# THE ROLE OF TUMOR STROMA AND CANCER ASSOCIATED FIBROBLASTS IN TUMOR GROWTH THROUGH CYTOKINES

by

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THE ROLE OF TUMOR STROMA AND CANCER ASSOCIATED FIBROBLASTS IN TUMOR GROWTH THROUGH CYTOKINES

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**ABSTRACT**

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**Keywords:** Tumor stroma, cancer associated fibroblasts, cytokine, tumor growth.

Development of a tumor is a parallel event with the expansion of tumor microenvironment. Although most of the treatment strategies target only malignant cells, advanced understanding of the stromal contribution in cancer progression may enhance our knowledge and allow us to develop better treatments. Tumor stroma consists of different types of cells such as a heterogenous population of fibroblasts, immune cells, pericytes, endothelial cells and other noncellular components like extracellular matrix. Studies have shown that the interaction between malignant cells and stromal components through cytokine secretion has a crucial role in cancer progression. Moreover, these stromal cells and components can be recruited, manipulated, and altered by cancer cells to provide a permissive environment rather than a defensive one. One of the major components of tumor stroma is cancer associated fibroblasts (CAFs). In normal tissues, fibroblasts are found in a quiescent state and they become activated upon stimulations such as wound healing and fibrosis. Their activation is a reversible process and most of the activated fibroblasts are removed by apoptosis after wound healing. However, recent studies showed that CAFs differ from normal activated fibroblasts in terms of proliferation, migratory capacity, and secretory phenotype. In vitro and animal studies reveal that they enhance tumor growth and progression.

In this study, we present that CAFs can enhance the growth of MCF7 and T47D breast cancer cells in vitro when they are co-cultured. Our co-culture experiments showed that this tumor promoting effect of CAFs is not dependent on cell-to-cell contact and it is caused by their crosstalk through cytokine secretion. We found that Activin A, Interleukin 5 and Angiogenin are present in higher levels in the cell culture media of CAF co-culture and similarly their expression in human breast cancer tumor samples are increased. Moreover, we showed that tumor growth due to CAFs can be reversed using specific neutralizing antibodies. Additionally, recombinantly produced cytokines were able to enhance the growth of MCF7 and T47D cells without the presence of CAFs in the culture. Therefore, we propose that CAFs induce tumor growth by their interaction with malignant cells through the secretion of specific cytokines and targeting this interaction can be developed as a new strategy in cancer treatment.

# ÖZET

## TÜMÖR STROMASI VE KANSERLE İLİŞKİLİ FİBROBLASTLARIN SİTOKİNLER ARACILIĞI İLE TÜMÖR BÜYÜMESİNDEKİ ROLÜ

BİRAN MUSUL

Master Tezi, Temmuz 2018

Tez Danışmanı: Prof. Devrim Gözüaçık

**Anahtar Kelimeler:** Tümör stroması, kanserle ilişkili fibroblastlar, sitokin, tümör büyümesi

Bir tümörün gelişmesi, tümörün mikroçevresinin genişlemesiyle paralel gerçekleşen bir olaydır. Çoğu tedavi stratejisi kötü huylu hücreleri hedeflemekte olsa da, kanser gelişimindeki stroma katkısını anlamak, bilgimizi genişletip daha iyi tedaviler geliştirmemize yardımcı olabilir. Heterojen fibroblast popülasyonu, bağışıklık sistemi hücreleri, perisitler ve endotelyal hücreler gibi farklı hücre türlerine ek olarak ektrasellüler matriks gibi hücresel olmayan bileşenler tümör stromasını oluştururlar. Çalışmalar göstermiştir ki, sitokinler üzerinden gerçekleşen, kötü huylu hücrelerle stroma bileşenleri arasındaki etkileşimin, kanser gelişimi üzerinde çok önemli bir rolü bulunmaktadır. Dahası, bu stromal hücreler, kanser hücreleri tarafından düzenlenerek, yönetilerek ve değiştirilerek daha elverişli bir çevre oluşturmaktadır. Tümör stromasının en büyük bileşenlerinden bir tanesi kanserle ilişkili fibroblastlardır. Normal dokularda, fibroblastlar sessiz durumda bulunurlar ve yara iyileşmesi ve fibröz gibi uyaranlarla aktif hale gelirler. Aktivasyonları geri çevirilebilir bir işlemdir ve çoğu aktif fibroblast yara iyileşmesi sonrasında apoptoz ile uzaklaştırılırlar. Fakat son çalışmalar göstermiştir ki, kanserle ilişkili fibroblastlar çoğalma, hareket kabiliyeti ve salgılama fenotipleri açısından normal aktif fibroblastlardan farklılaşmaktadır. İn vitro ve hayvan deneyleri, bu hücrelerin tümör büyümesi ve gelişmesini arttırdığını ortaya çıkarmıştır.

Biz bu çalışmada, birlikte kültür edildikleri şartlarda, kanserle ilişkili fibroblastların, MCF7 and T47D meme kanseri hücrelerinin in vitro ortamda büyümelerini arttırdığını sunmaktayız. Bizim birlikte kültür deneylerimiz göstermiştir ki, kanserle ilişkili fibroblastların bu tümör büyütücü etkisi hücrelerin temasından çok, sitokinler üzerinden gerçekleşen karşılıklı iletişim sebebiyle gerçekleşmektedir. Kanserle ilişkili hücrelerin birlikte kültürlerinde Activin A, Interleukin 5 ve Angiogenin faktörlerinin daha yüksek seviyelerde bulunduğunu ve benzer şekilde meme kanseri hastalarının tümör örneklerinde de bu faktörlerin ifadelerinin yükselmiş olduğunu bulduk. Dahası, özel nötralize antikorların kullanımı ile kanserle ilişkili hücre kaynaklı tümör büyümesini tersine çevirebileceğimizi gösterdik. Ek olarak, rekombinant olarak üretilen sitokinler, MCF7 ve T47D hücrelerinin büyümesini, kanserle ilişkili fibroblastların varlığı bulunmasa da arttırmıştır. Bu sebeple, kanserle ilişkili fibroblastların tümör büyümesini sitokinler aracılığıyla gerçekleştirdiklerini ve bu etkileşimin hedeflenerek yeni kanser tedavi stratejilerinin geliştirilebileceğini öne sürmekteyiz.

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**CAF:** Cancer associated fibroblast

**NAF:** Normal activated fibroblast

**INHBA:** Activin A

**IL5:** Interleukin 5

**ANG:** Angiogenin

**ECM:** Extracellular matrix

**LC3:** Microtubule-associated protein 1 light chain 3

**MMP:** Matrix metalloproteinase

**BM:** Basement membrane

**VEGF:** Vascular endothelial growth factor

**TGF-β:** Transforming growth factor β

**DMEM:** Dulbecco's modified eagle medium

**RFP:** Red fluorescent protein

**LUC:** Luciferase

# 1. INTRODUCTION

## 1.1 Cancer and Tumor Microenvironment

Despite extensive cancer research, advanced molecular biology techniques, medical treatments, and accumulating knowledge for many years there is still a gap between cancer biology in the experimental and clinical outcomes. This is mostly because until recent years cancer studies have mostly centered upon only malignant cells. However, today, researchers came to realization and it is a widely accepted that tumor microenvironment and the interactions among its components have a crucial role in cancer progression as well as cancer treatments.

Tumors do not only consist of uncontrollably dividing cells, but they are also composed of several different cell types including endothelial and mesenchymal cells, immune cells, fibroblasts as well as other non-cellular constituents such as extracellular matrix (ECM). In addition to this, researchers identified that malignant cells can recruit and manipulate these cells in tumor microenvironment, modify extracellular matrix and change the environment in a way that all these changes support tumor formation, progression, invasion, and metastasis. Although "The Seed and Soil Hypothesis" was first proposed by Stephen Paget in 1889 [1] and it has been more than hundred years now, we recently started to comprehend the important role of this complex interaction between the tumor cells and the tumor stroma. Today we know that to create a permissive environment rather than a defensive one, cancer cells can transform and alter the tumor stroma and the connective tissue in which they reside [2]. Additionally, one of the key features of malignant cancer cells is that they are capable of invasion and metastasis [3,4]. For this reason, the communication and interaction between cancer cells and other constituents of tumor stroma have gained an essential role in the understanding and the prevention of cancer progression [4-9].

## 1.2 Tumor Stroma

During cancer progression, host tissue stroma provides the maintenance of both normal epithelial tissues and malignant counterparts. Other than cancer cells, the tumor stroma mainly consists of basement membrane, extracellular matrix consisting of structural proteins like collagen, elastin, fibrilin, and proteoglycans, vasculature, and other nonmalignant cells such as fibroblasts, distinctive mesenchymal cell types, innate and adaptive immune cells, endothelial cells, and pericytes (Figure 1.1).

Angiogenesis and newly formed vasculature is also essential tumor formation and survival. Initiation of angiogenesis requires degradation of the basement membrane (BM) induced by matrix metalloproteinases (MMPs), formation of new endothelial cells and pericyte attachment. Collective work of other components of stromal compartments also has a crucial role in orchestrating these events by the secretion of various ECM molecules and growth factors such as fibroblast growth factor (FGF2), transforming growth factor (TGF-beta), and vascular endothelial growth factor (VEGF) [9].

