A Dissertation
for the Degree of Doctor of Philosophy

Studies on molecular understanding of endometrial diseases and development of graphene-based biotechnology

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ABSTRACT

Endometrial cancer and adenomyosis are well-known gynecological disorders of the female reproductive system. The endometrial cancer and adenomyosis are ovarian steroid hormone-dependent gynecological disorders and are related to unopposed E2 exposure. These disease are associated with several risk factors, such as infertility, pelvic pain, menorrhagia, and dysmenorrhea. Nevertheless, molecular mechanisms leading to endometrial cancer and adenomyosis remain unclear.

Hormone therapy, radiation therapy, and chemotherapy are variously implemented to treat gynecological disorders such as endometrial cancer, endometriosis, and adenomyosis. In particular, there are reports of bone damage such as osteoporosis, osteolysis, and osteosarcoma after radiation therapy. Also, Research has been reported that osteoporotic disease occurs in postmenopausal women and endometrial cancer patients. The occurrence of these bone disease can lead to huge losses in health care costs and reduce the quality of life and life expectancy of patients. Recently, the field of regenerative medicine using stem cells and biomaterials has been actively studied to prevent bone damage.

The main purpose of the first study was to understand the role of epithelial Mig-6 in the uterus. Progesterone (P4) has been used for several decades in endometrial cancer treatment, especially

in women who wish to retain fertility. However, it is unpredictable which patients will respond to P4 treatment and which may have a P4-resistant cancer. Therefore, identifying the mechanism of P4 resistance is essential to improve the therapies for endometrial cancer. Mitogen-inducible gene 6 (Mig-6) is a critical mediator of progesterone receptor (PGR) action in the uterus. In order to study the function of Mig-6 in P4 resistance, I generated a mouse model in which I specifically ablated Mig-6 in uterine epithelial cells using Sprr2f-cre mice (*Sprr2f*$^{cre+}$*Mig-6*$^{f/f}$). Female mutant mice develop endometrial hyperplasia due to aberrant phosphorylation of signal transducers and activators of transcription 3 (STAT3) and proliferation of the endometrial epithelial cells. The results from my immunoprecipitation and cell culture experiments showed that MIG-6 inhibited phosphorylation of STAT3 protein interactions. Our previous study showed P4 resistance in mice with *Mig-6* ablation in Pgr-positive cells (*Pgr*$^{cre+}$*Mig-6*$^{f/f}$). However, *Sprr2f*$^{cre+}$*Mig-6*$^{f/f}$ mice were P4-responsive. P4 treatment significantly decreased STAT3 phosphorylation and epithelial proliferation in the uterus of mutant mice. I showed that Mig-6 has an important function of tumor suppressor via inhibition of STAT3 phosphorylation in uterine epithelial cells, and the antitumor effects of P4 are mediated by the endometrial stroma. These data help to develop a new signaling pathway in the regulation of steroid hormones in the uterus, and to overcome P4 resistance in human reproductive diseases, such as endometrial cancer.

The second and third studies were to understand the pathophysiological function of β-catenin in adenomyosis development. Previous study showed that aberrant activation of β-catenin develops adenomyosis through EMT. To identify the molecular pathways regulated by aberrant activation of β-catenin, I performed DNA microarray and ChIP-seq analysis in the uteri of mutant mice which expressed a dominant stabilized β-catenin in the uterus. Pathway analysis of the microarray and ChIP-seq identified activation of TGF-β signaling in the mutant mice (*$Pgr^{cre/+}$ and $Ctnnb1^{f(ex3)/+}$*). Further ChIP analysis revealed *Tgf-β2* as a direct transcriptional β-catenin target gene in the uterus. Immunohistochemistry analysis also showed aberrant overexpression of TGF-β2 in epithelial cells of mutant mice as well as women with adenomyosis. There is a strong positive correlation between β-catenin and TGF-β2 proteins in women with adenomyosis. TGF-β2 positive epithelial cells of eutopic endometrium and adenomyosis lesions showed lower expression of E-cadherin compared to control epithelial cells in both mouse and human adenomyosis. Ishikawa cells with nuclear β-catenin induced the expression of TGF-β2 and vimentin but decreased the expression of E-cadherin. Interestingly, a cell invasion assay showed that nuclear β-catenin expression significantly increased invasiveness compared to the control group. Furthermore, Pirfenidone, a TGF-β inhibitor, treatment increased E-cadherin expression and reduced cell invasiveness in Ishikawa cells with

nuclear β-catenin.

Also pathway analysis of the microarray and ChIP-seq revealed dysregulation of Wnt/β-catenin signaling in the mutant mice compared to control mice. Lymphoid enhancer-binding factor 1 (LEF-1) major transcription factor of Wnt/β-catenin pathway, was up-regulated in mutant uterus compared to control. Immunohistochemistry analysis showed that LEF-1 levels are remarkably increased in the uterine of mutant mice compared to control during development of adenomyosis. Further the ChIP analysis revealed *Lef-1* as a direct transcriptional β-catenin target gene in the murine uterus. In human adenomyosis, the levels of LEF-1 were higher in women with adenomyosis compared to women without adenomyosis. HEC1A cells with nuclear β-catenin induced the expression of LEF-1 and ZEB1 but decreased the expression of E-cadherin. These data show that TGF-β2 and LEF-1 can be novel targets and biomarkers for treating adenomyosis.

The fourth study conducted research on study of regenerative medicine using graphene biomaterials and human adipose-derived mesenchymal stromal cells (hASCs) to treat bone disease after radiation therapy or in menopausal women, endometrial cancer patients. Recently, biomaterial graphene and stem cells have been actively studied i n the field of regenerative medicine for bone regeneration. So, this study was conducted to examine the effect of multilayer graphene consisting of various layers on the osteogenic differentiation of hASCs. Various layers (1 to 7) of graphene film,

which contained wrinkles between layers with 30 to 50 nm in wi dth, were attached with chemical vapor deposition on a cell cultu re glass. hASCs were cultured on the multilayer graphene films, which consisted of different number of the wrinkles. Three prima ry hASC lines subpassaged different times were provided and ce ll activity and osteogenic differentiation was subsequently monito red. The osteogenic differentiation of the hASCs, which was conf irmed by Alizarin red staining, were significantly promoted by th e culture of multilayer graphene films compared with the control (no layer). The graphene with three layers yielded optimal differ entiation. When the hASCs were cultured on the three-layer gra phene films for 24 hours, expression of cell adhesion molecule (F-actin) and FAK, ERK and RUNX2 were activated compared with the control. Real-time RT-PCR analysis showed significant upregulation of *ALP* and *OPN*, pRUNX2-targeted mRNA genes in the cells cultured on the multilayer graphene films for 24 hours. These results suggest that changes in the physical environment due to multilayer graphene films promote osteogenic differentiati on of hASCs through activation of the FAK-ERK

These studies will greatly contribute to our understanding of the molecular mechanism and to the development of new biomarkers and therapeutic approaches in endometrial cancer and adenomyosis. In addition, it may help to understand the field of regenerative medicine to treat bone disease that can occur after traditional treatments such as radiation therapy, hormone therapy of

gynecological disorders.

Keywords: Endometrial cancer, Adenomyosis, Gynecological disease, Uterus, β-catenin, MIG-6, TGF-β2, LEF-1, Regenerative medicine, Mesenchymal stromal cell, Graphene

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LIST OF ABBREVIATIONS

MIG-6 : Mitogen inducible gene 6

MRI : Magnetic resonance imaging

CT : Computed tomography

PTEN : Phosphatase and tensin homolog

GSK3β : Kinases glycogen synthase kinase-3 alpha/beta

APC : Adenomatous polyposis coli

CKI : Casein kinase-1

TP53 : Tumor protein p53

MDM2 : Mouse double minute 2 homolog

CDH1 : Cadherin-1

EMT : Epithelial to mesenchymal transition

ARID1A : AT-rich interacting domain-containing protein 1A

KRAS : V-Ki-ras2 Kirsten rat sarcoma viral oncogenes homologue

EZH2 : Enhancer of zeste homolog 2

PRC2 : Polycomb repressive complex 2

NICD : Notch intracellular domain

TGF-β : Tansforming growth factor beta

H3K27me3 : Histone H3 Lysine 27 methyltransferase

CBP : CREB binding protein

HDACs : Class I histone deacetylases

DNMT : DNA methyltransferase

lncRNA : Long non-coding RNA

E2 : Estrogen

P4 : Progesterone

PGR : Progesterone receptor

RALT : Receptor-Associated Late Transducer

ERRFI1 : ERBB receptor feedback inhibitor 1

EGF : Epidermal growth factor

STAT3 : Signal transducers and activators of transcription 3

SPRR2F : The small proline-rich protein 2F

TCF : T-cell factor

LEF-1 : Lymphoid Enhancing Factor 1

FAK : Focal adhesion kinase

ERK : Extracellular signal-regulated kinases

RUNX2 : Runt-related transcription factor 2

ECM : Extracellular matrix

ERα : Estrogen receptor alpha

hASCs : Human adipose-derived stromal cells

GAGs : Glycosaminoglycans

HA : Hydroxyapatite

PGA : Polyglycolic acid

PLGA : Polylactic-co-glycolic acid

PCL : Polycaprolactone

PLLA : Poly L-lactic acid

pGO : Porous graphene oxide

MSCs : Mesenchymal stromal cells

MAPK : Mitogen-activated protein kinase

PMMA : Poly methyl methacrylate

CVD : Chemical vapor deposition

SEM : Scanning electron microscope

AFM : Atomic force microscopy

OPN : Osteopontin

ALP : Alkaline phosphatase

OCN : Osteocalcin

OSX : Osterix

CHAPTER 1

GENERAL INTRODUCTION

Gynecological diseases have recently been attracting much attention with the objective of improving the quality of life of women. Thus, there has been a great increase in the number of molecular studies investigating the causes of gynecological diseases. Although many studies on endometrial cancer and adenomyosis have been performed, the underlying molecular mechanisms is difficult to understand as the altered molecular pathways are very complex and have shown different specificities in different cell types. Identification and understanding of regulatory factors shared among various gynecological diseases is essential to the design of future clinical trials and the implementation of diseases therapies.

Endometrial cancer is caused by excessive stimulation of the endometrium due to the decrease of nonresistant estrogen and progesterone (Kim and Chapman-Davis 2010; Yang, Thiel *et al.* 2011). Endometrial cancer is associated with obstetrics and gynecology disease and is frequently reported in women in their 60s or older (Lee, Kim *et al.* 2012). The risk factors for endometrial cancer are obesity, hypertension and diabetes, and other factors such as nulliparity, late menopause are all associated with increased female hormone levels (Burbos, Musonda *et al.* 2010; Braun, Overbeek-Wager *et al.* 2016). Endometrial cancer can be cured by surgical treatment at the time of initial detection However, when endometrial cancer is suspected in younger patients, hormone therapy is used rather than surgical treatment to preserve fertility (Carneiro, Lamaita *et al.* 2016). It is well known that progesterone

and its analogues prevent proliferation of endometrial cancer (Kim, Kurita *et al*. 2013). However, there are many studies that limit the use of progesterone therapy due to low response rates in endometrial cancer (Leslie, Thiel *et al*. 2012; Mehasseb and Latimer 2012). Despite previous research on progesterone therapy, the underlying mechanism of progesterone resistance is still poorly understood. In a previous study, it was confirmed that the mRNA and Protein levels of Mitogen inducible gene 6 (MIG-6) were reduced in patients with endometrial cancer (Jeong, Lee *et al*. 2009). Interestingly, there are studies that MIG-6 is known to modulate endometrial genes and progesterone signaling pathway (Yoo, Kim *et al*. 2015). Over 80% of endometrial cancers are caused by abnormal proliferation of epithelial cells. So, in chapter 4, I investigated the role of *Mig-6* in the endometrial epithelium.

Adenomyosis is an abnormally thickening of the uterine wall and enlargement of the uterus as the endometrial tissue digs into the uterine muscle layer (Bulman-Fleming 2008; Koike, Tsunemi *et al*. 2013). Symptoms of adenomyosis are menstrual irregularities, menstrual cramps, and long term persistent pelvic pain (Bernardi, Lazzeri *et al*. 2017) (Koike, Tsunemi *et al*. 2013). Research has also shown that adenomyosis can cause infertility. (Campo, Campo *et al*. 2012). Although accurate diagnosis is difficult before surgery, it can be confirmed indirectly through vaginal ultrasound and magnetic resonance imaging (MRI), and can be confirmed by Computed Tomography (CT) (Agostinho, Cruz *et al*. 2017; Bonatti, Vezzali *et*

al. 2017). Some studies showed that adenomyosis is an ovarian steroid hormone-dependent disease caused by high estrogen levels unopposed by progesterone (Levy, Dehaene *et al*. 2013). However, the precise etiology and pathophysiology of adenomyosis has not been well understood. Previous study has shown that protein levels of β-catenin are overexpressed in patients with adenomyosis. Previously, our team confirmed that mice with uterine conditional activation β-catenin cause adenomyosis and ChIP-seq and DNA microarray were performed to investigate genetic and molecular events associated with development of adenomyosis from normal uterine structures. Therefore, I found that *Tgf-β2* and *Lef-1* directly regulated by activated β-catenin in the murine uterus. In chapters 5-6, I investigated the role of *Tgf-β2* and *Lef-1* activated by uterine conditional activation β-catenin in the development of uterine adenomyosis.

It has been reported that bone disease such as osteoporosis, osteolysis and osteosarcoma are observed in radiation therapy for the treatment of gynecological diseases, in women before and after menopause, and in patients with endometrial cancer (Henry, Lachmann *et al*. 1996; Lee, Kim *et al*. 2015; Oh, Yoon *et al*. 2015; Ottanelli 2015). The main reason is that female hormones play a key role in bone formation and maintenance (Novack 2007; Seifert-Klauss and Prior 2010; Manolagas, O'Brien *et al*. 2013). Although hormone therapy is used to treat bone disease, prolonged use is not recommended because long-term hormone therapy increases the

risk of cardiovascular disease and breast cancer (Prentice 2014). Recently, the field of regenerative medicine has been developed to treat such bone loss. Stem cells, which have the potential to produce cells that make up the human body, are considered to be an indispensable material in the field of regenerative medicine (Liras 2010; Stoltz, de Isla *et al.* 2015). Therefore, it is very important to understand the process of inducing the differentiation of stem cells to obtain the desired cells. For this purpose, a method of inducing differentiation of stem cells by giving an artificial stimulus to the outside of the cell and a method of creating an environment in which the inherent self-forming ability is maximized are used. For this reason, research on regenerative medicine using biomaterials has been actively conducted recently. One of biomaterials, graphene, is growing in interest in tissue engineering and biomedical fields (Chung, Kim *et al.* 2013; Tan, Thompson *et al.* 2015; Zhang, Wang *et al.* 2016; Banerjee 2018). In chapter 7, In order to develop materials that can help regenerate damaged bone, I investigated the effect of stem cells on osteogenic differentiation on the monolayer and multilayer graphene films synthesized by chemical vapor deposition.

CHAPTER 2

LITERATURE REVIEW

1. Endometrial cancer

1.1. General aspects of endometrial cancer

In the United States, endometrial cancer is the 4th most diagnosed gynecologic cancer in women (Denschlag, Ulrich *et al*. 2010) and 8th leading cause of female cancer death (Jemal, Siegel *et al*. 2010). Approximately, 60,050 cases are diagnosed and caused 10,470 deaths with endometrial cancer in 2016 (Siegel, Miller *et al*. 2016). According to world cancer report in 2014, approximately 40% of endometrial cancer linked to obesity (McGuire 2016). Because developmental obesity by hormonal imbalance affects endometrium. Some reports showed that low-active women with obesity have a higher risk in endometrial cancer development.

Most common type of endometrial cancer is adenocarcinoma which originated in endometrial epithelial cells (Di Cristofano and Ellenson 2007). Endometrial cancer is classified into two types (type I and type II) depending on its histology and clinical features. Type I is the most common type which is related with unopposed estrogen levels, obesity and endometrial hyperplasia. It shows more slowly growing and less spreading than type II. Type II endometrial cancer most occur elderly postmenopausal women and are not associated with estrogen levels, obesity. It generally seems to be aggressive growth and more spreading than type I. Endometrial cancer stage is

from 0 to IV depending on spread of the tumor from its primary site in the endometrium to other parts of the body. It is important to know the stage of endometrial cancer for treatment planning (Setiawan, Yang *et al*. 2013; Aristizabal, Graesslin *et al*. 2014; Lobo and Thomas 2016).

Currently, endometrial cancer is nearly diagnosed by endometrial biopsy, transvaginal ultrasound and curettage (Goldstein 2009). But these methods are difficult to diagnosis endometrial cancer early. Because, there are various technical limitation including operator dependence, patient intolerance, small fields of view (Faria, Sagebiel *et al*. 2015). Therefore, women who were diagnosed at late stage needs to hysterectomy. For these reasons, studies of exact etiology and pathology in endometrial cancer are required for diagnosis and treatments. Many research revealed that several factors such as genetic mutation, hormone, and obesity related to risk of endometrial cancer development (Ziel 1982; Kim and Chapman-Davis 2010; Schmandt, Iglesias *et al*. 2011; O'Hara and Bell 2012; Onstad, Schmandt *et al*. 2016).

1.2. Genetic mutation

1.2.1. *PTEN*

Phosphatase and tensin homolog (*PTEN*) which is mapped to chromosome 10q23 has been well-known tumor suppressor that negatively regulates PI3K/Akt signaling pathway (Keniry and Parsons 2008). About 50% of endometrial cancer showed mutated or lost *PTEN* (Vivanco and Sawyers 2002). *PTEN* usually is associated with tumor suppressor, cell motility and cell survival (Leslie and Downes 2004). *PTEN* mutation were found in several cancer including endometrial, breast, brain, prostate and renal (Alimov, Li *et al*. 1999; Che, Yao *et al*. 2002; Endersby and Baker 2008; Yang, Ren *et al*. 2010; Gao, Mei *et al*. 2016). Mutation of *PTEN* activates PI3K/Akt signaling pathway which results in dysregulation of other signaling such as estrogen receptor-dependent signaling that may play an important role in endometrial cancer development (Kim, Yoo *et al*. 2014). In endometrial cancer, Mutation of *PTEN* gene were most common codon 130 encoded by exon5, which involves phosphatase domain (Minaguchi, Yoshikawa *et al*. 2001). Also, for the etiological study of endometrial cancer, several *PTEN* knock-out mice models were developed. These mice develop endometrial cancer (Stambolic, Tsao *et al*. 2000; Kim, Kim *et al*. 2013). Therefore, *PTEN* mutation may play a role in endometrial cancer development and *PTEN* is a potential therapeutic target for endometrial cancer.

1.2.2. *β-catenin*

β-catenin, also known as *CTNNB1*, which is mapped to chromosome 3p21, has various functions including cell proliferation, stem cell renewal, cell to cell adhesion and epithelial-mesenchymal transition (Kraus, Liehr *et al.* 1994; MacDonald, Tamai *et al.* 2009; Sanchez-Tillo, de Barrios *et al.* 2011). β-catenin is a key factor in canonical Wnt signaling pathway, several reports revealed that stabilization of β-catenin expressions are associated with many different cancer involving ovarian cancer, colon cancer, endometrial cancer, which abnormally accumulates in the nucleus (Polakis 2000). Generally, without Wnt signaling, cytoplasmic β-catenin is degraded through phosphorylation by β-catenin destruction complex, including kinases glycogen synthase kinase-3 alpha/beta (GSK3β), adenomatous polyposis coli (APC) and casein kinase-1 (CKI). But, with Wnt signaling, GSK3β, APC, CKI binds to LRP5/6 receptor which results in inhibition of GSK3β, APC, and CKI mediated β-catenin phosphorylation. Therefore, stabilization of β-catenin translocate into nucleus and activates Wnt signaling target genes such as *c-MYC*, *Cyclin D1* (He, Sparks *et al.* 1998; Shtutman, Zhurinsky *et al.* 1999). In endometrial cancer case, exon 3 mutation β-catenin is commonly found at an early-stage. Because Exon3 mutation of β-catenin which contains serine and threonine residues phosphorylated by GSK3β is commonly detected in endometrial hyperplasia (Machin, Catasus *et al.* 2002; Kurnit, Kim

et al. 2017). Although β-catenin molecular mechanisms in endometrial cancer development have not been completely elucidated, β-catenin expression pattern will be a favorable prognostic factor.

1.2.3. *TP53*

Tumor protein p53 (*TP53*), also well known as p53, is located on chromosome 17p13.1 in human (Cousin, Billotte *et al*. 2000). TP53 is most well-known tumor suppressor protein. Wild type TP53 has various functions including DNA repair, holding the cell cycle and initiating apoptosis (Zilfou and Lowe 2009). In normal cells, TP53 binds to its negative regulator, Mouse double minute 2 homolog (MDM2) protein, results in inactivation. But when cells are damaged, MDM2-TP53 complex is dissociated by various signaling and subsequent increase the amount of TP53 (Moll and Petrenko 2003). Increased TP53 acts as a transcription factor stimulating genes related with cell cycle arrest, DNA repair, apoptosis such as *p21*, *GADD45*, *FAS/APO-1* (Benchimol 2001). In human cancer, inactivation and mutation of *TP53* genes are common feature and linked closely to different types of cancer including ovarian cancer, breast cancer, and pancreatic cancer (Gasco, Shami *et al*. 2002; Corney, Flesken-Nikitin *et al*. 2008; Weissmueller, Manchado *et al*. 2014). In endometrial cancer, DNA-binding domain mutation encoded by exon5-8 of *TP53* is frequently detected (Tashiro, Isacson *et al*. 1997). Recently studies suggest that *TP53* Arg72pro

polymorphisms is associated with endometrial cancer development. *TP53* Arg72pro polymorphisms is the conversion of guanine to cytosine nucleotide, leading to arginine to proline. Therefore, this mutation induced different biological characteristics compared with wild-type TP53 (Jiang, Yao *et al.* 2011). But, more studies will be necessary to understand role of TP53 in endometrial cancer.

1.2.4. *CDH1*

Cadherin-1(*CDH1*) also known as E-cadherin is calcium dependent cell adhesion protein and also found in epithelial cells. CDH1 proteins are the product of gene located on chromosome16q22 and located in the cell membrane. Usually, CDH1 forms complexes with catenin which result in regulating cell polarity, integration, and cell to cell adhesion (Li, Wu *et al.* 2014). Dysregulation of CDH1 has been reported in many cancer research involving ovarian, breast, and endometrial cancer (Sundfeldt, Piontkewitz *et al.* 1997; Yalta, Atay *et al.* 2009; Singhai, Patil *et al.* 2011). Because, the common feature of down-regulated CDH1 is epithelial to mesenchymal transition (EMT), resulting in disruption of adhesion and increased invasion or migration (Katoh 2005). In endometrial cancer, mutation of CDH1 is rarely detected. But some reports showed that loss of heterozygosity at chromosome 16q22 cause low-expression of e-cadherin, resulting in endometrial cancer development and loss of CDH1 correlates with endometrial cancer development (Moreno-Bueno,

Hardisson *et al*. 2003). Also low-expressions of CDH1 were found more frequently in type II than type I endometrial cancer (Liu 2007). Therefore, CDH1 will be good prognostic factor for type II endometrial cancer.

1.2.5. Other genes

The AT-rich interacting domain-containing protein 1A (*ARID1A*) is a switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex that regulates gene expressions by altering the chromatin structure. *ARID1A* gene is located on chromosome 1p36.11 and its product act as a tumor suppressor in human (Wu, Wang *et al*. 2014). *ARID1A* gene mutation is often found in many cancer involving gastric cancer, ovarian cancer, pancreatic cancer and endometrial cancer (Birnbaum, Adelaide *et al*. 2011; Wang, Kan *et al*. 2011; Takeda, Banno *et al*. 2016). Actually, many research revealed that chromatin remodeling factors have been associated with cancer development. Some reports showed that low-protein levels and higher frequency of mutation of *ARID1A* are detected in endometrial cancer (McConechy, Ding *et al*. 2012; Werner, Berg *et al*. 2013). However, ARID1A gene mutation is rarely studied in endometrial cancer. Therefore, further studies are needed to clarify molecular mechanisms of ARID1A in endometrial cancer.

V-Ki-ras2 Kirsten rat sarcoma viral oncogenes homologue (*KRAS*), a member of RAS family, is a well-known proto-oncogene

that has functions in normal cells such as cell division, cell differentiation, and GTPase (Nussinov, Tsai *et al*. 2016). Aberrant expression of *KRAS* are frequently observed in many cancer. Some reports showed that Point mutations at the 12th codon and overexpressed *KRAS* are found in endometrial cancer (Ito, Watanabe *et al*. 1996; Pappa, Choleza *et al*. 2006; Birkeland, Wik *et al*. 2012). Also, *KRAS* knockdown in HEC1A cells, human endometrial adenocarcinoma cell, led to reducing of migration (Vartanian, Bentley *et al*. 2013). Therefore, *KRAS* alterations involving point mutation, silencing are associated with endometrial cancer development. KRAS also is a significant prognostic factor in endometrial cancer.

Mitogen-inducible gene 6 (*MIG-6*), a negative regulator of EGFR signaling, is a non-kinase scaffolding adaptor protein that perform the function of maintaining homeostasis and acting as tumor suppressor (Izumchenko and Sidransky 2015). MIG-6 is regulated by various factors including hormones, growth factors and stresses (Zhang and Vande Woude 2007). One report showed that Protein and mRNA levels of *MIG-6* are decreased in patients who are diagnosed with endometrial cancer. Also, *MIG-6* gene ablation was shown to induce endometrial hyperplasia development in mice model (Jeong, Lee *et al*. 2009; Yoo, Yang *et al*. 2018). Therefore, MIG-6 also may play an important role in endometrial cancer development.

1.3. Epigenetic change

Aberrant Epigenetic events have been thought to progress cancer development Therefore, Recently, various studies have been conducted to understand epigenetic mechanisms in cancer research. Epigenetic changes involving methylation of DNA, histone modification and non-coding RNA were found in many cancer involving breast cancer, prostate cancer, ovarian cancer and endometrial cancer (Balch, Fang *et al.* 2009; Wu, Sarkissyan *et al.* 2015; Stampoliou, Arapantoni-Dadioti *et al.* 2016). So, Understanding of epigenetic change may contribute to provide useful biomarkers and prevention for cancer development.

Several research showed that silenced genes by DNA promoter methylation were documented in endometrial cancer development. These Silenced genes are frequently associated with DNA repair, tumor suppressor and growth regulation of cells. The most well-known example is low expression of *MLH1*, which involved in meiotic crossing over, which plays a role in repair of DNA mismatches (Ellison, Lofing *et al.* 2004). Promoter hyper-methylation of *MLH1* is frequently found in endometrial cancer. One report revealed that Promoter hyper-methylation status of the *MLH1* gene is detected in normal endometrium regions of patient with endometrial cancer (Kanaya, Kyo *et al.* 2003). Another silenced gene is negative β-catenin regulator adenomatous polyposis coli (*APC*)

in endometrial cancer (Ignatov, Bischoff *et al*. 2010; Tao and Freudenheim 2010). Hyper-methylation seen on *APC* promoter is not detected on endometrial hyperplasia or normal endometrium. Interestingly, in atypical endometrial hyperplasia and early-stage endometrial cancer, promoter hyper-methylation of *APC* is found. But, late-stage endometrial cancer showed that a low frequency of *APC* promoter hyper-methylation compared to atypical endometrial hyperplasia and early-stage endometrial cancer (Ignatov, Bischoff *et al*. 2010). These results suggest that epigenetic change may play a role as an initiation of endometrial cancer progression.

Histone modification is a phenomenon that can change functions of gene without altering sequence of DNA. To form the nucleosome, DNA wraps around the special proteins called histone. The structure of the nucleosome can be modified by various methods such as acetylation, phosphorylation, ubiquitination and methylation of histone protein. These modifications alter the accessibility of transcription factors to DNA (Marino-Ramirez, Kann *et al*. 2005). Therefore, malfunction of histone modification may contribute to develop cancer progression. Alteration of histone modification has been reported in endometrial cancer development. Enhancer of zeste homolog 2 (EZH2), a histone-lysine N-methyltransferase enzyme, is the catalytic subunit of the polycomb repressive complex 2 (PRC2), which regulates chromatin-modifying, cell differentiation (Simon and Lange 2008). EZH2 has been reported to be overexpressed in

endometrial cancer (Oki, Sone *et al.* 2017). Also, it was confirmed that Histone H3 Lysine 27 methyltransferase (H3K27me3) regulating EZH2 did not normally express in endometrial cancer. Recently, one report suggested that H3K4me2 expression pattern might be used as prognostic factor of endometrial cancer progression (Li, Jia *et al.* 2017). In order to predict and prevent endometrial cancer development, research of genes associated with histone modification should be further studied

1.4. Obesity with hormonal effects in endometrial cancer development.

Endometrial tissue is tightly regulated by steroid sex hormones, which results in remodeling, growth, differentiation of endometrial cells (Kim and Chapman-Davis 2010). High levels of estrogen unopposed by progesterone are easily found in people who have been diagnosed with endometrial cancer (Allen, Key *et al.* 2008; Brinton and Felix 2014). High levels of estrogen are commonly induced by endogenous or exogenous. Estrogen is mainly produced in the ovary but also in adipose tissue (Siiteri 1987). Many research showed that Obesity is closely related with endometrial cancer risk by promoting estrogen metabolism (Arem and Irwin 2013; Zhang, Liu *et al.* 2014; Shaw, Farris *et al.* 2016). Women with obesity (defined as BMI > 30 and < 35 kg/m^2) and severe obesity (BMI > 35 kg/m^2) were, respectively, 2.6 fold and 4.7 fold more likely to have a higher

endometrial cancer risk than normal-weight women (BMI > 25 kg/m^2) (Shaw, Farris *et al*. 2016). Some reports suggest that increasing of estrogen due to adipose tissue causes an increase in bio-active IGF-I, which affects the development of endometrial cancer by activating the AKT and MAPK/ERK signaling pathway associated with proliferation and differentiation of cells (Schmandt, Iglesias *et al*. 2011; Bruchim, Sarfstein *et al*. 2014). However, Studies on the mechanism of relationship between endometrial cancer development and obesity are still lacking. Understanding of molecular mechanisms of obesity-endometrial cancer relation may reduce endometrial cancer risk.

2. Adenomyosis

2.1. General aspects of adenomyosis

Adenomyosis is a benign uterine condition characterized by the presence of heterotopic epithelial cells, endometrial glands, and stroma within the myometrium, which causes thickening or swelling of the uterus (Jain and Goel 2012; Harada, Khine *et al*. 2016). Adenomyosis development is associated with a hormonal imbalance. Common adenomyosis symptoms and signs include dysmenorrhea, chronic pelvic pain, and dyspareunia (Proctor and Farquhar 2006). Moreover, adenomyosis may cause infertility or subfertility (Harada, Khine *et al*. 2016). Several studies have shown that adenomyosis is associated with both hormonal and autoimmune factors (Ota, Igarashi *et al*. 1998; Garavaglia, Audrey *et al*. 2015), but the pathophysiology of adenomyosis is still largely unknown. Currently, adenomyosis is commonly diagnosed by endometrial biopsy, magnetic resonance imaging, and transvaginal ultrasonography (Tamai, Togashi *et al*. 2005; Dueholm 2006; Wortman 2008). However, these diagnostic methods have some limitations such as the limited field of view, low specificity, and difficulty in detecting early-stage disease (Reinhold, Tafazoli *et al*. 1998; Bazot, Darai *et al*. 2002; Tamai, Togashi *et al*. 2005). Therefore, more precise studies evaluating the molecular mechanisms involved in adenomyosis are required to design suitable

clinical trials and allow prevention, appropriate treatment, and prompt diagnosis.

