# The Role of Estrogen Receptor α in the Development and Maintenance of Uterine Epithelium in Mice

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in Biology

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Chair of Committee: Sanghyuk Chung, Ph.D

Committee Member: Frank McKeon, Ph.D

Committee Member: Tasneem Bawa-Khalfe, Ph.D

Committee Member: Sang Jun Han, Ph.D

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by

**Esra Unsal**

**University of Houston**

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## ABSTRACT

The uterus is composed of uterine cervix and uterine body. The cervical lumen is lined by the K14+/p63+/K8- squamous and K14-/p63-/K8+ columnar epithelium, which are demarcated at the squamocolumnar junction (SCJ). Location of SCJ changes which depends on several factors, including ovarian hormones, vaginal acidity, and age in women. The epithelial region that the SCJ moves is called the transformation zone (TZ), where most cervical cancers occur. In *K14E7* mice expressing HPV16 E7, SCJ shifts towards the uterine epithelium in an estrogen/ERα–dependent manner. We observed that SCJ moves toward the ectocervical epithelium in estrogen-depleted ovariectomized mice. (i.e., retraction of squamous epithelium). Estrogen administration expanded the squamous epithelium. These results suggest that HPV and estrogen/ERα promote squamous differentiation in the cervix. To better understand the role of ERα in this process, I first characterized genesis of cervical squamous epithelium and SCJ in postnatal mice. At birth, the entire cervical epithelium was composed of $K14^{-}/p63^{-}/K8^{+}$ cells. Several $K14^{+}/p63^{+}/K8^{-}$ epithelial cells appeared underneath $K14^{-}/p63^{-}/K8^{+}$ epithelial cells in the endocervix at postnatal day six (P6). They expanded to the posterior part of the cervix as aging. At P16 onwards, the endocervical epithelium became stratified. The location and epithelial marker expression of SCJ at P16 was similar to that in ovariectomized adults, indicating that squamous epithelium development was complete at P16. ERα expression pattern in the cervical epithelium at postnatal age coincided with expansion of K14+/p63+ cells. To study the role of epithelial ERα in squamous differentiation and SCJ formation, I used *Wnt7aCre/Esr1*$^{f/f}$ mice lacking expression of ERα specifically in the epithelium of the female reproductive tract including the cervix-they will be referred to as ER epithelium deletion (ED). Interestingly, ED mice had a delay in formation of SCJ. The location of SCJ in both ED and ERα knockout *(Esr1*$^{-/-}$*)* mice at prepubertal age (P21) was similar to that in *wild-type* mice. However, junction location was impaired in ED but not in *Esr1*$^{-/-}$ mice at adult age, suggesting that balance between epithelial and stromal ERα is required for maintenance of SCJ at adult age.

I found that, in epithelial ERα-deficient ED mice, $K14^{+}/p63^{+}/K8^{-}$ basal cells were present underneath the columnar epithelium in the entire uterus at adult age. I found that, although rare, these cells were also present in *wild-type* mice as early as postnatal day 14. Uterine basal cells expressed proteins that are not expressed in the other epithelial cell types in the cervix and uterine body. I found that K14 and p63 were expressed in human endometrium at the mRNA level. I did cell lineage tracing experiments in $Krt5^{CreER/+}/ROSA26^{mTmG/+}$ mice and found that uterine basal cells contribute to the columnar epithelium. Traced cells were mostly in the glandular epithelium. My results showed that the number of glands decreased in in ED mice but not in $Esr1^{-/-}$ mice. My results suggest that the balance between epithelial and stromal ERα signaling is crucial for the development and maintenance of cervical SCJ and uterine glandular genesis.

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# I. INTRODUCTION

A functional uterine epithelium is necessary for proper uterine homeostasis and function, which includes implantation of the blastocyst, support for the fetus to grow, and preparation for labor. Acquired or developmental abnormalities in the uterus lead to several complications and diseases, such as infertility, miscarriage, prematurity, endometrial cancer, and cervical cancer. Estrogen/ERα signaling has been implicated to regulate uterine growth and functional differentiation. ERαKO mice are infertile due to implantation defects and contain a hypoplastic uteri, suggesting the importance of ERα for proper uterine function. Uterus is connected to the vagina via cervix. Uterine columnar epithelial cells meet with squamous cells of cervix at squamocolumnar junction (SCJ) where most of the cervical cancers occur. Location of SCJ changes which depends on several factors, including ovarian hormones, vaginal acidity, and age in women. Studies propose SCJ as the site of embryonic cell population with susceptibility to infection by HPV and malignant transformation. To our interest, estrogen/ERα signaling has been implicated in maintenance of cervical squamous epithelium. Estrogen depletion and readministration leads to regression, and expansion of the squamous epithelium, respectively. We observed that SCJ moves toward the ectocervical epithelium in estrogen-depleted ovariectomized mice. In *K14E7* mice expressing HPV16 E7, SCJ shifts towards the uterine epithelium in an estrogen/ERα–dependent manner. These results suggest that estrogen/ERα promote squamous differentiation in the cervix. Understanding the formation and maintenance of SCJ will give insights into understanding the development of pathologies in that region which will open the door for new therapeutic approaches. In this study, I aimed to determine the role of ERα in development and maintenance of (1) cervical squamous epithelium and (2) uterine epithelium.

# II. BACKGROUND

## A. Development of female reproductive tract

The female reproductive tract (FRT) consists of the vagina, uterus, oviduct and the ovaries (Figure 1). The uterus is composed of uterine cervix and uterine body, which are referred to as cervix and uterus, respectively, throughout the text. The cervix is the entry of uterus, connecting the uterus to the vagina. The cervix accounts for the greater portion of FRT in mouse than in human (Figure 1). Müllerian duct (MD) originates from intermediate mesoderm adjacent to Wolffian duct. MD develops into the female genitalia including oviduct, uterine body, uterine cervix and upper part of vagina. Lower part of the vagina develops from endodermal urogenital sinus called sinus vagina. In embryonic days, MD epithelium is uniform and undifferentiated in the form of pseudostratified columnar cells. With the signals from the uterine and vaginal mesenchyme, each corresponding organ undergoes specific morphological changes (Cunha et al., 1976, Kurita et al., 2001a). During postnatal days of development, vaginal and the lower part of cervical epithelium differentiates into stratified squamous cell layer, whereas the upper part of the cervix and the uterus differentiate into columnar cells (Kurita et al., 2001a). Epithelial-mesenchymal interactions are important for the development of female reproductive tract. Tissue recombination of neonatal-uterine epithelium with vaginal mesenchyme induces squamous differentiation of uterine epithelial cells, which was replicated reciprocally with the combination of neonatal-vaginal epithelium with uterine mesenchyme. (Kurita, Cooke and Cunha, 2001). Tissue recombination of adult uterus and vagina, with the opposing mesenchyme failed to induce such effects, suggesting that developmental plasticity is lost in most of the vaginal and uterine epithelial cells at adult age (Kurita, Cooke and Cunha, 2001).

Squamous and columnar cells are different with respect to their morphology, function, and epithelial marker expression. Squamous cells stratify to form a barrier and protect the organ. Expression of epithelial markers such as cytokeratin 5 (K5) and cytokeratin 14 (K14) aids in maintaining the integrity of the squamous

epithelium. Columnar cells are involved in absorption and secretion which are essential to maintain the fertility and pregnancy. They express cytokeratin 7 (K7) and cytokeratin 8 (K8) and lack K5 and K14 expression. P63 is involved in the differentiation of squamous epithelium during development.

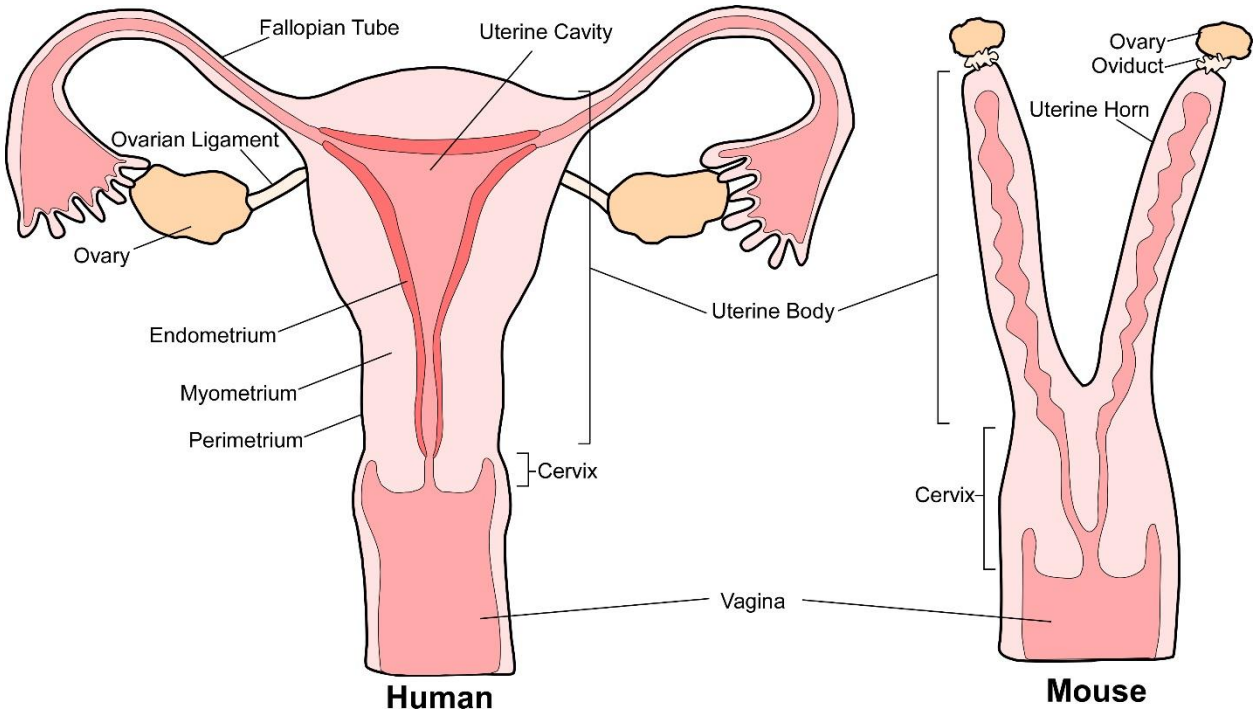


**Figure 1. Schematic of human and murine female reproductive tract.**

## B. Uterine and cervical epithelium

### Squamocolumnar junction and transformation zone

The cervical lumen is lined by the squamous and columnar epithelium, which are demarcated by the squamocolumnar junction (SCJ) (Figure 1 & 2). SCJ location shifts towards uterus or vagina depending on several factors, including ovarian hormones, vaginal acidity, and age in women. The epithelial region that the SCJ moves is called the transformation zone (TZ). The formation and dynamics of SCJ in the cervix are poorly understood, but TZ is exceptionally sensitive to cervical carcinogenesis. Estrogen levels increase at puberty, cycle until menopause, and stimulate glycogen deposition in the vaginal epithelium (Gregoire, Kandil&Ledger, 1971). Anaerobic metabolism of the vaginal glycogen to acidic products leads to increase in the vaginal acidity (Boskey et al., 1999). Acidic environment in the vagina stimulates replacement of columnar epithelium of SCJ with squamous epithelium (Elson, 2000).

Replacement of columnar epithelium by squamous epithelium is called squamous metaplasia. For unclear reasons, metaplastic squamous epithelium infected with HPV is more susceptible to dysplasia and cervical squamous cell carcinoma (Schiffman et al., 2007). Chronic estrogen treatment induces squamous metaplasia and carcinoma at SCJ in HPV transgenic mice expressing E7 but not in nontransgenic control mice (Brake & Lambert, 2005). These results suggest that HPV oncogenes trigger squamous metaplasia in the cervical epithelium in conjunction with estrogen-ERα signaling. Immunostaining of TZ biopsies with immature squamous metaplasia from young women exhibited a significantly higher density of ERα and PR positive cells compared to surrounding ectocervical epithelium, suggesting that the TZ area where metaplastic replacement occurs has higher sensitivity to sex hormones (Remoue et al., 2003). Squamous epithelium retracts toward ectocervical epithelium in ovariectomized (i.e., estrogen-depleted) mice. These findings point to the important role of estrogen and ERα in squamous differentiation in the cervix, which remains to be understood.

**Unique epithelial cell types at SCJ**

Reserve cells of SCJ (Figure 2), which are located underneath the columnar epithelium (Martens et al., 2007, Hoogduin et al., 2013), initially proliferate and form immature squamous metaplastic epithelium. Subsequently, these cells fully differentiate and form mature squamous epithelium (Reich et al, 2017), which is indistinguishable from the squamous epithelium of ectocervix (Reich et al, 2017).

A recent study has identified a discrete population of cells at SCJ of the human cervix (Herfs et al., 2012), which carry a unique gene expression profile and morphology comparing to surrounding cells. These cells were cuboidal-shaped, located at the interface of the squamous and columnar epithelium of SCJ (Figure 2), and were consistently present irrespective of age (Herfs et al., 2012). Some of the SCJ specific markers (Keratin 7, anterior gradient (AGR)2, matrix metalloproteinase (MMP)7, guanine deaminase (GDA), and cluster differentiation (CD)63) were shown to be expressed in the majority of high-grade CINs and HPV-induced cervical cancers comparing to low-grade CINs from ectocervical epithelium, suggesting that these cells might be involved in HPV-related cervical carcinogenesis (Herfs et al., 2013). These junction markers were expressed in both squamous cell carcinomas and adenocarcinomas arisen from the SCJ region, suggesting that these cells might be involved in maintenance of these two different cell types. (Crum et al., 2013). A mouse equivalent of these unique cell types still remains to be defined which could help establishing a mouse model to study the nature of the disease and find targeted therapy.

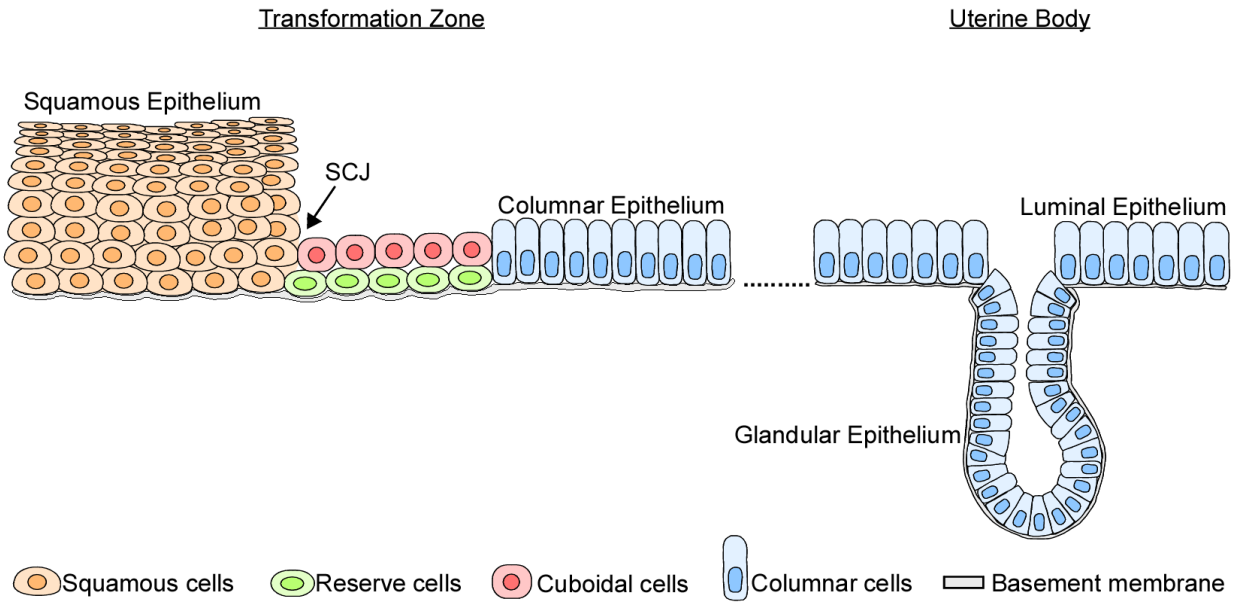


**Figure 2. Schematic of the cervical and uterine epithelial cell types.** Squamous and columnar epithelium of cervix meet at squamocolumnar junction (SCJ). SCJ moves to the right and left depending on various factors such as vaginal acidity, sex hormone cycle, and age.

**Maintenance of the uterine epithelium**

Human uterus consists of three layers: perimetrium, myometrium, and endometrium. Perimetrium covers the outer surface of the uterus. Myometrium is the middle layer of the uterine wall and consists of a thick layer of smooth muscle fibers, a supporting stroma and vascular tissue. The major function of myometrium is contraction of uterus during parturition (Lessey and Young, 2019). Endometrium is the innermost lining layer of the uterus and is composed of luminal and glandular columnar epithelium along with the surrounding stroma.

Luminal epithelium allows implantation of blastocyst via apical surface expression of cell adhesion molecules. During the window of receptivity, blastocyst implantation occurs at the site of luminal epithelium, followed by decidualization of stromal cells, and placental formation (Wetendorf &DeMayo, 2013). Glandular epithelium consists of ciliated columnar cells and secrete substances that are essential for these processes (Filant & Spencer, 2013, Lessey and Young, 2019). Anatomy of mouse uterus differs from human uterus. Humans have a pear-shaped uterus with a single triangular-shaped cavity whereas mice have a bicornuate uterus consisting of two tubular horns which are connected to separate cervical canals (Figure 1).

Uterus of a sexually mature female undergoes functional and structural changes during the menstrual cycle in human and estrous cycle in mice. These changes are dependent on ovarian hormones. Estrogen stimulates uterine epithelial proliferation in adult mice (Quarmby et al.,1984). Estrogen is required for uterine epithelial morphogenesis, cytodifferentiation, and secretory activity (Cooke, 1997).

Adult stem cells have been suggested to be responsible for maintenance of the uterine epithelium throughout reproductive lifespan. A recent study found traces of such cell population using cell lineage tracing techniques (Jin, 2019). Uterine epithelial cells were YFP labeled at a single cell level using low dosages of tamoxifen on a $Keratin19^{CreERT2}/Rosa26^{YFP/+}$ reporter mice and traced for their behavior and fate for up to a year. These labeled cells were persistently present in and expanded throughout the uterus of the mice and

regenerated both luminal and glandular uterine epithelium (Jin, 2019) suggesting that resident stem cells exist in the mouse uterus and support regeneration of epithelium. Problems in maintenance of the uterine epithelium are associated with various uterine pathologies including infertility, endometriosis, and endometrial cancer (Braun et al., 2016).

### C. **Estrogen and Estrogen Receptor Signaling**

Estrogens are steroid hormones synthesized mostly in ovaries as a circulating hormone. They are also produced in peripheral tissues including adipose tissue, bone, vascular endothelium and brain (Simpson, 2003&Rižner, 2009), but these estrogens act locally within corresponding tissues. Estrone, 17β-estradiol, and estriol are the three major estrogens, among which 17β-estradiol ($E_2$) is the most potent and abundant form. $E_2$ is involved in a variety of important physiological functions including development, growth, differentiation, and maintenance of female reproductive system and homeostasis of cardiovascular, immune, skeletal, and central nervous system. Estrogen-estrogen receptor (ER) signaling has been linked to different types of cancers including breast, ovarian, cervical, endometrial, and colorectal cancer. In addition, it has been implicated in the development of other diseases such as cardiovascular and neurodegenerative diseases, endometriosis, osteoporosis, obesity, and diabetes (Deroo et al., 2006).

Estrogen binds and activates ERα and ERβ, nuclear receptors, to modulate transcription of target genes and G protein-coupled estrogen receptor (GPER) on the plasma membrane for rapid responses. Activated ERs induce gene expression either directly via binding to estrogen-response elements (EREs) (classical pathway) or indirectly via binding to other transcription factors (non-classical pathway or tethering pathway). Estrogen receptors are distributed differently in different tissue types. ERα is predominantly expressed in the cervix, uterus, kidney, liver, and ovarian theca cells, while ERβ is expressed primarily in the prostate, bladder, lung, and ovarian granulosa cells. (Heldring et al., 2007). GPER is expressed broadly in most tissues, including breast, pancreatic, prostate, and uterine cervix (Feldman, R. and Limbird, L.

(2016). Structural differences between the ER subtypes and presence of other cofactors lead to various responses to estrogens. ERα is the dominant ER subtype in the majority of the female reproductive tissue in both human and mice, and expressed in both epithelium and stroma.

ERα consists of 5 different functional domains (Figure 3). N-terminal A/B domain contains ligand-independent activation function 1 (AF-1) domain. Zinc-finger DNA-binding domain (DBD) is responsible for specific binding of ERα to EREs. D domain is a flexible hinge region and connects the domains C and E. C-terminal ligand binding domain (LBD) encompassing E domain includes ligand-dependent activation function 2 (AF-2) domain and mediates ERα dimerization. F domain is located at the carboxyl-terminal and has been linked to the modulation of transcriptional activity, ligand binding, and dimerization of the estrogen receptor (Peters et al., 1999; Skafar et al., 2008). AF-1 function is independent of ligand binding and modulated by protein kinases and growth factor signaling pathways (Kato et al., 1995). AF-2 domain acquires conformational changes in its alpha helix 12 and recruits coregulator proteins when ERα is bound with a ligand (Brzozowski et al., 1997). ERα promotes proliferation of epithelial cells in cervix, uterus, vagina, and mammary glands.

