T.C.
REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES

THE ROLE OF REGNASE-1 PROTEIN IN SCULPTING OF TUMOR MICROENVIRONMENT

MSc. Göksu SARIOĞLU

TUMOR BIOLOGY AND IMMUNOLOGY PROGRAM

MASTER THESIS

ANKARA

2021

T.C.
REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
GRADUATE SCHOOL HEALTH SCIENCES

THE ROLE OF REGNASE-1 PROTEIN IN SCULPTING OF TUMOR MICROENVIRONMENT

Göksu SARIOĞLU (MSc.)

TUMOR BIOLOGY AND IMMUNOLOGY PROGRAM

MASTER THESIS

ADVISOR OF THE THESIS

ASSOCIATE PROFESSOR Gürcan GÜNAYDIN

ANKARA

2021

HACETTEPE UNIVERSITY

GRADUATE SCHOOL OF HEALTH SCIENCES

THE ROLE OF REGNASE-1 PROTEIN IN SCULPTING OF TUMOR MICROENVİRONMENT

Goksu SARIOGLU

Supervisor: Assoc. Prof. Gurcan GUNAYDIN

This thesis study has been approved and accepted as a Master dissertation in "Tumor Biology and Immunology Program" by the assesment committee, whose members are listed below, on 12.07.2021

Chairman of the Committee :	*Prof. Dr. A. Lale DOGAN* *Hacettepe University*
Advisor of the Dissertation :	*Assoc. Prof. Gurcan GUNAYDIN* *Hacettepe University*
Member :	*Prof. Dr. Aysegul ATAK YUCEL* *Gazi University*
Member :	*Assoc. Prof. Hande CANPINAR* *Hacettepe University*
Member :	*Assoc. Prof. Aysun Kilic SULOGLU* *Hacettepe University*

This dissertation has been approved by the above committee in conformity to the relatedissues of Hacettepe University Graduate Education and Examination Regulation.

28 Temmuz 2021

Prof. Diclehan ORHAN, MD, PhD

Director

YAYIMLAMA VE FİKRİ MÜLKİYET HAKLARI BEYANI

Enstitü tarafından onaylanan lisansüstü tezimin/raporumun tamamını veya herhangi bir kısmını, basılı (kağıt) ve elektronik formatta arşivleme ve aşağıda verilen koşullarla kullanıma açma iznini Hacettepe Üniversitesine verdiğimi bildiririm. Bu izinle Üniversiteye verilen kullanım hakları dışındaki tüm fikri mülkiyet haklarım bende kalacak, tezimin tamamının ya da bir bölümünün gelecekteki çalışmalarda (makale, kitap, lisans ve patent vb.) kullanım hakları bana ait olacaktır.

Tezin kendi orijinal çalışmam olduğunu, başkalarının haklarını ihlal etmediğimi ve tezimin tek yetkili sahibi olduğumu beyan ve taahhüt ederim. Tezimde yer alan telif hakkı bulunan ve sahiplerinden yazılı izin alınarak kullanılması zorunlu metinlerin yazılı izin alınarak kullandığımı ve istenildiğinde suretlerini Üniversiteye teslim etmeyi taahhüt ederim.

Yükseköğretim Kurulu tarafından yayınlanan ***"Lisansüstü Tezlerin Elektronik Ortamda Toplanması, Düzenlenmesi ve Erişime Açılmasına İlişkin Yönerge"*** kapsamında tezim aşağıda belirtilen koşullar haricince YÖK Ulusal Tez Merkezi / H.Ü. Kütüphaneleri Açık Erişim Sisteminde erişime açılır.

- o Enstitü / Fakülte yönetim kurulu kararı ile tezimin erişime açılması mezuniyet tarihimden itibaren 2 yıl ertelenmiştir. (1)

X Enstitü / Fakülte yönetim kurulunun gerekçeli kararı ile tezimin erişime açılması mezuniyet tarihimden itibaren 6 ay ertelenmiştir. (2)

- o Tezimle ilgili gizlilik kararı verilmiştir. (3)

…. /…/……

Göksu SARIOĞLU

---

[1] *"Lisansüstü Tezlerin Elektronik Ortamda Toplanması, Düzenlenmesi ve Erişime Açılmasına İlişkin Yönerge"*

*(1) Madde 6. 1. Lisansüstü tezle ilgili patent başvurusu yapılması veya patent alma sürecinin devam etmesi durumunda, tez **danışmanının** önerisi ve **enstitü anabilim dalının** uygun görüşü üzerine **enstitü** veya **fakülte yönetim kurulu** iki yıl süre ile tezin erişime açılmasının ertelenmesine karar verebilir.*

*(2) Madde 6. 2. Yeni teknik, materyal ve metotların kullanıldığı, henüz makaleye dönüşmemiş veya patent gibi yöntemlerle korunmamış ve internetten paylaşılması durumunda 3. şahıslara veya kurumlara haksız kazanç imkanı oluşturabilecek bilgi ve bulguları içeren tezler hakkında tez **danışmanının** önerisi ve **enstitü anabilim dalının** uygun görüşü üzerine **enstitü** veya **fakülte yönetim kurulunun** gerekçeli kararı ile altı ayı aşmamak üzere tezin erişime açılması engellenebilir.*

*(3) Madde 7. 1. Ulusal çıkarları veya güvenliği ilgilendiren, emniyet, istihbarat, savunma ve güvenlik, sağlık vb. konulara ilişkin lisansüstü tezlerle ilgili gizlilik kararı, **tezin yapıldığı kurum** tarafından verilir *. Kurum ve kuruluşlarla yapılan işbirliği protokolü çerçevesinde hazırlanan lisansüstü tezlere ilişkin gizlilik kararı ise, **ilgili kurum ve kuruluşun önerisi** ile **enstitü** veya **fakültenin** uygun görüşü üzerine **üniversite yönetim kurulu** tarafından verilir. Gizlilik kararı verilen tezler Yükseköğretim Kuruluna bildirilir.*
Madde 7.2. Gizlilik kararı verilen tezler gizlilik süresince enstitü veya fakülte tarafından gizlilik kuralları çerçevesinde muhafaza edilir, gizlilik kararının kaldırılması halinde Tez Otomasyon Sistemine yüklenir

***** *Tez **danışmanının** önerisi ve **enstitü anabilim dalının** uygun görüşü üzerine **enstitü** veya **fakülte yönetim kurulu tarafından karar verilir.***

ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisor Assoc. Prof. Gurcan GUNAYDIN and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences.

Goksu SARIOGLU

ACKNOWLEDGEMENT

It has been a year with so many sufferings and loss… I would like to start by pledging my gratitude towards healthcare professionals and everybody who is fighting a battle against the global pandemic in the frontline.

It was difficult to gather my strength to do my experiments during these difficult times but my advisor whom I am deeply grateful, Assoc. Prof. Gurcan Gunaydin shed a light on me and encouraged me to keep going. I am deeply thankful for his guidance and support during my studies.

I cordially thank Prof. Dr. Lale Dogan and Assoc. Prof. Hande Canpinar for their teachings and warm support for the past two years and all the of the faculty members and staff members of the Department of Basic Oncology.

I am completely indebted to Dr. Mustafa Emre Gedik and Elif Cakan for their advice and help in my experiments and I would like to thank every MSc and PhD student in the Basic Oncology Department for their helps and friendship.

I am deeply grateful to Assoc. Prof. Kemal Kosemehmetoglu and all the doctors in Hacettepe University Department of Medical Pathology for their helps in immunocytochemistry experiments and obtaining breast cancer tissue from patients. I would like to thank Prof. Dr. Ali Konan and Assoc. Prof. Ahmet Bulent Dogrul for all the help in surgical steps. In addition, I am very grateful for Dr. Sinan Efe Satici for his patience and helping me with information about the patients and surgeries.

Special thanks to my friends Meltem Celik, Eda Kusan, Basak Dalkiran and Mert Cagil Ceyhan for supporting me mentally and emotionally no matter how I decided to pursue my life and how badly I have become, they always hold my hand so that I can survive.

Lastly, I am beyond grateful and thankful to my parents and my brother for supporting me no matter where I am or what I am doing; I always know they will be beside me. I dedicate this thesis to them.

ABSTRACT

Sarioglu G., The Role of Regnase-1 Protein in Sculpting Tumor Microenvironment, Hacettepe University Graduate School Health Sciences Department of Basic Oncology Tumor Biology and Immunology Program Master's Thesis, Ankara, 2021, The tumor microenvironment (TME) contains cancer-associated fibroblasts (CAFs) and their tumor-supportive role helps to build an environment in favor of cancer cell progression. Recent researches have revealed that targeting the tumor microenvironment, as well as the cancer cells, inhibits cancer progression far more effectively, while also overcoming the development of drug resistance. Macrophage polarization also helps cancer cells transform the microenvironment into a pro-tumoral environment. In previous studies, Gunaydin et al. have shown that CAFs play a major role in shaping the TME, thereby inducing the accumulation of monocytes in the TME and their differentiation into M2-like macrophages, enabling them to transform into a subtype that can perform immunosuppressive functions. However, the mechanisms underlying monocyte accumulation in the TME of CAFs are not yet known. The fact that Regnase-1 (MCP-1 Induced Protein) is induced by MCP-1, the major regulator of monocyte recruitment, and control in inflammatory processes prompted us to investigate the effects of this protein on monocyte recruitment by CAFs to the TME. In this study, α-SMA (α-smooth muscle actin) positive CAFs were obtained from patients with breast tumors and Regnase-1 protein expression in these cells was investigated. According to our study results, it was observed that CAFs obtained from breast tumors expressed Regnase-1 and MCP-1 protein. Our study results showed that there is a statistically significant correlation between MCP-1 and Regnase-1 protein expressions in CAFs, as well as between these protein expressions and CAF grade.

Key words: Tumor microenvironment, breast cancer, macrophage polarization, cancer-associated fibroblast, Regnase-1.

ÖZET

Sarıoğlu G., Tümör Mikroçevresinin Şekillendirilmesinde Regnase-1 Proteininin Rolü, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Tümör Biyolojisi ve İmmünolojisi Programı Yüksek Lisans Tezi, Ankara, 2021, Kanserle-ilişkili fibroblastlar (KİF'ler), tümör mikroçevresinin bileşenlerinden biridir ve pro-tümöral etkileri, kanser hücresi gelişimi lehine bir ortam oluşturmaya yardımcı olur. Yakın zamanda yapılan araştırmalar, kanser hücrelerinin yanı sıra tümör mikroçevresi hedeflemenin, kanserin ilerlemesini çok daha etkili bir şekilde baskıladığını ve aynı zamanda ilaç direnci gelişimini engellediğini göstermiştir. Ayrıca makrofaj polarizasyonu da kanser hücrelerinin ortamı pro-tümöral bir mikroçevreye dönüştürmesine yardımcı olur. Günaydın ve arkadaşlarının daha önceki çalışmalarında, KİF'lerin tümör mikroçevresinin şekillendirmesinde büyük bir rol oynadığı, böylece monositlerin tümör mikroçevresinde birikmesi ile M2 benzeri makrofajlara farklılaşmalarını indükleyerek monositlerin immünosupresif işlevlerini yerine getirebilen bir alt tipe dönüşmelerini sağladıklarını göstermişlerdir. Ancak tümör mikroçevresindeki monosit birikimine neden olan KİF aracılı mekanizmalar henüz bilinmemektedir. Regnase-1'in (MCP-1 ile İndüklenmiş Protein), monosit çağrımının ana düzenleyicisi olan MCP-1 tarafından indüklenmesi gerçeği ve inflamatuar süreçlerdeki rolü, bu proteinin KİF'ler tarafından TME'ye monosit çağrımı üzerindeki etkilerini araştırmamıza neden oldu. Bu çalışmada, meme kanseri hastalarından α-SMA (smooth muscle actin) pozitif kanserle ilişkili fibroblastlar elde edilmiş ve bu hücrelerde Regnase-1 protein ekspresyonu araştırılmıştır. Çalışma sonuçlarımıza göre, meme kanseri hastalarından elde edilen KİF'lerin Regnase-1 proteinini eksprese ettiği gözlenmiştir. Çalışma sonuçlarımız, KİF'lerde MCP-1 ve Regnase-1 protein ifadeleri arasında ve aynı zamanda bu proteinler ile KİF grade arasında istatistiksel olarak anlamlı olan korelasyon gösterdiğini açıklamaktadır.

Anahtar Kelimeler: Tümör mikroçevresi, meme kanseri, makrofaj polarizasyonu, kanserle-ilişkili fibroblast, Regnase-1.

TABLE OF CONTENT

LIST OF ABBREVATIONS

BCA	Bicinchoninic Acid
BM-HSC	Bone Marrow-derived Hematopoietic Stem Cells
BM-MSC	Bone Marrow-derived Mesenchymal Stem Cells
CAF	Cancer-associated Fibroblasts
CCCH	Cys-Cys-Cys-His
CTD	C-terminal Domain
CTL	Cytotoxic T lymphocyte
CXCL	C X C Motif Chemokine Ligand
DC	Dentritic Cell
DCIS	Ductal Carcinoma In Situ
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
EMT	Epithelial-mesenchymal Transition
EndMT	Endothelial-mesenchymal Transition
ER	Estrogen Receptor
FAP	Fibroblast Activation Protein
FEC	Cyclophosphomide
FGF	Fibroblast Growth Factor
FSP	Fibroblast Specific Protein
GPER	G protein Coupled Estrogen Receptor
HER	Human Epidermal Growth Factor Receptor
HGF	Hepatocyte Growth Factor
Hh	Hedgehog

HIF-1	Hypoxia-inducible Factor
HRP	Horseradish Peroxidase
HSC	Hematopoietic Stem Cells
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Adhesion Molecule
IDC	Invasive Ductal Carcinoma
IDO	Indoleamine-2,3-dioxygenase
IGF	Insulin-like Growth Factor
IL	Interleukin
ILC	Invasive Lobular Carcinoma
iNOS	Inducible Nitric Oxide Synthase
kDA	Kilodaltons
LPS	Lipopolysaccharides
MCP-1	Monocyte Chemotactic Protein 1
MCPIP-1	Monocyte Chemotactic Protein Induced Protein 1
MDSC	Myeloid-derived Suppressor Cell
MET	Mesenchymal-to-epithelial Transition
MMP	Matrix Metalloproteins
MRC-5	Medical Research Council Cell Strain 5
MSC	Mesenchymal Stem Cells
NF	Normal Fibroblasts
NFκB	Nuclear Factor-κB
NG2	Neuron Glial Antigen
NTD	N-terminal Domain
OD	Optical Density
OPN	Osteopontin
OPN-MZF	Osteopontin-myeloid Zinc Finger
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor Receptor

PI3K	Phosphatidylinositol 3-kinase
PIN	Pilt-N-terminus
PVDF	Polyvinylidene Difluoride
REGNASE-1	Regulatory RNase-1
ROS	Reactive Oxygen Species
SDF-1	Stromal Cell-derived Factor
SDS	Sodium Dodecyl Sulfate
SG	Stress Granules
SMA	Smooth Muscle Actin
SMOi	Smoothed-inhibitor
SR-A	Class A Scavenger Receptors
SV40	Simian Virus 40
TAA	Tumor-associated Antigen
TAM	Tumor-associated Macrophages
TBS	Tris-buffered Saline
TGF	Transforming Growth Factor
TGS	Tris/Glycine/SDS
TGX	Tris-Glycine Extended
Th	T helper
TME	Tumor Microenvironment
TNBC	Triple-negative Breast Cancer
TNF	Tumor Necrosis Factor
TRAF	TNF Receptor-associated Factor
UTR	Untranslated Region
UV	Ultra-violet
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
ZF	Zinc Finger

LIST OF FIGURES

LIST OF TABLES

1.INTRODUCTION

In 2012, breast cancer was one of the major reasons for cancer losses worldwide with 324,000 casualties. Moreover, with 197,000 deaths, it was the second leading reason for the losses behind advanced lung cancer (1). A leading concern in this disease is recurrence and metastasis to other organs.

According to the literature about breast cancer, satisfactory results could not be obtained in situations where only the tumor cells were targeted (2). Considering the pro-tumoral consequences of the tumor milieu on carcinogenesis, it is predicted that targeting the microenvironment with cancer cells synergistically will give favorable responses in terms of therapy and prevent metastasis (3).

Therefore, experiments in this study will be conducted to decipher the role of CAFs in the tumor microenvironment.

Fibroblasts are the cells that form collagen, a type of protein that is the main ingredient in connective tissue and is involved in wound healing (4). These cells are activated and transform into CAFs in the TME (4). Factors derived/secreted from CAFs can contribute to forming a microenvironment that allows tumor formation and the metastatic properties of cancer cells. It has been observed that CAFs produce α-SMA unlike normal fibroblasts and these cause rearrangements in the tumor milieu (5).

It has been shown that CAFs have altered the TME by some pro-tumoral cytokines to escape from immune responses (6). This may be one of the main reasons for the recurrence of cancer and resistance to therapeutic agents.

Gok Yavuz et al. demonstrated that CAFs are influential in the transformation of monocyte/macrophage cells from M1 phenotype to M2 phenotype and in the monocyte/macrophage cells gaining an immunosuppressive character (7). CAFs' recruitment of monocytes is controlled by MCP-1 and stromal cell-derived factor-1 (SDF-1) (7). CAFs can differentiate monocytes, which are recruited in the tumor site, into M2-like macrophages and these cells are able to perform immunosuppressive roles (7).

However, determining the mechanisms underlying this effect is of great importance in order to develop therapeutic approaches to target this mechanism.

Regnase-1, which called Zc3h12a has been a well-studied inflammation response controller and is the founding member of ribonucleases that has proteins linked to the involvement of the NYN domain (8).

Additionally, Zc3h12a was reported to be the protein that was primarily induced by MCP-1. Hence, it was called MCP-1 induced protein 1 (MCPIP1); and was later called Regnase-1. Regnase-1 (Regulatory RNase-1 = MCPIP1) is a ribonuclease and its mRNA decay activity would affect inflammation (9).

In our study, we hypothesized that the Regnase-1 protein may be involved in the polarization of macrophages in cancer. The results were gathered by Western Blot analyses in protein lysates of CAFs that were obtained from breast cancer patients' cancer tissues. Protein levels of Regnase-1 in CAFs were documented.

2.GENERAL INFORMATION

2.1.Breast Cancer

Cancer is a disease that may result in death worldwide and is predicted to cause 13 million cancer-related deaths in 2030 (10). Among women, breast cancer mainly forms a risk of 10% mortality (11). In the early 1900's Halsted indicated that breast cancer is an illness that generates locally and lymphatics can spread it to other tissues (12). Breast cancer might be classified into several types based on pathology and invasiveness (13). Based on the origin of the cell involved, it might be divided into carcinomas and sarcomas (13). Carcinomas derive from epithelial cells of the breast which are a part of lobules and terminal ducts. Sarcomas derive from myofibroblasts and blood vessels, which is an uncommon form of breast cancer (13). Breast cancers are usually carcinomas and they have several subtypes based on the status of invasiveness regarding the tumor's initiation points (14). Distinguishing the tumor types by their pathological features and invasiveness is crucial for treatment because each one has its own specific response (14). We can divide breast cancer into three groups by this mean; non-invasive (in situ), invasive and metastatic breast cancer (Figure 2.1.) (14).

