

## Global Deletion of *Trp53* Reverts Ovarian Tumor Phenotype of the Germ Cell–Deficient White Spotting Variant (Wv) Mice<sup>1</sup>

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### Abstract

White spotting variant (Wv) mice are spontaneous mutants attributed to a point mutation in the *c-Kit* gene, which reduces the tyrosine kinase activity to around 1% and affects the development of melanocytes, mast cells, and germ cells. Homozygous mutant mice are sterile but can live nearly a normal life span. The female Wv mice have a greatly reduced ovarian germ cell and follicle reserve at birth, and the remaining follicles are largely depleted soon after the females reach reproductive stage at around 7 weeks of age. Consequently, ovarian epithelial tumors develop in 100% of Wv females by 3 to 4 months of age. These tumors, called tubular adenomas, are benign but can become invasive in older Wv mice.

We tested if additional genetic mutation(s) could convert the benign ovarian epithelial tumors to malignant tumors by crossing the Wv mutant into the *Trp53* knockout background. Surprisingly, we found that global deletion of *Trp53* suppressed the development of ovarian tubular adenomas in Wv mice. The ovaries of Wv/Wv; *Trp53* (–/–) mice were covered by a single layer of surface epithelium and lacked excessive epithelial proliferation. Rather, the ovaries contained a small number of follicles. The presence of ovarian follicles and granulosa cells, as indicated by *Pgc7* and inhibin- $\alpha$  expression, correlated with the absence of epithelial lesions. A reduction of *Pten* gene dosage, as in Wv/Wv; *Pten* (+/–) mice, produced a similar, though less dramatic, phenotype. We conclude that deletion of *Trp53* prolongs the survival of ovarian follicles in Wv mice and consequently prevents the proliferation of ovarian epithelial cells and development of ovarian tubular adenomas. The results suggest that various cell types within the ovary communicate and mutually modulate, and an intact tissue environment is required to ensure homeostasis of ovarian surface epithelial cells. Especially, the current finding emphasizes the importance of ovarian follicles in suppressing the hyperplastic growth of ovarian epithelial cells, dominating over the loss of p53.

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## Introduction

The white spotting (W) and variant (Wv) mice are spontaneous mutants derived from selective breeding to follow their unique coat color patterning, and several strains were identified to be caused by mutations in the receptor tyrosine kinase c-Kit [1–3]. Inactivating mutations of c-Kit affect most significantly the development of melanocyte, mast cell, and germ cell lineages and have pleiotropic effects on both embryonic development and hematopoiesis [4]. Homozygous W mutation in mice often leads to perinatal death resulting from a severe anemia [4]. A unique variant, Wv, harbors a c-Kit point mutation that reduces the c-Kit tyrosine kinase activity to around 1%, and the developmental phenotypes are less severe [2,3]. The heterozygous Wv mice in the C57BL/6 J(B6) background have dorsal and/or ventral white spots, whereas the homozygous mutant mice have a completely white fur coat [4,5]. The homozygous mutant mice have about 1% to 5% of oocytes/follicles compared with wild type at birth [1,6], and the follicles are depleted by about 2 months of age when the unaffected (wild type or heterozygous) mice become reproductively mature. Depletion of germ cells/ovarian follicles in females associates with elevation of gonadotropins and development of epithelial lesions [5–7]. Both male and female Wv/Wv mice are sterile but have a similar life span as wild type. The homozygous Wv female mice present several physiological aspects of menopause found in women [7,8].

In female mammals, the primordial germ cells are mitotically active during embryogenesis but are stationary following meiosis to produce primary oocytes immediately before birth [9]. Oocytes recruit and become surrounded by granulosa cells to form follicles in late embryogenesis for humans and the immediate neonatal period for mice [9]. Thus, a finite population of oocytes is established at birth, although this paradigm has been challenged and the controversy has not yet been resolved [10–12]. Nevertheless, after birth, the majority of the oocytes in mammals are progressively lost by atresia, and only a fraction of the follicles in the ovarian reserve develop to maturity and are used in ovulation [13,14]. The *Tp53* family proteins play critical roles in the development and attrition of ovarian follicles [15,16]. The impact of *Tp53* in female reproductive function has been recognized in both mice and women, although the main mechanism is thought to affect embryo implantation [17]. Several reports also noted the influence of Trp53 on oocyte development and attrition [16,18]. *Pten* is another tumor suppressor gene known to affect oocyte development and survival [19,20]. For most mammals in their natural environment, the oocyte reserve and thus the reproductive lifetime generally correlate with life span [21]. Women, especially in modern times, are unique in that they experience an extended life span following reproductive senescence due to ovarian follicle depletion, a phenomenon known as menopause. In this aspect, the female Wv mutant mice also have an extended life span following ovarian follicle depletion and thus bear its biological consequences. The reduced germ cell reserve and a normal life span of the mutant mice make the Wv mice a suitable laboratory model of menopause [8].

The germ cell-deficient phenotype of the W series of mice was characterized in the 1950s before the c-Kit mutation was identified in the strains [1]. It was found that germ cells fail to survive and migrate into the gonadal ridge during embryonic development [1]; for females, the outcome is that few ovarian follicles are present in adults [6]. Ovarian tumors arise following the depletion of germ cell/follicles in the ovaries [5,6]. The tumors are classified as tubular adenoma, a benign epithelial neoplasm [6]. A fraction of the ovaries develop

granulosa cell tumors, which are likely promoted by the elevated gonadotropins in the Wv/Wv mice following follicle depletion [6,7].

Although activating mutations or gene amplification of c-Kit receptor tyrosine kinase have been found in some cancer types in humans, c-Kit is not commonly expressed or involved in epithelial ovarian cancer [22,23]. Rather, the formation of ovarian tubular adenomas is attributed to the loss of function of c-Kit in the oocytes and subsequent follicle depletion [5,6].

More recently, the ovarian tumor phenotype of the Wv mice was studied to understand the relationship between reproductive etiological factors such as menopausal biology and increased ovarian cancer risk [5,24]. It was observed that the ovarian surface epithelia proliferate and invaginate to form the tubular adenomas following ovarian follicle depletion [5]. A reduction of cyclooxygenase (Cox)-2 gene dosage in the Wv/Wv mice leads to a reduced formation of the tubular adenomas; however, complete Cox-2 knockout does not, because of a compensatory expression of Cox-1 [5]. Suppression of Cox-1 by either genetic knockout or pharmacologic inhibitors prolongs postnatal ovarian follicle life span and also reduces the development of ovarian tubular adenomas in the Wv mice [25].

The benign tubular adenomas in the Wv mice resemble, in morphology, preneoplastic epithelial changes, such as surface invaginations, inclusion cysts, and papillomatosis, found in aged human ovaries [26,27]. These epithelial changes in ovaries precede cancer development [28], and the increased ovarian epithelial remodeling likely promotes the selection and expansion of cells that have acquired oncogenic mutations such as the inactivation of *Trp53* or activation of Ras. The oncogenic mutation(s) confers growth advantage, and ultimately the further expansion and selection of the mutant cells lead to carcinomas. The ovarian tubular adenomas in the ovary of the Wv mice resemble the low-grade ovarian cancer tumors (type I, low-malignant potential tumors), which usually have a wild-type *Trp53* and KRas or Raf-1 activating mutation [29]. In high-grade serous ovarian cancer, the tumor suppressor gene *TP53* is commonly mutated [29–32], which presumably allows the cell-autonomous survival and growth of ovarian cancer cells.

In the current study, we tested if the addition of an oncogenic mutation, such as *Trp53* deletion or reduction of *Pten* gene dosage, may convert the benign ovarian epithelial tumors into malignant cancer in this epithelial ovarian tumor-bearing mouse model. The results of the experiments were unexpected, and the findings provide further insight to the etiology and mechanism of ovarian cancer, especially related to cell-cell communication within the ovary and the increased ovarian cancer risk found in peri- and postmenopausal women.