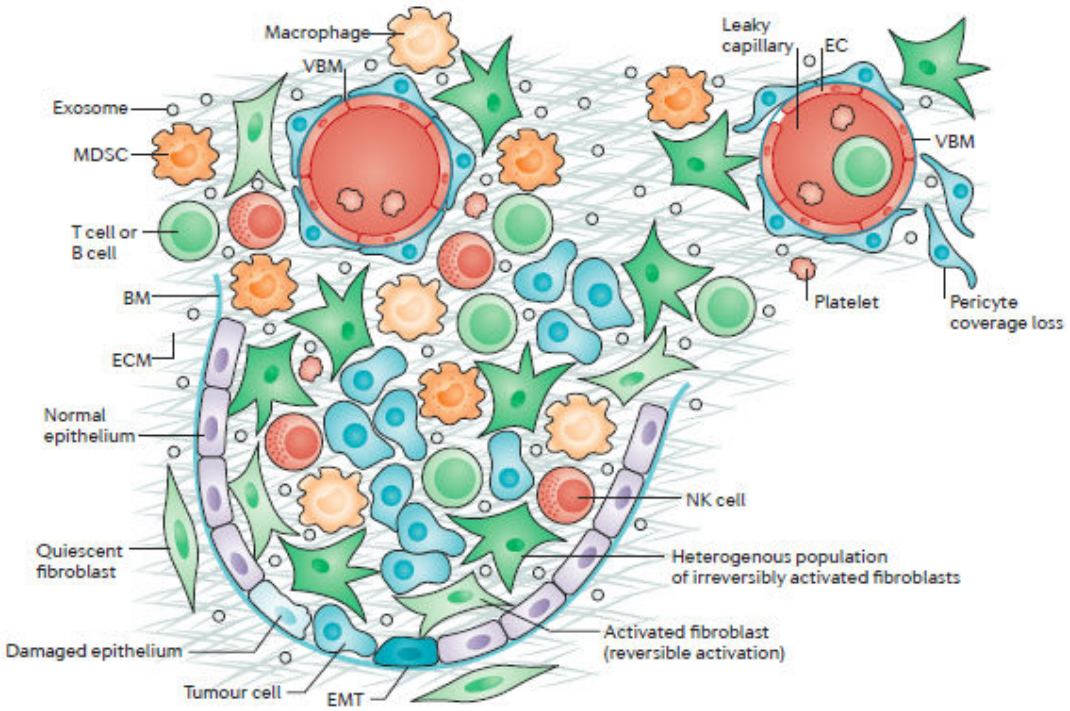


***Figure 1.1*** *Heterogenous population and components of tumor stroma [17].*

### 1.2.1 From Normal Stroma to Tumor Stroma

The normal stroma has a crucial role in providing epithelial tissue maintenance and integrity. It also sustains tissue homeostasis by the collaborative work of several cell types that it contains. These stromal cells have a continuous and constant crosstalk with normal epithelial cells, and this crosstalk is mainly mediated by either direct cell-to-cell contacts or secreted factors (Figure 1.2). Since these interactions are essential for tissue homeostasis, even the smallest changes in one part of this network may cause dramatic alterations in the whole system.

To allow a permissive and supportive environment for the cancer cells, transition from normal stroma to tumor stroma is achieved by the consequent changes in the host stromal compartments as a result of the formation of malignant cells and cancer development by the accumulation of genetic and epigenetic alterations [11]. During the initiation and early stages of tumor formation, progression and invasion, the basement membrane structure is disrupted, tissue stroma (containing fibroblasts, inflammatory infiltrates, capillaries) is altered, and tumor cells come to a direct contact with stromal compartments. These modifications also determine cytokine interactions between tumor cells and fibroblasts [12]. As a result of all these cancer-induced alterations and modifications in tissue stroma, it becomes a contribution to cancer development, invasion and metastasis [13].

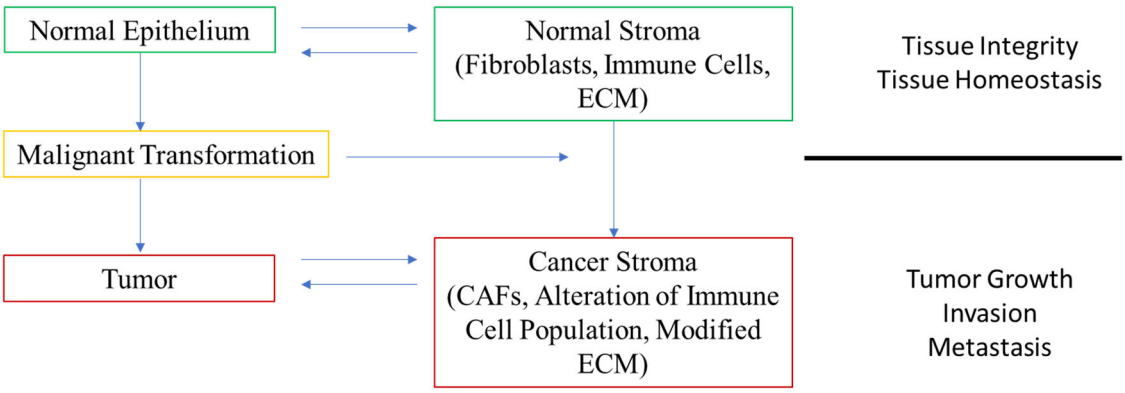


***Figure 1.2*** *Continuous crosstalk between epithelial cells/cancer cells and normal stroma/tumor stroma.*

Animal studies have shown that activation of stroma through various factors provides oncogenic signals and consequently facilitated tumorigenesis [14]. One of the most dramatic changes happen in ECM. Normal stroma in most organs consists of a low number of fibroblasts associated with ECM production whereas the activated stroma contains more ECM producing fibroblasts and as a result more ECM production [15]. This stromal expansion with the increased number of fibroblasts and densely deposited ECM is called desmoplastic reaction (morphologically desmoplasia) [16]. This reaction was initially considered as a defense mechanism against tumor growth, however, quite oppositely, researchers have shown that it takes parts in angiogenesis, migration, invasion, and metastasis in established tumors [17]. Other studies showed that collaboration of fibroblasts and cancer cells can enhance tissue growth and cancer

progression by both the secretion and degradation of ECM components inside the tumor stroma [18].

### 1.2.2 Angiogenic and profibrotic growth factors derived from cancer cells

Cancer cells have been shown to have a role in the determination of the volume and composition of tumor stroma by secreting several profibrotic growth factors such as TGF-beta, FGF2, and platelet-derived growth factor (PDGF) as they mediate fibroblast activation and tissue fibrosis [5]. Additionally, angiogenic factors like VEGF family, has a crucial role in activated stroma [19]. Although VEGF family proteins are mostly secreted by fibroblasts and inflammatory cells, malignant cells were also shown to release VEGF [20]. VEGF is known to increase neovascularization and vascular permeability which results in extravasation of plasma proteins. Then, fibroblasts, inflammatory and endothelial cells are attracted to these plasma proteins such as fibrin [21]. These cells, in return, enhance tumor fibrosis and angiogenesis.

## 1.3 Fibroblasts and their activation

Fibroblasts have quite distinct characteristics based on the tissue from which they derive. They were identified nearly hundred years ago, and gene analysis studies have shown that they have specific roles [22]. Normal fibroblasts are found in quiescent state embedded in ECM in tissue homeostasis and they interact with their neighbors through cell receptors such as integrins [17]. However, certain signals such as wound healing and fibrosis can stimulate fibroblasts to activate. Upon this activation, they are called myofibroblasts, they undergo some morphological and functional differentiation, they become capable of producing and secreting relevant mediators such as growth factors, cytokines, and immune signals [23]. However, this activation is reversible and after the signal that caused activation disappeared, like wound healing is completed, these activated fibroblasts either go back to their quiescent state or they are removed from the tissue by apoptosis [24]. This process considered to be different in cancers. One of the reasons why scientists define cancer as “a wound that never heals” is that activated fibroblasts stay in their activated state and they are not removed by apoptosis as in wound healing. Instead of this reversible process, they become prominent actors of carcinogenesis.

In cancer, these irreversibly activated fibroblasts are called cancer associated fibroblasts (CAFs). Like other activated fibroblasts, they are thought to reside in a highly heterogeneous population. Although local normal fibroblasts are considered to be the

progenitors of CAFs, studies have shown that they can also be derived from pericytes, muscle cells, and mesenchymal cells [25]. Studies have also showed that CAFs differ from normal fibroblasts by epigenetic and possibly some genetic changes [26]. CAFs are usually recognized by increased expression of alpha-smooth muscle actin (α-SMA) but other CAF markers such as fibroblast-specific protein 1 (FSP 1), fibroblast activation protein (FAP), and vimentin are also being used to define them [27].

In response to formation of cancer cells and tumorigenesis, normal fibroblasts get activated by secreted molecules such as TGF-β, MCP 1, and MMPs which modify ECM. Although several studies proposed an inhibitory effect of normal fibroblasts to cancer progression, cancer promoting role of CAFs is widely accepted. As a matter of fact, in breast cancers, 80% of this heterogeneous fibroblast population is considered to be CAFs and have activated phenotype [28].

### 1.3.1 CAFs as positive regulators of cancer

Cancer associated fibroblasts that are isolated from human tumors differ from normal fibroblasts from healthy tissue in many ways such as increased proliferative and migratory capacity, autocrine signaling, and increased secretory phenotype (Figure 1.3) [29]. However, interestingly, these changes that are observed in CAFs and that could be because of epigenetic changes which caused their activation, are not found in normal activated fibroblasts [30]. Moreover, many co-culture experiments have shown that CAFs have an ability to promote tumorigenesis whereas NAFs lack this ability []. Similarly, it is also observed that CAFs can induce invasion of cancer cells [31]. All these imply that, differently from normal fibroblast activation, CAFs have distinct and crucial roles in cancer development.

In addition, a part of the tumor promoting effects of CAFs is thought to be their ability to induce angiogenesis by secreting stromal cell derived factor 1 (SDF1) and recruiting endothelial cells. Similarly, CAFs contribute to tumorigenesis by secreting other modulators such as heat shock factor 1 (HSF1) that can complement this program in cancer cells and Yes-associated protein 1 (YAP1) that can alter ECM structure and increase cancer cell invasion [32]. Moreover, deregulations in Notch and p53 signaling in cancer associated fibroblasts enhance their proliferative capacity and makes cancer development more complex and difficult to understand [30,32]. However, to understand all these pathways, deregulations, and alterations more work is needed.

