Targeting progesterone signaling prevents metastatic ovarian cancer

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Effective cancer prevention requires the discovery and intervention of a factor critical to cancer development. Here we show that ovarian progesterone is a crucial endogenous factor inducing the development of primary tumors progressing to metastatic ovarian cancer in a mouse model of high-grade serous carcinoma (HGSC), the most common and deadliest ovarian cancer type. Blocking progesterone signaling by the pharmacologic inhibitor mifepristone or by genetic deletion of the progesterone receptor (PR) effectively suppressed HGSC development and its peritoneal metastases. Strikingly, mifepristone treatment profoundly improved mouse survival (~18 human years). Hence, targeting progesterone/PR signaling could offer an effective chemopreventive strategy, particularly in high-risk populations of women carrying a deleterious mutation in the *BRCA* gene.

progesterone | antiprogestins | hormone | ovarian cancer | BRCA

It is a mystery why women who inherit a deleterious germline *BRCA1* mutation are prone primarily to ovarian and breast cancer, despite the presence of a *BRCA1* mutation in every single cell of their body and ubiquitous expression of the mutant *BRCA1* gene (1, 2). One likely explanation would have been ovarian hormones, as both tissues are naturally targeted by ovarian hormones (1, 3). However, *BRCA1*-mutation carriers develop predominantly triple-negative breast cancer (TNBC), a breast cancer type lacking the expression of hormone receptors—the estrogen receptor (ER) and the progesterone receptor (PR) (4, 5). Hence, TNBC is considered a hormone-independent malignancy, in which tumor progression is not fueled by ovarian hormones, such as estrogen, and therefore does not respond to antiestrogen therapies (6–8). Extending this notion, ovarian hormones were thought to not be involved in the development of TNBC (9).

Intriguingly, however, when *BRCA1*-mutation carriers undergo prophylactic removal of their ovaries (and fallopian tubes) to reduce the risk of ovarian cancer, not only does this preventive surgery decrease ovarian cancer risk, but ovary removal (oophorectomy) also lowers the risk of breast cancer (by 37 to 62%) in multiple, albeit not all (10, 11), clinical studies (5, 12). This unexpected risk reduction of breast cancer suggests that ovarian factors or hormones likely play a role in the development, if not progression, of TNBC (13). Fittingly, steroid hormone levels are significantly elevated during the menstrual cycle in the majority of *BRCA1*-mutation carriers (14). Also, the predominant type of ovarian cancer among *BRCA1*-mutation carriers is high-grade serous ovarian cancer (4, 5), also known as high-grade serous carcinoma (HGSC), the most common and deadliest ovarian cancer type (15–19). Curiously, HGSC and TNBC, albeit arising from disparate tissues, are genomically similar malignances (20, 21), raising the possibility of a similar mechanism of development. Emerging collectively from these intriguing clinical

Significance

Why women carrying a pathogenic germline *BRCA1* mutation are predisposed to ovarian and breast cancer remains elusive. This study points to ovarian progesterone as a culprit. Generally, *BRCA1*-mutation carriers exhibit high yet individually varying levels of progesterone during the menstrual cycle. Although not all *BRCA1*-mutation carriers develop these cancers, all of them are advised to undergo prophylactic surgeries at a young age (under 40 y to 45 y) to prevent ovarian and breast cancer. Insights from robust in vivo findings in this study offer a novel concept: Targeting progesterone signaling with antiprogestins could be an effective nonsurgical prophylactic option for ovarian and breast cancer prevention for these high-risk women.

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Competing interest statement: J.K. and K.D.M. are listed as inventors on patent applications filed by Indiana University related to targeting progesterone signaling.

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observations is a compelling hypothesis that ovarian hormones or factors may play a significant role in the development of HGSC and TNBC in *BRCA1*-mutation carriers.

Elucidating the role of ovarian hormones in the development of ovarian and breast cancer would be vitally important for risk assessment and management for high-risk women. Women carrying a deleterious germline *BRCA1* or *BRCA2* mutation have extraordinarily high lifetime risks of developing ovarian (10 to 60%) and breast cancer (60 to 80%), yet not every *BRCA*-mutation carrier develops these cancers (12, 22). This high yet incomplete penetrance also suggests that *BRCA* cancer risk can be modified by other genetic elements or nongenetic factors (13). Currently, as there is no precise way to assess individual cancer risks, all *BRCA*-mutation carriers are advised to undergo prophylactic surgeries at a young age (under 40 y to 45 y) to reduce ovarian and breast cancer risks (5, 23). Thus, there is a pressing need to improve individual risk assessment and devise a nonsurgical preventive therapy for these high-risk women. Effective prevention requires the identification and intervention of a specific factor essential for cancer development. Generally, use of oral contraceptive pills and pregnancy have been consistently associated with a reduced risk of ovarian cancer (24–27). Yet no specific endogenous or environmental factor has been identified for intervention to prevent or reduce ovarian cancer risk.

Recently, we developed a unique mouse model of ovarian cancer that robustly mimics the clinical metastases of human HGSC (28, 29), which is responsible for over 70% of ovarian cancer cases and deaths (15, 19, 21). Although named "ovarian cancer," a large proportion of HGSCs may arise from the fallopian tube (28, 30–35), and, to a lesser extent, from the ovary or the peritoneum (36–38). To model human HGSC, we developed a genetically engineered mouse model by inactivation of two genes, *Dicer1* and *Pten*, in the reproductive tissues, including the fallopian tube and ovary (39, 40): *Dicer1* $^{flox/flox}$ *Pten* $^{flox/flox}$ *Amhr2* $^{cre/+}$ mice (28). These *Dicer1-Pten* double-knockout (DKO) mice develop metastatic HGSCs arising from the fallopian tube with 100% penetrance (28, 29). Like human ovarian cancer, this mouse HGSC invades the ovaries and spreads along the peritoneal lining across the peritoneal cavity—most notably, to the omentum, as well as to the diaphragm, mesentery, and peritoneal surfaces—all accompanied by ascites (28, 29). All DKO mice die of widespread peritoneal metastases. Besides strikingly mirroring the clinical features of human HGSC (41), these mouse HGSCs closely resemble human HGSC with histopathological, molecular, and genomic similarities (28, 29).

Additionally, although not harboring a *Brca1* or *BRCA2* mutation, HGSCs of these mice exhibit widespread genomic instability and markedly dysregulated signaling in DNA repair and homologous recombination, indicating defective homologous recombination repair (HRR) (29). The molecular hallmark of *BRCA* mutation-harboring cancers, an HRR defect is also observed in tumors lacking a germline *BRCA1/2* mutation, which is known as "BRCAness" (42). Exhibiting BRCAness, therefore, DKO HGSCs would phenocopy HGSC harboring a germline *BRCA* mutation. Accordingly, DKO mice would serve as a useful, relevant model for elucidating vital factors involved in cancer development among *BRCA1*-mutation carriers.

Harnessing this model, the current study has uncovered that 1) ovarian progesterone is a crucial endogenous factor inducing the development of primary HGSC harboring full metastatic capability, and 2) pharmacologic inhibition or genetic inactivation of progesterone signaling effectively suppresses HGSC development and its peritoneal metastases. Hence, targeting progesterone signaling represents a potentially effective nonsurgical prophylactic strategy for prevention of ovarian cancer—and, by extension, breast cancer—in *BRCA1*-mutation carriers.

Results

The Ovary Is Critical to the Development of Metastatic HGSC Originating in the Fallopian Tube. HGSC forms and progresses in the fallopian tube before spreading to the ovaries and metastasizing throughout the peritoneal cavity in DKO mice (*Dicer1* $^{flox/flox}$ *Pten* $^{flox/flox}$ *Amhr2* $^{cre/+}$) (Fig. 1). The fallopian tube—not the ovary—is the origin of HGSC in DKO mice (28). While the ovaries form no tumors after removing premalignant fallopian tubes (at 4 wk to 10 wk of age), surgical removal of both ovaries at a premalignant stage did not prevent tumors from developing in the fallopian tubes (28) (Fig. 2). Intriguingly, however, absence of the ovaries significantly prolonged the survival of these mice. Ovary-deficient DKO mice lived 4.5 mo (~13.5 human years) (43) longer than intact DKO mice (median survival, 13.2 mo of age [6.9 to 17.4] vs. 8.7 mo [5.9 to 12.4]; $n = 27$ and 33 mice; hazard ratio [HR], 0.26; 95% CI, 0.14 to 0.50; $P < 0.0001$) (Fig. 2*A*). Thus, the ovary, although not the tumor origin, may play a role in HGSC in this model.

With intact ovaries, DKO mice developed predominantly HGSC in the fallopian tube, also leading to extensive peritoneal HGSC metastases with complete penetrance (28) (Fig. 1). In contrast, ovariectomized DKO mice still formed fallopian tube tumors, but with markedly reduced or little peritoneal metastasis (Fig. 2 *B* and *C*). Also, these primary tumors were generally large, heterogeneous, and composed mainly of cystic and fibrous tissues as well as stromal tumors with only a small segment of HGSC (Fig. 2 *D–I*). Thus, the ovary appears to influence the type of tumor that develops in the fallopian tube of DKO mice. Absence of the ovaries profoundly diminishes the ability of DKO mice to develop HGSC in the fallopian tube, resulting in nearly absent or sporadic peritoneal metastasis, which leads to longer survival in ovary-deficient DKO mice. Hence, presence of the ovary is vital to the development of HGSC equipped with metastatic potential.

