Cell–Type–Specific Requirement of Progesterone Receptor for Suppression of Cell Proliferation and Carcinogenesis in the Cervix

by

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ABSTRACT

Cervical cancer is the third most leading cause of cancer deaths in women worldwide. Persistent infection with high–risk human papillomaviruses (HPVs) is causally associated with cervical cancer. High–risk HPV E6 and E7 oncoproteins promote cell proliferation by inactivating p53 and pRb cellular tumor suppressors, respectively, which accelerates cervical carcinogenesis. However, HPV is not sufficient for cervical cancer. Epidemiologic evidence suggests that females sex hormones contribute to cervical carcinogenesis. Consistently, chronic estrogen (E_2) treatment promotes cervical carcinogenesis in HPV transgenic mouse models expressing *HPV16–E6* and/or *HPV16–E7*. Our lab has demonstrated that synthetic progestin medroxyprogesterone acetate (MPA) prevents and regresses cervical cancer in the HPV transgenic mouse model. Our lab also has demonstrated that stromal estrogen receptor alpha (ERα) mainly mediates pro–tumorigenic action of E_2.

The goal of my study is to determine roles of epithelial and stromal progesterone receptor (PR) in suppression of cell proliferation and cervical cancer. In Chapter 1, I investigate whether deletion of PR promotes cervical carcinogenesis without chronic E_2 treatment. I found that ablation of PR expression in the cervical epithelium sensitizes HPV transgenic mice to spontaneous cervical carcinogenesis. I also found that spontaneous cervical cancers mimic ERα and PR status in cervical cancer patients, which provides a platform to study ERα–negative and PR–negative cervical cancer. In Chapter 2, I determine roles of stromal and epithelial PR in regulation of cervical epithelial cell proliferation and survival in different E_2 concentrations. I found that both epithelial and stromal PR are required for P_4 to exert its anti–proliferative and pro–apoptotic actions regardless of E_2 concentrations. Our lab has demonstrated that epithelial PR is required for cervical

cancer regression by MPA. These observations suggest that stromal PR is also required for progestin therapy to work. In other words, MPA would be effective in treating cervical cancer expressing PR in both cancer and surrounding stroma. My findings further support that PR is a tumor suppressor in cervical cancer and provide a biomarker for patient selection for a potential clinical trial.

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I. INTRODUCTION

HPV16 contributes to more than 50 percent of cervical cancer cases (1,2). While persistent infection with high–risk HPV is necessary, it is not sufficient and other cofactors are required for cervical cancer (3,4). Several lines of evidence implicate female hormones in the devastating gynecological cervical cancer (5-7). Individual roles of E_2 and P_4 in the malignancy, however, are poorly understood. Studies using HPV transgenic mice have provided clues (8).

We have been using *K14E6* and *K14E7* HPV–transgenic mouse models that are engineered to express *HPV16 E6* and *E7* oncogenes, respectively, in stratified squamous epithelia. In *K14E6/K14E7* double transgenic mice, chronic E_2 treatment and its estrogen receptor α (ERα) are necessary for cervical carcinogenesis (8-11). Maintaining the cancer–bearing mice for 3 additional months without E_2 treatment after E_2 treatment for 6 months has resulted in decreased tumor multiplicity and tumor size, indicating that exogenous E_2 is required for continued growth of cervical cancer (11). Selective ER downregulator ICI 182,780 (i.e., fulvestrant) or selective ER modulator raloxifene is efficient in treating cervical intraepithelial neoplasia (CIN, cervical precancer) and cervical cancer in *K14E6/K14E7* mice (12). Unlike ERα–positive breast cancer, stromal ERα, rather than epithelial ERα, mainly mediates the oncogenic functions of E_2 in the cervix (13,14). In opposition to E_2, P_4 inhibits epithelial cell proliferation and promotes apoptosis in a PR–dependent manner (15,16). In the cervix, transcriptional activation of *Pgr* requires E_2 and ERα (16). Epithelial PR is required for P_4 to antagonize E_2–induced cell proliferation and cell survival in cervical epithelium (16). While a treatment of *K14E6/K14E7* mice with MPA regresses cervical cancer in a PR–dependent manner, cervical cancer recurs when MPA treatment is discontinued (15,17). The recurrent cancers retain PR expression but do not regress upon MPA

therapy (17). MPA also prevents CIN from progressing to invasive cervical carcinoma, which requires PR (18). These data suggest that E_2 and ERα are pro–tumorigenic, but P_4 and epithelial PR are anti–tumorigenic in cervical cancer. Goals of my studies are [1] to determine whether the ablation of PR function promotes spontaneous cervical carcinogenesis in the absence of exogenous E_2 and [2] to characterize roles of stromal PR in P_4–mediated suppression of cervical epithelial cell proliferation and survival.

II. BACKGROUND

A. Human Papillomaviruses

HPV and its life cycle

Human Papillomaviruses (HPVs) are small, non–enveloped viruses with a circular dsDNA genome that is approximately 8 kilobase pairs in size (19,20). More than 200 HPV types have been identified, and they are classified into cutaneous and mucosal types (21). Cutaneous HPVs cause benign warts and non–melanoma skin cancers. Mucosal HPVs are transmitted through sexual contacts (19,21). The majority of sexually active women and men are likely to be infected with a mucosal HPV type during their lifetime (22). Mucosal HPV types are further divided into low–risk and high–risk depending on their propensity to cause cancer. The low–risk HPVs such as HPV6 and HPV11 cause genital warts. High–risk HPVs such HPV16 and HPV18 cause invasive cancers (19,21). High–risk HPVs are responsible for the vast majority of cervical and oropharyngeal cancers and approximately 50% of anal cancer, penile cancer, vaginal cancer and vulvar cancer (20). In particular HPV16 causes almost all oropharyngeal cancers and approximately 50% of cervical cancers (1).

High–risk HPVs encode 8 open reading frames including 6 early genes and 2 late genes and a long control region (LCR) (Figure 1). In undifferentiated basal epithelial cells, six viral regulatory proteins (i.e., E1, E2, E4, E5, E6, and E7) are expressed from the early region, while two structural viral capsid proteins (i.e., L1 and L2) are expressed from the late region in differentiated suprabasal epithelial cells (19). The LCR region contains the origin of replication and multiple transcription binding sites that include four binding sites for the viral E2 protein (19). E1 is a DNA helicase necessary for viral DNA replication: it interacts with cellular DNA

replication factors such as DNA polymerase α, replication A proteins (RPA), topoisomerases I and II, nuclear proliferation cell antigen (PCNA), and the replication factor C (RFC) (23). E2 is a transcription factor regulating transcription of viral and cellular genes. It also regulates viral DNA replication. Binding of E2 to the promoter–proximal elements inhibits E6 and E7 expression. E2 tethers HPV genome to the host chromosome by interacting with the cellular bromodomain protein Brd4 to segregate HPV genome during mitosis (24). *E2* gene is usually deleted during an integration of the HPV genome into the host chromosome in cervical cancer cells (25). E4 is associated with cytokeratin filament collapse, which facilitates the release of viral particles (26). E5 induces mitogen activated protein kinase activity, which enhances cellular responses to growth factors (20,21). E5 is also involved in transformation and immortalization in cooperation with E6 and E7 oncoproteins. E6 and E7 oncoproteins associate with the tumor suppressor p53 and pRB, respectively (27,28). Their detailed functions will be described in a separate section. L1 and L2 encapsulate the viral genome and interact with heparan sulfate proteoglycans and $\alpha_v\beta_4$ integrin on the basement membrane for viral entry into host cells (21,29).

The cervical squamous epithelium consists of a single undifferentiated, proliferating basal cell layers, non–proliferating suprabasal cell layers, and terminally differentiated, keratinized cell layers (30). HPV life cycle is closely associated with the differentiation of the infected host cells (Figure 2). HPV particles get an access to the stratified squamous epithelium through minor abrasions and exclusively target mitotic basal epithelial cells for productive infection. The viral episome is maintained in the nucleus of basal cells at a low copy number. In the suprabasl layers of infected epithelium, E7 promotes cell proliferation to amplify the viral genome (HPV uses cellular DNA polymerase for replications) at high levels. The amplified viral genomes are packaged into virions, and they are released (19,31).

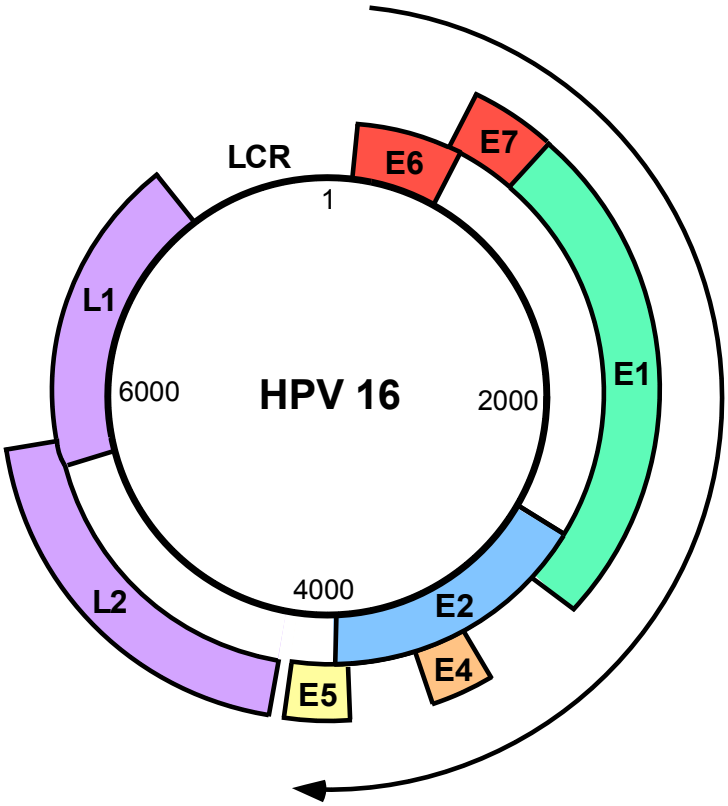


Figure 1. Schematic representation of the *HPV16* genome
The early genes (E) occupies about 4 kilobase pairs of the genome, while the late genes (L) are about 3 kilobase pairs. Noncoding long control region (LCR) occupies about 1 kilobase pairs of the genome. The viral genes are transcribed in a single direction indicated by an arrow. Modified from (32).

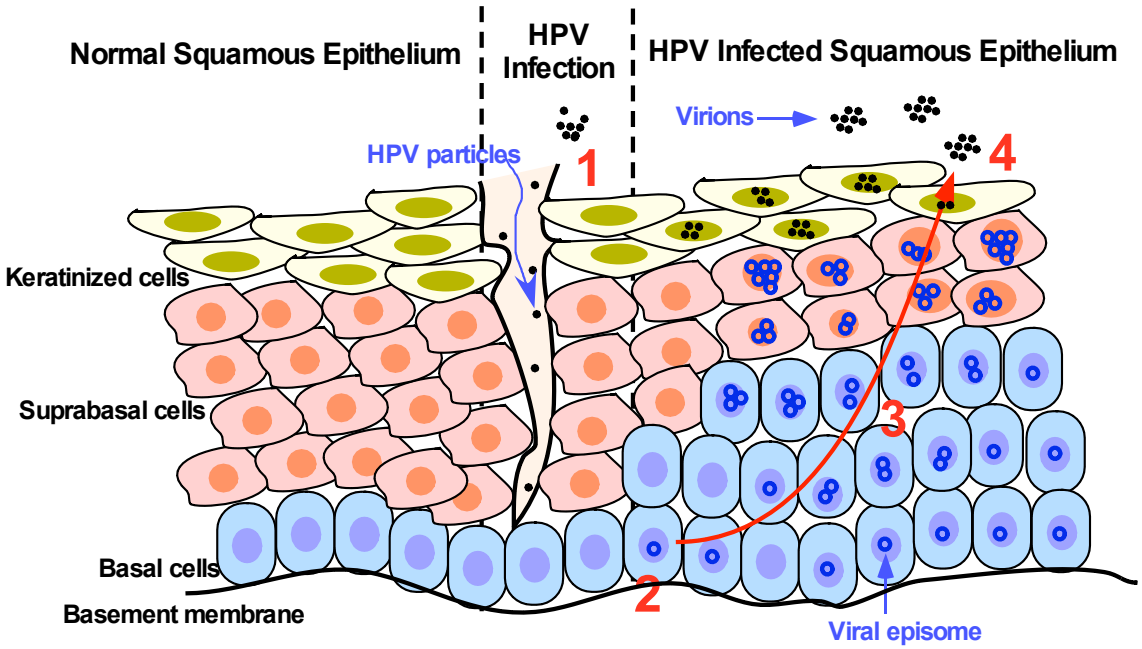


Figure 2. HPV life cycle
HPV life cycle is depicted: (1) HPV infects the basal epithelial cells though micro evasions. (2) Viral episomes are maintained at a low copy number in the basal epithelium. (3) Viral episomes are amplified in aberrantly proliferating suprabasal cells. (4) Episomes are encapsulated in virions, and virions are released. Blue circles and black dots indicate viral genomes and HPV virions, respectively. Modified from (31).

Cellular functions of HPV E6 and HPV E7 oncoproteins

High–risk HPV E6 proteins consist of approximately 150 amino acids and have two zinc–finger motifs (28). Among E6–interacting cellular proteins, p53 tumor suppressor is the most well–known target. The p53 protein promotes cell apoptosis and cell cycle arrest in response to cellular stresses. The cellular activity of p53 is tightly regulated by one of its transcriptional targets, E3–ubiquitin ligase Mdm2, which targets p53 for proteasome–mediated degradation (33). High–risk HPV E6 interacts with E6AP ubiquitin ligase, recruits p53 to the complex, and induces proteasomal degradation of p53 (28,34). Both high–risk and low–risk HPV E6 can interact with p53, but via different domains on p53. The binding of low–risk HPV11–E6 and p53 itself does not induce the degradation of p53 due to weak and rare interaction with E6AP, but rather it can sequester p53 in the cytoplasm and promote cell apoptosis (35,36). HPV E7 promotes aberrant proliferation by inhibiting pRB, which results in accumulation of p53 if E6 is not expressed (8,20). Thus, both E6 and E7 are required for continued cell proliferation. In the absence of direct interaction with p53, HPV E6 can regulate gene expressions of transcriptional targets of p53 by composing a complex with transcriptional co-activators p300/CBP (37,38). HPV E6–induced malignant progression is not limited to the disruption of p53–dependent pathways. Cellular immortalization is a characteristic of malignant cells. Normal somatic cells do not express telomerase, thus they have limited proliferation capacity. However, HPV16–E6 activates telomerase in host cells by transcriptionally upregulating hTRET, a catalytic subunit of telomerase enzyme complex, and eventually promotes immortalization of human epithelial cells (39). Upregulated telomerase activity is notable in cervical carcinomas and high–grade cervical intraepithelial lesions (CINs), cervical precancer (40). Oncogenic HPV E6 also disrupts cell apoptosis pathways. Bak and Bax, the Bcl–2 apoptotic protein family members, induce cell

apoptosis through mitochondrial pathways. Not only high–risk HPV16–E6 and HPV18–E6 but also low–risk HPV11–E6 binds to Bak, which promotes proteasome–mediated degradation of Bak protein *in vivo* (41,42). However, high–risk HPV E6 shows more efficient interaction with Bak than HPV11–E6 does (42). Ultraviolet B irradiation induces apoptosis in human epidermal keratinocytes by upregulating Bak. Ultraviolet B–induced apoptosis is inhibited by cutaneous HPV E6, a potential mechanism of HPV–induced skin cancer (43). Targeted inhibition of HPV18–E6 in cervical cancer cells activates Bax, inducing cell apoptosis in a PUMA–dependent mechanism *in vitro* (44).

HPV E7 proteins are about 100 amino acids long. The major function of HPV E7 is reactivating DNA synthesis in differentiated host cells to aid viral DNA replication (28,45). HPV E7 expression itself is enough to reactivate DNA synthesis of host cells in differentiated keratinocytes by promoting the expression of proliferating–cell nuclear antigen (PCNA) (45). HPV E7 physically interacts with Smad proteins regulating transforming growth factor–β (TGF–β) signaling, which prevents TGF–β mediated inhibition of cell proliferation *in vitro* (46). E7–defective HPV 16 fails viral replication, in part, because expression of capsid protein L1 is suppressed (47). HPV E7 also induces the transformation of primary keratinocytes and rodent fibroblast cell lines (48,49). The transformation of primary rodent cells requires HPV E7 in cooperation with activated Ras oncoprotein (50). HPV E7 contributes to anchorage–independent growth by associated with 600 kDa retinoblastoma protein associated factor, p600 (51). For cellular regulations, HPV needs to evade host cellular immunity. HPV E7 interacts with interferon regulatory factor–1 (IRF–1), by which *IFN–β* transcription is impeded by recruiting histone deacetylases (HDACs) to the promoter region (52). Murine keratinocytes expressing HPV16–E7 show reduced transcriptions of genes associated with antigen processing and presentation such as

Irf–1, *Tap–1*, and *Pa28* by impairing transcription activity of IFN–γ. Reduced antigen processing and presentation make E7–expressing keratinocytes less susceptible to T–cell mediated lysis and refractory to host immune responses *in vitro* (53). While HPV E7 interacts with various cellular proteins, its main target is pRB that is a key regulator of cell cycle. HPV E7 binds to pRB and its family members, p130 and p107 through conserved LXCXE motif (48,54,55). Although both high–risk HPV E7 (e.g., HPV16 and HPV18) and low–risk HPV E7 (e.g., HPV11 and HPV6) interact with pRB proteins, high–risk HPV E7 has higher affinities to pRB than low–risk HPV E7 (48). In the normal context, pRB interacts with and inhibits E2F transcription factors that control DNA synthesis. Upon HPV infections, HPV E7 occupies pRB, which liberates E2F and leads to uncontrolled cell proliferation. High–risk HPV E7 bound pRB is targeted for Cullin2 ubiquitin ligase–mediated degradation (56). E7 activity regarding inactivation of pRB is evaluated with overexpressed cyclin–dependent kinase inhibitor $p16^{INK4a}$ which normally inhibits phosphorylation of pRB family (48,57). Thus, $p16^{INK4a}$ is used as a HPV biomarker in cervical carcinoma (58).

Though HPV E6 and HPV E7 have distinct cellular targets and functions, they synergistically promote carcinogenesis. In head and neck carcinoma and cervical cancer, the development of cancer is mainly driven by E7, and E6 promotes their growth (8,59). Either HPV 16–E6 or HPV 16–E7 alone is not sufficient to suppress transcriptions of innate immune genes (IIG), suggesting HPV E6 and E7 synergistically block IIG-mediated cellular senescence *in vitro* (60).

HPV vaccines

In the United States, 34,800 women and men are estimated to be diagnosed with HPV-driven cancer every year (61). HPV vaccines have been developed to protect against initial infection from the certain types of HPV and development of HPV–related diseases. About 90 percent of HPV–associated cancers are preventable with timely HPV vaccination (62). Current HPV vaccines are derived from recombinant L1 capsid proteins which spontaneously self–assemble into immunogenic viral–like particles (VLPs). These VLPs have morphologic and antigenic similarities to authentic virions. Each vaccine is made up of combination of L1 VLPs of different HPV types. Currently available HPV vaccines are prophylactic, but not therapeutic (63,64).

The Food and Drug administration (FDA) has approved three multivalent HPV vaccines: Gardasil, Gardasil 9, and Cervarix. Gardasil (Merek&Co, NY, USA), the first HPV vaccine, was licensed in June 2006. Gardasil targets two high–risk types (i.e., HPV16 and 18) and two low–risk types (i.e., HPV6 and 11). High–risk type HPV16 and HPV18 cause about 70% of cervical cancer and 90% of anal cancers. Low–risk HPV6 and 11 cause 90% of anogenital warts, which are not life–threatening but cause pains and bleeding. Cervarix (GlaxoSmithKlin, Rixensart, Belgium) prevents infection by HPV16 and HPV18. Gardasil 9 (Merek&Co, NY, USA) has replaced Gardasil because it covers five additional high–risk types (i.e., HPV 31, 33, 45, 52, and 58). The five high–risk types cause about 20% of HPV–related cancer cases (65). Since 2017, only Gardasil 9 is available in the United States. The Advisory Committee on Immunization Practices (ACIP) recommends routine HPV vaccination be started for children at age 11 and 12. If needed, children can be vaccinated as early as age 9. In 2016, ACIP revised HPV vaccine schedule from 3 to 2 doses for children aged under 15 if the second dose is administrated within 6 to 12 months after

the first dose. Several clinical trials have supported that 2-dose series present comparable efficacy and immunogenicity to 3–dose series (66). Since mid–2019, 3–dose series catch-up vaccination is recommended for both men and women aged 13 to 26 years old who have not been vaccinated before or who have not fulfilled the vaccine series. For adults who are 27 – 45 years of age, catch-up vaccination is not routinely recommended and covered by insurance, while it is recommended for those who have been minimally exposed to HPV infection (i.e., had no or limited sexual partners) but have a chance for future HPV infection (i.e., have new sexual partners) (67).

Though HPV vaccines are effective against HPV–related diseases, there are a few challenges to overcome. First, the vaccination rate is still low. As of 2018, 51.5 percent of adolescents had completed HPV vaccination as FDA recommended, and 68.1 percent had received at least 1 dose of vaccine in the United States. Though the vaccination coverage rate has been increased about 5 percent from 2017, it is still inferior to the immunization goal of Healthy People 2020 Initiative pursuing 80 percent of HPV vaccination rate for female adolescents aged 13 to 18 years. (67,68). Second, this low vaccination rate reflects parental hesitancy against HPV vaccines about safety and necessity (69). For parents, health providers and clinicians need to fully explain the safety and efficacy of the vaccines and actively recommend HPV vaccination to reduce HPV-related cancer burden. The minimal availability of the vaccines in developing countries is most pronounced. High price of the vaccines and lack of strong health infrastructure have to be resolved in those countries (70). Some post–hoc trials and a national cohort analysis have suggested that single dose of bivalent and quadrivalent vaccines is as effective as two or three doses of the vaccine in terms of prevention of HPV infection and high–grade cervical disease (71,72). Efficacy of single dose of 9–valent vaccine still needs to be validated with independent study.

B. Cervical cancer

Statistics and prevention of cervical cancer

Cervical cancer is caused by persistent infection with high–risk HPV and ranked the third most leading cause of cancer deaths in women worldwide. As described above, HPV vaccination is the primary prevention of cervical cancer. In addition to the HPV vaccines, a secondary prevention method called the Pap test is also effective in preventing cervical cancer. This cytologic screening allows early detection of cervical precancer that can be easily treated to prevent its progression to cancer. An alternative screening is HPV DNA test to detect infections by high–risk HPVs (73,74). There were approximately 570,000 new cervical cancer cases and 311,000 deaths worldwide in 2018. The global incidence of cervical cancer has not decreased since 2000 (75). Although these preventive methods are effective, they are not readily available to women in developing countries. Consequently, about 80 percent of cervical cancer cases are occurred in developing countries (70,76). In the United States, cervical cancer is the second leading cause of cancer death in women aged 20 to 39 years in the United States (77).

