Conditional Inactivation of Brca1, p53 and Rb in Mouse Ovaries Results in the Development of Leiomyosarcomas

Katherine V. Clark-Knowles¹, Mary K. Senterman², Olga Collins³, Barbara C. Vanderhyden¹,⁴

¹Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada, ²Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa, Ontario, Canada, ³Department of Obstetrics and Gynecology, University of Ottawa, Ottawa, Ontario, Canada, ⁴Ottawa Hospital Research Institute, Ottawa, Ontario, Canada

Abstract

Epithelial ovarian cancer (EOC) is thought to arise in part from the ovarian surface epithelium (OSE); however, the molecular events underlying this transformation are poorly understood. Germline mutations in the BRCA1 tumor suppressor gene result in a significantly increased risk of developing EOC and a large proportion of sporadic EOCs display some sort of BRCA1 dysfunction. To generate a model in which Brca1-mediated transformation can be studied, we previously inactivated Brca1 alone in murine OSE, which resulted in an increased accumulation of premalignant changes, but no tumor formation. In this study, we examined tumor formation in mice with conditionally expressed alleles of Brca1, p53 and Rb, alone or in combination. Intrabursal injection of adenovirus expressing Cre recombinase to inactivate p53 resulted in tumors in 100% of mice. Tumor progression was accelerated in mice with concomitant inactivation of Brca1 and p53, but not Rb and p53. Immunohistologic analyses classified the tumors as leiomyosarcomas that may be arising from the ovarian bursa. Brca1 inactivation in primary cultures of murine OSE cells led to a suppression of proliferation that could be rescued by concomitant inactivation of p53 and/or Rb. Brca1-deficient OSE cells displayed an increased sensitivity to the DNA damaging agent cisplatin, and this effect could be modulated by inactivation of p53 and/or Rb. These results indicate that Brca1 deficiency can accelerate tumor development and alter the sensitivity of OSE cells to chemotherapeutic agents. Intrabursal delivery of adenovirus intended to alter gene expression in the ovarian surface epithelium may, in some strains of mice, result in more rapid transformation of adjacent cells, resulting in leiomyosarcomas.

Introduction

Epithelial ovarian cancer (EOC) is thought to arise from the ovarian surface epithelium (OSE) [1], although recent evidence implicates the fallopian tube as a potential tissue of origin of EOC of the serous histotype [2–5]. Embryonically derived from the coelomic epithelium, the OSE is a single layer of squamous to cuboidal cells that demonstrates a plastic phenotype reflecting its ability to undergo epithelio-mesenchymal transition [6]. With age and repeated ovulatory cycles, the OSE assumes a more irregular contour and forms invaginations or clefts into the stroma, which may pinch off completely and form epithelial inclusion cysts within the ovary. These crypts and cysts often show evidence of early metaplastic changes in cell shape and express markers up-regulated in ovarian tumors, such as E-cadherin [7] and CA125 [1], which suggests that these premalignant lesions may give rise to ovarian neoplasias. Prophylactic oophorectomy specimens from women at high risk for developing ovarian cancer, due to a strong family history of the disease or the presence of a germline mutation in BRCA1, have more of these morphological changes in the OSE than ovaries removed incidentally during total abdominal hysterectomy [8–10]. In one study, 6% of prophylactically removed ovaries from BRCA1 mutation carriers were found to harbor microscopic ovarian carcinomas [11].

Five to fifteen percent of ovarian cancers are thought to be due to hereditary factors and the majority of these can be attributed to germline mutations in the BRCA1 gene [12,13]. These germline mutations confer a lifetime risk of ovarian cancer up to 60% compared to ~2% in the general population [14,15]. Although somatic mutations in BRCA1 are rare, reduced or absent protein expression has been observed in up to 90% of sporadic ovarian tumors indicating that epigenetic factors, mainly promoter hypermethylation, are also involved in its regulation [16–18]. The BRCA1 gene has been implicated in a wide variety of cellular processes, including maintenance of genome integrity [19], DNA damage recognition and repair [20,21], cell cycle checkpoint control [22,23], and apoptosis [24].

Up to 60% of BRCA1 mutation-associated ovarian tumors also display mutations in the p53 tumor suppressor gene [25,26]. Mouse models of mammary tumorigenesis have revealed a role for p53 in Brca1-related transformation [27–29]. In mice in which Brca1 was inactivated in the mammary epithelium, the latency of tumor formation could be shortened by the concomitant inactivation of the p53 tumor suppressor gene [30,31]. Down-regulation of BRCA1 results in increased p53 and p21 expression [31], which may represent a significant obstacle to tumorigenesis that can be overcome by somatic mutation of the p53 gene.
The ability of BRCA1 to suppress cellular proliferation may depend, at least in part, on its association with the retinoblastoma tumor suppressor (RB), since RB preferentially binds to exon 11 of BRCA1 [32]. RB has also been shown to modify BRCA1 expression via its modulation of E2F transcriptional activity, with BRCA1 being an in vivo target of E2F [33]. And overexpression of BRCA1 inhibits the expression of RB and the RB family members p107 and p130 [34]. No association has yet been made between RB and BRCA1 dysfunction in ovarian cancer.