## Materials and Methods

### Sources, Genotyping, and Characteristics of Genetically Modified Mice

All of the mouse strains used in the study have been kept in the C57BL/6 J-background. Wv mouse (C57BL/6 J-KitW-v) breeding pairs were originally obtained from Jackson Laboratory in 2002. The Wv mouse colony has been maintained by inbreeding in our mouse facility for the last 12 years. The Wv genotypes of the resulting progeny were identified by coat color: white, gray with dorsal and/or ventral spots, or black/agouti represents Wv/Wv homozygous mutant, Wv/+ heterozygous mutant, or wild type, respectively.

The *Trp53* (+/–) mice were purchased from Taconic (Hudson, NY) [33,34]. Inactivation of the *Trp53* gene in the mutant allele was

achieved by replacing exons 2 to 6, including the start codon, with a neomycin-resistance gene (neo) cassette. The following primer sets were used for polymerase chain reaction (PCR) genotyping that amplifies the wild-type and targeted mutant *Trp53* allele: 5'-TGGTG CTTGG ACAAT GTGTT-3'; 5'-CTCCG TCATG TGCTG TGAAT-3'; 5'-GGATG ATCTG GACGA AGAGC-3'. All three primers were used simultaneously in the PCR, yielding a 450-base pair wild-type and/or 650-base pair *p53* mutant band.

A pair of *Trp53* floxed (*Trp53* [fl/fl]) mice was provided by Dr Denise Connolly (Fox Chase Cancer Center, Philadelphia, PA). The conditional mutant mice contain a functional *Trp53* gene in which exons 2 to 10 are flanked by loxP sites, and the breeding pair was originally obtained from MMRRC (The Mutant Mouse Regional Resource Center, [www.mmrrc.org](http://www.mmrrc.org)) [35,36]. The *Pten* (+/-) mice were a gift from Dr Antony Di Cristofano and were bred and genotyped following the published protocols [37]. The *Pten* allele was inactivated by replacing exons 4 and 5 with a neo cassette [37]. All animal studies were approved first by the Fox Chase Cancer Center and then the University of Miami Institutional Animal Care and Use Committee, and all experimental procedures were performed following NIH guidelines.

### Histology and Immunohistochemistry

Entire ovaries as well as the attached uterine horns from Wv ovarian tumor models were dissected, fixed in 10% formalin, paraffin embedded, and sectioned into 6  $\mu\text{m}$ -thick slices collected on positively charged glass slides. The sections were dewaxed in xylene and hydrated through graded ethanol. Heat-induced antigen retrieval was then carried out in 10-mM sodium citrate (pH 6.0) buffer in a microwave initially set at high-power setting (#10) for 2 minutes, followed by 10 minutes on low-power setting (#2). The endogenous peroxidase activity was blocked by immersing the slides in 3%  $\text{H}_2\text{O}_2$  in methanol for 15 minutes. After a 30-minute incubation in blocking serum, slides were incubated with primary antibodies at 4°C overnight at the following dilutions: rat monoclonal anti-Troma-1/cytokeratin-8 (CK8) (Developmental Studies Hybridoma Bank from The University of Iowa, line SP2/0) at 1:600 and mouse monoclonal anti-inhibin-alpha (Serotec, clone 5070) at 1:200. Alternatively, slides were blocked overnight at 4°C and incubated with goat polyclonal anti-Pgc7 (R & D Systems, Cat#: AF2566) at 1:1000 for 1 to 2 hours at room temperature. After extensive washing, the slides were incubated with horseradish peroxidase-labeled secondary antibodies (BD Pharmingen) at 1:100 dilution for CK8 or anti-rabbit labeled polymer horseradish peroxidase (Dako) for 35 minutes at room temperature. Diaminobenzidine was used as the chromogen for the immunoperoxidase reaction. The slides were counterstained with hematoxylin and mounted in 50:50 xylene/Permount.

### Preparation and Culturing of Mouse Primary Ovarian Epithelial Cells

Primary cultures of mouse ovarian surface epithelial (MOSE) cells were generated using a slightly modified protocol as described previously [38,39]. Briefly, ovaries from 2- to 6-month-old female mice were carefully dissected using sterilized surgical instruments. These ovaries were then washed with PBS and incubated in 0.25% Trypsin-EDTA solutions (Invitrogen) at 37°C for 1 hour. The surface cells were washed off from the ovaries by repeated pipetting through a 1-ml pipette with the tip cut to fit the size of the ovaries, and then the ovaries were removed and discarded. Dissociated MOSE cells

suspended in the medium were collected by a brief centrifugation and cultured on six-well plates previously coated with 0.1% gelatin. MOSE cells were maintained as monolayers in high-glucose DMEM/F12 medium supplemented with 10% fetal bovine serum, 1 mM glutamine, 10 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, 5  $\mu\text{g}/\text{ml}$  insulin, and 5  $\mu\text{g}/\text{ml}$  transferrin. For Wv/Wv ovaries, the epithelial tumor cells were isolated by incubation with DMEM/F12 medium containing 0.5% collagenase (Invitrogen) for 2 hours at 37°C, and the isolated epithelial cells were cultured under the same conditions as wild-type MOSE cells. By analysis of cytokeratin expression using immunofluorescence microscopy, the cell preparations were verified to be more than 90% epithelial cells.

For gene deletion, primary cultures MOSE cells isolated from *Trp53* (f/f) or Wv/Wv; *Trp53* (f/f) mice were treated with replication-deficient adenovirus carrying Cre coding insert (adenoviral Cre [Adv-Cre]) by addition of the viral vector (200 MOI) to the cells. Adenovirus expressing LacZ was used as a control, and LacZ expression indicated that high efficiency of infection (>90%) was achieved at 200 MOI. The viral expression vectors were purchased from the Vector Core at the University of Iowa and were used according to the manufacturer's protocol.

### Cell Growth Assay

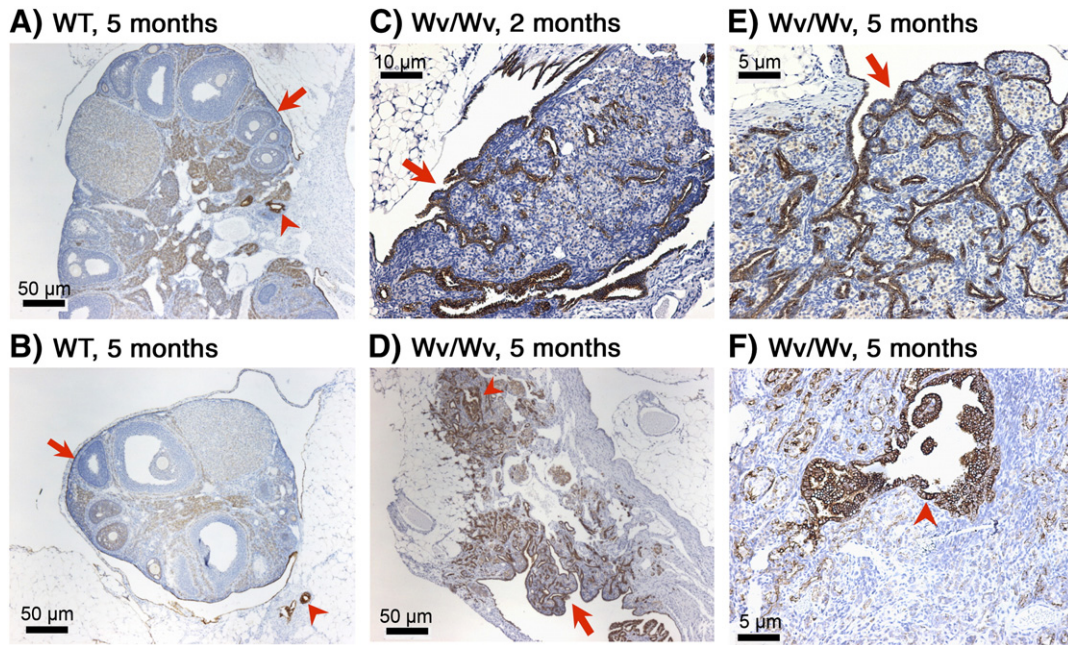
The growth rate of MOSE cells was analyzed using an MTT-like WST-1 cell proliferation kit from Roche. Briefly,  $2 \times 10^4$  MOSE cells of various genotypes were seeded on 96-well plates previously coated with 0.1% gelatin. These cells were allowed to grow for 24 to 96 hours. At each time point (24-hour intervals), the cell culture medium was replaced with freshly prepared reaction buffer composed of DMEM/F12 media containing 10% WST-1 solution. These cells were then incubated for 1 hour at 37°C, and the accumulation of the soluble formazan dye produced by viable cells was measured for absorbance at 450 nm using a standard multiwell spectrophotometer. The cell number correlates with the amount of soluble formazan dye.

