure 2A). Squamous intraepithelial lesions were not found in *Pgr*$^{+/+}$ mice (Figure 2B). As early as 8 weeks, 10% (1 of 10) of AKP mice exhibited LSILs. The nuclei in the epithelium were enlarged with variable size, irregular nuclear contours, and increased nuclear/cytoplasmic ratios (Figure 2C). Most frequently (7 of 10), AKP mice at 8 weeks had HSILs. The epithelium exhibited a full-thickness proliferation of abnormal parabasal-like cells with loss of maturation and increased nuclear/cytoplasmic ratios (Figure 2D). As early as 8 weeks, AKP mice (2 of 10) had invasive squamous cell carcinoma (Figure 2E). Additional magnification views are in Supplemental Figure S5. By 12 weeks, nearly 70% (16 of 23) of AKP mice developed squamous cell carcinoma (Figure 2A and Supplemental Table S2). At 16 weeks, 100% (11 of 11) AKP mice exhibited squamous cell carcinoma (Figure 2A and Supplemental Table S2).

Because previous studies examined the 2-month time point for KP mice,[29] KP mice were examined for precancerous lesion development. At 8 weeks, only one of six KP mice exhibited HSILs (Supplemental Figure S6 and Supplemental Table S3). The frequency of abnormal squamous histologic findings was higher in AKP mice than in KP mice at 8 weeks (Fisher's exact test = 0.0014, $P < 0.01$).

Atypical Lesions Outside the Vagina

Pgr$^{Cre/+}$ results in *Cre*-mediated recombination in the uterus, oviduct, and ovaries.[40] However, no malignant tumors were identified in the AKP mice besides the vagina. AKP mice had normal ovaries, with normal follicular development (Supplemental Figure S7). Consistent with normal follicular steroid hormone feedback, no significant differences were observed between *Pgr*$^{Cre/+}$ and AKP mice in follicle-stimulating hormone levels (*Pgr*$^{+/+}$: 3.607 ± 0.363 ng/mL, $n = 10$; AKP: 4.952 ± 1.857 ng/mL, $n = 10$; unpaired, two-tailed *t*-test, $P = 0.487$) or luteinizing hormone levels (*Pgr*$^{+/+}$: 0.230 ± 0.060 ng/mL, $n = 12$; AKP: 0.200 ± 0.031 ng/mL, $n = 10$; unpaired, two-tailed *t*-test, $P = 0.666$).

In some cases (5 of 14 mice), the oviductal epithelium in AKP mice at 16 weeks contained atypical epithelium with nuclear stratification and multiple layers of epithelial cells with nuclear atypia in the form of nuclear enlargement and rounding (Supplemental Figure S7 and Supplemental Table S2). There was no difference in body weight between AKP and *Pgr*$^{+/+}$ mice at any time point (data not shown). Uterine weight per body weight in AKP mice at 16 weeks was more than that in control mice (AKP: 4.2 ± 0.29 mg/g; control: 3.2 ± 0.29 mg/g; multiple *t*-test, $P < 0.05$), but no

