ONCOGENIC ROLE OF CANCER-ASSOCIATED FIBROBLAST SECRETION IN ENDOMETRIAL CANCER PROGRESSION *IN VITRO* AND *IN VIVO*

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ABSTRACT

Cancer-associated fibroblasts (CAFs) have demonstrated tumor-promoting roles in various cancers, yet their implication in endometrial cancer (EC) has not been fully explored. CAFs and its epithelial counterpart were isolated from human EC tissues using CD90- and CD326-antibodies conjugated magnetic beads, respectively. Isolated cell populations were characterized using morphology assessment, flow cytometry, and real-time PCR. CAFs secretion increased EC cell proliferation (195.3 ± 12.9%) and enhanced motility (134.9 ± 6.5%) while benign endometrial fibroblasts inhibited proliferation (62.4 ± 21.2%) and suppressed motility (15.2 ± 8.2%). Antibody array and ELISA showed higher GRO-α, IL-6, MCP-1, VEGF and RANTES cytokines levels in CAFs, than in benign fibroblast secretion. Of note, interleukin-6 (IL-6) was secreted 4.17-folds greater in CAFs; neutralizing its effect reduced EC proliferation (36.1 ± 4.2%) while treatment of EC cells with IL-6 recombinant protein alone increased cell proliferation. Interestingly, EC cells but not CAFs expressed IL-6 receptors (IL-6R and gp-130). Upon treatment with CAFs secretion, IL-6 receptor signaling was activated as evidenced with increased phosphorylation of Erk, Akt, JAK-3 and STAT-3 proteins. Suppression of these pathways using chemical inhibitors led to reduced CAFs-mediated EC cell proliferation. Further, STAT-3 target genes (TIMP-1, PIM-1, c-Myc, SOCS-3, NFκB1 and NFκB2) were upregulated. c-Myc mRNA level was elevated 138-folds higher in CAFs-treated EC cells and c-Myc knockdown with shRNA reduced EC cell proliferation (52.7 ± 1.8%). Surprisingly, treatment with CAFs secretion did not significantly increase the cell proliferation (64.1 ± 4.7%). To observe whether CAFs modulates EC growth in vivo, epithelial and fibroblast cells were inoculated subcutaneously into nude mice. Fibroblast cells alone and co-injections of benign fibroblast with EC cells showed no induction of tumor growth. However, co-injection of CAFs with EC cells showed increased growth (1166.2 ± 59.3 mm3) compared to EC cells alone (497.6 ± 36.7 mm3). EC cells with

reduced c-Myc expression grew into smaller tumors (293.9 ± 7.02 mm3) compared to those with high c-Myc level (748.4 ± 8.2 mm3), despite exposure to CAFs (361.9 ± 38.7 mm3). These suggest that CAFs exerts tumor-promoting effects in EC via c-Myc expression both in vitro and in vivo. Subsequent immunohistochemical analysis on human tissue specimens showed high expression of IL-6 receptors, phosphorylated STAT-3 and c-Myc protein in EC (n=9) but not in benign endometrial tissues (n=8). We further tested IL-6 receptors specific inhibitors (raloxifene and bazedoxifene acetate) and PI3K inhibitor (rapamycin) on CAFs-treated EC cells, and observed inhibition of proliferation (44.3 ± 2.6%, 46.2 ± 2.9% and 30.7 ± 12.1%, respectively), with evidence of apoptosis-mediated cell death. Our data shows that CAFs exert a growth-promoting role in EC tumor microenvironment, partly through activation of IL-6 receptors in EC cells to induce expression of c-Myc. Our study provides proof-of-principle evidence that CAFs-mediated IL-6 signaling has a direct role in EC tumorigenesis, which can be employed for novel EC therapeutic utility.

ABSTRAK

Fibroblas-berkaitan kanser (FBK) memainkan peranan dalam peningkatan pertumbuhan pelbagai kanser, namun implikasinya pada kanser uterus (KU) belum dikaji secara meyeluruh. FBK dan sel epiteliumnya diasingkan daripada tisu KU manusia menggunakan antibodi CD90 dan CD326 yang dikonjugasikan dengan manik magnet. Ciri-ciri sel fibroblas dan epitelium disahkan orijin asalnya dengan menggunakan kaedah morfologi, sitometri aliran dan reaksi polimerasi rantai. Rawatan rembesan FBK meningkatkan proliferasi (195.3 ± 12.9%) dan pergerakan sel KU (134.9 ± 6.5%) manakala rembesan dari fibroblas uterus bukan kanser membantutkan pertumbuhan (62.4 ± 21.2%) dan mengurangkan pergerakan sel KU (15.2 ± 8.2%). Profil cytokine dan ELISA menunjukkan FBK merembes dengan tinggi tahap GRO-α, IL-6, MCP-1, VEGF dan RANTES berbanding fibroblas bukan kanser. FBK merembes 4.17-kali lebih tinggi tahap interleukin-6 dan apabila dineutralkan, mengurangkan pertumbuhan KU (36.1 ± 4.2%). Peningkatan proliferasi sel KU setelah dirawat dengan protin rekombinan IL-6 melambangkan peranan IL-6 dalam pertumbuhan KU. Menariknya, sel KU, dan bukan sel FBK mempunyai reseptor IL-6 (IL-6R dan gp-130). Apabila dirawat dengan rembesan FBK, laluan-laluan reseptor IL-6 diaktifkan melalui fosfolirasi protin Erk, Akt, JAK-3 dan STAT-3. Penyekatan laluan-laluan tersebut dengan inhibitor yang spesifik menunjukkan pengurangan dalam pertumbuhan sel KU. Tambahan pula, ekspresi gen target STAT-3 (TIMP-1, PIM-1, c-Myc, SOCS-3, NFκB1 and NFκB2) ditingkatkan. Tahap mRNA c-Myc ditingkatkan sebanyak 138-kali dalam sel KU yang dirawat dengan rembesan FBK dan perencatan gen c-Myc dalam sel KU menurunkan kadar proliferasi (52.7 ± 1.8%) serta tidak tumbuh walaupun dirawat dengan rembesan FBK (64.1 ± 4.7%). Untuk menentukan sekiranya FBK mendorong pertumbuhan EC secara ‘in vivo’, sel epithelia dan fibroblas diinokulasi secara subkutan ke dalam tikus ‘nude’. Inokulasi cell fibroblas sahaja secara berasingan serta gabungan sel KU dan fibroblas bukan kanser

tidak menggalakkan sebarang pertumbuhan tumor di dalam tikus ‘nude’. Tetapi, inokulasi sel KU bersama dengan FBK menunjukkan kadar pertumbuhan yang tinggi (1166.2 ± 59.3 mm3) berbanding sel KU sahaja (497.6 ± 36.7 mm3). Sel KU yang menunjukkan ekspresi c-Myc yang rendah menghasilkan tumor yang lebih kecil (293.9 ± 7.02 mm3) berbanding dengan sel KU yang memperolehi ekspresi c-Myc yang tinggi (748.4 ± 8.2 mm3), malah, sel KU tidak mengalami proliferasi walaupun didedahkan kepada sel FBK (361.9 ± 38.7 mm3). Ini menunjukkan bahawa FBK dan bukan fibroblas bukan kanser mempunyai keupayaan untuk mendorong pertumbuhan KU in vitro dan in vivo melalui ekspresi gen c-Myc. Analisi imunohistokimia menunjukkan ekpresi tinggi reseptor IL-6, fosforilasi-STAT-3 dan protin c-Myc dalam tisu KU manusia (n=9) berbanding bukan kanser (n=8). Maka, inhibitor spesifik reseptor IL-6 (raloxifene dan bazedoxifene acetate) dan inhibitor PI3K (rapamycin), menunjukkan penurunan proliferasi sel KU yang dirawat rembesan FBK (44.3 ± 2.6%, 46.2 ± 2.9% dan 30.7 ± 12.1%, masing-masing), dengan bukti berlakunya kematian cell secara apoptosis. Data projek ini menunjukkan bahawa FBK mempunyai keupayaan untuk membantu pertumbuhan KU dalam persekitaran mikro kanser uterus. Sebahagian daripada pertumbuhan KU didapati berlaku melalui rembesan IL-6 dan laluan intraselular sel KU yang yang menginduksi ekspresi c-Myc. Projek ini memberi bukti dasar bahawa FBK melalui haluan IL-6 mempunyai peranan secara langsung dalam pertumbuhan KU dan boleh disasar untuk kegunaan terapeutik pada pesakit KU.

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LIST OF SYMBOLS AND ABBREVIATIONS

µM	:	Micromolar
µL	:	Microliter
µm	:	Micrometer / micron
µg	:	Microgram
kD	:	KiloDalton
mM	:	Milimolar
pg	:	Picogram
EH	:	Endometrial hyperplasia
EIN	:	Endometrial intraepithelial neoplasia
EC	:	Endometrial cancer
CAFs	:	Cancer-associated fibroblasts
Ep	:	Epithelial
Fib	:	Fibroblast
EpCAM	:	Epithelial cell adhesion molecule
α- SMA	:	alpha-smooth muscle actin
PTEN	:	Phosphatase and tensin homolog
PI3K	:	Phosphatidylinositol-3
AKT/PKB	:	Protein kinase B
KRAS	:	Kirsten Rat Sarcoma Viral Oncogene Homolog
ERK	:	Extracellular signal-regulated kinases
MAPK	:	Mitogen-activated protein kinases
JAK	:	Janus kinase
STAT	:	Signal transducer and activator of transcription
c-Myc	:	Myc proto oncogene

GM-CSF	:	Granulocyte monocyte-colony stimulating factor
GRO-α	:	Growth regulated oncogene-alpha
IFNγ	:	Interferon-gamma
IL-1α	:	Interleukin-1-alpha
IL-1β	:	Interleukin-1-beta
IL-2	:	Interleukin-2
IL-4	:	Interleukin-4
IL-5	:	Interleukin-5
IL-6	:	Interleukin-6
IL-8	:	Interleukin-8
IL-10	:	Interleukin-10
IL12p70	:	Interleukin-12-p-70
IL-13	:	Interleukin-13
MCP-1	:	Macrophage chemoattractant protein-1
MIP-I α	:	Macrophage inflammatory protein 1 alpha
MMP	:	Matrix metalloproteinase
NFκB	:	Nuclear factor kappa-light-chain-enhancer of activated B cells
PIM-1	:	Proto oncogene serine/threonine
RANTES	:	Regulated on activation, normal T expressed and secreted
SOCS-3	:	Suppressor of cytokine signaling-3
TIMP-1	:	TIMP Metallopeptidase Inhibitor 1
TNF-α	:	Tumor necrosis factor-alpha
VEGF	:	Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

The American Cancer Society estimates about 60,000 new cases of endometrial cancer (EC) diagnosed and about 10,000 deaths resulting from this malignancy for the year 2016. There is a rise in both incidence and death rates since 2001, resulting in a clinical burden to women worldwide ("American Cancer Society: Cancer Facts and Figures 2016," 2016).

Globally, EC has been affecting 318,000 women per year (Jemal et al., 2011). It is the most common gynecologic malignancy and the sixth most commonly diagnosed cancer among women ("6 Common Cancers - Gynecologic Cancers Cervical, Endometrial, and Ovarian," 2007). It is estimated that at some point in their lifetime, 2.7% of women would be diagnosed with this tumor, based on the data obtained from the 2004-2008 Surveillance Epidemiology and End Results (SEER) on endometrial cancer burden worldwide ("Cancer Trends Progress Report – 2011/2012 Update," 2012).

EC is classified into four stages with stage I corresponds to the cancer being confined to endometrium while stage IV indicates spread into the bladder or bowel wall (Han & Kohn, 2010). Surgery, radiation, and/or chemotherapy are advised as treatment modality depending on the tumor stages (Buchanan, Weinstein, & Hillson, 2009). The survival rate for early stages of EC is high. However, despite the availability of multiple treatment options, there is still a challenge in finding the most effective treatment especially in patients with advanced, therapy-resistant and recurrent disease (Cote, Ruterbusc, Olson, Lu, & Ali-Fehmi, 2015; Evans et al., 2011).

The endometrium is heavily regulated by hormonal exposures, mainly estrogen. Endometrial hyperplasia (EH), is non-cancerous condition characterized by excessive endometrium lining thickening. It is perceived that high exposure to estrogen would drive to the progression of a normal endometrium to become hyperplastic (C. L. Trimble et al., 2012). EH tissues consist mainly of stromal cells mostly fibroblast (Sivridis, Koutsougeras, & Giatromanolaki, 2013; C. Stewart, Michie, & Kennedy, 1998). Considered as a precancerous lesion, EH has a 29% risk of progressing to EC (Ellenson, Ronnett, & Kurman, 2010; Kurman, Kaminski, & Norris, 1985). In fact, EC was shown to concurrently occur in 42.6% of previously diagnosed patients with EH (Giede, Yen, Chibbar, & Pierson, 2008; C. Trimble et al., 2006). This highly suggests that the fibroblast-mediated effects in the endometrial microenvironment modulate the behavior of the endometrium of either remaining in a non-cancerous stage or progressing to EC. What remains unknown, however, is whether fibroblasts in the endometrial tumor microenvironment would attenuate or further progress EC carcinogenesis.

Endometrial intraepithelial neoplasia (EIN) is a type of non-invasive, pre-malignant lesion which is highly prone to transformation into EC (C. L. Trimble et al., 2012). One in three patients diagnosed with EIN is predicted to progress to carcinoma within the first year of diagnosis (Mutter, Kauderer, Baak, & Alberts, 2008). It was also predicted that patients who are cancer-free after diagnosis in the first year are at a 45-fold risk to progress to EC compared to patients with other types of benign lesions (Lacey et al., 2008; Owings & Quick, 2014). Excessive estrogen exposure in the endometrium, in addition to accumulating mutations results to the development of endometrial adenocarcinoma (C. Trimble et al., 2006; C. L. Trimble et al., 2012).

The tumor microenvironment is a complex system of non-malignant cells that supports and sustains the tumor growth(Tsai, Chang, Huang, & Kuo, 2014). One of the major players in the tumor microenvironment is cancer-associated fibroblasts (CAFs). CAFs are activated form of fibroblasts, existing within the tumor microenvironment. These cells communicate with the surrounding cancer cells through direct cell contact or indirect paracrine/ exocrine signaling and modulation of the extracellular matrix. (H. Fang & DeClerck, 2013; H. Li, Fan, & Houghton, 2007; Tsai et al., 2014).

Such complex communication network has a pivotal role in supporting tumorigenesis, angiogenesis, and metastasis (Cirri & Chiarugi, 2012), and has profound impact on the development and behavior of breast (E. Liu, 2007), colon (Bosman et al., 1993), prostate (Basanta et al., 2012) and gastric cancer (Zhi, Shen, Zhang, & Bi, 2010). In ovarian cancers, CAFs was shown to aid the neoplastic growth in normal tissues while encouraging migration and tumor growth (Y Zhang et al., 2011). The presence of fibroblast-like stromal cells in ovarian carcinoma resulted in an unfavorable clinical outcome indication where women with a high proportion of ovarian tumor stroma had decreased overall survival (Huang et al., 2014).

Interaction of CAFs and its effect on EC oncogenesis is relatively unknown at the inception of this study. Arnold et al showed that proliferation of a type of human EC cell line (the Ishikawa cells) was inhibited upon exposure to secretion from normal endometrial fibroblast (Arnold, Lessey, Seppälä, & Kaufman, 2002). This observation was further supported by a follow-up study that suggests the anti-proliferative effect was due to inhibition of PI3K signaling (Shi et al., 2011). It was unclear, at that time, about the role of CAFs in EC, whether these cells will demonstrate inhibiting effects as with the normal counterpart, or exert pro-tumorigenic activity similar to CAFs observed in other cancer types.

CAFs support tumor growth *via* various mechanisms. In addition to remodeling the architecture of the microenvironment to allow cancer cells to invade and metastasize, it also provides signals for cancer cell differentiation and progression (Karagiannis et al., 2012). One such mechanism that occurs during the tumor-fibroblast crosstalk is *via* production of secretary factors from CAFs, especially cytokines. CAFs in the tumor microenvironment are triggered to release cytokines due to the nature of cancer being in a continuously inflamed state. The continuous exposure to pro-inflammatory cytokines leads to sustained activation of signaling pathways which are required to drive carcinogenesis (Landskron, Fuente, Thuwajit, Thuwajit, & Hermoso, 2014).

In EC, the detection of pro-inflammatory cytokines in patients is linked with an advanced stage and worse prognosis (H. O. Smith et al., 2013). Despite strong correlation of cytokine levels with EC stages, the molecular mechanism of pro-tumorigenic cytokines secreted by CAFs in driving EC progression is lacking.

Hence, in this study, it was demonstrated that CAFs isolated from human tissues displayed growth-promoting effects on EC both *in vitro* and *in vivo*. This effect was shown to occur through secretion of pro-tumorigenic factors and activation of downstream signaling pathways to sustain EC carcinogenesis. These findings could eventually lead to CAFs being targeted for possible future therapies in relieving the clinical burden of EC.

1.1 Hypothesis

Secretion from cancer-associated fibroblast contributes to the progression of endometrial cancer.

1.2 Research Objectives

- To establish and characterize primary cultures of human endometrial fibroblast and epithelial cells from endometrial cancer tissues.
- To investigate the effect of normal and cancer fibroblasts on endometrial cancer cells proliferation, migration and invasion *in vitro*
- To determine the cytokines secreted by cancer-associated fibroblast that could modulate endometrial cancer proliferation
- To study the possible signaling pathways activated through cancer-associated fibroblast interaction in the endometrial cancer tumor microenvironment
- To investigate role of cancer-associated fibroblast in endometrial cancer by establishing a mouse model of tumor xenograft

CHAPTER 2: LITERATURE REVIEW

2.1 The endometrium

The human uterus is a part of the female reproductive system. The organ is pear-shaped, hollow, thick-walled, muscular and primarily consists of smooth muscle cells (Wray, 2007).

The outermost layer of the uterus is the perimetrium layer, enveloping the womb and is firmly attached to the underlying muscular layer. The second layer is the thick myometrium layer composed mainly of smooth muscle cells. The innermost endometrium layer is lined with simple columnar epithelial cells with many tubular glands **(Figure 2.1)**. The endometrium is responsible for menstrual and reproductive capabilities (Maruyama, Masuda, Ono, Kajitani, & Yoshimura, 2010).

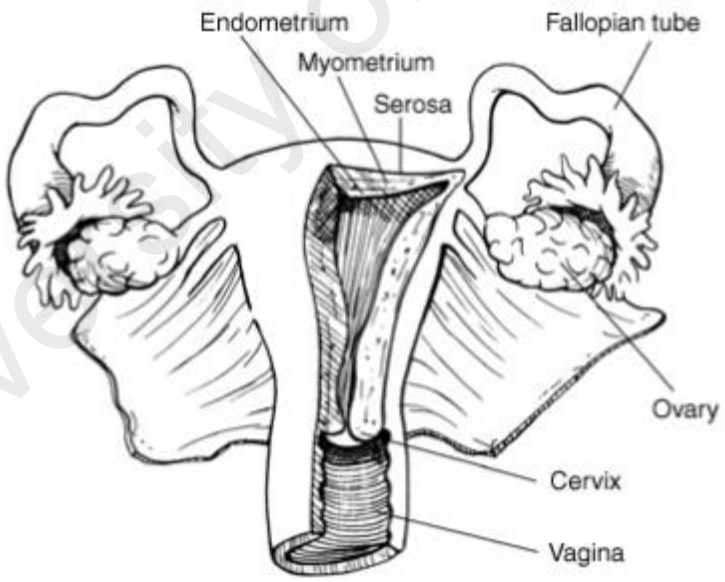


Figure 2.1. The endometrium lining in the uterus

("American Cancer Society: Cancer Facts and Figures 2016," 2016)

The endometrium layer constantly changes with each menstrual cycle by regenerating, differentiating and regressing under hormonal control. While it remains dormant in the prepubescent and postmenopausal years, the endometrium is otherwise a very hormone responsive organ towards progesterone and estrogen. The uterine cycle starts with an increase in estrogen level leading to regrowth and differentiation of endometrium, to prepare for possibilities of pregnancy. Upon release of the ovum from the ovary, the uterine lining enters the ovarian cycle preparing for possible implantation under the progesterone influence (Scanlon & Sanders, 2007). When pregnancy fails to occur, both the hormones are temporarily halted, causing the endometrial lining to shed and sloughed off. This cycle continues throughout a woman's reproductive lifetime (Strauss & Lessey, 2004).

A normal endometrium consists of stromal and epithelial cells **(Figure 2.2)**. The endometrial stromal cells are mesodermal cells in origin and mainly are fibroblastic in nature. The epithelial cells are columnar or cylindrical with changes in morphology from the proliferative to the secretory phase (Deligdisch, 2000).

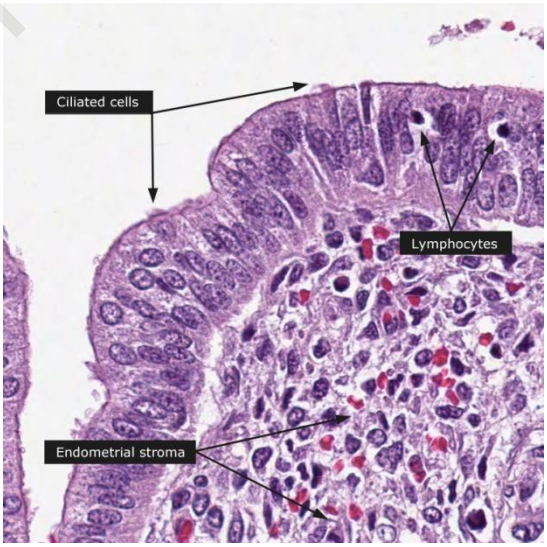


Figure 2.2. Histology of a normal endometrium

(Uhlén et al., 2015)

2.2 Benign endometrial conditions

Gynecological disorders are abnormal conditions occurring in one or more of female reproductive organs. Some of the common benign gynecology conditions include uterine fibroids (growth in the myometrium layer), polyps (growth in the endometrium layer), endometrial hyperplasia (excessive proliferation and thickening of the endometrium layer), endometriosis (appearance of endometrial tissue outside of the uterus), ovarian cysts (fluid-filled sac in the ovary) and adenomyosis (endometrial tissue grows into the myometrium) (Brinton et al., 2005).

Most gynecology disorders present with similar symptoms prior diagnosis, such as back or abdominal pain, feeling lethargic all the time, bloating of the abdomen, vaginal discomfort as well as irregular bleeding between menstruation (Cooper, Polonec, Stewart, & Gelb, 2013). While the exact cause for gynecological-related conditions has not been clearly understood, genetic predisposition, an imbalance in estrogens and progesterone in the reproductive system, early menarche, and null parity are among risk factors contributing to the etiology of gynecological disorders (Savelli et al., 2003; E. A. Stewart, 2001).

Of note, one of the common benign gynecology disorders is endometrial hyperplasia. This is a condition where an overgrowth of normal cells lining the uterus occurs, causing the endometrial lining to thicken (Montgomery, Daum, & Dunton, 2004). Often, hyperplasia occurs when the endometrial lining continues to grow in response to excessive estrogen without growth termination effect by progesterone. It is estimated that one-third of women with endometrial hyperplasia will progress to endometrial cancer (Sutter et al., 2004). While most benign conditions do not progress into cancer stages, hormonal imbalance or pre-existing mutation could drive the transformation from pre-cancerous to cancerous condition (Sankaranarayanan & Ferlay, 2006).

2.3 Endometrial cancer

2.3.1 Endometrial cancer epidemiology

Endometrial cancer (EC), is the most common gynecological-related carcinoma among women worldwide and is the sixth most common cancer overall. It occurs primarily in postmenopausal women (Sankaranarayanan & Ferlay, 2006). Global incidence of EC in 2012 recorded 319,605 new cases, representing 5% of all new cases for cancer in women or 25.1 per 100,000 women (Garg & Mutch, 2012).

The incidence and death rates of this disease have increased up to 21% since 2008 and to more than 100% over the last two decades (Sorosky, 2012). Global statistics in 2012 showed that Barbados had the highest rate for EC and that 53% of the cases occurs in the developed countries (Ferlay et al., 2014). In the United States, about 47,000 new cases of EC were estimated for the year 2012 and almost 60,000 new cases to be reported in 2016. This sharp increase in about 12,000 new cases in just under 4 years indicates the growing concern to address the unmet medical needs for EC ("American Cancer Society: Cancer Facts and Figures 2016," 2016). Analysis on the death rates due to EC showed an increase of 1.2% every year from 2004 until 2013 (Howlader et al., 2015).

EC is most commonly diagnosed among women in the postmenopausal age between 50 and 65 years old. While the average age at diagnosis is 61 years old, the risk of developing EC increases with older age (Jemal et al., 2011). Globally, it was observed that though Caucasian women have a higher risk of being diagnosed with EC, African-American women are more likely to die from it compared to any other ethnicity (Ferlay et al., 2014). Although the 5-year survival for this disease is high (81.7%), recurrence and treatment failure present as the main challenge in the management of this disease (Sorosky, 2012). In Malaysia, EC remains as the fourth most common cancer among women, and as the third cause of gynecological cancer death (Lim, 2002).

2.3.2 Endometrial cancer subtypes and grading

Generally, EC is classified to type 1 and type 2. Type 1 EC is more common, attributing to 80% of total EC cases with favorable prognosis. Type 1 EC is believed to be estrogen-dependent with endometrial adenocarcinoma accounting almost 85% of its histology subtype. Endometrial adenocarcinoma is a subtype which is composed mainly of malignant glandular epithelial cells (Jemal et al., 2011). In addition, type 1 EC usually follows a clear development route, commonly starts with endometrial hyperplasia before progressing into malignancy (Amant et al., 2005).

Type 2 is a lesser common type, making up 10% of EC cases. It is estrogen-independent and consists of more aggressive subtypes such as clear-cell and serous carcinomas. Type 2 EC has the tendency to metastasize and it has a lesser favorable prognosis compared to Type 1 EC (Amant et al., 2005). It was also observed that almost 50% of all EC relapse incidence occur in patients with type 2 EC (Holman & Lu, 2012).

The International Federation of Gynaecologists and Obstetricians (FIGO) recommends surgical staging, known as the FIGO staging system to assess the disease extent (Creasman et al., 1987). The assessments include evaluating the depth of myometrial invasion, cervical involvement, tumor size and location, an extension of tumor to fallopian tubes and ovaries, grading and subtypes, lymphovascular space invasion and lymph node status. These will collectively place patients across FIGO staging I-IV (Plataniotis & Castiglione, 2010). For example, patients presenting with 5% or lesser non-squamous solid tumor are categorized as grade 1, those with 6-50% solid growth as grade two and anything beyond 50% as grade 3 (Holman & Lu, 2012). Depending on the EC type and grade, one of the four basic treatment options will be recommended : surgery, radiation therapy, hormonal therapy or chemotherapy ("American Cancer Society: Cancer Facts and Figures 2016," 2016).

2.3.3 Endometrial cancer risk factors

Factors attributed to the development and progression of EC are mainly classified into external environment factors or genetic mutations. Some of the environmental factors thought to contribute to EC are unopposed estrogen therapy (10-20%), estrogen-producing tumor (>5%), usage of tamoxifen (2.5-7%), obesity (2-5%) and null parity (2-3%).

There is a 2-20-fold increase in risk among women who used estrogen replacement therapy to control menopausal symptoms (Pike et al., 1997). The first case of EC related to estrogen replacement therapy was reported in the 1960s among women who received estrogen treatment for menopausal symptoms (Gusberg & Hall, 1961). The incidence of endometrial hyperplasia, which is the precursor of EC, increases with the dose of estrogen used (Shapiro et al., 1985). Tamoxifen, which is used as an estrogen antagonist in the treatment of breast cancer, acts as an agonist in endometrial tissues, contributing to 6- to 8-fold increase EC incidence (Fisher et al., 1994).

Increasing incidence of obesity among women also has been implicated with the increase of EC incidence as there have been reports correlating obesity with 17-46% of all EC cases (Kaaks, Lukanova, & Kurzer, 2002). This happens when the endometrium is exposed to endogenous estrogen produced through aromatization in the adipose tissue and the rate of peripheral conversion of estrogen precursors to active estrogen is enhanced (Renehan, Tyson, Egger, Heller, & Zwahlen, 2008). This, in turn, increases 16α-hydroxylation of estrogen and increases serum levels of free estrogen (Carlson, Thiel, Yang, & Leslie, 2012). Such increase in levels of estrogen would cause an imbalance in the hormonal level of obese women thus contributing to increased risk of EC.

2.3.4 Endometrial cancer molecular pathogenesis

Like many cancer types, EC tumorigenesis is also driven by genetic mutations. A large genome-wide association study identified that locus rs1202525 near the CAPN9 gene on chromosome 1q42.2 showed constant association with risk of EC (Gallo & Bell, 2014; Long et al., 2012). Furthermore, the most recent data on whole-exome sequencing of EC and analysis based on The Cancer Genome Atlas (TCGA) revealed mutation in the estrogen receptor cofactor gene, NRIP1 in 12% of EC patients (Gibson et al., 2016). Smith's group further identified that forkhead box protein A2 (FOXA2) gene is frequently mutated in EC. The results also suggested that the pattern of the FOXA2 mutations points to a tumor suppressive role in EC (B. Smith et al., 2016). Patients with mutation in the mismatch repair (MMR) gene had been reported to have worse progression-free survival (McMeekin et al., 2016). A small subset of EC incidence are associated with genetic mutations and microsatellite instability that also occurs in colorectal cancer (Gallo & Bell, 2014). In fact, ring finger protein 43 (RNF43) gene mutation is one of the most common gene mutation, with almost 18% occurrence reported in both colorectal and endometrial cancers (Giannakis et al., 2014; TM Kim, Laird, & Park, 2013).

TCGA also reported frequently occurred mutations of tumor suppressors and oncogenes in EC ("Integrated genomic characterization of endometrial carcinoma," 2013). One of which is through the mutation of the phosphatase and tensin homolog (PTEN) gene. Using immunohistochemistry, PTEN mutation was found to occur in 83% of EC tissue tumor samples (25 of 30) and in 55% of endometrial hyperplasia samples (16 of 29) (Mutter, 2001; Mutter, Lin, & Fitzgerald, 2000). PTEN, a gene located on chromosome 10q23 functions as a tumor suppressor. Upon mutation, it loses the function of arresting cell cycle at G/S checkpoint (Bansal, Yendluri, & Wenham, 2009). PTEN mutation is frequently reported to co-exist with the mutated phosphatidylinositol-3-kinase catalytic subunit alpha (PI3KCA) gene in 36% of EC cases. Mutation of PTEN increases

the PI3KCA activation, resulting in phosphorylation of AKT pathway. Increase in AKT pathway phosphorylation would lead to the promotion of cancer cells survival and proliferation (Velasco et al., 2006).

The KRAS (Kirsten-RAS) gene, often mutated at the codon 12 or 13 is also found to contribute to EC occurrence. Association of KRAS mutation with 18% of endometrioid adenocarcinoma cases was first described in the early 1990s. KRAS mutation was also shown in cases of endometrial hyperplasia (Enomoto et al., 1990; Lax, Kendall, Tashiro, Slebos, & Hedrick, 2000; Lester & Cauchi, 1990). It is shown that KRAS mutations can coexist with mutations in PTEN, PIK3CA, and PIK3R1 genes. KRAS mutations were also associated with increased phosphorylation of MEK1/2, ERK1/2, and p38MAPK (O'Hara & Bell, 2012). Conditional mouse model of EC with ablated PTEN and activated KRAS in the reproductive tract resulted in a shorter time taken for EC to develop compared to mice with only single lesion (TH Kim et al., 2010). It is important to note that co-existence of different mutations in EC could lead to different therapy outcomes. A study done by Britta's group showed that EC cells lines harboring PIK3CA and/or PTEN and KRAS gene mutations responded to PI3K pathway inhibition (Weigelt, Warne, Lambros, Reis-Filho, & Downward, 2013).

Tumor suppressor TP53 gene mutations are shown to occur in 80-86% of serous EC but only in 17% of grade 3 endometrioid type (Lax et al., 2000). The p53 gene, located on chromosome 17 is important in preventing the growth of cells with damaged DNA. In response to DNA damage, p53 would accumulate causing cell cycle arrest and promote apoptosis. A mutated p53 however will result in inhibition of cell cycle arrest and apoptosis leading to the uncontrolled cell growth (George, 2011). Co-existence of p53 and PTEN gene mutations in EC suggests that both genes participate in the development

of this tumor, which would affect patients' response to treatment (Janiec-Jankowska, Konopka, Goluda, & Najmoła, 2010).

2.3.5 Endometrial cancer therapy and challenges

About 80% of stage I EC can be treated through surgery with total hysterectomy and bilateral salpingo-oophorectomy (Creasman et al., 1987). For patients who cannot undergo surgeries following diagnosis, adjuvant postoperative treatment including radiation or brachytherapy for locoregional control are as alternative treatment options. Adjuvant radiation therapy has been associated with a reduction in local area recurrence but does not impact overall survival of EC patients (Straughn et al., 2003).

Vaginal brachytherapy, on the other hand, was shown to reduce the risk of local recurrence while having good disease-specific and overall survival in patients with high to intermediate risk EC (Nout et al., 2010). This method is also associated with a much lower rate of gastrointestinal toxic effects leading to a better quality of life (Nout et al., 2009).

Patients with high-risk disease stages (stage III-IV) are often treated using chemotherapy, as chances for disease recurrence outside the pelvic region is greater in more advanced disease stages. Some significant clinical trial results showed improved survival in stage III-IV patients treated with doxorubicin and cisplatin when compared to whole abdominal radiotherapy (Randall et al., 2006). In another retrospective analysis, carboplatin and paclitaxel have shown good efficacy with minimal toxicity for advanced stage diseases (Sovak et al., 2006). The sandwich technique of first starting chemotherapy followed by radiation and reverting back to chemotherapy was used in a multicenter retrospective cohort of patients with advanced stages of EC. Results showed 69% of the patients had progress-free survival and 88% of them had overall survival for 3 years, highest of what has been seen thus far (Secord et al., 2007).

Despite advances in treatment modalities, treatment remains a challenge in patients who have a recurrence of EC when the disease becomes too aggressive and patients do not respond to chemotherapy, as there could be an alteration in genetic mutations, rendering treatment failure. This results in a low overall survival rate (Wright, Medel, Jalid Sehouli, Fujiwara, & Herzog, 2012).

Treatment is also a challenge among women who develop EC at a pre-menopausal age. The incidence in this particular cohort of women is projected to increase in the upcoming years. Due to their young age and the possibility to start or expanding a family, these women might not opt for a surgical procedure to remove their womb as part of EC treatment. This increases the risk of EC relapse if they do not respond to other treatment options such hormonal therapy or radiation therapy (Nogami et al., 2013).

2.4 Tumor microenvironment

2.4.1 Components of tumor microenvironment

The tumor microenvironment is defined as the environment surrounding the tumor cells and is recognized as the next generation hallmark of cancer. It consists of non-cancerous compartments including fibroblast, epithelial, immune cells as well as vasculatures and extracellular matrix (D Hanahan & Weinberg, 2011). Owing to Stephan Paget's 'Seed and Soil Hypothesis' postulated in 1889 (Paget, 1889), it is widely accepted that the microenvironment plays a crucial role in cancer development and progression. Tumor does not progress independently but is involved in a matrix of the interconnected network (Ribatti, Mangialardi, & Vacca, 2006) **(Figure 2.3)**. The microenvironment is also associated with regulation of tumor cell growth as the players involved has the potential to sustain the growth of localized tumors and to drive metastasis **(Figure 2.4)** (Douglas Hanahan & Coussens, 2012).

The composition of tumor microenvironment can be divided into three categories based on the cell origin. The first are cells of hematopoietic origin. This consists of cells originating from the bone marrow including T cells, B cells, natural killer (NK) cells, macrophages, neutrophils and myeloid-derived suppressor cells. Cells of this origin play a role in tumor development, promotion and progression. These cells have been the key target in developments of novel immunotherapies for cancer inhibition (Gkretsi, Stylianou, Papageorgis, Polydorou, & Stylianopoulos, 2015).

The next component is cells of the mesenchymal origin, comprising of fibroblasts, myofibroblasts, mesenchymal stem cells, adipocytes and endothelial cells. Bone marrow-derived myofibroblasts and mesenchymal stem cells were shown to support cancer stem cells and facilitate cancer initiation and development. This is believed to occur *via* a complex signal crosstalk between the cancer cells and these cells in the tumor microenvironment. Endothelial cells which make up the walls of blood vessels play a major role in vascular formation and angiogenesis to sustain tumorigenesis (Salo et al., 2014).

The third category consists of non-cellular components, including the extracellular matrix which holds and maintains tissue architecture of the microenvironment (Pattabirama & Weinberg, 2014). The extracellular matrix provides an adhesion platform for the cells and acts as a reservoir of growth factors for cancer sustainability (Gkretsi et al., 2015).

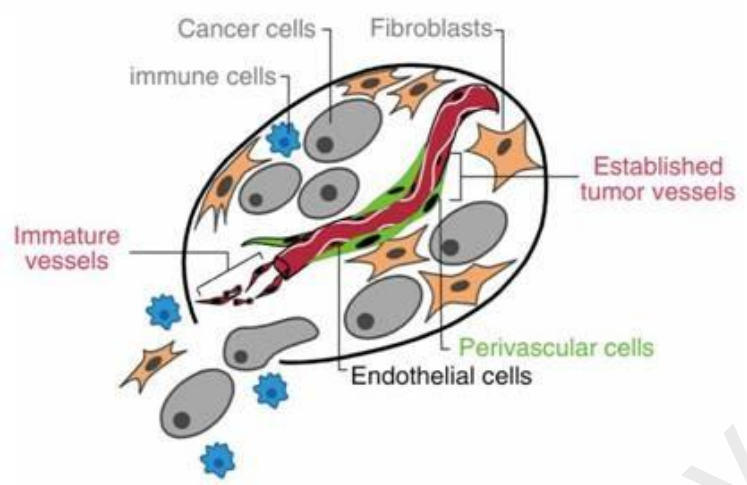


Figure 2.3. Players in the tumor microenvironment

(D Hanahan & Weinberg, 2000)

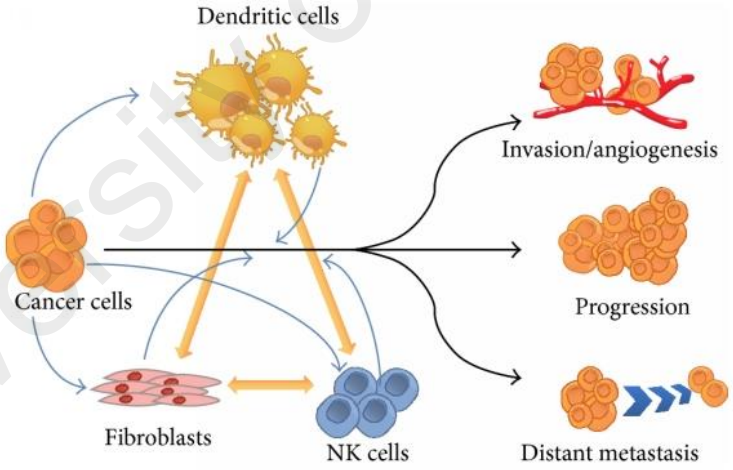


Figure 2.4. Interaction of the components in the tumor microenvironment contributing to tumorigenesis

(Tsai et al., 2014)

2.4.2 Role of tumor microenvironment in cancer

The relationship between tumor cells and host cells in the microenvironment is thought to be the underlying factor in disease initiation, progression, and tumorigenesis. The microenvironment is believed to promote carcinogenesis by several mechanisms, which broadly fall into two categories: contact-dependent and contact-independent mechanisms. The contact-dependent action involves interaction between cells and extracellular matrix adhesion molecules while contact-independent involves cross-talks between soluble molecules such as cytokines, chemokines and growth factors (H. Fang & DeClerck, 2013).

For example, in pancreatic adenocarcinoma that is surrounded by a thick desmoplastic region, tumor microenvironment plays a significant role where pancreatic stellate cells promote perineural invasion, angiogenesis, cancer cell growth and invasion (X. Sun, Jiang, Zhang, & Mao, 2016). In prostate cancer, stromal cells from tumor microenvironment have also demonstrated an increase in extracellular matrix remodeling, increased angiogenesis and a spike in inflammatory cells (Rowley, 1998).

Myofibroblasts in the tumor microenvironment have also been implicated as a source of signaling that facilitates tumorigenicity, where co-culture experiments showed myofibroblasts stimulate tumor formation from a nontumorigenic prostate cell line (Barclay, Woodruff, Hall, & Cramer, 2005). Reversal of tumor progression was observed in non-activated fibroblast in prostate cancer (Hayashi & Cunha, 1991), emphasizing the importance of considering tumor microenvironment in drug therapy.

In gynecological cancers, studies on the role of tumor microenvironment in cancer progression are rather limited. One group had shown that ovarian stromal fibroblast plays a synergistic role in the tumor microenvironment,in which secretions from the fibroblast promoted ovarian cancer proliferation, migration, and invasion (Shilong Fu et al., 2013).

Another very recent finding suggests that tumor microenvironment could be the key contributor to ovarian cancer cell invasion and metastasis (Z. Luo et al., 2016).

The role of tumor microenvironment was also observed in cervical cancer, where the presence of CXCL12, a pro-inflammatory cytokine was reported to be associated with reduced metastatic potential of the disease. The functional role of this cytokine was observed when silencing of CXCL12 gene in cervical cancer cell line resulted in increased migration and invasion of the cells suggesting tumor suppressor function of this cytokine.

2.4.3 Role of tumor microenvironment in endometrial cancer

While there have been plenty of studies on the role of tumor microenvironment in other common cancers, these mechanisms have been poorly explored in endometrial cancer. Occurrence of APC gene mutation in the stromal endometrial was sufficient to induce tumorigenesis of endometrial hyperplasia and EC. The mechanism by which it occurs were reportedly similar to the mechanism of unopposed estrogen signaling in the epithelial cells from the endometrium (Tanwar, Zhang, Roberts, & Teixeira, 2011). Janzen's group showed that stromal fibroblast mutations in influences EC's response to therapy. Their study results indicated that progesterone therapy works by paracrine signaling of PR in endometrial cancer microenvironment and that loss of stromal PR induced progesterone resistance. It was long thought that progesterone acts by binding to the PR present in epithelial cells. However, this study elegantly showed that stromal PR plays a pivotal role and that loss of the receptor could result in resistance towards progesterone therapies (Janzen et al., 2013).

In fact, exposure of estrogen and progestin on primary stromal cells isolated from EC patients showed differential effect on EC proliferation compared to stromal cells isolated from benign endometrium. Benign stromal cells exposed to both hormones decreased EC cells proliferation and induced high secretion of IGFBP-1, which was not observed from cancer stromal cells. Through a subcutaneous xenograft model, results showed that adding endometrial stroma from patients with or without EC influenced growth of EC *in vivo* (Pineda, Lu, Cao, & Kim, 2015). Estrogen was also shown to promote endometrial cancer invasion by stimulating tumor necrosis factor-alpha expression in cancer cells which in turn induces stromal expression of hepatocyte growth factor (Choi et al., 2009). In fact, endometrial CAFs overexpressing mircoRNA miR-148a exhibited lowered migration rate in five different EC cell lines upon co-culture (Aprelikova et al., 2013). A

very recent study by Teng's group showed that endometrial cancer fibroblast from tumor tissues promoted cancer progression via the SDF-1/CXCR4 axis in a paracrine- or autocrine dependent manner (Teng et al., 2016).

Taken together however, the study on the role of tumor microenvironment in endometrial cancer is still in the early stages. Thus, this pushes the need to understand the paracrine interaction of cancer cells and their surrounding host cells in this tissue. This will yield new insight into the underlying mechanism, which will lead to the discovery of new therapy intervention.