**Western Blot and Antibodies.** Cultured MOSE cells were washed with cold PBS and then lysed in SDS gel loading buffer. Lysates were collected and denatured in boiling water bath for 10 minutes. The protein extracts were separated on 8% or 10% SDS polyacrylamide gels, transferred onto nitrocellulose membrane, and subjected to immunoblotting according to standard procedures. The primary antibodies used were: anti-N-cadherin (mouse monoclonal, clone: 32/N-Cadherin, BD Bioscience), anti-E-cadherin (mouse monoclonal, clone: 34/E-Cadherin, BD Bioscience), anti-beta-catenin (mouse monoclonal, clone: 14/Beta-Catenin, BD Bioscience), anti-COX1 (#160105, #160110, Cayman Chemical), anti-COX2 (#160106, Cayman Chemical), anti-claudin-3 (Invitrogen), anti-PCNA (SC56, Santa Cruz), anti-p21 (mouse monoclonal, clone: SXM30, BD Bioscience), anti-phospho-AKT (#9276, Cell Signaling), anti-AKT (#9272, Cell Signaling), anti-phospho-Erk1/2 (mouse monoclonal, clone: E10, Cell Signaling), anti-Erk1/2 (#9102, Cell Signaling), and anti-beta-actin (clone AC-15, Sigma). Chemoluminescence detection was performed using the Super-Signal West Extended Duration Substrate detection kit (Pierce Biotech).

## Results

### Development of Epithelial Lesions and Tubular Adenomas from Surface and Rete Ovarri in the Ovary of Wv Mice

We have maintained a colony of Wv mice in C57BL/6 J background by inbreeding for the last 12 years. Of the more than 300



**Figure 1.** Development of tubular adenomas in Wv/Wv mice. Ovarian morphology was compared between wild-type littermates (A and B) and Wv/Wv (C to F) mice. CK8 (Troma-1) staining was used to highlight epithelial cells. (A, B) Two examples are shown of ovaries from wild-type controls at 5 months of age. Arrows indicate a layer of CK8-positive surface epithelium. Arrowheads indicate the presence of CK8-positive epithelial cells of rete ovarii in the hilus (A) or mesosalpinx (B) areas. (C) Representative examples of ovaries from Wv/Wv mice at the age of 2 months and (D) 5 months. The slides shown are selected from section near the middle of the tissues. An arrow in (C) indicates that the CK8-positive tubular adenoma lesion is contiguous with the ovarian surface epithelium. An arrow in (D) shows peripheral lesions that are likely derived from the surface, and an arrowhead indicates distinct lesions that are likely derived from rete ovarii, which are shown at a higher magnification in (E) and (F), respectively. A detailed time course of the development of the ovarian tubular adenomas has been previously published [5].

mature (3 months or older) Wv/Wv females examined, all exhibited ovarian tubular adenomas, whereas none were observed in the wild-type littermates.

The representative ovarian tumor morphology is shown in Figure 1. At 5 months of age, the wild-type ovary displayed a complex structure composed of various cell types. A single layer of Troma-1/CK8-positive ovarian surface epithelial cells enclosed the ovarian cortex containing follicles at all different stages of development (Figure 1, A and B, arrow). In addition to the ovarian surface, CK8-positive epithelia of rete ovarii were found in the hilus region (Figure 1A, arrowhead) or the mesosalpinx areas (Figure 1B, arrowhead). In the Wv/Wv ovary starting at around 2 months of age, the tissue was replaced with tubular adenomas, shown as CK8-positive epithelial layers mixed with stroma (Figure 1C). At this earlier stage, the epithelial lesions were contiguous with the surface epithelium (Figure 1C, arrow), suggesting that the infolding and invagination of the surface epithelia gave rise to the benign tumor. The tubular adenomas became enlarged for both epithelial and stromal components and appeared more invasive (spreading into stromal area) at later ages, as shown by an example from a 5-month-old Wv/Wv female (Figure 1D).

Previously, we observed that, in cases of early ovarian lesions in younger (7 to 10 weeks) Wv/Wv mice, the tubular structure is contiguous with the monolayer of the surface epithelium, indicating that the tubular epithelial lesions originate from the surface epithelium [5]. Closer observation showed that the epithelial lesions appeared to be of two different types (Figure 1D). The main tubular

adenomas have flat epithelial layers and distribute throughout the ovarian stroma (Figure 1, D and E, arrow). These lesions resemble surface deep invaginations/papillomatosis and likely originate from the surface epithelium. Another distinctive type of CK8-positive lesion was found often around the stem or hilus ligament area (Figure 1, D and F, arrowhead). The cells are columnar-to-stratified epithelial in appearance and often locate deep within the tissues. This second type of lesion likely is derived from rete ovarii because rete ovarii structure and cysts are often observed in the area in the wild-type or younger Wv/Wv ovaries.

Although dysplastic morphology is evident in some epithelial components of the tubular adenomas, the ovarian epithelial tubular structures in the Wv mice are considered nonmalignant tumors, and the lesions are confined to ovarian tissues and do not become metastatic. Histopathological evaluation also indicated their low-grade cytological morphologies without evident mitotic figures.

The oviducts in Wv/Wv mice were not affected because we consistently observed normal morphology of the ducts at all ages despite the persistent presence of ovarian tubular adenomas in the associated ovaries.

#### *Suppression of Ovarian Tubular Adenomas in Wv Mice upon Trp53 Deletion*

We attempted to convert the benign ovarian tubular adenomas into more neoplastic tumors by adding additional oncogenic mutations into the Wv model. Because TP53 inactivation is common in high-grade serous ovarian cancer, we first crossed and obtained

female Wv/Wv; *Trp53* (-/-) mice to analyze for possible ovarian tumor phenotypes.

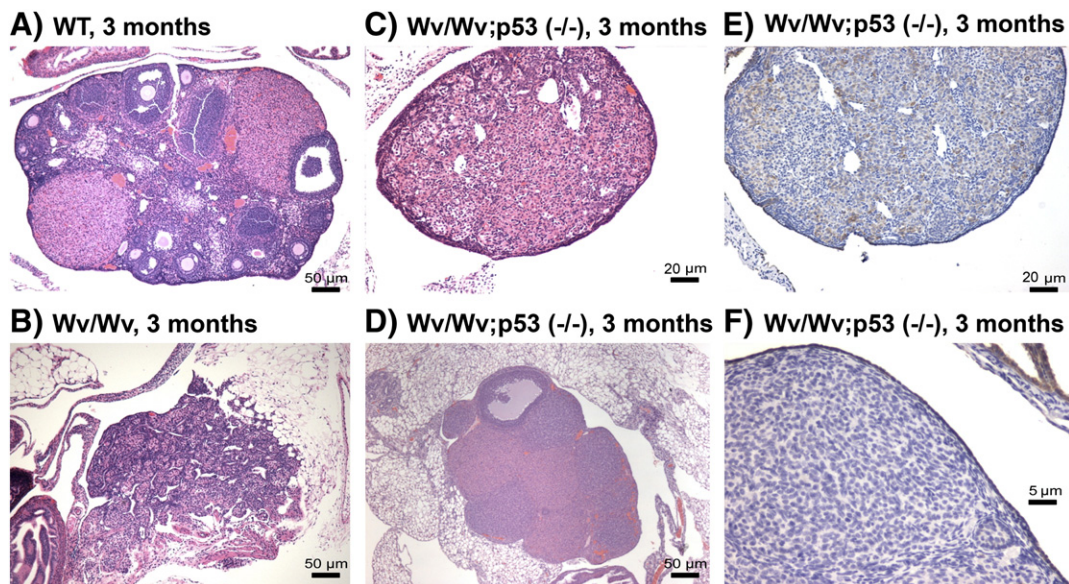
Our experience in breeding *Trp53* mutant mice was similar to that reported [33]. We observed a reduction of the expected ratio of *Trp53* (-/-) pups from matings between parents of *Trp53* (+/-) genotype to approximately 55% of the expected number. Also, a sex-biased ratio of 5:3 for male-to-female pups was observed. The genotype distribution among progenies was also similar after crossing into the Wv heterozygous background. Because Wv/Wv are sterile and *Trp53* (-/-) are poor breeders, we initiated breeding of Wv/+; *Trp53* (+/-) parents and kept any Wv/Wv; *Trp53* (-/-) female progenies for further analysis. In tabulating the breeding scheme over a 6-year period, of about 1000 progenies genotyped, we only identified 9 females and 14 males of Wv/Wv; *Trp53* (-/-) genotype. Thus, the Wv/Wv; *Trp53* (-/-) genotypes exhibit an increased embryonic lethality, with a 37% survival rate based on the expected Mendelian ratio. The female Wv/Wv; *Trp53* (-/-) mice needed for analysis were even more difficult to generate + because of sex-biased embryonic lethality, and only 29% of the expected number was produced.