Cervical anatomy

Cervix is the entry of the uterus that plays a crucial role for maintenance of pregnancy and delivery. Cervical stroma is composed of fibroblasts and smooth muscle cells. Cervical epithelium consists of two distinct epithelial cell types: columnar epithelium and stratified squamous epithelium. The upper part of cervix is lined by a single layer of columnar epithelium, and the lower part of cervix has stratified squamous epithelium. These two epithelia meet at the squamocolumnar junction (SCJ). It moves back and forth within the transformation zone (TZ) depending on hormonal status and vaginal acidity (Figure 3) (78,79).

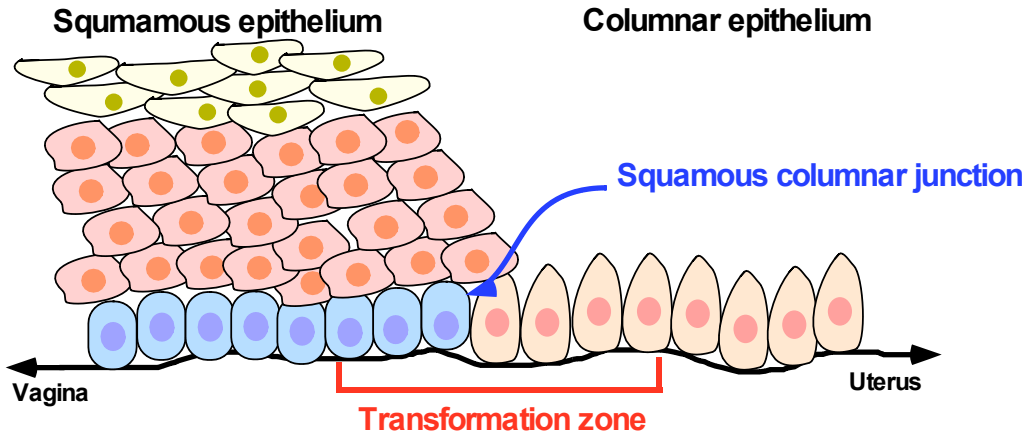


Figure 3. The transformation zone
Squamous epithelium meets columnar epithelium at squamous columnar junction (SCJ) in cervix. The SCJ moves back and forth in a region called transformation zone.

Progression, diagnosis, and treatment of cervical neoplasia

There are two main histological types of cervical cancer: squamous cell carcinoma and adenocarcinoma. The former accounts for 90% of cervical cancer cases. Herein, cervical cancer refers to squamous cell carcinoma, otherwise specified (21,80). Persistent infection with high–risk HPV first causes precancerous lesion called cervical intraepithelial neoplasia (CIN), which is graded as CIN1, CIN2, and CIN3 depending on its severity. Because 90% of CIN1 spontaneously regresses within 2 years, no treatment is recommended (81-83). Nonetheless, there are the desires of a drug against CIN1 (84). High–grade CIN2 and CIN3 have a higher potential to progress to cervical carcinoma if untreated (83). They are removed by simple surgery such as loop electrical excision procedure (LEEP) (85). In low– and mid–income countries where the Pap test is not readily available, visual inspection with acetic acid is proven effective in detecting CIN and cervical cancer. Detection of abnormalities from screenings is followed by colposcopy and cervical biopsy to confirm diseases (86).

Cervical cancer is confined to the cervix in stage I, and it spreads to the upper vagina and uterus in stage II. In stages III and IV, cancer has commonly extended to adjacent organs (e.g., pelvis, bladder, rectum, or local lymph nodes) and distant organs (e.g., liver, lungs, or bones). The most common treatment for early stage cancers is radical hysterectomy, surgically removing the cervix and uterus. If cancer is small, cervical conization is also available. As an adjuvant to surgery, radiation therapy and/or cisplatin–based chemotherapy are given to maximize treatment success (85,87). Metastatic and recurrent cervical cancer poorly responds to radiation and chemotherapy, and surgical approach is not amenable. In addition, median survival time of metastatic and recurrent cervical cancer patients is only a year. A group of stage III cervical cancer patients treated with cisplatin in addition to radiation therapy have superior disease–free survival and overall

survival rate than a group treated with radiation–only, while toxicity levels between the two regimens are similar (87,88). A phase III trial has supported that the combination of chemotherapy with an anti–angiogenesis drug bevacizumab (vascular endothelial growth factor inhibitor) is associated with significantly higher response and increased overall survival than chemotherapy alone; however, chemo-bevacizumab group is associated with additional adverse events such as hypertension and thromboembolism (89). Cervical cancer patients with metastatic and recurrent squamous cell carcinoma have responded to bevacizumab with disease–free survival for 6 months (90). FDA approved the use of bevacizumab to treat patients with recurrent or metastatic cervical cancer. Because a majority of cervical cancer is diagnosed before the age of 45, patients need to confront high chance of infertility, pre–term birth in future pregnancy, and postmenopausal symptoms following current treatment (radiation therapy often destroys the ovary). The development of more efficient treatment for cervical cancer is urgently needed (87).

Risk factors for cervical cancer

Chronic infection with high–risk HPV is necessary but not sufficient for the development of cervical cancer. Only 14% to 40% of CIN3 progress to invasive cervical carcinoma with a 3–year probability, implicating the involvement of other co-factors in cervical cancer development (3,4,91,92). Individuals infected with human immunodeficiency virus (HIV) had 10–fold increased chance of cervical cancer (93). Also, transplant recipients showed elevated incidence of cervical carcinoma *in situ* (94). These observations suggest that an impaired immune system accelerates the risk of cervical carcinoma. It is likely due to the increased risk of persistent HPV infection. Behavioral factors such as smoking has been suggested as a co–factor for cervical carcinogenesis. While the effects of smoking acquisition and clearance of HPV infection are

controversial, it is associated with the progression of precancerous lesions to cervical carcinoma (95-97). Tobacco carcinogens have been detected in the cervical mucus (98). Women who have experienced first sexual intercourse at an early age or had multiple sexual partners during lifetime are more susceptible to cervical cancer (99,100). The former is intriguing in that the transformation zone of the cervix goes through cellular alternations during puberty.

Among HPV–infected women, long–term (>5 years) use of oral contraceptives and multiple parities increase the risk of CIN and invasive cervical carcinoma (101). The association between cervical cancer and oral contraceptive use disappears if the use is ceased. These associations are not consistent if subjects are not stratified based on HPV infection. Multiple full–term pregnancies and early age at first full–term pregnancy are associated with a risk of CIN3 and invasive cervical carcinoma (102). These observations suggest a potential involvement of estrogen and progesterone in cervical carcinogenesis. A synthetic estrogen called diethylstilbestrol (DES) had been used to prevent preterm birth until 1970's. Daughters of women who took DES during pregnancy are more likely to be diagnosed with CIN lesions and clear cell adenocarcinoma (5). In addition, HPV–positive breast cancer patients who have used an aromatase inhibitor blocking estrogen synthesis have a reduced risk of cervical neoplasia compared to breast cancer patients who have not used the same drug (6). These results suggest that estrogen and HPV cooperate to promote cervical cancer. The effects of progesterone in cervical cancer are much less studied and controversial. Better understandings of functions and mechanisms of progesterone in cervical carcinoma are needed.

C. Progesterone signaling

Progesterone receptor isoforms

17β–estradiol (E_2, estrogen refers to E_2 herein) and progesterone (P_4) function via estrogen receptor (ER) and progesterone receptor (PR), respectively. They are ligand–dependent transcription factors belonging to the nuclear receptor superfamily. PR is encoded by the *Pgr* gene. Similar to other nuclear receptors, PR consists of DNA binding domain (DBD), C–terminal ligand binding domain (LBD) encompassing an activation function domain (AF), a hinge region between DBD and LBD, and N–terminal region containing AF (Figure 4). PR–A (94 kDa) and PR–B (116 kDa) are the two main isoforms of PR that are expressed in the female lower reproductive tract. They are transcribed from the same gene from different promoters in a human and mouse or translated from alternative start codons located on a single mRNA transcript in a chicken (103-105). Absence of AF–3 domain at the N–terminal region of PR–A contributes to its distinct functions from PR–B.

Upon ligand binding, PR dimerizes in the cytoplasm and translocates into the nucleus to activate transcription of target genes. PR isoforms have been known to regulate different transcriptional targets by interacting with different coactivators, which renders unique functions of the two isoforms (104,105). Several *In vitro* studies using artificial reporters controlled by the canonical progesterone receptor response element (PRE) suggests that PR–B is a stronger transactivator of gene transcription than PR–A. In certain conditions, ligand–bound PR–A inhibits transcriptional activation of targets gens by PR–B and ERα (106,107).

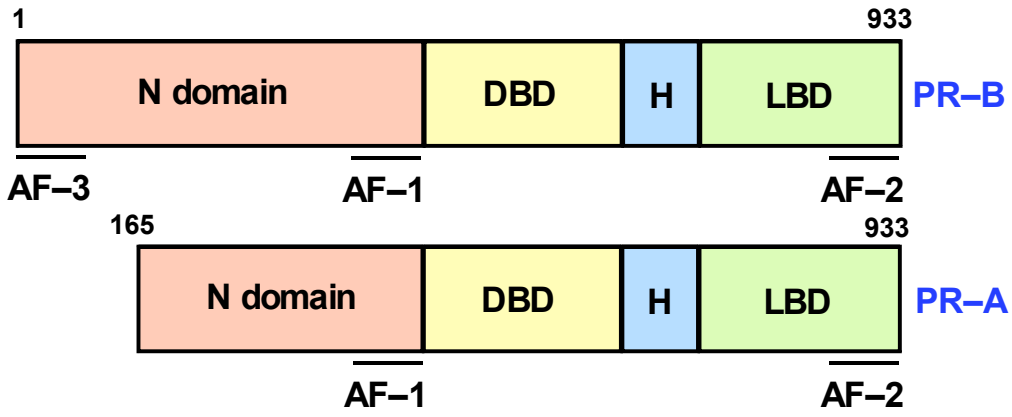


Figure 4. Schematics of progesterone receptor isoform A (PR–A) and B (PR–B)
Progesterone receptor isoforms consist of N–terminus domain, DNA binding domain (DBD), a hinge region (H), and ligand–binding domain (LBD). PR–A and PR–B have multiple activation function domains (AF). The numbers indicate amino acid residues in PR–B.

Progesterone in reproduction

P_4 is essential for the functions of the female lower reproductive tract (ovary, uterus, cervix, and vagina) and mainly produced by the ovary. Concentrations of endogenous E_2 and P_4 vary widely throughout the menstrual cycle. The average duration of human menstrual cycle is 28 days. During follicular phase (i.e., proliferative phase before ovulation), the E_2 level is elevated, and the P_4 level remains low. This condition promotes ERα–mediated proliferation of endometrium and increases the amount of cervical mucus. During luteal phase (i.e., secretory phase after ovulation), the E_2 level wanes, and the P_4 level increases (79,108,109). This hormonal change induces PR–mediated endometrial stroma remodeling known as decidualization (110). Moreover, cervical mucus becomes thick and viscous to prevent the passage of sperm (111). If conception and implantation occur, the E_2 and P_4 levels increase gradually throughout pregnancy. High levels of E_2 and P_4 are essential for the maintenance of pregnancy (112). However, P_4 decreases in the absence of conception, which induces shedding of uterine lining and eventually menses. After menopause, women become infertile due to ablated production of E_2 and P_4. Oral contraceptives provide high levels of E_2 and P_4 and prevent ovulation. P_4 makes crucial contributions to the development of mammary gland. In addition to prolactin, P_4 promotes extensive side branching and alveologenesis for milk secretion during lactation (113,114). Consequently, PR–knockout mice fails to develop the pregnancy–associated ductal production and lobuloalveolar differentiation despite normal mammary gland morphogenesis of the virgin mice (114). In a mouse model, PR–null mice are infertile because they fail to ovulate (114). Moreover, treatment of mice with progesterone antagonist mifepristone (RU486) inhibits ovulation (115). Selective ablation of PR–A fails to inhibits P_4–mediated suppression of E_2–induced epithelial cell proliferation and results in endometrial hyperplasia. In addition, PR–A is sufficient and necessary for ovulation and

decidualization of the stroma prior to implantation (116,117). An increased ratio of PR–A to PR–B results in endometrial hyperplasia and atypia with upregulated expression of uterine epithelial growth factors such as amphiregulin (118). The cervix serves as a gatekeeper for the birth. An expression level of PR is decreased in the cervix after parturition (119). Labor is induced with antiprogesterone treatment (e.g., mifepristone) in women and rodents by blocking PR activity (120,121). PR–B in myometrial cells is responsible for P_4 to exert its anti–inflammatory effects to maintain pregnancy (122). During parturition, PR–A levels becomes dominant over PR–B levels in the myometrial cells, in which PR–A suppresses the anti–inflammatory effects of PR–B and augments pro–inflammatory effects of P_4 (122,123).

Progesterone signaling in gynecological cancers

Women with multiple pregnancy, especially a history of twin pregnancies have a lower risk of ovarian cancer (124,125). P_4 treatment induces cell apoptosis in ovarian cancer *in vitro* (126). Consistently, PR positivity is associated with low stage and better survival of ovarian cancer patients (127). Insufficient progesterone actions also lead to endometrial cancer as well as endometrial hyperplasia. Reduced or undetectable level of PR is found in endometrial cancer (128). PR–positive tumors are associated with improved overall survival and better responses to progestin treatment than PR–negative tumors (129). Several studies done with P_4 treatment in PR–positive endometrial cancer *in vitro* support tumor suppressive functions of P_4/PR signaling. PR inhibits cell proliferation by blocking G_1 to S transition and induces cell apoptosis (130). P_4 decreases the metabolic potential of endometrial cancer cells by downregulating expression of cellular adhesion molecules including fibronectin, integrins α3, β1, and β3, and cadherin-6 invasion (130). However, the roles of P_4/PR in breast cancer are controversial. The majority of early breast cancer lesions

express PR, and its expression is retained in 60% of advanced cancers (131). Full–term pregnancy in early life is linked to a reduce risk of breast cancer (132). P_4 treatment in a PR–positive breast cancer cell line T47D inhibits breast cancer cell proliferation by upregulating MAPK phosphatase 1 (MKP1), suggesting anti–tumorigenic functions of P_4 in breast cancer (133). On the other hand, the use of P_4–containing oral contraceptives and hormone replacement therapy for the treatment of post–menopausal symptoms are associated with an increased risk of breast cancer (134). P_4 promotes cell proliferation in PR–positive breast cancer cell lines such as MCF7, suggesting P_4 is pro–tumorigenic in breast cancer (135). The role of P_4 and PR in breast cancer remains to be determined.

Progesterone signaling in cervical cancer

PR is expressed in both epithelium and stroma of the cervix. However, only 20 to 40% of cervical cancer express PR (136,137). PR–B expression in cervical cancer and surrounding stroma is associated with better prognosis (138). Total PR is not a prognostic marker in cervical adenocarcinoma (138-140). In a retrospective study with a small number of subjects, the use of medroxyprogesterone acetate (MPA), a synthetic progestin, is associated with a decreased risk of cervical cancer in HPV–infected women (7). Other studies that have not stratified data based on HPV status are not conclusive (141,142).

D. Cervical cancer mouse model

Transgenic mouse models expressing HPV16–E6 *(K14E6)* or HPV16–E7 *(K14E7)*, or both HPV16–E6/ HPV16–E7 *(K14E6/K14E7)* have been used to study HPV–associated cancers including cervical cancer (143,144). Expressions of the transgenes are regulated by the human keratin 14 (K14) promoter, which drives transgene expression in squamous epithelial cells, the natural target of HPV infection. Cervical cancer in *K14E6* and *K14E7* mice recapitulate pathogenesis of human cancer. Cervical cancer develops progressively from precancerous lesions (CIN1, CIN2, and CIN3) to invasive cervical cancer (8). Moreover, the majority of cancers develop in the transformation zone (145). They also express biomarkers including p16 and MCM7 similar to human cervical cancer (58,146). Six–month E_2 treatment promotes the development of CIN and cervical cancer in all *K14E7* and *K14E6/K14E7* mice (11). After 9 months of E_2 treatment, cervical cancer develops only in 40% of *K14E6* mice and 100% of *K14E7* mice (147). The E_2 treatment augments the number of proliferating cells in squamous epithelium in *K14E7* and *K14E6/K14E7* mice compared to that in *K14E6* mice (8). Likewise, tumor multiplicity and the size of cancers are reduced in *K14E6* mice compared to *K14E7* mice (147). These results indicate that E7 is more potent to induce cervical cancer. Cancer multiplicity and size in *K14E6/K14E7* mice are significantly greater than those in *K14E7* mice, indicating that E6 contributes to malignant progression and cancer growth (11). Cervical cancer developed in *K14E6* mice has increased pRB expression and no upregulation of p16, which is opposite to the cancers in *K14E7* mice (148).

Deletion of Rb in cervical epithelium and E_2 treatment in *K14E7* mice fail to induce cervical carcinogenesis (149). E_2 treatment of mice with deletion of all three pocket proteins (i.e., pRb, p107, and p130) does not fully recapitulate cervical cancer phenotypes of E_2–treated *K14E7* mice (150). In addition, *K14E7* mice expressing mutant pRb that cannot bind to E7 still succumb

to cervical cancer (149). These results indicate that pRb inactivation is not necessary for E7's tumorigenic potential and that other cellular targets are essential for cervical carcinogenesis. The cyclin–dependent kinase (CDK) inhibitor $p21^{CipI}$ is another cellular target of E7. Six–month of E_2 treatment in p21–null mice have an increased incidence of cervical cancer compared to p21–wildtype mice, and $K14E7^{CVQ}$ mutant mice that are unable to inactivate p21 have a reduced susceptibility to cervical cancer compared to $K14E7^{WT}$ mice (151). These observations suggest that p21 inactivation by E7 contributes to E7's oncogenic potentials in cervical carcinogenesis. Nine–month of E_2 treatment has decreased cervical carcinogenesis in $K14E6^{I128T}$ mutant mice that are defective in binding α–helix partners compared to that in $K14E6^{WT}$ mice, and cancer incidence in $K14E6^{\ delta146–151}$ mutant mice that are defective in binding PDZ partners is comparable to that in $K14E6^{WT}$ mice, which suggests the ability of E6 binds to α–helix partners contributes to cervical carcinogenesis (148). E6 promotes degradation of p53 by interacting with an α–helix partner, E6AP ubiquitin ligase (28,34). The loss of E6AP in $K14E6/E6AP^{-/-}$ mice in cooperation with 9 months of E_2 treatment have reduced cervical cancer incidence and cancer severity compared to the *K14E6* mice, and the cancers in $K14E6/E6AP^{-/-}$ mice have an increased expression of p53, suggesting that E6AP is a critical cellular target for E6's tumorigenic potentials (147). These results have shed new light on mechanisms of E6 and E7 and confirmed their known mechanisms in cervical carcinogenesis. They further validate the relevance of HPV transgenic mouse models.

With regard to a mechanism of E_2 signaling in cervical cancer, studies using the HPV transgenic mouse model have demonstrated that $K14E7/ER\alpha^{-/-}$ mice are resistant to E_2–induced cervical cancer, identifying ERα as the main mediator of pro–tumorigenic E_2 functions in the cervix (10). Following a serendipitous finding that the *CMV–CreER* transgene allele induces a deletion of ERα in the cervical stroma but not epithelium. Dr. Chung and his colleagues have

determined that stromal ERα is absolutely required for maintenance of CIN and its progression to cervical cancer (13). On the contrary, E_2 treatment promotes CIN or cervical cancer in all epithelial ERα–deficient *K14E7/Wnt7aCre/ERα$^{f/f}$* mice (14). These results strongly support that stromal ERα rather than epithelial ERα cooperates with HPV oncogenes in promoting cervical cancer.

P_4 inhibits proliferation of cervical epithelium in PR–dependent manner (15,16). Cervical cancer incidence in *K14E7/Pgr$^{-/-}$* mice is similar to *K14E7/Pgr$^{+/+}$* mice after 6 months of E_2 treatment (15). We have speculated that it is because P_4 levels are low in the experimental condition (i.e., chronic E_2 treatment) and thus PR is not so active in *K14E7/Pgr$^{+/+}$* mice. Consistently, the treatment of cancer–bearing *K14E6/K14E7/Pgr$^{+/+}$* mice with MPA promotes complete regression of cervical cancer (17). The therapeutic effect of MPA requires PR expression in cervical cancer cells and is dramatically dampened by simultaneous E_2 treatment (Mehta et al., unpublished). Interestingly, cervical cancer recurs even in the absence of exogenous E_2 (17). In addition, recurrent cervical cancer is refractory to MPA, suggesting that PR signaling is dysregulated. These results raise an intriguing possibility that endogenous E_2 is sufficient to support cervical carcinogenesis and continued growth of cancer if PR pathway is ablated. Treating *K14E6/K14E7* mice with MPA for the last 3 months of 6–month E_2 treatment completely prevents the development of cervical cancer (18), suggesting that MPA is an effective chemo–prevention reagent for cervical cancer. The preventive effect of MPA in the mouse model is dependent on PR (18).

III. MATERIALS AND METHODS

Animals and hormone treatments

For Chapter 1, *K14E7* [FVB–Tg(KRT14–HPV16E7)2304Plam] hemizygotes *(K14E7)* were mated with floxed PR mice (*$Pgr^{f/f}$)* to generate *E7/$Pgr^{f/f}$* (143,152). *K14E6* [FVB–Tg(KRT14–HPV16E6)5737Plam] homozygotes *(K14E6)* were crossed to *Wnt7aCre/$Pgr^{f/f}$* to generate *E6/Wnt7aCre/$Pgr^{f/+}$* (16,144,153). *E7/$Pgr^{f/f}$* males then were mated with *E6/Wnt7aCre/$Pgr^{f/+}$* females to generate 9 different genotypes: *NTG/NTG/$Pgr^{f/+}$* (*NTG;* non–transgenic)*, NTG/Wnt7aCre/$Pgr^{f/+}$*, *NTG/Wnt7aCre/$Pgr^{f/f}$, K14E7/NTG/$Pgr^{f/+}$, K14E7/Wnt7aCre/$Pgr^{f/+}$*, *K14E7/Wnt7aCre/$Pgr^{f/f}$, K14E6/K14E7/NTG/$Pgr^{f/+}$, K14E6/K14E7/Wnt7aCre/$Pgr^{f/+}$,* and *K14E6/K14E7/Wnt7aCre/$Pgr^{f/f}$*. They were aged without any treatment and euthanized when skin irritation or inguinal hernia is severe.

For Chapter 2, *$Amhr2^{cre/+}$* ($Amhr2^{tm3(cre)Bhr}$) knock–in mice were mated with *$Pgr^{f/f}$* to generate *$Amhr2^{cre/+}$/$Pgr^{f/f}$ ($Pgr^{sd/sd}$)*. *Wnt7aCre* mice were crossed to *$Pgr^{f/f}$* to generate *Wnt7aCre/$Pgr^{f/f}$* (*$Pgr^{ed/ed}$)* (16,153-155). *NTG/$Pgr^{f/f}$* littermates *($Pgr^{f/f}$)* were used as controls. Mice were ovariectomized at the age of 6–8 weeks and recovered for 2 weeks. All of the mice were then intraperitoneally injected with E_2 (1 μg) for 7 consecutive days. One group of mice were then daily treated with high concentration of E_2 (1 μg/day) or low concentration of E_2 (0.2 μg/day). Along with E_2, mice were daily treated with P_4 (1 mg/day) for 16 hrs, 3 days, 4 days, 5 days, or 7 days. A group of mice was only treated with vehicle (corn oil) along with E_2 (1 μg/day or 0.2 μg/day) for 3 days following 7 days of E_2 (1 μg) treatment. All mice were genotyped by PCR. All mouse strains were maintained on FVB/N genetic background. All procedures were approved by the University of Houston Institutional Animal Care and Use Committee.

