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7,12-Dimethylbenz[A]Anthracene Induces Sertoli–Leydig-Cell Tumors in the Follicle-Depleted Ovaries of Mice Treated with 4-Vinylcyclohexene Diepoxide

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Ovarian cancer is associated with high mortality due to its late onset of symptoms and lack of reliable screening methods for early detection. Furthermore, the incidence of ovarian cancer is higher in postmenopausal women. Mice rendered follicle-depleted through treatment with 4-vinylcyclohexene diepoxide (VCD) are a model of ovary-intact menopause. The present study was designed to induce ovarian neoplasia in this model by treating mice with 7,12-dimethylbenz[a]anthracene (DMBA). Female B6C3F1 mice (age, 28 d) received intraperitoneal sesame oil (vehicle; VCD– groups) as a control or VCD (160 mg/kg; VCD+ groups) daily for 20 d to cause ovarian failure. Four months after the onset of dosing, mice from each group received a single injection of DMBA (VCD–DMBA+ and VCD+DMBA+ groups, *n* = 15 per group) or vehicle control (VCD–DMBA–, *n* = 15; VCD+ DMBA–, *n* = 14) under the bursa of the right ovary. Ovaries were collected 3 or 5 mo after injection and processed for histologic evaluation. Immunohistochemistry was used to confirm classification of neoplasms. None of the animals in the VCD–DMBA– and VCD–DMBA+ groups (that is, mice still undergoing estrus) had tumors at either time point. At the 3-mo time point, 12.5% of the VCD+DMBA+ mice had ovarian tumors; at 5 mo, 57.1% of the VCD+DMBA+ and 14.3% of VCD+DMBA– ovaries had neoplasms. Neoplasms stained positively for inhibin α (granulosa cells) and negatively for keratin 7 (surface epithelium), thus confirming classification of the lesions as Sertoli–Leydig cell tumors. These findings provide evidence for an increased incidence of DMBA-induced ovarian neoplasms in the ovaries of follicle-depleted mice compared with that in age-matched cycling controls.

Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; OSE, ovarian surface epithelium; VCD, 4-vinylcyclohexene diepoxide.

Approximately 20,000 women are diagnosed with ovarian cancer annually, of whom 15,000 are anticipated to die of the disease. Ovarian cancer ranks fifth in deaths by all cancers and first in cancers of the reproductive system.[12] The survival rate of ovarian cancer patients improves greatly when the disease is detected early,[2] but fewer than 20% of ovarian cancers are found at an early stage due to the lack of reliable screening methods for early detection. Because approximately two-thirds of ovarian cancer cases are diagnosed in women older than 55 y, the incidence of ovarian cancer is increased in peri- and postmenopausal women.[12] For this reason, research using relevant animal models of menopause is needed to advance the understanding of the biology of neoplasms in the postmenopausal ovary.

Ovarian cancer can be due to transformation of surface epithelial cells, germ cells, or sex cord and stromal cells. Almost 90% of all ovarian cancers are thought to be derived from the flat-to-cuboidal epithelial cells (that is, the ovarian surface epithelium [OSE]) that cover the ovary.[6,49] Alternatively, fewer than 5% of ovarian cancers are classified as sex cord–stromal tumors, which include granulosa cell tumors, and Sertoli–Leydig cell tumors.[18] The incidence of sex cord–stromal ovarian cancers is highest in women older than 50 y, but has also been diagnosed in premenopausal women.[18] The etiology of ovarian cancer is not completely understood, but factors associated with development of the disease include ovulation, altered levels of gonadotropins (luteinizing and follicle-stimulating hormones) and steroid hormones (estrogens and androgens), germ-cell or follicle depletion, altered expression of oncogenes and tumor suppressor genes, altered levels of growth factors and cytokines, and exposure to environmental agents.[41]

Recently, an ovary-intact mouse model of menopause was developed by using the occupational chemical 4-vinylcyclohexene diepoxide (VCD).[24,25,27] Repeated daily dosing of mice and rats with VCD selectively destroys ovarian primordial and primary follicles by accelerating the natural process of follicular atresia.[14,15,42,44] Because VCD does not target larger follicles, the animal continues to ovulate normally until no more follicles can be recruited. Thus, ovarian follicular depletion in the VCD-treated mouse is gradual. As with women undergoing perimenopause, VCD-treated mice show increased levels of follicle-stimulating hormone,[27] irregular estrous cycles, and declining levels of estrogen[24] as they become follicle-depleted. In addition, residual ovarian tissue is retained after ovarian failure. Therefore, preservation of residual ovarian tissue in the VCD-treated follicle-depleted mouse makes this model ideal for studying the physiology of the postmenopausal ovary. The VCD-treated mouse model of peri- and postmenopause has been used to study several menopause-related disorders including atherosclerotic lesion develop-

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ment,[28] diabetic kidney disease,[20] osteoporosis,[51] and metabolic syndrome.[39] Because the VCD-treated mouse has been shown to be relevant for studies related to both perimenopausal and postmenopausal stages,[50] it is a useful candidate for studies of ovarian cancer.

