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Neoplastic change of squamo-columnar junction in uterine cervix and vaginal epithelium by exogenous estrogen in HPV-18 *URR E6/E7* transgenic mice☆

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Abstract

Objective. The goal of this study was to study whether estrogen could induce progression of cervical neoplasia by the influence of direct hormonal transactivation of the viral genes.

Methods. We examined the in vivo effect of estrogen on HPV-18 *URR E6/E7* transgenic mice. We analyzed the growth stimulation of epithelial cells at squamo-columnar junction and vagina and the expression of HPV *E6/E7* in transgenic mice. The promoter activity of HPV-18 *URR* after treatment of estrogen was also evaluated by in vitro transient transfection assay.

Results. The dysplastic lesions of lower genital tract were more frequently seen in the HPV-18 *URR E6/E7* transgenic mice and estrogen-treated mice, when compared to those of control group ($P < 0.05$). The majority of cells in whole epithelial layer of cervix and vagina were proliferating cell nuclear antigen-positive (PCNA) by immunohistochemical staining in the estrogen-treated transgenic mice. Hyperplastic glandular lesions were also identified in estrogen-treated HPV-18 *URR E6/E7* transgenic mice (5 of 21) and estrogen-treated nontransgenic mice (2 of 10). The level of *E6/E7* transcripts in transgenic mouse was increased in the presence of estradiol. Treatment with 0.5×10^{-6} M estradiol in the presence of cotransfection with the estrogen receptor expression vector and *URR*-CAT showed an additive effect of CAT activity (4.8-fold increase).

Conclusion. The HPV *E6* and *E7* oncogenes implicated in cervical cancer are indeed capable of potentiating tumor formation in animal model. Continual estrogen-induced proliferation might be viewed by in vivo and in vitro mechanisms by which squamous cells as well as glandular cells in cervix and vagina became permissive for neoplastic progression in HPV-18 *URR E6/E7* transgenic mice and their molecular systems.
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Keywords: Cervical cancer; Human papillomavirus (HPV); Estrogen; *URR*; *E6*; *E7*

Introduction

Invasive cervical cancer is preceded by a progressive spectrum of abnormalities of the cervical epithelium, which are considered precancerous lesions, such as cervical intra-epithelial neoplasm (CIN). Infection with high-risk human papillomavirus (HPV) types, such as HPV-16 and HPV-18, is strongly correlated with development of cervical cancer

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[1]. High-risk HPV types encode two oncoproteins, E6 and E7, which inactivate the function of the retinoblastoma and p53 tumor suppressor proteins and which have been shown to immortalize and transform cervical epithelial cells in vitro. However, most cervical lesions containing high-risk HPVs do not progress to invasive cervical cancers, implicating other cofactors in those rare cases where progression occurs. One significant cofactor that has been associated with cervical neoplastic lesions is exposure to estrogen. Estrogen has been shown to be a direct carcinogen, an effect apparently linked to a specific pathway of oxidative hormone metabolism [2]. Moreover, HPV infection has been shown to markedly increase the formation of these potentially carcinogenic estrogen metabolites [3]. Pregnancy appears to be a permissive environment for persistent HPV infection, while prolonged use of oral contraceptives (OCPs), most of which contain estrogen, has been shown to double the risk of HPV-induced cervical neoplasia [4]. More controversial are the data surrounding the relationship between hormonal use and cervical adenocarcinoma. The epidemiologic and clinicopathologic studies generally but not uniformly have demonstrated a 1.5- to 5-fold increase in the odds ratio for use of oral contraceptives among women with cervical adenocarcinoma compared with controls [5,6]. However, these studies have not been adjusted for HPV infection. Some case–control studies demonstrated a strong relationship between adenocarcinoma and infection with HPV-16, -31, and more frequently with HPV-18 [7,8]. With sensitive molecular techniques, these HPV types have been identified in adenocarcinoma and adenosquamous carcinomas with frequency of 80% or more [9,10].

Transcription of HPV *E6/E7* gene is controlled by the regulation of the upstream regulatory region (*URR*) in HPV. It has been proposed that a number of transcriptional factors, including NF 1, AP-1, SP1, YY1, and KRF-1, participate in the positive or negative regulation of *URR* activity [11,12]. Furthermore, the *URR* has been shown to contain response elements for progesterone and glucocorticoid. There is also speculation about the existence an estrogen response element (ERE) based on the activation the HPV-16 *URR* by estrogen in SiHa cells [13]. A current hypothesis is that estrogen may contribute to HPV persistence and subsequent neoplastic progression by increasing viral gene expression. But the precise mechanisms by which estrogen might contribute to HPV-induced neoplasia are still obscure. In vitro culture systems have provided important insights into the biological activities of viral oncogenes, but such studies do not provide an accurate picture of oncogenic role in development of cervical cancer [14]. Better understanding of the changes in cervical epithelium that contribute to cervical neoplastic progression will be an important contribution to women's health. Studying the process in human subjects is difficult; thus investigators have turned to the use of transgenic animals to better assess the contribution of individual oncogenes to cervical carcinogenesis.

