

Induction of Carcinogenesis by Concurrent Inactivation of *p53* and *Rb1* in the Mouse Ovarian Surface Epithelium¹

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Abstract

Alterations in *p53* and *Rb* pathways are observed frequently in epithelial ovarian cancer (EOC). However, their roles in EOC initiation remain uncertain. Using a single intrabursal administration of recombinant adenovirus expressing Cre, we demonstrate that concurrent inactivation of *p53* and *Rb1* is sufficient for reproducible induction of ovarian epithelial carcinogenesis in mice homozygous for conditional gene alleles. Similarly to progression of disease in women, ovarian neoplasms spread *i.p.*, forming ascites, and metastasize to the contralateral ovary, the lung, and the liver. These results establish critical interactions between *p53* and *Rb1* pathways in EOC pathogenesis, and provide a genetically defined immunocompetent mouse model of sporadic EOC.

Introduction

Ovarian cancer is the fifth leading female cancer among both new cancer cases and deaths in the United States (1). Almost 90% of cases are believed to be derived from the OSE,⁵ with serous adenocarcinomas comprising ~80% of EOC (2). By the time of diagnosis ~70% of tumors have spread beyond the ovary, and such cases are rarely curable by existing treatment schemes. Accordingly, over the past three decades the incidence and survival of ovarian cancer have remained relatively constant (1). Unfortunately, development of accurate genetic mammalian models of EOC has been delayed significantly, in large part because of absence of promoters with expression limited to the OSE. Two approaches have been reported recently. The first is based on the avian retroviral receptor TVA-replication-competent avian leukosis virus-derived vector (TVA-RCAS) gene delivery technique for introduction of defined alterations in explanted OSE with subsequent induction of tumor formation after injection of infected cells at *s.c.*, *i.p.*, or ovarian sites of immunodeficient mice (3). The second uses the MISIR receptor regulatory element for expression of the SV40 T antigen. Although insightful, these approaches either require *ex vivo* manipulations or result in transgene expression during embryonic development, dissimilar to the expected natural history of EOC in women. Mutations of *p53* gene are reported to be the most frequent alterations in sporadic EOC (4, 5), but initiate few, if any, carcinomas alone (3). *p53* signaling is interconnected frequently with *Rb*-mediated pathways (6, 7). Aberrations in the *Rb* pathway in EOC have been suggested by a number of recent studies (8–11); however, direct genetic evidence for their contribution to EOC

formation has been missing. To circumvent promoter-related limitations we have established a procedure for intrabursal administration of the recombinant adenovirus, taking advantage of the enclosed anatomical location of the mouse ovary within the bursa, which allows for selective exposure of the OSE to inducing agents. Using this approach in conjunction with Cre-*loxP*-mediated gene inactivation we have directly evaluated the role of *p53* and *Rb1* inactivation in the initiation of ovarian epithelial carcinogenesis.

Materials and Methods

Experimental Animals. Mice with floxed copies of *p53* and *Rb1* genes were described elsewhere (12, 13). *Rosa26STOPfloxLacZ* reporter mice [B6; 129-Gt(ROSA)26Sor^{T^{M1sor}}; Refs. 14–16] were purchased from the Jackson Laboratory. In these mice, expression of bacterial β -galactosidase is possible only after deletion of a stop codon flanked by *loxP* sites. All of the mice were maintained identically, following recommendations of the Institutional Laboratory Animal Use and Care Committee.

Genotyping. All of the mice with floxed alleles were maintained in homozygous status. If necessary, PCR genotyping was performed essentially as described (17). *p53^{floxP}* mice were identified with primers 10FM5' (5' AAG CTG AAG ACA GAA AAG GGG AGG G 3') and 10RM3' (5' AAG CTA AGG GGT ATG AGG GAC AAG G 3') or 10RM23' (5' ACA GAA AAG GGG AGG GAT GAA GTG A3'). PCR amplification of wild-type and floxed *p53* gene sequences results in 163-bp and 316-bp DNA fragments, respectively, using 10FM5' and 10RM3' or in 432-bp and 585-bp, respectively, using 10FM5' and 10RM23'. Cre-mediated excision was detected as either 198-bp or 467-bp DNA fragment on amplification with primers 1FM5' (5' GTG CCC TCC GTC CTT TTT CGC AAT C 3') and 10RM3' or 10RM23', respectively. *Rb1^{floxP}* mice were identified with primers Rb18M3' (5'-GGA ATT CCG GCG TGT GCC ATC AAT G 3') and Rb19EM5' (5' AGC TCT CAA GAG CTC AGA CTC ATG G 3'). PCR amplification of wild-type and floxed *Rb1* gene sequences results in 247-bp and 295-bp DNA fragments, respectively. Cre-mediated excision was detected as a 269-bp DNA fragment on amplification with primers Rb212M5' (5' CGA AAG GAA AGT CAG GGA CAT TGG G 3') and Rb18M3'.