Even though spontaneous ovarian tumors in rodents have been reported,[36] the paucity of these cases precludes their use in modeling ovarian cancer. Therefore, much effort has been put into developing relevant animal models for ovarian cancers. One such model involves the use of the carcinogen 7,12-dimethylbenz[a] anthracene (DMBA),[8,21,23,43] a polycyclic aromatic hydrocarbon that induces carcinogenic mutations by forming DNA adducts.[9] Recently, the DMBA model of carcinogenesis has been combined with the VCD model of menopause to cause ovarian cancer in F344 rats.[13,19] However, no studies have characterized the combined use of both chemicals in mice. Developing this combined model in mice is important because of the existence of various genetically engineered mice that have potential relevance to enhancing our understanding of the biology of ovarian cancer.

The present study was designed to determine whether ovarian failure affects susceptibility to the development of ovarian neoplasms in mice and to model DMBA-induced ovarian neoplasia in VCD-treated follicle-depleted mice. VCD-treated follicle-depleted mice and cycling controls received ovarian injections with DMBA to induce neoplasia. The incidence of neoplasms was determined by histologic evaluation, and the lesions were classified through immunostaining for keratin 7 and inhibin α.

Materials and Methods

Animals. Female B6C3F1 mice (age, 21 d) were purchased from Jackson Laboratories (Bar Harbor, ME). On arrival, mice were housed under SPF conditions in polycarbonate plastic cages at a room temperature of 22 ± 2 °C and on 12:12-h light:dark cycles and were fed ad libitum. Animals were certified free of *Mycoplasma pulmonis*, ecto- and endoparasites, and mouse hepatitis virus, minute virus of mice, mouse parvovirus, murine norovirus, rotavirus, Theiler murine encephalomyelitis virus, and Sendai virus. All mice were allowed to acclimate to the animal facilities for 1 wk before the start of the experiment. All experiments and methods were approved by the University of Arizona Institutional Animal Care and Use Committee and conformed to the *Guide for the Care and Use of Experimental Animals*.[16]

VCD-induced ovarian failure. Mice were allocated randomly into treatment groups and received daily intraperitoneal injections of VCD (in sesame oil; 160 mg/kg; Sigma–Aldrich, St Louis, MO) or sesame oil only (2.5 mL/kg; Sigma-Aldrich) for 20 d.

Intrabursal injections. Mice received intraperitoneal ketamine–xylazine (16 µL/g body weight; Ketaject, Phoenix Pharmaceutical, St Joseph, MO, and AnaSed, Lloyd Pharmaceuticals, Shenandoah, IA), and anesthesia was defined by cessation of movement, recumbency, and lack of response to aversive stimulation of paw and tail. The right flank of each animal was shaved and the skin cleaned with povidone iodine and 70% ethanol. An incision was made through the skin, fascia, and abdominal wall, and the ovary was externalized. DMBA (in sesame oil; 50 µg; Sigma–Aldrich) or an equivalent volume of sesame oil only (Sigma-Aldrich) was injected directly under the bursa, the thin membrane that surrounds the ovary. The muscle wall was closed by using 6-0 braided silk suture, and the skin was closed by using 3-0 braided silk suture. Surgical glue (Tissumend II, Veterinary Products Laboratories, Phoenix, AZ) was applied over the incision to prevent reopening of the wound in case the sutures became undone. The left ovary was not injected and was used as an untreated contralateral control.

Tissue collection. Animals were euthanized by CO_2 inhalation followed by cervical dislocation. Both ovaries were dissected, trimmed of fat, and separately fixed in 4% formalin for subsequent embedding, sectioning, and staining.

Hematoxylin and eosin staining. Ovaries were trimmed, placed in 4% formalin for 4 h, transferred to 70% ethanol, and paraffin-embedded. Each ovary was sectioned (4 to 5 µm) and mounted, and every 10th section was saved and stained with hematoxylin and eosin. Additional unstained ovarian sections were used for immunohistochemical staining.

Immunohistochemistry. Immunohistochemistry was performed by incubating sections in an automatic immunostainer (ES immunostainer, Ventana Medical Systems, Tucson, AZ) for 24 min. A keratin 7 stain (Dako, Carpenteria, CA) was used to label ovarian surface epithelial cells, and inhibin α (anti-inhibin clone R1, Dako) was used to stain steroidogenic sex-cord–stromal cellular components within tumors. Antibody application was followed by treatment with a biotin-conjugated goat antimouse antibody, avidin-D–conjugated horseradish peroxidase, and then 3′,3′-diaminobenzidine tetrahydrochloride with copper enhancement as color substrate. Hematoxylin was used as the counterstain. After clearing in graded alcohols and xylene, slides were coverslipped. Dark brown structures indicated positive staining.

Statistical analysis. Data for the incidence of ovarian neoplasms were analyzed by using the Fisher exact test (SPSS Statistics v17.0, Chicago, IL), with significance set at a P value of less than 0.05.