In this study, we sought to determine whether estrogen could cooperate to induce cervical neoplasia by the influence of direct hormonal transactivation of the viral genes. We used the endogenous *URR* of HPV-18, which is regulated by the natural promoters and repressors. We analyzed the growth stimulation of epithelial cells at squamo-columnar junction and vagina and the expression of HPV *E6/E7* in transgenic mice and in vitro promoter activity of HPV-18 *URR* by estrogen treatment.

Materials and methods

Gene construction and production of transgenic mice

The amplified sequence from HPV-18 plasmid was used for the generation of the transgene containing a 1.6-kb fragment of HPV-18 consisting of the intact *URR* and functional *E6* and *E7* open reading frames, using primers from HPV-18 *URR* upstream portion (5′TATGTGTGTGTGTATATATAT-3′) to HPV-18 *E7* downstream portion (5′TTATTACTGCTGGGATGCACAC-3′). The PCR fragment was subcloned into pCEP4 (Invitrogen, CH Groningen. The Netherlands) carrying SV40 poly(A) sequence. A 2.0-kb DNA fragment including HPV and SV40 poly(A) sequences were gel-purified from 0.7% agarose gel and dialyzed against TE buffer before use. Transgenic mice expressing HPV *E6/E7* under control of the HPV-18 promoter were created in our previous experiment [15,16]. Briefly, the amplified DNA fragment concentrations were adjusted to 4 ng/ml and injected into fertilized eggs derived from hybrid C57BL/6 × CBA (supplied from KRIBB in Korea). Back-crosses with C57BL/6 or BALB/c were carried out to create transgenic lines, and the lineages were maintained as heterozygotes. Nontransgenic littermates were used as the control group. Transgenic mice were identified by PCR using a DNA template prepared from the tails of offspring at 4 weeks after birth.

Reverse transcription–PCR

RNA was isolated from pieces of whole organs, including brain, skin, heart, lung, cervix, and liver of tumor-forming transgenic mouse. One microgram of total RNA was reverse-transcribed, and the cDNA was amplified using primers spanning whole coding sequence from *E6* to *E7* ORF. PCR products were visualized by ethidium bromide staining of 2.0% agarose gels in skin and cervix (Fig. 1).

Estrogen treatment

Subcutaneous continuous release pellets that deliver 17β-estradiol at dose of 0.25 mg over 60 days (Innorvative Research of America, Sarasota, FL, USA) were implanted in the dorsal back skin of heterozygous 1-month-old HPV-18 *URR E6/E7* transgenic mice. Groups of mice were treated with estrogenic hormone for 4 months. The trans-

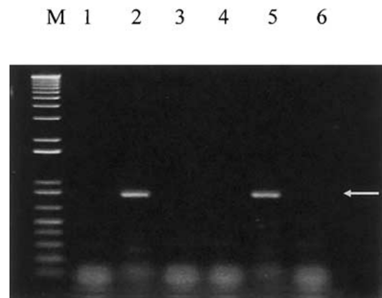


Fig. 1. RT-PCR analysis of *E6/E7* gene in transgenic mouse. Total RNAs were purified from brain (lane 1), skin (lane 2), heart (lane 3), lung (lane 4), cervix (lane 5), and liver (lane 6) of tumor-forming transgenic mouse and subjected to RT-PCR. Expressed *E6/E7* cDNA band is indicated by arrow.

genic mice and the control group were treated with one additional pellet insertion after 2 months. The live experimental mice were sacrificed at 4 months, if no morbidity occurred. The dead mice, which were survived at least 2 months after pellet insertion, were included in the study. Mice were housed in a pathogen-free barrier facility, and all procedures were approved by the Committee on Animal Research in The Catholic University of Korea.

