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Studies on β-catenin Signaling in Adenomyosis Development through Epithelial-Mesenchymal Transition

β-catenin Signaling과 Epithelial-Mesenchymal Transition 현상이 Adenomyosis 발병에 미치는 연구

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ABSTRACT

Adenomyosis is a common gynecological disorder defined by the presence of endometrial glands and stromal within the myometrium. Despite its frequent occurrence, the precise etiology and physiopathology of adenomyosis is still unknown. WNT/β-catenin signaling molecules are important and should be tightly regulated for uterine function. Abnormal activation of β-catenin signaling by mutations in CTNNB1 is a causative factor of endometrial cancer. To investigate the role of β-catenin signaling in adenomyosis, the expression of β-catenin was examined in human adenomyosis by immunohistochemical analysis. The expression of nuclear and cytoplasmic β-catenin was significantly higher in epithelial cells of human adenomyosis compared to control endometrium. To determine if constitutive activation of β-catenin in the murine uterus leads to development of adenomyosis, mice were used which expressed a dominant stabilized β-catenin in the uterus by crossing the PR-Cre mouse with *Ctnnb1*$^{f(ex3)/+}$ mice. Uteri of *PR*$^{cre/+}$ *Ctnnb1*$^{f(ex3)/+}$ mice display an abnormal irregular structure and highly active proliferation in the myometrium, and subsequently developed adenomyosis. Interestingly, the expression of E-cadherin was repressed in epithelial cells of *PR*$^{cre/+}$ *Ctnnb1*$^{f(ex3)/+}$ mice compared to control mice. Repression of E-cadherin is one of the hallmarks of epithelial mesenchymal transition (EMT). The expression of SNAIL and ZEB1, which are transcriptional repressors of E-cadherin and an EMT marker, was observed in some epithelial cells of the uterusin *PR*$^{cre/+}$ *Ctnnb1*$^{f(ex3)/+}$ mice but not in control mice. Vimentin and COUP-TFII, mesenchymal cell markers, was expressed in some epithelial cells of *PR*$^{cre/+}$ *Ctnnb1*$^{f(ex3)/+}$ mice. In human adenomyosis, the expression of E-cadherin was decreased in epithelial cells compared to control endometrium, while CD10, endometrial stromal marker, was expressed in some epithelial cells of human adenomyosis. These results suggest that abnormal activation of β-catenin

contributes to adenomyosis development through the induction of EMT.

Keywords: β-catenin, Adenomyosis, Epithelial-mesenchymal transition, Uterus

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LIST OF ABBREVIATIONS

APC : adenomatosis polyposis coli

AVE : average

CK1 : caein kinase I

COUP-TFII : chicken ovalbumin upstream promoter II

Cre : cyclization recombination

CT : computered tomography

Dsh : dishelved

DMEM : Dulbecco’s modified Eagle’s medium

EGF : epidermal growth factor

ER : estrogen receptor

E2 : estrogen

EMT : epithelial-mesenchymal transition

FBS : fetal bovine serum

FGF : fibroblast growth factor

FSH : follicle-stimulating hormone

GFP : green fluorescence protein

GSK3β : glycogen synthase kinase 3 beta

HEC-1A : human endometrial epithelial cells

HGF : hepatocyte growth factor

HP : hyperprolactinemia

IF : immunofluorescence

IHC : immunohistochemistry

LEF : lymphocyte Enhancer binding Factor

LH : luteinizing hormone

loxP : locus of X-over P1

LRP : low density lipoprotein receptor

MET : mesenchymal to epithelial transition

MRI : magnetic resonance imaging

PLAP : placenta-like alkaline phosphatase

PR : progesterone receptor

P4 : progesterone

SE : standard error

Tcf : T-cell factor

TGF-β : transforming growth factor-β

WIF-1 : WNT inhibitory factor 1

WNT : wingless/integrated

α-SMA : α-smooth muscle actin

CHAPTER 1.

General introduction and literature review

1. General introduction

Adenomyosis is a common benign gynecological disorder and defined by the presence of endometrial glands and stromal deep within the myometrium associated with adjacent myometrial hypertrophy and hyperplasia (Ferenczy, 1998; Tamai et al., 2005; Wang et al., 2009a). Adenomyosis occurs when the normal boundary between the endometrial basal layer and the myometrium is disrupted (Uduwela et al., 2000; Vercellini et al., 2006; Brosens et al., 2012; Koike et al., 2012). Invasion of the basal endometrium leads to dysfunctional myometrial hyperperistalsis, increased intrauterine pressure, and impairment of the proper uterine function (Kunz et al., 2005). This condition can interfere with the normal implantation and can cause subfertility (de Souza et al., 1995; Devlieger et al., 2003; Matalliotakis et al., 2005; Campo et al., 2012; Louis et al., 2012). Adenomyosis is frequently diagnosed in infertility patients (de Souza et al., 1995; Salim et al., 2012). Prevalence of adenomyosis was 10 % to 66 % in women (Vercellini et al., 2006). Symptoms include menorrhagia, dyspareunia, dyschezia, dysmenorrhea and chronic pelvic pain (Levgur et al., 2000). The spread of adenomyosis also correlates significantly with pelvic pain (Sammour et al., 2002). However, early diagnosis of adenomyosis is difficult because of the absence of pathogenic symptoms and biomarkers. Therefore, most women are not diagnosed until later stages and severely symptomatic women who do not respond to pharmacological therapy require invasive surgical intervention which involves a hysterectomy. Despite its prevalence, the precise etiology and pathophysiology of adenomyosis is poorly understood.

Endometrial hyperplasia is observed more frequently among cases with

adenomyosis (Parazzini et al., 1997). Adenomyosis has been suggested to be an ovarian steroid hormone-dependent disorder. High concentrations of estrogen without appropriate protection from progesterone have been linked to endometrial cancer, endometrial hyperplasia and adenomyosis (Schindler, 2009). When the relationship between adenomyosis and endometrial carcinoma was evaluated in hysterectomy specimens, this disease was positively associated with endometrial carcinomas in 17 % of cases (Fukuchi et al., 1998; Kobayashi et al., 1999; Ikeda et al., 2000; Saegusa et al., 2001; Saegusa and Okayasu, 2001; Kazandi et al., 2010; Schindler, 2010). Interestingly, the β-catenin signaling was activated in endometrial cancer and induced by high levels of estrogen (Amant et al., 2005; Schindler, 2009).

β-catenin (*CTNNB1*) functions in a dual manner in epithelial cells, depending on its intracellular localization. At the plasma membrane, β-catenin is an essential component of the E-cadherin-catenin unit, and is important for cell differentiation and the maintenance of normal tissue architecture. β-catenin can also act as the main effector of canonical WNT signaling in the nucleus, which is critically involved in tissue differentiation during embryonic development. In the absence of WNT signals in normal cells, β-catenin forms a complex with glycogen synthase kinase 3β (GSK3β) and adenomatous polyposis coli (APC). GSK3β phosphorylates β-catenin which marks ubiquitin-dependent degradation by the proteasome, thereby maintaining low levels of free cytoplasmic β-catenin (Aberle et al., 1997). Whereas, mutations in exon 3 of β-catenin result in protein stabilization, cytoplasmic and nuclear accumulation, and participation in signal transcriptional activation through DNA binding (Palacios and Gamallo, 1998). In adenocarcinomas, elevated β-catenin levels caused by mutations in CTNNB1 or APC, result in the activation of the WNT/β-catenin pathway. Recent evidence

suggests that estrogen-dependent proliferation depends on activated WNT signaling since it was shown to induce WNT pathway components in the endometrium of estrogen-treated women. Additional facts also show that up to 30% of estrogen-associated cancers exhibit nuclear β-catenin expression, the hallmark of canonical WNT signaling (Moreno-Bueno et al., 2002; Cloke et al., 2008). Mutations of WNT/β-catenin pathway members result in aberrant activation of the target genes, including those encoding for activators of epithelial-mesenchymal transition (EMT) (Schmalhofer et al., 2009).

EMT is a programmed development of biological cells characterized by the loss of cell adhesion, a repression of E-cadherin expression, and an increase in cell mobility (Polyak and Weinberg, 2009). EMT endows cells with migratory and invasive properties and can be induced by estrogen. One of the signals initiating an EMT is the canonical WNT pathway whose stimulation triggers the translocation of the β-catenin to the nucleus (Clevers, 2006). A key initial step of EMT is the downregulation of E-cadherin, which is repressed by several factors; namely, ZEB1, ZEB2, SNAI1, SNAI2, Twist1, Twist2, and E12/E47 (Polyak and Weinberg, 2009). In inducing EMT, ZEB1 not only transcriptionally represses epithelial markers like E-cadherin but also activates mesenchymal genes (Sanchez-Tillo et al.). Nonetheless, these phenomena are poorly understood in reproductive biology. Hence, understanding the molecular mechanisms whereby activation of β-catenin induces EMT and contributes to the development of adenomyosis will provide insight into the physiology and pathophysiology of this disease.

In a previous study, we have generated a dominant stabilized β-catenin in the murine uterus ($PR^{cre/+}$ $Ctnnb1^{f(ex3)/+}$) which results in infertility, hormone

insensitivity, and endometrial glandular hyperplasia (Jeong et al., 2009). These analyses demonstrated that β-catenin has important roles in normal uterine function as well as in tumorigenesis. In this study, we utilized the conditionally stabilized β-catenin mouse model and human adenomyosis samples to demonstrate that β-catenin is an important molecule in the etiology and pathology of adenomyosis.

2. Literature review

1) Adenomyosis

(1) Definition and prevalence

Adenomyosis is a common benign gynecological disorder and is defined by the presence of endometrial glands and stroma deep within the myometrium (Ferenczy, 1998; Tamai et al., 2005). The name itself has Latin roots: adeno- refers to glands, myo- refers to muscle, and –osis refers to an abnormal condition (He et al., 2011). The disorder involves ectopic invasion of endometrial glands and stromal tissue, inducing a local inflammatory response that leads to hypertrophy and hyperplasia of the surrounding muscle fibers (Fernandes et al., 2008; Kim et al., 2010). The prevalence of adenomyosis ranges from 1% to 70% depending on the location and criteria used for diagnosis. The reported prevalence of histologically-confirmed adenomyosis in hysterectomies varies from 14% to as much as 66% (Vercellini et al., 2006). There are also considerable variations across geographic areas and ethnic groups (Vercellini et al., 2006).

(2) Etiology and symptoms

Although adenomyosis is a common disorder worldwide, its precise etiology remains unknown. A number of hypothesized models for the development of adenomyosis have been proposed, that include factors such as age, pregnancy-related events, and hormone disorders. Age is suggested to be an important contributor; adenomyosis is most often detected in women 30 to 50 years of age.

Menstrual and reproductive factors are also likely to be strongly associated with adenomyosis, while elevated levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) as well as a history of depression are also suspected. The hormonal changes that occur during pregnancy may increase the risk of ectopic endometrium development within the myometrium (Vercellini et al., 2006). High concentrations of estrogens without appropriate regulatory influences from progesterone have also been linked to endometrial hyperplasia, and there a positive association has been identified between endometrial hyperplasia and adenomyosis. Other risk factors include tissue injury and repair. Adenomyosis is observed in chronic myometrial peristalsis and hyperperistalsis due to microscopic trauma at the boundary between the endometrium and myometrium. During these processes, the levels of local estrogen increase, further promoting chronic inflammation and the growth of adenomyotic implants due to the process of tissue injury and repair.

Common symptoms of adenomyosis include menorrhagia, dyspareunia, dyschezia, dysmenorrhea and chronic pelvic pain. Adenomyosis has been observed in 35 to 55% of women with fibroids, and causes uterine enlargement. However, uterine enlargement may be a trivial factor in the absence of fibroids. More importantly, endometriosis and endometrial cancer are often co-observed with adenomyosis (Hayata et al., 1994; Hanley et al., 2010; Kazandi et al., 2010; Schindler, 2010). The condition can interfere with normal implantation and can be a cause of subfertility, and as a result, adenomyosis is more frequently diagnosed in infertility patients (de Souza et al., 1995).

(3) Diagnosis

Computed tomography (CT) is most commonly used for diagnosing adenomyosis because it allows precise differentiation between adenomyosis, normal myometrium and fibroids (Williams et al., 2007). However, some limitations are present. Ultrasound and magnetic resonance imaging (MRI) have also been frequently used to diagnose adenomyosis. Common ultrasound imaging techniques in women with adenomyosis can identify uterine enlargement, an irregular myometrium with poorly defined cystic areas, striations and an ill-defined endometrial-myomterial border (Amant et al., 2005; Williams et al., 2007; Fernandes et al., 2008; Williams et al., 2008). Transvaginal ultrasound is more accurate than common ultrasound because the restricted resolution of the abdominal approach gives rise to difficulties in distinguishing focal adenomyosis from fibroids and realizing other subtle features of adenomyosis (Williams et al., 2007; Basak and Saha, 2009). In high resolution imaging techniques for diagnosing adenomyosis, magnetic resonance imaging (MRI) has several advantages including outstanding resolution, high sensitivity and specificity that permits identification of invasiveness in soft tissue (Basak and Saha, 2009). Although there are several drawbacks in using MRI inlcuding expense and a lack of availability (Basak and Saha, 2009), clinicians generally prefer MRI due to its sensitivity and specificity.

In addition to imaging techniques, laboratory testing, biopsies, and laparoscopy have been recommended for diagnosing adenomyosis. In laboratory testing, CA-125 is commonly used as an adenomyosis marker. Women with adenomyosis have significantly higher CA-125 levels than women with fibroids or healthy control subjects. However, high CA-125 levels can also be due to related gynecologic diseases including endometriosis, ovarian mass, fibroids, ectopic pregnancy, and ovarian hyperstimulation. Some doctors have attempted to diagnose

adenomyosis though myometrial biopsy, because histopathology has been a mainstay criteria for adenomyosis diagnosis (Levgur et al., 2000). However, the biopsy approach has been limited due to several problems, including the difficulty in selecting an optimal number of biopsies and sampling devices, as well as physician inexperience.

(4) Treatment

Hormonal and non-hormonal treatments can help to relieve the symptoms of adenomyosis. Hormonal medications include combined oral contraceptives, various progesterone medications, and gonadotropin-releasing hormone agonists. Gonadotropin-releasing hormone agonists such as leuprolide have also been used for women with adenomyosis. These medications act to prevent adenomyosis by reducing estrogen levels, effectively reducing the adenomyotic area, and inducing menopause. These medications are effective during treatment, but the symptoms quickly return after cessation. Moreover, such medications induce side effects including hot flashes, vulvovaginal atrophy, mood changes, and lowered bone density. Consequently, gonadotropin-releasing hormone agonists are used for short periods, usually no more than six months (Farquhar and Brosens, 2006).

Non-hormonal medications include non-steroidal anti-inflammatory drugs (NSAIDs) as well as newer therapies such as tranexamic acid and valproic acid which are available to treat menorrhagia and dysmenorrhea as long-standing therapies. Tranexamic acid is useful for the treatment of menorrhagia in women who wish to avoid hormone therapy or who are not unsuitable for it. However, tranexamic acid also causes adverse effects such as headaches, sinus and nasal

discomfort, back pain, nausea, vomiting, diarrhea, disturbances in color vision, musculoskeletal pain, and fatigue (Lethaby et al., 2007; Philipp, 2011). Valproic acid, a histone deacetylase inhibitor that has been applied to care epilepsy and biopolar disorder, is another alternative that can relieve adenomyosis symptoms.

If hormonal and non-hormonal treatments are ineffective in treating adenomyosis, hysterectomy may be an appropriate choice, which is regarded as the most definitive treatment (Farquhar and Brosens, 2006). The decision to apply a surgical approach to treatment relies on factors such including the woman's age; future fertility desires; the size, site, and extent of the adenomyotic regions; and the surgeon's preferences and skills (Farquhar and Brosens, 2006). Surgical options include hysterectomy, uterine artery embolization, excision of focal adenomyotic regions, and endometrial removal. Hysterectomy has long been used to permanently treat adenomyosis. In addition, it is the only definitive way to diagnose adenomyosis, but also renders the patient infertile. The approach is often best for women with severe invasiveness in more than one-third of the myometrium (Levgur, 2007). Endometrial ablation has also been considered as a treatment for adenomyosis in superficial regions.

(5) Animal models

Mouse models

For the study of adenomyosis, experimental animals have long been employed. The mouse model is a common and convenient experimental model, and is easy to manipulate with hormonal alterations or pituitary grafts. Hormonal imbalances are known to enhance the development of adenomyosis.

Hyperprolactinemia (HP) is the presence of abnormally high levels of prolactin in the blood and prolonged treatment with estrogen administration has been associated with adenomyosis (Lipschutz et al., 1967; Ostrander et al., 1985). Unfortunately, the precise hormonal conditions present in many of these experiments have remained unclear. Mori et al., previously performed intrauterine implantation of isographs of anterior pituitary glands in mice to identify the pathogenesis of adenomyosis (Mori and Nagasawa, 1983; Matsuda et al., 2001). They used SHN or SLN strainz for performing isologous graft in the anterior pituitary glands in the lumen of the right anterior uterine horn at seven weeks. At various time points, mice were sacrificed after implantation. Adenomyosis was widely observed implantation 90 days after implantation. It was concluded that the prolactin induced adenomyosis and could be prevented by bromocriptine (a suppressor of pituitary prolactin), or the presence of increased prolactin receptor mRNA in the affected uterus (Mori et al., 1991; Yamashita et al., 1999).

Using the same model, other groups have shown that increased matrix metalloproteinase mRNA in uterine tissues are observed in mainly luminal, glandular epithelial and myometrial cells (Yamashita et al., 1999). Moreover, it was also observed that apoptosis increased in the myometrial cells of mice with adenomyosis. In this model system, probucol, a cholesterol-lowering drug, was shown to be effective in inhibiting the development of adenomyosis (Zhou et al., 2004). Moreover, a number of experiments involving treatment with various estrogenic agents have reported the induction of adenomyosis (Guttner, 1980). However, estrogenic effects are often complicated by the presence of other uterine diseases, such as endometrial hyperplasia, squamous metaplasia and uterine carcinomas. Lipschutz et al., showed that a distinct and dose-dependent increase in

adenomyosis occurred when BALB/c mice were exposed to progesterone over 12 or 18 months (Lipschutz et al., 1967). The synthetic progestins, norethindrone and norethynodrel also induce adenomyosis, although a lesser extent than progesterone. Ostrander et al., determined that adenomyosis developed after prolonged progesterone treatment of both controls and mice treated with diethylstilboestrol during the neonatal period (Ostrander et al., 1985). Recently, oral administration of tamoxifen and toremifene during the neonatal period has been found to give rise to the development of adenomyosis in adults. These studies have revealed critical details regarding the etiology of adenomyosis involving hormonal perturbations in mice.

Rat models

Some researchers prefer rat models over mice for the study of adenomyosis, suggesting hormonal perturbations are the central pathophysiology. Rats also develop adenomyosis after pituitary grafting (Mori et al., 1998) similar to mouse models. Treatment with fluoxetine, a serotonin reuptake inhibitor, has been found to induce adenomyosis along with high circulating levels of prolactin.

Rabbit models

The rabbit also has been used for studying adenomyosis because the condition spontaneously develops and its prevalence can be increased by estrogen treatment over one or two years (Meissner et al., 1957). However, adenomyosis development following estrogen administration is not ideal for the study of adenomyosis as with mice. Furthermore, it is frequently complicated by the associated development of endometrial hyperplasia and endometrial carcinoma.

2) WNT/β-catenin signaling pathway

WNT signaling represents an important signaling pathway in development and disease (Clevers, 2006). At present, 20 WNT proteins have been identified that bind to cell surface receptors of the Frizzled family (Clevers, 2006), and three different pathways can be activated: the canonical WNT/13-catenin signaling pathway (Clevers, 2006), the non-canonical WNT/Planar cell polarity pathway (D'Onofrio et al., 2006) and the non-canonical WNT/Ca^{2+} pathway (Kukla et al., 2006). The canonical WNT/13-catenin signaling pathway is associated with female reproductive function.

(1) Canonical WNT/β-catenin signaling pathways

Central to activated canonical WNT/13-catenin signaling is the nuclear accumulation of 13-catenin. Binding of the WNT ligand to the Frizzled receptor involves members of the LRP family (Miele et al., 2006b). As a result , via an interaction with a protein called Dishevelled, as well as the degradation complex consisting of the scaffold proteins AXIN1 and AXIN2 (conductin), the tumour suppressor APC (adenomatosis polyposis coli) and the Ser-Thr kinases CK1 (caein kinase I) and GSK313 (glycogen synthase kinase 3 beta) it dissociates from 13-catenin (CTNNB1) and 13-catenin is therefore no longer targeted for degradation (Curry et al., 2006).

The WNT pathway consists of more than 30 extracellular WNT-ligands, which interact with receptors of the frizzled family (Logan and Nusse, 2004). Disheveled (Dsh) inhibits the Glycogen-Activated Kinase-3 (GSK3), which, in the

absence of WNT signaling phosphorylates and targets the 14 -catenin-Adenomatous Polyposis Coli (APC) complex for ubiquitination and proteolytic degradation. Upon WNT signaling activation, 14-catenin is stabilized, accumulates in the cytoplasm and translocates to the nucleus, where it interacts with DNA-binding proteins of the T-cell factor/lymphocyte enhancer binding factor (TCF/LEF) family. In the presence of 14-catenin, TCF/LEF acts as a transcriptional activator of proliferation stimulating genes such as c-myc and cyclin D1 (Tetsu and McCormick, 1999).

WNT receptors

Frizzled (Fz) seven-pass transmembrane receptors and LRP5/6 are critical for WNT/β-catenin signaling. The mammalian genome contains 10 Fz genes, most of which have variable capacities to activate β-catenin signaling when co-overexpressed with WNT and LRP5/6 (Binnerts et al., 2007). Specific WNT family members can activate β-catenin or non-canonical pathways depending on their receptor complement (van Amerongen et al., 2008). Fz function is related to both β-catenin and noncanonical pathways. The Fz-LRP5/6 co-receptor is essential for the activation of WNT/β-catenin signaling (He, 2004). However, some reports suggest that LRP6 antagonizes non-canonical WNT/β-catenin signaling, via competition for WNT ligands or an unknown mechanism (Tahinci et al., 2007; Bryja et al., 2009).

WNT antagonists and agonists

Several secreted protein families antagonize or modulate WNT/β-catenin signaling. The WNT inhibitory protein (WIF) binds to WNT, and functions as a WNT antagonist for both β-catenin and noncanonical signaling (Bovolenta et al.,

2008). There are two distinct classes of WNT inhibitors comprised of the Dickkopf (DKK) family and the WISE/SOST family. DKK proteins are LRP5/6 ligands/antagonists and are considered specific inhibitors of WNT/β-catenin signaling. WISE and SOST proteins constitute another family of LRP5/6 ligands/antagonists (Itasaki et al., 2003; Li et al., 2005b; Semenov et al., 2005). Like DKK1, SOST is able to disrupt the WNT-induced Fz-LRP6 complex *in vitro* (Semenov et al., 2005). Both DKK1 and SOST are strongly implicated in human diseases.

WNT pathway self-regulation

WNT/β-catenin signaling plays a critical role in cell proliferation, differentiation, and fate specification in the developmental period and in maintaining adult tissue homeostasis. A larger number of WNT target genes exist (Vlad et al., 2008) and are cell specific (Logan and Nusse, 2004). WNT signaling components, including Fz, LRP6, Axin2, TCF/LEF, and DKK1 are often regulated positively or negatively by TCF/β-catenin (Logan and Nusse, 2004) WNT induction of Axin2, DKK1, and suppression of Fz and LRP6 cause negative feedback that reduces WNT signaling. In contrast, WNT induction of TCF/LEF genes induces positive circuits that activate WNT signaling, a feature that can be exploited during tumorgenesis (Arce et al., 2006). These WNT pathway self-regulatory loops are cell-specific, and afford complexity in several responses.

(2) WNT/β-catenin signaling in uterine development

In the early embryonic stage of the anterior area of the coelomic cavity, Lim1-expressing epithelial cells are induced and undergo invagination by WNT 4,

which is expressed in the mesonephros or coleomic epithelium (Kobayashi et al., 2004). The primitive anlage of the Műllerian duct continuously extends toward and interacts with the Wolffian duct. This is followed by posterior elongation mediated by WNT9b expressing epithelial cells from the Wolffian duct. If the Wolffian duct or WNT9b is absent, the Műllerian duct does not develop further (Carroll et al., 2005). Elongation of the Műllerian duct is accomplished by the proliferation of coelomic epithelial cells, similar to mesoepithelial cells at the disral tip (Guioli et al., 2007). The end of the outgrowth will fuse to form the uterovaginal tube, which then joins the urogenital sinus. WNT7a is involved in the correct patterning of the Mllerian duct into the vagina, cervix, uterus and oviduct and uterus, and WNT5a is expressed in mesenchymal cells of the uterus, cervix, and vagina (Mericskay et al., 2004).

In mice, by embryonic Day 11.5, the Műllerian duct is formed via the folding of WNT4-expressing epithelial cells from the coelomic wall followed by posterior elongation into the colacal area (Kobayashi et al., 2004; Carroll et al., 2005). When the Műllerian duct is generated, WNT4 is detected at high levels in the mesenchymal cells surrounding the duct. Moreover, WNT4a is required for suppressing male differentiation of the female gonad (Vainio et al., 1999). Carroll et al., determined that WNT9b is expressed in the Wolffian ducts during early embryonic development when both Wolffian and Műllerian ducts exist and the initial Műllerian analage is present (E9.5-E14.5). WNT9b knockout mice do not exhibit elongation of the Műllerian duct. This result indicates that WNT9b is necessary for posterior outgrowth during Műllerian duct formation (Carroll et al., 2005).

WNT7a is expressed in the Műllerian ducts before birth and in oviduct and uterine luminal epithelium after birth (Miller et al., 1998). The disruption of WNT7a causes oviducts to be absent in most mice and, when present, they exist in uncoiled shapes similar to uterus morphology. Moreover, uterus physiology shows marked resemblances to the vagina, with thickening of the surrounding musculature, a relatively thin stroma, pronounced loss of glands and a luminal epithelium with a clear aquamous aspect. These results suggest that the loss of WNT7a induces posterioriation of the female reproductive tract, and plays an important role in patterning the Műllerian duct (Miller and Sassoon, 1998).

WNT5a is first observed in mesenchymal cells, surrounding the Műllerian ducts and later in mesenchymal cells of the uterus, cervix and vagina (Miller et al., 1998). WNT5a knockout mice exhibit normal oviducts and anterior uterine horns, but lack the more posterior cervical and vaginal structures. These mice have severely coiled uterine horns that are either fused at the midline or remain separated as sightless ending sacs. Moreover, wild type mice have high levels of WNT5a in the stromal regions of the endometrium, and WNT5a and WNT7a appear to be required, and cooperate in the formation of normal glands (Miller et al., 1998).

(3) WNT/β-catenin signaling in uterine function

The human uterus is composed of two major layers: the outer myometrial layer (myometrium) and the inner endometrial layer (endometrium). The endometrium is a dynamic tissue, and is involved in implantation, development and

outgrowth of the embryo. The endometrium is also composed of two sublayers: a functional layer and a basal layer. The functional layer, which is divested every month during menses, is replenished by the basal layer during the proliferative phase of the menstrual cycle. After menses, and during the first two weeks of the menstrual cycle, estrogen induces proliferation of the endometrium and thereby generates a new functional layer. During the second half of the menstrual cycle (known as the secretory phase), this functional layer will differentiate in order to prepare for implantation of the fertilized ovum. During this phase, progesterone, produced by the corpus luteum, counterbalances the proliferative effects of estrogen and is responsible for the induction of differentiation (Arumugam et al., 2006).

Analogous to the situation in the gastrointestinal tract, in proliferating epithelial cells, WNT/β-catenin signaling is activated while in differentiated cells WNT/β-catenin signaling is diminished (Clevers, 2006). Based on these observations, a critical role for WNT/β-catenin signaling was hypothesized for the endometrium. In short, during the proliferative phase of the menstrual cycle, estrogen enhances WNT/β-catenin signaling. However progesterone inhibits estrogen-induced proliferation by prevention of WNT/β-catenin signaling, thus inducing differentiation during the secretory phase. Over time, a number of studies have been reported evidence in support of this hypothesis. Nei et al., investigated nuclear localization of 18-catenin during the proliferative phase of the menstrual cycle, when estrogen levels are elevated and unopposed by progesterone (Nei et al., 1999). During the secretory phase of the menstrual cycle, when progesterone levels increase and estrogen levels decrease, nuclear β-catenin accumulation was also found to decrease. Further data in support of the suggested involvement of WNT/

β-catenin signaling in regulation of the menstrual cycle has been obtained from gene expression profiling studies (Talbi et al., 2006; Cloke et al., 2008; Sahlgren et al., 2008; Wang et al., 2009c; Kuokkanen et al., 2010). In summary, sex hormones regulate WNT/β-catenin signaling in the endometrium to maintain the balance between estrogen-induced proliferation and progesterone-induced differentiation throughout the menstrual cycle.