Ovariectomy

Mice were anesthetized under 2% isoflurane. The dorsal part of the anesthetized mouse was shaved and cleaned with alcohol swabs. A 1 cm–long midline dorsal skin incision was made between the middle of the back and the base of the tail. The right ovary and associated fat pad were located, and a small (~2 mm) musculature incision was made in the location. The right ovary and surrounding fat pat were carefully pulled out. A homeostatic clamp was placed right under the ovary. Using a nature chromic gut absorbable suture with needle (Stoelting, IL; Cat No. 50491), two knots were made right under the clamped site. The ovary was excised, and the uterine horn was returned to the cavity. It was repeated on the left side. The skin incision was closed with the suture and surgical glue (Penn veterinary supply, Inc., PA; Cat No. ABT3204604).

Vaginal cytology

Vaginal cells were collected by gently washing the vaginal canal with 20 µl of sterile PBS. Cell cytology was examined under light microscope to determine cell types present. Mostly nucleated and some cornified epithelial cells were present in proestrus, and mostly cornified epithelial cells were present in estrus. Cornified epithelial cells, nucleated epithelial cells, and leukocytes were present in metestrus, and mostly leukocytes were present in diestrus (156).

Tissue processing, histological staining and analyses

Mouse cervical and uterine tissues were harvested, fixed in 4 % (w/v) paraformaldehyde in PBS for 18–24 hrs, and paraffin–embedded. Tissues were serially sectioned throughout the cervix at 5 µm thickness. For disease scoring in Chapter 1, every tenth slide was subjected to H&E staining, hematoxylin (Thermo Fisher Scientific, Fremont, CA; Cat. No. 6765015) and eosin (Sigma

Aldrich, St. Louis, MO; Cat. No. E4382) and histopathologically analyzed to score the worst neoplastic disease. Criteria for histopathological analyses were the thickness of basal–like epithelial cell layer, frequency of nuclear atypia with enlarged nucleus, and stromal invasion as described previously (8). A mouse typically had multiple neoplastic diseases, and the worst disease was scored for each mouse. In Chapter 2, sections with fully opened cervix were selected and subjected to H&E staining.

Immunohistochemistry (IHC)

Sections were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was achieved by incubating in pepsin solution (Thermo Fisher Scientific; Cat. No. AP9007005) for 5 min for $p16^{Ink4a}$ IHC or microwaving sections for 20 min in 10 mM sodium citrate buffer (pH 6.0) for the others. Blocking was performed in 5% goat serum + 0.5% skim milk for MCM7 or in 5% goat serum for the others for 1 hr at room temperature before primary antibody incubation. Primary antibodies were manufactured from Immunotech [ERα (cat# IM1545, clone 1D5)], Thermo Scientific [Ki67 (cat# RM 9106, clone SP6)], Lab vision [MCM7 (cat# MS–862, clone 47DC141, discontinued)], Sigma–Aldrich [PR (cat# SAB5500165, clone SP2), and Rockland Immunochemicals [CDKN2A (p16) (cat# 600–401–AJ9). Primary antibodies were treated in blocking buffers (ERα, 1:100; Ki67, 1:100; MCM7, 1:200; PR, 1:1000, $p16^{Ink4a}$, 1:200) at 4 °C overnight. After extensive washes in 1X PBS, primary antibodies were followed by secondary antibodies. Secondary antibodies were purchased from Life Technologies [Alexa Fluor 488–conjugated anti–rabbit IgG (cat# A11008), Alexa Fluor 488–conjugated anti–mouse IgG (cat# A11001), and Alexa Fluor 594–conjugated anti–mouse IgG (cat# A11005)]. Sections were incubated with secondary antibodies diluted in the blocking buffer for 1 hr at room temperature.

Nuclei was stained with Hoechst 33258 solution (10 μg/mL; Sigma-Aldrich; cat# B2883) for 30 sec. Homemade gelvatol mounting medium was used to mount slides.

TUNEL assay

ApopTag Fluorescein in situ apoptosis detection kit (EMD Millipore, Burlington, MA; cat# S7110) was used for TUNEL assay. Cervical sections were deparaffinized, rehydrated and washed with PBS for 5 min. The sections then treated with proteinase K (20 μg/ml) diluted in PBS for 15 min at room temperature and washed with PBS twice for 2 min each. The sections were applied with equilibration buffer (10 sec < incubation time < 1 hr), while the working solution for TdT enzyme was prepared. The sections were then incubated with TdT enzyme prepared in reaction buffer for 1 hr 30 min at 37 °C. Incubation was stopped by applying stop/wash buffer diluted in ddH20 for 10 min at room temperature, and the sections were washed with PBS 3 times for 1 min each. The sections were incubated with α–digoxigenin conjugate prepared in blocking solution for 30 min at room temperature and washed with PBS 4 times for 2 min each. Nuclei was stained with Hoechst 33258 solution (10 μg/mL; Sigma-Aldrich; cat# B2883) for 30 sec. Homemade gelvatol mounting medium was used to mount slides.

Microscopy and digital image analyses

After staining, sections were visualized with an Eclipse Ti2 microscope (Nikon Instruments Inc., Melville, NY). Images were obtained with a Nikon DS-Qi2 monochrome CMOS camera or a DS-Ri2 color CMOS camera using a 20X objective lens. The size of tumors was measured, and nuclei was pseudo-colored using Nikon NIS-Elements imaging software. For the quantification of Ki67 and TUNEL positive cells, random microscopic field of views per cancer in Chapter 1 or images

acquired from endocervix areas in Chapter 2 were subjected to counting manually using the NIH ImageJ software.

Statistical analyses

For Chapter 1, MSTAT software (version 6.4.2) was used to carry out one-sided Wilcoxon rank sum test (comparison of cancer burden between E7 and E6E7) and Fisher's exact test (cancer incidence and correlation of ERα/PR expression). MSTAT software was also used for paired t–test (comparison of cell proliferation and apoptosis between PR–negative and PR–positive cancers found in the same mouse) and unpaired t–test (comparison of cell proliferation and apoptosis between PR–negative cancers found in PR–het and PR–null). One–way ANOVA with post–hoc Tukey HSD test was performed to compare cancer burden among different epithelial and/or cancer PR status using Prism 6 software. The P–value equal to or less than 0.05 was considered to be significant in all experiments.

IV. CHAPTER 1:

PGR Heterozygosity Promotes Spontaneous Cervical Carcinogenesis in HPV–Transgenic Mice

A. Rationale

Infection with HPV itself is not sufficient to cause cervical cancer, and other factors are also required for cancer development and progression (137,157,158). In HPV–infected women, multiple full–term pregnancies and prolonged use of the oral contraceptives increase the risk of cervical cancer, implicating the involvement of estrogen (E_2) and progesterone (P_4) in the cancer (159,160). These hormones function by binding to their cognate receptors, estrogen receptor (ER) and progesterone receptor (PR), which belong to the nuclear receptor superfamily of ligand–activated transcription factors (137). Breast cancer patients who have treated with aromatase inhibitors, a class of drugs blocking the estrogen synthesis, show lower risk of cervical cancer than non–treated patients (6). Women who are prenatally exposed to synthetic estrogen DES are at increased risk of high–grade cervical intraepithelial neoplasia (CIN) than non–exposed group (5). These findings imply that estrogen is a risk factor for cervical cancer. There are a few epidemiological studies on roles of progesterone in cervical cancer, but they are not conclusive due to low number of study subjects and lack of data stratification based on HPV infection (137,141,161). One epidemiological study suggests that the use of synthetic progestin, medroxyprogesterone acetate (MPA) in HPV–infected women is inversely associated with cervical cancer, implicating progesterone inhibits cervical cancer (7). Limitations of the study are

that the number of MPA injection during the time has not been considered and that young population has been added in the analysis.

A HPV–transgenic mouse model expressing HPV16 E6 and E7 oncoproteins has been used to understand molecular mechanisms underlying cervical cancer (8). Though the reproductive cycle in a mouse lasts 4 to 5 days which is much shorter than that in a woman having cycle in every 28 to 29 days, they are similar in hormone fluctuations (162). The level of estrogen surges in estrus stage of mouse and follicular phase of a woman (i.e., before ovulation) at which progesterone levels are low. In diestrus (mouse) and luteal phase (human), progesterone level goes up, and estrogen level decreases (162). Treating the HPV–transgenic mouse with low–dosage exogenous E_2 significantly increases cervical carcinogenesis (13,14). Administration of MPA followed by chronic estrogen treatment in the same mouse model regresses and prevents cervical cancer in epithelial PR–dependent manner, implicating anti–tumorigenic actions of PR signaling in cervical carcinogenesis (15,17,18). It is notable that the serum level of estrogen in this mouse is not so high as that in a mouse in estrus stage (145). An outstanding question has been a reason that exogenous E_2 is required. Prolonged exogenous E_2 keeps mice in continuous estrus–like stage, suggesting that P_4 levels remain low (8,108,137).

As PR activation suppresses cervical cancer, I postulated that chronic E_2 treatment promotes cervical cancer, at least in part, by preventing P_4 surges (i.e., inhibition of PR). If this was correct, cervical cancer would efficiently develop when P_4 surges are blocked by another means or PR expression is ablated. In the present study, we deleted *Pgr* gene in epithelial cells of HPV–transgenic mice and found that cervical cancer incidence significantly increased in *Pgr*–deficient *K14E7* and *K14E6/K14E7* mice. We also found that cervical cancer incidence similarly increased in HPV–transgenic mice with one *Pgr* allele, indicating that *Pgr* is haploinsufficient.

B. Results

Deletion of epithelial Pgr sensitizes HPV transgenic mice to spontaneous cervical cancer.

I postulated that PR deletion would ablate effects of P_4 surges, and E_2 surges would induce cervical cancer efficiently. To determine whether the loss of *Pgr* promotes cervical carcinogenesis, we generated HPV negative *(NTG)*, *K14E7 (E7)*, and *K14E6/K14E7 (E6E7)* transgenic mice on *NTG/Pgr*$^{f/+(f/f)}$, *Wnt7aCre/Pgr*$^{f/+}$, and *Wnt7aCre/Pgr*$^{f/f}$. *NTG/Pgr*$^{f/f}$ and *NTG/Pgr*$^{f/+}$ mice were used as controls in *NTG*, *K14E7*, and *K14E6/K14E7* background. These mice were confirmed that they were in regular estrous cycle; Five mice were randomly selected from *K14E7* and *NTG* group, in which at least two *K14E7/Wnt7aCre/Pgr*$^{f/f}$ and *NTG/Wnt7aCre/Pgr*$^{f/f}$ mice were included. Vaginal smear was collected daily for 5 consecutive days, and cell cytology was analyzed. In *Wnt7aCre/Pgr*$^{f/f}$ mice (n=5), the estrus stage was completed in 4–5 days similar to *NTG/Pgr*$^{f/f}$ control mice, demonstrating that *Wnt7aCre/Pgr*$^{f/f}$ mice cycled normally. They were aged without any exogenous treatment and subjected to histopathological analyses (Table 1).

In *NTG* groups, cervical neoplastic disease was barely found regardless of PR status, confirming that HPV is required for cervical cancer. One *NTG/Wnt7aCre/Pgr*$^{f/+}$ mouse (5.0%) developed cervical cancer which is likely to be an outlier, which mimics rare *HPV*–negative cervical cancer in women. Fourteen of forty–two *K14E7/NTG/Pgr*$^{f/+}$ mice (33.3%) developed cervical cancer. Twenty–two mice had CIN lesions, and six mice had no disease (Table 1). Cancer incidence was significantly higher than *NTG/NTG/Pgr*$^{f/+}$ control ($P = 0.0004$). As anticipated, the incidence significantly increased in *K14E7/Wnt7aCre/Pgr*$^{f/f}$ ($P = 0.009$). Thirty–seven of sixty *K14E7/Wnt7aCre/Pgr*$^{f/f}$ mice (61.7%) carried cervical cancer. Thirty–one of fifty *K14E7/Wnt7aCre/Pgr*$^{f/+}$ mice (62.0%) developed cervical cancer, while the others had CIN lesions. Surprisingly, cancer incidence (31 of 50; 62.0%) was also significantly higher in

K14E7/Wnt7aCre/Pgr$^{f/+}$ mice (P = 0.007), and it was not different from *K14E7/Wnt7aCre/Pgr*$^{f/f}$ (P = 1.00). Similar trends were found in *E6E7* mice. Eleven of fourteen *K14E6/K14E7/Wnt7aCre/Pgr*$^{f/f}$ mice (78.6%; P = 3.21 x 10^{-6}) and nine of fourteen *K14E6/K14E7/Wnt7aCre/Pgr*$^{f/+}$ mice (64.3%; P = 0.04) developed cervical cancer, which were significantly higher than the cancer incidence found in *K14E6/K14E7/NTG/Pgr*$^{f/+}$ (28.6%). Although there was an increase in *K14E6/K14E7/Wnt7aCre/Pgr*$^{f/f}$ (11 of 14; 78.6%) compared to *K14E6/K14E7/Wnt7aCre/Pgr*$^{f/+}$ (9 of 14; 64.3%), it did not reach statistical significance (P = 0.68). Expression of *E6* in *K14E7* mice did not increase cancer incidence, confirming that *E7* is the major oncogene for cervical cancer. These results indicated that one allele of epithelial *Pgr* is not sufficient to prevent cervical cancer.

Table 1. Summary of the worst neoplastic diseases in the lower reproductive tract of *K14E6/K14E7* mice

Genotypes	HPV oncogene	Epithelial PR status	Group size	No Disease	CIN	Cervical cancer	Incidence (%)
NTG/Pgr$^{f/+}$		WT	29	28	1	0	**0**
Wnt7aCre/Pgr$^{f/+}$	None	Het	20	17	2	1	**5**
Wnt7aCre/Pgr$^{f/f}$		Null	22	22	0	0	**0**
K14E7/NTG/Pgr$^{f/+}$		WT	42	6	22	14	**33.3**
K14E7/Wnt7aCre/Pgr$^{f/+}$	E7	Het	50	0	19	31	**62.0***
K14E7/Wnt7aCre/Pgr$^{f/f}$		Null	60	0	23	37	**61.7***
K14E6/K14E7/NTG/Pgr$^{f/+}$		WT	28	4	16	8	**28.6**
K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$	E6E7	Het	14	0	5	9	**64.3***
K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$		Null	14	0	3	11	**78.6***

Date indicate the numbers of mice. *f*; floxed allele, +; wild-type allele
NTG/Pgr$^{f/f}$ mice are included in *NTG/Pgr$^{f/+}$* group.
K14E7/NTG/Pgr$^{f/f}$ mice are included in *K14E7/NTG/Pgr$^{f/+}$* group.
K14E6/K14E7/NTG/Pgr$^{f/f}$ mice are included in *K14E6/K14E7/NTG/Pgr$^{f/+}$* group.

Two-sided fisher's exact test was done. P values are indicated below.

NTG/PR–WT vs. E7/PR–WT; **4.17×10^{-4}**
NTG/PR–Het vs. E7/PR–Het; **9.32×10^{-6}**
NTG/PR–Null vs. E7/PR–Null; **9.26×10^{-8}**

NTG/PR–WT vs. E6E7/PR–WT; **1.88×10^{-3}**
NTG/PR–Het vs. E6E7/PR–Het; **3.13×10^{-4}**
NTG/PR–Null vs. E6E7/PR–Null; **6.06×10^{-7}**

E7/PR–WT vs. E6E7/PR–WT; **0.79**
E7/PR–Het vs. E6E7/PR–Het; **1**
E7/PR–Null vs. E6E7/PR–Null; **0.35**

NTG/PR–WT vs. NTG/PR–Het; **0.41**
NTG/PR–WT vs. NTG/PR–Null; **1**
NTG/PR–Het vs. NTG/PR–Null; **0.48**

E7/PR–WT vs. E7/PR–Het; **7.16×10^{-3}**
E7/PR–WT vs. E7/PR–Null; **8.54×10^{-3}**
E7/PR–Het vs. E7/PR–Null; **1**

E6E7/PR–WT vs. E6E7/PR–Het; **4.48×10^{-2}**
E6E7/PR–WT vs. E6E7/PR–Null; **3.21×10^{-3}**
E6E7/PR–Het vs. E6E7/PR–Null; **0.68**

Deletions of epithelial Pgr result in more severe cancer phenotypes.

To determine whether the loss of PR increases tumor multiplicity, the number of microscopic tumors in each mouse was counted (Table 2). There was significantly fewer number of cancers per individual mouse in *K14E7/NTG/Pgr$^{f/+}$* (0.40 ± 0.10) than in *K14E7/Wnt7aCre/Pgr$^{f/+}$* (1.00 ± 0.13; P = 0.02) and *K14E7/Wnt7aCre/Pgr$^{f/f}$* (1.07 ± 0.13; P = 0.005). *K14E7/Wnt7aCre/Pgr$^{f/f}$* and *K14E7/Wnt7aCre/Pgr$^{f/+}$* had similar cancer multiplicity (P = 1.00). The trend was similar in *E6E7* mice. Though it was marginally significant, *K14E6/K14E7/NTG/Pgr$^{f/+}$* (0.46 ± 0.16) developed fewer cancers per mouse than *K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$* (1.29 ± 0.32; P = 0.07). *K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$* (1.07 ± 0.13) developed significantly more cancers than *K14E6/K14E7/NTG/Pgr$^{f/+}$* (P = 0.02), but similar to *K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$*(P = 1.00).

The size of the largest cancer developed in *K14E7/NTG/Pgr$^{f/+}$* (0.03 ± 0.01) was significantly smaller than cancers found in *K14E7/Wnt7aCre/Pgr$^{f/f}$* (0.07 ± 0.01; P = 0.03), but similar to the cancers found in *K14E7/Wnt7aCre/Pgr$^{f/+}$*(0.05 ± 0.01; P = 0.49). The tendency was maintained in *E6E7* mice, in which the largest cancer developed in *K14E6/K14E7/NTG/Pgr$^{f/+}$* (0.02 ± 0.01) was significantly smaller than cancers in *K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$* (0.08 ± 0.02; P = 0.04), but similar to the cancers in *K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$* (0.06 ± 0.02; P = 0.26). The sizes of the largest cancer in *Wnt7aCre/Pgr$^{f/+}$* was comparable to the cancers developed in *Wnt7aCre/Pgr$^{f/f}$* in *E7* (P = 0.79) and *E6E7* (P = 0.98) background. The total invasion area developed in *K14E7/NTG/Pgr$^{f/+}$* (0.04 ± 0.01) was marginally smaller than the cancer developed in *K14E7/Wnt7aCre/Pgr$^{f/f}$* (0.09 ± 0.01; P = 0.07), but similar to the cancer in *K14E7/Wnt7aCre/Pgr$^{f/+}$* (0.07 ± 0.01; P = 0.52). The total invasion area found in

K14E6/K14E7/NTG/Pgr$^{f/+}$ (0.03 ± 0.01) was similar to that in *K14E6/K14E7/Wnt7aCre/Pgr*$^{f/+}$ (0.09 ± 0.03; P = 0.21), but significantly smaller than that in *K14E6/K14E7/Wnt7aCre/Pgr*$^{f/f}$ (0.11 ± 0.03; P = 0.04). The total invasion area found in *Wnt7aCre/Pgr*$^{f/+}$ and *Wnt7aCre/Pgr*$^{f/f}$ in *E6E7* (P = 0.99) and *E7* (P = 0.92) background was comparable. Cancer multiplicity, the size of the largest cancer, and the total invasion area were similar between *E6E7* and *E7* mice when PR genotype is matched. These results indicate that the heterozygosity of *Pgr* increases the number of cancers, but not the size of cancer. In addition, *Pgr* null status promotes cancer growth.

However, all cancers were well–differentiated and histologically similar among the groups regardless of epithelial PR status or HPV oncogene (Figure 5). The cancer developed in *NTG/Wnt7aCre/Pgr*$^{f/+}$ group also had similar histology to the cancers developed in *E6E7* and *E7* mice.

Table 2. Summary of the disease severity in the lower reproductive tract of *K14E6/K14E7* mice

Genotypes	HPV oncogene	Epithelial PR status	Group size	Tumor multiplicity	The largest cancer	Total invasion area
K14E6/K14E7/NTG/Pgr$^{f/+}$		WT	28	**0.46 ± 0.16**	**0.02 ± 0.01**	**0.03 ± 0.01**
K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$	E6E7	Het	14	**1.29 ± 0.32**	**0.06 ± 0.02**	**0.09 ± 0.03**
K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$		Null	14	**1.43 ± 0.25**	**0.08 ± 0.02**	**0.11 ± 0.03**
K14E7/NTG/Pgr$^{f/+}$		WT	42	**0.40 ± 0.10**	**0.03 ± 0.01**	**0.04 ± 0.01**
K14E7/Wnt7aCre/Pgr$^{f/+}$	E7	Het	50	**1.00 ± 0.13**	**0.05 ± 0.01**	**0.07 ± 0.01**
K14E7/Wnt7aCre/Pgr$^{f/f}$		Null	60	**1.07 ± 0.13**	**0.07 ± 0.01**	**0.09 ± 0.01**

Individuals without cancers were valued as zero for both multiplicity and size.
Data indicate Mean ± S.E.M.
Cancer sizes are in mm^2.

Tukey's multiple comparisons test followed by one-way ANOVA test were done.
P-values are indicated below.

Tumor multiplicity

E6E7/PR–WT vs. E6E7/PR–Het; **0.07**
E6E7/PR–WT vs. E6E7/PR–Null; **0.02**
E6E7/PR–Het vs. E6E7/PR–Null; **1.00**

E7/PR–WT vs. E7/PR–Het; **0.02**
E7/PR–WT vs. E7/PR–Null; **0.005**
E7/PR–Het vs. E7/PR–Null; **1.00**

The largest cancer

E6E7/PR–WT vs. E6E7/PR–Het; **0.26**
E6E7/PR-WT vs. E6E7/PR–Null; **0.04**
E6E7/PR–Het vs. E6E7/PR–Null; **0.98**

E7/PR–WT vs. E7/PR–Het; **0.49**
E7/PR–WT vs. E7/PR–Null; **0.03**
E7/PR–Het vs. E7/PR–Null; **0.79**

Total invasion area

E6E7/PR–WT vs. E6E7/PR–Het; **0.21**
E6E7/PR–WT vs. E6E7/PR–Null; **0.04**
E6E7/PR–Het vs. E6E7/PR–Null; **0.99**

E7/PR–WT vs. E7/PR–Het; **0.52**
E7/PR–WT vs. E7/PR–Null; **0.07**
E7/PR–Het vs. E7/PR–Null; **0.92**

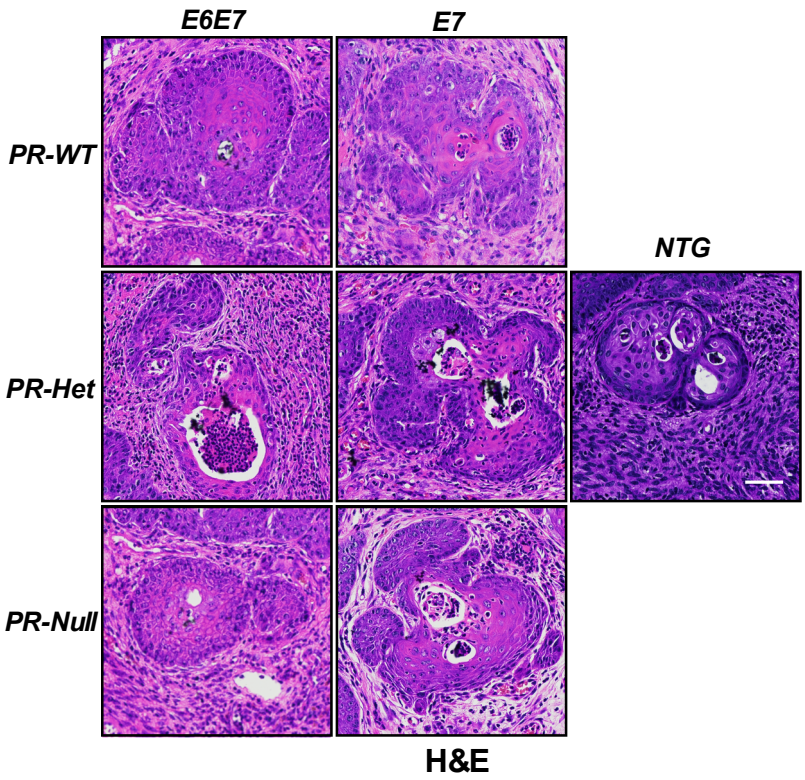


Figure 5. All cancers are well–differentiated.
Cancer histology is similar among cancers regardless of epithelial PR genotype or HPV status. Shown are representative images of H&E stained cervical cancer sections. Scale bar represents 50 μm.