Histology and immunohistochemical staining

Estrogen-treated transgenic offspring ($N = 30$) were sacrificed at 4 months. Nontransgenic littermates ($N = 10$) were also treated by subcutaneous continuous release pellets of 17β-estradiol at dose of 0.25 mg. Transgenic and nontransgenic mice (each group; $N = 10$) without estrogen treatment were used as negative controls for comparison with the estrogen-treated mice. After euthanasia, the vagina, cervix, both uterine horns, and other specific tissues were removed and immediately fixed in 10% formalin in PBS overnight. Tissues were dehydrated through graded alcohol and xylene and embedded in paraffin. Five micrometer sections through the full tissue were prepared, mounted, deparaffinized, and stained with hematoxylin and eosine. Two pathologists (H.S.K. and J.S.P.) blindly reviewed the lower genital tract biopsies of experimental mice. Pathologic lesions from squamo-columnar junction of cervix and vagina were classified by routine diagnostic criteria [17]. Briefly, these were as follows: cases of reserve cell hyperplasia showed multiple layers of small reserve cells covered by a residual layer of columnar cells on the surface; in all cases of intraepithelial neoplasia of the cervix and vagina (CIN) and Vain, nuclear abnormalities, such as nuclear enlargement, hyperchromatism and pleomorphism, were present; the presence of PCNA, a component of the δ-DNA polymerase, was used to determine which cells were proliferating. Tissues from nontransgenic mice without estrogen treatment were used for controls for PCNA expression. The results of immunostaining were evaluated independently without regard to the histologic diagnosis. The paraffin sections were air-dried overnight and rehydrated through graded alcohol and PBS. After endogenous peroxidase activity was blocked with 0.1% H_2O_2 for 15 min, sections were treated with proteinase K (Boehringer Mannheim, Indianapolis, IN, USA) using 20 μg/ml in PBS for 20 min to expose antigens. Horse serum (1.5% in PBS) was used to suppress nonspecific binding. Sections were incubated with a 1 : 200 dilution of PCNA mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C and developed using the avidin–biotin–immunoperoxidase (ABC) immunostaining method.

Transient transfection and CAT ELISA

In vitro transient transfection assay was carried out in C33A cells using HPV-18 *URR* chloramphenical acetyl transferase (CAT) reporter plasmid and estrogen receptor (ER) expression vector by the $CaPO_4$/DNA precipitation method. ER expression vectors were obtained from Dr. Pierre Chambon (Strasbourg, France). The whole sequence of HPV-18 *URR* was amplified from this plasmid and cloned into *Bam*HI and *Hind*II sites of pBLCAT8+ for in vitro transfection assay. To generate stable cell lines, 6 μg of the *URR*-CAT expression plasmid or SV40-derived β-galactosidase (β-Gal) internal control plasmid was cotransfected into the C33A cells by $CaPO_4$/DNA precipitation. C33A cells (10^6), maintained in phenol red-free DMEM with 10% FBS, were plated in 60-mm dishes for 5 h before transfection. After overnight incubation, cells were washed with PBS, complemented with estradiol, and then incubated for 24 h. Cells were washed twice with ice-cold PBS, collected, resuspended in 100 μl of 0.25 M Tris–HCl (pH 7.6), and subjected to three freeze–thaw cycles. Samples were cleared by centrifugation (12,000 rpm, 10 min, 4°C) and protein concentrations were determined using a Biorad protein assay dye reagent (Biorad, Hercules, CA, USA). β-Galactosidase was assayed according to standard protocol [18]. Fifty microliters of the clear lysates was tested for CAT concentration in the CAT ELISA according to the manufacturer's instructions (Boehringer Mannheim). The CAT concentration of each sample was normalized with respect to β-Gal activity. All the data presented in the text represent the mean of at least three independent transfections. Stimulation was calculated by dividing the amount of CAT enzyme in the treated cells by that in the untreated cells.

Statistical analysis

Comparisons between the estrogen-treated group and untreated group, transgenic mice, and nontransgenic mice were based on the chi-square test, because the numbers are too small for a test. In all cases a P value < 0.05 was considered statistically significant.