WNT/β-catenin signaling therefore plays an important role in the proliferation and differentiation of tissue that occurs during normal uterine physiology processes and plays an essential role in embryonic development, blastocyst implantation, endometrial decidualization and placenta formation. During implantation, hormonal signaling regulates receptivity of the endometrium. Levels of estrogen increase during the first 2 weeks of the menstrual cycle, supporting the outgrowth of the functional layer. From ovulation onwards, the inhibition of estrogenic effects further induces differentiation. Differentiation itself is primarily characterized by induction of secretory activity within the glands. Endometrial stromal cells initiate transformation into decidual cells (signaling the start of decidualization) and attract natural killer cells (Gellersen et al., 2007). In humans, such endometrial priming is different to the situation in mice in terms of invasion of the trophoblast and full decidualization of the uterine stroma (Dey et al., 2004).

(4) WNT/β-catenin signaling in uterine disorders

Prolonged high levels of estrogen are a major risk factor for endometrial cancer. During the secretory phase of the menstrual cycle, high estrogen levels are

counterbalanced by progesterone. When these progesterone levels are too low and estrogen levels are elevated, the proliferative effect of estrogen becomes dominant and will induce endometrial hyperplasia (Amant et al., 2005)

Endometriosis, a common and benign gynecological disorder, is identified by the appearance of endometrial glandular and stromal tissue outside the uterus (pelvic peritoneum, on the ovaries and in the rectovaginal septum). Symptoms of endometriosis include pelvic pain and infertility. Endometriosis has been referred to as an estrogen-dependent disease as it entails low progesterone receptor levels and resistance to progesterone therapy (Giudice and Kao, 2004; Burney et al., 2007; Bulun et al., 2010a,b). The etiology of the development and maintenance of this disease has also been proposed to involve WNT/β-catenin signaling.

Using gene expression profiling, results have shown that WNT/β-catenin signaling is indeed differentially regulated between the eutopic and ectopic endometrium (Burney et al., 2007; Kao et al., 2003; Wu et al., 2006). Furthermore, Gaetje et al. (2007), showed significantly higher expression of WNT7a was present in endometriotic tissues, most likely caused by reduced progesterone signaling (Zanatta et al., 2010). It has also been found that HOXA10 is a downstream target of PR and is decreased in endometriotic tissue from women and baboons (Taylor et al., 1999). In baboons, the decreased levels of HOXA10 expression decreases target genes including integrin B3. During endometriosis, the dysregulation of HOXA10 could be caused by methylation of related genes. In baboons, the F1 region of HOXA10 has been observed to be hypermethylated in comparison to endometriosis-free animals. In endometrial tissue during endometriosis, HOXA10 expression has been associated with hypermethylation of the HOXA10 gene. These

reports suggest that endometriosis results from post-transcriptional modifications, such as gene silencing or activation (Kim et al., 2007).

In addition to endometrial tissues homing towards the abdominal cavity, there is a special form of endometriosis that invades the myometrium, known as adenomyosis. Interestingly, using a mouse model in which WNT/β-catenin signaling was activated in the myometrium, endometrial glands and stroma were observed to be present in the myometrium (Tanwar et al., 2009; Wang et al., 2011). Whether these observations point towards an active process of endometrial tissue invading into the myometrium or the possibility that endometrial tissue is simply filling the gap generated by myometrial dystrophy is not entirely clear at this point. In summary, enhanced estrogen signaling relative to inhibited progesterone signaling in ectopic endometrium activates the WNT/β-catenin signaling pathway, and may be a mechanism for stimulating survival, proliferation and invasion of endometrial tissue outside its normal environment (Table 1).

A major risk factor for endometrial cancer is prolonged high levels of estrogen (Schindler, 2009). During the normal menstrual cycle, high estrogen levels are counterbalanced each month by progesterone during the secretory phase of the menstrual cycle. When these progesterone levels are too low, or when estrogen levels are too high, the proliferative effect of estrogen becomes dominant and will induce endometrial hyperplasia (Amant et al., 2005). Endometrial hyperplasia can, over time, develop further into Type I endometrial cancer, which constitutes 90% of endometrial cancer cases (Amant et al., 2005).

Jeong et al. (2009) has manipulated the progesterone receptor to drive Cre

expression in order to induce activating or inactivating mutations, or β-catenin in all uterine cell types (myometrium, stroma, glandular epithelium and luminal epithelium). Both β-catenin mutations led to severe sub-fertility or even infertility due to a failure to undergo decidualization during embryo implantation. Furthermore, Pgr-Cre induced constitutive β-catenin activation resulted in enlarged glands causing endometrial hyperplasia. The conditional ablation of β-catenin, however, results in a reduction in epithelial gland development and squamous cell metaplasia (Jeong et al., 2009).

Table 1. Summary of WNT/ β-catenin signaling in endometrial physiology and disease (van der Horst et al., 2012)

WNT/ β-catenin signaling in uterine development		
WNT4	WNT4 is identified in epithelial cells from the mesonephros or coelomic wall	(Kobayashi et al., 2004; Carroll et al., 2005)
	WNT4 is essential for Műllerian duct origination	(Stark et al., 1994; Vainio et al., 1999; Kobayashi et al., 2004; Carroll et al., 2005)
WNT5a	WNT5a is observed in mesenchymal cells of the Műllerian duct	(Miller et al., 1998)
	WNT5a knockout mice has the deficiency in cervical and vaginal structures	(Mericskay et al., 2004)
WNT7a	WNT7a is presented throughout the epithelium of Műllerian duct	(Miller and Sassoon, 1998)
	The loss of WNT7a outcomes posteriorization of the female reproductive tract	(Miller and Sassoon, 1998; Parr and McMahon, 1998)
WNT9b	WNT9b is present in epithelial cells of the Wolffian duct, when the Műllerian duct is present	(Carroll et al., 2005)
	WNT9b is essential for posterior outgrowth of the early Műllerian duct	(Carroll et al., 2005)
WNT/ β-catenin signaling in uterine physiology		
DKK1	DKK1 is progesterone induced and can inhibit WNT/β-catenin signaling	(Kao et al., 2002; Tulac et al., 2003; Tulac et al., 2006; Wang et al., 2009b)
FOXO1	FOXO1 is induced by progesterone and inhibit WNT/β-catenin signaling	(Takano et al., 2007; Ward et al., 2008; Wang et al., 2009b)
Gsk3β	Gsk3β inhibition activates WNT signaling and proliferation	(Gunin et al., 2004)

Sfrp2	Sfrp2, WNT antagonist, inhibits the proliferative effect of estrogen	(Nei et al., 1999)
WNT/β-catenin signaling in uterine function (during implantation)		
Dkk1	Dkk1 disturbs implantation in normal pseudopregnant recipients	(Xie et al., 2008)
	Dkk1 leads to reduce trophoblast migration and invasion	(Pollheimer et al., 2006)
Fzd5	Loss of Fzd5 is a reason of embryonic death through poor placental vascularization	(Ishikawa et al., 2001)
Lef1	Lef1 disruption gives rise to defects in placental formation	(Galceran et al., 1999)
Sfrp2	Sfrp2 interferes implantation in mice	(Mohamed et al., 2005)
Tcf1	Tcf1 disruption provokes defects in placental formation	(Galceran et al., 1999)
WNT2	The disruption of WNT2 promotes defective placental vascularization	(Monkley et al., 1996)
WNT3a	WNT3a treatment results in trophoblast migration and invasion	(Sonderegger et al., 2010)
WNT4	WNT4 is required for Bmp2 mediated decidualisation	(Lee et al., 2007; Li et al., 2007)
	WNT4 ablation has the defects of stromal cell survival, differentiation and progesterone responsiveness	(Franco et al., 2011)
WNT7a	WNT7a triggers WNT/β-catenin signaling in pseudopregnant mice	(Mohamed et al., 2005; Tanwar et al., 2011)

WNT7b	WNT7b disruption leads to embryonic death because of placental failure	(Mohamed et al., 2005; Tanwar et al., 2011)
WNT/β-catenin signaling in uterine disorders (endometriosis and endometrial carcinogenesis)		
Apc	Apc conditional knockdown induces endometrial hyperplasia and cancer	(Arango et al., 2005; Tanwar et al., 2011)
	Apc conditional knockdown drives myometrial loss and reduced gland numbers	(Wang et al., 2011)
β-catenin	Activating β-catenin mutations are associated with 15–40% of endometrial cancers	(Saegusa and Okayasu, 2001; Pijnenborg et al., 2004)
	Conditional activation of β-catenin in mice leads to tumor-like growths and multiple hemorrhagic sites at the uterine surface; increased myometrial area. Conditional knockdown of β-catenin in mice induces myometrial loss and areas of adipogenesis	(Jeong et al., 2009; Tanwar et al., 2009)
WNT7a	WNT7a is increased in endometriosis.	(Deutscher and Hung-Chang Yao, 2007; Gaetje et al., 2007)

3) Steroid hormone signaling in the uterus

The human uterus is a dynamic tissue responsible for the full menstrual cycle of proliferation, differentiation and shedding (Brosens et al., 2010). The endometrium itself consists of the functionalis layer and the basalis layer, of which the functionalis layer undergoes shedding during menses. The basalis remains intact during the menstrual cycle and generates a new functionalis during each menstrual cycle (Winterhager et al., 2009; Brosens et al., 2010). Balance within the endometrium is regulated by steroid hormones including estrogen and progesterone.

(1) Steroid hormone signaling in uterine disorders

Endometrial cancer is one of the most common types of female gynecological cancer (Jones et al., 2009) and can be delineated into two types. Among western countries, approximately 85% of all endometrial carcinomas are Type I endometrial cancers, which are associated with increased estrogen signaling (Amant et al., 2005). Type I endometrial cancer often exhibits mutations in *PTEN*, *KRAS* or *CTNNB1* (β-catenin) (Fukuchi et al., 1998; Kildal et al., 2005; Di Cristofano and Ellenson, 2007). In contrast, Type II endometrial cancer is predominantly observed in older post-menopausal women, and is not reliant upon estrogen. Type II endometrial cancers frequently contain alterations in *TP53* and *ERBB2* (Her-2/neu) (Amant et al., 2005; Kildal et al., 2005; Di Cristofano and Ellenson, 2007).

Increased levels of estrogen for prolonged periods and insufficient progesterone levels represent the core risk factors in endometrial cancer.

Progesterone prevents the proliferative action of estrogen by stimulating the differentiation of epithelial and stromal cells in the endometrium (Daniels and Weis, 2005; Modarresi et al., 2005). Although progesterone treatment can be applied for chronic endometrial cancer, the usage of this treatment is modest (15-25%) (Thigpen et al., 1999). Conversely, when progesterone is used as a primary treatment, response rates are noticeably improved (up to 60%) (Kim et al., 1997; Yahata et al., 2006).

(2) Relationship between steroid hormone signaling and WNT/β-catenin signaling *in utero*

Central to canonical WNT signaling is, a multi-protein complex called the destruction complex that consists of the scaffold proteins AXIN1 and AXIN2 (conductin), β-catenin (CTNNB1), the tumor suppressor APC (adenomatosis polyposis coli) and the Ser-Thr kinases CK1 (casein kinase I) and GSK3β (glycogen synthase kinase 3 beta) (Behrens et al., 1998). In the absence of WNT ligands, formation of the destruction complex triggers Thr/Ser-phosphorylation of β-catenin by CK1 and GSK3β, and its subsequent ubiquitination and proteasomal degradation. During WNT signaling, the formation of the destruction complex is inhibited, thus leading to cytoplasmic accumulation of β-catenin and its subsequent nuclear translocation (Clevers, 2006). In the nucleus, β-catenin interacts with members of the TCF/LEF transcription factor family, thereby regulating the expression of a broad spectrum of WNT downstream target genes (Huber et al., 1996; Cho and Dressler, 1998; Clevers, 2006). The latter include genes encoding proteins that play a central role in cell proliferation and survival such as cyclin D1 (*CCND1*) and *c-MYC*, in addition to factors involved in a broad spectrum of other

cellular functions including cellular migration (e.g. *CD44*), cell adhesion (*CDH1*), regulation of the extracellular matrix (*MMP7*) and many others (Clevers, 2006).

The central role of WNT/β-catenin signaling in the regulation of tissue homeostasis has been extensively investigated in the gut (Fodde et al., 2001; Clevers, 2006). Along the intestinal tract, stem cells are located at the bottom of the crypts of Lieberkuhn which give rise to new stem cells and proliferating progenitor cells (transient amplifying/TA cells) (Fodde et al., 2001). These progenitor cells actively divide and produce new cells that are pushed up along the flank of the crypt towards the villus and eventually differentiate into Goblet cells, enteroendocrine cells and absorptive epithelial cells (Fodde et al., 2001). A similar process appears to take place in the endometrium, where estrogen receptor activation in the basalis stimulates endometrial cell proliferation during the first two weeks of the menstrual cycle, thus giving rise to the functionalis. In Week 3 and 4 of the menstrual cycle, the corpus luteum produces progesterone, which reduces estrogen-driven proliferation and induces differentiation of the functionalis, thus preparing the endometrium for implantation of the fertilized oocyte at approximately Day 20 - 22 of the menstrual cycle. WNT/β-catenin signaling activity along the crypt-villus axis of the intestine follows a decreasing gradient from the stem cell (SC) and proliferative (TA) areas to the more differentiated regions (van de Wetering et al., 2002). In the endometrium WNT/β-catenin signaling has also been suggested to play a role in regulating proliferation and differentiation during the menstrual cycle. Nei et al., observed that in the human endometrium, nuclear β-catenin was clearly enhanced during the proliferative phase of the menstrual cycle, while it was mostly found in the cytoplasm during the secretory phase (Nei et al., 1999).

4) Epithelial-mesenchymal transition

(1) Definition

Epithelial–mesenchymal transition or transformation (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells. EMT is essential for numerous developmental processes including mesoderm formation and neural tube formation. EMT has also been shown to play a central role in wound healing, organ fibrosis and in the initiation of metastasis during cancer progression.

In normal development, several developmental milestones, including gastruation, neural crest formation and heart morphogenesis, rely on the plastic transition between epithelium and mesenchyme. Epithelial and mesenchymal cells represent the two major cell types in mammals. Epithelial cells are characterized by: (i) cohesive interactions among cells, facilitating the formation of continuous cell layers; (ii) existence of three membrane domains: apical, lateral and basal; (iii) presence of tight junctions between apical and lateral domains; (iv) apicobasal polarized distribution of the various organelles and cytoskeleton components; and (v) lack of individual epithelial cell mobility with respect to their local environment.

Multicelluar mesenchymal architectures differ from multicellular epithelial architectures in having: (i) loose or no interactions among cells, so that no continuous cell layer is formed; (ii) no clear apical and lateral membranes; (iii) no apicobasal polarized distribution of organelles or cytoskeletal components; and (iv) motile cells that may even have invasive properties. During development, certain

cells can switch from an epithelial to a mesenchymal status by means of a tightly regulated process defined as the EMT, which is associated with a number of cellular and molecular events. In some cases, EMT is reversible and cells undergo the reciprocal mesenchymal to epithelial transition (MET).). A schematic view of the molecular and structural features associated with epithelial and mesenchymal cells during EMT and MET (Fig. 1). Recently, several studies have reported that physiopathological transitions occur during the progression of epithelial tumor formation, endowing cancer cells with increased motility and invasiveness.

(2) EMT and related pathways

Multiple oncogenic signaling pathways including TGF-beta, FGF, EGF, HGF, WNT/β-catenin catenin and Notch are regulated by peptide growth factors including Src, Ras, Ets and integrin. These signaling pathways induce EMT via several types of proteins, ligands, receptors and effectors.

WNT/β-catenin signaling

The canonical WNT/β-catenin pathway plays critical roles in development and is frequently elevated in cancer. If WNT signals are absent, β-catenin is regulated by a complex together with APC and axin, and is phosphorylated by GSK3β and degraded by the ubiquitin/-proteasome pathway. In the presence of the WNT ligand, Frizzled receptors activate Disheveled (Dsh), and blocks β-catenin degradation. Accumulated cytoplasmic β-catenin translocates into the nucleus, and activates the transcription factor TCF/LEF, which promotes the expression of several target genes. Unsurprisingly, several studies have determined that WNT/β-catenin signaling-induced EMT plays a role in colorectal cancer metastasis and

squamous cell carcinoma progression (Taki et al., 2003). WNT/β-catenin signaling is induced by the activation of Slug, which is a direct transcriptional target of β-catenin/TCF (Conacci-Sorrell et al., 2003), and by the stabilization of Snail, via inhibition of its phosphorylation and degradation (Yook et al., 2005).

β-catenin plays a critical role in EMT regulation via two mechanisms. As a transcription factor, β-catenin regulates transcriptional modulators such as Slug, which induce the repression of E-cadherin. Another function of β-catenin is as a component of adherens junctions together with E-cadherin and α-catenin. Total β-catenin is involved in the regulation of adherens junctions and nuclear transcription while cytoplasmic β-catenin can be throught of as a pool that feeds both functions, and alternatively is phosphorylated and ubiquitinated for degradation by the proteasome. Activation of Slug by β-catenin promotes the establishment of EMT and represses E-cadherin. This action causes the dissolution of adherens junctions and further induces the movement of β-catenin toward the nucleus for transcriptional activity.

TGF-β signaling

TGF-β signaling plays important and sophisticated regulatory roles in many types of cancer. This signaling plays an important role in cell proliferation, differentiation, apoptosis, adhesion, invasion, and interactions with the cellular microenvironment. These mechanism have been reported in epithelial, endothelial, and hematopoietic cell lineages (Blobe et al., 2000). In the canonical TGF-β signaling pathway, TGF-β consists of homo- or heterodimers and is secreted into the extracellular matrix as part of a complex known as the large latency complex (LLC). When this complex is released, TGF-β is active. This TGF-β signaling

pathway was first identified as EMT signaling in 1994 (Miettinen et al., 1994). Moreover, the transcriptional activation of Snail, Slug, Zeb1, and Twist has been observed as a critical process in EMT-related TGF-β signaling (Xu et al., 2009; Wendt et al., 2012). In TGF-β signaling, the TGF-β receptors are located at the membrane and exhibit serine-threonine kinase activity. TGF-β binds to the receptor, and then phosphorylates receptor-regulated Smads (R-Smads). There are at least eight known variants of R-Smads, the principal of which are Smads 2 and 3,considered canonical to TGF-β signaling (Wendt et al., 2012). The R-Smads then form a complex with Smad 4, and the newly-formed complex translocates into the nucleus to function as a transcriptional regulator. Recently, in colon cancer and papillary thyroid cancer, Smad 4 mutations have been identified. Such mutations promote nuclear localization, and induce reductions in E-cadherin while increasing N-cadherin and fibroblastic phenotypes (D'Inzeo et al., 2012). Activated TGF-β signaling has been considered as the downstream effectors of this change, along with myc signaling, with potential links to the WNT and Notch signaling pathways. During this process, cell-cell adhesion is broken and the actin cytoskeleton is rearranged, which heavily influences the EMT process (Tian et al., 2011). Additionally, since TGF-β signaling is activated, downstream events such as myc signaling, WNT pathway activation and Notch signaling are promoted. In a clinical study, cholangiocarcinoma cell migration, invasion, fibroblastic phenotype and cadherin switch (a change from E-cadherin repression to N-cadherin upregulation) was observed after TGF-β treatment (Tian et al., 2011). TGF-β signaling was then investigated during chemotherapy and radiation. However, both radiation treatment and ionizing radiation led to increased TGF-β levels and increased circulating tumor cells and lung metastatic burden. The authors of the study suggested that these effects can be reduced with treatment by a small molecule inhibitor of TGF-β.

TGF-β has been considered as a critical factor for cancer cell survival and recovery after radiation and chemotherapy.

Notch Signaling

The Notch pathway is related to cell proliferation, survival, apoptosis, and differentiation, critical for the embryogenesis and function of many organs. The Notch receptors are typically located at the cell membrane as heterodimers. Notch ligands include the Delta and Jagged families that are membrane-bound ligands. Notch signaling is most frequently activated by the binding of ligands to the receptor as a result of contact with neighboring cells. Upon ligand binding, the intracellular domain of the receptor is then ubiquitinated, and protease and gamma-secretase enzymes cleave the transmembrane domain of the receptor. These events cause the release of the Notch intracellular domain (NICD) into the cytoplasm, which translocate into the nucleus and binds CSL (CBF1/RBP-Jκ/Suppressor of Hairless/LAG-1) transcription factors (Weerkamp et al., 2006). The Notch-CSL complex activates the transcription of Notch target genes, which include helix-loop-helix transcription factors, and HRT/Herp transcription factors that are involved in regulating a diverse cellular decisions (Bolos et al., 2007). The Notch signaling pathway has been identified as overexpressed in many human malignancies (Miele and Osborne, 1999; Miele et al., 2006a). It has also been reported that Notch is a critical regulator of the EMT process (Niessen et al., 2008; Sahlgren et al., 2008). Notch activation induces endothelial cells to undergo morphological, phenotypic, and functional changes consistent with mesenchymal transformation. During EMT induction, endothelial markers such as E-cadherin, Tie1, Tie2, and platelet-endothelial cell adhesion molecule-1 are downregulated and mesenchymal markers (α-SMA, fibronectin, and platelet-derived growth factor

receptors) are upregulated. Moreover, several reports have suggested that Notch-2 and its ligand, Jagged-1, are highly up-regulated in such cells, which is consistent with the acquisition of EMT. Subsequently, the loss of Notch signaling by siRNA silencing led to inhibition of the EMT process and mesenchymal-epithelial transition, associated with decreased expression of vimentin, ZEB1, Slug, Snail, and NF-κB (Wang et al., 2009c). These results provide support for therapeutic strategies that involve Notch signaling regulation for chemotherapy, whereby the inhibition of Notch signaling could be a potential targeted therapeutic approach for preventing tumor progression.

(3) EMT and metastasis

The invasion of surrounding tissues and metastasis to distant sites are the core features of malignancy. Recent reports indicate that both invasion and metastasis may critically rely upon the acquisition of EMT features by cancer cells. Metastasis involves a multistep process that includes dissociation of tumor cells from the epithelial layer, penetration through the basement membrane into adjacent connective tissue, intravasation, survival in the bloodstream, growth of metastatic cells in distant organs and the stimulation of neoangiogenesis.

The initiation of metastasis requires an invasive cell phenotype typical to the EMT process. Carcinoma cells first lose cell-cell adhesion, mediated by E-cadherin repression, break through the basement membrane with increased invasive properties, and enter the bloodstream through intravasation. Later, when these circulating tumor cells (CTCs) exit the bloodstream to form micrometastases, they undergo MET for clonal development at these metastatic sites. Thus, EMT and

MET are required for the initiation and completion of the invasion-metastasis cascade.

Like other cancer types, the heterogeneity of invasive breast carcinomas is reflected in their histological features and reaction to treatment. Histological features include the presence of differentiated tubules, enhanced proliferative ability (mitotic index) and anisokaryosis (Come et al., 2006). Hormonal receptor status also correlates with disease progression and is used as a marker for diagnostic and prognostic purposes (Lacroix et al., 2004). For these diagnoses, limitations are present in clinical studies because it is impossible to identify an undifferentiated phenotype that reflects a lack of differentiation or an active EMT process during tumor progression. However, EMT phenotypes based on cell-to-cell adhesion status have been proposed for the presumption of tumor progression (Klymkowsky and Savagner, 2009). In carcinosarcoma or metaplastic carcinomas, identifying the expression of cytokeratins or vimentin intermediate filaments can aid in the recognition of epithelial or mesenchymal areas. Evidence from cytogenetic studies strongly suggests that these epithelial and mesenchymal compartments arise from a mutual precursor cell population that undergoes EMT (Zhuang et al., 1997). Recent studies have identified Snail genes that are overexpressed in these tumors, involving the activation of Akt and β-catenin pathways (Saegusa et al., 2009). Some mammary tumors, infiltrating the lobular carcinoma, exhibit E-cadherin repression and significantly higher levels of the EMT master gene, Twist (Yang et al., 2004), but interestingly still maintain cytokeratin expression. This finding suggests that individualized cells have partial EMT completion. More recently, several techniques for expression profiling have provided new approaches for studying tumorigenesis. These expression profiles,

histological features and tumor phenotypes are standard for categorizing tumor stages.

5) Cre–loxP technology for the generation of mouse models

Cre–loxP technology involves homologous recombination between DNA sequences in the chromosome and introduced DNA to mutate genes of interest in the mouse genome (Capecchi, 1989). Cre–loxP technology allows for specific manipulation of the mammalian genome, and has generated numerous strains of mutant mice for research. Studies of these mutant mice have produced valuable data in many biological fields. While Cre–loxP technology can be used to generate mutant mice for any genes, many are necessary for embryonic development. In such cases, germline mutations often induce embryonic, neonatal, or preadult lethality, which confounds studies of gene function in later stages of development and during tumorigenesis (Weinstein et al., 2000). More recently, Cre–loxP technology involving inducible systems has become popular to overcome the problem of embryonic and early postnatal lethality (Le and Sauer, 2000; Nagy, 2000). Tissue or site-specific Cre–loxP-mediated recombination has been employed to study the spatial and temporal function of oncogenes and tumor suppressors.

(1) Cre-loxP system

The Cre–loxP site-specific recombination system is relatively simple and robust (Argos et al., 1986; Sternberg et al., 1986), with the conditional knockout of target genes achieved with the use of Cre recombinase (Nagy, 2000). The *Cre*

(cyclization recombination) gene encodes a 38-kDa site-specific DNA recombinase, which recognizes loxP 34-bp sites and catalyzes the recombination between two loxP (locus of X-over P1) sites. The loxP site contains an 8-bp non-palindromic core region flanked by two 13-bp inverted repeats. Cre–loxP recombination between two loxP sites excises all DNA sequences located within the two flanking loxP sites. Because the efficiency of Cre–loxP-mediated recombination is high and does not require any additional factors, it is very useful for the creation of experimental model systems. Usually, loxP sites are located within the same chromosome, allowing for deletion of the intervening DNA sequence. The loxP sites can also be located in different chromosomes for recombination between different chromosomes.

(2) Cre-loxP mediated gene inactivation or activation

By inserting the Cre protein either as a transgene or combined under the promoter of the tissue specific gene, one can express the recombinase at sufficient levels for genetic ablation when crossed into a mouse containing a gene of interest flanked by two loxP sites (floxed).

The vector itself can be constructed through multiple established procedures (Zhang et al., 2002; Deng and Xu, 2004). Commonly, a selectable marker and a nonselectable marker are transferred (Deng et al., 1993). For example, a neomycin (*neo*) gene for positive selection and a thymidine kinase (*tk*) gene for negative selection are introduced in a vector (Mansour et al., 1988). The gene of interest is flanked by loxP sites and can be deleted by Cre–loxP mediated recombination.

After introducing the construct containing the gene of interest into ES cells, Southern blots are performed to identify the cells containing the anticipated homologous recombination and these are injected into the embryo for germline transmission. Next, the removal procedure of the recombinant *neo* gene in ES cells is important and several methods such as transient Cre expression either in ES cells or mutant mice can be used, because the *neo* gene if present in an intron often disturbs endogenous gene expression and leads to reduction in expression or inactivation of the floxed genes (Hirotsune et al., 1998; Xu et al., 2001).

The Cre–loxP system is therefore very useful for cancer research as it allows for the study of gene activation. Carcinogenesis relies upon activation of numerous oncogenes and the inhibition of tumor suppressors. For example, activation of *KRAS* is found in a number of cancers, including pancreas (90%), colon (50%), and lung (30%) (Rodenhuis et al., 1988; Mills et al., 1995; Huncharek et al., 1999). Meuwissen et al., (2001) generated a mouse model with an activated K-Ras (K-ras G12V) mutation that specific to lung epithelial cells (Meuwissen et al., 2001). The construct harboring the conditional K-ras G12V transgene contained a broadly active beta-actin promoter, a green fluorescence protein (GFP) expression cassette, and a K-ras G12V cDNA sequence combined with a human placenta-like alkaline phosphatase (PLAP) expression construct. The GFP gene functions as a reportor for the presence of the target gene and also functions to inhibit the expression of K-ras G12V.