Progesterone Induces HGSC with Metastatic Potential in Ovary-Deficient DKO Mice. Observing the potent impact of the ovary on tumor formation and mouse survival, we postulated that ovarian hormones may be responsible for HGSC development harboring metastatic potential in the DKO model. To test this hypothesis, ovary-deficient DKO mice were treated with steroid hormones. In this experiment, upon surgical removal of the ovaries at 5 wk to 6 wk of age (premalignant stage), ovariectomized DKO mice were implanted subcutaneously with a pellet of 1) progesterone (P4; total 25 mg), 2) 17β-estradiol (E2; total 0.72 mg), 3) P4+E2, or 4) placebo for 3 mo. Typically, the majority of DKO mice (66.7%) form fallopian tube HGSC by 5 mo of age with limited metastasis (29). Therefore, 3-mo treatment of hormones (i.e., lasting until four and a half months of age) would provide a sufficient duration of hormone exposure to determine whether ovarian hormones are vital to the development of fallopian tube HGSC with metastatic potential. After hormone treatment, tumor development and mouse survival were monitored.

Strikingly, with progesterone (P4) treatment alone, all ovariectomized DKO mice developed predominantly HGSC in the fallopian tube, with widespread and abundant peritoneal metastases accompanied by ascites (100%: 32/32 mice) (Fig. 3 *A–C*). These primary and metastatic tumors were histopathologically confirmed as HGSC (Fig. 3 *D–G*). As expected, P4 treatment elevated serum progesterone levels, while not affecting estrogen and testosterone levels in ovariectomized DKO mice (*SI Appendix*, Fig. S1). Thus, these results support that P4 drives the development of primary and metastatic HGSCs. In contrast, ovariectomized DKO mice treated with a placebo generally formed large heterogeneous fallopian tube tumors with scant peritoneal metastasis (Fig. 3*H*), as observed similarly in ovariectomized DKO mice (with no placebo) (Fig. 2 *B* and *C*).

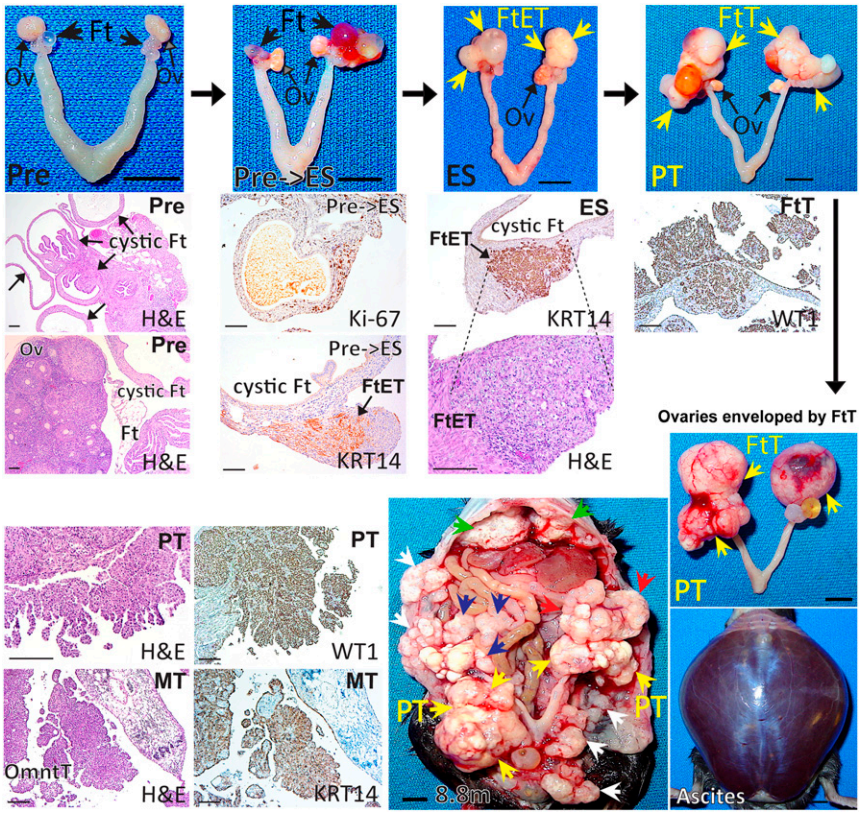


Fig. 1. HGSC development and metastatic progression in DKO mice. At a premalignant stage (Pre) (<4 mo to 5 mo of age), DKO mice exhibit multiple small cystic fallopian tubes with largely normal-looking ovaries. During tumor initiation, a small segment of cells in the fallopian tube stroma arises and begins to proliferate, as indicated by the proliferation marker Ki-67 staining. Some of the proliferating cells transform into early-stage HGSC in the fallopian tube (FtET), distinctively positive for KRT14. This early-stage HGSC grows and progresses in the fallopian tube (FtT) (4 mo to 6 mo of age). In parallel, equipped with metastatic potential, this growing fallopian tube HGSC invades and envelops the ovaries—and also metastasizes throughout the peritoneal cavity (6 mo to 10 mo of age). Typically, at an advanced stage, DKO mice present with primary fallopian tube tumors (PT, yellow arrows) with extensive peritoneal metastases to the omentum (red arrows), mesentery (blue arrows), peritoneum (white arrows), and diaphragm (green arrows), invariably accompanied by hemorrhagic ascites. Histologically, primary tumors (PT) and peritoneal metastases (MT, omentum tumors) exhibit characteristic histopathology of HGSC including positive staining for WT1 and KRT14. Hematoxylin & eosin (H&E) staining. Pre, premalignant stage; ES, early stage; Ft, fallopian tube; Ov, ovary; FtET, early-stage fallopian tube tumor (HGSC); FtT, fallopian tube tumor (HGSC); PT, primary (fallopian tube) tumor (HGSC); MT, metastatic tumors (HGSC); OmntT, omentum metastasis (HGSC); KRT14, cytokeratin 14; WT1, Wilms tumor 1; DKO, *Dicer1* $^{flox/flox}$ *Pten* $^{flox/flox}$ *Amhr2* $^{cre/+}$ mice. Scale bars in mouse morphological pictures, 0.5 cm; scale bars in histological pictures, 100 μm.

Likewise, histologically, primary fallopian tube tumors from placebo-treated ovariectomized DKO mice comprised mostly non-HGSC tissues—stromal tumors and cystic, fibrous tissues (Fig. 3 *I* and *J*)—with sporadic presence of HGSC cells.

Predictably, HGSCs with extensive peritoneal metastases significantly shortened the survival of P4-treated ovariectomized DKO mice, compared with placebo-treated ovariectomized DKO mice (median survival, 6.5 mo of age [5.1 mo to 13.5 mo] [$n = 32$ mice] vs. 12.2 mo [6.8 mo to 19.6 mo] [$n = 21$ mice]; HR, 3.01; 95% CI, 1.7 to 5.4; $P < 0.0001$) (Fig. 3*K*). Thus, these in vivo findings offer robust evidence that progesterone (P4) is the key ovarian factor enabling the development of HGSC with metastatic potential, leading to a poor prognosis and decreased survival in DKO mice.

Additionally, to determine whether a shorter period of progesterone exposure is sufficient to induce HGSC development leading to full-blown metastatic disease, ovariectomized DKO mice were treated with P4 for 1 wk (total 2 mg) or 3 wk (total 6 mg). A week of progesterone treatment (2 mg) did not appear to be fully adequate for metastatic HGSC development (66.7%: 12/18 mice), with survival (median survival, 11.0 mo of age [5.0 mo to 17.1 mo], $n = 18$ mice) similar to that of placebo-treated mice (12.2 mo [6.8 mo to 19.6 mo]; $n = 21$ mice; HR, 1.7; 95% CI, 0.87 to 3.3; $P = 0.08$). However, a 3-wk treatment of progesterone (6 mg) was sufficient for metastatic HGSC development (100%: 19/19 mice), with significantly shortened survival compared with placebo-treated mice (median survival, 7.2 mo of age [4.9 mo to 14.3 mo], $n = 19$ mice; HR, 2.5; 95% CI, 1.2 to 5.0; $P = 0.0016$). Also, mouse survival was comparable for 3 mo (25 mg) versus 3 wk (6 mg) of progesterone (median survival, 6.5 mo vs. 7.2 mo of age; $n = 32$ and $n = 19$; HR, 1.50; 95% CI, 0.86 to 2.6; $P = 0.13$; *SI Appendix*, Fig. S2). Thus, short-term premalignant exposure (minimum 3 wk) of progesterone is enough to drive the development of HGSC with full metastatic potential. Importantly, these findings suggest that a short period of progesterone exposure at a premalignant stage may be critical and sufficient to determine the tumor type and therefore the course of tumor progression.

Estrogen May Oppose HGSC-Inducing Effects of Progesterone. Estrogen (E2) preferentially impacts the uterus in ovariectomized DKO mice. The proliferative effect of estrogen on the uterus is well established in both humans and rodents (7, 44–46). Consistently, 17β-estradiol (E2) alone overwhelmingly produced uterine tumors or dilation, accompanied by acute inflammation, in ovariectomized DKO mice (90.9%: 10/11 mice) (*SI Appendix*, Fig. S3). This heightened estrogen-induced uterine response is attributable to a lack of progesterone, which normally opposes estrogen in the uterus (47). Histologically, uterine tumors were adenocarcinoma or squamous cell carcinoma.

Generally, there was a lack, or a reduced development, of fallopian tube tumors in ovariectomized DKO mice with E2 alone. Interestingly, some fallopian tube tumors exhibited a histology of low-grade serous carcinoma (*SI Appendix*, Fig. S3*J*), suggesting that E2-alone treatment may have altered tumor lineage in the fallopian tube. Also, as E2-induced uterine tumor development was rapid and dominant, the effect of E2 on the fallopian tube was not clear in DKO mice. When ovariectomized DKO mice were treated with both progesterone and E2, progesterone, as expected, counteracted the E2-induced development of uterine tumors or uterine swelling (*SI Appendix*, Fig. S3 *K–N*).