Table 1
Number of mice treated with or without 0.25 mg 17β-estradiol for 4 months

	No. of mice	Mortality (%)	No. of evaluable mice
Treated with estradiol			
Transgenic mice	30	13 (43)	22
Nontransgenic mice	10	6 (60)	4
Treated without estradiol			
Transgenic mice	10	0	10
Nontransgenic mice	10	0	10

Results

Histopathologic findings of cervico-vaginal epithelium

Mice treated with the dose of 0.25 mg 17β-estradiol were euthanized when morbidity was apparent, as detected by difficulty in walking. Estrogen treatment caused mortality due to urinary bladder dysfunction in both transgenic and nontransgenic mice. These mice were lost by obstructive urologic condition because of hypertrophy in the bladder neck. The other mice died of unknown causes. The cervical preparations were examined histologically. If no morbidity was apparent, mice were sacrificed at 24 weeks after insertion of estrogen pellet. A summary of this analysis is shown in Table 1. Dysplastic lesions of the lower genital tract were more frequently seen in the HPV-18 *URR E6/E7* transgenic mice (20/22), compared the nontransgenic mice (4/10) ($P < 0.05$) (Table 2). The incidence of dysplasia was significantly increased in estrogen-treated mice (22/22), compared to nonexposed mice (2/10) ($P < 0.05$) (Table 3). We also evaluated tissue sections for cells PCNA-positive in representative cervical epithelium. Such cells would be in late G1 or the S phase of the cell cycle. In transgenic mice without estrogen, PCNA was detectable only in the basal layer of the cervical epithelium (Fig. 2A), whereas the majority of cells in whole epithelial layer of cervix (Fig. 2B) and vagina (Fig. 2C) were PCNA-positive in the estrogen-treated transgenic mice. Hyperplastic glandular lesions were also identified in transgenic mice (5 of 22) and nontransgenic mice (2 of 10) (Table 4). An atypical glandular hyperplastic lesion mimicking adenocarcinoma in situ was detected in one estrogen-treated transgenic mouse (Fig. 3). Most mice with hyperplastic glandular lesions had coexisting squamous intraepithelial lesions.

Table 2
Histological grade of pathologic lesion in HPV-18 *URR E6/E7* transgenic mice and control mice

Histological grade	Transgenic mice[a] ($N = 22$)	Nontransgenic mice ($N = 10$)
Reserve cell hyperplasia	2	2
CIN 1 and/or VAIN 1 (LGSIL)	13	3
CIN 2 and/or VAIN 3 (HGSIL)	7	1

Note. CIN, cervical intraepithelial neoplasia; VAIN, vaginal intraepithelial neoplasia; LGSIL, low-grade squamous intraepithelial lesion; HGSIL, high-grade squamous intraepithelial lesion.

[a] The dysplastic lesions of lower genital tract were more frequently seen in the HPV-18 *URR E6/E7* transgenic mice (20/22), compared to the nontransgenic mice (4/10) ($P < 0.05$).

Table 3
Histological grade of pathologic lesion in mice treated with or without estrogen

Histological grade	Mice treated with estrogen[a] ($N = 22$)	Mice treated without estrogen ($N = 10$)
Reserve cell hyperplasia	0	4
CIN 1 and/or VAIN 1 (LGSIL)	14	2
CIN 2 and/or VAIN 3 (HGSIL)	8	0

Note. CIN, cervical intraepithelial neoplasia; VAIN, vaginal intraepithelial neoplasia; LGSIL, low-grade squamous intraepithelial lesion; HGSIL, high-grade squamous intraepithelial lesion.

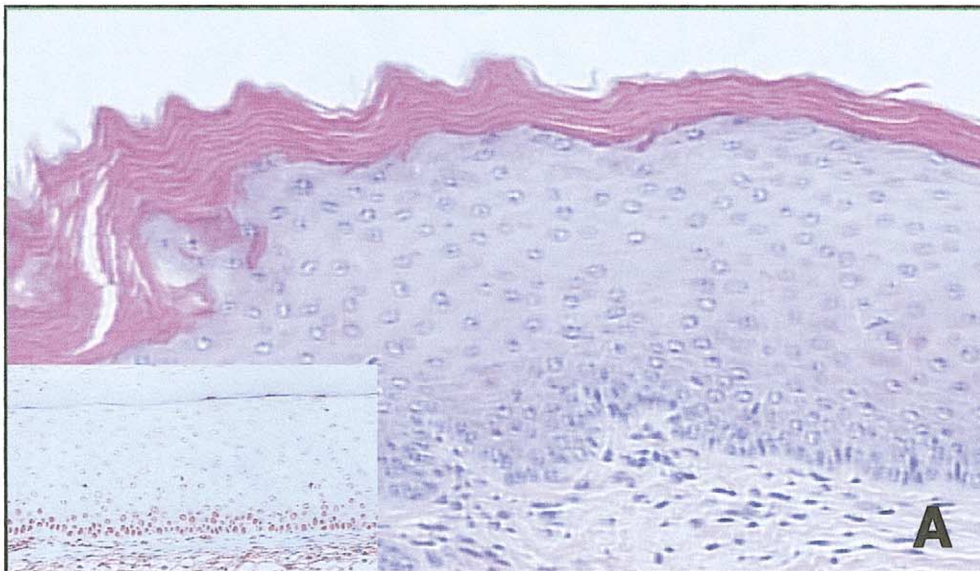
[a] The incidence of dysplasia was significantly increased in estrogen treated mice (22/22), when compared to those of nonexposed mice (2/10) ($P < 0.05$).