Increased cancer incidence in Pgr–deleted mice is not due to age.

It was impossible to elucidate whether the loss of *Pgr* or the presence of *E6* oncogene sensitizes development of cancers in younger ages, because the mice were euthanized at different time points due to non–cancerous reasons (e.g., skin irradiation, inguinal hernia). Instead, we wanted to exclude the possibility that the higher cancer incidence found in *Wnt7aCre/Pgr$^{f/f}$* and *Wnt7aCre/Pgr$^{f/+}$* than *NTG/Pgr$^{f/+}$* in *E6E7* and *E7* background is not caused by that *NTG/Pgr$^{f/+}$* mice were euthanized earlier than *Wnt7aCre/Pgr$^{f/+}$* and *Wnt7aCre/Pgr$^{f/f}$*. We compared ages of the mice at the time of euthanasia among genotypes (Table 3).

NTG/ NTG/Pgr$^{f/+}$ mice (329 ± 9.2) were sacrificed at similar ages to *NTG/ Wnt7aCre/Pgr$^{f/+}$* (314 ± 9.3; P = 0.99) and *NTG/Wnt7aCre/Pgr$^{f/f}$* (310 ± 13; P = 0.97) mice (Table 3). There was no significant difference between *NTG/NTG/Pgr$^{f/+}$* and *NTG/Wnt7aCre/Pgr$^{f/+}$* (P = 1.00). *K14E7/ NTG/Pgr$^{f/+}$* (227 ± 9.8; P = 1.52 x 10^{-9}) and *K14E6/K14E7/NTG/Pgr$^{f/+}$*(219 ± 13; P = 3.95 x 10^{-8}) mice were sacrificed later than *NTG/NTG/Pgr$^{f/+}$*. Consistently, *K14E7/Wnt7aCre/Pgr$^{f/+}$* (282 ± 7.5; P = 0.008) and *K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$* (231 ± 13; 9.13 x 10^{-6}) mice were sacrificed later than *NTG/Wnt7aCre/Pgr$^{f/+}$,* and *K14E7/Wnt7aCre/Pgr$^{f/f}$* (248 ± 7.7; P = 4.47 x 10^{-5}) and *K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$* (242 ± 13; P = 6.42 x 10^{-4}) mice were sacrificed later than *NTG/Wnt7aCre/Pgr$^{f/f}$. E7* and *E6E7* mice lived longer than *NTG* mice, which suggests that the increased cancer incidence in *E7* and *E6E7* compared to *NTG* mice is not due to age (Table 3).

K14E7/NTG/Pgr$^{f/+}$ (227 ± 9.8) mice lived significantly shorter than *K14E7/Wnt7aCre/Pgr$^{f/+}$* (282 ± 7.5; P = 0.0002), but similar to *K14E7/Wnt7aCre/Pgr$^{f/f}$* (248 ± 7.7; P = 0.63) (Table 3). The age of *K14E7/Wnt7aCre/Pgr$^{f/+}$* was older than that of *K14E7/Wnt7aCre/Pgr$^{f/f}$*, but the difference was marginal (P = 0.06). However, the age of *K14E6/K14E7/NTG/Pgr$^{f/+}$* (219 ± 13) was similar to that of *K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$* (231

± 13; P = 1.00) and *K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$* (242 ± 13; P = 0.95). *K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$* was sacrificed at similar age to *K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$* (P = 1.00). Though *K14E7/NTG/Pgr$^{f/+}$* mice were sacrificed earlier than *K14E7/Wnt7aCre/Pgr$^{f/+}$*, the increased cancer incidence in *K14E7/Wnt7aCre/Pgr$^{f/+}$* than *K14E7/Wnt7aCre/Pgr$^{f/f}$* is unlikely to be due to age. It is because the age of *K14E7/NTG/Pgr$^{f/+}$* is similar to that of *K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$,* and the age of *K14E6/K14E7/NTG/Pgr$^{f/+}$* was similar to that of *K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$* and *K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$* (Table 3). In addition to that, cancer–bearing *K14E7/Wnt7aCre/Pgr$^{f/+}$* mice (296 ± 8.8) were significantly sacrificed earlier than cancer–free *K14E7/Wnt7aCre/Pgr$^{f/+}$* mice (259 ± 7.3; P = 0.02, data not known). The ages of *E7* and *E6E7* mice were similar in *NTG/Pgr$^{f/+}$* (P = 0.54) and in *Wnt7aCre/Pgr$^{f/f}$* (P = 0.76), while *K14E7/Wnt7aCre/Pgr$^{f/+}$* lived significantly longer than *K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$* (P = 0.007), suggesting that minimal effect of *E6* in cancer incidence is not due to age (Table 3).

Table 3. Summary of the ages of mice at the time of euthanasia

Genotypes	HPV oncogene	Epithelial PR status	Age (days)
NTG/Pgr$^{f/+}$	None	WT	**329 ± 9.2**
Wnt7aCre/Pgr$^{f/+}$		Het	**314 ± 9.3**
Wnt7aCre/Pgr$^{f/f}$		Null	**310 ± 13**
K14E7/NTG/Pgr$^{f/+}$	E7	WT	**227 ± 9.8**
K14E7/Wnt7aCre/Pgr$^{f/+}$		Het	**282 ± 7.5**
K14E7/Wnt7aCre/Pgr$^{f/f}$		Null	**248 ± 7.7**
K14E6/K14E7/NTG/Pgr$^{f/+}$	E6E7	WT	**219 ± 13**
K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$		Het	**231 ± 13**
K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$		Null	**242 ±13**

Group sizes are indicated in Table 1.
Data indicate Mean ± S.E.M.

Tukey's multiple comparisons test followed by one-way ANOVA test were done.
P-values are indicated below.

NTG/PR–WT vs. NTG/PR–Het; **0.99**
NTG/PR–WT vs. NTG/PR–Null; **0.97**
NTG/PR–Het vs. NTG/PR–Null; **1.00**

E7/PR–WT vs. E7/PR–Het; **0.0002**
E7/PR–WT vs. E7/PR–Null; **0.06**
E7/PR–Het vs. E7/PR–Null; **0.63**

E6E7/PR–WT vs. E6E7/PR–Het; **1.00**
E6E7/PR–WT vs. E6E7/PR–Null; **0.95**
E6E7/PR–Het vs. E6E7/PR–Null; **1.00**

Two–sided Wilcoxon rank sum test was performed.
P-values are indicated below.

NTG/PR–WT vs. E7/PR–WT; **1.52×10^{-9}**
NTG/PR–Het vs. E7/PR–Het; **0.008**
NTG/PR–Null vs. E7/PR–Null; **4.47×10^{-5}**

NTG/PR–WT vs. E6E7/PR–WT; **$3{,}95 \times 10^{-8}$**
NTG/PR–Het vs. E6E7/PR–Het; **9.13×10^{-6}**
NTG/PR–Null vs. E6E7/PR–Null; **6.42×10^{-4}**

E7/PR–WT vs. E6E7/PR–WT; **0.54**
E7/PR–Het vs. E6E7/PR–Het; **0.007**
E7/PR–Null vs. E6E7/PR–Null; **0.76**

PR expression is not detectable in the majority of cancers in Pgr–sufficient genotypes.

Cancers are originated from epithelium. We postulated that PR expression on the cancers might be consistent with PR expression in epithelium, and PR IHC was performed on all the cancers found in *K14E7/NTG/Pgr$^{f/+}$* (n = 17), *K14E7/Wnt7aCre/Pgr$^{f/+}$* (n = 51), *K14E6/K14E7/NTG/Pgr$^{f/+}$* (n = 13), and *K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$* (n = 18) (Figure 6A). Cancers were either PR–positive or PR–negative. The expression of PR in the epithelium close by the cancers was consistent with the PR in cancer, whereas the epithelium distant from cancers were invariably PR–positive. Five mice were randomly selected from each *K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$* and *K14E7/Wnt7aCre/Pgr$^{f/f}$* and subjected to PR IHC. PR expression was undetectable in the cancers (data not shown). Regardless of PR expression in the epithelium and cancers, stromal PR was retained in all cancers.

Eight of thirteen cancers (61.5%) in *K14E6/K14E7/NTG/Pgr$^{f/+}$* mice and fifteen of eighteen cancers (83.3%) in *K14E6/K14E7/Wnt7aCre/Pgr$^{f/+}$* mice were PR–negative (Figure 6B). Ten of seventeen cancers (58.8%) in *K14E7/NTG/Pgr$^{f/+}$* and Twenty–nine of fifty–one cancers (56.9%) in *K14E7/Wnt7aCre/Pgr$^{f/+}$* lost PR expression. The fractions of PR–negative cancers were similar between *Wnt7aCre/Pgr$^{f/+}$* and *NTG/Pgr$^{f/+}$* in both *E6E7* and *E7* mice. It suggests that the lack of PR expression unlikely due to loss of heterozygosity.

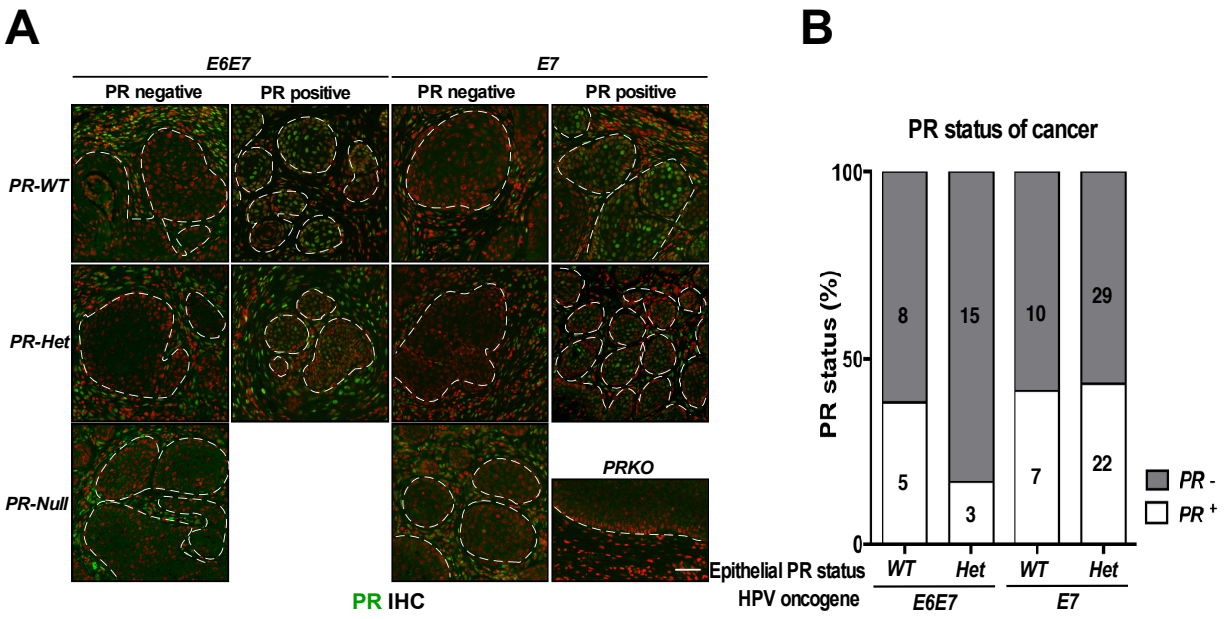


Figure 6. The majority of cancers in *Wnt7aCre/Pgr*$^{f/+}$ and *NTG/Pgr*$^{f/+}$ lose PR expression. **(A)** Both PR–negative and PR–positive cancers are found in *Wnt7aCre/Pgr*$^{f/+}$ and *NTG/Pgr*$^{f/+}$. Cervical cancer sections were stained for PR (green). Nuclei (red) was stained with Hoechst 33258. Cervical section from PR knock–out mice (*Pgr* $^{-/-}$) was used as negative control. White dotted lines separate cancers from stroma. Scale bar represents 50 μm. **(B)** More than a half of the cancers found in *Wnt7aCre/Pgr*$^{f/+}$ and *NTG/Pgr*$^{f/+}$ are PR–negative. Bar graph represents PR status of cancers developed in each genotype. The portion of PR–negative and PR–positive cancers in each group is depicted in gray and white box respectively. The number of stained cervical cancers is marked on each box.

The major mechanism of the loss of PR expression is the loss of ERα expression.

Expression of ERα is required for PR expression in cervix (16). To understand a mechanism of the loss of PR expression in cervical cancers in *Pgr*–sufficient genotypes, PR–negative and PR–positive cancers were randomly selected from *Wnt7aCre/Pgr*$^{f/+}$ and *NTG/Pgr*$^{f/+}$ in *E6E7* and *E7* background and subjected to ERα IHC (Figure 7). Stromal ERα was detectable in all cancers. All twenty PR–positive cancers (100%) were ERα–positive, and twenty–nine out of thirty–three PR–negative cancers (87.9%) were ERα–negative. PR expression was significantly associated with ERα expression. Both ERα and PR expression are declined in cervical cancer compared to normal cervix in women (136,163). To determine the loss of PR expression is correlated with the loss of ERα expression in cervical cancer in women, the GEPIA2 database was used. I first investigated the expression of *PGR* in cervical cancer (n = 306) compared to the adjacent normal tissues (n = 13). The level of *PGR* expression was significantly lower in cervical cancer than normal cervix ($P < 0.01$) (Figure 8A). In consistent with cancers in mice, *PGR* downregulation in human cervical cancer was significantly correlated with *ESR1* downregulation ($P < 2.2 \times 10^{-16}$) (Figure 8B). These results suggest that the main mechanism of the loss of PR expression in cervical cancer is the loss of ERα. PR–negative/low, ERα–positive/high cancers in both mouse and women suggest ERα–independent mechanism of the loss of PR expression.

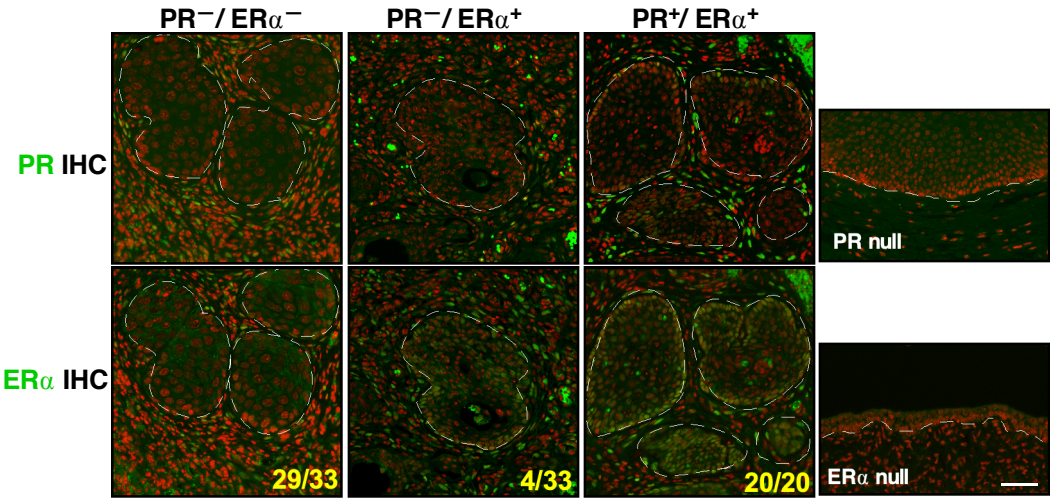


Figure 7. PR expression is correlated to ERα expression in cervical cancer.
ERα expression is undetectable in the most of PR–negative cancers, but positive in PR–positive cancers. Some PR–negative and PR–positive cervical cancers from *Wnt7aCre/Pgr*$^{f/+}$ and *NTG/Pgr*$^{f/+}$ were subjected to ERα (green in bottom panels) immunohistochemistry. Cervical section from *Esr1* knock–out mouse (ERα null) was used as negative control. PR (green in upper panels) staining was performed on the same cancers. Cervical section from *Pgr* knock–out mouse (PR null) was used as a negative control. Nuclei was pseudo–colored in red in both ERα and PR staining. White dotted lines isolate cancers from surrounding stroma. In controls, the lines separate epithelium (upper) from stroma (bottom). The numbers indicated in yellow indicate the number of ERα–positive or –negative cancers/ the number of PR–negative or PR–positive cancers analyzed. Scale bar represents 50 μm.

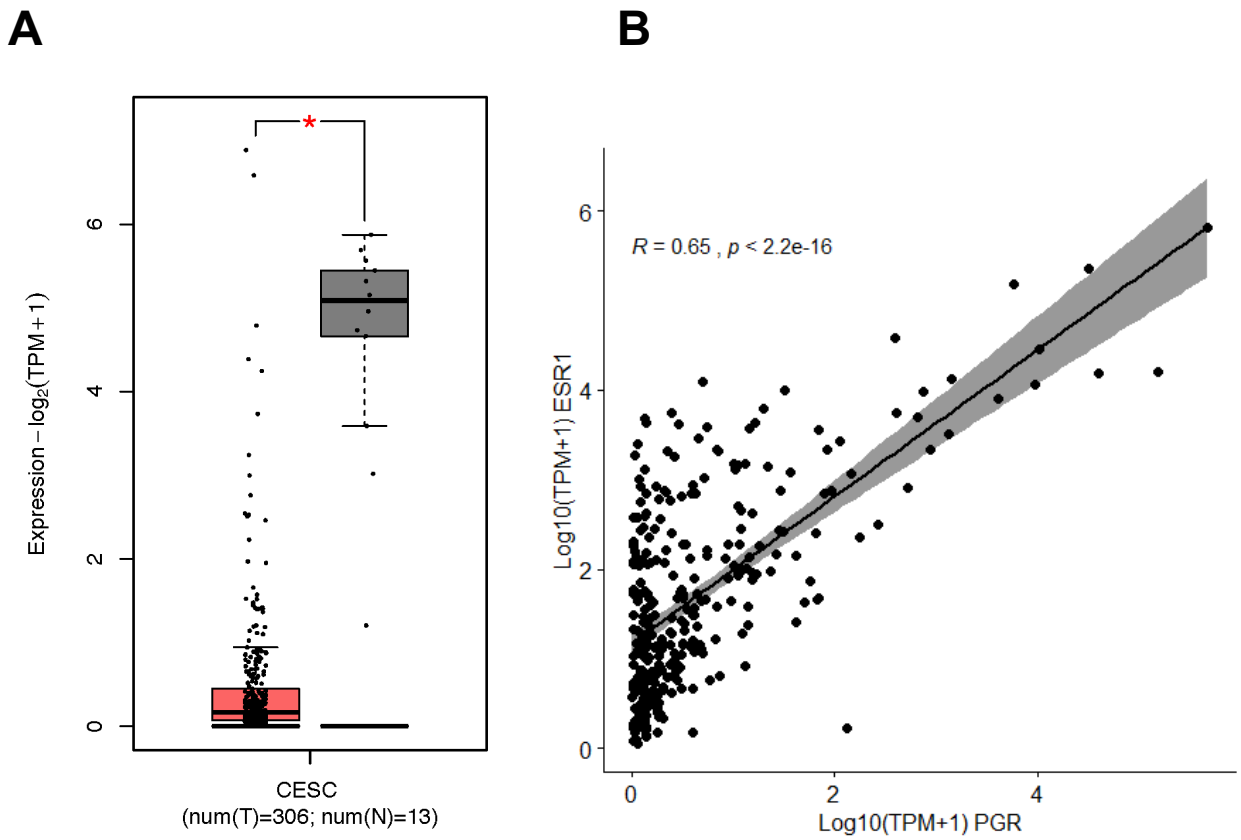


Figure 8. Downregulated *PGR* in cervical cancer is due to downregulated *ESR1* in women. Data were obtained from GEPIA2 (http://gepia2.cancer-pku.cn). **(A)** *PGR* is downregulated in cervical cancer (red; n = 306) than corresponding normal samples (gray; n = 13). Anova test was done. *, P<0.01. TPM, Transcripts per million; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma. **(B)** *ESR1* expression is directly correlated to *Pgr* expression. Pearson's correlation coefficient was used.

PR–negative cancers are larger than PR–positive cancers.

Cancer sizes were not different between *NTG/Pgr*$^{f/+}$ and *Wnt7aCre/Pgr*$^{f/+}$ and between *Wnt7aCre/Pgr*$^{f/+}$ and *Wnt7aCre/Pgr*$^{f/f}$ (Table 2). It was probably because *Pgr* genotype did not correlate with PR expression status (Figure 6). To determine cancer size correlates with PR expression status, the size of PR–negative cancer was compared to PR–positive cancer (Figure 9). All cancers developed in *K14E7/Wnt7aCre/Pgr*$^{f/f}$ and *K14E6/K14E7/Wnt7aCre/Pgr*$^{f/f}$ were included in PR–negative cancer group. The size of individual PR–negative cancer was significantly larger than PR–positive cancer developed in *K14E7/NTG/Pgr*$^{f/+}$ ($P = 0.02$) and *K14E7/Wnt7aCre/Pgr*$^{f/+}$ ($P = 0.01$) (Figure 9A). The size of the largest PR–negative cancer found in each mouse was significantly larger than the largest PR–positive cancer in *K14E7/NTG/Pgr*$^{f/+}$ ($P = 0.02$) and *K14E7/Wnt7aCre/Pgr*$^{f/+}$ ($P = 0.004$) (Figure 9B). Total invasion area of PR–negative cancers was significantly larger than that of PR–positive cancers in *K14E7/NTG/Pgr*$^{f/+}$ ($P = 0.01$) and *K14E7/Wnt7aCre/Pgr*$^{f/+}$ ($P = 0.002$) (Figure 9C). The trend was maintained in *E6E7* mice, but it did not reach at statistical significance due to small group sizes. These results indicate that the presence of PR in the cancers but not in epithelium is the major factor deciding cancer size. So far, the results suggest that the loss of PR expression suppresses cancer growth.