Interestingly, estrogen appeared to attenuate the effect of progesterone on HGSC development and mouse survival (Fig. 3*K*). Ovariectomized DKO mice treated with progesterone plus 17β-estradiol (P4+E2) were capable of developing primary and metastatic HGSCs, yet with seemingly diminished capacity. In ovariectomized DKO mice with P4+E2, primary fallopian tube HGSCs tended to be small or often heterogeneous,

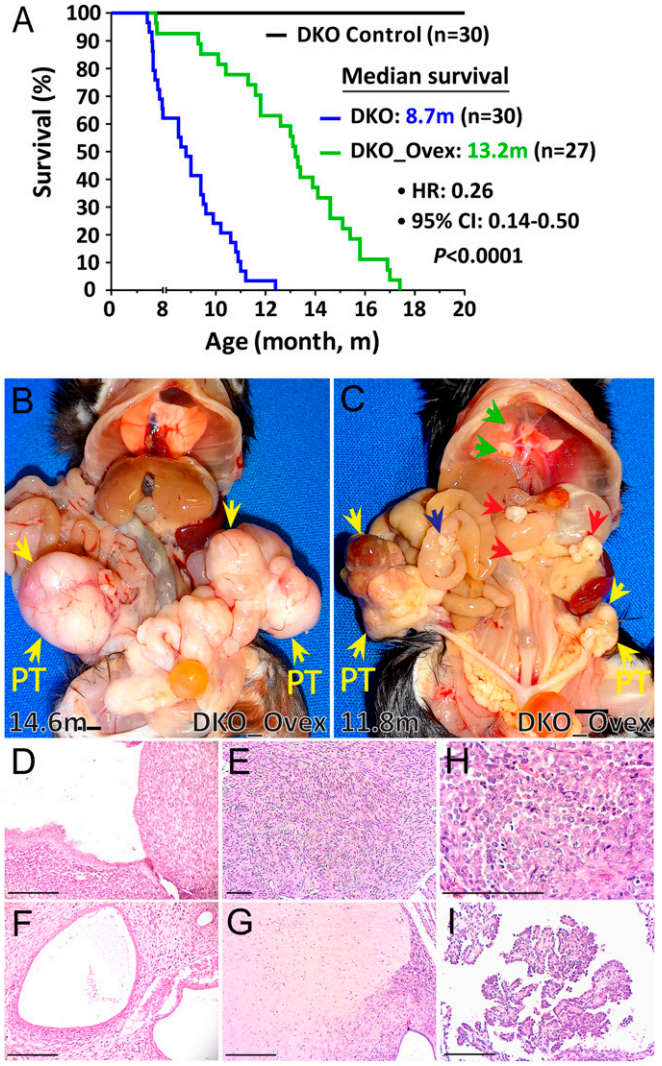


Fig. 2. Ovary removal at a premalignant stage significantly extends survival of DKO mice. (*A*) Survival curves of DKO mice with and without ovaries. Ovariectomized DKO mice (DKO_Ovex, 27 mice, green line) live significantly longer than DKO mice with the ovaries (DKO, 30 mice, blue line): median survival, 13.2 mo of age (range: 6.9 mo to 17.4 mo) vs. 8.7 (5.9 mo to 12.4 mo); HR, 0.26; 95% CI, 0.14 to 0.50; log-rank test, $P < 0.0001$. The black line refers to the survival curve of DKO control mice (*Dicer1* $^{flox/flox}$ *Pten* $^{flox/flox}$ *Amhr2* $^{+/+}$), which do not develop any tumors. (*B* and *C*) Tumor phenotype of ovariectomized DKO mice (DKO_Ovex). Ovary-deficient DKO mice still form primary fallopian tube tumors (PT, yellow arrows) with scant (*B*) or markedly reduced (*C*) peritoneal metastasis: peritoneal metastases to the omentum (red arrows), mesentery (blue arrows), and diaphragm (green arrows). Although not extensive, peritoneal metastasis was observed in 74.1% (20/27 mice) of ovariectomized DKO mice. (Scale bars, 0.5 cm.) (*D–I*) Histopathologic characterization of tumors from ovariectomized DKO mice (H&E). Primary tumors from ovariectomized DKO mice primarily comprise stromal tumors (*D* and *E*) and cystic (*F*) and fibrous (*G*) components, along with a segment of histologically HGSC cells (*H*). Sporadic peritoneal metastatic tumors typically exhibit a characteristic HGSC histology (*I*). (Scale bar, 100 μm.)

harboring non-HGSC components, accompanied by reduced or sporadic peritoneal metastases (*SI Appendix*, Fig. S3 *K–N*). Accordingly, P4+E2 tended to extend mouse survival (median survival, 9.9 mo of age [5.8 mo to 13.5 mo]; $n = 10$ mice; HR, 0.56; 95% CI, 0.30 to 1.06; $P = 0.09$), compared with P4 alone (6.5 mo [5.1 mo to 13.5 mo]; $n = 32$ mice). While requiring further study, these data raise an intriguing possibility that estrogen may suppress HGSC development by opposing or attenuating the effect of progesterone.

Active Ovarian Endocrine Function in DKO Mice. Collectively, these hormone supplement experiments indicate that the ovaries of DKO mice produce progesterone sufficient to induce fallopian tube HGSC possessing metastatic potential. *Amhr2*-Cre is expressed in the reproductive tract, including the ovary (primarily in granulosa cells) and the fallopian tube (stroma) (39, 48). Therefore, *Dicer1* and *Pten* would be deleted in ovarian granulosa cells as well as in fallopian tube stromal cells in DKO mice. At a premalignant stage (<4 mo to 5 mo of age) in DKO mice (29), multiple cysts form inside the lumen of fallopian tubes, which grow into large, bulging sacs, occasionally containing trapped ovulated eggs, likely leading to infertility (49, 50). Inside these enlarged fallopian tube cysts, cells in the tubal stroma begin to proliferate and transform into HGSC cells, which develop into fallopian tube HGSC and lead to peritoneal metastases (Fig. 1 and refs. 28 and 29).

Premalignant ovaries of DKO mice, however, look morphologically and histologically normal, with different stages of follicles and the presence of corpora lutea (Fig. 1). Apparently normal development of ovarian follicles and corpora lutea, along with presence of ovulated eggs in the fallopian tube, is indicative of a functional estrous cycle in DKO mice. In addition, the morphology and histology of premalignant ovaries and fallopian tubes in DKO mice are indistinguishable from those of mice lacking *Dicer1* alone (*Dicer1* $^{flox/flox}$ *Amhr2* $^{cre/+}$) (49, 50). Despite the apparently normal development and distribution of follicles and corpora lutea in their ovaries, *Dicer1-Amhr2*-Cre mice ovulated a lower number of eggs and exhibited aberrant estrous cycles, but produced a largely normal range of progesterone and 17β-estradiol (49, 50).

To further examine the steroidogenic function of the ovary in DKO mice, the estrous cycle was enhanced by treatment of gonadotropin hormones: pregnant mare serum gonadotropin (PMSG) and human CG (hCG). In this experiment, premalignant DKO mice at 4 wk to 5 wk of age were treated with PMSG and, 48 h later, with hCG—a procedure inducing "superovulation" (51). This superovulation regimen of PMSG and hCG was repeated every 3 d for 3 mo (i.e., 30 cycles). As indicated by the name, this sequential treatment of gonadotropins enhances the recruitment, growth, and ovulation of follicles, as well as corpus luteum formation, in the ovary (52). Naturally, therefore, this augmented estrous cycle would also amplify ovarian endocrine function, with an increased synthesis and release of progesterone and 17β-estradiol (53, 54) in the ovaries of superovulated premalignant DKO mice. If so, enhanced ovarian steroidogenesis of progesterone would accelerate HGSC development and progression. As predicted, premalignant DKO mice with repeated superovulation showed significantly more rapid HGSC development and progression than nonsuperovulated DKO mice (median survival: 6.2 mo vs. 8.7 mo of age; $n = 12$ and 30 mice; HR, 5.5; 95% CI, 1.7 to 18.2; $P < 0.0001$) (*SI Appendix*, Fig. S4*A*). Thus, superovulation-driven HGSC enhancement supports the expectation that premalignant DKO ovaries retain active endocrine function enabling HGSC development. Beyond the premalignant stage, DKO mice still appear to maintain ovarian endocrine function throughout tumor progression, as progesterone levels at an advanced stage were higher than those in ovariectomized mice (*SI Appendix*, Fig. S5).

Together, these findings reinforce the notion that ovarian progesterone is an endogenous factor necessary for DKO mice (lacking *Dicer1* and *Pten*) to develop HGSC with metastatic potential, suggesting that targeting progesterone signaling may be a viable approach to block HGSC development.