**Estrogen and Estrogen Receptors in FRT**

Tissue-recombination of epithelium and stroma from ERα knockout and ERα intact mice has shown that $E_2$ induced mitogenesis of uterine epithelium requires stromal ERα but not epithelial ERα (Cooke et al., 1997), suggesting that uterine epithelial maintenance is regulated $E_2$ via epithelial-stromal tissue interactions. These results were later confirmed by a study done on transgenic epithelial ERα deficient mice. Loss of epithelial ERα in *Wnt7a-Cre/Esr1*$^{f/f}$ mice has no effect on $E_2$ induced proliferation of uterine columnar epithelial cells (Winuthayanon et al., 2010). Partial loss of stromal ERα prevents proliferation of nearby epithelial cells in the uterus of *Amhr2*$^{Cre/+}$*/Esr1*$^{f/f}$ mice, suggesting a juxtacrine mechanism between stromal ERα and neighboring epithelial cells (Winuthayanon et al., 2017) for uterine epithelial maintenance. These results strongly support that $E_2$ promotes uterine epithelial cell proliferation via paracrine mechanism of stromal ERα. Observational and mouse model studies have indicated that estrogen signaling is crucial

during the development of FRT. Diethylstilbestrol (DES) is a synthetic non-steroidal estrogen that was prescribed to pregnant women between 1940 to 1971 to prevent miscarriages and preterm birth (Marselos &Tomatis, 1992). The Food and Drug Administration (FDA) banned its use in 1971 because it has been found to increase incidence of clear cell adenocarcinoma in cervix and vagina in daughters of DES users (referred to as DES daughters) (Herbst et al, 1971). In addition to the cancer risk, DES daughters exhibit developmental abnormalities in the reproductive tract, including squamous metaplasia in the uterus and adenosis in the cervix and vagina (Herbst et al., 2000). They also have the increased rate of infertility, miscarriage, and stillbirth (Hoover et al., 2011). Squamous metaplasia is the propagation of stratified squamous cells in the uterine epithelium. Adenosis is replacement of squamous cells of cervix and vagina with columnar cells. Female mice that are perinatally exposed to DES develop vaginal adenosis and uterine squamous metaplasia (Forsberg et al., 1976, McLachlan, Newbold&Bullock, 1980). DES fails to induce these phenotypes in *Esr1*$^{-/-}$ mice, demonstrating that ERα is essential for aforementioned effects of DES. (Couse et al. 2001).

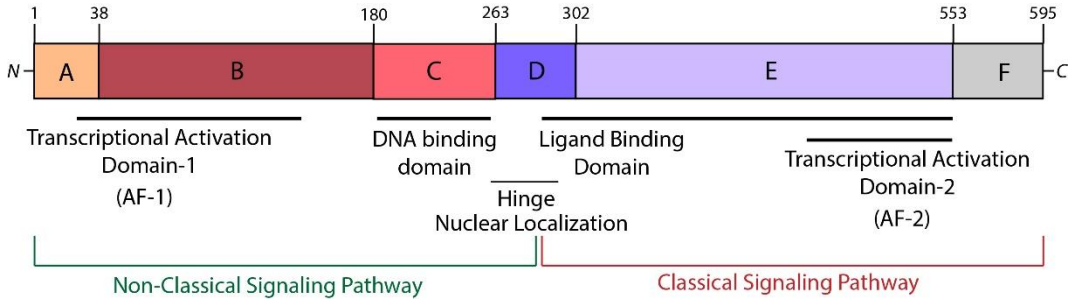


**Figure 3. Schematic of estrogen receptor α (ERα) domains.**

## III. MATERIALS AND METHODS

### Animals

*Wnt7a-Cre* transgenic mice, *Ltf-iCre* knock-in mice, and *Krt5*$^{CreER/+}$ knock-in (referred to as *K5CreER* hearafter) mice were used to recombine floxed alleles. *Esr1*$^{f/f}$ mice harbor *Esr1* alleles with exon 3 flanked by loxP sites (Hewitt et al., 2010). *Wnt7a-Cre* mice express Cre recombinase gene under the control of epithelium-specific mouse Wnt7a promoter (Winuthayanon et al., 2010). In Chapter 1 and 2, *Wnt7a-Cre* mice were crossed with *Esr1*$^{f/f}$ to generate *Wnt7a-Cre/Esr1*$^{f/f}$ mice in which the expression of ER$\alpha$ was ablated only in the epithelium. The tissues were harvested at various time points of postnatal and adult age. Some of these mice were ovariectomized at 6-8 weeks of age, which were either rested, or injected with 17β-estradiol or vehicle intraperitoneally before harvesting the female reproductive tissue. *NTG/Esr1*$^{f/+}$ and *Wnt7a-Cre/Esr1*$^{f/+}$ mice were used as control. ER$\alpha$ IF was performed to confirm deletion of ER$\alpha$ from the epithelium. *Ltf-iCre* mice expresses Cre recombinase in the uterine epithelium after puberty or 17β-estradiol injection (Daikoku, 2014). In Chapter 2, I generated *Ltf-iCre*/*Esr1*$^{f/f}$ mice by crossing *Ltf-iCre* with *Esr1*$^{f/f}$ in order to remove ER$\alpha$ allele specifically in uterine epithelium after developmental age. *K5CreER*/+ mice expresses tamoxifen-inducible CreER recombinase under the control of mouse keratin 5 promoter (Van Keymeulen, et al., 2011). CreER is a fusion protein of Cre recombinase and a mutated ligand-binding domain (LBD) of estrogen receptor. It is retained in the membrane until bound with tamoxifen, which leads to translocation of the CreER to the nucleus where it removes the floxed target allele. *ROSA26*$^{mTmG/+}$ is a two-color fluorescent Cre-reporter allele (Muzumdar et al., 2007). Mice express tdTomato gene under the control of ROSA26 locus in the majority of the cells. Floxed tdTomato (mT) cassette will be deleted when cre recombinase is present, enabling expression of EGFP (mG). In Chapter 2, I generated *K5CreER*/*ROSA26*$^{mTmG/+}$ mice by crossing *K5CreER* with *ROSA26*$^{mTmG/+}$ in order to label K5 expressing epithelial cells with EGFP protein permanently when treated with tamoxifen. Mice were given tamoxifen (3mg/day) for three days to activate CreER recombinase at 6-8 weeks of age. Tissue was harvested after one day and 3 weeks of rest. In Chapter 1, I used *Esr1*$^{-/-}$ germline ERα knockout mice to study the role of

ERα in development of SCJ. (Hewitt, S. C., 2010). In Chapter 1, to study the role of different domains of ERα in the development of cervical and uterine epithelium, I used *Esr1*$^{AF1/+}$, *Esr1*$^{AF2/+}$, *and Esr1*$^{DBD/+}$ mice. AF1 mutant allele lacks exon3 in ligand-independent activation function domain. AF2 allele has a substitution mutation in AF2 domain and lacks ligand-dependent function of ERα. DBD allele has a substitution mutation which abolishes interaction of ERα with the ERE within DNA (Ahlbory-Dieker et al., 2009). I mated each one of these mice with *Wnt7a-Cre/Esr1*$^{f/+}$ mice to generate *Wnt7a-Cre/Esr1*$^{M/f}$ *(*M stands for either of *AF1, AF2, or DBD* mutant alleles) which enables expression of only the mutant allele in the cervical epithelium. The mice were harvested at 8 weeks of age at diestrus stage. All mice genotypes were verified by PCR from genomic DNA extracted from the tail. All of the animals were housed in the animal housing facility of University of Houston. All of the animal procedures in this study were performed according to a protocol approved by the University of Houston Institutional Animal Care and Use Committee.

**Ovariectomy**

In Chapter 2, for some of the mice, ovaries were surgically excised as follows. Animals were anesthetized under 2% isoflurane according to the protocol approved by UH IACUC. Hair over the dorsum of the animal was removed using a clipper and the skin was carefully wiped with 70% ethanol-soaked cotton swaps. Approximately 1 cm of midline incision was made in the mid-dorsum of the mouse. The skin was separated from the underlying layer using fine forceps. Each ovary was located by spotting the white fat tissue surrounding it. A second incision was made over it and the ovary was taken out using fine forceps. Ovary was clamped from below and underneath the clamp was sutured using a chromic gut absorbable suture followed by careful removal of the ovary and placing the uterus back into the abdominal cavity. The same procedure was repeated with the other ovary. Incision site was hold together with forceps and closed using chromic gut absorbable suture with needle and a surgical glue (Penn veterinary supply, Inc., Lancaster, PA, USA; Cat# ABT3204604). Mice were monitored for recovery.

**Chemical and Hormone Treatment**

In Chapter 2, for long term $E_2$ treatments, continuous-release $E_2$ tablet (17β-estradiol; 0.05 mg/60 days, Innovative Research of America, Sarasota, FL) was inserted subcutaneously under the dorsal skin of female mice. For short term $E_2$ treatments, 17β-estradiol powder (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 100% EtOH at 1 mg/mL concentration and diluted in PBS prior to injection with 10 µg/mL concentration. Injections to mice were done intraperitoneally with 0.1 mL volume. Tamoxifen was prepared freshly prior to injections. Tamoxifen powder (Sigma Aldrich, St. Louis, MO) was dissolved in corn oil at 37°C by vortexing frequently and stored in a light-blocking vessel since it is light sensitive. Injections were done intraperitoneally with 0.1mL of Tamoxifen (3mg/day for three consecutive days) to activate K5CreER and delete targeted floxed allele.

**Vaginal Cytology**

The vaginal canal of mice was flushed with 20 µl of sterile PBS to collect the vaginal cells. Collected cells were smeared on a slide and observed under the microscope to determine the estrous stage of the mice. Mostly cornified cells were present in estrus stage. A mixture of cornified cells, nucleated cells and leukocytes were observed in metestrus. Majority of cells were leukocytes in diestrus. Mostly nucleated and some cornified epithelial cells were present in proestrus stage (Byers et al., 2012).

**Tissue processing and histological analyses**

Female reproductive tracts were harvested and fixed in 4% (w/v) paraformaldehyde for 24 hours. Fixed tissues were processed in Excelsior AS tissue processor (Thermo-Fisher Scientific, Waltham, MA, USA) and embedded in paraffin using Shandon Histocentre 3 (Thermo-Fisher Scientific). Serial tissue sections were obtained at 5-µm thickness throughout the cervix and uterus using HM 355S microtome (Thermo-Fisher Scientific). For histological analyses, representative tissue sections were stained with hematoxylin (Thermo-Fisher Scientific, Cat. No. 6765015) and eosin (Sigma Aldrich, St. Louis, MO, USA; Cat. No.

E4382) and mounted with Cytoseal XYL (Richard-Allan Scientific, San Diego, CA, USA; Cat# 8312-4). Sections with fully open cervix and uterus were selected for H&E staining.

**Immunohistochemistry**

Tissue sections were deparaffinized in xylene, rehydrated in gradually decreasing percentages of ethanol, and washed in PBS. Antigen retrieval was done by boiling sections in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes. Slides were then treated with a blocking buffer of 5% goat serum in PBS for 1 hr at RT to prevent non-specific binding. Sections were then incubated with primary antibody diluted in blocking buffer overnight at 4°C. Sections were then washed in PBS extensively and treated with a fluorescence-conjugated secondary antibody prepared in blocking buffer for 1 hour at RT. They were further washed in PBS thoroughly. Nuclei staining was done by incubation with Hoechst 33258 (10 μg/mL; Sigma-Aldrich, Cat# B2883) for 30 seconds. A detailed list with antibody information and dilutions is described in Table 1. In Chapter 2, commercial EMC1021 human endometrial cancer tissue microarray was purchased from Biomax (Derwood, MD, USA) for immunohistochemistry analyzes.

**Microscopy and digital image analyses**

Stained tissue sections were visualized by an Eclipse Ti2 microscope (Nikon Instruments Inc., Melville, NY, USA). Representative images were taken by Nikon DS-Qi2 monochrome CMOS camera (for fluorescence imaging) or DS-Ri2 color CMOS camera (for brightfield imaging) using Nikon NIS-Elements AR imaging software. Images were taken with the same exposure time for each staining and merged using the same software at 10, 20, or 40X magnifications. In some images, the nuclei were pseudo-colored using the Nikon NIS-Elements AR imaging software. For quantification of uterine epithelial cells for Ki67 and Caspase-3 in Chapter 2, images were taken from 2-3 random microscopic fields per tissue and quantified manually using ImageJ software. The number of total counted cells per uterine tissue was 600-800.

**RNA extraction and RT-PCR**

In Chapter 2, total RNA was isolated from mouse uterine tissue, and HeLa cells with Trizol (Life Technologies, Carlsbad, CA, USA; Cat# 15596018). RNA quantity and quality was assessed using a NanoDrop-1000 spectrophotometer (Thermo-Fisher Scientific). Human uterus total RNA was purchased from Takara (Shiga, Japan; Cat# 636551). One to two micrograms of total RNA were subjected to cDNA synthesis using m-MuLV reverse transcriptase (Promega, Madison, WI, USA; Cat# M1705) and oligo (dT) primer. cDNA products were subjected to polymerase chain reaction (PCR). GAPDH and Ppia genes were used as internal control. Primers were designed using Primer-Blast tool of NCBI. (Ye et al., 2012) and purchased from Integrated DNA Technologies (Coralville, IA, USA). *Krt14* primers were (F) 5' – CAGTCCCAGCTCAGCATGAA – 3' and (R) 5' – TGAGCAGCATGTAGCAGCTT– 3'. *Trp63* primers were (F) 5' – AGCAGCAAGTATCGGACAGC – 3' and (R) 5' – CTCCACAAGCTCATTCCTGAAG – 3'. *TP63* primers were (F) 5' – CCATGAGCTGAGCCGTGAAT – 3' and (R) 5' GGACTTGCCCTCTC TGGTT – 3'. *KRT5* primers were (F) 5'– GTCCTGGTATCAGACCAAGTATGAG – 3' and (R) 5' – ACTGCTGCTGGAGTAGTAGCTT – 3', *Ppia* primers were (F) 5' – CAGACGCCACTGTCGCTTT – 3' and (R) 5' – TGTCTTTGGAACTTTGTCTGCAA– 3' and *GAPDH* primers were (F) 5' – ACCACA GTCCATGCCATCAC – 3' and (R) 5' – TCCACCACCCTGTTGCTGTA– 3'. PCR products were run on 1.5% agarose gel and the bands were located using 100 bp ivDye DNA ladder (Gendepot, Katy, TX, USA; Cat# V1002-250).

**Statistical analyses**

MSTAT software (version 6.6) was downloaded from https://mcardle.wisc.edu/mstat and used to carry out all the statistical analyses. One-sided Wilcoxon rank sum test was used for proliferative and apoptotic indices, and comparison of gland numbers between groups in Chapter 2. The *p*-value equal or less than 0.05 was considered as statistically significant in these analyses.

**Table 2. List of antibodies and their dilutions**

| Antibody | Manufacturer | Catalog # | Host | Dilution |
|---|---|---|---|---|
| Agr2 | Proteintech | 12275-1-AP | Rabbit | 1:100 |
| Cleaved Caspase3 | Cell Signaling Technology | 9664S | Rabbit | 1:400 |
| ERα | Thermo Fisher | MA513191 | Mouse | 1:100 |
| Fascin | Abcam | Ab126772 | Rabbit | 1:100 |
| FoxA2 | Cell Signaling Technology | 8186S | Rabbit | 1:400 |
| GFP | Santa Cruz | Sc-9996 | Mouse | 1:100 |
| K5 | Biolegend | 905501 | Rabbit | 1:100 |
| K7 | Thermo Fisher | MA106315 | Mouse | 1:100 |
| K8 | Developmental Studies, Hybridoma Bank | TROMA-I | Rat | 1:100 |
| K14 | Biolegend | 905301 | Rabbit | 1:1000 |
| K19 | Proteintech | 10712-1-AP | Rabbit | 1:1000 |
| Ki67 | Thermo Fisher | RM-9106-S0 | Rabbit | 1:100 |
| p63 | SantaCruz | Sc-8431 | Mouse | 1:100 |
| Anti-rabbit IgG-Alexa Fluor 405 | Life Technologies | A31556 | Goat | 1:100 |
| Anti-mouse IgG-Alexa Fluor 488 | Life Technologies | A11001 | Goat | 1:100 |
| Anti-rabbit IgG-Alexa Fluor 488 | Life Technologies | A11008 | Goat | 1:100 |
| Anti-mouse IgG-Alexa Fluor 594 | Life Technologies | A11005 | Goat | 1:100 |
| Anti-rabbit IgG-Alexa Fluor 594 | Life Technologies | A11012 | Goat | 1:100 |
| Anti-rat IgG-Alexa Fluor 594 | Life Technologies | A11007 | Goat | 1:100 |
| Anti-goat IgG-FITC | Santa Cruz | Sc-2024 | Donkey | 1:100 |

## IV. CHAPTER 1:

### Epithelial Estrogen Receptor α is Required for the Maintenance of Squamocolumnar Junction in the Murine Cervix

### A. Rationale

The cervix is unique in that it is composed of squamous epithelium and columnar epithelium. These two different epithelia are demarcated at the sqaumocolumnar junction (SCJ) whose location changes within the transformation zone (TZ). The formation, maintenance, and dynamics of SCJ are poorly understood. TZ is exceptionally sensitive to human papillomavirus-induced cervical carcinogenesis in human and mouse models (Brake & Lambert, 2005). Chronic treatment of HPV16 E7-expressing mice (*K14E7*) with $E_2$ results in the movement of SCJ toward the uterus (i.e., the expansion of squamous epithelium) (Brake & Lambert, 2005). This phenotype is absent in *K14E7/Esr1*$^{-/-}$ mice (Chung et al., 2008). These results support that $E_2$ and ERα are involved in the maintenance of SCJ and that the HPV E7 oncoprotein disrupts the normal dynamics of SCJ. The exposure to DES at postnatal day one induces the development of columnar epithelium in the middle of squamous epithelium in the murine cervix and vagina. This effect is absent in ERα knockout mice (Couse et al. 2001). It is also absent when mice are treated with DES at adult ages. These results indicate that ERα plays a role in the normal development of cervical squamous epithelium. Determining the role of $E_2$/ERα in the development of squamous epithelium and maintenance of SCJ will not only help understand mechanisms of SCJ dynamics but also reveal information relevant to cervical neoplastic diseases.

Vaginal epithelium from neonates recombined with uterine mesenchyme differentiates into columnar epithelium. Similarly, uterine epithelium from neonates exhibits stratified squamous epithelium differentiation when recombined with vaginal mesenchyme (Cunha, 1976). This effect is absent when epithelium from adult mice are recombined with the stroma. These results indicate that underlying stroma can induce epithelial cell-fate determination in the uterus and vagina during postnatal development in mice.

In order to determine cell type-specific role of ERα in the formation and maintenance of SCJ, I chose to use *Wnt7a-Cre/Esr1*$^{f/f}$ mice (ERα is expressed in cervical stroma but not in epithelium), *Esr1*$^{-/-}$ mice (ERα is expressed in neither cervical stroma nor epithelium), and *Esr1*$^{+/+}$ mice (ERα is expressed in both cervical stroma and epithelium).

## B. Results

### *Development of cervical epithelium is complete by postnatal day 16*

Reproductive tract of mouse is composed of vagina, uterus, ovaries, and oviduct (Figure 4A). Murine cervix consists of three regions: ectocervix is at the lower end of the cervix and connected to the vagina; endocervical canal is a single tubular structure and is located in between ectocervix and endocervical septum; endocervical septum is the inner part of the cervix where squamocolumnar junction (SCJ) is located. The endocervical septum has two canals that are connected to two separate tubular horns of uterus. At birth, cervical epithelium is in the form of undifferentiated pseudostratified columnar epithelium (Forsberg, 1969). At adult age, cervical epithelium consists of four different cell types: squamous epithelial cells, reserve cells residing underneath the columnar cells at the SCJ, luminal columnar cells, and glandular columnar cells budding from the luminal epithelium.

To evaluate development of cervical epithelium and SCJ, I assessed morphological changes in the cervix throughout postnatal days. Figure 4B shows representative H&E images from ectocervix (region 1), endocervical canal (region 2), and endocervical septum (region 3). At birth (postnatal day 0, P0), entire cervical epithelium was undifferentiated and composed of two-layers of columnar cells. At P2, cuboidal cells started appearing underneath the columnar epithelium in ectocervix and expanded throughout canal and septum by P4, and P5, respectively. As the cuboilda-shaped cells expanded throughout the bottom layer, cells at the upper layer of the cervical epithelium became cuboidal by P5. It was unclear whether columnar cells transdifferentiated into cuboidal cells or pre-existing cuboidal cells expanded. Stratification of cuboidal cells started at P7, P8, and P16, for ectocervix, endocervical canal, and endocervical septum, respectively (Figure 4B). The entire cervical squamous epithelium was stratified at P16, and there was little change at P21, the weaning age.