2.5 Cancer-associated fibroblasts (CAFs)

2.5.1 Definition of CAFs

Fibroblasts surrounding the cancer cells play an important role in development and behavior of tumors (Cirri & Chiarugi, 2011). In contrast to resting fibroblasts, cancer-associated fibroblasts (CAFs) are identified as activated form of fibroblasts, communicating with surrounding cancer, inflammatory, and immune cells through direct cell contact, indirect paracrine/ exocrine signaling and modulation of the extracellular matrix.

As tumors are equated to wound that does not heal, fibroblasts in a cancerous environment are continuously activated and do not revert to normal phenotype nor decrease in quantity. This is due to the continuous signaling exerted by the tumor tissues on these fibroblasts. These continuously activated fibroblasts are known as CAFs (Douglas Hanahan & Coussens, 2012).

2.5.2 Characterizations and origin of CAFs

CAFs produce several specific proteins such as fibroblast-specific protein (FSP-1), fibroblast-activating protein (FAP), vimentin, α-smooth muscle actin and platelet-derived growth factor receptor beta. These markers have been used and corroborated in various studies to characterize CAFs isolated from primary tumor tissues (Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). Nevertheless, there has been no marker so far that is described as "CAFs-specific", owing to the apparent heterogeneity of CAFs (Martin Augsten, 2014).

The heterogeneity of CAFs markers is thought to result from the difference in its origin. CAFs have been shown to differentiate from at least four different lineages. CAFs are primarily thought to originate from normal fibroblasts or fibroblast precursors, which differentiated into CAFs upon stimulation with paracrine signals such as TGF-β. Secondly, CAFs are also thought to develop from mesenchymal stem cells (MSC), as both CAFs and MSC shows similarities in expression of cell surface markers such as HLA-DR, CD29, and CD90. In the tumor microenvironment, MSCs not only differentiate into CAFs but also into endothelial and various immune cells. Thirdly, CAFs could also originate from other mesenchymal cells such as vascular smooth muscle cells, which transdifferentiated in CAFs. Lastly, CAFs can transition from endothelial and epithelial *via* endothelial-mesenchymal transition and epithelial-mesenchymal transition, respectively (Madar, Goldstein, & Rotter, 2013) **(Figure 2.5)**.

While it is important to understand that different sources of CAFs contribute to the heterogeneity, little is known if different tumor types share certain sources for CAFs.

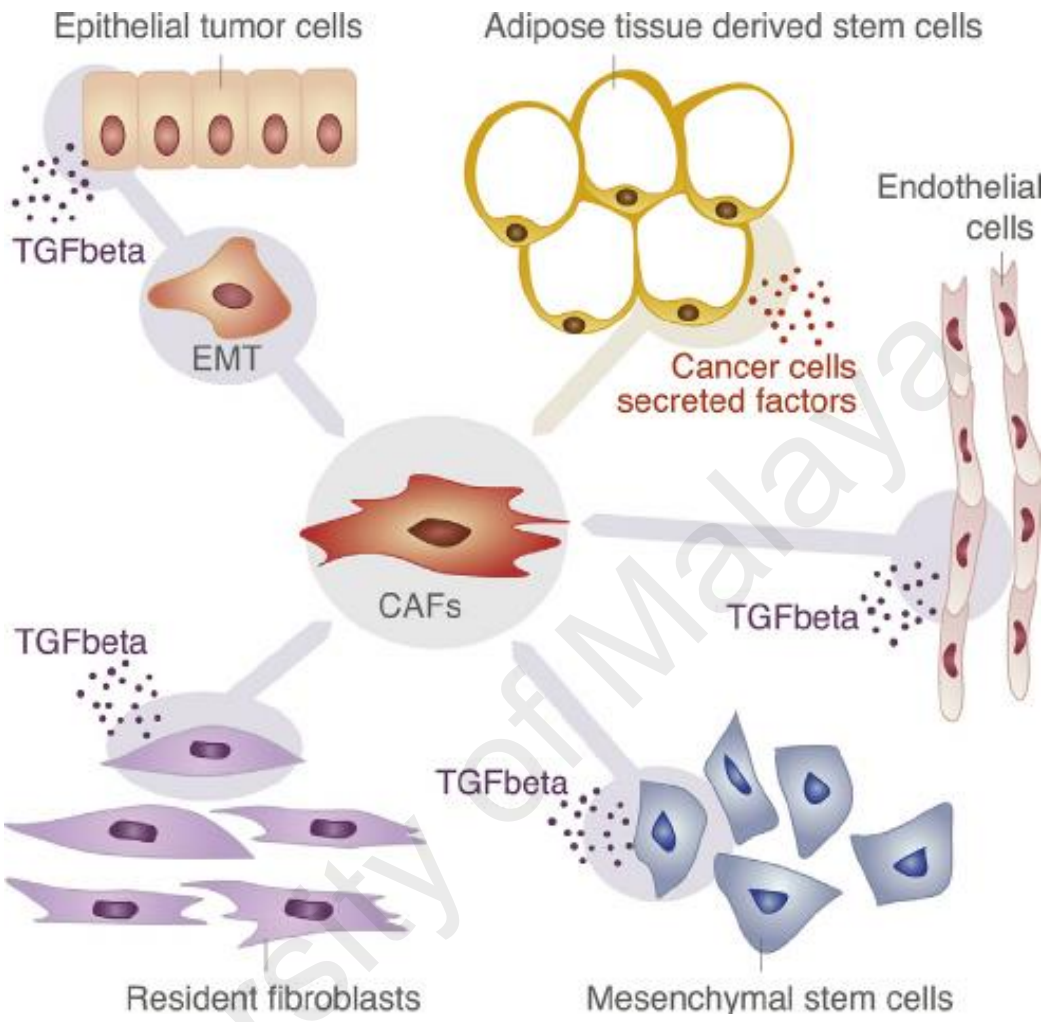


Figure 2.5. Known origins of CAFs in the tumor microenvironment

(A Calon, Tauriello, & Batlle, 2014)

2.5.3 Role of CAFs in tumorigenesis

Within a normal tissue, fibroblasts are the most common cell type of the stroma and is responsible for the deposition of fibrillary, which leads to the formation of tissue basement membranes (H. Fang & DeClerck, 2013; H. Li et al., 2007; Tsai et al., 2014). In the event of a wound repair, fibroblasts respond by activating signals that allow cells to migrate into damaged areas. The activated fibroblast is involved a unique crosstalk between the non-activated fibroblast and the injured epithelial involving adhesion molecules ICAM1, VCAM1, fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), epidermal growth factors (EGF) and transforming growth factor-beta (TGF-β) (H. Li et al., 2007). Upon completion of wound healing, the quantity of activated fibroblast will diminish. However, this is not the situation in CAFs, as the activated form of fibroblast is constantly present in the microenvironment of cancer.

Role of CAFs in carcinogenesis have been widely reported in other types of cancers and been implicated in advanced disease stages as well as poorer overall prognosis of disease outcome, for example in colorectal carcinoma (Alexandre Calon et al., 2015), and esophageal carcinoma (Ha, Yeo, Xuan, & Kim, 2014). In pancreatic adenocarcinoma, the activated fibroblast surrounding the tumor called pancreatic stellate cells was shown to upregulate cancer cell proliferation both *in vitro* and in an orthotopic model, migration, and invasiveness. The cancer cells treated with secretion from stellate cells also showed a low response to treatment of gemcitabine and radiation therapy, which is a clear indication of the essential role played by stellate cells in pancreatic cancer chemoresistance (Hwang et al., 2008). Similarly, in prostate cancer, CAFs were able to drive the transformation of benign prostate epithelial cells into exhibiting tumorigenic behavior, further emphasizing the critical role played by CAFs in cancer progression (Cunha, Hayward, & Wang, 2002; Cunha, Hayward, Wang, & Ricke, 2003). In breast cancer carcinogenesis, presence of CAFs was associated with the metastatic capability of

the tumour cells (Gangadhara, Barrett-Lee, Nicholson, & Hiscox, 2012; Khamis, Sahab, & Sang, 2012), similar to that in ovarian cancer where metastasis is shown to happen through increase in angiogenesis and tumour cells invasion (Y Zhang et al., 2011).

2.5.4 Role of CAFs in EC progression

Fibroblast cells separate the epithelium layer from the myometrium layer of the endometrium (Kobayashi & Behringer, 2003). Fibroblast cells in the endometrium have the ability to promote epithelial development and to reprogram differentiation of the overlying layer of epithelial cells. (Arnold, Kaufman, Seppälä, & Lessey, 2001; Arnold et al., 2002). Due to the close proximity of the fibroblast with the epithelial cells in the endometrium, it is postulated that transformation of this fibroblast to CAFs would impact significantly on endometrial cancer tumorigenesis (Orimo et al., 2001). Evidence on the role of CAFs in endometrial cancer prior to this study was very limited.

One of the earliest studies on the role of fibroblast in the endometrial cancer pathogenesis was by Arnold's group. Using primary cultures from endometrial tissues, Arnold's group demonstrated that the secretion from normal endometrial fibroblast cells inhibited the proliferation of Ishikawa cells, a human EC cell line (Arnold et al., 2002). This observation was further supported by Min Shi's group in which it was observed that such anti-proliferative effect could be due to inhibition of PI3K signaling (Shi et al., 2011). A more recent study showed that cancer fibroblast from tumor tissues promoted cancer progression via the SDF-1/CXCR4 axis in a paracrine- or autocrine dependent manner (Teng et al., 2016). The growing number of reports and studies on endometrial cancer tumor microenvironment indicate the recognition of CAFs role in the endometrial cancer tumorigenesis.

2.6 Tumor-fibroblast interaction

2.6.1 The effect of tumor-fibroblast interaction on tumorigenesis

Functional studies in various cancer types, including breast, colon, prostate and lung cancer, have confirmed the concept that fibroblasts can determine the fate of the epithelial cell since they are able to promote malignant conversion as well as to revert tumor cells to a normal phenotype (H. Li et al., 2007).

Data obtained by examining fibroblast from patients with a variety of cancers compared to patients without cancer show that the presence of CAFs is associated with the prediction of tumor existence and maintenance (Ostman & Augsten, 2009). *In vivo* model of cancer using fibroblast with activated hepatocyte growth factor or transformation growth factor beta showed that interaction between CAFs and tumor resulted in cancer initiation at various sites including the stomach and prostate (Bhowmick et al., 2004; Kuperwasser et al., 2004). Moreover, once a pre-cancer lesion is formed, the tumor-fibroblast interaction is shown to have a significant impact on cancer proliferation, invasion, and metastasis, through the production of growth factors and chemotactic factors, angiogenesis factors, and matrix metalloproteinases (Erez, Truitt, Olson, Arron, & Hanahan, 2010; Hwang et al., 2008; Kaplan et al., 2005; Karnoub et al., 2007; Ostman & Augsten, 2009).

2.6.2 Means of tumor-fibroblast communication

The crosstalk of fibroblast and tumor cells within the tumor microenvironment to promote carcinogenesis is thought to occur through several methods including *via* epigenetic changes and paracrine signaling (Dey, 2013; Swartz et al., 2012). Studies have shown that fibroblasts modulate cancer initiation in the epithelium. For example, it was demonstrated in prostate cancer that CAFs but not normal fibroblasts induced the transformation of epithelial cells (Hayward et al., 2001).

Moreover, using a transgenic mouse model, it was observed that Wnt1 overexpression in fibroblast of mammary leads to transformation of mammary epithelial cells in a C57BL/6 mouse model (Jue, Bradley, Rudnicki, Varmus, & Brown, 1992). One study, in fact, used fibroblast specific protein-1-deficient mice and showed a significant reduction of tumor growth and inhibition of metastatic potential in a highly metastatic murine mammary carcinoma cell line (Grum-Schwensen et al., 2005). Knockout of TGFβRII in fibroblast specific protein-1-positive fibroblast cells enhanced prostate and stomach cell carcinoma (Bhowmick et al., 2004)

In another elegant *in vivo* study using mice with conditional alleles of PTEN and a Fsp-cre transgene, it was observed that PTEN inactivation, particularly in mammary fibroblast, increased the occurrence of progression to adenocarcinoma of MMTV-Erb B2/Neu-driven tumors. What was more astounding was the PTEN-specific gene signature of the pre-neoplastic fibroblast was similar to stromal signatures found in patients with breast cancer (Trimboli et al., 2009).

Apart from inducing epigenetic changes, CAFs are shown to exert a pro-tumorigenic effect on cancer cells through paracrine cytokine signaling. Cytokines are defined as a soluble factor produced by one cell and act on another cell to bring change in function of the target cell (Dinarello, 2007). The profile of cytokines in the tumor microenvironment, mainly secreted by CAFs, is highly dynamic and subject to a multitude of changes at

various stages of tumor development. Elevated cytokine levels are commonly observed in various cancer types.

Most tumors are known to modulate their cytokine secretion in response to stresses like chemotherapeutic drugs, ionizing radiation, and hypoxia, hence govern the therapeutic outcome of cancer. Moreover, these soluble factors also interact with non-targeted cells surrounding the tumor tissue, and in turn, may determine the magnitude of damage to non-targeted tissues *via* bystander effect during therapeutic conditions (Dranoff, 2004). Cytokines may belong to the 4 types: tumor necrosis factor (TNF) family, chemokine family, interferon family and hematopoietin family (Paradkar, Joshi, Mertia, Agashe, & Vaidya, 2014).

Stromal cells in the tumor microenvironment are shown to synthesize cytokines which regulate proliferation, cell survival, differentiation, cell migration and induction of apoptosis (Quail & Joyce, 2013). Depending on the condition of the microenvironment, cytokines can modulate either anti-tumor or pro-malignancy response (Zamarron & Chen, 2011). This is highly dependent on the state of fibroblast in the microenvironment (DeRuiter et al., 1997; Landskron et al., 2014; Midgley et al., 2013; Untergasser et al., 2005).

The differential effects of CAFs and normal fibroblast on the phenotypes of EC cells are postulated due to the difference in soluble factors present in the secretion of the two different fibroblast populations. Cytokines are shown to directly modulate pro-tumor characteristics which induce cell transformation and malignant property of surrounding cells in the tumor microenvironment *via* various growth factors, hormones, and cytokines (Bhowmick et al., 2004). For example, hepatocyte growth factor produced by CAFs in lung cancer tissue activates c-Met, leading to resistance to tyrosine kinase inhibitors (W. Wang et al., 2009). Fibroblast-derived SDF-1 is shown to enhance invasiveness in

pancreatic cancers, working in synergy with IL-8 to promote angiogenesis of endothelial cells (Y Matsuo et al., 2009). CXCL14 secreted by CAFs in prostate cancer has shown to increase the growth and migration of fibroblasts leading to increased tumor growth, angiogenesis and macrophage infiltration (M Augsten et al., 2009). In oral squamous cell carcinoma, CCL7 secretion by CAFs induces IL-1α secretion by the cancer cells (Jung et al., 2010).

In breast cancer, oxidative stress is shown to activate hypoxia-inducible factor-1 which causes CAFs to secrete SDF-1 (Toullec et al., 2010). CAFs-secreted matrix metalloproteinases such as MMP-13 releases vascular endothelial growth factor (VEGF) from the extracellular matrix, causing promotion of angiogenesis. This leads to increased in a number of vasculatures and degradation of the extracellular matrix, which translated to an increase in squamous cell carcinoma and melanoma invasiveness property (Lederle et al., 2010).

In a chronic inflammatory process, TNF-α and IL-6 are among the most studied inflammatory cytokines in the tumor microenvironment (Mantovani, Allavena, Sica, & Balkwill, 2008). Malignant cells are shown to constantly produce small amounts of TNF-α with evidence from animal models that malignant cell-derived TNF-α enhances the tumorigenicity of skin, ovary, and pancreatic cancers (Balkwill, 2009). In ovarian cancer, elevated levels of TNF-α are found in the plasma of patients with advanced cancer disease. High levels of TNF-α was also associated with 11 gene signatures that correlated with poor prognostic significance in stage 1 lung cancer (W. Wang et al., 2009).

Thus, it is important to note that although there are many cytokines are found to contribute to carcinogenesis, their pro- or antitumoral roles depend on the balance of different inflammatory mediators and the stage of tumor development.

2.6.3 Cytokine signaling in the endometrial cancer tumor-fibroblast communication

While progress has been made in the understanding of the mechanisms of cytokines in the other common tumorigenic process, establishing a relationship between cytokines expression and disease progression, survival, as well as response to therapy in endometrial cancer remains relatively understudied.

Of note, prior to this study, Smith's group utilized primary endometrial cancer epithelial and stromal cell fraction to study if specific patterns of cytokine expression could be used to predict adverse outcome in endometrial cancer. The result showed that high levels of CSF-1, TNF-α and IL-6 correlated with low survival rates among patients (H. O. Smith et al., 2013).

Another group showed that the expression of stromal fibroblast growth factor was high in the presence of hepatocyte growth factor (HGF) expression. This, in turn, related to better overall survival of endometrial cancer patients with positive expression of HGF and stromal fibroblast growth factor (Felix, Weissfeld, Edwards, & Linkov, 2010).

One very recent finding reported that coculture of CAFs with endometrial cancer cells promoted proliferation, migration, and invasion both *in vitro* and *in vivo* via SDF-1α/CXCR4 axis. Disease progression was observed to occur *via* activation of the PI3K/Akt and MAPK/Erk signalings in a paracrine-dependent manner. This correlated with poor endometrial cancer prognosis where high levels of SDF-1α was associated with deep myometrial invasion and lymph node metastasis (Teng et al., 2016).

2.7 Interleukin-6 (IL-6)

2.7.1 IL-6 structure and function

IL-6 acts as a single-chain protein with a molecular mass ranging from 21 to 28 kD. The source of IL-6 has been documented to originate from various cells including fibroblasts, endothelial cells, keratinocytes and monocytes (Snick, 1990). The IL-6 family consists of a ligand-specific receptor α chain known as IL-6Rα and combined with a shared receptor chain, glycoprotein 130 (gp130) (X. Wang, Lupardus, LaPorte, & Garcia, 2009). The IL-6R co-dimerization with gp130 receptor ligand are often the precursors to activate signals including the mitogen-activated protein kinase (MAPK), phosphotidyl-inositol 3-kinase (PI3K) and the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathways (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011).

As a cytokine, IL-6 is involved in inflammation, infection responses and in the regulation of metabolic, regenerative, and neural processes. In a stable state, IL-6 is usually not continuously produced by normal cells but is induced by viral infections or lipopolysaccharide (Snick, 1990). IL-6 is essential for the control of metabolism and is secreted by muscle cells upon strenuous exercise. It is also secreted by adipocytes present in obese individuals (Greenberg & Obin, 2006; Pedersen, Steensberg, & Schjerling, 2001). When the IL-6R gene is deleted in hepatocytes, there was a reduction in insulin sensitivity and glucose tolerance but the concurrent occurrence of liver inflammation was also reported (B. Sun & Karin, 2012). Additionally, in the metabolism of the bone, IL-6 in the presence of sIL-6R triggers the formation of osteoclast which forms the formation and modeling of the human skeletal system (Franchimont, Wertz, & Malaise, 2005). This evidence showcases the importance of IL6-R in modulating downstream signaling activations.

2.7.2 Presence of IL-6 in inflammatory diseases and in cancer

Overproduction of IL-6 is reported in non-cancerous conditions as well as in inflammatory related cancers (Kishimoto, 2010). IL-6 have been described as the pleiotropic cytokine with many roles including driving inflammation-mediated diseases (Barnes, Anderson, & Moots, 2011). It is often linked to inflammatory diseases such as rheumatoid arthritis, irritable bowel syndrome and autoimmune diseases along with strong influence in cancer progression (Scheller et al., 2011).

IL-6 has been shown to promote the growth of various tumor types including colon, multiple myelomas and non-small cell lung cancer (Yuqi Guo, Feng Xu, TianJian Lu, Zhenfeng Duan, & Zhan Zhang, 2012). Inhibition of IL-6 pathway abrogated STAT3-mediated cell survival of gastric cancer and osteosarcoma (B. Fang, 2014), suggesting the importance of IL-6 in promoting tumor growth. It was also reported that epithelial ovarian cancer secrete high levels of IL-6 in the peritoneal ascites (Offner et al., 1995). Additionally, it was also reported that IL-6 secretion and its mRNA levels were high in squamous cells and adenocarcinoma of the uterine cervix. This correlated to a clinical finding where one-third patients with squamous cell carcinoma were reported having raised serum IL-6 levels (Takano et al., 1996).

2.7.3 Evidence of IL-6 in the endometrium and role in endometrial cancer

IL-6 is produced at sites of inflamed gynecological diseases such as in non-cancerous cysts, endometriosis or pre-eclampsia as well as endometrial and ovarian cancers (Borzychowski, Sargent, & Redman, 2006; Macciò & Madeddu, 2012; Schrepf et al., 2013). Studies have found that preeclamptic women tend to have IL-6 secreted in the plasma and not in placenta, prompting an invasion of decidual which leads to low blood flow to the developing fetus (Lockwood et al., 2008).

IL-6 also acts as a contributing factor to both endometriomas as well as cystic malignant ovarian tumors (Darai, Detchev, Hugol, & Quang, 2003). In fact, IL-6 has also been suggested to be a reliable marker of minimal-mild endometriosis condition that does not require non-invasive detection methods (Martínez et al., 2007).

Patients with EC have shown to have a high level of serum IL-6 which is associated with poor clinical outcomes (Bellone et al., 2005). Data obtained from an *in vitro* study showed that IL-6 promotes EC through an autocrine regulatory loop of the ERK/NK-κB pathway (Che et al., 2014). This indicated that IL-6 could possibly play a role in tumorigenesis of EC. Of note, significantly high levels of IL-6 were found in patients with malignant gynecological tumors who are unresponsive to chemotherapy (Scambia et al., 1994).

Even more recently, it was shown that inhibition of IL-6 receptors and downstream effectors JAK1 and STAT3 significantly reduced tumor growth, suggesting that IL-6 or its downstream pathways could be novel therapeutic targets for EC patients (Zee et al., 2015).

2.7.4 IL-6 downstream signaling pathways

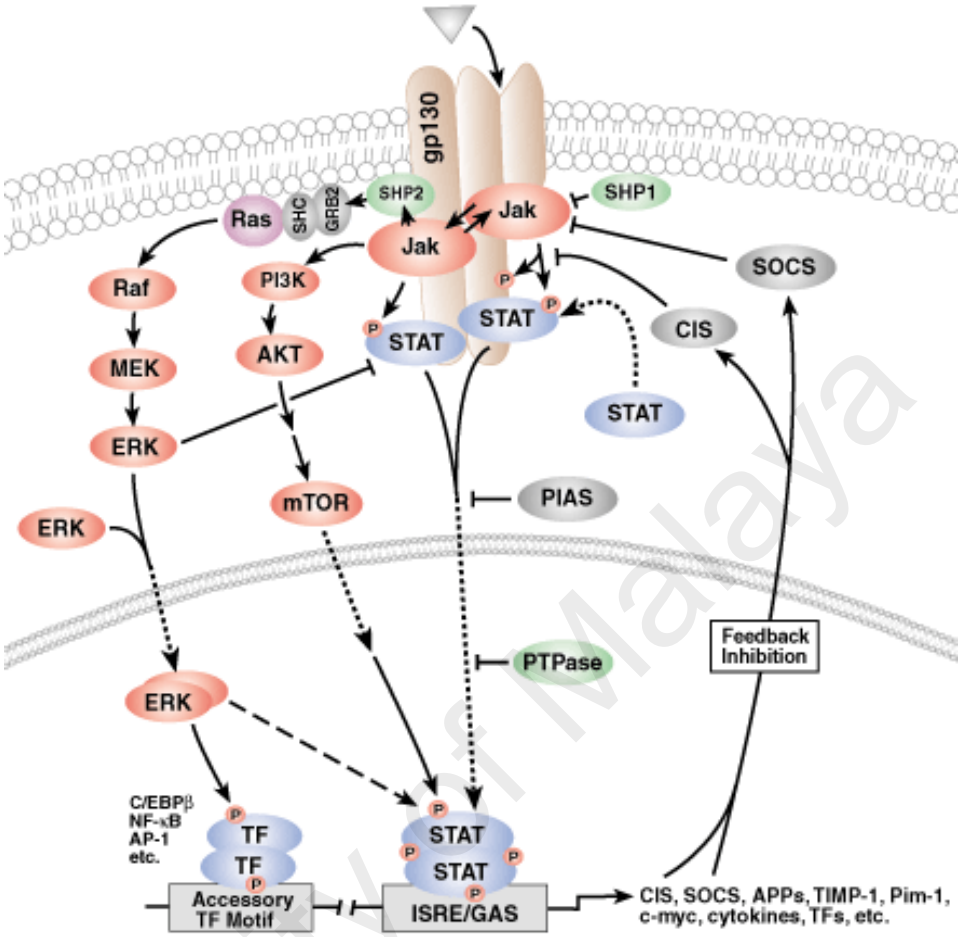


Figure 2.6. Activation of IL-6 downstream pathway cascades (Focosi, 2014)

IL-6 signals through the IL-6 receptor, consisting of two different subunits, IL-6Rα that produces ligand specificity, and signal transducer gp130, a receptor subunit shared with other cytokines in the IL-6 group.

In general, binding of IL-6 to its receptor activates a multitude of cellular events including activation of the PI3K/AKT/mTOR, the MAPK/ERK and the JAK/STAT3 pathways. Upregulation of suppressor of cytokine signaling 3 (SOCS3) gene transcription by STAT3 results in the termination of IL-6 cytokine signaling (C. Johnson et al., 2012; S. Jones, J. Scheller, & S. Rose-John, 2011) **(Figure 2.6)**.

2.7.4.1 The JAK/STAT pathway

The JAK/STAT pathway is the principal signaling mechanism for IL-6. JAK activation stimulates cell proliferation, differentiation, migration, and apoptosis. The JAK family comprises four members: JAK1, JAK2, JAK 3 and Tyk2. JAK activation occurs upon ligand-mediated receptor multimerization because two JAKs are brought into close proximity, allowing trans-phosphorylation (Rawlings, Rosler, & Harrison, 2004).

The activated JAKs subsequently phosphorylate additional targets, including the receptors and major substrates, STATs. STATs are latent transcription factors that reside in the cytoplasm until activated. The seven mammalian STATs has a conserved tyrosine residue near the c-terminus that is phosphorylated by JAKs (David et al., 1995). This phosphotyrosine allows the dimerization of STATs through interaction with a conserved SH2 domain. Phosphorylated STATs enter the nucleus, dimerizes STATs and bind specific regulatory sequences to activate or repress transcription of target genes (Winston & Hunter, 1995). Thus, the JAK/STAT cascade provides a direct mechanism to translate an extracellular signal into a transcriptional response (Rawlings et al., 2004).

STATs target genes that are upregulated upon IL-6 induction includes a plentitude of downstream genes such as C-reactive protein, lipopolysaccharide-binding protein, tissue inhibitor of metalloproteinases (TIMP)-1, Jun-B, c-Fos and interferon regulatory factor (IRF)-1, Sox2, NANOG, c-MYC, TWIST and ZEB-1 that contributes to overall progression of cancer (Carpenter & Lo, 2014). It was shown that in head and neck carcinoma, phosphorylation of STAT3 was a direct consequence of the increased production of IL-6 by tumor cells (Sriuranpong et al., 2003).

2.7.4.2 The PI3K/AKT/mTOR pathway

PI3K/AKT/mTOR pathway integrates both intracellular and extracellular signals and serves as a central regulator of basic biological processes of cells. This includes metabolism, growth, proliferation, survival and angiogenesis (Vara et al., 2004). High-frequency mutation of PTEN within this pathway was detected from type I EC and accompanied the PI3K/AKT/mTOR activation (UI Ali, Schriml, & Dean, 1999). Molecular alterations in AKT are rare, but both type I and type II EC showed a high rate of mutations in the PIK3CA gene (Okuda et al., 2010). Loss of PTEN activity in EC is associated with increased PI3K activity with resultant phosphorylation of its downstream substrate AKT. In turn, AKT upregulates mTOR activity, and hyperactivation of mTOR signaling enhances translation of mRNAs encoding growth factors, cell cycle regulators, survival proteins, and angiogenic factors, therefore, dysregulation of the PI3K/AKT/ mTOR pathway as a result of genetic mutations and amplifications has been thought to be a precursor for carcinogenesis (Paradkar et al., 2014).

Interestingly, in a study of metastatic EC, frequent mutations of KRAS, FGFR2 and PIK3CA were identified (Krakstad et al., 2012; O'Hara & Bell, 2012). The cellular location of the AKT activity is important in the behavior of cancer, the nuclear p-AKT level was significantly higher in grade 1 (well-differentiated,G1) than in grade 3 (poorly differentiated,G3) cancers and associated with estrogen receptor (ER-alpha) expression (Markowska, Pawałowska, Lubin, & Markowska, 2014).

IL-6 has been shown to regulate the expression of anti-apoptotic protein Mcl-1 through PI3K/Akt-dependent pathway in human cervical carcinoma (C. Johnson et al., 2012) and drives oncogenesis by inhibiting cellular apoptosis. Activation of PI3K/Akt pathway through IL-6 induction was observed to cause enhanced proliferation in multiple myeloma cells and human Hep3B hepatoma cells (Hsu et al., 2002).

2.7.4.3 The MAPK/ERK pathway

The MEK/ERK pathway (MAP kinase pathway) is an essential signal transduction pathway which transmits signals from cell surfaces to transcription factors in the nucleus. The pathway, also known as RAS-RAF-MEK-ERK signaling pathway, regulates cell proliferation and differentiation via RAF-MEK-ERK signaling cascade (F. Chang, Steelman, Shelton, et al., 2003).

MAPK is activated by the upstream growth factors and their receptors. A constitutive activation of MAPK signaling is often induced by the overexpression of growth factors and their receptors in cancer K-RAS gene encodes a protein that is a member of the small GTPase superfamily and is involved in signal transduction pathways between cell surface receptors and the nucleus (F. Chang, Steelman, Lee, et al., 2003).

K-RAS mutations have been identified in the 10% - 30% of endometrioid endometrial carcinomas (Lester & Cauchi, 1990). While some investigators have reported an almost complete absence of K-RAS mutations in serous and clear cell carcinomas of the endometrium, other researchers found a higher frequency of K-RAS mutations in microsatellite instability (MSI)-positive carcinomas than in MSI-negative tumors (Lax et al., 2000). Moreover, K-RAS mutations were detected in endometrial hyperplasia at a similar rate compared to that observed in endometrioid endometrial carcinomas, suggesting that K-RAS mutations could be early events during endometrial carcinogenesis (Markowska et al., 2014). IL-6 induced MAPK activation is thought to occur through recruitment of the protein tyrosine phosphatase SHP2 to Tyr in the central region of gp130 which then recruits Grb2/Shc/Ras/Mek1/2/Erk1/2 (Fischer & Hilfiker-Kleiner, 2008).

2.7.5 Targeted therapies

2.7.5.1 Targeting the players in tumor microenvironment

Many strategies are currently in different stages of clinical development to target components of tumor microenvironment as therapy approach for cancer treatment. Antiangiogenic and anti-inflammatory approaches revolving around cellular components of the tumor microenvironment has been hailed as the new and promising way to develop new inhibitors and targeted therapy against carcinomas (Sounni & Noel, 2013).

There have been four different therapeutic strategies proposed to target the tumor microenvironment, namely: targeting the tumor vasculatures to address the issue of angiogenesis, targeting the cancer-associated fibroblast to address inflammation, targeting the non-cellular environment in terms of ECM remodeling and targeting the metabolic feature of hypoxic tumor microenvironment (H. Fang & DeClerck, 2013; Sounni & Noel, 2013).

When a tumor grows to a certain size the formation of blood vessels is regulated by proangiogenic factors such as vascular endothelial growth factor-A, fibroblast growth factor, platelet-derived growth factor and epidermal growth factor (K. E. Johnson & Wilgus, 2014). Bevacizumab, a recombinant humanized vascular endothelial growth factor-neutralizing monoclonal antibody, was one of the first effective agents in targeting angiogenesis (Niu & Chen, 2013). Vascular endothelial growth factor signaling can also be inhibited using targeted using small molecule receptor tyrosine kinase inhibitors such as sunitinib, sorafenib, pazopanib which are approved for renal cell carcinoma indication (Gupta & Spiess, 2013). Ramucirumab, which is a monoclonal antibody against vascular endothelial growth factor-2 had shown early evidence in phase I trial of advanced solid tumors (Spratlin, Mulder, & Mackey, 2010) and is undergoing phase III clinical trial for breast and gastric cancer indications (H. Fang & DeClerck, 2013). In EC, overexpression

of vascular endothelial growth factor has been associated with poor prognosis as disease progression would have involved to the extent of deep myometrial invasion and lymph node metastasis (Westin & Broaddus, 2012). Bevacizumab which was studied in a phase II trial of recurrent EC showed a favorable response as a single agent with 13.5% response rate for a median of 6 months and overall survival median of 10.5 months (Aghajanian et al., 2011).

Cancer-associated fibroblasts (CAFs) expresses fibroblast activation protein alpha (FAP) which is unique to CAFs. Moreover, presence of this protein is associated with poorer prognosis in breast (Yu, Yang, Li, & Jiao, 2015) colon (Wikberg et al., 2013), ovarian (Mhawech-Fauceglia et al., 2015), pancreatic (Shi et al., 2012) and hepatocellular carcinoma (Ju et al., 2009) and is found to be mainly present in stroma of cells beside tumor cells making it an excellent candidate to target for carcinomas (Roberts et al., 2013).

Nevertheless, some phase I and II studies using sibrotuzumab, a humanized FAP monoclonal antibody, failed to yield clinical benefits in the colon and non-small cell lung cancer. The combination of small molecules with docetaxel instead lowered survival rates among lung cancer patients (Scott et al., 2003). Due to these issues, researchers are now trying to directly target FAP localized in tumor stroma which activates cytotoxic prodrugs which are believed to enhance drug delivery efficacy in the TME (Sounni & Noel, 2013). In EC, there has yet to be evidence implicating successful inhibition of cancer growth with FAP inhibitors.

ECM degradation and remodeling is a phenomenon commonly occurring in metastatic cancer where the tumor expands and breaks off to travel to new sites either within the organ or to other sites of the body. Cilengitide, which was the first potent integrin inhibitor, was found to be well tolerated in patients with recurrent glioblastoma

multiforme in a phase II clinical trial. Intetumumab, also known as CNTO 95, is a monoclonal antibody against human α_v integrin.

Upon testing in xenograft tumors of nude rats, intetumumab potentiates the efficacy of fractionated radiation therapy in human cancer without increased toxicity (Ning, Tian, Marshall, & Knox, 2010). In addition, intetumumab has shown a good safety profile and improved overall survival in phase II trial of metastatic melanoma indication (H. Fang & DeClerck, 2013). The combination of intetumumab to docetaxel resulted in shorter progression-free survival among castration-resistant prostate cancer patients without additional toxicity (Heidenreich et al., 2013).

Hypoxia is a major metabolic feature of the tumor microenvironment. It is a condition where tumor cells could adapt to the hypoxic environment and promote genomic instability as well as expression of proto-oncogenes which will eventually lead to therapeutic resistance (Wilson & Hay, 2011). A hypoxic environment provides the advantage of using bioreductive prodrugs which are activated by the enzyme into the toxic product in hypoxic tissues.

One of such drug was tirapazamine, which despite worked well in preclinical and early-phase clinical trials, had to be halted due to no benefit indications upon combination with chemotherapy or radiotherapy in non-small cell lung, head and neck cancer patients (Reddy & Williamson, 2009) and cervical cancer patients (DiSilvestro et al., 2014). TH-302 and AQ4N which are hypoxia-activated prodrugs in phase I/II trials are currently being investigated in patients with advanced malignancies and have shown minimal toxicity in phase I clinical trials (Guise et al., 2014; Q. Liu et al., 2012).

Another crucial target for inhibition of hypoxic signaling is the HIF-1α pathway with EZN-2968 which is an HIF-1α antisense mRNA has currently completed phase I clinical

trial (H. Fang & DeClerck, 2013; Tsai et al., 2014). Prognostic value of HIF-1α overexpression in EC, however, has yielded contradictory results thus far. It showed significantly higher expression in recurrent EC compared to the primary tumor but it was not an independent predictor for recurrent EC (Jijnenborg, Wijnakker, Hagelstein, Delvoux, & Groothuis, 2007; Pansare et al., 2007).

Targeted therapy in EC is still in its infancy. What is currently done is to target the various pathways thought to contribute to EC progression (Nogami et al., 2013). This includes bevacizumab, aflibercept, and thalidomide as antiangiogenic agents. Use of epidermal growth factor receptor inhibitors such as gefitinib, erlotinib, and cetuximab to inhibit EGFR activation lead to inhibition of EC cell proliferation in mice xenografts (Takahashi et al., 2009). As mutation in PTEN gene contributes to majority occurrence of type 1 EC, inhibitors targeting the mTOR complex such as temsirolimus and ridaforolimus could be effective (Slomovitz et al., 2010).

2.7.5.2 Targeting IL-6

Apart from targeting activated pathways, IL-6 inhibition also presents as a viable option as a therapeutic target in EC. The first IL-6 antibody approved by the Food and Drug Administration FDA and European Medical Association EMA to target IL-6 is siltuximab (CNTO 328). It was approved as in the treatment of multicentric Castleman's disease (Trikha, Corringham, Klein, & Rossi, 2003).

Siltuximab has been previously described for the treatment of cancer, including gynecological cancers, and had since underwent trials as an intervention to various cancers including Phase II trials for ovarian neoplasm and cancer (Guo et al., 2010; Trikha et al., 2003).

Sirukumab, a humanized anti-IL-6 monoclonal antibody has been tested in healthy subjects to determine pharmacokinetics/pharmacodynamics and safety (Xu et al., 2011). Tocilizumab, another antibody targeting IL-6 which has been recently approved is a mouse monoclonal antibody against the IL6R which has been used for rheumatoid arthritis, systemic juvenile idiopathic arthritis, and juvenile idiopathic polyarthritis indications (Rossi, Lu, Jourdan, & Klein, 2015).

While there are not any trials ongoing to target IL-6 in EC patients thus far, emerging evidence of good progress free survivals and improved overall survival in other indications could push the idea forward (Rossi et al., 2005).

It should also be understood that the use of molecular-targeted drugs in EC faces challenges including the various activation pathway and upregulation of target genes. Thus, development of drugs with multiple targets, combinations of molecular-targeted drugs and use of additive with current anticancer agents or hormone drugs may be required (Rossi et al., 2015).

CHAPTER 3: METHODOLOGY

3.1 Establishment and characterization of primary cultures of human endometrial cancer fibroblast and epithelial cells

3.1.1 Ethics statement

This study was approved by the Ethical Committee of University Malaya Medical Center (Ref No. 865.19). Written informed consent was obtained from all participants.

3.1.2 Collection of endometrial benign and cancerous tissues

Benign and cancerous human endometrial tissues were collected from women undergoing surgery at University Malaya Medical Center. Women above the age of 18 years old with histologically diagnosed endometrial conditions were included in this study. Pregnant women and those under the age of 18 were excluded. The tissue origin and disease stages were verified from the clinical histopathological report. Tissues were also collected from women undergoing hysterectomy, laparoscopy for subfertility or tubal ligation procedure. Endometrial cancer tissues included in this study consist of type 1 endometrial adenocarcinoma. Benign endometrial tissues included endometrial hyperplasia, endometrial fibroid, and endometrial cyst tissue types.

3.1.3 Establishment of human endometrial primary cultures

Tissues of sizes between 200 mg to 1 g were transported from the surgical theater to the laboratory in RPMI1640 media (Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and 1% penicillin/streptomycin (Life Technologies) (complete media). Tissues were minced to smaller sizes of about 1 mm^3 before digested with one of the collagenase types 1, 2, 3 or 4 (2 mg/ml) (Worthington, NJ, USA). Tissues were then placed in a rotator for 1 hour at 37 ˚C. Post-digestion, the cell suspension was separated from undigested tissues using a 100 µm sieve (BD

Biosciences, CA, USA). The suspension was centrifuged at 1,200 rpm and resulting cell pellet was washed with phosphate buffered saline (Life Technologies). To determine which collagenase that yield the best cell viability for each tissue type, cells were stained with Trypan blue (Life Technologies) and counted. Our result showed that cells from benign endometrial tissues thrive upon digestion with collagenase I or III while cells from EC tissues attached and grew better after digestion with collagenase II or IV. After digestion, centrifugation, and washing the resulting cell pellet, the cells were cultured in complete media at 37 ˚C. Cultures were maintained by media change every 72 hours and sub-cultured after reaching confluency.

3.1.4 Isolation of primary epithelial and stromal cells

Established primary cells contained mixed epithelial- and fibroblast-looking cells. To separate these cells, human CD90 anti-fibroblast and human CD326 (EpCAM) magnetic microbeads (Miltenyi Biotech, Cologne, Germany) were used to isolate fibroblast and epithelial cells, respectively. Briefly, $1x10^6$ cells were first centrifuged at 300 x *g* for 10 minutes. Cell pellets were then resuspended in 100 µl of buffer containing a final concentration of 0.5% bovine serum albumin and 2 mM ethylenediaminetetraacetic acid dissolved in pH 7.2, calcium and magnesium free phosphate buffered saline. Next, resuspended cells were incubated with either 20 µl of human CD90 anti-fibroblast or 100 µl of human CD326 (EpCAM) microbeads antibody for 1 hour. Cells were then separated using MiniMACS™ cell separator and MACS columns (Miltenyi Biotech). The separators contain powerful permanent magnets that induce a magnetic field within MACS column, allowing positively selected cells to retain in the column, until being flushed out into a microcentrifuge tube. Positively selected and isolated cells were then cultured in the media composition as described in Subchapter 3.1.3. Post 24 hours of isolation, cell morphology was observed under phase contrast microscope at 4x and 10x magnifications to ensure proper labeling as ‘epithelial’ or ‘fibroblast’. Successful

isolation and culture of primary epithelial cells were designated as 'Ep' and fibroblast cells as 'Fib'. Cells used in this project were:- 1) endometrial cancer cells : EC6-Ep, EC6-Fib, EC7-Fib, EC11-Fib, EC14-Fib, EC14-Ep; and 2) endometrial hyperplasia EH-Fib. All primary cultures used were at passage 10 and below to maintain the closest phenotype to its original excised tissue.

3.1.5 Observation of fibroblast marker using immunofluorescent staining

To observe if the isolated fibroblast cells expressed fibroblast marker alpha-smooth muscle actin (α-SMA), immunofluorescence staining was performed on all five isolated fibroblast cells used in this project: EC6-Fib, EC7-Fib, EC11-Fib, EC14-Fib and, EH-Fib. Round glass coverslips fitting 24-well plates were coated with fibronectin (BD Biosciences) and incubated for 1 hours before seeded with $5x10^3$ cells per well. On the following day, cells were fixed with 4% formaldehyde for 15 minutes. After washing with cold PBS, cells were permeabilized with 0.1% Triton X-100 for 1 hour at 4˚C. Then, cells were washed again with cold PBS and incubated with α-SMA primary antibody. The primary antibody was diluted at 1:100 ratio in PBS containing 0.1% Triton X-100. Incubation was done for 1 hour at 4˚C. Following washing, cells were then incubated with secondary antibody conjugated with TRIT-C. The secondary antibody was diluted at 1:1000 ratio in PBS containing 0.1% Triton X-100. The cells were incubated for 1 hour at 4˚C. After final washing, coverslips were mounted onto glass slides in DAPI medium and cells were viewed under a fluorescence microscope (Nikon ECLIPSE TI-S, NY, USA).