The ovaries from mice with the *Trp53* (-/-) mutation alone were not noticeably different in appearance and histology compared with those of wild type, as reported previously [40]. We found that crossing Wv mice into mutant p53 did not lead to the development of large ovarian tumors or a malignant tumor phenotype. Instead, surprisingly, the addition of the p53 mutation/deletion suppressed the formation of tubular adenomas in the Wv/Wv mice. The mice were analyzed before reaching 6 months of age because p53 null mice often develop sarcomas and lymphomas after this age [34]. In the 18 ovaries collected from all 9 Wv/Wv; *Trp53* (-/-) females at 2 to 6 months of age, we observed an intact ovarian structure without the presence of tumor. Compared with wild-type ovaries that contained follicles of a range of developmental stages (Figure 2A) or to Wv/Wv

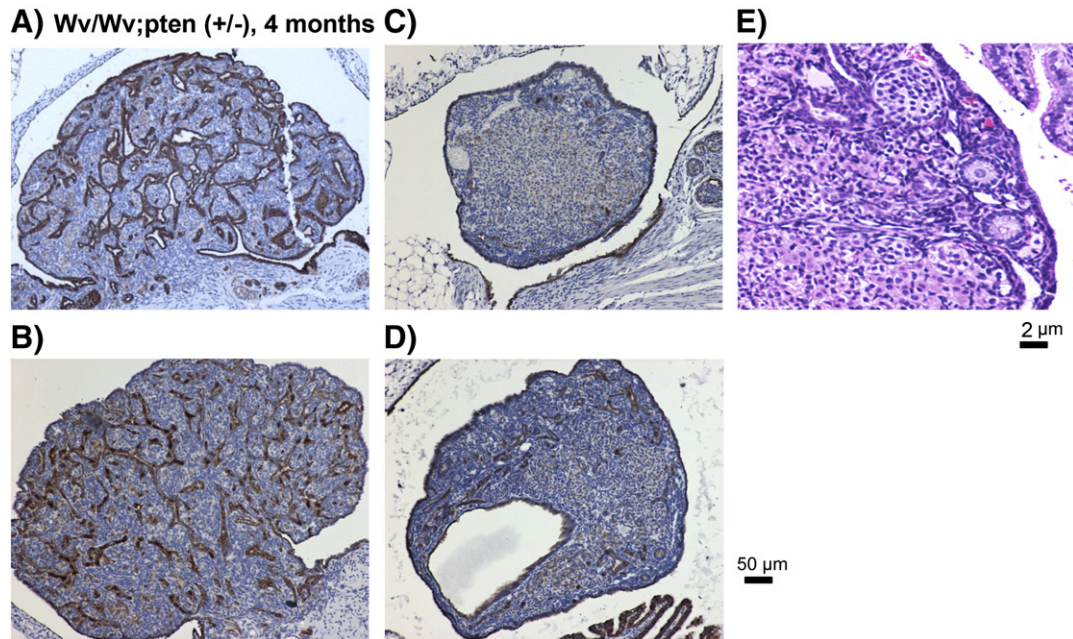
ovaries that were entirely permeated with tubular adenomas (Figure 2B), the Wv/Wv; *Trp53* (-/-) ovaries included no tumor structure, exhibited a more homogeneous cortex, and contained occasional follicles (Figure 2, C and D). Here, two examples with distinctive morphologies are shown. The ovary in (Figure 2D) has a few follicles yet contains corpora lutea-like structures. We speculate that the ovary was harvested immediately following gonadotropin stimulation and ovulation. Staining with CK8 shows that the representative ovary has a smooth layer of epithelium on the surface of the ovaries and no epithelium locates within the ovary (Figure 2, E and F).

#### Subtle Influence on Wv/Wv Ovarian Tumor Phenotypes with Reduction of Pten Gene Dosage

We also undertook another cross to analyze ovaries from Wv/Wv; *Pten* (+/-) mice with a goal to determine if a reduction of *Pten* tumor suppressor gene might increase the malignant features of the tubular adenomas in the Wv/Wv mice. *Pten* is often mutated or its expression is lost in ovarian cancer, though most frequently in the endometrioid subtype [41]. Homozygous *Pten* deletion causes early embryonic lethality in mice; however, heterozygous *Pten* mutant mice are predisposed to develop various epithelial tumors, most often the endometrial type [37,42,43]. We produced and analyzed 10 Wv/Wv; *Pten* (+/-) female mice at ages ranging from 3 to 12 months. The addition of *Pten* mutation into Wv/Wv mice did not significantly alter the tumor phenotype in most of the cases (Figure 3); rather, it reduced the presence of epithelial tumor lesions in about one third of the ovaries (6 out of 20 ovaries analyzed), especially at a younger age (3 to 4 months). Four representative ovaries from the Wv/Wv; *Pten* (+/-) mice are shown (Figure 3); two represent subtle changes (Figure 3, A and B), and the other two represent a reduction in tumor lesions (Figure 3, C and D). Upon close inspection, we observed the presence of follicles in the Wv/Wv; *Pten* (+/-) ovaries that contain no tubular epithelial lesions (Figure 3E). Consistently, the presence of



**Figure 2.** Suppression of tubular adenomas in Wv/Wv; *Trp53* (-/-) ovaries. H&E staining of ovaries from 3-month-old wild type (A), Wv/Wv (B), and Wv/Wv; *Trp53* (-/-) (C, D) are shown. An immunostaining of the epithelial cell marker CK8 of the ovary in (C) is shown in (E) from a 3-month-old Wv/Wv; *Trp53* (-/-) mouse. A higher magnification of the ovary in (E) is shown in (F). The images shown were selected from a section near the middle of the tissue. The staining shows a monolayer of CK8-positive epithelial cells enveloping a representative Wv/Wv; *Trp53* (-/-) ovary (C), and no tumor or epithelial lesions were observed in the ovarian cortex.



**Figure 3.** Ovarian morphological features of *Wv/Wv; Pten (+/-)* mutant mice. Representative CK8 staining (of epithelial cells) of four examples of ovaries from four 4-month-old *Wv/Wv; Pten (+/-)* mice is shown. Two ovaries (A, B) are similar to those of *Wv/Wv* mice, and the other two ovaries shown (C, D) exhibit a reduced tumor lesion. An example of H&E staining of the ovary with reduced tumor lesion is shown at higher magnification to visualize the presence of follicle structures (E).

follicles correlated with the absence of tubular adenomas in all the *Wv/Wv; Pten (+/-)* ovaries, and no follicle structures were ever observed in areas containing tubular epithelial structures.

Thus, again in contrast to our expectation, a reduction of the *Pten* gene dosage in the *Wv* mice only had subtle effects on the development of tubular adenomas. Rather, reduced *Pten* prevented/delayed the development of tubular adenomas in *Wv/Wv* mice, although the effect was not as dramatic as in the case of *Trp53* deletion because only 6 out of 20 ovaries showed a reduced epithelial tubular adenoma lesions compared with 18 out of 18 ovaries from the *Wv/Wv; Trp53 (-/-)* mice.