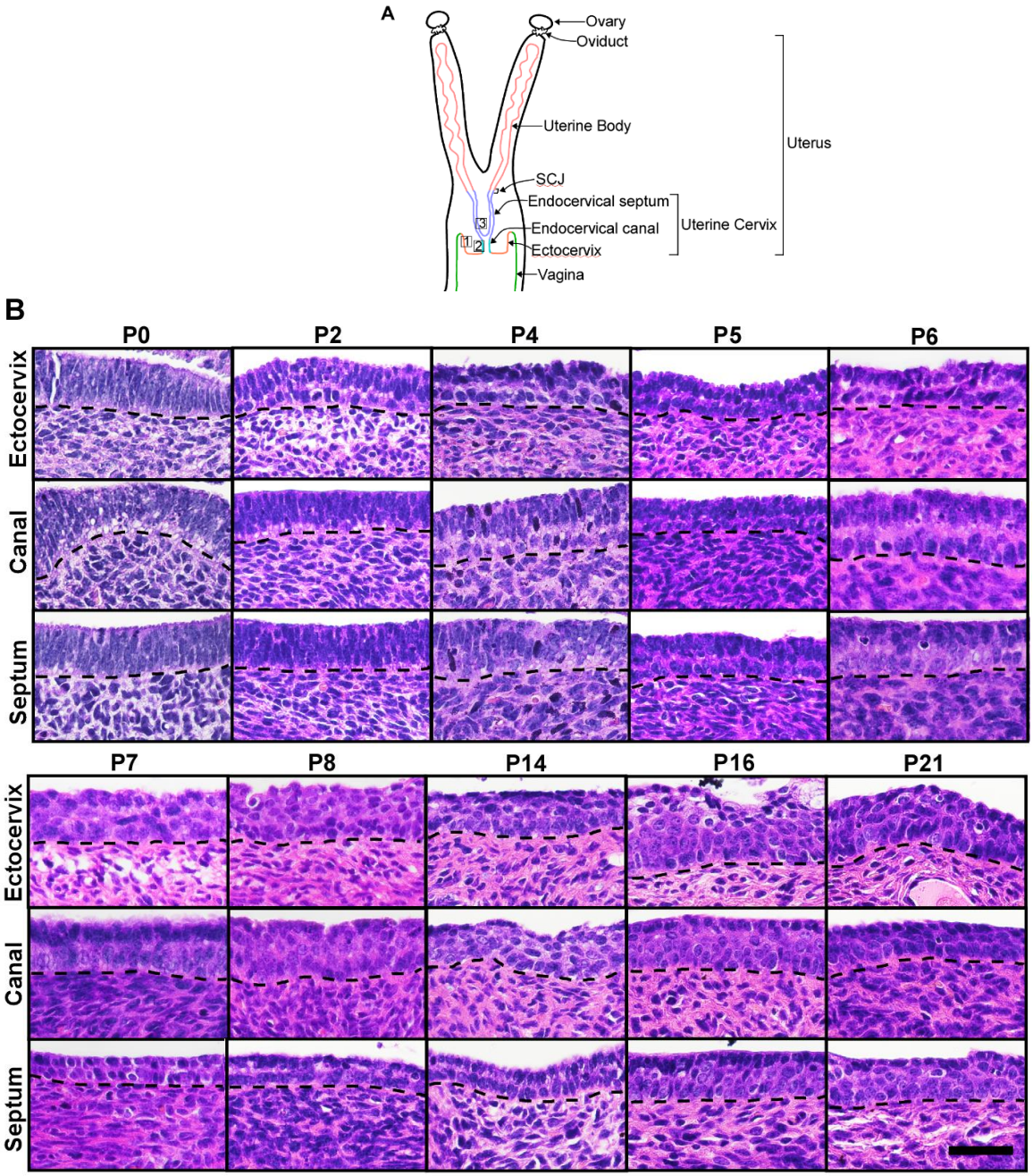


**Figure 4. Cell differentiation follows a direction from ectocervix to endocervix during postnatal development of the cervix.**

**(A)** Schematic of murine female reproductive tract. Pink, purple, light blue, orange, and green lines indicate the epithelium of uterine body, endocervical septum, endocervical canal, ectocervix, and vagina, respectively. **(B)** Representative H&E images of ectocervix (region 1), endocervical canal (region 2), and endocervical septum (region 3) from postnatal days (P) 0, 2, 4-8, and 16. Black lines separate epithelium from stroma. Scale bar represents 40 μm.

To characterize the development of cervical epithelium throughout postnatal ages at molecular level, I stained cervical tissue sections shown in Figure 5B for epithelial markers K8, K14, p63, and FoxA2. K8 is a columnar cell marker, K14 and p63 are squamous cell markers, and FoxA2 is a glandular epithelium marker (Kurita et al., 2001b, Kurita et al., 2005, Kelleher et al., 2017). It is also expressed in undifferentiated top most layer of cervical epithelial cells in ovariectomized mice. In our observations, we have detected its expression in the cervical epithelium at postnatal age, suggesting its possible role in cervical epithelial development.

At P0, entire cervical epithelium was K8+ (Figure 5B). At P2 onwards, undifferentiated columnar epithelium of cervix was both FoxA2+/K8+ (Figure5A, 5B). As the cuboidal cells appeared underneath the undifferentiated columnar epithelium of cervix, they were p63+(Figure 5A), which was followed by K14 expression (Figure 5B). At P5, cervical epithelium was still two layered: K8+/FoxA2+ columnar cells at the upper layer and K14+/p63+ cuboidal cells at the bottom layer of cervix (Figure 5A, 5B). At P7, K8+/FoxA2+ upper layer became cuboidal-shaped. Squamous epithelium started to stratify at ectocervix, canal, and septum, at P7, P8, and P16, respectively. Most of the parabasal cells which were located above K14+/p63+/ K8-/FoxA2- bottom layer cells were also K14+/p63+/K8-/FoxA2-. Topmost layer, on the other hand, was K14-/p63-/K8+/FoxA2+. I also found cells that are p63+/FoxA2+ in the parabasal cells of stratified squamous epithelium (Figure 5A, yellow arrows). By P16, bottom and parabasal cells were uniformly K14+/P63+ throughout the cervix. Top most layer of the epithelium was K14-/p63-/K8+/FoxA2+ and undifferentiated. FoxA2 is normally reported to be expressed in the glandular epithelium of uterus at adult age. Cervical glands were absent at P16, yet, FoxA2 was expressed in the cervical epithelium as early as P2. These results raise a possibility that FoxA2 is involved in cell-fate determination during the development of cervical epithelium.

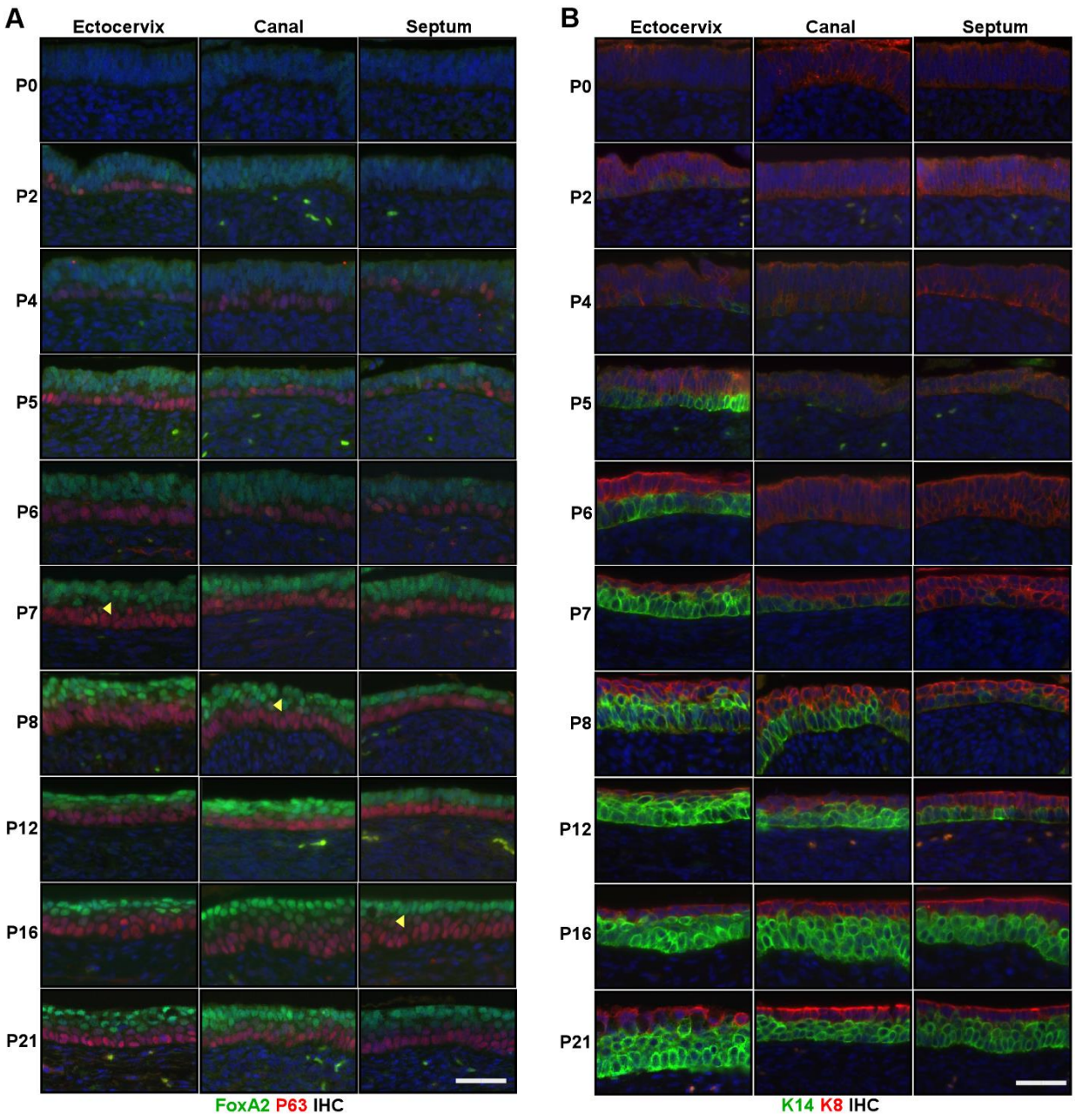


**Figure 5. Expression of epithelial cell markers in the cervix throughout postnatal development.** Cervical tissue sections from *wild-type* mice at selected postnatal ages were double stained for **(A)** FoxA2 (green), p63 (red), and nuclei (blue) and **(B)** K14 (green), K8 (red), and nuclei (blue). Yellow arrow in **(A)** indicates p63+/FoxA2+ cells. P: Postnatal day. Scale bar represents 40 µm.

***Cervical Squamocolumnar Junction Forms by P16***

To evaluate when squamocolumnar junction forms, I analyzed postnatal cervical tissue sections for expression of squamous cell marker K14 in the transformation zone (TZ). At P6, there were only 1-2 K14+ epithelial cells, supporting that squamous differentiation in upper end of endocervix was not complete (Figure 6). At P14, K14+ cells propagated in the most TZ, but they were not continuous. By P16, K14+ cells stopped expanding towards the uterus, and cells at the top layer remained negative for K14 (Figure 6). The cervical epithelium morphology became similar to that of P21 mice (Figure 6). These phenotypes were similar to those in the cervical epithelium in ovariectomized adult mouse (data not shown). These results suggest that cervical SCJ was formed by P16. At adult age, squamous epithelium stratifies with a varying level depending on estrus stage, and there was no K14- cells (Figure 6). Squamous epithelium reaches the full thickness and differentiation state at estrus stage. A small population of K14+ reserve cells was observed underneath the columnar cells of the SCJ in adult mouse at estrus stage (Figure 6).

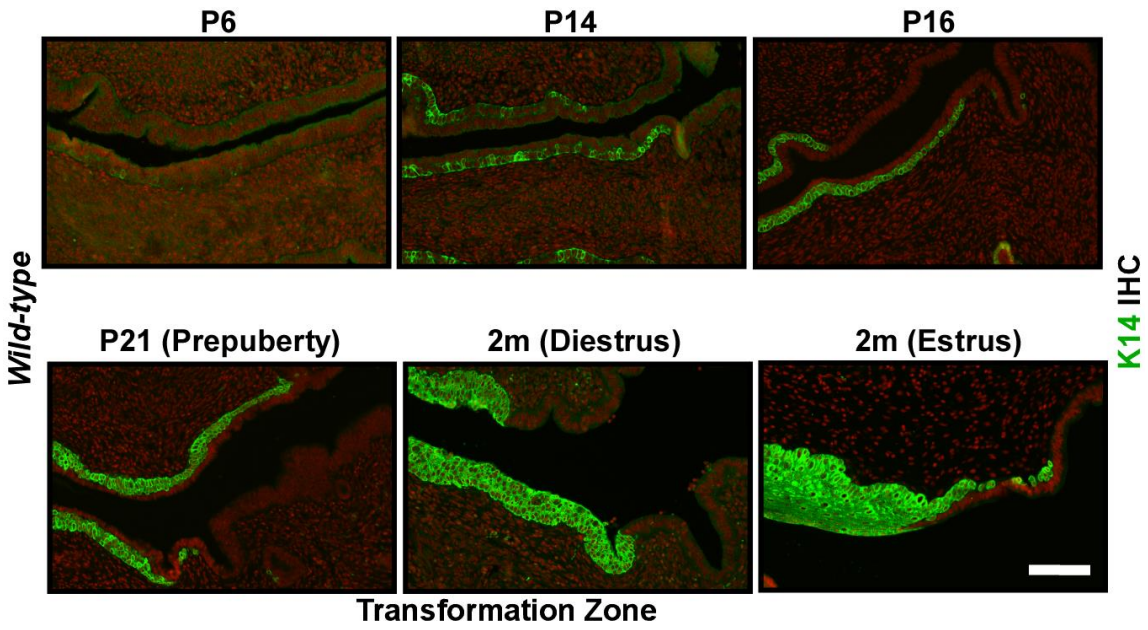


**Figure 6. Cervical squamocolumnar junction forms by postnatal day 16.**
Cervical tissue sections from *wild-type* mice at postnatal and adult age were stained for H&E (upper panel) and K14 (green), and nuclei(red) (lower panel). Scale bar represents 100 μm.

***Human and Murine Squamocolumnar Junction Have Different Epithelial Marker Expression Profile***

Human cervical SCJ-specific markers (e.g., AGR2, MMP7, and K7) have been reported (Herfs, 2012). Expression of these marker proteins was maintained in the cervical cancers originated from transformation zone, comparing to ectocervix, supporting their correlation with cervical squamous cell carcinoma. I sought to determine whether these protein markers were present in the mouse cervical SCJ. I investigated the expression of Agr2, Mmp7, and K7, together with FoxA2, K5, K19, and Fascin to characterize SCJ in adults. Agr2 was absent at the junction, but expressed in the glandular epithelium of cervix (Figure 7A). K7 was expressed throughout the luminal and glandular epithelium, but not reserve cells of SCJ (Figure 7B). FoxA2 was expressed in the glandular epithelium and in a short-stretch of columnar cells at the SCJ region (Figure 7C). K5 was expressed throughout squamous epithelium and reserve cells of SCJ (Figure 7D). K19 was expressed in all epithelial cell types (Figure 7E). Fascin, which is known as squamous basal cell marker in other tissues, was expressed in cervical cells at the basal and parabasal layers, and reserve cells (cyan arrow) (Figure 7F). Mmp7 was not located throughout the cervical and uterine epithelium (Data not shown). These findings suggest that human SCJ markers are not expressed in mouse SCJ cells.

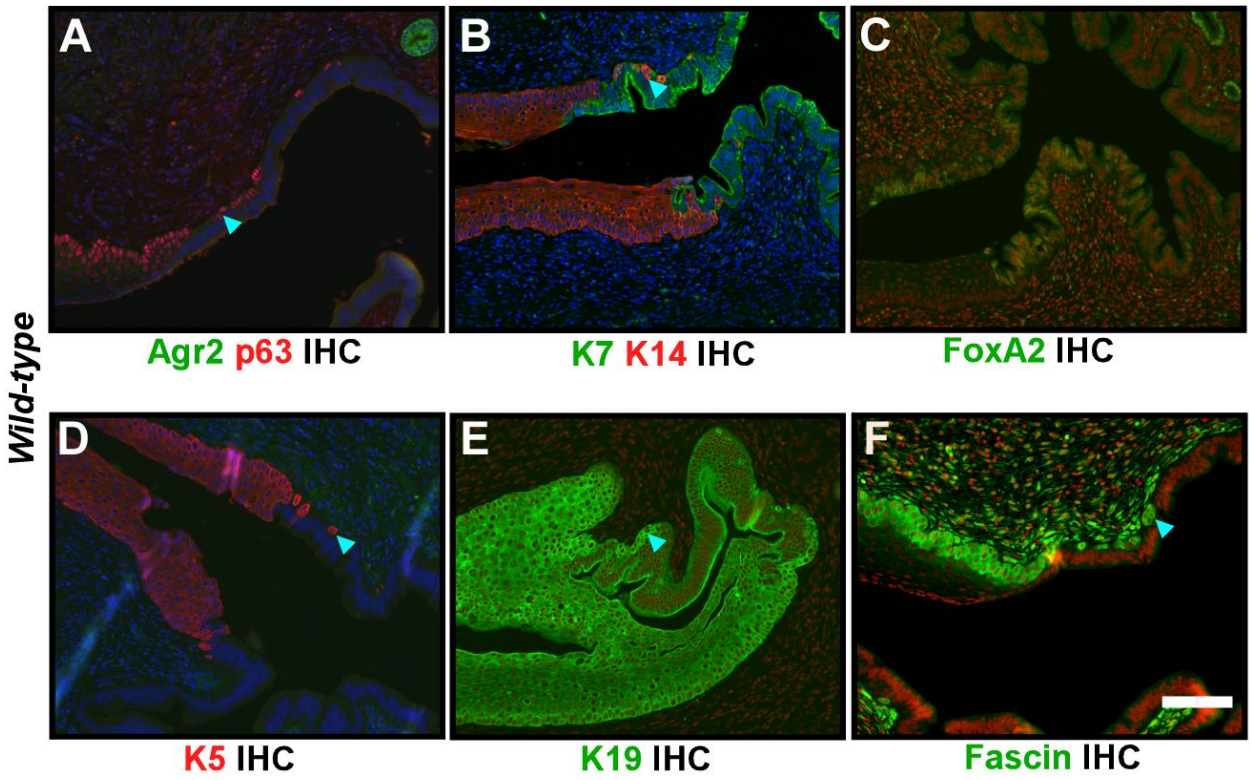


**Figure 7. Human and murine SCJ have different epithelial marker expression profile.** Cervical tissue sections from *wild-type* mice at adult age were stained for **(A)** AGR2 (green), p63 (red), and nuclei (blue); **(B)** K7 (green), K14 (red), and nuclei (blue); **(C)** FoxA2 (green), and nuclei (red); **(D)** K5 (red), and nuclei (blue); **(E)** K19 (green), and nuclei (red); and **(F)** Fascin (green), and nuclei (red). Cyan arrow indicates reserve cells. Scale bar represents 100 µm.

***Epithelial ERα expression coincides with the expansion of cuboidal cells during postnatal development***

DES exposure at postnatal age causes developmental defects, including adenosis in cervix and squamous metaplasia in the uterus in *wild-type* mice. ERα knockout *(Esr1*$^{-/-}$*)* mice are resistant to those DES-induced anomalies, suggesting its important roles on postnatal development of cervix. In this regard, I evaluated ERα expression in the cervix during postnatal days of development. ERα was present in the stroma as early as P0 (Figure 8). From P2 onwards, most of the stromal cells were ERα+. ERα was absent in the epithelium until P5. Patches of ERα positive cells started to appear in the cervical epithelium at P5 as the cuboidal cells expanded in the same region (Figure 8). By P10, almost all epithelial cells were ERα+. ERα expression in the cervical epithelium followed a similar pattern with p63 and K14, which is from vaginal to uterine body direction and coincided with the appearance and expansion of cuboidal cells in the cervix during postnatal development. This observation suggested its possible role in squamous epithelium development and formation of SCJ.

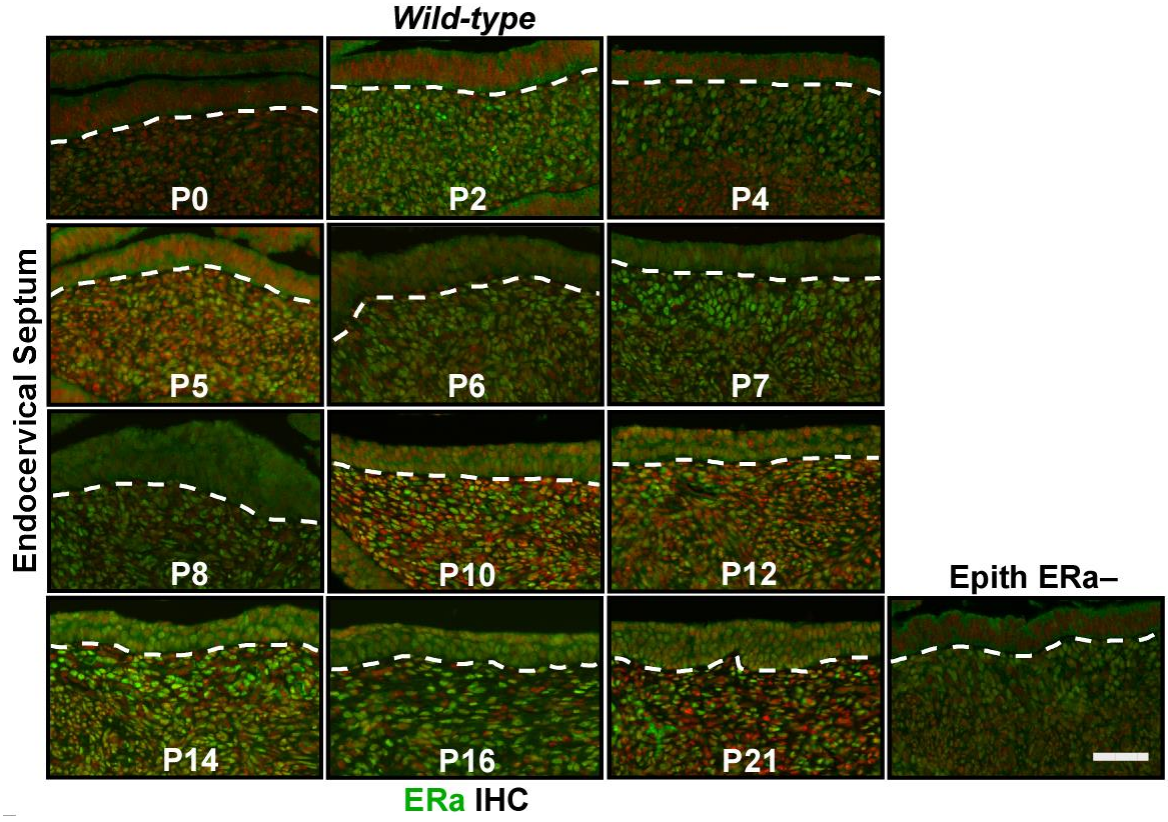


**Figure 8. Epithelial ERα+ cells expand throughout the cervix as the SCJ forms during postnatal development.**

Cervical tissue sections at selected postnatal ages were stained for ERα (green), and nuclei (red). White lines separate epithelium from stroma. P: Postnatal day. Scale bar represents 50 μm.