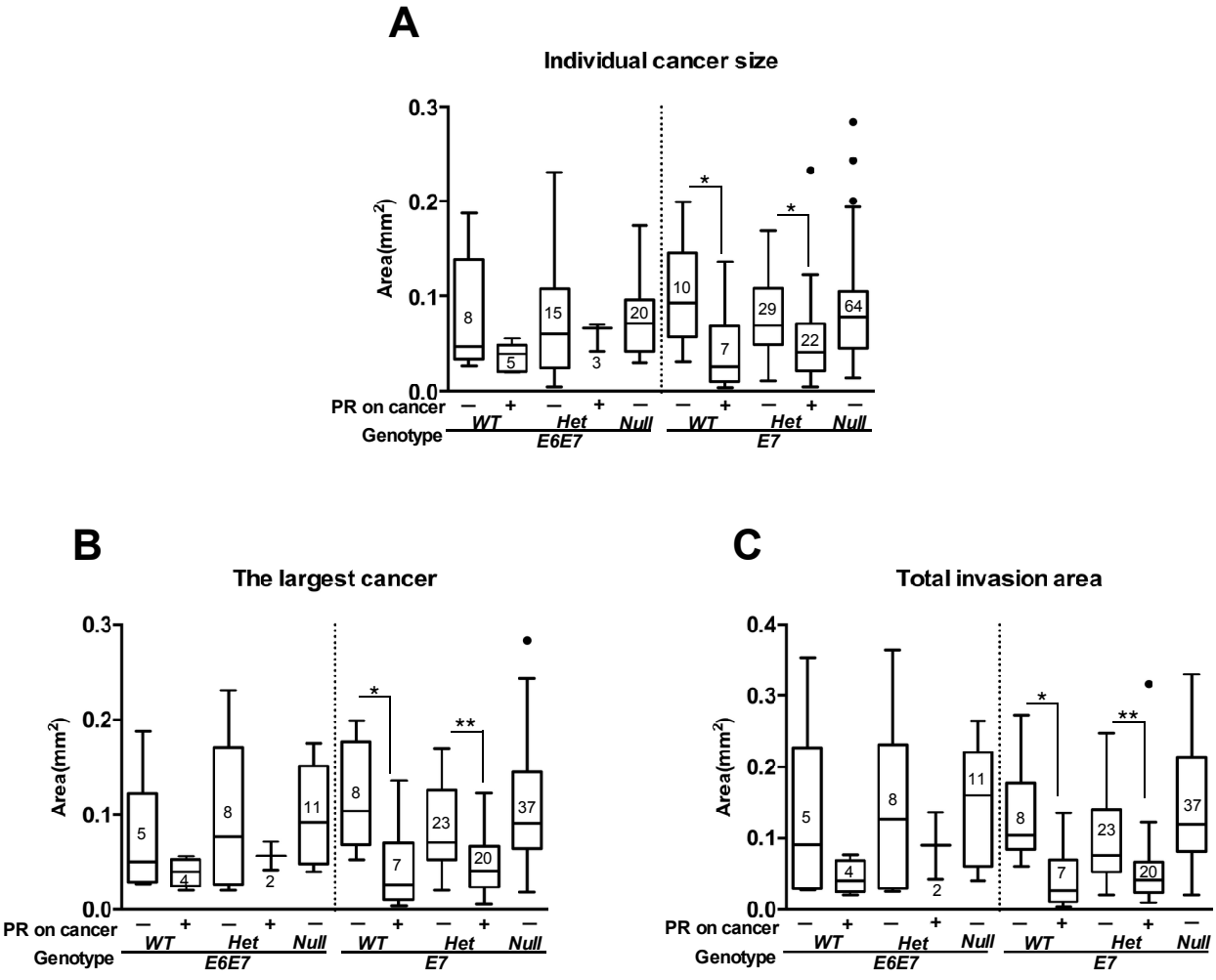


Figure 9. Sizes of PR–negative cancers are significantly larger than PR–positive cancers. **(A)** The size of Individual cancer size is plotted in box–and–whisker plot based on its PR status and genotype. Midlines of the box indicates medians, and lower and upper box limits represent the 25th and 75th percentiles respectively. Whiskers extend 1.5 times the interquartile range. The black dots show outliers. The numbers in the boxes indicate group size. **(B)** The largest cancer size in each mouse is plotted as described in (A). **(C)** The sum of cancer areas found in each mouse is plotted as described in (A). Two–sided Wilcoxon test was carried out. *, P≤0.02. **, P≤0.004.

PR–negative cancers are more proliferative and but less apoptotic than PR–positive cancers.

To determine a mechanism by which PR suppresses tumor growth, five mice bearing both PR–negative and PR–positive cancers were selected from *K14E7/Wnt7aCre/Pgr*$^{f/+}$ (Figure 10). This allowed us to compare PR–negative and PR–positive cancers under same hormone conditions. Ki67 was used as a proliferation marker. Proliferation indices (i.e., % Ki67^{+} cells) in all PR–negative cancers were higher than those in matching PR–positive cancers (Figure 10A). This correlation was highly significant (P = 0.006). The same pairs of cancers were analyzed for apoptosis by TUNEL assay. PR–negative cancer was significantly less TUNEL–positive that PR–positive cancer on the (P = 0.03) (Figure 10B). On the contrary, proliferation and apoptotic indices were similar in PR–negative cancer from *K14E7/Wnt7aCre/Pgr*$^{f/+}$ and *K14E7/Wnt7aCre/Pgr*$^{f/f}$ (Figure 11). These results indicate that PR inhibits cervical cancer growth by blocking proliferation and inducing death.

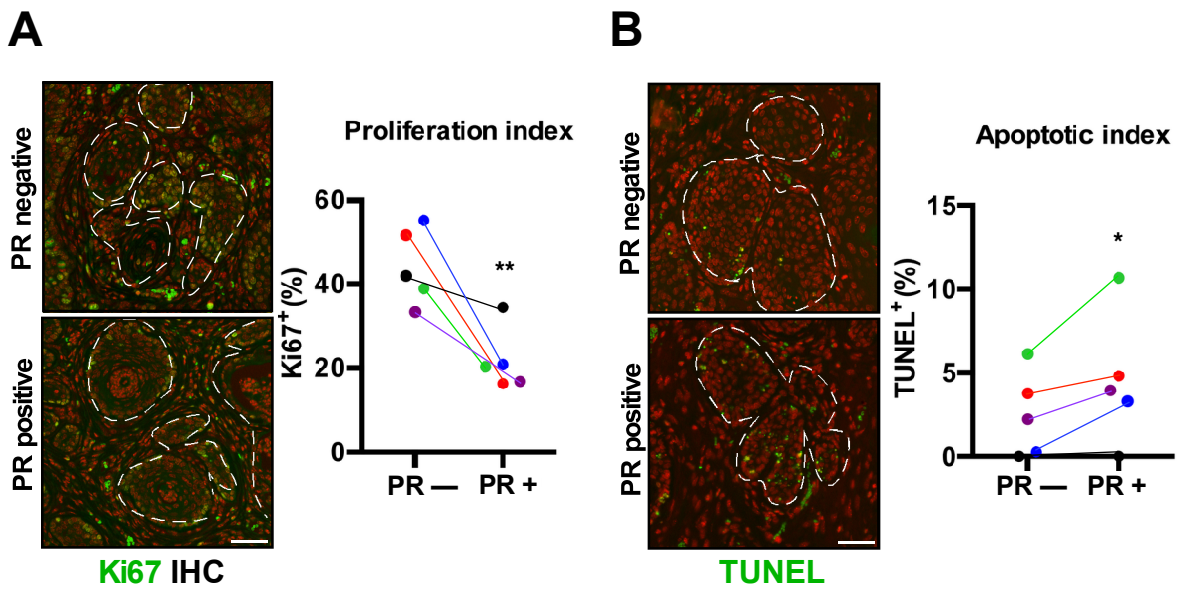


Figure 10. PR–negative cancers are more proliferative and less apoptotic than PR–positive cancers.

(A) PR–negative cancer is more proliferative than PR–positive cancer. Five mice bearing both PR–negative and PR–positive cancers were selected from *K14E7/Wnt7aCre/Pgr$^{f/+}$*, and their cervical sections were stained for Ki67 (green). Nuclei (red) was stained with Hoechst 33258. Each colored dot indicates individual mouse. White dotted lines separate cancers from stroma. Results were quantified and plotted in the dotted plot. Each colored dot indicates individual mouse. Paired one-sided t-test was carried out. **(B)** PR–negative cancer is less apoptotic than PR–positive cancer. The same five mice used in (A) were subjected to TUNEL staining. TUNEL positive cells are shown in green, and nuclei was pseudocolored in red. White dotted lines distinguish cancers from stroma. Results were quantified, and the same colored dot was used for the same individual in (A). Paired one-sided t-test was carried out. Scale bar indicates 50 μm. *P=0.03, **P=0.006.

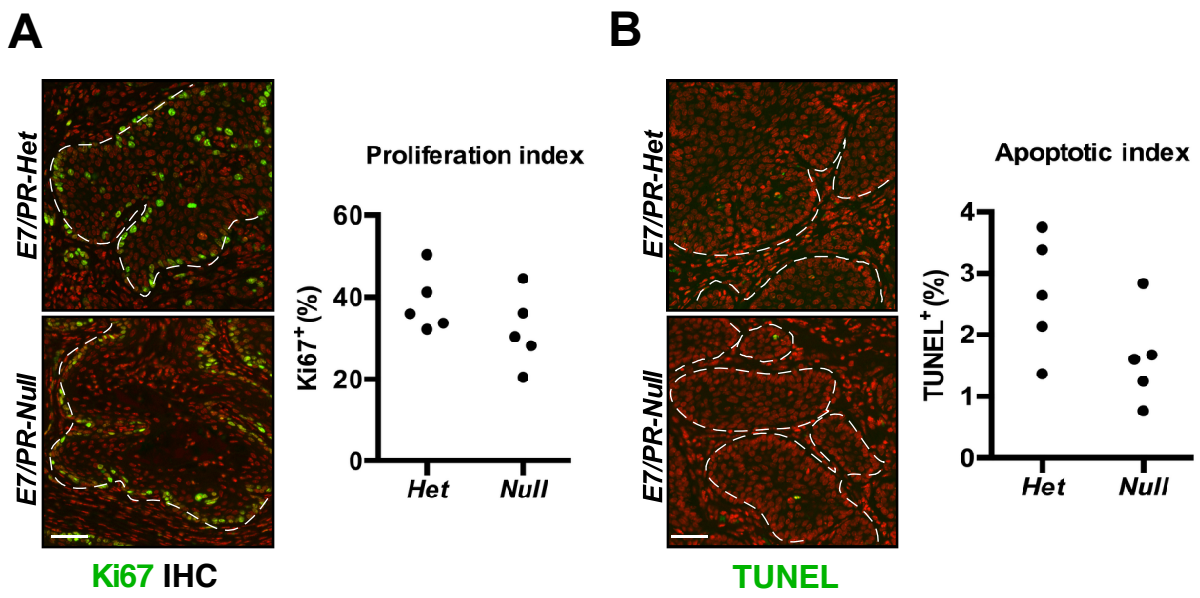


Figure 11. Epithelial PR genotype does not affect cell proliferation and apoptosis of cancers. **(A)** PR–negative cancers found in different epithelial PR genotypes are similarly proliferative. Five PR–negative cancers in similar sizes were randomly selected from *K14E7/Wnt7aCre/Pgr*$^{f/f}$ and *K14E7/Wnt7aCre/Pgr*$^{f/+}$. The selected cervical sections were stained for Ki67 (green) and nuclei (red). Lines separate cancers from stroma. Results were quantified and plotted in the dotted plot. Each dot indicates individual mouse. Unpaired one-sided t-test was carried out. No significance was found. **(B)** The index of cell apoptosis is similar between PR–negative cancers found in different epithelial PR–status. The same five PR–negative cancers mentioned in (A) were subjected to TUNEL (green) staining. Nuclei was pseudo–colored in red. Lines separate cancers from stroma. Results were quantified and plotted in the dotted plot. Each dot indicates individual mouse. Unpaired one–sided t–test was carried out. No significance was found. Scale bar represents 50 μm.

PR status does not affect expression patterns of cervical cancer biomarkers.

Both p16 and mcm7 have been used as biomarkers for E7 function in HPV–associated cervical cancer in human and mouse (58,146). To determine whether the loss of PR in cancers has an effect on *E7* biomarker expressions, p16 and mcm7 expressions were examined. Expression of p16 and mcm7 was upregulated in cancers in *K14E7* and *K14E6E7* mice compared to *NTG* control in which p16 was undetectable and mcm7 was only expressed in the outermost layer of the cancer. Expression patterns of p16 and mcm7 in cancers were similar between *K14E7* and *K14E6E7* mice. These suggest that cervical biomarker expressions are mainly due to E7 function (Figure 12). p16 and mcm7 were similarly expressed between PR–negative and PR–positive cancers developed in *K14E7* and in *K14E6/K14E7* mice. The expression patterns were also comparable between *K14E7/NTG/Pgr*$^{f/+}$ and *K14E7/Wnt7aCre/Pgr*$^{f/+}$ and between *K14E6/K14E7/NTG/Pgr*$^{f/+}$ and *K14E6/K14E7/Wnt7aCre/Pgr*$^{f/+}$, which suggests that the PR loss has no influence on E7 function (Figure 12).

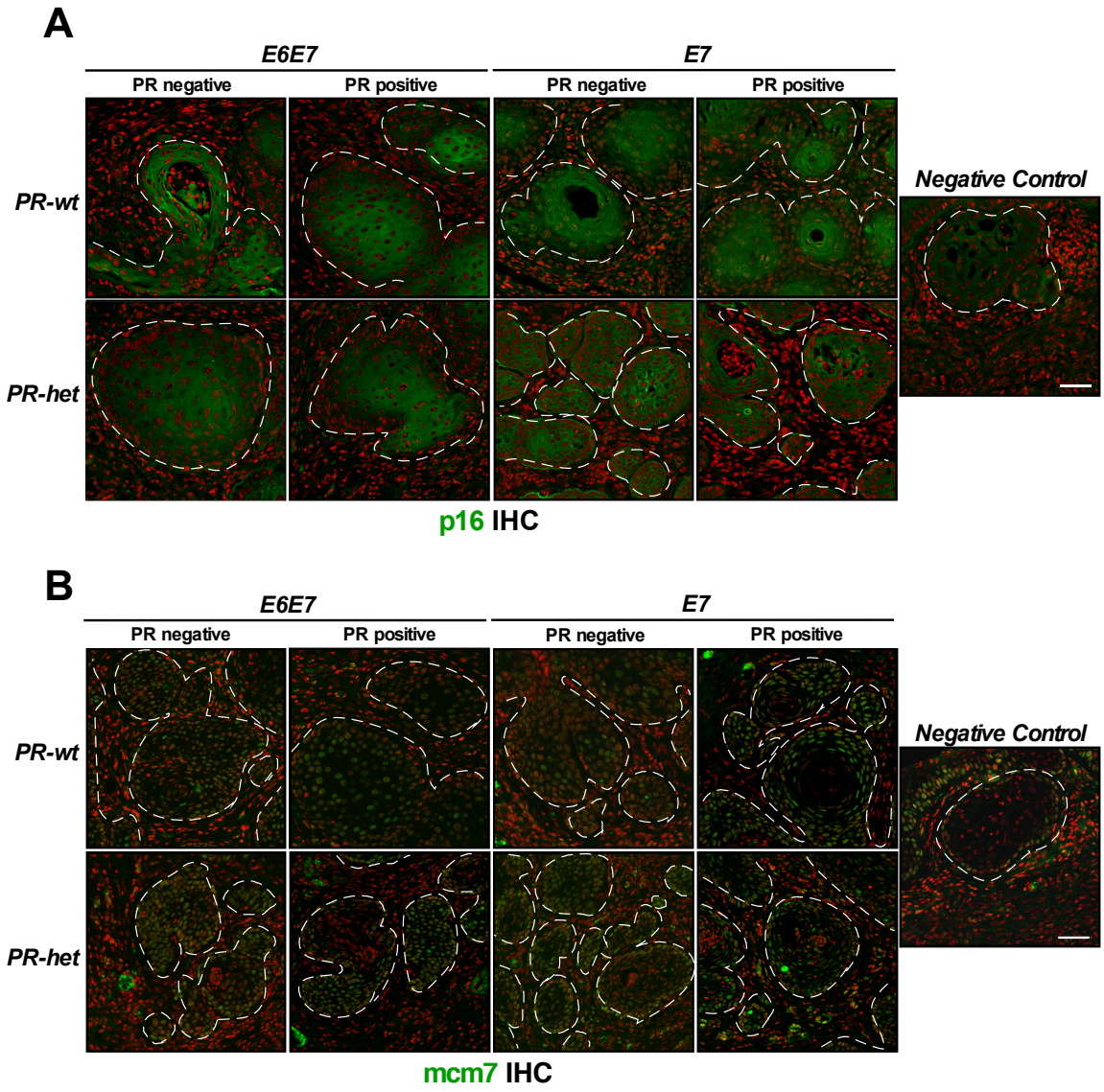


Figure 12. E7 biomarkers are similarly expressed in all HPV–positive cancers.
(A) All cancers express p16 in similar level. At least three PR–negative and PR–positive cervical cancers developed in *Wnt7aCre/Pgr*$^{f/+}$ and *NTG/Pgr*$^{f/+}$ mice in *E6E7* and *E7* background were stained for p16^{Ink4a} (green) and nuclei (red), and representative images are shown. Lines separate cancers from stroma. Scale bar represents 50 μm. **(B)** mcm7 expression levels are similar among cancers. At least three PR–negative and PR–positive cervical cancers developed in *Wnt7aCre/Pgr*$^{f/+}$ and *NTG/Pgr*$^{f/+}$ mice in *E6E7* and *E7* background were stained for E7 biomarker mcm7 (green) and nuclei (red), and representative images are shown. Lines distinguish cancers from surrounding stroma. Scale bar represents 50 μm. The cancer developed in *NTG/Wnt7aCre/Pgr*$^{f/+}$ was used as a negative control for p16 and mcm7 IHC.

C. Discussion

Chronic E_2 treatment promotes cervical cancer in HPV transgenic mouse model, which clearly shows that E_2 cooperates with *HPV* oncogenes for cervical carcinogenesis (8,10,13,14). While E_2 surge activates ERα and increases PR expression in cervix, anti–tumorigenic PR activity remains minimal because P_4 levels are low (15,16,137). However, this hormone condition is rare in women. In female menstrual cycle, E_2 and P_4 surges in turn, which alternately activates pro–tumorigenic ER α and anti–tumorigenic PR and consequently maintains homeostasis. We hypothesized that the deletion of epithelial PR ablates anti–tumorigenic actions of P_4 surge, in which E_2 surge induces pro–tumorigenic actions in the development and maintenance of cervical cancer.

Deletions of epithelial Pgr promotes spontaneous cervical carcinogenesis.

The cancer incidence was significantly higher in *K14E7/Wnt7aCre/Pgr$^{f/f}$* (61.7%) and *K14E6/K14E7/Wnt7aCre/Pgr$^{f/f}$* (78.6%) than in *K14E7/NTG/Pgr$^{f/+}$* (33.3%) and *K14E6/K14E7/NTG/Pgr$^{f/+}$* (28.6%), respectively (Table 1). These results support our hypothesis that *Pgr* is a tumor suppressor gene in cervical cancer. In previous findings, cancer incidence was 100% when the mice are treated with exogenous E_2 for 6 months (164). However, the mice only develop CIN lesions when they were exposed to E_2 for 3 months, implying cancer develops after 4 to 6 months of the E_2 treatment (18). The higher cancer incidence in exogenous E_2–treated mice compared to untreated mice in this study confirms that exogenous E_2 promotes cervical cancer. In addition to cancer incidence, tumor multiplicity and cancer size were significantly greater in *Wnt7aCre/Pgr$^{f/f}$* than in *NTG/Pgr$^{f/+}$* background (Table 2).

It was surprising that the cancer incidence in *Wnt7aCre/Pgr*$^{f/+}$ was similar to that in *Wnt7aCre/Pgr*$^{f/f}$ (Table 1). This suggests haploinsufficiency of *Pgr* in cervical cancer suppression. Haploinsufficiency of tumor suppressor genes has been identified (165). The CDK inhibitor *p27*^{Kip1} is an example of haploinsufficient tumor suppressor genes. *p27*$^{+/-}$ and *p27*$^{-/-}$ mice are more susceptible to intestine, lung, and pituitary cancers than *p27*$^{+/+}$ mice when they are stimulated with γ–irradiation and the chemical carcinogen ENU (165,166). The wild–type allele is retained in the cancers developed in *p27*$^{+/-}$ mice, suggesting that p27 is haploinsufficient for tumor suppression (166). The development of spontaneous carcinomas is significantly increased in *p53*$^{+/-}$ and *p53*$^{-/-}$ compared to *p53*$^{+/+}$ mice. More than half of cancers in *p53*$^{+/-}$ mice retain an intact wild–type *p53* allele, suggesting *p53* haploinsufficiency (167,168). It was worth noting that cancer incidence further increases in *p27*$^{-/-}$ and *p53*$^{-/-}$, but it did not in *Wnt7aCre/Pgr*$^{f/f}$. ENU promotes T–cell lymphomas in *Egr1*$^{+/-}$ and *Egr1*$^{-/-}$ mice with the similar penetrance (169). Of note, our results suggest for the first time that women who have only one functional *PGR* allele is more vulnerable to cervical cancer.

Although incidence was low, *K14E7/NTG/Pgr*$^{f/+}$ and *K14E6/K14E7/NTG/Pgr*$^{f/+}$ mice had spontaneous cervical cancer (Figure 1). It was unexpected because a report briefly mentions that none of the *K14–HPV16* transgenic mice develop spontaneous cervical cancer by 1.5 years of age without presenting data (9). These mice harbor the entire early region of HPV16 genome and thus express E6 and E7 and potentially other early genes. In addition, E_2 treatment induces invasive cancers in only 6 out of 11 mice (54.5%) unlike 100% in our *K14E6/K14E7* mice. According to the description in the published paper, it is highly likely that these mice are not on the pure FVB background, most susceptible to cervical carcinogenesis (13,15) Furthermore, it is reasonable to

speculate that only a small number of mice are analyzed based on a small group size (n=11) for the experiment.

Development of PR–negative cancer in Pgr–sufficient mice.

It was surprising that spontaneous cervical cancer occurred in mice on the *NTG/Pgr*$^{f/+}$ and *Wnt7aCre/Pgr*$^{f/+}$ background. We postulated that PR was not expressed in those cancers. Indeed, 56.9% – 83.3% of cancers were PR–negative (Figure 6). However, the remainder were PR–positive. Therefore, a loss of PR expression is not a sole mechanism of spontaneous cervical cancer in *Pgr*–sufficient HPV transgenic mice. Nonetheless, it is significant that PR–negative cancers arise spontaneously because a majority of human cervical cancers do not express PR (20–40% are positive for PR) (136,170). All cervical cancers arising in HPV transgenic mice treated with low–dose of E_2 are PR–positive (15,16). Therefore, PR–positive cervical cancer patients have longer disease–free survival than PR–negative cancer patients (171).