2.2. Molecular events in adenomyosis development

2.2.1. Genetic mutations

It has been suggested that genetic mutations in endometrial cells also induce adenomyosis. However, to date, the role of genetic mutations in adenomyosis has not been fully documented. Only a limited number of experimental studies confirming the mutational status of genes have been conducted to date. One study reported that mutations of estrogen receptor alpha (*ERα*) gene (*ESR1*) were found in adenomyosis (Oehler, Greschik *et al*. 2004). The *ERα* has been mapped to chromosome 6q25.1 and is encoded by the ESR1 gene. ERα is a ligand-dependent transcription factor whose expression is commonly regulated by estrogen and is functionally implicated in cell growth and integrity (Chen, Hsieh *et al*. 2014). In the uterus, ERα protein has been known to contribute to fertility, mammary gland maturation, and induction of ovulation (Lee, Kim *et al*. 2012). Another report indicated that DNA-binding domain mutations of *ESR1* can lead to defective transcriptional activation. In adenomyosis, the presence of two point mutations (P129R, M427I/L429 in *ESR1* has been confirmed (Oehler, Greschik *et al*. 2004). Although the above study identified only a 5% prevalence of

ESR1 mutations, this finding implies that the mutations may induce development of adenomyosis.

2.2.2. Changes in cellular phenotype

Epithelial to mesenchymal transition (EMT) refers to the phenotypic transition of epithelial cells to mesenchymal cells, which ultimately results in the conversion of epithelial cells into cells with metastatic and invasive potential (Son and Moon 2010). Although the transition of these cells is essential during development (Kalluri and Weinberg 2009), it is also considered an undesirable phenomenon in relation to progression in various diseases. Reprogramming of cells by EMT mechanisms is closely linked to changes in different regulatory networks. The dysregulation of epithelial cells is caused by changes in various regulatory steps, including transcription and translation. One of the major molecular changes occurring during EMT is a reduction in CDH1 expression (Kalluri and Weinberg 2009). Also, several reports have indicated that transcription factors such as Snail, Slug, and Twist can induce EMT in cancer (de Herreros, Peiro *et al*. 2010; Wang, Shi *et al*. 2013; Lamouille, Xu *et al*. 2014). Determining the relationship between EMT control mechanisms and various signaling systems will help in better understanding of adenomyosis development

2.2.2.1. Wnt/β-catenin signaling

Several reports have described Wnt/β-catenin signaling to play an important role in EMT progression (Jiang, Luo *et al.* 2007; Shan, Lv *et al.* 2015). β-catenin is a key factor in the canonical Wnt signaling pathway. Several reports have indicated that stabilization of β-catenin expression is associated with many different cancers, including ovarian, colon, and endometrial cancer. Cancer pathogenesis likely involves the abnormal accumulation of β-catenin in the nucleus (Polakis 2000). In general, in the absence of Wnt signaling, cytoplasmic β-catenin is degraded through phosphorylation by a β-catenin destruction complex, including kinases glycogen synthase kinase-3 beta (GSK3β), adenomatous polyposis coli (APC), and casein kinase-1 (CKI). However, in the presence of Wnt signaling, GSK3β, APC, and CKI bind to the Low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) receptor, which leads to inhibition of GSK3β-, APC-, and CKI-mediated β-catenin phosphorylation. Therefore, stabilization of β-catenin leads to translocation of the protein into the nucleus, which activates Wnt signaling and target genes involved in cell proliferation, such as *c-MYC* and *Cyclin D1* (He, Sparks *et al.* 1998; Shtutman, Zhurinsky *et al.* 1999). One study revealed that the stabilization of β-catenin leads to the activation of mesenchymal cell markers such as ZEB1 and SNAIL in endometrial epithelial cells. In addition, CDH1 protein expression has been shown to be reduced

in endometrial epithelial cells (Oh, Shin *et al.* 2013). These results suggest that a dominant stabilized β-catenin expression may play an important role in the pathogenesis of adenomyosis.

2.2.2.2. Notch signaling

Notch signaling also contributes to adenomyosis development by EMT progression (Qi, Zhao *et al.* 2015). It is well known that notch signaling regulates the growth of cells and developmental processes of various organs (Lai 2004). The Notch receptor is a membrane protein that includes Notch1, Notch2, Notch3, and Notch4. Delta-like and Jagged are the most well-known ligands of the Notch receptor. Theses ligands commonly have a Delta/Serrate/LAG-2 (Tang, Lee *et al.*) domain that can bind to the Notch receptor. Upon ligand binding, the Notch intracellular domain (NICD) is cleaved by several factors such as protease and gamma-secretase enzymes, which results in its release into the nucleus to regulate gene transcription (Yamamoto, Schulze *et al.* 2014). It has been reported that genes associated with EMT induction are also regulated by Notch signaling (Wang, Li *et al.* 2010). One study showed that Notch1 levels in adenomyosis are higher than those found in the normal endometrium. In addition, expression of Numb, a negative regulator of Notch signaling, is reduced, while the expression of mesenchymal cell markers such as N-cadherin, Slug, and Snail is also increased (Qi, Zhao *et al.* 2015). These data indicated that the Notch signaling

pathway may contribute to the development and pathogenesis of adenomyosis.

2.2.2.3. TGF−β signaling

Another important pathway that causes adenomyosis development is the transforming growth factor beta (TGF−β) signaling pathway (Shen, Liu *et al.* 2016; Yen, Huang *et al.* 2017). TGF−β is a member of the TGF superfamily that plays an important role in cell regulation of apoptosis, blocking of the cell cycle, and differentiation (Wu, Bitzer *et al.* 2005). Thus, TGF−β signaling is closely associated with cell homeostasis and various human diseases. TGF−β activates the TGF−β receptor II through the formation of a heteromeric complex, leading to the TGF−β receptor II−induced phosphorylation of TGF−β receptor I. Subsequently, activated TGF−β receptor I phosphorylates the C−terminal of serine residues of the Receptor activated−Smad (R−SMAD) transcription factors. Thus, phosphorylated R−SMADs can translocate into the nucleus after complex formation with Smad4. Here, the Smad complex induces the expression of target genes through interaction with co−activators such as CREB binding protein (CBP)/p300 (Verrecchia and Mauviel 2002; Zi, Chapnick *et al.* 2012). Some reports have indicated that TGF−β signaling induces expression of Snail, Zeb1 and HMGA2 proteins, which are known to induce factors involved in EMT (Miyazono 2009; Xu, Lamouille *et al.* 2009). Results

from microarray-based gene expression studies have determined that TGF-β is strongly expressed in adenomyosis-induced mouse models (Shen, Liu *et al.* 2016). These results suggest that the TGF-β signaling pathway may be an important target in the study of adenomyosis development.

2.3. Epigenetic changes.

Epigenetic changes in DNA expression have been studied in the development of adenomyosis. Histone modification is a phenomenon that can alter gene function without altering DNA sequences. To form the nucleosome, DNA wraps around special proteins called histones. The overall structure of the nucleosome can be modified by different modifications including acetylation, phosphorylation, ubiquitination, and methylation of histone proteins. These modifications alter the accessibility of transcription factors to the target DNA sequences (Marino-Ramirez, Kann *et al.* 2005). It has been suggested that an aberrant histone modification may contribute to adenomyosis progression. Class I histone deacetylases (HDACs) have been shown to be involved in the development of adenomyosis. HDACs remove the acetyl groups from histone proteins, which results in the promotion of gene transcription (Seto and Yoshida 2014). In adenomyosis, the expression of both HDAC1 and HDAC3 isoforms is higher in the eutopic and ectopic endometrium than in the normal endometrium (Liu, Nie *et al.* 2012).

It has also been reported that DNA methyltransferase (DNMT) proteins involved in DNA methylation may be implicated in adenomyosis development. Levels of DNMT1 and DNMT3B isoforms were found to be higher in the ectopic endometrium than in the normal endometrium, whereas DNMT3A levels were lower in the eutopic and ectopic endometrium (Liu and Guo 2012). Another study reported that aberrant expression of long non-coding RNA (lncRNA) also contributes to the development of adenomyosis, which was a new finding. The lncRNA is known to play an important role in gene regulation such as transcription and translation (Cao 2014). One study reported a total of 165 lncRNAs were abnormally expressed in the eutopic endometrium with adenomyosis; whereas, no abnormal lncRNA expression was found in endometrium without adenomyosis (Jiang, Sun *et al.* 2016). Similarly, a study evaluating lncRNA expression patterns showed that 576 lncRNAs were abnormally expressed in the ectopic endometrium with adenomyosis (Zhou, Zhang *et al.* 2016). Therefore, these results suggest that abnormal epigenetic modifications may be critical factors in adenomyosis development. Evaluation of these epigenetic changes may be helpful in defining treatment, prevention, and diagnosis of adenomyosis.

3. Regenerative medicine

3.1. General aspects of regenerative medicine

Regenerative medicine is intended to replace or regenerate damaged tissue or organ. In particular, regenerative medicine is expected to provide a new treatment method for diseases that cannot be treated by existing treatment methods, and it is attracting attention as a hope of future medicine (Mao and Mooney 2015). The body has the ability to regenerate through its inherent stem cells and restore the function of damaged tissue (Richardson, Kalamegam *et al.* 2016). However, if the degree of damage exceeds the ability of the inherent stem cell to regenerate, the function of the tissue can be restored only by replacing the stem cell transplanted from the outside (Mahla 2016). So, the most popular and interesting field in regenerative medicine is stem cells. Stem cells have become an important key to the treatment of various diseases due to their plasticity (Filip, Mokry *et al.* 2005). However, for regenerative medicine, studies on proliferation, survival, adhesion and differentiation of stem cells should be given priority. The development of biomaterials that can regulate the growth, regulation, attachment, and behavior of stem cells is important in future regenerative medicine. Therefore, the development of biomaterials capable of controlling the behavior of stem cells should be actively studied.

3.2. Biomaterials using stem cells in regenerative medicine

Stem cells have the ability to differentiate into various types of body tissues, that is, undifferentiated cells (Filip, Mokry *et al.* 2005). In this undifferentiated state, when appropriate conditions are met, differentiation into various tissue cells is possible, and studies are being conducted for application to therapy such as regeneration of damaged tissues (Stoltz, de Isla *et al.* 2015). In order to regenerate damaged tissues and organs using these characteristics, many transplantations of cells differentiated from stem cells or stem cells have been studied (Mahla 2016). In recent years, researches have been carried out to create a microenvironment that induces cell growth and differentiation by using various biomaterials for stem cell culture (Owen, Kline *et al.* 1981; Lutolf, Gilbert *et al.* 2009). The supporter using biomaterial is the most important tool to cultivate stem cells or to create a three dimensional environment (Meng, Leslie *et al.* 2014). This structure is known to be an ideal platform for communication between cells and cells, between cells and materials, and to assist in cell proliferation and differentiation (Gao, Peng *et al.* 2017; Kumar 2018). Biomaterials for regenerative medicine should basically have biocompatibility, biofunctional and minimal immune response, smooth interaction between materials and cells, and appropriate mechanical strength (Morais, Papadimitrakopoulos *et al.* 2010; Qi, Yan *et al.* 2015). Biomaterials include natural polymers derived from humans, animals and plants,

and synthetic polymers are widely used. Natural polymers include collagen, fibrinogen, hyaluronic acid, glycosaminoglycans (GAGs), chitosan, hydroxyapatite (HA) and cellulose found in extracellular matrix (ECM). They have the advantage of having mechanical similarity with biologically active, biocompatible natural tissues (Mano, Silva *et al.* 2007; Yunus Basha, Sampath Kumar *et al.* 2015). The synthetic polymeric materials are polyglycolic acid (PGA), polylactic-co-glycolic acid (PLGA), polycaprolactone (PCL), and poly L-lactic acid (PLLA). Synthetic polymers can be obtained cheaply through synthesis and easy to control mechanical properties, but they are less biocompatible and biofunctional than natural polymers (Kim, Sun Park *et al.* 2006; Gentile, Chiono *et al.* 2014). While these various biomaterials are being studied, graphene, which is a biomaterial used in this paper, has been recently recognized as a biological material for biological tissue engineering.

3.2.1. Biomedical applications of graphene.

Graphene is a two-dimensional material made of carbon atoms and has a honeycomb structure. It is the thinnest material in the existing material, and it shows superior properties in terms of electrical, mechanical, thermal and optical properties (Zhu, Duan *et al.* 2014; Dubey, Bentini *et al.* 2015; Adeel, Bilal *et al.* 2018; Kim, Cho *et al.* 2018). Graphene materials can be applied to the entire industry including transparent electrodes, next-generation

semiconductors, energy electrodes, ultra-light materials, heat dissipation, and printing electronics (Flores-de-Jacoby 1985; Torrisi, Hasan *et al*. 2012; Chaichi, Wang *et al*. 2018; Kim 2018; Lee, Park *et al*. 2018; Zhao, Yu *et al*. 2018). Potential availability of graphene is not only limited to applications using physicochemical properties, but also for biological and engineering applications, and is likely to be applied as a support and functional material for tissue engineering or drug delivery (Shin, Li *et al*. 2016; Lakshmanan and Maulik 2018).

For this reason, many researches related to the regulation and growth of stem cells using graphene have been conducted

3.2.1.1. Control of cell behavior

The nanotopography used to control stem cell behavior can affect the proliferation and adhesion of stem cells as well as differentiation into nerve, osteo, and muscle cells (Ravichandran, Liao *et al*. 2009). One of effective tissue engineering techniques is the regulation of cell behavior using various properties of extracellular matrix, the regulation of cell attachment pattern, and the regulation of cell growth and tissue regeneration ability through shape micro patterning. In order to provide the material functionality required for such tissue engineering techniques, a biomaterial composite of natural background material and graphene can be used. Graphene's excellent physical properties and biocompatible natural

composite materials can be used as intelligent materials with bioactivity, biocompatible materials. The most commonly used stem cell culture study using graphene is to coat graphene on a culture dish and grow cells. There have been studies in which various stem cells are cultured on graphene and differentiated into various cell lineage while changing the culture method. Two-dimensional film-like graphene film synthesized by chemical vapor deposition shows low toxicity and excellent biocompatibility in the test of cell viability of human mesenchymal stem cells (Gurunathan and Kim 2016). Human mesenchymal stem cells have been promoted to osteogenic differentiation on various substrates coated with graphene, and the effect of BMP-2, which is a commonly used bone morphogenetic growth factor, has been confirmed (Nayak, Andersen *et al.* 2011). Another study showed that nanoparticles of porous graphene oxide (pGO) and graphene helped to form and maturing cartilage tissue (Lee, Lim *et al.* 2015). There is also research showing that graphene further stimulates the differentiation of neural stem cells into neurons instead of glial cells. Relatively neural stem cells are more difficult to differentiate into neurons than glial cells. However, when cultured on graphene, the neural stem cells were more differentiated into neurons (Park, Park *et al.* 2011). There is also research that binds fluorine functional groups to graphene to promote the differentiation of mesenchymal stem cells into neurons. This result suggests that fluorine-bound graphene improves cell adhesion and proliferation, and also induces cell polarization spontaneously, affecting cell shape

and skeleton (Wang, Lee *et al.* 2012). One research team found that graphene quantum dots not only prevent non-ideal fibrous aggregation due to denaturation of α-synuclein, a protein that is a cause of Parkinson's disease, but also have a remarkable therapeutic effect on Parkinson's disease by releasing already formed aggregates into normal protein monomers.(Kim, Yoo *et al.* 2018). These studies suggest the infinite possibilities of biomaterial graphene for the application of regenerative medicine by modulating cell behavior

3.2.1.2. Drug delivery system

The drug delivery system is intended to control the release and absorption of drugs over time, to target drugs that deliver drugs to specific sites, and to early diagnosis (Singh and Lillard 2009). Applications of graphene drug delivery systems are possible because of their low toxicity, biocompatibility, stability, thickness and size control of graphene, and surface functionalization due to pi-pi bonding (Lakshmanan and Maulik 2018). A team of researchers have developed PEGylated nano-graphene oxide (NGO-SS-mPEG), which is mediated by disulfide linkage, to deliver intracellular drug. In drug delivery, there is a problem of diffusion barrier in the cell, which has been developed to release rapidly from tumor-relevant glutathione (GSH) levels. NGO-SS-mPEG is a complex with high solubility, stability and circulation properties in physiological environment and it has been proved that it can selectively release

drugs from NGO according to intracellular GSH stimulation (Wen, Dong *et al*. 2012). There have also been researches on cancer therapy using graphene, which has functionalized nanoscale graphene oxide (NGO) as sulfonic acid groups as a nanocomposite for the control and targeting of anticancer drugs. They then targeted human breast cancer cells with FA receptors after covalent attachment to folic acid (FA) molecules. Based on graphene-based nano-carriers, proven effects of controlled loading and target delivery of combined chemotherapeutic agents (Zhang, Xia *et al*. 2010). Graphene drug delivery system report shows similar results such as thermodynamic, optical, and geometrical properties compared with the previously reported carbon nanotubes. However, graphene has better dispersibility, smaller size control efficiency, lower cost than carbon nanotubes and has been shown to be more effective in drug delivery system and cancer treatment research.

CHAPTER 3

GENERAL MATERIAL

AND METHODS

Quantitative real-time PCR Analysis

cDNA was produced from 1㎍ of total RNA using random hexamers and MMLV Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). Real-time PCR was performed using RT-PCR SYBR Green detection system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions (PE Applied Biosystems, Foster City, CA). mRNA quantities were normalized against the housekeeping gene.

Immunohistochemistry analysis

Samples were pre-incubated with 10% normal serum in phosphate-buffered saline (PBS; pH 7.5) and then incubated with primary antibody in 10% normal serum in PBS overnight at 4° C. On the next day, samples were washed in PBS and incubated with a secondary antibody conjugated to biotin (Vector BA-1000, Laboratories, Burlingame, CA) for 1 hour at room temperature and then samples were washed in PBS and incubated with Horseradish Peroxidase (Invitrogen Corp., Carlsbad, CA) for 45 min at room temperature and then immunoreactivity was detected using DAB (Vector Laboratories, Burlingame, CA). Analysis of protein expression was observed under a light microscopy.

Western blot analysis

Cellular proteins were extracted using lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, and 0.125% Nonidet P-40 (volume/volume) in distilled water supplemented with both a protease inhibitor cocktail (Roche, Indianapolis, IN) and a phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO). Fifteen μg of protein lysates were electrophoresed using SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). Casein (0.5% volume/volume) was used to block the membrane prior to exposure to primary antibody immunoblotting. Immunoreactivity was visualized by incubation with a horseradish peroxidase-linked secondary antibody followed by exposure to ECL reagents according to manufacturer's instructions (GE Healthcare Biosciences, Piscataway, NJ).

Immunofluorescence analysis

Cells were washed with PBS, fixed with 4% paraformaldehyde for 20min at room temperature and permeabilized with 0.1% of Triton X-100 (Sigma-Aldrich, St. Louis, MO). After further washing, Cells were exposed to primary antibody overnight at 4° C and secondary antibodies for 2 hour at room temperature. Washed coverslips were then mounted onto microscope slides with a DAPI-impregnated mounting media (Vector Laboratories, Burlingame, CA). Images were

captured with a confocal microscope (LSM 700 confocal microscope; Carl Zeiss, Thornwood, NY, USA)

Invasion assay

For the transwell invasion assay, post-transfected cells (24 hours) were trypsinized and seeded at a density of 2.5 x 105 per 200 μl serum-free culture medium to insert chamber of a BioCoat (24-well insert; pore size, 8 μm; BD Biosciences, San Jose, CA). The cells were incubated at 37° C in 5% CO2 according to the experiment and noninvading cells were removed with cotton swab. Invading cells on the lower surface of the membrane were fixed with 100% methanol and stained with 1% crystal violet (Sigma-Aldrich, St. Louis, MO). Stained cells were captured via light microscopy using software from NIS Elements, Inc. (Nikon, Melville, NY).

CHAPTER 4

THE FUNCTIONAL ANALYSIS OF EPITHELIAL MIG-6 IN ENDOMETRIUM

1. Introduction

Endometrial cancer is a well-known gynecologic malignancy of the female reproductive tract. In the United States, endometrial cancer affected 60,050 women and caused 10,470 deaths in 2016. It comprises 7% of all cancer in women (Siegel, Miller *et al.* 2016). The majority of endometrial cancer is endometrioid adenocarcinoma, which is derived from epithelial cells of the endometrium (Di Cristofano and Ellenson 2007). The development of endometrial hyperplasia, a proliferative process in the epithelium, is a critical risk factor of endometrioid carcinoma (Kurman, Kaminski *et al.* 1985). The regulation of uterine epithelial cell and stromal cell proliferation is controlled by estrogen (E2) and progesterone (P4), both of which are ovarian steroid hormones (Critchley and Saunders 2009).

P4 is a steroid hormone produced by the ovaries. Luteinizing hormone and chorionic gonadotropin regulate the synthesis and secretion of P4 during the menstrual cycle and pregnancy (Graham and Clarke 1997). Coordinated actions of the progesterone receptor (PGR) mediate the P4 response in the endometrium (Rubel, Jeong *et al.* 2010). Stromal-epithelial communication is important for uterine function (Lee, Kim *et al.* 2013). PGR inhibits E2-mediated epithelial cell proliferation via mediating epithelial-stromal cross talk (Rubel, Jeong *et al.* 2010; Franco, Rubel *et al.* 2012). P4 lessens E2 stimulated uterine epithelial proliferation by modulating the gene expression in the uterine stromal cells (Kurita, Lee *et al.* 2000).

While the effect of P4 on uterine function is mediated by epithelial–stromal cross–talk, the exact molecular mechanism of epithelial–stromal cross–talk remains elucidated (Kurita, Lee *et al*. 2000).

A steroid hormone imbalance could lead to aberrant endometrial proliferation and endometrial cancer. P4 therapy is used against endometrial hyperplasia and early endometrial cancer in patients who want to preserve fertility (Hahn, Yoon *et al*. 2009; Kim, Kurita *et al*. 2013). P4 and its analogues can have an effect on suppression of endometrial cancer proliferation (Yang, Thiel *et al*. 2011). However, many studies suggest limiting the use of P4 therapy due to its low response rates in endometrial cancer (Decruze and Green 2007). Despite previous studies on P4 therapy, the underlying mechanisms of P4 resistance are still poorly understood.

Mitogen–inducible gene 6 (*MIG–6*; also referred to as Receptor–Associated Late Transducer (RALT), ERBB receptor feedback inhibitor 1 (*ERRFI1*), and gene 33) is a 50 kDa cytoplasmic protein. MIG–6 is identified as an early–response gene that can be transcriptionally regulated by epidermal growth factor (EGF), transforming growth factor alpha (TGF–α), and stress factors (van Laar, Schouten *et al*. 2001; Zhang and Vande Woude 2007). MIG–6 is also induced by mitogenic stimuli in a cell cycle–dependent manner (Wick, Burger *et al*. 1995). MIG–6 exhibits important tumor suppressor capabilities by regulating migration and invasion, cell proliferation, and the rate of G1–S phase progression (Fiorini, Ballaro *et al*. 2002; Reschke, Ferby *et al*. 2010; Ying, Zheng *et al*.

2010; Lin, Du *et al*. 2011). The low level of *Mig-6* is observed in human hepatocellular carcinoma (Reschke, Ferby *et al*. 2010), breast carcinomas (Anastasi, Sala *et al*. 2005), papillary thyroid cancer (Lin, Du *et al*. 2011), glioblastoma (Duncan, Killela *et al*. 2010), non-small cell lung cancer (Zhang, Staal *et al*. 2007), and endometrial cancer (Jeong, Lee *et al*. 2009).

Previously, my research team demonstrated that *Mig-6* has a critical function in the development of endometrial hyperplasia and E2-induced endometrial cancer as a mediator of PGR functions to suppress E2 signaling in the uterus (Jeong, Lee *et al*. 2009; Kim, Lee *et al*. 2013). *Mig-6* suppress tumorigenesis of endometrial cancer that is related with *Pten* deficiency and ERK activation in endometrial cancer (Kim, Franco *et al*. 2010). MIG-6 is identified as an adaptor protein that consists of important protein-protein interaction domains, an EGFR binding domain, an src homology 3 (SH3)-binding motif, a 14-3-3-binding domain, and a Cdc42- and Rac-interactive binding (CRIB) domain (Makkinje, Quinn *et al*. 2000; Pirone, Carter *et al*. 2001), but it does not have a domain with enzymatic activity (Zhang and Vande Woude 2007). My team identified signal transducers and activators of transcription 3 (STAT3) as a MIG-6 associated protein (Kim, Franco *et al*. 2010). Inappropriate expression of phosphorylation of STAT3 leads to tumorigenesis (Furtek, Backos *et al*. 2016). STAT3 is phosphorylated by receptor-associated Janus kinases in response to growth factors and cytokines, and is subsequently translocated to the cell nucleus where it acts as

a transcriptional activator (Xiong, Yang *et al*. 2014). STAT3 is a key signal transducer and regulator of gene expression that is critical to routine cellular processes including cell proliferation, development, angiogenesis, differentiation, survival, and immune function (Yu, Kortylewski *et al*. 2007). It is reported that STAT3 is associated with tumorigenesis and acts as an oncogene (Bromberg, Wrzeszczynska *et al*. 1999). Aberrant activation of STAT3 was identified in human endometrial cancer tissues as well as endometrial cancer cells (Chen, Hsieh *et al*. 2007). Additionally, STAT3 has been used as a cancer therapeutic target because it plays a pivotal role in oncogenic function and immunosuppression (Wang, Crowe *et al*. 2012). The functional relationship between MIG-6 and STAT3 in endometrial cancer development, however, remains elusive.

I developed uterine epithelium specific *Mig-6* knockout mice by crossbreeding floxed *Mig-6* (*Mig-6*$^{f/f}$) mice with Sprr2fcre mice to analyze the function of epithelial *Mig-6* for endometrial tumorigenesis (Contreras, Akbay *et al*. 2010). I demonstrated that *Mig-6* plays an important role during the development of endometrial hyperplasia. In addition, P4 treatment prevents the development of endometrial hyperplasia in mutant mice. Furthermore, *Mig-6* signaling has a critical role in regulating epithelial proliferation by mediating phosphorylation of STAT3. These results demonstrate that activation of endometrial stromal P4 signaling, including stromal *Mig-6*, prevents endometrial hyperplasia of mutant mice by regulating STAT3 activity.

2. Materials and methods

Mouse tissue samples

All mouse experiments were cared for according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University. The mice with epithelial-cell-specific *Mig-6* knockout in the uterus were generated using the *Sprr2f-cre* mouse model (Contreras, Akbay *et al.* 2010). To determine the endometrial hyperplasia development and P4 effects, vehicle (beeswax) or P4 (40 mg/pellet) pellet was injected subcutaneously into female control and mutant mice respectively, beginning at 9 weeks of age for 1 week before euthanization.

Immunohistochemistry and immunofluorescence analyses

Immunostaining analyses were performed as previously described (Kim, Yoo *et al.* 2015). Briefly, uterine sections were incubated with appropriate primary antibodies, anti-MIG-6 (Customized antibody by Dr. Jeong Lab), anti-pSTAT3 (CS-9131; Cell Signaling, Danvers, MA), anti-STAT3 (CS-4904; Cell Signaling, Danvers, MA), and anti-Ki67 (ab15580; Abcam, Cambridge, MA), in 10% normal goat serum in PBS overnight at 4° C. For immunohistochemistry, sections were incubated with secondary

antibody (Vector Laboratories, Burlingame, CA) and detected using the Vectastain Elite DAB kit (Vector Laboratories, Burlingame, CA). For immunofluorescence, sections were incubated with secondary antibody conjugated to Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen Crop., Carlsbad, CA) for 2 hours at RT. Then, sections were mounted with DAPI (Vector Laboratories, Burlingame, CA) to enable nuclear visualization. The immunohistochemical staining intensities were graded by H-Score. The H-score was calculated as previous reported (Ishibashi, Suzuki *et al.* 2003).

Cell culture and transient transfection

Ishikawa cells were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12; Gibco BRL, Gaithersburg, MD) with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD), and 1% (v/v) penicillin streptomycin (P/S; Gibco BRL, Gaithersburg, MD) at 37° C under 5% CO2. FLAG-tagging MIG-6 and V5-tagging STAT3 expression vectors were transfected using Lipofectamine 2000 reagent (Invitrogen Crop., Carlsbad, CA) in accordance with the manufacturer's instructions.

Immunoprecipitation

Immunoprecipitation was performed as described previously (Lee, Kim *et al.* 2013). Briefly, 0.5 μg of lysates were

immunoprecipitated with 1 μg of antibodies to FLAG (F1804; Sigma-Aldrich, St. Louis, MO), STAT3 (CS-4904; Cell Signaling, Danvers, MA), or MIG-6 (Customized antibody by Dr. Jeong Lab) with 30 μl of resuspended protein A-agarose (Pierce Biotechnology, Rockford, IL) and incubated overnight at 4° C. Immunocomplexes were applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was exposed to anti-V5 (A190-220A; Bethyl Laboratories, Montgomery, TX), anti-FLAG, and anti-STAT3 antibodies.

Statistical analysis

For all animal experiments, the samples were not predetermined using any statistical method. Based on our previous studies, 5 mice per group were used for all experiments to attain proper statistical power. A balance in sample size across groups were ensured by block randomization. To evaluate the result variations in group, the investigators were blinded to the group. There are no excluded samples and animals. In vitro experiments were conducted three times, and results are presented as the mean ± s.e.m. of three biological replicates. Student's t test was used for two groups. An analysis of variance (ANOVA) test was used for more than two groups, followed by Tukey or Bonferroni test for pairwise t-test. All

statistical tests were analyzed by the GraphPad Prism 5(San Diego, CA). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

The ablation of Mig-6 in the endometrial epithelial cells of mouse.

In the previous study, our research team found that epithelial *Mig-6* is a critical tumor suppressor in the uterus of $Wnt7a^{cre+}Mig\text{-}6^{f/f}$ mice (Kim, Lee *et al.* 2013). However, *Mig-6* is also expressed in skin, and deletion of *Mig-6* results in skin tumor formation over a wound (Ferby, Reschke *et al.* 2006; Zhang and Vande Woude 2007). *Wnt7a-Cre* activity was not only detected in uterine epithelia, but also in the ovary and skin. The ablation of *Mig-6* by *Wnt7a-Cre* leads to tumor formation at any surgical wounds in the skin, which limits surgical applications, including ovariectomy and subcutaneous injection of steroid hormone pellets for endometrial cancer studies in mice (Ferby, Reschke *et al.* 2006). Therefore, I generated a mouse model in which I specifically ablated endometrial epithelial *Mig-6* using *Sprr2f-cre* mice (Wang, Crowe *et al.* 2012) ($Sprr2f^{cre+}Mig\text{-}6^{f/f}$) to study the function of epithelial *Mig-6* in the uterus. The epithelium specific deletion of *Mig-6* in mutant mice was proven by immunofluorescence analysis (Figure 1). MIG-6 was expressed in all compartments of the uterus in control mice. Mutant mice showed that MIG-6 levels were identified in the stromal cells but not the epithelial cells of the uterus, while MIG-6 was not observed in the epithelial cells or the stromal cells of the $Pgr^{cre/+}Mig\text{-}6^{f/f}$ mice uterus.

This immunofluorescence analysis demonstrate my successful generation of uterine epithelial specific *Mig-6* ablated mice.

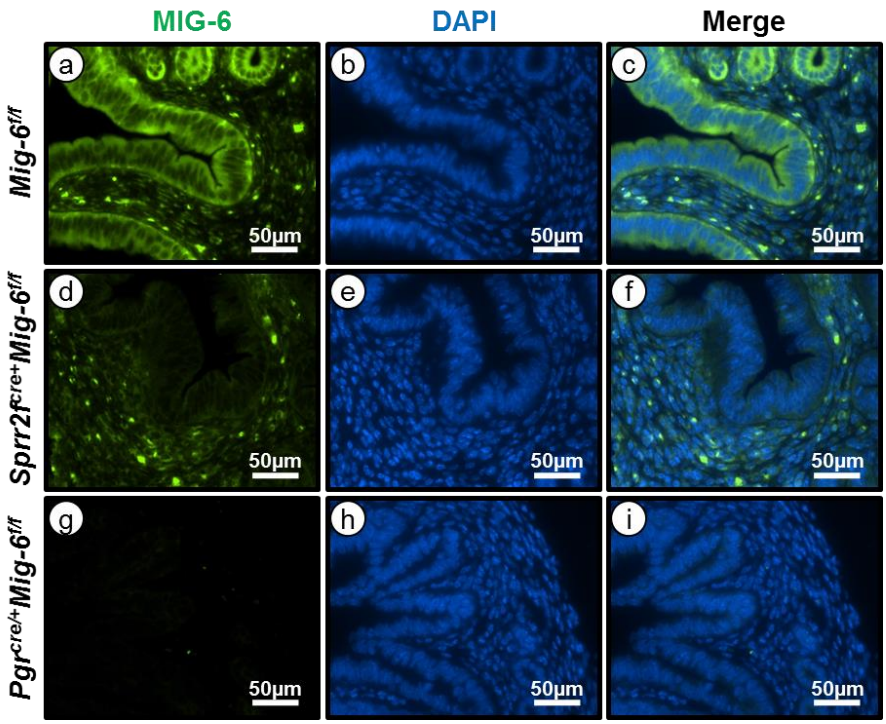


Figure 1. Generation of *Sprr2f*$^{cre+}$*Mig-6*$^{f/f}$ mice.