***Postnatal Development of Cervical Epithelium is Delayed in Epithelial ERα-Deficient Mice***

The expression of ERα in the cervical epithelium in postnatal ages coincided with the expression of K14 (Figure 8). To determine whether epithelial ERα was crucial for the development of SCJ, I used *Wnt7a-Cre/Esr1*$^{f/f}$ mice to ablate ER*α* expression specifically in the epithelium but not stroma. *Wnt7a* promoter is activated during embryogenesis, and thus *Wnt7a-Cre* transgenic allele deletes a floxed allele before birth. I confirmed that epithelial ERα was absent in *Wnt7a-Cre/Esr1*$^{f/f}$ mice at P10 (Figure 9A). I noticed that cuboidal-shaped cells were partially present in endocervical canal and septum at P6 in *Wnt7a-Cre/Esr1*$^{f/f}$ mice (Figure 9B), which was similar to that of P3 in *wild-type* mice (data not shown). From P8 onwards, cuboidal cells expanded continuously throughout the cervical epithelium (Figure 9B). These results showed a delay in the development of K14+ cuboidal cells when epithelial ERα was absent.

Stratification of ectocervix was observed at P7 in *wild-type* mice (Figure 4B), but partial stratification was observed at P8 and completed by P12 in epithelial ERα-deficient ectocervix (Figure 9B). Endocervical canal was not stratified as late as P14 (Figure 9B). By P21, both ectocervix and endocervical canal were stratified, but to the lesser extent compared to *wild-type* mice. Septum, on the other hand, never formed fully stratified epithelium. Taken together, I conclude that epithelial ERα is required for stratification of cervical epithelium.

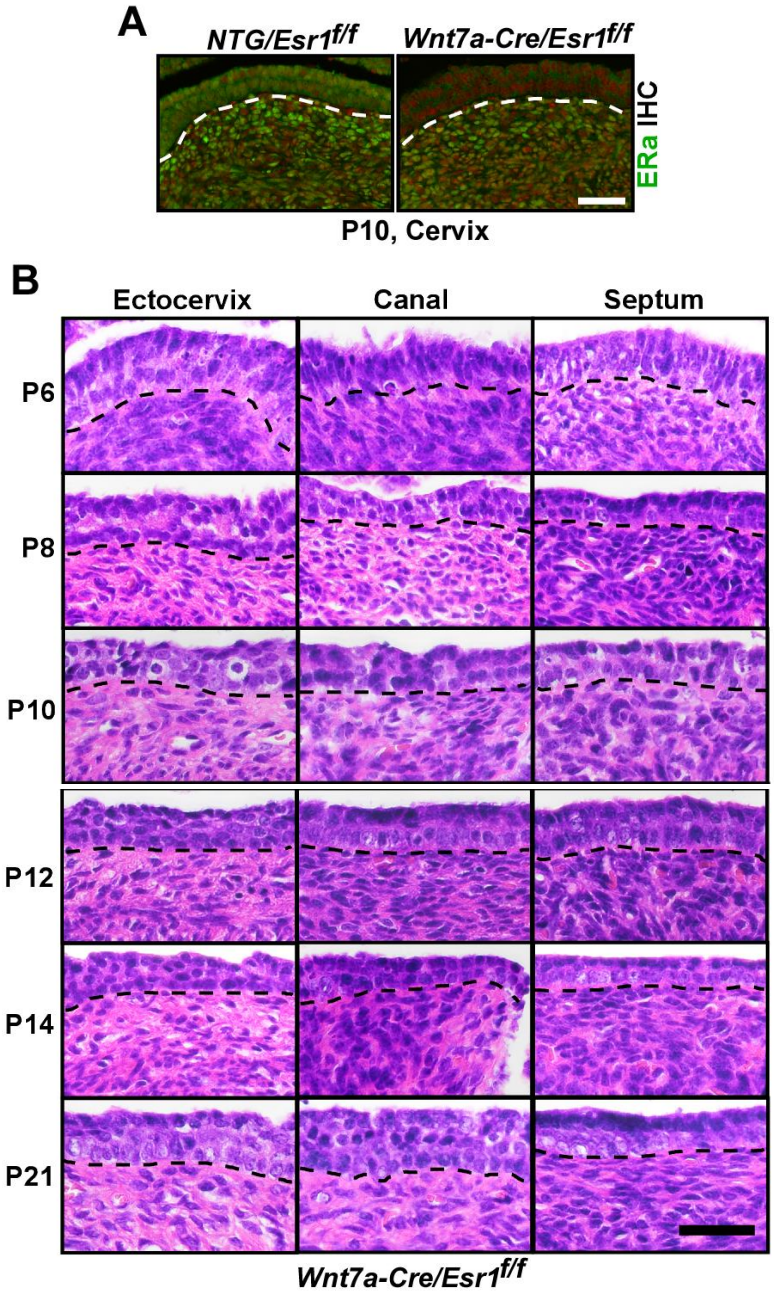


**Figure 9. Development of cervical epithelium is delayed when epithelial ERα is absent.**
**(A)** Cervical tissue sections from *NTG/Esr1*$^{f/f}$ and *Wnt7a-Cre/Esr1*$^{f/f}$ mice at P10 were stained for ERα (green), and nuclei (red). White lines separate epithelium from stroma. Scale bar represents 50 μm. **(B)** Cervical tissue was collected from *Wnt7a-Cre/Esr1*$^{f/f}$ mice at postnatal days (P) 6, 8, 12, 14, and 21 and stained with H&E. Representative images of ectocervix, endocervical canal, and endocervical septum are shown. Black lines separate epithelium from stroma. Scale bar represents 40 μm.

To further investigate the morphological delay in the development of cervical epithelium when epithelial ERα is absent, I analyzed expression of epithelial cell markers K8, K14, FoxA2, and p63 in *Wnt7a-Cre/Esr1*$^{f/f}$ mice at postnatal ages. There were patches of p63+ cells underneath the endocervical canal and septum in *Wnt7a-Cre/Esr1*$^{f/f}$ mice at P6 (Figure 10A), comparing to *wild-type* mice which has continuous layer of p63+ cells at the same age (Figure 5A). From P8 onwards, p63+ cuboidal cells were present throughout the cervical epithelium. The delay in development of cervical epithelium was more prominent in K14 expression pattern in *Wnt7a-Cre/Esr1*$^{f/f}$ mice. K14 expression was still partial in ectocervix and absent in both canal and septum at P8 (Figure 10B). K14 expansion in septum was not complete at P14 (Figure 10B). By P21, entire epithelium was K14+, although septum lacked stratification.

FoxA2 and K8 expression patterns (Figure 10A, 10B) were similar to those of *wild-type* mice (Figure 5A, 5B). FoxA2+/K8+ cells were taken over by p63+/K14+ cuboidal cells as the cervical epithelium developed. Overall, expansion of p63+K14+ cuboidal cells throughout the cervical epithelium, and therefore the formation of SCJ was delayed in epithelial ERα deficient mice (Figure 10B) comparing to *wild-type* mice.

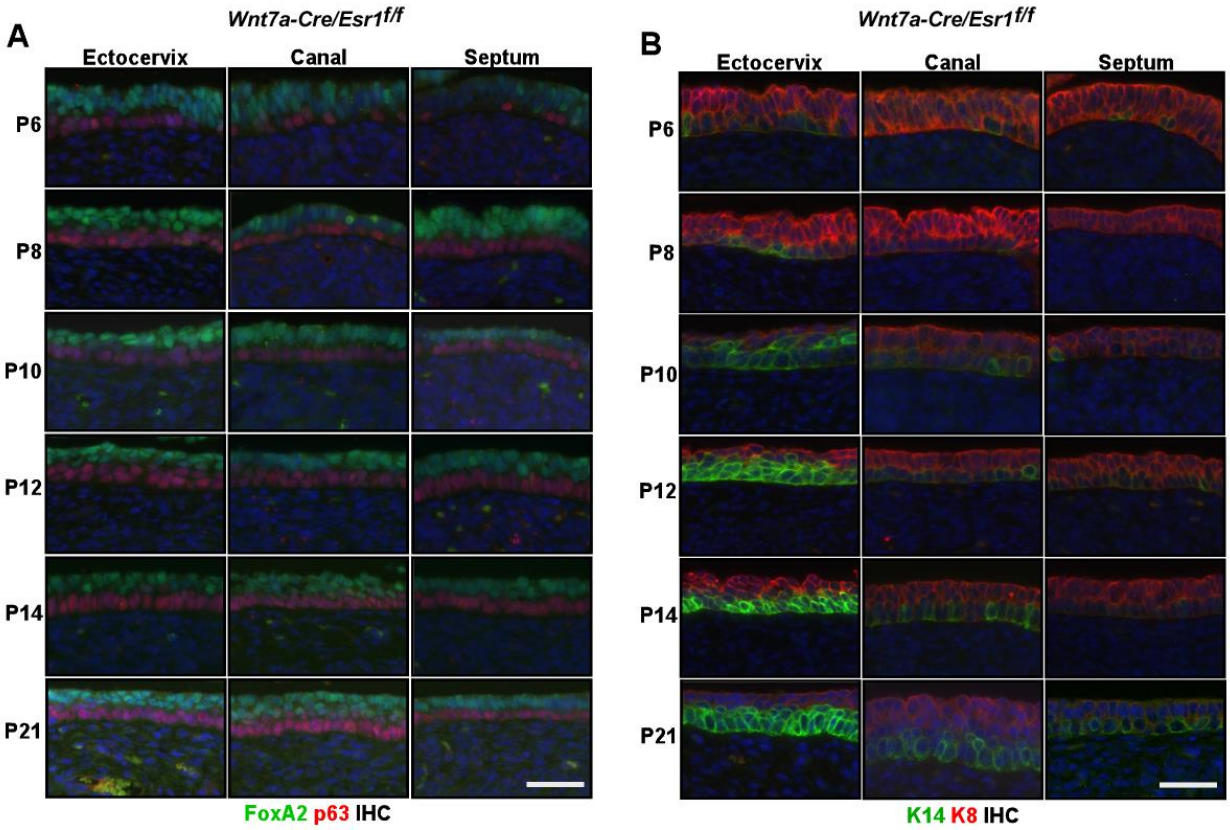


**Figure 10. Expression of epithelial cell markers in the cervix throughout postnatal development is delayed in epithelial ERα deficient mice.**
Cervical tissue sections at selected postnatal ages were double stained for **(A)** FoxA2 (green), P63 (red), and nuclei (blue) and **(B)** K14 (green), K8 (red), and nuclei (blue). P: Postnatal day. Scale bar represents 40 µm.

***Epithelial ERα is required for maintenance of cervical SCJ at adult age.***

SCJ was present in ERα knockout mice but lacked stratification, indicating that ERα is not required for formation of it. It also implied the role of ERα on full differentiation of epithelium. I analyzed K14 expression in transformation zone of *Wnt7a-Cre/Esr1*$^{f/f}$ mice throughout postnatal development and adult age to better understand the maintenance of cervical and SCJ epithelium when epithelial ERα is absent. At P6, K14 was not present (Figure 11), similar to *wild-type* mice. At P14, K14+ cell expansion was delayed (Figure 11). By P21, location where K14+ cells stopped expanding towards the uterus (Figure 11) was comparable to age-matching *wild-type* mice indicating that although delayed, SCJ formation was complete by prepubertal age P21 in epithelial ERα deficient mice. These findings suggest that epithelial ERα is not required for formation of SCJ. I analyzed aging mice for K14 expression to see if the junction is maintained properly. At six, and seven weeks of age, junction still lacked stratification. Interestingly, at 8 weeks of age, K14+ cells expanded from the original SCJ location towards the uterine body, underneath the columnar epithelium (Figure 11), indicating that SCJ maintenance is disrupted at adult age when epithelial ERα is ablated.

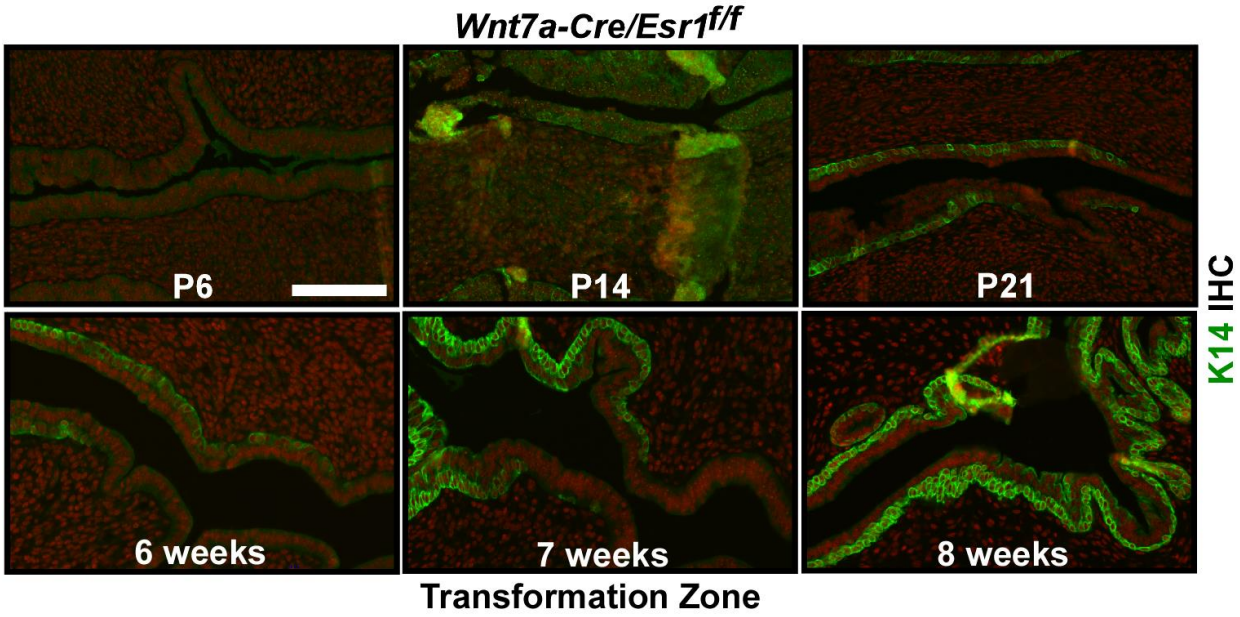


**Figure 11. SCJ maintenance is disrupted at adult age in epithelial ERα deficient mice.**
Cervical tissue sections from *Wnt7a-Cre/Esr1*$^{f/f}$ mice at selected ages were stained for K14 (green), and nuclei (red). P: Postnatal day. Scale bar represents 100 µm.

***Disruption of SCJ Maintenance Could be via a Failure in Cell-Fate Determination***

FoxA2 is known as glandular epithelium specific protein. It is involved in gland formation. Throughout my observations, I have located a small population of FoxA2+ columnar cells, localized at the SCJ region at adult age (Figure 7C). These cells might be the mouse counterpart of AGR2+/K7+/MMP7+/GDA+/CD63+ cuboidal cells of SCJ in human (Herfs et al., 2012).

SCJ maintenance is disrupted in epithelial ERα deficient mice at adult age, possibly via imbalance in stromal and epithelial ERα activity. To better understand the mechanism of disruption, I determined expression of FoxA2 and p63 in *NTG/Esr1*$^{f/f}$ and *Wnt7a-Cre/Esr1*$^{f/f}$ mice at P21 and 2-month-old adults. At P21, both *NTG/Esr1*$^{f/f}$ and *Wnt7a-Cre/Esr1*$^{f/f}$ mice had a two layered squamous epithelium: p63+/FoxA2- bottom layer and p63-/FoxA2+ upper layer (Figure 12). At adult age in *NTG/Esr1*$^{f/f}$ mice, basal, and suprabasal cells of squamous epithelium were p63+. Some of the upper layers of squamous epithelium were FoxA2+ (Figure 12). The fully differentiated top-most layer of the squamous epithelium was p63-/FoxA2-. There was a short stretch of FoxA2+/p63- columnar cells at the SCJ (yellow arrow). FoxA2 was also present in the glandular cells. On the other hand, *Wnt7a-Cre/Esr1*$^{f/f}$ mice had a continuation of the p63 and FoxA2 expression towards the uterine body, suggesting a failure in the cell-fate determination in SCJ region when epithelial ERα was absent at adult age (Figure 12).

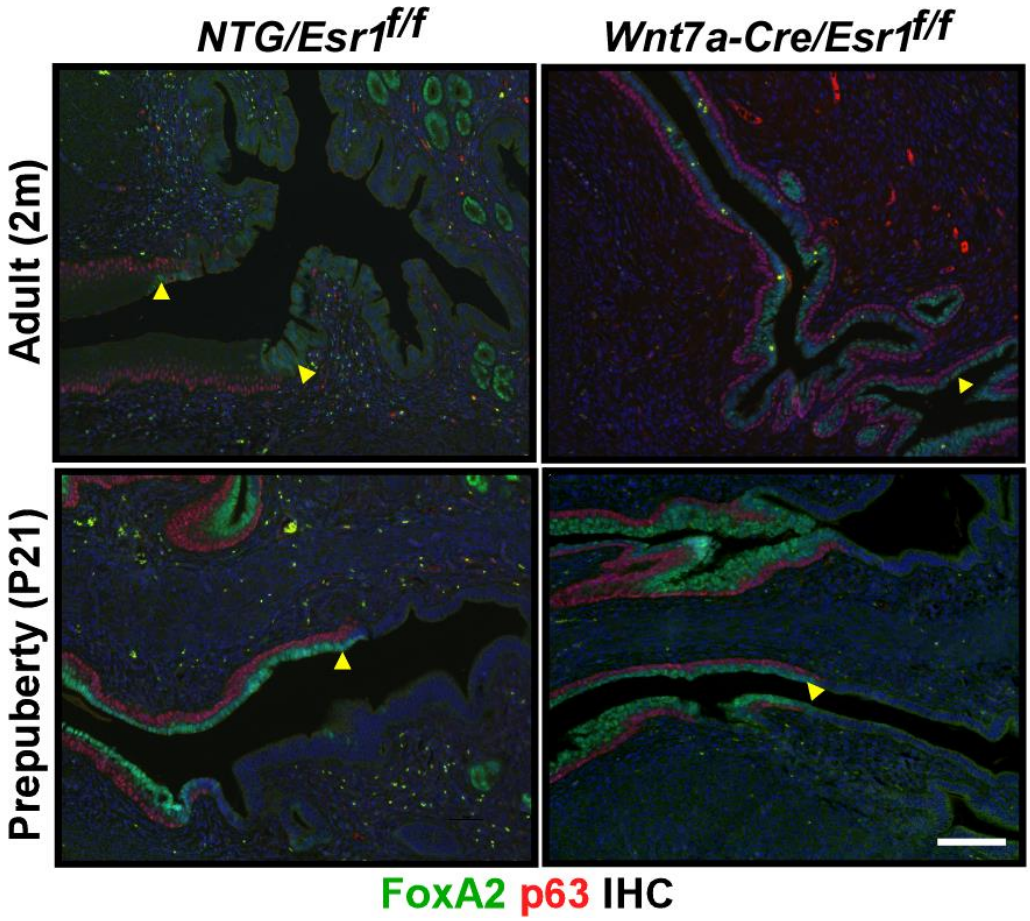


**Figure 12. Epithelial marker expression in SCJ is disrupted at adult age in epithelial ERα deficient mice.**

Cervical tissue sections from *Wnt7a-Cre/Esr1$^{f/f}$* and *wild-type* mice at prepubertal (P21) and adult (2-month-old) age were double stained for FoxA2 (green), P63 (red), and nuclei (blue). P: Postnatal day. Scale bar represents 100 µm.