#### ***NO Increased Cell Proliferation in the Ovaries of Wv/Wv; Trp53 (-/-) Mice***

Suppression rather than promotion of tumor development by *Trp53* deletion or *Pten* gene dosage reduction was unexpected. Thus, we examined by immunohistochemistry the effect of *Trp53* deletion on cell proliferation in the ovaries (Figure 4A).

We used Ki67 staining as an indication of cell proliferation. Because *Trp53* protein is difficult to be assayed in normal tissues because of its low abundance, we used the cyclin inhibitor p21, a *Trp53*-transcriptional target, as a reporter of *Trp53* activity. In wild-type ovaries, Ki67-positive cells were limited to a fraction of the granulosa cells (Figure 4A, left panel, arrow), and most of the cell populations, including surface epithelial and granulosa cells and oocytes, were positive for nuclear p21 (Figure 4A, left panel). In the *Wv/Wv* ovaries, Ki67-positive proliferating cells were abundant in both epithelial and stromal compartments (Figure 4A, middle panel, arrows). The tumor also contained widespread p21-positive cells, which consisted about half of the cell population (Figure 4A, middle panel). Interestingly, few cells in the *Wv/Wv; Trp53 (-/-)* ovary were positive for Ki67, although the ovary had a much reduced p21

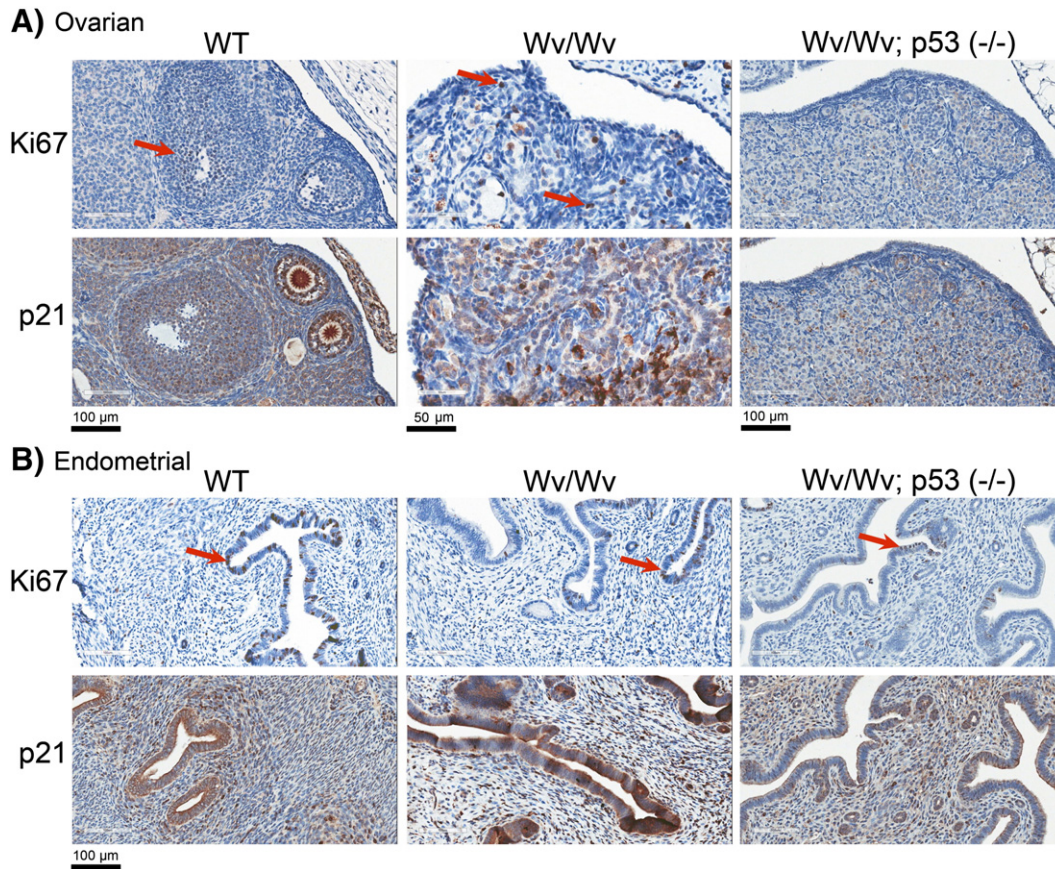
staining, consistent with the loss of p53 activity (Figure 4A, right panel).

The lack of cell proliferation did not apply to the endometrial tissues of the *Wv/Wv; Trp53 (-/-)* mice (Figure 4B). The uterus of the *Wv/Wv; Trp53 (-/-)* mice showed a similar extent of Ki67-staining compared with those from wild-type or *Wv/Wv* mice (Figure 4B, arrows). p21 staining of the endometrial epithelial cells of *Wv/Wv; Trp53 (-/-)* was slightly reduced compared with those of wild type or *Wv/Wv*, although the staining appeared largely cytoplasmic. The stromal cells showed similar nuclear staining of p21, suggesting that p21 expression in these cells was not regulated by *Trp53*. Thus, we conclude that *Trp53* deletion suppresses proliferation of all cell types in the *Wv/Wv; Trp53 (-/-)* ovaries but does not significantly affect uterine cells in the same mice.

#### ***Increased Growth of Ovarian Surface Epithelial Cells in Cultures upon Trp53 Deletion***

Next, we examined if p53 deletion alters ovarian surface epithelial cell survival and proliferation in culture to determine the possibility that the effect of *Trp53* deletion is non-cell autonomous.

We observed that newly isolated epithelial cells from *Wv/Wv* ovaries had a greater growth rate than ovarian surface epithelial cells isolated from wild-type controls at early passages (Figure 5A). Nevertheless, the growth rate of the *Wv/Wv* epithelial cells was reduced to the same level as that of the wild type at a later time, after being maintained for four passages, or about 2 weeks, in culture (Figure 5B). Western blot analysis indicated that, in early passages, the cells had similar levels of N-cadherin, E-cadherin, and beta-catenin, but the *Wv/Wv* epithelial cells had increased COX1, COX2, and claudin-3 expression (Figure 5C). In these cases, the ovarian surface epithelial cells from both the control (wild type) and *Wv/Wv* mice were kept in a background of *Trp53 (fl/fl)*. The addition of



**Figure 4.** Proliferation and p21 expression in ovarian and endometrial tissues. Ovaries (A) and associated uteri (B) were harvested from 3-month-old wild-type (WT), Wv/Wv, and Wv/Wv; *Trp53* (-/-) mice and used for immunostaining with Ki67 as a proliferative index and with p21 as a reporter of Trp53 activity. Adjacent sections were used for p21 and Ki67 stainings, and both the uterine horns and associated ovaries were analyzed together on same slides. Representative images are shown. Arrows indicate examples of Ki67-positive cells.

*Trp53* (f/f) made no observable difference in either wild-type or Wv/Wv mice or cells, but it allowed for *Trp53* gene deletion upon introducing the expression of Cre. Both Adv-LacZ and reverse transcription PCR of *Trp53* alleles indicated high efficiency of infection and gene deletion in the cultured cells. Adv-Cre-mediated *Trp53* gene deletion enhanced the growth rates of both Wv/Wv and wild-type ovarian epithelial cells (Figure 5, D and E). The enhanced growth of ovarian surface epithelial cells in culture upon *Trp53* deletion has been previously reported [39,44]. The cells were further analyzed by Western blot for cell cycle markers and activation of signaling pathways (Figure 5F). Proliferating cell nuclear antigen protein levels, an indicator of DNA replication and cell proliferation, were consistent with results obtained from the WST-1 cell growth assay (Figure 5, A, B, D, E, and F). The loss of p21, a *Trp53* transcription target, indicated the complete inactivation of *Trp53* following the addition of Adv-Cre (Figure 5F). However, *Trp53* deletion did not significantly alter Akt or Erk1/2 activation, in either wild-type or Wv/Wv cells, although the level of phospho-Erk1/2 was slightly lower in Wv/Wv than the wild-type cells (Figure 5F).