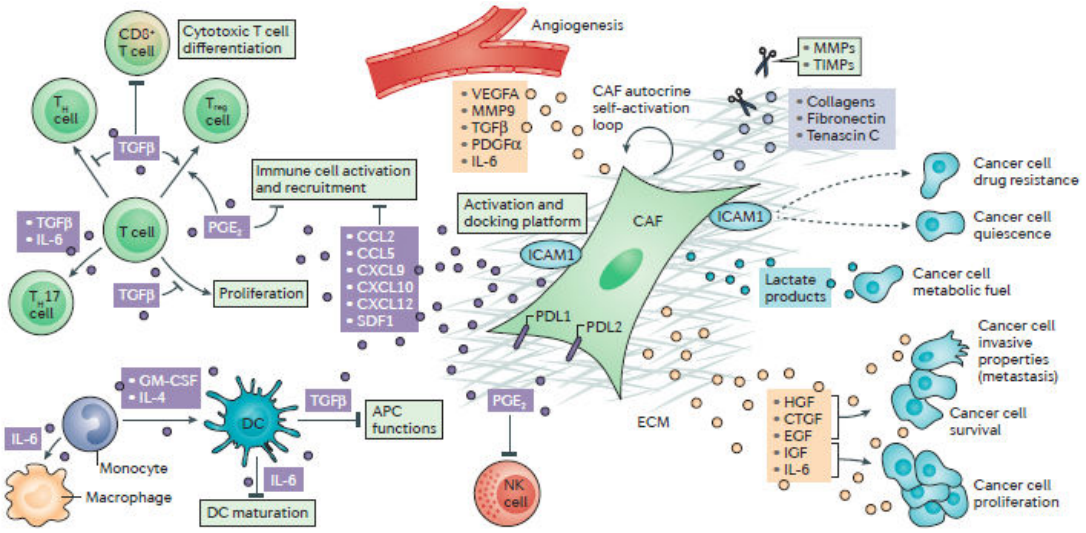


***Figure 1.3*** *CAFs secretory phenotype and their cross-talk with neighboring cells [17].*

### 1.3.2 CAFs and cancer stem cell niche

The generation, maintenance and promotion of cancer stem cells and cancer stem cell niche may be elicited by the cooperative work CAFs and cancer cells in remodeling ECM. Stromal remodeling and maintenance of cancer stem cell niche by WNT signaling may be regulated by fibroblasts which express Periostin (POSTN) [35]. Studies have shown elevated WNT signaling in colon cancer stem cells which are near CAFs implicating that the role of CAF-derived HGF in the formation of cancer stem cell niche. Similarly, in lung cancer, cancer stem cells have been shown to induce fibroblast activation by Thrombospondin 2 (THBS2) expression which in turn enhance metastasis. These signaling events between cancer stem cells and fibroblasts, activates fibroblast-derived IGF2 and IGF1 receptor signaling in cancer stem cells which induces the activation of Nanog expression and stemness in cancer cells [36].

### 1.3.3 CAFs in metastasis

CAFs are considered as one of the important members of metastatic growth at a secondary site. Release of mediators such as growth factors and cytokines into the circulation by CAFs from the primary site may directly or indirectly support metastasis and growth and cancer cells may gain invasive characteristics at a distant site [37,38]. ECM remodeling role of CAFs at the primary site may enhance invasion and CAFs may form ECM tracks to guide cancer cells. Studies demonstrated that metastasis associated

fibroblasts (MAFs) mediate breast cancer metastasis to the lung by expression of Tenascin C and VEGF-A [39]. In addition, CAFs induces angiogenesis in melanoma metastasis [40]. Similarly, IL-11 secretion from CAFs that are stimulated by TGF-β1 increases the survival and organ colonization in colorectal cancers. Researchers also reported that CAFs induce intravasation and metastasis colorectal cancer cells via STC1 secretion [41]. In animal studies, Fsp1-knockout mice presented impaired fibroblast mobility and reduced metastasis [42].

MAFs may emerge as a result of distant tissue fibroblast activation by metastatic cancer cells or they may be recruited from other tumor sites. Possibility of multiple origins may contribute to various functional properties.

### 1.4 Cytokines

Cytokines is a broad definition of small proteins (5-30 kDa) that have important roles in cell signaling and they affect the behavior of neighboring cells around them. They are involved in autocrine and paracrine signaling as modulating agents. Cytokines can be grouped as chemokines, lymphokines, interleukins and interferons. Despite some overlap, generally, growth factors are not considered as cytokines. They can be produces and secreted by a range of cells such as immune cells, endothelial cells, and fibroblasts [43].

Cytokines act through receptors and regulate cell-based responses. They can modulate the growth and maturation of certain populations of cells in health and diseases such as infection, inflammation, cancer, and reproduction [44]. For example, oxidative stress induces several inflammatory cytokines [45,46] and they themselves activate the secretion of other cytokines [47-49].

Cytokines circulate in picomolar concentrations and the variability in cellular sources differentiates them from hormones [50]. Each cytokine has a cell surface receptor and binding induces a subsequent intracellular signaling which leads to alterations in cellular function. These alterations may include deregulation of gene expressions, cellular response to a certain stimulus, and growth. The effect of a cytokine depends on the cytokine, its abundance, matching receptor, and downstream signaling.

During embryogenesis and immune responses, cytokines have a crucial role [51-53]. However, they can be dysregulated under certain pathologic conditions and they are linked to many diseases such as Alzheimer's disease [54] and cancer [55]. Especially, in

cancer which threatens normal tissue integrity and homeostasis, studies showed disrupted interactions and feedbacks of secreted cytokines [56].

Using recombinant DNA technology, some recombinant cytokines have been developed to be used as drugs [57]. For example; Erythropoietin, used to treat anemia; Interferon alpha and beta, used to treat hepatitis C and multiple sclerosis; Interleukin 2; used to treat cancer.

### 1.5 Cytokines and Cancer

Cytokines that are produced in tumor stroma have an important role in cancer progression. Some cytokines that are secreted in response to inflammation or immunity can inhibit cancer development whereas some cytokines can promote tumor growth, invasion and metastasis (Table 1.1). In addition, therapies based on stromal cytokines, their composition and effects on both cancer and stromal cells, have a potential to be used as effective treatment methods (Table 1.2).

***Table 1.1*** Cytokines and their roles in cancer formation

| | |
|---|---|
| IL-1 | Tumor invasion and angiogenesis |
| IL-6 | Chemically induced lymphomas |
| IL-12 | Inhibition of chemical carcinogenesis |
| IL-15 | NK cell leukaemias |
| IFN-γ | Inhibition of lymphomas |
| M-CSF | Breast cancer invasion |
| GM-CSF | Inhibition of lymphomas and carcinomas |
| TNF-α | Chemically-induced skin cancer |
| MIF | Inhibition of p53 |
| TGF-β | Inhibition of colon carcinomas |
| Fas-Fas ligand | Inhibition of lymphomagenesis |

***Table 1.2*** Cytokines as cancer therapy

| | |
|---|---|
| IL-2 | Enhances NK cell and CD8 T cell function; increases vascular permeabilty |
| IL-3 | Enhances cancer antigen presentation |
| IL-4 | Enhances eosinophil function and T-cell activation |
| IL-6 | Enhances T-cell and B-cell function; inhibition of IL-6 reduces lymphoproliferation |
| IL-7 | Enhances T-cell function |
| IL-10 | Inhibits cancer antigen presentation |
| IL-12 | Enhances Th1 immunity and cytotoxicity; anhibits angiogensis |
| IL-13 | Inhibits cytotoxicity against viral neoplasms |
| IL-15 | Enhances cytotoxicity |
| IL-18 | Enhances Th1 immunity and cytotoxicity; inhibits angiogenesis |
| M-CSF | Enhances macrophage function |
| GM-CSF | Enhances cancer antigen presentation |
| IFN-α | Enhances cancer antigen presentation and cytotoxicity |
| IFN-γ | Enhances cancer antigen presentation and cytotoxicity |
| TNF-α | Induces tumor-cell apoptosis; activates andothelium and granulocytes |
| TRAIL | Induces tumor-cell apoptosis |
| FLT3 ligand | Stimulates dendritic-cell and NK-cell function |
| Lymphotactin | Enhances T-cell recruitment |
| TGF-β | Inhibits T-cell effector function |

In this study, we present that CAFs can enhance the growth of MCF7 and T47D breast cancer cells in vitro when they are co-cultured. Our co-culture experiments showed that this tumor promoting effect of CAFs is not dependent on cell-to-cell contact and it is caused by their crosstalk through cytokine secretion. We found that Activin A, Interleukin 5 and Angiogenin are present in higher levels in the cell culture media of CAF co-culture and similarly their expression in human breast cancer tumor samples are increased. Moreover, we showed that tumor growth due to CAFs can be reversed using specific neutralizing antibodies. Additionally, recombinantly produced cytokines were able to enhance the growth of MCF7 and T47D cells without the presence of CAFs in the culture. Therefore, we propose that CAFs induce tumor growth by their interaction with malignant cells through the secretion of specific cytokines and targeting this interaction can be developed as a new strategy in cancer treatment.

## 2. MATERIALS AND METHODS

### 2.1 Isolation of fibroblasts and CAFs from human breast tissue samples

After receiving tissue samples in sterile DMEM media, they were washed with PBS. Then, they were minced into small pieces and treated with Liberase TL (Roche, 05401020001) at 37 ºC for 1-2 hours depending on the size of the sample. Samples were vortex every 30 minutes allowing a homogeneous mixture. After we obtained single cell suspensions, we washed the samples with DMEM and PBS and seeded them on 6-well culture plates.

### 2.2 Cell culture and transfection

MCF7, T47D, HEK293T, fibroblasts and CAFs were cultured in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, #BI01-050-1A). Culture media was supplemented with 10% (v/v) fetal bovine serum (FBS, PAN, #P30-3302) (3%FBS supplementation for co-culture and other experiments), antibiotics (Penicillin/streptomycin, Biological Industries, #BI03-031-1B) and L-Glutamine (Biological Industries, #BI03-020-1B) Cells were stored in 5% $CO_2$ humidified incubator at 37°C. HEK293T cells were transiently transfected with calcium phosphate transfection method following the standard protocols.

