The American Journal of Pathology, Vol. 183, No. 5, November 2013

The American Journal of
PATHOLOGY
ajp.amjpathol.org

TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Progesterone Signaling Inhibits Cervical Carcinogenesis in Mice

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Accepted for publication
July 18, 2013.

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Human papillomavirus is the main cause of cervical cancer, yet other nonviral cofactors are also required for the disease. The uterine cervix is a hormone-responsive tissue, and female hormones have been implicated in cervical carcinogenesis. A transgenic mouse model expressing human papillomavirus oncogenes *E6* and/or *E7* has proven useful to study a mechanism of hormone actions in the context of this common malignancy. Estrogen and estrogen receptor α are required for the development of cervical cancer in this mouse model. Estrogen receptor α is known to up-regulate expression of the progesterone receptor, which, on activation by its ligands, either promotes or inhibits carcinogenesis, depending on the tissue context. Here, we report that progesterone receptor inhibits cervical and vaginal epithelial cell proliferation in a ligand-dependent manner. We also report that synthetic progestin medroxyprogesterone acetate promotes regression of cancers and precancerous lesions in the female lower reproductive tracts (ie, cervix and vagina) in the human papillomavirus transgenic mouse model. Our results provide the first experimental evidence that supports the hypothesis that progesterone signaling is inhibitory for cervical carcinogenesis *in vivo*. *(Am J Pathol 2013, 183: 1679–1687; http://dx.doi.org/10.1016/j.ajpath.2013.07.026)*

High-risk human papillomavirus (HPV) is a small DNA tumor virus that infects the mucosal squamous epithelium and causes various malignant diseases in humans, including cancers of the cervix and oral cavity.[1,2] HPV-associated cancers are mainly caused by the oncogenic activities of E6 and E7, which interact with key cellular proteins that regulate various cellular processes, including, but not limited to, cell cycle and apoptosis.[1] Most notably, E6 promotes proteasome-mediated degradation of p53 and E7 inactivates pRb. Among more than a dozen high-risk HPV types, HPV16 is most commonly associated with cancers.[1] Transgenic mice expressing HPV16 E6 (*K14E6* mice) or E7 (*K14E7* mice) have been valuable to study a molecular mechanism of an individual viral oncoprotein in promoting cancers *in vivo*.[3,4] The expression of E6 and E7 in these mice is under the transcriptional control of human keratin 14 (*K14*) promoter, which drives transgene expression in the stratified squamous epithelia, the cell type normally infected by HPV in humans.[5,6]

Cervical cancer is the third most frequent malignancy and the fourth leading cause of cancer death in women worldwide.[7] Most of the cancer is associated with high-risk HPVs. Papanicolaou smear and HPV vaccines are effective in preventing the disease.[8] These preventive methods, however, are not readily available to women in developing countries where cervical cancer is most prevalent. The traditional treatments for the cancer (ie, surgery, chemotherapy, and radiation) have limited efficacy in treating late-stage or recurrent diseases.[9] Better understanding of cervical cancer pathogenesis will help to develop an effective therapy for the late-stage diseases.

Despite the potent tumorigenic activities of E6 and E7, HPV-positive dysplastic cervical lesions (ie, cervical intraepithelial neoplasia; CIN) often regress spontaneously, and only a minor fraction of HPV-infected women succumb to cervical cancer.[10] These observations suggest that HPV alone is not sufficient and that other cofactors are involved

Supported by University of Houston Small Grant Program and University of Houston New Faculty Award (S.-H.C.); NIH grants CA141583 (P.F.L.), R01 CA-77530 (J.P.L.), and U54 HD-0077495 (F.J.D.); and the Texas Emerging Technology Fund (Center for Nuclear Receptors and Cell Signaling).

http://dx.doi.org/10.1016/j.ajpath.2013.07.026

in cervical carcinogenesis. Studies that use the HPV transgenic mouse models have identified estrogen (estradiol, E_2) as a cofactor for HPV-associated cervical cancer.[11,12] On chronic treatment with E_2, the HPV transgenic mice but not nontransgenic control mice develop cervical cancer. This is consistent with an epidemiological finding that the long-term use of oral contraceptives increases the risk of cervical cancer in HPV-infected women.[13] Using the same mouse model, we have further found that estrogen receptor α (ERα) is necessary for the genesis and continued growth of the cancer[14,15] and that ERα expressed in stromal cells is required for the progression of the disease.[16]

Progesterone receptor (PR) is activated by its ligand progesterone (P4), which is another major female hormone. PR can be also activated by synthetic progestins such as medroxyprogesterone acetate (MPA) that is commonly used as an injectable contraceptive. PR plays a crucial role in normal physiology and pathophysiology in the hormone-responsive tissues such as the breast and the uterus.[17,18] Unlike the universal mitogenic function of ERα in those tissues, PR promotes breast cancer, whereas it inhibits endometrial cancer. The *PR* gene is also implicated as a tumor suppressor in ovarian cancer.[19] The cervix is a P_4-responsive tissue.[20] Epidemiological studies that looked at the association between the use of PR agonist MPA and the risk of cervical diseases have yielded conflicting results. Several case−control studies have shown that MPA is not a risk factor for the disease.[21−23] Other studies have suggested that the use of MPA increases the risk of the neoplastic disease in the cervix.[24−26] It has also been shown that MPA use is inversely associated with the cervical disease in HPV-positive women.[27] Therefore, a role of the P_4 signaling in human cervical cancer remains elusive. In the present study, we evaluated the effect of MPA or *PR* ablation on cancers of the cervix with the use of the HPV transgenic mice. Our results support the hypothesis that P_4 signaling inhibits carcinogenesis in the female lower reproductive tract.