Primary Culture of OSE. Individual ovaries were dissected and placed in DMEM/F12 (Ham's) medium containing Collagenase-Dispase at 5% CO₂ for 1 h. OSE were placed in six-well plates covered with 0.1% gelatin, maintained in Ham's medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, 5 μ g/ml insulin, 5 μ g/ml transferrin, and 5 ng/ml sodium selenite, and passaged on confluence. For estimation of proliferation, index cells were incubated with 100 μ M BrdUrd for 2 h in cell culture incubator (37°C), washed with PBS three times for 1 min each on ice, fixed with 4% paraformaldehyde for 30 min on ice, washed with PBS, 2 \times 3 min, and processed for BrdUrd staining starting with ddH₂O rinse followed by 4 N HCl as described in the Pathological Analyses.

Adenoviruses. Recombinant adenoviruses AdCMVLacZ, AdCMVEGFP, and AdCMVCre are modifications of the adenovirus-5 genome, from which the *ela* and *elb* regions required for viral replication had been deleted and replaced with *Escherichia coli LacZ* or enhanced green fluorescent protein (EGFP) or Cre driven by the CMV immediate early regulatory sequence (AdCMVLacZ, AdCMVEGFP, and AdCMVCre, respectively). Viruses purified as described (18) were titered at 10¹¹–10¹² infectious particles/ml, and frozen in small aliquots.

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⁵ The abbreviations used are: OSE, ovarian surface epithelium; EOC, epithelial ovarian cancer; Rb, retinoblastoma; CMV, cytomegalovirus; MOI, multiplicity of infection; pfu, plaque-forming unit(s); ABC, avidin-biotin-peroxidase; BrdUrd, bromodeoxyuridine.