### 2.3 Immunoblotting and antibodies

Cells were lysed at indicated time points in RIPA buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate) supplemented with a complete protease inhibitor cocktail (Roche, 04-693-131-001) and 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, P7626). Protein extracts (30 µg per well) were separated using 10-15% SDS-polyacrylamide gels (SDS-PAGE), and then transferred onto nitrocellulose membranes (Millipore, #IPVH00010). Membranes were blocked in 5% nonfat milk (Applichem, #A0830) in PBST (3.2 mM $Na_2HPO_4$, 0.5 mM $KH_2PO_4$, 1.3 mM KCl, 135 mM NaCl, and 0.05% Tween 20, pH 7.4) for 1 h and then incubated with primary antibodies in a 3% BSA PBST solution: anti-Flag (Sigma, A494842), anti-LC3 (CST, #2775S) and anti-β-ACTIN (Sigma-Aldrich, A5441) antibody as loading control. Following PBST washes, membranes were incubated with horseradish peroxidase (HRP)-coupled secondary anti-mouse (Jackson Immunoresearch laboratories, #115035003) or anti-rabbit antibodies (Jackson Immunoresearch Laboratories, #111035144). Band intensities were quantified using the ImageJ software.

**2.4 RNA isolation and qRT-PCR analyses**

Total RNA was extracted using TRIzol reagent (Sigma-Aldrich, #T9424) according to the manufacturer's instructions. cDNA was reverse transcribed from DNase-treated total RNA using M-MuLV reverse transcriptase (Fermentas, #EP0351), and random hexamers (Invitrogen, #48190-011).

For single-step qRT-PCR reaction, SYBR Green Quantitative RT-PCR kit (Roche, #04-913-914-001) and LightCycler 480 (Roche) were used. To activate the SYBR green, an initial cycle of 95°C, 10 min was performed followed by PCR reactions: 40 cycles of 95°C for 15 sec. and 60°C for 1 min. Then a thermal denaturation protocol was used to generate the dissociation curves for the verification of amplification specificity (a single cycle of 95°C for 60 sec., 55°C for 60 sec. and 80 cycles of 55°C for 10 sec). Changes in mRNA levels were quantified with the $2^{-\Delta\Delta CT}$ method and normalized to *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) mRNA.

**2.5 Immunofluorescence Analyses**

Cells were cultured on coverslides and fixed in ice-cold 4% paraformaldehyde/PBS. For indirect immunostaining experiments, following fixation, cells were permeabilized in PBS containing 0.1% BSA (Sigma, #A4503) and 0.1% saponin (Sigma, #84510). As primary antibodies, anti-αSMA (Abcam, ab5694), anti-Vimentin (Abcam, ab92547) were used. Anti-mouse Alexa Fluor 488 (Invitrogen, #A32723) and anti-rabbit Alexa Fluor 488 (Invitrogen, #A11034) were used as secondary antibodies. Coverslides were mounted onto glass slides, and samples were analyzed using Carl Zeiss LSM 710 confocal microscope (Zeiss, Germany).

**2.6 Flow cytometry analysis**

To confirm growth analyses, MCF7-RFP cells were co-cultured with fibroblasts and CAFs. At the end of co-culture, cells were washed 2 times with PBS and trypsinized. Cells were fixed by using ice-cold 70% ethanol and then resuspended in PBS. RFP positive cells were detected using BD FACSCanto™ instrument and analyzed using Flow Jo software (Tree Star Inc). A minimum of 10000 events per sample corresponded to gated population were collected, and cellular debris was not counted.

### 2.7 Cytokine Array

72 hours after fibroblast and CAF co-cultures, conditioned media used to detect differentially secreted cytokines. For this purpose, we used Human Cytokine Array C4000 (RayBiotech, AAH-CYT-4000) and followed manufacturer's instructions.

### 2.8 Luciferase Activity Analysis

MCF7 and T47D cells which express luciferase were treated with Chris Buffer at 37 ºC for 5 minutes. After that cell lysates were collected and centrifuged for 5 minutes at 1000 g. Supernatant was kept on ice and later mixed with homemade luciferase reagent to detect luciferase activity with luminometric measurement.

# 3. RESULTS

## 3.1 Characterization of Fibroblasts and CAFs isolated from tissue samples

CAFs are key components of tumor stroma and previous studies identified their tumor promoting capacity compared to fibroblasts and NAFs. In addition, in breast cancers, around 80% of the heterogeneous fibroblast population inside the tumor stroma is shown to be CAFs. One of the distinctive characteristics of CAFs is their elevated expression level of α-SMA and Vimentin, and the formation of stress fibers. Upon receiving healthy and tumor breast tissue samples in sterile conditions, they were minced into smaller pieces and treated with Liberase TL to obtain single cell suspensions. After that, these primary cell suspensions were cultured in DMEM (10% FBS) until we obtained normal fibroblasts and CAFs in cell culture conditions. Characterization of fibroblasts and CAFs was done using immunofluorescence staining using α-SMA and Vimentin antibodies. Confocal microscopy images showed the distinct morphological changes, increased level of expression of both α-SMA and Vimentin as well as the formation of stress fibers in CAFs which were isolated from tumor samples compared to fibroblasts from healthy breast tissue sample of the same patient (Figure 3.1).

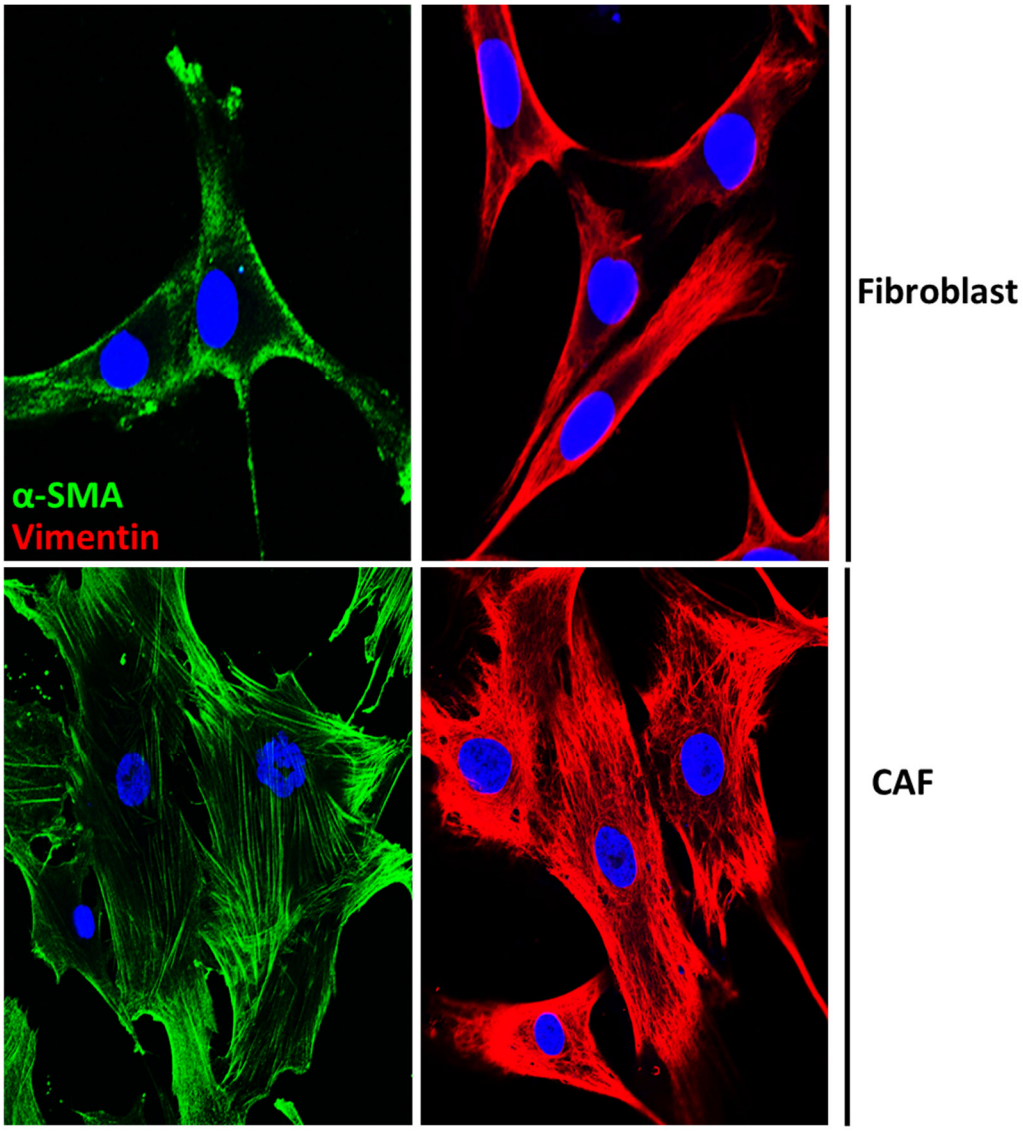


***Figure 3.1*** *Characterization of fibroblasts and CAFs. α-SMA and Vimentin stainings and confocal microscopy analyses showed morphological changes, increased expression levels of both proteins and the formation of stress fibers in CAFs (below) compared to fibroblasts (above) from the healthy tissue samples of the same patient.*

### 3.2 Differential effects of fibroblasts and CAFs on cancer cell growth in vitro

Although CAFs are similar to normal activated fibroblasts, their roles in tumorigenesis and cancer progression are not fully identified. However, several studies have shown their pro-tumorigenic roles and demonstrated that they enhance tumor growth. To see the effect of CAFs in cancer cell proliferation, we co-cultured MCF7 and T47D breast cancer cells with either fibroblasts or CAFs. MCF7 and T47D alone without the presence of fibroblasts or CAFs were used as control and after 72 hours of co-culture

in DMEM (3% FBS) their growths were compared. In the first part, we co-cultured fibroblasts and CAFs together with either MCF7 or T47D cells which stably expressing luciferase. After 72 hours, cells were lysed using Chris buffer and cell lysates were used to measure their luciferase activity using luminometric analyses. Both intensities of fibroblast and CAF co-cultures were normalized to MCF7 and T47D alone conditions. As a result of luciferase activity assays, co-culture of cancer cells with CAFs showed significantly increased proliferation compared to both control and fibroblast co-culture conditions (Figure 3.2A and 3.2B). Similarly, we confirmed these results by using microscopy images (Figure 3.2C) and FACS analysis (Figure 3.3D) of co-cultures of fibroblasts and CAFs with MCF7 cells which stably express RFP protein.