***ERα AF1, AF2, and DBD are Required for the Maintenance of the Cervical SCJ***

Balance between stromal and epithelial ERα was required for maintenance of SCJ at adult age. To find out which domain of epithelial ERα is involved, I utilized mice expressing *AF1*, *AF2*, or *DBD* mutant alleles of ERα (Figure 13A). *AF1* allele lacks exon3 in ligand-independent activation function domain. *AF2* allele has a substitution mutation in AF2 domain, which lacks ligand-dependent function of ERα (Arao, 2011). *DBD* allele has a substitution mutation which abolishes interaction of ERα with the ERE within DNA (Ahlbory-Dieker, 2009) (Figure 13A). To generate mice expressing only the mutant allele in the epithelium, I mated $Esr1^{AF1/+}$, $Esr1^{AF2/+}$, *and* $Esr1^{DBD/+}$ mice with $Wnt7aCre/Esr1^{f/+}$ mice. The resultant *Wnt7a-Cre/*$Esr1^{f/AF1}$, *Wnt7a-Cre/*$Esr1^{f/AF1}$, and *Wnt7a-Cre/*$Esr1^{f/DBD}$ mice express mutant and intact allele of ERα in stroma and only the mutant allele in epithelium. As a control, I generated *NTG/*$Esr1^{f/AF1}$, *NTG/*$Esr1^{f/AF1}$, and *NTG/*$Esr1^{f/DBD}$ mice which express mutant and intact allele of ERα in both epithelium and stroma. I collected cervical tissue at 2-month-old adult mice. To prevent hormone-and estrous cycle related variations, I did vaginal cytology and confirmed that all the mice were in diestrus stage before collection of the tissue.

K14 IHC showed that K14+ cells of squamous epithelium extended towards the uterus and the squamous and columnar cell boundary was absent in all three of the *Wnt7a-Cre/*$Esr1^{f/AF1}$, *Wnt7a-Cre/*$Esr1^{f/AF1}$, and *Wnt7a-Cre/*$Esr1^{f/DBD}$ mice, comparing to *NTG* age matching control mice (Figure 13B). SCJ maintenance was not restored when ERα mutant allele was expressed in the epithelium. This observation suggested that ERα AF1, AF2, and DBD are required for maintenance of cervical SCJ.

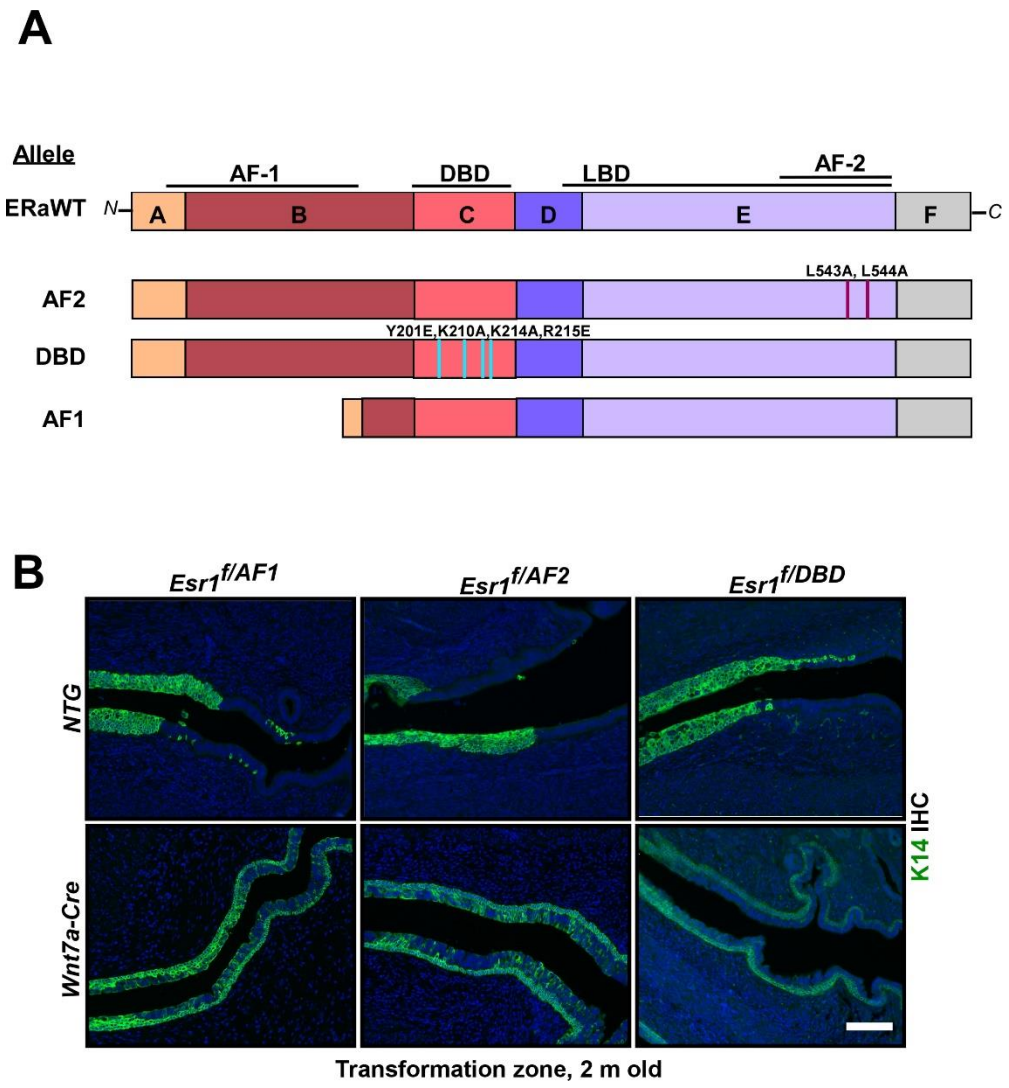


**Figure 13. AF2, DBD, and AF1 domains of ERα are required for maintenance of squamocolumnar junction at adult age.**
**(A)** Schematic of ERα mutant alleles *AF2*, *DBD*, and *AF1*, in comparison with *wild-type* allele. *AF2* and *DBD* alleles have substitution mutations in C, and E domains, respectively. *AF1* is a truncated allele which lacks exon 2 located in B domain. **(B)** Cervical tissue was collected at 2-month-old from *Wnt7a-Cre/Esr1*$^{f/AF1}$, *Wnt7a-Cre/Esr1*$^{f/DBD}$, and *Wnt7a-Cre/Esr1*$^{f/AF2}$ mice which express only the corresponding mutant allele in the epithelium and NTG mice as control. Vaginal cytology was done to collect the mice at diestrus stage. Sections were stained for K14 (green) and nuclei (blue) (n=6 per genotype). Scale bar represents 100 µm.

***ERα is not required for formation of SCJ formation***

Ablation of epithelial ERα led to delay in SCJ formation but SCJ was present by prepubertal age (P21), Stromal ERα is expressed in the cervix as early as postnatal day 0. Stromal ERα is required for proliferation of the cervical epithelium at adult age, suggesting its role in maintenance of epithelium. I questioned if ablation of ERα in both epithelium and stroma impacts squamous epithelium development and SCJ formation. In this regard, I determined the expression of K14 in the transformation zone in ERα knockout mice at P21 and 2-month-old adults. Transformation zone epithelium consisted of a K14+ bottom layer and K14- upper layer in both ages (Figure 14). Topographical location where K14+ cells stopped was similar to that of P16 *wild-type* mice, indicating that SCJ was formed even when ERα was absent in both epithelium and stroma. These results support that ERα is not required for the formation of SCJ. K14+ cells formed a single cell layer in adult ERα knockout mice (Figure 14), demonstrating that ERα is required for stratification of cervical epithelium.

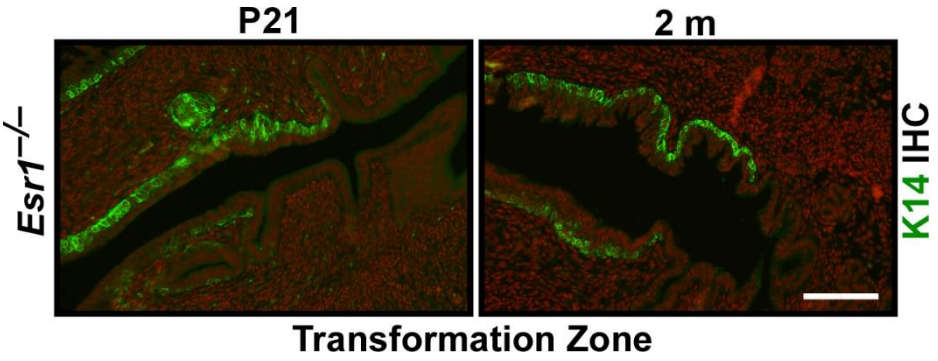


**Figure 14. ERα is not required for formation of squamocolumnar junction.** Cervical tissue sections from prepubertal (P21), and adult (2-month-old) ERα knockout mice (*Esr1*$^{-/-}$) were stained for K14 (green), and nuclei (red). Scale bar represents 100 µm.

## C. Discussion

In this study, I investigated role of ERα on development and maintenance of cervical SCJ. For that purpose, I initially assessed when exactly SCJ forms. K14+/p63+ cuboidal cells expanded underneath the undifferentiated FoxA2/K8+ columnar epithelium in a direction from ectocervix to endocervix at postnatal age. Development of cervical epithelium was completed at postnatal day 16. Morphology, epithelial marker expression profile, and the location where K14+/p63+ cuboidal-shaped cells stopped expanding towards the uterus at the upper part of endocervical septum at P16 was similar to that of ovariectomized mice. This observation suggested that cervical squamocolumnar junction is formed by P16. Human SCJ cuboidal cell marker K7 expression was not specific to junction in mouse. It was present in the entire columnar epithelium. Agr2 was expressed only in the glandular epithelium, indicating that human SCJ markers are not expressed in murine SCJ.

I found that stromal, and epithelial ERα expression in the endocervical septum starts at P0, and P5, respectively, proposing possible involvement of ERα in postnatal development of cervix. There was a cytoplasmic staining in the epithelium as early as P0, which was also present in epithelial ERα deficient mice, indicating that it was background staining. Development of squamous epithelium was delayed in epithelial ERα deficient mice, suggesting that epithelial ERα is required for timely development of squamous epithelium (Figure 15A). At adult age, squamous epithelium transitions between undifferentiated and fully differentiated state depending on ovarian hormone levels. Squamous epithelium remained undifferentiated in both ERα knockout and epithelial ERα deficient mice at adult age, regardless of estrus stage, suggesting that both epithelial and stromal ERα are required for full differentiation of squamous epithelium (Figure 15A).

SCJ formation was not impacted in ERα knockout mice, indicating that ERα is not required for formation of SCJ. Consistent with that, SCJ was formed by prepuberty (P21) in epithelial ERα deficient mice. However, in adult-age epithelial ERα deficient mice, squamous and columnar cell boundary was missing. Instead, a p63+/K14+ bottom-, and FoxA2+/K8+ upper-cell layer expanded towards the uterus. This

abnormality was absent in ERα knockout mice at adult age, which suggests that balance between stromal and epithelial ERα is required for proper maintenance of SCJ at adult age (Figure 15B). Disruption of junction occured after seven weeks old. I confirmed with vaginal cytology that *Wnt7a-Cre/Esr1*$^{f/f}$ mice started cycling at 28 days old, similar to *wild-type* mice (data not shown), meaning that ovarian hormones were present as early as 28 days old in epithelial ERα deficient mice. SCJ morphology, location and epithelial marker expression in ovariectomized epithelial ERα deficient mice was similar to P21 (data not shown). These findings indicate that other factors together with ovarian hormones are involved in disruption of SCJ after seven weeks old.

FoxA2 is normally involved in gland genesis in the uterus (Kelleher et al., 2017). There is no previous report showing FoxA2 expression in the SCJ. In my observations, FoxA2 was expressed in the cervical epithelium of mice as early as postnatal day two. During postnatal days of cervical epithelial development, FoxA2 was expressed at the upper layer of the cervical epithelium. At postnatal day 21, when postnatal development of cervical epithelium was complete, FoxA2 remained to be present at the undifferentiated-upper layer of cervical epithelium (Figure 13), which was similar to that of ovariectomized mice (data not shown). At adult age, FoxA2+ cell layer was replaced by fully differentiated squamous epithelium. I have detected a short stretch of FoxA2+ columnar cells at the SCJ of *wild-type* mice at adult age (Figure 13), located above the reserve cells. These cells might be the mouse counterpart of AGR2+/K7+/MMP7+/GDA+/CD63+ cuboidal cells of SCJ in human (Herfs et al., 2012), which are located above reserve cells. FoxA2+ cells might have a different epithelial marker expression profile but with similar function. Further study is needed to characterize FoxA2+ columnar cell population in SCJ. I propose that FoxA2 could be involved in maintenance of SCJ epithelium.

Epithelial ERα deficient mice had expansion of FoxA2+ upper layer and K14+/p63+ bottom layer epithelium towards the uterine epithelium at adult age. I speculate that loss in the balance of epithelial and stromal ERα somehow disrupts cell-fate determination in SCJ towards the expansion of these cells. Other factors, together with ovarian hormones might have triggered that expansion towards the uterine body at

adult age. I propose that stromal ERα inhibits and epithelial ERα promotes expansion of K14+/p63+ reserve, and FoxA2+ columnar cells of murine SCJ at adult age (Figure 15B).

Stratification of epithelium is essential for protection of the organ against chemical and mechanical damage. HPV can only infect basal cells, likely through microabrasions or microtrauma. It can be deducted that thin and undifferentiated epithelium which lacks stratification is more exposed and susceptible to HPV infection. Further study is required to understand the functional consequences of lack of SCJ boundary when epithelial and stromal ERα balance is shifted.

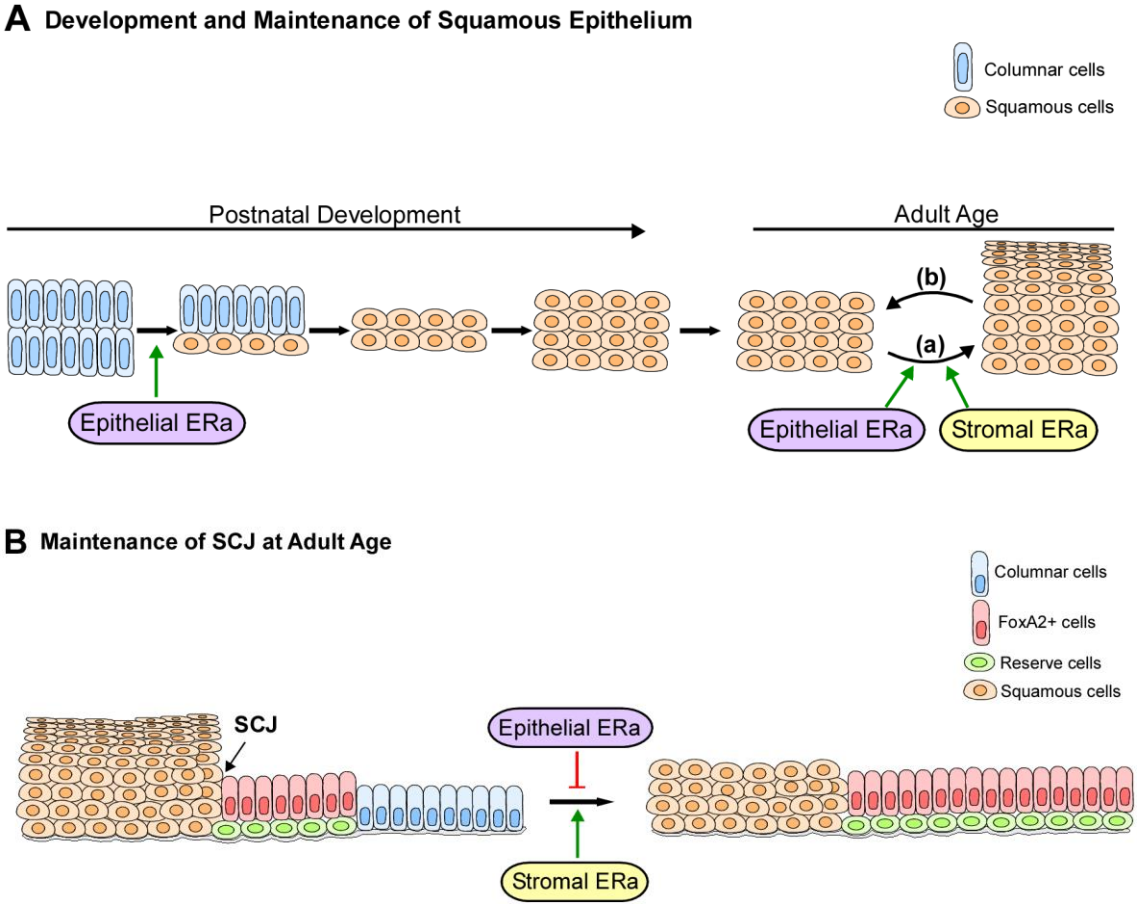


**Figure 15. Proposed model of ERα mechanisms in squamous epithelium and squamocolumnar junction. (A)** Epithelial ERα is required for timely development of squamous epithelium. At adult age, epithelium transitions between **(a)** undifferentiated and **(b)** fully differentiated state during estrus cycle. Both epithelial and stromal ERα are required for full differentiation of squamous epithelium. **(B)** Balance between squamous end epithelial ERα is required for maintenance of squamocolumnar junction (SCJ) at adult age. Epithelial ERα inhibits and stromal ERα promotes expansion of K14+/p63+ reserve and FoxA2+ columnar cells of SCJ at adult age.

## V. CHAPTER 2:

### Identification and Characterization of a New Epithelial Cell Type in the Uterus

#### A. Rationale

Uterus undergoes functional and structural changes during the menstrual cycle in human and estrous cycle in mice in an ovarian hormone dependent manner. Estrogen is the major hormone, and ERα is the primary receptor subtype in uterus (Hamilton, 2017). It has been shown that estrogen stimulates uterine epithelial proliferation in adult mice (Quarmby, 1984). Estrogen is implicated in many uterine diseases including endometrial cancers, endometriosis, and uterine fibroids (Deroo and Korach, 2006). Neonatal exposure of female mice to DES (a synthetic form of estrogen) results in high incidence of endometrial cancer at adult age (Newbold, 2006). ERα knockout mice has normal uterine development, but are infertile due to ovarian dysfunction and estrogen dependent-implantation defects (Hewit, 2002). Wild-type mice respond to estrogen treatment with an increase in uterine wet-weight up to four fold, which was not the case for ERα knockout mice (Lubahn et al., 1993) suggesting the role of ERα in uterine maintenance. Partial loss of stromal ERα prevents proliferation of nearby epithelial cells in the uterus of *Amhr2*$^{Cre/+}$*/Esr1*$^{f/f}$ mice, suggesting a juxtacrine mechanism between stromal ERα and neighboring epithelial cells (Winuthayanon et al., 2017) for uterine epithelial maintenance. Loss of epithelial ERα in *Wnt7a-Cre/Esr1*$^{f/f}$ mice had no effect on $E_2$ induced proliferation of uterine columnar epithelial cells. However, epithelial ERα was required for a full growth response of endometrial hyperplasia by actively inhibiting epithelial apoptosis in the uterus (Winuthayanon et al., 2010). These findings suggest that estrogen-ERα signaling is important for uterine epithelium maintenance. Further needs to be found about tissue compartment specific roles of ERα in uterus to understand and prevent uterine related abnormalities and diseases.

To study the tissue compartment specific role of ERα in the uterine epithelial maintenance, I generated *Wnt7a-Cre/Esr1*$^{f/f}$ mice which lacks epithelial ERα expression in the uterus. I have detected K14+/p63+ cell population underneath the uterine columnar epithelium when epithelial ERα was absent. *Esr1*$^{-/-}$ mice

lacked expansion of K14+/p63+ cells in the uterus. Deprivation and of estrogen via ovariectomy depleted these cells, whereas re-administration of estrogen propagated them suggesting that estrogen-stromal ERα signaling is involved in maintenance of these cells. With thorough analysis, I found that a small population of K14+/p63+ cells are present underneath the columnar epithelium in the wild-type mice as early as postnatal day 14. These findings suggest that K14+/p63+ cells (will be named as uterine basal cells) are normally present in wild-type mouse and their maintenance was disrupted when epithelial ERα was absent. Uterine basal cells expanded throughout the uterus when I deleted ERα from the uterine epithelium at adult age using *Ltf-iCre/Esr1*$^{f/f}$ mice, indicating that epithelial ERα is required for maintenance of uterine epithelium at adult age. Uterine basal cells had a unique epithelial marker expression profile, differing from squamous cells and reserve cells of cervix and columnar cells of uterus. To evaluate the potential function of basal cells in the uterine epithelium, I did cell lineage tracing experiments using tamoxifen inducible *Krt5*$^{CreER/+}$/*ROSA26*$^{mTmG/+}$ reporter mice. I found that GFP labeled cells transdifferentiated into glandular columnar epithelium. *Wnt7a-Cre/Esr1*$^{f/f}$ mice had a significant reduction in gland formation and had abnormal expression of glandular epithelium marker FoxA2 throughout the luminal columnar epithelium. I suggest that uterine basal cells might contribute to maintenance of glandular columnar epithelium. My data also implies that stromal and epithelial ERα have opposing roles in maintenance of uterine basal cells, where stromal ERα promotes, epithelial ERα inhibits their expansion in the uterus.