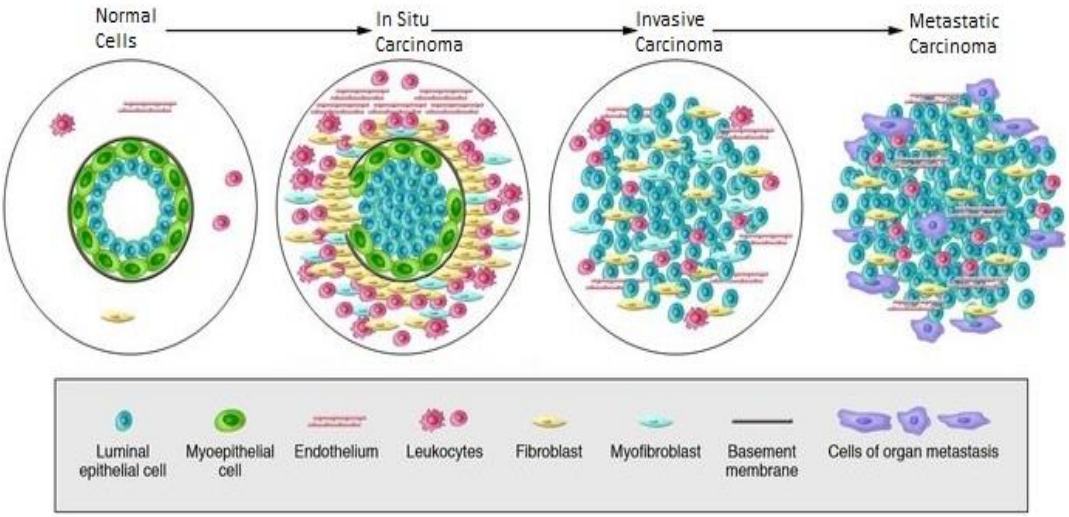

Figure 2.1. Subtypes of breast cancer by pathological features and invasiveness adapted by Polyak K. *et al.* (15).

- Ductal carcinoma in situ (DCIS) is one of the typical types of breast tumor with the potential to develop inside existing ducts. DCIS is not invasive but has the potential to develop invasive features (15).
- Invasive or infiltrative breast cancers spread to the other parts of the breast and surrounding stromal tissue. When a woman is diagnosed with an aggressive form of breast cancer, she is usually 50 or older (15).
- Metastatic breast cancers are the ones with the potential to spread to other organs like lymph nodes and uterus etc. (15).

Invasive carcinoma is divided into two groups as invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) according to the sites of the breast that they derive from (16). Several histological subtypes of invasive ductal carcinoma can be distinguished based on a variety of factors such as cell type (apocrine), quantity, architectural features (papillary, tubular), type and location of secretion (mucinous), and immunohistochemical profile (17). The invasive lobular carcinoma occurs predominantly later in life than invasive ductal carcinomas (after 50-60 years of age) (16). These classifications are very critical because of the different prognoses and treatments of each one of them (16).

The molecular subtypes of breast cancer are being used for determing the prognosis and treatment modality. Human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), progesterone receptor (PR) are usually investigated. (17). Breast cancer could be classified into four subtypes according to differential gene expression. HER2-enriched (HER2+, ER/PR-), luminal A, luminal B, and TNBC are the mentioned subtypes (18). Triple-negative breast cancer (TNBC) means the patient tumor tissue doesn't express HER2, ER, PR. Such expressions may be related with poor or good prognosis of the disease as well as the aggressiveness of the tumor (19). Luminal subtypes are more likely to have a better prognosis than the HER2-enriched and TNBC types (20).

2.1.1. Breast Cancer Epidemiology

Every year, the American Cancer Society (ACS) publishes a statistical paper of new cases and deaths estimated to occur in the United States the following year. ACS estimated that there would be approximately 2,650 new cases of males and 281,550 new cases of females in 2021, and a total of 44,130 deaths of both sexes (530 males and 43,600 females) (21).

According to statistical analyses, nearly one out of eight women (13%) will be diagnosed with invasive breast cancer in their lifetime. In the 2012-2016 period, the incidence rate of breast cancer expanded by 0.3% per year (22). This is thought to be due to an increase in hormone receptor-positive illness rates. Unlike this, death rates of breast cancer are declining by the year, from 1989 to 2017 approximately 375,900 deaths have been prevented (23).

2.2. Breast Cancer Microenvironment

Tumor and metastasis initiation is a complicated pathological process (24). Progression of cancer starts with increased motility and migration of cells, accompanied by stromal invasion. Tumor cells then infiltrate arteries or lymph vessels, adapting their functions to the circulatory system. They then extravasate, invade distant organs, and eventually develop into extreme metastases, usually after several years of dormancy (25).

Most studies focus on investigating cancer cell driven changes in tumor milieu. (26). Recently, TME has also gained attention for cancer research and treatment. (26). Several non-cancer cells, cancer-associated specific cell forms, and matrix molecules have an interaction with the tumors at both the primary and metastatic sites and affect their biological properties (27).

One of the crucial features of cancer is the contribution of the TME (24). Cancer cells are encircled by various cell types which help to alter the microenvironment in favor of tumor progression (Figure 2.2.) (24).

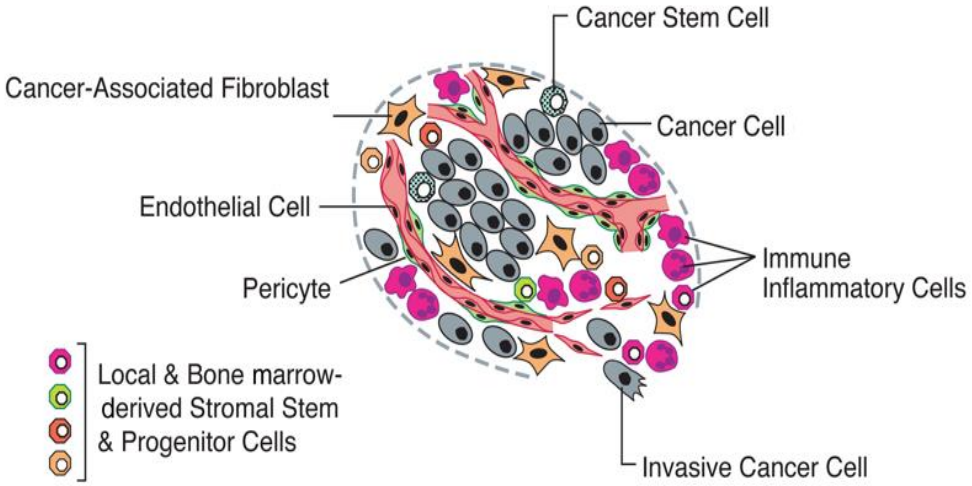

Figure 2.2. Tumor microenvironment components. Adapted from Hanahan D. and Weinberg R. (24).

Tumor initiation, development and distant metastasis are now known to be affected by exosomes, cytokines, and other soluble factors provided by the tumor as well as stromal cells (28). Tumor cells depend on epithelial-mesenchymal transition (EMT) during the multistep metastatic process (29). Such a transition is prominent in epithelial cells that are dedifferentiating and losing cell adhesions; resulting in higher mobility that may display more stem cell traits. This transition program is hijacked by cancer cells to detach from the epithelial tissues, enter vessels, and acquire the properties of self-renewal and cellular plasticity essential in the metastatic formation phase (30).

Cancer-associated stromal cells have a reaction to molecular and morphological changes in cancerous cells by transforming their secretomes and undergoing epigenetic modifications (31). By recruiting other cells, which are non-indigenous to breast tissue, the released factors may directly and/or indirectly influence tumor progression. Bone marrow-derived mesenchymal stem cells, and innate and adaptive immune cells are among the examples, which then become involved in stimulating a vicious cell-to-cell and factor-to-cell interaction loop (31).

In breast cancer, adipocytes, vasculature, fibroblasts, mesenchymal stem cells, and numerous immune cells, as well as their secretome, contribute to the formation of a tumor permissive microenvironment (32).

2.3. Normal Fibroblasts

Initially, Virchow (33) and then Duvall (34) used classical anatomy and microscopy techniques to describe connective tissue cells, with fibroblasts being the first to be classified as cells that produced collagen in the connective tissue (27).

Fibroblasts are single cells that are found in the extracellular space or near arteries in tissues, with no connection to the basement membrane but lodged in the interstitial fibrillar extracellular matrix (ECM) (35). Fibroblasts are non-epithelial and non-immune cells, potentially derived from mesenchymal lineage (35). Fibroblasts in normal tissue are normally regarded as inactive since they have low metabolic and transcriptomic activity. These cells are considered dormant, serene, or resting in this state. Mesenchymal stem cell (MSC) precursors and resting fibroblasts can share several characteristics (36).

The capability to activate and generate fibroblasts was initially found in wound healing environments, and then in circumstances including acute / chronic inflammation, and fibrosis. Fibroblasts display an elongated morphology with projecting features that have a fusiform or spindle-like outline (37).

An obstacle for studying fibroblasts *in vivo* is the absence of a sensitive and specific marker (2). There are a number of well-known fibroblast phenotypic indicators, although none of them is fibroblast-specific or present in all fibroblasts (Table 2.1.) (2). There is clearly a need to define better cellular markers with absolute precision for fibroblasts (2).

Table-2.1. Fibroblast Markers. Adapted from Kalluri R. (3).

Marker	Fibroblast types marker is found
Vimentin	Miscellaneous
α-Smooth-Muscle Actin	Myofibroblasts
Desmin	Skin fibroblasts
FSP1	Activated fibroblasts, Miscellaneous
FAP	Activated fibroblasts

Fibroblasts may be regarded as dormant mesenchymal cells that can be triggered into MSCs with adequate stimuli (35). These resting mesenchymal cells are uncommon interstitial cells that can replicate when stimulated by various growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF) as well as interleukin-6 (IL6) (38). Most of the characteristics attributed to fibroblasts are thought to be those of 'active' fibroblasts, myofibroblasts, and MSCs. Fibroblasts derived from the site of a healed wound or fibrotic tissue produce more ECM ingredients and proliferate more quickly than those derived from healthy organs. This form of enhanced activity is referred to as activation (35).

Their functions, once activated, include generating ECM, producing cytokines and chemokines, recruiting immune cells, as well as using physical force to alter the architecture of the tissue. Their morphology starts to change as well when they are activated (Figure 2.3.)

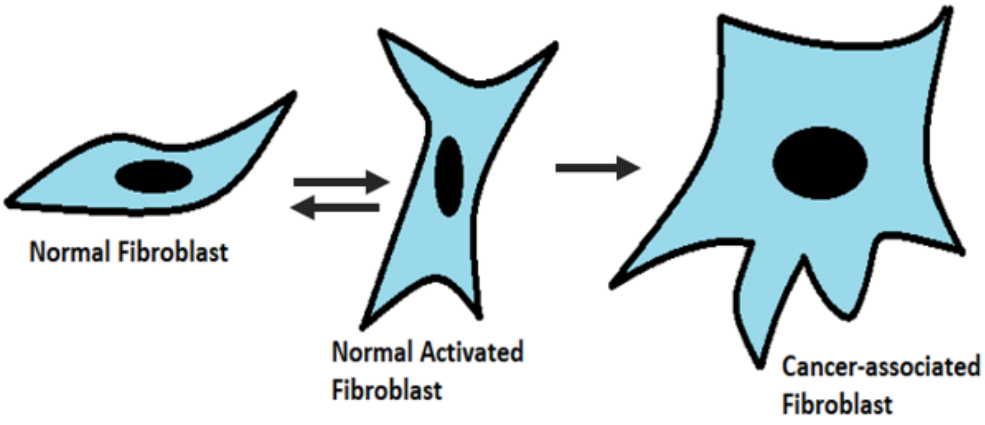

Figure 2.3. Fibroblast activation phases.

Quiescent fibroblasts are rarely found in embryonic tissue and are only found in mature tissues and organs (39). It is still not clear in adults whether MSCs or mesenchymal cells derived from monocyte precursors (fibrocytes) contribute to the activated fibroblast population (39). It was reported that the metastatic capabilities of breast cancer cells may be enhanced by bone marrow-derived MSCs (BM-MSCs) (39). For instance, in pancreatic cancer, BM-MSCs constitute a significant proportion of CAFs with pro-tumoral functions (40). One possibility stands that via secreted TGF-β, BM-MSCs can transform normal fibroblasts to CAF-like fibroblasts (40).

2.3.1. Cancer-Associated Fibroblasts

CAFs, which constitute the majority of the tumor stroma, are frequently faced with a variety of inflammatory cells and TME stimuli, allowing them to develop novel functions (41, 42) that are not observed in normal fibroblasts. These specific actions can play a part in sculpting the tumor's microenvironment and eventually exert an effect on the progression of the cancer cells. They may also demonstrate immunosuppressive properties (43).

Tumors have been known as “wounds that do not heal” (44). Fibroblasts are "educated" by the tumor during this phase to obtain tumor-supportive features.

Shred of evidence has shown that the growth of cancer cells is affected by CAFs (45). CAFs demonstrated an ability to suppress apoptosis of cancer cells, promote cancer cell proliferation, and stimulate angiogenesis in tumors (41). Cancer cells metastasize to other organs and tend to recruit normal fibroblasts (NF) to tumor sites. The active phenotype of fibroblasts in the tumor is caused by many genetic and epigenetic alterations that are driven by cancer cells (3).

TGFβ, epidermal growth factor (EGF), PDGF, fibroblast growth factor-2 (FGF2), and C X C motif chemokine ligand-12 (CXCL-12), all of which are secreted by cancer cells and other stromal cells, activate fibroblasts (46). Cell-cell contact via adhesion molecules may activate fibroblasts (e.g. intercellular adhesion molecule [ICAM1], vascular cell adhesion molecule-1 [VCAM1]) (47).

Myofibroblasts expressing α-SMA are observed in both tumors and tissue injury sites as activated fibroblasts. Myofibroblasts demonstrate fundamental effects in tissue repair via producing various cytokines, growth factors, and ECM proteins. Myofibroblasts also aid tissue regeneration by increasing angiogenesis and supporting the growth of neighboring epithelial cells. These myofibroblasts release SDF-1, an angiogenic chemokine, to assist tumor development and induce neoangiogenesis (41).

A particular marker is needed to detect CAFs in the tumor and the most frequently used one is α-smooth muscle actin. It has been identified as relevant for myofibroblasts (4). The tumor stroma includes both myofibroblasts (activated fibroblasts) as well as non-activated fibroblasts. A critical marker for determining myofibroblasts is fibroblast activation protein (FAP) (48). Orimo et al. reported that CAF populations can contain both myofibroblasts and fibroblasts. Moreover, unique markers for myofibroblasts could be α-SMA and FAP (41).

In previous studies, several other markers have also been identified (Table 2.2.), such as periostin (49), tenascin-C (50), neuron glial antigen-2 (NG2) (51), desmin, vimentin, fibroblast specific protein-1 (FSP-1) and PDGFR α and β (52).

Table 2.2. Markers of CAF adapted from An. Y *et al. (46).*

CAF MARKERS		NF MARKERS
Markers used commonly	**α-SMA** **FSP-1** **FAP**	**Vimentin**
Markers used rarely	**Tenascin-C** **Periostin** **NG-2** **Desmin** **PDGFR- α** **PDGFR- β**	
Negative markers	**Cytokeratin** **CD31**	

Myofibroblasts do not have their very own set of markers. However, because CAFs lack epithelial and endothelial characteristics, cytokeratin and CD31 are considered negative indicators. (53). No specific marker of CAFs has been identified, yet; however, several markers may help in identifying CAFs via combined utilization of such markers.

2.3.1.a Origin of Cancer-associated Fibroblasts

The subject of CAFs origin is still being debated and there are conflicting explanations about it. CAF has been linked to a variety of cells in a growing number of studies.

For instance, bone marrow-derived hematopoietic stem cells (HSC) and mesenchymal stem cells, resident tissue fibroblasts, epithelial-mesenchymal transition, and endothelial (endothelial-mesenchymal transition; EndMT) cells are also taken into consideration as the potential progenitors of CAFs (Figure 2.4.) (42). CAFs can stem from many types of cells, and thus, constitute a heterogeneous population (51).

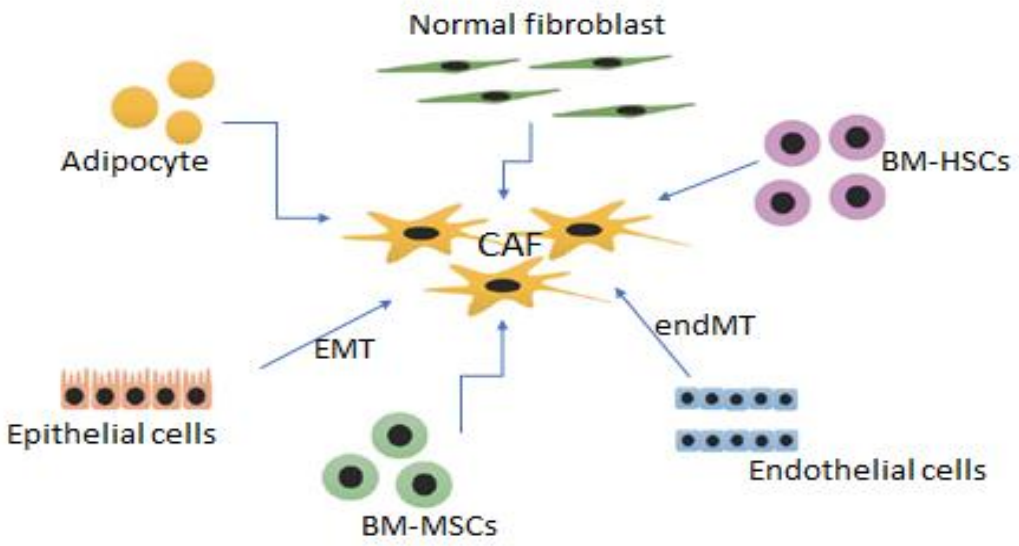

Figure 2.4. Potential origins of CAF. Adapted from Shiga K. *et al.* (4).

Resident fibroblasts of the tissue are thought to be the source of CAFs, according to one widespread explanation. A recent study has discovered that cancer cells change fibroblasts to become CAFs via activating miRNAs (miR-31, miR-214, and miR-155) (54).

It was reported that during tumor progression, resident human mammary fibroblasts can transform to CAFs. Kojima et al. showed this transformation by using a tumor xenograft model (55). The increases of hypoxia-inducible factor (HIF)-1 and CXCL12 by reactive oxygen species (ROS) also aid the transformation of fibroblasts into myofibroblasts with high migration ability (56). As a result, it is probable that

some cytokines generated by cancer cells aid the conversion of local fibroblasts into CAFs. However, some differences exist between CAFs and resident fibroblasts.

When the tumor stroma was studied in mice who had a bone marrow transplant and were xenotransplanted with pancreatic cell line, bone marrow-derived endothelial cells and α-SMA+ myofibroblasts were present in cancer sites (57). It has also been shown that at least 20% of CAFs are possibly originated from mesenchymal stem cells (58). Tumor-derived osteopontin (OPN) induces MSC-to-CAF transformation in the milieu to enhance tumor development and metastasis by the OPN-myeloid zinc finger 1 (MZF1)-TGF-1 axis (59). Hematopoietic stem cells originating from bone marrow, on the other hand, differentiate into hematopoietic cells such as leukocytes, erythrocytes, and thrombocytes. Some studies illustrated that not only can HSC distinguish between these hematopoietic cells, but also differentiate into CAFs (60).