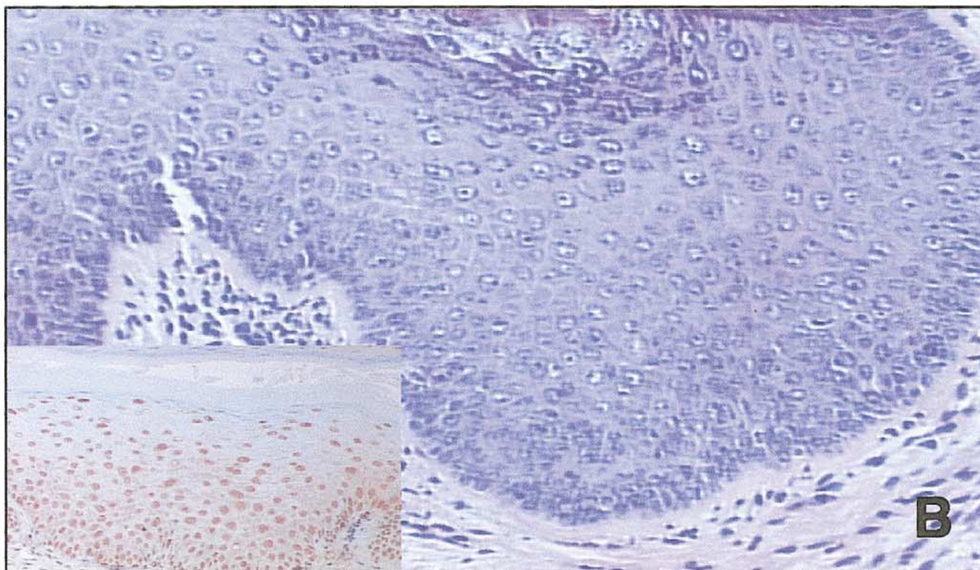
Expression of HPV-18 E6/E7 genes after estrogen treatment

To elucidate the relationship between up-regulation of HPV-18 *E6/E7* gene expression and the proliferation of cervical epithelial cells in transgenic mice by treatment with 17β-estradiol, we performed RT-PCR. The effects of estradiol were represented by the relative ratio of HPV-18 *E6/E7* to GAPDH mRNA expressed. The level of *E6/E7* transcripts in transgenic mouse was increased in the presence of estradiol, compared with those obtained from transgenic mouse in the absence of estradiol (Fig. 4).

HPV-18 URR activity by stimulation with estrogen in C33A cells

Based on the fact that the levels of HPV-18 *E6/E7* mRNA were increased by treatment with estradiol, we investigated the molecular effects of estradiol on the activity of HPV-18 *URR*. According to the results of the transient transfection experiments of *URR*-CAT expression plasmid in C33A cells, the expression of CAT enzyme was increased almost 2-fold after treatment with 0.5×10^{-6} M estradiol. CAT expression increased approximately 3-fold on cotransfection with the ER expression vector and *URR*-CAT. Treatment with 0.5×10^{-6} M estradiol on cotransfection with the ER expression vector and *URR*-CAT showed an additive effect of CAT activity (4.8-fold increase) (Fig. 5).