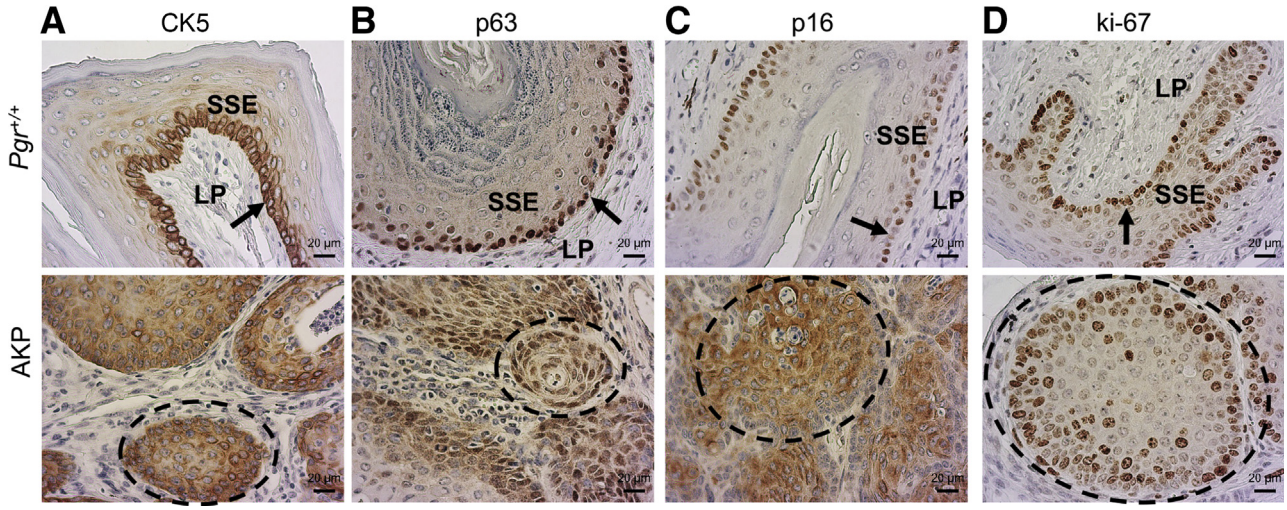

Figure 3 **A–D:** Molecular immunohistochemical staining for cytokeratin 5 (CK5) (**A**), tumor protein P63 (p63) (**B**), cyclin-dependent kinase inhibitor 2A (p16) (**C**), and marker of proliferation Ki-67 (**D**). **Arrow** indicates basal layer; **dashed circle,** nests of squamous cell carcinoma. Scale bar = 20 μm. SSE, stratified squamous epithelium, LP, lamina propria.

significant difference was found in uterine weight at 12 weeks (AKP: 3.5 ± 0.25 mg/g; $Pgr^{+/+}$: 3.4 ± 0.35 mg/g; multiple *t*-test, $P = 0.88$) or at 8 weeks (AKP: 2.9 ± 0.16 mg/g; $Pgr^{+/+}$: 3.5 ± 0.69 mg/g; multiple *t*-test, $P = 0.28$). Endometrial hyperplasia was observed in 21% (3 of 11) AKP mice at 16 weeks with enlarged glands separated by minimal amounts of stroma (Supplemental Figure S8 and Supplemental Table S2). Nuclear atypia was also observed with loss of polarity and rounding of the nuclei (Supplemental Figure S8). Endometrial adenocarcinoma was not observed in AKP mice. Squamous cell carcinoma was not detected in the cervix of AKP mice (Supplemental Table S2).

Molecular Characterization

Cytokeratin 5 (CK5) is a useful stain for squamous differentiation.[46,47] The basal epithelial cells of the $Pgr^{+/+}$ vagina showed intense perinuclear and moderate cytoplasmic CK5 expression with little to no staining in the lamina propria (Figure 3A and Supplemental Figure S9). Intense cytoplasmic and perinuclear CK5 staining was observed throughout the full thickness of the vaginal squamous cell carcinoma nests but sparing the underlying lamina propria (Figure 3A and Supplemental Figure S9). Intense nuclear staining for the basal epithelial cell marker tumor protein P63 (p63)[48] was demonstrated in the basal layer of the $Pgr^{+/+}$ vagina. Moderately intense nuclear p63 staining was observed across the full thickness of the nests of squamous cell carcinoma in AKP vaginas (Figure 3B and Supplemental Figure S9). Vaginal squamous cell carcinoma in women has diffuse staining for cyclin-dependent kinase inhibitor 2A (also known as p16).[46,49] The $Pgr^{+/+}$ vagina showed low-intensity, low-frequency nuclear staining for p16 which was limited to the basal layer. Nests of vaginal squamous cell carcinoma in AKP mice had intense, diffusely positive p16 staining (Figure 3C and Supplemental Figure S9). Marker of proliferation, Ki-67, staining in $Pgr^{+/+}$ mice was contained to a mostly single layer of the basal epithelium of the vagina. Ki-67 staining in AKP mice was seen in multiple layers in the nests of squamous cell carcinoma (Figure 3D and Supplemental Figure S9).