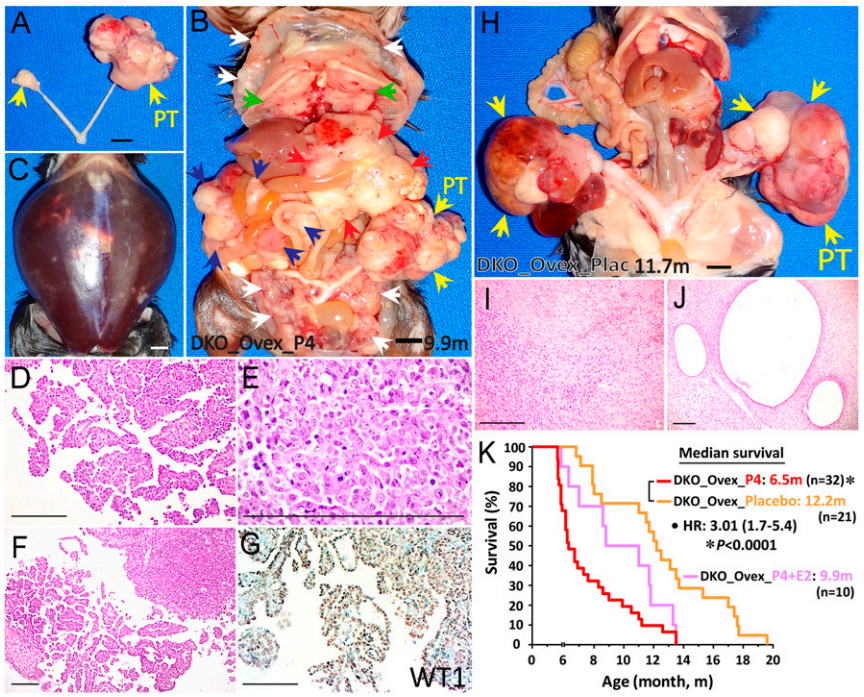


Fig. 3. Progesterone induces HGSC development with metastatic potential in DKO mice. (*A–C*) Tumor phenotype of ovariectomized DKO mice treated with progesterone (DKO_Ovex_P4). All ovariectomized DKO mice implanted with a P4 pellet (25 mg) for 3 mo develop primary fallopian tube tumors (PT, yellow arrows) (*A*), accompanied by widespread metastases throughout the pelvic and abdominal cavities, including the omentum (red arrows), peritoneum (white arrows), mesentery (blue arrows), and diaphragm (green arrows) (*B*), along with ascites (*C*). A mouse shown was killed at 9.9 mo of age. (Scale bars, 0.5 cm for *A–C*.) (*D–G*) Histopathologic characterization of HGSCs formed in P4-treated ovariectomized DKO mice. Primary and metastatic tumors exhibit structural and cellular features characteristic of HGSC: papillary, slit-like structure in primary tumors (*D*) and omentum metastatic tumors (*F* and *G*) with high-grade nuclear cellular features (*E*) and positive staining for Wilms tumor 1 (WT1), a HGSC marker. (Scale bars, 100 μm.) (*H–J*) Tumor phenotype and histopathology of placebo-treated ovariectomized DKO mice (DKO_Ovex_Plac). Large bilateral primary fallopian tube tumors (yellow arrows, PT) with little peritoneal metastasis in a mouse killed at 11.7 mo of age (*H*). (Scale bar, 0.5 cm for *H*.) Histologically, these fallopian tube tumors are typically stromal tumors with necrotic lesions (*I*) and also harbor multiple cysts lined with benign epithelium as well as fibrous tissue (*J*). Histopathology examination with H&E staining (*D–F*, *I*, and *J*). (Scale bars, 100 μm for *D–F*, *I*, and *J*.) (*K*) Survival curves. Significantly reduced survival in P4-treated ovariectomized DKO mice compared with placebo-treated DKO mice: median survival, 6.5 mo (5.1 mo to 13.5 mo) of age (n = 32 mice) vs. 12.2 mo (6.8 mo to 19.6 mo) (n = 21 mice); HR, 3.01; 95% CI, 1.7 to 5.4; log-rank test, $P < 0.0001$. DKO_Ovex_P4, ovariectomized DKO mice with P4 treatment (red line); DKO_Ovex_P4+E2, ovariectomized DKO mice with P4+E2 treatment (pink line); DKO_Ovex_Plac(ebo), ovariectomized DKO mice treated with placebo (orange line).

Mifepristone Inhibits HGSC Development and Significantly Extends Mouse Survival. Progesterone generates its biological effects through the PRs (55). Thus, to evaluate further whether progesterone signaling is critical to the development of HGSC with metastatic potential, DKO mice were treated with the PR antagonist mifepristone (RU486), an antiprogestin. Mifepristone acts by binding to PR and inhibiting the transcription of its target genes (56).

In this experiment, DKO mice (with intact ovaries) at 5 wk to 6 wk of age (premalignant stage) were implanted with mifepristone (3 mg/mo) or a placebo for 3 mo. As expected, DKO mice treated with a placebo developed primary HGSC coupled with abundant peritoneal HGSC metastases (Fig. 4 *A* and *B*). In contrast, as predicted, antiprogestin treatment effectively suppressed HGSC development in the fallopian tube and peritoneal metastasis, which was evidenced by a markedly low degree, or near absence, of peritoneal metastasis (Fig. 4 *C* and *D*). Histologically, the fallopian tubes from mifepristone-treated mice were composed of cystic and fibrous tissues as well as small segments of carcinoma cells or HGSC cells in the stroma, accompanied by limited peritoneal metastasis (Fig. 4). Interestingly, these sporadic primary HGSC cells and limited peritoneal tumors were histologically HGSC, but lacked expression of KRT14, a mouse HGSC marker (Fig. 4*G*). Thus, blocking P4/PR signaling by mifepristone not only inhibits HGSC development but also may alter or reduce metastatic potential of HGSC.

Additionally, in some cases, low-grade endometrioid-type carcinomas were observed in the ovary, fallopian tube, or uterus (Fig. 4*H*). Plausibly, inhibition of progesterone signaling decreases HGSC formation in the fallopian tube and its metastasis, which leads to an increased tumorigenic propensity for another tumor type in these reproductive tissues, in which deletion of *Dicer1* and *Pten* also occurs. Also, one-third of DKO mice (33.3%: 7/21) in the low-dose (3 mg/mo) group still developed fallopian tube HGSC and widespread peritoneal metastases similar to placebo-treated DKO mice. However, a high dose of mifepristone (16.7 mg/mo for 6 mo) (57) effectively suppressed HGSC development and peritoneal metastasis: only 9.5% (2/21) of these DKO mice were not inhibited by mifepristone. Together, these findings provide sound evidence that targeting progesterone signaling by antiprogestins inhibits HGSC development and peritoneal metastasis in DKO mice.

Crucially, antiprogestin treatment markedly increases mouse survival. DKO mice treated with mifepristone (3 mg/mo) lived longer by nearly 4 mo (~12 human years) than DKO mice with a placebo (Fig. 4*I*; median survival, 11.2 mo of age [5.9 mo to 21.3 mo] vs. 7.3 mo [5.0 mo to 12.6 mo]; n = 21 mice each; HR, 0.40; 95% CI, 0.21 to 0.78; $P = 0.0007$). The low dose of mifepristone for 3 mo showed tumor inhibition and extended mouse survival. Hence, we decided to gain further insights into the effect of longer-term (6 mo) mifepristone treatment on tumor inhibition and survival extension, albeit with a higher dose of mifepristone (16.7 mg/mo). Predictably, an extended high-dose treatment with mifepristone (16.7 mg/mo) further improved survival (median survival, 13.1 mo of age [6.6 mo to 24.3 mo]; n = 21; HR, 0.13; 95% CI, 0.06 to 0.30; $P < 0.0001$), allowing the DKO mice to live nearly 6 mo (~18 human years) longer than placebo-treated DKO mice. Thus, by blocking PR and thus suppressing HGSC development and peritoneal metastases, antiprogestin treatment profoundly improves mouse survival. Collectively, these findings strengthen the notion that ovarian progesterone acting through PR signaling is vital to the development of HGSC possessing metastatic capability.

Genetic Inactivation of PR Suppresses HGSC Development and Its Metastatic Potential. Although mifepristone is a potent antiprogestin, it also acts as an antagonist for the glucocorticoid receptor (GR), thus exhibiting antiglucocorticoid activity (56).