Materials and Methods

Mice

K14E6, K14E7, and $PR^{LacZ/LacZ}$ (referred to as $PR^{-/-}$ herein) mice were described previously.[5,6,28] Experimental mice were generated by crossing $K14E7/PR^{+/-}$ and nontransgenic $(NTG)/PR^{+/-}$, which were obtained by mating *K14E7* (FVB) with $PR^{+/-}$ (C57BL/6 × 129Sv). Double transgenic mice were generated by mating *K14E7* males and *K14E6* females. Female progenies were genotyped by PCR. Mice were intraperitoneally injected with 0.3 mL of 12.5 mg/mL bromo-deoxyuridine (BrdU) 1 hour before euthanasia to measure cellular proliferation. All procedures were performed according to an animal protocol approved by the University of Houston Institutional Animal Care and Use Committee and the University of Wisconsin Medical School Institutional Animal Care and Use Committee.

Hormone Treatments

Groups of female mice underwent ovariectomy at 6 to 8 weeks of age and were allowed to recover for 2 weeks. They were then intraperitoneally injected with ethanol vehicle, 1 μg of E_2 alone, or 1 μg of E2 plus 1 mg of P4 for 10 days. For cancer studies, slow-release, 17β-estradiol tablets (0.05 mg for 60 days; Innovative Research of America, Sarasota, FL) were inserted subcutaneously under the dorsal skin every 2 months beginning at 4 to 6 weeks of age as previously described.[11] Groups of mice were intraperitoneally injected with 4.5 mg of MPA (SICOR Pharmaceuticals, Irvine, CA) once a month.

Tissue Processing and Histological Analysis

Female mouse reproductive tracts were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections (5 μm thick) were made throughout cervices. Every 10th slide was stained with H&E, and the worst stage of neoplastic disease in each mouse was determined as described previously.[12] Alcian Blue staining was performed according to the manufacturer's manual. Briefly, paraffin sections were deparaffinized, rehydrated, and then stained in 1% Alcian Blue solution (Electron Microscopy Sciences, Hatfield, PA) for 30 minutes and rinsed in water. Nuclei were counterstained with 0.1% nuclear fast red (Electron Microscopy Sciences) for 5 minutes.

Immunohistochemistry

Antibodies were purchased from Santa Cruz Biotechnology [Santa Cruz, CA; PR (H190), ERα (MC20)], Calbiochem (San Diego, CA; BrdU), NeoMarkers (Fremont, CA; MCM7), and Rockland Immunochemicals (Gilbertsville, PA; biotinylated anti-rabbit/mouse IgG). Immunohistochemical staining for MCM7 and BrdU was performed as previously described.[3,12,14] For PR staining, sections were blocked in 10% goat serum diluted in PBS for 30 minutes. The sections were then incubated with PR antibody (1:200 in blocking buffer) at 4°C overnight, washed in PBS, and incubated with 1:100 biotinylated goat anti-rabbit IgG for 30 minutes followed by a 30-minute incubation with ABC complex (Vector Laboratories, Burlington, CA) according to the manufacturer's instructions. The immune complex was visualized by an incubation in 3,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) dissolved in 0.5 mg/mL PBS.

Statistical Analysis

One-sided Fisher exact test and Wilcoxon rank sum test were performed with MSTAT software version 5.5 (*http://www.mcardle.wisc.edu/mstat*, last accessed April 30, 2013). Fisher exact test was used for cancer incidence, and Wilcoxon rank sum test was used for disease severity and proliferation indices.

Results

P_4 Inhibits Epithelial Cell Proliferation in the Lower Reproductive Tracts of Female Mice

The proliferation of epithelial cells in the mouse female lower reproductive tracts (ie, cervix and vagina) depends on E_2 and ERα, and PR is one of its transcriptional targets.[14,29] To determine a role of PR in epithelial cell proliferation in those tissues, we treated mice that have undergone ovariectomy with vehicle, E_2 alone, or E_2 plus P_4 for 10 days. Although the cervical epithelia of vehicle-treated mice were hypoplastic regardless of the *PR* status (Figure 1A), cervical epithelia of E_2-treated mice displayed physiological hyperplasia in both $PR^{+/+}$ and $PR^{-/-}$ mice (Figure 1A). Such hyperplasia was absent in $PR^{+/+}$ but was maintained in $PR^{-/-}$ mice when treated with E_2 plus P_4 (Figure 1A). We observed similar phenotypes in ovary-intact mice (data not shown). We also analyzed cell proliferation by measuring BrdU incorporation, which is indicative of DNA synthesis (ie, cells in S phase). Consistent with histological analyses, compared with vehicle-treated mice, E_2 significantly increased cervical epithelial cell proliferation in both $PR^{+/+}$ and $PR^{-/-}$ mice at the similar level (Figure 1, B and C). The E_2-induced epithelial cell proliferation was abrogated by P_4 in $PR^{+/+}$ but not in $PR^{-/-}$ cervices (Figure 1, B and C). Similar results were obtained in the vaginal epithelium (Figure 1D). These results indicate that the P_4—PR signaling axis inhibits E_2-induced epithelial cell proliferation in the female lower reproductive tracts.