Hormone-Dependent and Estradiol-Responsive Tumors

Ovaries were removed at 6 weeks of age, and AKP and $Pgr^{+/+}$ mice were examined 6 weeks later (at 12 weeks of age). Ovarian hormone–depleted AKP and $Pgr^{+/+}$ mice at 12 weeks had zero gross lesions ($n = 8$) (Supplemental Table S4), suggesting that AKP vaginal squamous cell carcinoma may be hormone dependent. To assess hormone responsiveness, ovariectomized AKP and ovariectomized $Pgr^{+/+}$ mice were treated with a 17-β-estradiol pellet or sham for 60 days. Both ovariectomized $Pgr^{+/+}$ sham and ovariectomized AKP sham mice had no gross vaginal or uterine lesions, and the uteri were of grossly smaller size than those of intact mice (ovariectomized $Pgr^{+/+}$ sham mice: 0.51 ± 0.06 mg/g; $Pgr^{+/+}$ mice with intact ovaries: 3.24 ± 0.29 mg/g; two-tailed *t*-test; $P < 0.0001$; and ovariectomized AKP sham mice: 0.48 ± 0.054 mg/g; AKP mice with intact ovaries: 4.25 ± 0.29 mg/g; two-tailed *t*-test; $P < 0.0001$) (Figure 4). Ovariectomized $Pgr^{+/+}$-E2 uteri were translucent and cystically dilated, consistent with the edematous effects of estradiol in the uterus. Ovariectomized $Pgr^{+/+}$-E2 mice had no gross vaginal lesions (Figure 4). Ovariectomized AKP-E2 uteri did not exhibit the characteristic cystic enlargement of estradiol treatment. Ovariectomized AKP-E2 uteri were larger than ovariectomized AKP-sham uteri (ovariectomized AKP-sham mice: 0.48 ± 0.054 mg/g; ovariectomized AKP-E2 mice: 24.6 ± 15.2 mg/g; two-tailed *t*-test; $P < 0.0001$).