Immunofluorescence analysis of MIG-6 in whole uterine of *Mig-6*$^{f/f}$, *Sprr2f*$^{cre+}$*Mig-6*$^{f/f}$, and *Pgr*$^{cre+}$*Mig-6*$^{f/f}$ mice at 6 weeks of age. Green fluorescent protein indicates MIG-6 protein expression.

Endometrial hyperplasia development by conditional epithelial Mig-6 ablation in the mouse uterus.

According to our previous research, $Pgr^{cre+}Mig\text{-}6^{f/f}$ and $Wnt7a^{cre+}Mig\text{-}6^{f/f}$ mice display endometrial hyperplasia and cancer due to dysregulation of E2 and P4 (Jeong, Lee *et al.* 2009; Kim, Lee *et al.* 2013). To examine the development and advancement of endometrial hyperplasia and cancer in the mutant mouse uterus, I investigated the uterine weight, gross appearance and histologic morphology in control and mutant mice at 9 weeks, 10 weeks, and 5 months of age. The weight of the mutant mouse uterus was significantly increased than in comparison to the control mice after 10 weeks of age (Figure 2a and b). Histological analysis of these uteri showed a development of endometrial hyperplasia in the uterus of mutant mice from 10 weeks of age (Figure 2c). The uteri revealed a higher number of endometrial epithelial cells and an increase in the epithelium/stroma ratio in the uterus of mutant mice. Endometrial hyperplasia is caused by excessive proliferation of endometrial glands cells (Ambros 2000). I next investigated whether endometrial hyperplasia in mutant mice is caused by excessive endometrial epithelial cell proliferation. The levels of Ki67, a proliferation marker, were examined in the uterus of control and mutant mice at 10 weeks of age by immunohistochemical staining. The level of Ki67 was significantly higher within the epithelium of mutant mice compared with control mice (Figure 3a and b), however, stromal proliferation

was not different between the mice. These results showed that the uterus of the epithelial specific *Mig-6* ablation mice develops endometrial hyperplasia caused by increased cell proliferation from 10 weeks of age. These microscopic anatomical changes indicate that the uterus of mutant mice exhibits endometrial hyperplasia, which can increase the chances of developing endometrial cancer in humans.

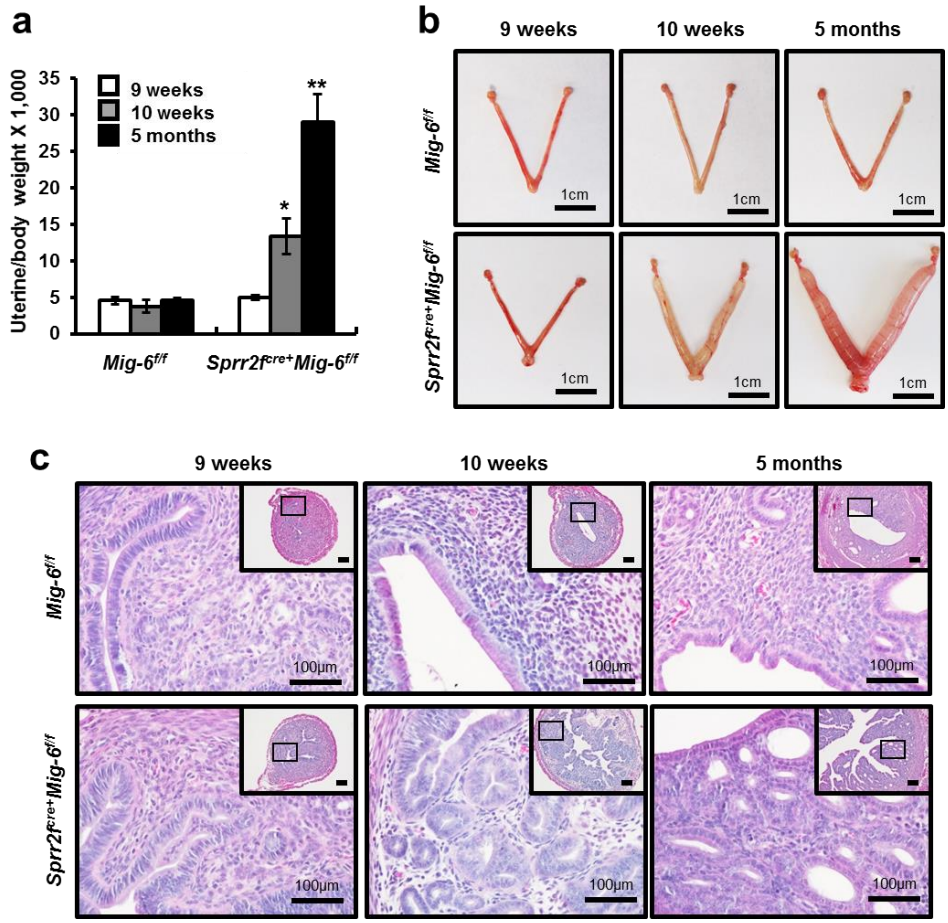


Figure 2. Development of endometrial hyperplasia in *Sprr2f^{cre+}Mig-6$^{f/f}$* mice uterus. (a) The ratio of uterine weight to body weight of *Mig-6$^{f/f}$* and *Sprr2f^{cre+}Mig-6$^{f/f}$* mice at weeks 9, 10 and 5 months. (b) Morphology *Mig-6$^{f/f}$* and *Sprr2f^{cre+}Mig-6$^{f/f}$* mice during endometrial hyperplasia development and progression. (c) Histology of uteri from mice with epithelial *Mig-6* ablation at weeks 9, 10 and 5 months. The results represent the mean ± SEM. *, $p < 0.05$ and **, $p < 0.01$.

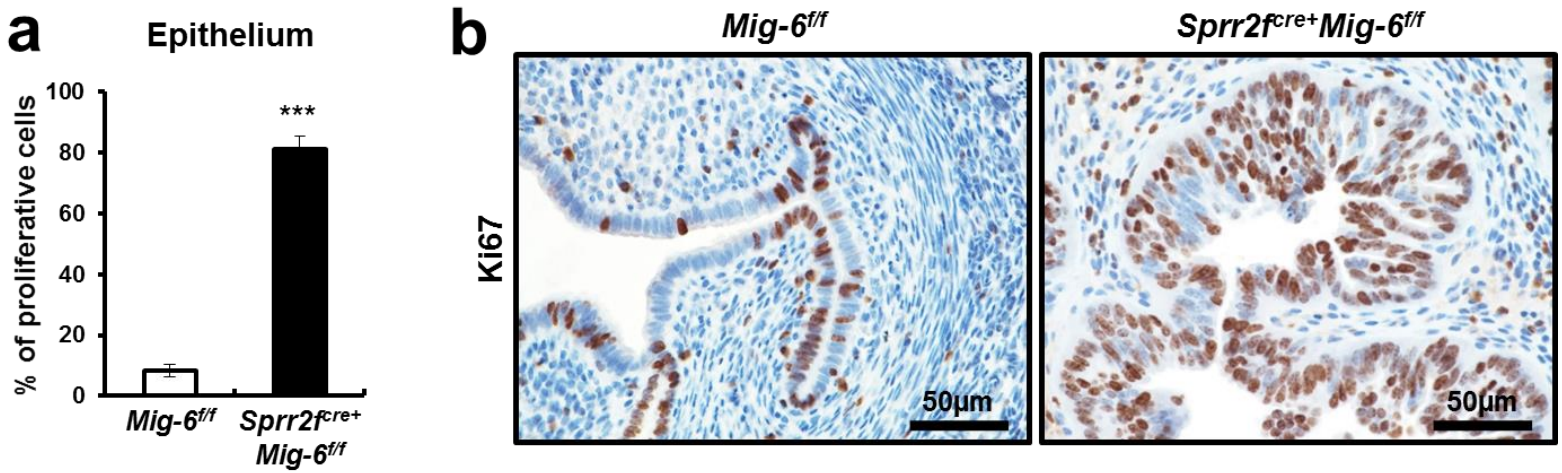

a
Epithelium
% of proliferative cells
100
80
60
40
20
0
***
Mig-6f/f
Sprr2fcre+ Mig-6f/f
b
Mig-6f/f
Sprr2fcre+Mig-6f/f
Ki67
50μm
50μm

Figure 3. Increase of epithelial cell proliferation by epithelial *Mig-6* ablation in the mouse uterus. (a) Quantification of Ki67 positive cells in epithelial cells of $Mig\text{-}6^{f/f}$ and $Sprr2f^{cre+}Mig\text{-}6^{f/f}$ mice. (b) Immunohistochemical analysis of Ki67 in $Mig\text{-}6^{f/f}$ and $Sprr2f^{cre+}Mig\text{-}6^{f/f}$ mice. The results represent the mean ± SEM. ***, $p < 0.001$.

Inhibition of STAT3 by interaction with MIG-6

STAT3 is a MIG-6-associated protein (Kim, Franco *et al.* 2010) and plays an important part in cell proliferation (Li and Shaw 2002; Yu, Kortylewski *et al.* 2007). Therefore, I examined the level of STAT3 by immunohistochemical analysis in the uterus of female control and mutant mice at 10 weeks of age. Levels of phosphorylated STAT3 were significantly higher in the uterine epithelium of mutant mice compared with control mice (Figure 4a and b), however, phosphorylated STAT3 in stromal cells of mutant mice showed no change. In addition, total STAT3 levels were not different in uterine stromal and epithelial cells of female control and mutant mice (Figure 4c and d). In order to analyze whether MIG-6 physically interacts with STAT3 to suppress its phosphorylation, I cotransfected FLAG-tagged MIG-6 and/or V5-tagged STAT3 expression vectors to Ishikawa human endometrial adenocarcinoma cell line and the cell lysates were immunoprecipitated with FLAG antibodies (Figure 4e). Next, I performed immunoprecipitation using protein lysates from the uteri of control and $Pgr^{cre/+}Mig\text{-}6^{f/f}$ mice. Immunoprecipitation was applied with anti-STAT3, anti-MIG-6, and anti-IgG antibodies, then examined by Western blot analysis to identify an interaction between MIG-6 and STAT3. I were able to demonstrate the interaction between MIG-6 and STAT3 in the mouse uterus (Figure 4f). The results showed that MIG-6 physically interacts with STAT3 protein.

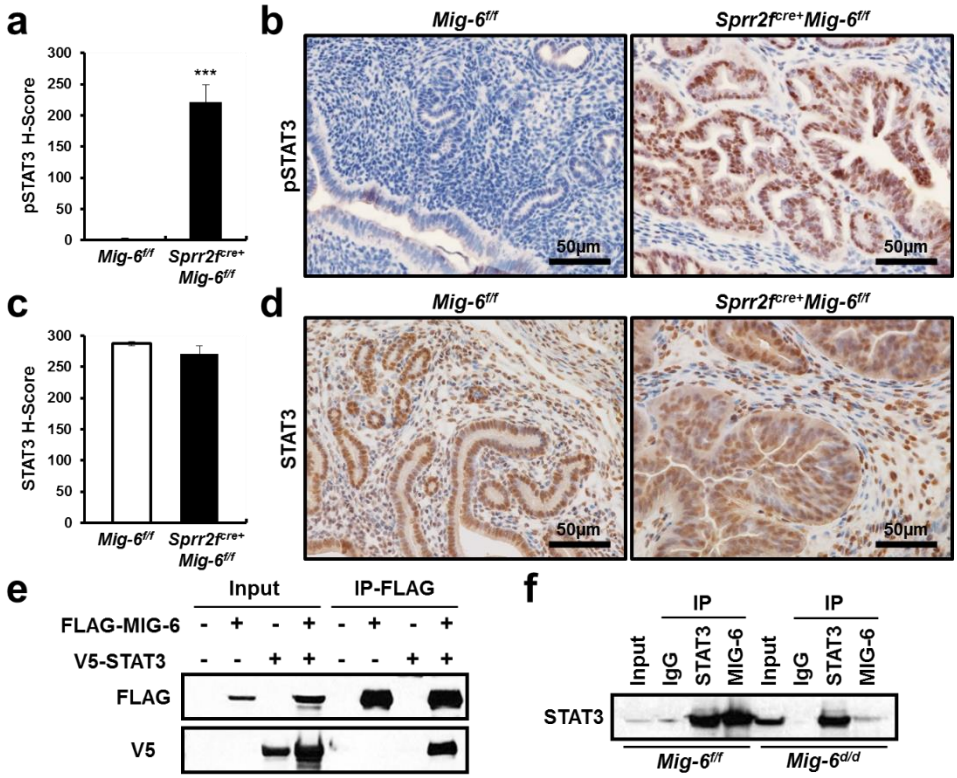


Figure 4. Inhibition of STAT3 phosphorylation by interacting with MIG-6. Quantification of pSTAT3 (a) and STAT3 (c) positive cells in epithelial cells of *Mig-6*$^{f/f}$ and *Sprr2f*$^{cre+}$*Mig-6*$^{f/f}$ mice. Immunohistochemical analysis of pSTAT3 (b) and STAT3 (d) in *Mig-6*$^{f/f}$ and *Sprr2f*$^{cre+}$*Mig-6*$^{f/f}$ mice. The protein interaction between MIG-6 and STAT3 by immunoprecipitation and Western blot analysis in vitro (e) and in vivo (f). The results represent the mean ± SEM. ***, $p < 0.001$.

MIG-6 suppresses STAT3 phosphorylation.

To investigate whether MIG-6 affects phosphorylation of STAT3, I cotransfected a MIG-6 expressed vector to Ishikawa cells, and treated with or without leukemia inhibitory factor (LIF), a known activator of STAT3, for 10 min (Cheng, Chen *et al.* 2001). Western blot analysis revealed that phosphorylation of STAT3 was increased by LIF. The MIG-6 overexpression significantly decreased STAT3 phosphorylation (Figure 5a and b). My results indicate that MIG-6 suppresses the phosphorylation of STAT3 in endometrial epithelial cells.

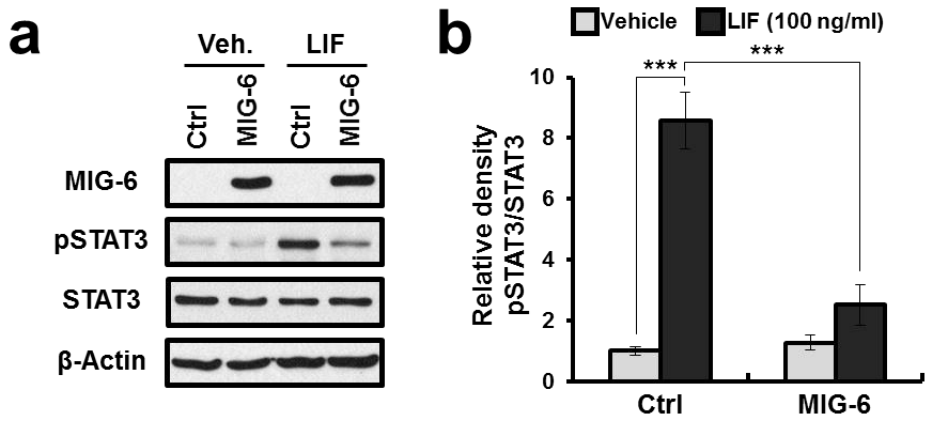


Figure 5. Regulation of STAT3 activity by MIG-6. (a) Flag-tagged MIG-6 transfected Ishikawa cell lysates were analyzed by Western blotting in the presence or absence of LIF (100 ng/ml) treatment for 10 mins. Intensity of pSTAT3 was obtained using Image J software for Western Blot analysis. The results represent the mean ± SEM. ***, $p < 0.001$.

Prevention of the development of endometrial hyperplasia in epithelial Mig-6 ablated mouse uterus by P4 treatment.

To determine the responsiveness of P4 on endometrial hyperplasia development in mutant mice, I treated 9-week old female control and mutant mice with vehicle or P4 for 1 week by subcutaneous injection. Mutant mice that were treated with vehicle exhibited a significantly higher uterine weight, and an increase in gross size compared to vehicle treated control mice.

The histological analysis showed endometrial hyperplasia in mutant mice treated with vehicle. However, there was no difference in uterine weight and gross size between female control and mutant mice after P4 treatment (Figure 6a and b). While mutant mice treated with vehicle developed endometrial hyperplasia in the uterus, P4 treated mice showed normal endometrium (Figure 6c). I could not observe any differences between female control and mutant mice after the P4 treatment. These data propose that mutant mice were responsive to P4 and that this prevented the development of endometrial hyperplasia.

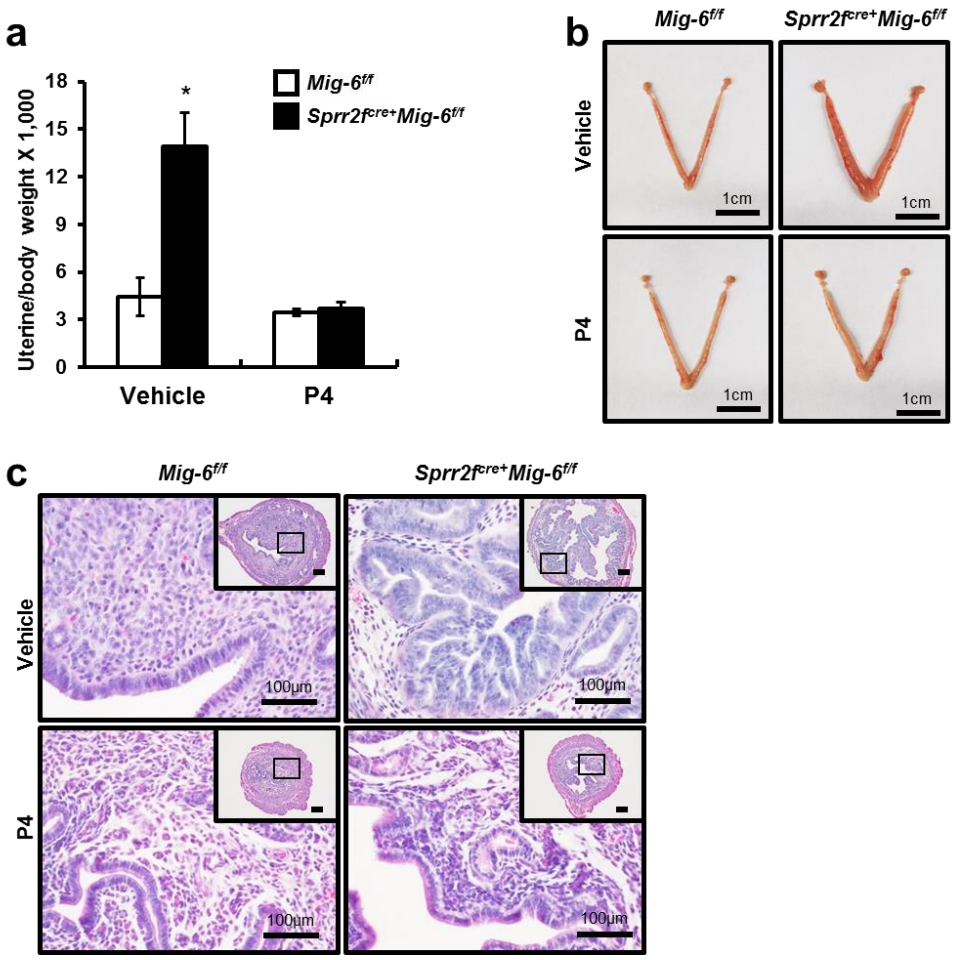


Figure 6. Prevention of endometrial hyperplasia in *Sprr2f^{cre+}Mig-6$^{f/f}$* mice uterus by progesterone treatment. (a) The ratio of uterine weight to body weight of *Mig-6$^{f/f}$* and *Sprr2f^{cre+}Mig-6$^{f/f}$* mice after P4 treatment. (b) Morphology *Mig-6$^{f/f}$* and *Sprr2f^{cre+}Mig-6$^{f/f}$* mice after P4 treatment. (c) Histology of uteri from *Mig-6$^{f/f}$* and *Sprr2f^{cre+}Mig-6$^{f/f}$* mice after P4 treatment. The results represent the mean ± SEM. *, $p < 0.05$.

Inhibition of active phosphorylation of STAT3 in epithelial Mig-6 ablated mice uterus by P4 treatment.

To analyze if the observed prevention of hyperplastic phenotype was in response to recovered STAT3 signaling and proliferation, I investigated the level of epithelial cell proliferation and phosphorylation of STAT3 in the uterus of mutant mice treated for 1 week with vehicle or P4 at 9 weeks of age. Immunohistochemistry analysis results showed that levels of proliferation were significantly lowered in the P4-treated mutant mice uterus in comparison to vehicle-treated mutant mice. In addition, phosphorylation levels of STAT3 were decreased in the uterus of mutant mice after P4 for 1 week as compared with vehicle. The level of total STAT3, however, was not affected by P4 treatment (Figure 7). These results demonstrate that P4 treatment prevents the endometrial hyperplasia development in uterine epithelial *Mig-6* ablation by inhibiting STAT3 phosphorylation and endometrial epithelial cell proliferation.

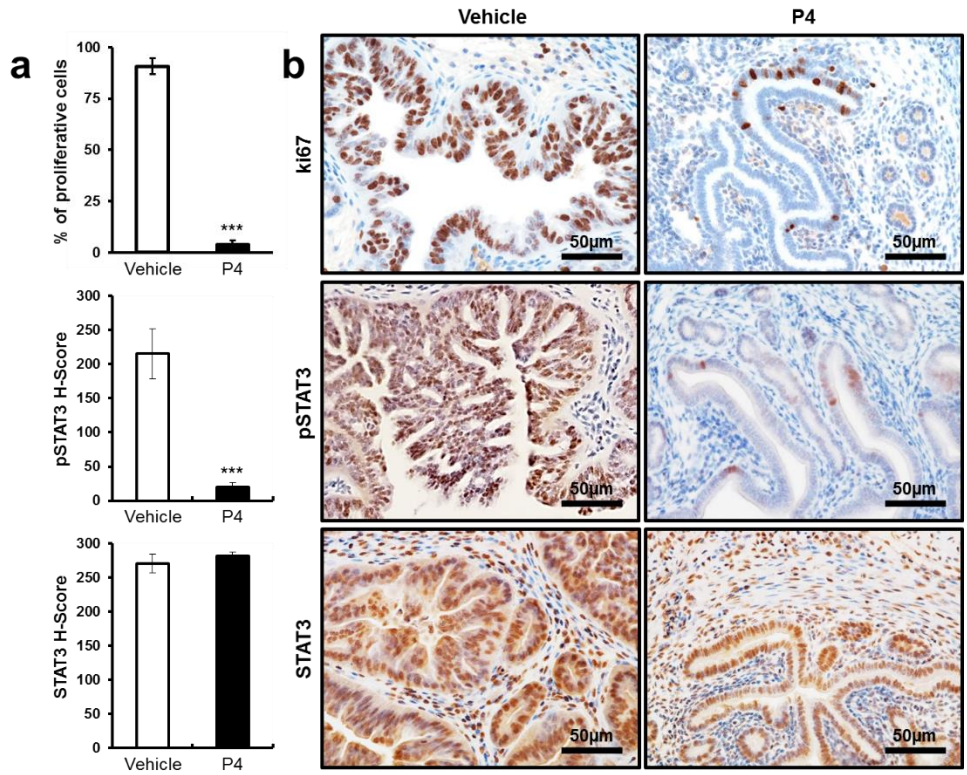


Figure 7. Inhibition of active epithelial proliferation in *Sprr2f*$^{cre+}$*Mig-6*$^{f/f}$ mice by progesterone treatment. (a) Quantification of Ki67, pSTAT3 and STAT3 positive cells in epithelial cells of *Mig-6*$^{f/f}$ and *Sprr2f*$^{cre+}$*Mig-6*$^{f/f}$ mice after P4 treatment. (b) Immunohistochemical analysis of Ki67, pSTAT3, and STAT3 in vehicle and P4 treated *Sprr2f*$^{cre+}$*Mig-6*$^{f/f}$ mice. The results represent the mean ± SEM. ***, $p < 0.001$.

4. Discussion

Mig-6 functions as a tumor suppressor through an anti-proliferative role in humans (Fiorini, Ballaro *et al.* 2002; Reschke, Ferby *et al.* 2010; Ying, Zheng *et al.* 2010; Lin, Du *et al.* 2011). Our team previously classified *Mig-6* as a target gene of the PGR (Jeong, Lee *et al.* 2009). Uterine specific ablation of *Mig-6* allows for the progression of endometrial hyperplasia and E2-dependent endometrial cancer due to an increase of endometrial epithelial cell proliferation by excessive E2 signaling in mice (Jeong, Lee *et al.* 2009). To comprehend the function of epithelial *Mig-6* in the uterus, our team created a mouse model in which *Mig-6* gene expression was ablated specifically in the *Wnt7a*-expressing cells (*Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice) (Kim, Lee *et al.* 2013). *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice revealed a higher level of epithelial cell proliferation and an increase in the progression of endometrial hyperplasia and E2-dependent endometrial cancer (Kim, Lee *et al.* 2013). However, *Wnt7a-Cre* mice showed cre recombinase activities in skin as well as in ovarian and uterine epithelium (Daikoku, Ogawa *et al.* 2014). *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice have the limitation to examine the pathophysiology and tumorigenesis using steroid hormone pellets because of tumor formation at any surgical wounds in the skin. In the present study, I generated another uterine epithelial specific *Mig-6* knockout mouse model to evaluate the function of epithelial *Mig-6* using a *Sprr2f-cre* mouse model (Contreras, Akbay *et al.* 2010). The

small proline-rich protein 2F (*Sprr2f*) gene is specifically expressed in endometrial epithelial cells including both the luminal and glandular compartments, but not in endometrial stroma, myometrium, and skin (Contreras, Akbay *et al.* 2010). *Sprr2f* $^{cre+}$*Mig-6*$^{f/f}$ mice can overcome the limitation of the cre recombinase expression in skin of *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice.

Sprr2f^{cre+}*Mig-6*$^{f/f}$ mice showed development of endometrial hyperplasia from 10 weeks of age as observed in *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice. Endometrioid-type endometrial adenocarcinoma and hyperplasia are associated with unopposed E2 exposure and continually increased proliferation of epithelial cells (Kurman, Kaminski *et al.* 1985; Ambros 2000). Levels of epithelial cell proliferation were significantly higher in the mutant mice compared with control mice at 10 weeks of age. These results suggest that increased proliferation in endometrial epithelial cells leads to the progression of endometrial hyperplasia and endometrial cancer.

Consistent activation of STAT3 leads to aberrant cell proliferation in carcinogenesis (Lin, Liu *et al.* 2011), indicating that STAT3 is a critical regulator of cancer cell proliferation and apoptosis. Here, I demonstrated that levels of STAT3 phosphorylation were significantly higher in the endometrial epithelial cells of mutant mice compared with control mice at the development of endometrial hyperplasia. I demonstrated that MIG-6 negatively regulates STAT3 phosphorylation through direct protein interactions in vivo and in vitro. Increased phosphorylation of STAT3

by LIF, which in turn induces further phosphorylation of STAT3, is significantly decreased by overexpressed MIG-6. These data indicate that MIG-6 inhibits uterine epithelial cell proliferation through inhibiting STAT3 phosphorylation. The progression and development of endometrial tumorigenesis is related to aberrant activation of STAT3 in endometrial epithelial cells of mutant mice.

P4 and E2, ovarian steroid hormones, are critical in the mediation of uterine events related to the establishment and maintenance of pregnancy (Lydon, DeMayo *et al.* 1995) as well as regulation of epithelial-stromal cross-talk through their cognate nuclear receptors (Rubel, Jeong *et al.* 2010). An imbalance of steroid hormones initiated by elevated levels of E2 and/or decreased P4 action can lead to aberrant endometrial proliferation and endometrial cancer (Jemal, Siegel *et al.* 2006). Clarifying the molecular mechanisms that regulate E2 and P4 in the uterus is paramount to understanding the pathophysiology of endometrial cancer.

There have been attempts for fertility preservation in premenopausal women with endometrial cancer through conservative treatment with high-dose P4 (Bovicelli, D'Andrilli *et al.* 2013). P4 can suppress the proliferation of endometrial cancer through inhibition of E2 action (Kaunitz 1998). The antagonistic effect of P4 on E2 supports the rationale for progestin-based therapy for endometrial cancer (Hahn, Yoon *et al.* 2009). To address the preventative role of P4 on endometrial hyperplasia, I treated mice with P4 for 1 week, beginning at 9 weeks of age. Female mutant mice

did not exhibit an endometrial hyperplasia phenotype after P4 treatment. Mutant mice treated with 1 week of P4 showed a decrease in epithelial cell proliferation and phosphorylation level of STAT3 in uterine epithelium. The uterus is made up of heterogeneous cell types that go through dynamic changes in order to support embryo development and implantation. These changes primarily rely on coordinated interactions mediated by P4 and E2. E2 induces epithelial proliferation in the murine uterus (Huet-Hudson, Andrews *et al.* 1989). Meanwhile, P4 inhibits E2-induced proliferation of the glandular and luminal epithelial cells. However, P4 or P4 with E2, leads to stromal cell proliferation in the uterus (Huet-Hudson, Andrews *et al.* 1989). P4 suppresses E2 stimulated epithelial proliferation via regulating stromal cell gene expressions (Kurita, Lee *et al.* 2000). However, the mediators involved in these regulatory cell-cell interactions have not been known. I have shown that activation of stromal P4 signaling including *Mig-6* impacts endometrial tumorigenesis. These indicate that stromal *Mig-6* is a mediator for the ability of P4 to regulate E2-induced uterine proliferation (Jeong, Lee *et al.* 2009). An understanding of the actions of hormones on the uterus requires elucidation of the mechanism of stromal and epithelial communication with each other and further, how this epithelial-stromal cross-talk is transformed by hormonal binding to stromal versus epithelial mediators. These results provide evidence that activated stromal P4 signaling along with *Mig-6* may play a role in the prevention of endometrial

hyperplasia of mutant mice by inhibition of STAT3 activity. Furthermore, these data suggest that treatment with a STAT3 inhibitor could be an alternative way to overcome epithelial proliferation in endometrial hyperplasia.

Overall, these findings show that loss of *Mig-6* in the endometrial epithelial cells results in endometrial hyperplasia in response to an increase of epithelial cell proliferation. MIG-6 negatively regulates the phosphorylation of STAT3 via direct protein interaction with STAT3. P4 treatment prevents the development of endometrial hyperplasia in mutant mice uteri through inhibition of epithelial cell proliferation and excessive activation of STAT3 by P4-induced stromal *Mig-6*. Therefore, this study provide a framework for understanding endometrial cancer development, and a useful animal model for studying new therapies in the treatment and prevention of endometrial cancer.

CHAPTER 5

The role of TGF−β2 activated by β−catenin in adenomyosis

1. Introduction

Adenomyosis is a common benign heterogenous gynecological disorder defined by the presence of endometrial glandular and stromal tissue found in the myometrium (Ferenczy 1998; Tamai, Togashi *et al.* 2005). It is associated with hypertrophy and hyperplasia of adjacent smooth muscle cells (Ferenczy 1998). Adenomyosis is diagnosed from 10% to 66% in women at the time of hysterectomy (Vercellini, Vigano *et al.* 2006). It shows symptoms of menorrhagia, dysmenorrhea, chronic pelvic pain, dyspareunia, and dyschezia (Levgur, Abadi *et al.* 2000; Sammour, Pirwany *et al.* 2002; Louis, Saso *et al.* 2012) and can interfere with implantation and causing subfertility (Devlieger, D'Hooghe *et al.* 2003; Matalliotakis, Katsikis *et al.* 2005; Campo, Campo *et al.* 2012; Louis, Saso *et al.* 2012). This disease is now being more frequently diagnosed in infertility patients by transvaginal ultrasonography and magnetic resonance imaging (Bazot, Cortez *et al.* 2001). However, early diagnosis of adenomyosis is difficult because of the absence of pathognomonic symptoms and biomarkers. Therefore, most women are not diagnosed until later stages of disease and severely symptomatic women who do not respond to pharmacological therapy require invasive surgical intervention (hysterectomy). It has been suggested that adenomyosis is an ovarian steroid hormone–dependent disorder, resulting from high estrogen levels unopposed by progesterone, similar to endometriosis, endometrial hyperplasia,

and endometrial cancer (Schindler 2009; Mehasseb, Panchal *et al*. 2011). However, the precise etiology and pathophysiology of adenomyosis is still unrevealed.