MPA Promotes Regression of Cervical and Vaginal Diseases in HPV Transgenic Mice

Antiproliferative effect of P_4 on cervical and vaginal epithelial cells prompted us to investigate if PR agonists are effective in treating cancers in the lower reproductive tracts of female mice. We first evaluated PR status in cervical cancers arising in our mouse model and found it was expressed in all cervical cancers we evaluated (Supplemental Figure S1). We chose to use depot-MPA that slowly releases PR agonist MPA and induces hypoplasia in the vaginal epithelium of mice.[30] We first performed dosing experiments and determined that 4.5 mg of MPA induces hypoplasia in the cervical epithelia for a month (Supplemental Figure S2). We then treated ovary-intact *K14E7* single transgenic mice with E_2 for 6 months and then with E_2 plus PBS (vehicle group) or E_2 plus MPA (MPA group) for an additional 2 months. Six-month treatment with E_2 resulted in cervical cancer or CIN lesions in all mice (Supplemental Table S1; control group) similar to previously published results.[16] The vehicle group treated with E_2 for 8 months had more severe cervical diseases than did the control group ($P = 0.012$), which is consistent with previously published results.[16] Strikingly, none of the 14 mice in the MPA group had cervical cancer or CIN lesions (Supplemental Table S1). The cancer incidence was significantly different from the vehicle group ($P = 0.006$). MPA showed similar effect on vaginal diseases (Supplemental Table S1). We also generated *K14E6/K14E7* double transgenic mice and divided them into three treatment groups (Figure 2A). All mice were treated with E_2 for 6 months, and the reproductive tracts were

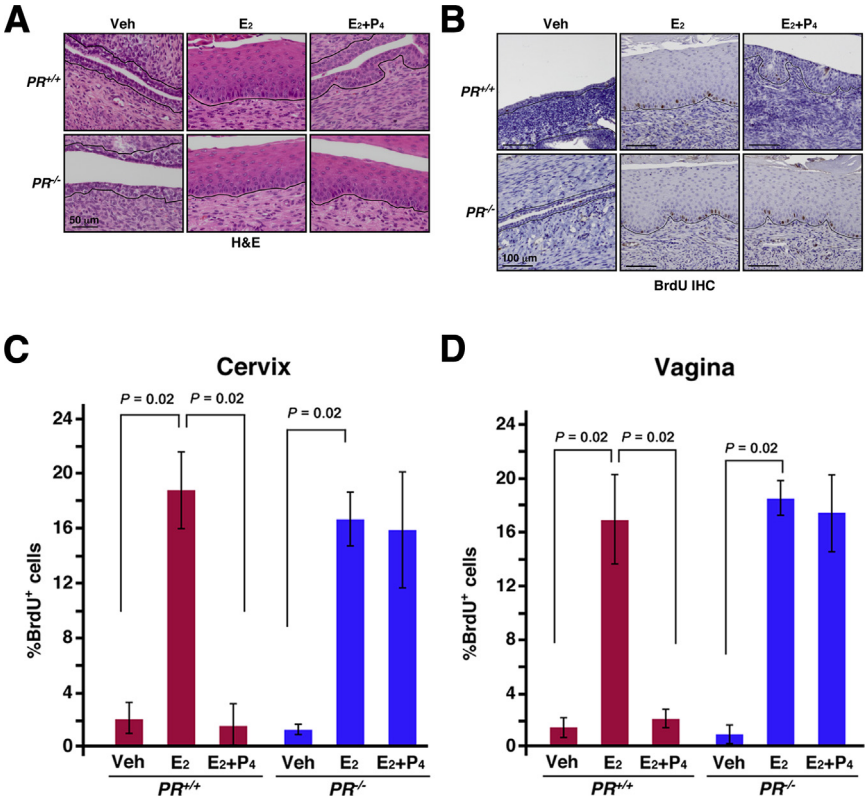

Figure 1 Activated PR inhibits E_2-induced epithelial cell proliferation in the female lower reproductive tracts. **A:** P_4 inhibits E_2-induced physiological hyperplasia in the cervical epithelia in a PR-dependent manner. Mice that underwent ovariectomy were treated with Veh, E_2, or $E_2 + P_4$. Shown are photomicrographs of representative H&E-stained cervical sections. Black lines indicate the basement membrane. **B:** E_2-induced DNA synthesis is inhibited by P_4 in $PR^{+/+}$ but not $PR^{-/-}$ cervices. Cervical sections described in **A** were stained for BrdU, and nuclei were counterstained with hematoxylin. **C:** Results shown in **B** were quantified. Five random microscopic fields per mouse cervical tissue were examined. Results are shown as means ± SEM ($PR^{-/-}$ $E_2 + P_4$ group, $n = 4$; the other groups, $n = 3$; (same number of mice were analyzed in **A**, **B**, and **D**). **D:** P_4 inhibits E_2-induced cell proliferation in the vaginal epithelia. Vaginal tissue sections obtained from mice described in **A** were stained for BrdU, quantified, and presented as described in **C**. $P = 0.02$ by Wilcoxon rank sum test. Scale bars: 50 μm (**A**); 100 μm (**B**). BrdU, bromo-deoxyuridine; E_2, estrogen; P_4, progesterone; PR, progesterone receptor; Veh, vehicle.