The Brca1, Rb and p53 tumor suppressors have been conditionally inactivated in mouse OSE to study their roles in ovarian epithelial cell transformation. Simultaneous inactivation of p53 and Rb in the mouse OSE led to the development of malignant ovarian tumors [35]. We inactivated Brca1 in the murine OSE which resulted in the increased accumulation of premalignant changes, although no tumor formation was observed after one year [36]. However, inactivation of Brca1 in cultured murine OSE (MOSE) cells resulted in a slowed proliferation that could be rescued by concurrent inactivation of p53 [36]. In the present study, a conditional and site-directed strategy was employed to test whether inactivation of Brca1, p53, and Rb in various combinations in the OSE is sufficient to establish a mouse model of Brca1-associated ovarian cancer. The ovaries of these mice were examined at various time points to determine if there were progressive alterations in epithelial morphology or tumor formation as has been observed in human prophylactic oophorectomy specimens. Primary cultures of OSE cells from these mice were also established to determine the impact of combined inactivation of these genes on their rate of proliferation and sensitivity to cisplatin.

Materials and Methods

Experimental Animals

Brca1loxP/loxP [FVB;129-Brca1tm28m] conditional knockout mice, bearing loxP sites in introns 4 and 13 of the Brca1 gene, and p53loxP/loxP [FVB;129-Tp53tm1Bom] [37] mice bearing loxP in introns 1 and 10 of the p53 gene were obtained from the Mouse Models of Human Cancers Consortium Mouse Repository (National Cancer Institute, Rockville, MD, USA). Rh1loxP/loxP mice [129sv-Rb1tm28n] [38], bearing loxP sites flanking exon 19 of the Rb gene, were obtained from Dr. Ruth Slack at the University of Ottawa (Ottawa, Ontario, Canada). The mice were intercrossed through multiple generations to create colonies of homozygous Brca1loxP/loxP/p53loxP/loxP, Rh1loxP/loxP/p53loxP/loxP, and Rh1loxP/loxP double conditional knockout mice. These mice were then intercrossed through successive generations in order to obtain Brca1loxP/loxP/Rh1loxP/loxP/p53loxP/loxP triple conditional knockout mice. All animal experiments described in this study were performed according to the Guidelines for the Care and Use of Animals established by the Canadian Council on Animal Care.

Genotyping and Detection of Recombination

Genomic DNA was extracted from ear punches or cultured cells as previously described [36]. Brca1loxP/loxP mice were genotyped as previously described [39]. p53loxP/loxP mice were genotyped using primers p53int1fwd (5′ CGA AAA AAC AGG TTA AAC GAG AGG 3′) and p53int1rev (5′ AGC AGA TAG GAG GCA GAG AGC 3′) to yield a 288 bp band for wild-type or a 370 bp band for floxed sequences or primers p53int1fwd (5′ AAG GGG TAT GAG GGA CAA GGG 3′) and p53int10rev (5′ GAA GAC AGA AAA GGG GAG GAG GAG 3′), which yield a 431 bp or a 584 bp fragment for wild-type and floxed sequences respectively. For detection of Cre-mediated excision of exons 2–10 of p53 [hereafter designated p53K2-10], primers p53int1fw and p53int10rev were used, yielding a 612 bp product. Remaining unrecombined sequence following Cre exposure was detected using primers p53int1fwd and p53int1rev or primers p53int10fw and p53int10rev in a separate amplification, Rh1loxP/loxP mice were genotyped using primers Rh1f (5′ GGC GTG TGC CAT CAA TG3′) and Rh1r (5′ AAC TCA AGG GAG ACC TG3′). DNA from wild-type mice yielded a 750 bp band, while floxed mice were identified by the presence of a 650 bp band. For detection of Cre-mediated excision of exon 19 of the Rb gene [hereafter designated Rb1A10], primers Rh1f and Rh1r were used, yielding a 260 bp product when recombination has occurred and a 750 bp product for the unrecombined fraction.

Tissue Collection

Animals were euthanized by CO2 asphyxiation at 60, 120, 180, or 240 days post-injection of AdGFP or AdCre. The ovaries (with the ovarian fat pad and bursa intact) were removed individually along with the attached oviduct and a portion of the uterus, fixed in formalin and paraffin-embedded. Five μm serial sections were cut for H&E staining and morphological changes to the OSE were assessed by examining five non-consecutive sections of each ovary at 200× magnification using an Olympus BX50 microscope (Olympus, Melville, NY, USA). Samples were blinded prior to examination. Sections were evaluated for the number of areas of columnar cells, areas of hyperplasia, and invaginations of the OSE, as described previously [36]. Mice that developed tumors were euthanized when they had reached a loss-of-wellness endpoint due to tumor burden, defined as any or all of the following: presence of respiratory distress, rapid weight loss or gain >5 g from the average body weight of control mice of the same age, and presence of a palpable mass or abdominal distention that impaired mobility. Tumors were removed, fixed in formalin and paraffin embedded, with a small piece of tissue reserved for genomic DNA isolation. Serial sections of tumor tissue with a thickness of 5 μm were cut for H&E staining and immunohistochemistry.