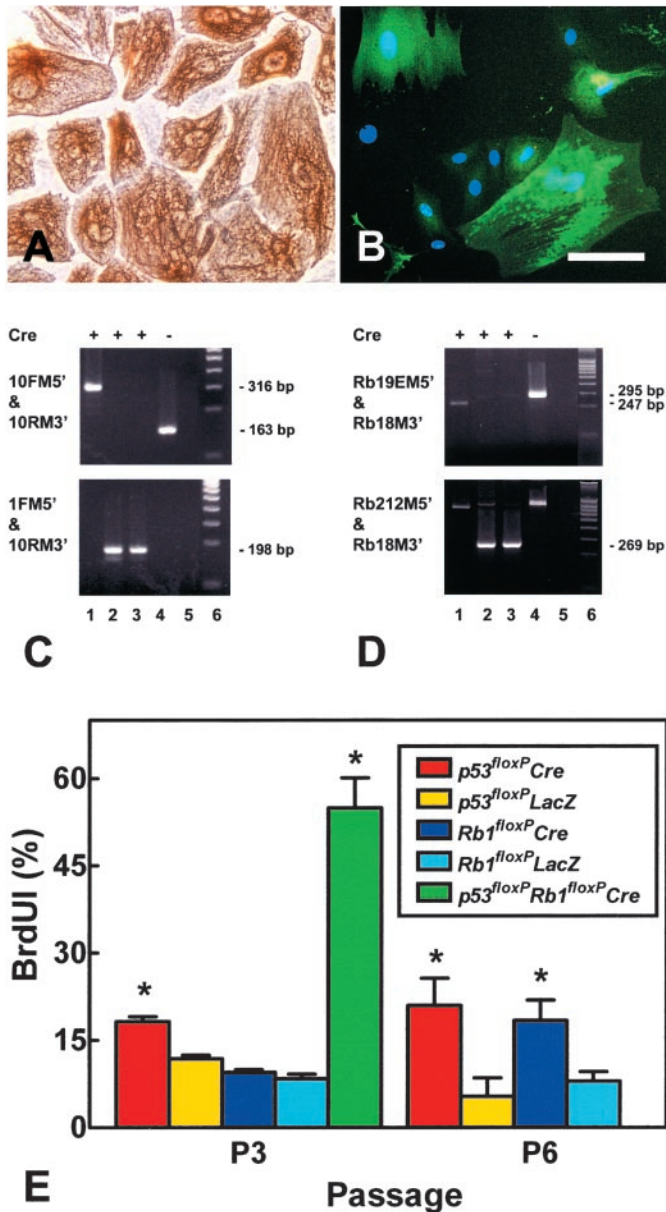


Fig. 1. Targeting OSE by recombinant adenovirus in the primary culture. **A**, cytokeratin 8 expression in OSE after three passages. TROMA-1 antibody, ABC elite method. **B**, EGFP expression (green fluorescence) in the majority of OSE 3 days after infection. Counterstaining with 4',6-diamidino-2-phenylindole (blue nuclear fluorescence). Calibration bar, **A**, 50 μ m, **B**, 100 μ m. **C** and **D**, PCR analysis of *p53* (**C**) and *Rb1* (**D**) gene structure in OSE containing either wild-type (*Lane 1*) or *flloxP/flloxP* (*Lanes 2–4*) gene 3 days after either AdCMVCre (*Cre +*) or mock (*Cre -*) infection. **C**, PCR with primers 10FM5' and 10RM3' results in 163-bp and 316-bp fragments for wild-type and floxed *p53* alleles, respectively. PCR with primers 1FM5' and 10RM3' detects 198-bp fragment diagnostic for Cre-mediated gene excision. **D**, PCR with primers Rb19EM5' and Rb18M3' result in 247-bp and 295-bp fragments for wild-type and floxed *Rb1* alleles, respectively. PCR with primers Rb212M5' and Rb18M3' detects 269-bp fragment diagnostic for Cre-mediated gene excision. The bands ~850 bp (*Lane 1*) and 900 bp (*Lanes 2* and *4*) are the result of specific amplification of exon 19 and surrounding intron sequences without and with inserted loxP sites, respectively. **C** and **D**, *Lane 5*, mock control. *Lane 6*, DNA marker. Three percent NuSieve agarose gels stained with ethidium bromide. **E**, proliferation of OSE cells containing $p53^{flloxP/flloxP}$ ($p53^{flloxP}$), or $Rb1^{flloxP/flloxP}$ ($Rb1^{flloxP}$), $p53^{flloxP/flloxP}$, or $Rb1^{flloxP/flloxP}$ ($p53^{flloxP}$, $Rb1^{flloxP}$), three (P3) and six (P6) passages after 2-h exposure to 200 MOI of either AdCMVCre (*Cre*) or AdCMVLacZ (*LacZ*). According to semiquantitative PCR analyses >90% of cells lost both functional copies of floxed genes 6 days after infection with AdCMVCre. Cells were treated with BrdUrd for 2 h and BrdUrd indices (% BrdUI; mean; bars, \pm SD) was determined. Mann-Whitney test yielded two-tailed *P*s of <0.0001 for $p53^{flloxP}Cre$ versus $p53^{flloxP}LacZ$ (18.25 \pm 0.82, *n* = 12 versus 11.84 \pm 0.62, *n* = 12) and $p53^{flloxP}Rb1^{flloxP}Cre$ versus $p53^{flloxP}LacZ$ (54.94 \pm 17.12, *n* = 11 versus 18.25 \pm 0.82, *n* = 12) at passage 3, and 0.0206 and 0.0028 for $p53^{flloxP}Cre$ versus $p53^{flloxP}LacZ$ (21.00 \pm 4.66, *n* = 11 versus 5.38 \pm 3.20, *n* = 11) and for $Rb1^{flloxP}Cre$ versus $Rb1^{flloxP}LacZ$ (18.40 \pm 3.50, *n* = 11, versus 8.00 \pm 1.6, *n* = 11), respectively, at passage 6. No significant differences were found among $p53^{flloxP}flloxP$, $Rb1^{flloxP}flloxP$, and $p53^{flloxP}Rb1^{flloxP}$ OSE, exposed to AdCMVLacZ, as well as wild-type OSE exposed to either AdCMVCre or AdCMVLacZ (data not shown).