One may ask why PR–positive cancers develop if PR is a tumor suppressor. One possibility is that PR is not functional. However, it is not favored because of less tumorigenic features of PR–positive cancers compared to PR–negative cancers (Figure 10). Nonetheless, it is possible that some of crucial PR downstream is disrupted and thus PR signaling is dampened. Another possibility is that constitutive activation of an unknown pathway overcomes PR's tumor–suppressive action. While PR target genes in cervix have not characterized, it is essential for PR to upregulate *Hand2* for progesterone–mediated inhibition of epithelial cell proliferation in the mouse uterus (172). Loss of heterozygosity (LOH) and epigenetic modifications such as promoter hypermethylation are common mechanisms involved in inactivation of tumor suppressor genes (173). If LOH is a main mechanism of the loss of PR expression, a percentage of PR–negative cancer would be higher in

K14E7/Wnt7aCre/Pgr$^{f/+}$ than *K14E7/NTG/Pgr*$^{f/+}$. However, they were similar between the two genotypes (Figure 6). ERα is required for PR expression in the murine cervix (16). We showed that 87.9% of PR–negative cancers were ERα–negative (Figure 7). PR expression was also correlated to ERα expression in human cervical cancer (Figure 8). Four PR–negative cancers expressed ERα, indicating ERα–mediated activation of *Pgr* expression is disrupted in those cancers (Figure 7). Promoter of *Pgr* is hypermethylated in cervical cancer tissues compared to normal cervical tissue specimens (174). HeLa cells do not express ERα and PR. Overexpression of ERα restores PR mRNA expression in HeLa, demonstrating that *PGR* is silenced, rather than deleted, in cervical cancer (175). ERα overexpression and treatment with a DNA demethylating agent 5'–AZA–dC activate PR expression in a PR–negative breast cancer cell line (176). These results strongly suggest that an epigenetic mechanism is responsible for the loss of PR expression in cervical cancer developing in *K14E7/Wnt7aCre/Pgr*$^{f/+}$.

In summary, our results suggest that spontaneous cervical carcinogenesis in *(K14E6)/K14E7/Wnt7aCre/Pgr*$^{f/+}$ mice better mimic the status of ERα and PR in cervical cancer patients. It also recapitulates the status of ERα and PR in cancer stroma in patients. Roles of PR in cervical cancer have been poorly understood because of the lack of a model recapitulating PR status in patients. We anticipated that further studies using our new genetic model will shed new light on the mechanism of hormone receptors in cervical cancer. It will be also useful to test whether selective ER modulators that inhibits ERα in the cervix are efficient in treating ERα–negative and PR–negative cancers by targeting stromal ERα.

V. CHAPTER 2:

Stromal PR is Required for P_4–mediated Suppression of Epithelial Cell Proliferation in Mouse Cervix

A. Rationale

E_2 and P_4 play important roles in the development and homeostasis of female reproductive tracts. In the cervix, ERα is required for PR expression (16). Upregulation of PR does not necessarily mean its high activity, because its activation is dependent on P_4 (177). PR is upregulated in estrus stage and fully activated in diestrus at which P_4 levels are highest.

It has been shown that regulation of epithelial cell proliferation by E_2 and P_4 in female lower reproductive tracts involves crosstalk between epithelia and stroma. In the cervix, E_2–induced cell proliferation of squamous epithelium is present in epithelial ERα–deficient mice but completely absent in stromal ERα–deficient mice, indicating that stromal ERα is more important (13,14). P_4 inhibits epithelial cell proliferation in a uterine tissue recombinant composed of PR–positive stroma and PR–negative epithelium, indicating that stromal PR, but not epithelial PR, mediates P_4 functions (178). P_4 also suppresses apoptosis in the uterine epithelium via stromal PR (179). We previously have demonstrated that epithelial PR is required for P_4–mediated inhibition of cell proliferation and promotion of apoptosis in the cervix (16). In the context of cervical cancer, epithelial PR is necessary of MPA's therapeutic effects (18). However, the role of stromal PR in cervical cancer suppression and treatment by MPA has not been characterized. Though it needs to be verified with independent study, stromal PR-B is known to be a good prognostic marker for squamous cell carcinoma (138).

To evaluate a potential role of stromal PR in cervical cancer, I determined whether stromal PR is required for P_4 to inhibit cell proliferation and induce apoptosis. I made of use *Amhr2*$^{Cre/+}$/*Pgr*$^{f/f}$ (referred to as *Pgr*$^{sd/sd}$ hereafter; sd, stromal deletion). To interrogate the role of stromal PR in physiological and pharmacological responses of cervical epithelial cells to P_4, mice were treated with P_4 under two different estrogen conditions, high E_2 (1μg/ml) or low E_2 (0.2 μg/ml). In the absence of stromal PR or epithelial PR, inhibition of cell proliferation and promotion of cell apoptosis by P_4 occurred only transiently in both E_2 conditions. These results indicate that both stromal and epithelial PR are required for continued suppression of cell proliferation and induction of cell apoptosis by P_4. My data also suggest both epithelial and stromal PR are required for P_4 to suppress cervical cancer.

B. Results

Incomplete deletion of stromal PR in Amhr2$^{cre/+}$/Pgr$^{f/f}$ mice

The *anti-Müllerian hormone receptor 2 (Amhr2)* gene is expressed in the stroma of the Müllerian ducts, which develop into the fallopian tubes, oviducts, uterus, cervix, and upper vagina of the female reproductive tract. We confirmed that *Amhr2$^{cre/+}$* mice activated *GFP* reporter gene expression in almost all stromal cells in the entire female reproductive tracts (Son and Chung, unpublished). To elucidate the role of stromal PR in cervical epithelial responses to P_4, I made use of *Amhr2$^{cre/+}$/Pgr$^{f/f}$* mice (referred to as *Pgr$^{sd/sd}$* hereafter; sd, stromal deletion). I evaluated the efficiency of stromal *Pgr* deletion by *Amhr2$^{cre/+}$* in endocervix (endocvx), ectocervix (ectocvx), and vagina (Figure 13A). *Pgr$^{f/f}$* control mice showed similar percentages of PR–positive stromal cells in endocvx (46.23 $\pm$ 1.56%), ectocvx (43.18 $\pm$ 2.97%), and vagina (43.27 $\pm$ 1.48%). *Pgr$^{sd/sd}$* mice showed significantly decreased percentages of PR–positive stromal cells in endocvx (5.76 $\pm$ 1.32%), ectocvx (14.99 $\pm$ 2.21%), and vagina (12.91 $\pm$ 2.70%) (Figure 13B). Stromal PR deletion was more efficient in the endocvx area (87.6%) compared to the ectocvx (65.3%) and vagina (70.2%). Epithelial PR expression was not affected as expected (Figure 13B). The efficiency of recombination was much lower in *Amhr2$^{cre/+}$/Pgr$^{f/f}$* compared to *Amhr2$^{cre/+}$/R26R$^{mTmG/+}$*. We do know a reason that the Cre activity for *Pgr* and *mTmG* GFP reporter alleles was different. It may be relevant to an observation that a *Cre* allele does not recombine all floxed alleles with the same efficiency (180). Although the deletion of *Pgr* was not perfect, I postulated that epithelial responses to P_4 would decrease according to the efficiency of stromal *Pgr* deletion if stromal PR is important. Unexpectedly, epithelial responses to P_4 were similarly diminished in the endocvx, ectocvx, and vagina. Only results from the endocvx area are described in this chapter.

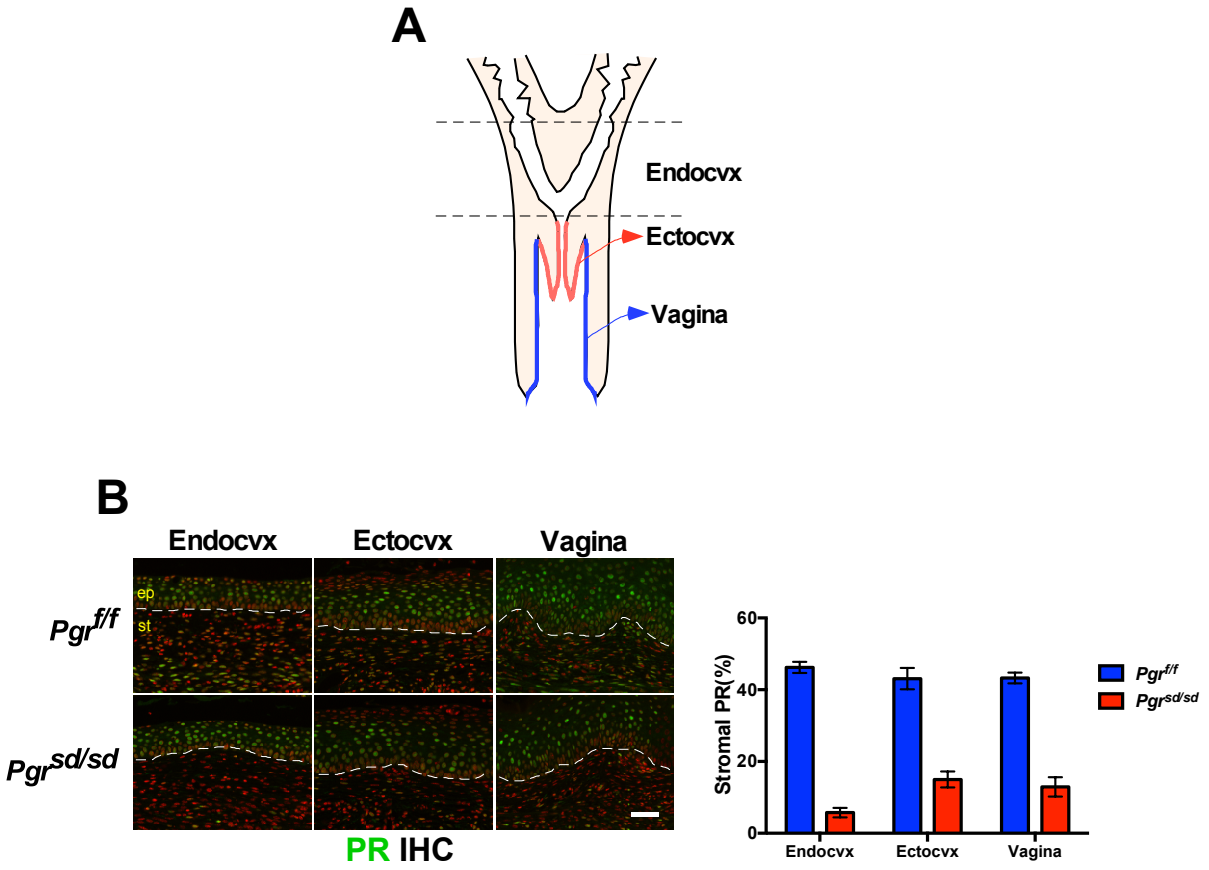


Figure 13. Incomplete but stroma−specific deletion of PR in *Pgr$^{sd/sd}$* mice
(A) Schematics of murine female reproductive tracts. Red and blue lines indicate ectocervical and vaginal epithelium, respectively. **(B)** The ranges of stromal PR deletion are varied in different locations of mouse cervix and vagina. Sections of cervix and vagina were stained for PR (green) and nuclei (red). White dotted lines separate upper epithelium (ep) from bottom stroma (st). Bar scale represents 50 μm. The number of stromal PR positive cells was quantified and normalized to the total number of cells per field of view (200−250 total cells/view)**.** Box and error bars indicate mean and S.E.M, respectively (n=3 per group).

Stromal PR is required for P_4–mediated suppression of E_2–induced cervical thickening.

E_2 and P_4 levels vary throughout menstrual cycle in women and estrus cycle in mice. During metestrus and diestrus (i.e., follicular phase in women), E_2 levels wane, and P_4 levels increase (109,181,182). The hormonal surges are absent during pregnancy and the use of oral contraceptives (183,184). To determine a role of stromal PR in P_4–mediated thinning of cervical epithelia, we first sought to determine the minimal dosage of E_2 to maintain fully thickened cervical epithelium. E_2 (1 μg/day) treatment for 7 days is required to reach the full thickness of cervical epithelium in an ovariectomized mouse (16). Thus, ovariectomized mice were initially treated with 1 μg of E_2 for 7 days and then with varying amounts of E_2 for 3 additional days. Fully stratified cervical epithelium was maintained in the mice treated with 0.2 μg, 0.3 μg, and 1 μg of E_2 (Figure 14A). The cervical epithelium got thin when treated 0.1 μg of E_2. I first treated all mice with 1 μg of E_2 for 7 days to induce the cervical epithelium with full thickness. To mimic hormonal conditions after estrus, I then treated them with low E_2 + high P_4 for 16 hrs and 3 days to mimic metestrus and diestrus, respectively (Figure 14B). To mimic a pharmacological condition, I used 1μg of E_2 and 1 mg of P_4 (i.e., high E_2+high P_4). *Wnt7aCre/Pgr$^{f/f}$ (Pgr$^{ed/ed}$)* and *NTG/Pgr$^{f/f}$ (Pgr$^{f/f}$)* described in Chapter 1 were used for as controls.

In the low E_2 condition, vehicle–treated (i.e., E_2 alone) *Pgr$^{f/f}$*, *Pgr$^{sd/sd}$*, and *Pgr$^{ed/ed}$* had fully thickened cervical epithelium (Figure 15A). Cervical epithelium was well–differentiated and composed of 8–10 cell layers in all genotypes. In *Pgr$^{f/f}$* control mice, cervical epithelium became thin after P_4 treatment for 16 hrs (7–8 cell layers) and 2 days P_4 (5–6 cell layers) (not shown). After 3–day P_4 treatment, cervical epithelium became thinner with only 3–5 cell layers. In *Pgr$^{sd/sd}$* mice, however, thick cervical epithelia were maintained even after 3–day P_4 treatment (8–10 cell layers). In *Pgr$^{ed/ed}$* mice, cervical epithelium was thinner in P_4–treated mice (5–8 cell layers) than in

vehicle–treated control (8–10 cell layers). However, it was still thicker than *Pgr*$^{f/f}$ control genotype treated with P_4 (3–5 cell layers). Similar results were observed in the high E_2 condition. In *Pgr*$^{f/f}$ control mice, cervical epithelium became hypoplastic (3–5 cell layers) upon 3–day P_4 treatment (Figure 15B). Treatment with P_4 for 3 days induced thin cervical epithelium in *Pgr*$^{ed/ed}$, but not in *Pgr*$^{sd/sd}$ mice. These results indicate that both stromal and epithelial PR are required for reducing the cervical epithelium thickness and that stromal PR is more important.

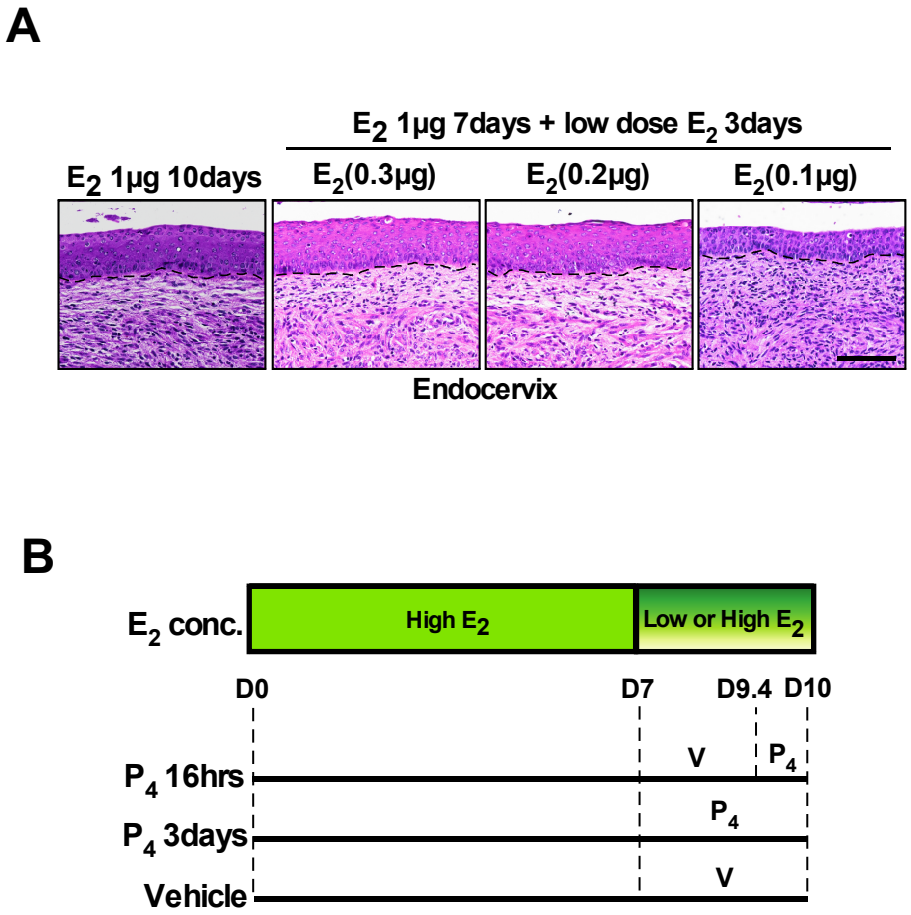


Figure 14. Treatment regimen to determine the roles of stromal PR in different E_2 conditions. **(A)** Low E_2 is enough to maintain high E_2–induced cervical hyperplasia. Wildtype mice were ovariectomized and recovered for 2 weeks. Then, the mice were treated with E_2 (1 μg/day) for 7 days followed by either 0.3 μg/day, 0.2 μg/day, or 0.1 μg/day of E_2 for 3 days. As a control, ovariectomized wildtype mice treated with 1 μg/day of E_2 for 10 days were used. Cervical sections were stained with H&E. Black lines separate epithelium (upper) from stroma (bottom). Bar scale represents 100 μm. **(B)** Treatment regimen of *Pgr*$^{sd/sd}$, *Pgr*$^{ed/ed}$, and *Pgr*$^{f/f}$ mice. Mice were ovariectomized at 6 to 8 weeks of age and recovered for 2 weeks. On day 0, all mice were treated with high E_2 (1 μg) daily for 7 days. On day 7, mice were randomly divided and given either high E_2 (1 μg) or low E_2 (0.2 μg) daily until day 10. In accompany with E_2, mice were treated with P_4 (1mg) for different periods of time: 16 hrs (on Day 9.4) or 3 days (on Day 7). As a control, mice were treated with high E_2 for 7 days followed by 3 days of E_2 with vehicle (v; corn oil). The overall treatment was 10–day long.

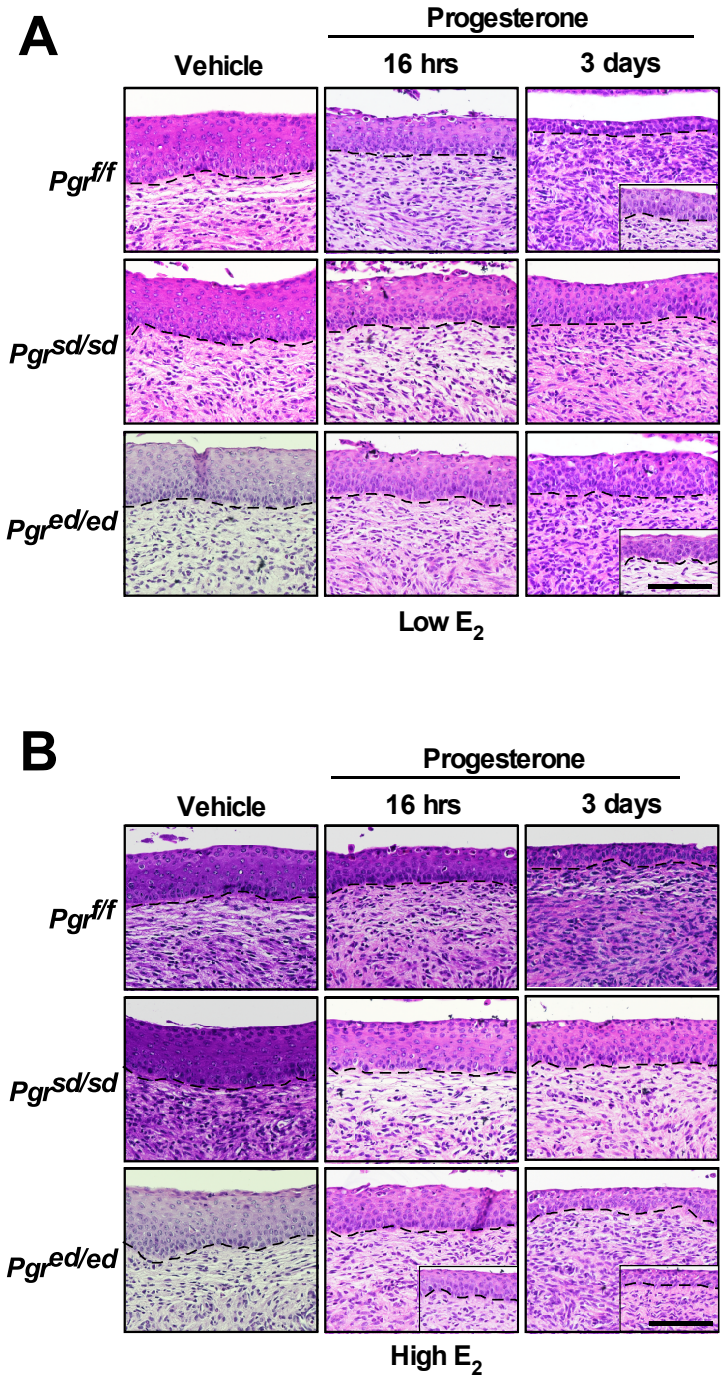


Figure 15. Stromal PR is required to antagonize E_2–induced cervical hyperplasia.
Ovariectomized mice were treated with P_4 for different periods of time **(A)** in Low E_2 (0.2 µg) or **(B)** in high E_2 (1 µg) condition. Their cervical sections were subjected to H&E staining. Representative images of each group are shown (n=1–5). Black lines separate epithelium (upper) from stroma (bottom). The insets are variations within the group. Bar scale represents 100 µm.

Stromal PR is necessary to inhibit cervical epithelial cell proliferation.

P_4 induces thinning of cervical epithelium, in part, by inhibiting epithelial cell proliferation (15,16). To determine whether stromal PR is required for P_4–mediated inhibition of epithelial cell proliferation, I analyzed the expression of Ki67 as a marker for cell proliferation. Under the low E_2 condition, cervical basal epithelial cells were similarly proliferative in vehicle–treated $Pgr^{f/f}$ (69.13 ± 6.20%), $Pgr^{sd/sd}$ (72.66%), and $Pgr^{ed/ed}$ (73.64%) mice (Figure 16). In $Pgr^{f/f}$ mice, cervical epithelial cell proliferation was decreased by treatment with P_4 for 16 hrs (31.61 ± 11.21%) and 3 days (31.39 ± 5.15%). Cervical epithelium was thinner after 3–day P_4 treatment compared to 16–hr treatment (Figure 15A). These results indicate that inhibition of cell proliferation is not the only mechanism of cervical hypoplasia. In $Pgr^{sd/sd}$ mice, cervical epithelial cell proliferation did not change upon P_4 treatment for 16 hrs (72.76 ± 4.68%) and 3 days (66.20 ± 8.05%). In $Pgr^{ed/ed}$ mice, P_4–mediated suppression of cell proliferation occurred at later time point; 3−day P_4 treatment inhibited cervical epithelial cell proliferation (22.71 ± 3.54%), but 16−hr (74.84 ± 11.43%) treatment did not. These results indicate that, under the low E_2 condition, stromal PR is necessary for P_4 to inhibit E_2−induced cervical cell proliferation, and epithelial PR is less much so.