Immunohistochemistry

Paraffin sections were deparaffinized in xylene and rehydrated in ethanol according to standard protocol. High temperature antigen retrieval was performed using sodium citrate buffer (pH 6.0) and endogenous peroxidase activity was blocked using 3% hydrogen peroxide in Stock PBS (S-PBS). All samples were blocked using an avidin/biotin blocking kit (DAKO, Cytomation, Carpentaria, CA, USA). Primary antibodies were diluted in S-PBS at the following concentrations: cat anti-CK19, 1:100 (TROMA-1, Developmental Studies Hybridoma Bank, University of Iowa, USA); rat anti-Ki67, 1:25 (DAKO); mouse anti-smooth muscle actin (SMA), 1:100 (DAKO); mouse anti-Desmin, 1:100 (DAKO); and rat anti-CD34, 1:50 (Novus Biologicals). For antibodies raised in rat, sections were incubated with primary antibody at room temperature for 2 hours or overnight. Following three 5 min washes in S-PBS, sections were stained with an anti-rabbit secondary antibody (1:200, DAKO) for 20 min followed by three 5 min washes and incubation with a streptavidin/horseradish peroxidase solution (1:200, DAKO) for 20 min. For antibodies raised in mice, the Vector® Mouse on Mouse™ immunodetection kit (Vector Laboratories, Burlingame, CA, USA) was used according to manufacturer specifications.
Developing was performed with diaminobenzidine (DAB) as a substrate (0.2% DAB, 0.001% H2O2; Sigma-Aldrich). Slides were counterstained with hematoxylin, dehydrated, and coverslipped with Permount mounting medium (Fisher Scientific, Ottawa, ON, Canada).

MOSE Proliferation and Sensitivity to Cisplatin

Primary cultures of MOSE cells were established and maintained as previously described [36]. For in vitro infections, 5.0 x 10⁵ MOSE cells of the various genotypes were infected with either AdCre or AdGFP following an established protocol [35], and experiments to evaluate proliferation and cisplatin sensitivity were performed following cell replating 72 hours later, as described previously [36]. Proliferation of MOSE cells was also assessed several passages (4–9) after infection. Experiments were performed three times in triplicate, with a separate infection for each replicate.

Statistical Analyses

Cell counts are expressed as the mean±SEM of three independent experiments performed in triplicate. The probability of significant differences was determined by Student’s t test (two groups) and by analysis of variance (ANOVA; multiple groups). Bonferroni’s post-test was used to determine significance between specific treatments when whole group differences were detected by ANOVA. Survival curves were compared using a Log-Rank test. For all analyses, significance was inferred at P < 0.05 and P values were two-sided. Analyses were performed using Graphpad Prism statistical software (Graphpad Software, San Diego, CA, USA).

Results

Conditional Inactivation of Brca1 in Conjunction with p53 and/or Rb Using Intrabursal Injection

We have previously shown that inactivation of Brca1 alone in the OSE resulted in an increase in the number of preneoplastic changes in the OSE after 240 days, but no tumor formation [36]. In this study we observed that no tumors arose in mice in which Rb alone was conditionally inactivated, even after one year following intrabursal injection of AdCre. There were no significant differences in the number of morphological changes in the OSE between the AdCre-treated Brca1loxP/loxP mice and the control Brca1loxP/loxP mice at any of the time points examined (Table 1). There was no significant age-related increase in the total number of changes, as was previously observed in the Brca1loxP/loxP mice. However, the mean number of morphological changes in the OSE doubled between 120 and 180 days in the Brca1loxP/loxP mice (4.32 ± 0.78 versus 9.52 ± 0.80 mean changes per section over five non-consecutive sections per ovary, P < 0.05), whereas this difference was not noted in the Brca1loxP/loxP mice.

Tumor formation was also not observed in mice in which both Rb and Brca1 were concomitantly inactivated via intrabursal injection of AdCre. The number of morphological changes in the OSE increased over time in both groups, however there were no significant differences between the Rbloxp/Brca1loxP mice and the RbloxP/Brca1loxP mice at any time point examined (Table 2). There was a significant increase in the number of areas of columnar cells between 60 and 240 days in the Brca1loxP/Brca1loxP mice (1.96 ± 0.45 versus 0.78 ± 0.70, P < 0.01) that was not seen in the Brca1loxP/Brca1loxP mice. As well, epithelial invaginations were noted only in the OSE of the Brca1loxP/Brca1loxP mice.

Tumor formation was observed following intrabursal injection of AdCre to generate cells with the following genotypes: p53LoxP/loxP, Brca1loxP/loxP, Brca1loxP/loxP, and Brca1loxP/loxP (Table 3). No tumors were observed in any of the mice injected with adenoviral GFP when followed to one year post-intrabursal injection. The presentation of the mice at endpoint was similar in all of the groups examined, with either a large, palpable dorsal mass(es) or severe abdominal distention. The period between onset of symptoms and loss-of-wellness endpoint was quite short, generally one week or less. Figure 1 illustrates the general appearance of the tumors upon necropsy for each genotype. The tumors were most often located at the end of the uterine horn involving the ovary and sometimes engulfing surrounding organs such as the pancreas and the spleen. Both ovaries were subjected to intrabursal injection in all animals, however bilateral tumors were only observed in the Brca1loxP/loxP mice, and only in 2 of 14 animals. The incidence, median survival, range of survival and presence of ascites