In the early 1980s, the term epithelial-mesenchymal transition was introduced by Greenburg et al. (61). EMT is the mechanism of transferring to mesenchymal cells with loose cell-cell contacts from epithelial cells with close junctions and obtaining mesenchymal properties. Breast cancer cells might transdifferentiate into myoepithelial cells under suitable conditions and ultimately become myofibroblasts (62).

The endothelial-mesenchymal transition was first detected in the embryonic phase during heart development (63). In the heart tissues, TGF signaling is implicated in the shift from endothelial cells to fibroblast-like cells (64). Several studies have shown that cancer stroma has a similar phenomenon. Once lung endothelial cells of mice were treated with TGF-ß1, the cells established a spindle-shaped fibroblast-like appearance. According to Zeisberg et al., CAFs derive from endothelial vascular cells (65). Expression of an endothelial marker, CD31, is down-regulated; whereas, mesenchymal markers are induced (e.g. FSP-1, α-SMA, and fibronectin) (65, 66).

These results indicated that resident fibroblasts, MSCs, and HSCs derived from the bone marrow could develop into CAFs via distinct mechanisms such as EMT and EndMT .

2.3.1.b. The Role of CAFs in Tumor Microenvironment

Tumor initiation; The tumor's growth is dependent on the dysregulated proliferation of cancer cells, as well as alterations in the microenvironment. There is mounting evidence that CAFs are targets and inducers of tumor activation signals (67). CAFs release cytokines (autocrine/paracrine) that help malignancies maintain their biological characteristics (Figure 2.5.)

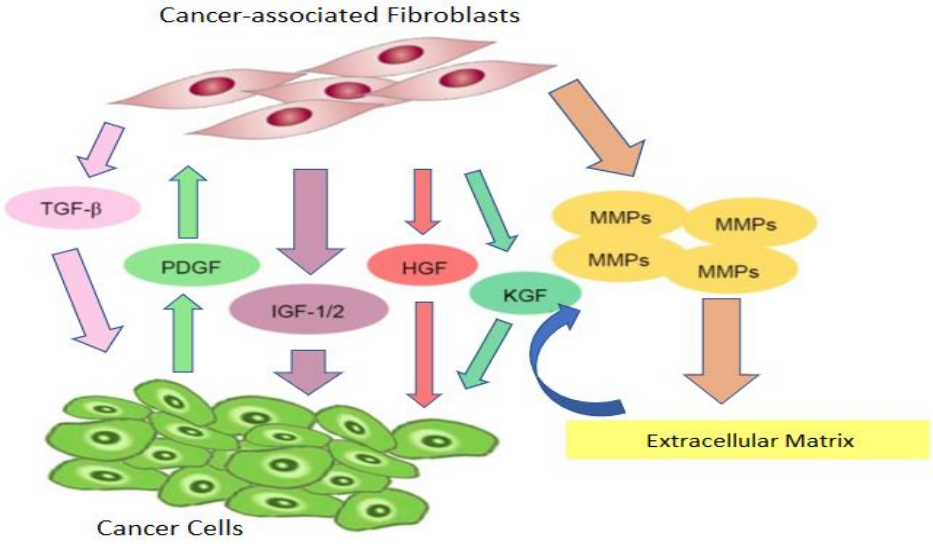

Figure 2.5. Factors secreted by CAF to promote tumor progression. Adapted from Tao L. *et al.* (47).

Novel proteins produced by CAF as membrane molecules (integrin α-11 and syndecan 1) and insulin-like growth factor (IGF) like family members 1 and 2 have been classified as having supporting roles for cancer cells, in addition to traditional growth factors like EGF and hepatocyte growth factor (HGF). These factors promote tumor growth and survival directly or indirectly or improve their migratory and invasive properties (68). Several experiments with genetically altered fibroblasts in

a mouse model have illustrated that resident fibroblasts are specifically related to cancer initiation.

Recently, a study employed human breast epithelial cells' capacity to establish ducts in the stroma of a mouse breast. It demonstrates that in mouse fibroblasts, overexpression of HGF and/or TGF promotes breast cancer in the human epithelium (69). Between fibroblast-derived TGFβ and HGF expression and tumor, initiation connection is shown in FSP1-Cre TGFβRIIRflox/flox mice, which do not have a TGFβ type II receptor on FSP1+ fibroblasts (these fibroblasts do not respond to TGFβ signaling) (70). These mice have increased prostate intraepithelial neoplasia and invasive forest carcinoma of squamous cells, combined with increased expression of HGF in the fibroblasts around them (70).

A series of investigations comparing the effects of normal fibroblasts and CAFs obtained from the primary tumor site revealed that there was a further link between growth factors and CAFs at tumor initiation (71, 72). In combination with either natural fibroblasts or CAFs, simian virus 40 (SV40)-transformed normal prostate epithelial cells were grafted onto mice, only the CAFs resulted in lesions resembling prostatic intraepithelial neoplasia (72). When immortalized prostate epithelial cells were utilized, the presence of CAFs caused enormous tumors to form in these mice, whereas tumors did not develop in the presence of normal fibroblasts. These findings suggest fibroblasts are needed to upkeep the homeostasis of epithelial cells, whereas CAFs are likely to originate and induce tumorigenic changes in epithelial cells (72).

Tumor progression; CAFs are also likely to facilitate tumor growth by specific contact with cancer cells. For instance, if co-injected into mice, CAFs help non-invasive tumor cells become more metastatic (73). In suspension with CAFs or regular fibroblasts, human MCF-7 breast cancer cells (which estrogen independently generates tumors and metastasizes) were injected together into nude mice (71). Xenografts containing CAFs grew more extensive than normal fibroblast-infused xenografts (71). Such increased growth was linked with increased proliferation of cancer cells (but not enhanced fibroblasts proliferation) and further angiogenesis,

suggesting that during tumorigenesis, according to the study, CAFs could have a bi-modal influence (71).

In addition to secreting growth hormones that directly regulate cell motion, myofibroblasts are a reservoir of ECM-degrading proteases such as matrix metalloproteins (MMPs) (74-76). MMPs are thought to allow cancer cells to breach tissue boundaries and escape from the tumor's initial site. The motility and invasiveness of cancer cells are also influenced directly by MMPs and other proteases. For MMP3, which is overexpressed in fibroblasts, such a direct effect has been explained. MMP3 specifically cleaves E-extracellular cadherin's domain, causing normal breast epithelia to dissect and initiate EMT, encouraging the invasiveness of cancer cells (77). MMP1 also demonstrates such a tumor-promoting effect.

Tumor metastasis; CAFs mediate the metastasis of tumors. Increasing evidence indicates that CAFs have a metastatic support function in tumors (78), although there is a data shortage on the involvement and role of CAFs in lymph nodes and distant metastases (79). In metastatic lymph nodes, stromal reactions include reactive and fibrotic tissue with improved vitronectin and fibronectin deposition, nodal fibrosis, desmoplasia, and hyaline stroma (80). One of these studies revealed immunohistochemical characterization of CAFs in metastatic lymph nodes from a female patient with uterine cervix cancer who had preoperative treatment (80). It has been indicated that the mesenchymal-like CAF phenotype is involved in improving cancer cell metastasis, whereas NFs with the epithelial-like phenotype inhibits breast cancer cell migration (81). Similarly, when co-injected with CAFs, normal prostate epithelial cells cause intraepithelial neoplasia in vivo, but not when injected alongside with NFs (72).

CAFs release pro-inflammatory cytokines which activate the signaling pathway of nuclear factor-κB (NF-κB) and promote cancer progression. CAFs have been shown to select for bone-metastatic cells in the stroma of TNBC samples (82). CAFs establish CXCL12 and IGF1, which are bone marrow relapse prognostic markers and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway activators

(74). In the CXCL12-rich microenvironment of the bone marrow, cancer cells are primed for metastasis, thus indicating CAFs' significant role in tumor metastasis.

Research recorded that the miR-26b downregulation in CAFs promotes the mobility of fibroblasts, which is a dominant trait of the CAFs. In addition, CAFs with decreased miR-26b expression facilitate the migration of human breast cancer cells and invasion (83). In addition, the PTEN/miR-320/ETS2 pathway releases proteins such as Emilin2 that distinguish between normal and cancerous stroma, and are linked to an increased risk of recurrence in breast cancer patients (84). This demonstrates that miR-320 regulates a key signaling pathway in fibroblasts associated with TME modulation.

2.3.2. CAF Targeted Therapy

In pre-clinical models, anti-CAF therapies, including several medications which target FAP, have been revealed to exert powerful anti-tumoral properties (85). It has been shown that high curcumin concentrations have cytotoxic effects on CAFs (86). Curcumin at low concentrations, on the other hand, had little influence on CAF production but did reduce vimentin and α-SMA expression, indicating that curcumin at low quantities can undo fibroblast activation (86). CAFs conditioned media boosted pancreatic cancer cells' migration and invasion potential, while curcumin-treated CAFs conditioned medium showed a negligible effect. CAF-conditioned media and flavonoid silibinin-conditioned medium were used to treat prostate tumor cells and results were gratifying (86).

A chemokine which plays a role in the promotion of cancer cell invasion is monocyte chemo-attractant protein-1 (87). CAFs stimulated with the flavonoid silibinin show lower expression of NF-κB, AP-1, and MCP-1 transcription modulators, thus reducing the invasiveness of cancer cells (87, 88) Studies have illustrated that strong expression of FAP-α stimulates tumor development and metastasis. Tumor development was blocked by treatment with anti-FAP-α antibodies or pulsed dendritic cells (DCs) (89). Xia et al. built a DNA

(Deoxyribonucleic Acid) vaccine expressing human FAP to kill unique CAFs (90). By developing a particular Cytotoxic T lymphocyte (CTL) response to FAP-α that destroyed CAFs. The vaccine effectively suppressed the progression of 4T1 tumors (90). In order to kill CAFs and destroy immunosuppressive elements in the TME, FAP- α -based vaccines can be used by inducing FAP-α specific CTLs (91).

The chance of immune escape can also be minimized by these vaccines, which is a benefit that tumor-associated antigen (TAA) does not have (92). Liao et al. demonstrated that in the fibroblasts in the tumor stroma, FAP was explicitly overexpressed (93). CD8+ T cells could affect cytotoxicity on the tumors by targeting the FAP antigen in CAFs (94). Thus, studies above have shown that targeting CAFs with the FAP antigen may inhibit tumor growth and progression, which may be mediated by T cell immunotherapy (95). An anti-tumor immune response was induced by a DNA vaccine that targeted the tumor antigen FAP-α, primarily mediated by CD8+ T cells (96).

Some clinical trials, in addition to basic researches, have targeted CAFs directly. Hedgehog (Hh) signaling activation is essential for the role of CAF. Aurélie et al. used smoothed-inhibitor (SMOi) docetaxel in phase I clinical trial (NCT02027376) for the treatment of TNBC patients that reduced hedgehog signaling in CAFs and led to substantial improvements (97). Some patients had a complete clinical response, even (i.e. disappearance of the tumor lesions without new lesions) (97). Targeting with FAP antibody in continuous sibrotuzumab infusions was found to be effective in the advanced FAP+ cancer patients' treatment in phase I clinical trial (98).

Drug resistance has emerged as a key barrier to the successful treatment of breast cancer patients, in spite of the development of novel anti-cancer treatments and the usage of existing medications in a more personalized manner (99). In addition to tumor intrinsic resistance mechanisms, it has become apparent that the microenvironment of the tumor plays a crucial role in response to therapy (99). Farmer et al. stated that resistance to 5-fluorouracil, epirubicin, and

cyclophosphamide (FEC) was predicted by a breast cancer patients' stromal gene expression pattern (100).

Nonetheless, separate studies indicate clear connections between CAF actions and treatment effectiveness against breast cancer cells. CAFs may facilitate therapy resistance by fostering tumor cell proliferation or providing supportive ECM that restricts drug penetration or causes adhesion-mediated drug resistance (101, 102). In addition, CAFs were reported to induce EMT in breast cancer cells, being the cause of the enhanced proliferation and invasion (103). G protein-coupled estrogen receptor (GPER)-integrin β1-dependently this phenotypic change might cause increased resistance to tamoxifen (104). It has been shown that conditioned medium derived from CAF improved resistance of doxorubicin to breast tumors by upregulation of HMGB1 (105) and the autophagy induction has consistently been identified to therapeutic resistance in osteosarcoma (106).

2.3.3. Immune Modulations by CAF

Due to their secretion of cytokines, CAFs may have a lot of effects on the immunity of tumors, thereby having a direct influence on immune cell functions and generating ECM molecules which may hinder the penetration of immune cells into tumors (107, 108). Tumor cells are able to prevent immune surveillance of anti-tumor T cells, macrophages, NK cells, and dendritic cells through direct immune-suppressing TGF-β1 functions. Via their development of (altered) ECM molecules, CAFs may also indirectly modulate immune cell activity. CAFs produce an immune-suppressive TME and this phenomenon also drives TGF-β1 (108). Multiple cytokines found in the CAF secretome alter the immune system to create an immune-suppressive TME to enable tumor development. IL-8, TGF-β1, IL-6, SDF-1, CCL2, CXCL1, VEGFs (Vascular endothelial growth factor), IL-10, PGE2, CSF-1, and indoleamine-2,3-dioxygenase (IDO) are among those mediators (109).

These mediators were shown to be the origin of the monocytes recruitment and M2 macrophage differentiation, change the Th1 (T-helper-1) to Th2 (T-helper-2)

balance, induce T-regulatory cell differentiation, decrease dendritic cell anti-tumor functions. And also, they increase myeloid-derived suppressor cell (MDSC) recruitment and differentiation, block NK cell activation, and decrease the function and viability of cytotoxic T cells (110, 111).

Numerous studies have shown in order to assist cancer progression, CAFs and macrophages interact (112). Some studies have documented that macrophage aggregation in TME is associated with poor patient prognosis (113). A study stated that post-prolyl peptidase, type I collagen cleaved from activated fibroblasts by FAP, might act as the macrophage substrate recognized by macrophage class A scavenger receptors (SR-A); hence it increased cancer adhesion of macrophages (114).

Two classes with distinct phenotypes consist of tumor-associated macrophages (TAMs). Via stimulating the immune system and releasing ROS, nitric oxide, and TNF (Tumor Necrosis Factor), M1 macrophages play an anti-tumor role. Immunosuppressive functions are carried out by M2 macrophages, fostering tumor growth, and angiogenesis, and ECM degradation (115). Accumulating evidence suggests that CAFs are the origin of EMT, interact with M2 macrophages to facilitate malignant tumor occurrence and development, and sustain cancer cell growth (Figure 2.6.) (116).

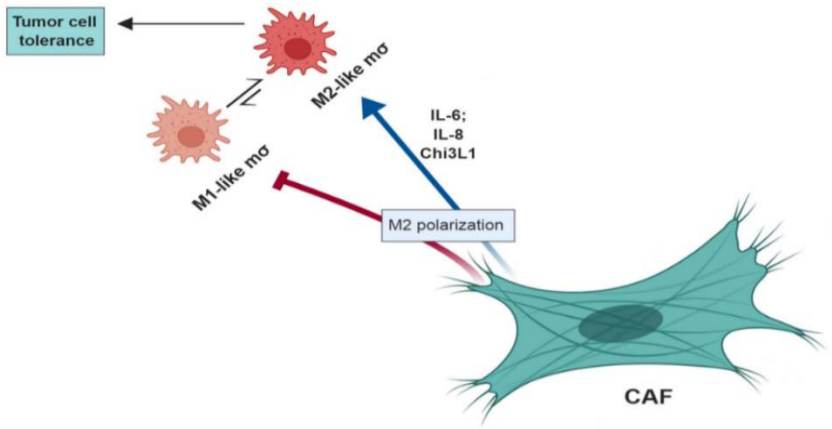

Figure 2.6. CAF affecting macrophage polarization and promoting tumor cell progression. Adapted from Monteran L. et al. (6).

Gok et al. showed that CAFs were not only capable of monocytes recruitment through SDF-1 and MCP-1 compared to NFs, but also polarized recruited monocytes into M2 macrophages with increased IL-10 expression, which had immunosuppressive effects in breast cancer (7).

2.4. Regnase-1

Regnase-1 has been described as a ribonuclease that through degrading transcripts, suppresses gene expression. By destabilizing cytokines related to inflammation, this protein serves as a negative regulator of inflammatory responses (8). Not only through its RNase role, MCPIP1 negatively regulates cellular inflammatory responses, but also by deubiquitinating TRAF (TNF receptor-associated factor) proteins (TRAF2, TRAF3, TRAF6) and interfering with the signaling of NF-κB (117). Via a close regulatory feedback-loop system, MCPIP1 and NF-κB control each other's activity (118).

Regnase-1 (Regulatory RNase 1), also known as ZC3H12A or MCPIP-1 (monocyte chemotactic protein-induced protein-1) (119) is a novel anti-inflammatory protein found in MCP-1-stimulated human blood monocytes and in IL-1β-stimulated *in vivo* human monocyte-derived macrophages (120). Together with Regnase-2, 3, and 4, MCPIP-1, also known as Zc3h12a, and the LPS (lipopolysaccharides)-inducible protein Regnase-1, comprises a protein family (121). These proteins have a single zinc finger domain of the Cys-Cys-Cys-His (CCCH) type and a Pilt-N-terminus (PIN)-like domain, both might be associated with the recognition and binding of RNA (122).

Four domains of Regnase-1 are an N-terminal (NTD) domain, a PilT N-terminus-like domain, a zinc finger (ZF) domain, and a C-terminal (CTD) domain (123, 124) (Figure 2.7). Also, the PIN-like domain includes the catalytic center of the RNase, but for complete enzymatic activity, it requires an intramolecular connection with NTD. mRNA recognition and direct binding are the responsibility of the ZF domain (125).

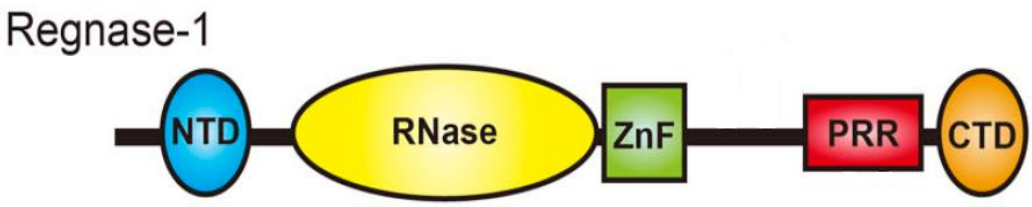

Figure 2.7. Domain schematic of Regnase-1. Adapted from Tanaka H. *et al.* (119).

Regnase-1 is found in a wide range of cells, and the target mRNAs of Regnase-1 have been discovered in a wide range of cell forms (126). There are cytokines, T-cell surface markers, inflammation-related genes, transcription factors, miRNAs, antiapoptotic genes, DNA demethylation genes and some viral RNAs among its targets (127).

Post-transcriptional gene regulation specifically regulates the efficiency of mRNA translation. In the mRNA stability adjustment, cis-acting elements located in the 3'-untranslated region (3'UTR) of mRNA play an important part (128, 129). These elements allow RNA-binding proteins to recognize target mRNA transcripts, and boost nuclease-dependent degradation (127, 130). Regnase-1 binds to target mRNA on the 3'UTR of these genes through reorganization of the conserved stem-loop structure, according to a recent investigation (131).