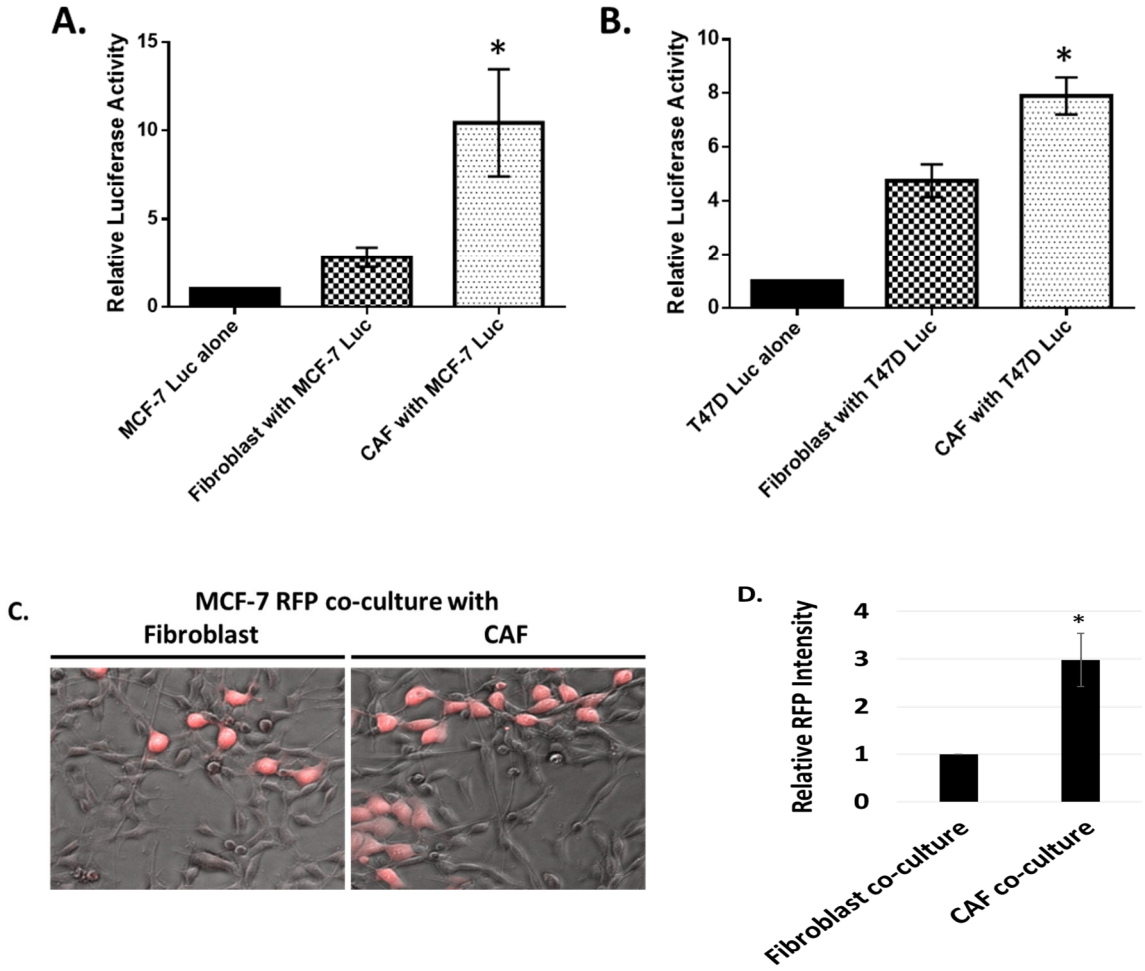


***Figure 3.2*** *Differential effects of fibroblasts and CAFs on cancer cell growth in vitro. A. Relative luciferase activity of MCF7 cells after 72 hours of either growing alone or co-culture with fibroblasts or CAFs (n=6, p<0.05). B. Relative luciferase activity of T47D cells after 72 hours of either growing alone or co-culture with fibroblasts or CAFs (n=3, p<0.05). C. Microscopy images of RFP expressing MCF7 cells after 72 hours of co-culture with fibroblasts or CAFs. D. Quantification of relative RFP intensities of fibroblast and CAF co-cultures of MCF7-RFP cells in FACS analysis.*

### 3.3 Analysis of secreted factors in fibroblast and CAF co-cultures

To determine whether tumor promoting effect of CAFs is dependent on cell-to-cell contact, we changed the co-culture conditions and provided a physical distance between fibroblasts/CAFs and cancer cells. For this purpose, after seeding either fibroblasts or CAFs onto cell culture plates, MCF7 cells were seeded on inserts which has pores (0.4μm) that will allow the passage of secreted factors but prevent a direct cellular contact (Figure 3.4A). Under these conditions, after 72 hours of co-culture or culturing

MCF7 cells alone, cancer cells were collected to measure their luciferase activity which reflect their proliferation. Relative luciferase activities in each group were measured and we observed a similar result that CAFs promoted the growth of cancer cells more than fibroblast co-culture and cancer cells alone conditions (Figure 3.4B). This result indicated that growth promoting effect of CAFs should be due to secreted factors such as cytokines, lymphokines, and/or growth factors. To determine which factors were secreted differentially in fibroblast and CAF co-culture conditions, co-culture media were collected and analyzed using RayBiotech cytokine array (Figure 3.3). As a result, we were able to identify three cytokines (Activin A, IL-5, and Angiogenin) which were secreted in significantly higher amounts (cutoff=1.5 fold) in CAF co-cultures compared to fibroblast co-cultures (Figure 3.4C and 3.4D).

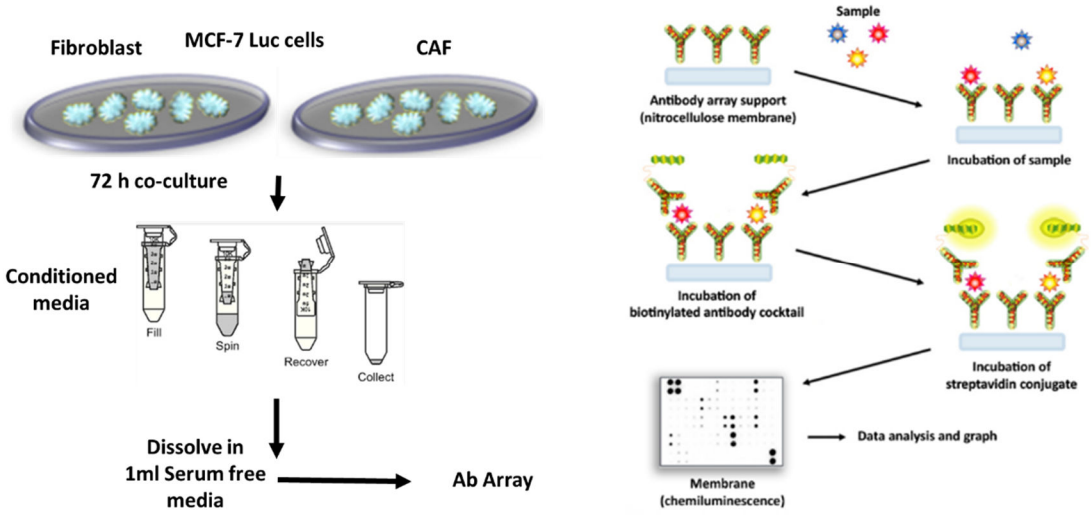


***Figure 3.3*** *Schematic representation of co-culture media collection and RayBiotech cytokine array.*

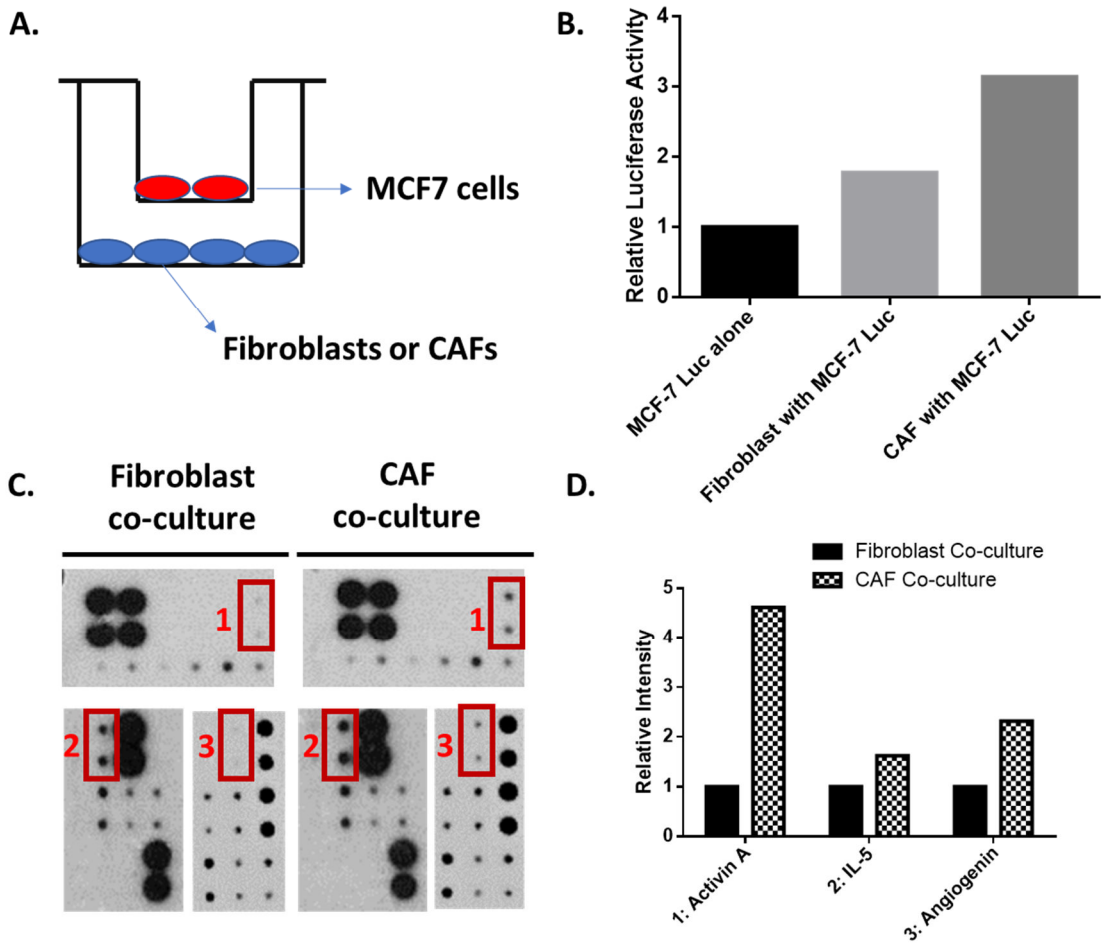


***Figure 3.4*** *Determination of differentially secreted factors in fibroblast and CAF co-cultures. A. Schematic representation of co-culture condition using inserts. B. Relative luciferase activities of MCF7 alone, fibroblast co-culture, and CAF co-culture after 72 hours using inserts. C. Representative image of two independent experiments of RayBiotech cytokine array results (1: Activin A, 2: IL-5, 3: Angiogenin). D. Quantification of cytokine array results. This graph represents the average of relative intensities of three cytokines that were 1.5-fold or higher in two independent experiments.*