### Table 1. Distribution of morphological features in the OSE over time following conditional inactivation of Rb.

| Areas of Columnar Cells | Time (Days) |
|-------------------------|-------------|
|                         | 60          | 120         | 180         | 240         |
| Brca1loxP/loxP          | 3.34 ± 0.33* (n = 10) | 2.38 ± 0.49* (n = 10) | 4.00 ± 0.53* (n = 10) | 4.33 ± 0.67* (n = 8) |
| Brca1loxP/loxP          | 2.48 ± 0.28* (n = 10) | 2.99 ± 0.53* (n = 8)   | 4.76 ± 0.53* (n = 10) | 4.02 ± 0.36* (n = 10) |

| Areas of Hyperplasia   | Time (Days) |
|------------------------|-------------|
|                        | 60          | 120         | 180         | 240         |
| Brca1loxP/loxP         | 2.60 ± 0.77* | 1.90 ± 0.54* | 4.52 ± 0.67* | 2.08 ± 0.38* |
| Brca1loxP/loxP         | 2.43 ± 0.69* | 2.96 ± 0.93* | 3.04 ± 0.70* | 2.32 ± 0.50* |

| Epithelial Invaginations | Time (Days) |
|--------------------------|-------------|
|                         | 60          | 120         | 180         | 240         |
| Brca1loxP/loxP           | 0.13 ± 0.07* | 0.04 ± 0.04* | 0*          | 0.06 ± 0.06* |
| Brca1loxP/loxP           | 0*          | 0.03 ± 0.03* | 0*          | 0*          |

| Total Changes            | Time (Days) |
|--------------------------|-------------|
|                         | 60          | 120         | 180         | 240         |
| Brca1loxP/loxP           | 6.05 ± 0.86* | 4.32 ± 0.78* | 8.52 ± 0.80* | 6.40 ± 0.80* |
| Brca1loxP/loxP           | 4.90 ± 0.79* | 5.98 ± 1.16* | 7.80 ± 1.10* | 6.40 ± 0.63* |

Numbers represent the mean±SEM number of morphological changes per section over five non-consecutive sections in n ovaries. Superscript letters denote a significant difference between time points for that treatment group.

doi:10.1371/journal.pone.0008534.t001
for these groups of mice are summarized in Table 3 with the Kaplan-”Meier survival plots depicted in Figure 2. When compiled, these data indicate that loss of p53 is necessary for tumor formation and that Brca1 deficiency accelerates tumor initiation and/or progression.

Recombination at the relevant loxP sites was confirmed in genomic DNA from all the tumor samples (Figure 3). Unrecombined DNA was also detectable in all tumor samples, which was not unexpected given that the tumors are likely composed of both tumor cells and the cells of the normal tissue they have invaded. Recombination at the relevant loxP sites was also detectable in genomic DNA from ascites cells isolated from tumor-bearing mice (data not shown).

Histopathologic Features of Tumors in Mice with Conditional Inactivation of Brca1, p53 and Rb

There was no obvious difference in the pathology of the tumors as related to their genotype. H&E stained sections revealed densely-packed and highly malignant cells with spindle-shape morphology, as well as the presence of anaplastic giant cells in all of the samples examined (Figure 4A). Further immunohistochemical analyses revealed that the tumors were predominantly negative for the epithelial marker cytokeratin (CK) 19, with the exception of some glandular structures present in a small number of the tumors (Figure 4B). These tumors were also probed for the presence of other epithelial markers such as CK8 and pancytokeratin and were found to be negative with the exception of the areas noted above. All of the tumors examined were positive for smooth muscle actin (SMA) (Figure 4C) and desmin (Figure 4D), both smooth muscle markers, and were negative for CD34, a hematological marker (Figure 4E). Pathologic review of tumor histology and immunohistochemical data indicates that these tumors are malignant leiomyosarcomas.

The gross pathology along with the H&E sections provided some clues as to the origin of the tumors. In some instances, normal ovary was visible at the edge of a tumor, such that the bursal membrane that surrounds the ovary was flush with the tumor (Figure 5A). In some cases, even when normal ovary was not grossly visible at necropsy, the ovary was found within the H&E sections of the tumor (Figure 5B). In these sections, the tumors also appeared to be connected to the bursal membrane (Figure 5C). Staining of sections of normal ovaries revealed that the bursa, which is an extension of the oviduct, is highly positive for SMA (Figure 5D). Staining also revealed that the inner lining

### Table 2. Distribution of morphological features in the OSE over time following conditional inactivation of Rb and Brca1.

| Epithelial Morphology | Time (Days) |
|-----------------------|-------------|
|                        | 60          | 120         | 180         | 240         |
| Areas of Columnar Cells |             |             |             |             |
| RbloxP/loxP/Brca1loxP/loxP | 1.96±0.45 (n = 10) | 4.00±0.59 (n = 10) | 4.27±0.49 (n = 12) | 5.18±0.70 (n = 10) |
| Areas of Hyperplasia |             |             |             |             |
| RbloxP/loxP/Brca1loxP/loxP | 2.64±0.68* (n = 10) | 2.70±0.84* (n = 10) | 4.23±0.75* (n = 10) | 4.14±0.84* (n = 10) |
| Epithelial Invaginations |             |             |             |             |
| RbloxP/loxP/Brca1loxP/loxP | 1.76±0.40* (n = 10) | 2.80±0.62* (n = 10) | 3.08±0.68* (n = 10) | 2.42±0.87* (n = 10) |