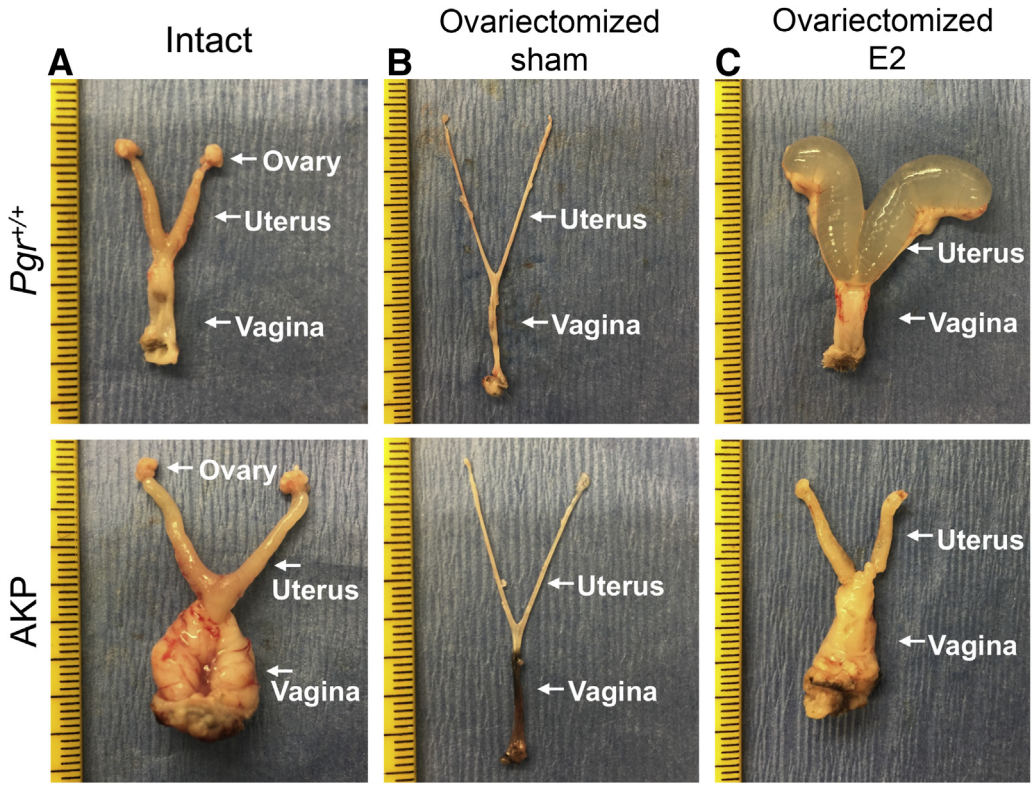

Figure 4 Gross lesions in *Arid1a*$^{flox/flox}$; *Kras*$^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$; *Pgr*$^{Cre/+}$ (AKP) mice with 17-β-estradiol treatment for 60 days. **A:** *Pgr*$^{+/+}$ intact mice were grossly normal. Intact AKP mice exhibited large gross vaginal lesions. **B:** Both ovariectomized *Pgr*$^{+/+}$ sham and ovariectomized AKP sham mice had tiny, thin uteri without gross vaginal lesions. **C:** Ovariectomized *Pgr*$^{+/+}$-E2 mice had enlarged fluid-filled uteri. Ovariectomized AKP-E2 mice exhibited gross vaginal lesions but without the edematous cystic nature of the uterus. Tick marks on the ruler indicate 1 mm.

However, the ovariectomized AKP-E2 uteri were similar to AKP-intact uteri in that they appeared smooth and solid. Most ovariectomized AKP-E2 mice (4 of 6) exhibited exophytic gross vaginal lesions (Figure 4 and Supplemental Table S5).

Both ovariectomized *Pgr*$^{+/+}$-sham and ovariectomized AKP-sham uteri were smaller than intact uteri (Supplemental Figure S10). Histologically, uteri from AKP intact mice had endometrial hyperplasia with an increased surface area of luminal epithelium, crowding of glandular epithelium, and little stroma between glands (Figure 5 and Supplemental Figure S10 and Supplemental Table S2). Neither ovariectomized *Pgr*$^{+/+}$-sham nor ovariectomized AKP-sham mice had any evidence of endometrial hyperplasia. Both contained a single layer of benign luminal epithelium and the expected number of endometrial glands without evidence of atypia (Figure 5 and Supplemental Figure S10 and Supplemental Table S5).

Ovariectomized *Pgr*$^{+/+}$ mice treated with 17-β-estradiol for 60 days (*Pgr*$^{+/+}$-E2) had edematous, enlarged uteri. Clear fluid resulted in dilated lumina. The luminal epithelium existed in a single layer that maintained polarity without evidence of nuclear atypia (Figure 5 and Supplemental Figure S10). Ovariectomized AKP-E2 uteri were not dilated by fluid. Instead, they were lined mainly by epithelium with squamous metaplasia without evidence of adenocarcinoma or nuclear atypia (Figure 5 and Supplemental Figure S10). Other nonmalignant phenotypes included endometrial hyperplasia (1 of 6 mice) and stump pyometra (2 of 6 mice) (Supplemental Table S5). The hyperplasia and metaplasia seen in ovariectomized AKP-E2 mice (4 of 6) was more penetrant than the hyperplasia observed in 16-week−old mice with intact ovaries (3 of 11).

The vaginal tissue was also hormone responsive. Histologically, hormone depletion in *Pgr*$^{+/+}$ vaginas (in ovariectomized *Pgr*$^{+/+}$-sham mice) resulted in a thin single epithelial layer, with decreased keratinization (Figure 6 and Supplemental Figure S11), consistent with models of vaginal atrophy in rodents.[50] Treatment with 17-β-estradiol pellets for 60 days (ovariectomized *Pgr*$^{+/+}$-E2 mice) reversed these effects, a finding similar to that of previous studies.[50] Ovariectomized *Pgr*$^{+/+}$-E2 vaginas exhibited normal keratinized stratified squamous epithelium and normal lamina propria (Figure 6 and Supplemental Figure S11). Similarly, ovariectomized AKP-sham vaginas revealed a thin layer of vaginal epithelium in more than a single layer. Treatment with estradiol reversed the benign, atrophic vaginal histologic findings in the ovariectomized AKP vaginas to squamous cell carcinoma (Figure 6 and Supplemental Figure S11). Histologic descriptions of each animal can be found in Supplemental Table S5.