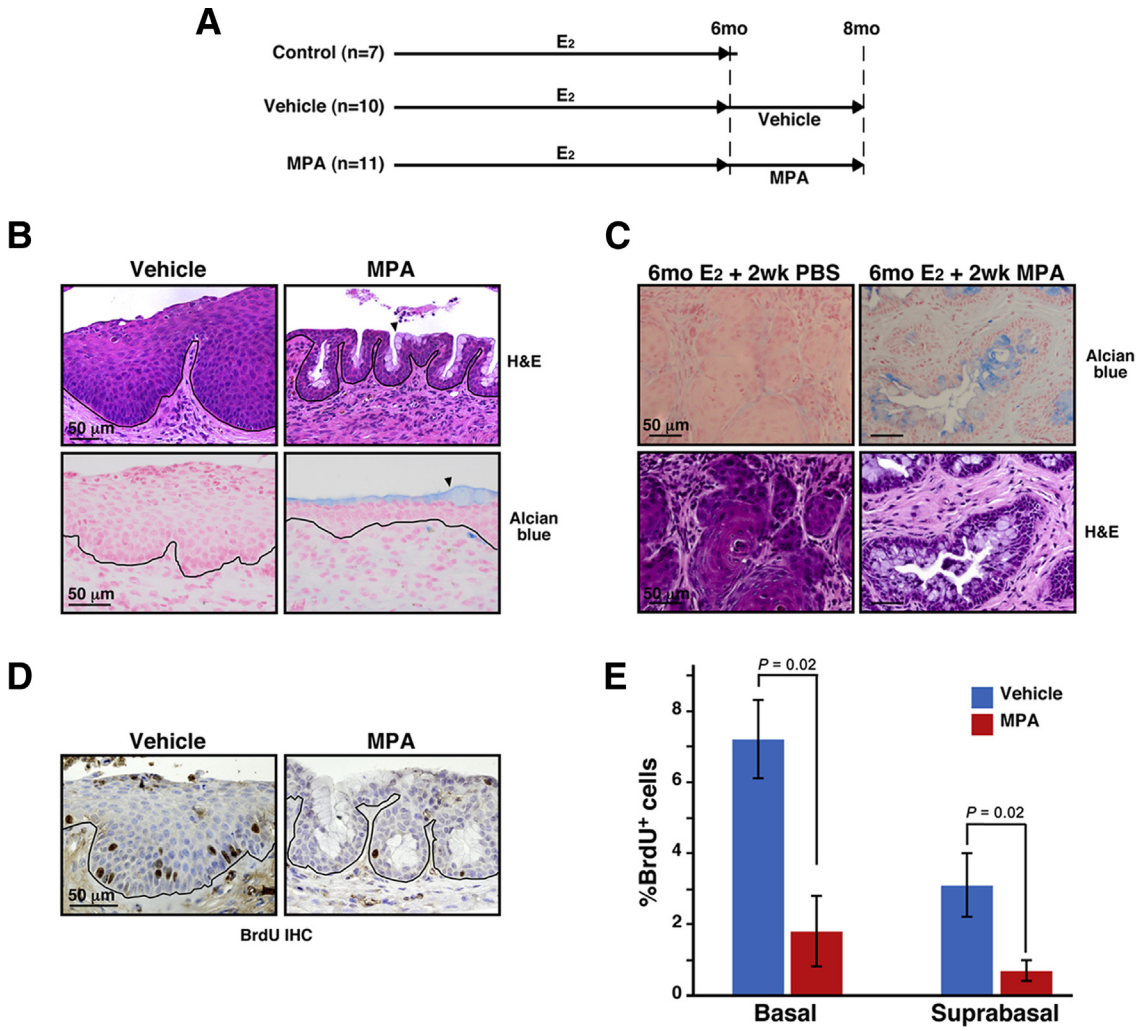

Figure 2 MPA promotes mucinification and inhibits cell proliferation in the cervical epithelium. **A**: *K14E6/K14E7* double transgenic mice were treated as depicted. **B**: MPA promotes hypoplasia and mucinification of epithelial cells in the cervix. Representative photomicrographs of H&E-stained or Alcian Blue-stained cervical sections are shown, and group size is indicated in **A**. **Arrowheads** point to mucinified cells. **Black lines** delineate the basement membrane. **C**: MPA promotes mucinification of cervical cancer. *K14E6/K14E7* double transgenic mice were treated with E_2 for 6 months and then with PBS vehicle or MPA for 2 weeks ($n = 3$ per treatment). Cancers in each group were stained with Alcian Blue. Note that Alcian Blue staining is evident in MPA-treated cancer. (**D**) MPA inhibits cervical epithelial cell proliferation. Cervical sections described in **A** and **B** were stained for BrdU, and nuclei were counterstained with hematoxylin. Black lines delineate the basement membrane. **E**: Results shown in **D** were quantified. BrdU-positive cells were counted in basal and suprabasal layers. Results are shown as means ± SEM ($n = 3$). $P = 0.02$ by Wilcoxon rank sum test. Scale bars: 50 μm (**B**—**D**). BrdU, bromo-deoxyuridine; E_2, estrogen; K14, keratin 14; MPA, medroxyprogesterone acetate.

isolated from one group immediately after 6-month treatment (control group). Another group was maintained for an additional 2 months with PBS vehicle treatment (vehicle group), and the third group was treated with MPA for 2 months (MPA group). Consistent with previously published results,[15] all mice (7 of 7) in the positive control (first) group developed cervical cancer and dysplasia (Table 1), indicating that all mice in the MPA group had the cancer as well as CIN at the time of the first MPA injection. Although the cancer was retained in 9 of 10 mice (90%) that were maintained for

Table 1 Summary of Lower Reproductive Tract Diseases in *K14E6/K14E7* Mice

Treatment group	Group size, No.	No disease, No.	Dysplasia only, No.			Cancer and dysplasia, No.	Cancer incidence, %
		Cervix (vagina)	CIN1 (VIN1)	CIN2 (VIN2)	CIN3 (VIN3)	Cervix (vagina)	Cervix (vagina)
Control	7	0 (0)	0 (1)	0 (2)	0 (1)	7 (3)	100 (42.8)
Vehicle	10	0 (0)	0 (2)	0 (2)	1 (3)	9 (3)	90 (30)
MPA	11	11 (10)	0 (0)	0 (0)	0 (0)	0 (1)	0 (9.1)

Mice were scored for the worst disease present in the cervix or, in parentheses, the vagina. Note: for Wilcoxon rank sum test (see *Statistical Analysis*), each lesion was given the following arbitrary score; no disease = 0; CIN1 (VIN1) = 1; CIN2 (VIN2) = 2; CIN3 (VIN3) = 3; cancer = 4.

CIN, cervical intraepithelial neoplasia; K14, keratin 14; MPA, medroxyprogesterone acetate; VIN, vaginal intraepithelial neoplasia.