Results

Incidence of ovarian neoplasms. The effect of direct application of vehicle or DMBA under the bursa of the right ovary of VCD-treated follicle-depleted mice and age-matched cycling controls was evaluated histologically at 3 or 5 mo after surgery (Figure 1). No tumors were identified in any of the cycling control mice (VCD–DMBA– and VCD–DMBA+ groups) at either time point. At 3 mo after injection, 1 of the 8 (12.5%) follicle-depleted mice that received DMBA (VCD+DMBA+ group) showed ovarian neoplasms in the injected (right) ovary. This number was increased to 4 of the 7 (57.1%) VCD+DMBA+ mice at 5 mo after injection. In comparison, 1 of the 7 (14.3%) VCD-treated follicle-depleted mice that received vehicle (VCD+DMBA– group) had ovarian neoplasia in the right ovary at the 5-mo time point. All ovarian tumors at the 5-mo time point occurred in the injected ovary. In addition, at 3 mo, there were 2 cases (1 each in the VCD+DMBA– and VCD+DMBA+ groups) of spontaneous (nonDMBA-induced) ovarian tumors in the contralateral, noninjected (left) ovary. Mice with neoplasms in the left ovary did not also have neoplasms in the right ovary and therefore were not included in the incidence data.

Morphologic features of ovaries. Sections from the ovaries of VCD-treated follicle-depleted mice and age-matched cycling controls collected at 3 and 5 mo after injection with vehicle or DMBA were stained with hematoxylin and eosin (Figures 2 to 4). At 3 mo, ovaries from VCD–DMBA– mice appeared normal, with evidence of ongoing folliculogenesis as demonstrated by the presence of preantral and antral follicles as well as corpora lutea and rare atretic follicles (Figure 2 A). In contrast, VCD–DMBA+

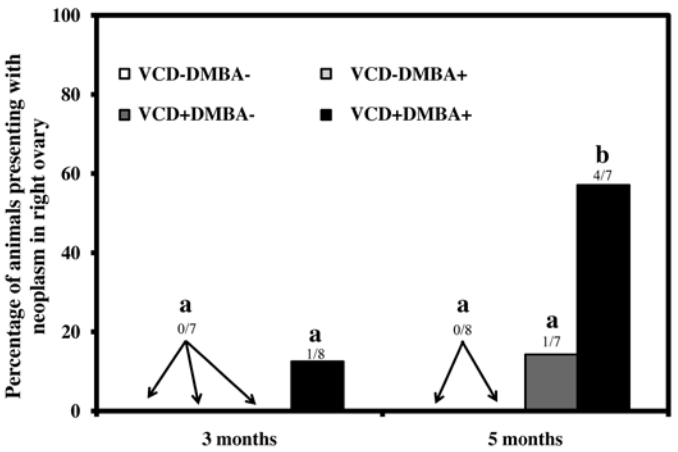

Figure 1. Incidence of ovarian neoplasms in VCD-treated follicle-depleted mice compared with age-matched cycling controls. Ovaries were removed 3 or 5 mo after injection and processed for histologic evaluation. Data are presented as percentage (%) of animals with neoplasms relative to the total number evaluated. The numbers over each bar represent the actual number of animals with neoplasms / actual total animals in the treatment group. Different letters indicate significant ($P < 0.05$; $n = 7$ to 8 per group) difference in incidence between groups.

ovaries showed markedly less folliculogenesis as evidenced by fewer follicles of all sizes, fewer corpora lutea, and more atretic follicles at both time points (Figure 2 B). At 5 mo after injection, VCD–DMBA+ ovaries showed no evidence of ovulation (no corpora lutea) and few follicles (Figure 2 C). As expected for follicle-depleted mice, VCD+DMBA– and VCD+DMBA+ ovaries showed no folliculogenesis and numerous luteinized stromal cells (Figure 2 D and E**)**. Ovaries from VCD+DMBA+ mice in which neoplasms were identified showed predominant clusters of Sertoli-like and Leydig-like cells, aggregates of larger luteinized cells forming sex cord tumors of intermediate differentiation which, in some cases, replaced normal ovarian tissue (Figure 3 A through E). In addition, both ovaries of all VCD-treated mice had numerous invaginations of the OSE and outer cortex of the ovary (also known as ovarian epithelial hyperplasia and benign tubular adenomas;[1] Figure 4 A through C).

Immunohistochemical staining. Whereas OSE-derived cancers stain positive for the intermediate filament protein keratin 7, sex-cord–stromal tumors stain positive for inhibin α.[29] To facilitate classification of tumor origin, immunohistochemistry for inhibin α (Figure 5 A) and keratin 7 (Figure 5 B) was performed. Positive staining for inhibin α was particularly strong in Sertoli-like cells and weak or absent in Leydig-like cells, thus confirming classification of tumors as Sertoli–Leydig cell tumors (Figure 5 A). Further, all neoplasms were negative for keratin 7 (data not shown). OSE and tubular adenomas were positive for keratin 7 (Figure 5 B) and negative for inhibin α (data not shown).