Likewise, the inactivation of tumor suppressor genes can induce carcinogenesis. For example, nearly 50% of all human cancers contain a dysfunctional p53 (Morgan and Kastan, 1997). However, it was previously unclear

whether constant inactivation of p53 was associated with tumor maintenance. To further investigate this notion, a reactivatable p53 knockout allele (p53-LSL) was generated using the Cre–loxP technology (Ventura et al., 2007). In this system, p53 expression was inhibited through a blocker inserted within the intron of the gene. The p53-LSL mice were crossed with mice containing a Cre recombinase–estrogen-receptor-T2 (Cre–ERT2) allele to allow for the reactivation of p53. To reactivate temporal expression of p53, tamoxifen administration was used to activate Cre recombinase (Indra et al., 1999) and permit recombination of the loxP sites.

(3) Uterine-specific conditional knockout mice

By generating the progesterone receptor Cre (PR^{cre}) mouse, in which the Cre recombinase is present in all PR positive cells, the function of genes through conditional knockout can be studied in the uterus (Soyal et al., 2005). PR sites can be chosen after the spatial and temporal expression is determined by inserting the bacterial betagalactosidase gene into the first exon of the PR site (Ismail et al., 2002). In the PR-LacZ mice, PR expression is present in the pituitary, ovary, uterus and mammary glands. In the uterus, PR expression is observed only in the luminal and glandular epithelium postnatally, and stromal expression after one month. During the postnatal period, successful PR^{cre} expression demonstrated that this system could be used for studying conditional ablation in the uterus without problems arising during pre-natal development. Furthermore, mice carrying specific Cre recombinases were generated to further study the roles of specific genes in different uterine compartments, such as the $WNT7a^{cre}$ and $Sprr2f^{cre}$ line

(Winuthayanon et al.; Contreras et al., 2010) in epithelial cells.

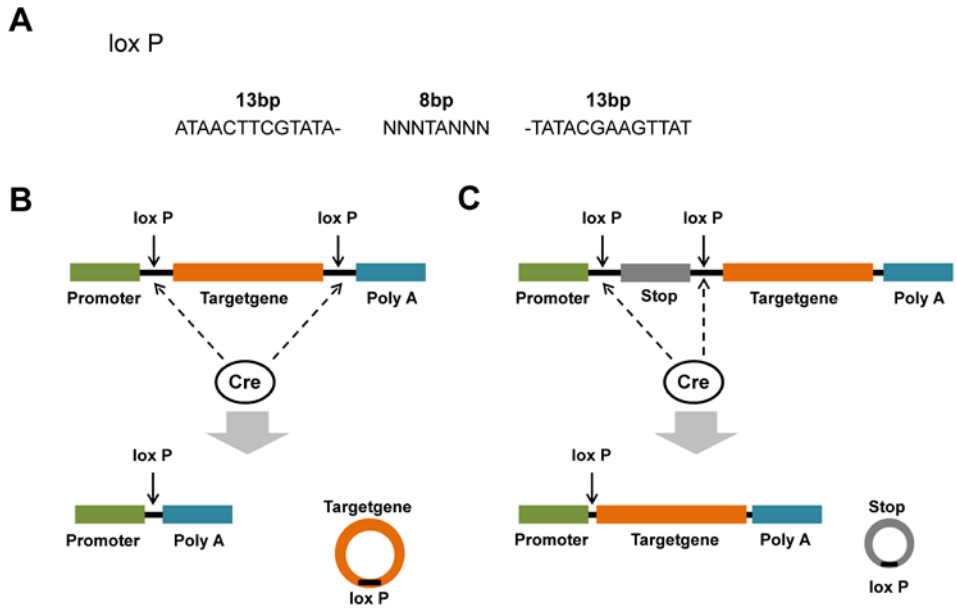

Figure 1. Schematic representation of the Cre-loxP system. (A) The loxP site contains an 8-bp nonpalindromic region flanked by two 13-bp inverted repeats. (B) Cre–loxP-mediated recombination can be applied for loss of function by deleting target gene between two lox P sites. (C) Cre/loxP system can be also used for activation of target genes.

CHAPTER 2.

General materials and methods

1. Human adenomyosis samples

Tissue samples of adenomyosis with their corresponding eutopic endometrium were collected from a total of 19 women with histologic evidence of adenomyosis. Adenomyosis samples were derived from hysterectomy surgical specimens. Controls comprised of 24 regularly cycling premenopausal women undergoing a hysterectomy for benign conditions with no history or evidence of adenomyosis, were documented not to be pregnant, and had not been on hormonal therapies for at least three months before tissue sampling. These samples were obtained by Michigan State University's Center for Women's Health Research Female Reproductive Tract Biorepository with MSUIRB approval and these samples were obtained with informed written consent from the patients. Full thickness endometrium was collected at Greenville Hospital System from hysterectomy samples using approved IRB protocols. Tissues were fixed in formalin and embedded in paraffin for histological analysis. Histological samples were examined by an independent pathologist, and phases were assigned according to the Noyes criteria (Noyes et al., 1975).

2. Animals and tissue collection

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University. Mice were cared for and used in the designated animal care facility according to the Michigan State University institutional guidelines. Control ($PR^{cre/+}$ and $Ctnnb1^{f(ex3)/+}$) mice and mutant ($PR^{cre/+}$ $Ctnnb1^{f(ex3)/+}$) mice were sacrificed at two, four, and six months of age. To collect the uteri, the mice were anesthetized with Avertin (2,2-tribromoethl

alcohol, Sigma-Aldrich, St. Louis, MO, USA) and euthanized by cervical dislocation. At the time of dissection, uterine tissues were fixed with 4 % paraformaldehyde (vol/vol) and paraffin embedded.

3. Immunohistochemistry

Uterine sections from paraffin-embedded tissue were cut at six μm and mounted on silane-coated slides, deparafinized and rehydrated in a graded alcohol series. Sections were preincubated with 10 % normal serum in PBS (pH 7.5). Then, uterine sections were incubated with primary antibodies in 10% normal serum overnight at 4 °C. Antibodies used are listed in Table 2. On the following day, sections were washed in PBS and incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) for one hour at room temperature. Immunoreactivity was detected using DAB (Vector Laboratories). In the uterine section of mice, immunohistochemical staining with anti-mouse antibody was carried out using the Vector Mouse on Mouse (MOM) kit (Vector Laboratories) following the manufacturer's instructions, to minimize cross reactivity between the mouse secondary antibody and mouse tissues. Briefly, sections were blocked overnight with MOM IgG blocking solution in PBS, washed twice for five minutes with PBS, incubated with MOM solution in PBS for 30 min, incubated with anti-β-catenin antibody overnight at 4 °C. On the following day, sections washed in PBS, incubated with secondary biotinylated anti-mouse (Vector Laboratories) for one hour at room temperature then washed, detected using the Vectastain Elite ABC system (Vector Laboratories). The sections were counterstained with hematoxylin, dehydrated, and mounted.

4. Immunofluorescence

Uterine sections from paraffin-embedded tissue were cut at six μm and mounted on silane-coated slides, deparafinized and rehydrated in a graded alcohol series. Sections were preincubated with 1% blocking solution from the TSA kit in PBS and 0.01% Triton X-100 (PBST). For maximal sensitivity of antibodies, we used tyramide signal amplification (TSA) immunofluorescence (TSA kit; PerkinElmer, Waltham, MA, USA) following the manufacturer's protocol. Sections were preincubated with 1% blocking solution from the TSA kit in PBS and 0.01% Triton X-100 (PBST), and then incubated with antibodies in PBST overnight at 4 °C. Antibodies used are listed in Table 2. On the following day, sections were washed in PBST and incubated with biotinylated secondary antibody (Vector Laboratories) for one hour at room temperature. Immunoreactivity was detected using the Vectastain Elite ABC system. After five -minute TSA reaction, slides were washed in PBST and then incubated the second antibodies, which we want to check the colocalization with primary antibodies, overnight at 4°C. On the third day, sections were washed in PBST and incubated with Alexafluor conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) for two hours at room temperature. Slides were counterstained by Vectashield mounting media with DAPI (Vector Laboratories). Images were captured with a confocal microscope (510 NLO confocal microscope with a META detector; Carl Zeiss, Inc., Thornwood, NY, USA) or fluorescent microscope (Nikon Instruments Inc., Melville, NY, USA) and processed using the image programs inherent in the microscope system (Carl Zeiss Meditec, Inc.).

5. Statistical analysis

Statistical analysis were performed using one-way ANOVA analysis followed by Tukey's post hoc multiple range test or Student's t tests using the Instat package from GraphPad (GraphPad Software, Inc., San Diego, CA). $p< 0.05$ was considered statistically significant.

Table 2.The lists used antibodies

Antibody name	Source	Clonality	Species	Dilution
APC	Abcam, Cambridge, MA, USA	monoclonal	rabbit	1:500
COUP-TFII	Perseus Proteomics, Tokyo, Japan	monoclonal	rabbit	1:1000
CD3	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	monoclonal	rat	1:500
CD4	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	polyclonal	goat	1:500
CD10	Novocastra, Burlingame, CA, USA	monoclonal	mouse	1:500
CD11b-FITC	Abcam, Cambridge, MA, USA	monoclonal	rat	1:200
Cyclin D1	Neomarkers, Fremont, CA, USA	monoclonal	mouse	1:500
E-cadherin	BD bioscience, San Jose, CA, USA	monoclonal	mouse	1:1000
GSK3β	Cell Signaling, Rockford, IL, USA	monoclonal	rabbit	1:500
Keratin 18	Proteintech, Chicago, IL, USA	polyclonal	rabbit	1:500
Ki67	BD bioscience, San Jose, CA, USA	monoclonal	mouse	1:500
phospho-histone H3 (Ser10)	Millipore, Billerica, MA, USA	polyclonal	rabbit	1:500
Snail	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	polyclonal	rabbit	1:500
Vimentin	Invitrogen, Carlsbad, CA, USA	monoclonal	mouse	1:500
WIF-1	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	polyclonal	goat	1:500
WNT4	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	polyclonal	goat	1:500
ZEB1	Dr. Dong S Darling		rabbit	1:1000
α-smooth muscle actin	Abcam, Cambridge, MA, USA	polyclonal	rabbit	1:500
β-catenin	BD bioscience, San Jose, CA, USA	monoclonal	mouse	1:500

CHAPTER 3.

Dysregulated WNT/β-catenin signaling plays an important role in the development of adenomyosis

1. Introduction

The accurate etiology and pathophysiology of adenomyosis is not known. Adenomyosis has been suggested to be an ovarian steroid hormone-dependent disorder. High concentrations of estrogen without appropriate protection from progesterone have been linked to endometrial cancer, endometrial hyperplasia and adenomyosis (Schindler, 2009). Interestingly, the β-catenin signaling was activated in endometrial cancer and induced by high levels of estrogen (Amant et al., 2005; Schindler, 2009).

β-catenin (Ctnnb1) is associated with E-cadherin, a transmembrane protein involved in cell-cell adhesion. Also, β-catenin is a component of the WNT signaling, which plays an important role in cell proliferation, differentiation during embryonic development. In the absence of WNT ligand in normal cells, β-catenin is phosphorylated by a complex including GSK3β, APC, axin, and CK1 (Fig. 2). In common cancers, these molecules are alterated or disrupted and β-catenin is activated.

In addition to its role in cancers, WNT/β-catenin signaling plays an important role in proliferation and differentiation during normal uterine physiology. Also, WNT/β-catenin signaling has been known as important pathways in disease (Clevers, 2006). Mutations in exon 3 of β-catenin leads the protein stabilization, cytoplasmic and nuclear accumulation, and participation in signal transcriptional activation through DNA binding (Palacios and Gamallo, 1998). Increased β-catenin level is cause of mutations in *CTNNB1* or *APC*, and brings the activation of the WNT/β-catenin pathway. Current data suggests that estrogen induces the

proliferation depends on WNT signaling beacause it was shown to increase WNT pathway molecules in the endometrium of estrogen-effected women.

In this study, we used the conditionally stabilized β-catenin mouse model in the uterus and human adenomyosis samples to investigate the association of WNT/β-catenin in the etiology and pathology of adenomyosis.

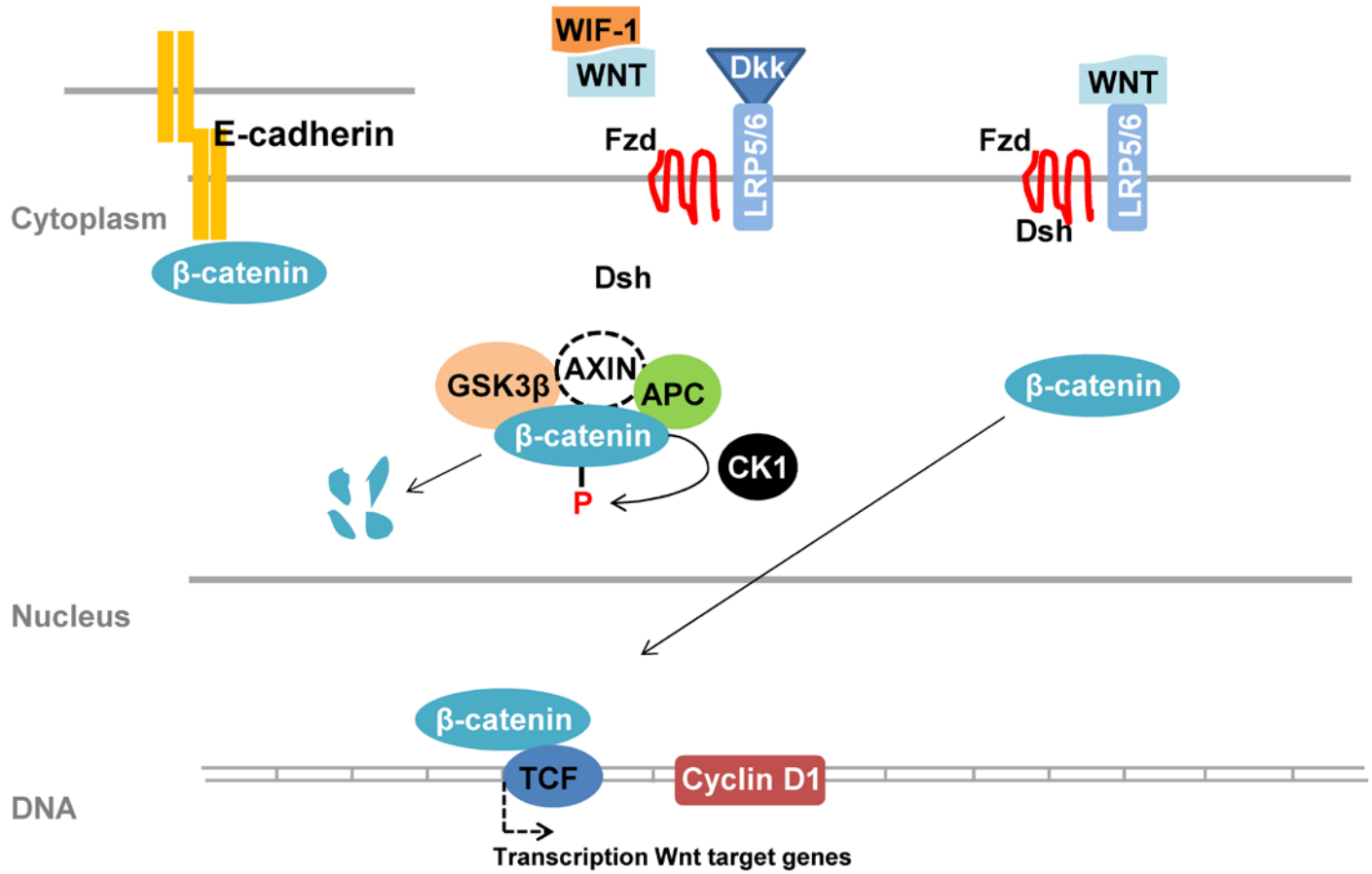
WIF-1
WNT
Dkk
WNT
E-cadherin
Fzd
LRP5/6
Fzd
LRP5/6
Cytoplasm
Dsh
β-catenin
Dsh
GSK3β
AXIN
APC
β-catenin
CK1
P
β-catenin
Nucleus
β-catenin
TCF
Cyclin D1
DNA
Transcription Wnt target genes

Figure 2. Overview of WNT/β-catenin signaling. In the absence of WNT, cytoplasmic β-catenin forms a complex with Axin, APC, GSK3β, and CK1, and is phosphorylated by CK1 and GSK3β. Phosphorylated β-catenin is degraded. In the presence of WNT signal, a receptor complex forms between Fzd and LRP5/6. This process disrupts a complex with Axin, APC, GSK3β, and CK1-mediated phosphorylation/degradation of β-catenin, allowing β-catenin to accumulate in the nucleus where it serves as a coactivator for TCF to activate WNT target genes such as Cyclin D1.

2. Materials and methods

1) Generation of the mice with uterine specific β-catenin conditionally stabilized

To investigate the function of the β-catenin activation, the phosphorylation sites in exon 3 were flanked by loxP in β-catenin (*Ctnnb1*$^{f(Ex3)}$) (Harada et al, 1999) because exon 3 of β-catenin is the site of phosphorylation and the mutations of the β-catenin exon 3 prevent its degradation and have been shown in several tumors (Fukuchi et al., 1998; Garcia rostan et al. 1999, Samowitz et al 1999). The *Ctnnb1*$^{f(Ex3)/+}$ mice were crossed with the *PR*^{cre} mouse to generate uterine specific stabilization of β-catenin (*PR*$^{cre/+}$ *Ctnnb1*$^{f(Ex3)/+}$) in PR-expressing cells (Soyal et al. 2005) (Fig. 3).

2) H&E staning

For Hematoxylin and Eosin (H&E) Staining, mice uterus was fixed overnight in 4% paraformaldehyde, followed by thorough washing in 70% ethanol. The tissues were processed, and embedded in paraffin. The six μm sections were cut and stained with hematoxylin and eosin by standard protocols.

3) Hormone treatments

The steroid hormone response was assessed in adult mice by ovariectomizing the mice at six weeks of age. Two weeks later, ovariectomized mice were treated with either vehicle (sesame oil), estrogen (E2) (0.1 μg/mouse),

progesterone (P4) (1mg/mouse in 100 μl) or E2 plus P4 and mice were sacrificed three month after the treatment by cervical dislocation while under anesthetic (Avertin; 2,2-tribromoethyl alcohol; Sigma-Aldrich). At the time of dissection, uterine tissues were fixed with 4 % paraformaldehyde (vol/vol) and paraffin embedded.

4) Immunohistochemistry

Uterine sections from paraffin-embedded tissue were cut at six μm and mounted on silane-coated slides, deparafinized and rehydrated in a graded alcohol series. Sections were preincubated with 10 % normal rabbit serum or 10 % normal goat serum in PBS (pH 7.5) and then incubated with WIF-1 (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), WNT4 (1:500; Santa Cruz Biotechnology Inc.), GSK3β (1:500; Cell Signaling, Rockford, IL, USA), APC (1:500; Abcam, Cambridge, MA, USA), Cyclin D1 (1:500; Neomarkers, Fremont, CA, USA), α-smooth muscle actin (α-SMA) (1:500; Abcam), and phospho-histone H3 (Ser10) (1:500; Millipore, Billerica, MA, USA) antibodies in 10% normal serum in PBS (pH 7.5) overnight at 4 °C. On the following day, sections were washed in PBS and incubated with biotinylated secondary antibody (Vector Laboratories) for one hour at room temperature. Immunoreactivity was detected using DAB (Vector Laboratories). Immunohistochemical staining with anti-β-catenin (1:500; BD bioscience, San Jose, CA, USA) was carried out using the Vector Mouse on Mouse (MOM) kit (Vector Laboratories) following the manufacturer's instructions, to minimize cross reactivity between the mouse secondary antibody and mouse tissues. Briefly, sections were blocked overnight with MOM IgG blocking solution in PBS, washed twice for five minutes with PBS,

incubated with MOM solution in PBS for 30 min, incubated with anti-β-catenin antibody overnight at 4 °C. On the following day, sections washed in PBS, incubated with secondary biotinylated anti-mouse (Vector Laboratories, Burlingame) for one hour at room temperature then washed, detected using the Vectastain Elite ABC system (Vector Laboratories). The sections were counterstained with hematoxylin, dehydrated, and mounted. Immunostained sections were examined by light microscopy and the intensity of β-catenin, WIF-1, WNT4, GSK3β, APC, and Cyclin D1 expressions scored according to the intensity of staining on a scale of zero (no staining) to five (strong staining).

4) Immunofluorescence

For observing the adenomyosis phenotype in the mutant ($PR^{cre/+}$ $Ctnnb1^{f(ex3)/+}$) mice, E-cadherin (1:1000; BD bioscience), Ki67 (1:500, BD bioscience) and α-smooth muscle actin (α-SMA) (1:500; Abcam) were incubated PBST overnight at 4 °C. On the following day, sections were washed in PBST and incubated with Alexafluor conjugated secondary antibodies (Sigma-Aldrich) for two hours at room temperature. Slides were counterstained by Vectashield mounting media with DAPI (Vector Laboratories). Images were captured with a confocal microscope or fluorescent microscope and processed using the image programs inherent in the microscope system.

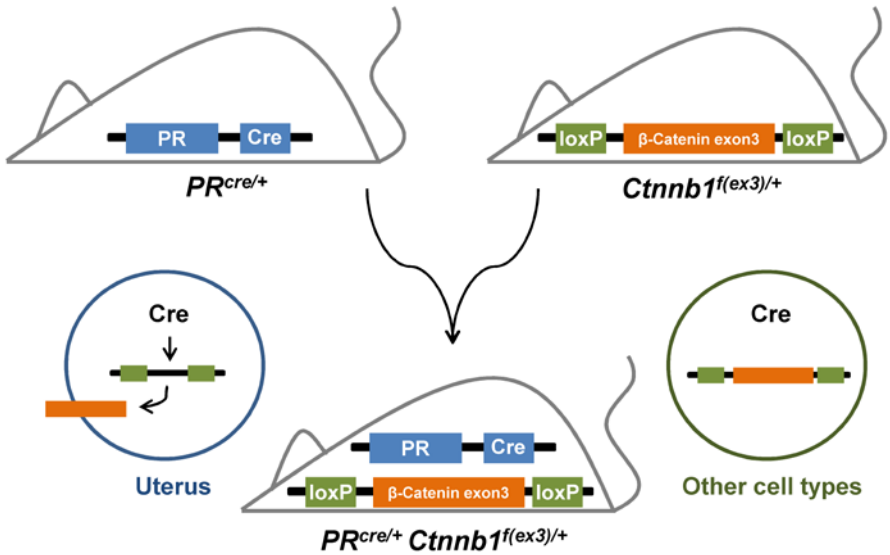

Figure 3. The breeding scheme for obtaining the uterine specific β-catenin stabilized mice. *PR*^{cre} mice and *Ctnnb1*$^{f(Ex3)/+}$ mice are crossed to generate uterine specific stabilization of β-catenin (*PR*$^{cre/+}$ *Ctnnb1*$^{f(Ex3)/+}$) in PR-expressing cells.

3. Results

1) Activation of β-catenin signaling in human adenomyosis

To determine if β-catenin signaling is dysregulated in adenomyosis, the expression of β-catenin was examined in endometrium from patients with and without adenomyosis by immunohistochemistry (Fig. 4, Table 3 and Table 4). The protein level of nuclear β-catenin was significantly higher in epithelial cells of eutopic endometrium (n=6) and adenomyosis region (n=8) compared to control endometrium without adenomyosis (n=13) in proliferative phase samples. In secretory phase samples, the level of nuclear β-catenin was also significantly higher in epithelial nucleus of eutopic endometrium compared with control endometrium. Higher level of nuclear β-catenin was observed in adenomyosis regions of secretory phase even though there is no significant difference (Fig. 5A). The expression of cytoplasmic β-catenin was not observed in control endometrium (n=13), but the level of cytoplasmic β-catenin in eutopic (n=6) and adenomyosis regions (n=8) was significantly higher compared with control endometrium in proliferative phase. In the epithelial cells of eutopic (n=7) and adenomyosis region (n=11) of secretory phase, the cytoplasmic β-catenin intensity was also significantly higher than control endometrium (n=11) (Fig. 5B). However, the expression of nuclear and cytoplasmic β-catenin was not different between proliferative and secretory endometrium in controls and adenomyosis. In addition, the level of membranous β-catenin expression was not different depending on menstrual cycles and adenomyosis disease (Fig. 5C). To better understand the integration of β-catenin in adenomyosis, we examined full thickness endometrium of patients with and without adenomyosis (Fig. 6). Weak expression of

membranous β-catenin was observed at full thickness endometrium of control patients. However, myometrium of control did not have any β-catenin staining (Fig. 6A). The expression of β-catenin was gradually increased at endometrium of the basal layer and adenomyosis regions of myometrium compared with the zona functionalis layer in adenomyosis (Fig. 6B).

To identify if WNT/β-catenin signaling molecules are dysregulated in adenomyosis, we also examined the expression profiles of WNT inhibitory factor-1 (WIF-1), WNT4, GSK3β, adenomatous polyposis coli (APC), and Cyclin D1 in normal endometrium (n=9) and eutopic (n=11) and adenomyosis region (n=13) of adenomyosis. Althogh there is no difference in WIF-1, and WNT4 expression between control endometrium and adenomyosis (Fig. 7), we observed lower level of GSK3β in eutopic and adenomiotic region of adenomyosis compare to control endometrium (Fig. 8A). GSK3β inactivation results in upregulation of β-catenin expression (Jope and Johnson, 2004). APC plays a central role in regulating the β-catenin level in the WNT/β-catenin signaling(Rubinfeld et al., 1993) and there is report that APC gene is mutated in endometrial cancer in women(Schlosshauer et al., 2000). However, APC levels were not much different between adenomyosis and normal group (Fig. 8B). Cyclin D1 is an important cell cycle regulator that is considered to be a downstream target of β-catenin (Tetsu and McCormick, 1999). Immunohistochemical staining in some adenomyosis (six among 11 eutopic region and six among 13 adenomyosis region) revealed strong nuclear staining of Cyclin D1 (Fig. 9). Although WNT/β-catenin signaling molecules such as WIF-1, WNT4, GSK3β, APC and Cyclin D1 do not have significantly difference between control and adenomyosis endometrium, the level of β-catenin expression were significantly increased in the nucleus and cytoplasm of adenomyosis endometrium. This result

suggests that the activation of β-catenin may play an important role in pathogenesis of adenomyosis.