Studies using animal models in many different species including mice, rats, rabbits, dogs, cats, and non-human primates have provided insight into this disease (Greaves and White 2006). Indeed, there have been several studies in mice using pituitary graft or hormonal treatment which have shown an increased incidence of adenomyosis (Guttner 1980; Mori and Nagasawa 1983; Ostrander, Mills *et al*. 1985; Parrott, Butterworth *et al*. 2001). Nevertheless, the molecular mechanism for the development and progression of adenomyosis is still unclear. Mice with uterine conditional activation of β-catenin ($Pgr^{cre/+}Ctnnb1^{f(ex3)/+}$) develop adenomyosis (Oh, Shin *et al*. 2013) and provide a novel model system to investigate the genetic and molecular events involved in the transition from normal uterine structure to adenomyosis.

The β-catenin has a dual function, regulating the coordination of cell-cell adhesion and gene transcription, depending on its localization in cell. At the plasma membrane, β-catenin is a component of the E-cadherin-catenin unit, thereby maintaining the cell differentiation and the maintenance of normal tissue architecture. The β-catenin nuclear translocation depends on Wnt signals and nuclear β-catenin can act as a transcriptional activator and regulates transcription of target genes responsible for cell proliferation and differentiation (Nelson and Nusse 2004). The

Wnt/β-catenin pathway is important on the tissue differentiation during embryonic development and tissue homeostasis and function in adults. In the absence of Wnt signals, β-catenin is degraded by the β-catenin destruction complex, including adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK-3β). The exon 3 of β-catenin is phosphorylated by GSK-3β and β-catenin is induced ubiquitin-dependent degradation by the proteasome (Rubinfeld, Souza *et al.* 1993; Miller and Moon 1996; Palacios and Gamallo 1998; Nelson and Nusse 2004). Mutations and excessive activation of β-catenin are associated with many cancers, including hepatocellular carcinoma, colorectal carcinoma, lung cancer, malignant breast tumors, ovarian and endometrial cancer (Morin 1999; Moreno-Bueno, Hardisson *et al.* 2002; Clevers 2006) as well as epithelial-mesenchymal transition (EMT) (Ilyas and Tomlinson 1997; Schmalhofer, Brabletz *et al.* 2009).

EMT is a biologic process of epithelial cells by loss of cell polarity and cell adhesion, and gain of migratory, invasive property, and cell mobility, and is programmed development of biological cells (Acloque, Adams *et al.* 2009). EMT is essential to occur in embryogenesis, in organ fibrosis, in wound healing and in the initiation of metastasis for cancer progression (Kalluri and Weinberg 2009), as well as in uterine diseases in women such as adenomyosis, endometriosis and endometrial cancer (Gaetje, Kotzian *et al.* 1997; Zeitvogel, Baumann *et al.* 2001; Chen, Li *et al.* 2010; Oh, Shin *et al.* 2013). Estrogen induces EMT in estrogen receptor (ESR)-positive

endometrial cells and serum E2 level was negatively correlated with E-cadherin expression levels in the epithelial components of the eutopic endometrium and adenomyotic lesions compared to controls (Chen, Li *et al.* 2010). 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). Furthermore, EMT is induced or regulated by growth and differentiation factors, including TGF-β (Moustakas and Heldin 2007). Crosstalk between TGF-β/Smad signaling and Wnt/β-catenin signaling is important in developmental and pathological events (Letamendia, Labbe *et al.* 2001; Minoo and Li 2010). TGF-β signaling influences on the Wnt pathway by regulating the activity of β-catenin through interaction of activated Smad complexes with APC/GSK-3β, β-catenin, and TCF/LEF1 (Xu, Lamouille *et al.* 2009; Minoo and Li 2010). In the context of EMT, Smad2 and Smad4 make a complex with LEF1 and suppressed the expression of E-cadherin by binding on *E-cadherin* promoter. These complexes induce the expression of mesenchymal markers, vimentin and fibronectin (Nawshad, Medici *et al.* 2007).

Here, I applied DNA microarray and ChIP-seq technology under in vivo conditions of aberrant β-catenin activation to identify the genome-wide β-catenin-binding regions in the mouse uterus. I found that *Tgf-β2* is directly regulated by activated β-catenin in the murine uterus through these analyses. The levels of TGF-β2 were higher in endometrial epithelial cells of β-catenin stabilized mice compared to control mice, as well as in epithelial cells of human

eutopic endometrium and adenomyosis lesions compared to control women. Furthermore, I found the correlation between β-catenin and TGF-β2 proteins in endometrium from women with and without adenomyosis. These results suggest that *Tgf-β2* has critical role in adenomyosis development as a direct target of β-catenin.

2. Materials and methods

Mouse tissue samples

Mice were maintained in the designated animal care facility according to the Michigan State University institutional guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. Uterine tissues were collected from control (*Pgr*$^{cre/+}$ *and Ctnnb1*$^{f(ex3)/+}$) and mutant (*Pgr*$^{cre/+}$*Ctnnb1*$^{f(ex3)/+}$) mice (Jeong, Lee *et al.* 2009). Uterine tissues were immediately frozen at the time of dissection and stored at −80° C for RNA extraction or ChIP analysis, or fixed with 4% (v/v) paraformaldehyde for histology and immunostaining analysis.

Human adenomyosis samples

Human adenomyosis samples were obtained from Michigan State University's Center for Women's Health Research Female Reproductive Tract Biorepository with the guideline set by the Institutional Review Boards of Michigan State University. Written informed consent was obtained from all participants. Tissue samples of adenomyosis with their corresponding eutopic endometrium were collected from surgical hysterectomy specimens. Controls comprised regularly cycling premenopausal women undergoing an endometrial

hysterectomy for benign conditions with no history or evidence of adenomyosis, who were documented not to be pregnant and who had not been on hormonal therapies for at least 3 months before tissue sampling. Histologic dating of endometrial samples was done based on the criteria of Noyes (Noyes, Hertig *et al.* 1975). Eutopic endometrium (4 proliferative and 4 secretory phase) and adenomyosis lesions (15 proliferative and 15 secretory phase) samples were from women with adenomyosis, and control endometrium (n=8 proliferative and n=13 secretory) samples were from women without adenomyosis. Samples used for immunostaining were fixed in 10% buffered formalin prior to embedding in paraffin wax.

RNA isolation and microarray analysis

Total RNA was extracted using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA). RNA was pooled from the uteri of 3 mice per genotype at 1 month of age. All RNA samples were analyzed with a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE) before microarray hybridization. Microarray data analysis was performed as described (Kim, Yoo *et al.* 2015). Differentially expressed genes were classified according to canonical pathway analyzed by Ingenuity System Software (Ingenuity Systems Inc., Redwood City, CA).

Chromatin Immunoprecipitation Sequencing (ChIP-Seq) analysis

β-catenin and Input ChIP were performed were performed by Active Motif, Inc. (Carlsbad, CA) on control and mutant mice uteri at 1 month of age. ChIP and input DNA were amplified using the Illumina ChIP-Seq DNA Sample Prep Kit (Illumina, San Diego, CA). DNA libraries were sequenced by Illumina's Hi-Seq Sequencing Service. The sequences were aligned to the mouse genome (NCBI Build 37, July 2007). Aligns were extended in silico (using Active Motif software) at their 3'-ends to a length of 150-250 bp and assigned to 32-nucleotide bins along the genome. The resulting histograms were stored in BAR (binary analysis results) files. Peak locations were determined by applying a threshold of 18 (five consecutive bins containing 18 aligns) and storing the resulting intervals in Browser Extensible Data (BED) files (BED, Affymetrix TAS software). These files were analyzed using Genpathway proprietary software that provides comprehensive information on genomic annotation, peak metrics, and sample comparisons for all peaks (intervals). The model based analysis of ChIP-Seq (MACS (Carroll, Meyer *et al.* 2006)) peak-finding algorithm was used to normalize ChIP against the input control. Genes associated with intervals were assessed using three increasingly less stringent requirements; if it was within 10 kb, 25 kb, or 50 kb upstream or downstream of a gene it was counted. Sequence conservation to

identify phastCons scores, analysis of enriched motifs, and cis-regulatory element annotation system (CEAS) were performed using the Cistrome Analysis Pipeline software (http://cistrome.org.ap/) under default settings (Lin, Vega *et al.* 2007). Ingenuity Systems Pathway Analysis (IPA) software (http://www.ingenuity.com) were used for gene functional annotations

ChIP assays

ChIP assays were performed in uteri of control and mutant mice at 4 weeks of age as previously described (Kim, Yoo *et al.* 2015). Briefly, for each ChIP reaction, 100 μg of chromatin was immunoprecipitated by 4 μg of antibodies against β-catenin (610154; BD biosciences, San Jose, CA). Eluted DNA was amplified with specific primers using SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). Primers used in PCR were as follows: BRE (forward: 5′-GTTACCGAGGGGCAGAATGC-3′; reverse: 5′-GGGGGTCATCATACAAGGCA-3′) and negative control (forward: 5′-CCGGAGCTCCTCAGATCCAC-3′; reverse: 5′-TGGATCCCTCTCCATCCCAC-3′). The resulting signals were normalized to input activity.

Quantitative real-time PCR Analysis

cDNA was produced from 1 μg of total RNA using random hexamers and MMLV Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). Real-time PCR was performed using RT-PCR SYBR Green detection system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions (PE Applied Biosystems, Foster City, CA). mRNA quantities were normalized against the housekeeping gene, *Rpl7* RNA. The sequences of the primers used for mouse *Tgf-β2* were 5'-TAAAATCGACATGCCGTCCC-3' and 5'-GAGACATCAAAGCGGACGAT-3' and for mouse *Rpl7* were 5'-TCAATGGAGTAAGCCCAAAG-3' and 5'-CAAGAGACCGAGCAATCAAG-3'.

Immunohistochemistry and immunofluorescence analysis

Immunohistochemistry and immunofluorescence analyses were performed as previously described (Kim, Lee *et al.* 2010; Franco, Dai *et al.* 2011). Uterine sections from paraffin-embedded tissue were preincubated with 10% normal serum in phosphate-buffered saline (PBS) and incubated with anti TGF-β2 (ab36495; Abcam, Cambridge, MA), anti-E-cadherine (610181; BD Bioscience, San Jose, CA), and anti-vimentin (ab92547; Abcam, Cambridge, MA) antibodies in 10% normal serum in PBS. On the following day, sections were washed in PBS and incubated with a secondary

antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Immunoreactivity was detected using the Vectastain Elite DAB kit (Vector Laboratories). Images were captured with a confocal microscope (510 NLO confocal microscope; Carl Zeiss, Thornwood, NY, USA) or fluorescent microscope (Nikon Instruments, Melville, NY).

Cell culture and transient transfections

The uterine endometrial epithelial cell line, Ishikawa (endometrial adenocarcinoma), were cultured in DMEM/F12 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 1% penicillin streptomycin (P/S; Gibco, Grand Island, NY). Cells were cultured in monolayer at 37° C in 5% CO2. For transient transfection of exon 3-deleted β-catenin vector (Palacios and Gamallo 1998) to Ishikawa cells, I performed using Lipofectamine 2000 (Invitrogen Crop., Carlsbad, CA).

Western blot analysis

Western blot analyses were performed described previously (Kim, Yoo *et al.* 2014). Samples, which were lysed in buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2.5 mM EDTA, 0.125% Nonidet P-40 (v/v), and protease inhibitors, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and

transferred to a polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were probed with anti-β-catenin (610154; BD biosciences, San Jose, CA), anti- TGF-β2 (ab36495; Abcam, Cambridge, MA), anti-E-cadherin (610181; BD biosciences, San Jose, CA), anti-vimentin (SC-6260; Santa Cruz Biotechnology), and anti-Actin (SC-1615; Santa Cruz Biotechnology) antibodies. Immunoreactivity was visualized by autoradiography.

Invasion assay

For the transwell invasion assay, post-transfected cells (24 hours) were trypsinized and seeded at a density of 2.5 x 105 per 200 μl serum-free culture medium to insert chamber of a BioCoat (24-well insert; pore size, 8 μm; BD Biosciences, San Jose, CA). The cells were incubated at 37° C in 5% CO2 for 48 hours and noninvading cells were removed with cotton swab. Invading cells on the lower surface of the membrane were fixed with 100% methanol and stained with 1% crystal violat (Sigma-Aldrich, St. Louis, MO). Stained cells were captured via light microscopy (Nikon Instruments Inc., Melville, NY) using software from NIS Elements, Inc. (Nikon, Melville, NY).

Statistical analysis

Statistical analyses were performed Student's t-test for data with two groups or Analysis of Variance (ANOVA) test for data containing more than two groups and then analyzed by Tukey test for pairwise t-test using Instat (GraphPad, San Diego, CA, USA). $p<0.05$ was considered statistically significant.

3. Results

Tgf-β2 is identified as a β-catenin target gene in the murine uterus

Previously, uterine specific β-catenin stabilized mice develop the adenomyosis through epithelial-mesenchymal transition (EMT) (Oh, Shin *et al.* 2013). To identify the molecular pathways regulated by activation of β-catenin in the uterus, I performed high-density DNA microarray analysis in the uteri of control ($Pgr^{cre/+}$ and $Ctnnb1^{f(ex3)/+}$) and mutant ($Pgr^{cre/+}Ctnnb1^{f(ex3)/+}$) mice at 4 weeks of age. The 2,079 genes (fold change greater than 1.5) were identified as differentially expressed in mice with uterine-specific stabilization of β-catenin compared to control. Of the 2,079 genes, 1,103 were increased, and 976 genes were decreased. To determine which pathways are regulated by β-catenin activation, I analyzed altered pathways using Ingenuity Systems Software. Among these, I focused on the increase genes necessary for stem cell progression in the mutant uterus compared to control. These results suggest that activation of β-catenin in the uterus has an important role of regulation in stem cell progression.

Furthermore, I performed chromatin immunoprecipitation sequencing (ChIP-seq) in the uteri of control and mutant mice at 4 weeks of age to identify direct target genes of β-catenin in uterus. The ChIP-seq data analysis identified 2,918 binding target sites by

activation of β-catenin in the uterus. From comparison of the ChIP-seq and microarray results, I identified 318 genes that not only contain β-catenin binding sites but those genes the expression of which is altered in the uterus of mutant mice as compared with control (Figure 8A; Yellow). Of the 318 genes, 189 were increased, and 129 genes were decreased. Interestingly, transforming growth factor beta 2 (*Tgf-β2*) was identified through those analysis. The microarray analysis result was validated by real-time PCR analysis that the expression of *Tgf-β2* was significantly increased in mice with uterine-specific stabilization of β-catenin compared to control mice (Figure 8B). From immunohistochemistry analyses, the mutant mice uterus exhibited an increase of TGF-β2 in epithelial cells at 4 weeks of age compared to control mice (Figure 8C). Additionally, the recruitment of stabilized β-catenin on promoter of *Tgf-β2* was also confirmed in the uterus of mutant mice uterus at 4 weeks of age by ChIP analysis (Figure 8D and 8E). These results suggest that *Tgf-β2* is responded by activated β-catenin signaling, and may have an important role in adenomyosis development of activated β-catenin uterus.

To determine whether TGF-β2 expression associated with EMT, I performed double immunofluorescence for TGF-β2 and E-cadherin, as an epithelial cell marker, and vimentin, as a mesenchymal cell marker, in uterus of control and mutant mice at 4 weeks of age. TGF-β2 levels were higher in endometrial epithelial cells of mutant mice compared to control (Figure 9). On the contrary, E-cadherin

levels were decreased in endometrial epithelial cells of mutant mice compared to control (Figure 9A). Interestingly, the expressions of vimentin were observed in some epithelial cells of the uterus in mutant mice and colocalized with TGF-β2 in these cells (Figure 9B).

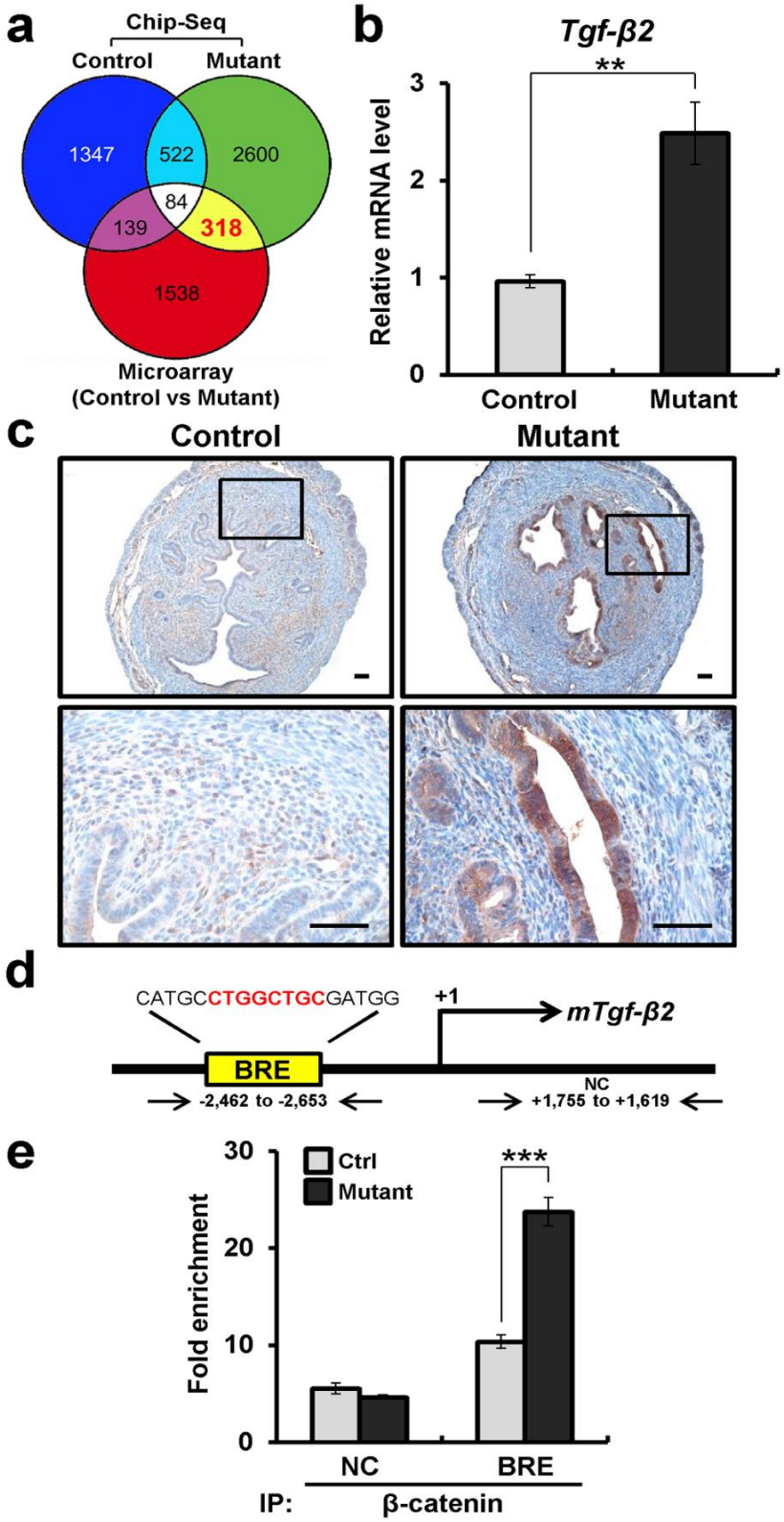

a
Chip-Seq
Control
Mutant
1347
522
2600
84
139
318
1538
Microarray
(Control vs Mutant)
b
Tgf-β2
**
Relative mRNA level
3
2
1
0
Control
Mutant
c
Control
Mutant
d
CATGCCTGGCTGCGATGG
+1
mTgf-β2
BRE
NC
-2,462 to -2,653
+1,755 to +1,619
e
30
20
10
0
Ctrl
Mutant
***
Fold enrichment
NC
BRE
IP:
β-catenin

Figure 8. Identification of *Tgf-β2* as a β-catenin target gene in the murine uterus. (A) Venn diagram illustrating the overlap between nonredundant genes bound by β-catenin as determined by ChIP-seq and those genes regulated by β-catenin by microarray (>1.5 fold) in control and mutant mice uteri. (B) The quantitative real-time PCR analysis of *Tgf-β2* in the uterus of control and mutant mice at 4 weeks of age. (C) The immunohistochemical staining of TGF-β2 in the uterus of control and mutant mice at 4 weeks of age. (D) Map of β-catenin response element (BRE) on the *Tgf-β2* promoter. (E) ChIP assay using anti-β-catenin antibody on *Tgf-β2* promoter in control and mutant mice uteri at 4 weeks of age. The results represent the mean ± SEM. ** $p<0.01$ and *** $p<0.001$.

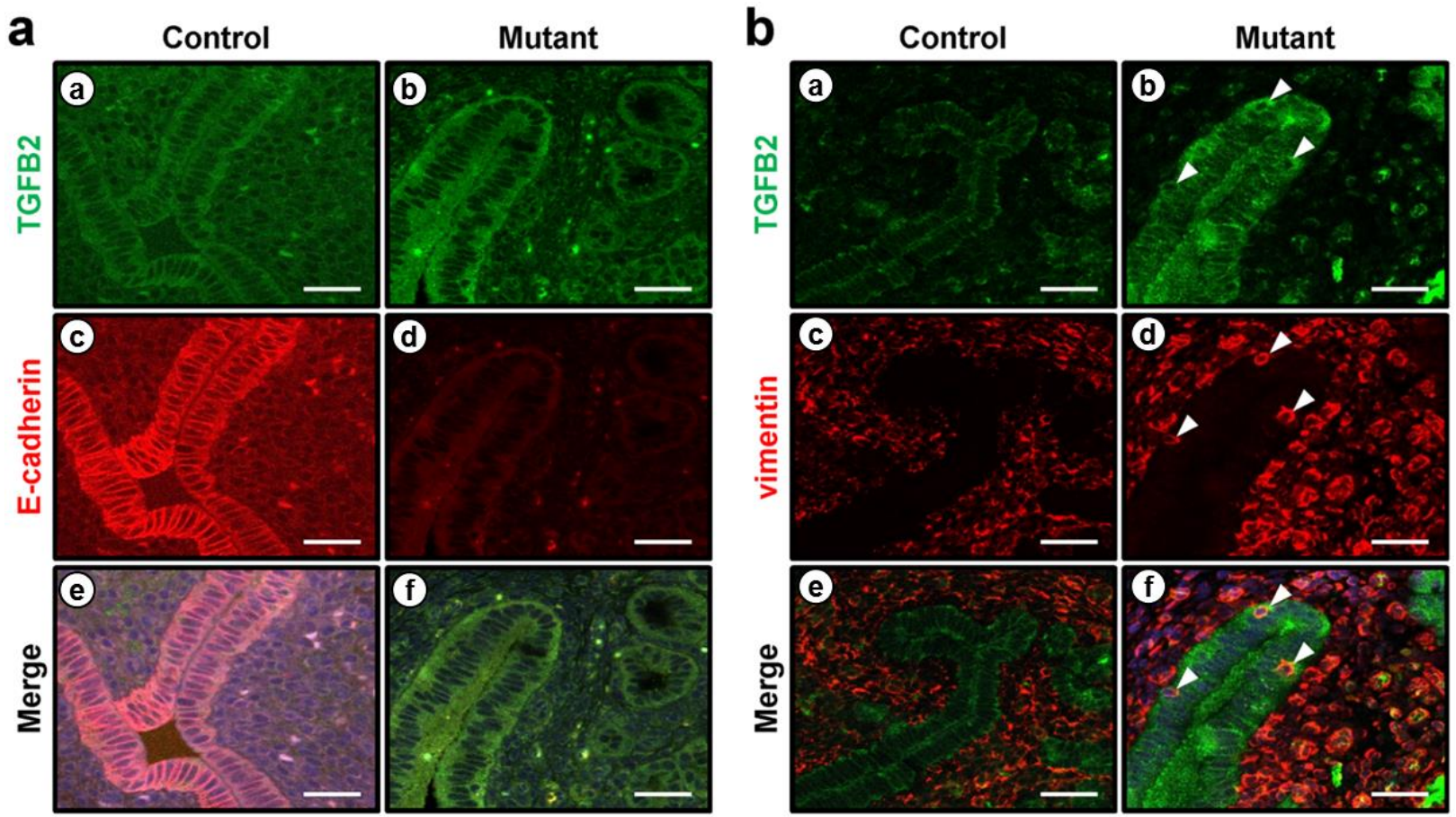

a
Control
Mutant
TGFB2
E-cadherin
Merge
a
b
c
d
e
f
b
Control
Mutant
TGFB2
vimentin
Merge
a
b
c
d
e
f

Figure 9. Correlation of TGF−β2 expression with EMT marker expression in mutant mouse uterus. The immunofluorescence analysis of TGF−β2 and E−cadherin (A) or vimentin (B) in the uterus of control (a, c, and e) and mutant (b, d, and f) mice at 4 weeks of age. Nuclei were counterstained with DAPI staining.

Levels of TGF-β2 is higher in adenomyosis of mice and human

As a previous our research, adenomyosis are developed in mice with uterine-specific stabilization of β-catenin from 4 months of age (Oh, Shin *et al.* 2013). Thus, to determine whether TGF-β2 is dysregulated in adenomyosis, I examined the level of TGF-β2 in the uteri of control and mutant mice at 2 months of age using immunohistochemistry analysis. The levels of TGF-β2 were higher in endometrial epithelial cells of mutant mice at 2 months of age compared to controls (Figure 10).

Additionally, I investigated the expression level of TGF-β2 in endometrium from women with and without adenomyosis by immunohistochemistry analysis. The expression levels of TGF-β2 were significantly higher in epithelial cells of eutopic endometrium (n=4 proliferative and n=4 secretory) and adenomyosis lesions (n=15 proliferative and n=15 secretory) compared to control endometrium without adenomyosis (n=8 proliferative and n=13 secretory) at proliferative phase as well as secretory phase (Figure 11). These results suggest that increase of TGF-β2 is important in the pathogenesis of adenomyosis.

Then, I analyzed the correlation of expression between β-catenin and TGF-β2 in women with and without adenomyosis from immunohistochemistry results. I showed the significant positive correlation between β-catenin and TGF-β2 in the endometrial epithelial cells (Spearman correlation coefficient $r=0.9136$, $p<0.0001$)

(Figure 12). Aditionally, I found that the high levels of TGF-β2 and low levels of E-cadherin in eutopic endometrium and adenomyosis lesions from women with adenomyosis compared to women without adenomyosis by double immunofluorescence staining (Figure 13). Taken together, these results suggest that TGF-β2 may play an important role in the development and progression of adenomyosis through correlation with β-catenin in the endometrium.

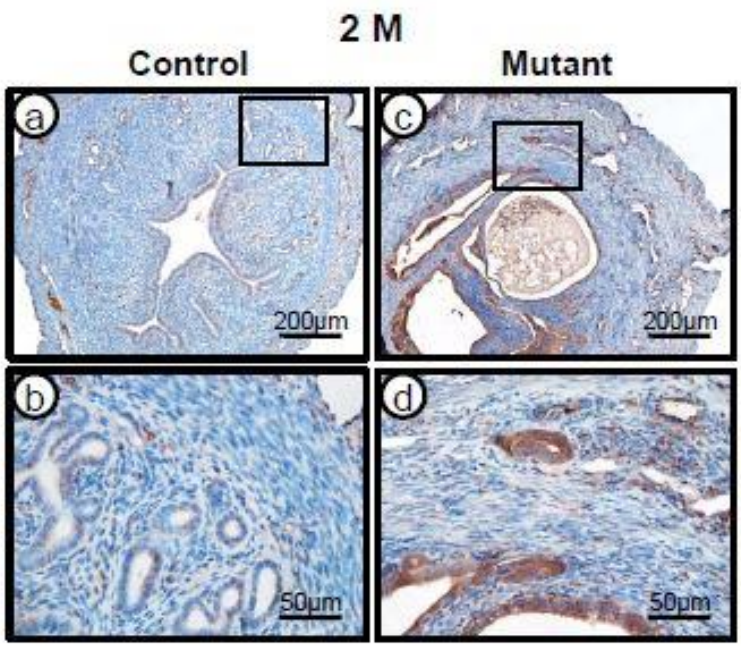


Figure 10. Levels of TGF-β2 in the murine uterus at 2 months of age.

The immunohistochemical staining of TGF-β2 in the uterus of control and mutant mice at 4 weeks of age.

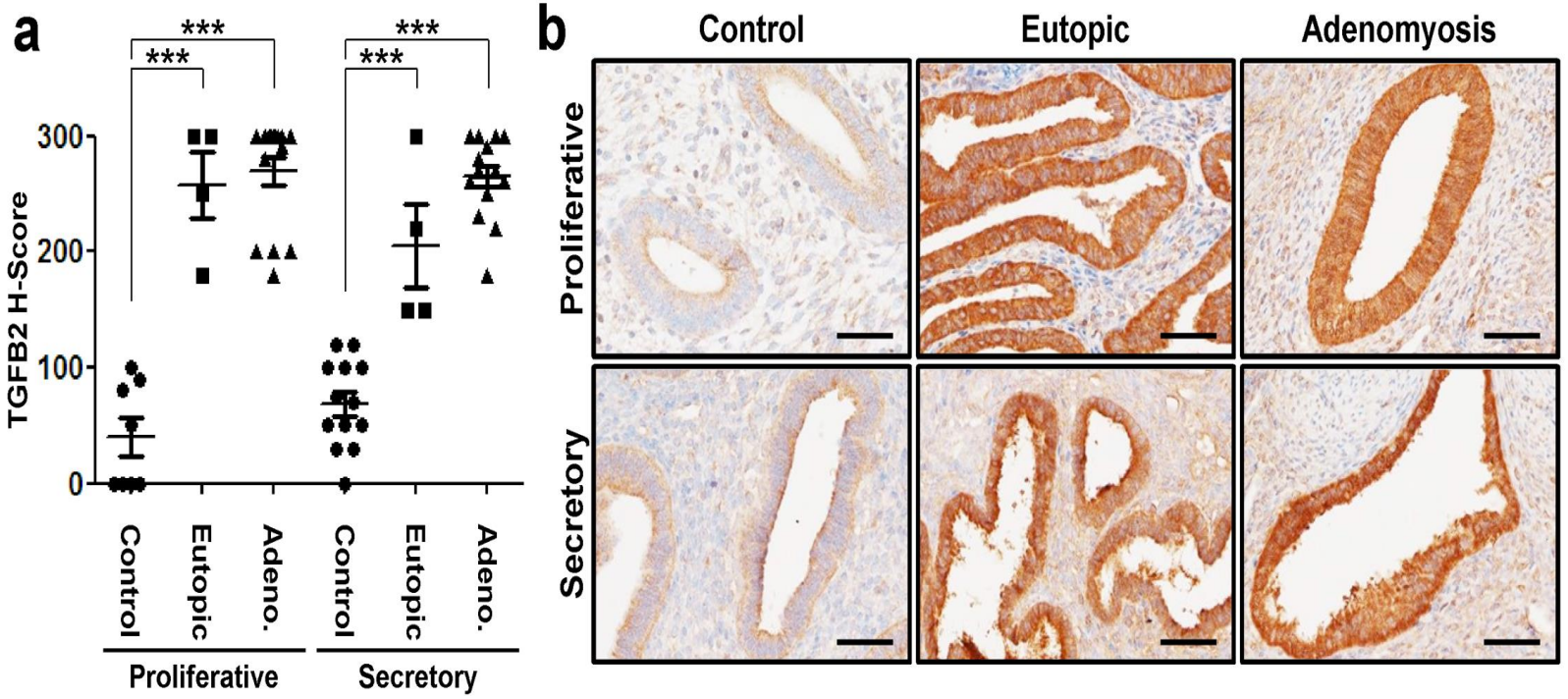

a
***
***
***
***
300
200
100
0
TGFB2 H-Score
Control
Eutopic
Adeno.
Control
Eutopic
Adeno.
Proliferative
Secretory
b
Control
Eutopic
Adenomyosis
Proliferative
Secretory

Figure 11. Level of TGF−β2 in endometrial tissue from women with adenomyosis. (A) Quantification of TGF−β2 protein levels by immunohistochemistry analysis in endometrium from proliferative and secretory phase in women with and without adenomyosis obtained by the immunohistochemical histological score (H−score). The results represent the mean ± SEM. *** p<0.001. (B) Representative photomicrograph of immunohistochemical staining of TGF−β2 in women endometrium with (a and b) and without (eutopic (b and e) and adenomyosis lesions (c and f)) adenomyosis at proliferative (a, b, and c) and secretory (d, e, and f) phase.