In the high E_2 condition, cervical basal epithelial cells were similarly proliferative in vehicle–treated $Pgr^{f/f}$ (66.31 ± 1.85%), $Pgr^{sd/sd}$ (70.88 ± 3.79%), and $Pgr^{ed/ed}$ (68.93%) mice (Figure 17). These proliferation indices were similar to those in the low E_2 condition described above. In $Pgr^{f/f}$ mice, E_2−induced cervical epithelial cell proliferation was decreased by the treatment with P_4 for 16 hrs (27.77 ± 2.26%) and 3 days (27.74 ± 4.27%). In $Pgr^{sd/sd}$ mice, 16−hr P_4 treatment (8.20 ± 1.12%) inhibited cervical epithelial cell proliferation, but 3−day P_4 (61.21%) treatment did not. In $Pgr^{ed/ed}$ mice, P_4 treatment for 16 hrs (44.60 ± 1.67%) and 3 days (33.30 ± 0.63%) suppressed cervical epithelial cell proliferation. 16–hour P_4 treatment suppressed cell

proliferation in *Pgr*$^{sd/sd}$ mice more than in *Pgr*$^{f/f}$ mice [8.20 ± 1.12% *(Pgr*$^{sd/sd}$*)* vs. 27.77 ± 2.26% (*Pgr*$^{f/f}$)]. These results indicate that stromal PR is more important than epithelial PR in P_4–mediated inhibition of cell proliferation, similar to the conclusion drawn above. However, it was intriguing that P_4 suppressed cell proliferation in *Pgr*$^{sd/sd}$ and *Pgr*$^{ed/ed}$ mice differently depending on the duration of P_4 treatment and E_2 doses. It will be discussed later.

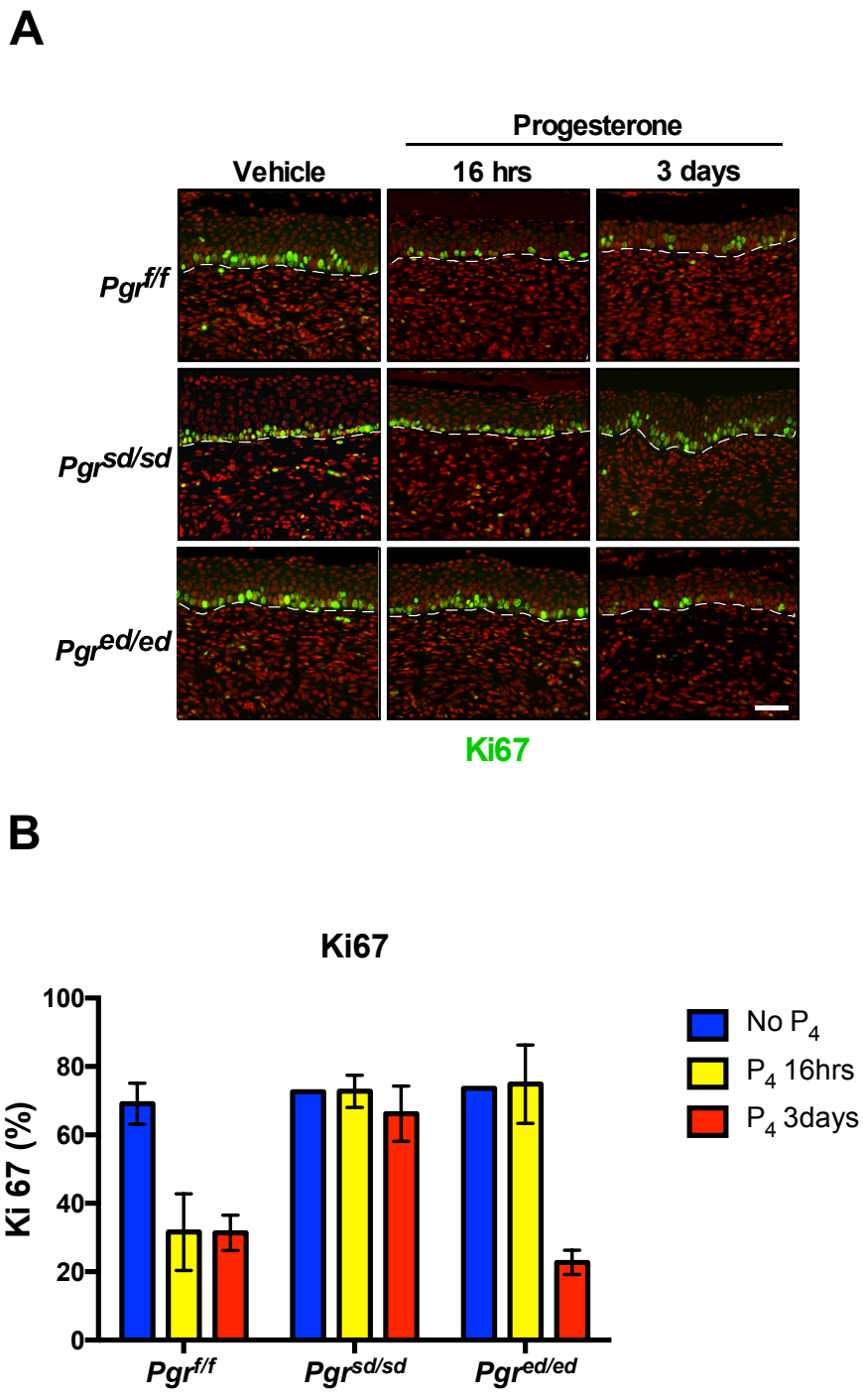


Figure 16. In low E_2 condition, P_4 fails to inhibit cell proliferation in *Pgr*$^{sd/sd}$ mice.
(A) Ovariectomized mice were treated with P_4 in low E_2 condition for different periods of time as indicated. Cervical sections were stained for Ki67 (green) and nuclei (red). Scale bar represents 50 μm. **(B)** Results in (A) were quantified and shown as mean ± S.E.M (n=1–3 per group).

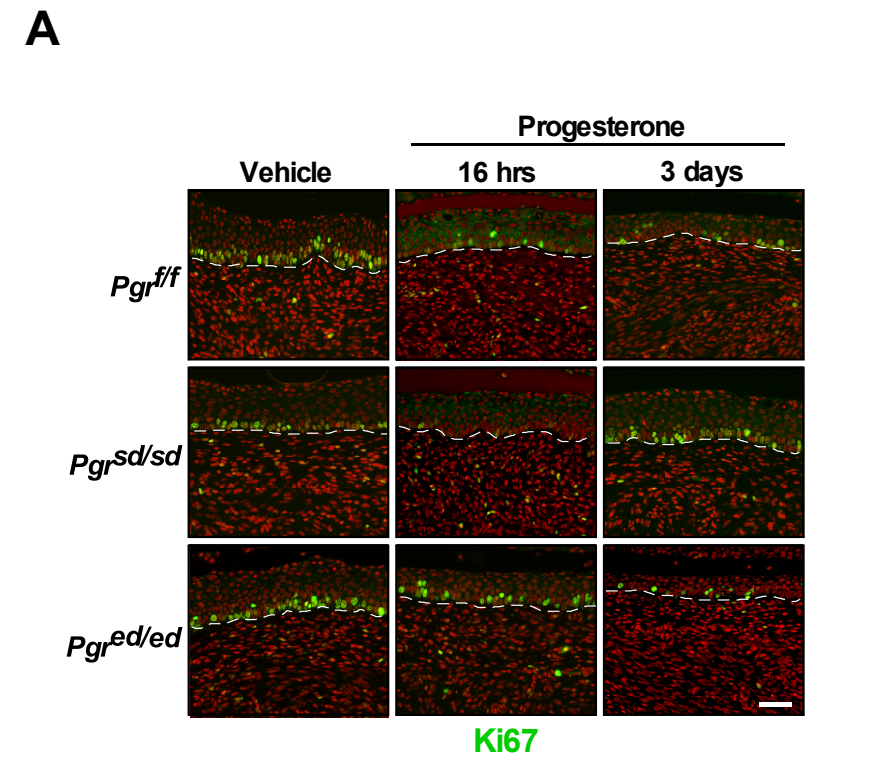


Figure 17. In high E_2 condition, P_4 temporarily inhibits cell proliferation in *Pgr*$^{sd/sd}$ mice. **(A)** Ovariectomized mice were treated with P_4 in high E_2 condition for different periods of time as indicated. Cervical sections were stained for Ki67 (green) and nuclei (red). Scale bar indicates 50 μm. **(B)** Results in (A) were quantified and shown as mean ± S.E.M (n=1–3 per group).

Stromal PR is required for P_4 to promote cervical epithelial cell apoptosis.

P_4 also induces thinning of cervical epithelium by promoting epithelial cell apoptosis (15,16). To determine stromal PR is necessary for P_4–mediated promotion of epithelial cell apoptosis, I analyzed cell apoptosis with TUNEL assay. Under the low E_2 condition, cervical epithelial cells were similarly apoptotic in vehicle–treated $Pgr^{f/f}$ (0.80 ± 0.17%), $Pgr^{sd/sd}$ (1.04%), and $Pgr^{ed/ed}$ (0.98%) mice (Figure 18). In $Pgr^{f/f}$ mice, cell apoptosis was increased by treatment with P_4 for 16 hrs (1.86 ± 0.57%) and 3 days (5.92 ± 0.68%). Cervical epithelium was thinner after 3–day treatment compared to 16–hr treatment (Figure 15A). P_4 inhibited cervical epithelial cell proliferation similarly after 16 hrs (31.61 ± 11.21%) and 3 days (31.39 ± 5.15%) (Figure 16). These results suggest that cell apoptosis is the main mechanism of cervical hypoplasia. In $Pgr^{sd/sd}$ mice, cervical epithelial cell apoptosis did not change upon P_4 treatment for 16 hrs (0.38 ± 0.16%) and 3 days (1.15 ± 0.18%). In $Pgr^{ed/ed}$ mice, 3−day P_4 treatment slightly increased cell apoptosis (2.38 ± 0.22%), but 16–hour P_4 treatment (1.05 ± 0.14%) did not. It was notable that 3−day P_4−mediated cell apoptosis was lower in $Pgr^{ed/ed}$ mice than in $Pgr^{f/f}$ [2.38 ± 0.22 % ($Pgr^{ed/ed}$) vs. 5.92 ± 0.68 % ($Pgr^{f/f}$)]. These results indicate that, under the low E_2 condition, both stromal PR and epithelial PR are necessary for P_4 to promote cell apoptosis in cervical epithelium.

Under the high E_2 condition, cervical basal epithelial cells were similarly apoptotic in vehicle–treated $Pgr^{f/f}$ (0.99 ± 0.09%), $Pgr^{sd/sd}$ (0.83 ± 0.16%), and $Pgr^{ed/ed}$ (0.93%) mice (Figure 19). These apoptotic indices were similar to those in the low E_2 condition described above. In $Pgr^{f/f}$ mice, 16–hour P_4 treatment (2.57 ± 0.45%) increased cell apoptosis, but 3−day P_4 treatment (0.77 ± 0.14%) did not. In $Pgr^{sd/sd}$ mice, 16–hour P_4 treatment (5.70 ± 0.10%) dramatically increased cervical epithelial cell apoptosis compared to the vehicle control (0.83 ± 0.16%), but 3−day P_4 treatment (1.54%) failed to promote cell apoptosis. In $Pgr^{ed/ed}$ mice, 16–hour P_4 treatment

increased cervical epithelial cell apoptosis (1.48 ± 0.03%), and 3−day P_4 treatment further promoted it (6.47 ± 0.99%). It was notable that 3−day P_4 treatment induced cell apoptosis more in *Pgr*$^{ed/ed}$ mice than in *Pgr*$^{f/f}$ mice [6.47 ± 0.99% (*Pgr*$^{ed/ed}$) vs. 0.77 ± 0.14% (*Pgr*$^{f/f}$)]. Also, 16−day P_4 treatment induced cell apoptosis more in *Pgr*$^{sd/sd}$ mice than in *Pgr*$^{f/f}$ mice [5.70 ± 0.10% (*Pgr*$^{sd/sd}$) vs. 2.57 ± 0.45% (*Pgr*$^{f/f}$)]. These results indicate that both stromal and epithelial PR are required for P_4 to induce cell apoptosis in cervical epithelium. However, it was intriguing that P_4 promoted cell apoptosis only transiently in *Pgr*$^{sd/sd}$ and *Pgr*$^{ed/ed}$ mice in the high E_2 condition, unlike in the low E_2 condition. It is possible that it might be similar under the low E_2 condition.

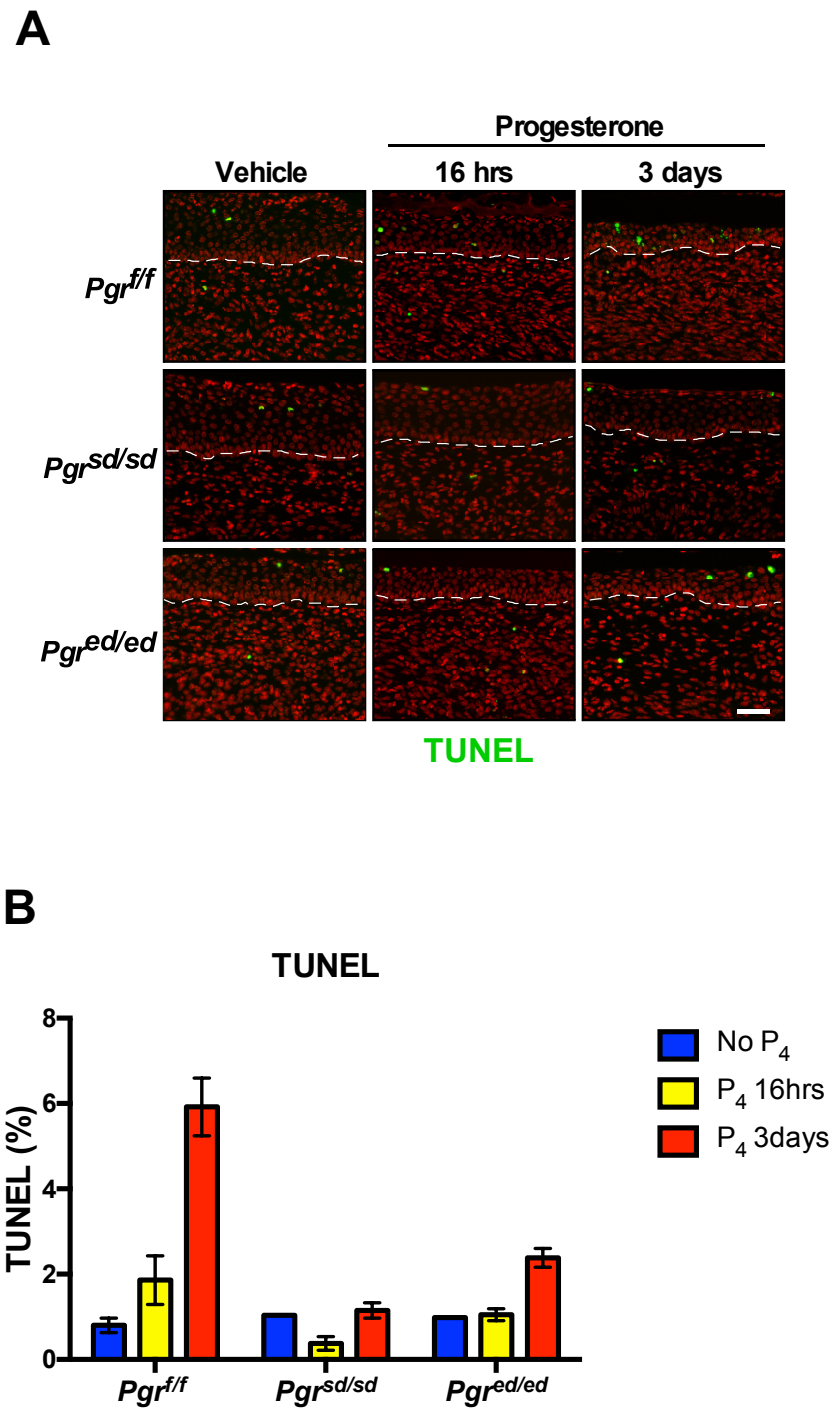
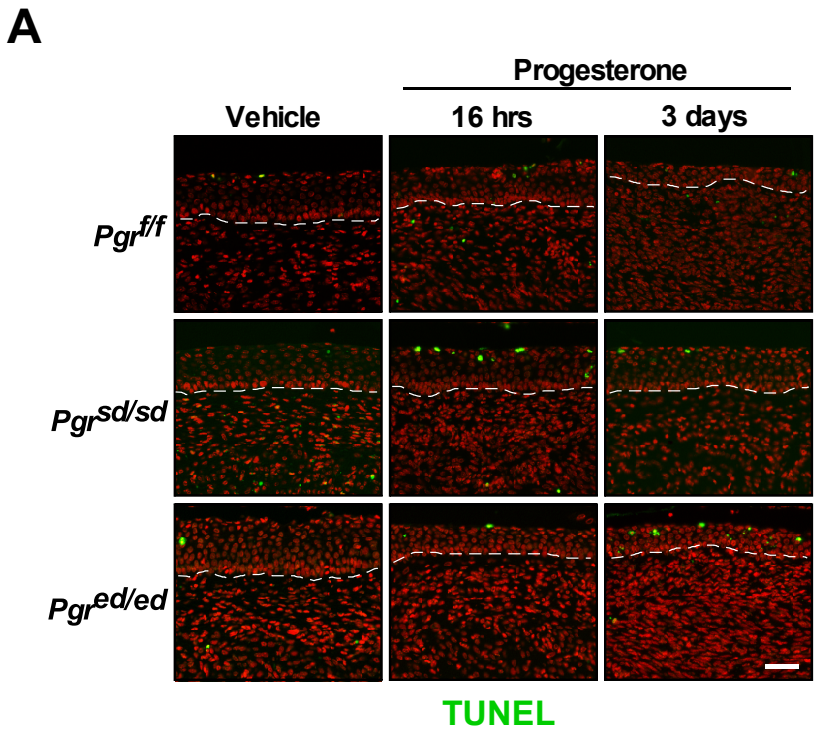


Figure 18. In low E_2 condition, P_4 fails to promote cell apoptosis in *Pgr*$^{sd/sd}$ mice.
(A) Ovariectomized mice were treated with P_4 in low E_2 condition for different periods of time as indicated. Cervical sections were stained for TUNEL (green) and nuclei (red). White lines separate epithelium (upper) from stroma (bottom). Scale bar represents 50 μm. **(B)** Results in (A) were quantified and shown as mean ± S.E.M (n=1–3 per group).



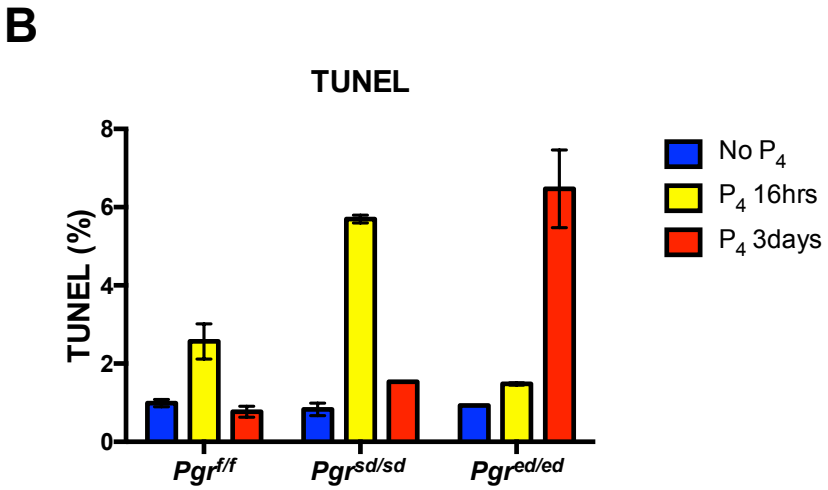


Figure 19. In high E_2 condition, P_4 temporarily promotes cell apoptosis in *Pgr*$^{sd/sd}$ mice.
(A) Ovariectomized mice were treated with P_4 in high E_2 condition for different periods of time as indicated. Cervical sections were stained for TUNEL (green) and nuclei (red). White lines separate epithelium (upper) from stroma (bottom). Scale bar represents 50 μm. **(B)** Results in (A) were quantified and shown as mean ± S.E.M (n=1–3 per group).

C. Preliminary data and future direction

I need to analyze more samples to draw a conclusion.

Both stromal and epithelial PR are required for prolonged inhibition of E_2–induced cervical hyperplasia.

In our previous study (16), we have shown that 7 days of P_4 treatment fails to inhibit epithelial proliferation and induce apoptosis in *Pgr*$^{ed/ed}$ mice under the high E_2 condition. It was inconsistent with my data that 3 days of P_4 treatment inhibited cell proliferation and promoted apoptosis under the same E_2 condition (Figures 17 and 19). Time–dependent effects of P_4 on proliferation and apoptosis under the low E_2 condition were different from those under the high E_2 condition (Figures 16 – 19). I postulated that P_4–mediated inhibition of proliferation and induction of apoptosis follow different kinetic curves in *Pgr*$^{sd/sd}$ and *Pgr*$^{ed/ed}$ mice from *Pgr*$^{f/f}$ control mice. To test this, I treated mice with P_4 for additional time periods (i.e., 4 days, 5 days, or 7 days) under the low E_2 condition (Figure 20A). I first analyzed histology of cervical sections. Longer periods of P_4 treatment failed to make the cervical epithelium thin in *Pgr*$^{sd/sd}$ mice (Figure 20B). In *Pgr*$^{ed/ed}$ mice, 4–day P_4 treatment decreased the thickness of cervical epithelium (5–8 cell layers), but 5–day and 7–day P_4 treatment did not (8–10 cell layers) (Figure 20B). These results suggest that P_4–mediated inhibition of cervical hyperplasia does not occur in the absence of stromal PR and occurs only transiently in the absence of epithelial PR.