Discussion

This study was designed to determine whether ovarian failure affects susceptibility to development of ovarian neoplasms in mice and to further characterize DMBA-induced ovarian neoplasia in the VCD-treated follicle-depleted mouse. To accomplish this goal, VCD-treated follicle-depleted mice and age-matched cycling controls received single unilateral intrabursal injections of DMBA (or vehicle only as a control), and the incidence of neoplasms was determined. In the present study, a single direct application of DMBA into the intrabursal space of the right ovary of VCD-treated follicle-depleted mice resulted in neoplasms in 12.5% and 57.1% of the mice at 3 and 5 mo after injection, respectively, whereas no neoplasms were identified in the right ovary of either VCD–DMBA+ or VCD–DMBA– cycling controls (at 3 or 5 mo) or VCD+DMBA– mice (3 mo). In addition, spontaneous tumors were identified in the right ovary only and left ovary only of 2 individual VCD+DMBA– mice (at 5 mo and 3 mo, respectively) and in the left ovary of a VCD+DMBA+ mouse (at 3 mo).

We performed immunohistochemical analysis to assist in classification of the tumors observed in this study. Keratin 7 was used as a marker for the OSE, and inhibin α was used to label sex-cord–stromal tumors.[29] All neoplasms were positive for inhibin α (both Sertoli- and Leydig-like components) and negative for keratin 7 (only present in OSE). Therefore, together with the morphologic data obtained from histologic evaluation, immunohistochemistry confirmed that the neoplasms in our mice were Sertoli–Leydig-cell tumors. These tumors are members of the sex-cord–stromal tumor family, which represents fewer than 5% of ovarian cancers in women.[18,22] Sertoli–Leydig-cell tumors have also been identified in VCD-treated follicle-depleted F344 rats treated with DMBA[13] and aging follicle-stimulating-hormone receptor knockout mice with ovarian failure (FORKO mice).[10] However, the present study is the first to describe the development of DMBA-induced Sertoli–Leydig-cell tumors in VCD-treated follicle-depleted wild-type mice.

Studies evaluating the DMBA model of ovarian carcinogenesis traditionally have focused on using rats rather than mice.[8,13,19,43] However, the few reports available on overall DMBA-induced carcinogenesis in mice have shown reproducible ovarian neoplasms despite differences in methods of delivery (gavage versus coated suture), dosage, length of exposure, and length of time before collection of tissues. In one study, C57BL6/129Sv mice received daily doses of DMBA by gavage for 3 wk; 71% of animals developed granulosa cell tumors after 1 y.[5] In another study, mice of a C57BL6/NCr;Sv129;FVB/NCr background received a weekly dose of DMBA by gavage for 6 wk; 6 mo after treatment, 27.3% (12 of 44) of mice developed ovarian neoplasms, of which 58% were granulosa cell tumors.[35] Recently, a study assessing the incidence of cancers in mice with a germline p53 mutation found that 80% of wild-type (AJ background) mice that received implants of DMBA-coated suture into their ovaries developed ovarian tumors, of which 50% were adenocarcinomas (malignant, epithelial-derived) when evaluated 3 mo after application of implants.[48]

In the present study, none of the cycling animals exposed to DMBA developed ovarian neoplasms but rather experienced follicle loss. DMBA targets ovarian follicles of all types and causes premature ovarian failure.[26] Therefore, decreased folliculogenesis in VCD–DMBA+ mice in the current study is likely the result of ovotoxicity from the single intrabursal injection of DMBA that they received. In contrast, other studies have documented the presence of DMBA-induced tumors in ovaries of cycling mice.[5,35,48] The reason for the lack of ovarian neoplasms in VCD–DMBA+ mice in the present study is not understood but may reflect the limited exposure to DMBA as compared with that of other studies. Perhaps neoplasms were more frequent in VCD+ mice because of the increased levels of luteinizing hormone (po-