### 3.3.1 Determination of the possible sources of cytokine secretion

To determine which cells predominantly secrete these three factors, MCF7 cells, fibroblasts, and CAFs were collected before and after co-culture of 72 hours using inserts. Total RNA from these cells were extracted using Trizol reagent and cDNAs were amplified using specific primers for Activin A, IL-5 and Angiogenin. qRT-PCR results demonstrated that CAFs are more likely to be the main sources of Activin A secretion due to the higher expression level compared to MCF7 cells and fibroblasts (Figure 3.5A)

whereas IL5 was secreted mostly by MCF7 cells with a little contribution of CAFs (Figure 3.5C). Our result also showed that both MCF7 cells and stromal cells contribute in Angiogenin secretion (Figure 3.5E). Moreover, our result also implies that the target of Activin A secreted from CAFs is cancer cells since MCF7 cells had higher expression level of Activin A receptor (Figure 3.5B) and the target of IL5 which was mostly secreted by MCF7 cells was stromal cells (Figure 3.5D) due to higher expression level of IL5RA.

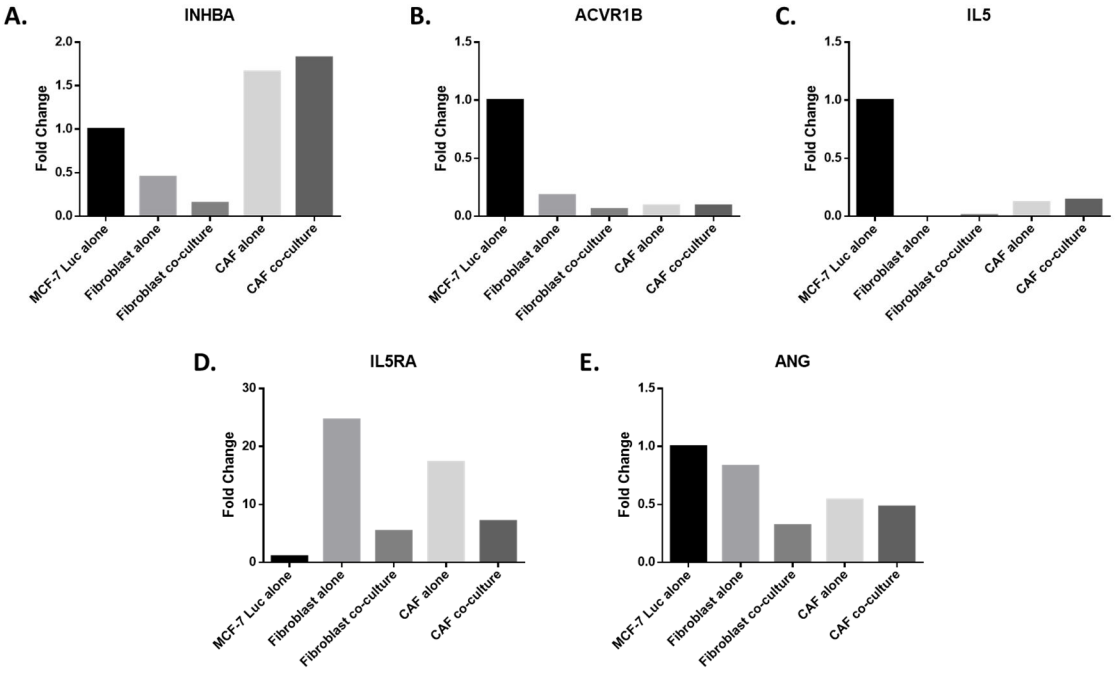


***Figure 3.5*** *Relative gene expression levels of Activin A, IL-5, Angiogenin and their receptors. These graphs represent the average relative gene expressions in two independent experiments. (INHBA: Activin A, ACVR1B: Activin A receptor 1B, IL5: Interleukin 5, IL5RA: Interleukin 5 receptor A, ANG: Angiogenin)*

### 3.4 Gene expression deregulations of cytokines and their receptors in human breast cancer tumors

To determine how the gene expression of these cytokines and their receptors are changed in breast cancer tumors, we extracted total RNA from healthy and tumor frozen samples of 17 breast cancer patients. The levels of the gene expressions in tumor samples were compared to their healthy tissue sample counterparts. We have found that all three cytokines and their receptors were expressed in elevated levels in most of the tumor

samples compared to healthy tissue samples (Figure 3.6). Furthermore, we analyzed these results to see if we could find a correlation between the tumor grade and gene expression levels. However, we were not able to identify a correlation (Table 3.1).

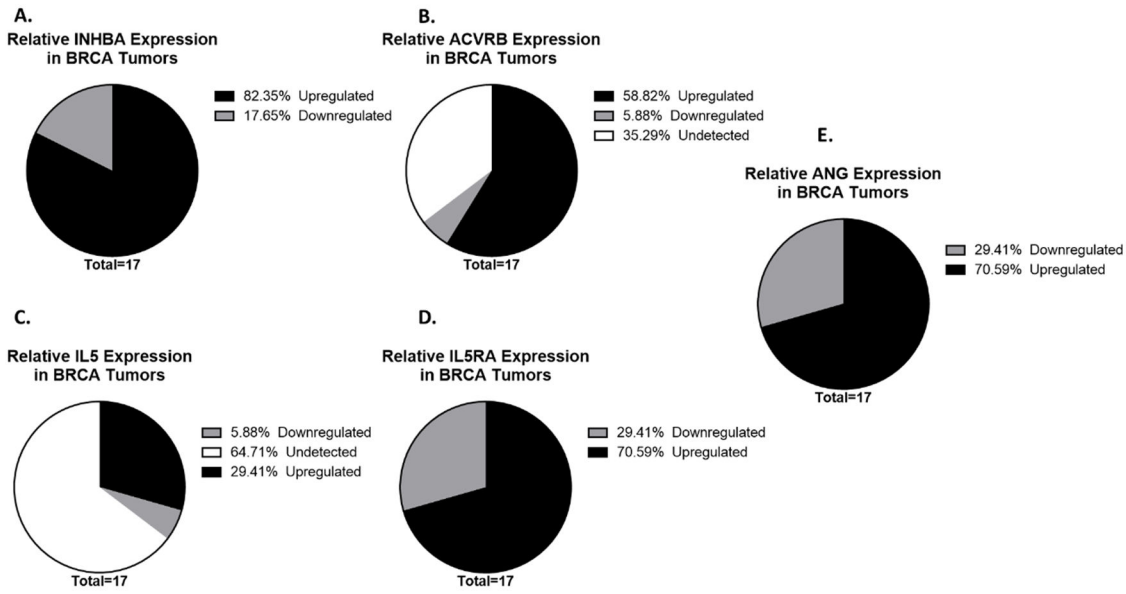


***Figure 3.6*** *Deregulation of Activin A, IL-5, Angiogenin and their receptor gene expressions in breast cancer tumors. Gene expression levels were identified using qRT-PCR after RNA extraction from frozen healthy and tumor tissue samples of 17 breast cancer patients.*

***Table 3.1*** *Tumor grades and gene expression regulations.*

| Grade | Patient | INHBA | ACVR1B | IL5 | IL5RA | ANG | BTC |
|---|---|---|---|---|---|---|---|
| T1 | BRCA4 | UP | UP | UNDETECTED | UP | UP | DOWN |
| | BRCA5 | UP | DOWN | UNDETECTED | UP | DOWN | UNDETECTED |
| | BRCA14 | UP | UNDETECTED | UNDETECTED | DOWN | UP | UP |
| | BRCA15 | DOWN | UNDETECTED | UNDETECTED | UP | UP | UP |
| | BRCA17 | UP | UP | UNDETECTED | DOWN | UP | DOWN |
| | BRCA20 | UP | UNDETECTED | UNDETECTED | DOWN | UP | UP |
| T2 | BRCA7 | UP | UNDETECTED | DOWN | DOWN | DOWN | UNDETECTED |
| | BRCA10 | UP | UNDETECTED | UNDETECTED | UP | UP | UP |
| | BRCA11 | UP | UP | UNDETECTED | UP | UP | DOWN |
| | BRCA16 | UP | UP | UNDETECTED | UP | UP | UP |
| T3 | BRCA19 | DOWN | UNDETECTED | UNDETECTED | DOWN | DOWN | UP |
| T4 | BRCA12 | UP | UP | UNDETECTED | UP | DOWN | UP |

### 3.5 Preventing the tumor promoting effect of CAFs using neutralizing antibodies

After we showed that CAFs are able to promote cancer cell proliferation, this effect does not necessarily require cell-to-cell contact and it can be achieved through secretion of certain cytokines differently than normal fibroblasts, we started to wonder if we could prevent this by using specific neutralizing antibodies, so it will stop the downstream signaling pathways. For this purpose, we commercially obtained neutralizing antibodies against Activin A, IL5 and Angiogenin.