3.1.6 Determination of cell surface marker expression of isolated cells

To determine the purity of the isolated epithelial and fibroblast cells, the expression of specific cell surface markers were determined using flow cytometry. Isolated cultured cells were trypsinized and $1x10^6$ single cell suspension was blocked with 10% normal goat serum (Biowest, Nuaillé, France). The single cell suspension was stained with AlexaFluor 647 (AF647)-conjugated human epithelial cell adhesion molecule (EpCAM) or PE-conjugated human CD90 antibodies (Biolegend, CA, USA). Isotype controls used were AF647 mouse IgG2b,κ and PE mouse IgG1,κ, respectively. ECC-1 and T-HESC cell lines were used as the positive control for epithelial and fibroblast cells, respectively. BD FACSCanto II flow cytometer was used to analyze the stained cells and the results were viewed using FACS DiVa software (BD Biosciences).

3.1.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

To observe the relative gene expression levels of epithelial, fibroblast, and endometrial-related markers, qRT-PCR was performed on RNA extracted from isolated cells. Total RNA was extracted from cultured cells using TRIzol (Invitrogen, California, USA) and one µg RNA was converted into cDNA using DyNAmo cDNA synthesis kit (Finnzymes, Vantaa, Finland). The sequence of primers used to detect epithelial (EpCAM, E-cadherin, cytokeratin 8) and fibroblast cell markers (α-SMA and vimentin) are listed in Table 3.1. The sequence of primers used to detect endometrial-related markers (estrogen receptor alpha and beta, progesterone receptor, progesterone-associated endometrial protein and matrix metallopeptidase one and nine) are listed in Table 3.2. GAPDH gene was used as endogenous control for all the reactions. ABI StepOne Plus (Applied Biosystem, CA, USA) was used to perform this experiment. The total reaction time was set to 35 cycles. The qRT-PCR reaction components final concentration consists of 5x HOT FIREPol EvaGreen qRT-PCR Mix (Solis Biodyne, Tartu, Estonia), 10 pmol/µl forward and reverse primer, 10 ng/µl cDNA template, and

PCR grade H_2O. Specificity profile was observed during each qRT-PCR reaction to ensure primer-specific gene amplification occurs. Relative gene expression for each cell was then calculated using comparative CT or $2^{-\Delta\Delta CT}$ method.

Table 3.1 Primer sequences for cell characterization genes

Gene (Gene ID)	Primers (5'-3')		Annealing Temperature (Ta)	Source
EpCAM ID: 4072	F	AATGTGTGTGCG TGGGA	55˚C	(Lu et al., 2010)
	R	TTCAAGATTGGT AAAGCCAGT		
E-cadherin ID: 999	F	TTTGTACAGATG GGGTCTTGC	55˚C	(Burke, Cao, Irving, & Skumatz, 1999)
	R	CAAGCCCACTTT TCATAGTTCC		
Cytokeratin 8 ID: 3856	F	CTGGTGGAGGA CTTCAAGAAC	55˚C	(Traweek, Liu, & Battifora, 1993)
	R	GACCTCAGCAAT GATGCTGTC		
α-sma ID: 58	F	GACGAAGCACA GAGCAAAAGAG	55˚C	(Szeto et al., 2005)
	R	TGGTGATGATGC CATGTTCTATCG		
Vimentin ID: 7431	F	TGGCACGTCTTG ACCTTGAA	60˚C	(Y. Luo et al., 2006)
	R	GGTCATCGTGAT GCTGAGAA		

Table 3.2 Primer sequences for functional endometrial-related markers

Gene (Gene ID)	Primers (5'-3')		Annealing Temperature (Ta)	Source
Estrogen receptor-alpha ID: 2099	F	GGGAAGTATGG CTATGGAATCTG	58˚C	(Z. H. Chen et al., 2014)
	R	TGGCTGGACACA TATAGTCGTT		
Estrogen receptor-beta ID: 2100	F	AGCACGGCTCCA TATACATACC	60˚C	(J. Chen et al., 2015)
	R	TGGACCACTAAA GGAGAAAGGT		
Progesterone receptor ID: 5241	F	CGCGCTCTACCC TGCACTC	58˚C	(Huggins, Wong, Hankinson, & Vivo, 2006)
	R	TGAATCCGGCCT CAGGTAGTT		
Progesterone-associated endometrial protein ID: 5047	F	GAGATCGTTCTG CACAGATGG	58˚C	(Kavlashvili et al., 2016)
	R	CGTTCGCCACCG TATAGTTGAT		
Matrix metallopeptidase 1 ID: 4312	F	TTGTGGCCAGAA AACAGAAA	60˚C	(Song et al., 2011)
	R	TTCGGGGAGAA GTGATGTTC		
Matrix metallopeptidase 9 ID: 4318	F	TGTACCGCTATG GTTACACTCG	60˚C	(Dai et al., 2014)
	R	GGCAGGGACAG TTGCTTCT		

3.1.8 Cell lines

Human endometrial cancer cell lines ECC-1 (CRL-2923), HEC-1A (HTB-112) and immortalized human normal endometrial fibroblast cell line, T-HESC (CRL-4003) were purchased from American Type Culture Collection (MD, USA). ECC-1 was cultured in RPMI1640 media while HEC-1A was cultured in McCoy's 5A Medium Modified media. Both media were supplemented with 10% FBS and 1% penicillin/streptomycin. ECC-1 and HEC-1A cell lines were subcultured in a ratio of 1:3 every three days. T-HESC was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 3.1 g/L glucose and 1mM sodium pyruvate without phenol red (Life Technologies), supplemented with 1.5 g/L sodium bicarbonate (Life Technologies), 1% ITS+ Premix (BD Biosciences), 500 ng/ml puromycin (Sigma-Aldrich, MO, USA), and 10% charcoal/dextran treated fetal bovine serum (Biowest). T-HESC cell line was subcultured in a ratio of 1:4 every five days.

3.2 The effect of benign and cancer-associated fibroblasts secretion on endometrial cancer cell proliferation, migration and invasion *in vitro*

3.2.1 Preparation of fibroblast conditioned media

To reduce the influence of serum on cell proliferation, fibroblast cells were cultured in media with a low percentage of FBS. Optimization was done by culturing cells in media supplemented with 1%, 2%, 5% and 10% of FBS. Post 72 hours, fibroblast cells cultured with 1% FBS were observed to have low viability while those cultured with 5% showed similar confluency with 10% FBS. Fibroblast cells that were cultured with 2% FBS were observed to have attached and survived without exceeding cell confluency. Thus, in the subsequent conditioned media collection, fibroblast cells were first cultured in complete media to ensure cell attachment and stability. Post 24-hours, media was changed to RPMI1640 containing 2% FBS. After 72 hours of culture, conditioned medium from each fibroblast cell was collected and centrifuged in Amicon ultra centrifugal filter unit (Merck Millipore, MA, USA). Centrifugation was done at 5000 x *g* for 1 hour at 4˚C. Protein levels in each concentrated media were quantified using Bradford assay and then stored in the freezer at -20˚C.

3.2.2 Assessment of cell viability by methyl thiazolyl tetrazolium (MTT) assay

Cell viability upon treatment with fibroblast concentrated media was assessed using MTT assay. Epithelial cells ECC-1, HEC-1A, EC6-Ep, and EC14-Ep cells were seeded at a concentration of $5x10^3$ cells per well in a 96-well plate for 24 hours. The epithelial cells were then treated with the concentrated fibroblast media at concentrations of 0.1 µg/µL, 0.5 µg/µL, 1 µg/µL and 2 µg/µL. Post 72 hours treatment, 100 µL of MTT solution at a concentration of 5 mg/ml was added. Following incubation for 4 hours at 37ºC in 5% CO_2 incubator, the formed purple crystal formazan was solubilized using 100 µL of 10% sodium dodecyl sulfate. The solubilization step was performed for an additional 4 hours on a shaker. After ensuring all the purple crystal formazan had solubilized, the absorbance value was measured using Spectramax M3 multimode plate reader (Molecular Devices, CA, USA) at a wavelength of 575 nm with 650 nm reference value.

3.2.3 Assessment of cell proliferation using 5-bromo-2-deoxyuridine (BrdU) assay

BrdU cell proliferation assay (Cell Signaling Technology, MA, USA) was used to quantify cell proliferation rate in addition to measuring cell viability *via* MTT assay. BrdU assay detects BrdU incorporation into cellular DNA during cell proliferation. Similar to the set up for MTT assay, ECC-1, HEC-1A, EC6-Ep, and EC14-Ep were seeded at a concentration of $5x10^3$ cells per well in the 96-well plate. Cells were treated with conditioned media collected from four CAFs (EC6, 7, 11, 14-Fib) and from T-HESC cell line. The epithelial cells were treated with the conditioned media at a concentration of 0.25 and 2 µg/µl for 72 hours. The BrdU incorporation was terminated with the addition of 10 µl of 1X BrdU solution. The treated epithelial cells were incubated for another 24 hours in an incubator. Next, the cells were fixed with the Fixing/Denaturing Solution that was provided with the kit for 30 minutes at room temperature. This was

followed by incubation with of 100 µl/well of 1X detection antibody solution for 1 hour at room temperature. After washing, 100 µl/well of 1X HRP-conjugated secondary antibody were added for 30 minutes at room temperature. Post-washing, 100 µl of TMB Substrate were added to each well. Once color changes occurred and stabilized, 100 µl of STOP solution were added to terminate the assay and plates were read within 30 minutes. Results were read using Spectramax M3 multimode plate reader (Molecular Devices) at an absorbance of 450 nm with a reference wavelength of 0 nm.

3.2.4 Assessment of cell motility and invasion using scratch-wound and transwell assay

To observe the migratory effect of epithelial cells in response to fibroblasts secretion, scratch-wound and transwell assay were performed. For the scratch-wound assay, ECC-1 cells were seeded at a concentration of $5x10^4$ cells per well in 24-wells plate before treated with serum-free media, complete media containing 10% FBS or media containing 1 µg/ml secretion from T-HESC, EC7-Fib or EC11-Fib. A fine scratch was made in each well using a 10 µl pipette tip. The treated epithelial cells were observed every day for 48 hours under phase contrast microscope for signs of cell junction migration to close the scratch gap.

For the transwell assay, $1x10^3$ ECC-1 cells were serum-starved for 24 hours before seeded in the transwell inserts per well. The bottom layer of the wells contained either serum-free media, complete media containing 10% FBS or 1 µg/ml condition media from T-HESC, EC7-Fib or EC11-Fib. The set-up was incubated in 5% CO_2 at 37ºC for 24 hours. Non-migrated cells at the top of the membrane were gently removed with cotton swabs and the migrated cells at the bottom layer of the membrane were fixed with 4% formaldehyde/PBS for 1 minute. Then, the inserts were stained with 1% crystal violet/ethanol for 5 minutes at room temperature. The stained membranes were then

washed by lowering the inserts into a 24-well plate containing distilled water for 5 minutes. This washing step was repeated for three times. Membranes of the transwell were cut out and mounted on glass slides using mounting media. A bright-field light microscope was used to observe the slides and to enumerate the number of positively stained cells. Representative images of five random fields within each insert were captured. Cell numbers from these five fields were counted, averaged and graphed.

Invasion capabilities of ECC-1 cells in response to fibroblasts secretion were determined using a similar transwell method as described above. Instead of using the transwell as provided, transwell inserts were coated with 100 µl of 1X Basement Membrane Extract (BME Matrigel) and incubated in 5% CO_2 at 37ºC for 24 hours. While similar to cell migration, cell invasion, however, requires the cells to enzymatically degrade the extracellular matrix to migrate and be established at a new location. Hence, ECC-1 cells at a concentration of $1x10^3$ were serum-starved for 24 hours before seeded on the transwell inserts. The bottom layer of the wells contained either serum-free media, complete media containing 10% FBS or 1 µg/ml condition media from T-HESC, EC7-Fib or EC11-Fib. The set-up was incubated in 5% CO_2 at 37ºC for 24 hours. Non-migrated cells and the remains of the BME Matrigel at the top layer of the membrane were gently removed with cotton swabs. The migrated cells at the bottom layer of the membrane were fixed with 4% formaldehyde/PBS for 1 minute. Then, the inserts were stained with 1% crystal violet/ethanol for 5 minutes at room temperature. The stained membranes were washed by lowering the inserts into a 24-well plate containing distilled water for 5 minutes. Finally, membranes of the transwell were cut out and mounted on glass slides using mounting media. A bright-field light microscope was used to observe the slides and to enumerate the number of positively stained cells. Representative images of five random fields within each insert were captured. Cell numbers from these five fields were counted, averaged and graphed.

3.3 Determination of soluble factors secreted by CAFs and their underlying mechanism of action in promoting EC proliferation

3.3.1 Identification and measurement of cytokine levels (Cytokine Array)

Raybiotech Quantibody® Human Cytokine Array (Raybiotech, GA, USA) was used to detect the levels of 20 cytokines secreted by normal fibroblasts (T-HESC) and cancer-associated fibroblasts (CAFs of EC6-Fib, EC7-Fib, and EC11-Fib). This technology employs an array-based multiplex ELISA system to simultaneously quantify 20 cytokines in a high-throughput manner with a sample amount of lesser than 50 µl. T-HESC cell line, EC6-Fib, EC7-Fib, and EC11-Fib were cultured in their respective complete media for 24 hours before cultured in media containing 2% FBS. Media containing only 2% FBS in the absence of cells were used as negative control. Post 72 hours, conditioned medium from the cells were collected and centrifuged in Amicon ultra centrifugal filter unit (Merck Millipore, MA, USA) at 5000 x *g* for 1 hour at 4˚C. The negative controls were also concentrated in a similar manner. Freshly collected 100 µg proteins from each fibroblast secretions were added into respective array well and incubated overnight at 4°C in a rotary movement. Each well was washed, incubated with reconstituted antibody cocktail for 2 hours at room temperature, washed again, before addition of Cy3-conjugated streptavidin. Following additional extensive washing, the fluorescence signal was measured using the Agilent High-Resolution Microarray Scanner (C-model) (Agilent Technologies, CA, USA) and raw signal data were extracted from TIFF image with GenePixPro 6.1 (Molecular Devices) before analyzed with Q-Analyzer (Raybiotech). Cytokine levels from each fibroblast secretion were compared with negative control. The fluorescence intensity from quadruplicate array wells was averaged and graphed.

3.3.2 Measurement of cytokine levels using enzyme-linked immunoabsorbent assay (ELISA)

To determine and quantitate the levels of selected cytokines in a larger number of fibroblasts samples, ELISA techniques were utilized. The selected cytokines were; growth regulated oncogene-alpha (#ELH-GRO-alpha), regulated on activation, normal T expressed and secreted (#ELH-RANTES), vascular endothelial growth factor (#ELH-VEGF) from Raybiotech, interleukin-6 (MAX™ Deluxe human IL-6) and monocyte chemotactic protein 1 (MAX™ Deluxe MCP-1/CCL2) from Biolegend (CA, USA). Capture antibody, diluted to 1:100 was coated on the provided 96-well plates and left overnight. The next day, plates were washed three times with wash buffer and blocked with blocking buffer for 2 hours. Post washing, conditioned media from ten different CAFs and benign endometrial fibroblasts were incubated for another 2 hours. The fibroblast secretions used were as follows; CAFs: EC6,7,11,14,16,22,24,25,26,27-Fib, benign fibroblasts: T-HESC cell line, normal endometrium 1 and 2 (NE1,2), endometrial hyperplasia 1 and 4 (EH1,4), endometrial fibroid (EF3,8), endometrioma (EM6,10) and endometrial cyst (Cyst 5). Post incubation, the detection antibody provided with each ELISA kit was diluted to a ratio of 1:100 and added to the plate. The plates were incubated for another 1 hour. Following washing, the secondary antibody was then diluted to a ratio of 1:1000 and added to the plate. The plates were then incubated for 30 minutes. TMB substrate was added and the solution in each well was observed until there were color changes. Finally, STOP solution was added to end the experiment. The plate was read using Spectramax M3 Multimode plate reader (Molecular Devices) at an absorbance value of 450 nm and 0 nm as a reference wavelength. Cytokines concentrations were determined by plotting a standard curve on log-log axis graph with cytokine concentration on the c-axis and absorbance on the y-axis. After getting the best-fit line through the

standard points, value for each sample was determined. Assay sensitivity was between 2 pg/ml to 10 pg/ml.

3.3.3 Determination of cytokine activity on EC proliferation

GRO-α and IL-6 were highly secreted by CAFs compared to secretion from benign endometrial fibroblasts. To determine if these two cytokines were responsible for EC cell proliferation, inhibition experiments using neutralizing antibodies were performed. ECC-1 cells were cultured in the presence of EC7-Fib and EC11-Fib conditioned media and treated with either purified anti-human IL-6 antibody (Biolegend) or GRO-α neutralizing antibody (R&D Systems, MN, USA) with their respective isotype controls. Treatment was done at concentrations of 0.25 to 1 µg/ml for 72 hours. Cell viability was then examined using MTT assay. ECC-1 cells were also treated with the neutralizing antibodies and isotype control without the presence of conditioned media.

ECC-1 cells cultured in complete medium were also treated with GRO-α (R&D Systems) or IL-6 (Biolegend) recombinant proteins to mimic the effect of CAFs-secreted cytokines on cell proliferation. ECC-1 cells were treated with the recombinant proteins and its isotype controls at doses from 0.0625 mg/ml to 2 mg/ml for 72 hours. Cell viability was assessed using MTT assay.

3.3.4 Extraction of total protein and Western blotting

Western blotting method was used to observe if treatment of CAFs conditioned media on EC cell could result in the upregulation of proteins in the intracellular signaling pathways. ECC-1 cells were treated with either 1 µg/ml fibroblast conditioned media or with a combination of 1 µg/ml CAFs conditioned media with neutralizing antibodies. Post-72 hours, cells were washed with cold PBS. Cell lysates were collected using chilled lysis buffer containing 1% Triton-X, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 1X phosphatase inhibitor and a 1X protease inhibitor. The protein concentration of lysates was determined using Bradford assay. Lysates containing 30 µg proteins were then run on 10% SDS-PAGE gels at 100V for 90 minutes and transferred to polyvinylidene difluoride membranes at 100V for 60 minutes. Membranes were then incubated with 5% (weight per volume) nonfat dry milk (Cell Signaling), prepared in 1X PBS-Tween20 0.1% for 1 hour at room temperature. Then, membranes were washed for 5 minutes with 1X PBS-Tween20 0.1%. The washing procedure was repeated for five times. The membranes were then incubated with primary antibody overnight at 4ºC with gentle shaking. Post 24 hours of incubation with primary antibody, the membranes were washed with 1X PBS-Tween20 0.1% for 5 minutes. The washing step was repeated for five times. Then, the membranes were incubated with respective secondary antibodies for 1 hour at room temperature with gentle shaking. Secondary antibodies were diluted at 1:1000 ratio of 5% (weight per volume) nonfat dry milk (Cell Signaling), prepared in 1X PBS-Tween20 0.1%. Post-incubation and washing, bands were visualized using ECL prime Western blotting detection reagent (Amersham, GE Healthcare Lifesciences, Sweden) using the chemiluminescent exposure at 30 seconds viewed through UVP BioSpectrum Imaging System (UVP, CA, USA). Primary antibodies used were, rabbit anti-human serine/threonine kinase Akt, phosphorylated-Akt, extracellular signal-regulated kinases (Erk), phosphorylated-Erk, Janus kinase 3 (JAK3), phosphorylated-JAK3, signal

transducer and activator of transcription 3 (STAT-3), phosphotylated-STAT3, interleukin-6 receptor alpha (IL-6Rα), glycoprotein-130 (gp-130), c-Myc and β-actin monoclonal antibodies (Cell Signaling Technology). All antibodies were diluted in 5% (weight per volume) solution of bovine serum albumin (Sigma-Aldrich) reconstituted with 1X PBS-Tween20 0.1%.

3.3.5 Observation on activation of IL-6 downstream signaling pathway

To determine the functional roles of activated proteins from the Western blotting experiment, epithelial cells were treated with pathway specific molecular inhibitors in the presence of fibroblast conditioned media. U0126 and LY294002 (Cell Signaling Technology) were used to specifically inhibit for Erk and Akt pathways while STATTIC and AD412 (Santa Cruz Biotechnology, CA, USA) were used to target JAK3 and STAT3 pathways. ECC-1, HEC-1A, EC6-Ep and EC14-Ep cells were seeded at the concentration of $1x10^3$ cells per well on a 96-well plate. In the presence of either 1 µg/ml EC6-Fib or EC11-Fib conditioned media, cells were treated with different doses of inhibitor; 5 and 10 µM for U0126 or 10 and 50 µM for LY294002 for 72 hours. Similarly, ECC-1 and EC6-Ep cells were treated with 10, 50 and 100 µM of STATTIC or AD412 in the presence of either 1 µg/ml EC7-Fib or EC11-Fib conditioned media. Cell viability was then assessed using MTT assay.

3.3.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

To evaluate the relative mRNA expressions of IL-6-pathways related genes, the qRT-PCR method was used. Total RNA was extracted from ECC-1 cells and ECC-1 cells treated with 1 µg/ml of either EC6-Fib, EC7-Fib, EC11-Fib or EC14-Fib conditioned media using TRIzol (Invitrogen, California, USA). RNA (1 µg) was converted into cDNA using DyNAmo cDNA synthesis kit (Finnzymes, Vantaa, Finland). Sequence of primers used to detect interleukin-6 receptor alpha (IL6-Rα), glycoprotein130 (gp130), c-Myc,

suppressor of cytokine signaling 3 (SOCS-3), TIMP metallopeptidase inhibitor 1 (TIMP-1), proto-oncogene serine/threonine-protein kinase (PIM-1), nuclear factor kappa-light-chain-enhancer of activated B cells 1 and 2 (NF-κB-1 and 2) are listed in Table 3.3. GAPDH gene was used as endogenous control for all the reactions. ABI StepOne Plus (Applied Biosystem, CA, USA) was used to perform this experiment. The total reaction time was set to 35 cycles. The qRT-PCR reaction components final concentration consists of 5x HOT FIREPol EvaGreen qRT-PCR Mix (Solis Biodyne, Tartu, Estonia), 10 pmol/µl forward and reverse primer, 10 ng/µl cDNA template, and PCR grade H_2O. Specificity profile was observed during each qRT-PCR reaction to ensure primer-specific gene amplification occurs. Relative gene expression for each cell was then calculated using comparative CT or $2^{-\Delta\Delta CT}$ method.

Table 3.3 Primer sequences for IL-6 activated genes

Gene	Primers (5'-3')		Annealing Temperature (Ta)	Source
IL-6Rα ID: 3570	F	ATTGCCATTGTTCTGAGGT	55˚C	(Jiang, Yang, L.Elliott, & Head, 2011)
	R	TAGTCTGTATTGCTGATGTC		
gp130 ID: 3572	F	ATTTGTGTGCTGAAGGAGGC	55˚C	(Kulikov, Sinyakova, Naumenko, Bazovkina, & Popova, 2010)
	R	AAAGGACAGGATGTTGCAGG		
c-Myc ID: 4609	F	AATGAAAAGGCCCCCAAGGTAGTTATCC	60˚C	(Zhao, Wang, & Qin, 2014)
	R	GTCGTTTCCGCAACAAGTCCTCTTC		
PIM-1 ID: 5292	F	AACTGGTCTTCCTTTTTGGTT	55˚C	(Zemskova, Sahakian, Bashkirova, & Lilly, 2008)
	R	TACCATGCCAACTGTACACAC		
SOCS-3 ID: 9021	F	AGACCTTCAGCTCCAAAAGC	55˚C	(Sriram, Benkovic, Hebert, Miller, & O'Callaghan, 2004)
	R	ACCAGCTTGAGTACACAGTCG		
TIMP-1 ID: 7076	F	CTGTTGTTGCTGTGGCTGATA	55˚C	(Collette, Maheux, Mailloux, & Akoum, 2006)
	R	CCGTCCACAAGCAATGAGT		
NF-κB-1 ID: 4790	F	AGGAAGAAAATGGCGGAGTT	55˚C	(Y.-E. Kim et al., 2008)
	R	GCATAAGCTTCTGGCGTTTC		
NF-κB-2 ID: 4791	F	CGTGAAAGACCCTCCTGTTC	55˚C	(David E. Nowak, Tian, & Brasier, 2005)
	R	AGAGCGAGATCCGGAGTTG		

3.3.7 Establishment of cell lines with low c-Myc expression

To determine if c-Myc gene expression contributed to CAFs-mediated EC proliferation, EC cells were transfected with short hairpin RNA (shRNA) vector targeting c-Myc or its scrambled control (Dharmacon, CO, USA). Briefly, $1x10^4$ ECC-1 cells in 24-well plate were cultured in complete media for 24 hours to achieve 80% confluency. The multiplicity of infection (MOI) 5 and 10 of the shRNA was tested on the EC cells. Since the lentiviral is tagged with fluorescence labeling, transfected cells were observed daily under fluorescence microscope for 48 hours to ensure maximum transfection efficiency was achieved. Both MOI 5 and 10 showed successful integration into the cell. Puromycin-resistant clones were then positively selected in complete media containing of 1 μg/mL puromycin (Sigma-Aldrich). ECC-1 cells were then continued to be cultured in complete media containing puromycin (1 μg/ml) to ensure constant selection of positively transfected cells. Knockdown of the c-Myc gene was verified by determining the mRNA and protein levels using RT-PCR and Western blotting, respectively.

3.3.8 Determination of CAFs secretion effect on EC cells with low c-Myc expression

The proliferation of EC cells with a low c-Myc expression upon treatment with CAFs secretion was assessed using cell viability assay. Individual ECC-1 clones that continuously express low c-Myc expression and scrambled shRNA construct were subjected to varying concentration of CAFs conditioned media for 72 hours. Cell viability was assessed post-treatment using MTT assay as described in Subchapter 3.2.2.

3.3.9 Establishment of in vivo mouse model for EC tumor xenograft

To evaluate the contribution of fibroblasts to the growth of human endometrial cancer *in vivo*, ECC-1 cells were implanted into athymic nude mice, with and without CAF cells. Athymic female nude mice purchased from Taconic (BALB/c, 4 weeks old, n=7 per group) (Cambridge, MA, USA) were used. Animals were maintained under specific pathogen-free conditions and were fed with Teklad Global 19% protein extruded rodent diet. This special diet was chosen to reduce the interference by estrogen expression during *in vivo* imaging of the mice tumors (Harlan Laboratories, WI, USA). IACUC committee of University Malaya (FAR/27/07/2012/IC (R)) approved all mice procedures.

In order to capture the image of endometrial and fibroblast cells *in vivo*, green fluorescent protein (GFP) and red fluorescent protein (RFP) T-antigen lentiviral particles (Gentarget, CA, USA) were transfected into EC11-Fib and ECC-1 cells, respectively. The selection was maintained by supplementing the cultures with puromycin with a final concentration of 1 µg/ml (Sigma-Aldrich) for a period of 2–8 weeks. ECC-1 cells transfected with RFP (5,000 cells) alone or in combination with fibroblast cells transfected with GFP (20,000 cells) were injected subcutaneously into the right flank of mice. Fibroblast and epithelial cells were also injected separately as controls. Tumor size was measured twice every week, using digital calipers. Tumor volume was calculated according to equation: tumor volume = length (L) x width (W) ^{2} / 2. Additionally, the fluorescently labeled tumors were imaged weekly using Carestream MS FX-PRO small rodent imager (Molecular Imaging, CT, USA). The experiment was terminated when the tumor size of mice in the control group reached an average of 1000 mm^3. All mice were euthanized at the end of the experiment using CO_2 chamber method. Tumors were excised and imaged using a digital camera.

In order to observe the effect of c-Myc in CAFs mediated tumor growth *in vivo*, RFP-tagged ECC-1 cells were transfected with shRNA viral particle targeting c-Myc gene and shRNA viral particle with a scrambled c-Myc gene sequence. The selection was further maintained by supplementing the cultures with puromycin with a final concentration of 1 µg/ml (Sigma-Aldrich) for a period of 2 weeks. Athymic female nude mice (BALB/c, 4 weeks-old, n=8 per group) were obtained from Taconic (Cambridge, MA, USA). Animals were subcutaneously injected with cells according to the following groups as described in Table 3.4.

Mice were monitored every other day and tumor size was measured twice every week, using digital calipers. Mice were also imaged once a week using Carestream MS FX-PRO small rodent imager (Molecular Imaging). The experiment was terminated when the tumor size of mice in the control group reached an average of 1000 mm^3. All mice were euthanized at the end of the experiment using CO_2 chamber. Tumors were excised and imaged using a digital camera.

Table 3.4 Cell combinations according to mice groups

Groups	Cell combinations
Group 1	ECC-1 cells only
Group 2	EC11-Fib cells only
Group 3	ECC-1 cells combined EC11-Fib cells (1:4 ratio)
Group 4	ECC-1 cells with c-Myc scrambled shRNA sequence
Group 5	ECC-1 cells with c-Myc scrambled shRNA sequence combined with EC11-Fib cells (1:4 ratio)
Group 6	ECC-1 cells with c-Myc knockdown shRNA sequence
Group 7	ECC-1 cells with c-Myc knockdown shRNA sequence combined with EC11-Fib cells (1:4 ratio)

3.4 Histopathological and immunohistochemical analysis

To observe the activation of c-Myc and Ki67 protein in mice tumors from the experiment described in Subchapter 3.3.9, immunohistochemical analysis was carried out. Tumor xenografts were removed from the nude mice, post-sacrifice. Collected tissues were fixed overnight in neutral buffered formalin prior to paraffin wax processing and embedding. Tissue sections were cut at 4 µm size and fixed on positively-charged SuperFrost™ Plus microscope slides (Thermo Fisher Scientific, MA, USA). Wax on the tissues was deparaffinized in the following sequences and duration; 3 times in xylene for 2 minutes each, 3 times in absolute alcohol for 2 minutes each, 1 time in 70% alcohol for 2 minutes. Following that, endogenous peroxidase present on tissues was blocked with 0.3% hydrogen peroxide for 10 minutes. Antigen retrieval method was done by placing the slides in sodium citrate buffer and heating at 100˚C for 30 minutes. Tissues were then incubated with either c-Myc (Cell Signaling Technology) or Ki-67 (Biolegend) antibodies for 1 hour at room temperature. A labeled streptavidin-biotin system with a horseradish peroxidase label (DAKO Corp., CA, USA) was used to detect the primary antibodies and visualized by incubation with 3,3'-diaminobenzidine chromogen and hydrogen peroxide substrate (DAKO Corp., CA, USA) for 10 minutes. The slides were then counterstained with hematoxylin and mounted with mounting media (Sigma-Aldrich). Images were viewed and captured using Nikon Eclipse 2000 and Nikon ES-Fi1, respectively (Nikon GamBH, Dusseldorf, Germany).

Similarly, to observe activation of IL-6 signaling markers in human endometrial tissues, eight benign and nine endometrial cancer tissues removed during debulking surgery were used for immunohistochemical analysis was done using the method described above. Primary antibodies used were IL-6Rα and gp130 (Santa Cruz Biotechnology), p-STAT3 and c-Myc (Cell Signaling Technology).

3.5 Inhibition of IL-6 pathway as treatment for endometrial cancer

3.5.1 Attenuation of CAFs-mediated EC proliferation with IL-6 pathway inhibitors

To observe if key molecules in the IL-6 downstream signaling pathway could be targeted with therapeutic agents, EC cells were treated with three inhibitors to reverse the CAFs-mediated EC proliferation. The three agents used were; rapamycin (Clearsynth Labs, Mumbai, India) which inhibits mTOR downstream of PI3K pathway, raloxifene hydrochloride, and bazedoxifene acetate (Clearsynth Labs) which targets IL-6 receptors, IL-6Rα and gp130 respectively. All three inhibitors were solubilized to a final concentration of 1% DMSO.

EC cell lines (ECC-1 and HEC-1A) and EC epithelial primary cultures (EC6-Ep and EC14-Ep) were treated with increasing doses of rapamycin from 0.25 to 2 μM. EC cell lines were also treated with raloxifene hydrochloride and bazedoxifene acetate with increasing doses from 0.195 to 100 μM. All EC cells that were treated with inhibitors were performed with and without the addition of 1 μg/ml CAFs conditioned media. To determine the specificity of IL-6 receptor inhibitors, ECC-1 cells were treated with a combination of IL-6 recombinant protein at 1 μg/ml and each inhibitor. Cell viability was assessed post 72 hours of treatment using MTT assay.

3.5.2 Evaluation of the action of IL-6 pathway related molecular inhibitors

To observe if rapamycin, raloxifene hydrochloride, and bazedoxifene acetate affect EC cell proliferation *via* induction of apoptosis in the presence of CAFs conditioned media, annexin V-PE/7-AAD double staining assay (BD Biosciences) was performed. ECC-1 cells with $1x10^3$ cell concentration were treated with either 2 µM of rapamycin, or with the IC_{50} values of raloxifene hydrochloride and bazedoxifene acetate. This experiment was performed with and without the presence of 1 µg/ml EC11-Fib conditioned media, for 72 hours. ECC-1 cells treated with either raloxifene or bazedoxifene acetate in the presence of IL-6 recombinant protein was included as a positive control in this set-up.

Treated ECC-1 cells and its spent media were collected into 15 ml tubes and centrifuged at 1500 x *g* for 10 minutes. The resulting cell pellets were washed twice with cold PBS and the cells were resuspended in 1X Binding buffer at a concentration of $1x10^6$ cells/ml. A solution containing 100 µl of the cell suspension ($1x10^5$ cells) were transferred to a 5 ml culture tube. Cells were then stained with 5 µl of Annexin V-PE and 5 µl of 7-AAD (BD Bioscience). Following gentle vortex and incubation for 15 minutes at room temperature in the dark, 400 µl of 1X Binding buffer were added to each tube. Evaluation on the percentages of apoptotic cells was done within 1 hour of staining using FACS Canto II flow cytometer (BD Bioscience) and results were analyzed using FACS DiVa software (BD Bioscience).

3.6 Statistical analysis

Statistical analysis that assessed the differences between means of control and test group was performed using Student's *t*-test while those differences among group means were done using analysis of variance (ANOVA). All statistical analysis for this project was performed using GraphPad Prism version 5 (GraphPad Software Inc, CA, USA). A *P*-value <0.05 was considered to be statistically significant.

CHAPTER 4: RESULTS

4.1 Establishment and characterization of epithelial and fibroblast cells from human endometrial tissues

Four human endometrial cancer tissues (EC6, EC7, EC11, and EC14) and one endometrial hyperplasia tissue (EH, as control tissue) were collected from patients undergoing surgeries. All five tissues were subjected to hematoxylin and eosin (H&E) staining. Two different areas from each tissue slide are shown in **Figure 4.1**. H&E staining for all five tissues showed glandular-like features of the endometrium lined with epithelial cells. Fibroblast cells can be seen adjacent to the epithelial cells lined-endometrium glands.

Staining of endometrial hyperplasia tissue displayed normal looking cuboidal epithelium in a single layer (black arrow) with dilated hyperplastic glands (white arrowhead). Stroma surrounding the glands could also be observed **(Figure 4.1 A)**.

In contrast, staining of endometrial cancer tissues showed irregular glandular structures displaying an increased cell density and clustered layers of malignant cells. The endometrial cancer tissues staining also displayed appearance of multiple lumens and reduced population of the stromal cell, a feature which was not observed in the hyperplasia tissue. The glandular structure of endometrial cancer tissues also appeared to be condensed and arranged narrowly **(black arrows, Figure 4.1 B, C)**. Furthermore, a total loss of glandular structure with significant increase in cell density was also observed **(black arrows, Figure 4.1 D, E)**.

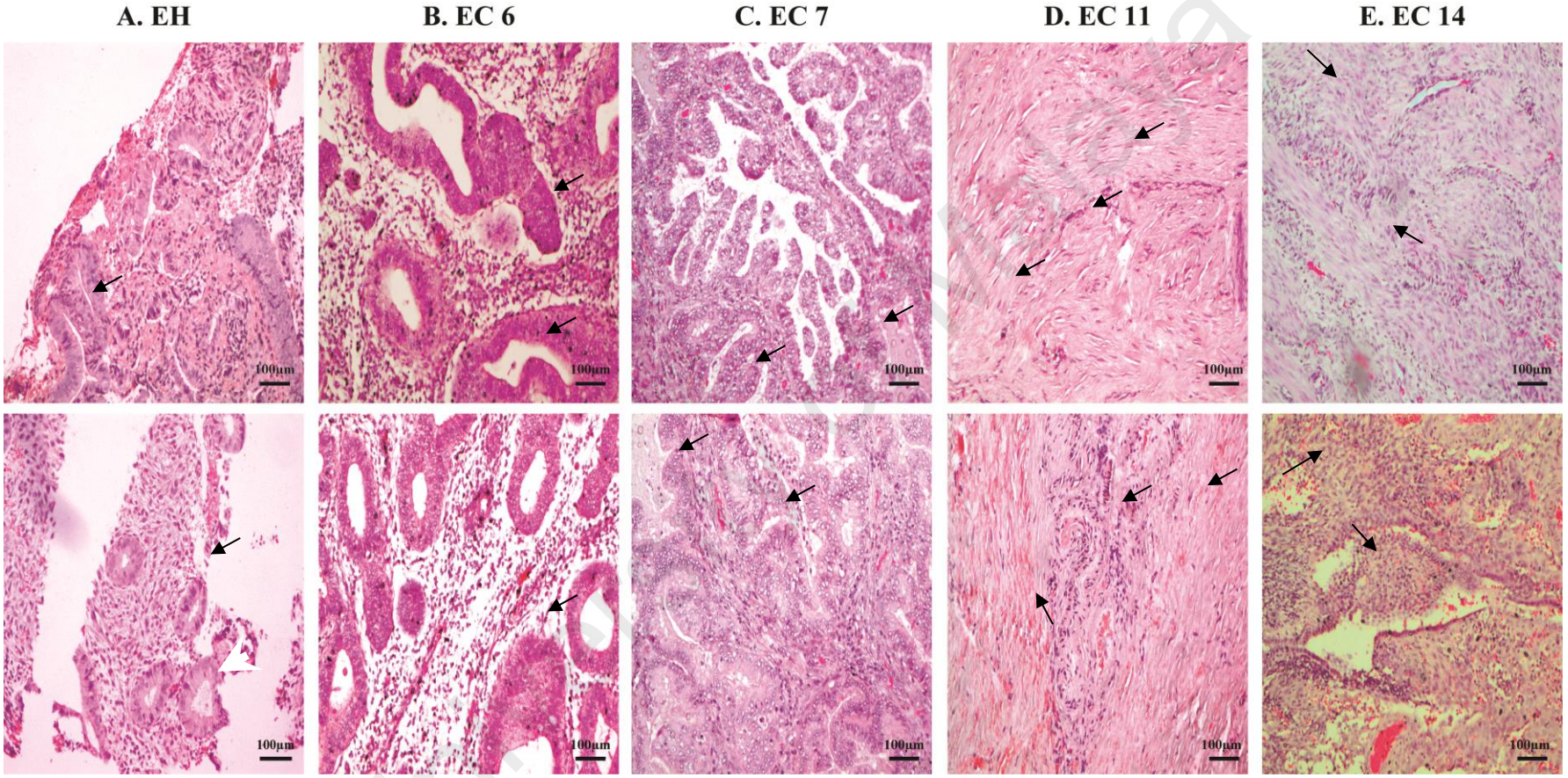


Figure 4.1. Hematoxylin & eosin (H&E) staining of human endometrial tissues.
Endometrial hyperplasia tissue (A) showed cuboidal epithelium (black arrow) with dilated glands (white arrowhead). Four endometrial cancer tissues (B-E) showed a lack of glandular structures with scattered cell bundles (black arrows). Images displayed are representative of two different areas of each slide. Magnification: 10x, scale bar: 100 µm.

To establish primary epithelial and fibroblast cells from endometrial tissues, the four human endometrial cancer (EC) tissues (EC6, EC7, EC11, and EC14) shown in **Figure 4.1** were digested with collagenase. Cell isolation using magnetic beads conjugated with anti-CD326 and CD90 antibodies was done to isolate the epithelial and fibroblast cells respectively. The isolated cells were designated as 'Ep' for the epithelial and 'Fib' for the fibroblast cell populations.

To determine the outcome of the primary cell isolation, three cell lines were used as positive cell controls, which were ECC-1, HEC-1A, and T-HESC. ECC-1 and HEC-1A are Type 1 human endometrial adenocarcinoma cell lines with epithelial origin and T-HESC is an immortalized normal human endometrial fibroblast cell line isolated from a benign endometrium. ECC-1 and HEC-1A cell lines displayed a monolayer sheet of evenly growing cuboidal-appearance cells with regular formations. Isolated primary epithelial cell (EC6-Ep and EC14-Ep) showed similar morphology to that of ECC-1 and HEC-1A with a tendency to grow in homogeneous colonies **(Figure 4.2)**.

The positive control cell line for fibroblast cells, T-HESC, displayed elongated filament-like features that are also termed as 'bipolar-looking' due to the two different ends of the cell structure. All five isolated fibroblast cells exhibited similar morphology with T-HESC cells **(Figure 4.3)**.

There was a clear difference in morphology between the elongated fibroblast cell and homogeneously distributed sheets of epithelium cells in our cultures. Using magnetic beads coupled with cell type-specific antibody, epithelial and fibroblast cells population were successfully separated into individual cultures that grew well and were expanded thereafter.

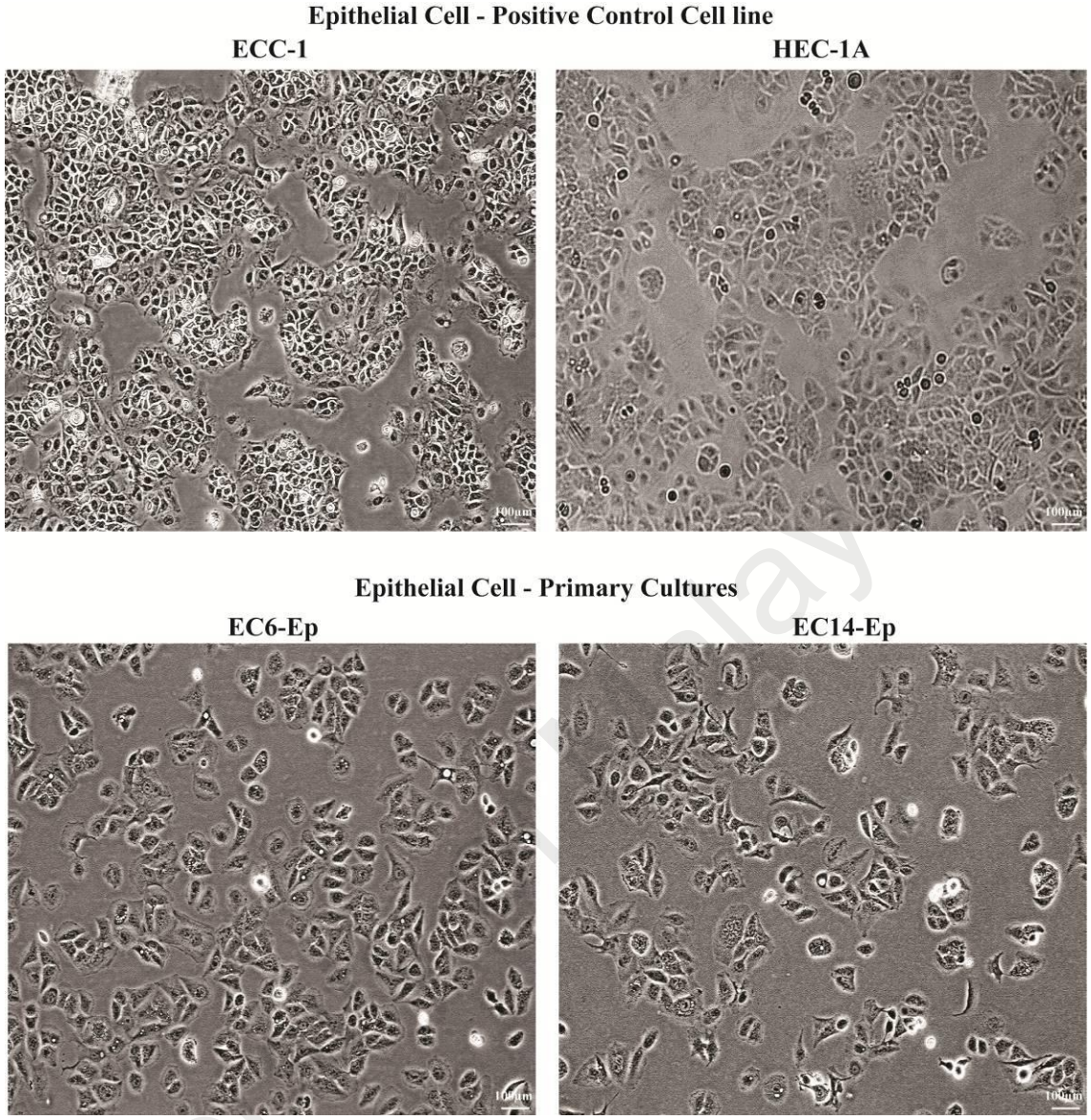


Figure 4.2. Establishment of epithelial cells from primary human endometrial tissues cultures.
Phase contrast images show ECC-1 and HEC-1A which are human epithelial endometrial cancer cell lines, as the positive control epithelial cells. EC6-Ep and EC14-Ep are the isolated epithelial primary cultures from endometrial cancer tissues EC6 and EC14. Magnification: 10x, scale bar: 100µm.