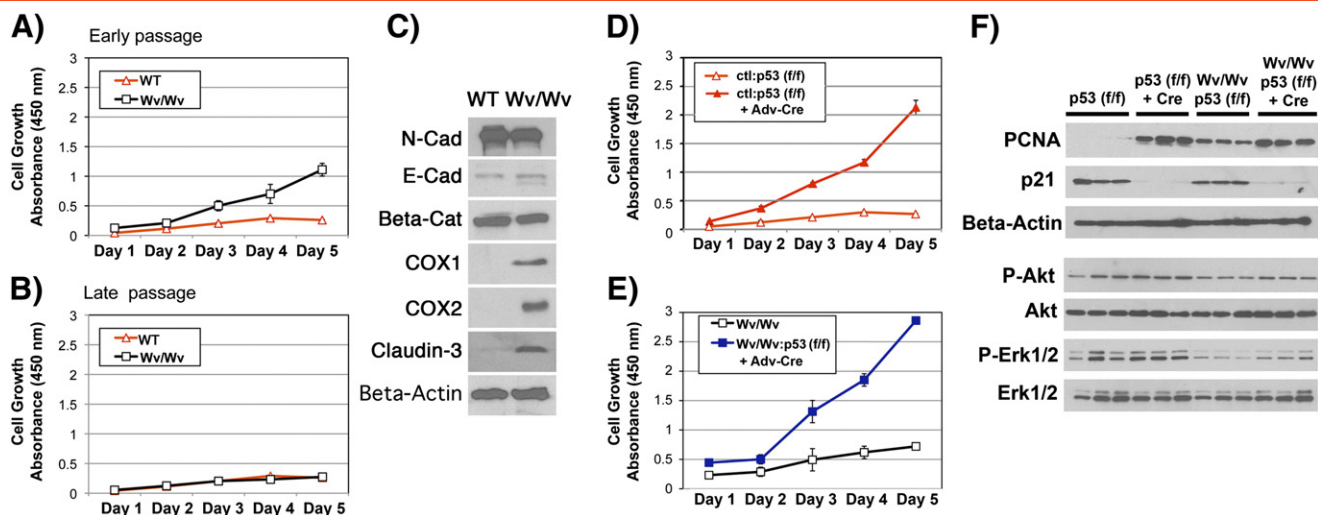
Thus, *Trp53* deletion itself does not impair or slow epithelial proliferation in the Wv/Wv ovarian surface epithelial cells. The lack of epithelial proliferation in the Wv/Wv; *Trp53* (-/-) ovaries likely was caused by a cell nonautonomous mechanism, such as growth suppression imposed by stromal cells. We reason that epithelial

proliferation was then restored in cultures, which were free of growth suppression imposed by the ovarian stroma.

#### *Prolonged Ovarian Follicle Life Span in Wv Mice with Trp53 Deletion*

A clue to the mechanism underlying the unexpected finding that addition of oncogenic mutation in Wv/Wv mice suppressed rather than increased epithelial tumor development was the observation that follicle structures were present in the tumor-free ovaries. Similar to those of Wv/Wv; *Pten* (+/-), in the Wv/Wv; *Trp53* (-/-) ovaries, a number of follicle structures consisting of a circle of granulosa cells surrounding an oocyte could be seen within the cortex (Figure 6). Occasionally, mature follicles were also observed (such as in Figure 2D). Immunostaining of PGC7, a germ cell and oocyte antigen, confirmed the presence of ovarian follicles (Figure 6).

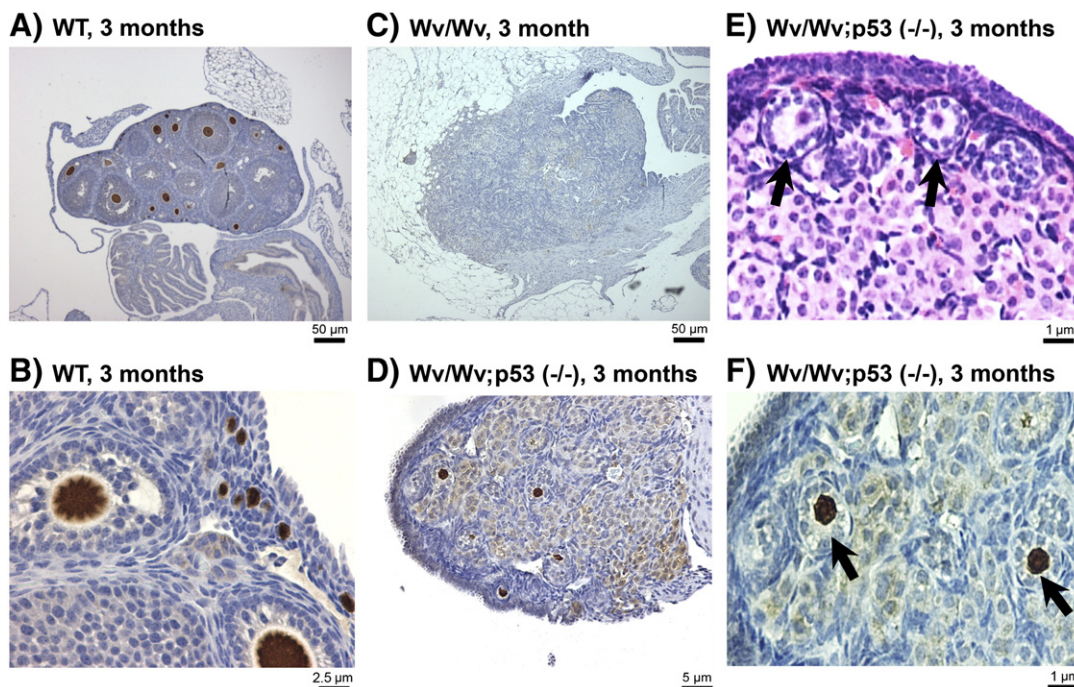
In the reproductively competent wild types of around 3 months of age, the ovaries contained a large number (hundreds) of follicles at various developmental stages, as revealed by PGC7 staining (Figure 6A). Seen at a higher magnification, the primary follicles were found located immediately underneath the surface epithelium (Figure 6B). However, no ovarian follicles, at germ cell, primary, or mature stage, were observed in the ovaries of Wv/Wv mice, which were occupied by tubular adenomas (Figure 6C). In the Wv/Wv; p53 (-/-) ovaries, PGC7 staining confirmed that the structures indeed



**Figure 5.** Proliferation and signaling of primary mouse ovarian surface epithelial cells in cultures. Primary ovarian surface epithelial cells were prepared from ovaries of mice of various genotypes. A total of six ovaries from three mice in each group were used to prepare the epithelial cells of wild-type ovarian surface or Wv/Wv ovarian tubular adenomas. The cells were pooled for experiments. The growth rates were measured by WST-1 assay ( $n = 3$ ) from day 1 to 5. (A) Freshly prepared wild-type control (WT) and Wv/Wv ovarian epithelial cells were compared. (B) Growth of WT and Wv/Wv ovarian epithelial cells was maintained in cultures for four passages or around 2 weeks. The growth rate was then determined by WST-1 assay. (C) Lysates prepared from the cells were analyzed by Western blot for N-cadherin (N-Cad), E-cadherin (E-Cad), beta-catenin (Beta-Cat), COX1, COX2, claudin-3, and beta-actin. (D, E) Ovarian surface epithelial cells were prepared from p53 (f/f) and Wv/Wv; *Trp53* (f/f) mice. The cells in primary cultures were treated with either Adv-Cre or Adv-LacZ (ctl), and the growth rate was measured. (F) Lysates were prepared from the cells following treatment with Adv-Cre or Adv-LacZ and were analyzed by Western blot. Three independent cell preparations per group were analyzed.