#### 3.5.1 Testing of neutralizing antibodies' effectiveness

To assess whether Activin A and Angiogenin neutralizing antibodies work efficiently we performed HUVEC tube formation assay and MDA-MB-231 wound healing assay with or without the presence of neutralizing antibodies. HUVEC cells have been shown to form tubes similar to vasculature when they are grown in Matrigel. However, when there is an anti-angiogenic factor like VEGF neutralizing antibody in the culture media they can no longer form these tubes. For this reason, we performed an experiment where we culture HUVEC cells in Matrigel with or without VEGF neutralizing antibody as positive and negative control. We tested the effectiveness of Angiogenin neutralizing antibody and compared the tube formation with controls. When we treated these cells with Angiogenin neutralizing antibody, we observed that HUVEC cells cannot form tubes and the number of nodes increase (Figure 3.7). This result was very similar to the effect of VEGF antibody, so we concluded that our antibody works efficiently.

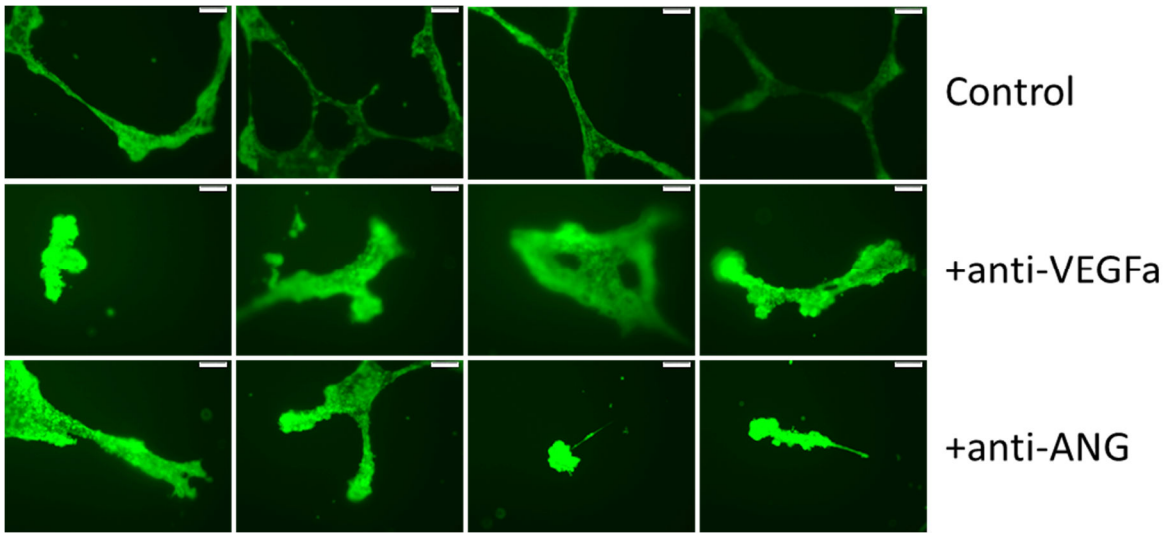


***Figure 3.7*** *Effect of Angiogenin neutralizing antibody on HUVEC tube formation.*

Similarly, to test the effectiveness of Activin A neutralizing antibody, we performed a wound healing assay. There are several studies showing that autocrine signaling of Activin A enhances the migratory capacity of MDA-MB-231 cells. Hence, we treated these cells with Activin A neutralizing antibody for 24 hours and observed wound healing activity. Compared to control, we observed a significant decrease in wound healing suggesting that the antibody works efficiently (Figure 3.8).

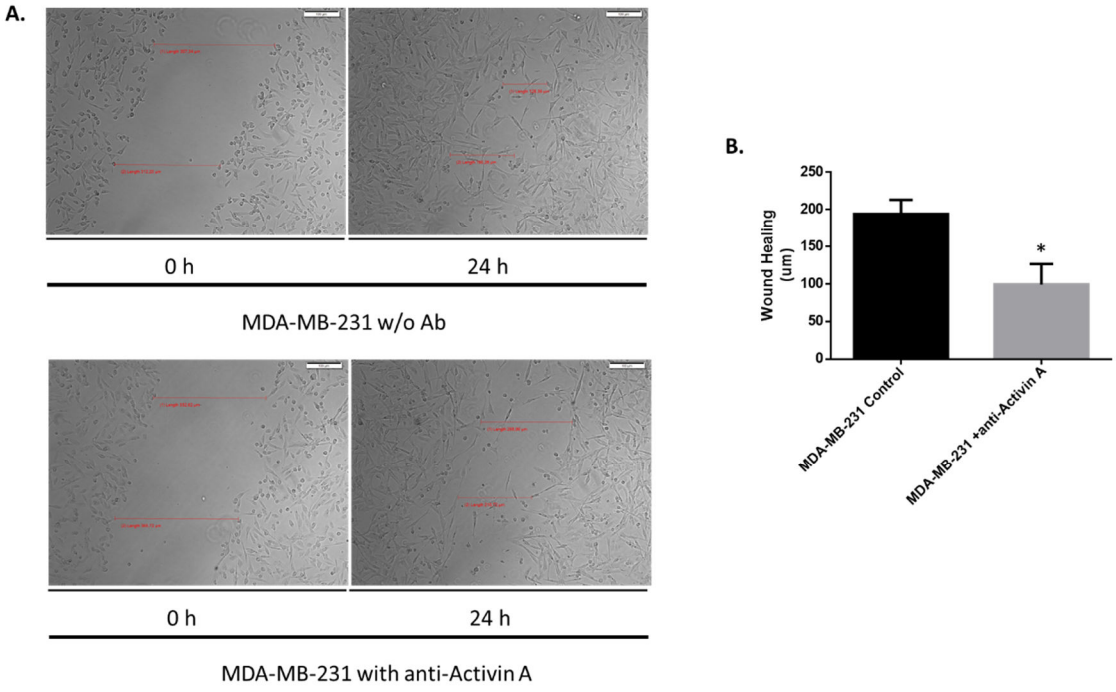


***Figure 3.8*** *Effect of Activin A neutralizing antibody on MDA-MB-231 wound healing. A. Microscopy images of 0 hour and 24 hours after the culture with or without Activin A neutralizing antibody. B. Quantification of MDA-MB-231 cells wound healing (n=3, $p<0.05$)*

### 3.5.2 Effect of Activin A, IL5 and Angiogenin neutralizing antibodies on the growth of MCF7 cells

After we showed that our antibodies work effectively, we wanted to see their effect on the growth of MCF7 cells when they are co-cultured with CAFs. To determine whether these antibodies will be able to reverse the tumor promoting role of CAFs in co-cultures, we checked luciferase activity of MCF7 cells after 72 hours of co-culture under the presence of each neutralizing antibody (1 µg). We compared these results with the

group where we didn't treat the cells with any of those antibodies. Relative luciferase activity results showed that both Activin A and Angiogenin neutralizing antibodies were able to significantly inhibit the growth of MCF7 cells compared to CAF co-culture, whereas although we also observed a decrease when we used IL5 antibody, it was not significant (p=0.0507) (Figure 3.9). This result suggested that pro-tumorigenic role effect of CAFs are through secretion of cytokines and this can be significantly reversed with the use of targeted neutralizing antibodies.

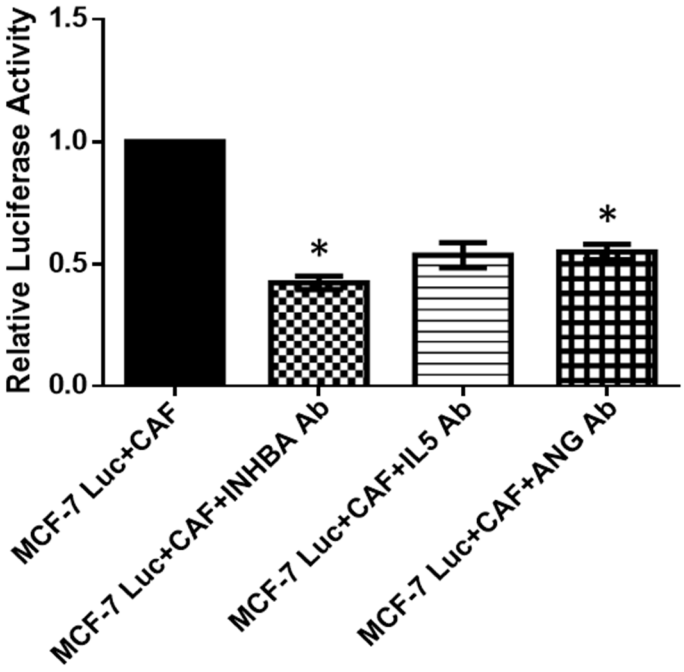


***Figure 3.9*** *Effect of neutralizing antibodies on MCF7 cell growth. Compared to CAF co-culture without any antibodies, both Activin A and Angiogenin neutralizing antibody treatments resulted in significant decreases in relative luciferase activity of MCF7 cells after 72 hours co-culture (n=3, p<0.05). Similarly, IL5 antibody considerably lowered the cell growth.*

### 3.6 Effect of recombinantly produced cytokines on cancer cell growth in vitro

To determine whether Activin A, IL5 and Angiogenin were sufficient to promote the growth of cancer cells in vitro, we transfected Hek293T cells with commercially obtained plasmids of these cytokines and produced flag tagged proteins. Since, all these three factors are secreted from cells, we expected to able to detect these proteins in the

culture media 72 hours after the transfection. After we collected the media and condition them, we used Western Blot using flag antibody to detect flag tagged proteins. Our results showed that the transfection was successful and Hek293T cells secreted these cytokines in abundant amounts. Later, we used these conditioned media to treat MCF7 and T47D cells for 72 hours without co-culturing them with CAFs. After 72 hours, these cells were collected, and their relative luciferase activities were compared to MCF7 and T47D alone conditions. Without CAFs, treatment of MCF7 (Figure 3.10A) and T47D (Figure 3.10B) cells with each of recombinantly produced Activin A, IL5 and ANG significantly increased their growth. This result suggested that the tumor promoting effect of CAFs in tumor stroma is achieved by the secretion of cytokines and presence of these proteins are sufficient to enhance tumor growth without the presence of CAFs.