In addition, Regnase-1 is related to numerous biological processes, as brain growth and adipogenesis by regulating differentiation of cells and apoptosis, in addition to its immune response functions (132). Targeting the myocardial MCPIP1 overexpression resulted in NF-κB activity inhibition and a decrease in proinflammatory cytokine development induced by LPS inducible nitric oxide synthase (iNOS) expression and activation of caspase-3 (133). Thus, MCPIP1 appears to be a potent negative inflammation regulator (134).

2.4.1. The Role of Regnase-1 in Cancer

Accumulating evidence suggests that MCPIP1 could be controlling inflammation, the development of angiogenic factors, the activity of transcription factors, and miRNA biosynthesis during the angiogenesis process. However, tumor cell studies indicate that under normal and pathological conditions, MCPIP1 can exhibit various effects (135) (Figure 2.8).

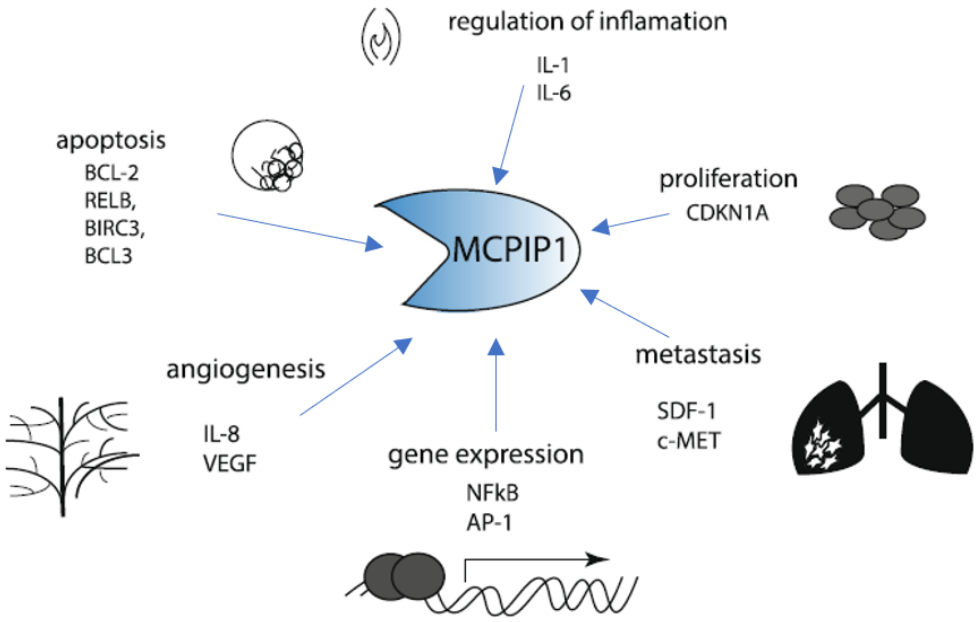

Figure 2.8. Varies functions of MCPIP1. Adapted from Miekus K. *et al.* (136).

MCPIP1 can both, directly and indirectly, control the apoptotic mechanism (136). MCPIP1's indirect impact on apoptosis is attributed to its effects on the stress granules (SGs) generation (137). Under stress circumstances, involving heat shock, arsenite-induced oxidative stress, and energy deprivation, the MCPIP1 expression blocks SG generation and promotes apoptosis of macrophages (138). MCPIP1 over-expression decreased cell viability, induced late apoptosis characteristic nuclear morphology, and enhanced activity of caspase 3/7 (139).

Apoptosis is also regulated by MCPIP1 directly via a mechanism that is linked to its enzymatic activity (140). A cell line of breast cancer MDA-MB-231 studies has

shown that MCPIP1 acts as a potent tumor inhibitor that enhances apoptosis by inducing the decay of mRNA transcripts of the antiapoptotic gene. 31 transcripts affected by MCPIP1 expression were identified by Lu et al., out of which 6 anti-apoptotic genes were downregulated and 25 pro-apoptotic genes were upregulated (140). MCPIP1 in particular binds and cleaves RelB, Bcl2A1, Bcl2L1, Birc3, and Bcl3 encoding mRNAs shown in immunoprecipitation experiments with RNA. Finally, human sample analysis showed that in breast tumor cells, MCPIP1 expression is inhibited, which in turn, can help those cells to escape apoptosis (140).

During tumor growth, inflammation is a significant inducer of angiogenesis, and inflammatory cytokines have been documented to promote a wide spectrum of tumor development processes (141). The proinflammatory cytokines MCP-1, IL-1, and IL-6 are required for angiogenesis and tumor formation, and they increase cancer cell invasion and metastasis in animal models. Treatment of human umbilical vein endothelial cells (HUVECs) with inflammatory agents IL-8, TNF-α, IL-1ß, and MCP-1 elevated MCPIP1 gene coding, resulting in angiogenesis-related characteristics and angiogenesis-related gene expression, resulting in the vascular tube-like morphology (142).

2.5. Aim

It is evident that CAFs have a puissant role in shaping the TME. We know that CAFs may be responsible from macrophage polarization to M2-type macrophages in TME, which means having a pro-tumoral niche and an poor prognosis. We aimed to discover a connection between CAFs and Regnase-1 protein in developing such a polarization by conducting western blot analyses.

3.MATERIALS AND METHODS

All experiments of this study were conducted between August 2019 — June 2021 at the Research Laboratories of the Department of Basic Oncology, Cancer Institute, Hacettepe University in Ankara, Turkey.

3.1.Materials

Table 3.1. List of materials used in the study

Phosphate buffered saline (PBS)	(Sigma-Aldrich,USA)
DMEM High Glucose Cell Medium (Dulbecco's Modified Eagle Medium)	(Biological Industries, Israel,)
Trypsin-EDTA (Solution B (0.25%), EDTA (0.05%)	(Biological Industries, Israel)
Trypan Blue	(Merck, Germany)
Serological Pipettes (5ml, 10ml and 25ml)	(Corning, USA)
Tissue Culture Flasks (75cm^2 and 25cm^2)	(Sarstedt, Germany)
Centrifuge Tubes (2ml, 15ml and 50ml)	(Sarstedt, Germany)
Culture Dish (100mm x 20mm)	(Corning, USA)
Tissue Culture Plates (6, 12, 96 well)	(Sarstedt, Germany)
L-glutamine	(Biological Industries, Israel)
Fetal Bovine Serum (FBS)	(Biological Industries, Israel)

Penicillin / Streptomycin	(Biological Industries, Israel)
Dimethyl sulfoxide (DMSO)	(Ambresca, USA)
Cryo-vial tubes, sterile	(Corning, USA)
Mycoplasma Detection Kit	(Biological Industries, Israel)
0.2 ml PCR tubes, sterile	(Corning, USA)
Sodium Borate	(Sigma- Aldrich, USA)
8 Well Chamber Slides	(Sarstedt, Germany)
Paraformaldehyde	(Serva, Germany)
Conventional staining kit (Bond Max)	(Leica, Germany)
RIPA Lysis and Extraction Buffer	(ThermoFisher Scientific, USA)
Halt Protease & Phosphatase Single-Use inhibitor cocktail	(ThermoFisher Scientific, USA)
EDTA (500 gr.)	(ThermoFisher Scientific, USA)
Cell Scraper	(CELLTREAT, USA)
Pierce™ BCA Protein Assay Kit (500 ml)	(ThermoFisher Scientific, USA)
β-mercaptoethanol	(MERCK-Germany)

10x Tris/Glycine/ SDS, 1 lt.,	(Bio-Rad, USA)
Mini-PROTEAN TGX Gels, 10 gels/box, 10-well, 50 microliters, 4-20% resolving gels	(Bio-Rad, USA)
Prot/Elec Pipet Tips, bulk	(Bio-Rad, USA)
Spectra™ Multicolor Broad Range Protein Ladder	(ThermoFisher Scientific, USA)
Tris-HCl	(Sigma-Aldrich, USA)
Bromophenol blue	(Applichem, Germany)
Glyserol	(GerBu, Germany)
Horizontal Electrophoresis System	(Bio-Rad, USA)
Trans-Blot Turbo RTA Transfer Kit, PVDF, 40 Blot	(Bio-Rad, USA)
Trans-Blot Turbo Transfer System	(Bio-Rad, USA)
10% Tween 20, 1 lt	(Bio-Rad, USA)
10x Tris Buffered Saline (TBS), 1 lt	(Bio-Rad, USA)
BlueBlock PF (10x), 1lt	(Serva, Germany)
Pierce™ ECL Western Blotting Substrate (500 ml)	(ThermoFisher Scientific, USA)
GAPDH (D16H11) Rabbit mAb	(Cell Signaling Technology, USA)

β-Actin (13E5) Rabbit mAb	(Cell Signaling Technology, USA)
Human/Mouse MCPIP1 Antibody (MAB7875)	(R&D Systems, USA)
LEAF™ Purified anti-mouse/rat/human MCP-1 Antibody	(BioLegend, USA)
Anti-rabbit IgG, HRP-linked Antibody	(Cell Signaling Technology, USA)
Goat Anti_Mouse IgG H&L (HRP)	(Abcam, USA).

3.1.1. Devices

Table 3.2. List of devices used in the study

Kodak Gel Logic 1500 Screening System	Carestream Health, USA
Phase-Contrast Microscope Olympus BX50	Olympus, England
Incubator	Heraeus, Germany
Shaker	Heidolphy, Germany
Microcentrifuge	Eppendorf, Germany
-80^{o}C Freezer	Bosch,Germany
Centrifuge	Heraeus,Germany
Horizontal Electrophoresis System	Bio-Rad Laboratories,USA

PowerPac Basic Power Supply	Bio-Rad Laboratories,USA
Trans-Blot Turbo Transfer System	Bio-Rad Laboratories,USA
Vortex	Vortex-Genie
Water Purification System	Milipore, USA
Heat Block	Techne Dri Bloc, England
Fume Hood	Unitest, Turkey
Laminar Flow Hood	Thermo-Electron, USA
Water Bath	GFL, Germany
+4^{o}C Cold Room	Alarko Carrier, Turkey
Bond Max, Immunocytochemistry staining	Leica Biosystems, Germany
-20^{o}C Freezer	Bosch, Germany
Haemocytometry	Neubauer,USA
Mr.Frosty	Nalgene-Thermo Scientific, USA
Spectrophotometer	SpectraMax,USA
Plate Shaker	Terra Universal, USA

3.1.2 Media, Solutions and Buffers

Full DMEM Medium (Fibroblast medium): DMEM High Glucose medium (with 1mM Sodium Pyruvate); supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin; stored at 4^{o}C. FBS bottles were heat-inactivated before use at 56^{o}C for 30 minutes.

1X PBS Solution: One PBS tablet was put to 100 mL of distilled water, which was carefully mixed and sterilized.

Trypan Blue: 0.4-gram Trypan blue was dissolved in 80 ml PBS then boiled slowly. When cooled to room temperature, aliquoted and stored at room temperature.

10X Sodium Borate Solution: 19.07-gram $Na_2B_4O_7$ was added to 1-liter distilled water and stirred until dissolved. It was filtered to a clean bottle and diluted to 1X with water before using in gel preparation.

4% paraformaldehyde: 800 ml 1X PBS was added to a glass beaker and heated to 60^{o}C while stirring. 40-gram paraformaldehyde powder was added to the heated PBS solution. NaOH was added to clear the solution. When the powder was dissolved the solution was left to cool and filter. The volume was increased to 1 L to 1X PBS and the pH was adjusted to 6.9 with HCl. The solution was aliquoted and stored at 4oC.

4X Laemmli Buffer: For 50 ml solution, 1.97-gram Tris-HCl (0.250 M) powder was added to 20 ml distilled water. 20 ml glycerol was added to the solution and 4-gram SDS was dissolved in a stirrer. 4-milligram bromphenol-blue was added to the solution and the volume was adjusted to 50 ml.

3.2 Methods

3.2.1. Investigations with Human Tissue Samples

In this study, patients undergoing surgery with a pre-diagnosis of breast cancer as a result of the clinical evaluation by Hacettepe University School of Medicine Department of General Surgery were included. During the pathological evaluations of the surgical materials; tissue samples were taken from 5 patients (4 females and 1 male) whose breast cancer diagnosis is confirmed. Within the scope of this study, there was no change in the routine treatment practices of the patients.

The sample size was aimed to provide 3 independent experiments. Considering the possible problems that may be encountered in the ex vivo isolation of primary CAFs from human breast cancer tissue samples, the number of patients participated in the study was 5 (4 female and 1 male patient). Research ethics approval for experimenting with human tissues was received from "Hacettepe University Non-interventional Clinical Researches Ethics Board, Ankara" before the initiation of the experiments (Approval Number: 2019/23-22).

3.2.2. Preparation of Tissues

The tissues were obtained from Hacettepe University Department of Medical Pathology in DMEM supplemented with 1% penicillin/streptomycin under sterile conditions. Later, the tissues were washed and cut to 1 mm^3 pieces (Figure 3.1.) by washing with the help of sterile PBS solution in a laminar flow hood. These pieces were then planted in 12-well culture plates (Figure 3.2.) and cultured in DMEM under optimum conditions (37°C, 5% CO_2 in an incubator). Each well was supplemented with 500 µl medium.

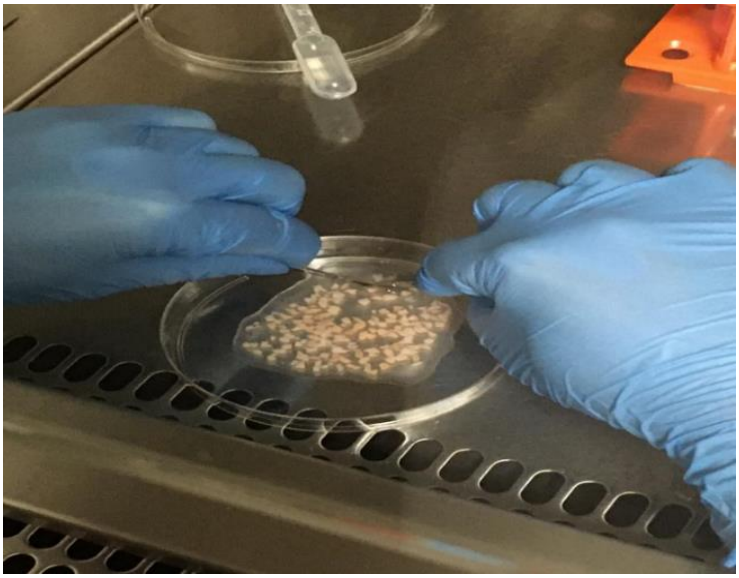

Figure 3.1. Cross-cutting the human breast cancer tissue

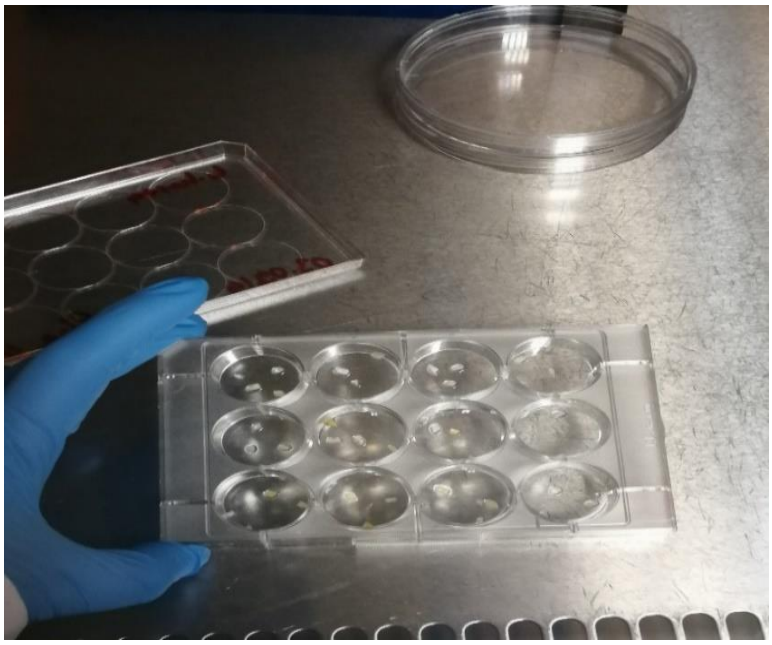

Figure 3.2. Planting the breast cancer tissue pieces to 12-well culture plate.

According to the literature, tissues, including human skin, mouse ear and tail ends might be used and put in culture medium on a flask or plate to obtain fibroblasts. The main reason behind this procedure is that other cell types can't be cultivated as easily as fibroblasts, giving them the well-deserved reputation of being easy to grow on plastic (35).

Every three days, the medium was aspirated and placed with 500 µl fresh medium in order to prevent any contamination and alteration from pH balance changes.

3.2.3.Fibroblast Isolation and Culture of Isolated Fibroblasts:

Approximately after two weeks of incubation of the tissues (Figure 3.3), fibroblasts in the culture medium were obtained by the passaging method. Fibroblasts were grown (Figure 3.4.) in DMEM prepared by adding 10% FBS (5% CO_2) and incubated at 37^oC in the incubator.

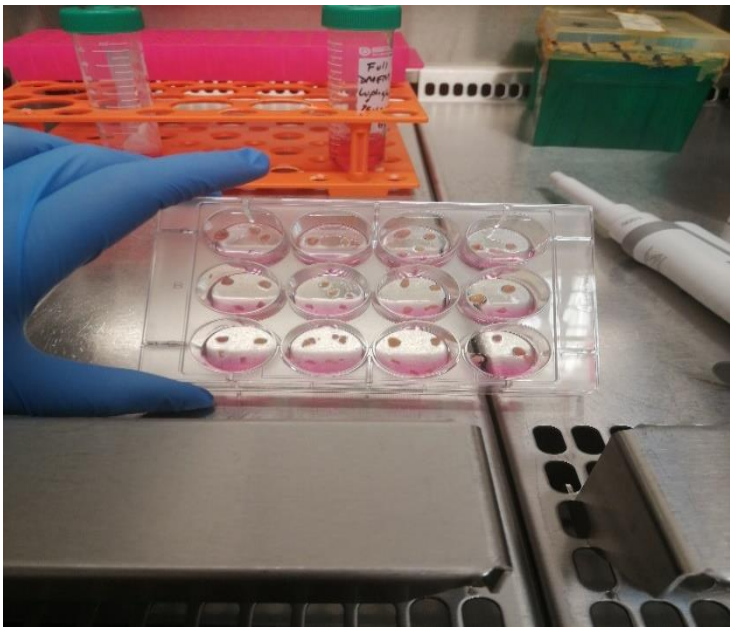

Figure 3.3. Fibroblast explantation on 12-well culture plate

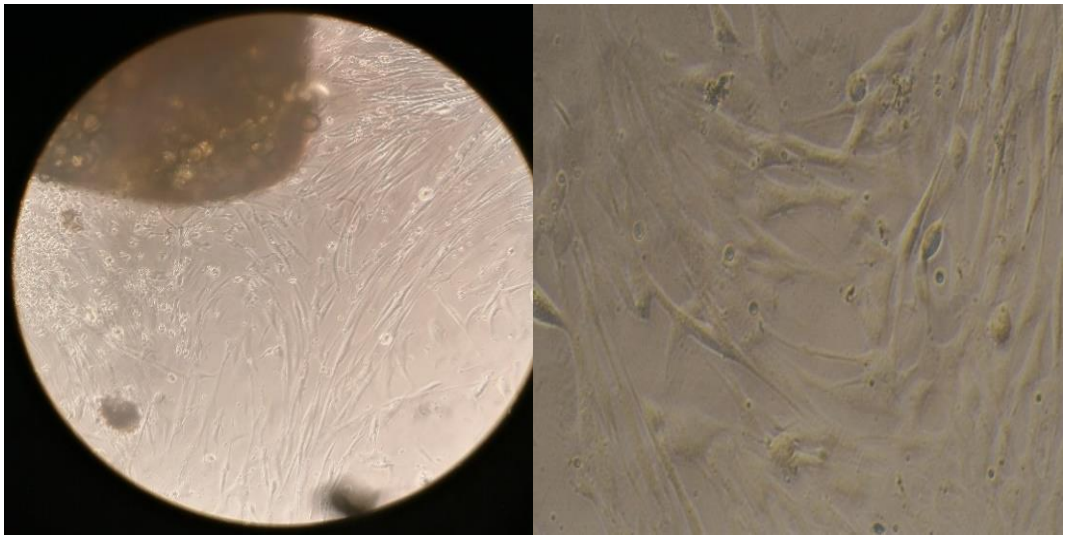

Figure 3.4. Low density of fibroblast explanted from breast cancer tissue

When the cells reach a 70-80% confluency (Figure 3.5.), the tissue pieces were removed with a Pasteur pipette and the medium was aspirated. Fresh medium was added to wells and placed back in the incubator. After 24 hours of incubation without the tissue pieces, trypsinization was performed on cells at the well surface to culture them on T25 culture plates. Cells were counted with Trypan Blue and cultured with DMEM.