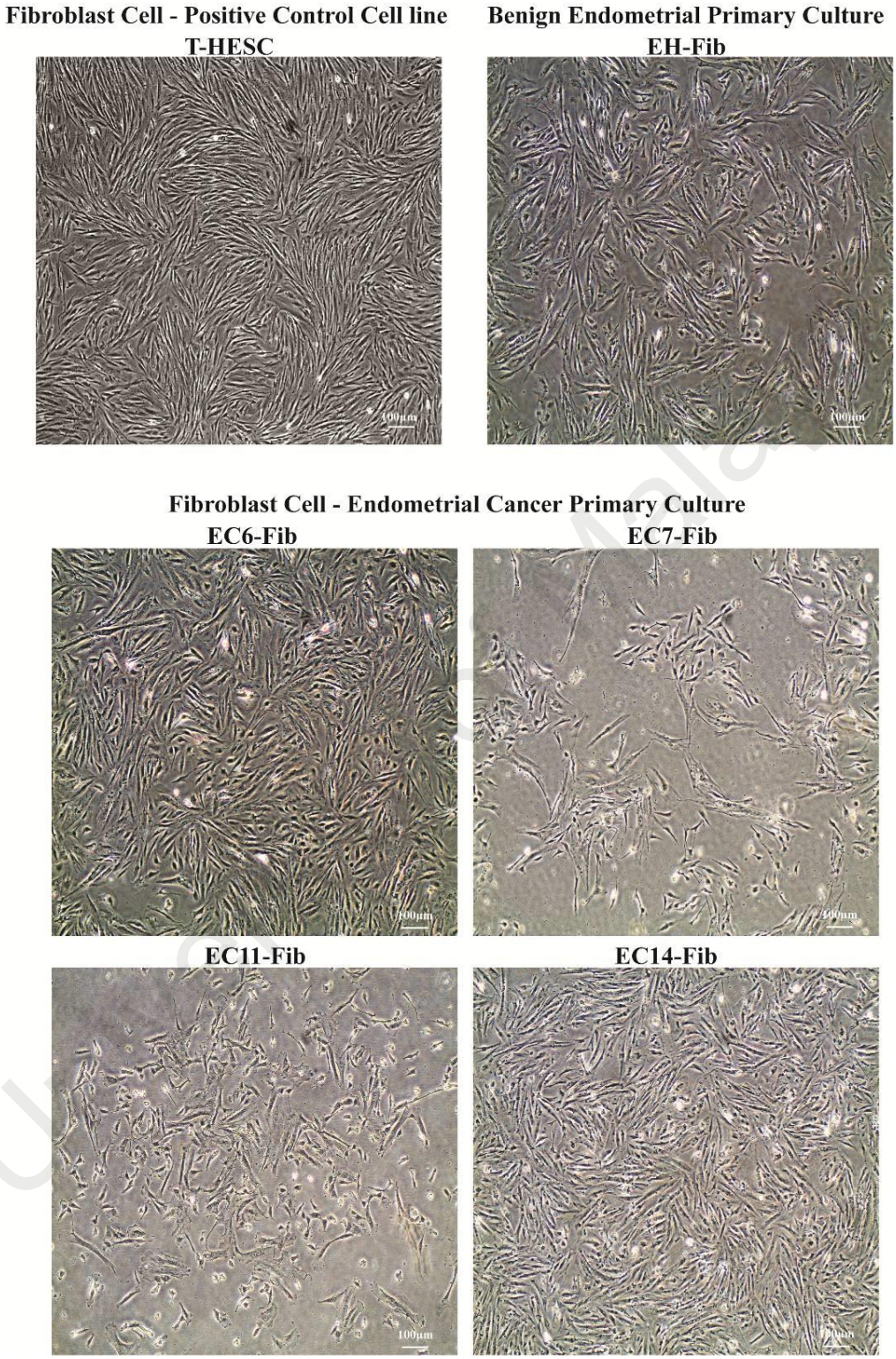


Figure 4.3. Establishment of fibroblast cells from primary human endometrial tissues cultures.

Phase contrast images T-HESC which is the benign human endometrial fibroblast cell line, as the positive control fibroblast cell. EH-Fib is the fibroblast cell isolated from benign endometrial hyperplasia tissue. EC6-Fib, EC7-Fib, EC11-Fib, and EC14-Fib are the isolated fibroblast primary cultures from endometrial cancer tissues EC6, EC7, EC11, and EC14. Magnification: 10x, scale bar: 100µm.

To characterize the isolated epithelial and fibroblast cultures, several validation assays were performed. Firstly, to detect if EpCAM or CD90 antibodies, which are cell-specific markers for epithelial and fibroblast cells respectively, are present in the isolated primary cultures, flow cytometry method was carried out. This method also allowed for quantification on the percentages of EpCAM and CD90 markers in our isolated primary cultures. Cells were stained with Alexa Fluor 647-conjugated EpCAM and PE-conjugated CD90 antibodies.

Positive controls endometrial cancer epithelial cell line, ECC-1 showed 98% positive staining for EpCAM with no cells expressing CD90 marker. Similarly, EC6-Ep and EC14-Ep showed 54.6% and 54.9% EpCAM expression with minimal CD90 staining **(Figure 4.4 A-C)**.

In contrast, T-HESC cell line demonstrated 73.8% positivity for CD90 fibroblast marker with no tangible EpCAM expression. Equivalently, all four cancer-associated fibroblast cells (CAFs) - EC6-Fib, EC7-Fib, EC11-Fib, and EC14-Fib showed at least 73% positive reaction for CD90 expression. Fibroblast cells isolated from endometrial hyperplasia tissue, EH-Fib, showed 64.8% positivity for CD90 expression. All fibroblast cells were negative for EpCAM expression **(Figure 4.4 D-I)**.

Both isolated epithelial and fibroblast cell population showed specific staining for their respective cell surface markers with minimal cross-contamination. The results indicate that although 100% positivity for EpCAM or CD90 markers cannot be achieved, it is important to note that there was no presence of EpCAM marker in the fibroblast cells or vice versa. All isolated cells were maintained under passage 10 to avoid cellular senescence that may result in morphological and molecular changes. Furthermore, throughout the experimental period, any signs of reduced growth rate in the primary cultures used for this study was not observed.

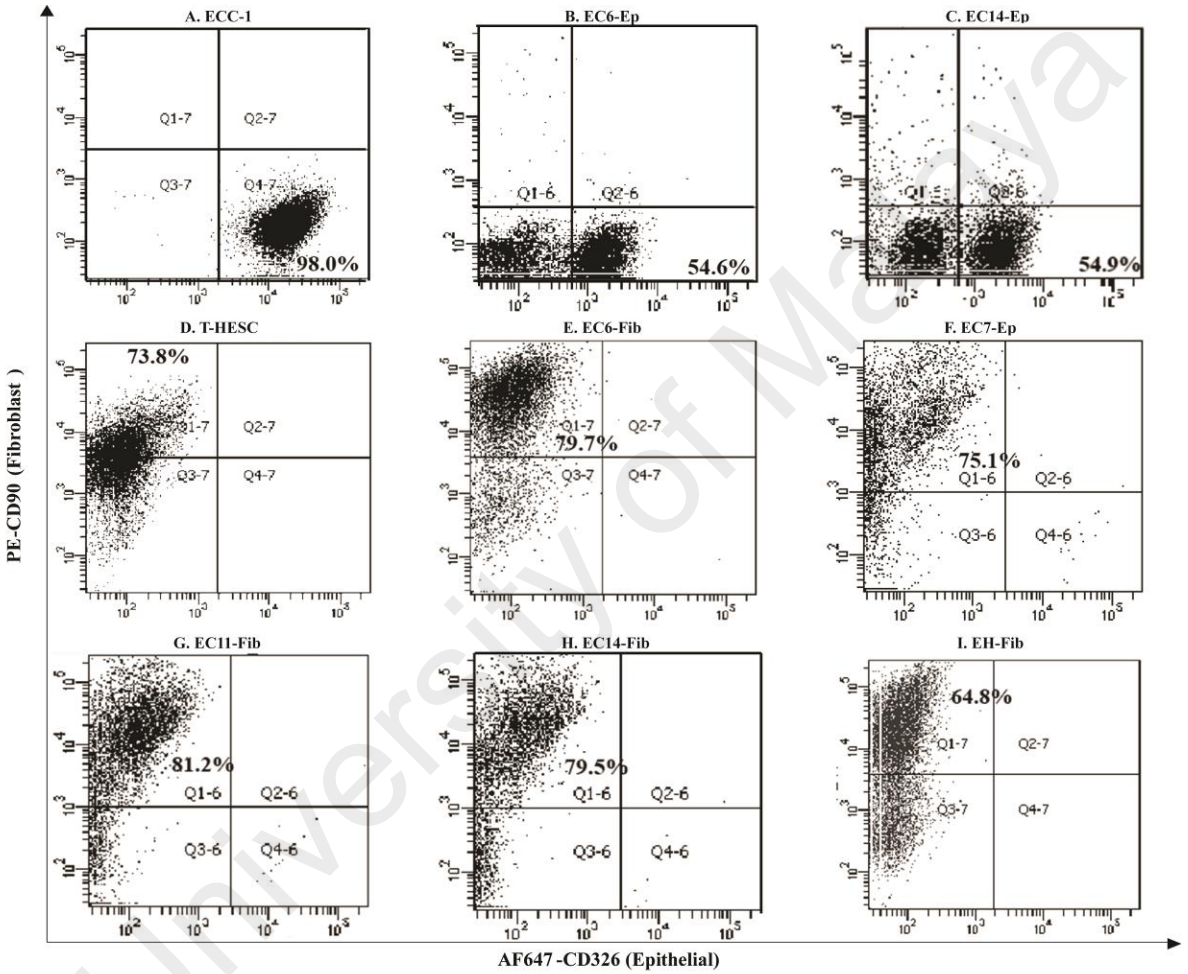


Figure 4.4. Expression of EpCAM and CD90 in isolated cells using flow cytometry.
AlexaFluor647-labeled CD326 and PE labeled-CD90 antibody were stained on the isolated epithelial and fibroblast cells to determine the percentage of the cell surface markers present. Results show the percentages of EpCAM and CD90 for (A) ECC-1, (B) EC6-Ep, (C) EC14-Ep, (D) T-HESC, (E) EC6-Fib, (F) EC7-Fib, (G) EC11-Fib, (H) EC14-Fib, (I) EH-Fib.

Next, to examine if the four cancer-associated fibroblasts (CAFs) cells (EC6,7,11 and 14-Fib) isolated from the primary endometrial cancer tissues express α-smooth muscle actin (α-SMA) protein, immunofluorescence staining was carried out. Expression of α-SMA marker is commonly expressed in fibroblasts that have transformed to myofibroblasts. Two primary epithelial cells, EC6-Ep, and EC14-Ep were included as a negative control for this experiment.

Upon staining, slides were observed under a fluorescence microscope at 10x magnifications using DAPI and TRITC filter. Results showed that all four CAFs strongly stained positive for α-SMA. Intense staining was observed on the elongated spindles in the cytoplasm **(Figure 4.5 A-D)**. Both EC6-Ep and EC14-Ep primary epithelial cells showed negative staining for α-SMA protein **(Figure 4.5 E-F)**. The results showed that all four isolated CAFs cells specifically stained for α-SMA, an indication that the cells have transformed into an activated form of fibroblast.

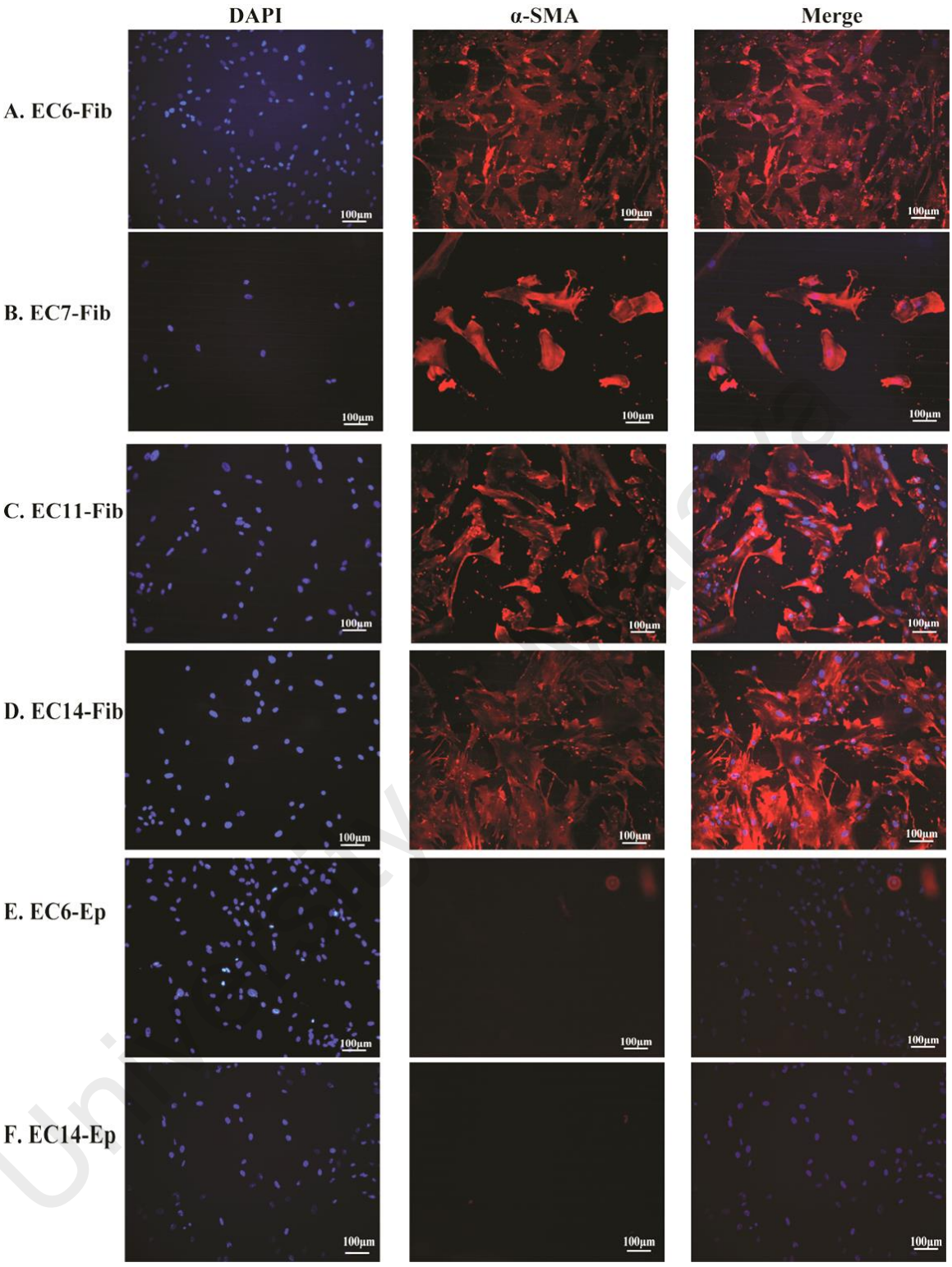


Figure 4.5. Evaluating the presence of α-SMA marker in isolated primary cell cultures.

Fibroblast cells isolated from primary endometrial hyperplasia (EH-Fib) and all four CAFs (EC6,7,11 and 14-Fib) were subjected to immunofluorescence staining to detect the presence of α-SMA protein. Two primary epithelial cells (EC6,14-Ep) were included as negative controls. Images on the left panel show cells stained with DAPI (blue). The middle panel shows cell images stained with α-SMA antibody conjugated with AlexaFlour588 secondary antibody (TRITC, red). Merged images are shown on the right panel. Magnification: 10x, scale bar: 100µm.

To evaluate the expression of other epithelial and fibroblast markers, quantitative RT-PCR method was employed. Epithelial cells EC6-Ep and EC14-Ep showed high relative expression of EpCAM, cytokeratin 8 and E-cadherin genes, with low expression of vimentin and α-SMA genes **(Figure 4.6 A, B)**. The expression level shown was normalized with the level of endogenous control GAPDH. In contrast, CAFs showed greater mRNA expression of vimentin and α-SMA, with low expression of EpCAM, E-cadherin, and cytokeratin 8 **(Figure 4.6 A-C)**.

In addition, quantitative RT-PCR was also used to observe if functional markers of the endometrium can be detected in the isolated primary cultures. All four EC tissues expressed varying degrees of estrogen and progesterone receptors (ERα, ERβ, and PR) **(Figure 4.6 D-F)**, consistent with the observation that EC is hormone-responsive tumors. Expression of three commonly secreted proteins by the endometrium, progesterone-associated endometrial protein (PAEP) and matrix metallopeptidase 1 and 9 (MMP1, 9) in these cells was also evaluated. PAEP was expressed mainly by fibroblasts (EC7-Fib and EC11-Fib). Greater MMP1 expression was observed compared to that of MMP9 in both epithelial and fibroblast cells. Taken together, our data strongly suggested that these primary epithelial and fibroblast cells were maintaining phenotypes closest to *in vivo* conditions.

Taken together, these data indicate that the isolated epithelial and fibroblast cells from human endometrial tissues were relatively homogenous culture with expected cell morphology and expression of specific cell markers, without significant presence of contaminating cells.

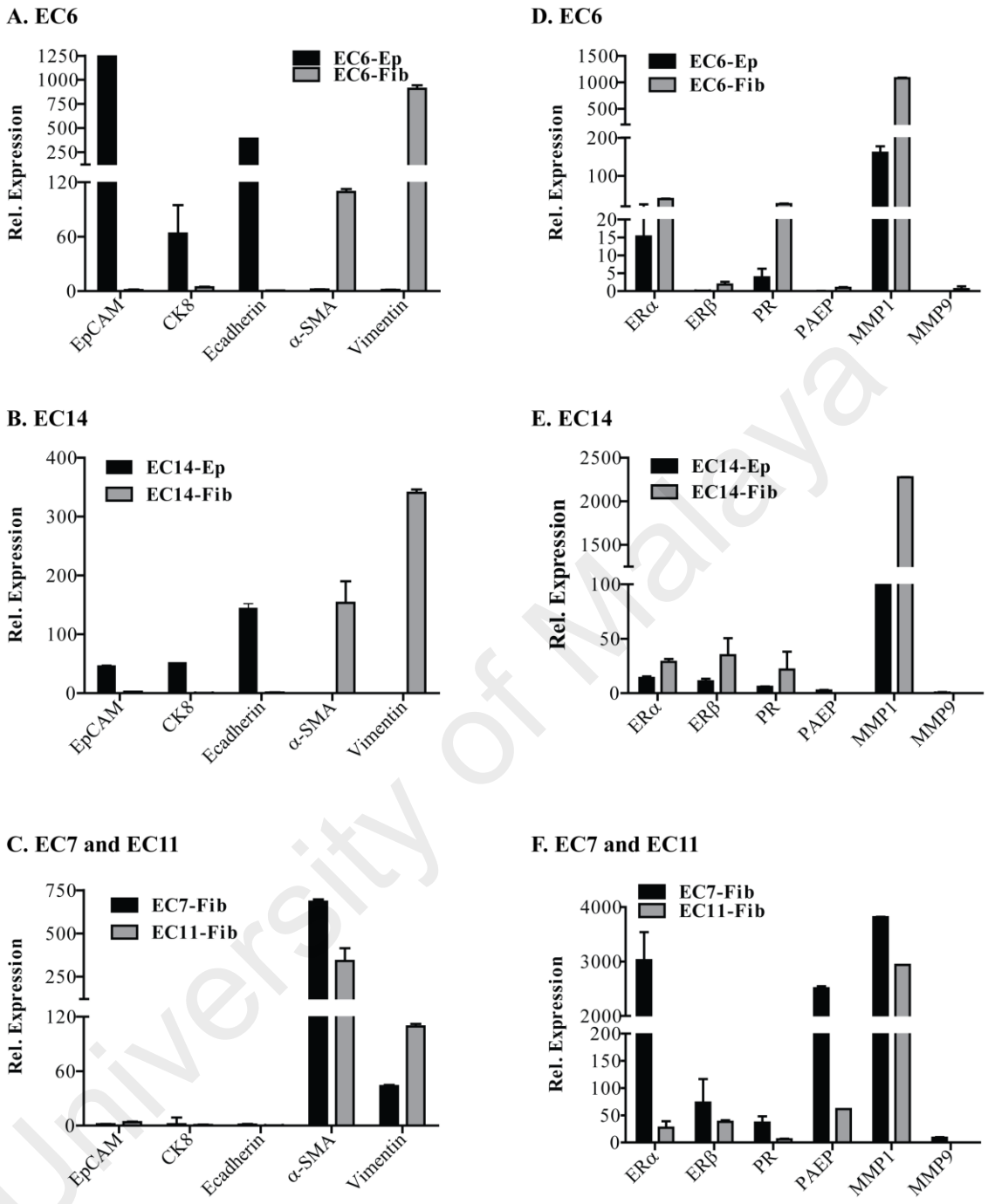


Figure 4.6. Expression of epithelial and fibroblast markers in established primary cultures from endometrial cancers.

Quantitative PCR analysis were done to evaluate the expressions of EpCAM, CK8, E-Cadherin, α-SMA, vimentin (A-C), estrogen receptor-α, estrogen receptor-β, progesterone receptor, progestagen-associated endometrial protein and matrix metallopeptidase 1 and 9 expression (D-F) in epithelial and fibroblast cell from EC6, EC14 and fibroblast cells from EC7 and EC11. Data, average; error bars, SD. Data shown are representative of three independent experiments.

4.2 Differential effect of normal fibroblast and cancer-associated fibroblasts (CAFs) on EC proliferation, motility, and invasion

4.2.1 Effect on EC cell proliferation

To determine and compare the effects of various fibroblast cell secretions on endometrial cancer cells proliferation, cell viability, and BrdU assays were performed. Conditioned media from fibroblast cells cultured in media containing 2% FBS were harvested, according to the methodology described in Subchapter 3.2.1. ECC-1 and HEC-1A human endometrial cancer cell lines and EC6-Ep and EC14-Ep epithelial primary cultures were treated with the conditioned media in doses of 0.1, 0.5, 1 and 2 µg/µl for 72 hours. Interestingly, supernatants from CAFs induced a promoting effect, in which the growths of all four EC epithelial cells were significantly enhanced when compared to those cultured in control media. All CAFs cells increased proliferation of EC6-Ep even at the lowest concentration. The greatest growth-promoting effects were seen with treatment of 1 µg/µl EC7-Fib and EC11-Fib, where at least 2-fold cell proliferation was observed in ECC-1 and HEC-1A cell lines **(Figure 4.7 A)**.

Conditioned media from normal endometrial fibroblast T-HESC cell line inhibited the proliferation of all EC epithelial cells, in a dose-dependent manner **(Figure 4.8 A-D)**. Data for CAFs are shown as the average of all four CAFs tested. Statistical analysis was done using the averaged CAFs data compared to the data of T-HESC. The greatest inhibitory effect was by T-HESC's conditioned media inhibited, where it inhibited almost 31% of HEC-1A's proliferation rate **(Figure 4.8 B)**. Previous studies used multiple primary cells established from normal human endometrium to demonstrate that normal fibroblast is growth inhibitory to endometrial cancer cells (Arnold et al., 2001; Min et al., 2011). Our data demonstrated that similar effects can be observed using secretion from normal endometrial fibroblast cell line (T-HESC).

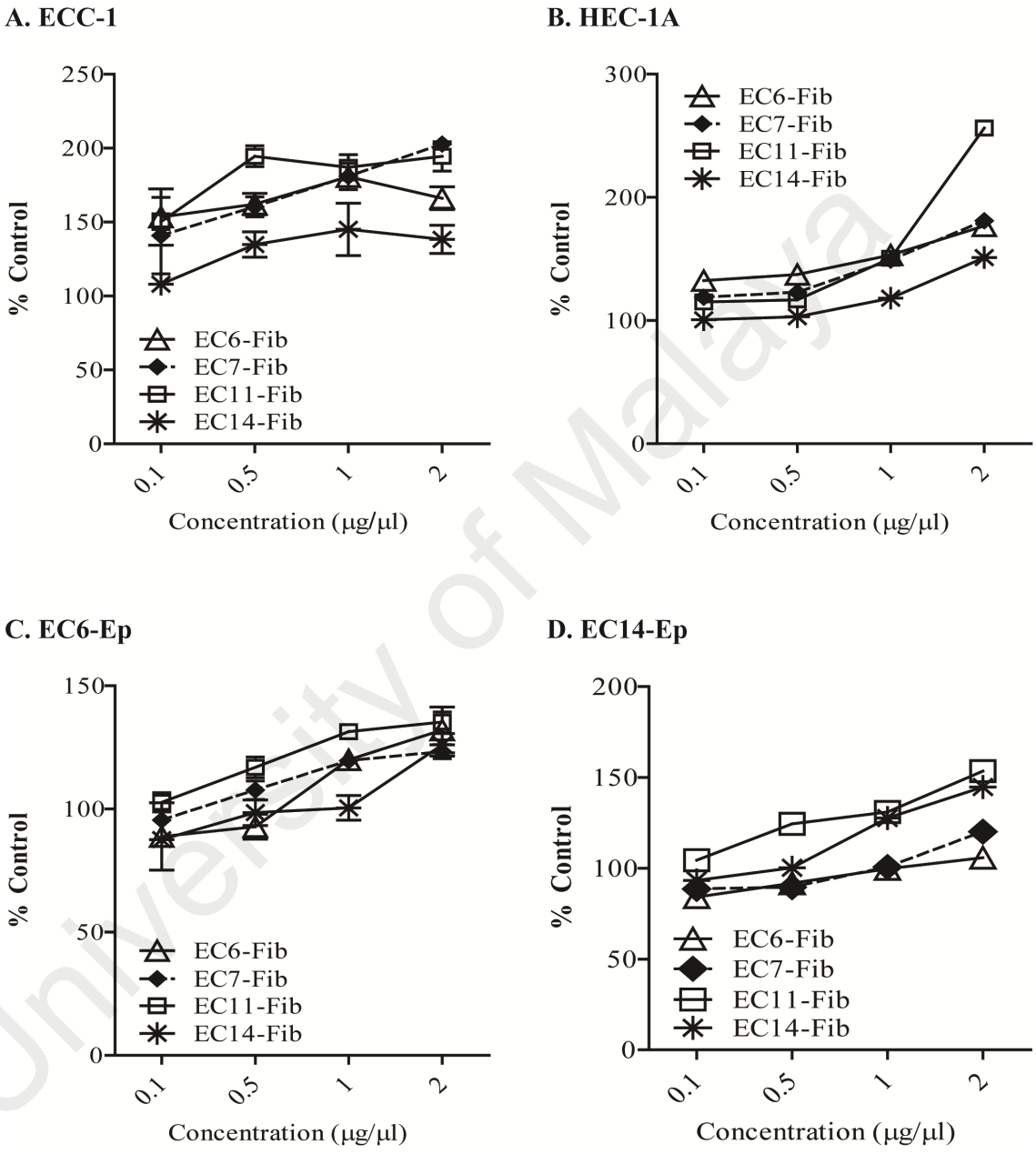


Figure 4.7. Differential effect of CAFs on EC cells.
Conditioned media from four different CAFs were treated on (A) ECC-1, (B) HEC-1A, (C) EC6-Ep and (D) EC14-Ep in increasing doses for 72 hours. After 72 hours treatment, cell viability was evaluated using MTT assay. Data obtained were normalized with control media that contains 2% FBS. Data shown are representative of three independent experiments. Data, average; error bars, SD.

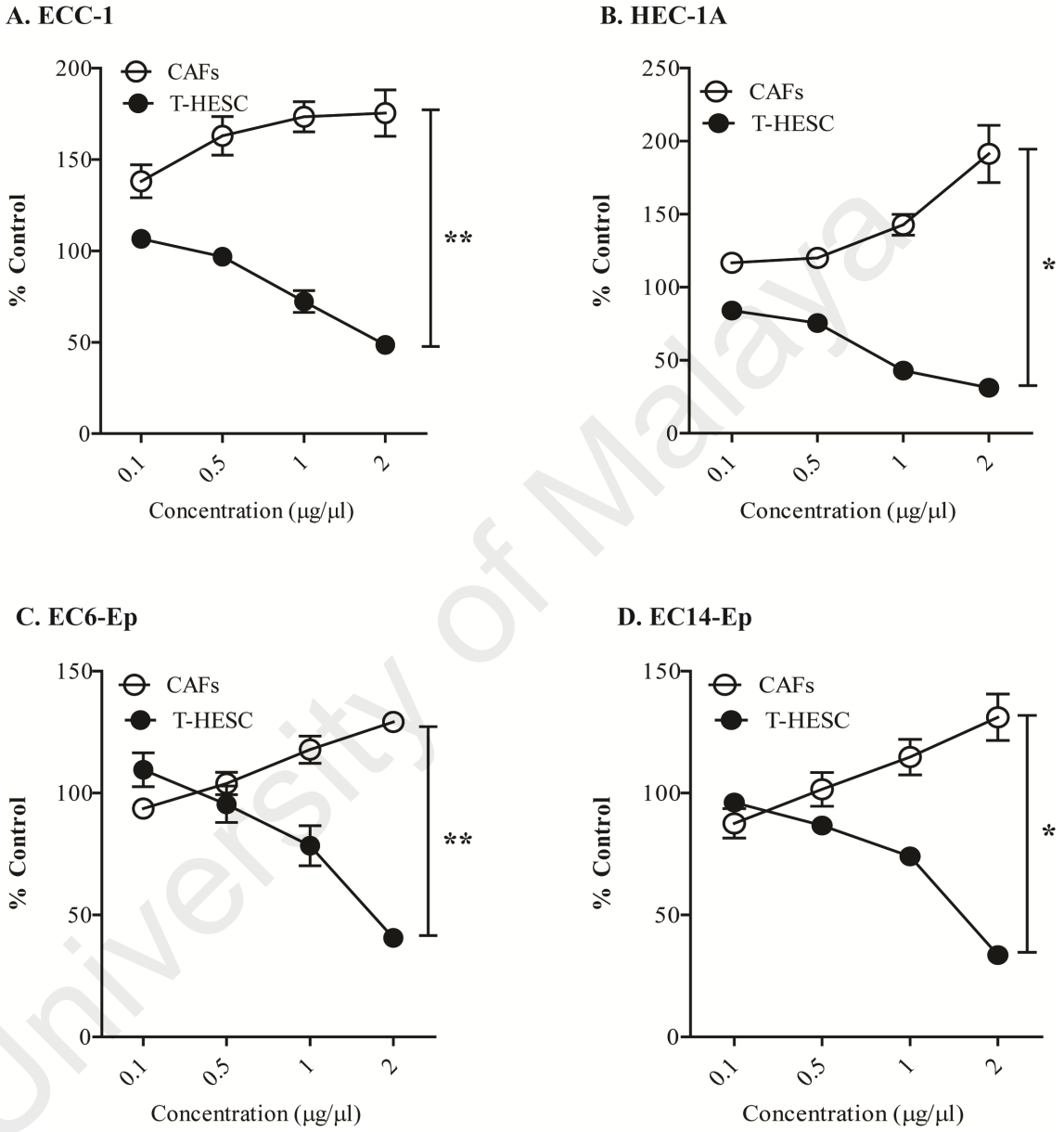


Figure 4.8. Differential effect of CAFs and normal fibroblast on EC cells.
Conditioned media T-HESC were treated on (A) ECC-1, (B) HEC-1A, (C) EC6-Ep and (D) EC14-Ep in increasing doses for 72 hours. After 72 hours treatment, cell viability was evaluated using MTT assay. Data obtained were normalized with control media that contains 2% FBS. Data for CAFs are shown the average of all four CAFs tested. *: $P<0.005$; **: $P<0.0001$. Data shown are representative of three independent experiments. Data, average; error bars, SD.

Conditioned media from benign endometrial (EH-Fib) was also collected and tested for their effects on EC cells proliferation. As shown in **Figure 4.9**, EH-Fib conditioned media did not significantly affect the proliferation of ECC-1 and HEC-1A cells. However, upon testing on primary epithelial cells EC6-Ep and EC14-Ep, EH-Fib conditioned media inhibited cell growth in a dose-dependent manner. This observation was similar to that of T-HESC in **Figure 4.8**. This data suggests that the growth-promoting effects by CAFs and growth-inhibitory effects by benign fibroblast were specific to endometrial cancer tissues and was not observed by chance.

Taken together, treatment of secretion from CAFs and T-HESC resulted in a differential proliferative effect on the growth of EC cells.

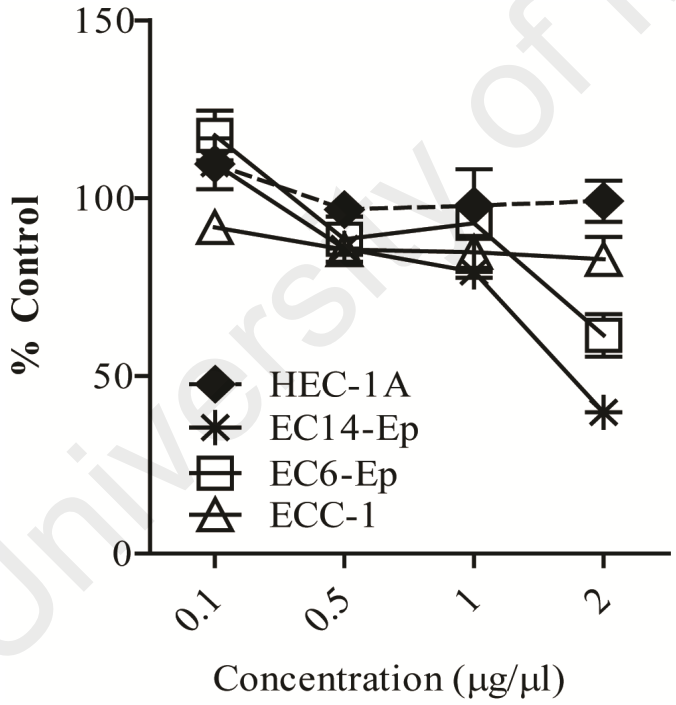


Figure 4.9. Effect of endometrial hyperplasia fibroblast secretion on EC cells. Conditioned media from EH-Fib were tested on ECC-1, HEC-1A, EC6-Ep, and EC14-Ep in increasing doses for 72 hours. Cell viability was evaluated using MTT assay at the end of the experiment. Data obtained were normalized with control media that contains 2% FBS. Data shown are representative of three independent experiments. Data, average; error bars, SD.

Increased cell viability by CAFs-conditioned media may result from increased cell proliferation. Hence, to evaluate the proliferation rate of cancer cells following exposure to CAFs and benign fibroblast, BrdU assay was performed. Similar to cell viability assay, all four CAFs increased BrdU in EC cells in a dose-dependent manner, with at least 50% greater proportion than that by control media. In contrast, T-HESC inhibited the proliferation with at least 2-folds, compared to that by control media **(Figure 4.10)**. This assay confirmed that CAFs effect on increased EC cell growth was indeed through increased cell proliferation.

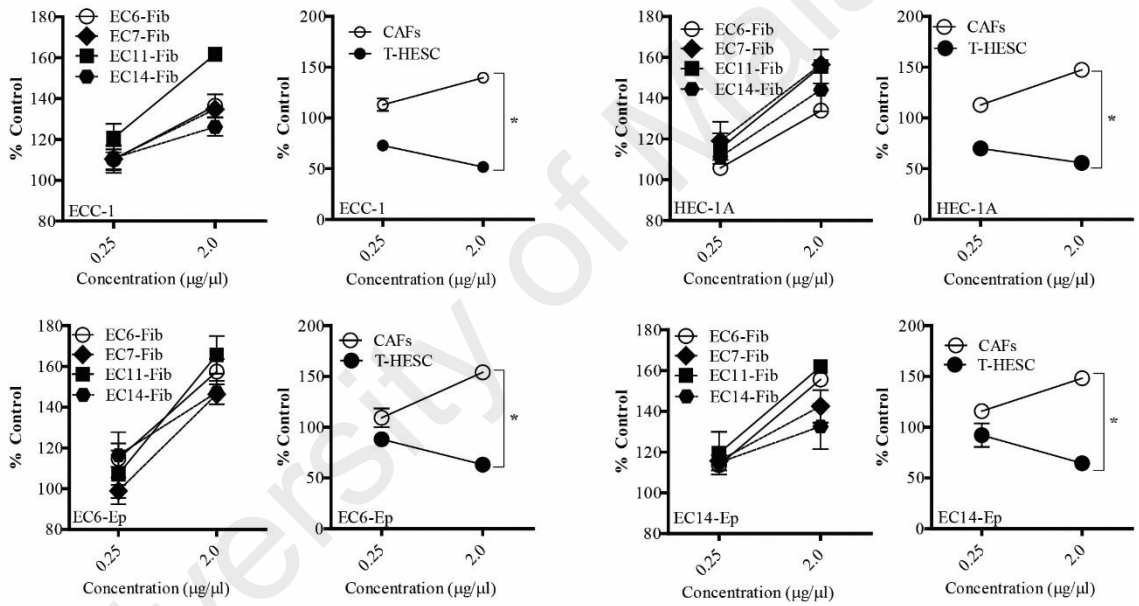


Figure 4.10. Differential effect of CAFs and T-HESC secretion on EC cell proliferation using BrdU cell proliferation assay.
Conditioned media from CAFs and T-HESC were treated on all four EC epithelial cells and proliferation rate was determined using BrdU assay. Treatment was carried out for 72 hours. *: $P<0.005$. Data obtained were normalized with control media that contains 2% FBS. Data shown are representative of three independent experiments. Data, average; error bars SD.

4.2.2 Effect on EC cell motility and invasion

To measure if CAFs secretion induced motility of cancer cells, scratch-wound **(Figure 4.11)** and transwell assay **(Figure 4.12)** were performed. The gap made on ECC-1 cells were closed to almost 100% in 48 hours, following exposure to EC11-Fib and closed more than 50% in distance after exposure to EC7-Fib. The gap distance upon exposure to T-HESC, however, did not close and remained relatively similar than it was at the beginning of the experiment **(Figure 4.11)**.

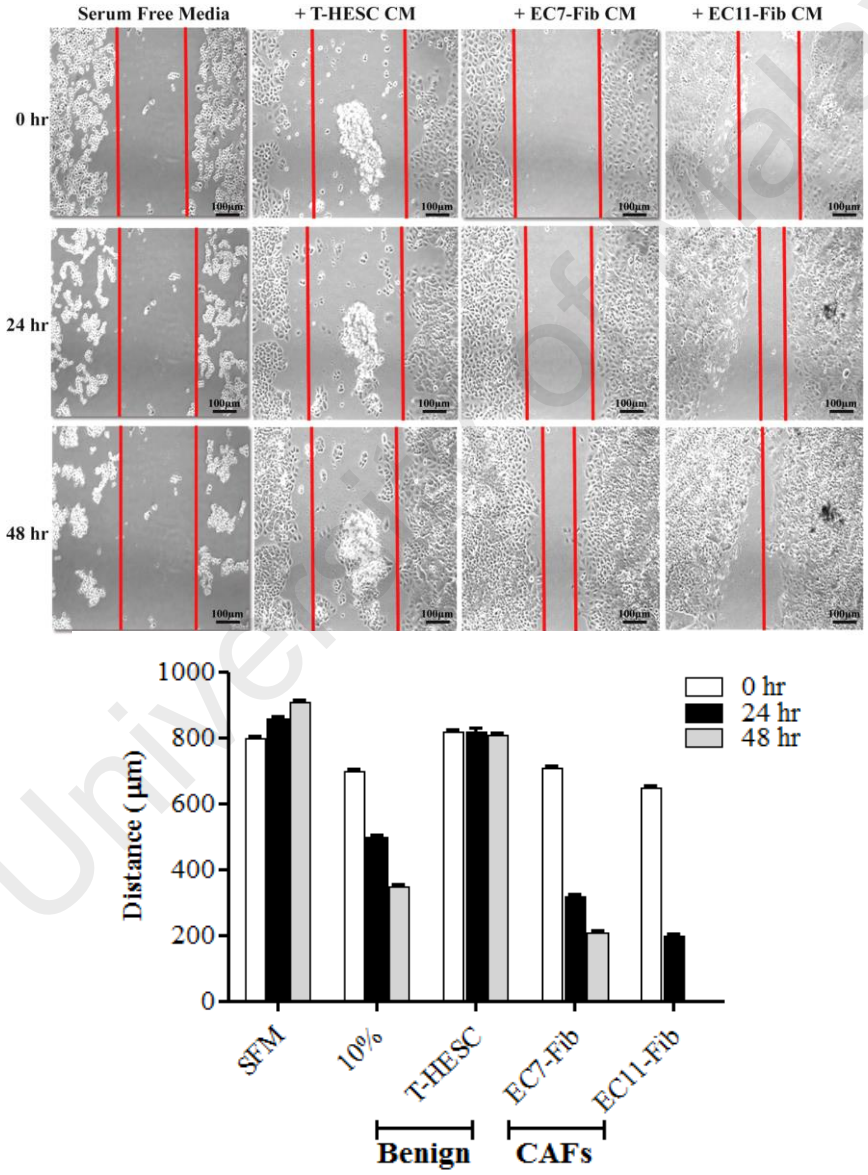


Figure 4.11. Differential effect of T-HESC and CAFs secretion on EC motility. ECC-1 cell line was treated with fibroblasts secretion for 48 hours and 10 µl pipette tip was used to make a fine scratch on the cell monolayer. Gap distance in µm shown is averaged from three different wells of the 24-well plate. Magnification: 10x, scale bar: 100µm.

Using Transwell assay, the number of ECC-1 and HEC-1A cells migrating towards EC7 and EC11-Fib secretion were at least 6-folds higher than towards serum-free media **(Figure 4.12)**. In contrast, the number of cells migrated towards T-HESC secretion was considerably lower than media containing 10% FBS (complete medium).

Using similar method, it was found that the number of ECC-1 cells migrating towards EC7 and EC11-Fib secretion by invading the basement matrix was significantly high, at least 5-folds higher than those cells towards serum-free media **(Figure 4.12)**. T-HESC secretion did not significantly cause ECC-1 cells to invade the basement matrix. This indicates that CAFs demonstrated specific property to induce migration and invasion of cancer cells, which was absent in normal fibroblasts.

Taken together, the data shows that apart from promoting cancer cell proliferation, CAFs secretion also contributes to cancer cells migration and invasive capabilities. The proliferative, migration and invasive capabilities were not seen with T-HESC secretion.