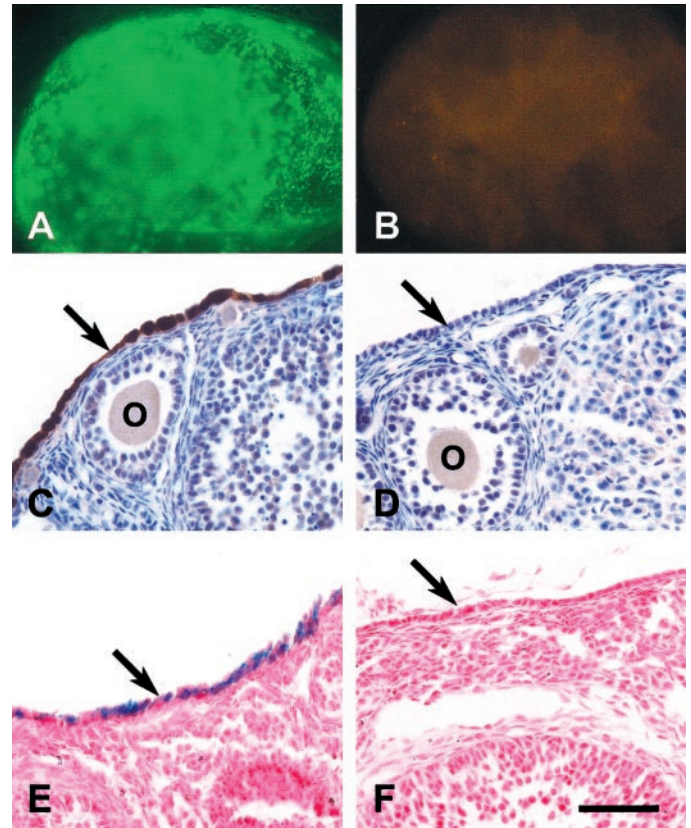


Fig. 2. Targeting OSE by intrabursal administration of the recombinant adenovirus in the mouse. Wild-type (**A–D**) and R26STOPflloxLacZ (**E** and **F**) mice were subjected to a single ovarian intrabursal injection with 5×10^7 pfu/ μ l of either AdCMVEGFP (**A**, **C**, and **F**), AdCMVLacZ (**B** and **D**) or AdCMVCre (**E**). Ovaries were collected 3 days after injection and processed immediately for fluorescence (**A** and **B**), ABC immunostaining with rabbit anti-EGFP antibody (**C** and **D**) or histochemical detection of β -galactosidase (**E** and **F**). Arrow, OSE; O, oocyte. Counterstaining with Hematoxylin (**C** and **D**), and Nuclear Fast Red (**E** and **F**). Calibration bar: (**A** and **B**) 500 μ m, (**C** and **D**) 50 μ m.

Adenovirus Administration. Pilot titration experiments demonstrated that infection at MOI 100 and 400–1000 resulted in lower infectivity or excessive cell damage, respectively (data not shown). Thus, for all of the subsequent cell culture experiments $\sim 2 \times 10^5$ OSE cells were incubated in 1 ml of serum-free medium with 4×10^7 AdCMVCre, AdCMVEGFP, or AdCMVLacZ pfu (MOI 200). After 2 h at 37°C, cells were washed twice with PBS and covered with complete medium containing 5% fetal bovine serum. Adenoviral delivery into the ovarian bursa was performed by injection with a Hamilton syringe and a 30-gauge beveled needle under the control of a dissection microscope after deep anesthesia with i.p. Avertin (2.5% v/v in 0.85% NaCl; 0.020 ml/g body weight). Ovaries were accessed via dorsal incision, the needle was inserted into the oviduct near the infundibulum toward the bursa, and adenovirus (5×10^7 pfu/ μ l in PBS) was injected. According to our preliminary titration experiments using trypan blue and AdCMVEGFP for injection to the bursa of 60-day-old FVB/N females, a 10 μ l volume is sufficient to fill the bursa entirely, yet without any significant leakage to the peritoneal cavity from the place of injection in >95% of cases. This volume was used in all of the subsequent experiments.

Ovulation and Carcinogenesis Studies. To synchronize ovulation, 60-day-old females were mated with vasectomized males, and the first day after ovulation was considered complete at midnight of the day after mating. Postnatal day 1 was considered completed 24 h after birth. AdCMVCre and AdCMVLacZ were injected into the bursa of the right and left ovary, respectively, of $p53^{flloxP/flloxP}$, $Rb1^{flloxP/flloxP}$, $p53^{flloxP/flloxP}Rb1^{flloxP/flloxP}$, and wild-type mice 1.5 days after ovulation. To exclude possible inadvertent effects of mixed genetic background, all of the mice were kept on FVB/N background.

Microdissection PCR. Frozen and paraffin sections were placed on foil attached to glass slides, stained with H&E, and evaluated under microscope. Identified lesions as well as single cells were dissected either manually or with UV laser (Laser Microdissection System; Leica) and collected into caps of