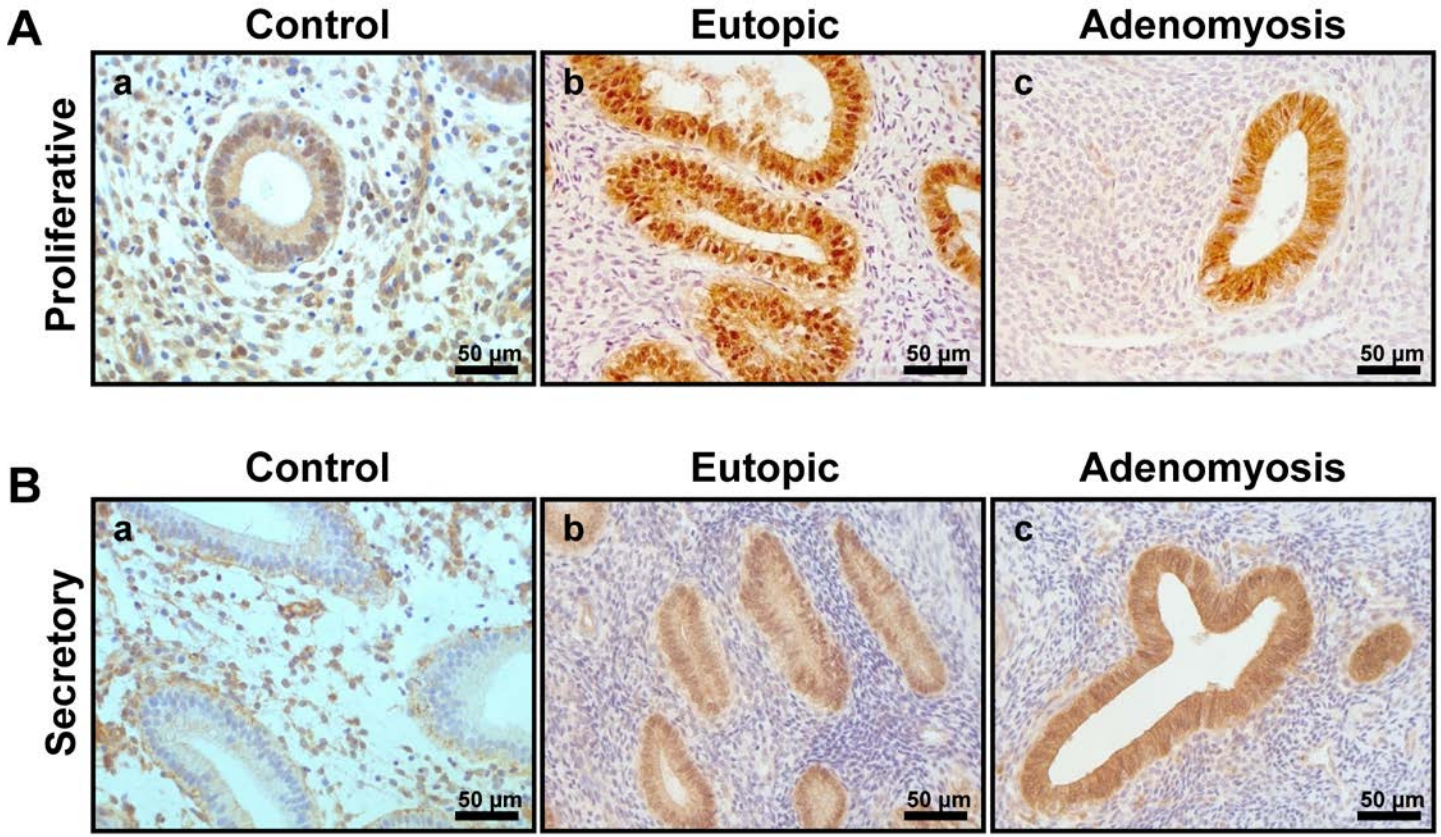
A
Control
Eutopic
Adenomyosis
Proliferative
a
b
c
50 μm
50 μm
50 μm
B
Control
Eutopic
Adenomyosis
Secretory
a
b
c
50 μm
50 μm
50 μm

Figure 4. Activation of β-catenin in endometrial tissue from women with adenomyosis. Photomicrographs represent immunostaining for β-catenin in human endometrium with (b and c) and without adenomyosis (a). β-catenin was confined to cell cytoplasm and intensity of β-catenin expression was not difference between proliferative and secretory phases (A and B). Nuclear accumulation of β-catenin was prominent in eutopic endometrium and adenomyosis region (b and c). Scale bar = 50 μm.

Table 3. The β-catenin expression intensity in control endometrium

Sample	Stage	Age	Cycle	Membrane	Nucleus	Cytoplasm
LB 11/19/04A	Normal	-	Proliferative	0	0	0
1-4-1 D23N	Normal	-	Proliferative	0	0	0
IHH 2/2/06	Normal	-	Proliferative	1	0	0
JF 2/14/06 d13	Normal	-	Proliferative	1	0	0
1-1-1 D7N	Normal	-	Proliferative	1	0	0
1-5-1 D9N	Normal	-	Proliferative	1	0	0
1-6-1 D10N	Normal	-	Proliferative	1	0	0
D813	Normal	-	Proliferative	2	0	0
D814	Normal	-	Proliferative	2	0	0
CD 112904A	Normal	-	Proliferative	2	0	0
1-8-1 D9	Normal	-	Proliferative	2	0	0
1-7-1 D10N	Normal	-	Proliferative	3	0	0
S06-13460F	Normal	-	Proliferative	3	0	0
AVE				1.46	0.00	0.00
SE				0.27	0.00	0.00
LF 5/2/06	Normal	-	Secretory	0	0	0
VT 111004C	Normal	-	Secretory	0	0	0
LF 5/2/06 d24	Normal	-	Secretory	0	0	0
IMH 110104C	Normal	-	Secretory	0	0	0
cv 2/16/06 d21	Normal	-	Secretory	1	0	0
YSC 120604	Normal	-	Secretory	3	0	0
N1	Normal	-	Secretory	3	0	0
N3	Normal	-	Secretory	3	0	0
N5	Normal	-	Secretory	3	0	0
S08-1076B	Normal	-	Secretory	3	0	1
J-D24	Normal	-	Secretory	2	3	0
AVE				1.60	0.00	0.10
SE				0.48	0.00	0.10

Table 4. The β-catenin expression intensity in eutopic region and adenomyotic region

Sample	Stage	Age	Cycle	Eutopic region			Adenomyotic region		
				Membrane	Nucleus	Cytoplasm	Membrane	Nucleus	Cytoplasm
SP08-10697	Adenomyosis	-	Proliferative	1	3	2	2	2	1
S08-17037B	Adenomyosis	-	Proliferative	1	3	3	1	3	3
S08-15085B	Adenomyosis	45	Proliferative	2	3	0	1	0	0
2445	Adenomyosis	46	Proliferative	2	4	2	3	1	2
7335	Adenomyosis	48	Proliferative	0	5	2	3	0	2
8285	Adenomyosis	47	Proliferative	3	5	3	4	1	3
SP08-10063	Adenomyosis	-	Proliferative	No Endometrium			2	1	0
2965	Adenomyosis	35	Proliferative	No Endometrium			4	0	3
AVE				1.50	3.83	2.00	2.50	1.00	1.75
SE				0.43	0.40	0.45	0.42	0.38	0.45
SP08-9494	Adenomyosis	-	Secretory	0	0	1	3	2	0
12215	Adenomyosis	33	Secretory	2	0	2	3	0	2
SP08-11402	Adenomyosis	-	Secretory	3	1	0	3	1	0
SP08-10050-1c	Adenomyosis	45	Secretory	3	2	1	0	0	1
SP08-11304	Adenomyosis	-	Secretory	2	3	0	2	3	3
14905	Adenomyosis	39	Secretory	2	3	4	5	0	2
11360	Adenomyosis	42	Secretory	1	5	2	4	0	3
SP08-9904	Adenomyosis	-	Secretory	No Endometrium			3	1	0
6010	Adenomyosis	44	Secretory	No Endometrium			3	3	2
8022	Adenomyosis	39	Secretory	No Endometrium			2	0	2
16200	Adenomyosis	38	Secretory	No Endometrium			3	0	1
AVE				1.86	2.00	1.43	2.82	0.91	1.45
SE				0.40	0.69	0.53	0.38	0.37	0.34

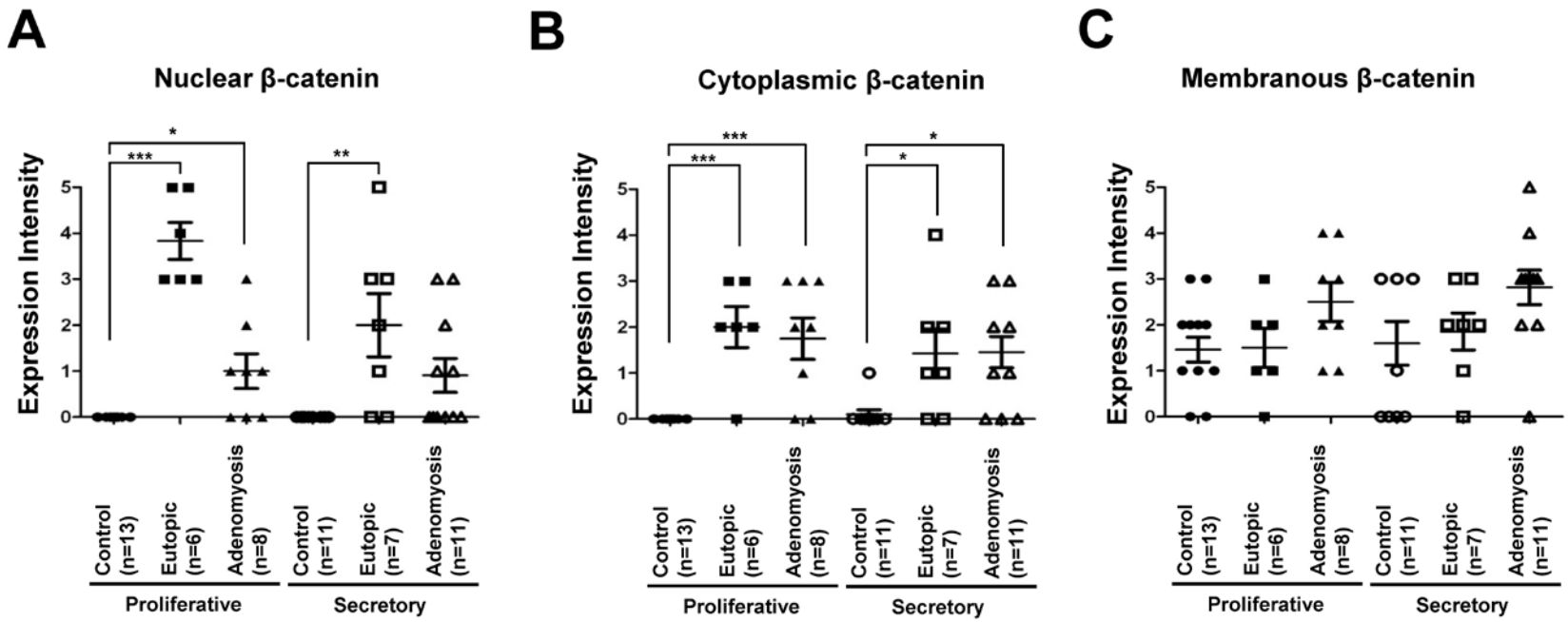
A
Nuclear β-catenin
Expression Intensity
Control (n=13)
Eutopic (n=6)
Adenomyosis (n=8)
Control (n=11)
Eutopic (n=7)
Adenomyosis (n=11)
Proliferative
Secretory
B
Cytoplasmic β-catenin
C
Membranous β-catenin

Figure 5. The intensity of β-catenin expression in endometrial tissue with and without adenomyosis. (A) Nuclear β-catenin was scored by measuring expression intensity of endometrial epithelial cells from control women (n=24), eutopic endometrium (n=13) and adenomyosis region (n=19). (B) Cytoplasmic β-catenin was scored by measuring expression intensity of endometrial epithelial cells from control women (n=24), eutopic endometrium (n=13) and adenomyosis region (n=19). (C) Membranous β-catenin was scored by measuring expression intensity of endometrial epithelial cells from control women (n=24), eutopic endometrium (n=13) and adenomyosis region (n=19). In membrane of epithelial cells, the intensity of β-catenin expression was not changed between controls, eutopic and adenomyosis regions at different menstrual phases. ***, $p< 0.001$; **, $p< 0.01$; *, $p< 0.05$, one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple range test.

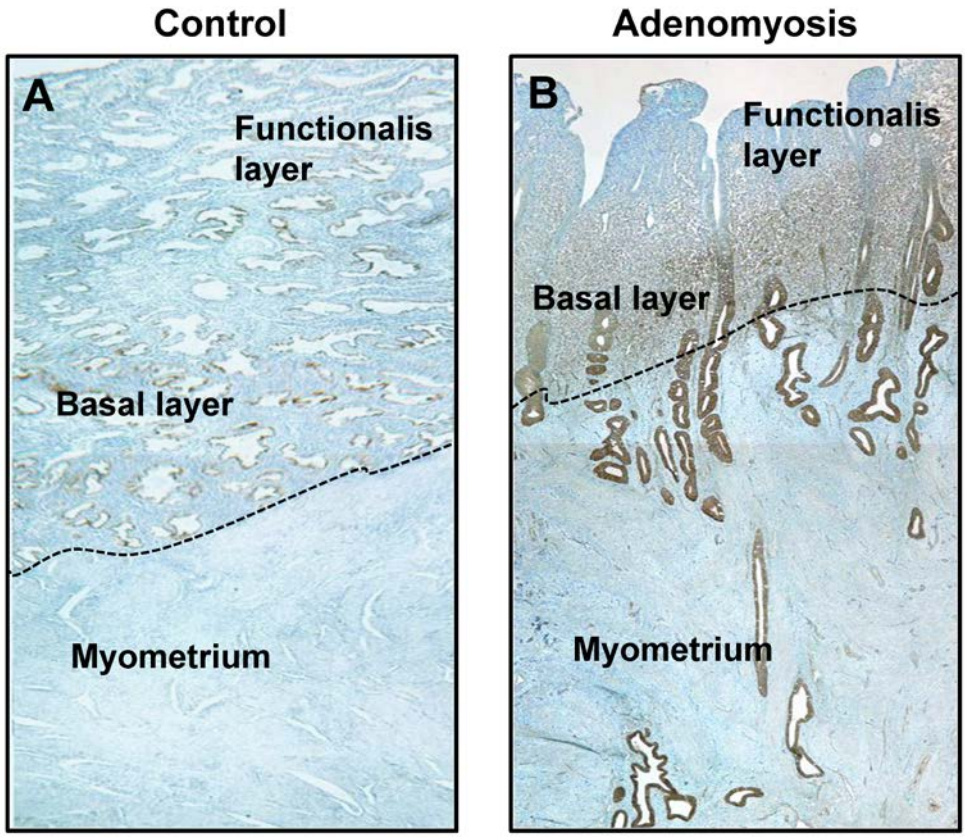

Figure 6. The expression of β-catenin was observed in full-thickness endometrium without (A) and with adenomyosis (B). Nuclear accumulation of β-catenin is observed in epithelial cells of endometrium and myometrium with adenomsyosis. The expression of β-catenin was gradually increased at endometrium of the basal layer and adenomyosis regions of myometrium (B).

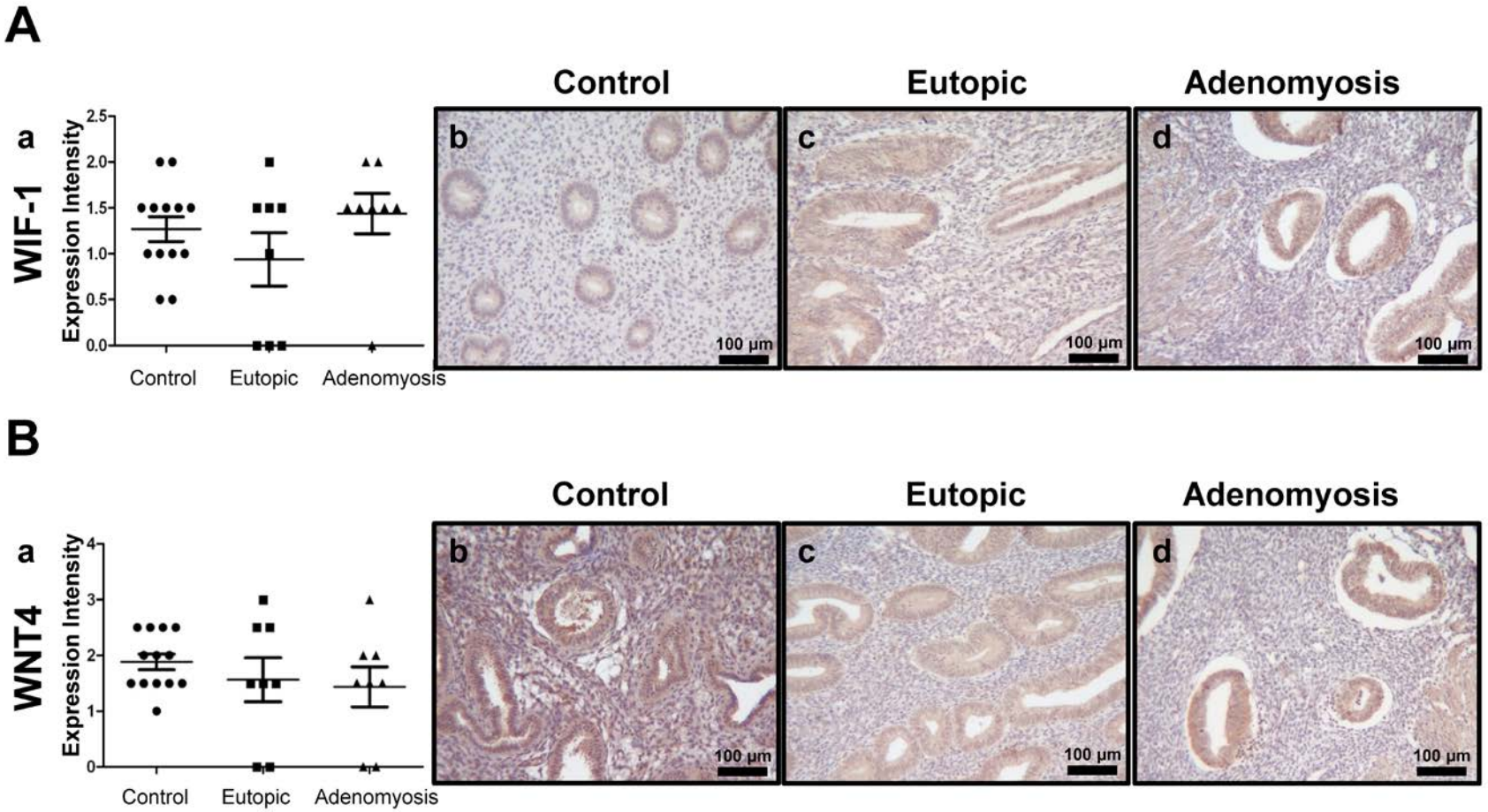
A
a
WIF-1
Expression Intensity
2.5
2.0
1.5
1.0
0.5
0.0
Control
Eutopic
Adenomyosis
Control
Eutopic
Adenomyosis
b
c
d
100 μm
100 μm
100 μm
B
a
WNT4
Expression Intensity
4
3
2
1
0
Control
Eutopic
Adenomyosis
Control
Eutopic
Adenomyosis
b
c
d
100 μm
100 μm
100 μm

Figure 7. The intensity of WIF-1 and WNT4 expressions in endometrial tissue with and without adenomyosis. (A) WIF-1 expression was not difference in human endometrium with (b and c) and without adenomyosis. (B) WNT4 expression was not also different in human endometrium with (b and c) and without adenomyosis. Scale bar = 100 μm.

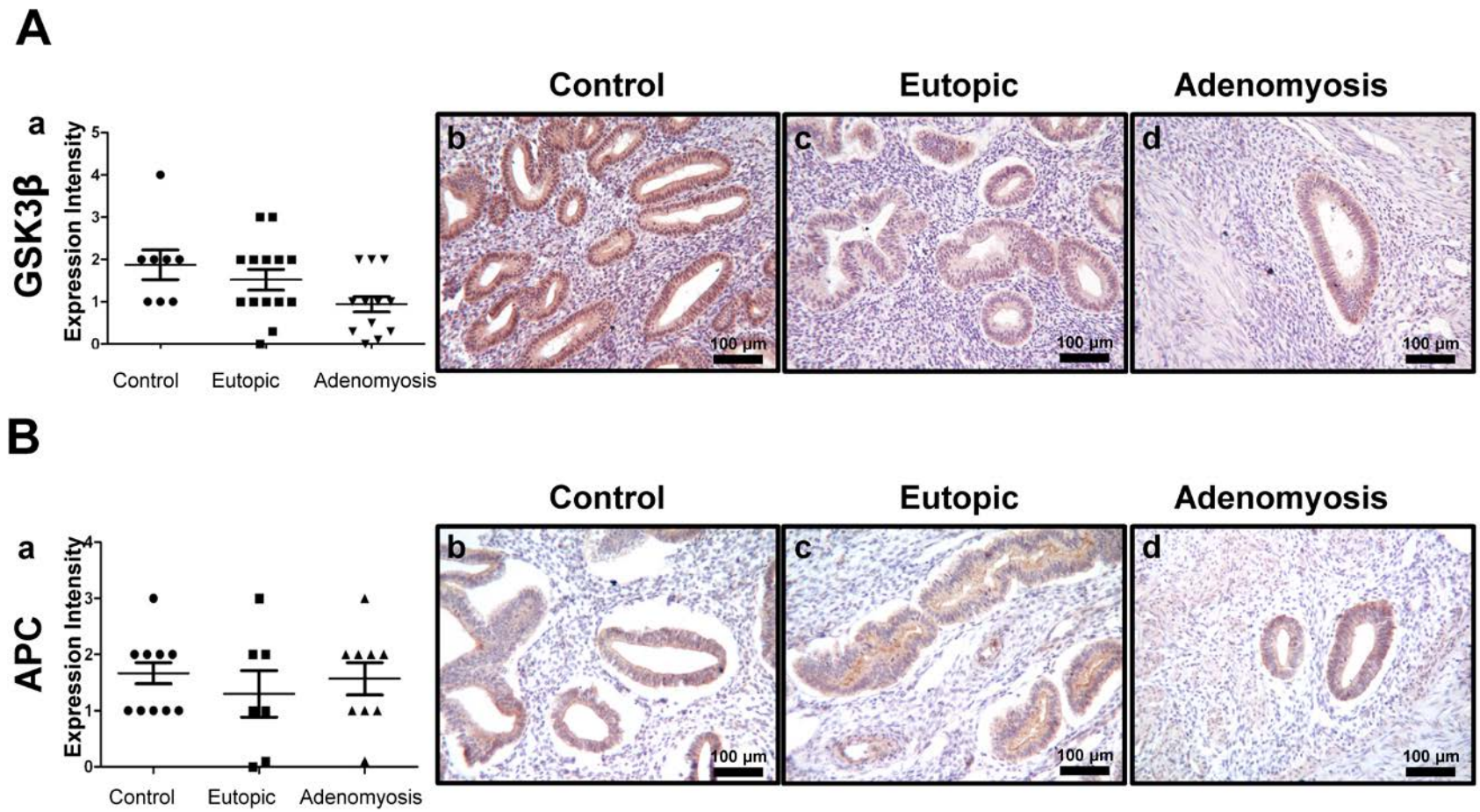
A
a
GSK3β
Expression Intensity
0
1
2
3
4
5
Control
Eutopic
Adenomyosis
Control
Eutopic
Adenomyosis
b
c
d
100 μm
100 μm
100 μm
B
a
APC
Expression Intensity
0
1
2
3
4
Control
Eutopic
Adenomyosis
Control
Eutopic
Adenomyosis
b
c
d
100 μm
100 μm
100 μm

Figure 8. The intensity of GSK3β and APC expressions in endometrial tissue with and without adenomyosis. (A) The intensity of GSK3β was not statistically significant difference in human endometrium with (b and c) and without adenomyosis, but was decreased in human endometrium with adenomyosis. (B) APC was not different in human endometrium with (b and c) and without adenomyosis. Scale bar = 100 μm.

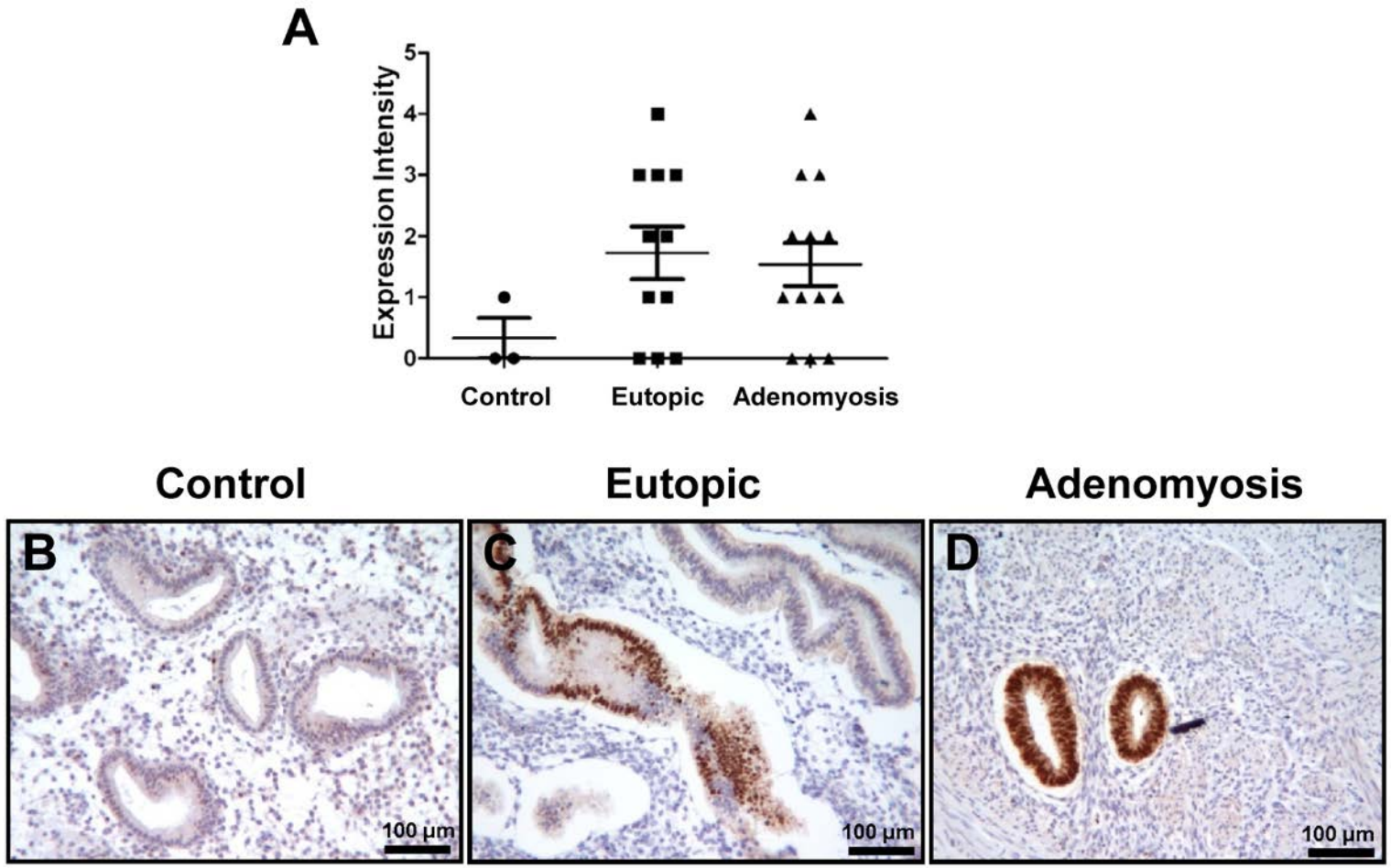
A
Expression Intensity
5
4
3
2
1
0
Control
Eutopic
Adenomyosis
Control
Eutopic
Adenomyosis
B
C
D
100 μm
100 μm
100 μm

Figure 9. The intensity of Cyclin D1 expression in endometrial tissue with and without adenomyosis. The intensity of Cyclin D1 is induced in adenomyosis although the result shows no statistically significant increases. Scale bar = 100 μm.

2) Myometrial defects in the mice with uterine specific stabilization of β-catenin

To determine if abnormal activation of β-catenin in the murine uterus develops adenomyosis, *Ctnnb1*$^{f(ex3)/+}$ mice were crossed with *PR*^{cre} mice in PR-expressing cells (*PR*$^{cre/+}$ *Ctnnb*$^{f(ex3)/+}$) (Jeong et al., 2009). Our previous data from showed that mutant mice (*PR*$^{cre/+}$ *Ctnnb*$^{f(ex3)/+}$) resulted in infertility, hormone insensitivity, and endometrial glandular hyperplasia (Jeong et al., 2009). In addition to endometrial hyperplasia, we observed an increase of β-catenin levels and nuclear localization in the myometrium and epithelium in the uterus of mutant mice compared to control mice (*PR*$^{cre/+}$ *Ctnnb*$^{f(ex3)/+}$) (Fig. 10). In the uterus of the mutant (*PR*$^{cre/+}$ *Ctnnb*$^{f(ex3)/+}$) mice, we observed an increase of β-catenin levels and nuclear localization in the myometrium and epithelium compared to control mice (*PR*$^{cre/+}$ and *Ctnnb*$^{f(ex3)/+}$) (Fig. 10). Histological assessment of uteri from two month old mutant mice revealed severe defects in the myometrium. To investigate these defects in more detail, smooth muscle actin (α-SMA) immunohistochemistry was employed to specifically mark the myometrium. Control mice showed regular concentric layers of smooth muscle cells (Fig. 11Aa and Fig. 12A). However, mutant mice exhibited an abnormal, irregular structure which exhibits an interwoven pattern and scattering of α-SMA positive cells in the myometrium (Fig. 11Ab and Fig. 12D). The thickness of myometrium area is significantly increased in mutant mice (189.85 ± 62.09 μm, n=11) compared with control mice (93.88 ± 34.6 μm, n=10) (Fig. 11Ac). In order to determine the extent of cell proliferation in the myometrium of mutant mice, we performed immunohistochemical staining for phospho-Histon H3, a mitotic marker, in control and mutant mice at two months of age. Immunohistochemical staining of phospho-Histone H3 demonstrated that

proliferation was significantly increased in the myometrium of mutant mice (4.43 ± 0.63 %, n=8) compared to control mice (0.25 ± 0.17 %, n=10) (Fig. 11Bc). We also observed the colocalization of myometrium and proliferative cells by using α-SMA and Ki67 as proliferative marker in control and mutant mice (Fig. 12). There are not positive-Ki67 cells in the myometrium region of control mice. However, we examined the positive-Ki67 cells in the myomterium of mutant mice, and these proliferative cells were not coexpressed with myometrial cells. These results suggest that activation of β-catenin in the uterus exhibited abnormal irregular structure and increased cell proliferation at the myometrium. The origin of proliferative cells is not from myometrium.