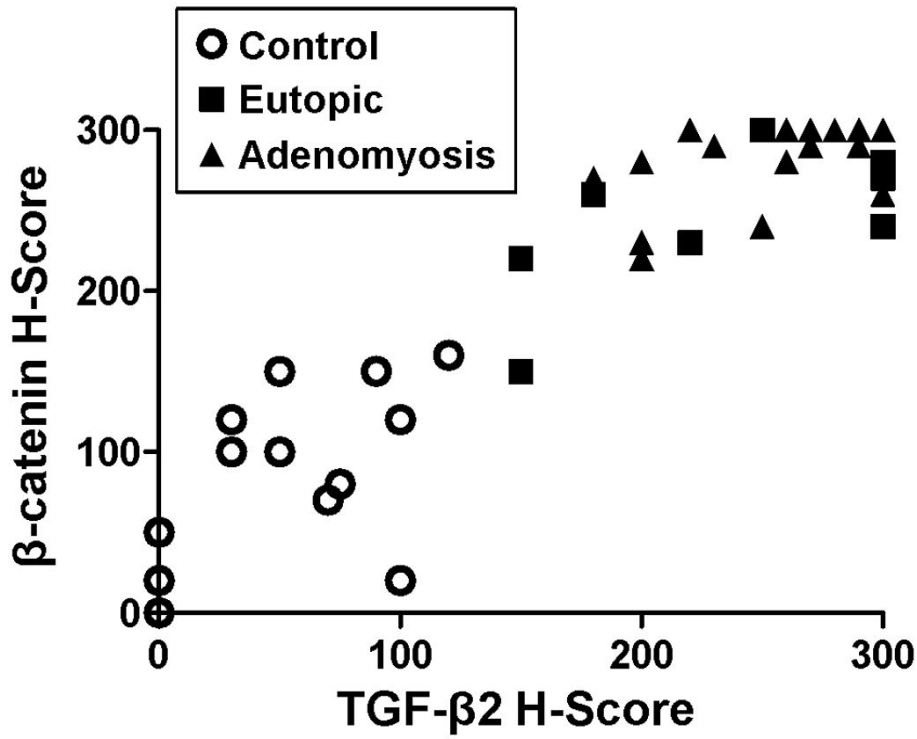


Figure 12. Positive correlation between TGF-β2 and β-catenin in human endometrium with and without adenomyosis throughout the menstrual cycle phases based on immunohistochemistry results (correlation coefficient = 0.9136, $p < 0.0001$).

Control
Eutopic
Adenomyosis
TGF-β2
E-cadherin
Merge
a
b
c
d
e
f
g
h
i

Figure 13. Conflicting expression between TGF−β2 and E−cadherin in epithelium of human endometrium with and without adenomyosis. The immunofluorescence analysis of TGF−β2 (a, b, and c) and E−cadherin (d, e, and f) in human endometrium with (a, d, and g) and without (eutopic (b, e, and h) and adenomyosis lesions (c, f, and i)) adenomyosis. Nuclei were counterstained with DAPI staining.

β-catenin leads to epithelial-mesenchymal transition in adenomyosis development through induction of TGF-β2

To determine whether activated β-catenin regulates EMT in endometrial epithelial cells, I investigated the EMT-associated protein levels in endometrial epithelial cell, Ishikawa, transfected with exon3-deleted *β-catenin* vector by western blot analysis. The exon3-deleted β-catenin is not phosphorylated by GSK-3β and results in consistent stabilization of the protein, nuclear accumulation, and participation in signal transduction and transcriptional activation through complex formation with DNA binding proteins (Palacios and Gamallo 1998). TGF-β2 levels were increased in exon3-deleted β-catenin transfected Ishikawa cells over time. The level of E-cadherin, an epithelial cell marker, was lower in Ishikawa cells activated β-catenin over time. In contrast, the level of vimentin, a mesenchymal cell marker, was increased in Ishikawa cells activated β-catenin over time (Figure 14A). Next, I examined an invasiveness of Ishikawa cells activated β-catenin. The gain of function of β-catenin exhibited a higher infiltration rate in the transwell invasion assay of Ishikawa cells compared to controls. Invasion ability of β-catenin-activated Ishikawa cells was increased by more than 1.7 fold compared to control cells (Figure 14B and C). These results suggest that activated β-catenin causes EMT via regulation of epithelial gene and mesenchymal gene expression as well as TGF-β2 expression.

To resolve the molecular mechanisms in which β-catenin induce EMT via TGF-β2, I treated a TGF-β inhibitor, Pirfenidone (5-methyl-1-2-[1H]-pyridone) to exon3-deleted β-catenin transfected Ishikawa cells. I examined the levels of E-cadherin after pirfenidone treatment during 18 and 48 hours by western blot analysis. Levels of E-cadherin were decreased in Ishikawa cells activated β-catenin at 48 hours, however its levels were overcome by pirfenidone treatment at 48 hours (Figure 14D). As well as, increase of invasiveness in Ishikawa cells by β-catenin-activation was recovered by pirfenidone treatment during 48 hours (Figure 14E and F). These results show that pirfenidone may suppress EMT by hyperactivated β-catenin via suppression of TGF-β signaling.

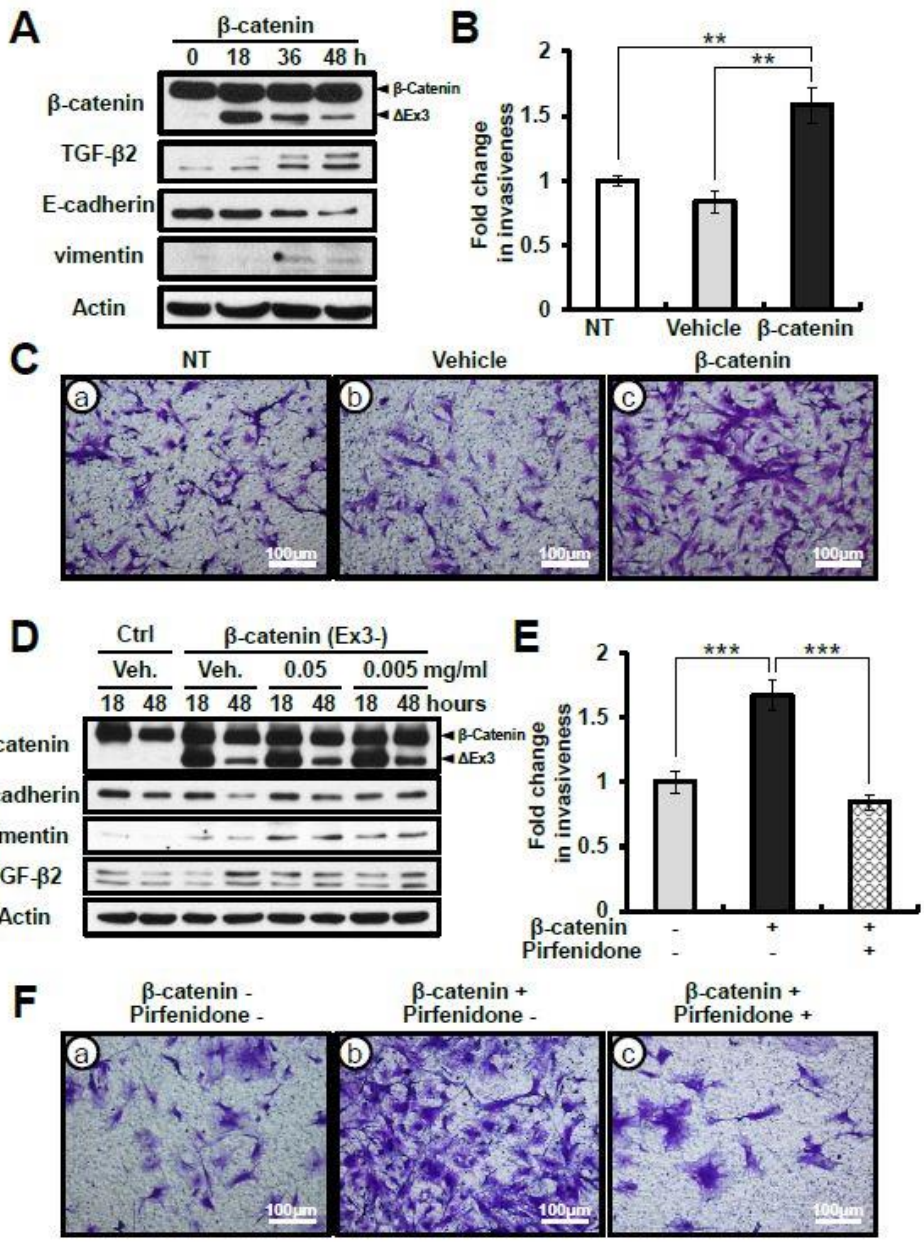

A
β-catenin
0 18 36 48 h
β-catenin
TGF-β2
E-cadherin
vimentin
Actin
β-Catenin
ΔEx3
B
Fold change in invasiveness
NT
Vehicle
β-catenin
C
NT
Vehicle
β-catenin
a
b
c
100μm
D
Ctrl
β-catenin (Ex3-)
Veh.
Veh.
0.05
0.005 mg/ml
18 48 18 48 18 48 18 48 hours
β-catenin
E-cadherin
vimentin
TGF-β2
Actin
β-Catenin
ΔEx3
E
Fold change in invasiveness
β-catenin
Pirfenidone
F
β-catenin -
Pirfenidone -
β-catenin +
Pirfenidone -
β-catenin +
Pirfenidone +

Figure 14. Increase of TGF-β2 by activated β-catenin leads to EMT in adenomyosis development. (A) Western blot analysis of β-catenin, TGF-β2, E-cadherin, and vimentin in Ishikawa cells transfected with exon3-deleted *β-catenin* vector for 0, 18, 36, and 48 hours. (B) Quantification of invaded through the matrigel and transwell membrane non-transfected Ishikawa (NT) and vehicle or exon3-deleted *β-catenin* vector transfected Ishikawa cells. (C) Representative result of transwell invasion assays of non-transfected Ishikawa (NT, a) and vehicle (b) or exon3-deleted β-catenin (c) vector transfected Ishikawa cells. (D) Western blot analysis of β-catenin, TGF-β2, E-cadherin, and vimentin in vehicle or exon3-deleted *β-catenin* vector transfected Ishikawa cells treated with or without pirfenidone for 18 and 48 hours. Actin was used as sample-loading control. (E) Quantification of invaded through the matrigel and transwell membrane vehicle or exon3-deleted β-catenin vector transfected Ishikawa cells treated with or without pirfenidone during 48 hours. (F) Representative result of transwell invasion assays of vehicle (a) or exon3-deleted β-catenin (b and c) vector transfected Ishikawa cells treated without (b) or with (c) pirfenidone for 48 hours. The results represent the mean ± SEM. ** $p<0.01$ and *** $p<0.001$.

4. Discussion

Adenomyosis is a common benign heterogenous gynecological disorder. Its symptoms include the menorrhagia, dysmenorrhea, chronic pelvic pain, dyspareunia, and dyschezia (Levgur, Abadi *et al*. 2000; Sammour, Pirwany *et al*. 2002). With the aid of transvaginal ultrasonography and MRI, this disease is now being more frequently diagnosed in infertility patients because adenomyosis can interfere with implantation, causing subfertility and miscarriage (de Souza, Brosens *et al*. 1995; Campo, Campo *et al*. 2012; Louis, Saso *et al*. 2012). However, most women are not diagnosed until later stages because early diagnosis of adenomyosis is difficult due to the absence of pathognomonic symptoms and biomarkers. Despite its prevalence, the etiology and pathophysiology of adenomyosis are still unknown. Therefore, identification of the mechanisms involved in the early pathogenesis of adenomyosis and strategic therapies for nonsurgical treatment are critical.

Normal WNT/β-catenin signaling is critical for uterine development and function (Arango, Szotek *et al*. 2005; Li, Zhang *et al*. 2005; Mohamed, Jonnaert *et al*. 2005; Rider, Isuzugawa *et al*. 2006; Jeong, Lee *et al*. 2009). However, our team showed that hyperactive β-catenin is a cause of adenomyosis development through the induction of EMT (Oh, Shin *et al*. 2013). The levels of E-cadherin, an epithelial cell marker, are repressed in endometrial epithelial cells of uterine specific β-catenin stabilized mice compared to control

mice. As well as, SNAIL and ZEB1, the transcriptional repressors of E-cadherin gene and mesenchymal cell markers, are observed in some epithelial cells during the pathogenesis of adenomyosis in β-catenin-activated mice uteri (Oh, Shin *et al.* 2013). However, the precise molecular mechanism of β-catenin in adenomyosis remains poorly understood.

In this study, I have identified hyperactive β-catenin-regulated uterine genes using the high-density DNA microarray analysis and ChIP-seq analysis on mutant ($Pgr^{cre/+}Ctnnb1^{f(ex3)/+}$) mice. DNA microarray analysis revealed 1,103 and 976 transcripts whose abundance significantly increased or decreased, respectively, in the mutant mice uteri as compared with controls. From the pathway analysis using Ingenuity Systems Software, I found altered pathways including stem cell progression such as Wnt/β-catenin signaling, human embryonic stem cell pluripotency, basal cell carcinoma signaling, TGF-β signaling, mouse embryonic stem cell pluripotency, ErbB2- ErbB3 signaling, ERK/MAPK signaling, and ErbB signaling. Additionally, I have identified 2,092 and 3,524 binding target sites for β-catenin via ChIP-seq analysis in the uteri of control and mutant mice, respectively. Interestingly, when the microarray expression data were coupled with ChIP-seq data, I identified 318 genes that are dysregulated and have β-catenin binding sites in mutant mice. Of the 318 genes, 189 were increased in mutant. Interestingly, Pathway Analysis of the DNA microarray identified activation of TGF-β signaling in the mutant mice and

ChIP-seq data revealed TGF-β2 as a direct transcriptional β-catenin target gene in the uterus (Figure 8).

TGF-β superfamily signaling plays a pleiotropic role in fundamental cellular and developmental processes (Massague 2012). TGF-β superfamily members are key regulators of female reproduction, including ovulation, uterine decidualization, and embryo development (Juengel and McNatty 2005; Li, Agno *et al.* 2011; Itoh, Kishore *et al.* 2012; Gao, Duran *et al.* 2015). Furthermore, TGF-β signaling has been shown to play an important role in EMT (Derynck, Muthusamy *et al.* 2014) and it influences on the Wnt/β-catenin signaling through interaction of activated Smad complexes with APC/GSK-3β, β-catenin, and TCF/LEF1 (Xu, Lamouille *et al.* 2009; Minoo and Li 2010). Therefore, the results suggest that TGF-β2 is a potentially novel mediator of β-catenin action in adenomyosis.

I found the higher levels of TGF-β2 in endometrial epithelial cells of mutant mice compared to controls by immunohistochemistry (Figure 10). Moreover, immunohistochemistry analysis also showed aberrant overexpression of TGF-β2 in endometrial epithelium from women with adenomyosis compared to women without adenomyosis (Figure 11). Interestingly, I showed a significantly positive correlation between β-catenin and TGF-β2 in the endometrial epithelial cells of women (Figure 12). Immunofluorescence results confirmed that TGF-β2 proteins are well colocalized with vimentin in the epithelial cells of the uterus in mutant mice (Figure 9). Taken

together, these results suggest that aberrant activation of β-catenin and TGF-β2 plays an important role in the pathogenesis of adenomyosis.

Previous our research has shown that hyperactivation of β-catenin in the *Pgr*-positive cells induces expression of vimentin, mesenchymal marker, in some epithelial cells and decreased expression of E-cadherin, epithelial marker, in epithelial cells compared to control endometrium (Oh, Shin *et al.* 2013). In this study, I found that the Ishikawa cells with nuclear β-catenin expression expressed TGF-β2 and vimentin but decreased the expression of E-cadherin. Furthermore, cell invasion assay showed that nuclear β-catenin expression significantly increased invasiveness compared to the control (Figure 14).

I treated a TGF-β inhibitor, Pirfenidone (5-methyl-1-2-[1H]-pyridone) to Ishikawa cells with nuclear β-catenin expression to confirm whether β-catenin induce EMT via TGFβ2. Pirfenidone (5-methyl-1-2-[1H]-pyridone; Shionogi & Co. Ltd.; MARNAC Inc.) is a Federal Drug Administration (FDA)-approved TGF-β inhibitor for the treatment of idiopathic pulmonary fibrosis (King, Bradford *et al.* 2014). Pirfenidone is a small synthetic molecule and inhibition of TGF-β production (Shimizu, Kuroda *et al.* 1998; Zhou, Latham *et al.* 2005). Results show that pirfenidone treatment increased E-cadherin expression in Ishikawa cells with nuclear β-catenin expression (Figure 14). These results suggest

that pirfenidone may suppress EMT and adenomyosis via suppression of TGF-β signaling.

In conclusion, I identified that *Tgf-β2* is direct transcriptional β-catenin target gene in the uterus by DNA microarray and ChIP-seq analysis. The levels of TGF-β2 are higher in endometrial epithelial cells of β-catenin stabilized mice compared to control mice, as well as in epithelial cells of human eutopic endometrium and adenomyosis lesions compared to women without adenomyosis. I demonstrate a strong positive correlation between β-catenin and TGFB2 proteins in women with adenomyosis. As well as, I found that activated β-catenin leads to EMT in endometrial epithelial cells through induction of TGF-β2 and TGF-β inhibitor suppresses the EMT related β-catenin. These results provide significant insights into our understanding of the pathophysiological function of β-catenin in adenomyosis development and suggest new therapeutic potential of TGF-β inhibitor for adenomyosis.

CHAPTER 6

DYSREGULATION OF LEF−1 IN ADENOMYOSIS

1. Introduction

Adenomyosis is a benign gynecological disorders characterized by the presence of heterotopic epithelial cells, ectopic endometrial glands and stroma within myometrium (Bird, McElin *et al*. 1972; Ferenczy 1998; Tamai, Togashi *et al*. 2005).

Adenomyosis is associated with ectopic invasion of stromal tissue and endometrial glands, leading to hypertrophy and hyperplasia of the surrounding myometrium (Ferenczy 1998). The prevalence of adenomyosis has been reported to range from 5% to 70%, depending on the diagnostic criteria (Taran, Stewart *et al*. 2013). In spite of its prevalence, the reason for aetiology and pathogenesis of adenomyosis is still unknown. The most symptoms of adenomyosis include heavy menstrual bleeding, menorrhagia, dysmenorrhea, longer menstrual cycles than normal (Levgur, Abadi *et al*. 2000; Sammour, Pirwany *et al*. 2002; Peric and Fraser 2006) and can interfere normal uterine function such as implantation and fertility (Campo, Campo *et al*. 2012; Louis, Saso *et al*. 2012). Consequently, adenomyosis can be often diagnosed in patients with infertility (de Souza, Brosens *et al*. 1995). Diagnosis of adenomyosis based on clinical findings is non-specific, therefore it is not easy to diagnosis of adenomyosis. Magnetic resonance images (MRI) have been commonly used for diagnosing adenomyosis, because MRI possess the advantage of outstanding resolution, high sensitivity that allows identification of invasiveness in soft tissue (Basak and Saha

2009). However, It is difficult to diagnosis the early stages of adenomyosis because the variable signs and absence of biomarkers. For these reason, the women who were diagnosed with late-Stage of adenomyosis requires hysterectomy. In spite of its prevalence, the precise etiology and physiopathology of adenomyosis is still unknown.

Several animal models for pathological study of adenomyosis were developed in mouse (Lipschutz, Iglesias *et al.* 1967; Zhou, Mori *et al.* 2004), rat (Mori, Kyokuwa *et al.* 1998), and rabbit (Meissner, Sommers *et al.* 1957). A novel mouse model of adenomyosis has been established (Oh, Shin *et al.* 2013) and revealed critical details regarding the physiopathology of adenomyosis.

Wnt/β-catenin signaling is known to play a key roles cell proliferation, cell fate specification during embryonic development and maintenance of tissue homeostasis (Logan and Nusse 2004). As a results, aberrant Wnt/β-catenin signaling is linked closely to many different cancer involving colon cancer (Lammi, Arte *et al.* 2004), hepatocellular carcinoma (Laurent-Puig and Zucman-Rossi 2006), ovarian cancer (Palacios and Gamallo 1998). It has been reported that Wnt/β-catenin signaling induce epithelial-mesenchymal transition (EMT) (Thiery and Sleeman 2006). In addition to, Wnt/β-catenin signaling is also important component of uterine function. Wnt/β-catenin signaling is critical role between the implanting blastocyst and the receptive uterus (Mohamed, Jonnaert *et al.* 2005) and in the mouse uterus during the decidualization (Herington, Bi *et al.* 2007). Without Wnt signaling, cytoplasmic β-catenin is degraded

by a β-catenin destruction complex involving adenomatous polyposis coli (APC) and the kinases glycogen synthase kinase-3 alpha/beta (GSK-3) and casein kinase-1 (CKI) (Gordon and Nusse 2006). In the presence of Wnt signaling, a β-catenin destruction complex function is disrupted. As a results, Wnt-induced stabilization of β-catenin translocates to the nucleus and binds to Tcf/Lef (T-cell factor/Lymphoid enhancing factor) transcription factors to activate Wnt target gene (Behrens, von Kries *et al*. 1996).

Lymphoid enhancer-binding factor 1 (LEF-1) is a member of the Tcf/Lef family of transcription factors. It has been well known that LEF-1 in Wnt/β-catenin signaling is essential component for the increased proliferation of pro-B cell (Reya, O'Riordan *et al*. 2000). LEF-1 in Wnt/β-catenin signaling acts as a transcriptional activator of proliferation stimulating genes such as *c-Myc*, *Mmp7* (Matrix Metallopeptidase 7), *Cyclin D1* (He, Sparks *et al*. 1998; Tetsu and McCormick 1999; Garcia and Isaacson 2011; Bucan, Mandel *et al*. 2012).And several evidence show the β-catenin /LEF-1 complex that induces EMT (Kim, Lu *et al*. 2002; Kato, Shimmura *et al*. 2007). Likewise, connection between β-catenin and LEF-1 is important for induction of proliferation and EMT. In the presence of Wnt signaling, β-Catenin interacts with LEF-1 and positively regulates its transcriptional activity (Korinek, Barker *et al*. 1997; Narasipura, Henderson *et al*. 2012). As a results, mutated β-catenin lead to increased expression of LEF-1 that cause

development of several cancer such as breast cancer, endometrial cancer (Machin, Catasus *et al*. 2002; Nguyen, Rosner *et al*. 2005).

Here, I found that Chip analysis demonstrated that β-catenin directly bind to BRE region of *Lef-1* promoter in murine uterus. Also Immunohistochemistry analysis showed that LEF-1 expression were significantly higher in human eutopic endometrium and adenomyosis lesions compared to control women as well as a dominant stabilized β-catenin mutant mice ($Pgr^{cre/+}$ $Ctnnb1^{f(ex3)/+}$) compared to control ($Pgr^{cre/+}$ and $Ctnnb1^{f(ex3)/+}$) mice. Furthermore, I found that activated β-catenin induced expression of LEF-1 and ZEB1 but decreased E-cadherin in endometrial adenocarcinoma cells. Also, it was confirmed that activated lef-1 reduced the expression of E-cadherin, but the expression pattern of ZEB1 was not changed in endometrial adenocarcinoma cells. These results suggest that *Lef-1* as a direct target gene of β-catenin may play an important role in development of uterine adenomyosis.

2. Materials and methods

Animals and tissue collection

Mice were cared in the designated animal care facility according to the Michigan State University institutional guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. Uterine tissues were collected from control (*Pgr*$^{cre/+}$ and *Ctnnb1*$^{f(ex3)/+}$) and mutant (*Pgr*$^{cre/+}$*Ctnnb1*$^{f(ex3)/+}$) mice to examine adenomyosis development as previously described (Oh, Shin *et al.* 2013). Uterine tissues were instantly frozen at the time of dissection and stored at −80° C for RNA or ChIP analysis, or fixed with 4% (volume/volume) paraformaldehyde for immunostaining analysis.

Human adenomyosis samples

Human Tissue samples of adenomyosis were obtained with informed consent using a guideline approved by the Institutional Review Boards of Michigan State University. Tissue samples of adenomyosis with eutopic endometrium were obtained from surgical hysterectomy specimens. Controls comprised regularly cycling premenopausal women undergoing an endometrial hysterectomy for benign conditions with no history or evidence of adenomyosis, who were documented not to be pregnant and who had not been on

hormonal therapies for at least 3 months before tissue sampling. Histologic dating of endometrial samples was done based on the criteria of Noyes (Noyes, Hertig *et al.* 1975). Eutopic endometrium (6 proliferative and 5 secretory phase) and adenomyosis lesions (8 proliferative and 6 secretory phase) samples were from women with adenomyosis, and control endometrium (n=6 proliferative and n=9 secretory) samples were from women without adenomyosis. Samples used for immunostaining were fixed in 10% buffered formalin prior to embedding in paraffin wax.

ChIP assays

ChIP assay were conducted by Active Motif (Carlsbad, CA, USA) using uterus of control and mutant mice at one month of age as previously described (Kim, Yoo *et al.* 2015). Briefly, for each ChIP reaction, 100 μg of chromatin was immunoprecipitated by 4 μg of antibodies against β-catenin (610154; BD biosciences, San Jose, CA). Eluted DNA was amplified with specific primers using SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). Primers used in PCR were as follows: BRE (forward: 5′-GTTACCGAGGGGCAGAATGC-3′; reverse: 5′-GGGGGTCATCATACAAGGCA-3′) and negative control (forward: 5′-CCGGAGCTCCTCAGATCCAC-3′; reverse: 5′-TGGATCCCTCTCCATCCCAC-3′). Immunoprecipitation was

performed as a negative control. The resulting signals were normalized to input DNA.

Quantitative real-time PCR Analysis

Total RNA was extracted using RNeasy total RNA isolation kit (Qiagen, Valencia, CA) according to manufacturer's instruction. As a template for quantitative PCR, cDNAs were synthesized using quantitative PCR random hexamers and MMLV Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). Expression levels of *lef-1*, *zeb1*, *e-cadherin* in control or mutant mice and human endometrial adenocarcinoma cells were measured by Real-time PCR was performed using Real-time PCR SYBR Green detection system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions (PE Applied Biosystems, Foster City, CA). Real-time PCR results for murine tissues were normalized against the housekeeping genes, *Rpl7*. The sequences of the primers used for mouse *Lef-1* were 5' - TATGAACAGCGACCCGTACA-3' and 5' -TCGTCGCTGTAGGTGATGAG -3' and for mouse *Rpl7* were 5' -TCAATGGAGTAAGCCCAAAG-3' and 5' -CAAGAGACCGAGCAATCAAG-3'. For human endometrial adenocarcinoma cells, Real-time PCR result were normalized against the housekeeping genes, *Gapdh*. The sequences of the primers used for human *Lef-1* were 5' -TGGAGGCCTCTACAACAAGG-3' and 5' -CCTGGAGAAAAGTGCTCGTC-3' and for human *Zeb1* were

5’-TCCCACACGACCACAGATAC-3’ and 5’-GAGGAGAACTGGTTGCCTGT-3’ and for human *E-cadherin* were 5’-GCCTCCTGAAAAGAGAGTGG-3’ and 5’-TAGGGCTGTGTACGTGCTGT-3’ and for *Gapdh* were 5’-CAAGAATTTGGCTACAGCA-3’ and 5’-TGTGAGGAGGGGAGATTCA-3’.

Immunohistochemistry analysis

Uterine sections were pre-incubated with 10% normal serum in phosphate-buffered saline (PBS; pH 7.5) and then incubated with anti LEF-1 (#2230; Cell signaling, Danvers, MA) antibody in 10% normal serum in PBS overnight at 4° C. On the next day, Uterine sections were washed in PBS and incubated with a secondary antibody conjugated to biotin (Vector BA-1000, Laboratories, Burlingame, CA) for 1 h at room temperature and then Uterine sections were washed in PBS and incubated with Horseradish Peroxidase (Invitrogen Corp., Carlsbad, CA) for 45 min at room temperature and then immunoreactivity was detected using DAB (Vector Laboratories, Burlingame, CA). Analysis of LEF-1 expression was observed under a light microscopy.

Cell culture and transient transfections

HEC1A cell, human endometrial adenocarcinoma cell line, were cultured in DMEM/F12 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). Cells were cultured in monolayer at 37° C in 5% CO2. For transient transfection of exon 3-deleted *β-catenin* vector and *Lef-1* Vector to HEC1A cells were grown on glass coverslips to 70%, I performed transfection using Lipofectamine 2000 (Invitrogen Crop., Carlsbad, CA).

Western blot analysis

Cellular proteins were extracted using lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, and 0.125% Nonidet P-40 (volume/volume) in distilled water supplemented with both a protease inhibitor cocktail (Roche, Indianapolis, IN) and a phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO). Fifteen μg of protein lysates were electrophoresed using SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). Casein (0.5% volume/volume) was used to block the membrane prior to exposure to anti-LEF-1 (Cell Signaling, Danvers, MA), anti-β-catenin (610154, BD Bioscience, San Diego, CA) or anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA), or E-cadherin (1:3000; 14472, Cell Signaling, Danvers, MA) or

ZEB1(1:500, sc-81428, Santa Cruz Biotechnology, Santa Cruz, CA) antibody immunoblotting. Immunoreactivity was visualized by incubation with a horseradish peroxidase-linked secondary antibody followed by exposure to ECL reagents according to manufacturer's instructions (GE Healthcare Biosciences, Piscataway, NJ). Quantification of all were performed using ImageJ software.

Immunofluorescence analysis

HEC1A cells were transfected using Exon-3-deleted *β-catenin* vector and *Lef-1* vector. Upon completion of transfection, coverslips were washed with PBS, fixed with 4% paraformaldehyde for 20min at room temperature and permeabilized with 0.1% of Triton X-100 (Sigma-Aldrich, St. Louis, MO). After further washing, Cells were exposed to anti-LEF-1 (1:250), anti- β-catenin (1:500; 610154, BD Bioscience, San Diego, CA), ZEB1 (1:500; sc-10572, Santa Cruz Biotechnology, Santa Cruz, CA) or E-cadherin (1:3000; 14472, Cell Signaling, Danvers, MA) antibodies overnight at 4° C and secondary antibodies for 1 hour at room temperature. Washed coverslips were then mounted onto microscope slides with a DAPI-impregnated mounting media (Vector Laboratories, Burlingame, CA). Images were captured with a confocal microscope (LSM 700 confocal microscope; Carl Zeiss, Thornwood, NY, USA). Quantification of all were performed using ImageJ software.

Statistical analysis

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

3. Results

LEF-1 levels are higher in dominant stabilized β-catenin in the murine uterus

Our previous research, uteri of stabilization of β-catenin at 4 months of age develops adenomyosis in mice (Oh, Shin *et al.* 2013). Based on this preliminary research, to determine whether LEF-1 expression is altered in animal model of uterine adenomyosis, I examined levels of *Lef-1* in control ($Pgr^{cre/+}$ *and* $Ctnnb1^{f(ex3)/+}$) or mutant uterus ($Pgr^{cre/+}Ctnnb1^{f(ex3)/+}$) at 4 weeks of age by real-time PCR and immunohistochemistry assay. Expression of *Lef-1* is significantly increased ($p<0.01$) in uteri of mutant compared to control mice (Figure 15A). Also immunohistochemistry showed that LEF-1 levels are increased in epithelial cells and stromal cells compared to control mice (Figure 15B). These results mean that LEF-1 may play an important role in developing adenomyosis induced by dominant stabilized β-catenin in the murine uterus.