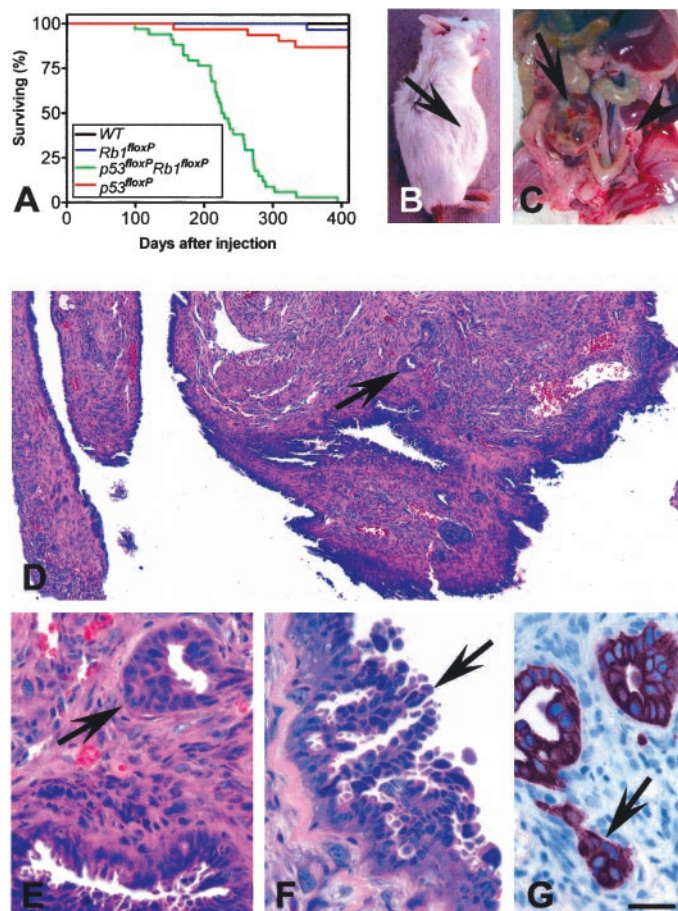


Fig. 3. Survival and pathology of mice with conditional inactivation of *p53* and *Rb1* in OSE. **A**, survival of *p53*^{floxP/floxP} ($n = 31$), *Rb1*^{floxP/floxP} ($n = 29$), *p53*^{floxP/floxP}*Rb1*^{floxP/floxP} ($n = 34$), and wild-type ($n = 32$) female mice after a single ovarian intrabursal injection of AdCMVCre. Median survival for *p53*^{floxP/floxP}*Rb1*^{floxP/floxP} mice was 227 days. **B**, abdominal distension (arrow) of the mouse with ovarian tumor. **C**, ovarian polycystic tumor (arrow) and normal ovary (arrowhead) of *p53*^{floxP/floxP}*Rb1*^{floxP/floxP} mouse 255 days after a single intrabursal administration of AdCMVCre and AdCMVLacZ, respectively, into the right and left ovary. Low (**D**) and high (**E**) magnification of ovarian serous cystic adenocarcinoma with invasive growth (arrow). **F**, neoplastic OSE cells form papillary structures (arrow). **G**, CK 8 is consistently detected in ovarian carcinoma cells arranged in invasive glandular structures and isolated groups (arrow) laying in dense fibrous tissue. **D–F**, H&E. **G**, immunohistochemical detection of CK 8 with TROMA-1 antibody, ABC elite method. Calibration bar: **B**, 21 mm; **C**, 68 mm; **D**, 180 μ m; **E–G**, 55 μ m.

Eppendorf tubes filled with lysis buffer. DNA was isolated and processed essentially as described earlier (17).

Pathological Analyses. Moribund mice in long-term experiments, as well as those to be sacrificed according to schedule, were anesthetized with avertin, and, after cardiac perfusion at 90 mm Hg with PBS followed by phosphate-buffered 4% paraformaldehyde, were subjected to gross pathology evaluation. Ovaries and other affected organs were removed, processed for preparing paraffin sections, and characterized by microscopic evaluation. Serial sections were prepared for confirmation of neoplastic invasion. Pathology studies were performed by one of us (A.Y.N.) according to existing human and mouse EOC classifications (3, 19, 20). Immunohistochemical analysis of paraffin sections of paraformaldehyde-fixed tissue was performed by a modified ABC technique (17). The antibodies to EGFP (Living colors A.v. rabbit peptide antibody; 1:100 dilution; Clontech), cytokeratin 8 (CK8 TROMA-1 antibody; 1:50, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), or BrdUrd (PharMingen; 1:100), were incubated with deparaffinized sections for 1 h at room temperature. Ten-min boiling in 10 mM citric buffer was used for antigen retrieval for detection of CK8. Rates of cell proliferation were evaluated by BrdUrd uptake according to earlier established protocols (21). At least 150 cells/field in 10 fields were scored for each tissue section. Detection of bacterial β -galactosidase was performed according to published protocols (22–24).

Statistical Analyses. InStat 3.03 and Prism 3.02 (GraphPad, Inc. San Diego, CA) software was used in this study. Survival fractions were calculated using the Kaplan-Meier method. Survival curves were compared by log rank Mantel-Haenszel tests. Means were compared by estimation of the two-tailed P with Mann-Whitney test.

Results and Discussion

To test the efficacy of recombinant adenovirus infection, primary cultures of OSE (Fig. 1A) were prepared, and cells were infected with adenovirus containing the EGFP encoding gene under control of the CMV promoter (AdCMVEGFP) at a MOI of 200 infectious particles per cell. More than 80% of OSE cells expressed EGFP 24 h after infection (Fig. 1B). Similar results were also observed in cultured OSE of *Rosa26STOP*^{floxP}*LacZ* [B6;129-Gt(ROSA)26Sor^{TM1sor}] reporter mice (14–16) after infection with AdCMVCre (data not shown). Accordingly, administration of AdCMVCre to OSE cells carrying *p53*^{floxP/floxP} and/or *Rb1*^{floxP/floxP} resulted in inactivation of the respective gene(s) (Fig. 1, C and D). According to BrdUrd incorporation assay, OSE proliferation was pronounced after the first passage after inactivation of both *p53* and *Rb1* (Fig. 1E; data not shown). Modest but statistically significant increase in proliferation of OSE was evident three and six passages after individual inactivation of *p53* and *Rb1*, respectively (Fig. 1E). Taken together, these results indicate that recombinant adenovirus efficiently infects OSE, the CMV promoter is adequate for Cre expression in these cells, Cre-mediated inactivation of floxed *p53* and *Rb1* occurs after a single administration of AdCMVCre, and simultaneous inactivation of both genes results in dramatic increase of OSE proliferation.