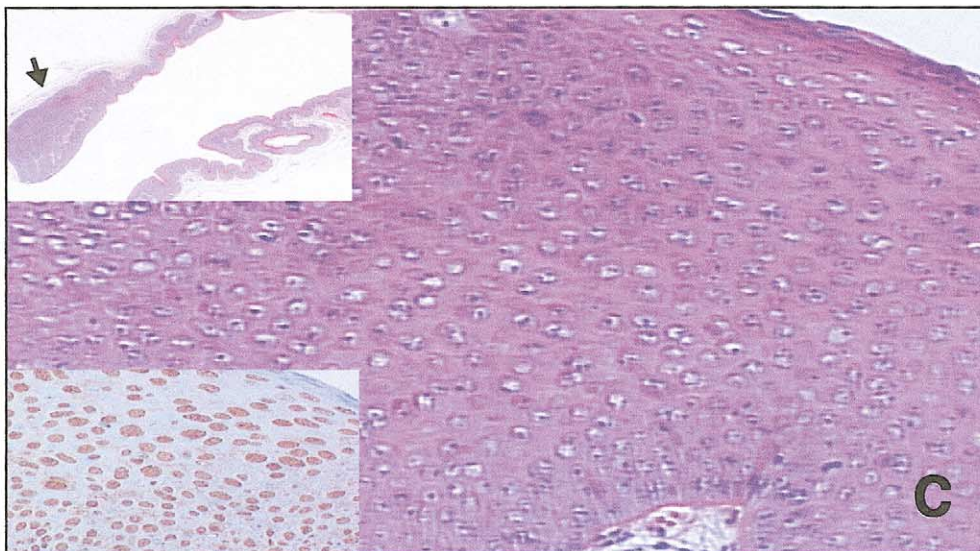


Fig. 2. (A) Cervical epithelium of non-estrogen-treated transgenic mouse, normal tissue; insert: positive cells for PCNA immunostaining only in basal layer. (B) Dysplastic cervical epithelium of estrogen-treated transgenic mouse, CIN 2; insert: positive cells for PCNA immunostaining in basal, suprabasal and intermediate layers. (C) Dysplastic vaginal epithelium of estrogen-treated transgenic mouse, VAIN 3; insert: (upper) whole section of the cervico-vaginal region; arrow indicates nodular tumor without invasion; (lower) positive cells for PCNA immunostaining throughout whole layers.

Table 4
Glandular lesions of the cervix and vagina in mice treated with or without 17β-estradiol

Mice	Transgenic mice or nontransgenic with estrogen	Coexisting squamous epithelial lesion	Glandular lesion
1	Transgenic	CIN 1	Glandular hyperplasia
2	Transgenic	CIN 1	Glandular hyperplasia
3	Transgenic	CIN 1	Glandular hyperplasia
4	Transgenic	VAIN 3	Glandular hyperplasia
5	Transgenic	CIN 1	Atypical glandular hyperplasia (adenocarcinoma in situ)
6	Nontransgenic	None	Glandular hyperplasia (high-grade glandular dysplasia)
7	Nontransgenic	CIN 1	Atypical glandular hyperplasia

Note. CIN, cervical intraepithelial neoplasia; VAIN, vaginal intraepithelial neoplasia.

Discussion

The most prominent feature of cervical carcinogenesis is a progression of epithelial lesions through distinctive histopathologic stages from HPV infection through carcinoma in situ to invasive cervical cancer [1]. Continuous HPV *E6* and *E7* gene expression can cause persistent cell cycle dysregulation and facilitate genetic instability. Earlier studies demonstrated that *E6* and *E7* act at different steps in the immortalization of primary human mammary epithelial cells; *E7* acts at an early step through inactivation of the p16-pRb cascades leading to an extended life span of primary cells, and *E6* acts at a late step through its activation of telomerase [19]. In a model of skin K14 HPV *E6* or HPV *E7* transgenic mouse treated with chemical carcinogens, *E6* was found to increase malignant progression of skin papillomas, whereas *E7* promoted only the formation of papillomas [20]. Most cervical lesions containing high-risk HPV types are not progressive to invasive cervical lesions, suggesting that environmental or genetic factors are linked to cervical carcinogenesis associated with HPV infection. Papillomavirus lesions are exacerbated during pregnancy when estrogen levels are elevated [21]. Prolonged use of estrogen has been shown to double the risk of HPV-induced cervical cancer and estrogen increases the transcription of HPV-16-transforming proteins in cervical carcinoma cells [4,13]. The benign hyperplasia induced by the combination of chronic estrogen and persistent HPV oncogene expression seems to be necessary to initiate neoplastic development, which would progress to invasive cervical carcinomas in the advance of time. The most compelling circumstantial evidence is that the most estrogen-sensitive genital site (transformation zone of the cervix) is the site where >90% of HPV-induced lesions and cervical cancers occur in women