Numbers represent the mean±SEM of the number of morphological changes per section over five non-consecutive sections in n ovaries. Letters are used to denote a significant difference between time points for that treatment group.

doi:10.1371/journal.pone.0008534.t002

### Table 3. Tumor formation following intrabursal injection of adenoviral Cre recombinase.

| Genotype | # of mice | # of mice with tumors | Median survival in days (range) | % with ascites |
|----------|-----------|----------------------|---------------------------------|--------------|
| Brca1loxP/loxP | 5 | 0 | | |
| RbloxP/loxP | 5 | 0 | | |
| RbloxP/loxP/Brca1loxP/loxP | 5 | 0 | | |
| p53loxP/loxP | 8 | 8 | 179.5 (101–200) | 50 |
| RbloxP/loxP/p53loxP/loxP | 10 | 10 | 170 (99–207) | 30 |
| Brca1loxP/loxP/p53loxP/loxP | 12 | 12 | 147.5 (105–166) | 42 |
| RbloxP/loxP/p53loxP/loxP/Brca1loxP/loxP | 14 | 14 | 118.5 (89–183) | 0 |

*Number of mice indicates the number of animals who received intrabursal injection of adenoviral Cre recombinase and were euthanized when they had reached a loss-of-wellness endpoint due to tumor burden or had reached the 240 day time point.

doi:10.1371/journal.pone.0008534.t003
Figure 1. Gross tumor presentation of mice at loss-of-wellness endpoint following intrabursal administration of AdCre. Representative images of tumors arising in mice in which Brca1, p53, and/or Rb had been inactivated in the OSE: p53$^{-12-10}$ (A), Brca1$^{AS-13}$/p53$^{-12-10}$ (B), Rb$^{A19}$/p53$^{-12-10}$ (C), and Rb$^{A19}$/p53$^{-12-10}$/Brca1$^{AS-13}$ (D).

doi:10.1371/journal.pone.0008534.g001

Figure 2. Survival of mice following inactivation of multiple tumor suppressors in the OSE in vivo. Kaplan-Meier plots showing the time to loss-of-wellness endpoint of the mice in which Brca1, p53, and/or Rb had been inactivated in the OSE. A) The median survival time of the Brca1$^{AS-13}$/p53$^{-12-10}$ mice (147.5 days) is significantly shorter than that of the p53$^{-12-10}$ mice (179.5 days), P<0.05. B) The median survival time of the Brca1$^{AS-13}$/p53$^{-12-10}$ mice (147.5 days) is significantly less than that of the Rb$^{A19}$/p53$^{-12-10}$ mice (170 days), P<0.001. C) The median survival time of the Rb$^{A19}$/p53$^{-12-10}$/Brca1$^{AS-13}$ mice (118.5 days) is significantly less than that of the Rb$^{A19}$/p53$^{-12-10}$ mice (170 days), P<0.05. D) The median survival time of the Rb$^{A19}$/p53$^{-12-10}$/Brca1$^{AS-13}$ mice (118.5 days) is less than that of the Brca1$^{AS-13}$/p53$^{-12-10}$ mice (147.5 days), although the difference in survival was not statistically significant.

doi:10.1371/journal.pone.0008534.g002
of the bursal membrane is positive for CK19 (Figure 5E). Ovaries from the p53D2-10, RbD19/p53D2-10, Brca1D5-13/p53D2-10, and RbD19/Brca1D5-13/p53D2-10 mice at early time points (60 and 120 days) were uninformative in terms of potential precursor lesions for these tumors. There were no advanced morphological changes (invaginations or inclusion cysts) noted in ovaries collected from the mice at 60 or 120 days post-AdCre injection (data not shown).

Effect of Concomitant Inactivation of Multiple Tumor Suppressor Genes on Proliferation of MOSE Cells In Vitro

The appearance of non-epithelial tumors in the mice limited the investigation of the consequences of disruption of the tumor suppressor genes in OSE cells in vivo. We therefore isolated MOSE cells from mice with conditional expression of these genes and have previously demonstrated that inactivation of Brca1 in MOSE cells resulted in decreased proliferation [36]. Inactivation of p53 in p53loxP/loxP MOSE cells had no effect on proliferation, but proliferation was dramatically increased when both Brca1 and p53 were inactivated [36]. Here we show that, when growth rates were examined 72 hours after exposure to AdGFP or AdCre, conditional inactivation of Rb in RbDloxP/loxP MOSE cells had no effect on proliferation over the subsequent 96 hour period (Figure 6A). When both Brca1 and Rb were inactivated in RbDloxP/loxP/Brca1DloxP/loxP MOSE cells, the proliferation defect seen when Brca1 was inactivated alone (Figure 6G) was eliminated; however there was no significant increase in the rate of proliferation of the Rb/Brca1-deficient cells.
Figure 6. MOSE cell proliferation following inactivation of tumor suppressors. Number of viable MOSE cells evaluated 72 hours (A–D, G) or several passages (E–F, H) after infection with AdCre or AdGFP. 