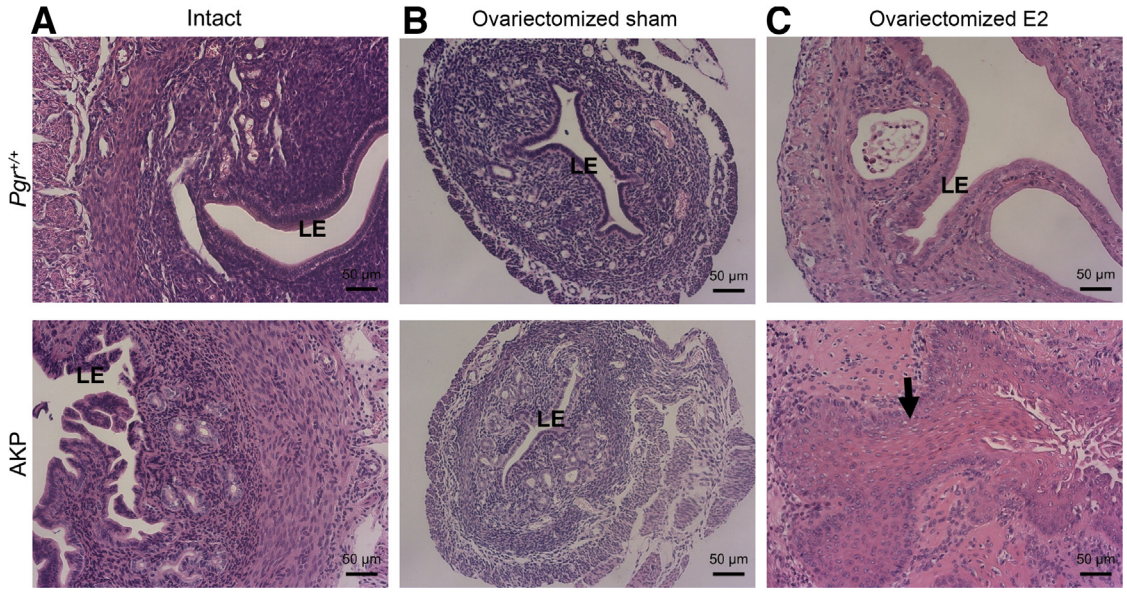

Figure 5 Uterine histologic analysis in $Pgr^{+/+}$ and $Arid1a^{flox/flox}$; $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$; $Pgr^{Cre/+}$ (AKP) mice with and without 17-β-estradiol (E2) treatment. **A:** 16-week–old intact $Pgr^{+/+}$ and AKP mouse uteri are shown for comparison. **B:** AKP intact uteri have endometrial hyperplasia. Ovariectomized AKP sham mice are similar in histologic findings to ovariectomized $Pgr^{+/+}$ sham mice. **C:** Ovariectomized $Pgr^{+/+}$-E2 mice had enlarge dilated uteri without evidence of endometrial hyperplasia or atypia. Ovariectomized AKP-E2 mice had squamous metaplasia of the endometrium (**black arrow**). Hematoxylin and eosin staining. Scale bar = 50 μm. LE, luminal epithelium.

Concurrent Mutations in Human Squamous Cells Lower Female Reproductive Tract Cancers

To examine the co-occurrence of mutations in *ARID1A* and *KRAS* in human cancer datasets, in silico analysis of existing datasets was performed. Vaginal squamous cell carcinoma was not selected for large-scale genomic sequencing by TCGA. Examination of the MSK-IMPACT Clinical Sequencing Cohort ($n = 10{,}945$) found that *ARID1A* and *KRAS* mutations tended to co-occur ($P < 0.001$) across all cancer types.[1] However, only 50 of the MSK-IMPACT Clinical Sequencing Cohort were cervical cancer samples, and none were vaginal or vulvar cancer samples.[1] Cervical cancer subset from the TCGA PanCancer dataset, containing 278 samples,[2] had *ARID1A* (20 of 278 or 7.2%) and *KRAS* (16 of 278 or 5.8%) mutations. Concurrent mutations in both *ARID1A* and *KRAS* were present (2 of 278 or <1%) with a co-occurrence tendency ($P = 0.322$). Limiting the dataset to 251 samples derived from cervical squamous cell carcinoma revealed 11 samples with mutations in *ARID1A* (11 of 251 or 4.4%). Of those 11 samples, two also had mutations in *KRAS* (2 of 11 or 18.2%). Both cervical squamous cell carcinoma samples with *ARID1A* and *KRAS* mutations were HPV negative.