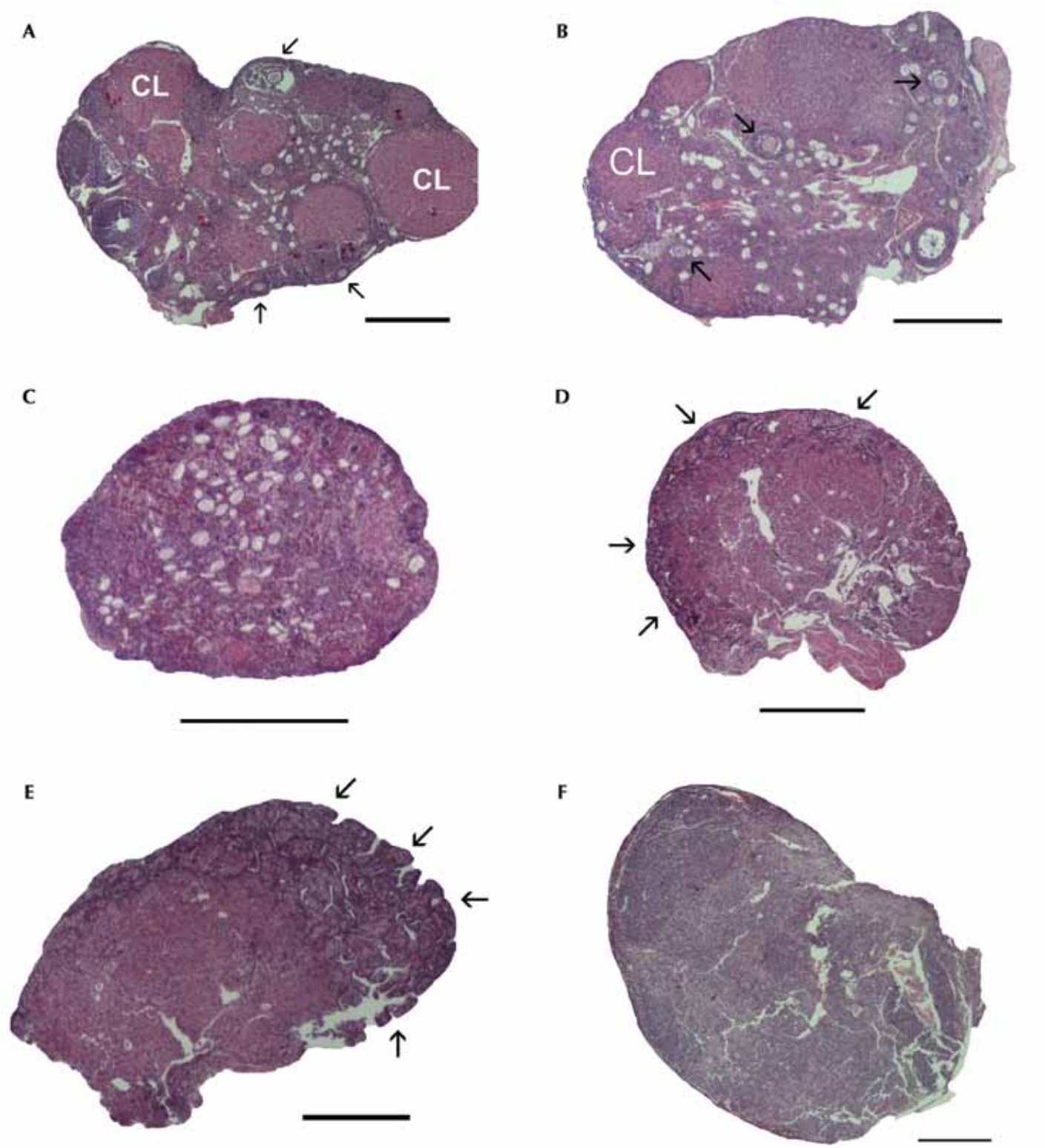

Figure 2. Ovaries collected at 3 and 5 mo after intrabursal injection with DMBA. Ovaries were removed 3 or 5 mo postinjection and processed for histological evaluation as described in Materials and Methods. (A) VCD–DMBA– ovary at 3 mo shows evidence of folliculogenesis (arrows, follicles) and ovulations (CL, corpus luteum). (B) VCD–DMBA+ ovary at 3 mo shows decreased number of follicles (arrows) and corpora lutea. (C) VCD–DMBA+ at 5 mo showing lack of follicles and corpora lutea. (D and E) VCD+DMBA– ovaries showing examples of tubular adenoma (black arrows) and luteinized stromal cells. (F) VCD+DMBA+ ovary showing a tumor that has replaced normal ovarian tissue. Hematoxylin and eosin stain; magnification, ×4 (bar, 500 µm). Panels A and F were constructed by combining overlapping images.