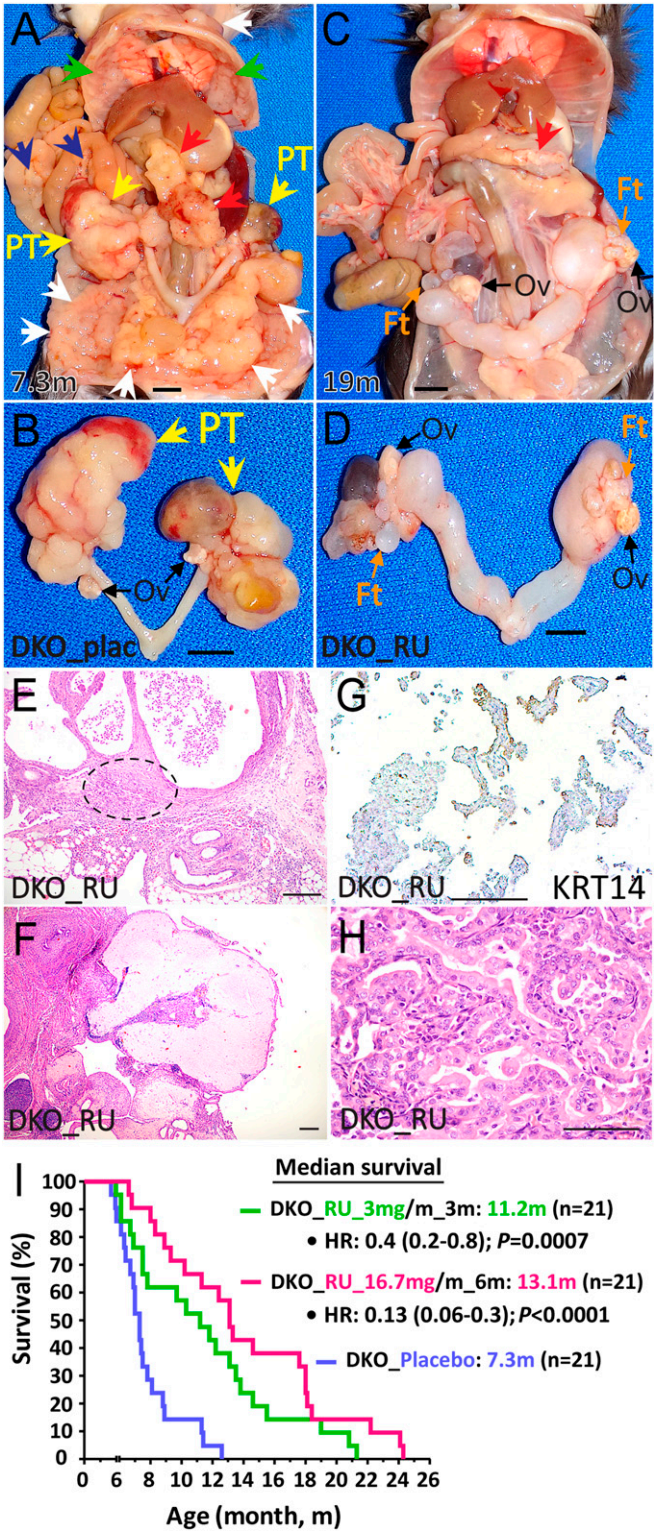


Fig. 4. The PR antagonist mifepristone suppresses HGSC development and significantly extends mouse survival. (*A* and *B*) Tumor phenotype of DKO mice treated with a placebo. As expected, placebo-treated DKO mice develop HGSC in the fallopian tube (PT, yellow arrows) along with abundant peritoneal metastases to the omentum (red arrows), mesentery (blue arrows), peritoneum (white arrows), and diaphragm (green arrows). A mouse shown was killed at 7.3 mo of age (*A* and *B*). (Scale bars, 0.5 cm.) (*C–H*) Tumor phenotype and histopathology of DKO mice treated with mifepristone (RU486). Typically, mifepristone-treated DKO mice develop little peritoneal metastasis: small tumor nodules in the omentum (a red arrow) from a 19-mo-old DKO mouse treated with a mifepristone pellet (9 mg for 3 mo). (Scale bars, 0.5 cm for *C* and *D*.) Histologically, the fallopian tubes contain multiple cysts (*E*), necrotic and fibrous tissues (*F*), proliferative stromal cells (*F*), and a small segment of carcinoma cells (*E*, cells inside a dotted circle). Also, a small group of tumor cells in the omentum was histologically HGSC but negative for KRT14, an HGSC marker (*G*). Low-grade endometrioid-type carcinoma in the ovary was also observed in mifepristone-treated mice. (*H*) Histopathology examination with H&E staining. (Scale bar, 100 μm.) (*I*) Survival curves of DKO mice treated with mifepristone (RU486) or placebo. Mifepristone treatment at 3 mg/mo for 3 mo (DKO_RU_3 mg/m_3m, 21 mice, green line) significantly extended survival of DKO mice, compared with placebo-treated group (DKO_Placebo, 21 mice, blue line): median survival, 11.2 mo (5.9 mo to 21.3 mo) vs. 7.3 mo (5.0 mo to 12.6 mo) of age; HR, 0.40; 95% CI, 0.21 to 0.78; log-rank test, $P = 0.0007$. Survival was further improved with a high-dose longer treatment of mifepristone (16.7 mg/mo for 6 mo) (DKO_RU_16.7 mg/m_6m, 21 mice, red line): median survival, 13.1 mo (6.6 mo to 24.3 mo) of age; $n = 21$; HR, 0.13; 95% CI, 0.06 to 0.30; log-rank test, $P < 0.0001$; compared with placebo-treated DKO mice. Survival extension for 6 mo (16.7 mg/mo) vs. 3 mo (3 mg/mo) was not statistically significant: HR, 0.59; 95 CI, 0.31 to 1.13; $P = 0.11$. DKO_plac, DKO mice treated with placebo; DKO_RU, DKO mice treated with mifepristone (RU486).

Therefore, to supplement the pharmacological inhibition of PR by mifepristone, the PR gene (*Pgr*) was genetically ablated in DKO mice to verify the impact of blocked PR signaling on HGSC development. To inactivate the PR gene in the fallopian tube, a conditional PR allele ($Pgr^{flox/flox}$) was bred into DKO mice ($Dicer1^{flox/flox}$ $Pten^{flox/flox}$ $Amhr2^{cre/+}$), generating cPR-DKO mice ($Pgr^{flox/flox}$ $Dicer1^{flox/flox}$ $Pten^{flox/flox}$ $Amhr2^{cre/+}$). In cPR-DKO mice, the PR gene would be deleted in the fallopian tube cells lacking *Dicer1* and *Pten*, from which HGSC arises, as well as in other reproductive tissues, including the ovary, uterus, and cervix (39).

As predicted, the genetic ablation of PR inhibited HGSC development and metastasis formation. Approximately one-third of cPR-DKO mice (32.5%: 13/40 mice) developed ovarian or fallopian tube tumors with limited peritoneal metastasis (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S6*A*). Mifepristone effectively blocked primary and metastatic tumor development, thus prolonging mouse survival (Fig. 4). These cPR-DKO mice, however, despite absent or markedly reduced peritoneal metastasis, generally formed large primary tumors—likely due to embryonic gene deletion driven by *Amhr2*-Cre (39), These large primary tumors led to an early termination of mice for humane reasons (median survival: 7.5 mo of age [4.0 mo to 11.8 mo]; $n = 13$ mice). Histologically, these primary tumors were eclectic and composed of poorly differentiated tumors/carcinoma or granulosa cell tumors or both, occasionally accompanied by a small segment of HGSC cells (Fig. 5 and *SI Appendix*, Fig. S6 *A–H*). Also, these histologically HGSC cells were negative for the HGSC marker KRT14 (*SI Appendix*, Fig. S6 *B–E*), similar to sporadic presence of HGSC in mifepristone-treated DKO mice (Fig. 4*G*). Therefore, like mifepristone treatment, PR deletion also decreases HGSC development and may alter metastatic potential of HGSC.

As also expected, PR deletion did not completely block HGSC development. Similar to the low-dose antiprogestin treatment, a portion of cPR-DKO mice (32.5%: 13/40 mice), resembling the DKO phenotype, developed primary fallopian tube HGSC with widespread peritoneal metastases (median survival: 8.7 mo of age [5.8 mo to 14.8 mo]; $n = 13$ mice; Fig. 2*A*, 8.7 mo for DKO mice), suggesting that HGSC can also form independently of PR signaling (*SI Appendix*, Fig. S6 *I–P*). In addition to ovarian and fallopian tube primary tumors, cPR-DKO mice formed primary cervical or uterine tumors, or both, with little metastasis (55.0%: 22/40 mice). Development of cervical, uterine, and ovarian tumor phenotypes, which were not observed in mifepristone-treated DKO mice, is likely due to genetic deletion

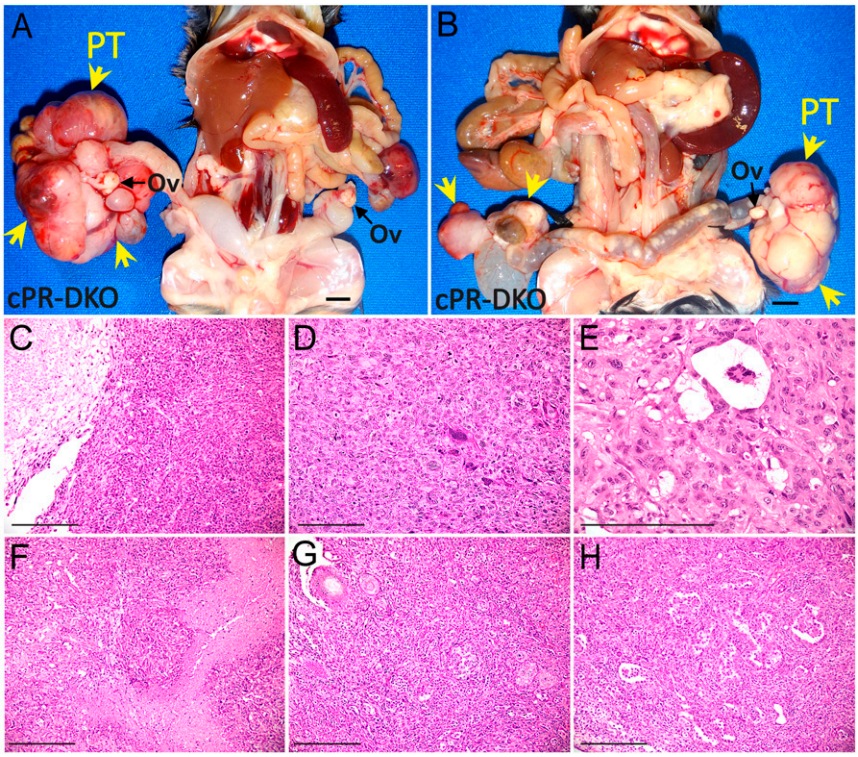


Fig. 5. Genetic ablation of PR inhibits HGSC development in cPR-DKO mice. (*A* and *B*) Phenotype of cPR-DKO mice with fallopian tube tumors. Typically, cPR-DKO mice (8.9 [*A*] and 6.6 [*B*] months of age) exhibit massive fallopian tube tumors (yellow arrows) with intact ovaries (black arrows), accompanied by scant peritoneal metastasis. (Scale bars, 0.5 cm.) (*C*–*H*) Histopathology of fallopian tube tumors (H&E). Poorly differentiated tumors (*C* and *D*) with a swath of necrotic lesions on the left (*C*). Poorly differentiated carcinoma with glands (*E*). Carcinoma with glandular differentiation (left) and necrosis (right) (*F*). Carcinoma with squamous (upper left) and glandular differentiation (*G*). Poorly differentiated carcinoma with glandular differentiation (*H*). (Scale bars, 100 μm.) No cPR-DKO control mice (*Pgr* $^{flox/flox}$ *Dicer1* $^{flox/flox}$ *Pten* $^{flox/flox}$ *Amhr2* $^{+/+}$) developed tumors (seven mice examined from 8.8 mo to 20.5 mo of age).

of *Pgr*, *Dicer1*, and *Pten* driven by *Amhr2*-Cre during embryonic development (39).

Collectively, and corroborating the antiprogestin results, PR deletion suppresses HGSC development and its peritoneal metastasis. Hence, these findings reinforce the notion that progesterone/PR signaling is crucial to the development of HGSC harboring metastatic potential.