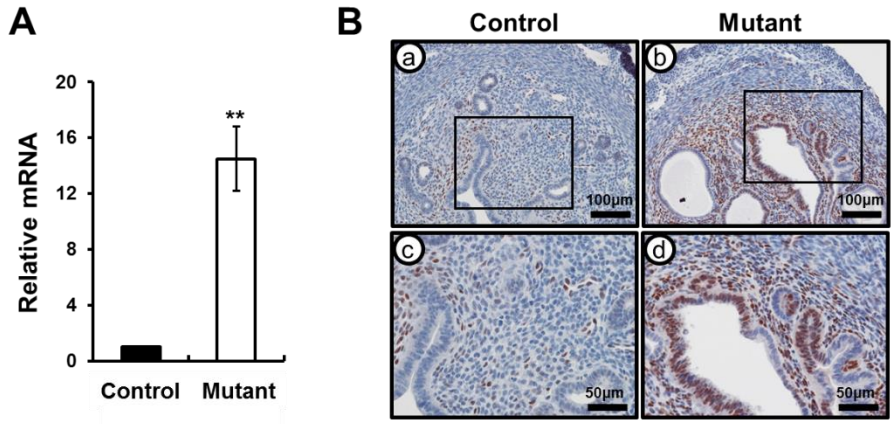


Figure 15. Expression levels of LEF-1 are increased in dominant stabilized β-catenin in the murine uterus. (A) The real-time PCR analysis of *lef-1* in the uteri of control and mutant mice at 4 weeks of age. The expression levels of LEF-1 are significantly increased in mutant mice compared to control mice. The result represents the mean ± SEM. ** $p<0.01$. (B) The immunohistochemical staining of LEF-1 in the uterus of control and mutant mice at 4 weeks of age.

Lef-1 is identified as β-catenin target gene in the murine uterus.

To determine whether β-catenin binds to the putative *Lef-1* promoter in the murine uterus, ChIP assay was performed on uterine of chromatin from control and mutant mice at 2 months of age. ChIP analysis showed that recruitment of β-catenin on BRE1 and BRE2 is significantly increased ($p<0.05$) in mutant uterus compared to control (Figure 16A and B). It indicates that *Lef-1* is directly regulated by β-catenin in murine uterus. These results suggest that adenomyosis is developed through β-catenin regulates transcriptional activation of *Lef-1* in the murine uterus.

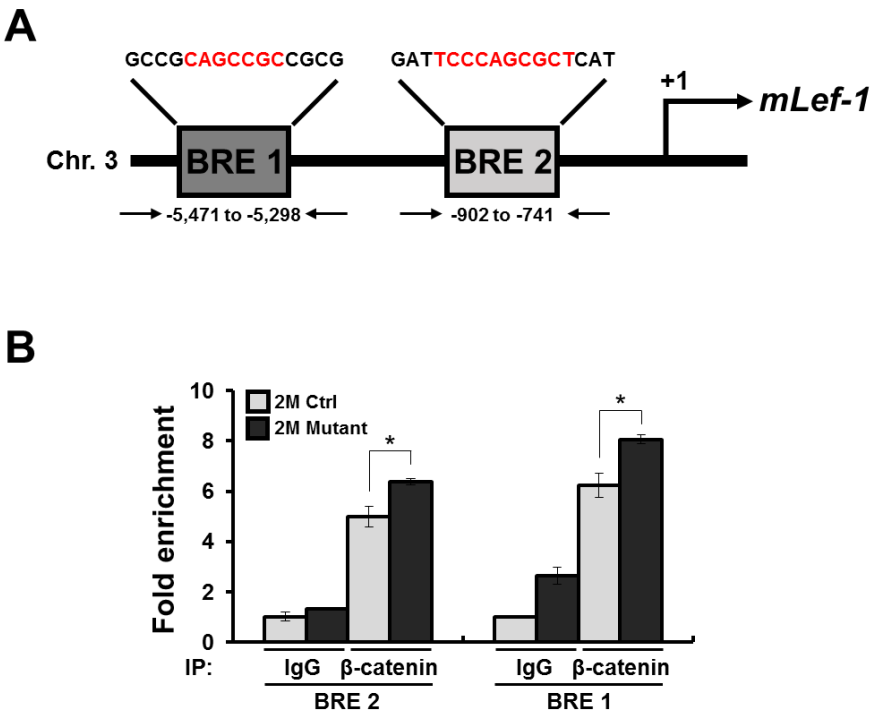


Figure 16. *Lef-1* is identified as a target gene of β-catenin in the murine uterus. (A) Map of β-catenin response element (BRE) 1 and 2 on the *Lef-1* promoter. (B) ChIP assay using anti-β-catenin antibody on *Lef-1* promoter in uteri of control and mutant mice at 8 weeks of age. The results represent the mean ± SEM. * $p<0.05$

Dysregulation of LEF-1 in activated β-catenin in the murine uterus

To identify the expression pattern of LEF-1 during progression of adenomyosis in uteri of mutant mice, control or mutant mice were sacrificed at 4 weeks, 2 months and 6 months.

LEF-1 levels were higher in epithelial cells of mutant uterus compared to control at 4 weeks, 2 months and 6months. But in the stromal compartment, LEF-1 levels were only higher in mutant uterus at 4 weeks of age than in the control (Figure 17 A, B). Also, the expression of LEF-1 protein was confirmed in epithelial cells of adenomyosis region at 2 months and 6 months (Figure 18). These results suggest that dysregulation of LEF-1 in the uterine epithelial cells and in the stromal cells of immature uterus may play a key role in developing adenomyosis.

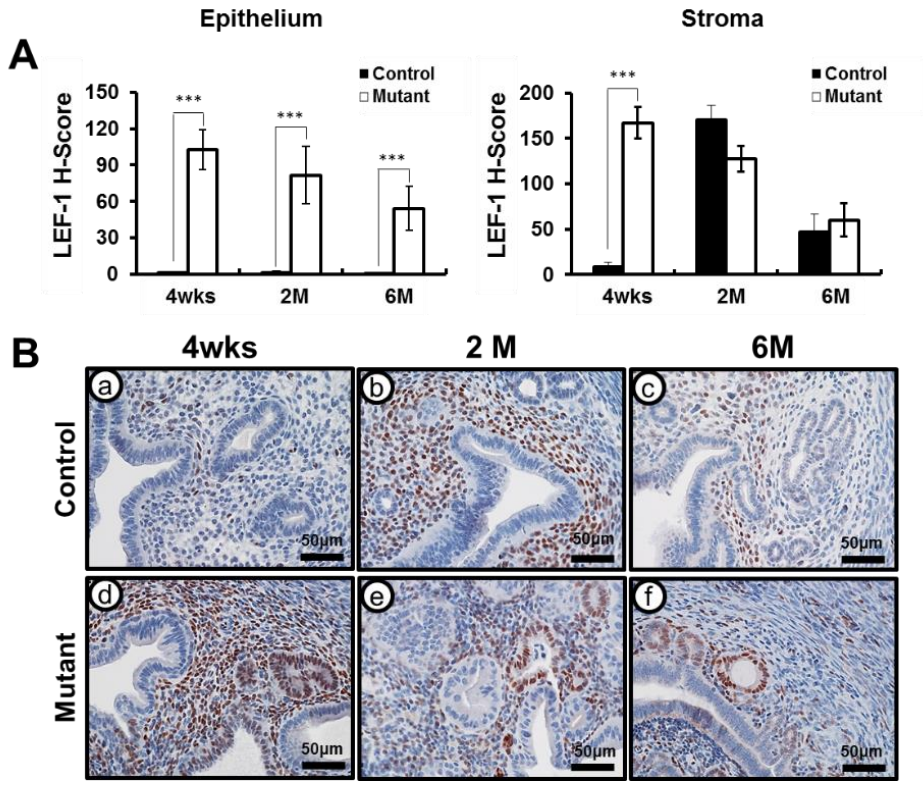


Figure 17. Expression pattern and time course of LEF-1 in murine uterus developing adenomyosis. (A) Control(a,b and c) and mutant(d,e and f) mice were sacrificed at 4 weeks, 2 months and 6 months of age. H-Score of LEF-1 in the uteri of control and mutant mice. Quantification of LEF-1 levels by immunohistochemistry analysis in uteri of control or mutant by the immunohistochemical histological score (H-score). LEF-1 expression in epithelium and stroma was significantly increased in mutant compared to control mice. The results represent the mean ± SEM. *** $p<0.001$, (B) The localization pattern of LEF-1 by immunohistochemistry in the control or Mutant uterus at 4 weeks, 8 weeks and 6 months of age. Nuclei were counterstained with hematoxylin.

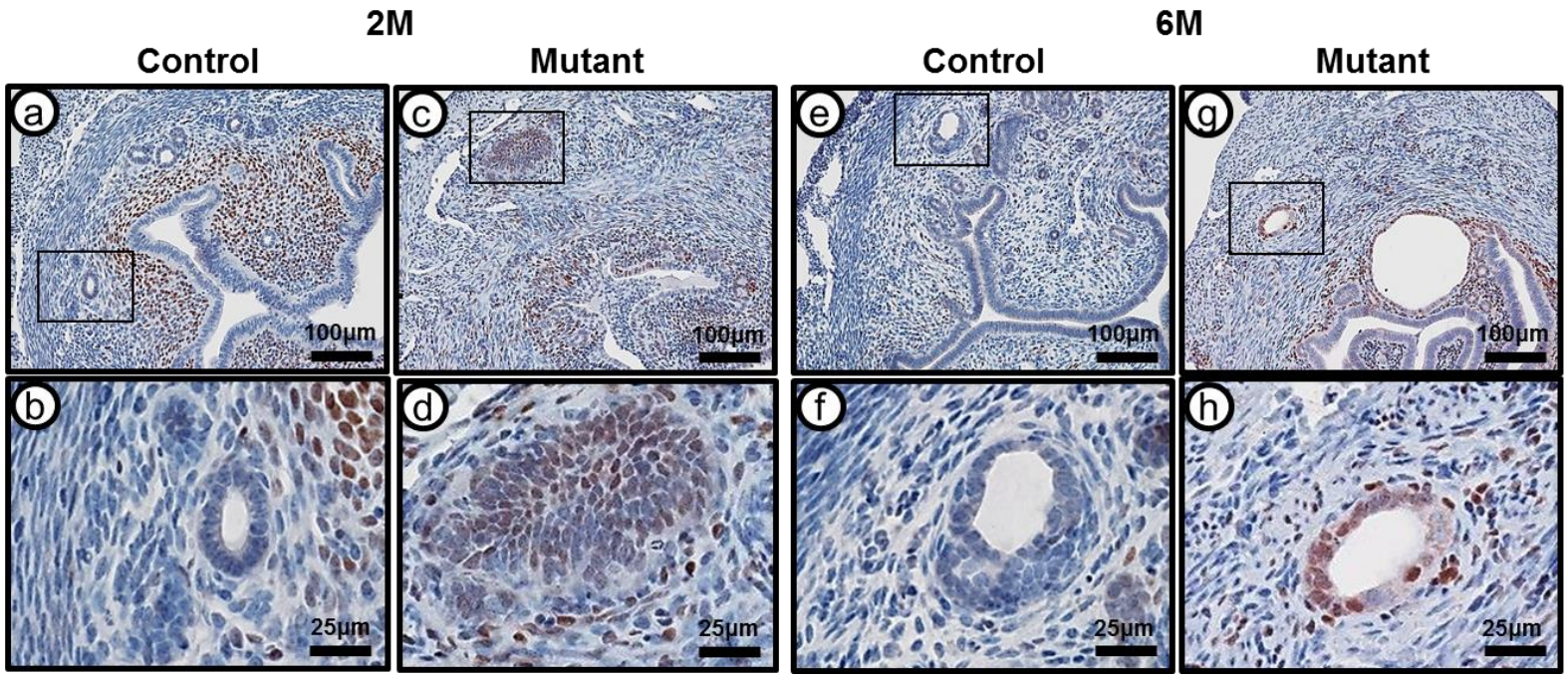

2M
6M
Control
Mutant
Control
Mutant
a
c
e
g
100μm
100μm
100μm
100μm
b
d
f
h
25μm
25μm
25μm
25μm

Figure 18. The expression of LEF-1 in adenomyosis in the myometrium. (A) The immunohistochemical staining of LEF-1 in the uterus of control (a, b, e and f) and mutant (c, d, g and h) mice at 2 and 6 months of age.

LEF-1 levels are higher in human endometrium from women with adenomyosis

To investigate that dysregulation of LEF-1 in human adenomyosis, protein levels of LEF-1 were examined by immunohistochemistry analysis in endometrium from women proliferative and secretory endometrium with or without adenomyosis (Figure 19 A, B). In the proliferative phase, the protein levels of LEF-1 were significantly higher in epithelial cells of eutopic endometrium (n = 6) compared to control endometrium without adenomyosis (n = 6) but not in the adenomyosis lesions (n = 8). In the secretory phase samples, there was no significant difference of protein levels of LEF-1.

In addition, there was no difference in stromal cells in proliferative phase and secretory phase (Figure 20). These results suggest that activation of LEF-1 in epithelial cells of proliferative endometrium may play important role in the development and progression of adenomyosis.

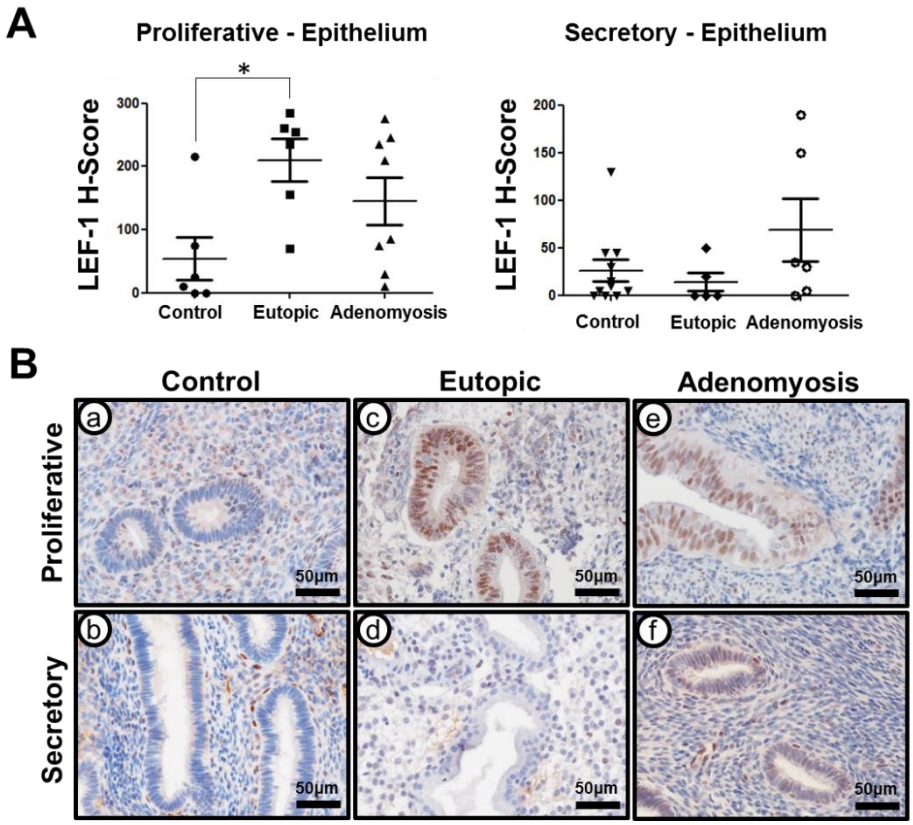


Figure 19. The expression of LEF-1 in proliferative and secretory endometrium from women with or without adenomyosis. (A) H-score of LEF-1 in human endometrium with or without adenomyosis. Levels of LEF-1 were significantly higher in human proliferative endometrium with adenomyosis. The results represent the mean ± SEM. * $p<0.05$. (B) The localization of LEF-1 in human endometrium with or without adenomyosis. LEF-1 expression was higher in human proliferative endometrium with adenomyosis. Nuclei were counterstained with hematoxylin.

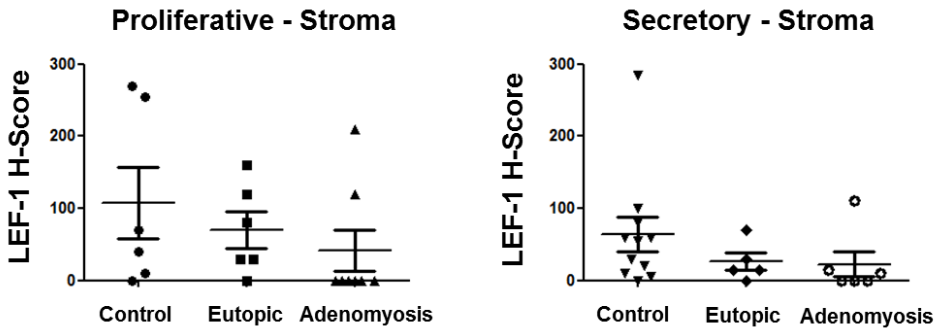
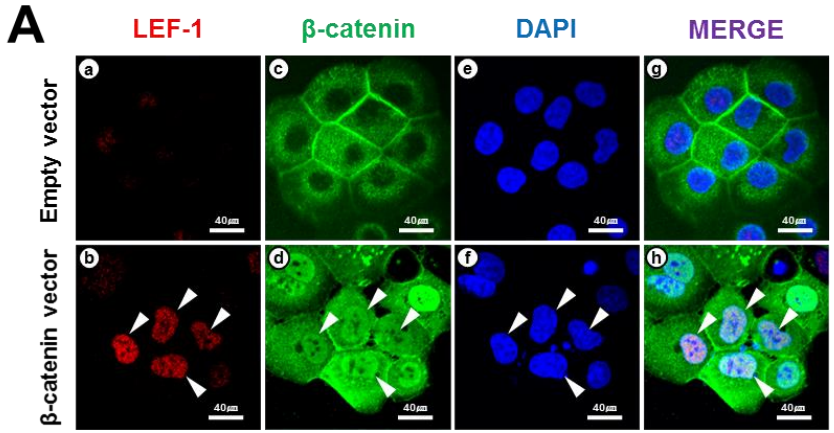


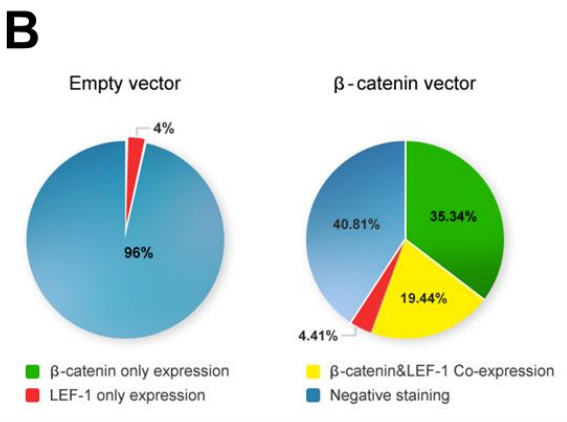
Figure 20. The expression levels of LEF-1 in stromal region of endometrium with or without adenomyosis. (A) H-score of LEF-1 in human endometrial stroma with or without adenomyosis. Stromal LEF-1 expression was not changed between controls, eutopic and adenomyosis lesions at different phases of menstrual cycle.

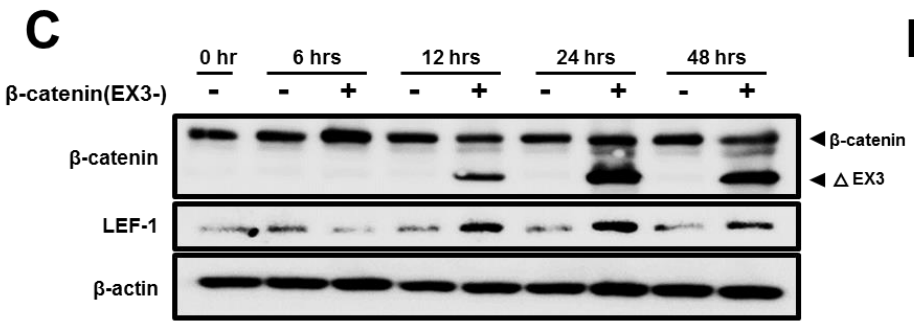
LEF-1 with activated β-catenin leads to induce EMT

To examine whether activated β-catenin induces LEF-1 expression in endometrial epithelial cells, HEC1A (endometrial adenocarcinoma), I performed double immunofluorescence for β-catenin and LEF-1 in HEC1A cells transfected using exon3-deleted *β-catenin* vector. Deletion of exon3 prevents β-catenin phosphorylation by GSK-3β, resultingly β-catenin becomes stabilization, and induce nuclear accumulation of β-catenin (Palacios and Gamallo 1998). Nuclear β-catenin and LEF-1 were co-localization in post-exon3-deleted β-catenin transfected HEC1A cells for 24hrs. (Figure 21 A). Pie chart represented numerical data of LEF-1 and nuclear β-catenin. (Figure 21 B). And also I investigated LEF-1 protein and mRNA levels. HEC1A cells were harvested after exon3-deleted β-catenin transfection for 6hr, 12hr, 24hr and 48hr and then assayed by western blot or qRT-PCR. Expression of LEF-1 was increased in exon3-deleted β-catenin transfected HEC1A cells compared to control (Figure 21 C, D). ZEB1 is widely known as an inducer of EMT (Liu, El-Naggar *et al.* 2008). Human *ZEB1* promoter has been reported to contain consensus-binding site for TCF/LEF factors (Sanchez-Tillo, Fanlo *et al.* 2014). And Loss of E- cadherin is also known to hallmark of EMT (Onder, Gupta *et al.* 2008). Nuclear β-catenin/LEF-1 complexes are well known to suppress *E-cadherin* promoter activity (Jamora, DasGupta *et al.* 2003). So I investigated expression patterns of ZEB1 and E-

cadherin in exon3-deleted β-catenin transfected HEC1A cells. I found that ZEB-1 proteins were co-localized with LEF-1 in exon3-deleted β-catenin transfected HEC1A cells. (Figure 22 A). The percentage of expression is indicated on the pie chart (Figure 22 B). And also I found that E-cadherin levels were lower in HEC1A cells activated β-catenin compared to control. (Figure 22 C, D). And I examined that protein and mRNA Levels of ZEB1 and E-cadherin. Protein, mRNA levels of ZEB1 were highly increased compared to control. And Protein, mRNA levels of E-cadherin were significantly decreased after post-exon3-deleted β-catenin transfection in HEC1A cells compared to control (Figure 22 E, F). These results suggest that activated β-catenin could lead to EMT through induction of LEF-1.


A
LEF-1
β-catenin
DAPI
MERGE
Empty vector
β-catenin vector
a
b
c
d
e
f
g
h
40㎛


B
Empty vector
β-catenin vector
4%
96%
40.81%
35.34%
19.44%
4.41%
β-catenin only expression
LEF-1 only expression
β-catenin&LEF-1 Co-expression
Negative staining


C
0 hr
6 hrs
12 hrs
24 hrs
48 hrs
β-catenin(EX3-)
- - + - + - + - +
β-catenin
LEF-1
β-actin
◀ β-catenin
◀ △EX3

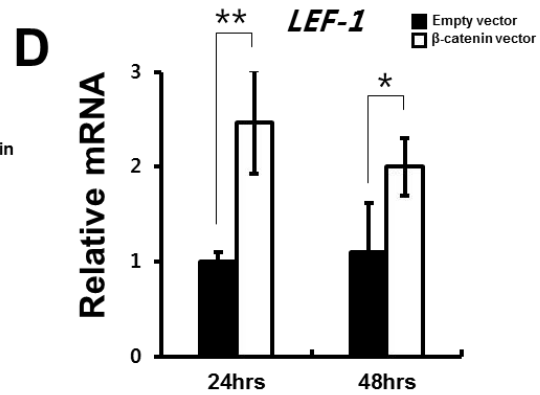

D
LEF-1
Empty vector
β-catenin vector
**
*
Relative mRNA
3
2
1
0
24hrs
48hrs

Figure 21. Activated β-catenin up-regulates expression of LEF-1.

(A) Double Immunofluorescence analysis of nuclear β-catenin and LEF-1 in empty vector and exon3-deleted *β-catenin* (⊿Ex3) vector transfected HEC1A cells. The expression of LEF-1 (a, b; red), β-catenin (c, d; green) were examined. Nuclei were counterstained with DAPI staining (e, f; blue). Images (g, h) were merged. (B) Pie chart represents the percentage of LEF-1 (red), nuclear β-catenin (green) and co-localization of LEF-1 and β-catenin (yellow). Protein levels and mRNA levels of LEF-1 were evaluated by (C) western blot and (D) qPCR in exon3-deleted *β-catenin* vector transfected HEC1A cells. The result represents the mean ± SEM. ** $p<0.01$ and SEM. * $p<0.05$

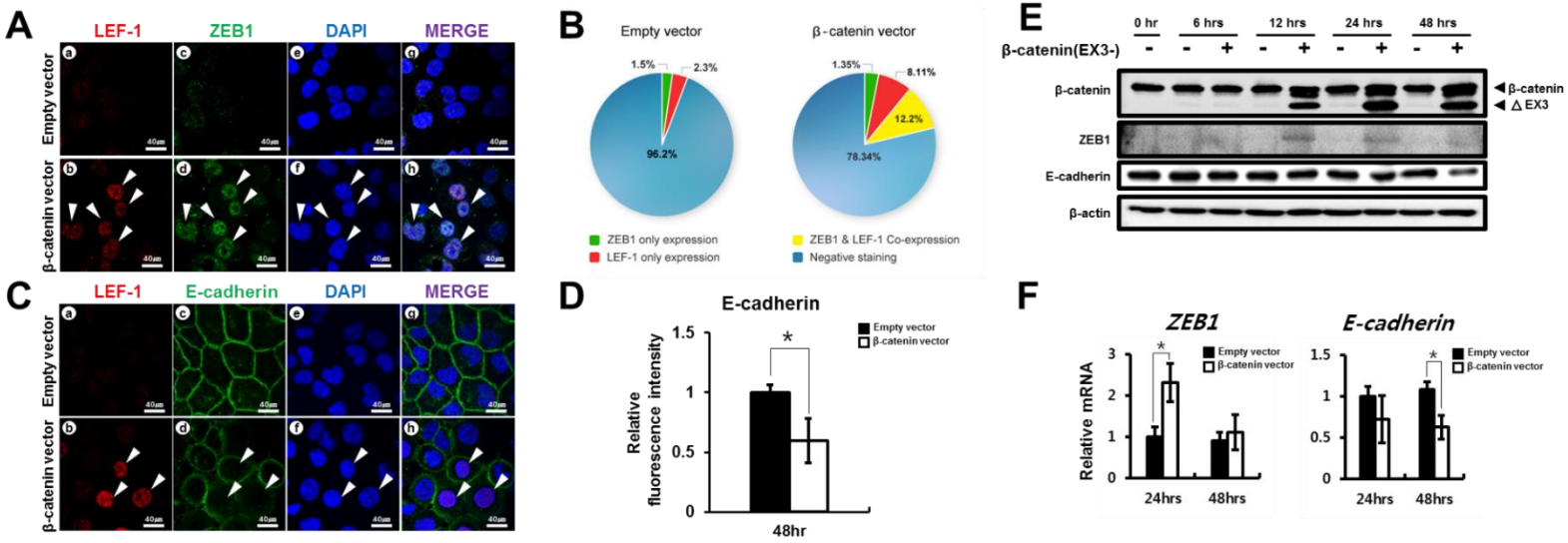

A
LEF-1
ZEB1
DAPI
MERGE
Empty vector
β-catenin vector
40μm
B
Empty vector
β-catenin vector
1.5%
2.3%
96.2%
1.35%
8.11%
12.2%
78.34%
ZEB1 only expression
LEF-1 only expression
ZEB1 & LEF-1 Co-expression
Negative staining
C
LEF-1
E-cadherin
DAPI
MERGE
Empty vector
β-catenin vector
D
E-cadherin
Relative fluorescence intensity
Empty vector
β-catenin vector
48hr
E
0 hr
6 hrs
12 hrs
24 hrs
48 hrs
β-catenin(EX3-)
β-catenin
ZEB1
E-cadherin
β-actin
β-catenin
△EX3
F
ZEB1
E-cadherin
Relative mRNA
Empty vector
β-catenin vector
24hrs
48hrs

Figure 22. Increased LEF-1 by activated β-catenin induces EMT. (A) Double Immunofluorescence analysis of ZEB1 and LEF-1 in empty vector and exon3-deleted *β-catenin* (⊿Ex3) vector transfected HEC1A cells. The expression of LEF-1 (a, b; red), ZEB1 (c, d; green) were examined. Nuclei were counterstained with DAPI staining (e, f; blue). Images (g, h) were merged. (B) Pie chart represents the percentage of LEF-1 (red), ZEB1 (green) and co-localization of LEF-1 and ZEB1 (yellow) (C) Expression pattern of E-cadherin and LEF-1 in empty vector and exon3-deleted *β-catenin* vector transfected HEC1A cells for 48hrs (D) Relative fluorescence intensity of E-cadherin in empty vector and exon3-deleted *β-catenin* vector transfected HEC1A cells for 48hrs. The result represents the mean ± SEM. * $p<0.05$ (E) Level of ZEB1 and E-cadherin protein was measured by Western blot. (F) The mRNA expression level of *ZEB1*, *E-cadherin* in exon3-deleted *β-catenin* vector transfected HEC1A cells for 24, 48 hours. The result represents the mean ± SEM. * $p<0.05$

Overexpression of LEF-1 without activated β-catenin leads to repress E-cadherin

To investigate whether LEF-1 overexpression changes the expression of EMT-related proteins in HEC1A (endometrial adenocarcinoma), HEC1A cells were transfected with *LEF-1* vector and double immunofluorescence and Western blot were performed. As a result, the protein expression of E-cadherin was decreased but protein levels of β-catenin and ZEB1 was not changed (Figure 23). These data confirmed that the E-cadherin protein, the epithelial cell protein, was reduced as a result of LEF-1 alone overexpression, but that the stromal related protein ZEB1 did not increase. These results suggest that LEF-1 protein induced by β-catenin further promotes EMT induction more than only expression.