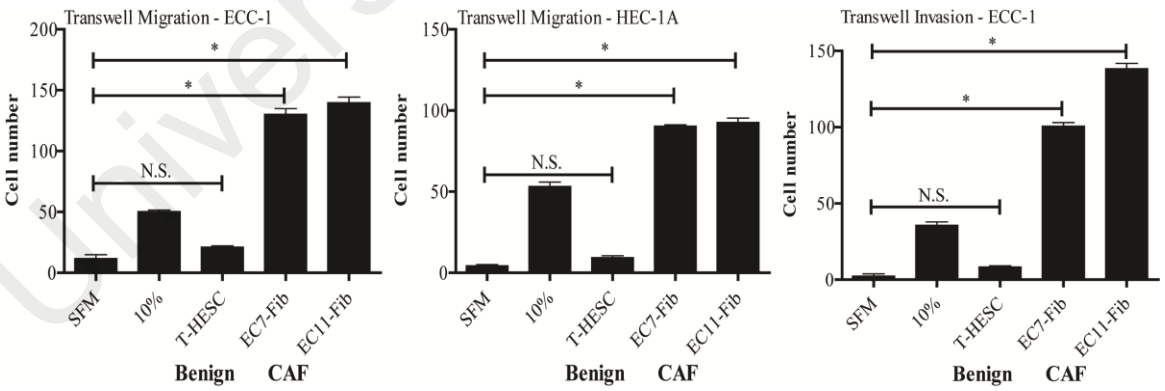


Figure 4.12. Differential effect of T-HESC and CAFs secretion on EC motility and invasion.
Transwell assay was performed using ECC-1 (migration and invasion) and HEC-1A (migration) cell lines in the presence of T-HESC and CAFs-conditioned media. The cell lines were treated with fibroblast secretions for 24 hours after which migrated cells were stained with crystal-violet and counted. Cell number shown are representative of three independent experiments. *: $P<0.05$; N.S.: not significant. Data: average, error bar: SD.

4.3 Mechanisms underlying CAFs-mediated EC cell proliferation

4.3.1 Identification of cytokines in secretion of endometrial fibroblasts

To determine the secretory factors responsible for CAFs-mediated cell proliferation, migration, and invasion, a 20-cytokine target array was performed, which compared the levels of different cytokines in the conditioned media harvested from three different CAFs and three types of benign fibroblasts **(Figure 4.13-4.18)**.

It was observed that eight cytokines were secreted higher in CAFs than benign fibroblast: growth-regulated oncogene alpha (GRO-α), interleukin-6 (IL-6), macrophage chemoattractant protein-1 (MCP-1), regulated on activation, normal T expressed and secreted (RANTES) and vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9), macrophage inflammatory protein 1 alpha (MIP-1α) and macrophage inflammatory protein 1 beta (MIP-1β) **(Figure 4.13-4.14)**.

Seven cytokines were found higher in the secretion of benign fibroblast than in CAFs: interleukin-1 beta (IL-1β), interleukin-13 (IL-13), interleukin-4 (IL-4), interleukin-5 (IL-5), tumor necrosis factor alpha (TNF-α), granulocyte-monocyte colony stimulating factor (GM-CSF) and interleukin-10 (IL-10) **(Figure 4.15-4.16)**.

Three cytokines were secreted in an almost equal amount between CAFs and benign fibroblasts: interleukin-8 (IL-8), interleukin-2 (IL-2), interleukin-1 alpha (IL-1α) **(Figure 4.17)**. The level of interleukin-12p70 (IL-12p70) could only be detected in a minuscule amount in EC11-Fib secretion while interferon gamma (IFNγ) remained undetermined in both benign and cancer fibroblast **(Figure 4.18)**.

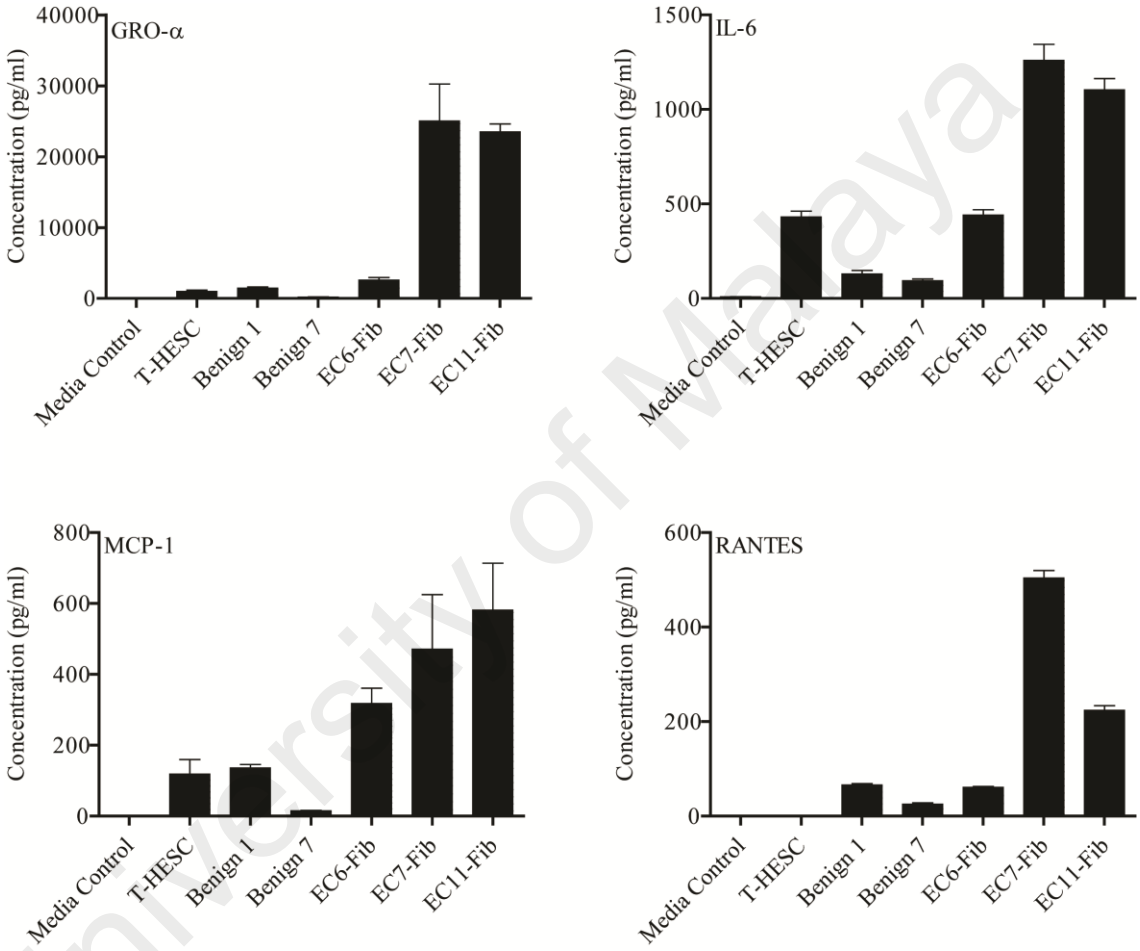


Figure 4.13. Identification and measurement of cytokines secreted by benign and cancer-associated fibroblasts.

A 20-target cytokine array (Raybiotech Quantibody Human Cytokine Array) was performed with secretion collected from three endometrial benign and three endometrial cancer fibroblasts which were cultured in media containing 2% FBS. Graphs show GRO-α, IL-6, MCP-1 and RANTES levels for each fibroblast secretion. Data, average; error bar, S.E.M.

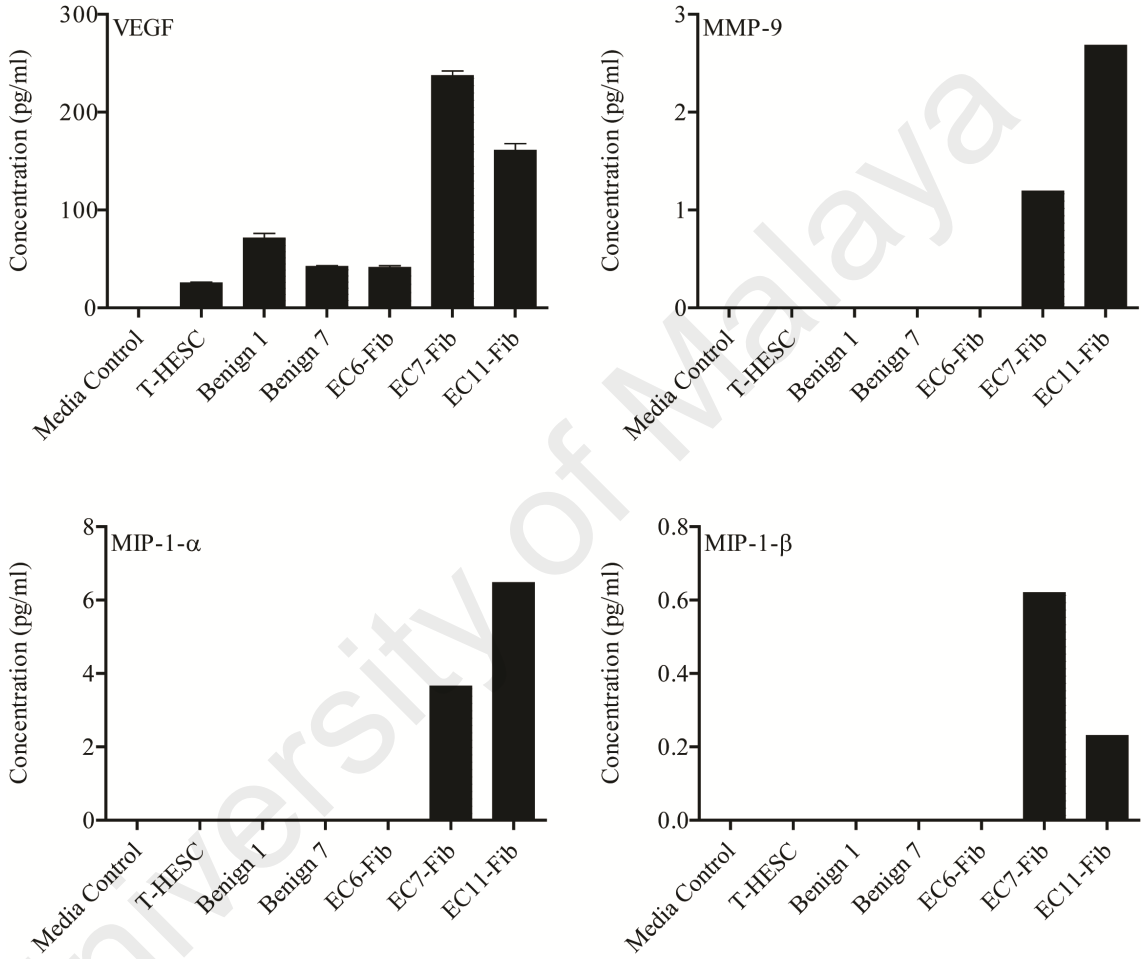


Figure 4.14. Identification and measurement of cytokines secreted by benign and cancer-associated fibroblasts.

A 20-target cytokine array (Raybiotech Quantibody Human Cytokine Array) was performed with secretion collected from three endometrial benign and three endometrial cancer fibroblasts which were cultured in media containing 2% FBS. Graphs show VEGF, MMP-9, MIP-1-α and MIP-1-β levels for each fibroblast secretion. Data, average; error bar, S.E.M.

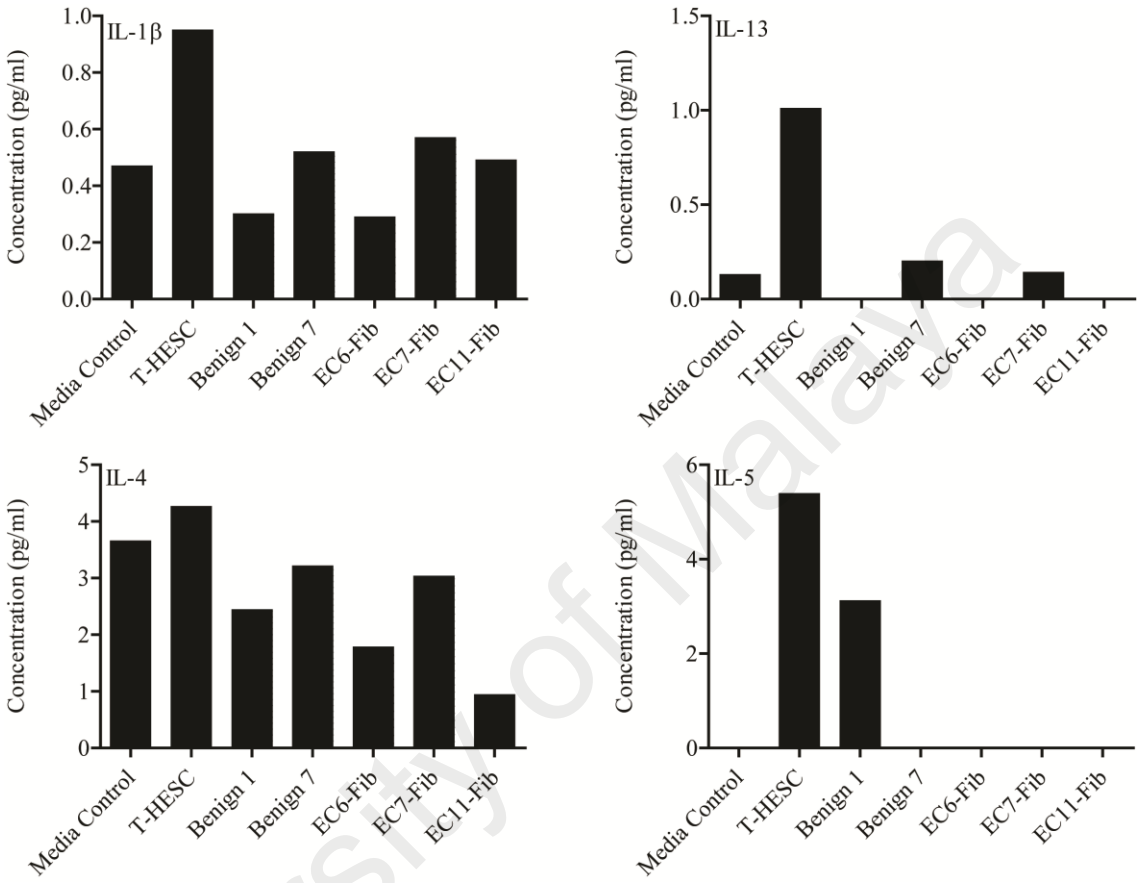


Figure 4.15. Identification and measurement of cytokines secreted by benign and cancer-associated fibroblasts.

A 20-target cytokine array (Raybiotech Quantibody Human Cytokine Array) was performed with secretion collected from three endometrial benign and three endometrial cancer fibroblasts which were cultured in media containing 2% FBS. Graphs show IL-1β, IL-13, IL-4, and IL-5 levels for each fibroblast secretion. Data, average; error bar, S.E.M.

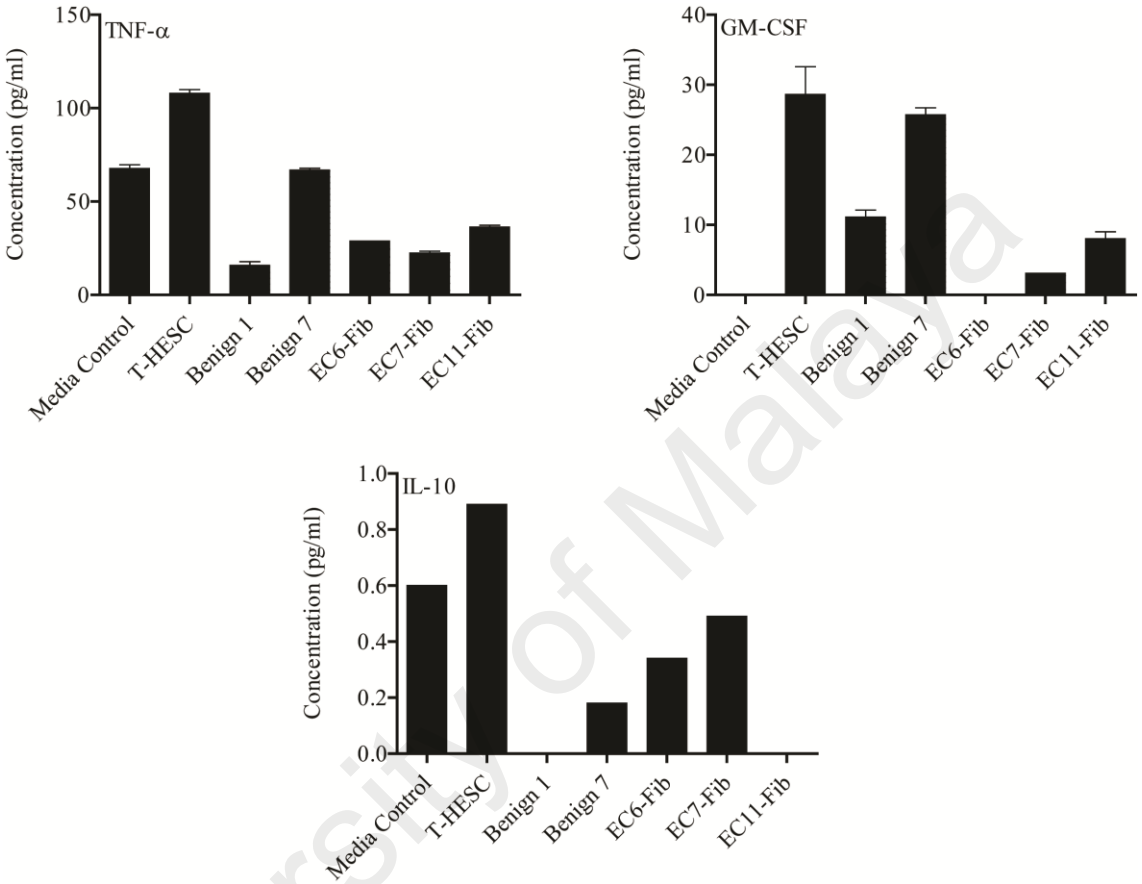


Figure 4.16. Identification and measurement of cytokines secreted by benign and cancer-associated fibroblasts.

A 20-target cytokine array (Raybiotech Quantibody Human Cytokine Array) was performed with secretion collected from three endometrial benign and three endometrial cancer fibroblasts which were cultured in media containing 2% FBS. Graphs show TNF-α, GM-CSF, and IL-10 levels for each fibroblast secretion. Data, average; error bar, S.E.M.

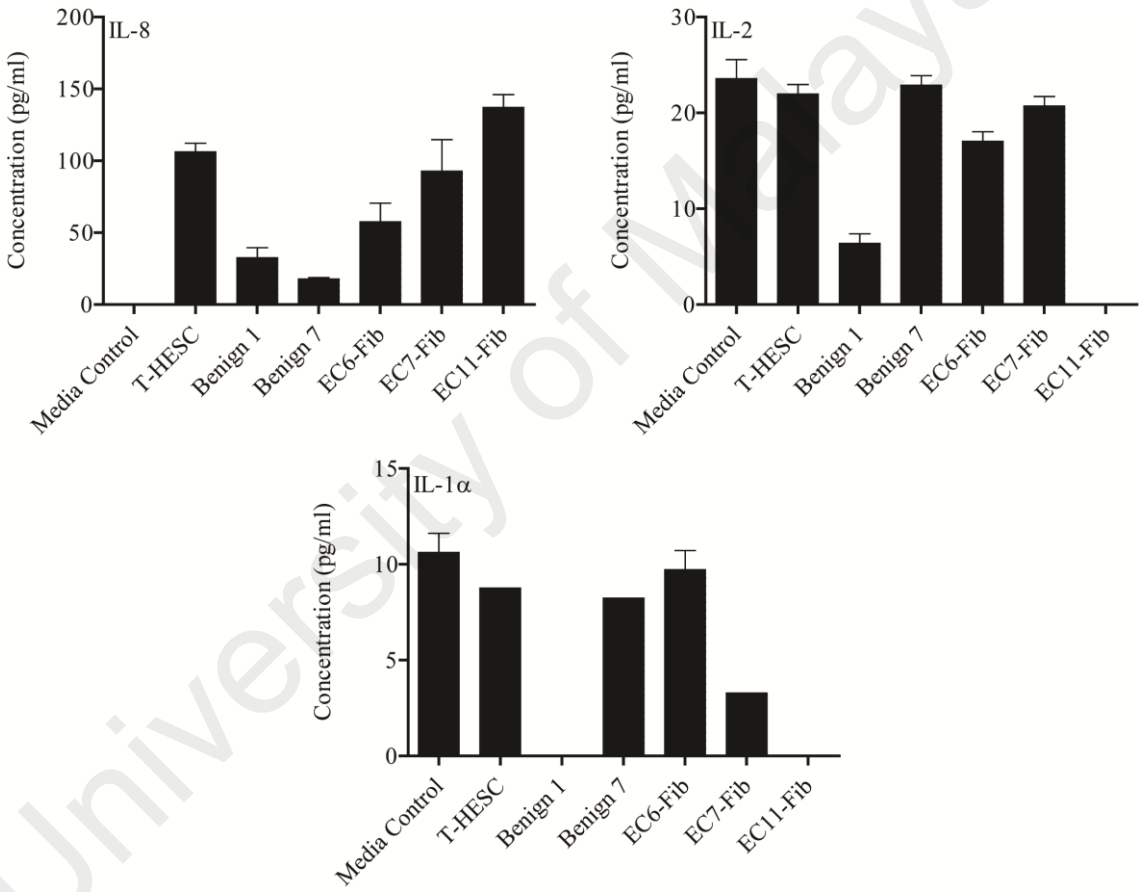


Figure 4.17. Identification and measurement of cytokines secreted by benign and cancer-associated fibroblasts.

A 20-target cytokine array (Raybiotech Quantibody Human Cytokine Array) was performed with secretion collected from three endometrial benign and three endometrial cancer fibroblasts which were cultured in media containing 2% FBS. Graphs show IL-8, IL-2 and IL-1-α levels for each fibroblast secretion. Data, average; error bar, S.E.M.

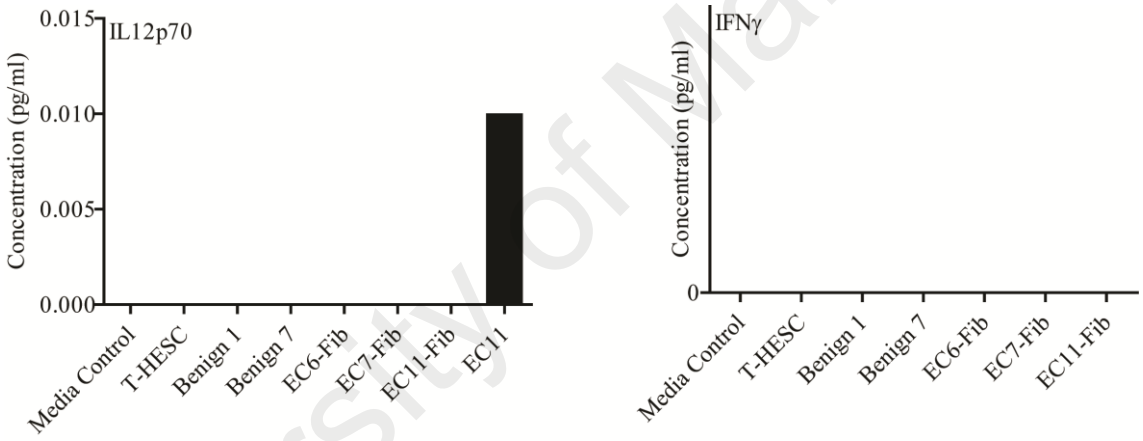


Figure 4.18. Identification and measurement of cytokines secreted by benign and cancer-associated fibroblasts.

A 20-target cytokine array (Raybiotech Quantibody Human Cytokine Array) was performed with secretion collected from three endometrial benign and three endometrial cancer fibroblasts which were cultured in media containing 2% FBS. Graphs show IL12p70 and IFNγ levels for each fibroblast secretion. Data, average; error bar, S.E.M.

From the array results graphed according to cytokine levels for each fibroblast secretion, the data was averaged to represent the mean of three benign fibroblasts compared to three CAFs. This step was done to evaluate which cytokines are highly secreted by CAFs compared to benign fibroblast.

Interestingly, five of those twenty cytokines: GRO-α, IL-6, MCP-1, RANTES and VEGF were found to be notably high in CAFs. GRO-α was secreted at a significantly high level by CAFs at a concentration of 9577 ± 2245 pg/ml, which was almost 11-fold more than that by benign fibroblast. CAFs secreted 4-folds more IL-6 at a concentration of 934.21 ± 58 pg/ml followed by MCP-1 at 456.55 ± 110 pg/ml (~4-folds) compared to secretions from benign fibroblast. RANTES, secreted at a concentration of 262 ± 9.3 pg/ml by CAFs were about 8-fold higher than its content detect in benign fibroblast. VEGF was observed to be about 5-fold higher in CAFs at concentrations of 146.35 ± 4.67 pg/ml than in benign fibroblast **(Figure 4.19)**.

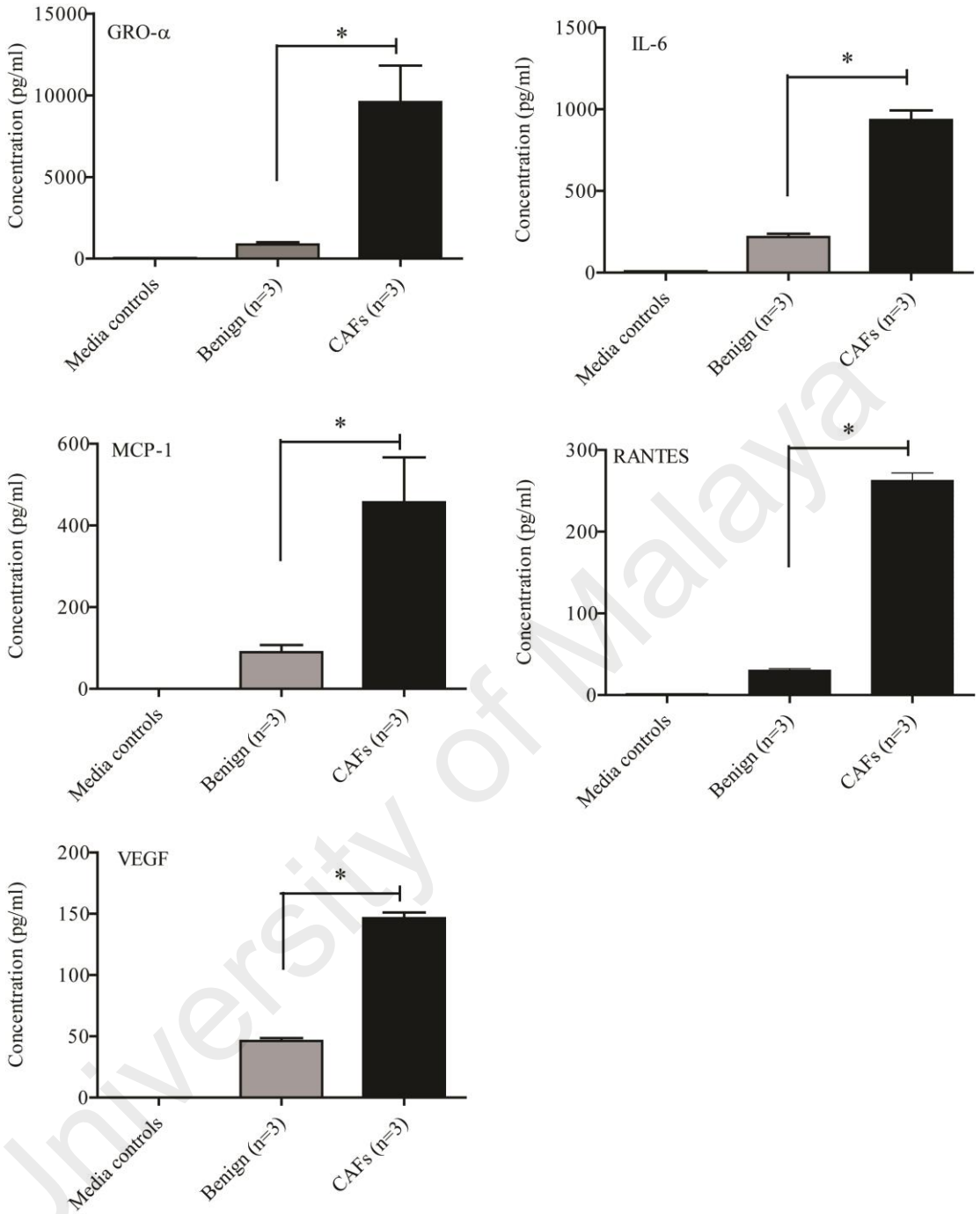


Figure 4.19. Identification and measurement of cytokines in CAFs.
Five cytokines: GRO-α, IL-6, MCP-1, RANTES, and VEGF were identified to be secreted by CAFs at a significantly high level compared to secretion from benign fibroblasts. *: $P<0.005$; Data, average; error bar, SD.

To validate results from the cytokine array, the levels of GRO-α, IL-6, MCP-1, VEGF, and RANTES were determined in secretion from 10 different CAFs compared to benign fibroblasts using ELISA. Cytokine levels were determined by plotting absorbance value against standard curve per cytokine. Similar to the array results, secretion from CAFs was significantly higher than control fibroblasts for all the five cytokines. Levels of GRO-α were at least 11 times more in CAFs compared to benign fibroblast. The IL-6 level was at least 10-fold higher in CAFs (897.6 pg/ml) than in benign fibroblasts (87.89 pg/ml) secretion. MCP-1 (5.6-fold), VEGF (3.3-fold) and RANTES (3-fold) were found elevated in CAFs secretion when compared to those from benign fibroblasts **(Figure 4.20)**. These data allowed further investigation on the highly secreted cytokine by CAFs in promoting EC cell proliferation.

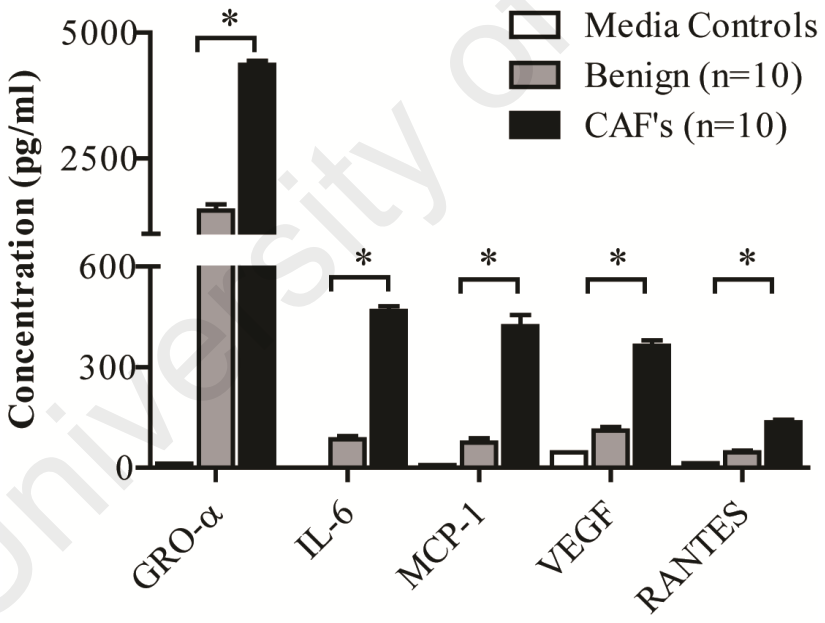


Figure 4.20. Identification and measurement of cytokines secreted by normal and cancer-associated fibroblasts.
Individual cytokines levels were measured using ELISA with secretion collected from ten endometrial cancer and ten benign endometrial fibroblasts. ELISA data shown are representative of two independent experiments. *: $P<0.005$; Data, average; error bar, SD.

4.3.2 Effect of GRO-α inhibition on CAFs-mediated EC proliferation

Cytokine array and ELISA results demonstrated that GRO-α was the highest present cytokine in CAFs secretion. To determine whether GRO-α contributes to EC cell proliferation, ECC-1 and EC6-Ep cells were treated with GRO-α neutralizing antibody in the presences of CAFs conditioned media for 72 hours, and cell viability was measured.

Even at the highest treatment dose of 1 µg/ ml of GRO-α neutralizing antibody in the presence of EC11-Fib or EC7-Fib, no inhibition of cell proliferation was observed **(Figure 4.21 A)**. In fact, the difference in cell viability between GRO-α IgG control antibody and GRO-α neutralizing antibody were not significant. Similar results were obtained when GRO-α neutralizing antibody was treated on EC6-Ep primary culture **(Figure 4.21 B)**. Hence, this data suggest that GRO-α is not involved in CAFs-mediated EC cell proliferation.

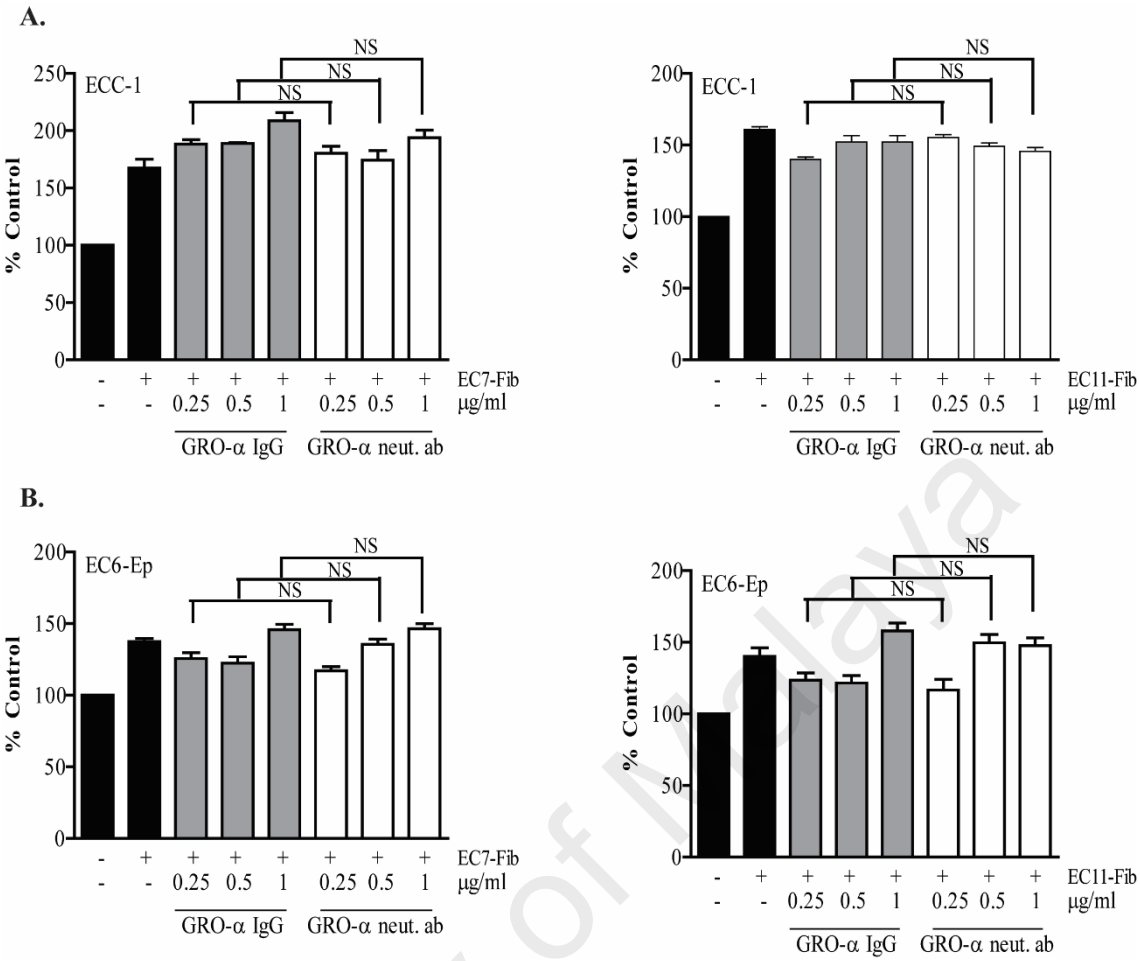


Figure 4.21. Effect of GRO-α on CAFs-mediated EC cell proliferation.
ECC-1 cell line (A) and EC6-Ep primary culture (B) were treated with GRO-α neutralizing antibody in the presence of EC7-Fib and EC11-Fib conditioned media along with its IgG isotype control doses of 0.25, 0.5 and 1 µg/ml. Cell viability was evaluated using MTT assay. Data were normalized with control media. Data shown are representative of three independent experiments. Data, average; error bar, SD. NS: not significant

To determine if GRO-α by itself could contribute to endometrial cancer cell proliferation, ECC-1, and EC6-Ep cells were treated with increasing doses of GRO-α recombinant protein. This was performed without the presence of CAFs-conditioned media. Both the EC cancer cells showed increased cell proliferation in response to increasing concentration of GRO-α recombinant protein. At 2μg/ml, cell proliferation was almost 2-fold higher than control media **(Figure 4.22 A)**.

Next, to confirm that the GRO-α neutralizing antibody used in this experiment is specific to inhibiting GRO-α cytokine, ECC-1 cells were concurrently treated with GRO-α recombinant protein and GRO-α neutralizing antibody. GRO-α IgG was used as isotype control. ECC-1 cells treated with recombinant GRO-α and its IgG control showed no decrease in cell proliferation for all three doses tested. However, ECC-1 cells treated with GRO-α recombinant protein and its neutralizing antibody showed proliferative inhibitory effect **(Figure 4.22 B)**. These accumulating data indicates that while GRO-α plays a role in promoting EC cell proliferation **(Figure 4.22)**, the progression effect is not due to CAFs-mediated secretion **(Figure 4.21)**. While GRO alpha directly induced EC cell proliferation, this cytokine may not be the key contributing factor in CAF-mediated effects.

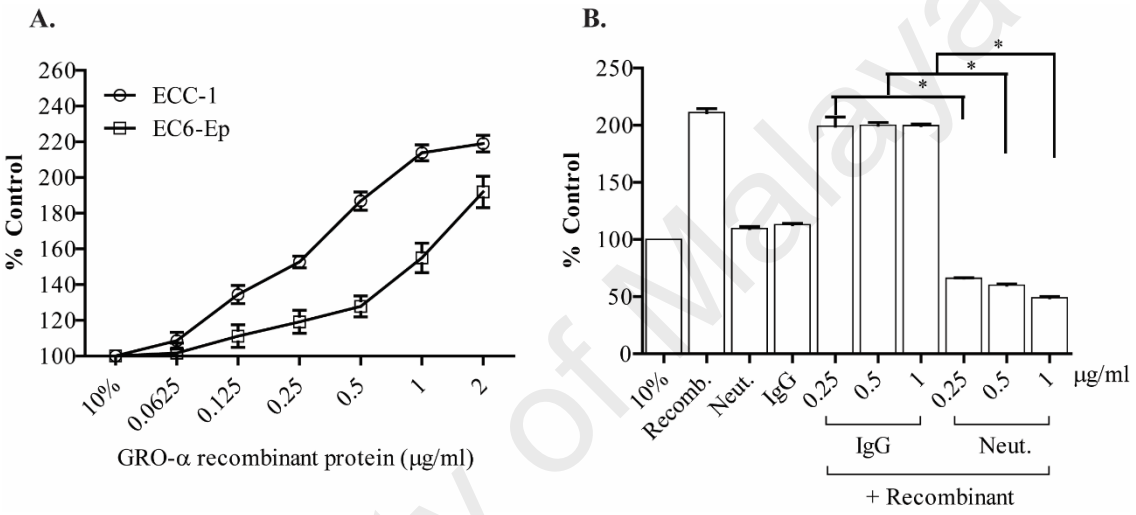


Figure 4.22. GRO-α in EC cell proliferation.
Increasing doses of GRO-α recombinant protein was treated on ECC-1 cell line and EC6-Ep primary cells (A). GRO-α neutralizing antibody in the presence of GRO-α recombinant protein was treated on ECC-1 cell line (B). Data obtained were normalized with control media. Data shown are representative of three independent experiments. *: $P<0.05$; Data, average; error bar, SD.

4.3.3 Effect of IL-6 inhibition in CAFs-mediated EC proliferation

Due to lack of evidence to support that GRO-α promotes CAFs-mediated EC cell proliferation, the effect of IL-6 on EC cells was considered next. ECC-1 and EC6-Ep cells were treated with IL-6 neutralizing antibody at doses of 0.25, 0.5 and 1 µg/ml. Treatment was done in the presence of CAFs (EC7-Fib and EC11-Fib) conditioned media. Treatment of all three doses of IL-6 neutralizing antibody resulted in growth inhibitory effects. Almost 50% inhibitory effect compared to isotype controls in ECC-1 **(Figure 4.23 A)** and EC6-Ep cells **(Figure 4.23 B)** was observed. This growth inhibitory effect of IL-6 neutralizing antibody was observed in the presence of both CAFs secretion.

Both ECC-1 and EC6-Ep cells were treated with IL-6 neutralizing antibody or isotype control alone, in the absence of CAFs secretion. No significant decrease in cell proliferation was observed both cells (~5% inhibition) **(Figure 4.23 C)**.

Next, to study if IL-6 is integral in EC cell proliferation, ECC-1 and EC-6 Ep cells were treated with increasing doses of IL-6 recombinant protein for 72 hours in the absence of CAFs conditioned media **(Figure 4.23 D)**. Both EC cells showed an increase in cell proliferation in a dose-dependent manner. Treatment of IL-6 recombinant protein at 2 µg/ml showed almost 250% increase in proliferation rate for both cells. The data suggests that IL-6 has a direct effect on promoting EC cell proliferation. Taken together, the data shows that IL-6 present in CAFs conditioned media is involved in mediating EC cell proliferation.

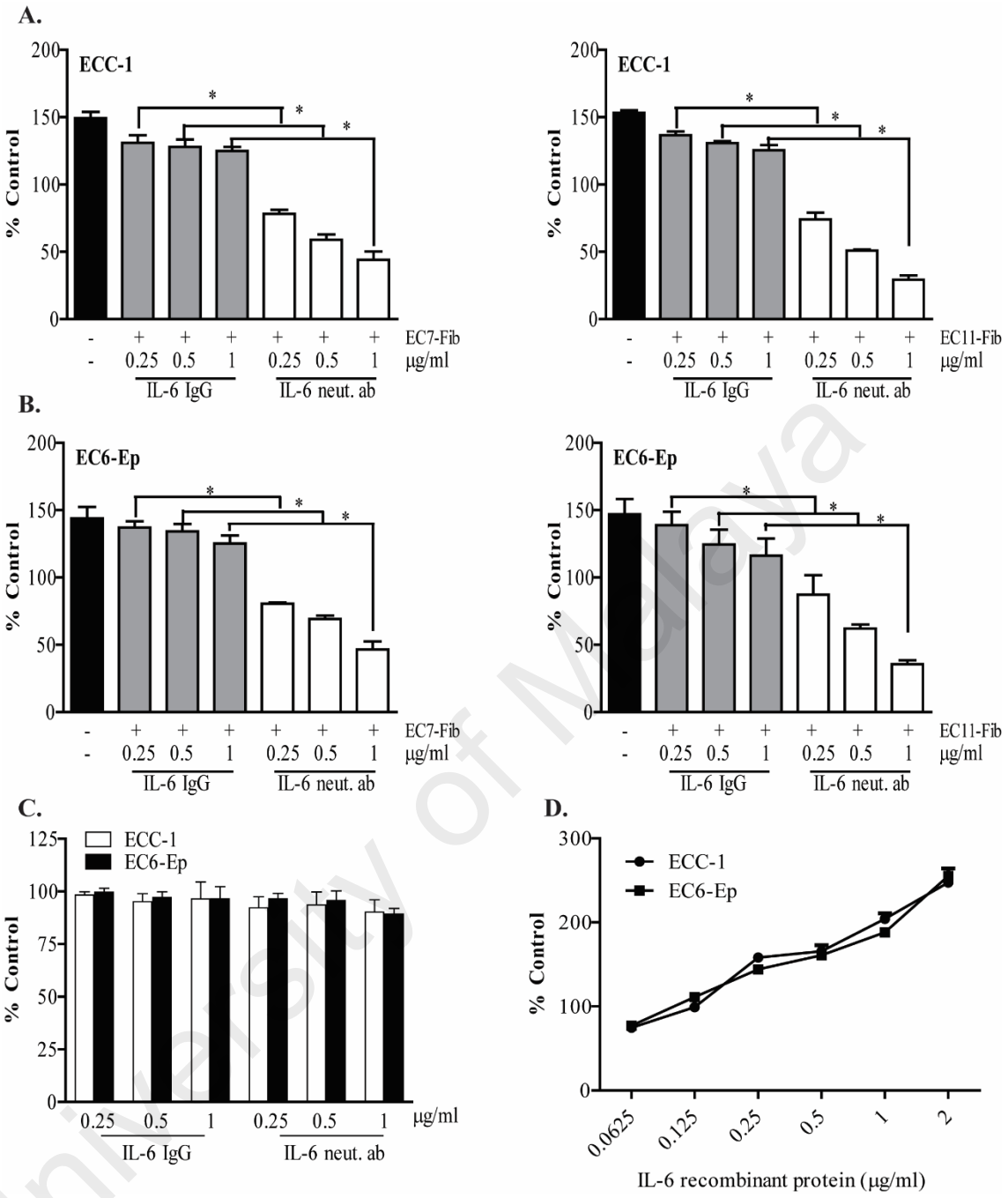


Figure 4.23. Inhibition of IL-6 reduces CAF-mediated EC proliferation.
ECC-1 cell line and EC6-Ep primary cell were treated with IL-6 neutralizing antibody and its IgG control in the presence CAFs conditioned media (A, B). ECC-1 and EC6-Ep cells were treated similarly with IL-6 neutralizing antibody and its IgG control in the absence of CAFs conditioned media (C). ECC-1 and EC6-Ep cells were treated with IL-6 recombinant protein only, without conditioned media (D). Cell viability was determined using MTT assay and normalized to control media (media containing 2% FBS). Data, average; error bars, SD. *,$P<0.05$. Data shown are representative of three independent experiments.