Loss of PR Expression in Mouse and Human HGSCs. These robust in vivo experimental findings provide compelling evidence that progesterone-induced HGSC development in DKO mice hinges on PR activation. Therefore, we sought to examine PR expression during the development and progression of HGSC. At a premalignant stage, PR expression, as well as ER alpha expression, was widespread in the epithelium and stroma of the fallopian tube in DKO mice (Fig. 6*A*). Surprisingly, however, while ER expression remained robust, PR expression was completely absent in all stages of mouse HGSCs, including early-stage HGSC (DKO_P4_ET), primary HGSC (DKO_HGSC_PT; DKO_P4_HGSC_PT), and metastatic HGSC (DKO_HGSC_MT) (Fig. 6*A*).

Similarly, in humans, PR and ER were abundantly expressed in the epithelium and stroma of normal fallopian tubes (5/5 cases)—as well as premalignant fallopian tubes obtained from risk-reducing salpingo-oophorectomy (RRSO) in *BRCA1*-mutation carriers (5/5 cases), who are at genetically high risk of developing HGSC (Fig. 6*B*). As in mouse HGSCs, however, PR expression was completely absent in HGSC tissues from *BRCA1*-mutation carriers who had developed ovarian cancer (5/5 cases) (Fig. 6*B*). Additionally, in human HGSCs from a population with undetermined BRCA status (i.e., mostly sporadic HGSCs as most would carry normal BRCA genes), PR expression was negative for the majority of HGSC cases (73.7%: 84/114 cases), whereas ER expression was positive for most HGSCs (86.0%: 98/114 cases) (Fig. 6*B* and *SI Appendix*, Fig. S7). Likewise, the majority of human HGSCs are known to be negative for PR expression (69 to 75%) and positive for ER expression (81 to 85%) (58, 59). Similarly, 59% of HGSCs are PR negative and 77% are ER positive in *BRCA1*-mutation carriers (60). In our study, however, ER was negative in HGSC tissues from *BRCA1*-mutation carriers (all five cases) (Fig. 6*B*), which may be due to a small sample size.

Collectively, absence of PR expression in HGSC tissue strongly suggests that, after progesterone has induced PR-expressing fallopian tube cells into HGSC, these HGSC cells then lose PR expression. Therefore, these findings lead to an intriguing possibility that progesterone/PR signaling is primarily involved in the initiation and formation of HGSC, but may not directly contribute to the progression of HGSC. Crucially, a similar pattern of PR expression and PR loss between DKO model and *BRCA1*-mutation carriers postulates that progesterone/PR signaling may be critical to HGSC development in these high-risk women.

Altered BRCA1 Signaling and Evident BRCAness in Progesterone-Induced HGSC. To examine the molecular signaling pathways underlying progesterone (P4)-induced HGSC development, we performed gene expression profiling using RNA sequencing and, subsequently, pathway analysis using genes whose expression was altered in early-stage HGSC (ET) from P4-treated ovariectomized DKO mice (P4-Ovex-DKO) (Fig. 7*A*). Among the 10 most significantly altered pathways for the up-regulated genes, DNA damage/repair regulation and BRCA1 signaling were notably altered (Fig. 7*B*). Dysregulated BRCA1/DNA repair signaling suggests that P4-induced HGSC development may be linked to molecular mechanisms implicated in ovarian cancer (HGSC) among *BRCA1*-mutation carriers. Also, significantly altered pathways for the down-regulated genes included those relating to immune and inflammatory response (Fig. 7*C*), suggesting a presumed role of progesterone in immune suppression and inflammation (61, 62). Additionally, as significant metabolome changes are evident during the development and progression of HGSC (63), progesterone may also elicit metabolomic alterations specific to HGSC development.

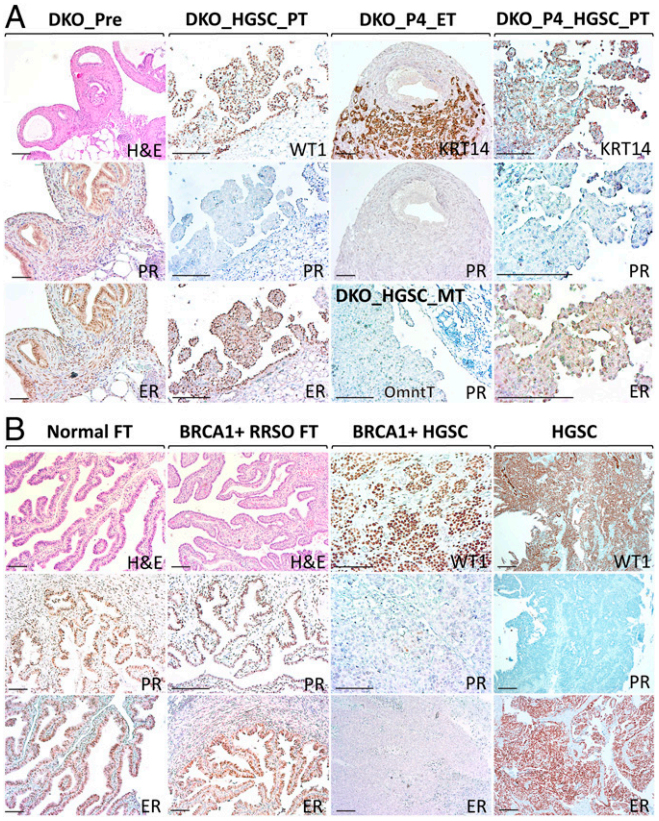


Fig. 6. Expression of PR and ER in mouse and human HGSCs. (*A*) Histopathology of premalignant mouse fallopian tubes and mouse HGSCs. PR and ER are abundantly expressed in the epithelium and stroma of premalignant fallopian tubes in DKO mice (DKO_Pre). In stark contrast, PR expression is completely absent—while ER expression still remains robust—in primary HGSC (DKO_HGSC_PT) and metastatic HGSC (DKO_HGSC_MT) from DKO mice with intact ovaries as well as early-stage HGSC (DKO_P4_ET) and primary HGSC (DKO_P4_HGSC_PT) from ovariectomized DKO mice with P4 treatment. HGSCs are positive for the mouse HGSC marker KRT14 (cytokeratin 14) as well as WT1, a human HGSC marker. (*B*) Normal and premalignant human fallopian tube tissues and human HGSCs. PR and ER expression are widespread in the epithelium and stroma of normal fallopian tubes from women with benign uterine abnormality (5/5 cases) (Normal FT), and fallopian tubes from prophylactic RRSO from *BRCA1*-mutation carriers (5/5 cases) (BRCA1+ RRSO FT). In contrast, PR expression, as well as ER expression, was absent in HGSCs (5/5 cases) from ovarian cancer patients positive for a pathogenic germline *BRCA1* mutation (BRCA1+ HGSC). PR expression was negative for the majority of HGSCs (73.7%: 84/114 cases) from ovarian cancer patients whose *BRCA1*-mutation status is undetermined, whereas ER expression was positive for most cases (86.0%: 98/114) in these largely sporadic HGSCs. All mouse and human HGSCs were histopathologically confirmed as HGSC. (Scale bars, 100 μm.)

Crucially, known as "BRCAness," tumors wild type for *BRCA1/2* can harbor molecular features similar to those in tumors from *BRCA*-mutation carriers (42). Although not carrying a *Brca1* or *Brca2* mutation, both P4-induced HGSC (DKO_Ovex_P4_ET) and DKO HGSC (DKO_ET) exhibit widespread molecular alterations in BRCA signaling and homologous recombination pathways (29) (Fig. 7*D*), amply demonstrating "BRCAness." Thus, the HGSC-inducing role of ovarian P4 could be closely relevant to *BRCA*-mutation carriers.