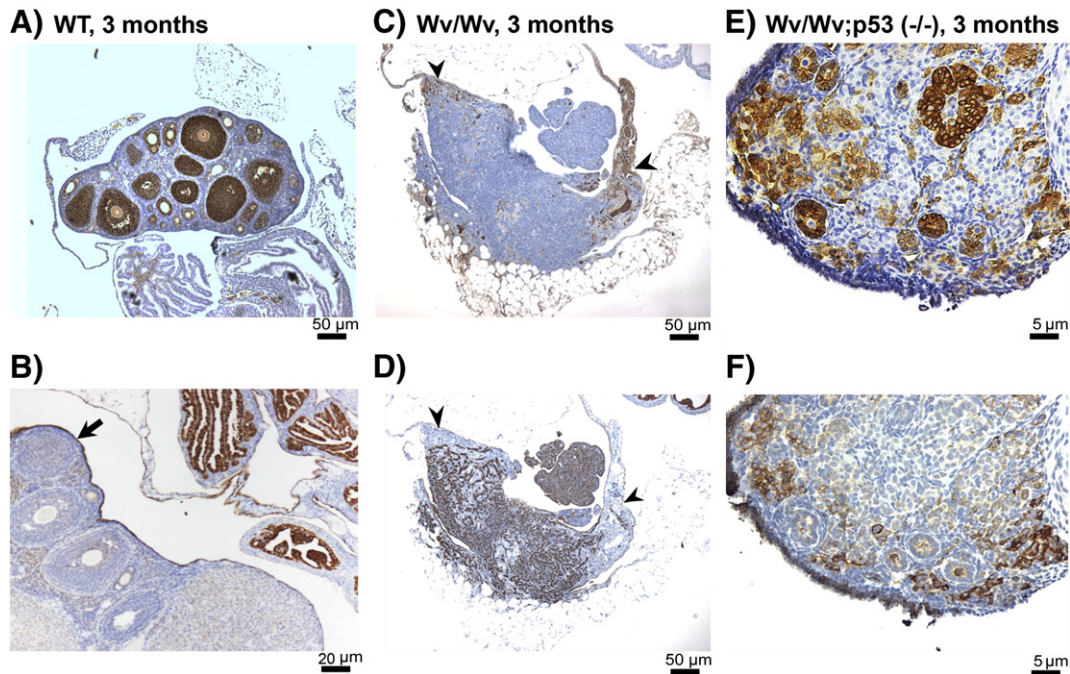
contained PGC7-positive oocytes, as shown by a representative image at or near the largest cross section of an ovary (Figure 6D). Similar to those of wild type, primary follicles appeared to align beneath the

surface epithelium of the Wv/Wv; *Trp53* ( $-/-$ ) ovaries, as seen by hematoxylin and eosin (H&E) (Figure 6E, arrows) or PGC7 (Figure 6F, arrows) staining shown at a high magnification. From



**Figure 6.** Rescue of germ cells/follicles in Wv/Wv; *Trp53* ( $-/-$ ) ovaries. Immunostaining of the germ cell marker PGC7 was performed on ovaries of various genotypes to identify germ cells and follicles. (A) Three-month-old wild type and (B) at a higher magnification. (C) Three-month-old Wv/Wv. (D) Three-month-old Wv/Wv; *Trp53* ( $-/-$ ). (E) An example of the Wv/Wv; *Trp53* ( $-/-$ ) ovaries is shown at a higher magnification to exemplify the follicle structures (arrow). (F) An example of PGC7 immunostaining of the Wv/Wv; *Trp53* ( $-/-$ ) ovary is shown in higher magnification. Arrows indicate examples of primary follicles.





**Figure 7.** Inverse correlation between the presence of granulosa cells and epithelial lesions in *Wv/Wv* ovaries. Representative examples of tissue sections stained with the granulosa cell marker inhibin- $\alpha$  (A, C, E) and epithelial marker CK8 (B, D, F) in adjacent sections of ovarian tissues from 3-month-old wild-type (A, B), *Wv/Wv* (C, D), and *Wv/Wv; Trp53 (-/-)* (E, F) mice. An arrow indicates the single layer of CK8-positive ovarian surface epithelial cells (B). To illustrate the inverse correlation between the presence of granulosa cells and epithelial cells, arrowheads indicate that, in an area which is positive for inhibin- $\alpha$  (C), CK8 staining is absent in the adjacent section (D).

tabulation of the ovaries analyzed, the largest cross section of *Wv/Wv; Trp53 (-/-)* ovaries had 12  $\pm$  5 PGC7-positive follicles compared with 300  $\pm$  56 identified in a section of control ovaries. In contrast, no ovarian follicles were detected in more than 200 *Wv/Wv* mice analyzed, which is consistent with another recent study from our laboratory reporting the time course of ovarian follicle depletion in the mutant mice [25].

We also confirmed that the tumor-free *Wv/Wv; Pten (+/-)* ovaries contained follicles (5  $\pm$  3 in a section), though to a lesser degree than those of *Wv/Wv; Trp53 (-/-)*. No follicles were observed in *Wv/Wv; Pten (+/-)* ovaries that showed adenoma structures.

Thus, preservation of ovarian follicles may be responsible for a reduced ovarian tumor phenotype in the *Wv/Wv* mice with *Trp53* knockout or *Pten* heterozygous deletion. The finding supports the hypothesis of follicle depletion as the cause of ovarian epithelial proliferation and formation of tubular adenomas [24].

#### *Inversed Correlation between Inhibin- $\alpha$ Expression and the Presence of Epithelial Tubular Adenoma in the *Wv* Ovaries*

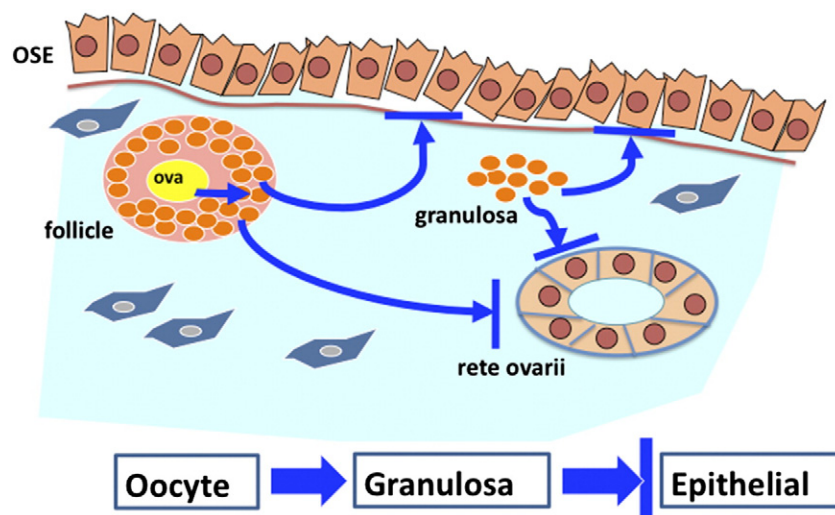
We further analyzed the ovarian follicles by staining granulosa cells for inhibin- $\alpha$ . In 3-month-old wild types, the ovaries contained follicles at various stages throughout the cortex, and a number of large and mature follicles were very prominent. These follicles contain multiple layers of the inhibin- $\alpha$ -positive granulosa cells (Figure 7A). Small primary follicles with only a single layer of inhibin- $\alpha$ -positive granulosa cells located mostly at the outer cortex, aligned along and immediately beneath the surface epithelium (Figures 6B and 7A), which showed as a single-layered CK8-positive cells (Figure 7B, arrow). In the follicle-depleted tubular adenomas of *Wv/Wv* ovaries, inhibin- $\alpha$ -positive granulosa cells were greatly

reduced (Figure 7C). However, small patches of unorganized inhibin- $\alpha$ -positive granulosa cells, which were remnants of degenerated follicles, could be observed (Figure 7C, arrowheads). We recognized a strict inverse correlation between the presence of inhibin- $\alpha$ -positive granulosa cells and CK8-positive tubular adenomas at the same location within an ovarian tubular adenoma (Figure 7, C and D, arrowheads). The inverse correlation was confirmed in all 12 cases of *Wv/Wv* ovaries analyzed for CK-8 and inhibin- $\alpha$ : where granulosa cells were present, tubular adenomas were absent. The ovaries from *Wv/Wv; Trp53 (-/-)* mice in which oocytes and follicles were preserved also had an increased presence of granulosa cells (Figure 7E) and suppression of epithelial tubular adenomas (Figure 7F).

Thus, the results suggest that ovarian epithelial cells and granulosa cells are mutually exclusive.