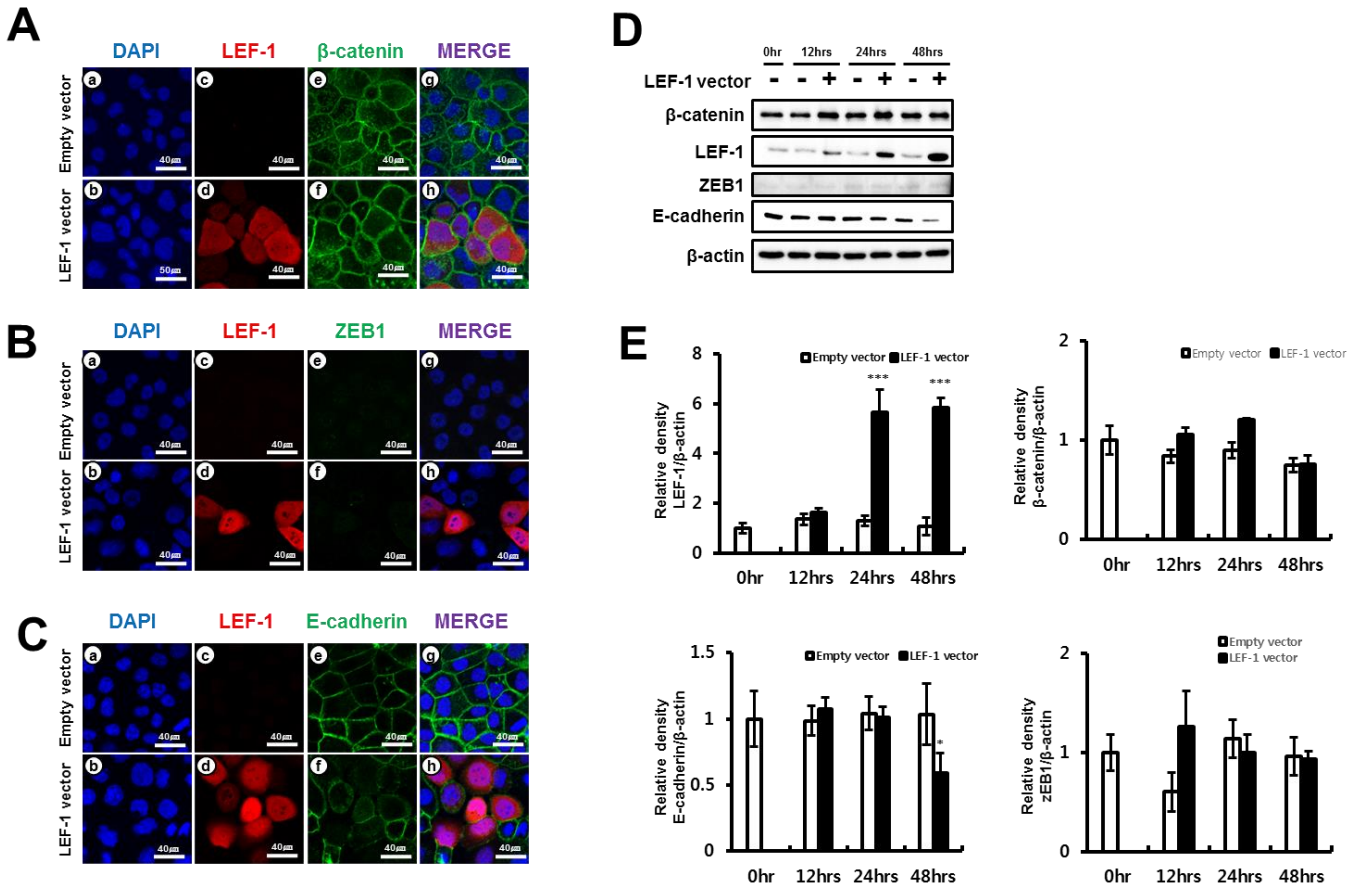

A
DAPI
LEF-1
β-catenin
MERGE
Empty vector
LEF-1 vector
B
DAPI
LEF-1
ZEB1
MERGE
Empty vector
LEF-1 vector
C
DAPI
LEF-1
E-cadherin
MERGE
Empty vector
LEF-1 vector
D
0hr
12hrs
24hrs
48hrs
LEF-1 vector
β-catenin
LEF-1
ZEB1
E-cadherin
β-actin
E
Empty vector
LEF-1 vector
Relative density LEF-1/β-actin
Relative density β-catenin/β-actin
Relative density E-cadherin/β-actin
Relative density zEB1/β-actin
0hr
12hrs
24hrs
48hrs

Figure 23. Increased LEF-1 induces the suppression of E-cadherin expression. (A, B, C) Double Immunofluorescence analysis of β-catenin and LEF-1 in empty vector and *Lef-1* vector transfected HEC1A cells at 48 hours. The expression of LEF-1, β-catenin, ZEB1 and E-cadherin were examined in *LEF-1* vector transfected HEC1A cells for 12, 24 and 48 hours. Nuclei were counterstained with DAPI staining. (D) Level of β-catenin, LEF-1, ZEB1 and E-cadherin protein was measured by Western blot. (E) Image J was used to quantify ZEB-1, β-catenin and E-cadherin protein levels. The result represents the mean ± SEM. * $p<0.05$, *** $p<0.001$

4. Discussion

Adenomyosis is common benign uterine pathology. Adenomyosis is accompanied by dysmenorrhea, chronic pelvic pain, and dyspareunia (Bird, McElin *et al*. 1972; Ferenczy 1998; Levgur, Abadi *et al*. 2000). Also adenomyosis can cause infertility (de Souza, Brosens *et al*. 1995). Adenomyosis has been frequently diagnosed using Computed tomography (CT), ultrasound and magnetic resonance imaging (MRI) (Basak and Saha 2009; Woodfield, Siegelman *et al*. 2009). However, adenomyosis is difficult to diagnose because sign and symptoms are unspecific. Although many women have lived with adenomyosis, its precise etiology remains still unknown.

Wnt/β-catenin signaling pathway has been reported diversity function such as embryonic development, endometrial decidualization, implantation and placenta formation in normal uterus (Sonderegger, Pollheimer *et al*. 2010; Zhang, Patterson *et al*. 2012; Tepekoy, Akkoyunlu *et al*. 2015). Previous study of our team has shown that Wnt/β-catenin signaling has an important role in development of adenomyosis through induction of EMT. Mesenchymal markers such as COUP-TFII, vimentin were observed in epithelial cells in mutant mouse. And also Expression of E-cadherin was down-regulation in epithelial cells in mutant mice

compared to control mice (Oh, Shin *et al*. 2013). However, the cause of adenomyosis by activated β-catenin has been poorly understood.

In this study, I used uteri of stabilization of β-catenin mice and human endometrium with adenomyosis to investigate molecular characterization of adenomyosis and identify pathology.

LEF-1 has been reported transcription factor of β-catenin. β-catenin/LEF-1 complex regulates the expression of Mmp7, Cyclin D1 (Behrens, von Kries *et al*. 1996; Bucan, Mandel *et al*. 2012). β-catenin/LEF-1 also are essential components for promoting survival and proliferation of pro-B cell (Reya, O'Riordan *et al*. 2000). In the uterus, LEF-1 is important play role in endometrial gland formation and development of endometrial carcinomas (Shelton, Fornalik *et al*. 2012). But molecular mechanism of LEF-1 in development of adenomyosis remains unclear. I found, the expression level of *Lef-1* is increased in endometrial cells of mutant mice compared to control by real-time PCR and immunohistochemistry (Figure 15A, B). Further ChIP analysis revealed that β-catenin bound to *Lef-1* promoter in murine uterus (Figure 16A, B). Therefore, these results suggest that *Lef-1* is an important target gene of β-catenin in the developing adenomyosis.

Interestingly, Expression of LEF-1 is higher in endometrial epithelial cells of mutant mice compared to control at 4 weeks, 2 months and 6 months of age. LEF-1 levels are higher in endometrial stromal cells of mutant mice compared to control at 4 weeks of age,

but LEF−1 levels are similar in endometrial stromal cells of mutant or control at 2 months, 6 months of age (Figure 17A, B). This result suggests that dysregulation of LEF−1 in the endometrial epithelial cells may play an important role in developing adenomyosis.

In this study, I identified dysregulation of *Lef−1* by activated β−catenin in mutant compared to control mice. To further investigate the LEF−1 in adenomyosis, I examined levels of LEF−1 in endometrium from women with and without adenomyosis. I observed that LEF−1 levels are higher in the endometrial uterine epithelial cells of women with adenomyosis compared to without adenomyosis in proliferative phase, but not in secretory phase (Figure 19 A, B). Expression of LEF−1 is similar to women with or without adenomyosis in endometrial stromal cells (Figure 20). Development of adenomyosis is related to high concentration of estrogen and ERα levels are higher in epithelial cells at proliferative phase in women with adenomyosis compared to without adenomyosis (Guo, Zhang *et al.* 2004). Also Estrogen−mediated interactions between ERα and β−catenin/LEF−1 has been reported (Ray, Xu *et al.* 2008). As a result, when high estrogen levels, it can cause the expression of LEF−1 in the epithelium of adenomyosis reached its highest expression.

Our previous data showed that a dominant stabilized β−catenin in the murine uterus, which results in the down−regulation of E−cadherin in epithelial cells as well as human endometrium

epithelial cells with adenomyosis (Oh, Shin *et al*. 2013). ZEB1 and E-cadherin are known as key markers for EMT development. One of typical marker for EMT is loss of E-cadherin expression (Baranwal and Alahari 2009). Several studies have shown that β-catenin/LEF-1 complex suppresses *E-cadherin* promoter activity (Huber, Korn *et al*. 1996; Jamora, DasGupta *et al*. 2003). In this study shown that exon3-deleted β-catenin transfected HEC1A cells increased LEF-1 and ZEB1 levels but decreased E-cadherin levels (Figure 21, 22). However, the expression of E-cadherin was reduced by overexpression of the LEF-1 in HEC1A cells and no difference in expression of ZEB1 was observed (Figure 23). These results suggest that β-catenin / LEF-1 complex, not LEF-1 alone, may increase ZEB1, inhibit E-cadherin, and induce the adenomyosis.

In conclusion, I demonstrated that expression of *Lef-1* is regulated by β-catenin directly binds to BRE region of *Lef-1* promoter in the murine uterus. The levels of LEF-1 are higher in murine uterus of aberrant activation of β-catenin compared to control and also human endometrium with adenomyosis compared to without adenomyosis. In addition, I identified that β-catenin activation induce induction of LEF-1, ZEB1 and repression of E-cadherin in endometrial epithelial cells. These results suggest that β-catenin leads to increasing ZEB1 and repression of E-cadherin in adenomyosis development through induction of LEF-1. The

results of these studies will help to understand pathophysiological role β-catenin/LEF-1 signaling in adenomyosis.

CHAPTER 7

THE ROLE OF MULTILAYER GRAPHENE ON HUMAN ADIPOSE-DERIVED STROMAL CELLS

1. Introduction

Stem cell refers to the concept of the ability to differentiate into various types of body tissue cells, that is, undifferentiated cells having stemness (Chagastelles and Nardi 2011). Stem cells include embryonic stem cells, adult stem cells, gamete cells, and cancer stem cells (Eve, Marty *et al.* 2008; Ciurea, Georgescu *et al.* 2014). Mesenchymal stem cells, one of these stem cells, are being actively studied as new cell therapy agents because they are able to differentiate into various cells as well as to inhibit excessive immune responses, to mediate gene therapy, and to produce various cytokine (Wei, Yang *et al.* 2013; Wang, Ting *et al.* 2016; Marofi, Vahedi *et al.* 2017).

Mesenchymal stromal cells (MSCs) are obtained from bone marrow, umbilical cord blood, adipose tissue, etc., and are attached to cultured dish and proliferate, and can be differentiated into various mesodermal cell types including osteoblasts, chondrocytes, adipocytes, muscle cells, and blood vessel cells (Marion and Mao 2006; Orbay, Tobita *et al.* 2012; Li, Bai *et al.* 2014; Ullah, Subbarao *et al.* 2015; Fellows, Matta *et al.* 2016). Because of this ability, it is also called mesenchymal stem cells (Lindner, Kramer *et al.* 2010). Because of this proliferation and differentiation potential of MSCs, tissue engineering studies are interested as a material for regeneration medicine (Lindner, Kramer *et al.* 2010).

Stem cells are an important technology in that they directly regenerate diseases and damaged tissues, but stem cells alone are not easy to reconstruct organs (Stoltz, de Isla *et al*. 2015). In order to overcome these technical limitations, biomaterial technology for transplantation and delivery of stem cells and tissue engineering technology that combines stem cells and biomaterials have been actively studied. Biomaterials are natural or synthetic biocompatible materials that have been used as an adjunct to the treatment of disease, either alone or in combination with other treatments (Shue, Yufeng *et al*. 2012; Banyard, Bourgeois *et al*. 2015; Morelli, Salerno *et al*. 2016).

Graphene is a simple structure of carbon crystals with a two-dimensional hexagonal honeycomb lattice structure and exhibits excellent physico-chemical properties such as high electrical conductivity and thermal conductivity, high mechanical strength and hardness, unique optical properties, and chemical stability (Zhu, Duan *et al*. 2014; Dubey, Bentini *et al*. 2015; Adeel, Bilal *et al*. 2018; Kim, Cho *et al*. 2018; Nasir, Hussein *et al*. 2018). Due to these characteristics, various studies have been carried out recently by combining with the bio-field. Recently, graphene quantum dots have been reported to inhibit aggregation and fibrosis of alpha-synuclein, confirming the possibility of treating Parkinson's disease (Kim, Yoo *et al*. 2018). Studies have also been carried out that graphene stimulates the differentiation of stem cells into osteoblast, neuronal

and myocardial cells (Nayak, Andersen *et al*. 2011; Crowder, Prasai *et al*. 2013; Ahadian, Zhou *et al*. 2016; Bouzid, Sinitskii *et al*. 2016; Ren, Li *et al*. 2017; Lee, Seo *et al*. 2018).

The extracellular matrix (ECM) is important in regeneration studies. ECM which allows cells to connect and function together, has been shown to play an important role as a cell-mediated regulator of cell division, differentiation and death (Lu, Takai *et al*. 2011; Gattazzo, Urciuolo *et al*. 2014). ECM stores and appropriately supplies the biochemical factors necessary for cell growth and differentiation (Yue 2014). For this reason, ECM has recently become more important for cell therapy and regenerative medicine coupled with stem cells.

Cell adhesion is regulated by cellular adhesion molecules located in the cytoplasm, and integrin is one of the most representative cell adhesion protein (Weber, Bjerke *et al*. 2011; Khalili and Ahmad 2015). Integrin is a major receptor involved in the interaction with other cells and ECM, as well as acting as a mediator of cell attachment, as well as the role of biochemical signaling through the cell membrane (Humphries, Travis *et al*. 2004; Streuli 2016). FAK (Focal adhesion kinase) is known to play a major role in cell signaling through integrin in cell migration, adhesion and cell growth (Schlaepfer, Hauck *et al*. 1999; Sieg, Hauck *et al*. 1999; Wozniak, Modzelewska *et al*. 2004; Zhao and Guan 2011). The FAK-mediated cell adhesion pathway contains several downstream molecules

including the extracellular signal–regulated kinase (ERK) of the mitogen–activated protein kinase (MAPK) pathway (Renshaw, Price *et al.* 1999; Yee, Weaver *et al.* 2008). Studies have shown that Runt–related transcription factor 2 (RUNX2), an important protein for osteogenic differentiation, is phosphorylated through the MAPK/ERK signaling pathway (Xiao, Jiang *et al.* 2000; Salasznyk, Klees *et al.* 2004). RUNX2 phosphorylation by MAPK/ERK has been reported to play an important role in the response of osteogenic differentiation (Li, Ge *et al.* 2017).

Here, I found that wrinkles were formed in the multilayer graphene films and osteogenic differentiation of human adipose–derived mesenchymal stromal cells (hASCs) was effectively promoted in the multilayer graphene films. Furthermore, I found that the FAK, ERK, and RUNX2 activation was increased by the wrinkle–shaped physical environment change caused by the multilayer graphene films, thereby increasing the expression of the osteogenic differentiation–related gene in hASCs. These results suggest that the development of a stem cell culture supporter capable of promoting the differentiation of stem cells into osteocyte in vitro can be applied to various stem cell and regenerative medicine application fields.

2. Materials and methods

Preparation of monolayer graphene

Graphene was synthesized by the chemical vapour deposition method on a high-purity copper foil (Alfa Aesar, 99.999%) with flowing 70 mTorr H2 and 650 mTorr CH4 gas. As grown graphene on Cu was spin-coated with poly methyl methacrylate (PMMA) and back-side graphene was etched using oxygen plasma. Remaining copper was etched in 1.8 wt% ammonium persulfate solution. Finally, the monolayer graphene was rinsed with distilled water several times, followed by transferring on a target substrate. Before final use, the PMMA layer on graphene was removed by acetone.

Preparation of multilayer graphene

Monolayer graphene film was synthesized on high-purity Cu foil using chemical vapor deposition (CVD) method. Continuous graphene films coated with a PMMA layer can be isolated from Cu foils and transferred to a target surface after wet chemical etching. The PMMA was removed by using acetone before the Cu etching.

The number of graphene layers was controlled by repeating this transfer process.

Human adipose-derived mesenchymal stromal cells

Human adult adipose tissue were obtained with informed consent using a guideline approved by the Institutional Review Boards of Seoul national university. Human adult adipose tissue was collected from five randomized patients who underwent gynecologic surgery through laparoscopic surgery after obtaining consent to isolate hASCs. In the operating room, adipose tissue from the intestinal reservoir was immediately transferred to PBS containing FBS and the hASCs were isolated.

Immunofluorescence analysis

hASCs were cultured on culture glass and multilayer graphene films in DMEM / F12 medium for 24 hours, washed with PBS, fixed with 4% paraformaldehyde for 20min at room temperature and permeabilized with 0.1% of Triton X-100 (Sigma-Aldrich, St. Louis, MO). After further washing, Cells were exposed to anti-F-actin (1:250; ab130935, Abcam, Cambridge, MA), anti-pRUNX2 (1:250; bs-5685R, Bioss, Woburn, MA), pERK (1:500; #4376, Cell signaling technology, Danvers, MA), pFAK (1:250; ab81298, Abcam,

Cambridge, MA) antibodies overnight at 4° C and secondary antibodies for 2 hour at room temperature. Washed coverslips were then mounted onto microscope slides with a DAPI-impregnated mounting media (Vector Laboratories, Burlingame, CA). Images were captured with a confocal microscope (LSM 700 confocal microscope; Carl Zeiss, Thornwood, NY, USA). Quantification of all were performed using ImageJ software.

Quantitative real-time PCR Analysis

Total RNA was extracted using RNeasy total RNA isolation kit (Qiagen, Valencia, CA) as described above. Expression levels of Runt-related transcription factor 2 (*RUNX2*), Osteocalcin (*OCN*), Osteopontin (*OPN*), Alkaline phosphatases (*ALP*), and Osterix (*OSX*) in cells cultured on culture glass or in monolayer or multilayer graphene films were measured by Real-time PCR was performed using Real-time PCR SYBR Green detection system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions (PE Applied Biosystems, Foster City, CA). Real-time PCR results for hASCs were normalized against the housekeeping genes, *GAPDH*. The sequences of the primers used for *RUNX2* were 5' - gacagccccaacttcctgt-3 and 5' - ccggagctcagcagaataat-3' and for *GAPDH* were 5' - gagtcaacggatttggtcgt-3' and 5' - ttgattttggagggatctcg-3' and for *OCN* 5' -agcaaaggtgcagcctttgt-3' and 5' -agcaaaggtgcagcctttgt-3' and for *OPN* were 5' -

actccattgactcgaacgac-3' and 5'-gcatcagggtactggatgtc-3' and for *ALP* were 5'-gacctcctcggaagacact-3' and 5'-tgaagggcttcttgtctgtg-3'.

Statistical analysis

Statistical analyses of measured data 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 (San Diego, CA). $p<0.05$ was considered statistically significant.

3. Results

Multilayer graphene film formed wrinkles and a height of several nanometers.

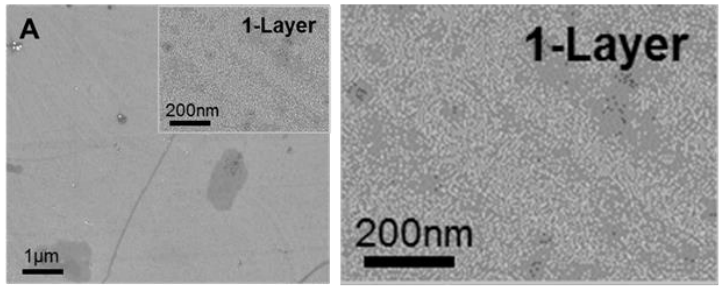
Multilayer graphene films were prepared by laminating layers 1, 3, 5, and 7 using CVD. The resulting graphene film was confirmed by scanning electron microscope (SEM) and Atomic force microscopic (AFM). I confirmed that the planned mono- or multi-layer graphene film was successfully made, and it was confirmed that as the number of graphene films increased, wrinkles of about 30 to 50 nm (Figure 24) and a height of several nanometers were formed on the graphene surface (Figure 25).

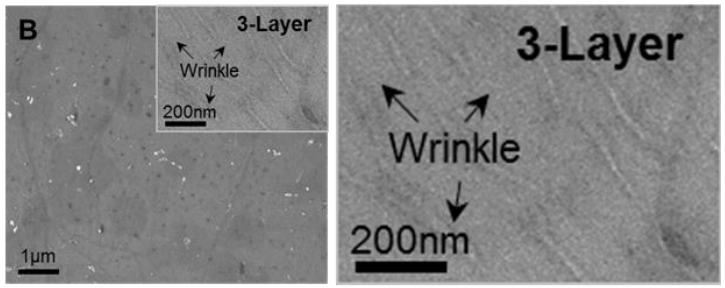
Effect of osteogenic differentiation of hASCs on multilayer graphene films

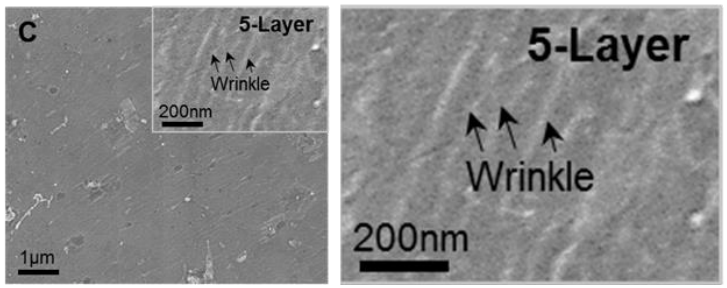
In order to confirm the optimal induction conditions of hASCs in the prepared multilayer graphene films, induction of osteogenic differentiation was carried out for 2 weeks in the graphene films with 1, 3, 5 and 7 layers. The degree of calcium deposits was measured using Alizarin Red S staining to determine the extent of osteogenic differentiation. As shown in Figure 26, osteogenic differentiation of hASCs was increased in a glass substrate coated with a graphene film as compared with a glass substrate (graphene film-uncoated glass

substrate; control group). On the coated substrate, it was confirmed that the differentiation into osteogenic cells was effectively increased. In addition, it was confirmed that the multilayer graphene film of three layers is effective for the osteogenic differentiation of the hASCs most, and when layers were stacked more than five layers, the degree of osteogenic differentiation of hASCs gradually decreased (Figure 26).

In order to analyze precisely the induction of osteogenic differentiation on multilayer graphene films, especially 2 and 3 layer glass substrates, the level of calcium deposits and the expression level of *RUNX2* and *OCN* genes were measured in hASCs induced by osteogenesis for 14 days. As shown in Figure 27, the degree of calcium deposits was the best in the three layer graphene films, the expression of *RUNX2* and *OCN* genes was quantitatively significantly increased in the two-layer and three-layer multilayer graphene films induced by osteogenic differentiation. From the above results, it was confirmed that the osteogenic differentiation of hASCs was effectively promoted by coating of the multilayer graphene films, and that the induction effect of differentiation was improved in 2 to 5 layers of graphene (Figure 27).


A
1-Layer
200nm
1μm
1-Layer
200nm


B
3-Layer
Wrinkle
200nm
1μm
3-Layer
Wrinkle
200nm


C
5-Layer
Wrinkle
200nm
1μm
5-Layer
Wrinkle
200nm

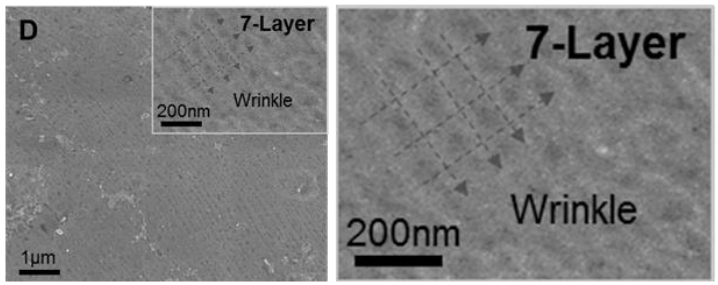
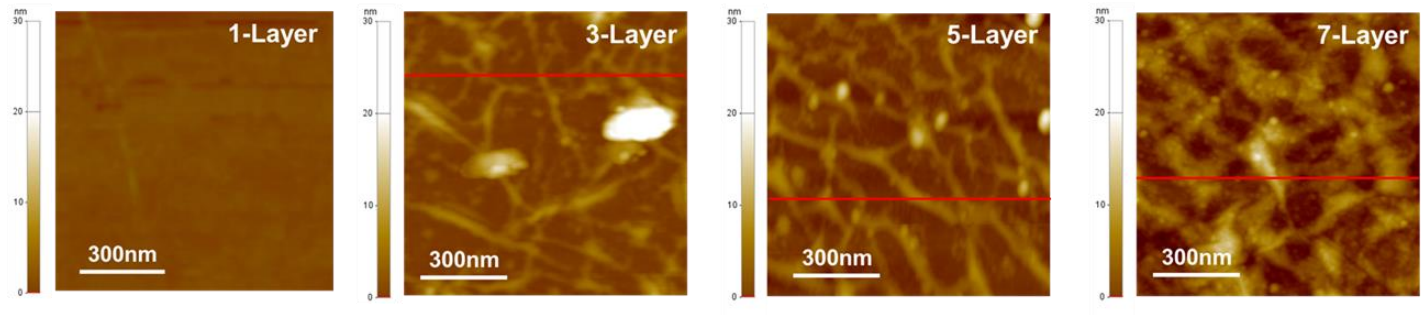
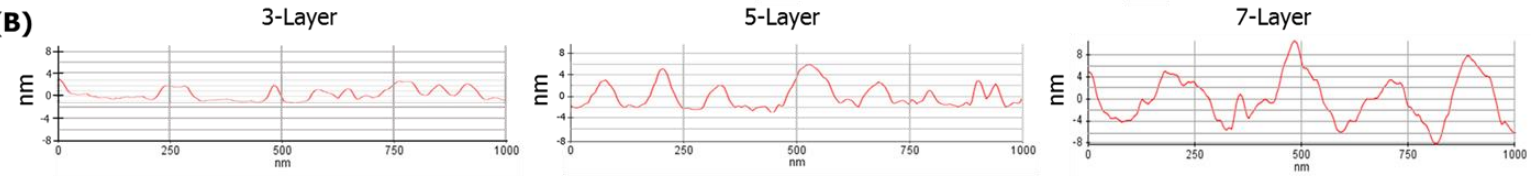

D
7-Layer
Wrinkle
200nm
1μm
7-Layer
Wrinkle
200nm

Figure 24. SEM image of various layer graphene. Using CVD technique, the multilayer graphene films were synthesized on the cell culture slide glass. (A, B, C, D) SEM images of mono layer and multilayer graphene films formed wrinkles as the number of graphene films increases.

(A)



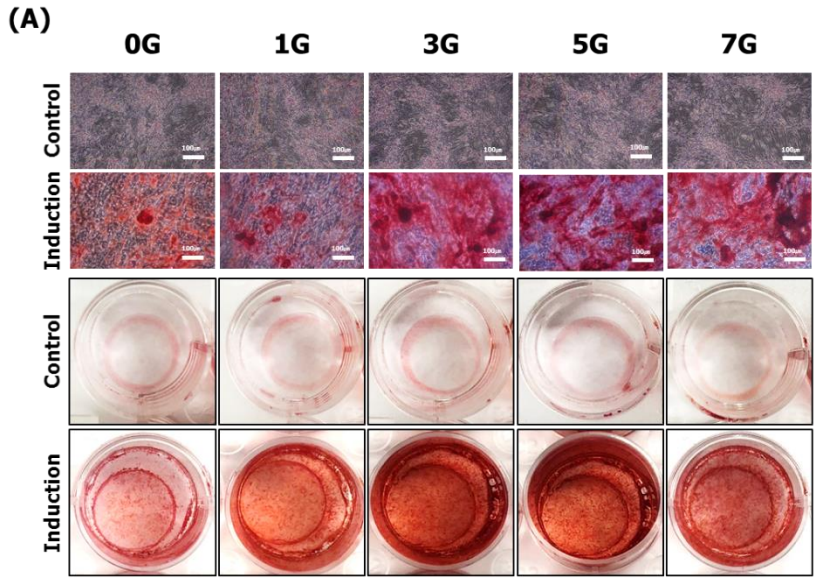
(B)



(C)

Line (RED)	Min(nm)	Max(nm)	Mid(nm)	Mean(nm)	Rpv(nm)	Rq(nm)	Ra(nm)	Rz(nm)	Rsk	Rku
3-Layer	-1.284	3.322	1.019	0.330	4.607	1.117	0.937	3.596	-0.628	2.366
5-Layer	-2.954	5.763	1.405	0.000	8.716	2.130	1.782	6.315	-0.957	3.002
7-Layer	-8.307	10.547	1.120	0.000	18.855	4.119	3.431	12.574	-0.283	2.393

Figure 25. AFM analysis of various layer graphene. Using CVD technique, the multilayer graphene films were synthesized on the cell culture slide glass. (A, B and C) AFM images confirmed the formation of a height of several nanometers in multilayer graphene films. (C) AFM has been used to analyze the surface roughness of various substrate graphene film coatings


(A)
0G
1G
3G
5G
7G
Control
Induction
Control
Induction

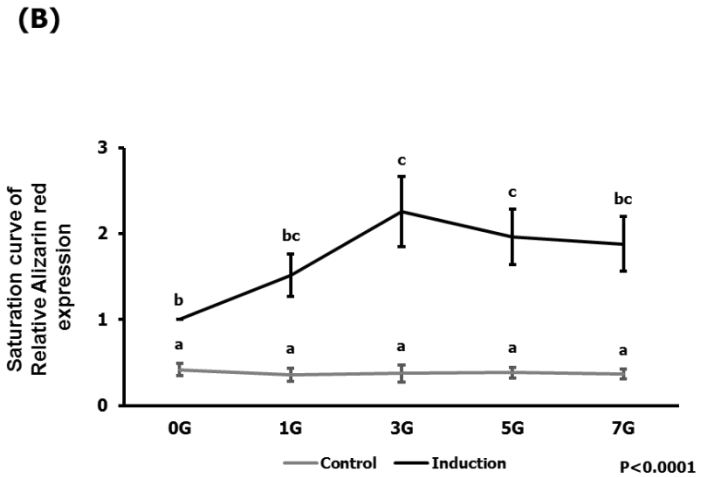

(B)
Saturation curve of Relative Alizarin red expression
3
2
1
0
b
bc
c
c
bc
a
a
a
a
a
0G
1G
3G
5G
7G
Control
Induction
P<0.0001

Figure 26. Determination of optimal hASCs osteogenic induction conditions in multilayer graphene films. Osteogenic differentiation of hASCs on cell culture slide glass, which is composed of layers of none, one, three, five and seven of each multilayer graphene. (A) Total $1x10^5$ hASCs were seeded into each layer graphene films containing an osteogenesis induction medium and were subsequently cultured during 14 days. hASCs are stained with the Alizarin Red S dye. hASCs after Alizarin Red S staining was monitored under an inverted microscope. Bar=100μm (B) Comparing of each extraction data from different layers of graphene film. A significant ($p<0.0001$) model effect was detected. Values in parentheses indicate model effect of treatment and bars with different letters differ significantly.