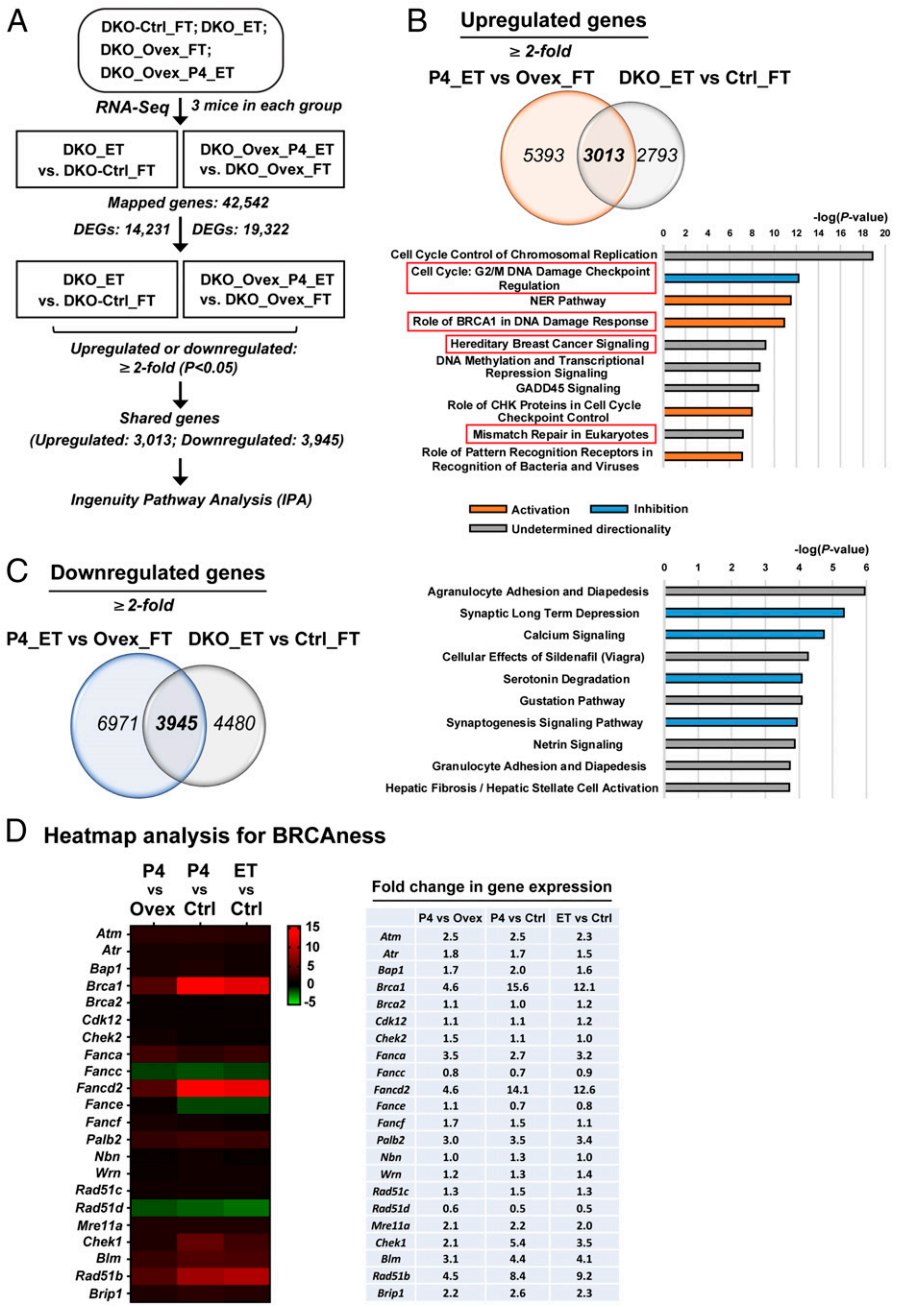
Discussion

This report shows that progesterone is a pivotal endogenous factor raising ovarian cancer risk—and inhibition of progesterone signaling profoundly reduces the risk. These conclusions drawn from robust in vivo findings directly oppose the conventional notion that progesterone protects against ovarian cancer (64–66). This traditional view was formed largely from observational studies, which showed a significant correlation between use of oral contraceptives or pregnancy and a reduced risk of ovarian cancer (24, 27). A synthetic progesterone (progestin) is the main component of combined oral contraceptive pills (COCPs) (67), and progesterone levels during pregnancy are ten times higher than in the luteal phase of a menstrual cycle (68). Therefore, progesterone has generally been viewed as a protective factor against ovarian cancer (64–66).

Distinct from observational studies, the present study has directly tested a causal relationship between ovarian progesterone and ovarian cancer risk by employing a robust mouse model, which unfailingly develops metastatic ovarian cancer faithful to the clinical disease of human HGSC. This study leads to an unambiguous, opposite conclusion: (Ovarian) progesterone elevates ovarian cancer risk. This study shows that primary HGSCs formed in this mouse model are primed with metastatic capability—and ovarian progesterone is a crucial endogenous factor determining the development of primary HGSC equipped with full metastatic potential. Crucially, blocking progesterone signaling by pharmacological inhibition or genetic inactivation effectively suppresses primary HGSC development and also appears to alter or reduce its metastatic potential, collectively leading to profound suppression of peritoneal metastasis. The pivotal role of ovarian progesterone in HGSC development also underscores that interactions between genes (e.g., *Dicer1* and *Pten*) and (endogenous) environment (e.g., ovarian progesterone) would be vital to cancer development, as evident in complex biological traits and diseases (69).

Our findings in genetically high-risk mice postulate that ovarian progesterone may be a vital factor elevating or determining the risk of ovarian cancer in humans. Generally, major drivers of ovarian cancer would be deleterious gene mutations accumulating during the premenopausal and postmenopausal years of a woman's life (70). Hence, in the general population of healthy women, whose lifetime risk of ovarian cancer is 1.3% (71), the risk posed by ovarian progesterone would be relatively low. On the other hand, ovarian progesterone may pose potentially significant risk to high-risk women, such as those positive for a pathogenic germline *BRCA1* or *BRCA2* mutation whose lifetime risk of ovarian cancer (predominantly HGSC) (4) is as high as 60% (12, 17). With high lifetime risk (10 to 60%) for HGSC, *BRCA1/2*-mutation carriers account for 10 to 15% of all HGSCs, with 85 to 90% of HGSC cases being sporadic (17). Clinically as well as genomically, HGSCs arising from *BRCA1/2*-mutation carriers are indistinguishable from sporadic HGSCs in the general population (21, 72). Moreover, many sporadic HGSCs, albeit not carrying germline *BRCA* mutations, harbor HR deficiency (i.e., BRCAness) and exhibit a treatment response to poly(ADP ribose) polymerase inhibitors, as do HGSCs positive for germline *BRCA1/2* mutations (73, 74). Similarly, DKO mice develop metastatic HGSC with striking phenotypic, histopathologic, and molecular similarities to human HGSC (29). Also, although not harboring a *Brca1* or *Brca2* mutation, DKO HGSCs exhibit widespread molecular alterations in BRCA signaling and homologous recombination pathways (29), demonstrating "BRCAness" (42) (Fig. 7*D*). Previously, it was postulated that BRCA mutations may influence endocrine factors affecting the menstrual cycle (75, 76). Therefore, the HGSC-inducing role of ovarian progesterone observed in the DKO model could be closely relevant to *BRCA1/2*-mutation carriers. Elevated levels of ovarian progesterone may interact with, or contribute to, *BRCA*-mutation–related events to promote the development of ovarian cancer (HGSC) in these high-risk women.

A crucial link to this progesterone hypothesis are the findings from a large clinical study of *BRCA1/2*-mutation carriers, in which steroid hormone levels were measured during the



Fold change in gene expression

	P4 vs Ovex	P4 vs Ctrl	ET vs Ctrl
Atm	2.5	2.5	2.3
Atr	1.8	1.7	1.5
Bap1	1.7	2.0	1.6
Brca1	4.6	15.6	12.1
Brca2	1.1	1.0	1.2
Cdk12	1.1	1.1	1.2
Chek2	1.5	1.1	1.0
Fanca	3.5	2.7	3.2
Fancc	0.8	0.7	0.9
Fancd2	4.6	14.1	12.6
Fance	1.1	0.7	0.8
Fancf	1.7	1.5	1.1
Palb2	3.0	3.5	3.4
Nbn	1.0	1.3	1.0
Wrn	1.2	1.3	1.4
Rad51c	1.3	1.5	1.3
Rad51d	0.6	0.5	0.5
Mre11a	2.1	2.2	2.0
Chek1	2.1	5.4	3.5
Blm	3.1	4.4	4.1
Rad51b	4.5	8.4	9.2
Brip1	2.2	2.6	2.3

Fig. 7. Molecular pathways altered in progesterone-induced HGSC development. (*A*) Pathway analysis workflow. RNA sequencing data from DKO-Ctrl_FT, DKO_ET, DKO_Ovex_FT, and DKO_Ovex_P4_ET (average values from a minimum of three individual mouse samples per each experimental group) are used for analysis. Comparison between DKO_ET and DKO-Ctrl_FT yields 14,231 differentially expressed genes (DEGs) whose expression is altered in early-stage HGSC (ET). In addition, comparison between DKO_Ovex_P4_ET and DKO_Ovex_FT produces 19,322 DEGs in ET from P4 treatment. From these two comparison analyses, shared up-regulated and down-regulated genes are selected, using twofold change ($P < 0.05$) as a cutoff, to produce DEGs that are regulated by P4 and altered in HGSC development. Overall, 3,013 up-regulated and 3,945 down-regulated genes are identified and used for Ingenuity Pathway Analysis (IPA). (*B*) Up-regulated genes. Venn diagram shows 3,013 up-regulated genes that are shared between 1) DKO_Ovex_P4_ET vs. DKO_Ovex_FT and 2) DKO_ET vs. DKO_Ctrl_FT comparisons. IPA of these shared up-regulated genes reveals top 10 altered pathways that may be critical to HGSC development regulated by progesterone. (*C*) Down-regulated genes. Venn diagram shows 3,945 down-regulated genes that are shared between 1) DKO_Ovex_P4_ET vs. DKO_Ovex_FT and 2) DKO_ET vs. DKO_Ctrl_FT comparisons. IPA of the shared down-regulated genes uncovers a distinct set of top 10 altered pathways that are potentially involved in P4-regulated HGSC development. Altered pathways are ranked by negative log of the *P* value for the enrichment score. The color scheme is based on Z scores, with activation in red, inhibition in blue, and undetermined directionality in gray. (*D*) Heatmap analysis for BRCAness. Altered expression of genes in HRR associated with germline *BRCA*-mutation tumors. Comparisons in gene expression: 1) DKO_Ovex_P4_ET vs. DKO_Ovex_FT (P4 vs. Ovex); 2) DKO_Ovex_P4_ET vs. DKO-Ctrl_FT (P4 vs. Ctrl); and 3) DKO_ET vs. DKO-Ctrl_FT (ET vs. Ctrl). For DKO_Ovex_FT, ovariectomy was performed at 4.9 wk to 5.4 wk of age, and fallopian tubes were sampled at 4.1 mo to 4.2 mo of age ($n = 3$; each sample is a pair of fallopian tubes from a single mouse). For DKO_Ovex_P4_ET, after ovariectomy and P4 pellet (25 mg) implantation at 5.1 wk to 5.9 wk of age, early-stage HGSC (ET) samples were collected at 4.4 mo to 4.6 mo of age, ~3 mo after P4 treatment ($n = 3$: 3 ETs from three individual mice). For DKO_Ctrl_FT, fallopian tubes were collected from 4.6 mo to 5.2 mo of age ($n = 3$; each sample consists of 10 pooled fallopian tubes from five mice, i.e., 15 mice total). For DKO_ET, early-stage HGSC (ET) samples were collected at 5.8 mo to 6.0 mo of age ($n = 3$: three ETs from three individual mice).

menstrual cycle (14). In this clinical study, the average progesterone level in *BRCA1/2*-mutation carriers was over 2 times (121%) higher than that in noncarriers. Additionally, 59% of these carriers exhibited progesterone levels above the top 75th percentile level of noncarriers. Despite high genetic risk, not all *BRCA1/2*-mutation carriers develop ovarian cancer. The penetrance rates of ovarian cancer are 40 to 60% for *BRCA1*-mutation carriers and 10 to 30% for *BRCA2*-mutation carriers (12, 22). Incomplete penetrance by gene mutations suggests that additional genetic factors or other nongenetic factors may be needed for cancer development (69). Thus, it is plausible that varying levels of menstrual progesterone may explain differential individual risks for ovarian cancer among *BRCA1/2*-mutation carriers. Crucially, our findings raise a hypothesis that *BRCA1/2*-mutation carriers with high progesterone levels may be at significantly greater risk of developing ovarian cancer than carriers with lower progesterone levels.