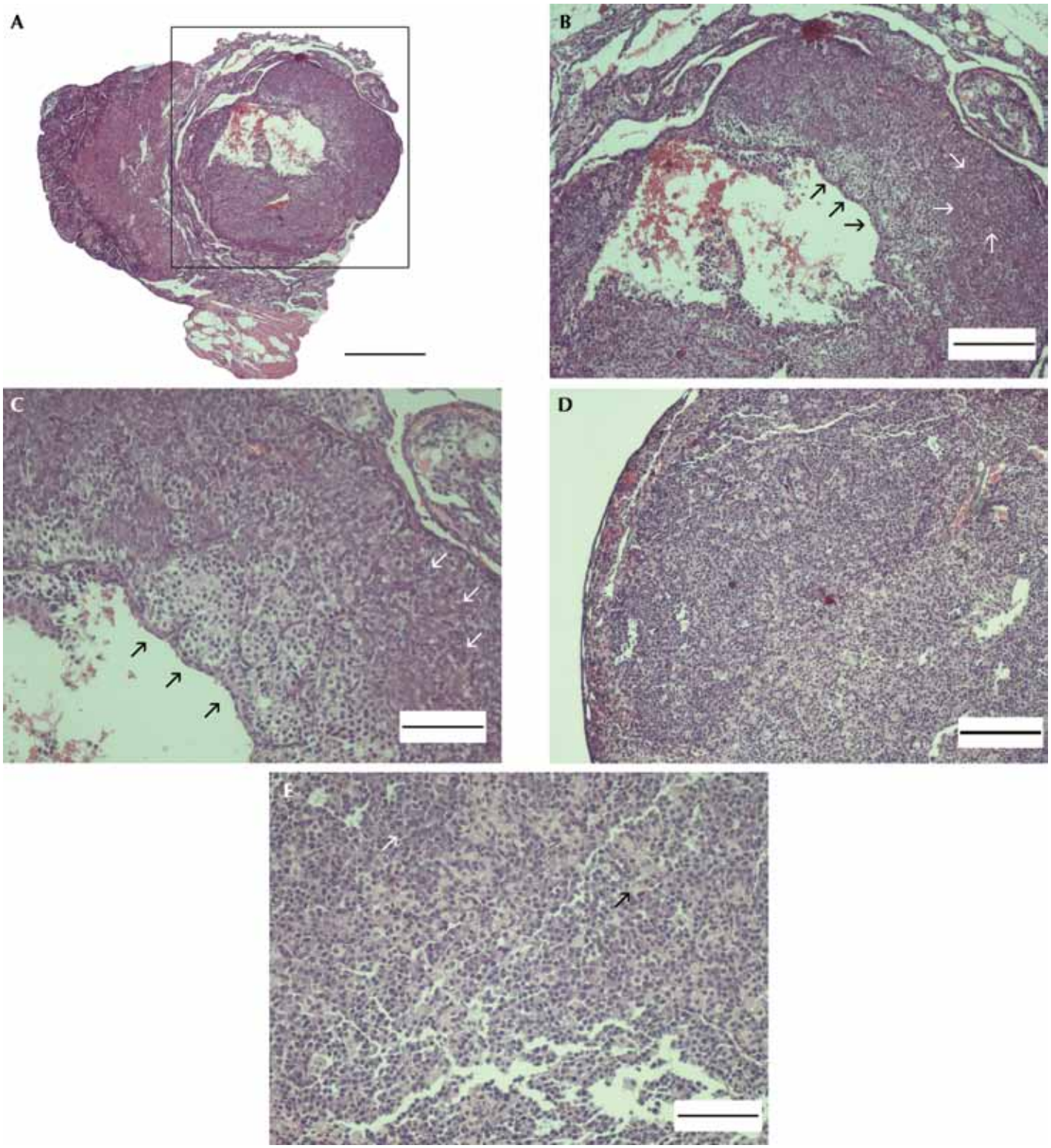

Figure 3. Sections from tumor-containing ovaries at 5 mo after injection. (A) VCD+DMBA+ ovary shows a large Sertoli–Leydig-cell tumor (black square; magnification, ×4). (B and C) Higher-magnification images (B, magnification: ×10; C, magnification, ×20) showing the Sertoli- (white arrows) and Leydig cell-like (black arrows) components of the tumor shown in panel A. (D) VCD+DMBA+ ovary with Sertoli–Leydig-cell tumor where Sertoli-like cells have invaded the entire ovary (same as shown in Figure 2 F). Magnification, ×10. (E) Higher-magnification image (×20) of ovary in panel D showing the Sertoli- (white arrows) and Leydig- (black arrows) like components. Hematoxylin and eosin stain. Scale bars: A, 500 µm; B and D, 200 µm; and C and E, 100 µm.