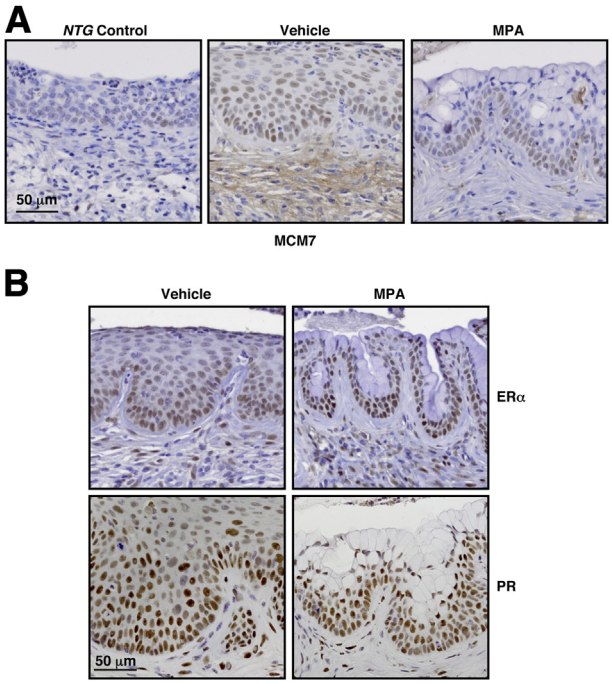

Figure 3 Function of E7 and ERα is retained in MPA-treated mice. **A:** Cervical sections from *K14E6/K14E7* double transgenic mice treated with vehicle or MPA were stained for MCM7 ($n = 3$). Nuclei were counterstained with hematoxylin. *NTG* cervical sections were used as control. **B:** Cervical sections shown in **A** were stained for ERα or PR. Nuclei were counterstained with hematoxylin. Representative photomicrographs of three tissues from each treatment are shown. ERα, estrogen receptor α; K14, keratin 14; MPA, medroxyprogesterone acetate; NTG, non-transgenic; PR, progesterone receptor.

2 months without MPA (vehicle group), none of the 11 mice that were treated with MPA (MPA group) had cervical cancer (Table 1). The difference in cervical cancer incidence between vehicle and MPA group was highly significant ($P = 3.4 \times 10^{-5}$). We also found that MPA-treated mice did not have CIN lesions (Table 1). We observed a similar therapeutic effect of MPA on neoplastic disease of the vagina (Table 1). These results indicate that MPA promotes regression of cervical/vaginal cancers as well as dysplastic lesions in the HPV transgenic mouse model.

MPA Induces Mucinification of Cervical Cancer Cells and Inhibits Cervical Epithelial Cell Proliferation

Histological analyses of the female reproductive tracts described in Table 1 found that the cervical epithelia in MPA-treated mice were hypoplastic compared with those in the vehicle control (Figure 2B). We also noted that cells at the top layer of the cervical epithelia in the MPA-treated mice had clear cytoplasm (Figure 2B). These cells were strongly stained with Alcian Blue (Figure 2B), indicating mucinification of epithelial cells. Mucinification was also evident in the vaginal epithelia of MPA-treated mice (Supplemental Figure S3). To determine whether MPA also promotes mucinification in cervical cancer cells, a subset of *K14E6/K14E7* mice treated with E_2 for 6 months was treated with MPA for 2 weeks. Although the cervical cancers in vehicle-treated mice were negative for Alcian Blue staining, those in MPA-treated mice were strongly stained (Figure 2C). The epithelial cell proliferation was significantly reduced in both basal and suprabasal layers in MPA-treated mice compared with the vehicle control (Figure 2, D and E). These results suggest that MPA induces terminal differentiation of cervical cancer cells and inhibits their proliferation.

MPA Does Not Affect Expression of HPV Oncogenes and ERα

K14 promoter drives expression of E6 and E7 in the transgenic mice.[5,6] E7 results in E2F activation by inactivating pRb and thus up-regulates E2F target genes such as minichromosome maintenance complex component 7 (*MCM7*).[3] To address whether MPA negatively affected K14 promoter in *K14E6/K14E7* double transgenic mice, we stained cervical sections for MCM7. It was evident that MCM7 expression was up-regulated in *K14E6/K14E7* mice compared with *NTG* control mice, and MPA did not alter the staining pattern (Figure 3A). ERα is required for the continued growth of cervical cancer.[15] ERα is also required for expression of PR in the murine cervix (J. Son, F.F. Mehta, S.H. Chung, unpublished data). We found that there was no appreciable difference in staining patterns of ERα or PR in the cervix of MPA-treated *K14E6/K14E7* double transgenic mice compared with vehicle-treated controls (Figure 3B). These results indicate that MPA has no effect on expression of HPV oncogenes or ERα.

Ablation of *PR* Has a Marginal Effect on Neoplasia of the Female Lower Reproductive Tracts in *K14E7* Transgenic Mice

We next sought to determine whether ablation of *PR* alone augments disease severity. We generated ovary-intact mice

Table 2 State of Lower Reproductive Tract Diseases in $PR^{+/+}$ and $PR^{-/-}$ Background

Genotypes	Group size, No.	No disease, No.	Dysplasia only, No.			Cancer and dysplasia, No.	Cancer incidence, %
		Cervix (vagina)	CIN1 (VIN1)	CIN2 (VIN2)	CIN3 (VIN3)	Cervix (vagina)	Cervix (vagina)
NTG/PR$^{-/-}$	29	19 (27)	8 (1)	2 (1)	0 (0)	0 (0)	0 (0)
NTG/PR$^{+/+}$	25	21 (25)	4 (0)	0 (0)	0 (0)	0 (0)	0 (0)
K14E7/PR$^{-/-}$	13	1 (5)	0 (5)	4 (1)	6 (0)	2 (2)	15.4 (15.4)
K14E7/PR$^{+/+}$	24	0 (6)	2 (7)	10 (9)	7 (2)	5 (0)	20.8 (0)

Mice were scored histopathologically for the worst disease present in the cervix or, in parentheses, the vagina.
CIN, cervical intraepithelial neoplasia; K14, keratin 14; NTG, non-transgenic; PR, progesterone receptor; VIN, vaginal intraepithelial neoplasia.