Accordingly, this notion suggests potential ways to assess and prevent ovarian cancer risk among *BRCA1/2*-mutation carriers. Currently, owing to high genetic risk (10 to 60%) of ovarian cancer, *BRCA1/2*-mutation carriers are advised to undergo prophylactic RRSO—surgical removal of the fallopian tubes and ovaries—between the ages of 35 and 40 y (5, 77). Despite high risk, many women (40 to 90%) with a germline *BRCA1/2* mutation do not develop ovarian cancer during their lifetimes. However, as individual risk prediction is not possible, all *BRCA1/2*-mutation carriers are generally subject to risk-reducing prophylactic surgery (78). Alternatively, the current study suggests

that monitoring progesterone levels may help assess and refine a *BRCA1/2*-mutation carrier's risk of developing ovarian cancer. If the risk is deemed high, an antiprogestin (progesterone antagonist), such as mifepristone (57) or ulipristal acetate (UPA) (79), could be clinically used as a nonsurgical option to prevent or at least significantly lower the risk of ovarian cancer in these high-risk women. Human trials are warranted to determine the impact of ovarian progesterone levels on HGSC development and the potential benefits of prophylactic antiprogestin therapy in high-risk populations of women.

Another intriguing finding from our study is an apparent loss of PR expression after HGSC development. Once progesterone transforms PR-expressing cells into HGSC, PR expression appears to vanish (Fig. 6). It is well established that ER and PR expression are associated, and PR expression is regulated by ER in most tissues targeted by estrogen and progesterone, including the breast and uterus, as well as in breast cancer (45, 80–82). In breast cancer, ER expression is a robust prognostic marker for the effective treatment response and marked survival improvement with an antiestrogen therapy (7, 8, 81). The therapeutic benefits of antiestrogens also affirm that estrogen signaling is vital to progression of ER-positive breast cancers (7, 8). In contrast, a close association between ER and PR expression and the prognostic value of ER expression do not seem to apply to HGSC. There are no significant associations between ER and PR expression in *BRCA1* mutation and sporadic HGSCs (60, 83), as also shown in our study of human HGSC cases (*SI Appendix*, Fig. S7*C*). Generally, despite abundant ER expression, patients with HGSCs do not benefit from an antiestrogen therapy (84), indicating that HGSC progression does not depend on estrogen signaling. Genomic similarity between HGSC and TNBC also suggests that estrogen/ER signaling may not be critical to HGSC progression (20). As ER status has little association with PR status in HGSC, the loss of ER observed in HGSCs from *BRCA1*-mutation carriers in our study (Fig. 6*B*) is thus likely independent of the loss of PR in these HGSCs. Although commonly expressing ER, HGSC may behave like an ER-negative cancer, such as TNBC.

Presence of PR expression in premalignant tissue and subsequent loss of PR expression in HGSC in our study suggest that progesterone/PR signaling is a critical determinant of HGSC development and its metastatic potential—but is likely not a primary factor for tumor progression. Alternatively, other possibilities exist in which progesterone could still exert its effects in the absence of PR. One is a paracrine mode of PR signaling (85, 86), in which progesterone may bind to PRs expressed in noncancer cells and subsequently impact the progression of HGSC devoid of PR expression. Paracrine signaling of PR still occurs through the transcriptional activation of PR, which is a classical genomic mechanism, the major mechanism of progesterone action (55). Another possibility is nongenomic signaling of progesterone via membrane-bound receptors responding to progesterone—a mechanism known to mediate the rapid effects of progesterone (87). It is also possible that progesterone may act on tumor progression through interaction with the GR (88).

Loss of PR, however, may represent a larger picture of cancer development and cancer progression. A general oncologic assumption may be that factors which spur the growth and progression of existing tumors would also stimulate their initiation and development. Contrasting with this notion, our findings suggest that progesterone/PR signaling would be needed for the initiation and development, but not the progression, of HGSC. In essence, progesterone can be envisioned as the spark that starts the wildfire, but once the fire started, the spark would no longer be needed for the spread. Hence, these unexpected findings of losing PR expression also postulate a larger principle in cancer development and progression: Biologically, cancer development and cancer progression are on a continuum, but, mechanistically, they may be under distinct controls and regulated by separate factors. The concept of distinct mechanistic controls of disease processes is also evident in an infectious disease such as COVID-19. In this coronavirus disease, immune response appears to play distinct, opposite roles in different phases of disease: it may be beneficial to a patient at an early phase of disease, but harmful at an advanced phase (89). Viewing cancer development and cancer progression as distinct processes may reconcile the contradicting observations and offer a conceptual framework to elucidate seemingly conflicting roles of hormones in cancer.

Beyond ovarian cancer, our findings also offer an intriguing perspective on the potential role of progesterone in development and prevention of breast cancer (57, 90). Although women who inherit a pathogenic *BRCA1* mutation are predisposed to ovarian and breast cancer, the reason for this particular tissue tropism remains unclear (1, 3, 91). Curiously, *BRCA1*-mutation carriers develop predominantly HGSC and TNBC (4, 5), distinct malignancies yet with marked genomic similarity (20). Therefore, despite the presumed absence of hormone involvement in TNBC, our findings on HGSC imply that ovarian progesterone may also be a hormonal factor critical to the development, if not progression, of TNBC. Together, our study postulates that ovarian progesterone could be a reason for *BRCA1*-mutation carriers' peculiar susceptibility to both breast (TNBC) and ovarian (HGSC) cancers. Hence, targeting progesterone signaling may reduce the risk of breast cancer as well as ovarian cancer in these high-risk women.

A tumor-inducing role of progesterone in high-risk women is supported by clinical observations, including a recent large clinical study of hormone replacement therapy (HRT) in *BRCA1*-mutation carriers (92), and also in the Women's Health Initiative studies on HRT among postmenopausal women in the general population (93–96). Consistently, these large clinical studies point to a potential breast cancer-inducing role of synthetic progesterone (progestin) and a possible protective effect of estrogen against breast cancer development. These notions are also consistent with our findings of an increased risk of death in ovariectomized DKO mice treated with progesterone alone (HR, 3.01; 95% CI, 1.7 to 5.4; $P < 0.0001$) and a reduced risk of death in ovariectomized DKO mice with progesterone plus 17β-estradiol (HR, 0.56; 95% CI, 0.30 to 1.06; $P = 0.09$). In line with our findings in a mouse model of HGSC harboring BRCAness, in which the antiprogestin mifepristone suppresses HGSC development and metastases (Fig. 4), additionally, mifepristone treatment also prevented mammary tumor development in a murine model lacking *Brca1* and *p53* (57). Collectively, these findings support the notion that ovarian progesterone could be a critical factor contributing to the development of HGSC and TNBC, particularly in *BRCA1*-mutation carriers.

Our present study may also offer fresh insights into the protective effects of COCPs against ovarian cancer. COCPs contain synthetic progesterone and estrogen, and effectively prevent pregnancy (97). It is well established that contraceptive effects of COCPs (including low-dose COCPs) are primarily achieved by suppressing the release of pituitary gonadotropins (luteinizing hormone and follicle-stimulating hormone) (67), which subsequently leads to the suppression and blockage of ovulation as well as the inhibition of ovarian progesterone and estrogen synthesis (98, 99). Essentially, COCPs shut down the menstrual cycle, effectively suppressing the endogenous synthesis of ovarian progesterone and estrogen (67, 98). Therefore, despite taking hormone pills (synthetic progesterone and estrogen), women on COCPs have lower blood levels of progesterone and estrogen than nonusers (100). It has long been thought that the protective benefit of COCPs (the Pill) against ovarian cancer would be owing to their effect on blocking ovulation (101). Our findings suggest that the protective effect of COCPs against ovarian cancer may be additionally driven by the effect of COCPs on

inhibiting the ovarian synthesis of progesterone (98). Consequently, women on COCPs would exhibit sustained lower levels of exposure to menstrual progesterone during their reproductive years, which would lead to a decreased risk of ovarian cancer, later, in their postmenopausal years.

Targeting progesterone signaling with antiprogestins offers a potentially promising therapy for ovarian cancer prevention. Importantly, our concept of antiprogestin prevention therapy will need to be further verified using a clinically more relevant antiprogestin, such as UPA (102, 103). Although outside the scope of this study, employing additional mouse models of ovarian cancer including one driven by a *Brca* mutation will likely be informative in further corroboration of the present study. Nevertheless, our robust in vivo findings present a compelling concept that ovarian progesterone is a vital endogenous factor raising the risk of ovarian cancer—and prophylactic antiprogestin therapy could offer a potentially effective nonsurgical preventive option for *BRCA*-mutation carriers.

Materials and Methods

Mouse use and experiments were approved by the Institutional Animal Care and Use Committee at Indiana University School of Medicine; human specimens were collected with informed patient consent and approval from respective Institutional Review Boards at the University of Kansas Medical Center and Keimyung University School of Medicine (South Korea). Experimental details of mouse generation, mouse bilateral ovariectomy, hormone pellet implantation, hormone measurements, immunohistochemistry, RNA sequencing, pathway analysis, and statistical analysis are available in *SI Appendix, Materials and Methods*.

Data Availability. The RNA sequencing data have been deposited in Gene Expression Omnibus (GEO) (accession no. GSE157960).

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