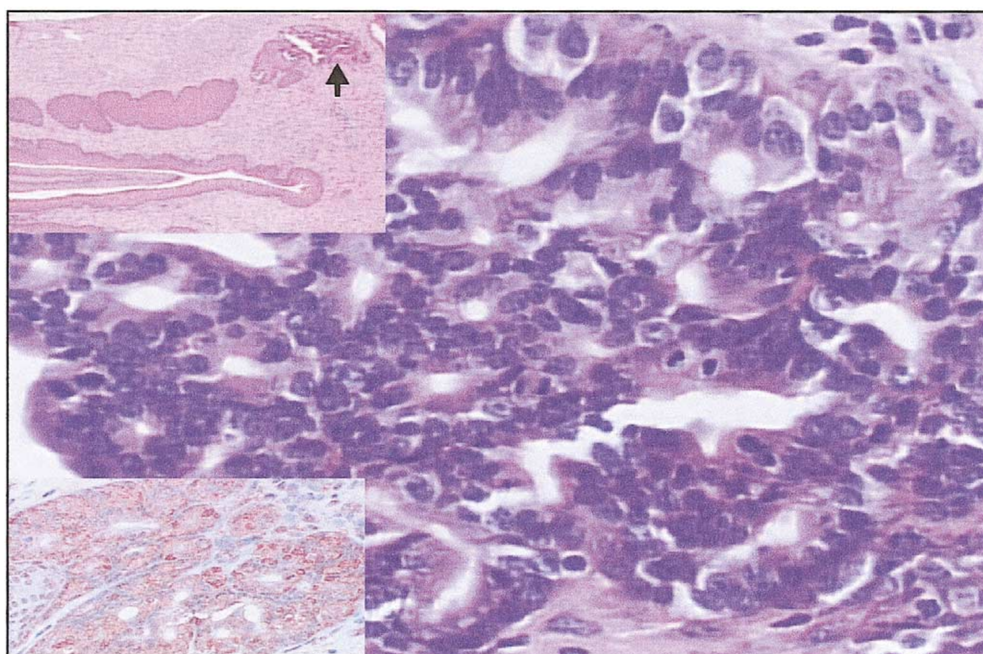


Fig. 3. Atypical glandular hyperplasia mimicking adenocarcinoma in situ (AIS) in estrogen-treated transgenic mouse. insert: (upper) whole section of transformation zone; arrow indicates a focus of atypical glandular proliferation; (lower) positive cells for PCNA immunostaining along nuclei of glandular epithelium.

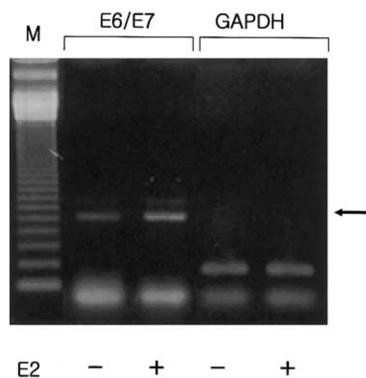


Fig. 4. RT-PCR analysis of HPV-18 *E6/E7* gene. The level of *E6/E7* transcripts in cervix of transgenic mouse was increased in the presence of estradiol (E2), compared with those obtained from transgenic mice in the absence of estradiol (arrow).