4.3.4 Activation of IL-6 downstream signaling pathways in CAF-mediated EC proliferation

To understand the underlying mechanism of IL-6-induced cell proliferation, signaling pathways activated by this cytokine were investigated. Firstly, the expression of the two IL-6 receptors (IL-6R and gp-130) were evaluated. Real-time PCR **(Figure 4.24 A)** and Western blotting **(Figure 4.24 B)** was performed on EC epithelial cells (ECC-1, HEC-1A, EC6-Ep, and EC14-Ep) and on four CAFs (EC6-Fib, EC7-Fib, EC11-Fib, EC14-Fib). As the aim was to detect if IL-6R and gp130 receptors protein were present on all eight cells tested using Western blot, no specific positive control was assigned. The immunoblotting was done using whole cell lysates. The molecular weight of IL-6R and gp-130 proteins were approximately at 52 kD and 103 kD, respectively.

Both assays showed that IL-6R and gp-130 were expressed only in EC epithelial cells but not in CAFs cells. This could be observed at both mRNA and protein level which led us to believe that IL-6 cytokines are secreted from CAFs to EC cells to activate a paracrine signaling action.

IL-6 was shown to initiate a signaling cascade that promotes cell proliferation. Thus, the role of IL-6 in CAFs secretion in the activation of three commonly implicated signaling pathways in EC tumorigenesis: the PI3K/Akt, MAPK/Erk and JAK/STAT3 pathways were investigated.

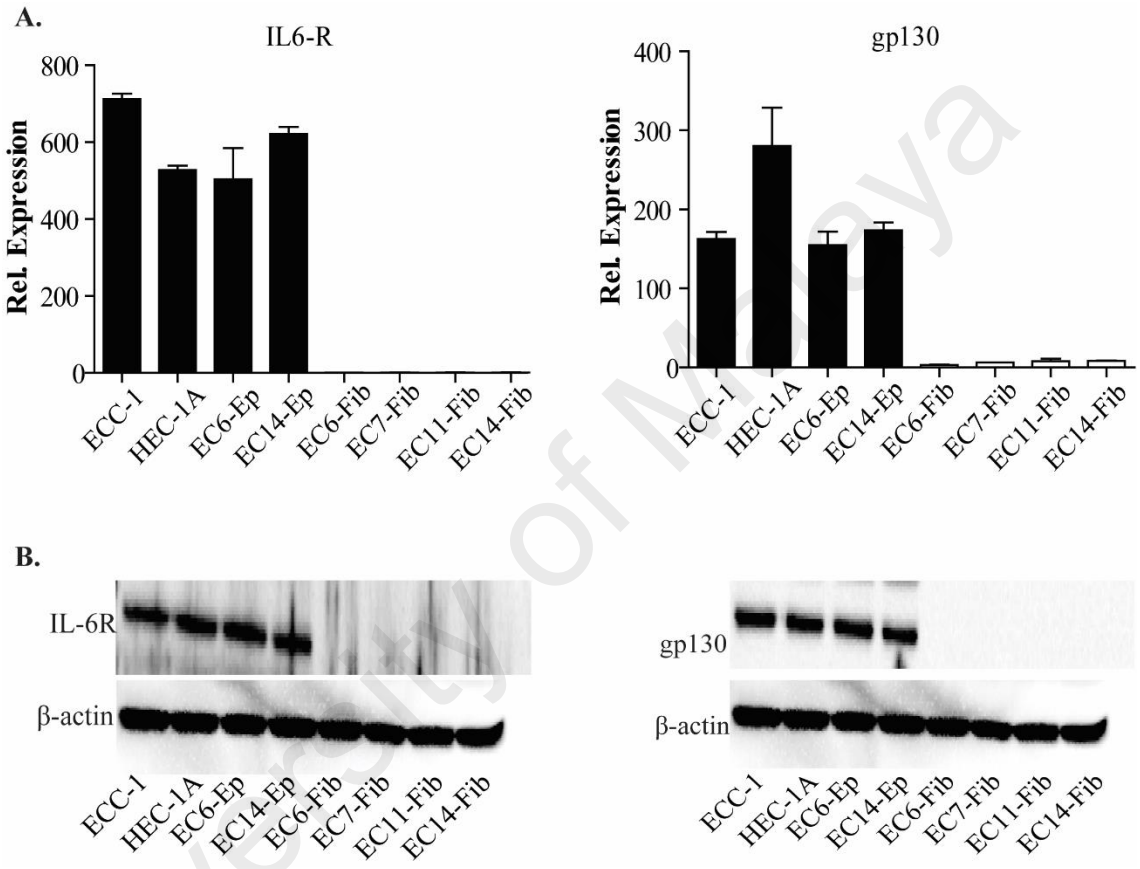


Figure 4.24. Evaluation of IL-6 receptors on epithelial and fibroblast cells.
Quantitative PCR analysis (A) was done to evaluate the expressions of IL-6R and gp130 in endometrial epithelial and fibroblast cells. Similarly, 30µg of protein extracted from all four epithelial and fibroblast cells was used to determine the presence of these receptors using Western blot analysis (B). Data shown are representative of three independent experiments.

4.3.5 Activation of PI3K/Akt and MAPK/Erk signaling pathways in CAFs-mediated EC proliferation

ECC-1 cells were treated with control media (media with 2% FBS) or conditioned media from T-HESC, EC6-Fib, EC7-Fib, EC11-Fib and EC14-Fib cells (2µg/µl) before harvesting the protein for Western blot and ELISA assays. Treatment with T-HESC secretion notably reduced expression of phospho-Akt and phospho-Erk protein in ECC-1 cells. Contrastingly, the phospho-Akt protein was slightly elevated upon treatment with the four CAFs conditioned media. Phospho-Erk protein level was significantly higher in the similar treatment condition. Upregulation of phospho-Akt and -Erk were seen in both Western blots and in the ELISA assay **(Figure 4.25)**. This indicated that the secretion from CAFs but not from benign fibroblast activates Akt and Erk pathways.

To further investigate the functional role of PI3K/Akt and MAPK/Erk pathways in CAFs-mediated cell proliferation, ECC-1, EC6-Ep, HEC-1A and EC14-Ep cells were treated with PI3K selective inhibitor (LY294002) and Erk selective inhibitor (U0126) in the presence of EC6-Fib and EC11-Fib conditioned media for 72 hours. Both LY294002 and U0126 significantly reduced CAFs-mediated cell proliferation in these cells ($P<0.0001$) **(Figure 4.26)**. U0126 caused a greater growth inhibitory effect in EC cells treated with EC11-Fib conditioned media.

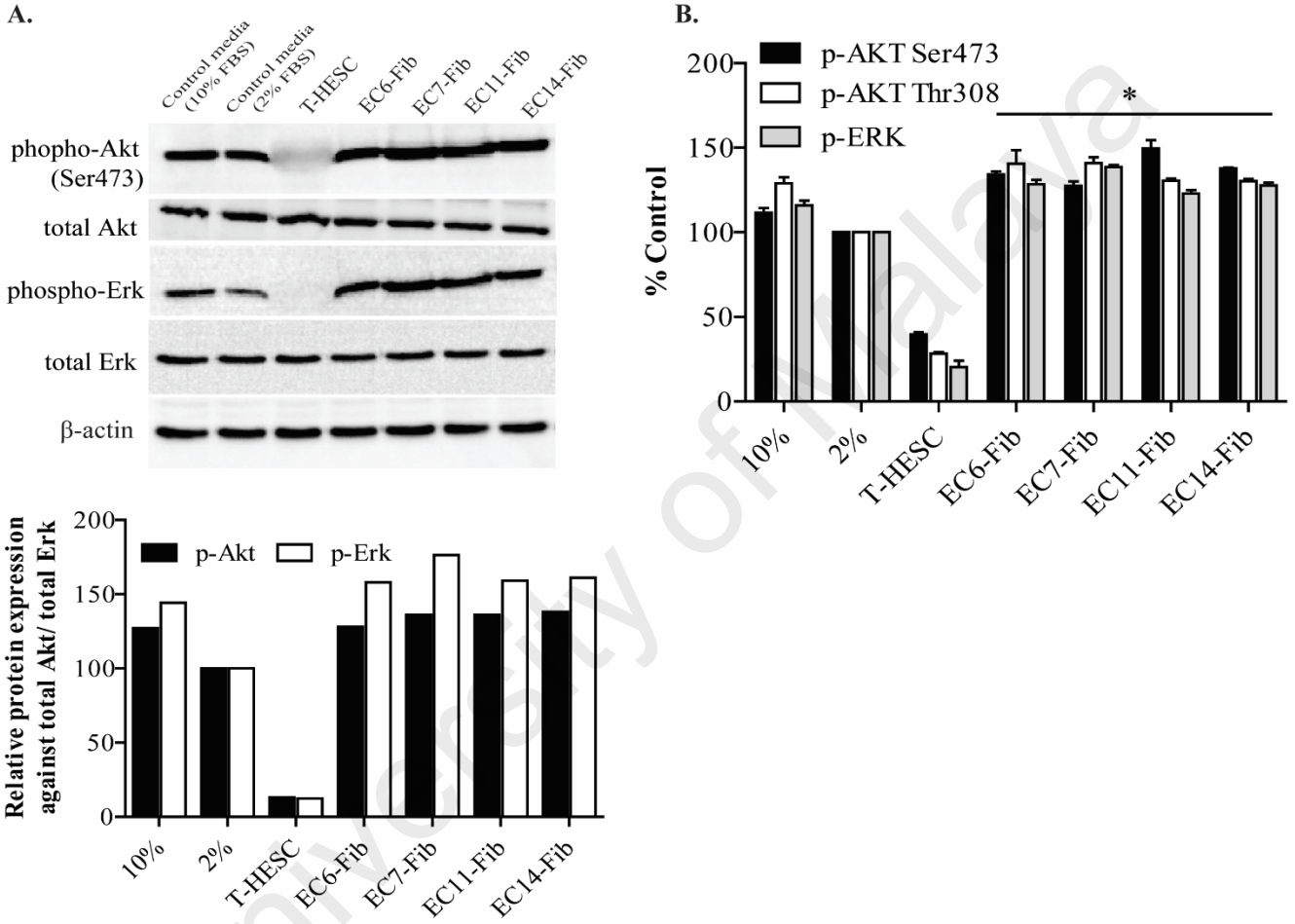


Figure 4.25. Activation of PI3K/Akt and MAP/Erk pathways.
Western blot analysis of phosphorylated-Akt (Ser473) and -Erk protein expression was evaluated in ECC-1 cells after treated with either T-HESC cells or cancer CAFs secretions. (A). Quantitative analysis using ELISA method measuring phosphorylated-Akt (Ser473 and Thr308) and phosphorylated-Erk levels in ECC-1 cells after treated with either T-HESC or CAFs, in comparison to cells treated with control (media containing 2% FBS) (B). Data, average; error bars, SD. *,$P<0.05$. Data shown are representative of three independent experiments.

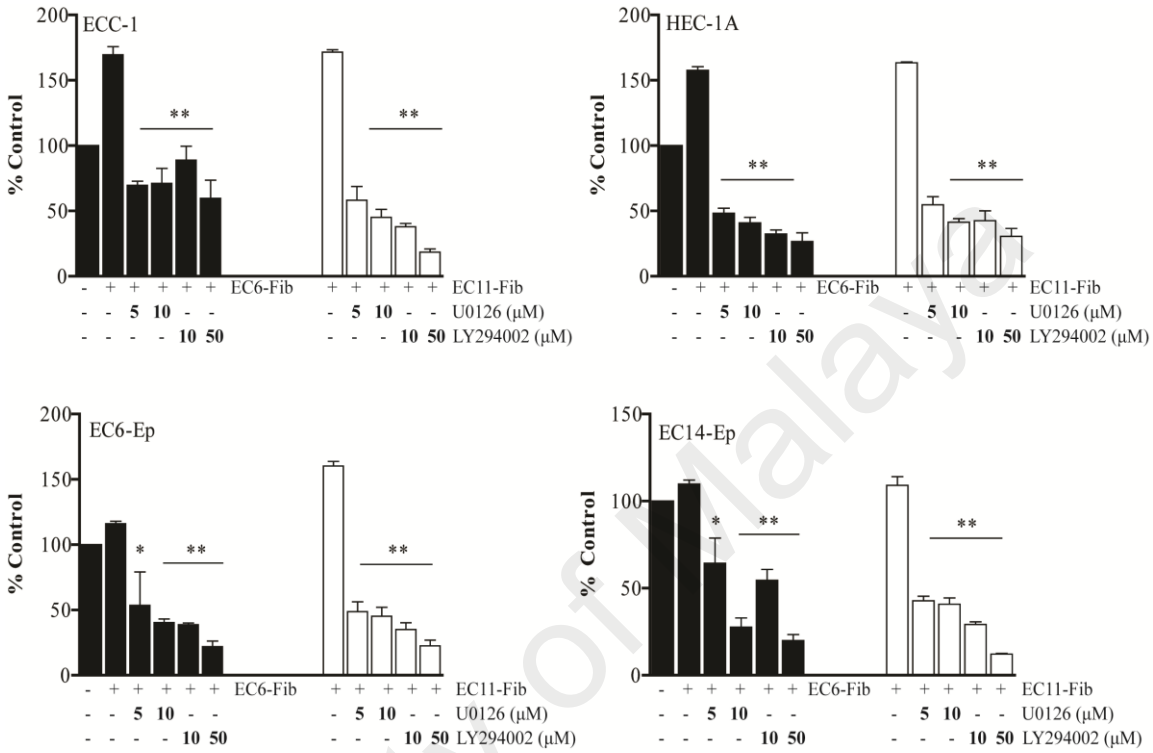


Figure 4.26. Inhibition of PI3K/Akt and MAPK/Erk using LY294002 and U0126 in the presence of CAFs-conditioned media affects endometrial cell proliferation.
ECC-1, EC6-Ep, HEC-1A and EC14-Ep cells were treated with either PI3K pathway selective inhibitor (LY294002) or Erk pathway selective inhibitor (U0126) in the presence of cancer-associated fibroblasts conditioned media (EC6-Fib or EC11-Fib) (1 μg/μl) for 72 hours. Data shown are cell viability after normalized with control (media containing 2% FBS). Data, average; error bars, SD. *, $P<0.05$; **, $P<0.0001$. Data shown are representative of three independent experiments.

These inhibitory effects were only observable in the presence of CAFs conditioned media. In the absence of CAFs conditioned media, cell proliferation was insignificantly affected **(Figure 4.27)**. These results indicate that both PI3K/Akt and MAPK/Erk pathways are involved in CAFs-mediated EC proliferation.

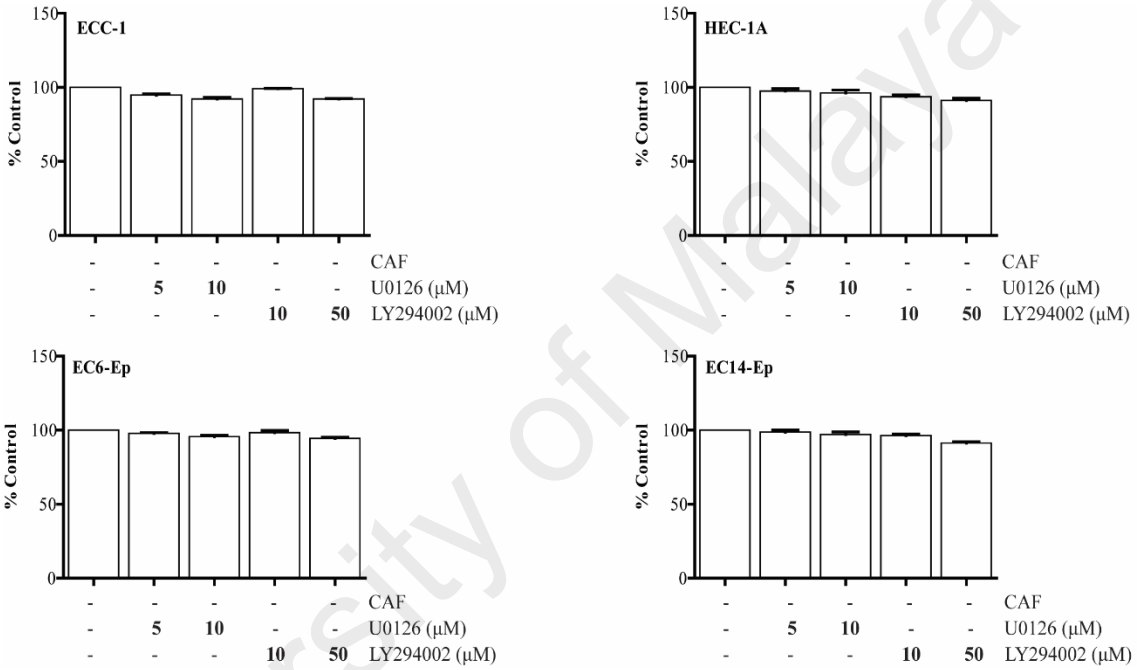


Figure 4.27. Inhibition of PI3K/Akt and MAPK/Erk using LY294002 and U0126 in the absence of CAFs-conditioned media.
ECC-1, EC6-Ep, HEC-1A and EC14-Ep cells were treated with either PI3K pathway selective inhibitor (LY294002) or Erk pathway selective inhibitor (U0126) in the absence of cancer-associated fibroblasts conditioned media for 72 hours. Data shown are cell viability after normalized with control (media containing 2% FBS). Data, average; error bars, SD. *, $P<0.05$; **, $P<0.0001$. Data shown are representative of three independent experiments.

4.3.6 Activation of JAK/STAT-3 in CAFs-mediated EC proliferation

Next, the effects of CAFs on the activation of JAK/STAT3 pathway were investigated. Results showed that the phospho-JAK3 and phospho-STAT3 levels were significantly elevated (~2-fold increase) compared to ECC-1 treated with control media **(Figure 4.28 A)**. Upregulation of both these pathways were attenuated by IL-6 neutralizing antibody even in the presence of CAFs conditioned media **(Figure 4.28 B)**. These observations indicate that IL-6 secreted by CAFs modulates activation of JAK/STAT3 pathway in EC cells.

To examine the functional mechanism of JAK/STAT3 pathway in IL-6 mediated tumor growth, ECC-1 cell line, and EC6-Ep primary culture were treated with JAK3 selective inhibitor (AD412) and STAT3 selective inhibitor (STATTIC). This was performed in the presence of either EC7-Fib or EC11-Fib conditioned media for 72 hours. Both inhibitors significantly reduced ECC-1 and EC-6Ep cell proliferation even at the lowest dose of 10 μM compared to control media (media with 2% FBS). Treatment with the inhibitors alone did not cause many changes in cell proliferation as indicated by the black bars **(Figure 4.28 C, D)**.

Taken together, these data indicate that IL-6 secreted by CAFs induced paracrine signaling in EC cells, resulting in activation of IL-6 receptor pathway. This, in turn, activated a cascade of downstream signaling pathways, including PI3K/Akt, MAPK/Erk, and JAK/STAT3

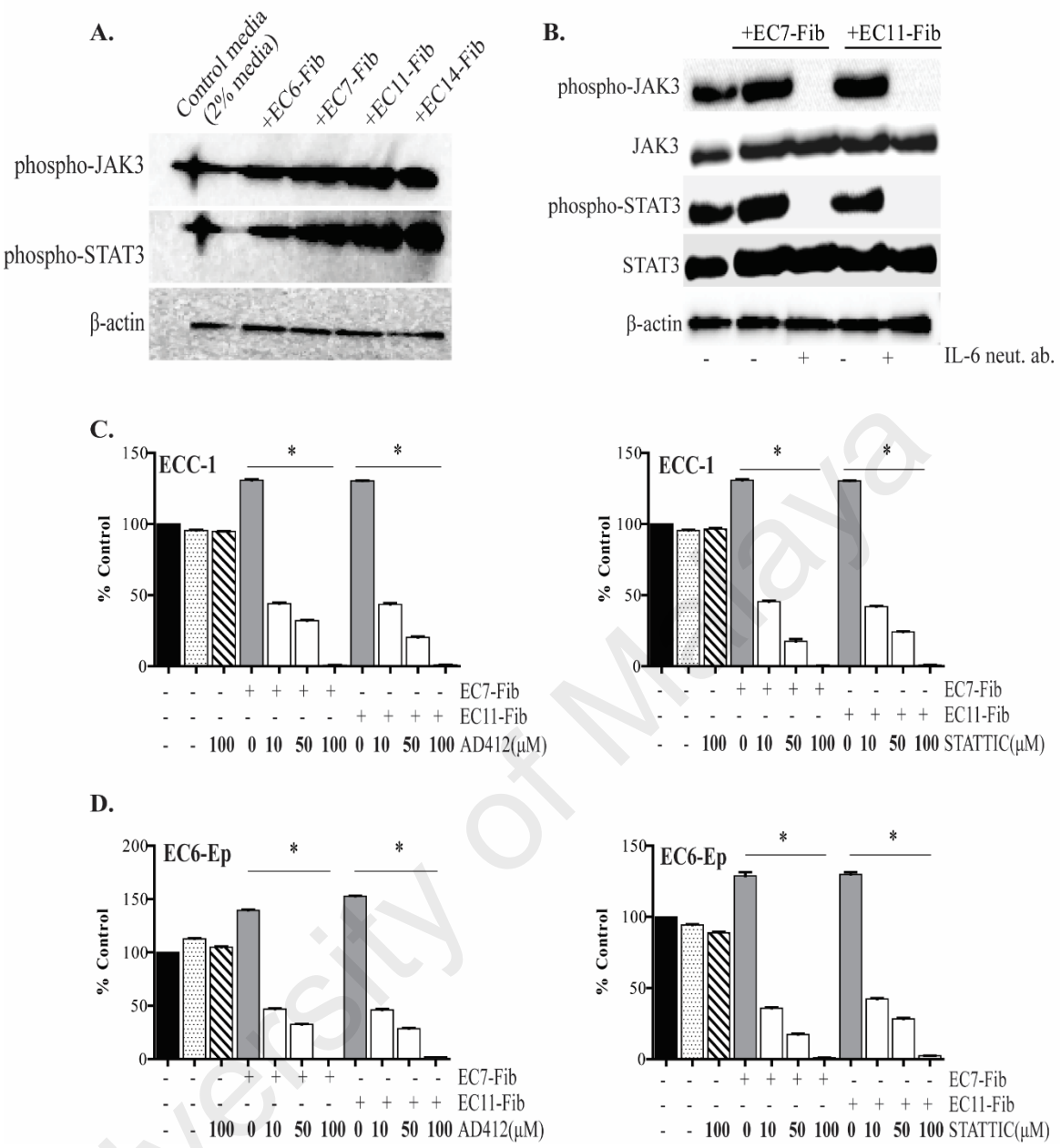


Figure 4.28. Inhibition of JAK3 and STAT3 pathways using AD412 and STATTIC with and without CAFs-conditioned media.

Western blot analysis of phosphorylated-JAK3 and –STAT3 protein expression in ECC-1 cells following treatment with cancer-associated fibroblast cells (EC6-Fib, EC7-Fib, EC11-Fib, and EC14-Fib). Expression of phospho-JAK3 and –STAT3 was determined in ECC-1 cells treated with IL-6 neutralizing antibody in the presence of CAFs conditioned media (A, B). ECC-1 cells and EC6-Ep were treated with either JAK3 selective inhibitor (AD 412) or STAT3 selective inhibitor (STATTIC) in the presence of CAFs conditioned media (EC7-Fib or EC11-Fib) (1 mg/ml) for 72 hours (C, D). Black bars indicate condition without the presence of CAFs secretion. Cell viability was examined using MTT assay and normalized to control media (media containing 2% FBS). Data, average; error bars, SD. *, $P<0.05$. Data shown are representative of three independent experiments.

4.3.7 Activation of STAT3, MAPK/Erk, and PI3K/Akt pathways downstream target genes

STAT-3 is a transcription factor that is often found to be phosphorylated in various malignancies upon induction of IL-6 and is often co-activated with Erk and Akt pathways. Thus, to determine if its downstream target genes were induced in our model, expression of six related target genes were measured. RNA extracted from ECC-1 cells, either alone or after treatment with four types of CAFs for 72 hours were subjected to real-time PCR **(Figure 4.29)**.

c-Myc expression level was significantly higher in ECC-1 cells treated with CAFs secretion compared to non-treated control. Secretions from EC6, 7, 11 and 14-Fib on ECC-1 cells each caused c-Myc levels to be upregulated to almost 100-fold, 116-fold, 302-fold and 124-fold higher than control media, respectively. TIMP-1, SOCS-3, and NFκB1 genes were also upregulated in treated ECC-1 cells, although at a much lower level.

TIMP-1 gene expression was higher in CAFs-treated cells than control media by at least 13-fold. SOCS-3 gene expression was about 5-fold higher in EC7-Fib treated ECC-1 cell. Treatment with other CAFs could only induce about 1-3 fold higher than control. Expression level NFκB1 gene was only about 0.5-fold higher than control media after EC14-Fib treatment. PIM1 was the only gene down-regulated in ECC-1 cells after treatment with different CAFs (<0.5 fold vs. control) while NFκB2 showed a mixed pattern of expressions. Of all six genes, c-Myc showed the highest induction indicating that it may be involved in CAFs-mediated EC proliferation.

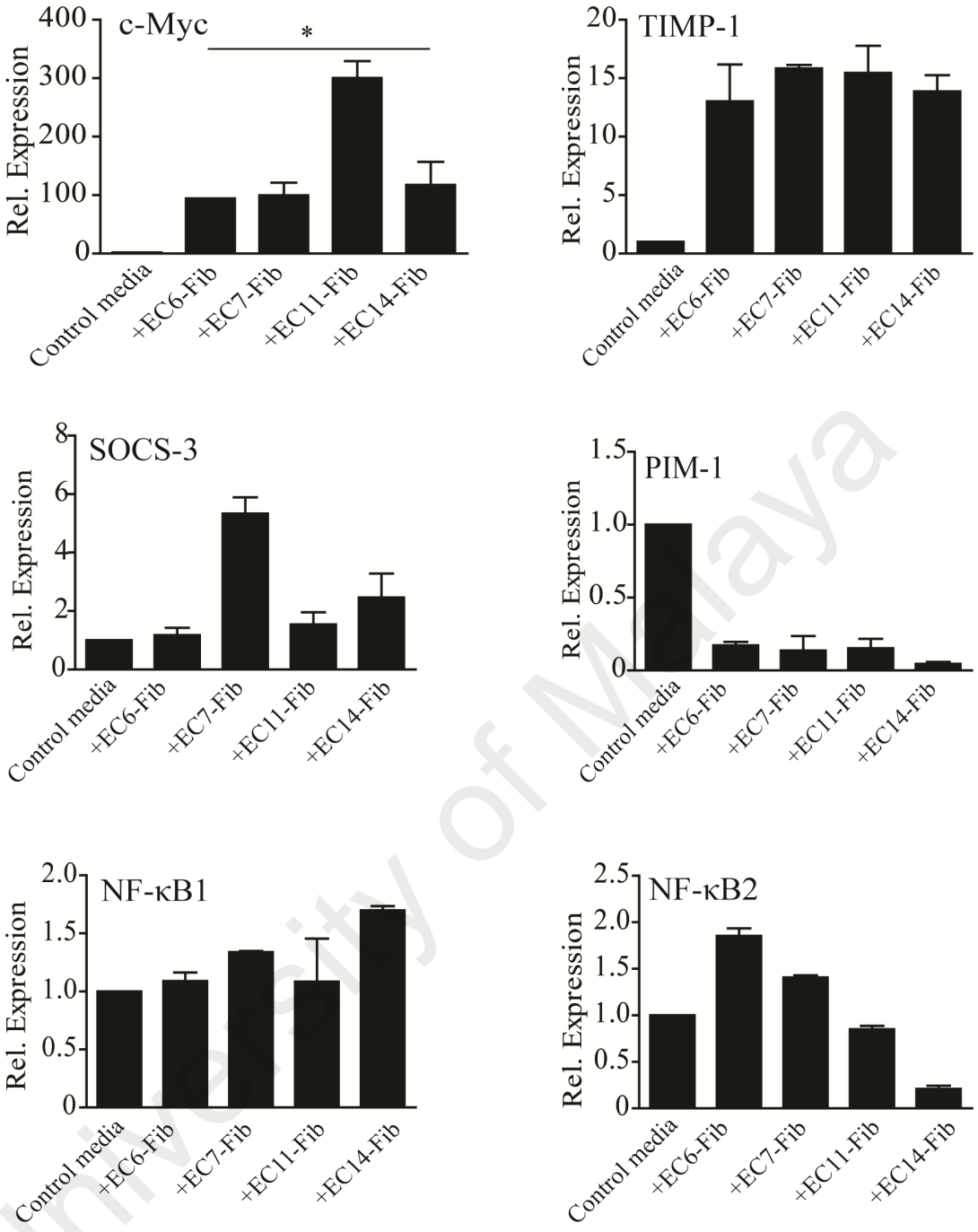


Figure 4.29. Activation of downstream target genes by STAT3, MAPK/Erk, and PI3K/Akt pathways.

ECC-1 cells after treated with CAFs for 72 hours was subjected to quantitative real-time PCR to examine the expression of STAT3 target genes (c-Myc, TIMP-1, PIM-1, SOCS-3, NFκB1, and NFκB2). Data were normalized to that in untreated ECC-1 cells. Data, average; error bars, SD. *, $P<0.05$. Data shown are representative of three independent experiments.

4.3.8 c-Myc regulates CAFs-mediated EC proliferation

Next, the role of c-Myc expression in EC-CAFs interaction was investigated. Western blotting showed c-Myc protein was upregulated upon treatment of ECC-1 cells with conditioned media from four CAFs (~2-4 folds higher) **(Figure 4.30 A)**.

Three individual shRNA constructs were used to downregulate the expression of c-Myc in ECC-1 cells. Expression of c-Myc protein was downregulated in ECC-1 cells to almost 50% upon transfection with c-Myc-specific shRNA constructs compared to c-Myc scramble construct **(Figure 4.30 B, C)**. ECC-1 cells with reduced c-Myc expression showed a notable decrease in cell proliferation (54% inhibition) compared to cells transfected with c-Myc non-targeting control (10% inhibition) **(Figure 4.30 C)**. Despite exposed to CAFs secretion, ECC-1 with reduced c-Myc expression did not increase their cell proliferation **(Figure 4.30 C)**. All three c-Myc-specific shRNA constructs successfully inhibited EC cell proliferation by at least 10-folds compared to scramble construct. This evidence indicates that c-Myc is an important mediator in EC proliferation and knocking down c-Myc in EC cells reversed CAFs proliferative effect.

To determine if CAFs effect on EC cells could be reversed by chemical inhibitors, 10058-F4 which is a c-Myc small molecule compound was used to treat ECC-1 cells in the presence of CAFs. While treatment with the highest dose of inhibitor alone did not cause much effect on EC cell proliferation, dose-dependent suppression of cell proliferation was observed when CAFs secretion was co-treated with this inhibitor **(Figure 4.30 D)**. The most notable effect was seen at 100 μM in which about 80% inhibition was observed in the presence of EC7- and 11-Fib conditioned media.

Taken together, our results show that CAFs-mediated EC proliferation could be reversed by either knockdown of c-Myc gene or by using c-Myc-specific small molecule inhibitor.

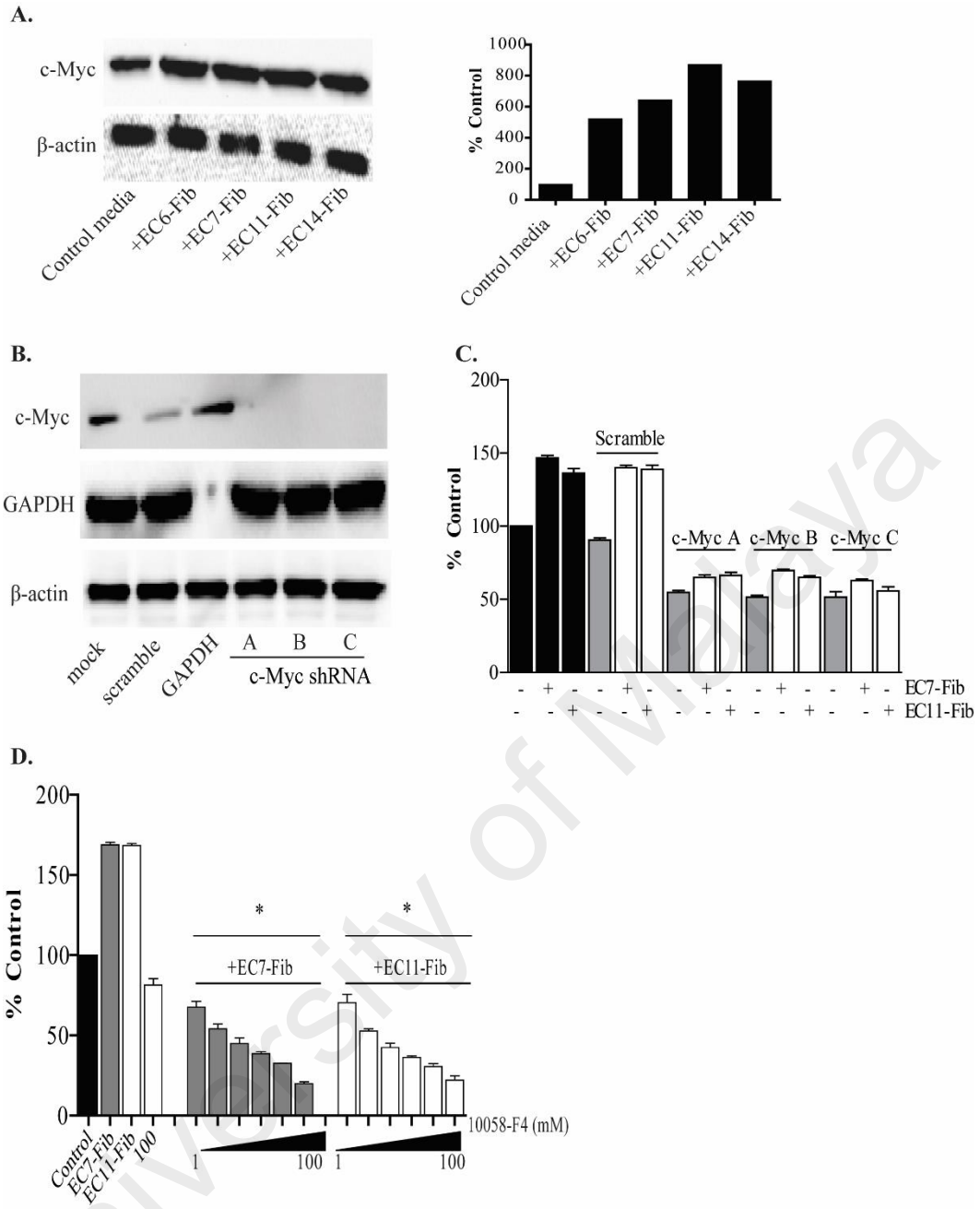


Figure 4.30. c-Myc regulates CAFs-mediated EC cell proliferation.
c-Myc protein expression was analyzed in ECC-1 cell after treated with CAFs. β-actin was used as an endogenous control. Bar graph shows densitometry analysis of c-Myc protein level (A). ECC-1 cells were transfected with a mock, shRNA construct targeting a scrambled c-Myc sequence, shRNA targeting GAPDH and three individual shRNA construct targeting c-Myc. Levels of c-Myc post-knockdown in ECC-1 cells were analyzed by western blotting with β-actin as an endogenous control. ECC-1 cells with low c-Myc levels was treated with CAFs secretion to examine cell viability (B, C). ECC-1 cells treated with c-Myc-specific inhibitor 10058-F4 in the presence of CAFs secretion (D). Cell viability was examined using MTT assay and normalized to its respective controls. Data, average; error bars, SD. *, $P<0.05$. Data shown are representative of three independent experiments.

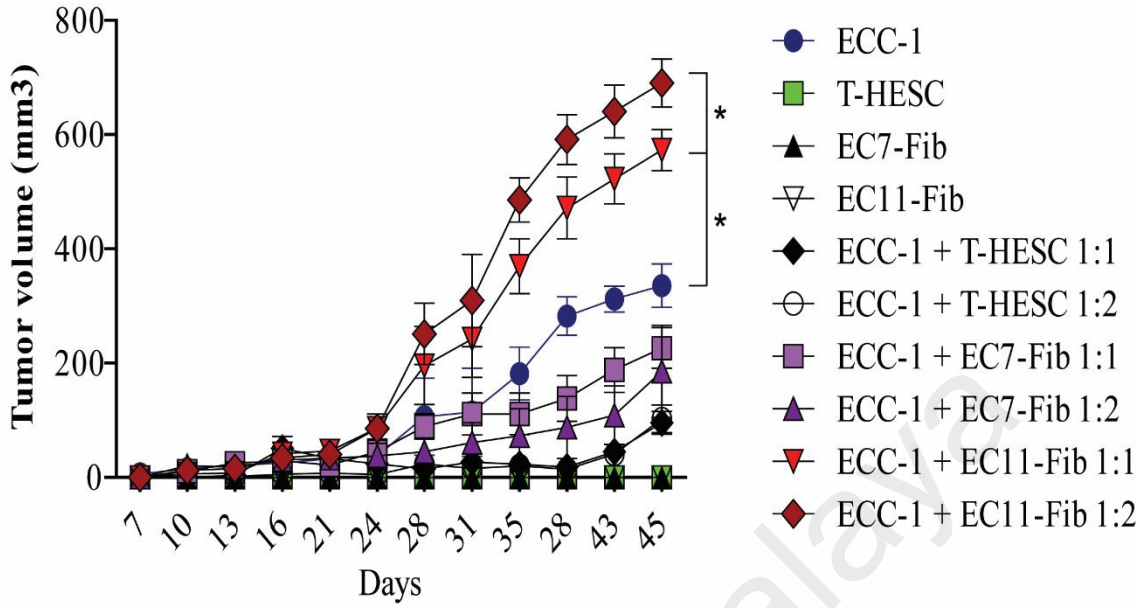
4.4 The role of CAFS in EC growth *in vivo*

4.4.1 CAFs modulate EC growth in vivo

To examine the role of CAFs-mediated EC growth *in vivo*, ECC-1 cells were inoculated subcutaneously into the right flank of 4-6 weeks of Balb/c nude mice either alone, or in combination with EC7-Fib, EC11-Fib or benign fibroblast T-HESC in the ratio of 1:1 and 1:2. The experiment started with three mice per group; however, a mouse in the ECC-1 only group died before the end of the experiment.

Co-injection of ECC-1 with EC7-Fib both in the ratio of 1:1 and 1:2 failed to induce growth above the control group (ECC-1 only). However, significant growth rate occurred in ECC-1 injected with EC11-Fib in the ratio of 1:1 and 1:2 compared to the ECC-1 only group where tumor sizes were 572.93 mm^3 and 690.14 mm^3, respectively. Interestingly, co-injection of ECC-1 with benign fibroblast, T-HESC both in the ratio of 1:1 and 1:2 grew in smaller tumor sizes (95.51 mm^3 and 102.61 mm^3) compared to ECC-1 only group (335.75 mm^3). Excised tumor images showed correlation with the observed tumor sizes **(Figure 4.31)**. Mice injected with CAFs cells only did not incur tumor growth until the end of the experiment.

A.



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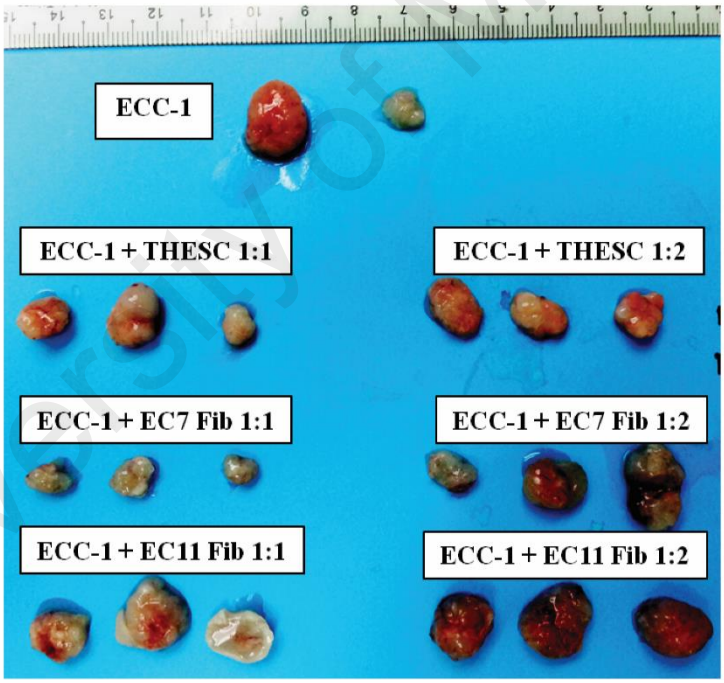

Figure 4.31. CAFs modulate EC growth *in vivo*.
To observe differential effect of CAFs and normal fibroblast *in vivo*, ECC-1 was first inoculated subcutaneously into the right flank of 4-6 weeks of Balb/c nude mice either alone, or in combination with EC7-Fib, EC11-Fib or benign fibroblast T-HESC in the ratio of 1:1 and 1:2 as a starting point (A). Image shows tumors removed from mice on the final day of measurement (B). Data, average; error bars, SD. *, $P<0.05$.

A second experiment was performed with five mice per group. This time, the experiment aimed to observe if a higher proportion of fibroblast cells would result in significant difference in EC tumor growth. ECC-1 cells were injected either alone or in the combination with EC11-Fib or benign fibroblast T-HESC in the ratio of 1:2 and 1:4.