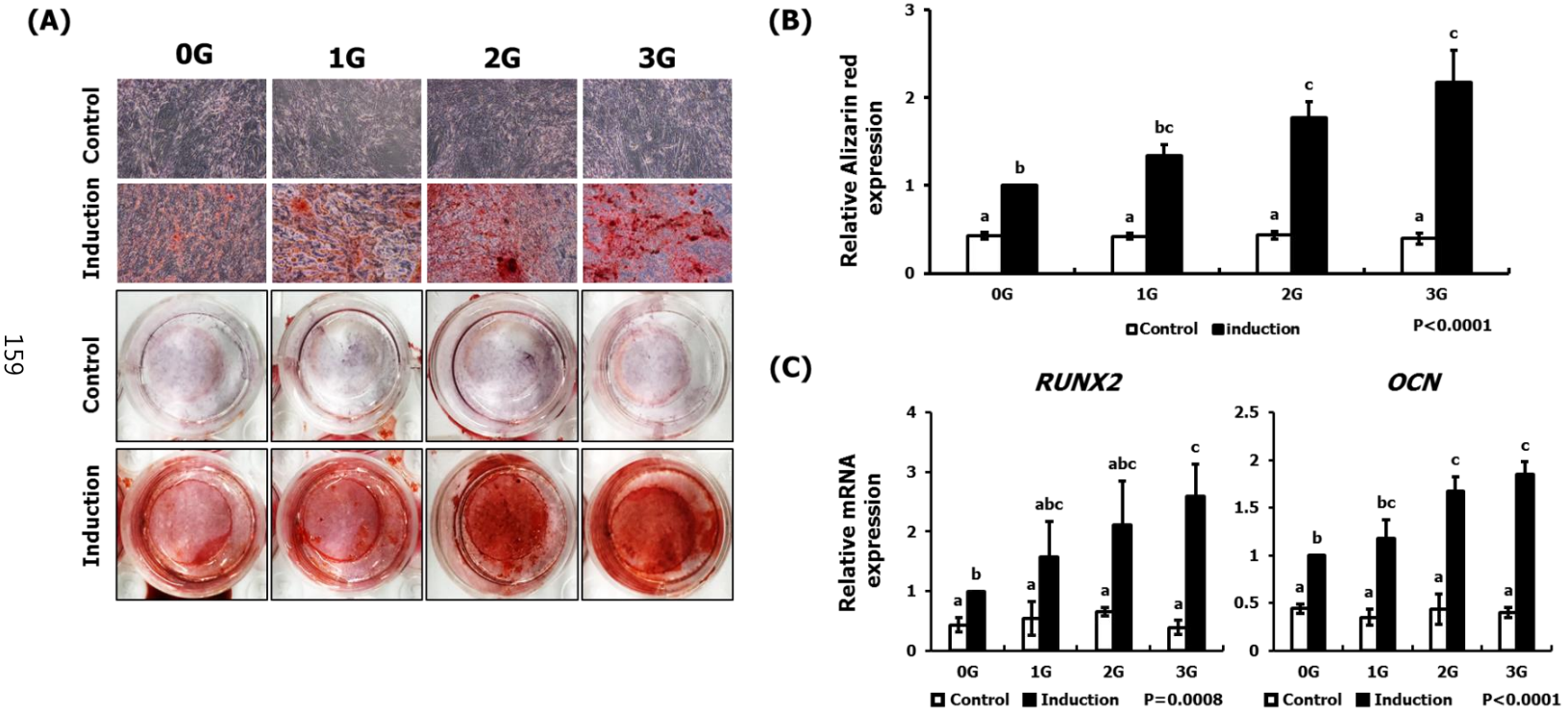

(A)
0G
1G
2G
3G
Control
Induction
Control
Induction
(B)
Relative Alizarin red expression
0G
1G
2G
3G
Control
induction
P<0.0001
(C)
RUNX2
OCN
Relative mRNA expression
Control
Induction
P=0.0008
Control
Induction
P<0.0001

Figure 27. Identification of osteogenic differentiation of hASCs in graphene (especially 1, 2 and 3 layers). (A) Total $1x10^5$ hASCs were cultured in osteogenic induction media during 14 days. hASCs are stained with the Alizarin Red S dye. hASCs after Alizarin Red S staining was monitored under an inverted microscope. Bar=100μm. (B) Comparing of each extraction data from different layers of graphene. The most effective osteogenic differentiation was observed in the three layer. (C) Real-time RT-PCR analysis of osteogenic differentiation-related genes such as *RUNX2* and *OCN* of osteogenic differentiation-induced hASCs in multilayer graphene films. The expression level of osteogenic differentiation-related genes are significantly increase on multilayer graphene group compared to control ($p=0.0008$ for *RUNX2*, $p<0.0001$ for *OCN*). Values in parentheses indicate model effect of treatment and bars with different letters differ significantly.

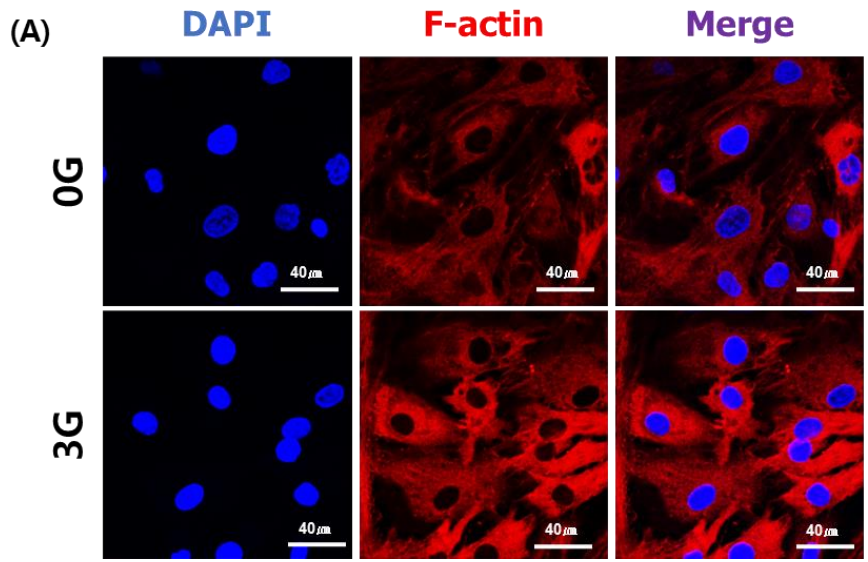
Effect of hASCs adhesion properties on multilayer graphene films

Changes in the physical environment affect the expression pattern of various molecules, and it is known that F-actin is affected by assembly depending on cell tension, cell-ECM adhesion, and cell surface area (Kim, Kim *et al.* 2018). F-Actin not only affects the active tension acting on cell adhesion, but also increased F-actin stress fibers have been shown to improve osteogenic differentiation (Maruthamuthu, Aratyn-Schaus *et al.* 2010; Sen, Xie *et al.* 2015). To investigate the effect of multilayer graphene films on the adhesion of hASCs, hASCs were cultured in multilayer graphene films for 24 hours, and the expression pattern of F-actin was confirmed by immunofluorescence. As a result, it was confirmed that the expression pattern of F-actin was stretched on the multilayer graphene film when compared with the general culture glass (Figure 28).This data suggests that multilayer graphene film affects the assembly of F-Actin in hASCs.

Multilayer graphene films enhanced osteogenic differentiation through FAK-ERK- RUNX2 activation.

Activated FAK protein activates ERK / MAPK signal and induces nuclear localization of pERK. As a result, the nuclear pERK phosphorylates RUNX2 and increases expression of osteogenic differentiation-related genes (Li, Ge *et al.* 2012). To investigate the

effect of hASCs on the FAK / ERK signaling pathway in multilayer graphene films, hASCs were cultured in multilayer graphene films for 24 hours and immunofluorescence staining and real-time RT-PCR were performed. As a result, the expression of phosphorylated FAK and phosphorylated nuclear ERK and RUNX2 was increased, and the expression of *OPN* (p=0.0211) and *ALP* (p=0.007), the target genes of activated RUNX2, was increased. However, there was no significant difference in *OCN* (p=0.2384) and *OSX* (p =0.5294). (Figure 29). These data suggest that wrinkles in multilayer graphene films increase expression of osteogenic differentiation-related genes expression by inducing FAK, ERK and RUNX2 activation of hASCs.


(A)
DAPI
F-actin
Merge
0G
3G
40㎛

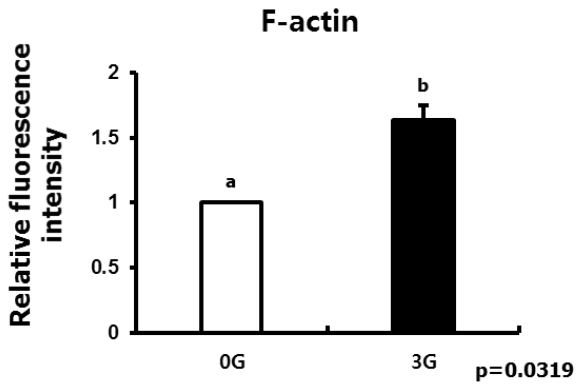

(B)
F-actin
Relative fluorescence intensity
2
1.5
1
0.5
0
a
b
0G
3G
p=0.0319

Figure 28. Effect of multilayer graphene films on adhesion of hASCs. Total $5x10^4$ hASCs were cultured on three layer graphene films or no-layer for 24 hours prior to staining. (A) Adhesion of hASCs was analyzed by immunofluorescence staining for F-actin (Red). (B) Strong intensity and stretched F-actin were observed when compared with the control group. Scale bar indicated 40μm.

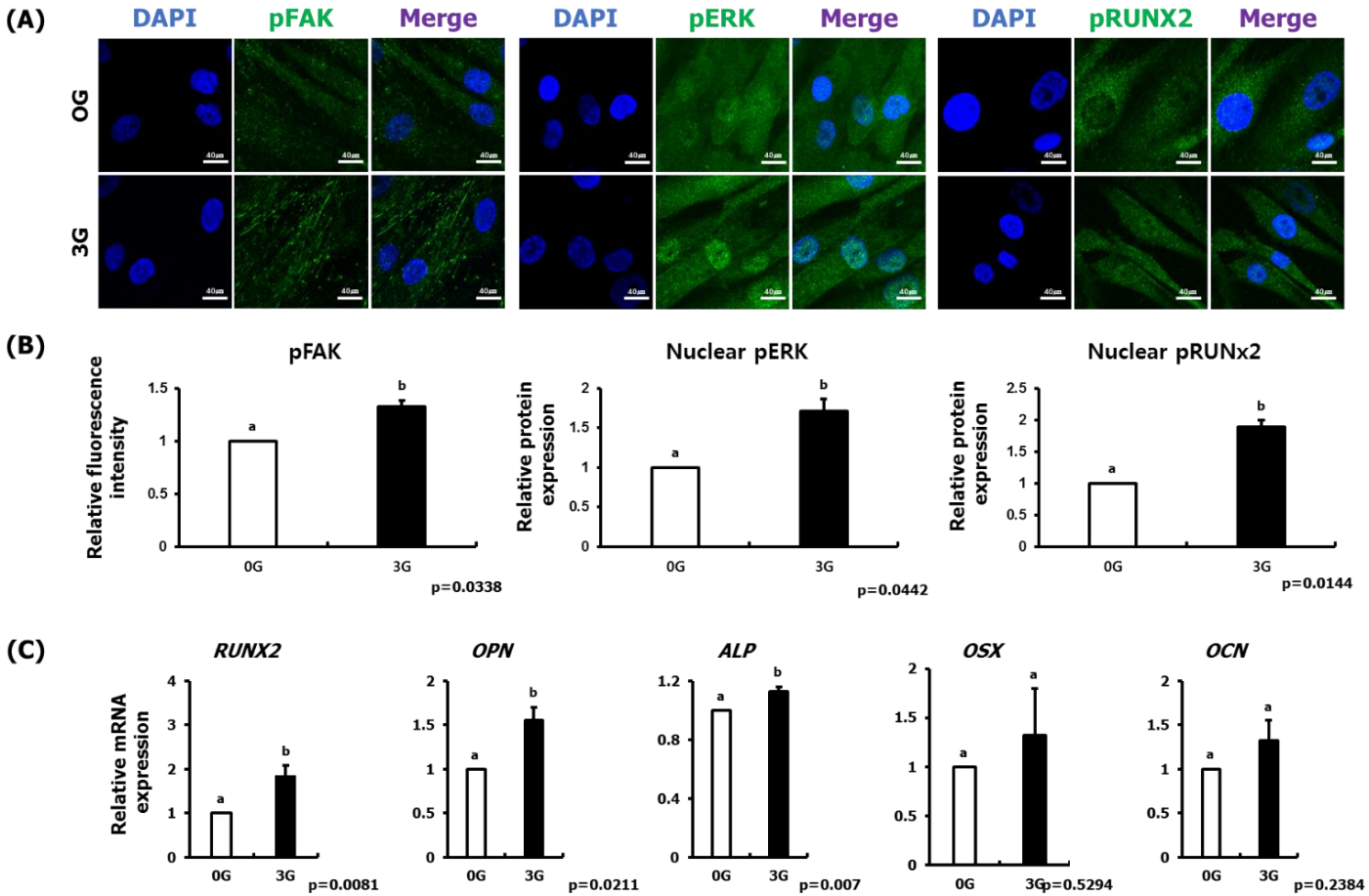

(A)
DAPI
pFAK
Merge
DAPI
pERK
Merge
DAPI
pRUNX2
Merge
OG
3G
40㎛
(B)
pFAK
Relative fluorescence intensity
p=0.0338
Nuclear pERK
Relative protein expression
p=0.0442
Nuclear pRUNx2
Relative protein expression
p=0.0144
0G
3G
(C)
RUNX2
OPN
ALP
OSX
OCN
Relative mRNA expression
p=0.0081
p=0.0211
p=0.007
p=0.5294
p=0.2384

Figure 29. Multilayer graphene promote osteogenic differentiation-related genes in hASCs through FAK-ERK-RUNX2 activation. Total $5x10^4$ hASCs were cultured on three-layer graphene or no-layer for 24 hours prior to staining. (A) The increase of pFAK, pERK and pRUNX2 were detected by immunofluorescence analysis compared with the control group. Scale bar indicated 40μm. Relative fluorescence intensity of pFAK and relative expression of pERK and pRUNX2 expressed in nuclei. (B) Real-time RT-PCR detected upregulated mRNA expression of osteogenic differentiation-related genes such as *RUNX2*, *ALP*, and *OPN* in the cells being cultured for 24 hours on the multilayer graphene group compared with the control ($p=0.0081$ for *RUNX2,* $p=0.007$ for *ALP* and $p=0.0211$ for *OPN*). However, *OCN* and *OSX* increased, but there was no significant difference ($p = 0.2384$ for *OCN* and $p = 0.5294$ for *OSX*). Values in parentheses indicate model effect of treatment and bars with different letters differ significantly.

4. Discussion

There are methods of controlling the differentiation direction and differentiation promoting degree of stem cells by using physical techniques and imparting chemical characteristics to the cell culture substrate. The most commonly used stem cell study in regenerative medicine using graphene is graphene coating on a culture plate and controlling cell differentiation and proliferation. There have been studies in which various stem cells are cultured on graphene and differentiated into various lines of cells while changing the culture method (Nayak, Andersen *et al.* 2011; Crowder, Prasai *et al.* 2013; Lee, Seo *et al.* 2018). The most representative is the osteogenic differentiation study using mesenchymal stem cells

Until recently, in vivo implantable inorganic or protein-based organic scaffolds have been studied to replace and treat tissue or bone damage (Henkel, Woodruff *et al.* 2013; Jafari, Paknejad *et al.* 2017). These scaffolds are physically easy to shape, are biodegradable and biodegradable, but are not effective in promoting osteogenic differentiation because of low bone conduction (Ciapetti, Granchi *et al.* 2012). For this reason, biological growth factors such as BMP-2, FGF-2 are further used to promote osteogenic differentiation (Lee, Kim *et al.* 2015; Marupanthorn, Tantrawatpan *et al.* 2017), but the use of biological growth factors have limitations such as immune response problems, contamination, and cost

(Berthold and Walter 1994; Gifre, Aris *et al*. 2017). The development of a culture support capable of promoting the differentiation of stem cells into a desired cell lineage can not only be safely applied to various stem cell applications, but also can provide a high therapeutic effect on tissue or bone damage.

In the present study, our team fabricated a multilayer graphene film using CVD technique and evaluated the degree of promoting the osteogenic differentiation of hASCs in this three dimensional multilayer graphene film. Several previous studies have confirmed wrinkles in the monolayer graphene film. So, our team investigated the shape of multilayer graphene film. I have found that the formation of graphenes containing 1, 3, 5 and 7 layers results in the formation and increase of 30 to 50 nm wrinkles and the formation of a height of several nanometers as the number of layers increases (Figure 24 and 25).

Stem cells are known to be affected by the proliferation and differentiation even with small changes in surface topography (Abagnale, Steger *et al*. 2015). Therefore, I examined the effect of multilayer graphene films on the osteogenic differentiation of hASCs and found that osteogenic differentiation of hASCs was better in multilayer graphene films than in general culture dishes and monolayer graphene films. In particular, it was confirmed that osteogenic differentiation of hASCs was effective in the three-layer graphene film (Figure 26 and 27).

In general, stem cell behavior, such as adherence, proliferation and specific lineage differentiation, is known to be influenced by stem cell–matrix interactions, ECM (Gattazzo, Urciuolo *et al*. 2014; Ahmed and Ffrench–Constant 2016). The branched expression of F–actin protein is known to be influenced by cell tension, cell–matrix interactions, and substrate topography (Bachir, Horwitz *et al*. 2017) (Hanein and Horwitz 2012). For this reason, the F–actin expression pattern was confirmed, and branched networks of F–actin were observed on the surface of multilayer graphene films changed by wrinkles and height (Figure 28).

It is known that stable cell attachment by physical and chemical environment changes promote osteogenic differentiation of stem cells (Hwang, Varghese *et al*. 2008). The FAK / ERK signaling pathway associated with cell adhesion is a signaling pathway known to activate RUNX2 and induce osteogenic differentiation (Salasznyk, Klees *et al*. 2007; Salasznyk, Klees *et al*. 2007). RUNX2 protein is known to play an important role in bone formation (Javed, Chen *et al*. 2010; Xu, Li *et al*. 2015). RUNX2, which is activated by phosphorylation in mouse and human cells, is known to directly regulate genes such as *OCN*, *OPN*, *ALP*, and *OSX* (Huang, Yang *et al*. 2007; Jensen, Gopalakrishnan *et al*. 2010). Thus, activation of the FAK / ERK protein in multilayer graphene films was investigated and the expression of the osteogenic differentiation–related genes activated by the phosphorylated RUNX2 was compared with the control group. As a result, I confirmed that FAK, ERK and RUNX2

activity was increased by immunofluorescence analysis, and that expression of *RUNX2*, *ALP*, and *OPN*, which are related to osteogenic differentiation-related genes, was significantly increased ($p=0.0081$ for *RUNX2*, $p=0.007$ for *ALP* and $p=0.0211$ for *OPN*). However, *OCN* and *OSX* were increased but not significant ($p=0.2384$ for *OCN*, $p=0.5294$ for *OSX*). These data suggest that activation of the FAK/ERK protein in hASCs cultured on multilayer graphene films for 24 hours phosphorylates RUNX2 and upregulates the osteogenic differentiation-related genes to promote osteogenic differentiation.

In conclusion, this study has developed a differentiation promoting substance that can promote osteogenic differentiation of hASCs in vitro and provides an understanding of physiological changes of hASCs caused by environmental changes through multilayer graphene films. It can be safely applied to a variety of stem cell applications, and it is expected to have a high therapeutic effect in tissue or bone damage.

In order to using the stem cells on regenerative medicine, the basic behavior control of stem cells such as cell proliferation, survival, adhesion and differentiation will be a priority In addition, physiochemical changes in the use of biomaterials to change the physiological signal of the stem cells to know that the treatment method can go a step further to improve.

CHAPTER 8

GENERAL DISCUSSION

AND CONCLUSION

In this study, I aimed to understand the mechanisms of development of endometrial cancer and adenomyosis, and to develop new biomarkers and therapeutic approaches, and to treat bone disease that may occur after treatment of gynecological diseases such as radiation therapy and hormone therapy

In chapter 4, the main purpose of first study was to understand the role of epithelial MIG-6 in the uterus. MIG-6 is known to act as a tumor suppressor through anti-proliferative action in humans. The results demonstrate that loss of endometrial epithelial cell *Mig-6* has been shown to induce endometrial hyperplasia through proliferation of endometrial epithelial cells. I also confirmed that MIG-6 protein blocks phosphorylation of STAT3 protein through binding with STAT3. In addition, P4 treatment inhibited endometrial epithelial cell proliferation and prevented the development of endometrial hyperplasia, and STAT3 activity was decreased by MIG-6 of P4-induced stromal cells. These results provide a pathological understanding of endometrial cancer development and a new animal model for the treatment and prevention of endometrial cancer.

In chapter 5, the purpose of the study was to understand the pathological function of adenomyosis developed by β-catenin. Normal Wnt/β-catenin signaling has been shown to be important for uterine development and function. However, our team found that hyperactive β-catenin was responsible for the development of

uterine adenomyosis through EMT induction. Thus, I have confirmed that *Tgf-β2* is a β-catenin target gene directly in the uterus by DNA microarray and ChIP-seq analysis. TGF-β2 levels were found to be higher in the endometrial epithelial cells of β-catenin activated mice than in the control group. In addition, TGF-β2 expression was higher in women with adenomyosis than women without adenomyosis in the endometrium and adenomyosis. It was also confirmed that TGF-β inhibitor inhibited EMT-related proteins. These results suggest a new understanding of the pathophysiology of β-catenin in the development of adenomyosis and the potential for TGF-β inhibitors as medicine for the treatment of adenomyosis.

In chapter 6, I have further studied the pathology and molecular characteristics of adenomyosis during the development of adenomyosis using a mouse model of adenomyosis and a patient tissue with adenomyosis. As a result, the expression of *Lef-1* has been shown to be regulated by the binding of β-catenin to the BRE region of the *Lef-1* promoter in the mouse uterus. The expression of LEF-1 was found to be abnormal in the mouse model of adenomyosis, compared with the control group, and it was confirmed that the expression of LEF-1 was also increased phase in women with adenomyosis compared to without adenomyosis. It was also confirmed that β-catenin activation induces LEF-1, ZEB1 and inhibits E-cadherin expression in endometrial adenocarcinoma. These results suggest that β-catenin induces the increase of ZEB1

and inhibition of E-cadherin in the development of adenomyosis through the induction of LEF-1. As a result of this study, I could understand the pathophysiological role of β-catenin/LEF-1 signaling in adenomyosis.

In chapter 7, regenerative medicine study using graphene and human adipose derived mesenchymal stromal cells were performed to treat bone diseases that occurred in patients with radiation therapy, hormone therapy, and endometrial cancer. It was confirmed that hASCs cultured in the multilayer graphene films prepared by the chemical vapor deposition method promoted osteogenic differentiation more than the general culture dish. I confirmed that wrinkles were formed in the multilayer graphene films and the FAK, ERK and RUNX2 were activated due to the physical environment change, thereby promoting osteogenic differentiation of hASCs. As a result of this study, it may help that the development of a stem cell culture dish that can effectively differentiate stem cells into various cells in vitro can be helpful in various stem cell and regenerative medicine fields. Also, it can be considered that the autologous mesenchymal stromal cells cultured in an optimal state for osteogenic differentiation in vitro can be used to treat bone disease.

In conclusion, these studies will greatly contribute to our understanding of molecular understanding of endometrial diseases and development of graphene-based biotechnology (Figure 30). It will also help in the field of regenerative medicine, which can be used

to treat bone disease and other diseases that can occur in gynecological diseases.

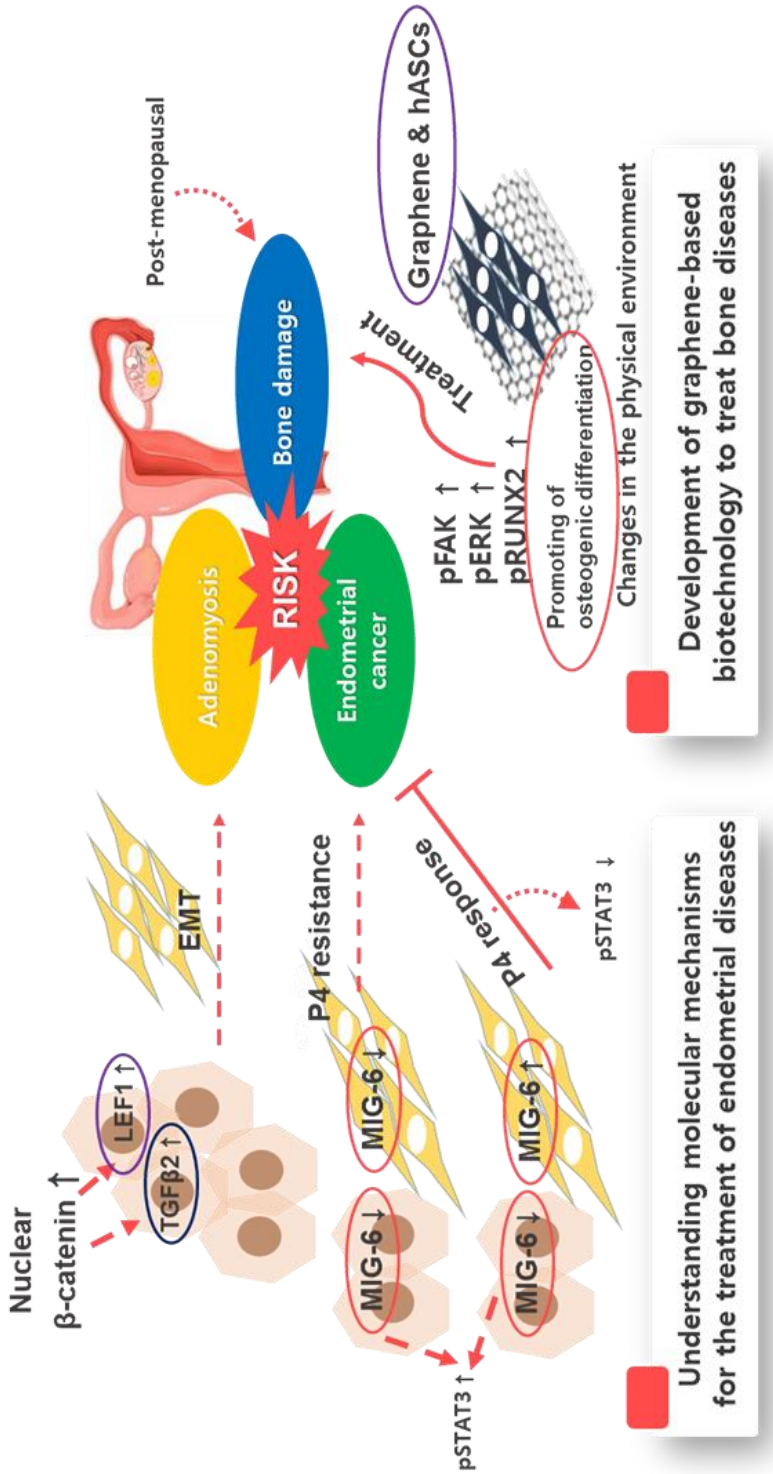


Figure 30. The scheme of molecular understanding of endometrial diseases and development of graphene-based biotechnology.

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SUMMARY IN KOREAN

본 논문에서는 자궁내막암과 자궁선근증 발달 분자메커니즘의 이해 및 새로운 바이오 마커, 치료 접근법의 개발에 대한 연구와 폐경 후 및 부인과 질환을 치료하기 위한 방사선 치료 후 발생할 수 있는 골 관련 질환을 치료하기 위해 인간 지방유래 기질세포와 생체소재 그래핀을 활용한 재생 의학 연구를 진행하였다

Chapter 4에서는, 자궁내막암의 암 치료법을 개선하기 위해 프로게스테론 내성의 기전에 대한 연구를 진행하였다. 자궁내막암은 부인과 질환에서 가장 흔한 악성 종양으로, 자궁내막암 치료를 위해 프로게스테론이 많이 사용되고 있다. 하지만, 프로게스테론 내성을 가지는 환자도 있기 때문에 이러한 내성의 기전을 밝히는 것이 중요하다. Mitogen-inducible gene 6 (*Mig-6*)는 자궁 내 progesterone receptor (PGR) 작용의 중요한 중개자로 알려져 있는데, 프로게스테론 내성에서 *Mig-6*의 기능을 연구하기 위해 Sprr2f-cre 마우스 ($Sprr2f^{cre/+}Mig\text{-}6^{f/f}$)를 사용하여 자궁 상피 세포에서 *Mig-6*를 특이 적으로 제거한 마우스 모델을 만들었다. 아 마우스 모델은 STAT3의 비정상적인 인산화와 자궁 내막 상피 세포의 증식으로 인해 자궁 내막 증식을 일으키는 것을 확인하였고 면역침전 및 세포 배양 실험을 통해 MIG-6가 단백질 상호 작용을 통해 STAT3 단백질의 인산화를 억제한다는 것을 확인하였다. 이전 연구에서 Pgr 양성 세포 에서 *Mig-6*를 특이적으로 제거한 마우스모델 ($Pgr^{cre/+}Mig\text{-}6^{f/f}$)에서는 프로게스테론 내성을 보였지만, $Sprr2f^{cre/+}Mig\text{-}6^{f/f}$ 마우스는 프로게스테론에 반응을 보였다. 프로게스테론 치료를 통해 돌연변이 마우스의 자궁에서 STAT3

인산화와 상피 세포 증식을 유의하게 감소시키는 것을 확인하였다. 위 결과를 통해 MIG-6가 자궁 상피 세포에서 STAT3 인산화의 저해를 통해 종양 억제 인자로서의 중요한 기능을 가지고 있음을 보여 주었고 프로게스테론의 항 종양 효과는 자궁내막기질세포의 MIG-6에 의해 조절된다는 것을 확인하였다. 이 연구를 통해 자궁 내 스테로이드 호르몬에 의한 새로운 신호 전달 경로를 발견하고 자궁 내막 암과 같은 부인과 질환에서의 프로게스테론 내성을 극복하는데 많은 도움을 줄 것으로 생각된다.

Chapter 5와 6에서는, 자궁선근증의 발달과정 중 β-catenin의 병리학 적 기능을 이해하기 위해 연구를 진행하였다. 이전 연구를 통해 자궁에서의 β-catenin의 비정상적인 활성화가 상피간엽이행을 통해 자궁선근증 발달을 유도한다는 것을 확인하였다. β-catenin의 비정상적인 활성화에 의해 조절되는 분자 경로를 확인하기 위해 자궁에서 β-catenin의 비정상적인 활성화가 발생하는 자궁선근증 마우스모델 자궁에서 DNA microarray와 ChIP-seq 분석을 수행하였다. Microarray 및 ChIP-seq 분석을 통해 자궁선근증 마우스모델 ($Pgr^{cre/+}Ctnnb1^{f(ex3)/+}$)에서 TGF-β 및 β-catenin 신호가 활성화 되는 것을 확인했다. 또한 ChIP-seq 분석 결과 *Tgf-β2*와 *Lef-1*이 자궁 내 전사인자 β-catenin의 표적 유전자로 확인되었다. 면역 조직 화학 분석을 통해 자궁선근증을 가진 환자뿐만 아니라 자궁선근증 마우스모델 상피세포에서 TGF-β2와 LEF-1의 비정상적인 과 발현이 되는 것을 확인하였다. 또한, β-catenin의 활성화가 유도된 자궁내막선암세포에서 TGF-β2와 LEF-1의 발현이 증가하는 것을 확인하였고, E-cadherin 단백질의 감소와 ZEB1 및 Vimentin 단백질이 증가하는 것을 확인하였다. 흥미롭게도, 세포 침윤 분석은 핵에서의 β-catenin 발현이 유도된 자궁내막선

암세포에서 대조군에 비해 침윤성을 유의하게 증가 시킨다는 것을 보여주었다. 또한 TGF-β 억제제인 pirfenidone이 핵에서 β-catenin 발현이 유도된 자궁내막선암세포의 E-cadherin 발현을 증가시고 세포 침윤성을 감소시키는 것을 확인하였다. 이 연구를 통해 β-catenin의 직접적인 타켓 유전자인 *Tgf-β2* 와 *Lef-1*이 자궁선근증 발달에 중요한 역할을 하는 것으로 확인하였다.

Chapter 7에서는 방사선 치료, 호르몬 치료와 같은 부인과 질환 치료 후에 발생할 수 있는 골 관련 질환을 치료기 위해 생체소재인 그래핀과 인간지방유래기질세포를 이용한 골 재생에 관한 연구를 진행하였다. 여러 층으로 구성된 다층 그래핀 필름을 제작 후 주사전자현미경 및 원자현미경을 이용하여 확인한 결과 30 내지 50nm의 주름 및 수 나노미터의 높이가 형성되는 것을 확인하였다. 인간 지방유래 기질세포를 이용하여 다층 그래핀 필름에서 골분화 유도한 후 확인한 결과 다층 그래핀 필름에서 일반배양용기와 비교하였을 때 골분화가 더 빨리 촉진되는 것을 확인하였다. 인간 지방유래 기질세포를 다층 그래핀 필름에서 하루 동안 배양한 후 세포부착 관련 단백질을 확인 한 결과 길게 뻗은 모양의 F-actin 단백질 발현 양상과 pFAK, pERK, pRUNX2 단백질이 증가하는 것을 확인하였다. 실시간 중합효소연쇄반응 분석을 통해 24시간 동안 다층그래핀에서 배양 된 세포에서 pRUNX2 표적 유전자인 *ALP* 및 *OSN*의 발현이 증가하는 것을 보였다. 이러한 결과는 다층 그래핀 필름으로 인한 물리적 환경 변화가 인간 지방유래 기질세포의 부착을 촉진하면서 FAK 및 ERK 단백질의 활성화로 인해 인산화 된 RUNX2가 전사인자로서 활성화 되어 골 관련 유전자의 발현을 증가시킴으로써 골 형성 분화를 촉진 한다는 결과를 얻었다.