## B. Results

***There is a small population of K5+/K14+/p63+ cells underneath the columnar epithelium in mice***

*Wnt7a-Cre/Esr1$^{f/f}$* mice which lacks epithelial ERα expression in the uterus had abnormal expansion of cells which express K5 (Figure 16A), K14, and p63 (data not shown) underneath the columnar epithelium of the uterus at adult age (Figure 16A, right panel), comparing to age matching *NTG/Esr1$^{f/f}$* (Figure 16A, left panel), and *Wnt7a-Cre/Esr1$^{f/+}$* (data not shown) mice. To find out if this abnormality was of developmental origin, I analyzed younger ages for K14 expression and observed that K14$^{+}$ cells were absent in the uterus of *Wnt7a-Cre/Esr1$^{f/f}$* mice at P21 and appeared in the uterus after seven weeks old (Figure 16B).

I questioned if K14+/p63+ cells which expanded in the uterus of epithelial ERα deficient mice are normally present in *wild-type* mouse. To find that out, I stained every fifth section and a total of seven sections per *wild-type* uterine tissue from adult mouse for K14 expression. I found these cells in all the *wild-type* mice I analyzed at a rare frequency (Figure 17A). To determine if these cells are not present in the uterus of *wild-type* mice due to age related abnormalities, I analyzed their presence as early as postnatal day 14 (P14). Considering the rarity of these cells, I initially determined expression of *K14* and *Trp63* at P14 at RNA level (Figure 17B). They both were expressed in total n=2 tissue I analyzed, suggesting that these cells could be present in the uterus at P14. To find that out, I stained ever third section and a total of seven sections per *wild-type* uterine tissue from P14 mouse for K14 expression. It was present underneath the glandular epithelium of the uterus (Figure 17C) in two of the three tissues I analyzed, similar to adult age. I concluded that there is a small population of K5+/K14+/p63+ cells underneath the columnar epithelium of *wild-type* uterus. These cells morphologically look like basal cells of squamous epithelium. Therefore, I named them as uterine basal cells.

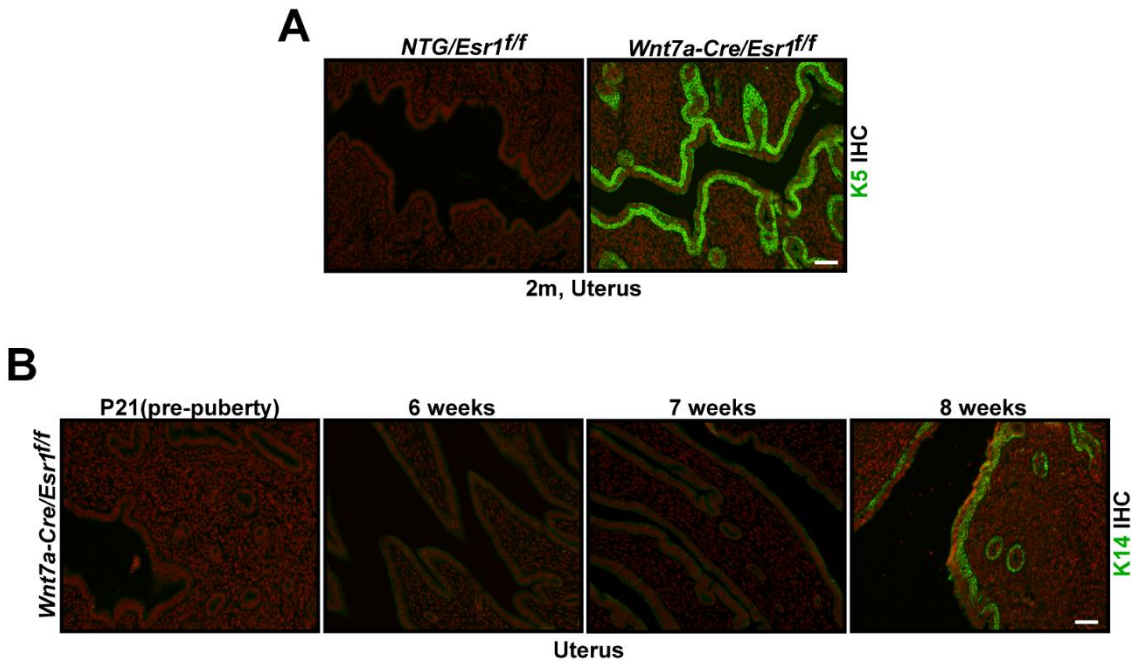


**Figure 16. K5+/K14+ cells expand throughout the uterus in epithelial ERα deficient mice at adult age.**

**(A)** Representative images of uterine sections from *Wnt7a-Cre/Esr1*$^{f/f}$ and *NTG/Esr1*$^{f/f}$ mice at two months old which were stained for K14 (green), and nuclei (red). n≥6 per genotype. Scale bars represent 50 μm **(B)** Representative images of uterine sections from *Wnt7a-Cre/Esr1*$^{f/f}$ mice at six, seven, and eight weeks of age which were stained for K14 (green), and nuclei (red). n=1–3 per genotype. Scale bars represent 50 μm.

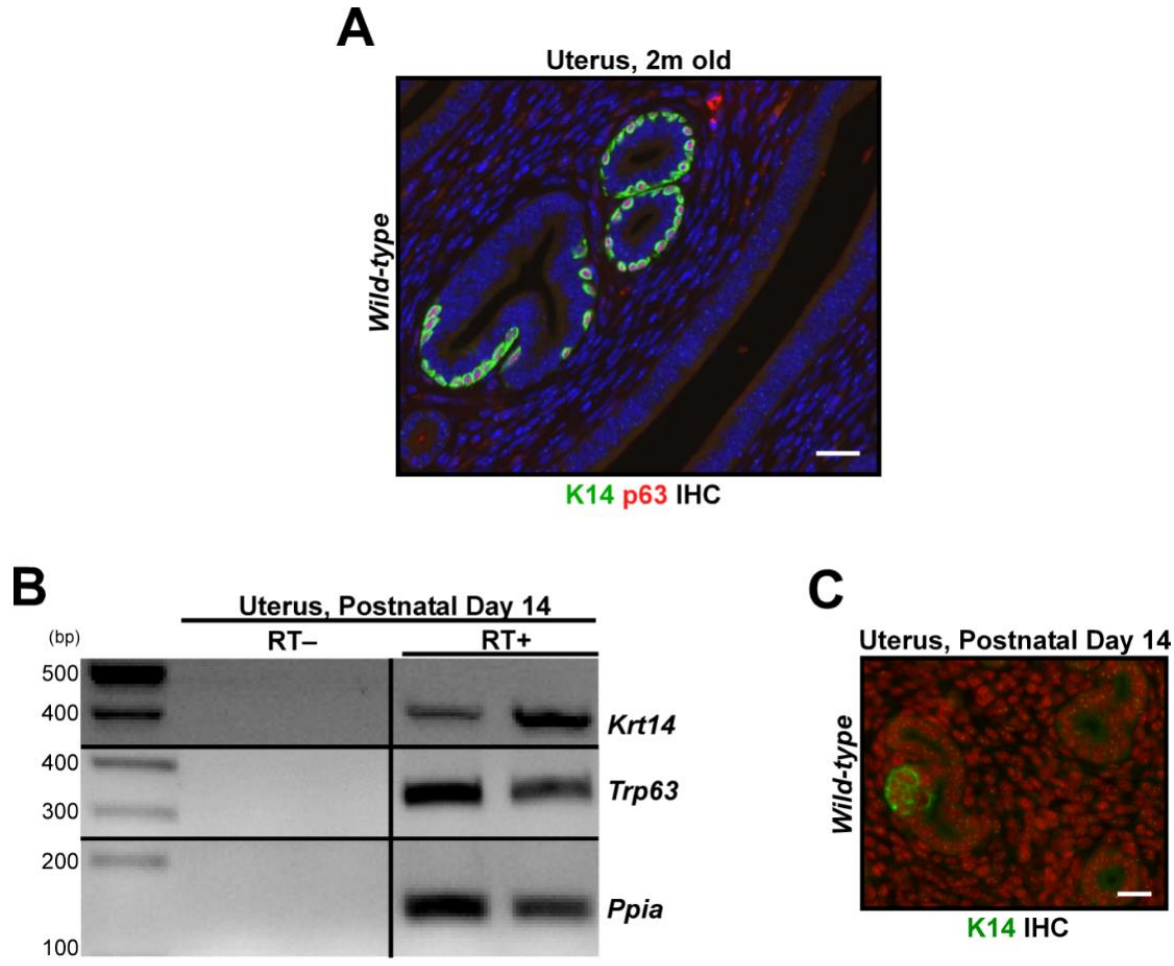


**Figure 17. Uterine basal cell markers are expressed in the murine uterus as young as postnatal day 14.**

**(A)** Uterine tissue sections from two months old mice were stained every fifth section for K14 (green), p63 (red), and nuclei (blue) (n=6). Scale bar represents 50 µm. **(B)** cDNA was synthesized from RNA extracted from postnatal day 14 uterus and subjected to PCR with primers against *Trp63*, *Krt14*, and *Ppia* genes. Intervening lanes between RT- and RT+ lanes were removed. 1.5% agarose gel. RT: Reverse transcriptase. **(C)** Uterine tissues from postnatal day 14 *wild-type* mice (n=2) were sectioned and stained every third section for K14 (green) and nuclei (red). Scale bar represents 20 µm.

***Uterine basal cells differ from cervical reserve cells and uterine columnar cells***

To characterize uterine basal cells at molecular level, I determined expression of different epithelial cell proteins in the wild-type uterus at adult age. Keratin 5, keratin 14 and p63 were expressed in the uterine basal cells together with squamous cells of cervix and reserve cells of SCJ. Keratin 7, which is known as columnar epithelial cell marker was also present in the uterine basal cells but not the reserve cells or squamous cells of cervix. Keratin 8 is normally expressed in the uterine columnar cells at adult age. Uterine basal cells lacked K8 expression (Figure 18A). Keratin 19 was expressed in all epithelial cell types in both cervix and uterus. These results suggest that basal cells of uterus have different epithelial marker expression profile and are different from squamous and reserve cells of cervix and columnar cells of uterus. Agr2 and FoxA2 are gland specific proteins and were absent in the uterine basal cells (Figure 18B, 18C). Interestingly, throughout my observations, I mostly located uterine basal cells underneath or nearby glandular epithelium, suggesting their possible role in maintenance of glandular epithelium. Table 2 summarizes the list of protein markers I stained and their expression in cervical and uterine epithelium.

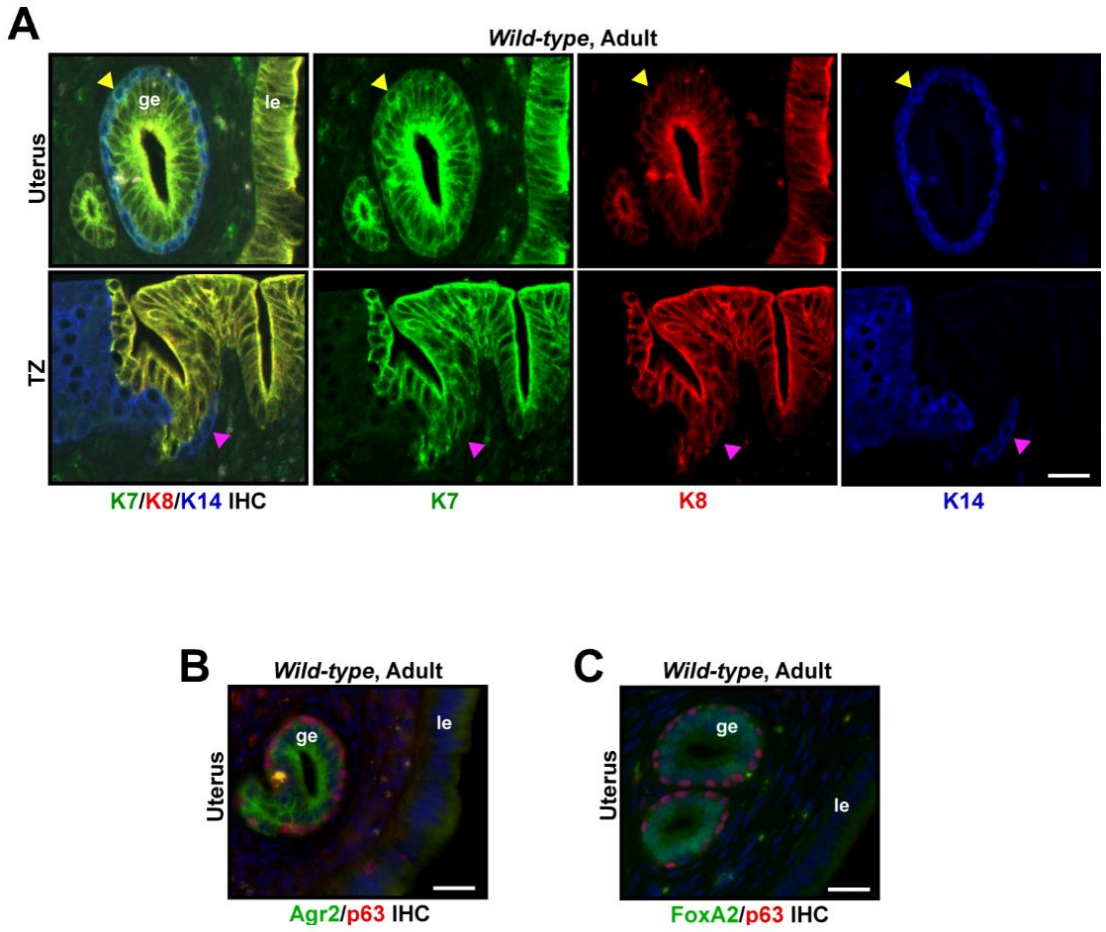


**Figure 18. Uterine basal cells differ from the other cell types in the cervix and the uterus.**
Female reproductive tissue was harvested from adult *wild-type* mice. **(A)** Uterine sections were stained for K7 (green), K8 (red) and K14 (blue). Yellow arrow indicates K7+/K14+/K8- uterine basal cells. Magenta arrow indicates K14+/K7-/K8- squamocolumnar junction reserve cells. Uterine sections were stained for **(B)** Agr2 (green), p63 (red), and nuclei (blue) and **(C)** FoxA2 (green), p63 (red), and nuclei (blue). TZ: Transformation Zone. Ge: Glandular epithelium. Le: Luminal epithelium. Scale bars represent 25 µm.

**Table 2. Expression of epithelial protein markers in the uterine and squamocolumnar junction epithelial cells**

| Protein | SCJ | | | | Uterus | | |
|---|---|---|---|---|---|---|---|
| | Squamous Cells | Reserve Cells | FoxA2+ Columnar Cells | FoxA2- Columnar Cells | Luminal Columnar Cells | Glandular Columnar Cells | Basal Cells |
| p63 | + | + | - | - | - | - | + |
| Fascin | + | + | - | - | - | - | + |
| Keratin 5 | + | + | - | - | - | - | + |
| Keratin 14 | + | + | - | - | - | - | + |
| Keratin 7 | - | - | + | + | + | + | + |
| Keratin 8 | - | - | + | + | + | + | - |
| Keratin 19 | + | + | + | + | + | + | + |
| Foxa2 | - | - | + | - | - | + | - |
| Agr2 | - | - | - | - | - | + | - |

***Epithelial ERα is required for maintenance of the uterine epithelium at adult age***

I questioned if the abnormal expansion of uterine basal cells in epithelial ERα deficient mice at adult age was due to a developmental defect or acquired at adult age. To find that out, I aimed to remove ERα from the epithelium at adult age using *Ltf-iCre* mice which express cre recombinase only in the uterine epithelium of adult or estrogen-administered prepubertal mice (Daikoku, 2014). I generated *Ltf-iCre/Esr1*$^{f/f}$ mice and determined the efficiency of uterine epithelial ERα deletion at various ages. ERα deletion was not complete at as late as 16 weeks old (Figure 19A). Lactoferrin (Ltf) is an estrogen inducible gene and it has been shown that its expression varies during estrous cycle. It is expressed at the highest level at proestrus stage when estrogen levels increase (Teng et al, 2002). I argue that ERα + cells could have been selected over ERα- cells when estrogen levels were reduced during estrous cycle. To overcome the possible impact of estrogen fluctuations on efficiency of ERα deletion, I gave slow release estrogen pellet to *Ltf-iCre/Esr1*$^{f/f}$ and *NTG/Esr1*$^{f/f}$ mice and led them rest for 2 weeks before collecting the uterine tissue (Figure 18B). Epithelial ERα deletion was not complete (Figure C, mid-panel) in *Ltf-iCre/Esr1*$^{f/f}$ mice. Nonetheless, I found that uterine basal cells expanded underneath the columnar epithelium of *Ltf-iCre/Esr1*$^{f/f}$ mice (n=5) comparing to age matching NTG mice (n=7) with the same treatment regimen (Figure 18C). Interestingly, K14+ cell population coincided with ERα- columnar epithelium (Figure C). These finding suggests that epithelial ERα is required for maintenance of uterine epithelium at adult age.

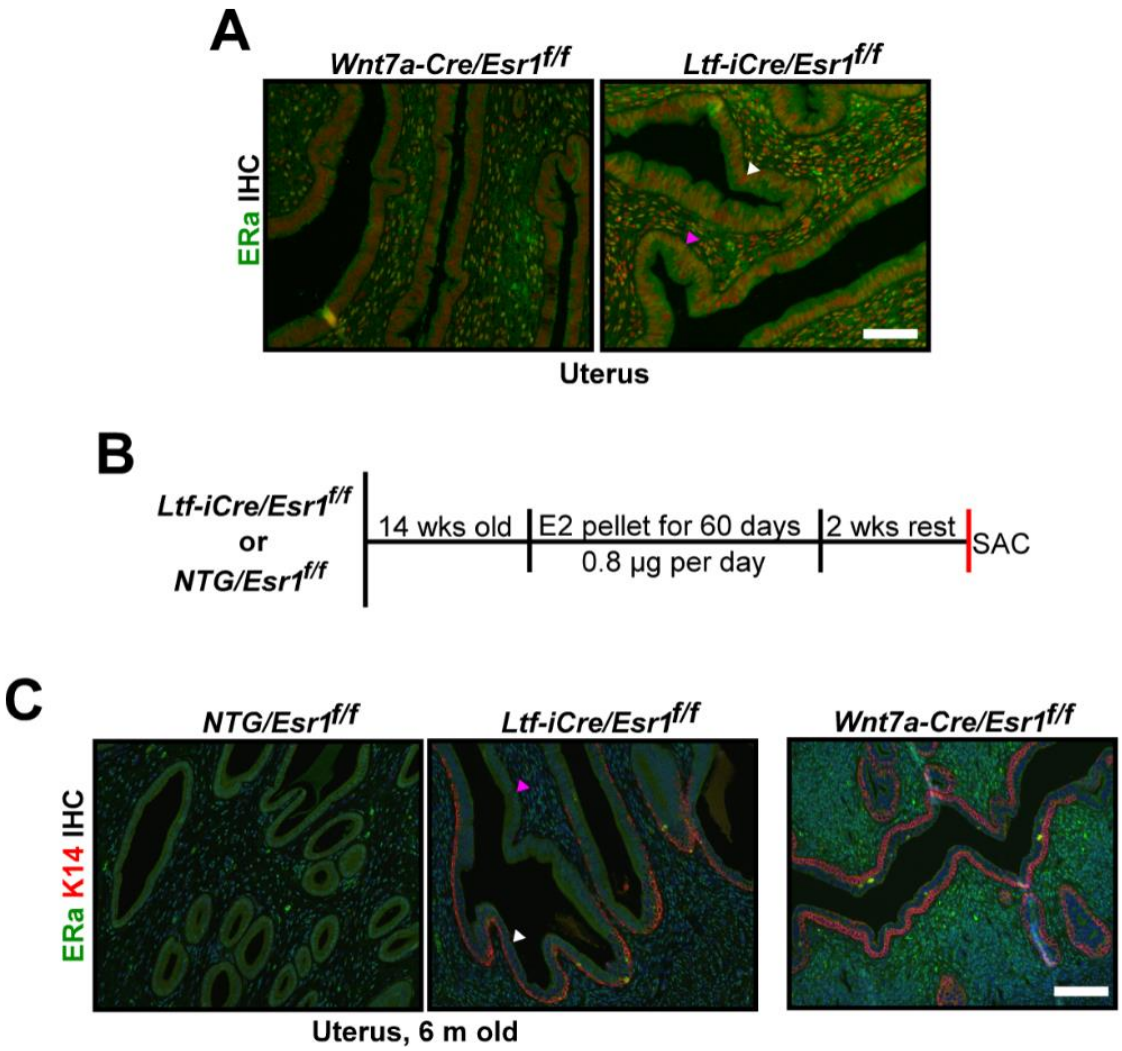


**Figure 19. Epithelial ERα is required for maintenance of the uterine epithelium at adult age. (A)** Treatment regimen. Mice were given slow release $E_2$ tablets (0.8 µg/day) for 60 days and rested for two weeks before harvest. **(B)** Uterine sections were stained for ERα (green), K14 (red), and nuclei (blue) (n=4 each). Magenta arrows, and white arrows in **(A)** and **(C)** indicate ERα+, and ERα- cells, respectively. Scale bars represent 100 µm.

***Estrogen is required for proliferation and survival of uterine basal cells***

Uterine basal cells did not expand throughout the uterus in *Esr1*$^{-/-}$ mice (Figure 20A). Basal cells in the uterus of *Wnt7a-Cre/Esr1*$^{f/f}$ mice were not detectable when estrogen was depleted by ovariectomy (Figure 20B). These cells repopulated the uterus when estrogen was readministered (Figure 20C). These findings suggest that estrogen is required for maintenance of these cells and stromal ERα may be involved in expansion of these cells when epithelial ERα is absent.