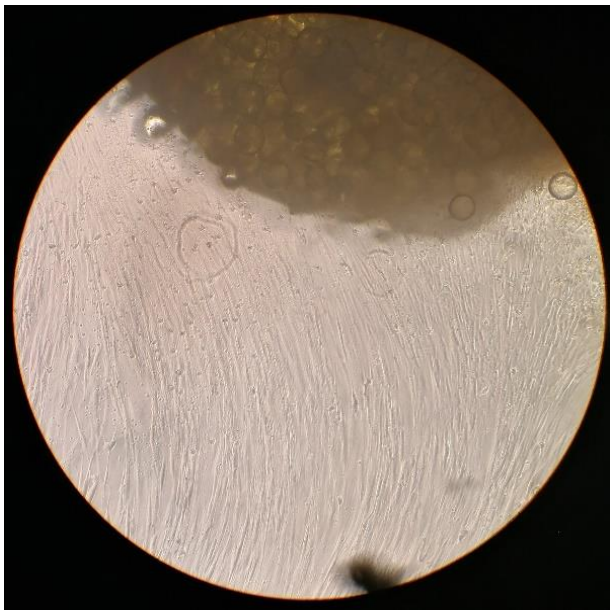

Figure 3.5. High density of fibroblast explanted from breast cancer tissue

Each plate and well were appointed a number in order to differentiate fibroblasts when characterizing them. The first three passages of fibroblasts were included in this study.

3.2.4.Immunocytochemistry and Characterization:

In the first passage of cells, characterizations were performed to determine whether the obtained fibroblast cells were CAF. Cells were planted in 8-well chamber slides (143) (Figure 3.6) and when they reached the desired density, the cells were fixed using 4% paraformaldehyde.

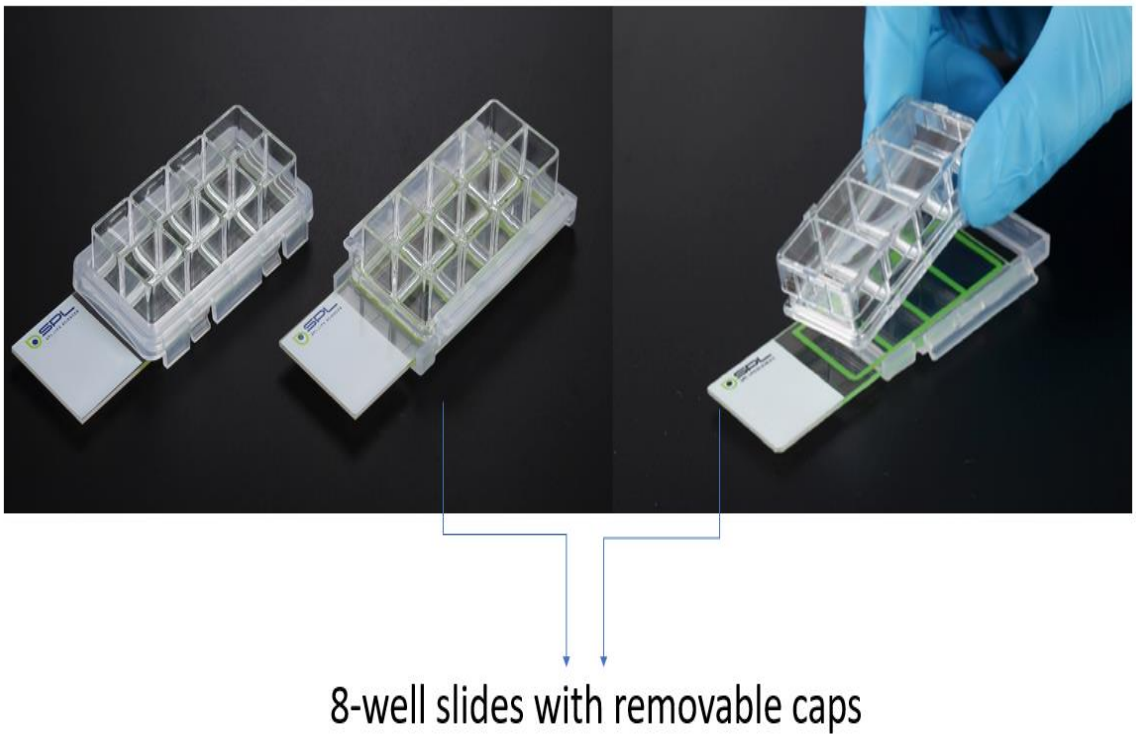

Figure 3.6. Glass slides used for characterization adapted from SPL Life Sciences (143).

For the fixation process, at room temperature, the medium was removed and the wells were washed with 500 µl non-sterile PBS for 5 minutes. 200 µl 4% paraformaldehyde was added to the wells for 15 minutes. After this, wells were washed with non-sterile PBS twice. Later, these cells were stained with

immunocytochemistry antibodies: vimentin, pan-cytokeratin, and α-SMA. Then, immunocytochemical/morphological analyses were performed. After the primary antibody incubation, the incubation phase started with the secondary antibody produced against the organism in which the HRP (Horseradish Peroxidase) the conjugated primary antibody is produced in order to visualize the primary antibody. Finally, the substrate of diaminobenzidine (DAB), which is the substrate of the peroxidase enzyme in HRP conjugated to the secondary antibody, was used in order to stain the proteins to which the secondary antibody is attached. Primary and secondary antibody staining was performed on the Leica Bond Max device with the conventional kit content. Images were viewed with an Olympus BX50 microscope.

Cells with less than 50-60% α-SMA positive staining were discarded after this stage.

3.2.5. Cell Culture:

Normal fibroblast cells were utilized as the control group in the experiments. Adherent MRC-5 (144)(Kind gift from Prof. Dr. Bedia CAKMAKOGLU) lung fibroblast cell line for use as normal fibroblasts were included in the study. Human normal lung cells MRC-5 (145)(Medical Research Council cell strain-5) are frequently used in cancer research Cells were grown in T25 and T75 flasks in sterile conditions in DMEM containing 1% penicillin/streptomycin and incubated under appropriate conditions (37^{o}C, 5% CO_2).

3.2.5.a. Cell Counting

The cell numbers were determined by counting the cells in a hemocytometer (146) (Figure 3.7.). In order to resuspend the cells thoroughly, the suspension of cells was mixed with the help of a pipet movement. 20 µl of 'Trypan Blue' (0.1 percent w/v Trypan Blue in PBS solution) was well combined with 20 µl of cell suspension, which stained blue on the non-viable cells. 20 µl of this mixture was filled with a capillary effect in the Neubauer hemocytometer chamber. A light

microscope was used to count viable cells (100x magnification) in the four corner squares.

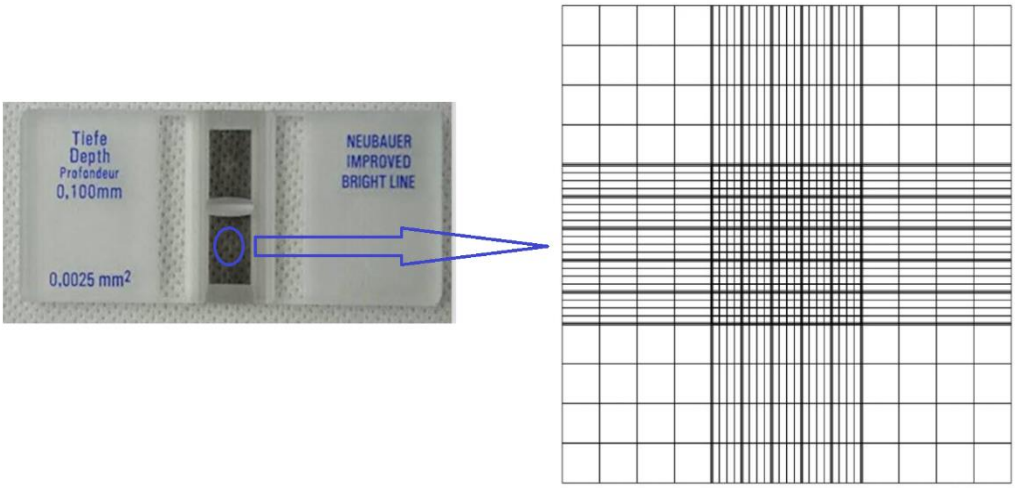

Figure 3.7. Zoomed hemocytometer displaying the Neubauer chamber with line counting adapted from Vembadi (146).

The arithmetic means count per four corner squares (Figure 3.7.) was determined for the total viable cells.

The total amount of cells was determined by precisely calculating the quantity volume of the cell suspension by the cell concentration per ml. Approximately more than 90% of viable cells were used in this study.

3.2.5.b Trypsinization

The cells were treated with trypsin in order to remove cells from the plate surface. Then the flask was placed in a 37^{o}C incubator so the trypsin could be activated. After 3 to 4 minutes, the flask was taken out of the incubator back to the laminar-flow hood and supplemented with DMEM to inactivate trypsinization. The medium and trypsin-containing cells were aspirated with serological pipets and

placed in a 50 ml centrifuge tube. Cells were centrifuged at 1800 rpm for 5 minutes and after that, the supernatant was removed and the pellet was resuspended with fresh DMEM. In this stage, cells were stained with Trypan Blue in order to count cells on a Thoma lam. Approximately 450.000-500.000 cells were cultured in T75 cm^2 flasks and placed in a 37^oC incubator for the cells to grow again. The first four passages of these cells will be used in the experimental steps.

3.2.5.c. Freezing Cells

Cells between passages were frozen for later usage. When trypsinization occurred, after counting the cells some of the cells remain without passaging. These cells were supplemented with 200 µl FBS and 100 µl DMSO and placed in a cryovial tube. Each cryovial contains 1 million cells in 700 µl DMEM. Then the tubes were placed in Mr.Frosty (Nalgene Freezing bottle) (Figure 3.8.) (147). To achieve the critical and continuous -1^oC/minute cooling rate required for effective cryopreservation and cell recovery, vials were frozen slowly. The cells were initially frozen at -80°C for 24 hours. After the vials waited 24 hours at -80^oC, they were removed from Mr. Frosty and placed in a separate container that kept them in the liquid nitrogen tank's gaseous phase (-150^oC to -180^oC).

Figure 3.8. Mr. Frosty™ Freezing Container adapted from Thermo Scientific (156). (147)

3.2.5.d. Thawing Cells

The vials were withdrawn from the nitrogen tank and placed in a beaker with sterile water in a water bath at 37^{o}C. When the suspensions had fully thawed, the cells were then placed in a 50 ml centrifuge tube with 5 ml of growth media. Then, the suspension of cells was centrifuged at 1800 rpm for 5 minutes. The DMSO-containing supernatant was discarded, and the cell pellet was resuspended and incubated at 37°C in a suitable growth medium.

3.2.5.e. Detection of Mycoplasma Contamination

In our laboratory, in order to avoid contamination, we regularly test cell lines for mycoplasma, therefore the MRC-5 cell line was tested for contamination regularly while using and before freezing.

Mycoplasmas were identified through the EZ-PCR (Polymerase chain reaction) Mycoplasma Test Kit (148). For the identification of mycoplasma in cell cultures, this is a ready-to-use PCR mix. With PCR checking, the results are obtained shortly. The presence of contaminated mycoplasma can be easily determined, by determining the bands of amplified DNA fragments in electrophoresis. The primer collection allows the identification of different types of mycoplasma as well as extremely sensitive and specific Acholeplasma and Spiroplasma species.

For the test protocol, the manufacturer's instructions were followed. Simply, test samples were prepared along with the positive and negative controls. The PCR amplification in the thermal cycler was then performed. Agarose gel and running buffer was prepared with diluting 10X Sodium Borate and by gel electrophoresis, amplified products have been analyzed. Finally, the results were observed with UV (ultra-violet) transillumination in Kodak Gel Logic 1500 Screening System.

3.2.6. Obtaining Protein Lysates

Medium of CAF cells and MRC-5 normal fibroblast cells were drawn to prepare lysates for Western Blot experiments. Later, Lysis Buffer containing RIPA, protease, and phosphatase inhibitor cocktail and EDTA (Ethylenediamine tetraacetic acid) was prepared for the usage of lysis buffer. This stage was carried out on the ice and under cold room (+ 4^{o}C) conditions due to the Lysis buffer usage conditions. Flasks were washed with cold 1X PBS and then cells on the T25 culture plate surface were treated with lysis buffer and scratched with a cell scraper in order to obtain proteins from cell culture. Cells and lysis buffer then aspirated with pipets to a 2 ml centrifuge tube and placed in a shaker for 10 minutes. The centrifuge tubes then vortexed for 30 seconds and this process was repeated 3 times. Then the tubes had been centrifuged at 13400 rpm for 10 minutes. The supernatant was taken carefully without disturbing the pellet on the bottom and placed in a new 2 ml centrifuge tube. These lysates either used directly or placed in -80^{o}C for later use.

3.2.7. Protein Quantitation

Protein quantitation was made for the determination of protein concentration of protein lysates to be used in the Western Blot experiment.

10 µl protein standards (which are 2 mg/ml, 1.5 mg/ml, 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml respectively) and 10 µl lysates from the samples prepared before were added to wells of 96-well plate separately. 50x solution of solvents A and B from the BCA kit (Bicinchoninic acid) was prepared and 200 µl from it was added to the samples in the wells. The plate was shortly shaken in a 96-well plate shaker and placed in a 37^{o}C incubator for 30 minutes. After this, in 562 nm wavelength, the Optical Density (OD) of each well was assessed with the help of a Spectrophotometer. Results were used for calculating the number of protein lysates that will be added to gels.

3.2.8. SDS Gel Electrophoresis

After protein quantitation, the lysates were diluted with lysis buffer and each sample was added β-mercaptoethanol and 4X Laemmli buffer (loading dye). Each sample was kept in a dry heating block at 95^{o}C for 5 minutes for denaturation of the proteins. After the protein denaturation step, the separation of proteins with different weights (kilodaltons-kDa) in the lysate was carried out by SDS (Sodium Dodecyl Sulfate) polyacrylamide gel electrophoresis. Samples were added to wells of TGX (Tris-Glycine Extended) gels and they were placed in an electrophoresis system. The tank was loaded with TGS buffer diluted to a 1x solution. The samples started to run for 5 minutes at 50 volts in order to align the samples to a starting point and then they run 90 minutes at 100 volts.

3.2.9. Western Blot

After the running stage is completed, the proteins that separate according to their weight in the gel were transferred from the gel to the PVDF (Polyvinylidene difluoride) membrane. This step was conducted with the help of the Trans-Blot Turbo Transfer System. Gels were placed between two filters and membrane (Figure 3.9.) (149). Filters were soaked in a cup contains 20 ml Transfer Buffer 20 ml ethanol 60 ml distilled water. The membrane was also soaked with 70% ethanol and then placed in the same cup containing transfer buffer briefly. The first filter was placed on the bottom layer of the cassette and the membrane was put on top of the filter. TGX gel was carefully removed from the plastic cover by breaking the layers and placed on top of the membrane. Lastly, the second filter was put on the upper side of these layers and with the help of the top cassette pressed to each other.

In the Turbo mix program, blotting was conducted in 3 minutes. When the blotting was over, the gel was transferred to the membrane.

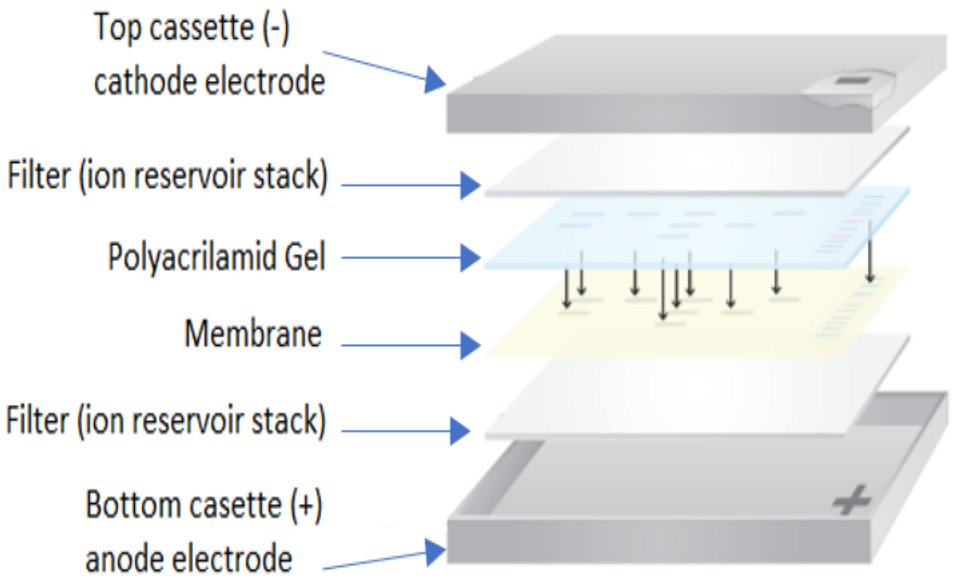

Figure 3.9. Western Blot schematic adapted from Bio-Rad Trans Blot Turbo Transfer System Quick Start Guide (149).

3.2.9.a. Primary Antibody Incubation

After the transfer step, the membrane was blocked in 1X BlueBlock solution for 1-1.5 hours on the shaker set at room temperature and speed of 100 rpm. Then, the blocking process is completed, at 4°C the membrane was placed in a box with primary antibody and incubated overnight. Regnase-1 and MCP-1 antibodies were prepared in a 1x Blueblock solution with a 1:1500 ratio while GAPDH and ß-actin were prepared with a 1:3000 ratio.