To evaluate the feasibility of direct targeting of OSE by recombinant adenoviruses in the mouse, we have administered AdCMVEGFP to the ovarian bursa. As determined by both fluorescence (Fig. 2, A and B) and immunostaining (Fig. 2, C and D), >90% of OSE cells expressed EGFP 1 day after injection with AdCMVEGFP. Notably no underlying stromal cells were infected. Expression ceased to be detected by the 21st day after administration, in agreement with the transient character of adenovirus infection. Similar results were obtained with the AdCMVLacZ reporter (data not shown). To evaluate Cre-loxP-mediated gene deletion, AdCMVCre was administered to OSE of *Rosa26STOP*^{floxP}*LacZ* mice (14–16). Consistent with Ad reporter experiments, *E. coli* β -galactosidase expression was detected only in OSE but to a somewhat lesser extent (about 60–80% of cells; Fig. 2, E and F). At the concentrations used no reactive hyperplasia or other pathological alterations were observed in OSE after infection with any of the applied recombinant adenoviruses. These results indicate that adenovirus can be used as an efficient vector for selective induction of temporal localized gene expression in OSE. Thus, in conjunction with the Cre-loxP system, this approach should be useful for OSE-targeted inactivation of floxed tumor suppressor genes, as well as for induction of oncogene expression in STOP^{floxP} designs such as *K-ras* (25) and *newErbB2* (26).

Both adenovirus infection efficacy and success in fixation of acquired genetic mutations increase in actively proliferating cells. Because in the adult mouse OSE proliferation is induced after ovulation, the peak of proliferation was determined in synchronized females (see “Materials and Methods”). Using BrdUrd incorporation assay, we identified the peak of proliferation at 1.5 days after ovulation (mean \pm SD, $2.5 \pm 0.3\%$; $n = 3$ versus $1.5 \pm 0.3\%$; $n = 3$ on day 1.5 and 3.5 after ovulation; $P = 0.0034$). Thus, in all of the subsequent experiments adenoviral administration was performed at 1.5 days after ovulation.

Thirty three of 34 mice (97%) with inactivation of both genes succumbed to ovarian tumors at a median of 227 days (Fig. 3A; Table 1). In agreement with our pilot experiments indicating possible leakage of AdCMVCre on injection in ~5% of cases (see “Materials and Methods”), one tumor was not associated with ovary and had features of undifferentiated neoplasm (Table 1). At the same time, 4 of 31 (13%) and 1 of 29

Table 1 Ovarian neoplasms induced by a single intrabursal administration of AdCMVCre to mice carrying conditional p53 and/or Rb1 alleles

Genotype	p53 ^{floxP/floxP}	p53 ^{floxP/floxP} Rb1 ^{floxP/floxP}	Rb1 ^{floxP/floxP}	Wild-type
Mice with neoplasms, total, % ^a	13 (4/31) ^b	100 (34/34)	3 (1/29)	0 (0/32)
Mice with ovarian neoplasms, total %	6 (2/31)	97 (33/34)	0 (0/29)	0 (0/32)
Well-differentiated serous epithelial neoplasms of the ovary, %	0 (0/2)	39 (13/33)	0 (0/0)	0 (0/32)
Poorly differentiated CK8-positive neoplasms of the ovary, %	100 (2/2)	45 (15/33)	0 (0/0)	0 (0/0)
Undifferentiated neoplasms of the ovary, % ^c	0 (0/2)	15 (5/33)	0 (0/0)	0 (0/0)
Peritoneal spreading, %	0 (0/2)	27 (9/33)	0 (0/0)	0 (0/0)
Ascites, %	0 (0/2)	24 (8/33)	0 (0/0)	0 (0/0)
Metastases in the contralateral ovary, %	0 (0/2)	15 (5/33)	0 (0/0)	0 (0/0)
Lung metastases, %	50 (1/2)	18 (6/33)	0 (0/0)	0 (0/0)
Liver metastases, %	0 (0/2)	3 (1/33)	0 (0/0)	0 (0/0)

^a Nonovarian neoplasms were not physically associated with the ovary and included poorly differentiated spindle and polymorphic cell neoplasms without any morphological or immunohistochemical features of specific differentiation, such as expression of epithelial (CK8 and Pan-cytokeratin), lymphoid (CD45R and CD3), smooth (smooth muscle actin), and striated (α -sarcomeric actin) muscle and histiocytic (F4/80) and schwannian (S100) markers. All of the neoplasms had no functional floxed gene(s) according to microdissection PCR genotyping and were likely a result of rare inadvertent leakage of the AdCre during intrabursal administration.