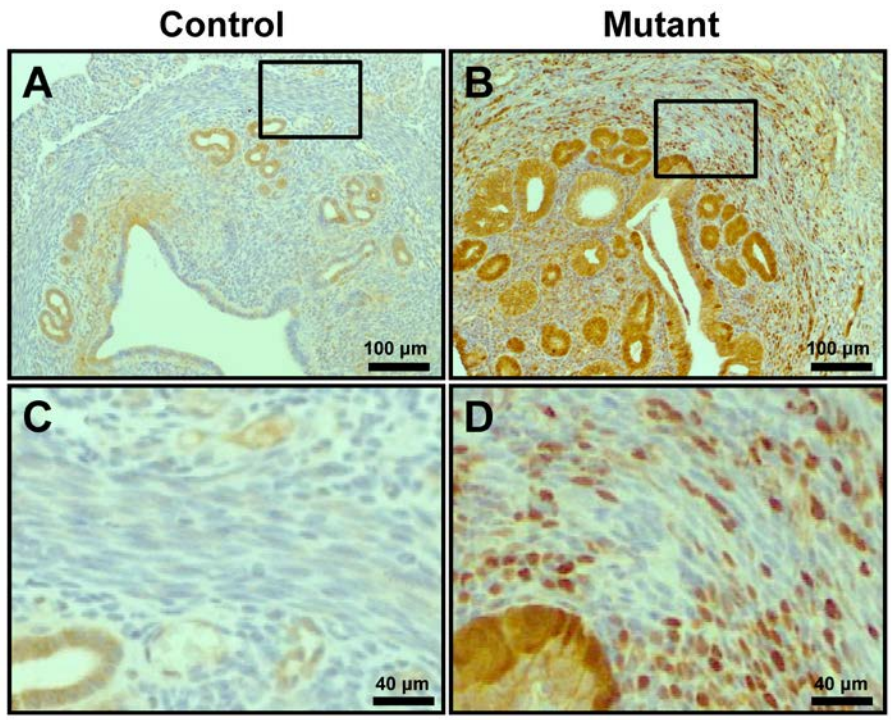

Figure 10. Analysis of conditionally dominant stabilized β-catenin in the murine uterus. Six week old control mice and mutant mice were sacrificed. Immunohistochemical analysis for β-catenin demonstrated cytoplasmic β-catenin was observed in control mice (A and C), but nuclear accumulation of β-catenin in the epithelium and myometrium of mutant mice uterus (B and D). C and D are high magnification pictures (Scale bar = 40 μm) of the boxed areas in A and B (Scale bar = 100 μm).

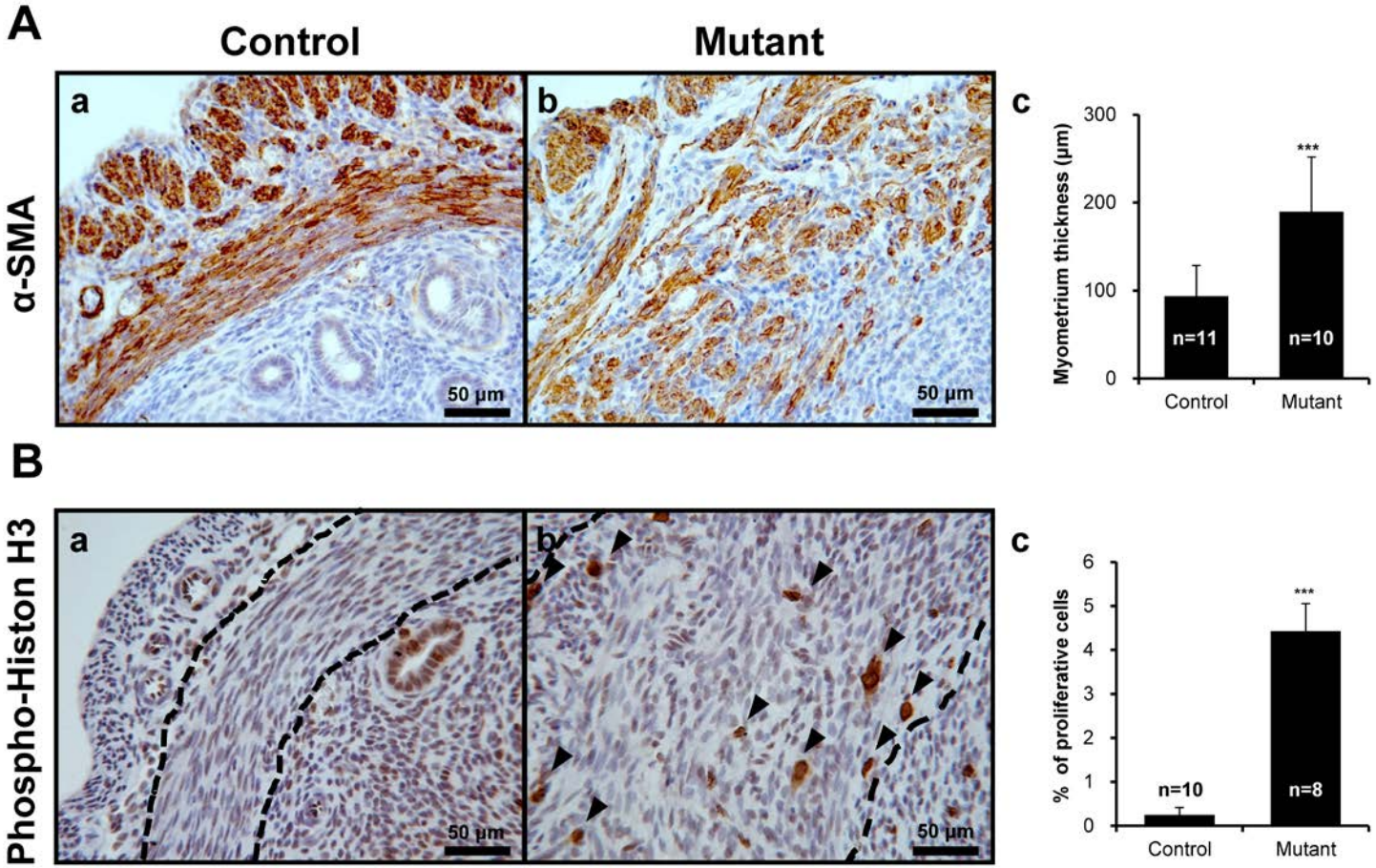
A
Control
Mutant
α-SMA
a
b
50 µm
50 µm
c
Myometrium thickness (µm)
300
200
100
0
***
n=11
n=10
Control
Mutant
B
Phospho-Histon H3
a
b
50 µm
50 µm
c
% of proliferative cells
6
5
4
3
2
1
0
***
n=10
n=8
Control
Mutant

Figure 11. Abnormal irregular structure and highly active proliferation in myometrium of uteri of mutant mice. (A) Immunohistochemical localization for smooth muscle actin (α-SMA), a smooth muscle cell marker, was examined in uterus of control (a) and mutant (b). The myometrial area of mutant mice was significantly increased (c) and revealed abnormal irregular structure. (B) Phospho-histone H3 was used for identification of proliferationin control (a) and mutant mice (b). Proliferative ability was increased in myometrium of mutant mice compared with control mice (c). Arrowheads indicate positive-phospho-histone H3 cells. Dashed lines indicate inner circular layer of myometrium. ***, $p< 0.001$, one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple range test. Scale bar = 50 μm.

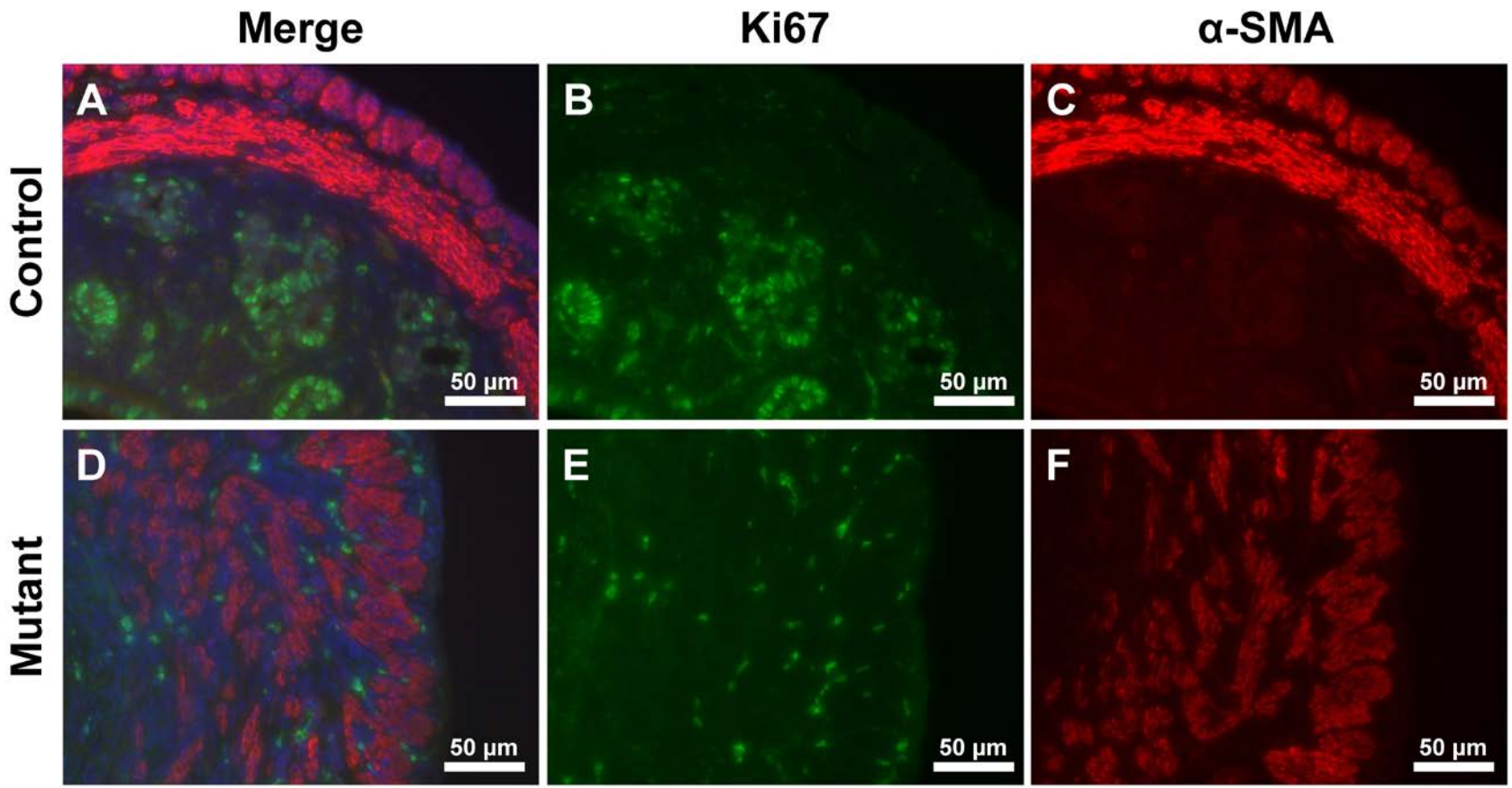
Merge
Ki67
α-SMA
Control
Mutant
A
B
C
D
E
F
50 μm
50 μm
50 μm
50 μm
50 μm
50 μm

Figure 12. Irregular structure and highly active proliferation in myometrium of uteri of mutant mice. α-smooth muscle actin (α-SMA; red) was used as a smooth muscle cell marker and Ki67 (green) was used as a proliferative cell marker. The myometrial area of mutant mice were increased and revealed abnormal irregular structure. The proliferative cells were not co-expressed with positive α-SMA cells in the myometrium of mutant mice. Scale bar = 50 μm.

3) Development of adenomyosis in the mice with uterine specific stabilization of β-catenin

In addition to the abnormal myometrial structure, mutant mice subsequently showed glands and stromal cells in the myometrium which is adenomyosis (Fig. 13). To identify the progression of adenomyosis in mutant mice, we sacrificed control mice and mutant mice at two, four and six months of age (n=20 per age per genotype), and investigate histology by using H&E staining. Histological analysis showed control mice had defined endometrium and regular myomterium but mutant mice exhibited abnormal endometrium, myomterium and adenomyosis development. In four month-old mutant mice, the incident of adenomyosis was 40% and endometrial glandular hyperplasia was observed in all mutant mice. At six months of age, 80% of mutant mice had abnormal glandular hyperplasia and adenomyosis in myomtrium. This histology of mutant mice having endometrial glandular hyperplasia and adenomyosis is very similar to human adenomyosis. Whereas human uterus of adenomyosis including hyperplasia of the smooth uterine musculature is 2-3 times the normal size (Devlieger et al., 2003), there were no significant difference between uterus/body weight ratio of control and mutant ($PR^{cre/+}Ctnnb1^{f(ex3)/+}$) mice at 4 month (3.95 ± 0.83 % vs. 3.15 ± 0.23 %) and 6 month (5.14 ± 0.87 % vs. 3.47 ± 0.28 %) of age although the length of mutant uteri was smaller compared to control mice. In histological results, the well-constructed endometrium and myometrium were seen in control mice but, enlarged glands and thickened myometrium were found in mutant mice. Another feature of mutant mice was adenomysis development in loose myometrium. In immunofluorescence analysis, we demonstrated the expression of E-cadherin as epithelial cells marker and smooth muscle actin (α-SMA) (Fig. 14). In these results, the epithelial cells

were located in myometrium region of mutant mice. These results suggest that β-catenin signaling plays an important role as a regulator of the endometrial growth homeostasis, and abnormal activation of β-catenin in the uterus results in adenomyosis development.

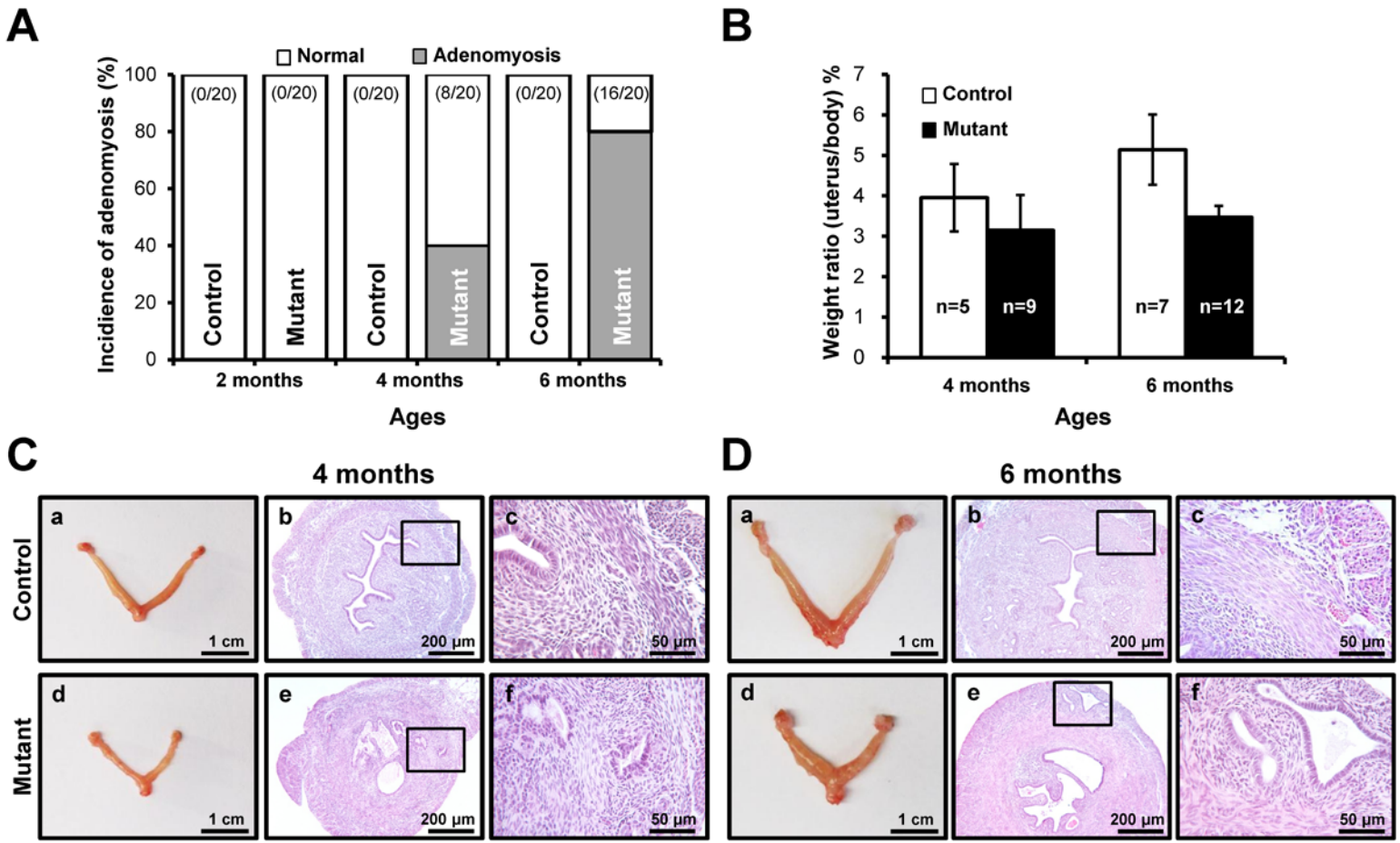
A
Normal
Adenomyosis
Incidience of adenomyosis (%)
100
80
60
40
20
0
(0/20)
(0/20)
(0/20)
(8/20)
(0/20)
(16/20)
Control
Mutant
Control
Mutant
Control
Mutant
2 months
4 months
6 months
Ages
B
Control
Mutant
Weight ratio (uterus/body) %
7
6
5
4
3
2
1
0
n=5
n=9
n=7
n=12
4 months
6 months
Ages
C
4 months
D
6 months
Control
Mutant
a
b
c
d
e
f
1 cm
200 μm
50 μm

Figure 13. Development of adenomyosis in the mice with uterine specific stabilization of β-catenin. (A) Incidence of adenomyosis development was scored in control mice and mutant mice. Control and mutant mice at two, four and six months of age were sacrificed. (B) Uterine weight was determined for control and mutant mice at 4 and 6 month of age. The uterine weight was not different between control and mutant mice. (C and D) Gross anatomy and histology of control and mutant mice uteri at four (C) and six (D) months of age. Uterine length of mutant mice was smaller than control mice but same thickness compared to control mice. In histology analysis, adenomyosis phenotype was observed in mutant mice. (a and d) Scale bar = 1 cm. (b and e) Scale bar = 200 μm. (c and f) Scale bar = 50 μm.

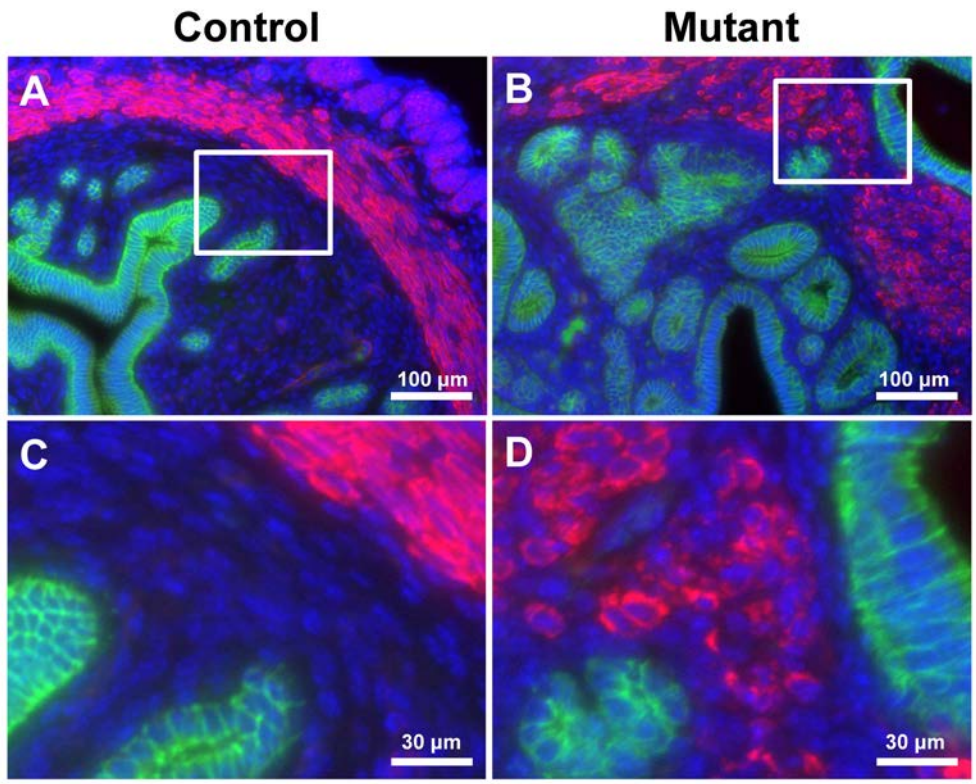

Figure 14. Abnormal structures in endometrium and myometrium of uteri of mutant mice. At four month of age, control mice and mutant mice were sacrificed. Immunofluorescence analysis for E-cadherin (green) and α-SMA (red) demonstrated E-cadherin expression was observed in myometrium of mutant mice (B and D). The image of control mice is A and C. B and D show the E-cadherin and α-SMA of mutant mice. C and D are high magnification pictures (Scale bar = 30 μm) of the boxed areas in A and B (Scale bar = 100 μm).

4. Discussion

Adenomyosis is one of gynecological disorder. Adenomyosis symptoms include menorrhagia, dyspareunia, dyschezia, dysmenorrhea and chronic pelvic pain. The prevalence of adenomyosis is 10 % to 66 % in women (Vercellini et al., 2006). Although the prevalence of adenomyosis is very frequent, the precise etiology and pathophysiology are still unknown. Moreover, there is no good treatment to date except hysterectomy. Hysterectomy for adenomyosis treatment results in costly health care and may not be an option for those women wishing to maintain future fertility.

In the previous study, we identified that activation of β-catenin plays a critical role in the uterine function and the development of endometrial hyperplasia and cancer (Jeong et al., 2009). These results suggest that the tight regulation of β-catenin is important in physiological uterine function. In this study, we identified that activation of WNT/β-catenin signal related molecules plays an important role in the pathogenesis of adenomyosis. In absence of WNT signal, a complex including GSK3β, Axin, adenomatous polyposis coli (APC) leads to degrade β-catenin. However, in the presence of WNT signal, GSK3β is inactivated and the cytoplasmic β-catenin is accumulated. As β-catenin is increased in the cytoplasm, β-catenin translocates into the nucleus and it binds with TCF/LEF and activates WNT target genes such as Cyclin D1 (Molenaar et al., 1996; van de Wetering et al., 2002; Daniels and Weis, 2005). The uterine specific β-catenin activation promoted abnormal irregular structure in the myometrium and proliferation was significantly increased in the myometrium of mutant mice compared to control mice (Fig. 11 and Fig. 12). The mice with conditional uterine activation of β-catenin developed

adenomyosis and provide a novel model system to investigate the genetic and molecular events involved in the transition from normal to adenomyosis. Constitutive activation of β-catenin in the uterine mesenchyme (*Amhr2*$^{cre/+}$ *Ctnnb*$^{f(ex3)/+}$) also shows myometrial hyperplasia, develop mesenchymal tumors and causes occasional adenomyosis (Tanwar et al., 2009). WNT/β-catenin signaling molecules are important and should be tightly regulated for uterine function (Arango et al., 2005; Li et al., 2005a; Mohamed et al., 2005; Rider et al., 2006; Jeong et al., 2009). Therefore, our results suggest that aberrant β-catenin activation is important to etiology of adenomyosis.

CHAPTER 4

Epithealial and mesenchymal transition is associated with adenomyosis development

1. Introduction

Dysregulation of WNT/β-catenin signaling leads to aberrant activation of the target genes, associated to epithelial-mesenchymal transition (EMT) (Schmalhofer et al., 2009). During the process of EMT, the characterization of epithelial cells is lost and the cells gain migratory and invasive properties to become mesenchymal cells (Polyak and Weinberg, 2009). EMT is a crucial for several developmental processes including mesoderm formation and neural tube formation. EMT has also been known as the necessary course for wound healing, and the initiation of metastasis for cancer progression. Metastasis undergoes a multistep process including dissociation of tumor cells from the epithelial layer, penetration through the basement membrane into the adjacent connective tissue, intravasation, survival in the bloodstream, and growth of metastatic cells in the distant organ with stimulation of neoangiogenesis.

β-catenin is associated in EMT regulation by two mechanisms. First, β-catenin regulates transcriptional factors such as ZEB2, SNAI1, SNAI2, Twist1, Twist2, and E12/E47 (Polyak and Weinberg, 2009), which repress E-cadherin. Another function of β-catenin is an element of adherens junctions with E-cadherin. Nonetheless, these phenomena are poorly known in reproductive biology. Specially, the etiology of adenomyosis is not well known. In the adenomyosis development, we hypothesis this EMT phenomena is involved in the process of adenomsyosis. Therefore, we tried to investigate the molecular mechanisms whereby activation of β-catenin induces EMT and contributes to the development of adenomyosis in human adenomyosis and uterine specific β-catenin activated mice.

2. Materials and methods

1) Immunohistochemistry

Uterine sections from paraffin-embedded tissue were cut at six μm and mounted on silane-coated slides, deparafinized and rehydrated in a graded alcohol series. Sections were preincubated with 10 % normal goat serum in PBS (pH 7.5) and then incubated with COUP-TFII (1:1000; Perseus Proteomics, Tokyo, Japan), and Snail (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibodies in 10% normal serum in PBS (pH 7.5) overnight at 4 °C. On the following day, sections were washed in PBS and incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) for one hour at room temperature. Immunoreactivity was detected using DAB (Vector Laboratories). Immunohistochemical staining with anti-E-cadherin (1:1000; BD bioscience, San Jose, CA, USA) was carried out using the Vector Mouse on Mouse (MOM) kit (Vector Laboratories) following the manufacturer's instructions, to minimize cross reactivity between the mouse secondary antibody and mouse tissues. Briefly, sections were blocked overnight with MOM IgG blocking solution in PBS, washed twice for five minutes with PBS, incubated with MOM solution in PBS for 30 min, incubated with anti-E-cadherin antibodies overnight at 4 °C. On the following day, sections washed in PBS, incubated with secondary biotinylated anti-mouse (Vector Laboratories) for one hour at room temperature then washed, detected using the Vectastain Elite ABC system (Vector Laboratories). The sections were counterstained with hematoxylin, dehydrated, and mounted. Immunostained sections were examined by light microscopy.

2) Immunofluorescence

Uterine sections from paraffin-embedded tissue were cut at six μm and mounted on silane-coated slides, deparafinized and rehydrated in a graded alcohol series. For maximal sensitivity of ZEB1 (1:2000), Vimentin (1:500; Invitrogen, Carlsbad, CA, USA), COUP-TFII (1:1000; Perseus Proteomics), or CD10 (1:500; Novocastra, Burlingame, CA, USA) we used tyramide signal amplification (TSA) immunofluorescence (TSA kit; PerkinElmer, Waltham, MA, USA) following the manufacturer's protocol. Sections were preincubated with 1% blocking solution from the TSA kit in PBS and 0.01% Triton X-100 (PBST), and then incubated with anti-ZEB1, anti-COUP-TFII and anti-CD10 antibodies in PBST overnight at 4 °C. On the following day, sections were washed in PBST and incubated with biotinylated secondary antibody (Vector Laboratories) for one hour at room temperature. Immunoreactivity was detected using the Vectastain Elite ABC system. After five -minute TSA reaction, slides were washed in PBST and then incubated E-cadherin (1:1000; BD bioscience), Keratin 18 (1:500; Proteintech, Chicago, IL, USA), CD3 (Santa Cruz Biotechnology Inc), CD4 (Santa Cruz Biotechnology Inc.) or CD11b-FITC (Abcam, Cambridge, MA, USA) overnight at 4°C. On the third day, sections were washed in PBST and incubated with Alexafluor conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) for two hours at room temperature. Slides were counterstained by Vectashield mounting media with DAPI (Vector Laboratories). Images were captured with a confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA) or fluorescent microscope (Nikon Instruments Inc., Melville, NY, USA) and processed using the image programs inherent in the microscope system (Carl Zeiss Meditec, Inc.).