A) $Rb^{loxP/loxP}$ MOSE cells evaluated 72 hours following infection with AdCre ($Rb^{\Delta19}$) to inactivate $Rb$ or with AdGFP ($Rb^{loxP/loxP}$).

B) $Rb^{loxP/loxP}/Brca1^{loxP/loxP}$ MOSE cells infected with AdCre ($Rb^{\Delta19}/Brca1^{\Delta5-13}$) to inactivate $Rb$ and $Brca1$ or with AdGFP ($Rb^{loxP/loxP}/Brca1^{loxP/loxP}$).

C) $Rb^{loxP/loxP}/p53^{loxP/loxP}$ MOSE infected with AdCre ($Rb^{\Delta19}/p53^{\Delta2-10}$) to inactivate $Rb$ and $p53$ or AdGFP ($Rb^{loxP/loxP}/p53^{loxP/loxP}$).

D) $Rb^{loxP/loxP}/p53^{loxP/loxP}/Brca1^{loxP/loxP}$ MOSE infected with AdCre ($Rb^{\Delta19}/p53^{\Delta2-10}/Brca1^{\Delta5-13}$) or AdGFP ($Rb^{loxP/loxP}/p53^{loxP/loxP}/Brca1^{loxP/loxP}$).

E–F) Proliferation of MOSE cells 4–9 passages following infection of AdCre or AdGFP. 

E) $p53^{loxP/loxP}$ vs. $p53^{\Delta2-10}$ MOSE.

F) $Rb^{loxP/loxP}$ vs. $Rb^{\Delta19}$ MOSE.

G) $Brca1^{loxP/loxP}$ vs. $Brca1^{\Delta5-13}$ MOSE evaluated 72 hours following infection with AdCre or AdGFP. 

H) $Brca1^{loxP/loxP}$ vs. $Brca1^{\Delta5-13}$ MOSE, 4–9 passages following infection with AdCre or AdGFP.

I) PCR of genomic DNA collected from cultured $Brca1^{loxP/loxP}$ OSE cells 1 or 6 passages following in vitro infection with AdCre ($Brca1^{\Delta5-13}$) or AdGFP ($Brca1^{loxP/loxP}$). Error bars represent the SEM of data obtained from three experiments performed in triplicate. *, ** and *** indicate a significant difference where $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

doi:10.1371/journal.pone.0008534.g006
When p53 was inactivated in conjunction with Rb in MOSE cells, the resulting Rb/p53-deficient cells showed a dramatic increase in proliferation as compared to their control (AdGFP-infected) counterparts, resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.

When the proliferation rate of p53A2−10 MOSE cells was examined several passages following AdCre infection, the p53-deficient MOSE had developed a significantly increased rate of proliferation compared to AdGFP-infected cells cultured for the same length of time (P<0.001; Figure 6E). After several passages, the RbA19 MOSE cells also had a significantly increased rate of proliferation, detectable as early as 48 hours (Figure 6F). In contrast, Braf-deficient MOSE, which showed a reduced rate of proliferation immediately after inactivation of Braf (Figure 6G), failed to show any difference from their AdGFP-infected counterparts after several passages (Figure 6H). While recombination at the loxP sites could still be detected in the AdCre-infected cells at parts after several passages (P<0.001; Figure 6D), resulting in a 2.7-fold increase in cell number after 96 hours.
Based on the work of Flesken-Nikitin et al. [35], it was anticipated that the Rb<sup>19/19</sup>/p53<sup>x2-10</sup> mice would develop adenocarcinomas with little to no tumor formation seen when either p53 or Rb was inactivated alone. In contrast to that study, the Rb<sup>19/19</sup>/p53<sup>x2-10</sup> mice in this study died of tumors that were classified as malignant leiomyosarcomas. Furthermore, conditional inactivation of p53 alone led to the development of leiomyosarcomas in 100% of the mice. Ovarian sarcomas are rare in women [40], although aberrations in both the p53 and Rb pathways have been implicated in the development and progression of leiomyosarcomas [41–43], and mice that are null or heterozygous for p53 are highly prone to developing lymphomas and sarcomas [44,45]. Several groups have shown the capacity for OSE to develop into epithelial tumors using the method of intrabursal injection of AdCre for the activation or inactivation of genes [35,36,46], indicating that these cells are capable of transformation to epithelial cancers. The reasons for the prevalence of leiomyosarcomas are therefore unclear, but there are several possible factors that should be considered. First, the OSE is a poorly differentiated epithelium that is known to undergo epithelial-mesenchymal transition during the peri-ovulatory period [1], and it is possible that the inactivation of p53 facilitated transformation more effectively in these mesenchymal cells. Second, although evidence from our lab and others [35,36,46] indicate that intrabursal delivery of adenoviruses results in infection of primarily surface epithelial cells, it may be possible that some underlying stromal cells are also infected and are more readily transformed than the epithelial cells.