rather in men [22]. Epidemiologic studies have demonstrated a significant increase in the odds ratio for use of oral contraceptives among women with cervical adenocarcinoma compared with controls [5,6]. Interestingly, case–control studies demonstrated a strong relationship between cervical adenocarcinoma and infection with HPV-18 [7,8]. Chronic estrogen exposure and HPV oncogenes cooperate to elicit proliferation of cervical epithelium by signaling through the estrogenic receptor [23]. The weak point of these studies is that they utilize an artificial system, keratin K14 enhancer promoter, instead of the endogenous natural *URR* promoter of the virus. It is almost impossible to conduct animal experiments with a physiologic dose of 17β-estradiol, where the blood level corresponds with the physiologic state in humans. The dose of estradiol was adapted from previous reports: transgenic mice treated with 0.25 mg 17β-estradiol for 60 days predominantly developed neoplastic changes in vulvo-vagina and cervix [24,25]. The cyclic expression of HPV-18 oncoproteins with sustained elevation of estrogen levels, associated with estrogenic stimulation at estrus, may be necessary to initiate cervical carcinogenesis in HPV-18 *URR E6/E7* transgenic mice. Chronic estrogenic exposure seems to be an essential cofactor required for elaboration of cervical carcinogenesis by the HPV-18 oncogenes. This hypothesis might be supported our findings that neoplastic glandular cells were coincidentally identified in squamous intraepithelial lesions from HPV-18 *URR E6/E7* transgenic mice treated with 17β-estradiol. The representative immunohistochemical tissue sections demonstrated that estrogen induced an increase PCNA-positive cells within the whole layer of the squamocolumnar epithelium of cervix in HPV-18 *URR E6/E7* transgenic mice compared with nontransgenic mice. Estrogen is thought to directly up-regulate viral transcription in HPV-induced cervical carcinogenesis, since the natural element of viral control region is included in the transgenic mice. We have shown that the *URR* gene of HPV-18 is up-regulated and the *E6/E7* gene is overexpressed by in vitro treatment with estrogen. Increased proliferation of cervical epithelial cells could be due to up-regulation of HPV-18 *E6/E7* transgene expression during estrogen-induced transformation zone carcinogenesis. In this study, we tried to link estrogen treatment and HPV-mediated cervical carcinogenesis by animal and cell line models. As also reported by other groups, such linkage appears to be acceptable for several reasons. Several articles have indicated that estrogen is clinically and experimentally linked to HPV carcinogenesis [2,24,25–27]. Similar to our results, another study paper reported that estrogen plays a role in cervical and vaginal squamous carcinogenesis in human papillomavirus type 16 transgenic mice [23]. At a molecular level, estrogen has been known to induce HPV E6/E7 oncogene expression by targeting the AP-1 site, a major determinant of *URR* [28]. Indole-3-carbinol, as an antagonist of estrogen, was experimentally used for the treatment of cervical carcinoma cells [29–30]. Overall, these data suggest that estrogen is linked to HPV carcinogenesis, as we found using our model systems. The reasons for estrogen promotion of cervical cancer are likely to be multiple. Estrogen also increases proliferation of estrogen-sensitive cells, including HPV-infected cells [31]. Infection with HPV increases 16-α-hydroxylation of estradiol, thereby increasing the amount of 16-α-hydroxyestrogen, a carcinogenic metabolite [1]. Hyperplastic cervical epithelium by chronic estrogen stimulation may be coordinated by transcription and release of growth factors from the underlying stroma in response to activation of estrogen receptor-α by ligands. The overexpressed HPV oncoproteins transform proliferating cells in estrogen-induced hyperplastic lesions in the genital epithelium through their ability to dysregulate genomic instability, evoking carcinogenesis. Squamous metaplasia seems to be the initial phenomenon of cervical carcinogenesis in the transgenic mice and clinical disease. Squamous metaplasia is more

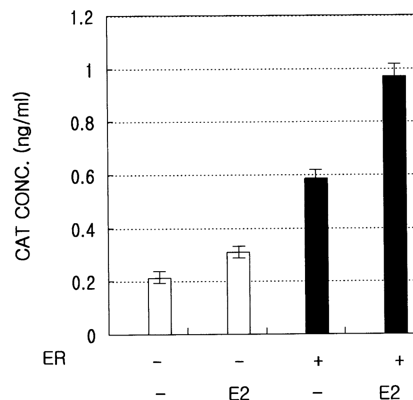


Fig. 5. Estradiol-dependent activation of HPV *URR*. Transient transfections were performed in C33A cervical cancer cell lines using HPV-18 *URR*-CAT reporter plasmid and ER expression vector in the absence or presence of estradiol (0.5 mM). After transfections, cells were harvested and extracted for determination of protein concentration and β-galactosidase activity was normalized for CAT assay. CAT activity was determined by CAT ELISA. Bars indicate SD. ER, estrogen receptor: E2, estradiol.

extensive in estrogen-treated transgenic versus nontransgenic mice or estrogen and indole-3-carbinol combination-treated transgenic mice. Similar to the case in K14–HPV-16 transgenic mice [25], chronic estrogen treatment stimulated development of hyperplastic changes in our transgenic mice using HPV-18 *URR E6/E7*. The cervical epithelial cells of the transformation zone become vulnerable and foster squamous metaplasia and dysplastic progression. Epithelial acidification occurs as a result of increased estrogen production, vaginal bacterial flora alterations, and epithelial wounding. Yeast two-hybrid assays have demonstrated binding of M2 pyruvate kinase to HPV-16 *E7* [32]. HPV *E7*-mediated intracellular acidosis due to lactate accumulation may be a factor that contributes to a squamous rather than a columnar fate in glandular reserve cells in estrogen-treated transgenic mice.

This study has some weakness due to the small numbers of animals, which may have biased the data. But, the study could demonstrate that the HPV *E6* and *E7* oncogenes implicated in cervical cancer are capable of tumor formation in animal models. In this context, continual estrogen-induced proliferation could be viewed as in vivo and in vitro mechanisms by which squamous cells as well as glandular cells in cervix and vagina became permissive for neoplastic progression. Synergistic activation of proliferation by viral oncoprotein cell cycle dysregulation and estrogen response, together with altered paracrine stromal–epithelial interactions, may contribute to the promotion of neoplastic progression in the squamo-columnar junction of the cervix and vaginal epithelium.

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