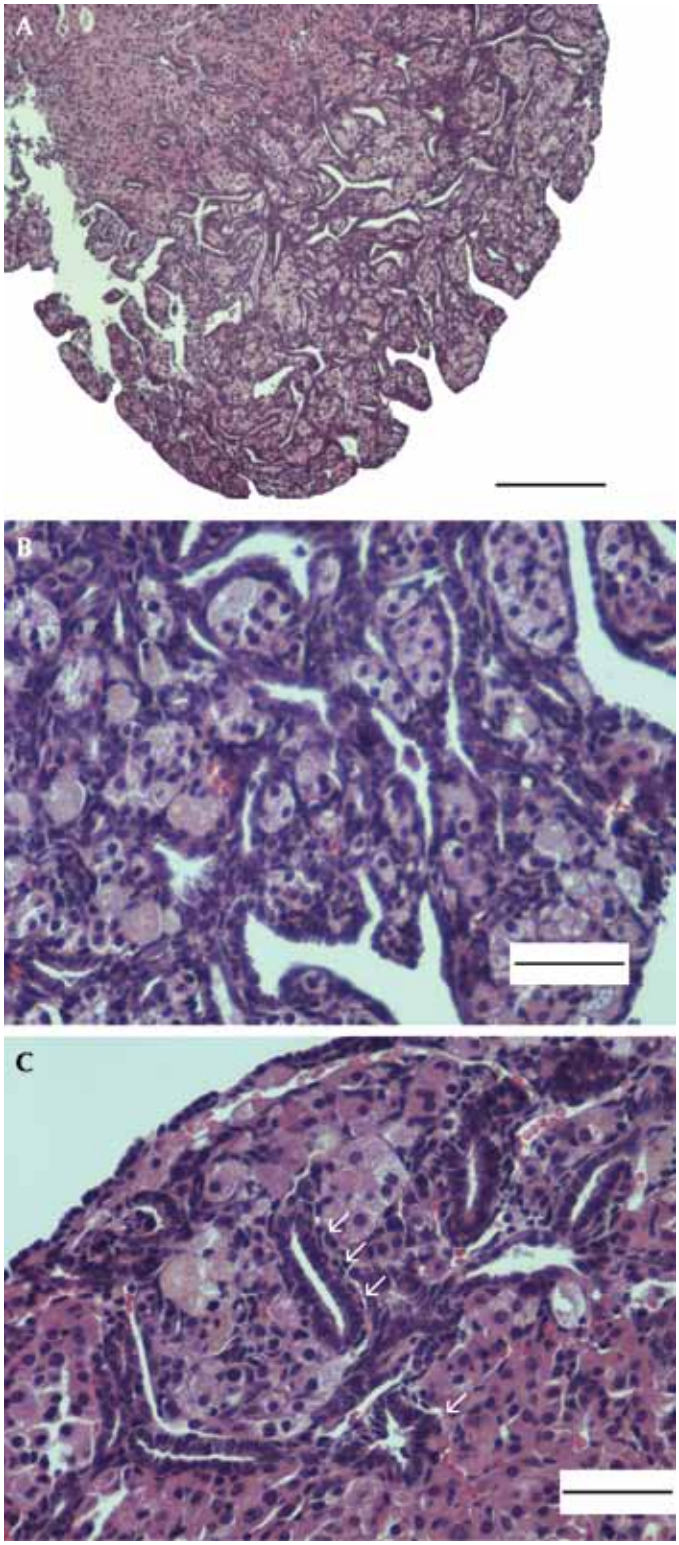

Figure 4. Sections from ovaries containing tubular adenomas at 3 mo after injection. (A) Ovary containing a tubular adenoma (magnification, ×10; bar, 200 µm). (B) Same tumor as in panel A (magnification, ×20; bar, 100 µm). (C) Tubular adenoma with inclusion cysts (magnification, ×40; bar, 100 µm). White arrowhead shows the presence of inclusions. The larger pink cells are background luteinized stromal cells. Hematoxylin and eosin stain.