3.2.9.b. Secondary Antibody Incubation

After the primary antibody incubation, the incubation stage will be started with the secondary antibody produced against the organism in which HRP (Horseradish Peroxidase) the conjugated primary antibody is produced in order to visualize the primary antibody.

The membrane was washed with 1X TBS-T (prepared with 50 ml TBS (tris-buffered saline) buffer 5 ml Tween buffer and 445 ml distilled water) 3 times for 10 minutes at 100 rpm in a shaker when the primary antibody incubation was over. In this way, unwanted residues will be cleaned from the membrane surface. After this step, the membrane was incubated with the secondary antibody at room temperature which was prepared in a 1X BlueBlock solution with the 1:3000 ratio.

After the process above, the membrane was washed with 1X TBS-T 3 times for 10 minutes again to prepare the membrane for the next step.

3.2.10. Chemiluminescence Imaging

Using the Western Blot method, expressions of Regnase-1 and MCP-1 were investigated in protein lysates extracted from CAF cells. Monoclonal antibodies that recognize a single determinant of denatured proteins with high affinity and specificity will be used for the determination of the proteins we target. As the control protein, GAPDH and ß-actin were used.

After the secondary antibody incubation, the ECL (Enhanced chemiluminescence) solution containing luminol, which is the substrate of the peroxidase enzyme contained in HRP conjugated to the secondary antibody, was used so that the proteins to which the secondary antibody is bound can emit chemiluminescence. Finally, the densitometric densities of the bands obtained by chemiluminescence, imaging were determined with the help of the Kodak Gel Logic 1500 Screening System.

3.2.11. Densitometric Analysis:

LI-COR® Image Studio Lite 5.2 application was used for semi-quantitative analyses of Western Blot bands. GAPDH and ß-actin bands were used for normalization in the analyses.

In the program, every band was assigned with the same square and their expression was calculated by the program. Each sample's expression band can be compared when normalized with GAPDH and ß-actin expressions.

Later on, the expression bands can be quantitatively analyzed and graphics can be drawn.

3.2.12. Optimization of House-Keeping Proteins

During the western blot experiments, GAPDH was being used as the control protein, the results and the research showed that using ß-actin for the control of Regnase-1 would be more suitable. Experiments conducted with two different housekeeping proteins.

3.3. Collection of Data:

Samples obtained as a result of cancerous breast tissue excision performed by Hacettepe University Faculty of Medicine Hospital Department of General Surgery. Research ethics approval for experimenting on human tissue was received from "Hacettepe University Ethics Board and Commissions Ankara" before starting the experiments with a permission number: 2019/23-22. Before the surgeries, all of the patients were enlightened with an Informed Consent Form. In the conduct of all experimental steps outlined in this thesis, the Declaration of Helsinki regulations were properly followed.

The tissues have taken in DMEM containing 10% FBS and 2% penicillin-streptomycin and CAF isolation was performed directly through the explantation protocol. While obtaining the tissue sample, no special intervention was made for the patient, except for the breast tissue biopsy/tumor excision protocol planned for diagnosis. The patients' clinical follow-up data, evaluation of the course of the disease together with the factors to be examined within the scope of the study; can provide data that can shed light on the disease prognosis.

3.4.Analysis of Data:

When comparing the two groups in the analysis of the data, the student's t-test was utilized (expression levels in reference control MRC-5 cells) and in order to evaluate correlation, Spearman's Correlation was utilized. The significance level was set at p-value <0.05. The statistical analysis of the study was done with IBM SPSS 23 software.

LI-COR® Image Studio Lite 5.2 was also used to analyze Western Blot bands quantitation.

4-RESULTS

4.1. Investigations with Human Tissue Samples

In this study, 5 patients with breast cancer diagnosis were included and the tissues were obtained from Hacettepe University School of Medicine Department of General Surgery.

The patients were chosen to have invasive carcinomas (Table 4.1) for experimental purposes.

Table 4.1. Patients' diagnoses

PATIENT CODE	DIAGNOSIS
Patient 1	Infiltrative ductal carcinoma
Patient 2	Infiltrative ductal carcinoma
Patient 3	Infiltrative ductal carcinoma
Patient 4	Infiltrative ductal carcinoma
Patient 5	Invasive lobular carcinoma

4.1.1. Determination of CAFs From Explanted Fibroblasts

The explantation process was performed as explained in sections 3.2.2 and 3.2.3. After the incubation of tissues in 12-well culture plate, fibroblasts started to explant to the culture plate. These cells were collected from the culture to use in further experiments.

Every patient and the wells in culture were appointed a specific number in order to distinguish them in the characterization stage (Table 4.2.).

Table 4.2. Fibroblasts used in the experiments

PATIENT CODE	PLATE NUMBER	WELL NUMBER	GIVEN CODE
1	1	3	CAF1
2	1	8	CAF2
3	1	11	CAF3
4	2	12	CAF4
5	1	9	CAF5

4.1.2. Determination of α-SMA, Vimentin, Cytokeratin Expressions in Explanted Fibroblasts

The cells obtained from the surgery were stained with α -SMA, vimentin and pancytoceratin in order to distinguish them as CAFs. Vimentin was used for positive control for fibroblast staining, Pan-cytoceratin was used for negative control and, α-SMA was used for differentiation. After the staining process, characterization of these cells was performed as positive staining with vimentin and negative staining with pan-cytokeratin (at least 50-60% α-SMA staining was aimed) (Table 4.3).

Table 4.3. α-SMA Positivity Percentage

GIVEN CODE	α -SMA POSITIVITY PERCENTAGE
CAF1	65%
CAF2	50%
CAF3	50%
CAF4	80%
CAF5	60%

With the help of a light microscope, the stained cells were examined. (Figure 4.1.,4.2.,4.3.,4.4.)

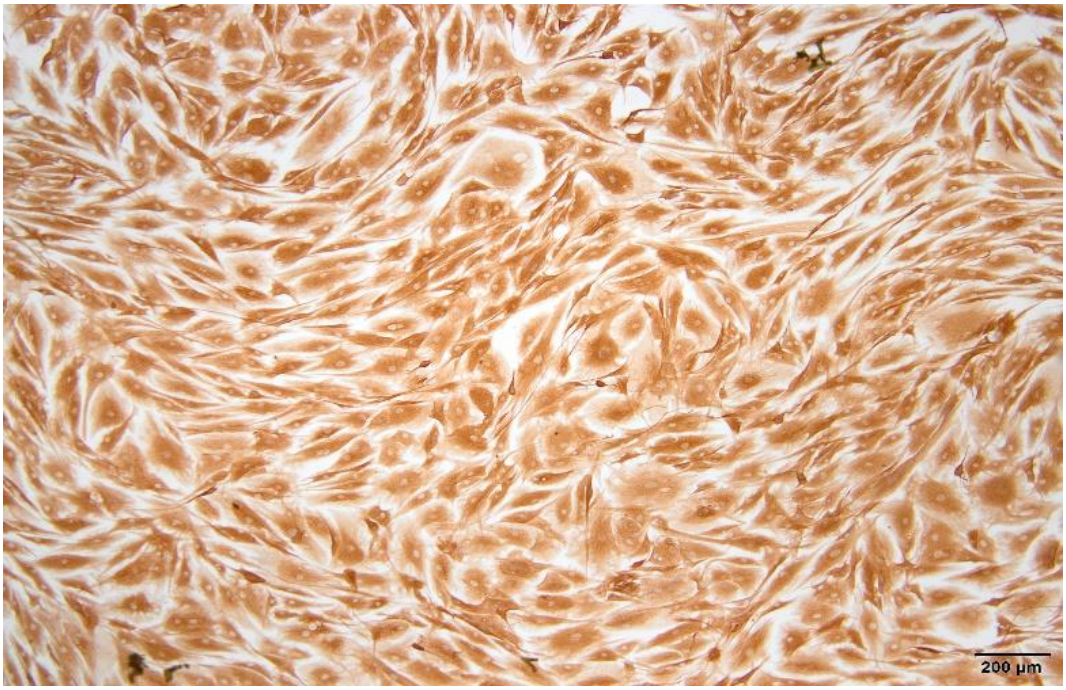

Figure 4.1. Vimentin Staining

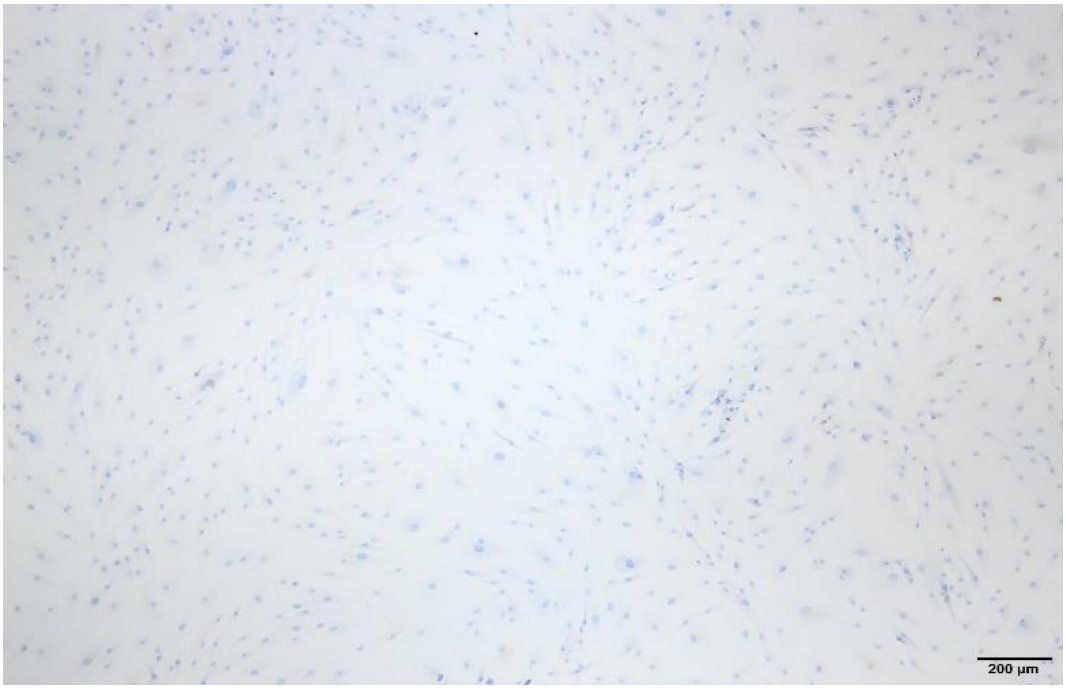

Figure 4.2. Cytoceratin Staining

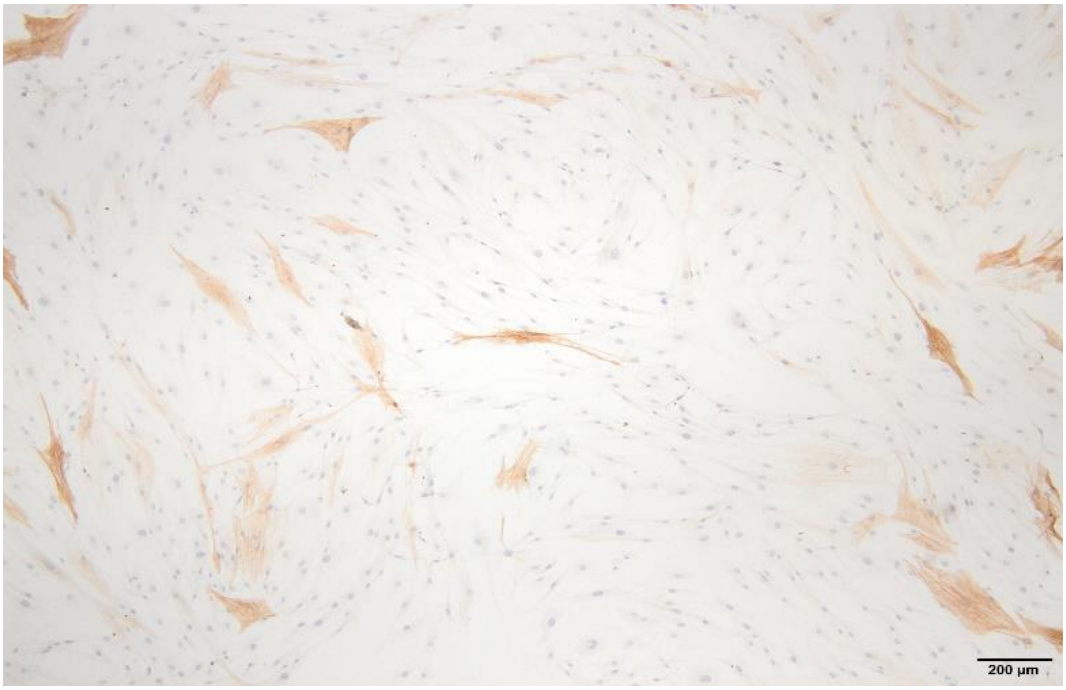

Figure 4.3. α-SMA Negatif Staining

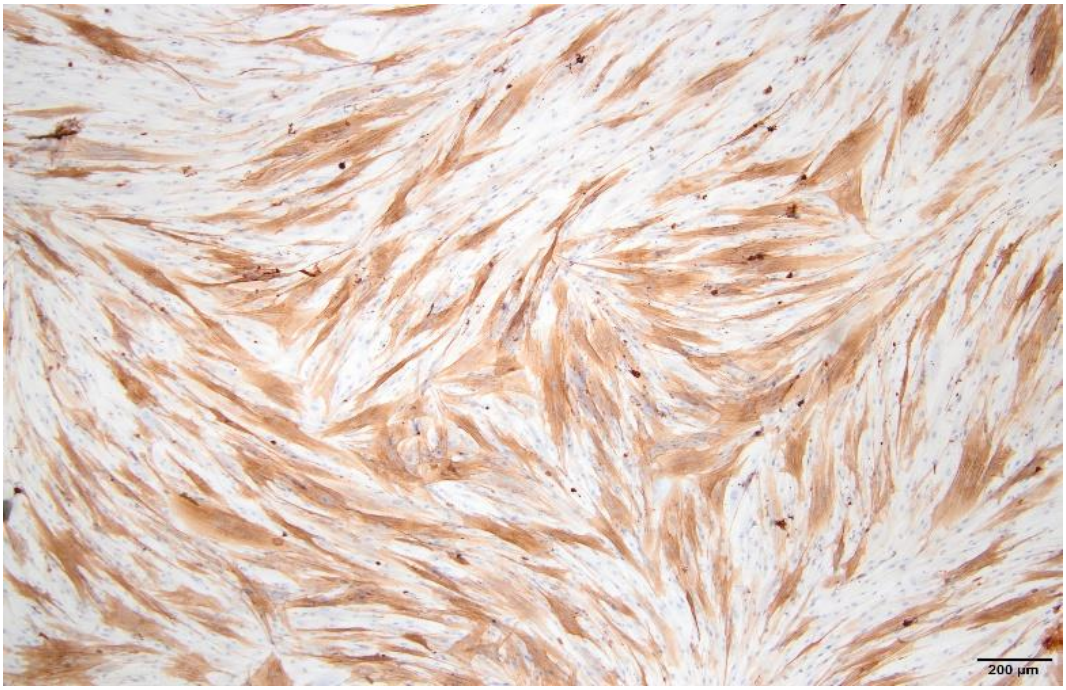

Figure 4.4. α-SMA Positive Staining

4.1.3 Correlation Between CAF Grade and Clinical / Pathological Features

After the characterizations of fibroblasts, patients' clinical and pathological features were used for evaluating the correlations between them and CAF grade of the isolated fibroblasts. We accepted the 60% α-SMA positivity as the limit for using high grade term. Statistically significant and positive correlation was found between Ki-67 grade and CAF grade. (Table 4.4.).

Table 4.4. Correlation between the isolated CAFs with clinical and pathological features.

		CAF Grade		Correlation
	Number of cases	Low %	High %	p value
Sex				0.495
Female	4	1	3	
Male	1	1	0	
Age				0.789
>50	2	1	1	
≤50	3	1	2	
Grade				0.219
2	2	1	1	
3	3	1	2	
Ki-67 Grade			**R= 0.899**	**0.044**
5-10%	2	1	1	
20-25%	1	0	1	
40-45%	1	1	0	
70-%	1	0	1	
Tumor Size				0.789
≤2	3	2	1	
>2	2	0	2	
Molecular Subtype				0.219
ER/PR(+)	5	2	3	
HER2(+)	2	0	2	
Triple Negative	0	0	0	
Lymph node involment				0.495
Positive	4	1	3	
Negative	1	1	0	

4.2. Determination of Mycoplasma in the Medium Obtained From CAF Cell Culture

The presence of mycoplasma was determined using the Biological Industries EZ-PCR Mycoplasma Test Kit. As part of the study's contamination control, all cell cultures were tested for mycoplasma. All of these tests came out negative, and there was no evidence of mycoplasma infection. One of these tests is illustrated in Figure 4.6. The study was conducted with the cells without infection. If any contamination was detected, infected cells were diminished and new cultures for those cells were used during the study.

Figure 4.5. Representitive for mycoplasma detection imaging.

4.3. Optimization of Protein Quantitation

In the first western blot experiments, there were differences between GAPDH bands which were loaded to the gel in order to have a control protein. To overcome this problem, some alterations applied to the protein quantitation process.

After the modifications, the coefficient of determination (R^2) value was evaluated as 1 (Figure 4.7.) and the stabilization for the efficiency of the experiment was ensured.

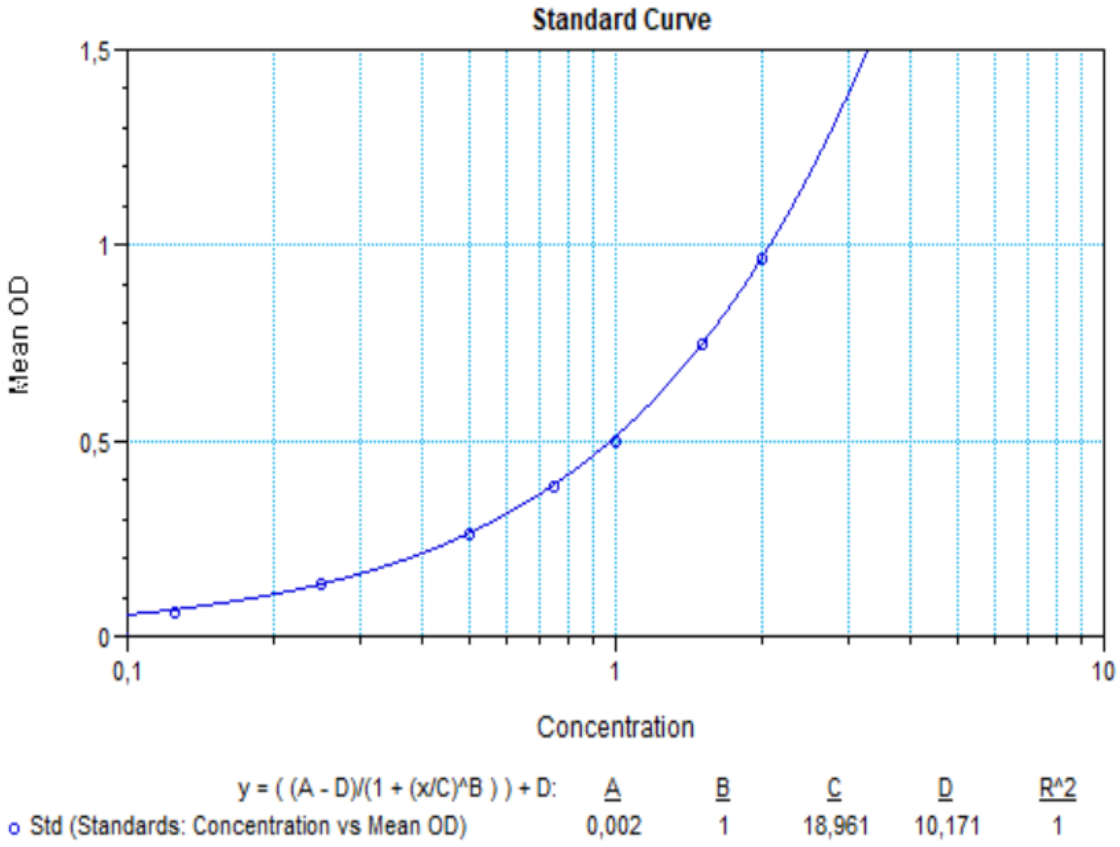

Figure 4.6. Standart curve of the protein quantitation.