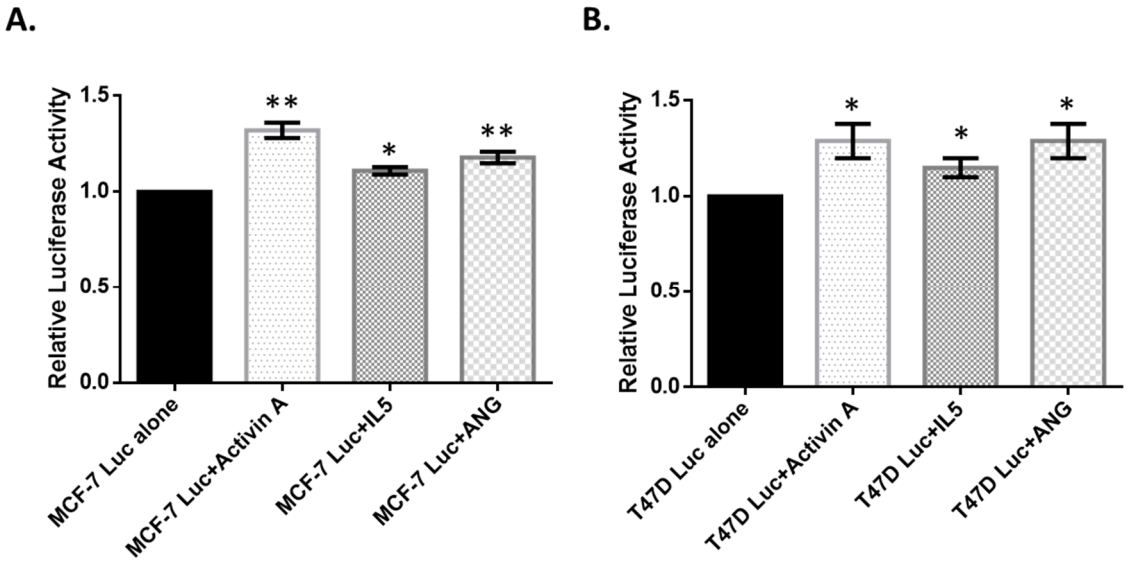


***Figure 3.10*** *Effect of recombinantly produced cytokines on MCF7 and T47D cell growth. A. Relative luciferase activity of MCF7 cells 72 hours after the treatment of Activin A ($p<0.005$), IL5 ($p<0.05$) and Angiogenin ($p=0.005$). B. Relative luciferase activity of T47D cells 72 hours after the treatment of Activin A ($p<0.05$), IL5 ($p<0.05$) and Angiogenin ($p=0.05$). ($n=3$ for each condition)*

### 3.7 Effect of cytokines on cancer cell autophagy

Autophagy has been reported to act as survival mechanism in established tumors and several studies have shown that it can also act as a chemotherapy resistance mechanism in tumors. Similarly, it helps cancer cells maintain their metabolic activities under stress conditions. Hence, we wanted to see whether Activin A, IL5 and Angiogenin

had any effect on cancer cell autophagy. For this reason, we treated MCF7 and T47D cells with recombinantly produced cytokines for 12 and 24 hours. We used LC3-I to LC3-II conversion to observe autophagic activation. We were able to show that these cytokines induced autophagy especially in T47D cells after 24 hours (Figure 3.11). This result implies that CAFs may induce tumor growth trough cytokine secretion which may alter autophagic activity of cancer cells.

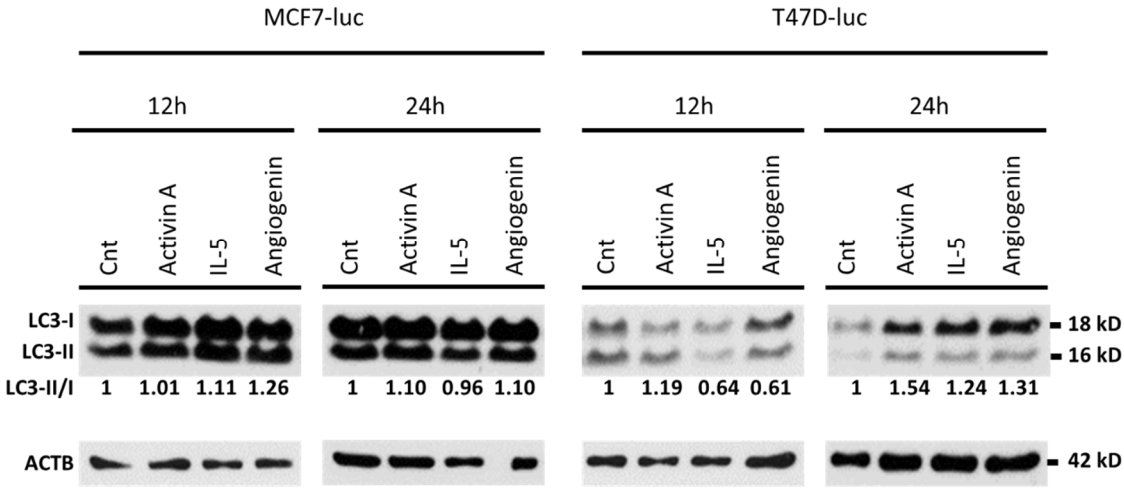


***Figure 3.11*** *Effects of recombinant proteins on autophagic activity of MCF7 and T47D cells. 12 and 24 hours after the treatment of these cells with recombinant proteins, their autophagic activities were measured LC3-II/I ratio.*

## 4. DISCUSSION

Cancer is one of the biggest challenges that the scientists are facing today. Despite extensive research and funding, unfortunately there is still variance between experimental and clinical outcomes. Although there are many approaches available to cure cancer, most of them target only cancer cells. However, we now know that these cancer cells reside in a microenvironment called tumor stroma and there is a constant crosstalk between cancer cells and neighboring cells. Thus, understanding the tumor stroma, its components, interactions, and crosstalk of cancer cells and stromal cells may have a crucial importance for treating cancer. Tumor stroma has been shown to be composed of different cell types and studies suggest that these cells can be recruited and manipulated by malignant cells to support tumor growth and metastasis. In addition, stromal cells can modify and transform the connective tissue so that the microenvironment becomes a permissive one for cancer progression. Although normal tissue stroma is essential for tissue homeostasis, even minor changes leading to tumor stroma may cause dramatic alterations. The tumor stroma basically consists of a heterogeneous population of fibroblasts, immune cells, vasculature, pericytes, endothelial cells and ECM. In this study, we focused on cancer associated fibroblasts which is one of the major components of tumor stroma. CAFs have been shown to have a tumor promoting role due to epigenetic and possibly genetic differences in their genome. In many co-culture experiments, results showed that CAFs enhance the growth of cancer cells when they are co-cultured together. Additionally, differently than fibroblasts and normal fibroblasts, CAFs proliferate robustly, gain enhanced migratory capacity and increased level of secretion.

In this study, we obtained fibroblasts and CAFs from healthy and tumor samples of breast cancer patients. First, we co-cultured MCF7 and T47D cells with either fibroblasts or CAFs to determine whether CAFs could enhance the proliferation of breast cancer cell lines in vitro compared to normal conditions and fibroblast co-culture. Our luciferase activity results after 72 hours of co-culture revealed that CAFs can increase the growth of MCF7 and T47D cells. Similarly, we confirmed this result with FACS analysis and microscopy images. Furthermore, to identify whether this tumor promoting effect of CAFs was due to cell-to-cell contact, we provided a physical distance in co-culture conditions which will allow them to communicate only through secreted molecules. Our results indicated that, even without cell-to-cell contact, CAFs are still able to enhance cancer cell growth through cytokine secretion. After 72 hours of co-culture with either fibroblasts or CAFs, we used RayBiotech cytokine array and we were able to identify

three cytokines (Activin A, IL5 and Angiogenin) that were present in higher amounts in CAF co-culture media. Today, we know that neighboring cells have a continuous communication and there is a crosstalk between cancer cells and stromal cells. To determine which cells provide these three cytokines that are present in the media, we separately collected cancer cells and CAFs and checked their expression levels. qRT-PCR results suggested that CAFs are the main source of Activin A, MCF7 cells are the main source of IL5 and they contribute similarly to the secretion of Angiogenin. Additionally, we wanted to see if the expressions of the cytokines were altered in human breast cancer tumor samples. Compared to healthy tissues, we observed higher expression of the cytokines in tumor samples.

Targeting tumor stroma and the crosstalk between cancer cells and CAFs may be used as another approach in treating cancer. An application of this can be the use of neutralizing antibodies which will block the communication and downstream signaling. For this reason, we co-cultured MCF7 cells with CAFs for 72 hours and treated them with neutralizing antibodies of Activin A, IL5 and Angiogenin. Especially Activin A and Angiogenin antibodies significantly reduced the growth of cancer cells. Given that most of Activin A was provided by CAFs in co-culture experiments, this result shows the potential of neutralizing antibodies for blocking the communication and the tumor promoting role of CAFs. On the other hand, we used recombinantly produced cytokines which we obtained from transfected Hek293T cell media. After 72 hours of treatment of both MCF7 and T47D cells with recombinant cytokines resulted in a significant enhancement in the growth of these cells.

According to recent studies, cancer cells develop several mechanisms to overcome stress conditions. Autophagy has also been proposed as one of these mechanisms to promote tumor growth by maintaining the metabolic activities and drug resistance in established tumors. To assess the autophagic activity in MCF7 and T47D cells, we treated them with recombinant cytokines for 12 and 24 hours. Our results suggested that, especially in T47D cells, cytokines induced autophagy.

A tumor cannot develop without the parallel expansion of tumor stroma. Although we still don’t know the exact role of tumor stroma in tumorigenesis and cancer progression, the available evidences suggest that cancer associated fibroblasts have a crucial role. In this study we present that CAFs may have a role in promoting cancer through cytokine secretion and they can be defined as potential targets for anticancer therapy.

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