3) Cell culture

Human endometrial epithelial cells (HEC-1A) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin (Gibco BRL), and 0.1 mg/ml streptomycin (Gibco BRL) at 37°C under 5% CO_2. Michigan Cancer Foundation-7 (MCF-7) cells were cultured in opti-MEM media (Gibco BRL) containing 10% fetal bovine serum (FBS; Gibco BRL), 1% Na-pyruvate (Gibco BRL), and 1% antibioitic-antimycotic (Gibco BRL).

4) Transfection

The plasmid constructor of exon 3-deleted β-catenin was kindly provided by Dr. John Lydon at Baylor College of Medicine, Houston, TX. β-catenin expression vectors were generated by PCR and cloned into the pcDNA3.1-V5 (Invitrogen Corp.) plasmid vectors. Overexpressing β-catenin were prepared by transfecting HEC-1A and MCF-7 cells cells with plasmid vectors containing exon 3-deleted β-catenin using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. After twenty-four hours post-transfection, the cells were harvested or used for doing immunofluorescence.

5) Immunofluorescence after transfection

Transfected cells were washed with PBS and were fixed in 4% PFA (vol/vol) for 30 minutes. Following three washes with PBS, the preparations were incubated at 37°C with anti-β-catenin (1:1000; BD bioscience) and ZEB1 (1:500; Santa Cruz

Biotechnology Inc.) with PBST in incubator for 2 hours. The cells were then washed in PBST another three times for 10 minutes each and incubated for 2 hours with Alexa fluor conjugated secondary antibodies (Sigma-Aldrich) diluted with PBST. After cells washed with PBST, the preparations were counterstained by Vectashield mounting media with DAPI (Vector Laboratories). Images were captured with a confocal microscope (Carl Zeiss, Inc.) and processed using the image programs inherent in the microscope system (Carl Zeiss Meditec, Inc.).

6) Western blot

Transfected cells were washed with cold PBS, scraped off, and harvested. Cells were then incubated for 20 min in lysis buffer containing 0.5% triton X-100, 20mM HEPES (pH7.4), 150mM NaCl, 2mM DTT, and 1mM PMSF. The lysates were centrifuged at 20 000 xg for 10 min at 4°C. The protein concentrations of clarified lysates were determined with the Bradford assay, with BSA as a reference. Samples containing 20 μg proteins were applied to SDS-PAGE. The separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membranes were blocked overnight with 0.5% casein (wt/vol) in PBS with 0.1% Tween 20 (vol/vol) (Sigma–Aldrich) and probed with anti-β-catenin (1:1000; BD bioscience), E-cadherin (1:3000; BD bioscience), ZEB1 (1:1000; Santa Cruz Biotechnology Inc.), and COUP-TFII (1:1000; Perseus Proteomics) antibodies. Immunoreactivity was visualized by incubation with peroxidase-labeled goat anti-mouse IgG, anti-rabbit IgG or anti-goat IgG and treatment with ECL reagents (Amersham, Piscataway, NJ, USA) followed by exposure to X-ray film. To control for loading, the membrane was stripped and probed with anti-actin (Santa Cruz Biotechnology Inc.) and developed again.

Image J software was used for quantification of western blot results.

3. Results

1) Epithelial to mesenchymal transition in adenomyosis development

In order to determine if the adenomyosis regions have endometrial epithelial cells in the mutant mice, we performed immunohistochemistry for E-cadherin, an epithelial cell marker (Kurihara et al., 2007; Lee et al., 2010). As shown in Figure 15, we detected E-cadherin positive epithelial cells in the adenomyosis regions of mutant mice but not the myometrium of control mice at six months. Interestingly, the expression of E-cadherin was lower in epithelial cells of eutopic (Fig. 15Ad) and adenomyosis regions (Fig. 15Ae) of mutant mice compared to control mice (Fig 15Aa and c). Repression of E-cadherin is one of the hallmarks of epithelial mesenchymal transition (EMT) (Polyak and Weinberg, 2009). EMT is a process by which epithelial cells lose their cell-cell adhesion and polarity, and they acquire mesenchymal and migratory properties. EMT plays an important role in embryogenesis, fibrosis, and tumor metastasis (Acloque et al., 2009). EMT is initiated by a number of transcription factors, including Snail, Slug, Twist, ZEB1, and SIP1, via the repression of E-cadherin expression (Thiery, 2002). Due to the invasive behavior and cytoskeletal rearrangement of endometrial epithelial cells during ectopic implantation, we reasoned that EMT might be involved in adenomyosis development observed in mutant mice. We performed immunohistochemistry to distinguish the expression of Snail between control and mutant mice (Fig. 15B). In our results, Snail-positive cells were not detected in control uteri (Fig 15Ba and c), but we observed Snail expression in the epithelial cells of mutant uteri but not stromal cells (Fig 15Bb, d and e).

Using immunofluorescence analysis, we demonstrated the expression of ZEB1 and E-cadherin in control and mutant mice. Interestingly, although the number of ZEB1-positive epithelial cells was sparse, the expression of ZEB1, which is a transcriptional repressor of E-cadherin, was observed in some epithelial cells of the uterus in mutant mice (Fig. 16E and G). The loss of E-cadherin expression was observed in ZEB1-positive epithelial cells (Figure 16E and F). In control mice, positive ZEB1 expression was limited in the majority of stromal and myometrium (Fig. 16A and C). These results indicated that EMT is related to the adenomyosis.

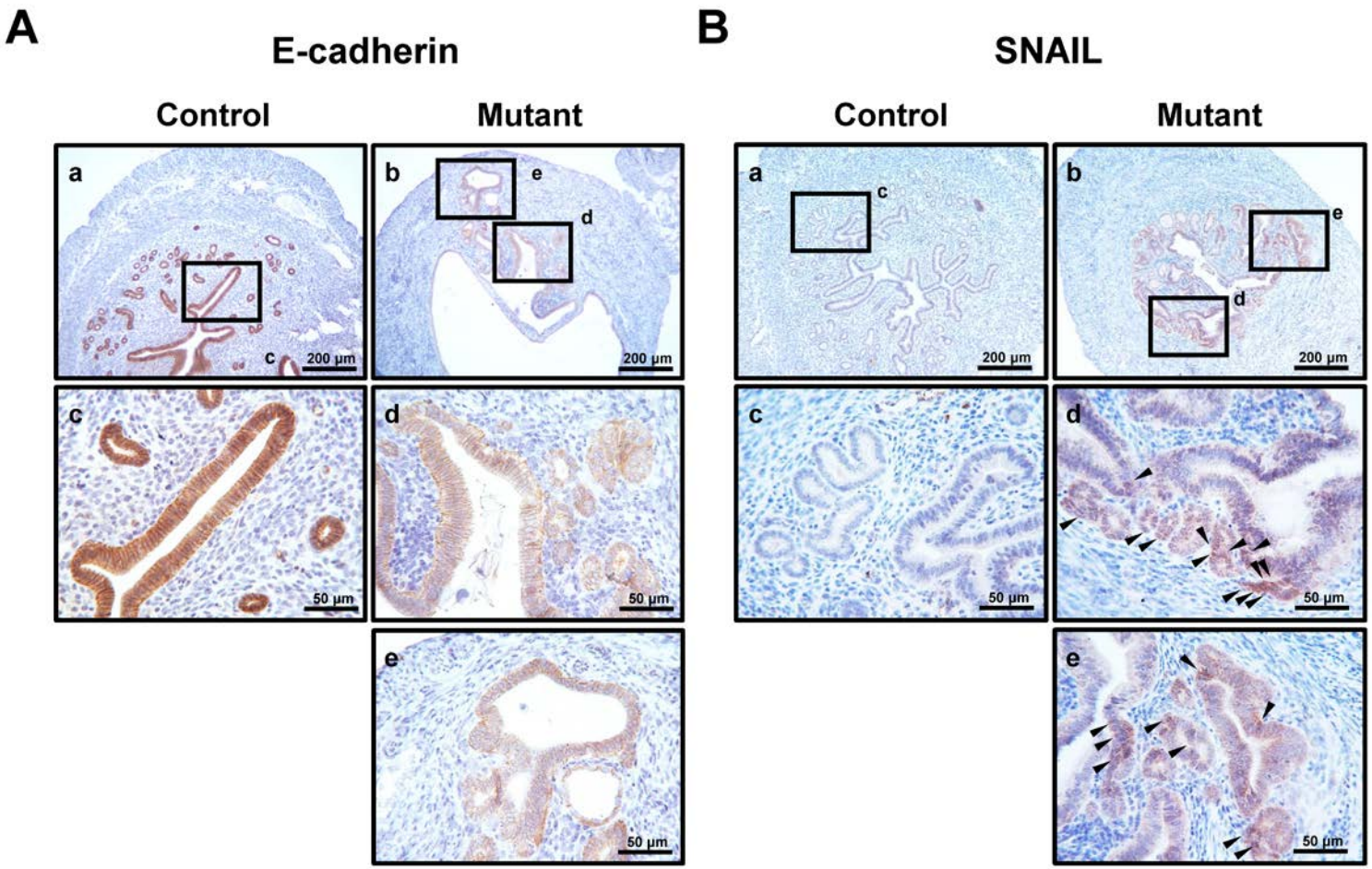
A
E-cadherin
Control
Mutant
a
b
c
d
e
200 µm
50 µm
B
SNAIL
Control
Mutant
a
b
c
d
e
200 µm
50 µm

Figure 15. The expressions of E-cadherin and Snail in the uterus of control and mutant mice. (A) Immunohistochemical analysis of E-cadherin demonstrated suppression of E-cadherin expression at epithelial cells in mutant mice (b, d and e) compared with control mice (a and c) at four months. (B) Immunohistochemical analysis of Snail demonstrated induction of Snail at epithelial cells in mutant mice (b, d and e) but not in control mice (a and c) at four months. (a and b) Scale bar = 200 μm. (c, d, and e) Scale bar = 50 μm.

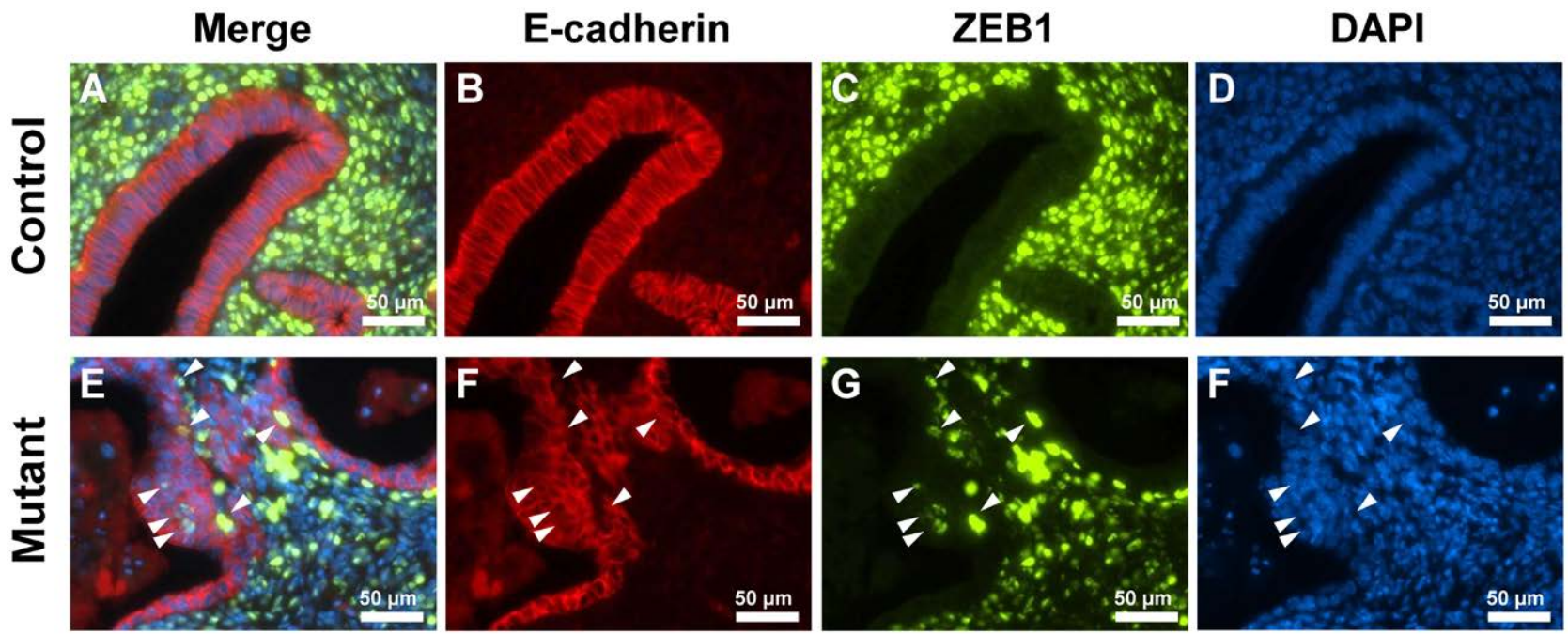

Figure 16. The expression of ZEB1 in uterus of control mice and mutant mice. Immunofluorescence analysis of E-cadherin (red; B and F) and ZEB1 (green; C and G) was performed in control mice (A-D) and mutant (E-H) mice. The expression of ZEB1 was observed in epithelial cells of the uterus in mutant mice but not control mice. Merged images (A and E) and DAPI images (D and F). Arrowheads indicate positive-ZEB1 cells. Scale bar = 50 μm.

3) The expression of mesenchymal cell markers in adenomyosis development

Vimentin is an intermediate filament protein and is ubiquitously found in a variety of mesenchymal cells. Several reports have investigated that Vimentin is overexpressed in various epithelial cancers, including prostate cancer, breast cancer, malignant melanoma, and lung cancer (Upton et al., 1986; Gilles et al., 2003; Zhao et al., 2008). In our data, Vimentin was limited in stromal cells of control mice, but Vimentin expression was observed and upregulated in some epithelial cells of mutant mice (Fig.17). Moreover, Cytokeratin 18 expression was decreased in the Vimentin-positive epithelial cells of mutant mice (Fig.17).

Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) is highly expressed in the mesenchymal cells and plays critical roles during mouse development (Lee et al., 2010). In the female uterus, COUP-TFII is expressed in uterine stromal cells and the myometrium but not epithelial cells (Kurihara et al., 2007). The expression of COUP-TFII was observed in 29.50 ± 2.24 % eutopic epithelial cells and 13.36 ± 4.95 % adenomyotic epithelial cells of mutant mice (n=7) (Fig. 18B and C) while its expression was limited in endometrial stromal cells and the myometirum of control mice (0.50 ± 0.26 %, n=9) (Fig. 18A). These results suggest that abnormal activation of β-catenin contributes to adenomyosis development through induction of mesenchymal markers in epithelial cells.

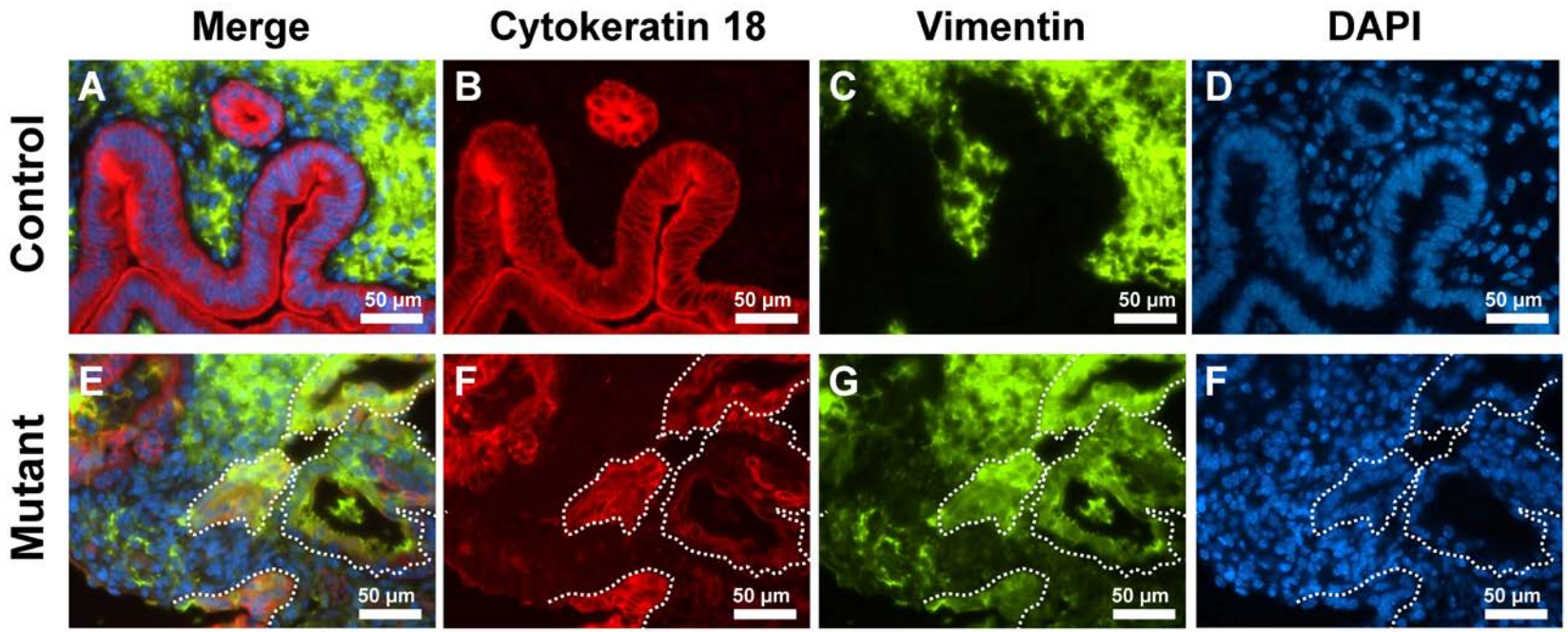

Figure 17. The expression of Vimentin in uterus of control mice and mutant mice. Immunofluorescence analysis of Cytoketratin 18 (red; B and F) and Vimentin (green; C and G) was performed in control mice (A-D) and mutant (E-H) mice. The expression of Vimentin was observed in epithelial cells of the uterus in mutant mice but not control mice. White dotted lines indicate positive-Vimentin cells in epithelial cells. Scale bar = 50 μm.

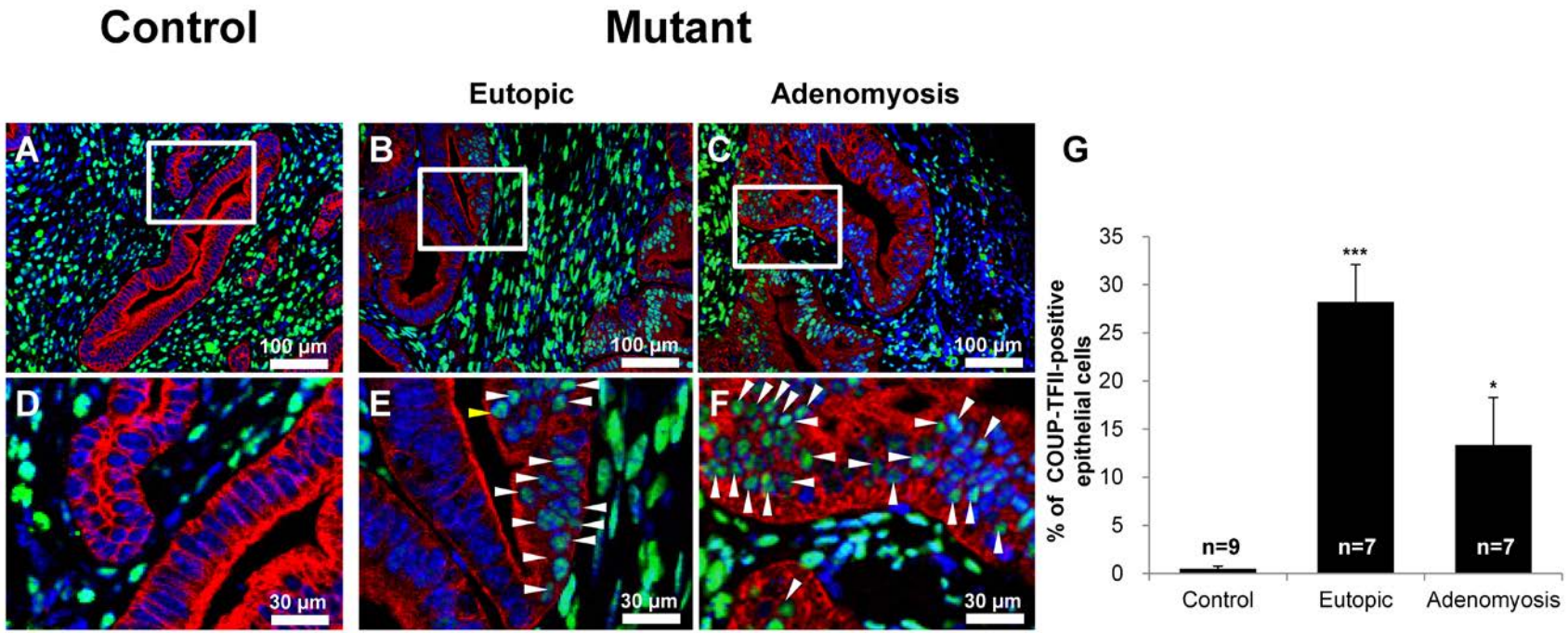
Control
Mutant
Eutopic
Adenomyosis
A
B
C
D
E
F
100 µm
100 µm
100 µm
30 µm
30 µm
30 µm
G
% of COUP-TFII-positive epithelial cells
35
30
25
20
15
10
5
0
***
*
n=9
n=7
n=7
Control
Eutopic
Adenomyosis

Figure 18. The expression of COUP-TFII in uterus of control and mutant mice. Immunofluorescence analysis of COUP-TFII (green) and Cytokeratin 18 (red) was performed in uterus of control (A and D) and mutant (B, C, E, and F) mice. COUP-TFII was observed in epithelial cells of mutant mice (B, C, E, and F) while its expression was limited in endometrial stromal cells and the myometrium of control mice (A and D). D, E, and F are high magnification pictures (Scale bar = 30 μm) of the boxed areas in A, B, and C (Scale bar = 30 μm). Arrowheads indicate positive-COUP-TFII cells. G showed COUP-TFII-positive cells were significantly increased in mutant mice. *, $p< 0.05$; ***, $p < 0.001$, one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple range test.

4) Epithelial to mesenchymal transition in human adenomyosis

To elucidate the impact of EMT in human adenomyosis, immunohistochemistry for E-cadherin, COUP-TFII and CD10 was performed with control endometrium (proliferative phase, n=13; secretory phase, n=11) and eutopic endometrium (proliferative phase, n=6; secretory phase, n=7) and adenomyosis region (proliferative phase, n=8; secretory phase, n=11) from patients with adenomyosis. As shown in the mutant mice (Fig. 15A), eutopic endometrium and adenomyosis region from patients with adenomyosis showed decreased E-cadherin level in epithelial cells compared to control endometrium (Fig. 19A). To evaluate the expression of CD10 in human endometrium with or without adenomyosis, we performed immunofluorescense analysis for CD10. The expression of CD10 was observed in some epithelial cells of eutopic endometrium and adenomyosis region, while its expression was limited in endometrial stromal cells in control endometrium (Fig. 19B). To confirm CD10-positive cells are not present in infiltrating lymphocytes rather than epithelial cells, we examined the expression of CD3, CD4 and CD11b in CD10 positive cells. CD3 is expressed on T helper and T cytotoxic lymphocytes (Chuang et al., 2001; Jedryka et al., 2012) and CD4 is expressed on T helper lymphocytes (Nelson, 2008; Yamagami et al., 2011). CD10 positive cells were neither CD3 nor CD4 positive cells in human endometrium with adenomyosis (Fig. 20 and Fig. 21). CD11b found on monocytes, macrophages, granulocytes, some B cells, dendritic cells, and natural killer cells (Khanh do et al., 2011). CD10 positive cells were also CD11b negative cells (Fig. 22). Lymph node was used as positive control of CD3, CD4 and CD11b immunostaining. These results confirmed that CD10-positive cells are epithelial cells but not infiltrating lymphocytes. We also performed immunohistochemistry analysis for COUP-TFII.

The expression of COUP-TFII was observed in some epithelial cells of eutopic and adenomyotic region (Fig. 23).

Moreover, several reports have associated to the loss of E-cadherin expression, and the increased nuclear β-catenin expression in tumorigenesis (Fukuchi et al., 1998; Clevers, 2006). To gain insight into the participation of activated β-catenin in EMT, we overexpressed β-catenin with transfection of exon 3-deleted β-catenin vector into MCF-7 cells using Lipofectamine 2000, and observed the expression of β-catenin and ZEB1. Deletion of exon 3 of the β-catenin resulted in nuclear β-catenin, and ZEB1 induction (Fig. 24). We also examined β-catenin, E-cadherin, ZEB1, and COUP-TFII by western blot in HEC1A cells after β-catenin activation. E-cadherin expression was downregulated in β-catenin overexpressed cells with deletion of β-catenin exon 3. By contrast, the expression of the mesenchymal maker ZEB1 and COUP-TFII increased (Fig. 25). These results suggest that EMT may occur within adenomyosis development in human.

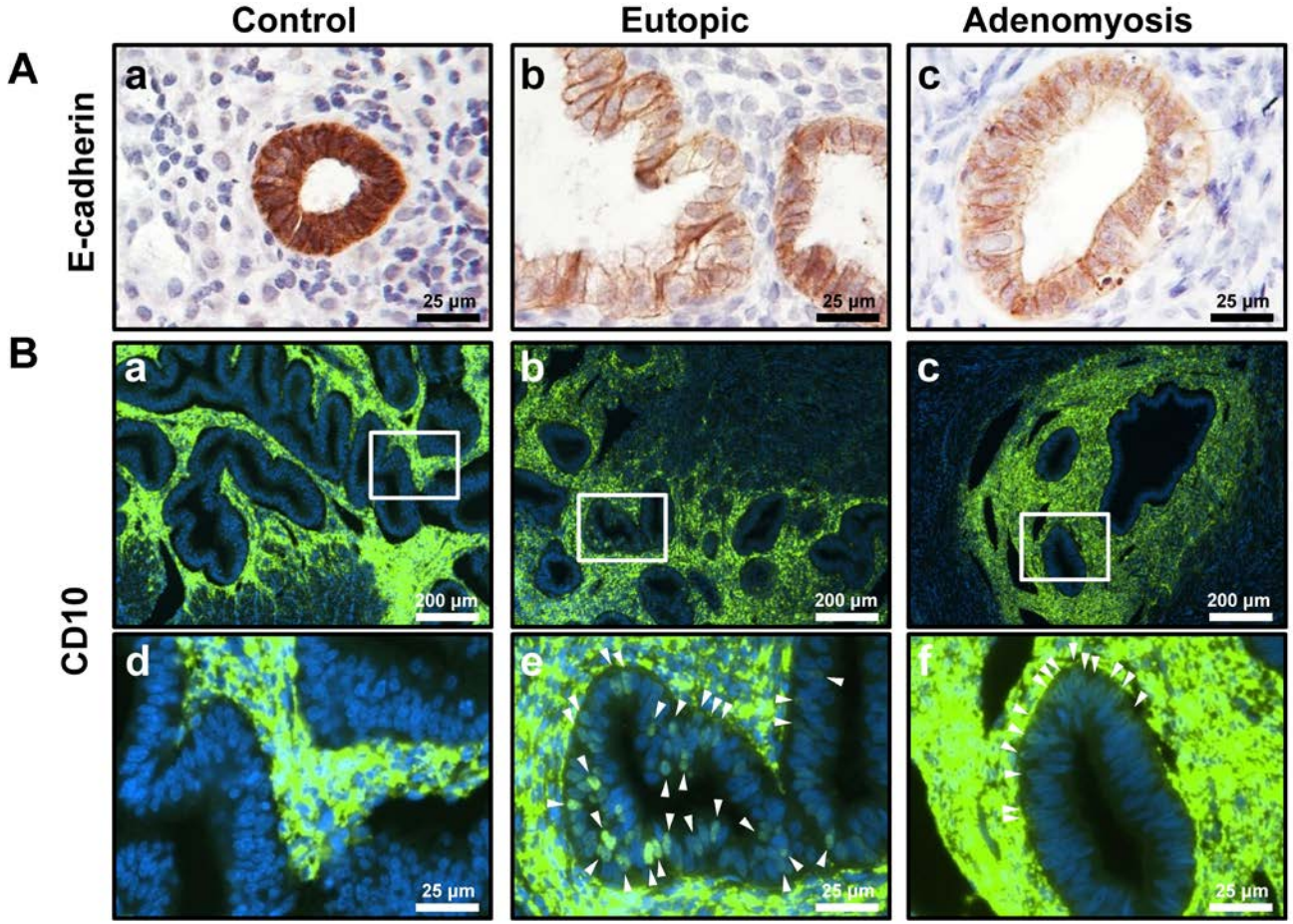
Control
Eutopic
Adenomyosis
A
E-cadherin
a
b
c
25 μm
B
CD10
a
b
c
200 μm
d
e
f
25 μm

Figure 19. Repression of E-cadherin and induction of CD10 expression in epithelial cells of human adenomyosis. (A) Immunohistochemical analysis of E-cadherin showed suppression of E-cadherin expression in epithelial cells of eutopic endometrium (b) and adenomyosis region (c) compared to normal endometrium (a). Scale bar = 25μm. (B) The expression of CD10 was detected in some epithelial cells of eutopic endometrium (b and e) and adenomyosis region (c and f) but not control endometrium (a and d). d, e, and f are high magnification pictures (Scale bar = 25 μm) of the boxed areas in a, b, and c (Scale bar = 200 μm).