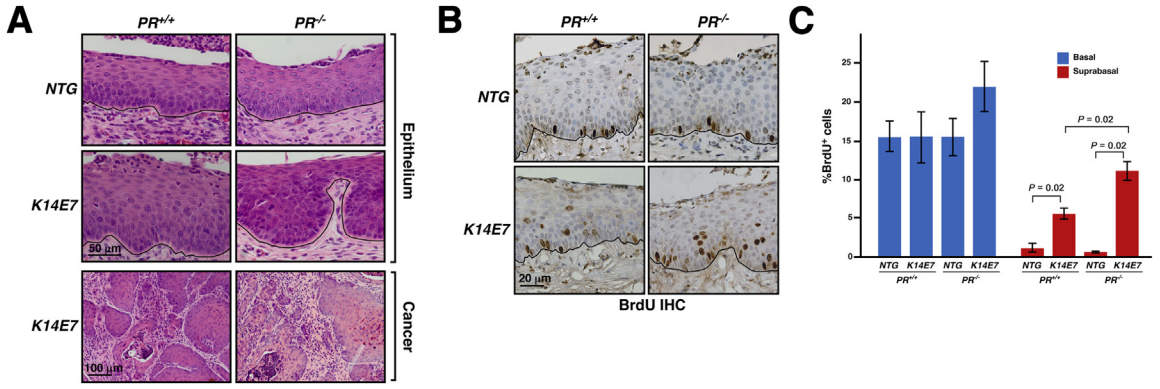

Figure 4 Evaluation of the cervical epithelia and cancer in mice treated with E_2 for 6 months. **A**: Photomicrographs of representative H&E-stained cervical epithelia and cancers in indicated genotypes. Number of mice is indicated in Table 2, and **black lines** indicate the basement membrane. **B**: *PR*-deficiency increases the suprabasal cell proliferation induced by E7. Cervical sections from indicated genotype were stained for BrdU, and nuclei were counterstained with hematoxylin. **Black lines** delineate the basement membrane. The suprabasal layer is above the basal layer, the bottom of the epithelia. **C**: Results shown in **B** were quantified by counting five random microscopic fields per mouse. BrdU-positive cells were quantified in basal and suprabasal layer separately. Results are shown as means ± SEM ($n = 3$). $P = 0.02$ by Wilcoxon rank sum test. Scale bars: 50 μm (*K14E7/PR*$^{+/+}$ epithelium, **A**); 100 μm (*K14E7/PR*$^{+/+}$ cancer, **A**); 20 μm (**B**). BrdU, bromo-deoxyuridine; E_2, estrogen; K14, keratin 14; NTG, non-transgenic; PR, progesterone receptor.

with four different genotypes (*NTG/PR*$^{+/+}$, *NTG/PR*$^{-/-}$, *K14E7/PR*$^{+/+}$, *K14E7/PR*$^{-/-}$) and treated them for 6 months with E_2. The severity of overall cervical disease state ($P = 2.6 \times 10^{-10}$) and the cancer incidence ($P = 0.02$) were significantly increased in *K14E7/PR*$^{+/+}$ mice compared with *NTG/PR*$^{+/+}$ control mice (Table 2). The cervical cancer incidence in *K14E7/PR*$^{+/+}$ mice was 20.8%, which is lower than that on FVB background[12] but similar to that on a mixed genetic background.[3,16] Although cervical cancer was absent, overall cervical disease severity in *NTG/PR*$^{-/-}$ mice was worse than that in *NTG/PR*$^{+/+}$ mice ($P = 0.05$), consistent with the idea that PR inhibits cervical neoplasia. Cervical cancer incidence (20.8% versus 15.4%; $P = 0.53$) and disease severity ($P = 0.46$), however, were comparable between *K14E7/PR*$^{+/+}$ and *K14E7/PR*$^{-/-}$ mice (Table 2). The severity of vaginal disease was comparable between *NTG/PR*$^{+/+}$ and *NTG/PR*$^{-/-}$ ($P = 0.09$) as well as between *K14E7/PR*$^{+/+}$ and *K14E7/PR*$^{-/-}$ ($P = 0.18$). Histopathology of cervical cancers and epithelia in *K14E7/PR*$^{+/+}$ and *K14E7/PR*$^{-/-}$ mice was indistinguishable (Figure 4A). These results indicate that the deletion of *PR* alone has a marginal effect on the development of cancers in the female lower reproductive tracts of *K14E7* mice under the condition used in this experiment (ie, E_2 treatment for 6 months).