Weekly tumor measurement showed that once again groups with fibroblast cells only did not induce any growth in mice. However, there was a marked increase in tumor growth of ECC-1 when co-injected with EC11-Fib in the ratio of 1:2 and 1:4 (623.72 mm^3 and 1166.19 mm^3 respectively). ECC-1 in combination with T-HESC at 1:4 showed total inhibition of tumor growth until the end of the experiment. Fibroblast cells injected alone did not show any signs of growth. At the end of the experiment, tumor removed from the mice showed a significant increase in size in ECC-1 with CAF (1:4) groups compared to ECC-1 alone **(Figure 4.32)**.

Collectively, CAFs promoted EC growth both *in vitro* and *in vivo*. In contrast, T-HESC inhibited the EC growth. Most importantly, mice harboring fibroblast cells only did not show any signs of tumor growth until the end of the experiment.

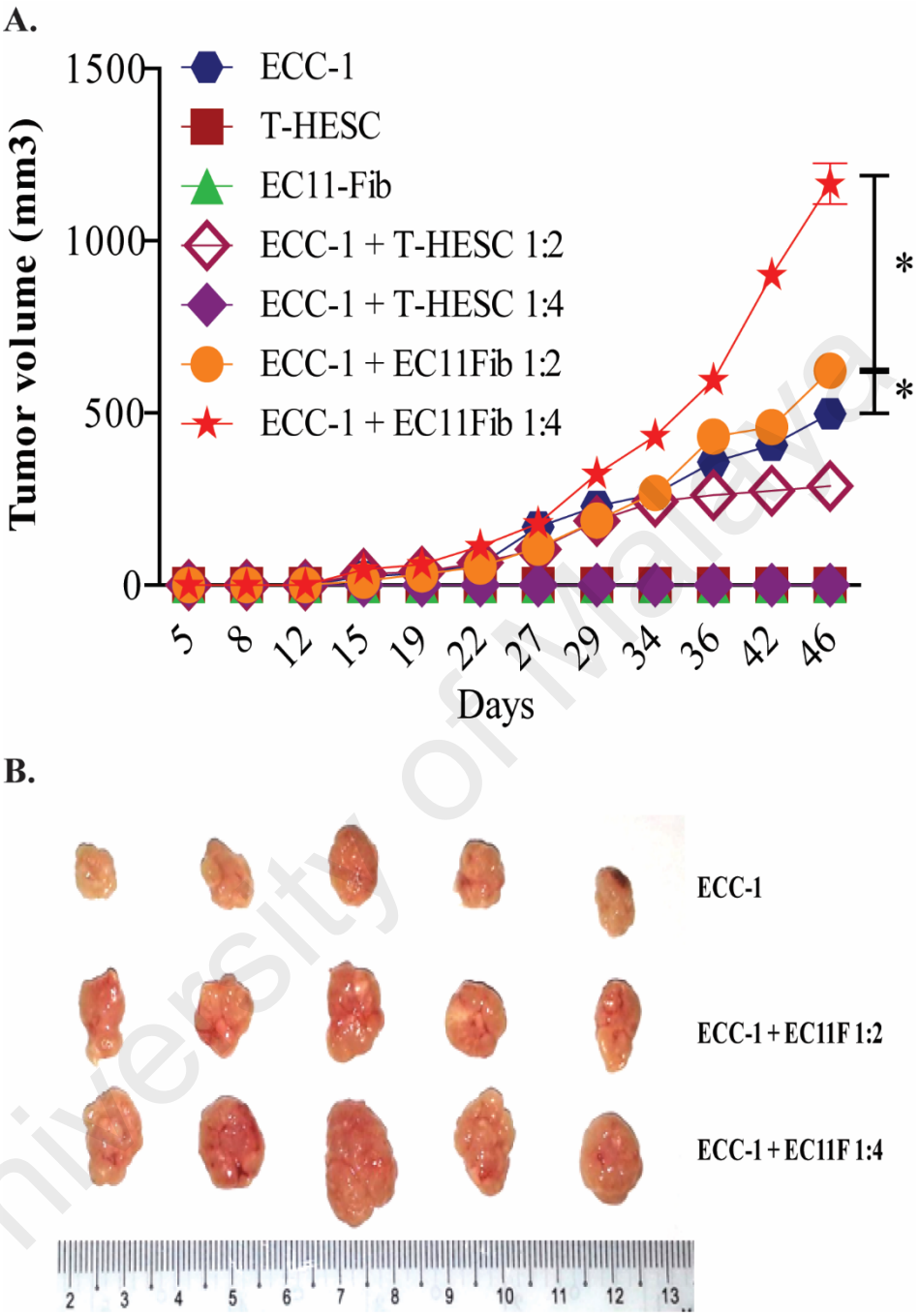

Figure 4.32. CAFs modulate EC growth *in vivo*.
To observe the effect of CAFs at a higher ratio, ECC-1 was inoculated either alone or in combination with EC11-Fib or benign fibroblast, T-HESC in the ratio of 1:2 and 1:4 (A). Image shows tumors removed from mice on the final day of measurement (B). Data, average; error bars, SD. *, $P<0.05$.

4.4.2 c-Myc regulates CAFs-mediated EC growth in vivo

To determine whether c-Myc also have a role in promoting EC growth *in vivo,* a subcutaneous human tumor xenograft mouse model was used. ECC-1 cells were transfected with red fluorescence protein (RFP) while EC11-Fib was transfected with green fluorescence protein (GFP).

ECC-1 tumors were about 736.8 ± 11.1 mm^3 while those mice injected with CAFs alone (EC11-Fib cells) alone did not show any signs of cell growth. Mice injected with a combination of ECC-1 and EC11F (1:4 ratio) showed at least 1.4 times greater tumor size (1042.2 ± 27 mm^3) compared to those injected with ECC-1 cells alone (P<0.0001). Interestingly, mice injected with ECC-1 with reduced c-Myc expression showed at least 2.5 times smaller tumor size (293.9 ± 7 mm^3) when compared to those in the scramble group (P<0.0001).

Co-injection with CAF failed to induce greater tumor growth in this group (361.9 ± 13.7 mm^3) (P=0.013). Notably, there was a small but significant difference in growth rate of ECC-1 transfected with scramble shRNA, with and without EC11-Fib (P=0.0118) **(Figure 4.33 A)**. The tumor size between the groups was also analyzed using *in vivo* fluorescence imaging and it was closely correlated to the tumor volume measured at the end of the experiment **(Figure 4.33 B, C)**.

Overall, reduction of c-Myc gene in the EC cells caused a markedly lower tumor growth, that was not further induced despite co-injection with CAFs cells. Thus, the accumulating data shows that c-Myc plays an important role in promoting EC growth *in vivo*.

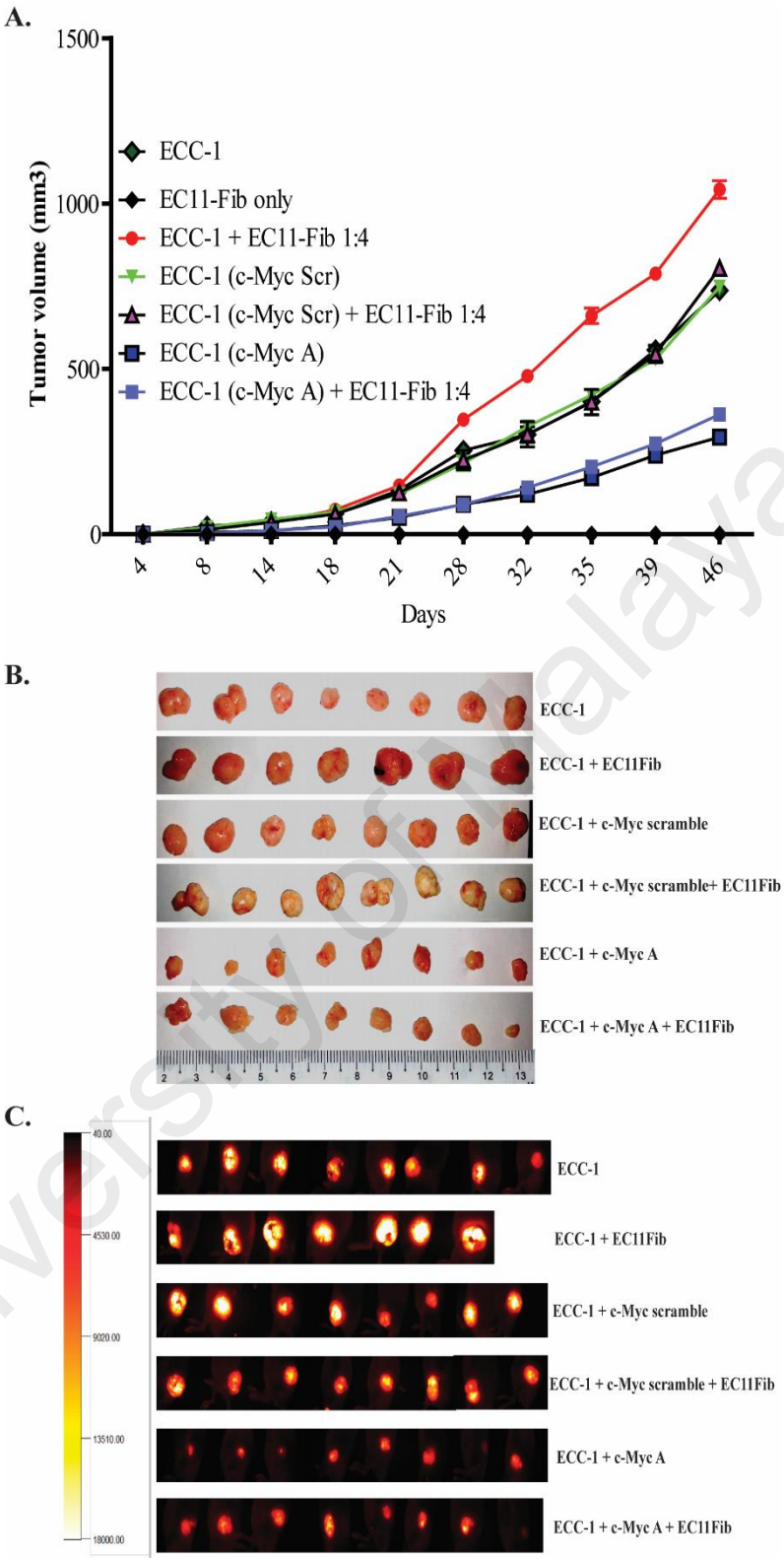

Figure 4.33. *In vivo* growth of EC tumor with c-Myc downregulation.
Stable clones of ECC-1 cells expressing c-Myc scramble or shRNA A constructs were established. RFP-transfected ECC-1 and GFP-transfected fibroblasts were inoculated subcutaneously into the right flank of 4-6 weeks of Balb/c nude mice. These cells were injected with or without EC11Fib cells, in a ratio of 1:4 (tumor:fibroblast). The growth of tumor cells was measured using caliper twice weekly until the day of termination (A). At the end of the experiment, tumor excision was performed (B) and was imaged using fluorescence imaging system (C). Data, average; error bars, SD.

4.4.3 Expression of ki67 and c-Myc protein in mice tumors

Upon termination of the experiment, tumors were excised and processed for immunohistochemical analysis, for the expression levels of c-Myc protein and ki67. Tumors from the group of ECC-1+ EC11Fib and ECC-1 with c-Myc scramble+EC11Fib showed more intense staining of nuclear ki67 expression, indicating more proliferative cells in these tumors Staining was weak to none in groups that had low levels of c-Myc, even in groups co-injected with EC11Fib. Groups with ECC-1 that expressed a base level of the c-Myc gene and co-injected with EC11Fib showed stronger c-Myc protein intensity than those group with reduced c-Myc level **(Figure 4.34)**. Our data indicated that collectively, c-Myc has a significant role in CAFs-mediated EC cell proliferation *in vivo*.

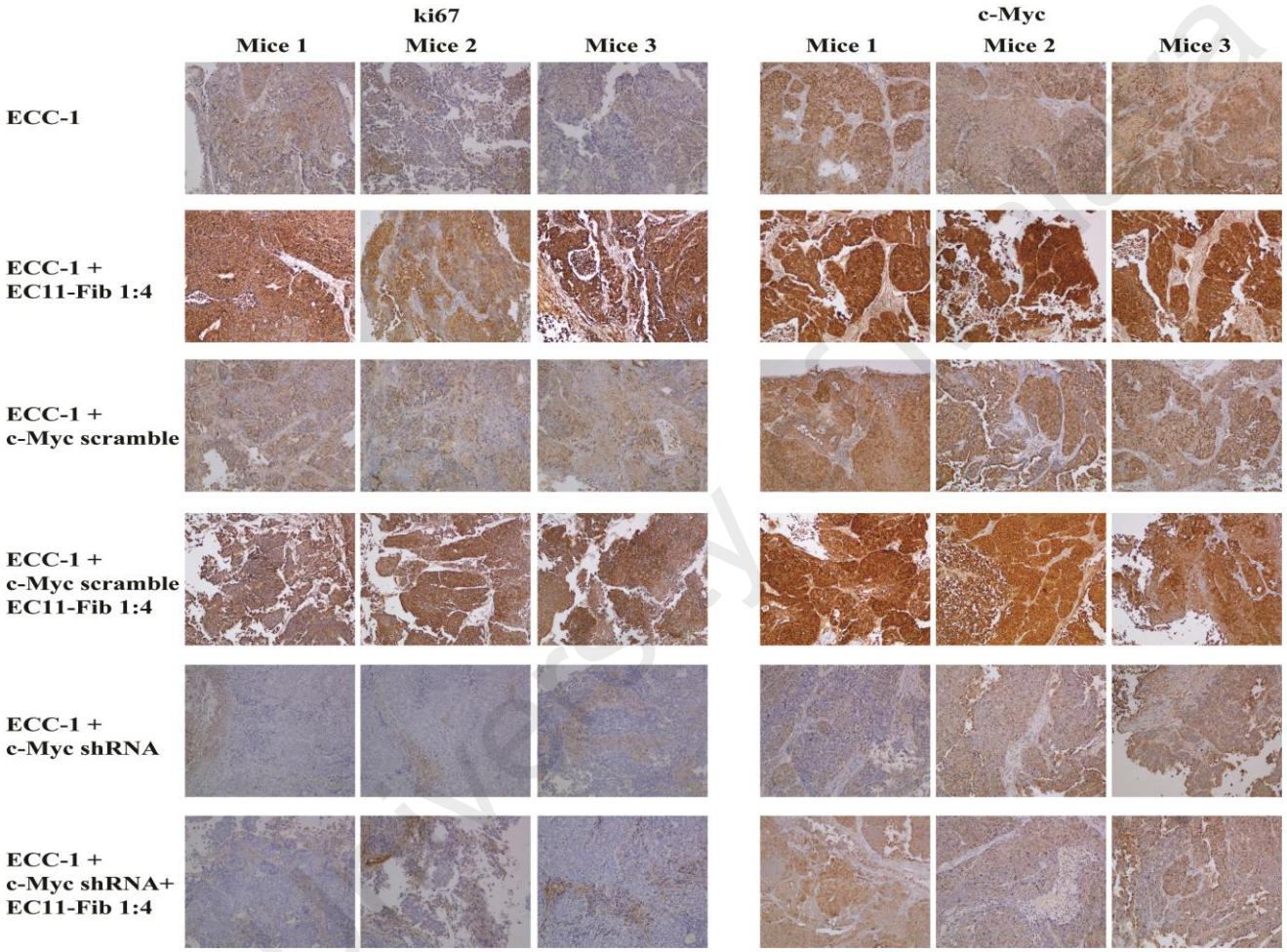

Figure 4.34. *In vivo* protein expression of ki-67 and c-Myc.
Mice tumors excised were subjected to immunohistochemistry to observe the presence of ki-67 and c-Myc protein. Three samples from each group are shown as a representative image. Magnification, 10x; Scale, 100 micron.

4.5 Activation of IL-6 signaling markers in human cancer tissues

Accumulating data thus far showed that activation of IL-6 signaling pathway by CAFs led to activation of phospho-STAT3 and c-Myc genes, which resulted in increased EC growth. To further examine whether this pathway is also activated in human EC tissues, the expression of IL-6R, gp130, phospho-STAT3 and c-Myc in eight benign **(Figure 4.35)** and nine cancerous tissues **(Figure 4.36)** of the human endometrium were examined.

Overall, positive staining was observed to be intense and uniform in the glandular linings of the cancer tissues compared to the benign tissues when observed at 10x magnification. Staining was also intense in the epithelial compartments compared to the surrounding stromas. Stronger IL-6R and gp130 staining were observed in the epithelial compartment of the cancerous tissues compared to in benign tissues. Phospho-STAT3 and c-Myc staining were also evident and predominantly confined to the nucleus in the cancer tissues although a rather weak and diffuse staining was seen in the cytoplasm of the benign tissues.

Further, it was seen that human endometrial cancer tissues expressed high levels of IL-6R, gp130, phospho-STAT3 and c-Myc proteins, which were minimally expressed in benign tissues. This suggests that IL-6 pathway was activated in human EC tissues.

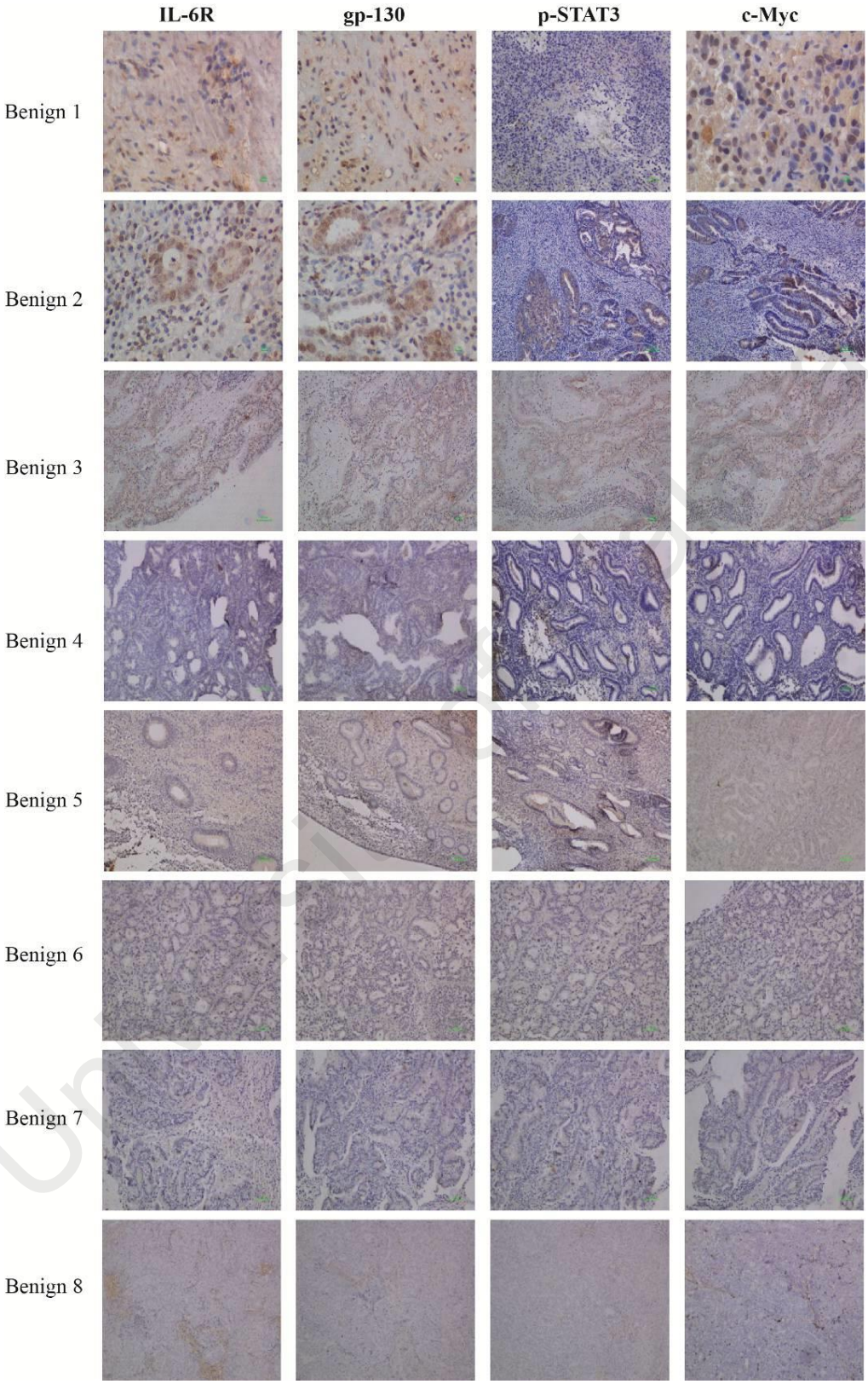

Figure 4.35. Expression of IL-6 receptors and key downstream signaling molecules in endometrial benign tissues.
Eight formalin-fixed paraffin embedded tissues from human endometrial benign conditions were stained with antibodies targeting human IL-6R, gp-130, phospho-STAT3 and c-Myc proteins. Detection was performed using light microscope at 10x magnification. Scale, 100 micron.

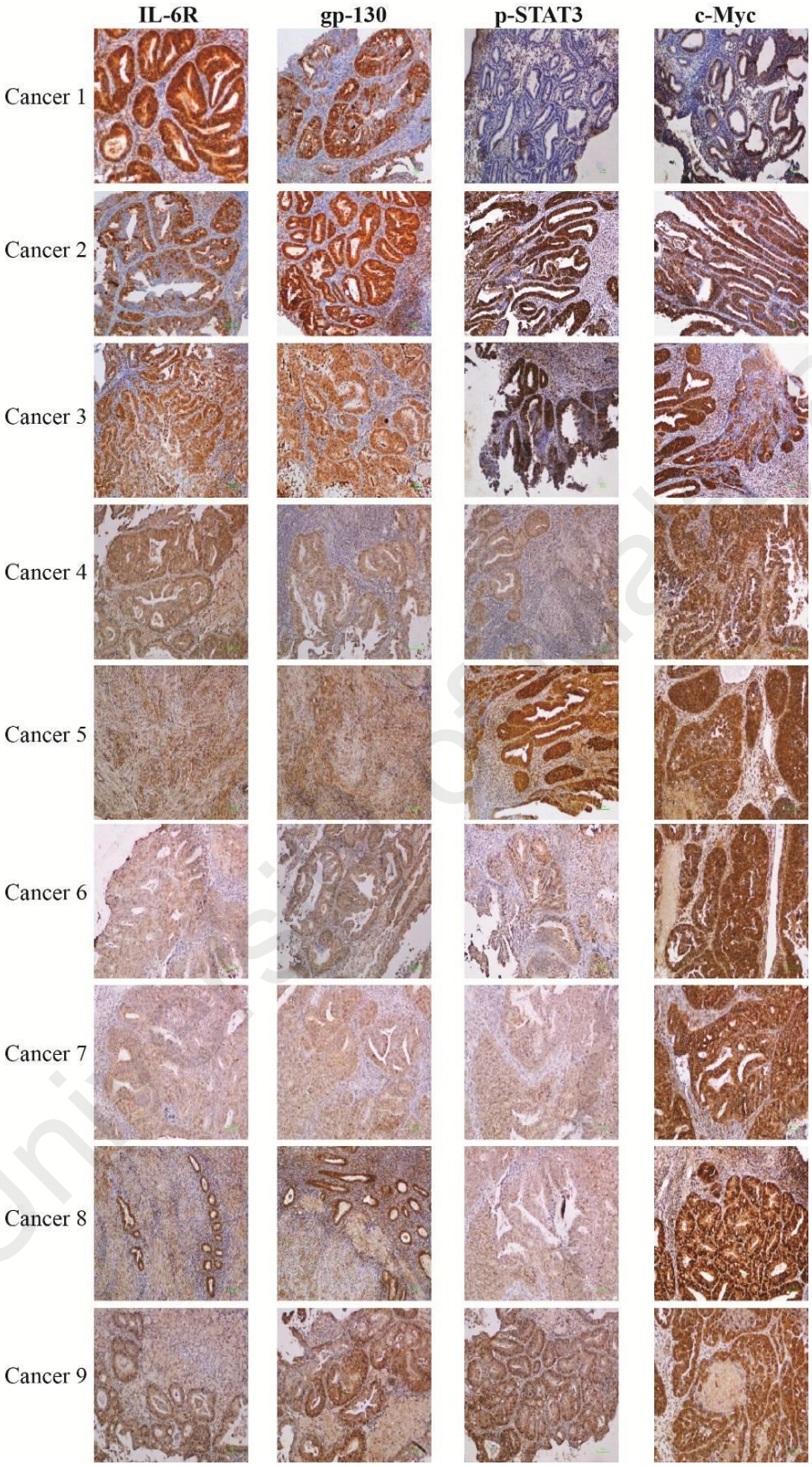

Figure 4.36. Expression of IL-6 receptors and key downstream signaling molecules in endometrial cancer tissues.

Nine formalin-fixed paraffin embedded tissues from human endometrial cancer conditions were stained with antibodies targeting human IL-6R, gp-130, phospho-STAT3 and c-Myc proteins. Detection was performed using light microscope at 10x magnification. Scale, 100 micron.

4.6 IL-6 mediated targeted therapy for endometrial cancer

4.6.1 Inhibitors action on CAFs-mediated EC proliferation

The data thus far suggests that IL-6 mediated pathways could be an important player in the pathogenesis of endometrial cancer and could potentially be targeted for future treatments. To test this hypothesis *in vitro*, IL-6 receptors-specific inhibitors, (raloxifene and bazedoxifene acetate) and PI3K inhibitor (rapamycin) were tested on CAFs-treated EC cells. These inhibitors are currently used for other therapeutic indications.

To evaluate whether rapamycin can be clinically useful in reversing CAFs-mediated EC cell proliferation, ECC-1, EC6-Ep, HEC-1A and EC14-Ep cells were treated with rapamycin in the presence of EC11-Fib conditioned media. Data showed significant inhibition of EC cells proliferation compared to control (P<0.0001) **(Figure 4.37)**. At the highest dose tested (2 μM), rapamycin reduced ECC-1 cells from 180% (conditioned media treatment alone) to 40% (conditioned media and rapamycin treatment) (P<0.0001), while minimal inhibition was observed when cells were cultured in control media.

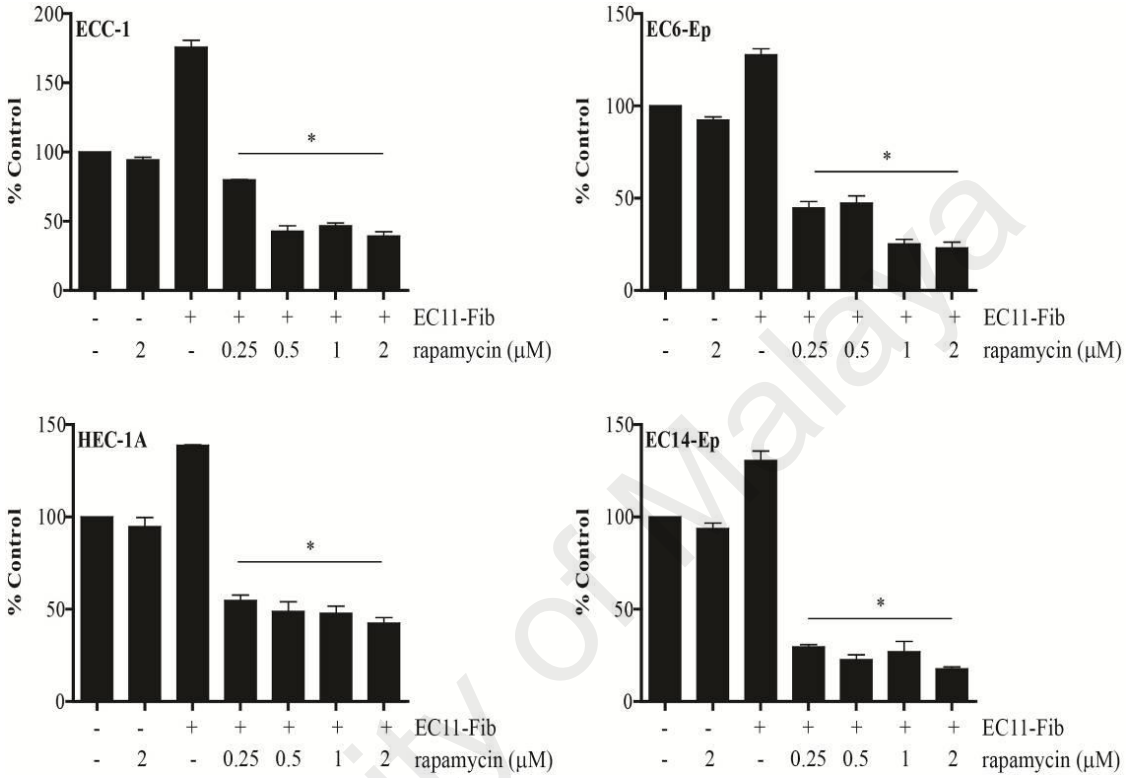

Figure 4.37. Rapamycin as PI3K/mTOR pathway inhibitor.
ECC-1 cell line, EC6-Ep cell, HEC1-A cell line and EC14-Ep cell were treated with increasing dose of rapamycin for 72 hours under the influence of control media (media containing 2% FBS) or 1 µg/µl EC11-Fib conditioned media or rapamycin alone. Data, average; error bars, SD. *, P<0.0001 compared to EC11-Fib treated cells. Data shown are representative of two independent experiments.

Two IL-6 receptor inhibitors (raloxifene and bazedoxifene acetate) were examined for their effects on abrogating IL-6 mediated cancer proliferation. ECC-1 and HEC-1A cells were treated with these two inhibitors in a dose-dependent manner, up to 100 µM for 72 hours, with and without EC6-Fib or EC11-Fib conditioned media. MTT results showed a dose-dependent decrease in proliferation in both cell lines, only when CAFs secretion was present **(Figure 4.38)**.

Raloxifene showed a rather low IC_{50} at 0.019 µM for ECC-1 and 0.038 µM for HEC-1A. The IC_{50} for bazedoxifene acetate was 0.223 µM and 0.297 µM for ECC-1 and HEC-1A respectively. Most importantly, in the absence of CAFs conditioned media, these two inhibitors showed no inhibiting nor promoting effects across all concentrations **(Figure 4.38 A).**

To demonstrate that these two inhibitors were specific for IL-6 actions, ECC-1 cells were treated with IL-6 recombinant protein prior to treatment with raloxifene or bazedoxifene acetate. MTT results showed a dose-dependent decrease in ECC-1 proliferation only in the presence of IL-6 recombinant protein **(Figure 4.38 B)**. These observations showed the significance of CAF as the source of IL-6 for such therapeutic inhibitors to inhibit EC proliferation.

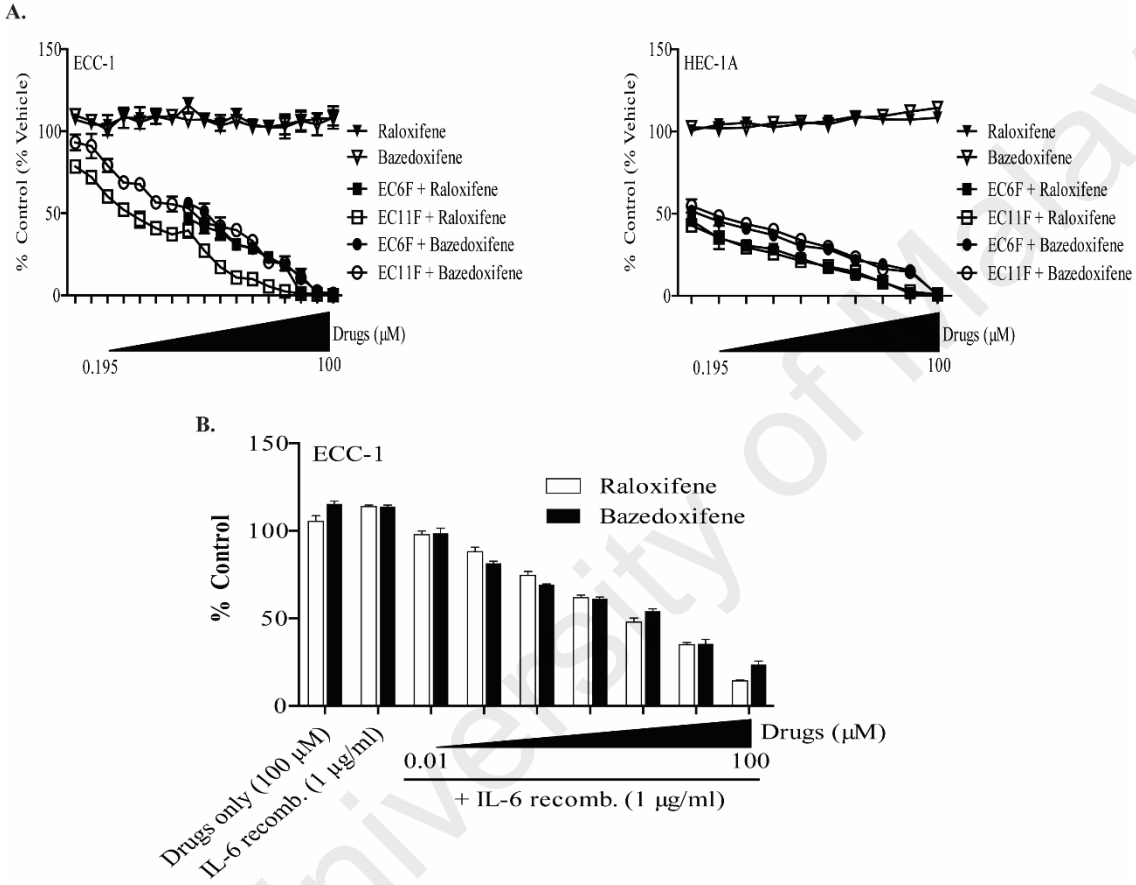

Figure 4.38. Inhibition of IL-6 receptors as a therapeutic option for endometrial cancer treatment.
ECC-1 and HEC-1A cells were treated in increasing doses of bazedoxifene acetate and raloxifene for 72 hours with and without the presence of EC6-Fib or EC11-Fib conditioned media (A). ECC-1 cells were exposed to IL-6 recombinant protein before treating with Bazedoxifene and Raloxifene in an increasing dose (B). Cells were treated for 72 hours and quantified by MTT assay. Data shown are representative of two independent experiments.

4.6.2 Inhibitors action on induction of apoptosis in CAFs-mediated EC proliferation

Annexin V labeling was performed to determine whether rapamycin affects EC cell proliferation *via* induction of apoptosis **(Figure 4.39)**. Treatment of ECC-1 with 1 µg/µl EC11-Fib conditioned media did not significantly affect the percentage of apoptotic cells; however, concurrent treatment with 2 µM rapamycin resulted in an increase of apoptotic cell population (annexin V-positive cells) from 4.8% to 21.1%. This suggests that rapamycin or its analogs may be useful in limiting CAFs-mediated EC cell proliferation in the clinical setting.

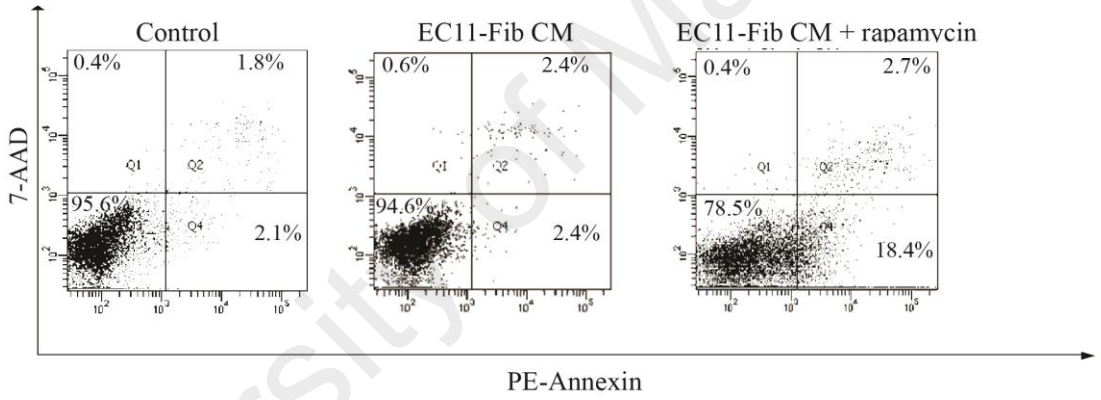

Figure 4.39. Mechanism of action of rapamycin as PI3K/mTOR pathway inhibitor. ECC-1 cells treated with 2 µM of rapamycin with or without 1 µg/µl EC11-Fib conditioned media for 72 hours, were stained with annexin V-PE and 7-AAD before analyzed with flow cytometry. Data shown are representative of two independent experiments.

Annexin V labeling was performed to determine whether raloxifene and bazedoxifene acetate affects EC cell proliferation *via* induction of apoptosis **(Figure 4.40)**. ECC-1 cells were treated with either raloxifene only, bazedoxifene only, IL-6 recombinant protein in combination with either inhibitors or EC11-Fib in combination with either inhibitor. Inhibitors were used at their IC_{50} values.

Treatment of ECC-1 cells with inhibitors only and IL-6 recombinant protein with inhibitors did not cause much increase in apoptotic cells (2-4%). However, ECC-1 cells treated with either raloxifene or bazedoxifene acetate showed almost 53-57% increase in apoptotic cells (PE-Annexin V-positive cells). These results indicate that rapamycin, raloxifene, and bazedoxifene acetate could be a potential therapeutic approach to inhibit the growth-promoting action of CAFs.

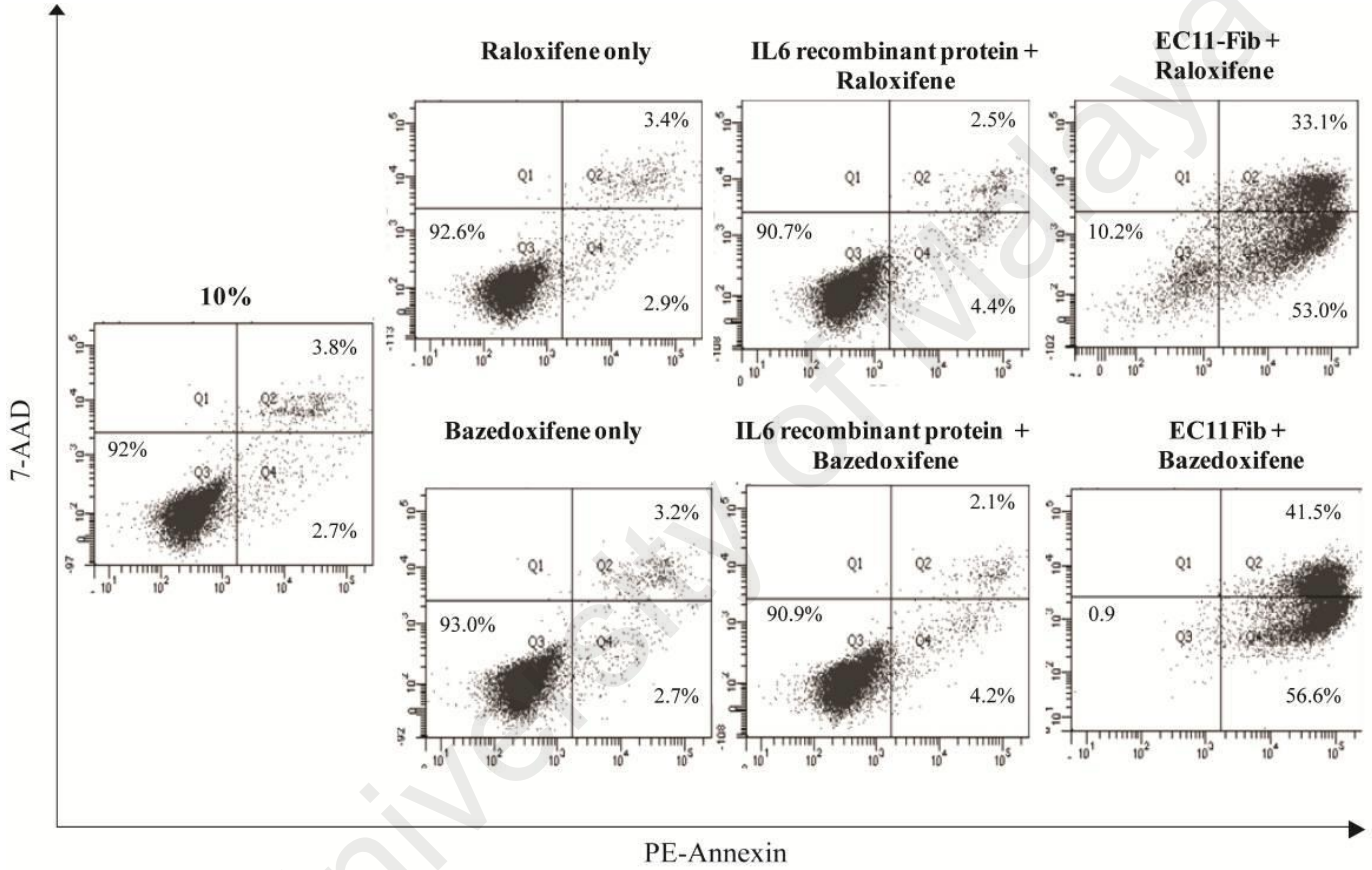

Figure 4.40. Mechanism of action of raloxifene and bazedoxifene acetate.
ECC-1 cells treated with IC_{50} of raloxifene or bazedoxifene acetate with or without 1mg/ml IL-6 recombinant protein or 1 µg/µl EC11-Fib conditioned media for 72 hours, were stained with annexin V-PE and 7-AAD before analyzed with flow cytometry. Data shown are representative of two independent experiments.

4.6.3 IL-6 receptors inhibitors in inhibiting downstream JAK/STAT3 signaling pathway

To determine whether both the IL-6 receptor inhibitors could inhibit JAK/STAT3 pathway, ECC-1 cells were treated with either inhibitor alone, IL-6 recombinant protein with inhibitors or EC11-Fib conditioned media with inhibitors before collecting cell lysates for Western blot.

Results showed that the inhibitors significantly reduced expression of IL6Rα, gp130, phospho-JAK3 and phospho-STAT3 protein levels during both treatments with IL-6 recombinant protein as well as with EC11-Fib secretion **(Figure 4.41)**. This indicates that by significant inhibition of these pathways, the reduction of CAFs-mediated EC proliferation could translate to overall reduced EC growth.