Discussion

A small subset of mutated genes is enriched in female reproductive tract malignant tumors,[5] with unclear functional role. The AKP mouse was created to study two such genes. In the current study, AKP mice developed highly penetrant, hormone-responsive squamous cell carcinoma in the vagina. Of note, the vaginal squamous cell carcinoma described here is HPV independent. In support of the translational implications of this combination of genetic changes, in silico analysis of large-scale genome sequencing studies indicated the presence of concurrent mutations in *ARID1A* and *KRAS* in HPV-negative squamous cell cervical carcinoma samples, the closest genome-wide sequencing dataset to vaginal squamous cell carcinoma. Therefore, this model potentially represents a unique HPV-negative molecular subset of vaginal squamous cell carcinoma.

Because the Pgr^{Cre} mouse is a powerful *Cre* recombinase for endometrial cancer modeling,[51] our original hypothesis was that AKP female mice would develop endometrial cancer. Oncogenic *KRAS* has been detected in up to 30% of endometrial cancers from women.[23–25] More than 40% of endometrial cancers from women have a mutation in *ARID1A*.[8–10] Loss of a proposed tumor suppressor and gain of an oncogene in the uterus leads to endometrial cancer.[13,15–17] There are a number of reasons why these mice did not develop endometrial cancer. First, the mice with aggressive early vaginal tumors were euthanized early for humane reasons. By 16 weeks, AKP mice had large, aggressive vaginal tumors, limiting the ability to study other gynecologic malignant tumors that may develop at older time points. Second, the molecular effects of ARID1A and KRAS in the vagina versus the uterus may be tissue-specific. The fact that neither deletion of *Arid1a* nor expression of oncogenic *Kras* alone is sufficient to drive cancer even with