#### Discussion

Although the p53 gene is the most prominent tumor suppressor, we found in the specific situation of the ovarian tubular adenomas from the *Wv* mice that deletion of *Trp53* actually suppressed the tumor phenotype. The germ cell-deficient *Wv/Wv* mice develop a benign epithelial ovarian tumor, and the initial goal of our study was to convert the benign tumors into malignant tumors by adding an oncogenic mutation. Instead, we observed that deletion of *Trp53* suppressed the ovarian tumor phenotype in the *Wv/Wv* mice. Although initially perplexed by the results, we observed and realized that the absence of p53 allows the prolonged survival of ovarian germ cells and oocytes. Subsequently, the presence of follicles is critical in maintaining the homeostasis of ovarian surface and rete ovarii epithelia and is essential in preserving the structural arrangement of the multiple cell types within an ovary (Figure 8).



**Figure 8.** Proposed mechanism for the suppression of epithelial proliferation by follicles in ovaries. A cartoon illustrates the interactions between cell types within the ovary in maintaining tissue homeostasis. In the premenopausal stage, oocytes (ova) stimulate the proliferation of granulosa cells and the maturation of the follicle structures, which suppress proliferation of both ovarian surface (OSE) and rete ovarii epithelial cells. Based on the inverse correlation, we also speculate that granulosa cells from the remnants of degenerated follicles can also suppress ovarian epithelial proliferation. The model suggests that, in the *Wv/Wv* ovaries, epithelial proliferation to form tubular adenomas results from the depletion of ovarian follicles and associated granulosa cells and consequently the loss of growth suppression imposed by a paracrine factor(s) produced by granulosa cells. Furthermore, *Trp53* deletion rescues oocytes in the *Wv/Wv* ovaries. Consequently, the oocytes stimulate granulosa cell growth, and the increased presence of granulosa cells suppresses epithelial proliferation and the development of ovarian tubular adenomas.

This finding suggests that *Trp53* is critical in controlling the survival and life span of ovarian germ cells and follicles in the *Wv* mice and that the depletion of follicles is a key causal factor for the ovarian tubular tumor phenotype. *Trp53* is known to regulate prenatal oogenesis and oocyte attrition [16] and acts in chemical-induced follicle loss [45]. *Trp53* deletion in the *Wv/Wv* mice presumably reduces oocyte apoptosis and prolongs follicle survival, and oocytes then recruit and stimulate granulosa cells. Alternatively, *Trp53* is also directly important for controlling the growth and survival of granulosa cells [46]. The impact of *Trp53* activity on life span of ovarian follicles observed in the current study adds to the recognized roles of p53 and its family members in female reproduction [15–17,47].

The tumor suppressor function of *Trp53* is generally executed through its cell autonomous regulation of gene expression of a large collection of effectors in both cell growth arrest and programmed cell death [48], and the tumor suppressor function and mechanisms have been well demonstrated in mouse models [49]. In the current study, as observed in culture, *Trp53* deletion enhances proliferation of ovarian surface epithelial cells, which is in agreement with previous reports [36,44]. However, when globally absent in tissues, *Trp53* deletion seems to suppress proliferation of ovarian epithelial cells and reverts the tumor phenotype in the *Wv* mice. We attribute the observation to a non-cell autonomous effect, that the presence of ovarian follicles due to preservation following the loss of *Trp53* in oocytes then suppresses growth of the epithelial cells, despite the simultaneous loss of *Trp53* in the epithelial compartment. Indeed, in a preliminary study, we also found that restricted *Trp53* deletion in the ovarian surface epithelial cells can greatly enhance tumor growth in the *Wv/Wv* mice. These findings will be completed and reported in a separate manuscript.

*Pten* is known to have an active role in controlling ovarian oocyte and follicle activation [19,20], which may explain our finding that a reduction of *Pten* gene dosage also lessened ovarian follicle depletion and tumor development in the *Wv/Wv*; *Pten* (+/–) mice. *Pten* deletion in oocytes, or elimination of the downstream transcription factor Foxo3a, also causes stage-specific follicular activation and subsequent follicle depletion; however, no ovarian tubular adenomas have been reported [19,20,50,51]. One possibility is that there are residual follicles in the Foxo3a and conditional *pten* mutant mice compared with the early and complete deletion in the *Wv* mice. Additionally, another possibility is that residual granulosa cells may be present following the degeneration of the large number of prematurely activated follicles, and the granulosa cells prevent epithelial proliferation to form tubular adenomas in the Foxo3a or *pten* mutant mice.

The idea that an intact ovarian tissue structure is required for cell homeostasis has been previously proposed [24,52]. One recognized mechanism for the presence of ovarian follicles in suppressing ovarian epithelial growth is the regulatory cycle of the ovarian-pituitary axis [53]. Hormones, activin, and inhibin produced from oocyte-associated granulosa cells suppress gonadotropins released from the pituitary [53]. Upon follicle depletion, the increased gonadotropins then stimulate epithelial proliferation, as proposed in the gonadotropin stimulation hypothesis [54–58]. However, transgenic mice overexpressing follicle-stimulating hormone and luteinizing hormone do not develop ovarian epithelial tumors [59,60], and systemic gonadotropin stimulation is not sufficient. The inverse relationship between the inhibin- $\alpha$ -positive granulosa and the CK8-positive epithelial cells in the same tissues, observed in the current study, suggests that granulosa cells may be part of a negative paracrine signal to suppress ovarian epithelial proliferation. A mutually exclusive

expression of inhibin- $\alpha$  and cytokeratin has been observed previously in mouse ovarian tumors induced by SV40 T-antigen (FvB/n-Tg(Amhr2-SV40TAg)1Bcv) [61]. Thus, the suppressive activity of granulosa cells, within either intact follicles or clusters from follicle remnants following oocyte degeneration, may be common and may not be limited to tubular adenomas in the Wv/Wv ovaries. Possibly, follicle depletion (and thus granulosa cell degeneration) causes ovarian epithelial outgrowth by both increased systemic gonadotropin stimulation and the loss of a local suppressing signal from granulosa cells.

Another study also reported that additional deletion of *Trp53* suppresses ovarian tumors in *Pten*-deficient and *Kras* mutant mice [49]. The authors attributed their unusual finding as a new paradigm, that wild-type *Trp53* can promote survival and differentiation of the *Pten* ( $-/-$ ); *Kras* mutant tumor cells [49,62,63]. From the images shown in the articles, it appears that *Trp53* deletion also restored ovarian follicles in the ovaries. However, the studies did not report or discuss the analysis of ovarian follicle status or consider a non-cell autonomous mechanism of *Trp53* action.

Based on these observations and analyses, we propose a model for the regulation of homeostasis of the ovarian cell types (Figure 8). It is established that oocytes provide stimulatory signal for proliferation of granulosa cells [9,64–66]. We propose that granulosa cells, either associated with intact follicles or as remnants of degenerated follicles, produce a paracrine factor that suppress proliferation of epithelial cells either at the surface or in rete ovarii. The obvious possibility is that the granulosa cell factor may be inhibin- $\alpha$ ; however, inhibin- $\alpha$  knockout does not stimulate epithelial proliferation in ovaries [67,68]. Transforming growth factor- $\beta$ , an epithelial growth suppressor, may be a good candidate, and identification of such factor will be critical to verify the current model. The model suggests that, in the Wv/Wv ovaries, depletion of ovarian follicles leads to the reduced presence of granulosa cells and consequently the absence of the paracrine factor to restrain the growth of both surface and rete ovarii epithelia and the formation of tubular adenomas.

Our finding emphasizes the importance of ovarian follicles in suppressing the hyperplastic growth and transformation of ovarian epithelial cells, dominating over the cell autonomous activity due to loss of *Trp53*. Perhaps, the follicle depletion hypothesis [24,69], comprising both the incessant ovulation hypothesis [70,71] and the gonadotropin stimulation theory [54–58], is most suitable to explain the epidemiological observation that ovarian cancer risk is associated with menopause (the depletion of ovarian follicle reserve) following incessant ovulation and menopausal-increased gonadotropins when the follicle reserve is depleted.

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