4.4. MCP-1 Protein Expression Levels in NF and CAFs

Comparison of MCP-1 protein expression levels in MRC-5 fibroblasts and CAF cells were shown by using Western Blot analysis. All of the samples were normalized with GAPDH protein bands which were used as control. Three independent experiments were performed for each condition. The differences in protein expression levels of MCP-1 in cells were analyzed with student's t-test. (Figure 4.7.)

Western blot analysis demonstrated that protein expression of MCP-1 in the MRC-5 fibroblast cell line is nearly the same as the CAF4 which has the highest α-SMA expression from all of the CAFs and has no statistical significance. (p-value, "*" is <0.05 and "**" is <0.005).

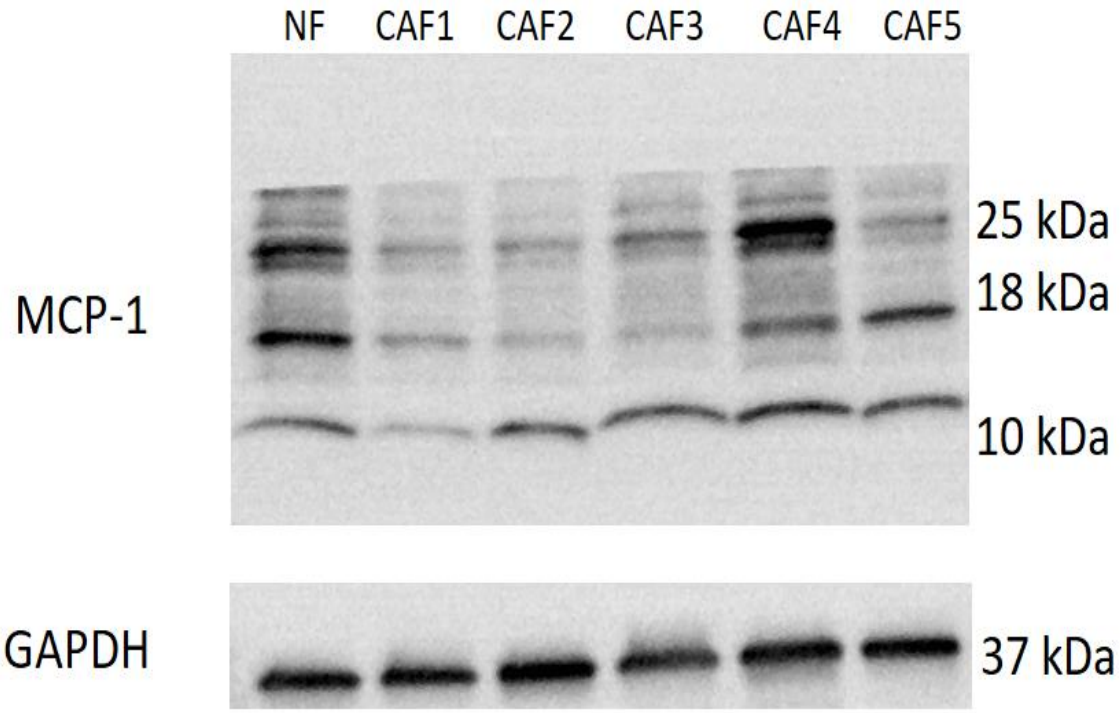

Figure 4.7. Images of MCP-1 and GAPDH protein western blots in MRC-5 cells and CAFs.

Table 4.5. Relative band density of MCP-1 protein expression in NF and CAF cells.

	Relative Band Density ± SEM
NF	1.356 ± 0.147
CAF1	0.433 ± 0.071
CAF2	0.295 ± 0.025
CAF3	0.634 ± 0.094
CAF4	1.498 ± 0.113
CAF5	0.636 ± 0.116

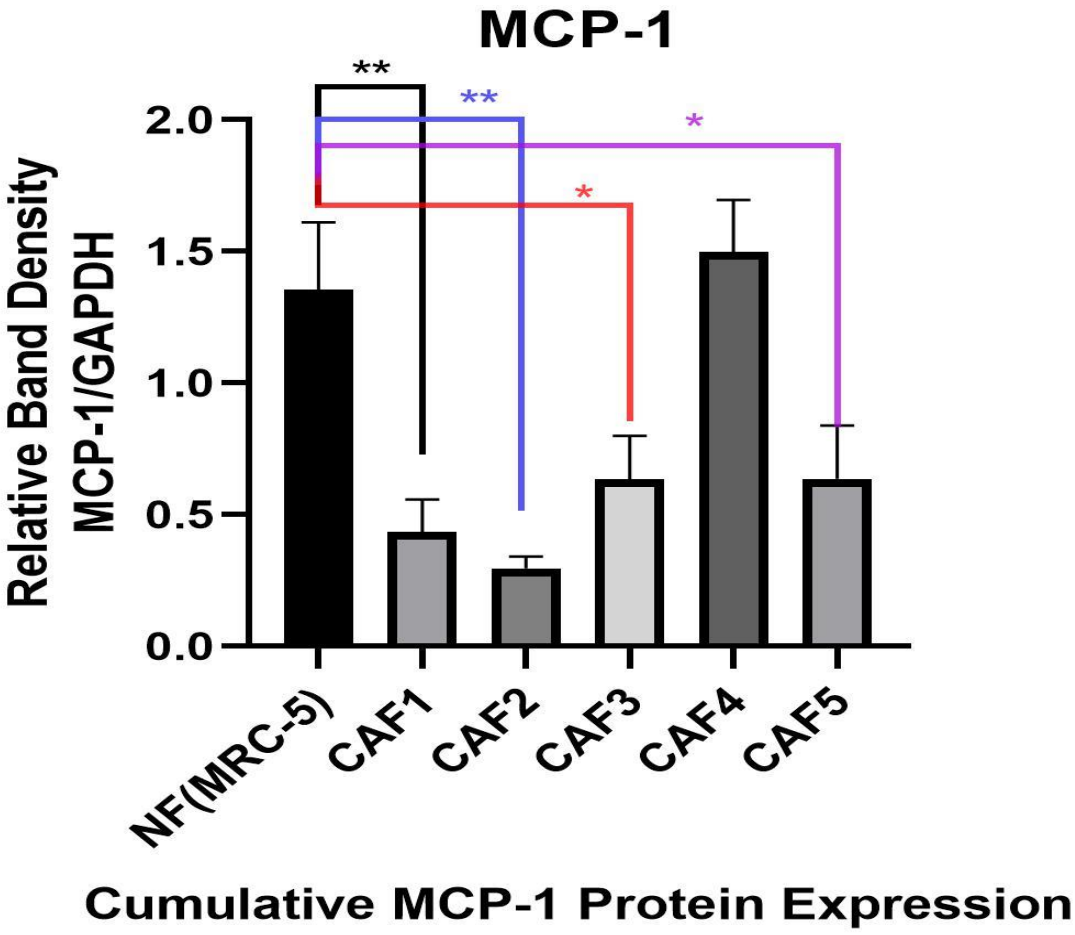

Figure 4.8. Analysis of MCP-1 protein expression.

4.5. Regnase-1 Protein Expression Levels in NF and CAFs

Comparison of Regnase-1 protein expression levels in MRC-5 fibroblasts and CAF cells were performed by using Western Blot analyses. All of the samples were normalized with GAPDH protein bands which were used as control. Three independent experiments were performed for each condition. The differences in protein expression levels of Regnase-1 in cells were analyzed with student's t-test. (Figure 4.9.)

We analyzed the western blots of 65 kDa bands and 50 kDa bands separately. Total band expression levels show no significant differences from each other except CAF3 and CAF4 but 50 kDa bands showed that protein expression of Regnase-1 in MRC-5 fibroblast cell line is nearly the same with the CAF4 which has the most α-SMA expression among all of the CAFs and has no statistical significance. Moreover, CAFs with low α-SMA expression show significantly different and low expression of Regnase-1 in 50 kDa (Figure 4.10, 4.11.) (p-value "*" is <0.05 and "**" is <0.005).

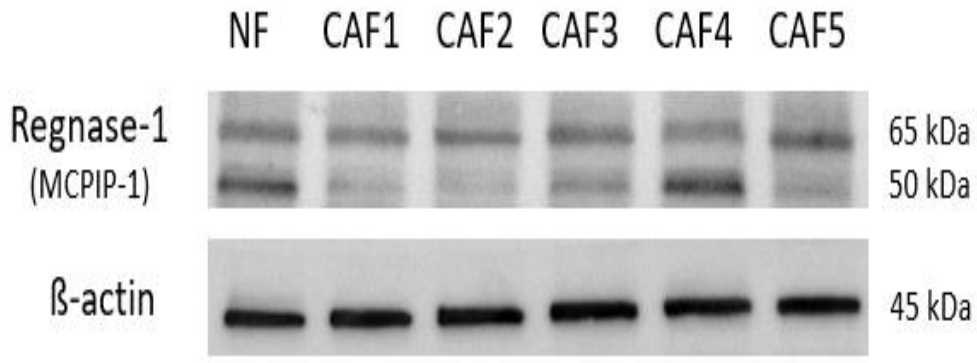

Figure 4.9. Images of Regnase-1 and ß-actin protein western blots in MRC-5 cells and CAFs.

Table 4.6. Relative band density of Regnase-1 (50 kDa) protein expression in NF and CAF cells.

	Relative Band Density ± SEM
NF	0.207 ± 0.020
CAF1	0.067 ± 0.011
CAF2	0.069 ± 0.034
CAF3	0.092 ± 0.028
CAF4	0.207 ± 0.036
CAF5	0.046 ± 0.023

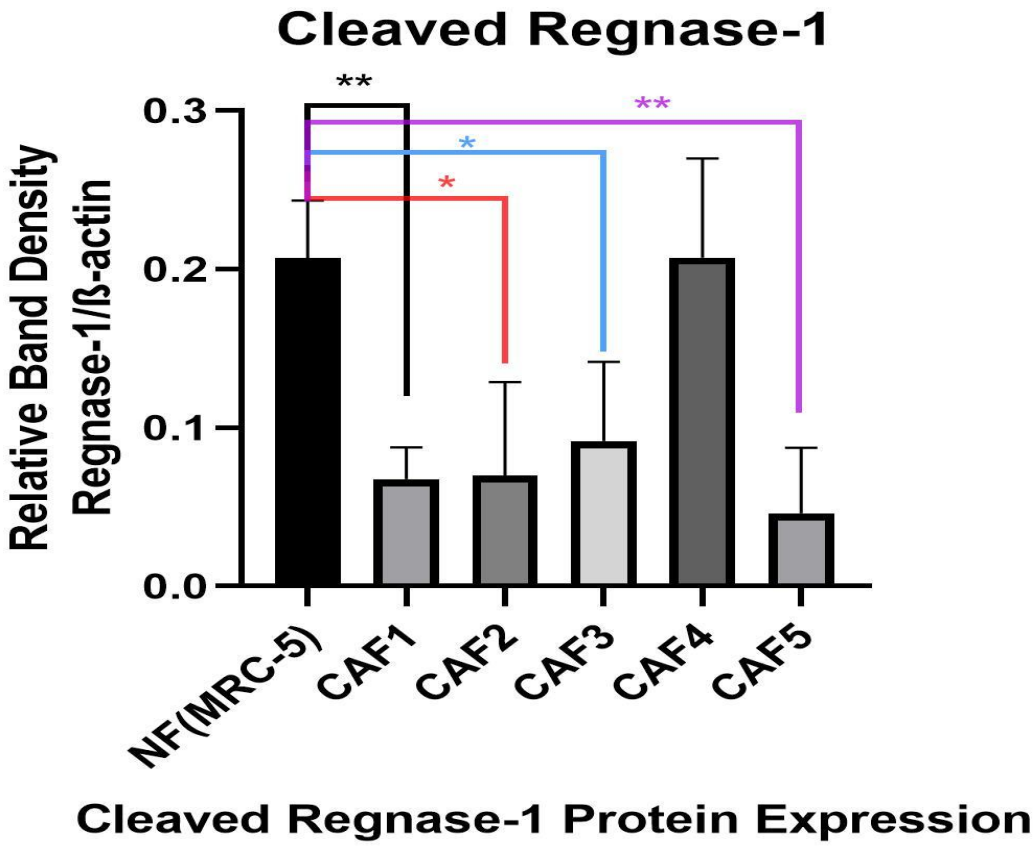

Figure 4.10. Analysis of Regnase-1 (50 kDa) protein expression.

Table 4.7. Relative band density of Regnase-1 (65 kDa) protein expression in NF and CAF cells.

	Relative Band Density ± SEM
NF	0.386 ± 0.02554
CAF1	0.343 ± 0.01779
CAF2	0.319 ± 0.01819
CAF3	0.276 ± 0.02791
CAF4	0.223 ± 0.03018
CAF5	0.331 ± 0.02869

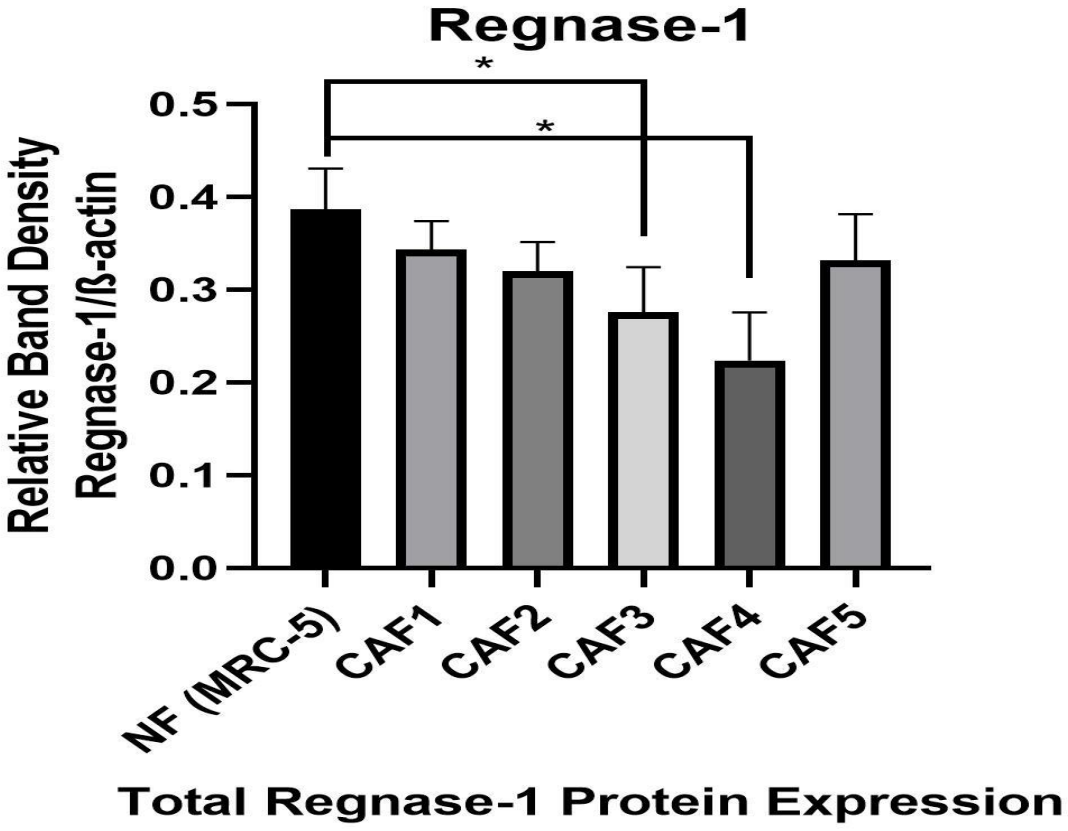

Figure 4.11. Analysis of Regnase-1 (65 kDa) protein expression.

4.6. Correlation Between MCP-1 and Regnase-1 Expression

The correlation between MCP-1 and Regnase-1 protein expression was evaluated with Spearman's correlation. There was a significant and strong correlation with p-value <0.05 in between MCP-1 expression and cleaved Regnase-1 expression, but no significant correlation was detected in between MCP-1 and total Regnase-1 protein expression (p-value =0.655).

4.7. Correlation Between CAF Grade and Protein Expressions of MCP-1 and Regnase-1

The correlation between MCP-1 and Regnase-1 protein expression was evaluated with Spearman's correlation. There was a significant and strong correlation with p-value <0.05 in between CAF grade and both MCP-1 expression and cleaved Regnase-1 expression.

5.DISCUSSION

In the 1990s, Teicher et al. discovered that therapy-resistant breast cancer cells in vivo no longer showed significant resistance to medicine when subjected to therapies *in vitro* (150). According to Jain et al., extracellular divisions (e.g. such as vascular and interstitial) are challenges to therapeutic medication delivery. (151). The causes of drug resistance in cancer cells are linked to both cell-autonomous processes, such as genetic and epigenetic alterations, and the TME, which includes CAFs, according to these researches. (152).

Here we investigated proteins expressed by breast cancer-CAFs which are related to a pro-tumoral environment and eventually cancer progression in order to develop a further understanding of a therapy target.

CAFs also have immune-modulatory mechanisms in TME and the suppression of macrophage functions in breast cancer is often described. The production of CCL2 and CXCL16 by CAFs can alter the recruitment of monocytes to breast tumors (153, 154) In vitro, inhibiting IL-6 reduced CCL2 production and subsequent monocyte recruitment. In vivo, CCL2 produced by tumor cells and stromal cells attracts monocytes to breast tumors and increases metastasis and breast cancer stemness in a CCR2 and NOTCH1-dependent way, according to Qian et al. and Tsuyada et al. (155). Liao et al. found that FAP+ CAF depletion resulted in increased IL-2 and IL-7 expression and decreased expression of CSF-1, IL6, VEGF, and IL-4. This resulted in the reduction of pro-tumoral macrophage recruitment and T-regulatory cells. Moreover, enhanced recruitment of anti-tumoral dendritic cells and cytotoxic T cells in a metastatic breast cancer model 4T1. (93).

In this study, we showed that CAFs could express both MCP-1 and Regnase-1 proteins. These proteins have roles in inflammation and their properties are linked to the immune-modulatory mechanism of CAFs.