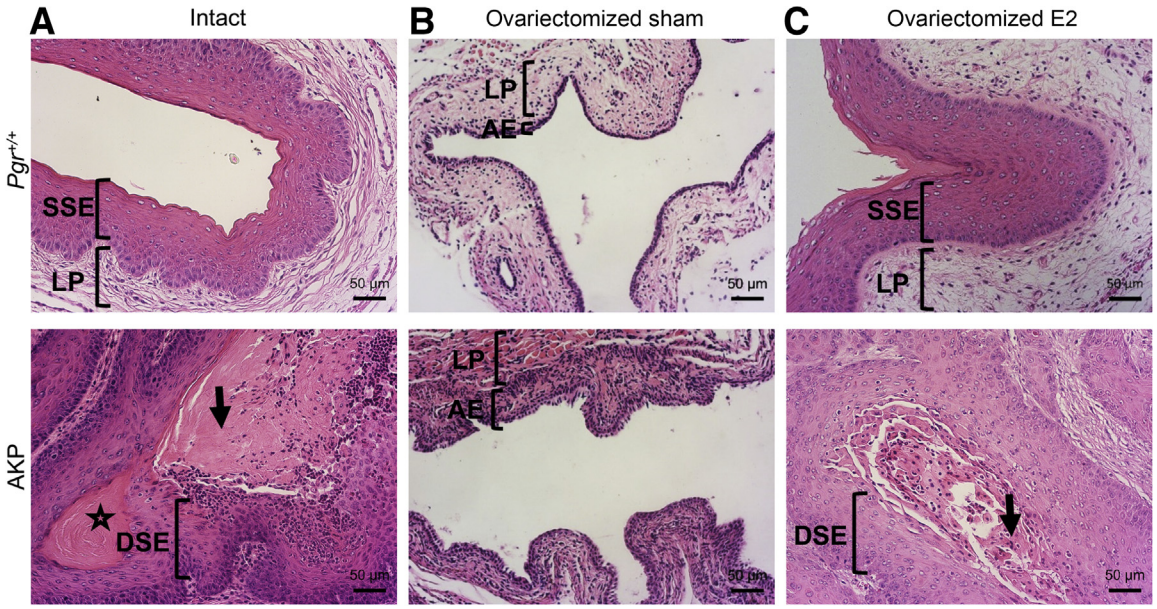

Figure 6 Vaginal histologic analysis in $Pgr^{+/+}$ and $Arid1a^{flox/flox}$; $Kras^{Lox\text{-}Stop\text{-}Lox\text{-}G12D/+}$; $Pgr^{Cre/+}$ (AKP) mice with and without 17-β-estradiol (E2) treatment. **A:** 16-week–old intact $Pgr^{+/+}$ and AKP mouse vaginas are shown for comparison. **B:** Intact $Pgr^{+/+}$ vaginas exhibited normal keratinized stratified squamous epithelium (SSE) with underlying lamina propria (LP). Intact AKP vaginas showed squamous cell carcinoma with central keratinization (**star**), eosinophilic cytoplasm (**arrow**), and dysplastic squamous epithelium (DSE). Ovariectomized $Pgr^{+/+}$ sham vaginas had signs of vaginal atrophy with thin atrophic epithelium (AE) and thin lamina propria. **C:** Hormone replacement resulted in a thickened epithelial layer with keratinization and normal lamina propria. **B:** Ovariectomized AKP sham mice had thinning of vaginal epithelium without evidence of carcinoma. **C:** Ovariectomized AKP-E2 mice exhibited squamous cell carcinoma with dysplastic squamous epithelium (DSE) and eosinophilic cytoplasm (**black arrow**). Hematoxylin and eosin staining. Scale bar = 50 μm.

multiple different *Cre* recombinases[13–18,26,28,29,32] supports the idea of dual genetic contributions being important in malignant transformation.

The AKP mouse described here has unique caveats compared with other mouse models with $Arid1a^{flox}$ allele. First, the $Arid1a^{flox}$ allele used in the AKP mice has a series of *LoxP* cassettes flanking exon 8, leading to deletion of the protein.[43] Other $Arid1a^{flox}$ alleles have a series of *LoxP* cassettes flanking exons 5 to 6, leading to deletion of the ARID DNA-binding domain or point mutations in the ARID domain, which allow protein expression without DNA binding.[14,52] Regardless of the $Arid1a^{flox}$ allele used, conditional deletion of *Arid1a* in the uterus did not result in endometrial cancer.[13–17] Development of endometrial cancer with conditional deletion of *Arid1a* requires combination with deletion of *Pten* or expression of an oncogenic $Pik3ca^{H1047R}$ allele.[13,14] The additional genetic changes led to the development of cancer in mice with two different genetic backgrounds (ie, CD-1 or C57BL/6/129S1/BALB/c mix).[13,14] Using lactoferrin *Cre* to delete *Arid1a* and express mutant *PIK3CA* in the epithelial cells of the uterus, mice developed invasive endometrial adenocarcinoma at 14 to 17 weeks.[14] This development of endometrial cancer at older age is consistent with the potential for AKP mice to develop endometrial cancer. However, it was not observed because of early euthanasia due to vaginal tumors. A detailed description of each genetically engineered female reproductive tract *Arid1a* mouse model is provided in Supplemental Table S6.[53]

Mechanistically, the early development of aggressive and highly penetrant vaginal tumors may be in part due to the suppression of oncogene-induced cellular senescence mediated by ARID1A loss. Cellular senescence is frequently found in aging or cancerous tissue, potentially because of oncogenic signaling.[54] Oncogenic $KRAS^{G12D}$ expression in pancreatic cancer cell lines induces cellular senescence.[55] Of note, ARID1A knockdown repressed oncogene-induced cellular senescence in $KRAS^{G12D}$ pancreatic cell lines, leading to cell cycle progression.[55] Consistent with this view, ARID1A promoter hypermethylation and decreased ARID1A expression led to increased squamous cell carcinoma progression *in vitro* and *in vivo*.[56] Cyclin-dependent kinase inhibitor 2A or p16 is a potential marker of cellular senescence. Specifically, as expected, p16 staining was localized to the nucleus[46,49] in the $Pgr^{+/+}$ mouse. The AKP vaginal squamous cell carcinoma expressed p16 in both the nucleus and the cytoplasm (Figure 3). Cytoplasmic p16 staining may represent the cellular mechanism in which p16 becomes inactivated and allows for tumor progression.[57,58] Both the functional role of *Kras*-mediated oncogenic cellular senescence in vaginal squamous cell carcinoma development and the mechanistic role of *Arid1a* loss in the translocation of p16 warrant further investigation.