To find out a mechanism of how uterine basal cells are maintained, I aimed to look at proliferation and apoptotic indices of them. Considering the rarity of these cells, I utilized *Wnt7a-Cre/Esr1*$^{f/f}$ mice to have enough number of cells for analysis. I have ovariectomized *Wnt7a-Cre/Esr1*$^{f/f}$ mice and did three days of $E_2$ treatment or no treatment on n=3-4 mice each. I analyzed expression of Ki-67 to assess proliferative status of uterine basal cells in response to estrogen, or no treatment. I stained for p63 as uterine basal cell marker (Figure 21A). I quantified Ki67+/p63+ cells in p63+ cell population. Proliferative P63+ cells were higher in three days $E_2$ treated *Wnt7a-Cre/Esr1*$^{f/f}$ mice (25.15 ± 9.53%) comparing to untreated *Wnt7a-Cre/Esr1*$^{f/f}$ mice (5.56 ± 0.98%) (Figure 21B). The difference was statistically significant (P=0.02857).

I analyzed expression of caspase-3 to assess apoptotic indices of uterine basal cells in response to estrogen in the same treatment conditions. I double stained the tissue sections for caspase-3 and p63 (Figure 21C). I quantified caspase-3+/p63+ cells in p63+ cell population. Apoptotic p63+ cells were less in three days $E_2$ treated *Wnt7a-Cre/Esr1*$^{f/f}$ mice (0.39 ± 0.13%) comparing to untreated *Wnt7a-Cre/Esr1*$^{f/f}$ mice (1.38 ± 0.45%) (Figure 21D). The difference was statistically significant (P=0.01695). These results suggest that estrogen is required for maintenance of uterine basal cells.

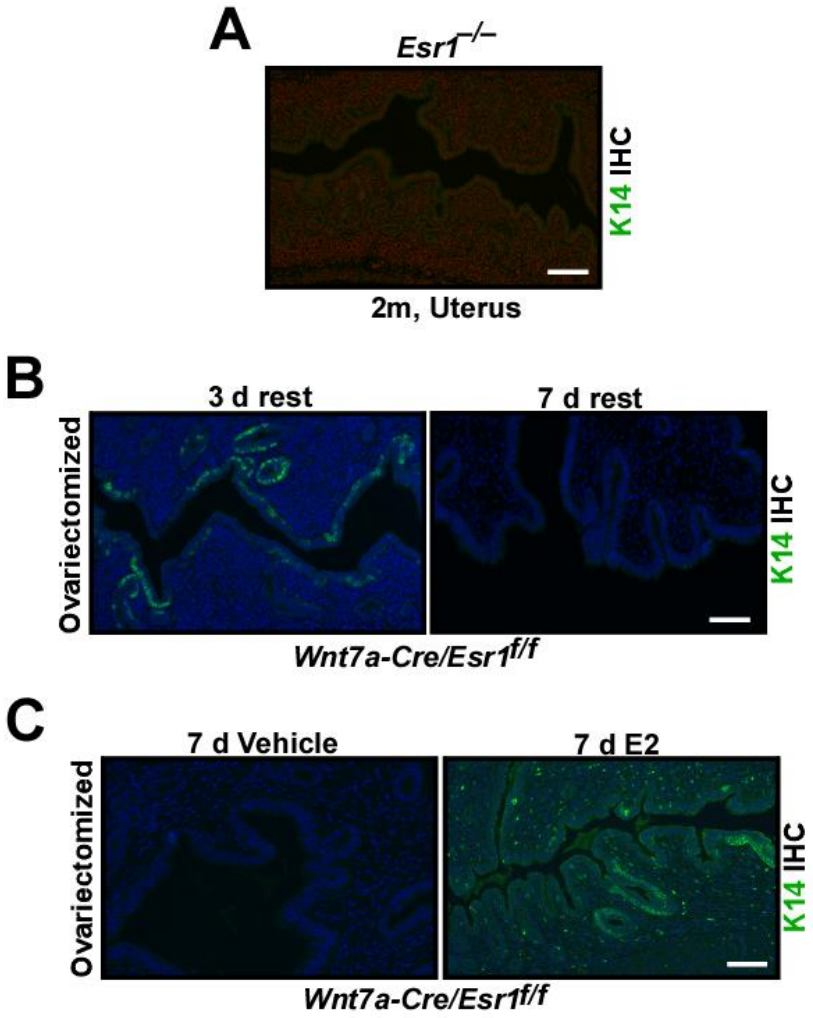


**Figure 20. Estrogen is required for maintenance of uterine basal cells.**
**(A)** Uterine tissue sections from *Esr1*$^{-/-}$ mice at two months of age (n=3) were stained for K14 (green), and nuclei (red). **(B)** Ovariectomized *Wnt7a-Cre/Esr1*$^{f/f}$ mice were rested for three, and seven days and uteri were harvested. Tissue sections were stained for K14 (green), and nuclei (blue) (n=3 each). **(C)** Ovariectomized *Wnt7a-Cre/Esr1*$^{f/f}$ mice were rested for two weeks and given vehicle or $E_2$ for seven days. Uterine tissue sections were stained for K14 (green), and nuclei (blue) (n=3 each). Scale bars represent 50 µm.

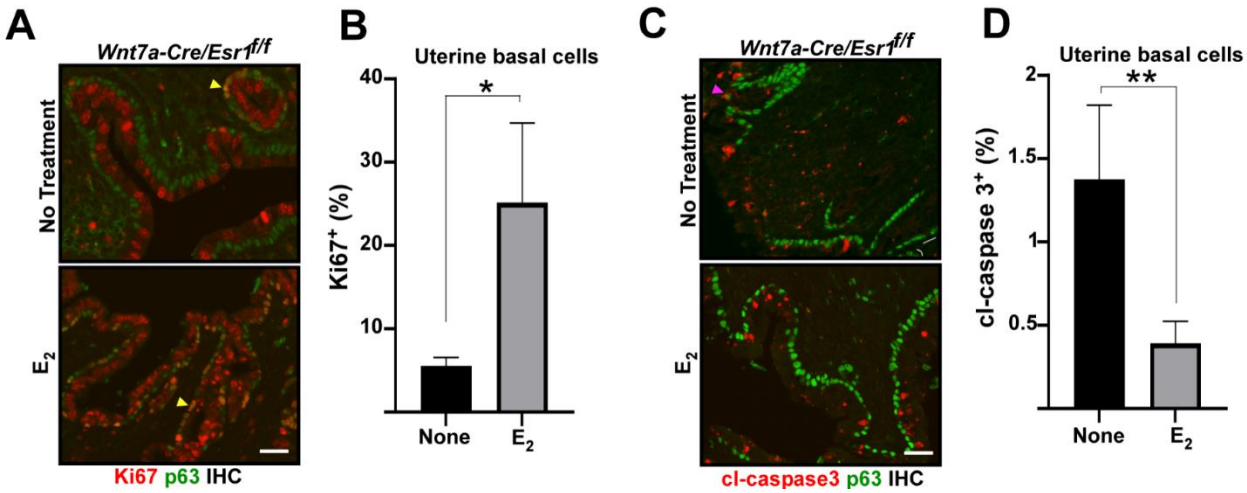


**Figure 21. Estrogen induces proliferative, and reduces apoptotic indices of uterine basal cells**
*Wnt7a-Cre/Esr1*$^{f/f}$ mice were ovariectomized at two months of age and given $E_2$ (n=4) or untreated (n=3) for three days. Representative immunohistochemistry images of **(A)** Ki67 (red), and p63 (green), and **(C)** caspase-3 (red), and p63 (green). Yellow, and magenta arrows indicate Ki67+/p63+ cells, and caspase-3+/p63+ cells, respectively. Scale bars represent 20 µm. Results were quantified for Ki67+ **(B)**, or caspase-3+ **(D)** uterine basal cells. P-values are *=0.02857, **=0.01695

***Uterine Basal Cells Transdifferentiate into Columnar Epithelial Cells***

To find out the function of the uterine basal cells, I designed cell lineage tracing experiments by crossing *ROSA26*$^{mTmG/+}$ double reporter mice with a tamoxifen-inducible *Krt5*$^{CreER/+}$ mice. *Krt5*$^{CreER/+}$*/ROSA26*$^{mTmG/+}$ mice which express reporter GFP allele in K5 expressing cells when upstream loxP flanked STOP codon is excised by tamoxifen-activated CreER recombinase (Figure 22A). I did tamoxifen treatment at 6-8 months old adult mice to label K5 expressing cells with GFP (Figure 22B.) and led them rest for one day to evaluate the labeling efficiency. One day rested mice (n=3) had K5+/GFP+ and K5+/GFP- cell populations underneath the columnar epithelium of the uterus (Figure 22C, left panel), indicating that K5+ cells in the uterus were successfully labeled with GFP, although not fully efficiently. This was expected since tamoxifen inducible CreER activity is highly dependent on the dosage of tamoxifen, duration of treatment, and tissue type. Then, I rested (n=6) mice for three weeks (Figure 22B) to trace fate of the GFP+ cells after estrous cycle triggered uterine epithelium regeneration. I found K5+/GFP+ and K5+/GFP- populations similarly with one day rested mice, as I expected. Interestingly, I also found a K5-/GFP+ columnar cell population (Figure 22C, right panel). These cells were usually present at the glandular epithelium, or at the glandular-luminal epithelium proximal regions. These findings support possible transdifferentiation of K5+/GFP+ uterine basal cells to K5-GFP+ columnar epithelium, suggesting that uterine basal cells may be involved in maintenance of uterine columnar epithelium.

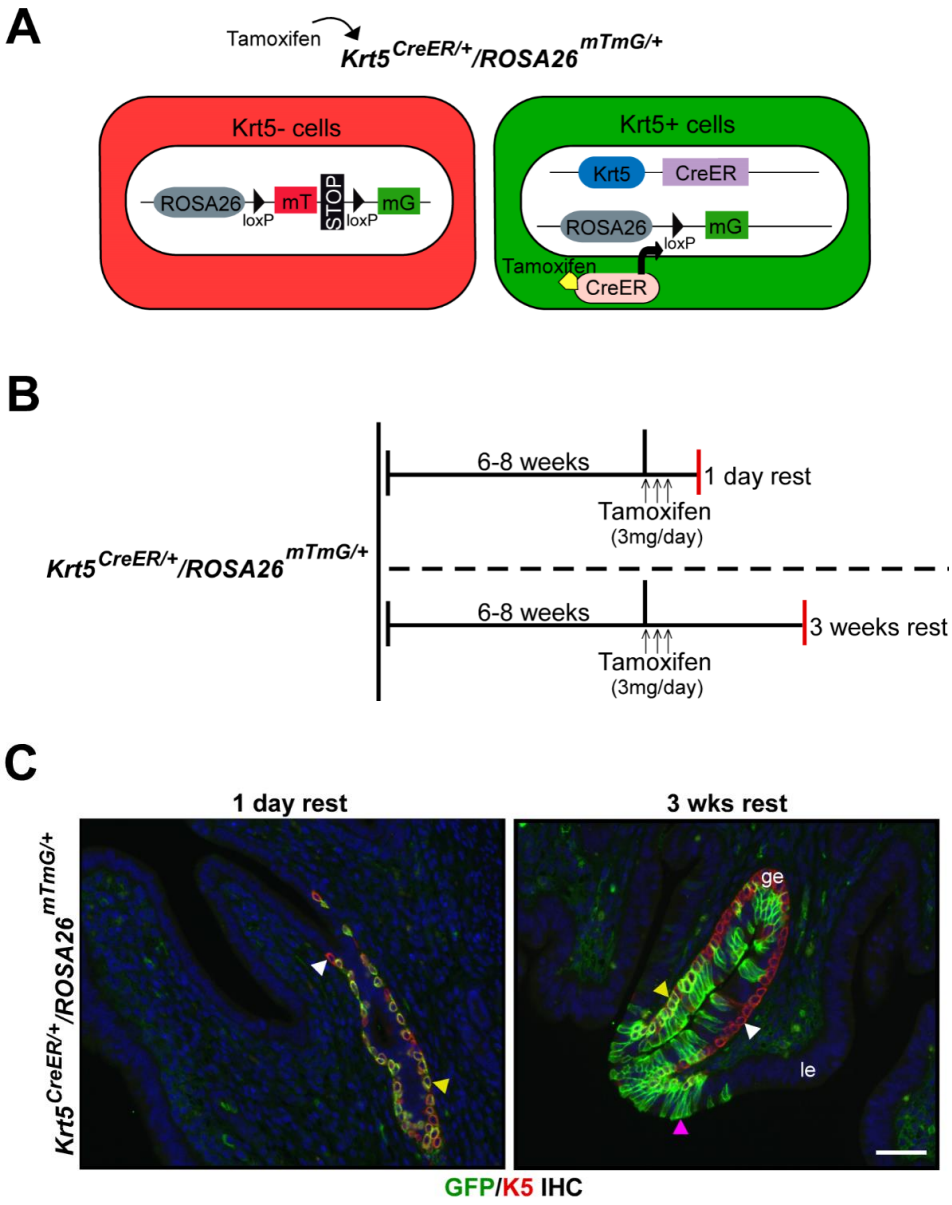


**Figure 22. Uterine basal cells might transdifferentiate into uterine columnar epithelium.** **(A)** Labeling K5 expressing cells using *Krt5*$^{CreER/+}$*/ROSA26*$^{mTmG/+}$ mice. CreER is expressed under Krt5 promoter and gets activated when bound with tamoxifen. It cleaves tdTomato (mT) and the following STOP codon which enables expression of GFP (mG). **(B)** Treatment regimen. Mice were given tamoxifen for three days (3mg/day) at 6-8 weeks of age and rested for one day and three weeks. (**C)** Uterine sections were stained for GFP (green), K5 (red) and nuclei (blue). White, yellow, and magenta arrows indicate K5+/GFP+, K5+/GFP-, and K5-/GFP+ cells, respectively. Dashed white line separates epithelium from the stroma. Ge: Glandular epithelium. Le: Luminal epithelium. Scale bars represent 50 µm.

### *Epithelial ERα-Deficient Mice Has Impaired Uterine Gland Formation*

Throughout my observations, I located uterine basal cells mostly underneath glandular epithelium. Uterine basal cells expand throughout the uterus when epithelial ERα is absent. I questioned if there was an abnormality in the maintenance of uterine glands in epithelial ERα deficient mice. In this regard, I analyzed uterine transverse tissue sections from nine weeks old *Wnt7a-Cre/Esr1*$^{f/f}$ and *NTG/Esr1*$^{f/f}$ mice (n=3 each) for gland formation. I did vaginal cytology to ensure that all the mice were in the same estrus stage. H&E staining showed that *Wnt7a-Cre/Esr1*$^{f/f}$ mice had lesser number of glands comparing to *NTG/Esr1*$^{f/f}$ mice (Figure 23A). Total number of glands in *Wnt7a-Cre/Esr1*$^{f/f}$ mice (18) was more than two-fold less than *NTG/Esr1*$^{f/f}$ mice (52) which was statistically significant (P=0.0247) (Figure 23B) indicating that epithelial ERα deficient mice has impaired uterine gland formation.

To find out if the already formed glands in *Wnt7a-Cre/Esr1*$^{f/f}$ mice have functional defects, I analyzed expression of gland-specific marker Agr2 in *Wnt7a-Cre/Esr1*$^{f/f}$ and *NTG/Esr1*$^{f/f}$ mice. Agr2 was absent in the glands of *Wnt-7aCre/Esr1*$^{f/f}$ mice (Figure 23C). There is no report on the connection between Agr2 and ERα in the uterus. My results support that epithelial ERα might be required for Agr2 expression in uterine glands. Further studies are required to confirm the connection between ERα and Agr2 in uterus.

FoxA2 is a transcription factor and a critical regulator of postnatal uterine gland differentiation in mice (Kelleher, 2017). It was expressed in both glandular and columnar epithelia of *Wnt7a-Cre/Esr1*$^{f/f}$ mice (Figure 23D). I interpreted this finding as impaired gland formation in epithelial ERα deficient mice could be via failure in FoxA2-induced formation of glands.

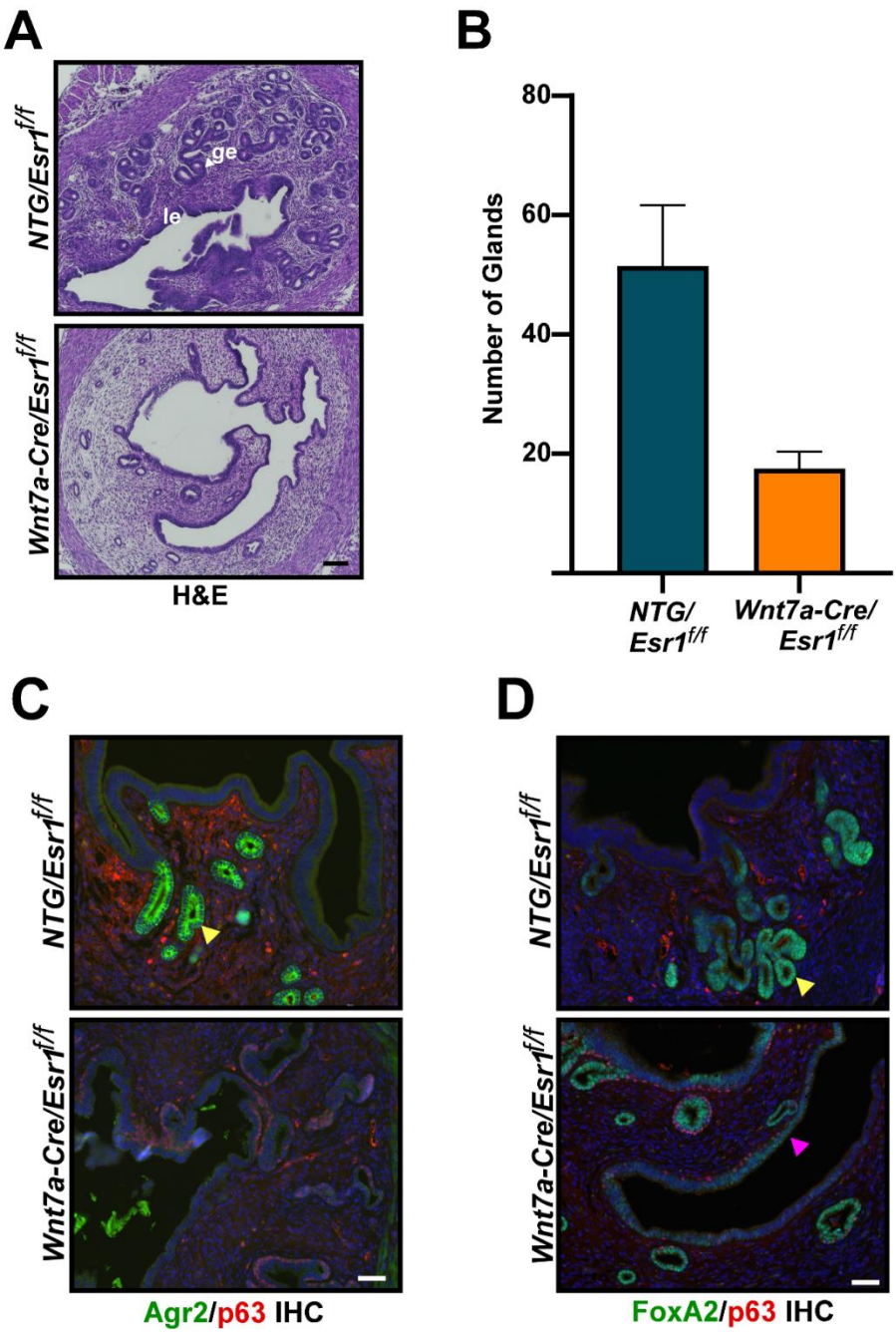


**Figure 23. Epithelial ERα-deficient mice have impaired uterine gland formation.**
Mice were harvested at nine weeks of age (n=3 per genotype) and transverse sections were made. **(A)** Representative H&E images. White arrow indicates glandular epithelium. Ge: Glandular epithelium. Le: Luminal epithelium. Scale bar represents 100 µm. **(B)** Number of glands in **(A)** were quantified and shown as mean ± S.E.M (n=3). $p < 0.05$ (One-sided Wilcoxon rank sum test). Sections were immunohistochemically stained for **(C)** Agr2 (green) and p63 (red) and **(D)** FoxA2 (green) and p63 (red). Yellow arrows indicate glandular epithelium. Magenta arrow indicates FoxA2+ cells in luminal epithelium. Scale bars represent 50 µm.

***Uterine basal cell markers are expressed in the human endometrium***

Presence of K14+/p63+ basal cells in the normal human endometrium has not been reported. To determine if uterine basal cells that I identified and characterized in mouse were relevant to human endometrium, I initially analyzed expression of uterine basal cell markers in human uterus at RNA level. I did reverse-transcriptase PCR of cDNA that I synthesized from human uterus total RNA and human adenocarcinoma HeLa cells for Krt14 and Trp63 genes. I have not detected Krt14 and Trp63 gene amplification in RNA from HeLa cells which are of columnar cell origin. However, both of these genes were expressed in human uterus (Figure 24), indicating that uterine basal cell markers are expressed in the human endometrium at RNA level.