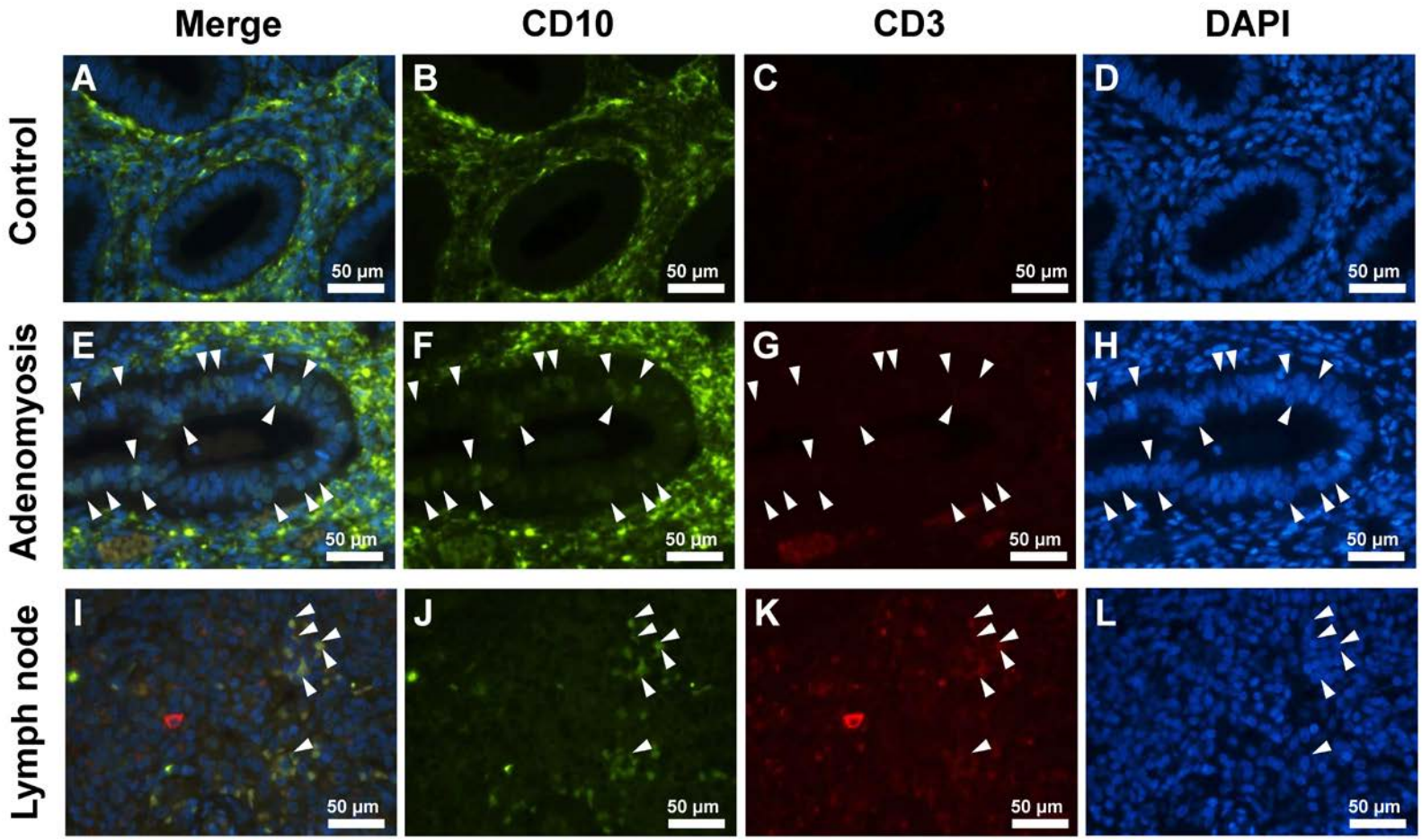
Merge
CD10
CD3
DAPI
Control
Adenomyosis
Lymph node
A
B
C
D
E
F
G
H
I
J
K
L
50 μm

Figure 20. The expressions of CD10 and CD3 in human endometrium with and without adenomyosis. Immunofluorescence analysis of CD10 (B, F and J; green) and CD3 (C, G and K; red) was performed with control endometrium (A, B, C and D), eutopic endometrium of adenomyosis (E, F, G and H) and baboon lymph node (I, J, K, and L). Merged images (A, E and I) and DAPI images (D, H, and L). Arrowheads indicate positive-CD10 cells. Scale bar = 50 μm.

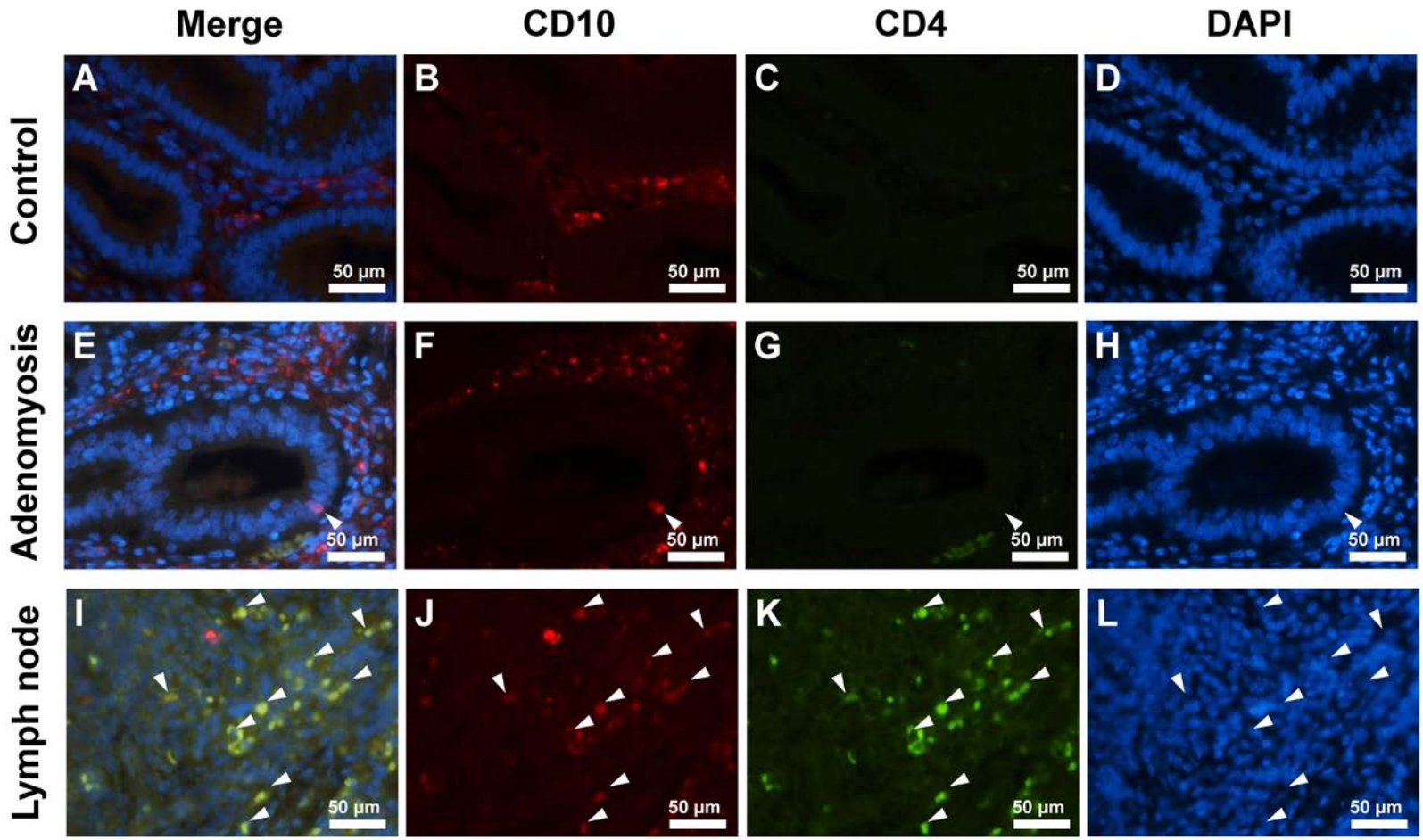
Merge
CD10
CD4
DAPI
Control
Adenomyosis
Lymph node
A
B
C
D
E
F
G
H
I
J
K
L
50 μm

Figure 21. The expressions of CD10 and CD4 in human endometrium with and without adenomyosis. Immunofluorescence analysis of CD10 (B, F and J; red) and CD4 (C, G and K; green) was performed with control endometrium (A, B, C and D), eutopic endometrium of adenomyosis (E, F, G and H) and baboon lymph node (I, J, K, and L). Merged images (A, E and I) and DAPI images (D, H, and L). Arrowheads indicate positive-CD10 cells. Scale bar = 50 μm.

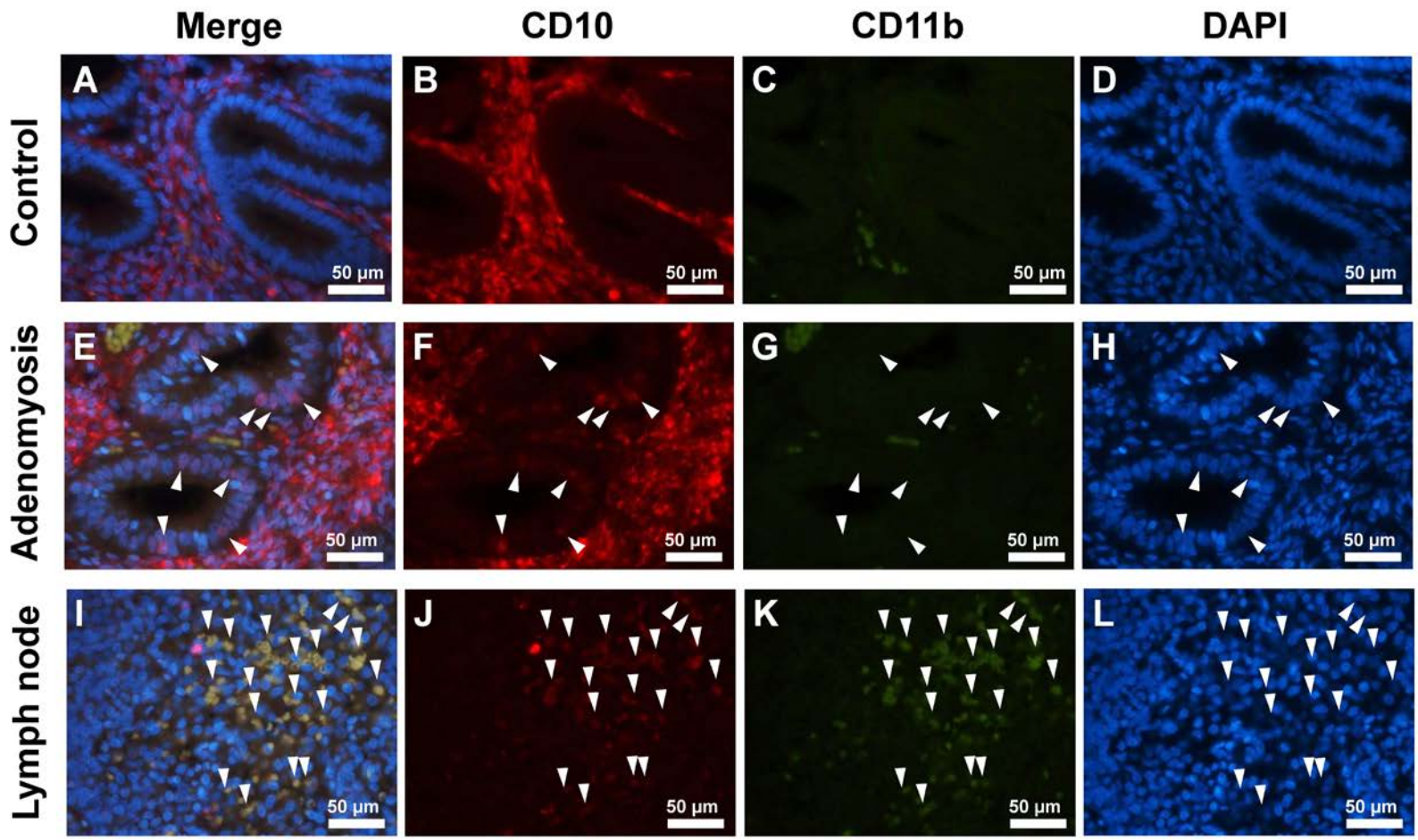
Merge
CD10
CD11b
DAPI
Control
Adenomyosis
Lymph node
A
B
C
D
E
F
G
H
I
J
K
L
50 μm

Figure 22. The expressions of CD10 and CD11b in human endometrium with and without adenomyosis. Immunofluorescence analysis of CD10 (B, F and J; red) and CD11b (C, G and K; green) was performed with control endometrium (A, B, C and D), eutopic endometrium of adenomyosis (E, F, G and H) and baboon lymph node (I, J, K, and L). Merged images (A, E and I) and DAPI images (D, H, and L). Arrowheads indicate positive-CD10 cells. Scale bar = 50 μm.

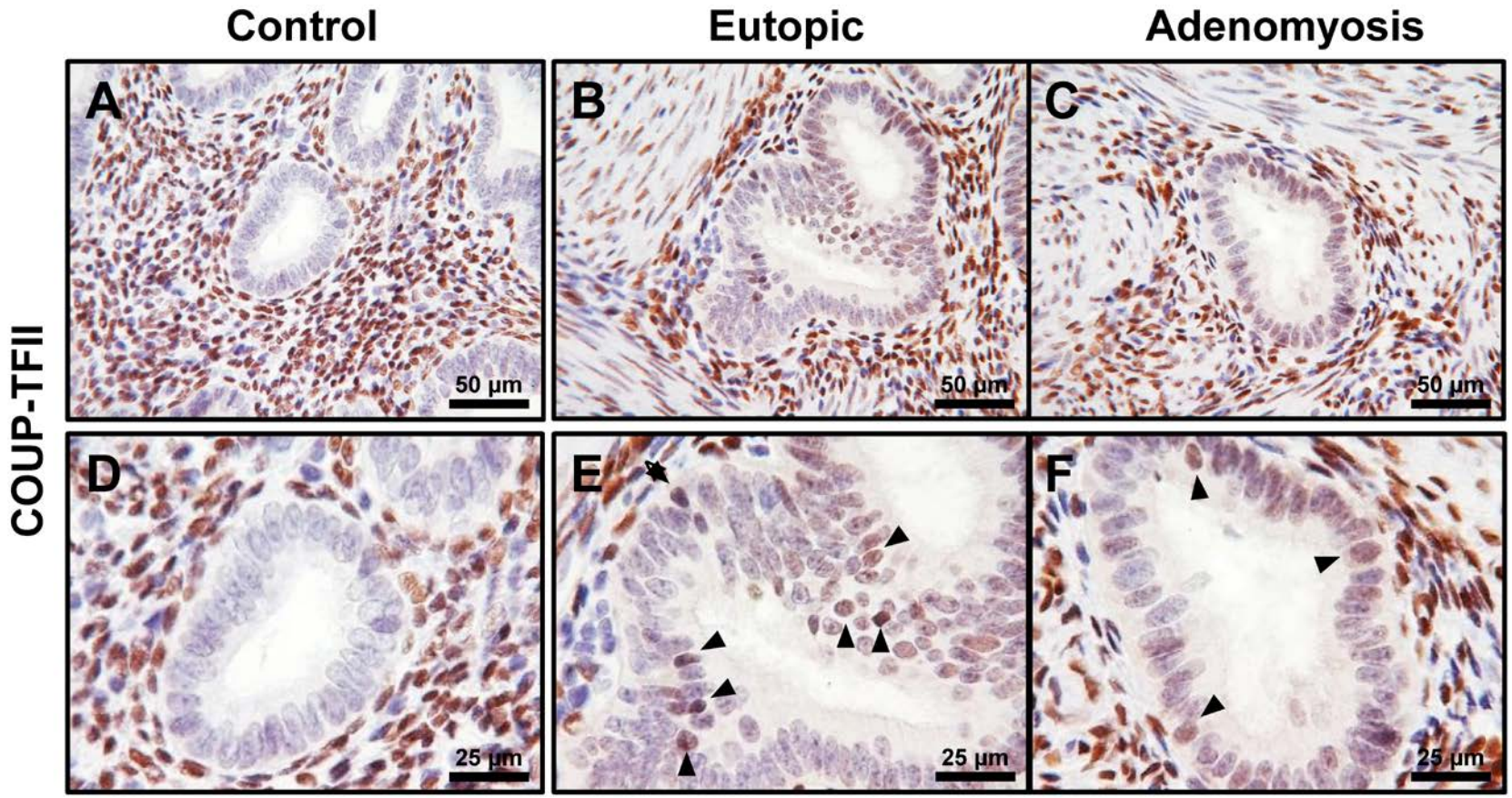
Control
Eutopic
Adenomyosis
COUP-TFII
A
B
C
D
E
F
50 μm
50 μm
50 μm
25 μm
25 μm
25 μm

Figure 23. The expression of COUP-TFII in in human endometrium with and without adenomyosis. COUP-TFII is detected in stromal cells in the control endometrium. (A and D) In epithelial cells of eutopic endometrium and adenomyosis region, COUP-TFII is detected in some epithelial cells (B, C, E and F). Arrowheads indicate positive-COUP-TFII epithelial cells. D, E, and F (Scale bar = 25 μm) are high magnification pictures of the boxed areas in A, B, and C (Scale bar = 200 μm).

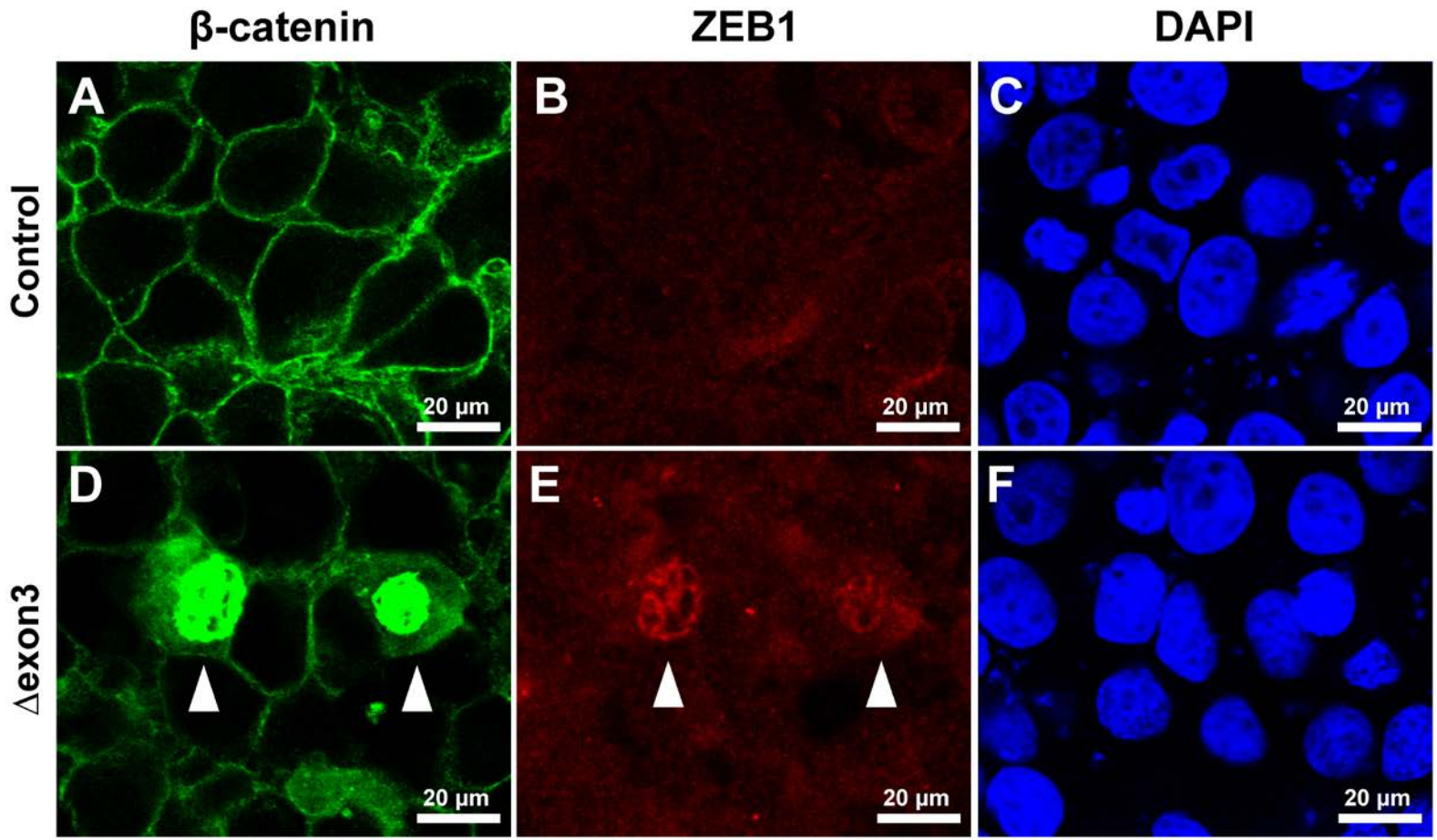
β-catenin
ZEB1
DAPI
Control
Δexon3
A
B
C
D
E
F
20 μm
20 μm
20 μm
20 μm
20 μm
20 μm

Figure 24. Immunofluorescense analysis of β-catenin and ZEB1 in MCF-7 cells after transfection of β-catenin expression vector. Control and β-catenin expression vector were transfected MCF-7 cells and the expression of β-catenin (A and D; green) and ZEB1 (B and E; red) were examined. When β-catenin was observed in nucleus of MCF-7 cells, the expression of ZEB1 was detected in epithelial cells. Scale bar = 20 μm.

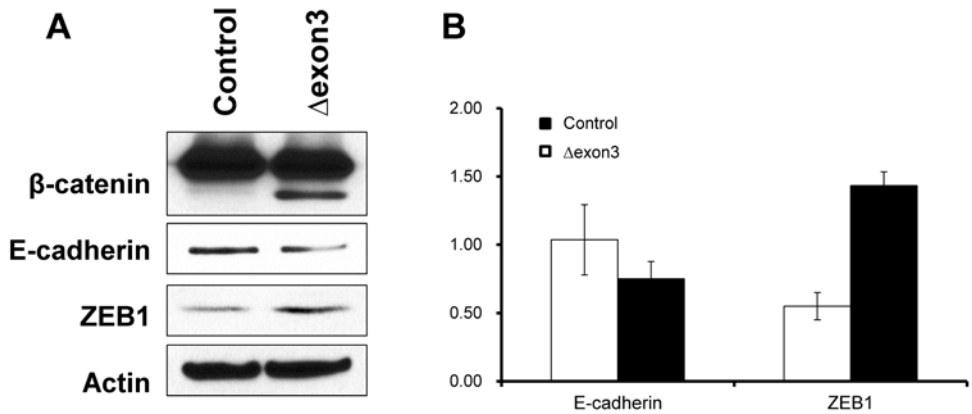

Figure 25. The expressions of E-cadherin, ZEB1, and Actin in HEC-1A epithelial cells after transfection of control or β-catenin expression vector. After β-catenin activation, E-cadherin is repressed and the expression of ZEB1 is increased. Actin was used as a loading control. (B) E-cadherin and ZEB1 quantification of western blot results is band densitometry/load control. The data shown are the means and error bars indicate SE.

4. Discussion

WNT/β-catenin signaling plays a critical role in the uterine function and the development of endometrial hyperplasia and cancer. Recently, it has been proposed that adenomyosis is an ovarian steroid hormone-dependent disorder, resulting from high estrogen levels unopposed by progesterone. Chen *et al.* have suggested estrogen-induced epithelial-mesenchymal transition of endometrial epithelial cells contributes to the development of adenomyosis (Chen et al., 2010). Changes of EMT markers including E-cadherin and Vimentin were linked to estrogen levels. In previous chapter, we identified that aberrant activation of β-catenin is an etiology of adenomyosis. EMT of the tumor cells has been shown with a nuclear accumulation of the transcriptional activator β-catenin. Activated β-catenin is one of EMT inducer in tumoriogenesis. Furthermore, increased level of β-catenin induces a loss of epithelial cell differentiation (Mariadason et al., 2001; Naishiro et al., 2001). E-cadherin is downregulated in the uterine epithelial cells of women and baboon with endometriosis compared with endometriosis-free controls (Zeitvogel et al., 2001; Braundmeier and Fazleabas, 2009). The most central marker of EMT is E-cadherin repression. The loss of E-cadherin is required for detachment, invasion, and metastasis which are associated with activated β-catenin signaling.

In this study, we observed E-cadherin, EMT markers (Snail and ZEB1) and mesenchymal markers (Vimentin and COUP-TFII) expression to identify the EMT process is involved in adenomyosis development. E-cadherin expression was repressed in the uterus of mutant mice and human adenomyosis (Fig. 15, and Fig. 19). The mark of EMT initiation is the induction of numerous transcription factors, Snail, Slug, Twist, ZEB1, and SIP1, and the repression of E-cadherin expression

(Thiery, 2002). Snail and ZEB1 expression has been related to tumor cell migration, invasion, and metastasis. We examined exciting patterns of Snail and ZEB1-positive cells and E-cadherin repression in some epithelial cells of the uterus in mutant mice (Fig. 15 and Fig. 16). Our results showed that loss of E-cadherin and aberrant expression of Snail and ZEB1 are related with EMT and induce the metastatic potential. Vimentin is also involved in an EMT and observed in epithelial cells involved in epithelial cell relocation (Gilles et al., 2003). In the expressions of Vimentin and Keratin 18 in mutant mice, Vimentin is aberrantly expressed and Keratin 18 is repressed in some epithelial cells of mutant mice (Fig. 17). We also found COUP-TFII-positive epithelial cells in mutant mice and human adenomyosis (Fig. 18 and Fig. 23) Therefore, our results clearly support an EMT process are involved in endometrial hyperplasia and adenomyosis development (Fig. 26).