$Kras^{G12D}$-induced vaginal papillomas have been reported in mice.[29,59] Spontaneous vaginal papillomas were noted when $Kras^{G12D}$ was expressed through the insulin promoter factor 1 *Cre* recombinase ($Ipf1^{Cre}$, also known as $Pdx1^{Cre}$) with a median age of 235 days.[59] When $Kras^{G12D}$ was expressed with the Pgr^{Cre} (KP mice), vaginal papillomas

began as early as 2 months. However, KP mice did not exhibit malignant tumors.[29] AKP female mice exhibited malignant vaginal tumors earlier (Figure 2) than KP female mice which developed nonmalignant lesions.[29] The benign nature of KP and the $Ipf1^{Cre}$ models suggests that a secondary hit may be necessary for transformation to malignancy. This hypothesis is supported by the presence of vaginal squamous cell carcinoma in $Kras^{G12D}$ mice with a *Pten*-inactivating mutation driven by vaginally delivered adenovirus *Cre*.[30] The vaginal lesions are exophytic, protruding externally from the vagina, and histologically resemble those described in AKP mice.[30]

The effects of 17-β-estradiol treatment on tumor growth were somewhat surprising. Clinically, vaginal squamous cell carcinoma is not treated as a hormone-responsive disease. Although 17-β-estradiol is clinically used to increase epithelial cell thickness and treat the symptoms associated with postmenopausal vaginal atrophy, the mechanisms involved in squamous cell proliferation and differentiation are not well studied. Furthermore, the effects of 17-β-estradiol treatment on progesterone receptor expression, and thus, the function of *Cre* recombinase in the vagina, warrant further study.

Clinically, 17,600 women were diagnosed with primary vaginal squamous cell carcinoma, and 8062 women died of vaginal squamous cell carcinoma worldwide in 2018.[4] The number of new cases of vaginal cancer in the United States for 2020 were 6230, with 1450 deaths.[60] Although vaginal cancer is not a common cancer, the incidence of vaginal squamous cell carcinoma disproportionately affects black women. The incidence of vaginal cancer is 72% higher in black women than white women, making vaginal cancer a disease of significant health disparity.[61] Because the HPV vaccine's impact decreases the prevalence of HPV-dependent squamous cell carcinoma, this model of HPV-negative squamous cell carcinoma becomes even more critical. Similar to vaginal, anal, vulvar, and cervical cancers, head and neck cancers are considered HPV-associated diseases. Survival of patients with HPV-positive head and neck cancers is significantly higher than that of those with HPV-negative tumors.[62] Mouse models with HPV-negative disease offer a significant opportunity to study these rarer tumors.

The genetically engineered mouse model that targets the knockout of the tumor suppressor *Arid1a* and the knock-in of oncogenic $Kras^{G12D}$ in the gynecologic tract leads to primary squamous cell carcinoma of the vagina. With age, the AKP mice develop preneoplastic lesions and eventually invasive squamous cell carcinoma. The role of hormone expression in primary squamous cell carcinoma of the vagina alludes to the potential mechanistic and therapeutic targets. Finally, we speculate the possible mechanism for tumor progression of repressed oncogene-induced cellular senescence via translocation of p16 from the nucleus to the cytoplasm. This genetically engineered mouse model of primary squamous cell carcinoma of the vagina may lead to advances in early detection, understanding of initiation and progression, and novel treatment options.

Author Contributions

X.W., M.P., and S.M.H. designed the study, collected and analyzed data, performed literature search, and generated figures. J.R.H.W. interpreted data, performed literature search, and generated figures. R.E.E. analyzed data, performed literature search, and generated figures. J.P.L. and F.J.D. contributed new reagents/analytic tools. All authors wrote the paper and approved the submitted and published versions. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated or resolved. S.M.H. is the guarantor of this work, and as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2021.03.013*.

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