Ablation of *PR* Increases E7-Induced Suprabasal Cell Proliferation and Squamous Cell Differentiation in the Female Reproductive Tracts

One of the hallmarks for E7 expression is the increased suprabasal cell proliferation in the squamous epithelia.[3] We compared cervical epithelial cell proliferation in all four groups described in Table 2. No significant differences were observed in basal cell proliferation among the study groups, and suprabasal cell proliferation was significantly increased in *K14E7/PR*$^{+/+}$ and *K14E7/PR*$^{-/-}$ compared with *NTG/PR*$^{+/+}$ and *NTG/PR*$^{-/-}$, respectively (Figure 4, B and C). More importantly, the suprabasal cell proliferation in *K14E7/PR*$^{-/-}$ mice was significantly increased compared with that in *K14E7/PR*$^{+/+}$ mice (Figure 4C). The cervical transformation zone within the endocervix is composed of squamous and columnar epithelial cells, and E7 augments aberrant squamous differentiation therein.[14] Both columnar and stratified squamous epithelia were present in the cervices of *NTG/PR*$^{+/+}$ and *K14E7/PR*$^{+/+}$ mice (Figure 5A). We noted, however, that squamous cells occupied the entire cervical epithelia in *NTG/PR*$^{-/-}$ and *K14E7/PR*$^{-/-}$ mice (Figure 5A). It is also worth noting that uterine luminal and glandular epithelia of *NTG/PR*$^{-/-}$ and *K14E7/PR*$^{-/-}$ mice also displayed extensive squamous differentiation (Figure 5B). No squamous epithelial cells were observed in the uteri of *NTG/PR*$^{+/+}$ and *K14E7/PR*$^{+/+}$ mice. These results indicate that PR inhibits E7-mediated suprabasal cell proliferation as well as squamous epithelial differentiation in the cervix, which are associated with cervical cancer.[3,14]

Discussion

E_2, P_4, and their respective receptors have been well documented to contribute to the pathogenesis of women's cancers, including those of the breast, the ovaries, and the endometrium.[18,31] They have been also suspected to modulate cervical carcinogenesis in conjunction with HPV.[32] It has been reported that E_2 and ERα are required for the development of cervical cancer in HPV transgenic mouse models.[12,14,16] On the contrary, a role of P_4 and PR in the pathogenesis of cervical disease is poorly understood. In this study, we provide experimental evidence to suggest that P_4 signaling inhibits cervical carcinogenesis. i) P_4

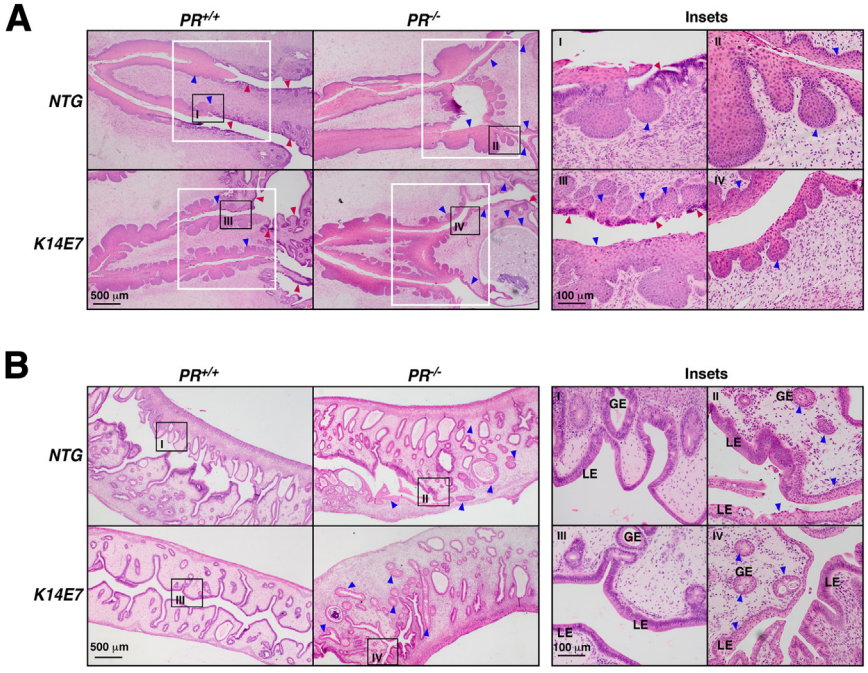

Figure 5 *PR*-deficiency promotes aberrant squamous differentiation in the female reproductive tracts. Representative photomicrographs of H&E-stained tissue sections from mice indicated in Table 2 are shown. **A:** Low-magnification photomicrographs of the endocervix and the lower uterus and the corresponding high-magnification photomicrographs of numbered **insets.** Equivalent endocervical area in each genotype is enclosed in **white boxes**. The **right side** of each **white box** is the uterus. **Blue** and **red arrowheads** point to squamous and columnar epithelia, respectively. Note that the columnar epithelia are absent in the endocervix and that the squamous epithelia are present in the uteri of *NTG/PR*$^{-/-}$ and *K14E7/PR*$^{-/-}$ mice. **B:** Low-magnification photomicrographs of the uteri and the corresponding high-magnification photomicrographs of numbered **insets**. **Blue arrowheads** indicate the squamous epithelium. Note that the squamous epithelia are evident in LE and GE of *NTG/PR*$^{-/-}$ and *K14E7/PR*$^{-/-}$ mice. Scale bars: 500 μm (*K14E7/PR*$^{+/+}$, **A** and **B**); 100 μm (**insets III**, **A** and **B**). GE, glandular epithelium; K14, keratin 14; LE, luminal epithelium; NTG, non-transgenic; PR, progesterone receptor.

inhibited proliferation of cervical epithelial cells (Figure 1). ii) A PR agonist MPA promoted regression of cervical cancer and dysplasia (Table 1 and Supplemental Table S1). The fact that *K14E7/PR*$^{-/-}$ mice had cervical cancer incidence similar to *K14E7/PR*$^{+/+}$ mice (Table 2) is not necessarily contradictory to this conclusion. Remember, that the extent of hormone receptor activation depends on the concentration of its ligands.[33] In other words, low concentration of a ligand activates it weakly, and high concentration does so more strongly. Ovary-intact mice produce P_4 at a low level when treated with E_2.[34] Consistently, mice that have undergone an ovariectomy and mice with intact ovaries display similar levels of basal epithelial cell proliferation in the cervix when treated with E_2 (S.H. Chung, unpublished results). E_2 treatment regimen used in this study keeps mice in continuous estrus in which endogenous P_4 levels are low.[11,35] It is conceivable that a weak PR activation under our cancer study condition (ie, chronic E_2 treatment) is not sufficient to suppress cervical carcinogenesis but can inhibit aberrant squamous differentiation (Table 2 and Figure 5).