A third reason for the occurrence of leiomyosarcomas in this study may be differences in the strain of mouse used. Genetic modifiers due to strain differences can influence the phenotype in mouse models of disease [47–49], so it is possible that strain differences may account for the discrepancies in both tumor incidence and histology. The animals utilized in the Flesken-Nikitin study [35] were backcrossed to the FVB/n strain, whereas the animals in this study were maintained on the FVB/n;129sv mixed strain background. Finally, it is possible that, in the strain used in this study, the smooth muscle cells of the bursal membrane, which would also be in contact with the adenoviral Cre recombinase, are more sensitive to the consequences of these recombination events than the OSE. The bursal membrane is largely composed of smooth muscle cells, much like the uterine and oviductal myometrium with which it is contiguous. Since the tumors that developed in these mice were generally found to be continuous with the bursal membrane, it may be that the tumors are arising from this tissue rather than the ovarian surface epithelium. We have recently found that Cre-mediated recombination can occur in cells in the bursal membrane and that cells carrying the recombined gene can still be found at least 65 days after intrabursal delivery of AdCre [50].

Despite this unanticipated phenotype, Brca1-deficiency in these tumors influenced the survival time of the mice, causing a significant reduction in the length of survival of p53 and p53/Rb-deficient mice, compared to the mice without Brca1 deficiency. Breast and ovarian cancers in women with BRCA1 mutations arise at an earlier age than sporadic cancers [51], so it was interesting to observe that, despite the difference in histologic phenotype, inactivation of Brca1 in mice resulted in tumors that developed more quickly. This action is in agreement with a recent report describing the ability of BRCA1 deficiency to accelerate uterine leiomyosarcoma development [52]. The mechanism of this acceleration in tumor initiation and/or progression is unknown, although loss of Brca1 function is associated with genomic instability [19].

When MOSE cell proliferation was examined in vitro, the concomitant inactivation of p53 rescued the growth suppression that was seen with inactivation of Brca1 alone [36] and led to a substantially increased growth rate as compared to control cells. Loss of p53 function has been demonstrated to partially rescue the embryonic lethality of Brca1 knockout mice and appears to play a critical role in mammary tumorigenesis in mice [27–31,53]. Concomitant inactivation of both p53 and Rb also resulted in a significant increase in proliferation in MOSE cells, an observation in agreement with results of similar studies reported previously [35]. Conditional inactivation of all three tumor suppressor genes, Brca1, p53, and Rb, simultaneously in MOSE cells resulted in a dramatic increase in proliferation with significant increases in cell number with time over any of the other genotypic combinations. This increased rate of proliferation may explain the acceleration in tumor progression in cells lacking all three genes.

Inactivation of p53 or Rb alone in MOSE cells had no immediate effect on proliferation, but after at least four passages, the growth characteristics became notably different, showing increased rates of proliferation relative to controls. These results suggest that, with prolonged culture, these cells may spontaneously acquire further genetic abnormalities that result in uncontrolled proliferation. The lack of any obvious morphological alterations in the OSE at 60 or 120 days after intrabursal AdCre injection in p53/Rb-deficient mice supports the hypothesis that tumor initiation requires additional genetic changes. The long latency in tumor development could be a result of the relatively low proliferative index of the OSE [54].

The increased sensitivity of MOSE to cisplatin treatment observed when only one tumor suppressor gene is inactivated appears to be lost when two or more are inactivated simultaneously. Cisplatin-resistant ovarian and cervical carcinoma cells have been shown to harbour multiple genetic changes, particularly affecting pathways leading to apoptosis that render the cells aberrantly tolerant of accumulated DNA damage [55,56]. In this regard, familial ovarian cancers have a higher frequency of p53 mutations than sporadic cancers [14], and it has been suggested that loss of p53 function is required for a cell to tolerate the loss of the Brca1 function [57–59]. Thus, loss of Brca1 may result in compensation in the cell by the p53 or Rb pathways, resulting in an increased apoptotic response to cisplatin. The concomitant loss of the p53 or Rb expression may enable the cells to better tolerate cellular stresses, including exposure to cisplatin.

These results show similarities to mouse models of mammary tumorigenesis and uterine leiomyosarcomas in that Brca1 inactivation can accelerate the initiation and/or progression of p53-mediated tumors. Brca1 status, in combination with that of other tumor suppressor pathways, influence sensitivity of OSE cells to chemotherapeutic agents. The combination of tumor suppressors inactivated in this study failed to yield epithelial cancers, indicating that further analyses are needed to determine the mechanisms by which BRCA1 mutations cause transformation of the ovarian epithelium.

Acknowledgments

We are grateful to Dr. Anton Berns and Dr. Jos Jonkers for providing the Brca1<sup>x2-10</sup>/p53<sup>x19/19</sup> mice ahead of publication and for providing the p53<sup>19/19</sup>/Rb<sup>x2-10</sup> mice. We would also like to thank Dr. Ruth Slack for providing the Rb<sup>19/19</sup>/p53<sup>x2-10</sup> mice, Kerri Courville and Colleen Crane for their technical assistance, and Dr. Ken Garson for his critical review of the manuscript.