^b Numbers in parentheses indicate number of mice with neoplasm out of total number of mice.

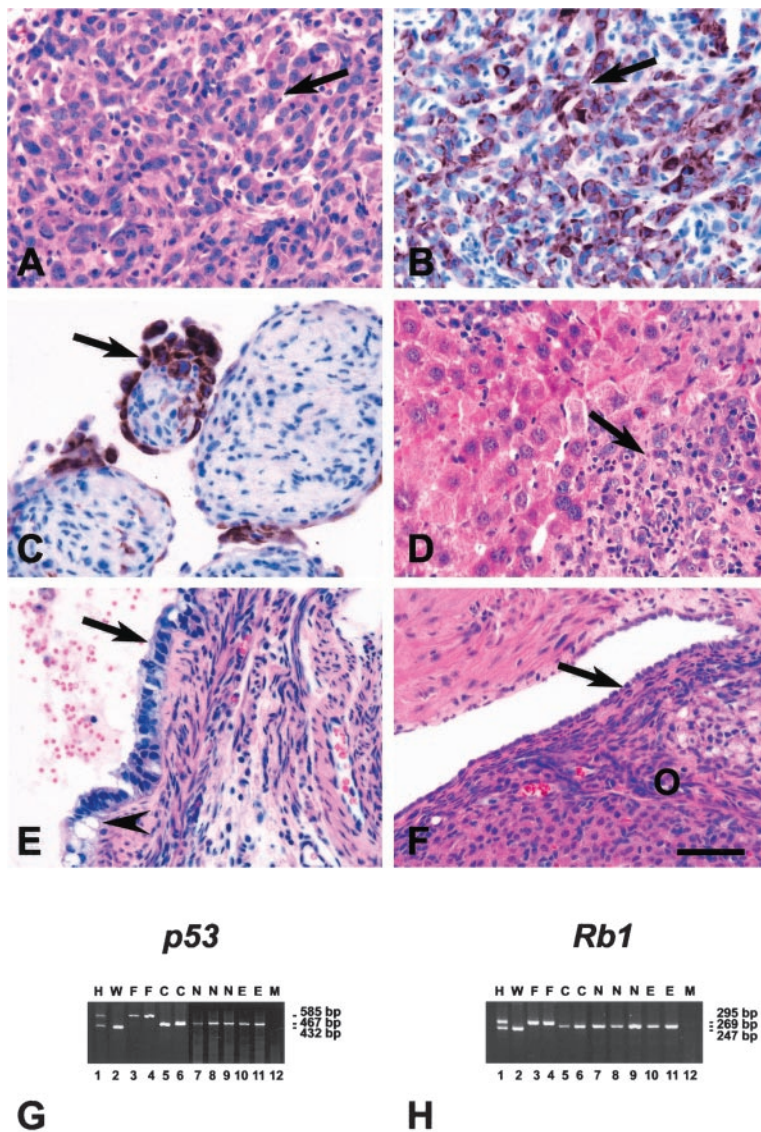
^c Neoplasms originating from the ovary but without any specific morphological or immunohistochemical features of differentiation (see comment to ^a). All of the neoplasms had loss of both floxed genes according to PCR genotyping.

(3%) mice developed tumors after Ad5Cre-mediated inactivation of floxed p53 or Rb1, respectively. Among those tumors only 2 tumors in p53^{floxP/floxP} mice arose from the ovary by 400 days after injection (Table 1). No tumors were observed after administration of AdCMVLacZ and AdCMVCre into the bursa of floxed (p53^{floxP/floxP}, Rb1^{floxP/floxP}, and p53^{floxP/floxP}Rb1^{floxP/floxP}) and wild-type mice, respectively. Grossly, the

mice had abdominal distension (Fig. 3B). On necropsy polycystic tumor masses substituted the ovary and invaded surrounding tissues (Fig. 3C). Hemorrhagic or serous ascites were observed in 24% of the mice with ovarian tumors (Table 1).