Abnormal Squamous Differentiation in the Female Reproductive Tracts of *PR*$^{-/-}$ Mice

The cervix is composed of columnar epithelium and stratified squamous epithelium, which are demarcated at the squamo-columnar junction in the transformation zone of the endocervix.[11] Although it is poorly understood how the two different epithelial cell types are maintained at the junction, an aberrant squamous differentiation called atypical squamous metaplasia is associated with cervical squamous carcinomas.[14,36] We found that *PR* ablation resulted in the absence of columnar epithelium in the cervix (Figure 5A). It also promoted squamous differentiation in the uterus (normal uteri are devoid of squamous cells) (Figure 5B). These results indicate that PR is crucial in determining the fate of epithelial cells in female upper reproductive tracts. In this regard, it is intriguing that postnatal deletion of wingless-type MMTV integration site family, member 4 (*Wnt4*) or ectopic expression of constitutively active smoothened homolog (*Smo*) results in squamous differentiation in the uterus.[37,38] It will be interesting to determine whether these genes are involved in the regulation of squamous differentiation in the cervical transformation zone and/or cervical carcinogenesis.

Tumor Suppressive Activity of PR in Cervical Cancer

A function of P_4 requires *PR*, which is an ERα target gene in the female reproductive tract.[29] Likewise, function of PR and ERα depends on their ligands, P_4 and E_2, respectively.[18,29] During the reproductive cycle in women and mice, an E_2 surge is followed by a P_4 surge; the former thickens the epithelium, and the latter wanes it in the female reproductive tract.[35,39] In other words, the ERα signaling promotes epithelial cell proliferation, and the PR signaling inhibits it in the same tissues.[18] We postulate that the P_4 surge (ie, maximal activation of PR) constantly counteracts cervical carcinogenesis in the context of the menstrual cycle. If this were correct, the loss of PR expression will be advantageous for cells to become cancerous. It has been shown that CIN grade III lesions are more likely to co-express ERα, PR, and Bcl-2 than cervical cancer, suggesting that CIN grade III positive

for these three factors is less likely progress to cervical cancer in women.[40] In addition, loss of heterogeneity at four microsatellite markers, one of which is at the vicinity of the *PR* locus, is significantly associated with CINs that are prone to persist or progress.[41] Furthermore, it has been observed that most human cervical cancers do not express PR.[42,43] Alternatively, persistent estrogenic stimulation (eg, E_2-only oral contraceptives) will inhibit the P_4 surge; thus, PR cannot suppress cervical cancer efficiently; perhaps *PR* loss will have no benefit to disease progression under such a condition. Consistently, PR expression was retained in cervical cancers arising in the HPV transgenic mice treated with E_2 for 6 months (Supplemental Figure S1). On the basis of the anti-cervical cancer effect of MPA in a mouse model (Table 1) and the published analyses on PR status in neoplastic disease of the cervix in women,[42,43] we hypothesize that PR functions as a tumor suppressor in the context of human cervical cancers such as in endometrial cancer.[18]

Potential PR Target Genes Crucial for Its Tumor Suppressor Function in the Cervix

P_4 is required for regulation of most PR target genes in the murine uterus.[44] Those target genes have been identified in mice that underwent an ovariectomy and then treated only with P_4.[44] However, PR is undetectable in the cervix of mice that underwent an ovariectomy, and E_2 treatment induces its expression in the same tissue (J. Son, F.F. Mehta, and S.H. Chung, unpublished results).[45] In other words, mice that underwent an ovariectomy need to be treated with E_2 plus P_4 to identify PR target genes in the cervix. Therefore, it is unclear which PR target genes identified in the uterus are relevant to the disease phenotype in the lower reproductive tracts. Nonetheless, we note several PR target genes [eg, heart and neural crest derivatives expressed transcript 2 (*Hand2*), mitogen-inducible gene 6 protein homolog (*Mig6*), myelocytomatosis oncogene (*Myc*)] that are linked to suppression of carcinogenesis. A transcription factor Hand2 represses the expression of stromal fibroblast growth factors that mediate E_2-induced cell proliferation in the uterine epithelia.[46] This finding is potentially relevant to our previous results showing that stromal ERα is required for cervical neoplasia.[16] Mig6 is a tumor suppressor that inhibits epidermal growth factor receptor-mediated skin cancer and E_2-dependent endometrial cancer.[47,48] It is intriguing that ERα is hyperactive in *Mig6*-deficient uterine tissues.[48] Although it is counterintuitive that PR activates *Myc*, it may be relevant to the tumor suppressive activity of PR in the lower reproductive tracts. It has been shown that hyperactivation of Myc promotes terminal differentiation of keratinocytes in a cyclin E-dependent manner.[49,50] MPA promoted mucinification of cervical epithelial cancer cells, which is a terminal differentiation (Figure 2B). We postulate that up-regulation of *Hand2* and/or hyperactivation of *Myc* contribute, at least in part, to suppression of cervical cancer by PR.

In summary, we showed that PR agonist MPA regresses PR-positive cervical cancers arising in the HPV transgenic mouse model. Although this may not be applicable to most patients with cervical cancer (most human cervical cancers are negative for PR), our results provide a unique model system to study cross talk between the E_2 signaling and the P_4 signaling in the context of a cancer *in vivo*.

Acknowledgments

We thank Dr. Paul F. Lambert (University of Wisconsin-Madison) for guidance and support for this project and Dr. Roger E. Price (Baylor College of Medicine) for his consultation on histopathology.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.07.026*.

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