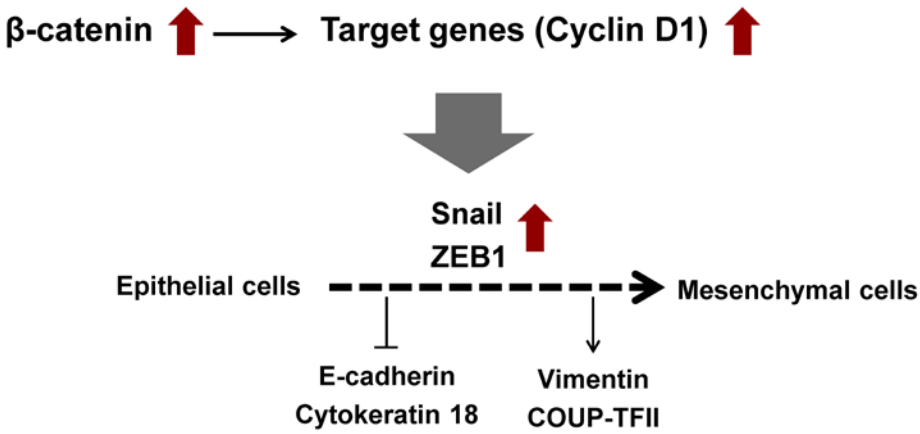

Figure 26. Schematic diagram indicating the EMT mechanism during the development of adenomyosis. In the mutant mice, loss of E-cadherin and aberrant expression of Snail and ZEB1 are observed. Moreover, mesenchymal markers such as Vimentin and COUP-TFII are expressed in some epithelial cell and induce the metastatic potential.

CHAPTER 5.

Hormone dysregulation is associated with the development of adenomyosis

1. Introduction

Adenomyosis is one of gynecological disorders, and characterized by the presence of endometrial glands and stroma cells within the myometrium. Most patients are premenopausal women between the age of 40 and 50. There are also reports that tamoxifen treated breast cancer patients have adenomyosis in postmenopause. Based on these cases, numorous scientists have suggested steroid hormone is associated with adenomyosis development, but the precise etiology is unclear and unexplained.

Female steroid hormones control proliferation and differentiation of uterus (Tsai and O'Malley, 1994). This ovarian steroid hormone-dependent disorder strongly has been suggested for the etiology of endometrial cancer. In the patients of endometrial cancer, endometrial hyperplasia and adenomyosis, high levels of estrogen without proper protection from progesterone have been reported (Schindler, 2009). To investigate the mechanism of adenomyosis adenomyosis, a lot of rodent models and primates have been used. Particulary, the experiments of the implantation of an anterior pituitary gland into the uterine lumen or hormonal alterations have been applied to induce the adenomyosis in mouse model. In this chapter, to address the role of ovarian steroid hormones of adenomyosis development in different condition of β-catenin expression, we used control ($PR^{cre/+}$ and $Ctnnb^{f(ex3)/+}$) and mutant mice ($PR^{cre/+}$ $Ctnnb^{f(ex3)/+}$) and treated the steroid hormones after ovariectomized the mice.

2. Material and method

1) Hormone treatments

The steroid hormone response was assessed in adult mice by ovariectomizing the mice at six weeks of age. Two weeks later, ovariectomized mice were treated with either vehicle (sesame oil), estrogen (E2) (0.1 μg/mouse), progesterone (P4) (1mg/mouse in 100 μl) or E2 plus P4 and mice were sacrificed three month after the treatment by cervical dislocation while under anesthetic (Avertin; 2,2-tribromoethyl alcohol; Sigma-Aldrich). At the time of dissection, uterine tissues were fixed with 4 % paraformaldehyde (vol/vol) and paraffin embedded.

2) H&E staning

For Hematoxylin and Eosin (H&E) Staining, mice uterus was fixed overnight in 4% paraformaldehyde, followed by thorough washing in 70% ethanol. The tissues were processed, and embedded in paraffin. The six μm sections were cut and stained with hematoxylin and eosin by standard protocols.

3. Results

Ovarian steroid hormone dependency of adenomyosis development

It has been suggested that adenomyosis is an estrogen-dependent disorder in females in which estrogen causes growth and progression of adenomyosis (Zhou et al., 2012). We examined the role of ovarian steroid hormones in the development of the adenomyosis phenotype in the mutant mice. Mutant and control mice were ovariectomized and treated with vehicle, estrogen (E2), estrogen plus progesterone (E2+P4) or P4 for 3 months and sacrificed at 6 months old of age. Ovariectomized mutant mice did not develop adenomyosis as observed in intact mutant mice (Fig. 27A). This demonstrates that the adenomyosis phenotype of mutant mice is dependent on ovarian hormone stimulation. Although the control mice showed endometrial hyperplasia as expected from chronic E2 treatment, they did not show adenomyosis phenotype. All of the mutant mice treated with E2 developed adenomyosis (Fig. 27B). This result supports that adenomyosis is an estrogen-dependent disease. Ovariectomized mutant mice treated with E2+P4 for 3 months developed adenomyosis as intact mutant mice (Fig. 27C). However, ovariectomized mutant mice treated with P4 for 3 months did not develop adenomyosis (Fig. 27D). Therefore, these results demonstrate that β-catenin activation and estrogen is important to etiology of adenomyosis.

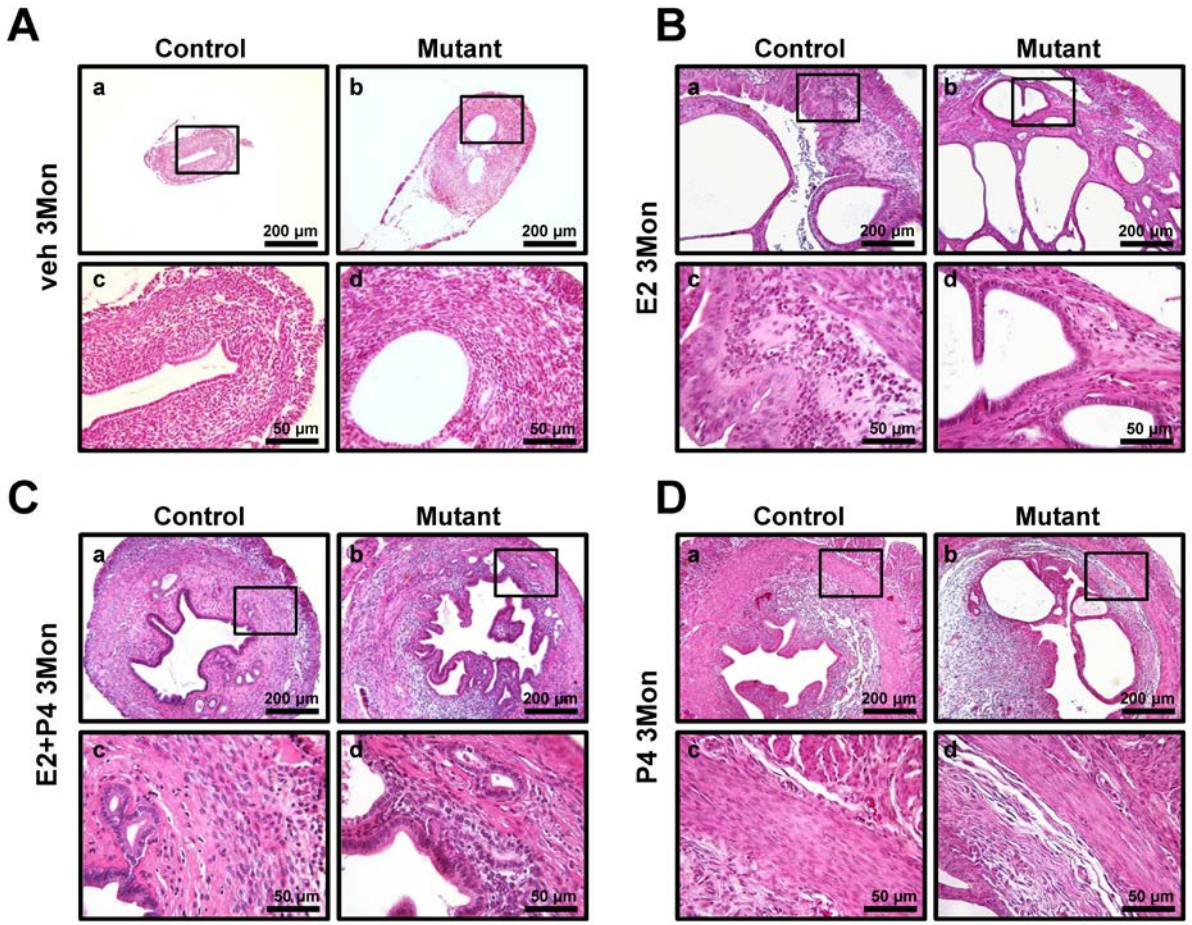
A
Control
Mutant
veh 3Mon
a
b
c
d
200 μm
50 μm
B
Control
Mutant
E2 3Mon
a
b
c
d
200 μm
50 μm
C
Control
Mutant
E2+P4 3Mon
a
b
c
d
200 μm
50 μm
D
Control
Mutant
P4 3Mon
a
b
c
d
200 μm
50 μm

Figure 27. Ovarian steroid hormone dependent development of adenomyosis in mutant mice. (A) Uteri of 6 month old ovariectomized control and mutant mice treat with vehicle. Histological analysis showed that mutant mice did not develop adenomyosis phenotype. (B) Formation of adenomyosis in ovariectomized mutant mice treated with E2. Adenomyosis induced in the uteri of ovariectomized mutant mice treated with E2 for 3 months. Histological analysis showed that mutant mice develop adenomyosis phenotype. (C) Uteri of 6 month old ovariectomized control and mutant mice treated with E2+P4 for 3 months. Histological analysis showing adenomyosis in the ovariectomized mutant mice treated with E2+P4 for 3 months. (D) Uteri of 6 month old ovariectomized control and mutant mice treat with P4. Histological analysis showed that mutant mice did not develop adenomyosis phenotype.

4. Discussion

Adenomyosis is charactedrized by the location of endometrial glands and stroma within the myometrium. Control myometrium without adenomyosis is consisted with uniform concentric layers around the endometrium, but the myometrium with adenomyosis comprised irregular smooth muscle layers. There are several hypotheses for the etiology of adenomyosis. One theory is that any damage may increase endometrial tissue and the uterine line becomes relocated. Second theory is that high levels of estrogen induce abnormal growth of the endometrium. Also there are the other theories, the barrier between the endometrium and myomterium is broken, and allows the endometrium to invade into the myometrium of the uterus. In spite of all theories, the pathogenesis of adenomyosis remains unknown.

To treat endometriosis or adenomyosis, clinics have used agonize or antagonize ER because they considered a potentially effective therapy. Moreover, high level of estrogen lacking the protection of progesterone have been suggested to endometrial cancer, endometrial hyperplasia and adenomyosis (Schindler, 2009). Previous studies have demonstrated that raloxifene, oral selective estrogen receptor modulator (SERM), results in the suppression of estrogen-induced endometrial cell migration (Chen et al., 2010). This finding suggests that raloxifene may be an effective therapy for endometrial disorders caused by oestrogen-induced cell adhesion alterations. Chen YJ et al. (2012) also confirmed estrogen induced epithelial-mesenchymal transition of endometrial epithelial cells and contributes to the development of adenomyosis (Chen et al., 2010).

In our results of hormone assay, the adenomyosis phenotype of mutant mice is dependent on ovarian hormone stimulation. All of the mutant mice treated with E2 or E2+P4 developed adenomyosis (Fig. 27B and C) although ovariectomized mutant treated with veh or P4 for 3 months did not develop adenomyosis (Fig. 27A and D). Therefore, our results suggest that aberrant β-catenin activation and estrogen is important to etiology of adenomyosis.

CHAPTER 6.

General discussion and future plan

Adenomyosis is a common benign gynecological disorder in women worldwide. Common symptoms include menorrhagia, dyspareunia, dyschezia, dysmenorrhea, and chronic pelvic pain. The majority of symptomatic women who are nonresponsive to pharmacological therapy require surgical intervention (Vercellini et al., 2006). With the aid of transvaginal ultrasonography and MRI this disease is now frequently diagnosed in infertility patients and can interfere with implantation, and cause subfertility and miscarriage (de Souza et al., 1995; Campo et al., 2012; Louis et al., 2012). Given the prevalence of adenomyosis and associated symptoms, there is, unfortunately, no good evidence-based treatment to date, except hysterectomy. Hysterectomy for adenomyosis treatment results in costly healthcare needs and may not be an option for women wishing to maintain future fertility. The precise etiology and pathophysiology of adenomyosis is still unknown. Our results suggest that activation of β-catenin plays an important role in the pathogenesis of adenomyosis.

To provide further insight into the development of adenomyosis, many different species including mice, rats, rabbits, dogs, cats and non-human primates have been studied (Greaves and White, 2006). To address the mechanism of disease development, several studies in the mouse model or hormonal treatment have demonstrated the ability to induce symptoms of adenomyosis (Guttner, 1980; Mori and Nagasawa, 1983; Ostrander et al., 1985; Parrott et al., 2001). However, the development of more accurate animal models is still required to understand the specific molecular mechanisms of the disease.

In order to investigate the pathophysiological role of β-catenin activation in adenomyosis, we used engineered mice in which β-catenin was stabilized in the

reproductive tract using previously generated mice harboring modified β-catenin alleles, in addition to the PR^{cre} mouse (Harada et al., 1999; Soyal et al., 2005; Jeong et al., 2009). In a previous study, we have shown that activation of β-catenin plays a potentially critical role in the regulation of uterine function as well as the development of endometrial hyperplasia and cancer in a steroid hormone-dependent manner (Jeong et al., 2009). In addition to those phenotypes, the uteruses of mutant mice exhibited abnormal irregular structures in the myometrium, and proliferation was significantly increased (Fig. 11 and Fig. 12). We have obtained robust evidence that mice with conditional uterine activation of β-catenin go on to develop adenomyosis and have created a novel model system to further investigate the genetic and molecular events involved in the transition from normal conditions to adenomyosis. Constitutive activation of β-catenin in the uterine mesenchyme (*Amhr2*$^{cre/+}$ *Ctnnb*$^{f(ex3)/+}$) can also induce myometrial hyperplasia, as well as cause the development of mesenchymal tumors and occasional adenomyosis (Tanwar et al., 2009). WNT/β-catenin signaling molecules are critical in these processes and should be tightly regulated during normal uterine function (Arango et al., 2005; Li et al., 2005a; Mohamed et al., 2005; Rider et al., 2006; Jeong et al., 2009). These findings suggest that tight regulation of β-catenin in uterine mesenchymal cells and epithelial cells is important in physiological uterine function and that the dysregulation of β-catenin can lead to several pathological conditions including endometrial hyperplasia, uterine tumors and adenomyosis.

β-catenin interacts with E-cadherin in cell-cell adherens junctions. Tumor cell EMT is associated with nuclear accumulation of the transcriptional activator β-catenin. β-catenin has been shown to induce EMT during gastrulation in sea urchins (Logan et al., 1999) and also in human cell culture systems (Eger et al.,

2000; Morali et al., 2001). Moreover, increased activity of β-catenin directly leads to a loss of epithelial cell differentiation (Mariadason et al., 2001; Naishiro et al., 2001). E-cadherin is reduced in the uterine epithelial cells of women and baboons with endometriosis, compared to endometriosis-free controls (Zeitvogel et al., 2001; Braundmeier and Fazleabas, 2009). The most important hallmarker of EMT is the loss of E-cadherin. Its loss is a requirement for detachment, invasion, distribution and metastasis, which are directly linked with activated β-catenin signaling. We observed that E-cadherin expression was decreased in the uterus of mutant mice as well as in human adenomyotic tissue (Fig. 15A, and Fig. 19). EMT is initiated by a number of transcription factors, including Snail, Twist, ZEB1, and SIP1, via the repression of E-cadherin expression (Thiery, 2002). ZEB1 expression has also been associated with tumor cell migration, invasion, and metastasis. Nicole S. et al (2006), identified ZEB1 as being overexpressed in tumor-associated stroma of low-grade uterine cancers, but it has also been observed as aberrantly expressed in the tumor epithelial cells of aggressive endometrial cancers (Spoelstra et al., 2006). We observed interesting expression patterns for Snail and ZEB1-positive cells and E-cadherin repression in some epithelial cells of the uterus in our mutant mice (Fig. 15B and Fig. 16). Our results suggest that the loss of E-cadherin and aberrant expression of Snail and ZEB1 are associated with EMT and promote metastatic potential. Vimentin is also associated with EMT and is expressed in epithelial cells undergoing migration (Gilles et al., 2003). We observed the expression patterns of vimentin and repression of keratin 18 in epithelial cells of mutant mice (Fig. 17).

COUP-TFII positive cells are observed in some epithelial regions of the uterus of mutant mice and human adenomyosis patients (Fig. 18 and Fig. 23). COUP-TFII is strongly expressed in stromal regions and plays critical roles in

normal development and tumor formation (Lee et al., 2010; Qin et al., 2010). COUP-TFII induces the extracellular matrix-degrading proteinases matrix metalloproteinase 2 (MMP2) and urokinase-type plasminogen activator (uPA), both of which are known to play critical roles in angiogenesis and metastasis (Navab et al., 2004). CD10 is also a prominent factor during tumor invasion. CD10 is a cell-surface zinc-dependent metalloproteinase, and its expression has been detected within the invasive areas of various cancers such as prostate, breast, colorectal, and skin carcinomas (Albrecht et al., 2002; Ogawa et al., 2002; Kesse-Adu and Shousha, 2004; Takahara et al., 2009). In our study, CD10 was found to be expressed in the epithelial cells of human adenomyotic tissue, while the control epithelial cells were negative for expression (Fig. 19B). Therefore, our findings clearly support the hypothesis that EMT is involved in endometrial hyperplasia and the development of adenomyosis.

Currently, two theories are generally understood for the development of adenomyosis. One is endomyometrial invagination of the endometrium and the other is the *de-novo* development of adenomyosis from Müllerian rests (Ferenczy, 1998). It has been proposed that adenomyosis is an ovarian steroid hormone-dependent disorder, resulting from high estrogen levels that are unopposed by the influences of progesterone. It positively correlates with endometrial hyperplasia, endometrial cancer and endometriosis, which are conditions also associated with unopposed estrogen levels (Parazzini et al., 1997; Bergholt et al., 2001). Chen *et al.* have suggested that estrogen-induced epithelial-mesenchymal transition of endometrial epithelial cells contributes to the development of adenomyosis (Chen et al., 2010). Changes in EMT marker expression including E-cadherin and vimentin were linked to estrogen expression. In order to verify the effect of steroid

hormone regulation om the development of adenomyosis, we observed the histology after ovariectomy. Mutant mice treated with E2 and E2+P4 were found to still develop adenomyosis. However, mutant mice treated with vehicle or P4 showed abnormal luminal epithelial structures but did not develop true adenomyosis. These results suggest that β-catenin is a critical factor associated with unopposed estrogen that contributes to the development of adenomyosis (Fig. 27).

In conclusion, our results have demonstrated a role for β-catenin activation in the development of adenomyosis using a $PR^{cre/+}$ $Ctnnb^{f(ex3)/+}$ mouse model and analysis of tissue from human adenomyosis patients. We observed that β-catenin activation caused induction of Snail, ZEB1 expression and repression of E-cadherin expression, as well as mesenchymal cell marker expression in epithelial cells during adenomyosis development.

To date, no effective medical therapies for adenomyosis exist. Some reports have identified a lack of progesterone receptors in the endometrium of patients with adenomyosis. This deficiency is hypothesized to cause a lack of responsiveness during subsequent hormonal therapy for adenomyosis. Nevertheless, for women who desire to maintain fertility, hormonal therapy remains the preferred option. The objective of such therapy is not to cure adenomyosis, but to attempt to control the symptoms. Therefore, a major problem is the return of symptoms after discontinuation of therapy, and this issue is exacerbated by the fact that these medications can be used for short periods.

In the studies presented in this thesis, we have identified mechanisms

involved and the pathophysiological role of β-catenin signaling in the development of adenomyosis. The results suggest that dysregulation of β-catenin signaling is associated with EMT and is critical for the development of adenomyosis. In addition to defining an association between β-catenin signaling and the development of adenomyosis, we demonstrated the important effects of hormones on adenomyosis development through the activation of β-catenin. These findings provide greater insights for the development of new therapeutic approaches for adenomyosis. Moreover, we propose that our uterine-specific β-catenin activated mice ($PR^{cre/+}$ $Ctnnb1^{f(ex3)/+}$) can be used for biomarker development in further adenomyosis research.

For future studies, we intend to further investigate the molecular mechanisms responsible for the development of adenomyosis using assays for hormone regulation. A team led by Virginia et al. (2006) have already identified the alteration of WNT/β-catenin signaling in accordance with steroid hormone response, and that progesterone downregulates GSK3-β expression leading to the accumulation of β-catenin in the stromal cytoplasm (Rider et al., 2006). Our proposed studies will also help to clarify the mechanisms involved in steroid hormone regulation during the development of adenomyosis.

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SUMMARY IN KOREAN

배경 및 목적: 자궁 선근증 (Adenomyosis)은 자궁 근층 (Myometirum) 내 자궁내막선 (endometrial gland)과 기질의 존재에 의해 정의된 부인과 질환이다. 발병률이 높음에도 불구하고, 정확한 병리학적 요인과 기전을 밝혀내지 못 하였다. 최근, 비정상적인 WNT/β-catenin 신호 전달이 중요한 부인과 질환에 관련되어 있음에 주목 받고 있다. 특히, CTNNB1의 돌연변이에 의한 β-catenin의 신호가 비정상적인 활성화 된다면, 자궁 내막 암의 원인이 될 수 있다는 보고가 있다. 따라서, 본 연구에서는 자궁 선근증 발생과정에 WNT/β-catenin 신호 전달의 역할 및 발병 메커니즘을 규명하고자 한다.

방법: 우선, 정상적인 인간의 자궁 조직과 자궁 선근증을 가진 조직에서 β-catenin 발현을 면역 조직 화학 분석에 의해 조사 하였다. 이 실험에서 자궁 선근증 조직에서 핵 및 세포질 β-catenin의 발현이 비정상적으로 활성화 되는 것을 관찰하였다. 이 실험결과를 토대로, 자궁 특이적 β-catenin이 활성화된 마우스를 생산하여 자궁 선근증의 발달에 관한 메카니즘을 확인하고자 하였다. 우선, *Ctnnb1*$^{f(ex3)/+}$ 마우스와 Progesteron receptor (PR) - Cre 마우스간 교배를 하여 형질 전환 마우스를 생산하였다. 그리고, 면역 조직 화학 분석법을 통하여 형질 전환 마우스의 조직학적 문제점 및 자궁 선근증 발달의 상피−중간엽 이행 (EMT) 관련성을 확인하였다.

결과: 2개월령 $PR^{cre/+}$ $Ctnnb1^{f(ex3)/+}$ 형질 전환 마우스는 불규칙한 근층 구조를 가지고 있으며, 증식 세포가 정상 마우스에 비해 자궁 근층에 많이 존재함을 확인하였다. 그리고 4개월, 6개월령 $PR^{cre/+}$ $Ctnnb1^{f(ex3)/+}$ 형질 전환 마우스의 자궁 조직내에서 자궁 선근증을 확인하였다. 또한, 흥미롭게도 면역 조직 화학 분석에서 E-cadherin의 발현이 정상 마우스에 비해 $PR^{cre/+}$ $Ctnnb1^{f(ex3)/+}$ 형질 전환 마우스에서 억제 되어 있음을 확인하였다. 이 결과를 바탕으로 상피-중간엽 이행의 관련성을 확인하기 위하여, E-cadherin 발현 감소를 유도하는 Snail, ZEB1 및 중간엽 세포 마커로 알려진 Vimentin와 COUP-TFII 발현을 면역 조직 화학 분석법으로 확인하였다. $PR^{cre/+}$ $Ctnnb1^{f(ex3)/+}$ 형질 전환 마우스 자궁의 일부 상피 세포에서 비정상적으로 Snail, ZEB1, Vimentin, 그리고 COUP-TFII 발현을 관찰하였다. 또한, 인간의 자궁 선근증 조직내에서도 상피-중간엽 이행이 이루어 지는지 확인하기 위해, E-cadherin, COUP-TFII 그리고 CD10 발현을 확인하였다. 마우스 실험결과와 동일하게 인간의 자궁 선근증에도 E-cadherin의 발현은 감소하였고, COUP-TFII 및 CD10이 비정상적으로 자궁 내막의 상피 세포에서 발현하였다. 위 결과들을 바탕으로, β-catenin의 비정상적인 활성화가 상피-중간엽 이행 (EMT)을 유도하여 자궁 선근증의 발전에 기여함을 확인하였다. 이 연구는 자궁 선근증 발달 현상을 이해함으로써, 자궁 선근증 진단법 및 치료제 개발에 응용 가능할 것으로 전망된다.

주요어 : β-catenin, 자궁선근증 (Adenomyosis), 상피-중간엽 이행 (Epithelial-mesenchymal transition), 자궁 (Uterus)

감사의 글

그동안 저를 응원하고, 도와주셨던 모든 분들께 감사의 인사를 드립니다.

오랜 기간 부족한 저를 질책과 격려로 이끌어주신 임정묵 교수님, 미국 땅에서 과학을 가르쳐 주고자 하셨던 정재욱 교수님... 제 박사학위과정 동안 두분의 지도 교수님을 만나, 값진 배움을 얻을 수 있었음에 감사드립니다. 그리고 바쁘신 가운데도 저의 논문심사에 참여해 주시어 많은 조언 해주신 한재용 교수님, **Asgi** 교수님 감사드립니다. 또한, 저의 사수셨던 이승태 교수님께서 제 박사학위 논문심사에 참여하시어 격려해 주심에 감사했습니다.

더불어 저와 함께 오랜 시간을 나누었던 실험실 식구 여러분들께 고마움을 전하고 싶습니다. 거침없는 충고를 해 주시는 4차원 이은주 교수님, 힘겨워 하는 저에게 많은 도움을 주셨던 박종흠 교수님, 박준홍 박사님, 안지연 박사님, 그리고 낯선 미국땅에서 엄마, 아빠처럼 돌보아주셨던 김홍님 박사님, 김태훈 박사님, 함께 해서 든든했던 나의 룸메이트 재희, 그리고 진정 과학을 사랑하시는 유정윤 박사님, 한국에서 온 저를 걱정하시며 챙겨주셨던 미세스 구, 졸업 후에도 저를 응원해주었던 장미선배, 정규오빠, 길아오빠, 승표오빠, 고단하고 힘들 때 손을 내밀어 주었던 분들... 박학다식한 준희오빠, 오랜 시간 함께 했던

채현이, 야무진 경은이, 동갑내기 보연씨, 말 안 듣지만 든든한 종일이, 품절녀인 화영이, 엄마가 된 아란이, 귀염둥이 보람이, 떠나가버린 정기, 쾡한 명욱이, 조용조용 윤미, 박박이라는 영현이... 모두들 감사했습니다. 그리고 내 험한 갈굼에도 꿋꿋한 효숙이, 동우, 꼬맹이 영임아 열심히 응원할께!!! 마지막으로 저를 격려 해주셨던, **WCU Biomodulation** 전공 교수님들과 힘들고 눈물 날 때, 저를 응원하고 감동시켜주었던 동료분들께 감사드립니다.

긴 대학원 시절 나의 애인이었던 상길이, 먼길 떠날 때마다 반찬 챙겨주셨던 그녀의 어머니, 대학원 생활을 너무라도 잘 알기에 내 이야기를 가장 많이 들어주었던 효정이, 가끔 만나 넋두리하며 세상 이야기 들려주었던 민정이와 성희, 멀리서 응원해 주었던 이나와 연경이 그리고 서진이 엄마 영륜이... 항상 내 결혼이 걱정인 정은이... 나의 소꿉 친구들과 대학 동기, 그리고 선후배님들... 제 곁에 있어 주어서 외롭지 않게 학위를 마칠 수 있었던 것 같습니다. 감사합니다.

우리 가족들!!! 제 긴 학위 과정동안 맛난 음식으로 응원해 주셨던 우리의 요리사 서울 작은 아빠, 엄마, 끊임없이 격려 해 주시고 사랑해주시는 창원 작은 아빠, 엄마, 친지분들께 감사함을 전하고 싶습니다. 그리고 가장 사랑하고 존경하는 아빠, 엄마!!! 제가 여기까지 오는 동안 모든 사랑과 정성을 다 해주심에 정말 감사드립니다. 또한 옆에서 묵묵히 지켜보며 격려해준 형부와 언니, 먼저 학위 받고 나를 보며 걱정이 많았던 영석이, 그 옆에서 함께 응원 해 주었던 효은이...

든든하고 힘이 되었습니다. 감사합니다. 그리고 사랑합니다.

그동안 너무나 많은 분들로부터 격려와 사랑을 받아 여기까지 올 수 있었습니다. 지금까지 받은 사랑을 밑바탕으로 누군가에 도움이 될 수 있는 자가 되도록 최선을 다 하겠습니다. 정말 감사했습니다.