To see if basal cell markers are present in human uterus at protein level, I determined for expression of mouse uterine basal cell marker K14 in a commercial human endometrial cancer tissue microarray (EMC1021) which had five normal tissues, and 97 endometrial carcinomas with different types. Of those, 88 were adenocarcinomas, four were adenosquamous carcinomas, and two were undifferentiated carcinomas from different patients (Figure 25A). Each tissue section (core) had a diameter of 1.5 mm and 4 μm thickness. I used K8 as columnar epithelial cell marker in the immunohistochemical staining. Adenosquamous carcinomas are known to carry both squamous and columnar cell phenotype and express markers of each cell type. K14 was expressed in those cancers (Figure 25B). None of the normal tissue and undifferentiated carcinoma cases had K14+ cells (Figure 25B). Uterine basal cells are very rare in *wild-type* adult mouse. Considering the fact that stained section was a very small portion of the total endometrium, this result was expected. On the other hand, of the total number of n=88 endometrial adenocarcinoma cases, 27 of the patients had K14+ cells (Figure 25B), which is more than 1/4 of cases, supporting that K14+ basal cells present in human uterus. I used a human endometrial cancer microarray containing grade I (n=16), II (n-23), and III (n=21) cancers as well as normal (n=5). The manufacturer provided H&E images of these samples (Figure 26A). I stained them for K14 and found that some adenocarcinoma and all adenosquamous carcinoma samples were positive for K14 (Figure 25B). It was

intriguing that the prevalence of K14+ tumors was higher in tumor grade III (57%) compared to grade I (13%) and grade II (22%) tumors (Figure 26). Taken together, I conclude that K14+ basal cells are present in the human endometrium.

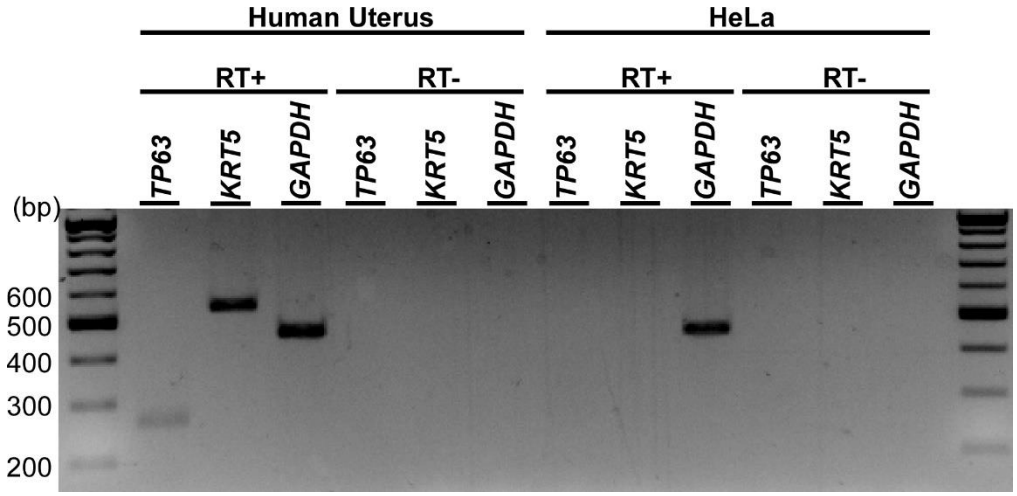


**Figure 24. Uterine basal cell markers are expressed in the human uterus at RNA level.** cDNA was synthesized from human uterus total RNA and HeLa cells and subjected to PCR with primers against *TP63*, *KRT5*, and *GAPDH* genes. 1.5% agarose gel. RT: Reverse transcriptase.

A

| Pathology Diagnosis | Number of Tissues |
|---|---|
| Normal | 5 |
| Undifferentiated carcinoma | 2 |
| Adenosquamous carcinoma | 4 |
| Adenocarcinoma | 88 |

B

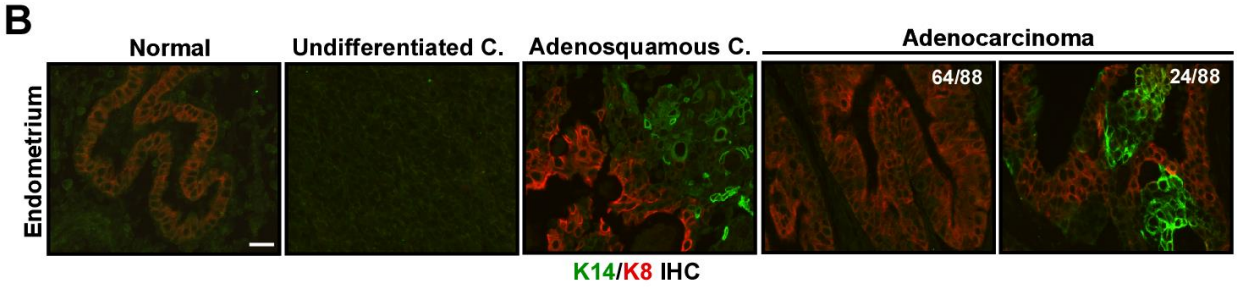


**Figure 25. Uterine basal cell markers are expressed in human endometrial adenocarcinomas.** **(A)** EMC1021 human endometrial cancer tissue microarray contains 97 cases of different types of endometrial carcinomas and 5 cases of normal tissue. **(B)** Human endometrial cancer tissue microarray was stained for K14 (green) and K8 (red). Data represents normal endometrium, undifferentiated carcinoma, adenosquamous carcinoma, K14-, and K14+ adenocarcinoma. Numbers on the top-right corner of adenocarcinoma panel indicate the number of K14-, or K14+ cancer cases per total number of endometrial adenocarcinomas. Scale bar represents 25 µm.

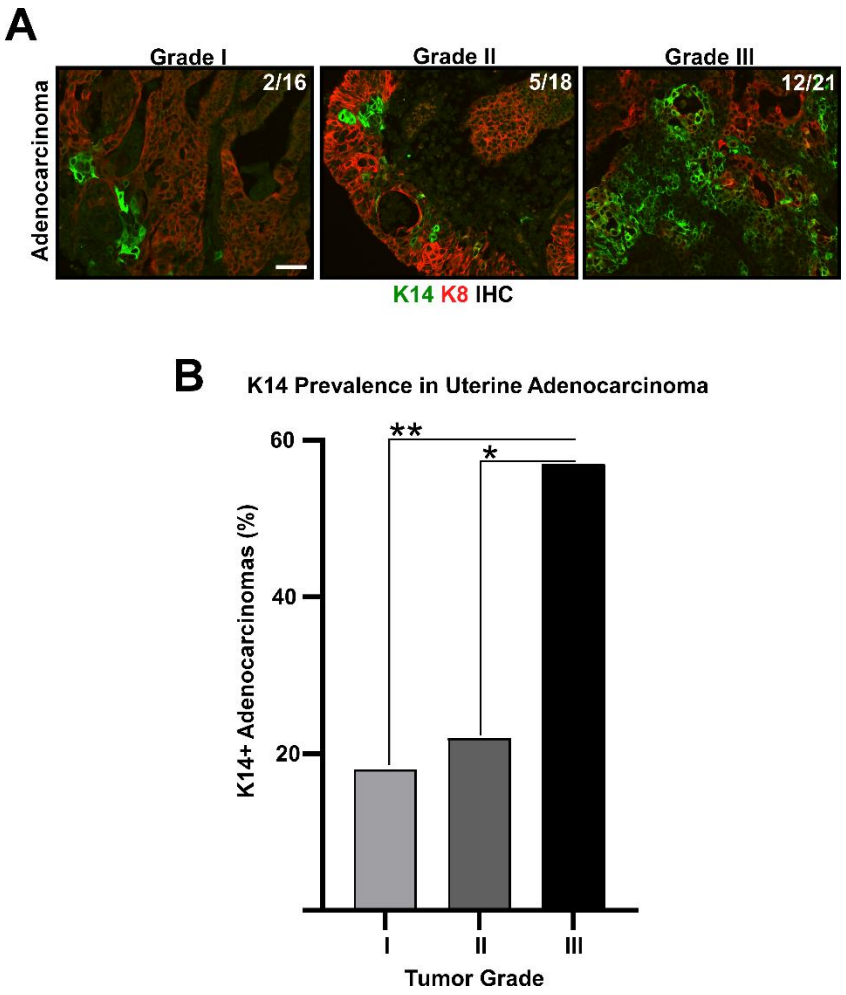


**Figure 26. K14+ phenotype is more prevalent as the tumor grade increases in human endometrial cancers.**
**(A)** Representative images of human endometrial adenocarcinoma cases from EMC1021 tissue microarray with tumor grades I, II, III; which were stained for K14 (green) and K8 (red). Scale bar represents 50 μm. **(B)** Graph shows percentage of K14+ endometrial adenocarcinomas from tumor grades I, II, and III. P-values are *: 0.037, and **: 0.006 (Fisher's exact test).

## C. Discussion

In this study, I identified a new cell population in the uterus (named as uterine basal cells), located underneath the columnar epithelium, that has unique epithelial protein expression profile comparing to squamous, and reserve cells of cervix and luminal, and glandular columnar cells of uterus. Uterine basal cells were present in the uterus of mice as young as postnatal day 14 (Figure 17), suggesting their function in the uterus. Uterine basal cells were mostly located underneath glandular epithelium. Cell lineage tracing experiments showed that uterine basal cells contribute to glandular columnar epithelium (Figure 22).

### *Mechanism of ERα in maintenance of uterine epithelium*

Uterine basal cells propagated throughout the uterus when epithelial ERα was absent. $E_2$ makes a difference in the frequency of these cells, even when epithelial ERα is absent, suggesting that ERα inhibits and stromal ERα promotes expansion of these cells (Figure 27). Uterine basal cells do not propagate the uterus in

*Esr1*$^{-/-}$ mice. These results suggest that balance between stromal and epithelial is crucial for maintenance of uterine basal cells (Figure 27). Epithelial ERα deficient mice had impaired gland formation supporting that ability of basal cells to become glandular epithelium was impacted when epithelial ERα was absent. My results suggest involvement or ERα in the maintenance of uterine epithelium.

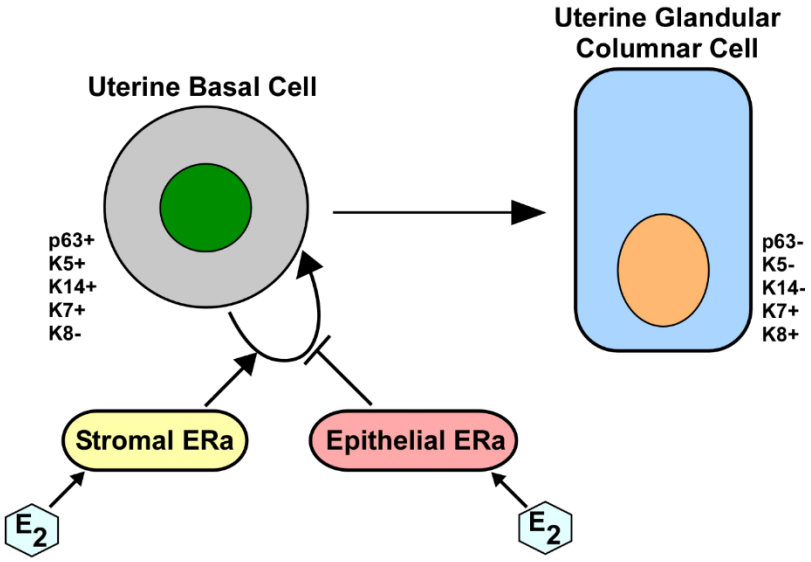


**Figure 27. Proposed model for the role of ERα on uterine epithelial maintenance.**

***Selective Deletion of Epithelial ERα in the Uterus at Adult Age***

Cre-mediated deletion of *Esr1* at adult age by *Ltf-iCre* mice was incomplete in the uterus (Figure 19A). One interpretation is that *Ltf* is not expressed in all epithelial cells in the uterus and accounts for partial deletion of *Esr1* by cre. However, *Ltf-iCre*/*Rosa26R* reporter mice (Daikoku, 2014) has LacZ expression in the entire luminal and glandular epithelium, demonstrating that *Ltf-iCre* is expressed throughout the uterus. *Ltf* is an estrogen inducible gene with fluctuating expression levels during estrous cycle. It is expressed at the highest level at proestrus stage when estrogen levels increase (Teng et al, 2002). One possibility is that not all epithelial cells will have ERα deletion at the same time. When estrogen levels reduced and *Ltf-iCre* had reduced activity, ERα+ cells were selected over ERα- cells in the uterus of *Ltf-iCre/Esr1*$^{f/f}$ mice. Partial Esr1 deletion might mean that epithelial ERα is preferred for uterine epithelial maintenance.

Although *Esr1* deletion was partial, uterine basal cells expanded underneath the columnar epithelium of *Ltf-iCre/Esr1*$^{f/f}$ mice (Figure 19C). Moreover, uterine basal cells expanded nearby, or underneath the ERα- columnar epithelium (Figure 19C). These finding suggests that epithelial ERα is required for maintenance of uterine epithelium at adult age.

***Potential Mechanisms of Uterine Basal Cell Maintenance***

Abnormal expansion of K14+/p63+ cells in the uterine epithelium has been previously reported to be correlated with abnormalities in expression of *Sox17, Wnt7a, Beta-catenin, Gata2, Wnt4,* and *Smo* genes in mice. The severity of the squamous metaplasia ranged from single layered to stratified K14+/p63+ cells. Ablation of Sox17 from uterine epithelium leads to expansion of one layered K14+/p63+ cells, similar to epithelial ERα deficient mice (Wang et al, Nature, 2018). Sox17 regulates IHH signaling pathway to govern uterine epithelial–stromal interactions during the window of receptivity (Wang et al, Nature, 2018). *Wnt-7a*$^{-/-}$ mice have fully differentiated squamous epithelium in the uterus and lacks uterine glands (Miller, 1998). *Wnt-7a*$^{-/-}$ mice have a postnatal loss of *Hoxa* genes which are important for anteroposterior

patterning in the female reproductive tract during development (glands (Miller, 1998). Loss of *Wnt-7a* may lead to vaginal-like fate in the uterus, possibly via expansion and stratification of uterine basal cells.

Uterine epithelium specific ablation of Wnt4 and Gata2 genes in $PR^{Cre}/Wnt4^{f/f}$ and $PR^{Cre}/Gata2^{f/f}$ mice leads to squamous cell metaplasia, which becomes severe with $E_2$ treatment, suggesting that *Wnt4 and Gata2* regulates epithelial differentiation in response to $E_2$ (Franco et al, 2010, Rubel et al, Cell Reports, 2016). $PR^{Cre}$/ $Ctnnb1^{f/f}$ mice which lacks *Beta-catenin* expression in the uterine epithelium also had a squamous cell metaplasia in the uterus (Jeong et al., 2009). Constitutive activation of *Smo* gene in the uterine epithelium leads to expansion of K14+/p63+ cells and reduction in the number of glands (Franco et al., 2010).

Abnormalities related to expression of these genes, leads to expansion of K14+/p63+ cells, in the uterus, either as a single, or stratified layer, suggesting that proper expression of these genes is important for regulation of uterine epithelial differentiation and maintenance. I speculate that expansion, and stratification of the uterine basal cells is modulated by a complex mechanism which involves these genes, together with ERα. More study is needed to find the correlation between ERα and these genes in the uterine basal cell maintenance.

***Maintenance of Uterine Glandular Epithelium***

Uterine glands are important for establishment of pregnancy in mice (Kelleher, 2018). FoxA2 is a regulator of uterine gland development in mice. Ablation of FoxA2 expression in the uterus leads to failure in gland formation in $Pg^{rCre/+}/FoxA2^{f/f}$ mice (Jeong et al, 2010). These mice are also infertile, due to loss of leukemia inhibitor factor (*Lif*) and implantation failure. Epithelial ERα deficient mice had impaired gland formation and abnormal FoxA2 expression at the luminal epithelium (Figure 23D). Gland formation is proposed to branch at the luminal-glandular neck regions (Jin, 2019). I speculate that FoxA2-induced gland genesis fails when epithelial ERα is absent.

Anterior gradient 2 (AGR2) is a member of protein disulfide isomerase (PDI) family (Persson et al., 2005), and reported to be involved in synthesis and secretion of membrane-bound mucin 2 (MUC2) protein (Park et al., 2009), particularly in intestines and other mucus secreting organs. AGR2 was first discovered in ERα+ breast cancer cells (Thompson et al., 1998). It was co-expressed with ERα in MCF-7 breast cancer cells and was absent or minimally expressed in ERα negative MDA-MB-231 breast cancer cells. It was later shown that AGR2 is upregulated by estrogen treatment in human breast tissue which was transplanted to athymic nude mice (Wilson et al., 2006). AGR2 is a marker for SCJ in human cervix (Herfs et al., 2012), It was shown to be expressed in the adenocarcinomas and squamous cell carcinomas originated from SCJ but absent in ectocervical CIN regions. I have detected its expression specifically in uterine glands but not SCJ in mice, suggesting that Agr2 is not a marker for murine SCJ. AGR2 target protein MUC2 is present in the endometrium and its levels increase in secretory phase of menstrual cycle. (Alameda et al., 2007), suggesting that AGR2 might be involved in glandular epithelium function. Agr2 was not detected at protein level in the uterine glands of epithelial ERα deficient mice (Figure 23C) and ERα knockout mice (data not shown) demonstrating that ERα signaling is required for expression of Agr2 in the uterus. Higher AGR2 expression was found in endometrial cancers that are developed in women treated with tamoxifen comparing to the ones who were not exposed to tamoxifen (Hrstka et al., 2017). In vitro analyzes showed the effect of AGR2 on cell proliferation (Hrstka et al., 2017). AGR2 is proposed as a biomarker for cancer detection (Kamal, 2018). It is worth to look into the mechanism of connection between AGR2 and ERα signaling in the uterus.

## D. Future Directions

Epithelial ERα deficient mice had impaired gland formation which might be due to failure in cell-fate determination in the process of uterine glandular genesis. I showed that uterine basal cells contribute to glandular epithelium. Abnormal expansion of uterine basal cells when epithelial ERα is ablated, suggests that epithelial ERα is involved in gland epithelium maintenance by uterine basal cells. To seek the correlation, I am generating *Krt5*$^{CreER/+}$*/ROSA26*$^{mTmG/+}$*/Esr1*$^{f/f}$ mice to trace ERα ablated and GFP labeled uterine basal cells and evaluate the efficiency of gland formation in the absence of ERα expression.

Ablation of ERα from the epithelium in mice led to expansion of uterine basal cells in mice. Based on that finding, I speculate that there might be a correlation between K14+ phenotype and ERα loss from the epithelium in human endometrial cancers. To find that out, I will analyze more endometrial cancer tissue microarrays for ERα and K14 expression.

Various genes have been implicated in squamous metaplasia in the uterus. I suggest that the origin of metaplasia is uterine basal cells. Currently, I am looking for the expression of Sox17, Gata2, Wnt4, and Wnt7a in the uterus of epithelial ERα deficient mice, comparing to *wild-type* mice to see the correlation of them with ERα.

## VI. SUMMARY AND SIGNIFICANCE

Problems in the uterine epithelial maintenance lead to various complications, such as infertility, miscarriage, prematurity, endometrial cancer, and cervical cancer. Endometrial and cervical cancers are the leading causes of cancer related death among women worldwide. Understanding the mechanisms of uterine epithelial maintenance is important to prevent or treatment of these pathologies.

Transformation zone of cervix which comprises squamocolumnar junction (SCJ) is where most of the cervical cancers originate from. Location of SCJ changes depending on several factors, including ovarian hormones. In K14E7 mice expressing HPV16 E7 oncogene, SCJ shifts towards the uterine epithelium in an estrogen/ERα–dependent manner, supporting that ERα is involved in the maintenance of SCJ. In this dissertation, I have used in vivo mouse models to demonstrate that ERα signaling is involved in SCJ maintenance. Formation of SCJ was delayed and its maintenance at adult age was disrupted in epithelial ERα deficient mice, which was not the case in ERα knockout mice, suggesting that the balance between epithelial and stromal ERα signaling is crucial for the development and maintenance of cervical SCJ. I suggest that stromal ERα is inhibitive, and epithelial ERα is promotive for the maintenance of squamous epithelium. Understanding the mechanism of estrogen-ERα signaling in the SCJ maintenance will give new insights into cervical carcinogenesis and enable new therapeutic approaches.

In this dissertation, I also identified a unique cell type (named as uterine basal cells) in the uterus underneath the columnar epithelium. My results suggest that estrogen-ERα signaling is involved in maintenance of these cells. I suggest that ERα inhibits and stromal ERα promotes proliferation uterine basal these cells. Cell lineage tracing experiments suggest that uterine basal cells contribute to glandular epithelial maintenance. Epithelial ERα deficient mice have expansion of these cells and impaired gland formation, suggesting that ERα is involved in uterine glandular genesis, via uterine basal cells.

This is the first study to find the role of ERα in maintenance of SCJ. Estrogen/ERα promote squamous differentiation in the cervix. An imbalance between stromal and epithelial ERα leads to impaired SCJ maintenance. Problems in SCJ maintenance may be linked to increased risk of cervical cancer. This is also the first study reporting uterine basal cells which are involved in columnar epithelial maintenance. I also found that uterine basal cell markers are expressed in 27% of human endometrial adenocarcinomas, supporting their presence in human endometrium. In addition, my results imply a correlation of basal cell marker expression with the worse disease phenotype. Understanding the mechanisms of uterine epithelial maintenance by uterine basal cells will give more insights into uterine epithelium related diseases, including infertility and endometrial cancer.

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