Author Contributions

Conceived and designed the experiments: BCV. Performed the experiments: KVCK OC. Analyzed the data: KVCK MKS BCV. Contributed reagents/materials/analysis tools: MKS. Wrote the paper: KVCK BCV.
References

1. Auersperg N, Edelson MI, Mok SC, Johnson SW, Hamilton TC (1998) The biology of ovarian cancer. Semin Oncol 25: 281–304.

2. Hurst JE, Gard GB, McIlroy K, Nevell D, Field M (2009) High rates of occult fallopian tube and the dominant ovary mass: clues to serum tumor origin. J Surg Pathol 33: 376–383.

3. Shaw PA, Rouzbehman H, Pasr ES, Pintilie M, Begley H (2009) Candidate serous cancer precursors in fallopian epithelium of BRCA1/2 mutation carriers. Mod Pathol 22: 1153–1162.

4. Levannon K, Crum C, Drapkin R (2008) New insights into the pathogenesis of serous ovarian cancer and its clinical impact. J Clin Oncol 26: 5284–5293.

5. Roh MH, Kindelberger C, Crum CP (2009) Serous tubal intraepithelial carcinomas and the dominant ovarian mass: clues to serum tumor origin. Am J Surg Pathol 33: 376–383.

6. Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC (2001) Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr Rev 22: 255–280.

7. Sandfeld K, Poutiakewiz Y, Ivarsson K, Nilsson O, Hellberg P, et al. (1997) Cell cycle control in human epithelial ovarian cancer and normal ovary. J Int J Cancer 74: 275–280.

8. Salazar H, Godwin AK, Daly MB, Leub PB, Hogan WM, et al. (1996) Microscopic benign and invasive malignant neoplasms and a cancer-prene phenotype in prophylactic oophorectomies. J Natl Cancer Inst 88: 1810–1820.

9. Schlosshauer CW, Cohen CJ, Pennault-Licora F, Miranda CR, Bignon YJ, et al. (2003) Prophylactic oophorectomy: a morphologic and immunohistochemical study. Cancer 98: 2599–2606.

10. Werness BA, Alify AM, Bielat KL, Eltabbakh GH, Piver MS, et al. (1999) Altered surface and cyst epithelium of ovaries removed prophylactically from women with a family history of ovarian cancer. Hum Pathol 30: 151–157.

11. Finch A, Shaw P, Rosen B, Murphy J, Naredo SA, et al. (2005) Clinical and pathologic findings of prophylactic prophylactic oophorectomies in 139 BRCA1 and BRCA2 carriers. Gynecol Oncol.

12. Berchuck A, Heron KA, Carney ME, Lancaster JM, Fraser ER, et al. (1998) Frequency of germline and somatic BRCA1 mutations in ovarian cancer. Clin Cancer Res 4: 24–25.

13. Pal T, Purnmuth-Wey J, Bets JA, Krueger JP, Fiorica J, et al. (2005) BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases. Cancer.

14. Aunoble B, Sanches R, Didier E, Bignon YJ (2000) Major oncogenes and tumor suppressor genes controlling the BRCA1 and BRCA2 genes in sporadic ovarian cancer. Int J Cancer 87: 317–321.

15. Finch A, Shaw P, Rosen B, Murphy J, Naredo SA, et al. (2005) Clinical and pathologic findings of prophylactic prophylactic oophorectomies in 139 BRCA1 and BRCA2 carriers. Gynecol Oncol.

16. Berchuck A, Heron KA, Carney ME, Lancaster JM, Fraser ER, et al. (1998) Frequency of germline and somatic BRCA1 mutations in ovarian cancer. Clin Cancer Res 4: 24–25.

17. Finch A, Shaw P, Rosen B, Murphy J, Naredo SA, et al. (2005) Clinical and pathologic findings of prophylactic prophylactic oophorectomies in 139 BRCA1 and BRCA2 carriers. Gynecol Oncol.

18. Chan KY, Ozcelik H, Cheung AN, Scully R, Visco AP, et al. (1997) E- cadherin in human epithelial ovarian cancer and normal ovary. J Int J Cancer 74: 275–280.

19. Salazar H, Godwin AK, Daly MB, Leub PB, Hogan WM, et al. (1996) Microscopic benign and invasive malignant neoplasms and a cancer-prene phenotype in prophylactic oophorectomies. J Natl Cancer Inst 88: 1810–1820.
56. Perego P, Romanello S, Carenini N, Magianni I, Leone R, et al. (1998) Ovarian cancer cisplatin-resistant cell lines: multiple changes including collateral sensitivity to Taxol. Ann Oncol 9: 423–430.

57. Brugarolas J, Jacks T (1997) Double indemnity: p53, BRCA and cancer. p53 mutation partially rescues developmental arrest in Brca1 and Brca2 null mice, suggesting a role for familial breast cancer genes in DNA damage repair. Nat Med 3: 721–722.

58. Cao L, Li W, Kim S, Brodie SG, Deng CX (2003) Senescence, aging, and malignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. Genes Dev 17: 201–213.

59. Xu X, Qiao W, Linke SP, Cao L, Li WM, et al. (2001) Genetic interactions between tumor suppressors Brca1 and p53 in apoptosis, cell cycle and tumorigenesis. Nat Genet 28: 266–271.