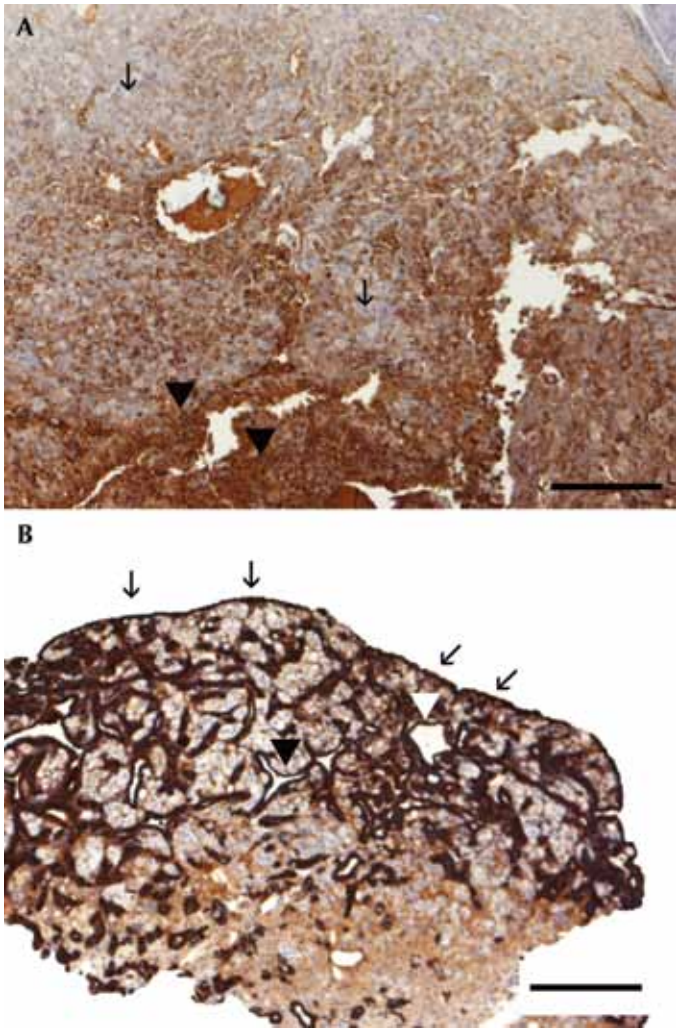

Figure 5. Immunohistochemical staining of ovarian neoplasms present in VCD-treated follicle-depleted ovaries 5 mo after intrabursal injection with DMBA. All ovaries are from VCD+DMBA+ mice with Sertoli–Leydig-cell tumors. (A) Tumor with strong dark-brown staining for inhibin α in Sertoli-like cells (black arrowheads) and weak or absent staining in Leydig-like cells (black thin arrows). (B) Tubular adenoma showing dark-brown positive staining for keratin 7 (black thin arrows) and background luteinized stromal cells lacking staining but with inclusions (arrowheads). Magnification, ×10; bar, 200 µm.

tential stimulation to the ovarian stroma) that accompany ovarian failure. Depletion of ovarian follicles and oocytes has been hypothesized to cause ovarian tumors in animal models of premature ovarian failure.[46] Therefore, because the current study represents the first time that DMBA-induced ovarian neoplasms have been induced in follicle-depleted mice, the number of tumor cases among VCD+DMBA– mice may represent a baseline incidence for ovarian neoplasms in mice with ovarian failure rather than as a direct effect of previous VCD exposure.

Alternatively, increased expression of microsomal epoxide hydrolase mRNA (*Ephx1* or *mEH*) is highly enriched in the residual ovarian tissue of VCD-treated follicle-depleted mice when compared with age-matched cycling controls.[38] The epoxide hydrolase 1 enzyme, together with CYP1A1 and CYP1B1, is involved in the bioactivation pathway of DMBA to its final ovotoxic 3,4-diol-

1,2-epoxide metabolite.[11,31] Although epoxide hydrolase 1 is not the rate-limiting enzyme in this pathway, its increased levels in VCD-treated follicle-depleted ovaries may impart increased susceptibility to toxicity by DMBA. Furthermore, the possibility that VCD acts as an initiating agent that makes ovarian tissue more susceptible to developing neoplasms must also be considered.

Ovarian cancer has been termed a disease of the postmenopausal stage[22] because the incidence increases with age and peaks during the postmenopausal years.[12] Among the risk factors for ovarian cancer are germ cell depletion, increased levels of gonadotropins, and altered levels of estrogens and androgens.[41] These risk factors develop or undergo marked changes during and after the menopausal transition[3,4,41] and occur in VCD-treated mice as they undergo ovarian failure.[24,25,27] One interesting finding from the current study is the presence of invaginations of the ovarian surface epithelium in both ovaries of all VCD-treated follicle-depleted animals (benign tubular adenomas). This phenomenon has been described previously in both humans[40] and animal models with premature ovarian failure.[17,32,34,47] Studies in W^x/W^v (which have altered *Kit* genes[30,37]) and Sl/Sl^t and Sl/Sl^d mice (which have mutant *Kitl* genes[7,17]) show that these mice undergo premature ovarian failure accompanied by changes in OSE morphology and increased stromal invagination[17,32,47] leading to tubular adenomas. In those animals, lack of follicles leads to decreased levels of estrogen and progesterone, resulting in decreased negative feedback on the secretion of luteinizing and follicle-stimulating hormones and causing their levels to rise.[33] High levels of luteinizing hormone can lead to increased stimulation of the stromal compartment, stromal cell hypertrophy, and luteinization. Furthermore, advanced ovarian failure in mice leads to migration of OSE cells into the ovarian stroma and altered epithelial–stromal cell interactions. These changes are thought to result in increased susceptibility to malignant transformation[46] by stimulation of OSE cells by androgens produced from tubular adenomas.[45] Moreover, an increased incidence of OSE pseudostratification, papillomatosis, deep cortical invaginations, epithelial inclusion cysts, and increased stromal activity has been noted in women with a family history of ovarian cancer when compared with women with no history of the disease.[40] Therefore, the observation of benign tubular adenomas in both ovaries of VCD-treated follicle-depleted mice (with or without DMBA treatment) supports the idea that this phenomenon is associated with ovarian failure and that these changes may precede ovarian neoplasia in these mice.

In summary, results of this study support previous reports predicting an increased risk for the development of ovarian neoplasms after ovarian failure. Compared with that in cycling animals, follicle depletion and the resulting altered hormonal milieu may predispose mice to the development of ovarian neoplasms. Although our mice did not develop neoplasms of epithelial origin (the most common ovarian tumors in humans), the marked stratification of OSE cells and invaginations of OSE cells into the stromal compartment (tubular adenomas) present in VCD-treated follicle-depleted mice indicate potential early changes leading to future neoplasms. All tumors observed in this study were Sertoli–Leydig-cell tumors, thus providing a mouse model for postmenopausal sex-cord–stromal tumors of the Sertoli–Leydig type. Therefore, the present study supports a relevant contribution of the VCD-treated mouse model to the study of ovarian cancer as a menopause-related pathology.

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