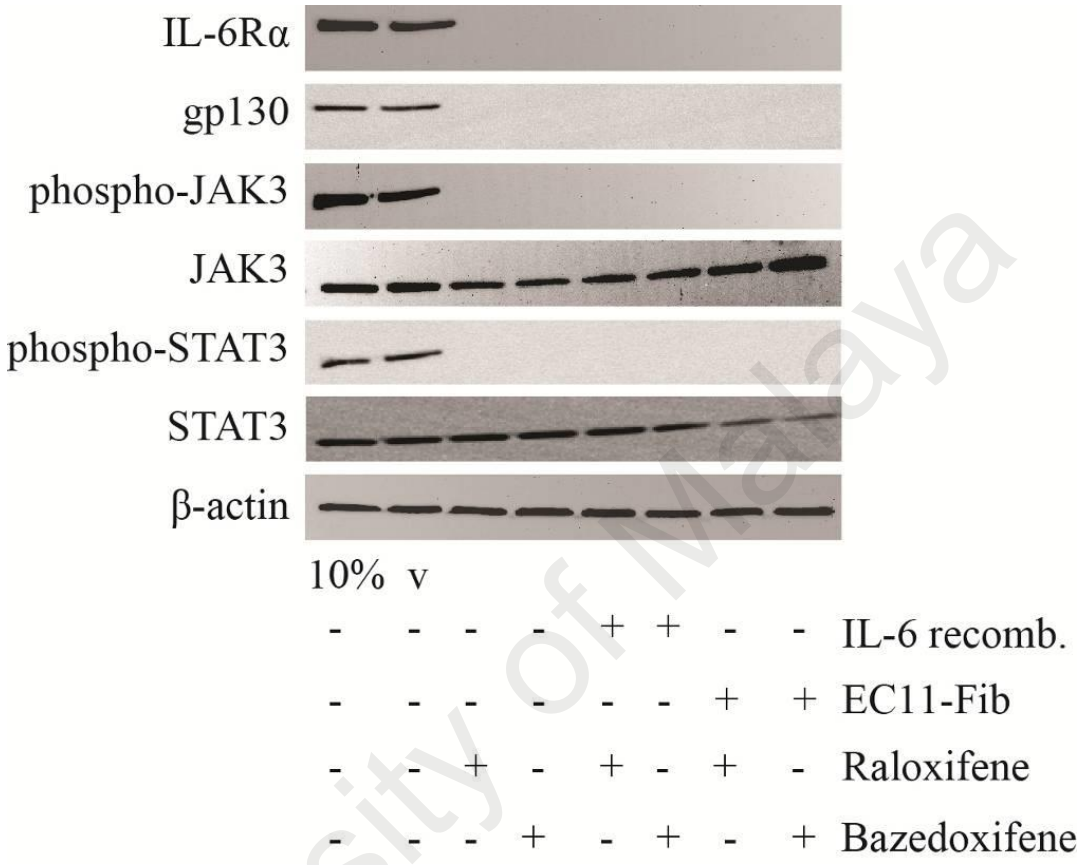

Figure 4.41. Inhibition of JAK/STAT3 pathway upon raloxifene and bazedoxifene treatment.
ECC-1 cells treated with either inhibitors alone, IL-6 recombinant protein with inhibitors or EC11-Fib conditioned media with inhibitors before collecting cell lysates. Data shown are representative of two independent experiments.

Taken together, our findings show that IL-6 plays a major role in CAF-mediated endometrial cancer proliferation mainly by activating cancer survival pathways including PI3K/Akt, MAPK/Erk, and JAK/STAT3, which in turn activates the c-Myc gene. Targeting IL-6 receptors in endometrial cancer may be a promising therapeutic approach for patients with this malignancy.

CHAPTER 5: DISCUSSION

5.1 Tumor microenvironment in gynecological cancer

For decades, cancer research was focused only on the malignant cells without much attention to the other surrounding elements. With increasing evidence that cancer is not a stand-alone disease but largely dependent on its microenvironment, the paradigm for the cancer therapy approaches has shifted from tumor-centered to a more complex tumor-ecosystem view (D Hanahan & Weinberg, 2000). Components of tumor microenvironment are now recognized as factors in determining the success of a therapy (Sounni & Noel, 2013). One of the earliest examples is the development of anti-angiogenic inhibitors to target neo-angiogenesis, a process that allows tumor cells to overcome deficiency of oxygen and nutrients during rapid growth (Folkman, 1971; Jain, 2013). The approval of bevacizumab (Avastin), a humanized monoclonal antibody against vascular endothelial growth factor (VEGF) for metastatic colorectal carcinoma as a combinatory agent with 5-fluorouracil (Hurwitz et al., 2004), signifies that tumor microenvironment can be an effective target in cancer drug development.

The present study focused on a different component of tumor microenvironment, the cancer-associated fibroblasts (CAFs), and their functional role in endometrial cancer progression. Using primary fibroblast cultures from human endometrial cancer tissues, CAFs were shown to exihibit pro-tumorigenic effects by inducing EC cell proliferation *in vitro* and *in vivo*. These effects were not observed with fibroblasts from non-cancerous endometrial tissues. The putative role of CAFs' tumor-promoting effect has been observed in other gynecology malignancy but was relatively new in EC, at the time this study was initiated. It was only recently that others made similar observation, and they showed that CAFs promote EC progression *via* the SDF-1/CXCR4 axis in a paracrine- or autocrine-dependent manner (Teng et al., 2016).

In other gynecological malignancies, it was reported that CAFs in the ovarian cancer caused more cancer cells to migrate and invade *in vitro*, indicating a role of CAFs in ovarian cancer metastasis (Yuan Zhang et al., 2011). The interaction of stromal fibroblasts and tumor cells was mediated by soluble factors present in the fibroblasts secretion (Shilong Fu et al., 2013). Further, in a cervical cancer model, CAFs also exhibited radiation protective effects during the treatment (Chu, Yang, Huang, & Liu, 2014). In fact, secretion from CAFs also induced the epithelial-mesenchymal transition of cervical cancer through the IL-6/STAT3/Snail pathway, an observation not seen with secretion from normal cervical fibroblasts (Ren et al., 2014).

Prior to this study, there were very minimal evidence to indicate whether CAFs from EC exhibit pro-malignant or anti-malignant properties. In fact, not all soluble factors produced by fibroblasts were shown to stimulate proliferation as some may inhibit growth and induce apoptosis (Rodrigues-Lisoni et al., 2010). Two studies in EC showed that stroma secretion from non-cancerous endometrium reversed the effect of EC progression (Arnold et al., 2002; Shi et al., 2011). While this study provided one of the earliest evidence on the pro-malignant role of CAFs in EC, much investigation are to be done in understanding the mechanism of CAFs-mediated carcinogenesis. Similar to the cervical cancer study, this study demonstrated that CAFs-mediated EC tumorigenesis occurred through IL-6 signaling activation. Abrogation of the IL-6 pathway in the model used here showed a significant reduction in EC growth. However, the question remains if a total tumor eradication can be achieved by targeting the CAFs as the source of pro-tumorigenic cytokines.

Multiple compounds that inhibit CAFs or its crosstalk signals with cancer cells are in various pre-clinical and clinical development stages. These agents can be categorized by their specific targets; 1) signal transduction pathways involved in CAF and tumor-CAF

crosstalks such as inhibitors of TGF-β, PDGFR, VEGF, VEGFR, HGF/MET or IGF-1R; 2) unique target of CAFs and its by-product such as inhibitors of MMPs and serine proteases or 3) agents that do not fit into either of the above two categories (Takebe et al., 2013).

5.2 Human primary cultures as model to study tumor microenvironment

In cancer research, tumor cell cultures using immortalized cell lines are frequently used models to understand the biology, to develop new anticancer drugs, and to delineate their mechanism of action. These models provide certain levels of consistency in reproducing experimental results, due to the relatively stable generic drift. However, the prolonged culture of immortalized cell lines increases cell adaptability to tissue culture conditions, which may lead to genetic and epigenetic alterations over time (DeRose et al., 2013). This is because cancer cell lines are usually established with extensive chromosomal rearrangements, oncogene mutations, allelic loss, and gene amplifications. As such, loss in phenotypic properties, molecular changes and alteration in signaling pathways could incur if the cells are continuously cultured for a long time (Staveren et al., 2009).

The heterogeneity of tumor microenvironment leads to the question if usage of conventional cell lines is sufficient when studying cancer pathogenesis, which involves a plentitude of pro-tumor players and soluble factors. Conventional cell lines usage is restricted in the ability to mimic the complexity of tumor microenvironment, which could be one of the reasons why novel anti-cancer compounds are frequently effective *in vitro* but fails in preclinical and clinical studies. Nevertheless, due to this complexity, it is difficult to reproduce cell culture models that exactly represents the tumor biology *in vivo* (Staveren et al., 2009).

In an effort to overcome these limitations, one of the common strategies used is to establish patients-derived primary culture as it is a closer representation of the human tumor characteristics. Establishment and characterization of primary cultures in cancer studies have started with culture of primary pancreatic carcinoma cells *in vitro* before implanted into nude mice. Tumorigenicity was determined with the growth of the cells on soft agar as well as in the nude mice (Grant, Duke, & Hermon-Taylor, 1979). Following this, many other carcinomas, including breast, prostate, lung and oral cancers began to have its own primary cancer models mainly to explore pharmacological effects of drug candidates. This allow development of prospective anti-cancer drugs utilizing primary cultures to study the agent's inhibitory effect and its mechanisms of action (Graziano et al., 1987; Lee et al., 2005; Pandrangi et al., 2014; Peehl, 2005).

In ovarian cancer research, there are two recently established and characterized primary culture models for ovarian cancer derived either from tumor tissues or ascites fluids. Both models were established through enzymatic and mechanical removal of epithelial cells from ovarian biopsies (Shepherd, Thériault, Campbell, & Nachtigal, 2006; Thériault, Portelance, Mes-Masson, & Nachtigal, 2013). This model was used to investigate the initial response of the ovarian cancer cells to cis-platinum treatment *in vitro*, suggesting the usefulness of primary cultures in identifying appropriate chemotherapeutic agents, prior to treatment on patients (Balconi et al., 2006).

To date, *in vivo* subcutaneous and orthotopic endometrial cancer models exist primarily to study effects of distant metastasis (Doll et al., 2009), gene loss in downregulating tumor growth (Peña et al., 2015) and efficacy of single agent drug in attenuating endometrial cancer growth (C. Schwab et al., 2014). However, primary culture models that reflect the tumor microenvironment in EC are lacking, and this issue was relatively addressed through findings from this study.

In the present study, a primary culture of endometrial cancer model was established. The model was derived from human endometrial cancer tissue by tissue digestion, culturing to ensure cell stability, and selection of cell subpopulations using specific pre-determined markers. Successful isolation of the tumor and fibroblasts cells with a suitable method is critically dependent on the tissue condition itself along with the ability of the method to disrupt extracellular matrix. Upon successful isolation, cell culture has to be maintained and monitored constantly to ensure no cross-contamination from other cell types occurs (McCallum & Lowther, 1996).

In this study, pre-determined cell surface markers were used to isolate cells from the primary culture in this study. This may result in a selection of specific cell lineage for the downstream functional investigations. While the successfully isolated cells express the intended cell surface marker, there could be a subset of the intended cells that did not express the marker. Hence, there will be a sub-population of intended cells not included in the study. In addition, this study also did not utilize basement membrane extracts in the propagation of isolated cell, as the cells showed healthy culture conditions. It is worth noting, however, that there are many pre-coated dishes with basement membrane extract available to provide a conducive environment for isolated cells to grow (Dhanabal et al., 1999). It is unknown, if the phenotypes shown by the primary cells will differ if grown in specific basement membrane proteins.

In this study, primary endometrial cancer tissues were degradation using enzymes prior to selection using cell surface markers. While enzymatic method shows great success, one drawback, however, is the difficulty to completely remove epithelial and stromal cells causing some heterogeneity in the culture. Therefore, in order to maximize the cell isolation success and ensuring continuity of homogenous culture, this study used a combination of mechanical digestion method to break down tissues to smaller sizes, with

the enzymatic method using collagenase to degrade extracellular matrix and ensuring single cell suspension is obtained. Post-digesting, the protocol is continued with by enriching the mixed population by cell isolation method using cell-specific surface markers. This combinatory method had shown higher successful rate which required shorted cell growth time with longer cell attachment time compared to solely using mechanical digestion method (R. Wang, He, Liang, Zeng, & Li, 2010). Cell viability post-digestion using enzymatic method is shown to be much higher than mechanical technique (Raouf & Sun, 2013).

There are other various methods to isolate a subpopulation of cells from primary culture, including tissue explants techniques and 3D organoids culture methods (Alberts et al., 2002). While 3D cultures are easy to establish from single cell suspension epithelial cells, it is difficult to assess the effect of treatment to target non-epithelial cells, in the tumor microenvironment (Ohta & Sato, 2014). Although explants cell culture method addresses this issue, this technique is not good for soft tissues and it requires serial subculturing to enrich the required cell populations (Pei et al., 2004). Therefore, despite the existence of various culture methods, it is worth to note that endometrial cancer tissues used in this study were successfully isolated and cultured *via* a combinatory mechanical and enzymatic digestion method.

5.3 Selection of cell surface markers in fibroblasts isolation

In this study, after stabilizing the primary cells growing conditions using mechanical and enzymatic methods, fibroblasts cells were selected from the mixed culture of endometrial cancer. CD90 antibodies conjugated with magnetic beads were utilized to isolate out the fibroblasts cells. CD90, a cell adhesion molecule known as the thymocyte differentiation antigen-1 (Thy-1) is often expressed in mesenchymal stem cell. Isolation of fibroblast cell using CD90 is not new and has been commonly used to enrich the

fibroblasts cells from a mixed cell population (Kisselbach, Merges, Bossie, & Boyd, 2009; K. Schwab, Hutchinson, & Gargett, 2008). In fact, in the current study, flow cytometry showed that at least 70% of the cells expressed positively for CD90 post-isolation. Moreover, the epithelial counterpart exhibited very minimal expression of this marker. This indicates that isolation for CD90-specific fibroblast cells was successfully achieved.

In addition to CD90, other common markers used in isolation and characterization of fibroblasts are α-smooth muscle actin (α-SMA) and vimentin. CAFs are often identified using the α-SMA marker, a marker of activated form of myofibroblasts (Nagamoto, Eguchi, & Beebe, 2000). Vimentin is the most frequently found intracellular marker in the intermediate filament of fibroblasts, making it a reliable marker to detect this cell subpopulation. In this study, a relatively high mRNA expression of α-SMA and vimentin in CD90-positive cells were observed, indicating a specific and homogenous population of fibroblasts cells. The markers used to characterize CAFs in this study are also seen to be expressed by benign fibroblasts. To date, there are no 'CAFs-specific' markers. Hence, cells were characterized with both markers of targeted and unwanted cell population. In this study, cells demonstrating high CAFs-positive marker expression with a low epithelial marker were considered as the targeted cell population. This type of modified characterization method was also used in another study, whereby endometrial fibroblasts cells were verified using fibroblasts markers such as vimentin and α-SMA to show positive cells isolation. In addition, the group used CD31 marker to demonstrate a lack of endothelial cells contamination and cytokeratin marker to exclude epithelial components (Teng et al., 2016).

In the present study, EpCAM was used to isolate the epithelial compartment from the primary endometrial culture. Using flow cytometry, it was observed that all fibroblasts

cells isolated with CD90 in the culture system expressed an almost negligible level of EpCAM expression. Primary endometrial epithelial cells expressed at least 54% specificity for EpCAM. High level of EpCAM expression had also been detected in many carcinomas and cancer stem cells (Patriarca, Macchi, Marschner, & Mellstedt, 2012). EpCAM overexpression is associated with advanced disease stage and worse overall survival (Gastl, Spizzo, Obrist, Dünser, & Mikuz, 2000; Spizzo et al., 2004). Since EpCAM is expressed exclusively in epithelial and epithelial-derived neoplasms, it may be one of the appropriate markers to select and isolate epithelial cells from a mixed population of primary culture. While there are other epithelial cell surface markers such as E-cadherin and cytokeratins (H. Chen et al., 2011), EpCAM has thus far been the marker most widely utilized for isolation as well as epithelial cell characterization in many cancers (Barriere et al., 2014; Kimura et al., 2010; Trzpis, McLaughlin, Leij, & Harmsen, 2007).

Taken together, it was shown in this study that by using CD90 and EpCAM antibodies, a relatively homogenous population of fibroblasts and epithelial cells were isolated. Characterization using flow cytometry and real-time PCR showed the success in marker-specific isolation. Nevertheless, it is understood that for this project, CAFs and epithelial cells used are the positive fraction after isolation using the pre-determined markers. Thus, it is possible that there could be fibroblasts and epithelial cells fractions in the primary culture that were not selected due to the absence of such markers in these cells.

5.4 Origins of CAFs

In the present study, CAFs from different primary endometrial culture displayed varying levels of fibroblast markers expression. This effect could be due to the difference in the CAFs origin, as the CAFs used in this study were established from different study subject. The origin and initiation of CAFs remain unsolved with the possibility of multiple emergence and variations between tumor histotypes and in different areas of individual tumors (Cirri & Chiarugi, 2011).

CAFs have been shown to differentiate from at least four different lineages. Firstly, CAFs are primarily thought to originate from normal fibroblasts or fibroblasts precursors, through a differentiation process upon stimulation with paracrine signals such as TGF-β (Kalluri & Zeisberg, 2006), cell adhesion molecules or even microRNAs (Mitra et al., 2012). Secondly, CAFs are thought to derive from mesenchymal stem cells (MSC). Both CAFs and MSC show many similarities including sharing similar expression of cell surface markers (HLA-DR, CD29, and CD90) and intracellular markers (vimentin and α-SMAs). CAFs are considered to be more differentiated cells than MSC, as they show a higher proliferation rate and secrete higher level of cytokines, when compared to MSC (Paunescu et al., 2011). Lastly, CAFs are also thought to originate from the endothelial-mesenchymal transition (EndMT) and epithelial-mesenchymal transition (EMT) (Kalluri & Zeisberg, 2006; Ostman & Augsten, 2009). The importance of EMT was recently recognized as a potential mechanism of epithelial cancer metastasis. Transdifferentiation of myofibroblasts from epithelial cells is a special case of EMT, which only generates CAFs instead of malignant cancer cells (Forino et al., 2006). Although CAFs generated through EMT may not present as aggressive as cancer cells, the ability to promote growth and metastasis is still profound.

While it is important to understand that different sources of CAFs contribute to the heterogeneity, little is known if different tumor types share certain sources for CAFs. Nevertheless, it is essential to investigate the origins of CAFs as it could provide better understanding of their functional and biological contribution to EC tumorigenesis.

5.5 Pro-tumorigenic role of CAFs in EC progression

Fibroblasts cells have a profound role in regulating the growth and differentiation of epithelial layer (Cirri & Chiarugi, 2012), and hence they have been widely documented as an underlying factor in tumor growth promotion, migration, and invasiveness.

In the present study, supernatants from the four established CAFs were shown to exert a growth promoting effects on EC cells *in vitro* and *in vivo*. Subcutaneous co-inoculation of EC cells with CAFs in nude mice led to significant increase of tumor size when compared to injection of EC cells alone. Similar observation was reported very recently, in which CAFs were shown to enhance tumor growth, migration and invasion in EC cells both *in vitro* and *in vivo* through SDF-1/CXCR4 axis (Teng et al., 2016). This study led by Teng's group is the only other study apart from the current work that examined the effects of CAFs in EC *in vivo* using a subcutaneous mice xenograft model. Interestingly, they observed a contrasting phenotype of benign fibroblasts when co-injected with EC in mice. They observed a slight growth of EC tumors in the presence of normal fibroblasts (Teng et al., 2016), while our study showed a total inhibition of tumor growth. Nevertheless, there was no growth in mice from inoculation of CAFs or benign fibroblasts alone in both studies.

While this study showed preliminary evidence that secretion from CAFs and not benign fibroblast promoted EC motility and invasion, further investigation is needed to determine the exact molecular mechanisms driving these phenotypes. This is because

specific soluble factor in the secretion of CAFs could be the key player in promoting CAFs-mediated EC motility and invasion. Perhaps, with the identification of such molecule, the actin cytoskeleton rearrangement and mechanisms of invasive protrusions by tumor cells could be further evaluated (Yamaguchi & Condeelis, 2007).

Data from this study concurred with previously reports by Arnold's and Shi's group, that secretion from normal endometrial fibroblasts attenuated EC growth. It was also established that the tumor-promoting effect by CAFs was specific, as fibroblasts established from benign endometrial hyperplasia did not induce tumor growth instead showed reduced cell proliferation. (Arnold et al., 2001; Arnold et al., 2002; Shi et al., 2011). More importantly, this observation was similar to Arnold's study where fibroblasts from normal foreskin failed to exhibit similar growth inhibitory effect. In fact, the growth promoting effect by endometrial CAFs was also shown specific to endometrial proliferation, as it failed to induce tumor growth on pancreatic cancer cells (Wu, Looi, Subramaniam, Masamune, & Chung, 2016), indicating the specificity of CAFs phenotypes in endometrial cancer proliferation.

5.6 The effect of CAFs soluble factors in EC progression

Fibroblasts secrete various soluble factors in their environment, and it is evident that differential secretion content derived from CAFs and benign fibroblast are responsible for the opposing effects seen in EC cells. The tumor-enabling CAFs are thought to secrete more inflammatory and pro-tumorigenic factors compared to benign fibroblasts, which translate into progression and increased the growth of EC. In this study, an array of twenty cytokines upon analysis with CAFs and benign fibroblasts secretion showed several prominent cytokines that were highly secreted by CAFs, which were otherwise absent in the secretion of normal fibroblasts.

Of note, growth regulated oncogene-α (GRO-α), which is also known as the chemokine (C-X-C motif) ligand, was found to be exorbitantly expressed in the CAFs secretion, leading to further investigation on this chemokine. Neutralizing GRO-α in the CAFs secretion, however, showed no effect in inhibiting EC proliferation. This was despite the observation that GRO-α recombinant protein displayed dose-dependent growth promotion. These data suggest that although GRO-α levels are elevated in gynecological cancer, its role might be limited to be used as a prognostic marker for cancer detection, and may not be suitable to be a target as a therapeutic approach (Nishikawa et al., 2012). In one study, however, it was shown that high levels of GRO-α expression correlated with increased proliferation and invasiveness of colon carcinoma cells (A. Li, Varney, & Singh, 2004). Interestingly in ovarian cancer, it was shown that GRO-α expression is activated by RAS and is essential for cell survival and malignant transformation of ovarian epithelial cells. More importantly, it was observed that senescent fibroblasts induced by GRO-α can promote tumor growth, while depletion of senescence through immortalization resulted in loss of such tumor promoting activity (Yang et al., 2006). In a non-cancerous endometrium, GRO-α has been found in the cycling endometrium, mainly in the stroma compartment. The functional role of this chemokine still remains unknown (Nasu et al., 2001) and its role in EC have yet to be validated.

Contrastingly, neutralizing IL-6 in the conditioned media resulted in significant inhibition of EC proliferation in both EC cell line and primary culture. Treatment with recombinant IL-6 protein on EC cells showed an increase in cell proliferation. Further study on the IL-6 pathway showed that the IL-6 receptors are only expressed in EC epithelial cells but not in CAFs, indicating a one-way paracrine signaling. Observation on the mechanism underlying CAF's growth promotion showed that PI3K/Akt, MAPK/Erk, and JAK/STAT3 signaling were upregulated in the presence of CAFs-

secretion. Treatment with pathway-specific inhibitors effectively inhibited CAFs-mediated EC proliferation. These data, therefore, indicate that IL-6 in CAFs secretion plays a role in EC cells proliferation through activation of PI3K/Akt, MAPK/Erk, and JAK/STAT3 signaling pathways.

IL-6 has long been shown to promote the growth of various tumor types including colorectal cancer (Becker et al., 2005), multiple myeloma (Gadó, Domján, Hegyesi, & Falus, 2000), non-small cell lung cancer in which patients with high circulating IL-6 was associated with low survival outcome (C. Chang et al., 2013), prostate cancer (Nguyen, Li, & Tewari, 2014), pancreatic cancer (Y Zhang et al., 2013) and breast cancer where IL-6 triggered malignant features in mammospheres (Sansone et al., 2007). Similarly, inhibition of IL-6 pathway abrogated STAT3-mediated cell survival of gastric cancer and osteosarcoma (B Tu, Du, Fan, Tang, & Tang, 2012; Kinoshita et al., 2013) suggesting the importance of IL-6 in promoting tumor growth. Even more recently, phosphorylated-STAT3 expression in the tumor stroma, an indication of IL-6-JAK pathway activation, was thought to be a critical contributor to cancer progression and response to therapy by modulating PI3K pathway (Abell & Watson, 2005; Bournazou, 2013).

In EC, it was reported that IL-6 levels were elevated in the serum of EC patients (H. O. Smith et al., 2013) and was associated with chemotherapy resistance and poor prognostic outcome (Bellone et al., 2005). Our results showing the absence of IL-6 receptors at both mRNA and protein level from the CAFs could mean that EC promoting effect observed was specific to the one-way paracrine signaling of CAFs secreted IL-6. This, in turn, translates to CAF's role as a key player in the tumor microenvironment, modulating the behavior of cancer cells through its secretion of inflammatory cytokines to communicate within the cancer environment.

5.7 Crosstalks between IL-6 activated downstream pathways

Data from this study suggest that regulation of PI3K/Akt, MAPK/Erk, and JAK/STAT3 pathways through induction of IL-6 may be a key factor in the fibroblasts effects on EC cell proliferation. Activation of these pathways resulted in an increase in EC cell proliferation, but this effect was inhibited by treatment with pathway-specific inhibitors.

Both PI3K and MAPK pathways have been associated with stimulation by external growth factors and cytokines (Aksamitiene, Kiyatkin, & Kholodenko, 2012; Gentilini et al., 2007), which can be found in both CAFs and normal fibroblasts. AKT is involved in cell survival pathway (PI3K/Akt/mTOR) and it has the ability to inhibit apoptosis and induce proliferation in its phosphorylated form, phospho-akt (p-akt). EC has been implicated in activation of PI3K pathway in up to 83% of its cases, usually triggered by the loss of function by PTEN gene. Activated Akt modulates the function of numerous factors involved in the regulation of cell survival, cell cycle progression and cellular growth (Vara et al., 2004). Endometrial cancer patients are usually given hormonal replacement therapy as part of mainstay treatment. Inhibition of PI3K/AKT/mTOR pathway is of therapeutic interest recently and has been implicated in conferring resistance to conventional therapies, and so PI3K/AKT/mTOR pathway inhibitors in combination with hormonal and/or cytotoxic agents are being evaluated.

Additionally, SDF-1α has been shown to mediate the activation of PI3K/Akt pathway but not the MAPK/Erk pathway, after treated with supernatants from uterine muscle cells (Tsukamoto et al., 2007). Involvement of PI3K/Akt and Erk pathways have been implicated in cardioprotection and chemotherapy resistance mediated by the SDF-1α/CXCR4 axis (Singh, Srivastava, Bhardwaj, Owen, & Singh, 2010; Teng et al., 2016). Furthermore, as activation of both pathways *via* SDF-1α/CXCR4 was shown to affect cell

growth, migration, and invasion, it was suggested that SDF-1α in CAFs secretion promotes EC development through a paracrine dependent manner (Teng et al., 2016).

IL-6 can activate PI3K/Akt pathway and regulate cyclin A1 in order to sustain prostate cancer survival (Wegiel, Bjartell, Culig, & Persson, 2008). It was also shown through another study that IL-6 can promote EC growth through an autocrine regulatory loop with ERK-NF-κB as a critical mediator of IL-6 production (Che et al., 2014). Additionally, aberrant activation of JAK/STAT downstream of the IL-6 pathway has been identified as an underlying factor mediating tumor progression and metastasis in various cancers (Quintás-Cardama & Verstovsek, 2013), yet its implication and role in EC are unclear.

One study reported that presence of IL-6 in the microenvironment of colorectal cancer stimulated crosstalk between the cancer cells and immune cells via miRNAs miR-21 and miR-29b, allowing cancer cells to sustain inflammation-like conditions and to promote prometastatic behavior (SA Patel & NJ Gooderham, 2015). In ovarian cancer, it was shown that IL-6 enhances tumor survival and increases the resistance of the tumor to chemotherapy *via* JAK/STAT signaling (Duan et al., 2006). IL-6 is also shown to be a part of a malignant cell autocrine network comprises of TNF-α, IL-1α, CCL2, CXCL12 and VEGF in the ovarian cancer tumor microenvironment (Kulbe et al., 2007).

In the present study, c-Myc was highly upregulated in EC cells upon treatment with CAFs secretion, at both mRNA and protein levels. RNAi method to knockdown c-Myc gene in EC cell as well as c-Myc-specific inhibitor both showed significant tumor cell proliferation. Despite re-exposure of CAFs secretion to EC cells expressing low levels of c-Myc, an increase of EC cell proliferation did not occur, both *in vitro* and *in vivo*. Analysis of IL-6 receptors, phosphorylated-STAT3, and c-Myc on human endometrial tissues showed high positive expression in the cancer tissues compared to those from the benign endometrium.

The induction of STAT3 by CAFs-secreted IL-6 was observed to be similar to Bromberg's observation in other cancers (J. Bromberg, 2002; J. F. Bromberg et al., 1999; Ott, Rosenwald, & Campo, 2013). Activation of STAT3 then induced various oncogenic proteins including c-Myc, and STAT3 activation was shown to be a pre-requisite in mediating rapid activation of c-Myc gene (Kiuchi et al., 1999). c-Myc is found activated in many human tumors with poor disease prognosis outcome, including EC (Dang, 1999; Geisler et al., 2004; Pelengaris, Khan, & Evan, 2002). Normal cells in culture have been shown to express just a few thousand molecules of c-Myc protein per cell (Waters, Littlewood, Hancock, Moore, & Evan, 1991), yet this can be over two times higher in cancer cell lines grown under similar conditions (Dang, 1999). Inhibition of c-Myc prevents excessive cell proliferation by inducing apoptosis to regain cellular homeostasis as well as by controlling the cell physiology (Dang, 1999).

Overall, our data suggests that secretion from CAFs, mainly IL-6 contributes to the progression of EC *via* activation of Akt, Erk and STAT3 pathways. c-Myc gene is shown to be a critical regulator in promoting EC progression and that targeting IL-6 could be a novel therapeutic method for EC treatment.

5.8 Development of targeted therapy for EC-CAFs interaction

Multiple compounds that inhibit CAFs or its crosstalk signals with cancer cells are in pre-clinical and clinical development. These agents can be categorized by their specific target; 1) if they inhibit signal transduction pathways involved in CAF and tumor-CAF crosstalks such as inhibitors of TGF-β, PDGFR, VEGF, VEGFR, HGF/MET or IGF-1R, 2) unique target of CAFs and its by-product such as inhibitors of MMPs and serine proteases or 3) agents that do not fit into either of the above two categories (Takebe et al., 2013).

In this study, three inhibitors of the IL-6 pathway were used. These includes rapamycin (inhibiting mTOR), raloxifene and bazedoxifene acetate (IL-6 receptor inhibitors). Rapamycin exerts its biological activity by inhibiting the kinase mammalian target of rapamycin (mTOR), which regulates important cellular processes such as control of cell cycle and cell size, translation initiation, and transcription. A highly specific mTOR inhibitor, rapamycin arrests cells in the G_1 phase and has shown antitumor activities *in vivo* as well as *in vitro*. The ability of rapamycin to inhibit cancer cell proliferation has led to efforts to develop rapamycin and related mTOR inhibitors as anticancer agents (Bae-Jump, Zhou, Boggess, & Gehrig, 2009). Results from the present study showed a significant reduction in cell proliferation upon treatment with rapamycin in the presence of CAFs secretion. The anti-proliferative effect was seen to occur through induction of apoptosis. As a single agent, the orally bioavailable derivative of rapamycin, RAD001 (Everolimus) has been showing significant success in attenuation of tumor cells growth both *in vitro* and *in vivo* (Boulay et al., 2004). As a treatment option for EC, RAD001 showed positive results in a phase II clinical trial with patients previously treated with progressive or recurrent EC (Slomovitz et al., 2010).

Apart from rapamycin, some other mTOR inhibitors that have been used clinically include temsirolimus and ridaforolimus. In a phase II trial of temsirolimus as first-line treatment for recurrent endometrial cancer previously untreated with chemotherapy, 5 of 19 patients (26%) had a partial response and 12 (63%) had stable disease (A. M. Oza et al., 2011). In a phase II trial of temsirolimus as a second-line treatment for recurrent endometrial cancer that had been previously treated with chemotherapy, the partial response and stable disease rates were 7 and 44%, respectively (A. Oza et al., 2008). With ridaforolimus, 13 of 45 patients (28.9%) achieved a clinically beneficial response for ≥16 weeks (Colombo et al., 2007). A phase III trial of ridaforolimus is currently being planned.

Effects of two IL-6 receptor inhibitor in abrogating IL-6 mediated cancer proliferation were examined; Raloxifene, marketed as EVISTA is currently used to treat post-menopausal women with rheumatoid arthritis (Mokuda et al., 2012), while bazedoxifene acetate, marketed as DUAVEE, is investigated as an agent to treat post-menopausal related osteoporosis (Hiligsmann, Sedrine, & Reginster, 2013). Results from this study showed a promising effect in inhibition of EC cells upon treatment with these two inhibitors only in the presence of CAFs, indicating that presence of IL-6 is required in order for these inhibitors to successfully attenuate EC proliferation.

There are currently on-going efforts to develop IL-6 specific targets. Tocilizumab, a humanized IL-6R specific monoclonal antibody that was previously approved for rheumatoid arthritis is now proposed to alleviate cancer cachexia (Ando et al., 2013; Berti, Boccalatte, Sabbadini, & Dagna, 2013). Additionally, REGN88 which is a fully humanized IL-6R monoclonal antibody is currently undergoing phase III clinical trial in rheumatoid arthritis with future indication possibility in solid tumor (S. A. Jones, J. Scheller, & S. Rose-John, 2011). Apart from that, siltuximab or CNTO 328 that

neutralizes IL-6, demonstrated promising safety and tolerability outcome in phase I/II clinical trial in advanced or refractory solid tumors (R. Chen & Chen, 2015). It is worth to note however, there have yet to be a conclusive targeted therapy for EC and what has been tried so far are treatment modalities from other cancers adapted to treat EC (Dong et al., 2013).

Challenges faced in failure to obtain a good response rate for targeted therapy in EC related clinical trials could relate to the various carcinogenetic pathways in EC, rendering inhibition of a single molecule insufficient for anticancer activity. Thus, development of drugs with multiple targets including to target CAFs could be effective in reversing CAFs-mediated EC growth (Nogami et al., 2013)

Taken together, our study suggests that the IL-6 pathway is also a novel target for EC especially in the context of the tumor microenvironment, and hence, application of IL-6 pathway inhibitors may provide a new avenue for treating aggressive EC. It is also possible that IL-6 could be in synergy with other inflammatory cytokines in the tumor microenvironment and further investigations on the crosstalk are crucial to understanding the role of CAFs in EC, to design better therapy approaches for this disease.

CHAPTER 6: CONCLUSION AND FUTURE STUDIES

In summary, this study demonstrated that CAFs derived from human EC tissues exert growth-promoting effects on EC tumor cells while fibroblast from benign endometrium inhibited EC proliferation *in vitro* and *in vivo*. It was also established that CAFs-mediated EC proliferation was regulated through IL-6 pathway activation and c-Myc overexpression. Knockdown of c-Myc gene resulted in reduced EC proliferation *in vitro* and *in vivo* and targeting IL-6 pathway using molecular inhibitors resulted in attenuation of EC proliferation, possibly through induction of apoptosis.

Data from this study indicates that CAFs-mediated IL-6 signaling pathway could be a potential target in EC therapeutic approach. Findings from this study suggest that CAFs may be an appropriate model to study the role of fibroblast in EC progression as well as provide clues on how they may be a critical regulator in the interaction of cancer cell in the microenvironment. This work presents as one of the earliest evidence to show that c-Myc overexpression can be induced *via* in a CAFs-mediated IL-6R/STAT3 pathway activation in EC progression.

Data from our study showed that IL-6 receptors are only present on the EC epithelial cells but not on CAFs cells, indication a one-way paracrine signaling. However, further investigation is needed to study the nature of IL-6 in cancer promotion, if it acts as a single cytokine or through crosstalk with other inflammatory cytokines in the tumor microenvironment. This is because, being in an inflamed state of fibroblast, CAFs have been shown to produce a variety of secretory factors to promote carcinogenesis. Previous studies have shown cytokines such as CSF-1, TNF-α, IL-6 to be implicated in EC progression and with low survival rates among patients (Öhlund, Elyada, & Tuveson, 2014). However, minimal evidence can be found in determining if the cytokines responsible for EC progression originated from CAFs.

Although most studies focus on the impact of a single cytokine on disease progression, the fact remains that crosstalks between molecules are essential in the tumor microenvironment to drive carcinogenesis. IL-6, for example, was shown to stimulate crosstalks in the colorectal cancer tumor microenvironment to sustain inflammation and to promote pro-metastatic behavior (S Patel & N Gooderham, 2015). IL-6 also stimulated receptor activator in bone cells causing enhancement of metastatic breast cancer growth within the bone (Zheng et al., 2014). Hence, blocking the IL-6 signaling could present as a potential therapy option for treatment of EC. In fact, targeted therapy of anti-IL-6 have been in clinical trials and found to be tolerated well (Y Guo, F Xu, T Lu, Z Duan, & Z Zhang, 2012). Data from the current study on the inhibitory effect of raloxifene and bazedoxife in CAFs-mediated environment reinforced the notion that certain small molecule inhibitors yield significant therapeutic response in the presence of specific ligands. As such, agents to block IL-6 through means of targeted therapy could be investigated as a therapy potential for EC.

While this study only focused on the role of IL-6 in CAFs-mediated EC progression, it cannot be denied that there are several other key cytokines involved as well. In depth study on roles of other cytokines and its activated crosstalks in EC is could warrant a significant lead in term of EC disease progression. For example, a group recently showed that TNF-α activated estrogen signaling in epithelial endometrial cells which was postulated to be the stepwise accumulation in EC initiation (Gori et al., 2011). Similar to other malignancies, EC disease stability is thought be maintained by a plethora of soluble factors. Thus, it would be worthwhile to study the downstream effect of cytokines crosstalk in EC to further understand the possible molecular cues to aid in therapy design. This evidence shows that crosstalk of cytokines in the microenvironment could have heightened carcinogenesis effect.

In this study, it was observed that secretion from non-malignant fibroblast attenuated the growth of EC, similar to two previously reported works (Arnold et al., 2002; Shi et al., 2011). Differential cytokine profiling from this study showed higher levels of TNF-α, GM-CSF and several cytokines in the interleukin family. The profound growth inhibitory outcome by normal endometrial fibroblast on EC progression leads to the question if there is a possibility for a 'naturally occurring' inhibitor already at play that which could be harnessed for EC therapy. Thus, an in-depth study on these cytokines could yield a better understanding of the mechanism underlying effects of normal fibroblast in EC cancer inhibition. A comprehensive cytokine profiling between non-cancerous and cancerous fibroblast could lead to an understanding of the players in the transformation towards becoming CAFs. It is also worth to note that growth reduction effect by normal fibroblast might not only occur through soluble factors but could also be through molecular mechanism such as miRNA mediated through exosomes. Naturally present in the cell secretions, exosomes are equated to cellular nanoparticles that could be used to target cancer cells through transportation of miRNAs into the cancer cells. This would lead to the inhibition of pro-tumorigenic mRNA in the cancer cells, which would eventually translate to reduction in cancer occurrence (Milane, Singh, Mattheolabakis, Suresh, & Amiji, 2015). Similarly, an elegant study done on myeloid cell lines showed that agonistic antibody exerted reverse signaling on the gene expression of several cytokines and intracellular signaling leading to an overall regression of disease (Sipos, Török, Kalic, Duda, & Filkor, 2015). Whether such effect is also occurring in normal fibroblast mediated EC inhibition remains to be further investigated.

It was observed from this project, that generally, CAFs behaves differently from benign fibroblast. The differential effect exerted on EC progression lead to the question of the possibility if fibroblasts from different endometrial state could affect EC tissues differently. Even more interesting would be to look at the stepwise molecular changes

that causes benign fibroblast to become CAFs. Once such study in fact reported that a pro-inflammatory cytokine epigenetically reprogrammed fibroblast in a pro-invasive phenotype which led to invasion of cancer cell (Albrengues et al., 2015). This emphasizes that certain factors present in the tumor microenvironment could affect the surrounding fibroblasts which results in the transformation to become CAFs. A contrasting approach is also thought as mean to study the different states of CAFs in cancer. For example, in liver cancer, hepatic stellate cells which play a critical role in liver fibrosis was de-differentiated by treatment with vitamin D. As a result, the dedifferentiated stellate cells were normalized back to normal state and halted fibrosis, suggesting the vitamin D receptor ligand as a potential therapy for liver fibrosis (Ding et al., 2013). Another way to deactivate CAFs, is by utilizing therapies to enhance miRNA expressions as used in prostate cancer (Musumeci et al., 2011) and ovarian cancer (Mitra et al., 2012). A comprehensive study detailing the differential states of fibroblast may increase our understanding of the types of therapies to administer for different EC patients based on their fibroblast profiling. Which such a wide area for investigation, the impact on EC is yet to be understood, giving it a niche area to study on.

Finally, going along the same line of delineating functional properties of different fibroblast on cancer progression, one other research avenue that can be studied on is to further characterize the origins of CAFs and its effect on EC progression. Of note, it has been widely described that CAFs may originate from four different mechanisms: 1) transition from normal fibroblast upon stimulation by paracrine signaling; 2) differentiation of mesenchymal stem cells; 3) endothelial-mesenchymal transition; and 4) epithelial-mesenchymal transition (Öhlund et al., 2014; Veirman et al., 2014). It is important to understand the molecular drivers of CAFs formation and the relative contribution of each source type. This is because CAFs from different origin could behave differently in the tumorigenesis of EC. Perhaps, specific markers exclusive for each origin

type need to be outlined that yield in specific subtypes of CAFs. Discovery of such markers would aid on the study on how they might affect EC progression. It is yet to be determined if CAFs in EC are from one origin only, or from multiple lineage. Adding on to the complexity, CAFs from different patients affected EC growth rate differently, albeit in the similar growth-promoting pattern. This further pushes the need to establish origin-specific markers to characterize CAFs in depth, as it would translate to the efficacy for a possible an anti-CAFs targeted therapy.

Taken together, it is evident that CAFs positively regulate the EC progression and their secretion may impede the effectiveness of current therapy. This study showed that IL-6 in CAFs-mediated secretion has a significant impact on EC progression and that targeting IL-6 signaling pathway may present as a potential therapeutic approach for EC.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS

1. **Subramaniam KS**, Omar IS, Kwong SC, Mohamed Z, Woo YL, Mat Adenan NA, Chung I. (2016) Cancer-associated fibroblasts promote endometrial cancer growth via activation of interleukin-6/STAT-3/c-Myc pathway. American Journal of Cancer Research. American Journal of Cancer Research 6(2):200-213

2. **Subramaniam KS**, Tham ST, Mohamed Z, Woo YL, Mat Adenan NA, Chung I. (2013) Cancer-Associated Fibroblasts Promote Proliferation of Endometrial Cancer Cells. PLoS ONE 8(7): e68923

3. **Subramaniam KS**, Wong MS, Woo YL, Mat Adenan NA, Mohamed Z, Chung I. (2013) Preliminary Study on the Occurrence of PTEN and PIK3CA Gene Mutations in Endometrial Cancer among Malaysian Women. Journal of Health and Translational Medicine, 16(1), pp 1-5

4. Wu YS, Looi CY, **Subramaniam KS**, Masamune A and Chung I. (2016) Stellate cells secretion induces pancreatic cancer cell proliferation via Nrf2-mediated metabolic reprogramming and ROS detoxification. Oncotarget. Epub ahead.

PAPERS PRESENTED

a) As poster presentation:

1. Interleukin-6 (IL-6) Secreted by Cancer-Associated Fibroblasts Promote Endometrial Cancer Cell Proliferation.
   Faculty of Medicine Research Week (FOMRC),
   May 11-15 2015, University Malaya, Kuala Lumpur, Malaysia.

2. Cancer-Associated Fibroblasts Promote Endometrial Cancer Cell Proliferation *in vitro* and *in vivo.*
   American Association for Cancer Research Annual Meeting (AACR),
   05 Apr 2014 to 09 Apr 2014, San Diego Convention Center, San Diego, USA.

3. Cancer-Associated Fibroblasts Promote Endometrial Cancer Proliferation.
   University of Malaya Researchers Conference (UMRC),
   November 19-20 2013, University Malaya, Kuala Lumpur, Malaysia.

b) As oral presentation:

1. Effects of Cancer-Associated Fibroblast on Endometrial Cancer Cells.
   International Conference on Advances in Medical Science (ICAMS),
   April 16-18 2013, Impiana KLCC, Kuala Lumpur, Malaysia.

2. Interleukin-6 (IL-6) secretion by cancer-associated fibroblasts induces endometrial cancer cell proliferation *via* JAK/STAT3 signaling.
   -Awarded Best Oral Presenter-
   Pharmacology & Physiology International Scientific Congress (PPISC),
   August 22-24 2014, Putra World Trade Centre, Kuala Lumpur, Malaysia.