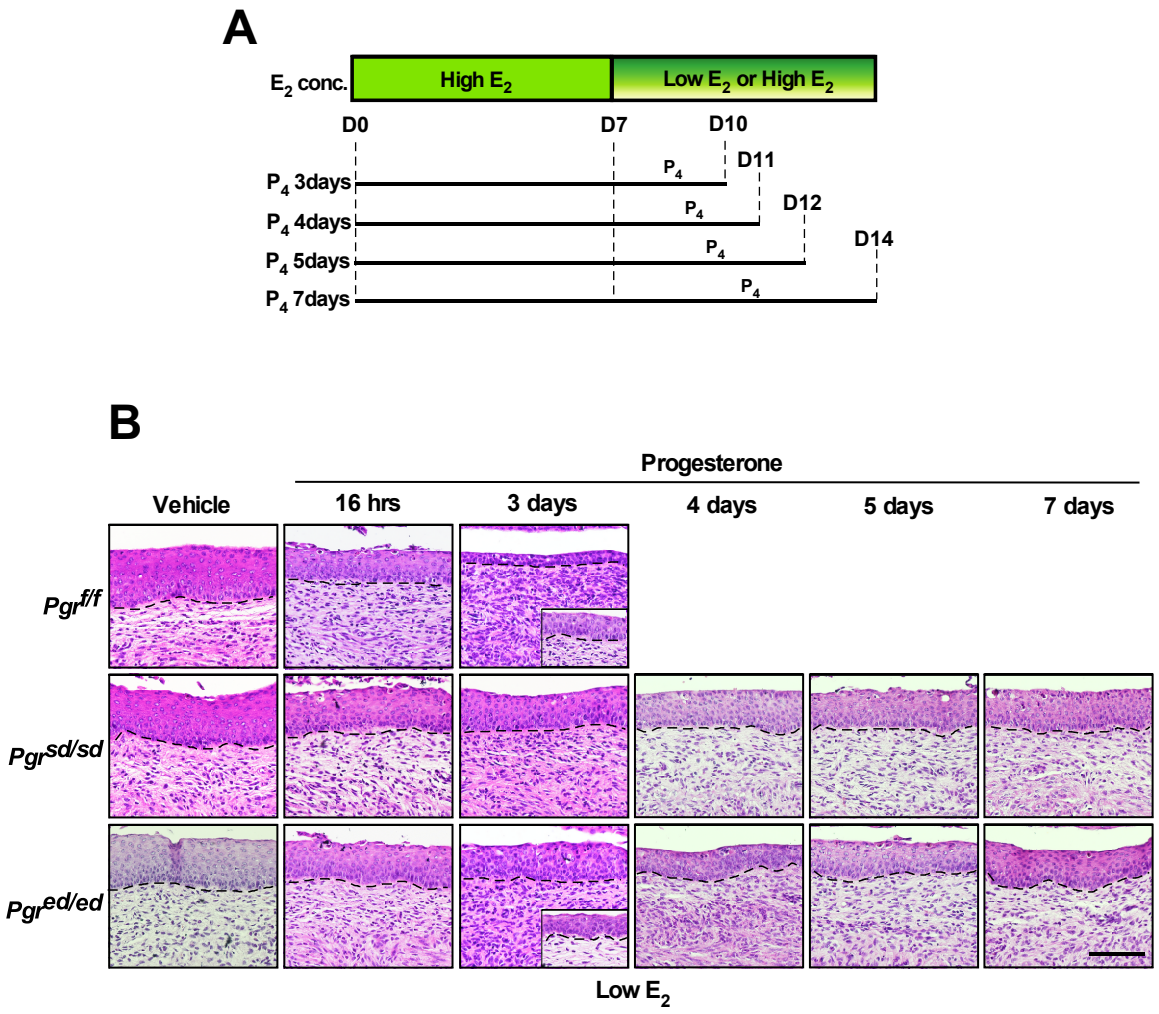


Figure 20. Both stromal and epithelial PR are required for prolonged suppression of cervical hyperplasia.

(A) Treatment Regimen. Mice were ovariectomized at 6 to 8 weeks of age and recovered for 2 weeks. On day 0, all mice were treated with high E_2 (1 μg) daily for 7 days. On day 7, mice were randomly divided and given either high E_2 (1 μg) or low E_2 (0.2 μg) daily. In accompany with E_2, mice were treated with P_4 (1mg) for different periods of time: 3 days, 4 days, 5 days, and 7 days. End points were different for different treatment: 10 days (3 days P_4), 11 days (4 days P_4), 12 days (5 days P_4), and 14 days (7 days P_4). **(B)** Cervical sections from the mice described in (A) were subjected to H&E staining. Images for vehicle, 16 hrs, and 3 days were brought from Figure 15A. Representative images of each group are shown (n=1–5). Black lines separate epithelium (upper) from stroma (bottom). Scale bar represents 100 μm.

Both stromal and epithelial PR may be necessary for P_4 to inhibit E_2–induced cell proliferation and to promote apoptosis in the cervix.

16–hour P_4 treatment temporally inhibited cervical cell proliferation in the high E_2 condition, but the temporary P_4 effect was absent in the low E_2 condition (Figure 16, Figure 17). To determine whether the temporary effect occurs at later time point, I analyzed samples described in the previous section for Ki67 expression, a proliferation marker. In $Pgr^{sd/sd}$ mice, 4−day P_4 treatment (39.48%) inhibited cervical cell proliferation, but the P_4 inhibitory effect was gradually disappeared as the treatment was prolonged to 5 days (52.22%) and 7 days (78.15%) (Figure 21). In $Pgr^{ed/ed}$ mice, 4−day (29.89%) P_4 treatment inhibited cervical epithelial cell proliferation, but the effect also gradually diminished as the treatment was prolonged to 5 days (53.50%) and 7 days (78.15%), similar to $Pgr^{sd/sd}$ mice. Proliferation is inhibited in 7−day P_4 treated $Pgr^{f/f}$ mice (16). These results suggest that stromal PR and epithelial PR are required for P_4 to continuously inhibit cell proliferation in cervical epithelium.

I also determined apoptotic indices in these tissues. In $Pgr^{sd/sd}$ mice, although small, 4−day P_4 treatment (2.35%) increased cell apoptosis in the cervix compared to vehicle treatment (1.04%). The apoptotic indices remained higher than vehicle control after P_4 treatment for 5 days (1.66%) and 7 days (2.11%) (Figure 22). In $Pgr^{ed/ed}$ mice, 4−day P_4 treatment (6.44%) significantly increased the apoptotic index compared to the vehicle control (0.98%) (Figure 22B). As P_4 treatment was continued, P_4–mediated cell apoptosis in the cervical epithelium was decreased by approximately 2–fold (3.10% at 5 days, 3.58% at 7 days). These results suggest that both stromal PR and epithelial PR are required for P_4−mediated promotion of cell apoptosis at the level similar to wild–type mice.

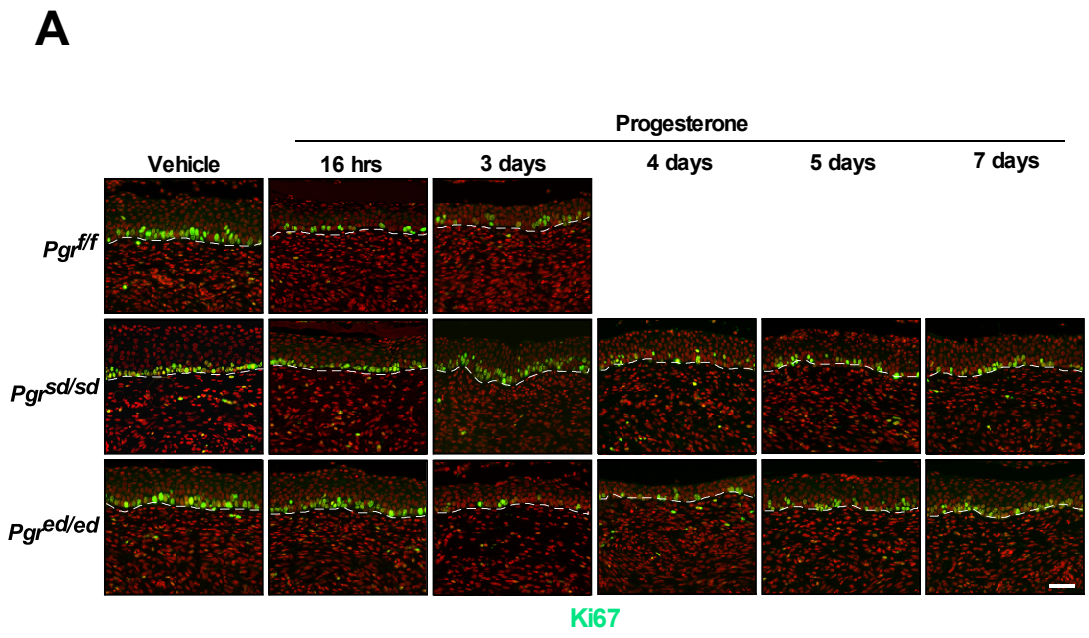


Figure 21. Both stromal and epithelial PR are required to suppress E_2–induced cell proliferation in cervix.

(A) Ovariectomized mice were treated with P_4 in low E_2 condition for different periods of time as indicated. Cervical sections were stained for Ki67 (green) and nuclei (red). White lines separate epithelium (upper) from stroma (bottom). Scale bar represents 50 μm. Data for vehicle, 16 hrs, and 3 days were brought from Figure 16. **(B)** Results in (A) were quantified and shown as mean ± S.E.M (n=1–3 per group).

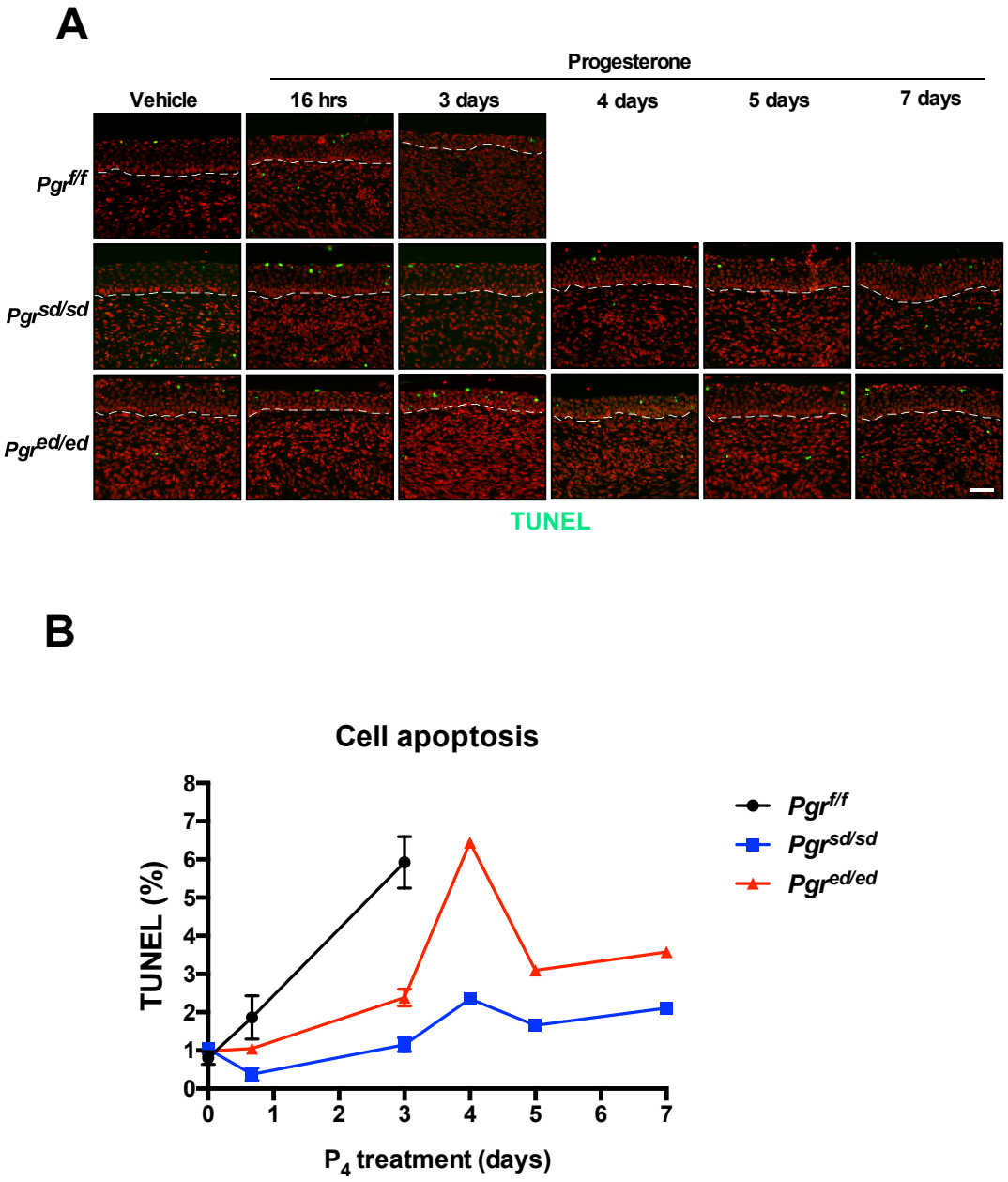


Figure 22. Both stromal and epithelial PR may be required to induce cell apoptosis in cervix. **(A)** Ovariectomized mice were treated with P_4 in low E_2 condition for different periods of time as indicated. Cervical sections were stained for TUNEL (green) and nuclei (red). White lines separate epithelium (upper) from stroma (bottom). Scale bar indicates 50 μm. Data for vehicle, 16 hrs, and 3 days were brought from Figure 18. **(B)** Results in (A) were quantified and shown as mean ± S.E.M (n=1–3 per group).

D. Discussion

In the present study, I investigated a role of stromal PR and epithelial PR in P_4–mediated suppression of proliferation and survival of cervical epithelial cells. Although the deletion was not complete, I was able to specifically delete *Pgr* in cervical and vaginal stroma using $Amhr2^{cre/+}$ knock−in mouse (Figure 13B). Stromal PR was required for P_4 to inhibit cervical hyperplasia and cell proliferation in the cervix (Figure 21). In addition, it was also essential to promote cell apoptosis in cervical epithelium (Figure 22). Consistent with our previous study, we found that epithelial PR is also necessary to suppress cell proliferation and to promote cell apoptosis in cervical epithelium (Figure 21, Figure 22). Taken together, both stromal and epithelial PR are required to fully suppress cell proliferation and induce cell apoptosis in cervical epithelium. A mechanism by which stromal and epithelial PR exerts these activities remains to be determined.

Selective deletion of stromal PR in the cervix

The *Cre*−mediated *Pgr* deletion by $Amhr2^{Cre/+}$ mice was incomplete in the cervix (Figure 13). The partial activity of $Amhr2^{Cre/+}$ has been discussed in the uterus in which the *Cre* activity is recombining a flowed *Esr1* allele is observed only in anti−mesometrium (185,186). Here, $Amhr2^{Cre/+}$ mice exhibited a higher *Cre* activity in endocervical stromal cells than in ectocervical and vaginal stromal (Figure 13B). The *Amhr2*−*Cre* allele is not effective in myometrial cells. Thus, stromal PR effect is likely through fibroblasts. Anti−proliferative signaling from the remaining PR–positive stromal cells in $Pgr^{sd/sd}$ mice may be insufficient.

Potential mechanisms of stromal PR for P_4 in the cervix

In the current study, we have demonstrated for the first time that stromal PR is necessary to antagonize E_2–induced cervical hyperplasia and cell proliferation (Figure 20, Figure 21). P_4 also induced cell apoptosis in a stromal PR–dependent manner (Figure 22). Stromal PR alone, however, was not enough to exert full P_4 effects. Epithelial PR was also required to inhibit E_2−promoted epithelial cell proliferation and survival in the mouse cervix, consistent with our published results (15,16). Collectively, these data strongly support that both stromal and epithelial PR are necessary for P_4 to impede E_2−mediated cell proliferation and survival. One possible mechanism is that stromal PR secretes paracrine factors that inhibits epithelial cell proliferation in the cervix. The fact that incomplete deletion of stromal PR abrogates P_4 effects suggests that such factors need be at a certain concentration; i.e., secretion from a smaller number of stromal cells is not sufficient to mediated P_4 effects to the full extent. Several paracrine signaling pathways have been identified as PR downstream in the mouse uterus. Stromal PR is necessary to induce epithelial Indian hedgehog (IHH) expression in the uterus (187-189). IHH binds to its receptor patched homolog 1 (PTCH1) located on the surface of the stromal cells and inhibits expression of fibroblast growth factors (FGF) through a PTCH1–Hand2 complex, which in turn disrupts uterine epithelial cell proliferation (172,189,190). Dickkopf–1 (Dkk1) and forkhead box O1 (FOXO1), known as PR cellular targets in the uterine stroma, inhibit oncogenic Wnt/β–catenin signaling in uterine epithelial cells through paracrine mechanisms (187,191,192). Epithelial and stromal PR–specific target genes in the cervix need to be elucidated to understand paracrine signaling mediated via P_4/PR. Another possible mechanism is that stromal PR represses pro–proliferative and pro–survival activity of stromal ERα. Stromal ERα is required for E_2–induced epithelial cell

proliferation in the cervix (13,14). It has shown that PR physically interacts with ERα and modulates its function in breast cancer cells (193).

Different kinetics of P_4 effects depending on E_2 concentrations

In the *Pgr*$^{sd/sd}$ and *Pgr*$^{ed/ed}$ mice, we have observed that inhibition of epithelial cell proliferation and promotion of cell apoptosis by P_4 were transient, while the effects are likely prolonged in the *Pgr*$^{f/f}$ mice (16). It was unexpected that the transient P_4 responses in the *Pgr*$^{sd/sd}$ and *Pgr*$^{ed/ed}$ mice were faster in the high E_2 condition than in the low E_2 condition. In the *Pgr*$^{sd/sd}$ mice, 16–hour P_4 treatment temporally suppressed E_2–induced cervical epithelial cell proliferation in the high E_2 condition, but 4–day P_4 treatment was required in the low E_2 condition. In the *Pgr*$^{ed/ed}$, 16–hour P_4 treatment inhibited epithelial cell proliferation under the high E_2 condition, but 3–day P_4 treatment was necessary under the low E_2 condition. Similarly, 16–day P_4 treatment induced apoptosis in the *Pgr*$^{sd/sd}$ mice under the high E_2 condition, but 4–day P_4 treatment was required under the low E_2 condition. In the *Pgr*$^{ed/ed}$ mice, 3–day P_4 treatment induced cell apoptosis in the high E_2 condition, but 4–day P_4 treatment was required in the low E_2 condition. These phenotypes may be detected in *Pgr*$^{f/f}$ mice if we analyze more time points. It is possible that significant promotion of cell apoptosis in the *Pgr*$^{f/f}$ earlier than 3–day P_4 treatment in the high E_2 condition. In summary, my results indicate that epithelial and stromal PR are required for P_4 to inhibit cell proliferation. They also suggest that progestin therapy would be effective only if PR is expressed in cancer cells and cancer stroma.

Potential mechanism of ERα and PR in cervical cancer

Ablation of ERα in stromal cells results in complete regression of cervical neoplastic disease in *K14E7* transgenic mouse model (13). Deletion of epithelial ERα delays, but does not abrogate, cervical carcinogenesis in *K14E7* mice (14). These results indicate that stromal, but not epithelial, ERα is required for maintenance of CIN and its progression to cervical cancer (Figure 23). It is likely that stromal ERα promtes cervical cancer through a paracrine mechanism. Genes encoding pro–inflammatory cytokine and chemokines such as *CXCL1* and *CXCL5* are upregulated in cervical stroma of E_2–treated *K14E6/K14E7* mice compared to normally cycling mice (194). In addition, two ER antagonists (i.e., ICI 182,780 and Methyl Piperidino Pyrazole) suppress expression of several pro–tumorigenic genes such as *FGF1*, *VEGF–C*, and *EREG* in *ex vivo* cultured cervical cancer–associated fibroblasts (195). Results from our lab demonstrate that epithelial PR is required for cervical cancer regression by MPA (Baik *et al*., unpublished). My results suggest that stromal PR is also necessary (Figure 23). If PR is not expressed in the cervical stroma, MPA would be inefficient in treating cervical cancer regardless of PR status in cancer, and the risk of cervical cancer would increase. It is a possibility that stromal PR suppresses stromal ERα–mediated upregulation of pro–tumorigenic secretory factor–coding genes. Because epithelial ERα is required for PR expression, it is possible that the loss of epithelial ERα expression promotes cervical cancer.

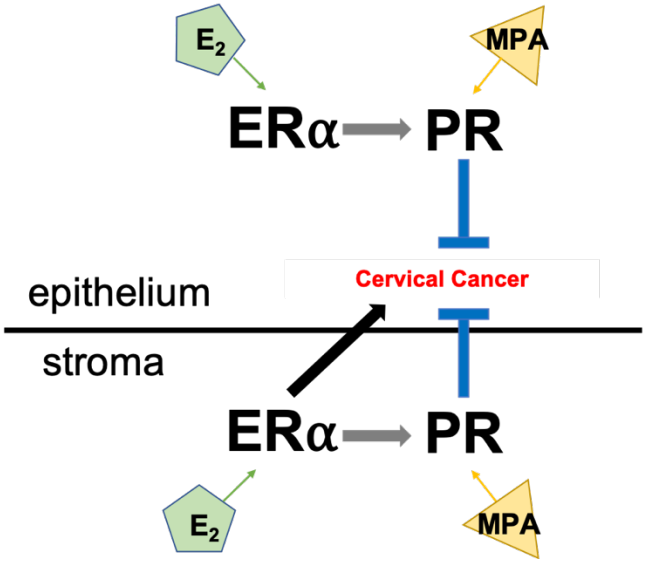


Figure 23. A proposed model of ERα and PR mechanisms in cervical cancer.
ERα activates PR expression in the cervical epithelium and stroma. Epithelial PR and stromal PR cooperate to regress cervical cancer.

VI. SUMMARY AND SIGNIFICANCE

The global burden of cervical cancer has not been decreased even in the presence of effective vaccines, which emphasizes the importance of better understandings of the cervix and the disease. PR has been hypothesized as a tumor suppressor in cervical cancer in a ligand–dependent manner. Elucidating the underlying mechanisms of PR has been limited due to lack of PR expression in cervical cancer cell lines. *In vivo*, the HPV–transgenic mouse model has been used a powerful model to demonstrate that PR signaling is tumor suppressive in cervical cancer. Though the HPV–transgenic mouse model that highly recapitulates cervical cancer patients in terms of pathogenesis, some limitations have had to be overcome for better clinical relevance: the necessity of chronic estrogen treatment for cervical carcinogenesis and uniformed cancer population with positive ERα and PR expression. In this dissertation, I have generated the mouse model which develops cervical cancer spontaneously. The development of PR–negative and PR–positive cancers in the mouse model helps us to understand the pathways involved in the loss of PR expression in the patients. In our previous study, MPA has been suggested as an effective prevention and treatment for cervical cancer driven with exogenous estrogen treatment. It could be determined in my mouse model that MPA can reduce cancer incidence and prevent the loss of PR expression, which may support the effectiveness of MPA or other PR agonists in cervical cancer. It may also answer whether the PR–positive cancers are still active and responsive to the treatment. Therefore, the mouse model that I generated in this dissertation highly recapitulates cervical cancer patients than the original model. The tumor suppressive functions of PR have been mostly determined by focusing on epithelial PR rather than stromal PR because cervical cancer is originated from epithelium. Roles of stromal PR in cervical cancer have been underestimated. As many evidences

support epithelia–stroma interplay in carcinogenesis, the necessity of understandings of stromal PR in cervical carcinogenesis has been emerged. In this dissertation, for the first time, I have suggested that stromal PR is required for P_4 to suppress cell proliferation and to induce cell death in the cervix under physiological and pharmacological conditions, suggesting possible involvements of stromal PR in cervical carcinogenesis, which needs to be determined with independent study. The manifest anti–proliferative and pro–apoptotic effects of stromal PR with incomplete deletion also support significance of stromal PR in cervix. The fact that hormone levels alter kinetics of P_4 responses suggests the necessity of thoughtful considerations of patients' hormone status for diagnosis and treatment. So far, my dissertation supports that PR is a ligand–dependent tumor suppressor in cervical cancer with cell–type–specific functions.

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