Thirteen of 33 neoplasms that developed in the ovaries of p53^{floxP/floxP}Rb1^{floxP/floxP} mice (39%) were composed of multiple serous

Fig. 4. Characterization of ovarian tumors induced by inactivation of p53 and Rb1. A and B, poorly differentiated carcinoma consisting of epithelioid cells in loose trabecular formation (arrow) containing CK8 (B). C, i.p. spreading of CK8 positive (arrow) neoplastic cells. D, liver metastasis (arrow) of poorly differentiated ovarian carcinoma. E, early dysplastic OSE lesions consisting of disorganized, ciliated (arrow) and vacuolated (arrow-head) atypical cells. F, flat OSE (arrow) 180 days after exposure to a single administration of AdCMVLacZ. A, and D–F, H&E. B and C, immunohistochemical detection of CK 8 with TROMA-1 antibody, ABC elite method. Calibration bar: A–F, 50 μ m. G and H, PCR analysis of p53 (G) and Rb1 (H) gene structure in the same sets of tissues (Lanes 1, 4, and 7–11) and primary cell cultures (Lanes 2, 3, 5, 6, F and C) samples collected from mice heterozygous (Lane 1, H), wild-type (Lane 2, W), and homozygous (Lanes 3–11) for both floxed gene without (Lanes 3 and 4, F) or with previous exposure to AdCMVCre (Lanes 5–11, C, N, and E). N and E, cells from ovarian neoplasms and early neoplastic lesions, respectively, collected by microdissection. M (Lane 12), mock control. PCR primers are as described in legend to the Fig. 1, with exception that all three primers were used simultaneously and 10RM23' primer was used instead 10RM3', resulting in 585-, 467-, and 433-bp fragments diagnostic for floxed, excised, and wild-type alleles of p53 gene, respectively.



cysts (Fig. 3D). These cysts were lined by crowded neoplastic epithelial cells with large hyperchromatic nuclei and small round to polygonal cytoplasm (Fig. 3, E and F). Neoplastic cells formed papillary structures (Fig. 3F), invaded underlying tissues (Fig. 3, D–G), and contained simple epithelium-specific cytokeratin 8 (CK8; Fig. 3G). Fifteen neoplasms (45%) were poorly differentiated and consisted of CK 8-positive epithelioid cells, which sometimes formed trabecular structures (Fig. 4, A and B). Tumors expanded i.p. (27%; Fig. 4C), formed ascites containing groups of neoplastic cells (24% of cases), and metastasized to the opposite ovary (15%), the lung (18%), and the liver (6%; Fig. 4D). Taken together, both pathology and biological behavior of induced neoplasms were remarkably similar to those of human EOC, particularly to its most common type, serous adenocarcinoma. Furthermore, in close concordance with human EOC, these neoplasms are induced in adult mice.

Microdissection PCR confirmed loss of both copies of both *Rb1* and *p53* in the neoplastic cells of all 19 of the cases tested (Fig. 4, G and H; data not shown). Notably, loss of both genes was detected in early dysplastic lesions (Fig. 4, E, G, and H). No similar alterations were observed in the other ovary exposed to *AdCMVLacZ* (Fig. 4F). Simultaneous Cre-loxP-mediated recombination may result in rearrangements between chromosomes 11 (*p53*) and 14 (*Rb1*) with potential influence on carcinogenesis. No such alterations were detected after PCR with four combinations of primers flanking *Rb1* (Rb212 and Rb18M3') and *p53* (1FM5' and 10RM3') loxP sites (data not shown). Thus, respective interchromosomal rearrangements are unlikely to have a selective advantage in this model.

Formation of a few epithelial tumors in *p53^{loxP/loxP}* mice alone indicates that p53 may be sufficient for initiation of OSE carcinogenesis. However, the complete effect of its inactivation is evident only in cooperation with alterations in the Rb pathway. These results support earlier studies which indicated that oncogenic transformation of OSE is easily achieved by cooperation of *p53* inactivation with activation of *c-myc*, *K-ras*, and *Akt* (3), or by inactivation of *p53*, *Rb*, and *PP2A* pathways by SV40 T antigen (11). Notably, both Akt and K-ras are involved in RB network signaling by regulating cyclin D1 activity (reviewed in Ref. 6). It is of note that some genetic alterations preferentially occur in specific EOC subtypes such as serous (*p53*; Refs. 4, 27), mucinous (*ras*; Refs. 27, 28), and endometrioid (*CTNNB1/β-catenin* and *P TEN*; Refs. 4, 5, 10). Interestingly, carcinomas of the serous subtype more frequently have *Rb1* loss of heterozygosity and aberrant immunostaining (8), in close agreement with the observation of serous neoplasms in our model. Additional studies shall allow understanding of particular involvement of other genetic pathways, including *c-MYC*, *AKT-2*, *PIK3CA*, *HER2/NEU*, and *EEF1A2*. A small subset of hereditary cases are associated with alterations in tumor susceptibility genes *BRCA1* and *BRCA2* (reviewed in Ref. 29). Mutations in *BRCA1* and *BRCA2* are rare in sporadic cases. However, recent studies indicate that Rb1 interacts with *BRCA1* (reviewed in Ref. 30). Taken together, our experiments have established a genetically defined and phenotypically accurate model of EOC and have provided direct genetic evidence that defects in p53 and Rb1-mediated pathways may cooperate in ovarian carcinogenesis. These results furnish both rationale and suitable modeling means for development and testing of diagnostic, therapeutic, and preventive approaches aimed at p53 and Rb1 signaling circuits in EOC.

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