MCP-1 (CCL2) is a chemotactic factor for monocytes that belongs to the C-C chemokine family (156). MCP-1 is thought to be the same as JE, a gene whose expression in mouse fibroblasts is activated by a PDGF. (157). However, because of

its monocyte chemoattractant features, the human analog known as CCL2 was first isolated from human cells (156).

Monocytic, endothelial, epithelial, smooth muscle, fibroblasts, astrocytic, microglial and, mesangial cells all produce CCL2 (158). These cells are essential for antiviral immune responses in the bloodstream and tissues. However, CCL2 is discovered to be primarily produced by monocytes and macrophages (159). Natural killer (NK) cells, monocytes and, memory T lymphocytes migrate and infiltrate the body in response to CCL2. CCL2 is one of the most researched members of the chemokine family, with evidence that it could be used to treat a variety of disorders (156).

Cancer cells could be affected on a wide range including tumor progression, angiogenesis and, invasion by the CAF secreted cytokines like HGF, SDF-1 and, IL-6 and this can be employed instead of ECM. (4). TAMs, which are also one of the most encountered cell kinds in the tumor environment, are considered to make up to half of all cancer tissue mass. Classically activated (M1) macrophages stimulate immune responses, and alternatively activated (M2) macrophages supports tumor growth, which are the two types of polarized macrophages. TAMs have features like M2-polarized macrophages (160). CAFs increased macrophage M2 polarization, which worked in tandem with CAF suppression rather than NK cell recruitment. TAMs and CAFs work together to adjust NK cells in colorectal cancer, and a unique mechanism for this function was discovered (161).

In this research, we found that CAFs express MCP-1 protein which could lead to recruiting monocytes in breast cancer TME. The control fibroblast cell line (MRC-5) was found to express higher MCP-1 than primary CAF cells. We speculate that the higher expression of MCP-1 in MRC-5 cells might be due to the fact that MRC-5 cells are indeed a cancer cell line in contrast to CAFs, which are primary cells. Even though cancer cells lines can mostly be utilized for cancer research, their inherent characteristics that are different from primary cells are undeniable, as they keep dividing and growing over time.

In a previous study, our group discovered that CAFs shape the TME by attracting monocytes and promoting immunosuppressive PD-1-positive TAMs, as well as transforming recruited monocytes into M2-like macrophages having the capability of inhibiting the immune responses through the PD-1 axis (7).

Unlike NF-educated monocytes, CAF-educated monocytes showed severe immune suppression and increased the breast cancer cells' motility, as well as raising the expression of EMT-related genes and vimentin protein in cancer cells. When compared to control M1 macrophages, CAF-educated M1 macrophages showed elevated levels of M2 markers and production of anti-inflammatory cytokine IL-10, but decreased formation of pro-inflammatory cytokine IL-12, implying that CAFs can also elicit M1 macrophages to transdifferentiate into M2 macrophages. (7). In this study, we searched for the proteins underlying the effects of CAFs on macrophage polarization and explore the possible pathway for this activity.

They also used tissue samples from breast cancer patients and looked into the link between CAF invasion and TAMs. TAM levels in human breast cancer samples taken were found to be highly correlated with CAF grade. It was also linked to a greater Ki-67 proliferation index as well as a larger tumor size (7). Unfortunately, due to having an inadequate number of patients for a correlation test, in this study, there were no correlations between CAF grade and the given parameters.

Natural cytokines and chemokines undergo posttranslational changes that impact their biochemical and biological properties. As a posttranslational modification, glycosylation of the MCP-1 protein could be detected using the western blot.

In a study, MCP-1 (12 and 13.5 kDa), which was glycosylated, was shown to be two to three times reduced capability of chemotacticity for monocytes and THP-1 cells (10 kDa). MCP-1(5–76) and MCP-1(6–76) demonstrated essentially no bioactivity when NH2-terminally truncated, however, COOH-terminally modified

MCP-1(1–69) retained all of its chemotactic and Ca2+-inducing properties. The agonistic potency of natural MCP-1 variants was linked to their ability to immunize the Ca2+ response induced by intact MCP-1 in THP-1 cells. (162).

The effectiveness of neutrophil chemoattractant is unaffected by removing a small number of residues from the NH2 terminus of human granulocyte chemotactic protein-2. The situation is completely different for C-C chemokines such as MCPs. These chemokines can be totally inactivated by changing or removing amino acids in the NH2-terminal region (162).

In our study, all of the MCP-1 protein bands having triple bands (in 18 kDa and 25 kDa) was interesting. From the researches described above, we interpret this as MCP-1 showing glycosylation bands between 25 kDa and 15 kDa which is consentient with the MCP-1 western blot result of Ji et.al. (163).

The biological activity of some cytokines is known to be modulated by glycosylation. Depending on the producer cell type, heterogeneity leading to posttranslational modification has been identified for human MCP-1. However, the impact of glycosylation on human MCP-1 biologic activity has yet to be identified (162).

A transcriptome investigation of human monocyte-derived cells treated with IL-1ß or MCP-1 in 2011 discovered ZC3H12A, a new inflammatory gene that encodes a 599-amino-acid chain that corresponds to a 66-kDa protein. MCPIP1 is a protein that is expressed in a variety of human and mouse organs, including bone marrow, placenta, spleen and, heart (164). It is later called Regnase-1 (Regulatory-RNase). Regnase-1 is an RNase that degrades immune-related mRNAs to modulate immunological homeostasis post-transcriptionally.

In this study, Regnase-1 protein was detected with western blot in all of the CAFs and NF control at 66 kDa.

Regnase-1 cleaves the 3' UTRs of a group of mRNAs, including IL6 and IL12b, to destabilize them (165). Although inactivating Regnase-1 results in an autoimmune disease in mice that includes T cell activation and

hyperimmunoglobulinemia, Regnase-1-mediated immune control mechanism remains unknown. Uehata et al. demonstrated that Regnase-1 is required for the autonomous production of abnormal effector CD4+ T cells (165). Regnase-1 also controls the RNAs of a number of genes in T cells, such as Ox40, c-Rel and, Il2, by cleaving their 3' UTRs. They also found TCR stimulation causes Malt1/paracaspase to cleave Regnase-1 at R111, releasing T cells from inhibition mediated by Regnase-1. Moreover, they showed that the activity of the Malt1 protease is required for maintaining T cell effector genes mRNA stability. These findings suggest that dynamic modulation of Regnase-1 expression in T cells is crucial for T cell activation control (165).

Following that, Rosenbaum et al. demonstrated in their work that MALT1 activity among mature Tregs is essential for their immune-suppressive properties. In the activated Tregs, the RNA-degrading enzymes Regnase-1 and Roquins are inactivated by the MALT1 paracaspase cleavage. Large sets of immune regulatory mRNAs can be controlled post-transcriptionally by these mechanisms. They discovered that Tregs with BCL10 deficiency or MALT1 protease inhibition are unable of Regnase-1 and Roquin degradation, as well as to upregulate CTLA4 they employed it as an indicator for the Treg immune-suppressive characteristic, as well as a variety of other factors that interact together to mediate Treg-suppression, which is currently poorly understood. (166).

In our study, all of the MCPIP-1 protein bands having double bands (in 50 kDa and 65 kDa) was interesting. We believe this shows cleavage of Regnase-1 in all of the samples except NF. This could be explained by both cell line and primary cell differences or the fact that normal fibroblast Regnase-1 protein isn’t cleaved in contrast to CAFs and this could be utilized as a target for treatment of tumors.

Recently, Wei et al. targeted Regnase-1 for cancer treatment by programming long-lived effector T cells. They found that, through a critical gene target BATF, Regnase-1 inhibited mitochondrial metabolism and effector responses. BATF operated as a limiting factor for anti-tumor responses and mitochondrial metabolic programming. They also found that the genome-wide CRISPR screening,

in combination with Regnase-1 deletion, finds SOCS1 and PTPN2 as potential targets for boosting anti-tumor immunity. (167).

From our previous experiences, we know that CAFs can turn macrophages to the M2 phenotype which has pro-tumoral properties. Because Regnase-1 is the protein that is induced by MCP-1, we searched for a link between CAFs and Regnase-1 to learn more about a potential target. Regnase-1 is being explored as a cancer treatment target, primarily in T cell immunity, and CAFs have recently become one of the most studied subjects due to tumor cell resistance to therapy when targeted alone. Their impacts on the TME and inflammation have sparked interest in a number of studies, but we are the first to study Regnase-1 in CAFs. We believe the MCP-1/MCPIP-1 activity is related to the macrophage polarization induced by CAFs in TME.

In contrast, a study group found that, through the induction of PERK and CNX expression, MCP-1/MCPIP-1 signaling, which is indirectly produced by IL-1 stimulation, may enhance ER stress-induced apoptotic sensitivity in renal cell carcinoma cells. As a result, MCP-1 and MCPIP may help to reduce the carcinogenic effect of IL-1 in renal cell carcinomas (168).

According to our findings, we found all of the CAFs express both MCP-1 and Regnase-1. Their expression levels are varied by their α-SMA expression levels. In the MCP-1 protein levels, we found there is a significant difference between MRC-5 normal fibroblast levels and the CAFs except CAF4. In the Regnase-1 protein levels, no significant changes between CAFs and normal fibroblast control cells detected except CAF3 and CAF4. We propose that having a high percentage of α-SMA expression could be the reason that CAF4 has significantly different expression levels of these proteins.

We suggest, in breast cancer, that CAFs and M2 macrophages are vital in sculpting the TME. Therefore, therapeutic approaches to alter the immunosuppressive microenvironment that is mediated by CAFs should be further examined.

6-RESULTS AND RECOMMENDATIONS

1- Breast tumor tissues were obtained from 4 female and 1 male breast cancer patients.

2- Fibroblasts were isolated from breast tumor tissues by the explantation method.

3- Immunocytochemistry results showed that the cells isolated from tumors were not stained for the chosen negative control “pan-cytokeratin” and stained for the chosen positive control “vimentin”. Most cells were stained for the “α-SMA” and these cells were accepted as CAFs during this study. A combination of negative staining for cytokeratin and positive staining for both vimentin and α-SMA was used.

4- Normal lung fibroblast cell line MRC-5 was used as the normal fibroblast control.

5- Protein lysates were prepared from both CAFs and MRC-5 cell line.

6- Optimization of protein quantitation was performed with different amounts of proteins in lysates. Some of the samples were diluted in order to achieve a standard curve R^2 evaluation as 1.

7- Western blot analyses showed that MRC-5 cell line had a higher expression of both MCP-1 and Regnase-1 proteins. We suggest using primary normal fibroblast isolation from healthy people who had breast reduction surgery for a better comparison.

8- While experimenting with Regnase-1 antibody, there were non-specific binding at 37 kDa which could not be washed with TBS-T or blocked by the blocking solution. Hence, GAPDH couldn’t be used as the control protein. We used ß-actin as house-keeping protein in Regnase-1 protein expression analyses.

9- From our results, we showed CAFs express both MCP-1 and Regnase-1 proteins which could be related to their ability of monocyte recruitment and

polarization of macrophages. We suggest using RT-PCR for further investigations at an mRNA level in order to see the differences between mRNA expression levels and protein expression levels and to investigate posttranscriptional and posttranslational modifications occurred in these processes in CAFs.

10- We suggest conducting similar investigations in monocytes which were educated by CAFs in order to decipher the role of these proteins in monocytes in the TME.

11- We suggest analyzing related proteins such as MALT-1 and NFκ-B for further investigations of Regnase-1 associated pathways in CAFs in order to see if the NFκ-B is related to Regnase-1 activation and the MALT-1 is responsible for the cleavage of Regnase-1.

12- Western blot analyses of this study showed, there is a positive correlation between Regnase-1 and MCP-1 protein expressions and also between CAF grade and both Regnase-1 and MCP-1 protein expressions.

13- We showed a positive and statistically significant correlation between CAF grade and Ki-67 index.

REFERENCES

1. Momenimovahed Z, Salehiniya H. Epidemiological characteristics of and risk factors for breast cancer in the world. Breast Cancer (Dove Med Press). 2019;11:151-64.

2. Sun Y. Translational horizons in the tumor microenvironment: harnessing breakthroughs and targeting cures. Med Res Rev. 2015;35(2):408-36.

3. Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer. 2006;6(5):392-401.

4. Shiga K, Hara M, Nagasaki T, Sato T, Takahashi H, Takeyama H. Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. Cancers (Basel). 2015;7(4):2443-58.

5. LeBleu VS, Kalluri R. A peek into cancer-associated fibroblasts: origins, functions and translational impact. Dis Model Mech. 2018;11(4).

6. Monteran L, Erez N. The Dark Side of Fibroblasts: Cancer-Associated Fibroblasts as Mediators of Immunosuppression in the Tumor Microenvironment. Front Immunol. 2019;10:1835.

7. Gok Yavuz B, Gunaydin G, Gedik ME, Kosemehmetoglu K, Karakoc D, Ozgur F, et al. Cancer associated fibroblasts sculpt tumour microenvironment by recruiting monocytes and inducing immunosuppressive PD-1(+) TAMs. Sci Rep. 2019;9(1):3172.

8. Habacher C, Ciosk R. ZC3H12A/MCPIP1/Regnase-1-related endonucleases: An evolutionary perspective on molecular mechanisms and biological functions. Bioessays. 2017;39(9).

9. Iwasaki H, Takeuchi O, Teraguchi S, Matsushita K, Uehata T, Kuniyoshi K, et al. The IkappaB kinase complex regulates the stability of cytokine-encoding mRNA induced by TLR-IL-1R by controlling degradation of regnase-1. Nat Immunol. 2011;12(12):1167-75.

10. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, et al. Breast cancer. Nat Rev Dis Primers. 2019;5(1):66.

11. Smith RA, Andrews KS, Brooks D, Fedewa SA, Manassaram-Baptiste D, Saslow D, et al. Cancer screening in the United States, 2018: A review of current American Cancer

Society guidelines and current issues in cancer screening. CA Cancer J Clin. 2018;68(4):297-316.

12. Halsted WS. I. The Results of Radical Operations for the Cure of Carcinoma of the Breast. Ann Surg. 1907;46(1):1-19.

13. Veronesi U, Boyle P, Goldhirsch A, Orecchia R, Viale G. Breast cancer. Lancet. 2005;365(9472):1727-41.

14. Colditz GA, Kaphingst KA, Hankinson SE, Rosner B. Family history and risk of breast cancer: nurses' health study. Breast Cancer Res Treat. 2012;133(3):1097-104.

15. Polyak K. Breast cancer: origins and evolution. J Clin Invest. 2007;117(11):3155-63.

16. Allison KH. Molecular pathology of breast cancer: what a pathologist needs to know. Am J Clin Pathol. 2012;138(6):770-80.

17. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B, et al. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol. 2013;24(9):2206-23.

18. Kalinowski L, Saunus JM, McCart Reed AE, Lakhani SR. Breast Cancer Heterogeneity in Primary and Metastatic Disease. Adv Exp Med Biol. 2019;1152:75-104.

19. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature. 2012;486(7403):346-52.

20. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747-52.

21. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer Statistics, 2021. CA Cancer J Clin. 2021;71(1):7-33.

22. DeSantis CE, Ma J, Gaudet MM, Newman LA, Miller KD, Goding Sauer A, et al. Breast cancer statistics, 2019. CA Cancer J Clin. 2019;69(6):438-51.

23. Feng Y, Spezia M, Huang S, Yuan C, Zeng Z, Zhang L, et al. Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. Genes Dis. 2018;5(2):77-106.

24. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

25. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell. 2011;147(2):275-92.

26. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer. 2009;9(4):239-52.

27. Place AE, Jin Huh S, Polyak K. The microenvironment in breast cancer progression: biology and implications for treatment. Breast Cancer Res. 2011;13(6):227.

28. Massague J, Obenauf AC. Metastatic colonization by circulating tumour cells. Nature. 2016;529(7586):298-306.

29. Hay ED. An overview of epithelio-mesenchymal transformation. Acta Anat (Basel). 1995;154(1):8-20.

30. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer. 2009;9(4):265-73.

31. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19(11):1423-37.

32. Sflomos G. BC. Breast Cancer Microenvironment and the Metastatic Process. Springer Cham.2017.

33. Virchow R. Die Cellularpathologie in Ihrer Begruendung auf Physiologische und Pathologische Gewebelehre. Hirschwald A, editor1858.

34. Fernandes IR, Russo FB, Pignatari GC, Evangelinellis MM, Tavolari S, Muotri AR, et al. Fibroblast sources: Where can we get them? Cytotechnology. 2016;68(2):223-8.

35. Kalluri R. The biology and function of fibroblasts in cancer. Nat Rev Cancer. 2016;16(9):582-98.

36. Micallef L, Vedrenne N, Billet F, Coulomb B, Darby IA, Desmouliere A. The myofibroblast, multiple origins for major roles in normal and pathological tissue repair. Fibrogenesis Tissue Repair. 2012;5(Suppl 1):S5.

37. Lynne A. Murray DAK, Geoffrey J. Laurent. Fibroblasts. In: Peter J. Barnes JMD, Stephen I. Rennard, Neil C. Thomson, editor. Asthma and COPD: Basic Mechanisms and Clinical Management: Academic Press; 2009. p. 193-200.

38. Ohlund D, Elyada E, Tuveson D. Fibroblast heterogeneity in the cancer wound. J Exp Med. 2014;211(8):1503-23.

39. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature. 2007;449(7162):557-63.

40. Wen S, Niu Y, Yeh S, Chang C. BM-MSCs promote prostate cancer progression via the conversion of normal fibroblasts to cancer-associated fibroblasts. Int J Oncol. 2015;47(2):719-27.

41. Orimo A, Weinberg RA. Heterogeneity of stromal fibroblasts in tumors. Cancer Biol Ther. 2007;6(4):618-9.

42. Deng Y, Cheng J, Fu B, Liu W, Chen G, Zhang Q, et al. Hepatic carcinoma-associated fibroblasts enhance immune suppression by facilitating the generation of myeloid-derived suppressor cells. Oncogene. 2017;36(8):1090-101.

43. Gunaydin G, Kesikli SA, Guc D. Cancer associated fibroblasts have phenotypic and functional characteristics similar to the fibrocytes that represent a novel MDSC subset. Oncoimmunology. 2015;4(9):e1034918.

44. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene. 2008;27(45):5904-12.

45. Cheng Y, Li H, Deng Y, Tai Y, Zeng K, Zhang Y, et al. Cancer-associated fibroblasts induce PDL1+ neutrophils through the IL6-STAT3 pathway that foster immune suppression in hepatocellular carcinoma. Cell Death Dis. 2018;9(4):422.

46. An Y, Liu F, Chen Y, Yang Q. Crosstalk between cancer-associated fibroblasts and immune cells in cancer. J Cell Mol Med. 2020;24(1):13-24.

47. Tao L, Huang G, Song H, Chen Y, Chen L. Cancer associated fibroblasts: An essential role in the tumor microenvironment. Oncol Lett. 2017;14(3):2611-20.

48. Park JE, Lenter MC, Zimmermann RN, Garin-Chesa P, Old LJ, Rettig WJ. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. J Biol Chem. 1999;274(51):36505-12.

49. Kikuchi Y, Kashima TG, Nishiyama T, Shimazu K, Morishita Y, Shimazaki M, et al. Periostin is expressed in pericryptal fibroblasts and cancer-associated fibroblasts in the colon. J Histochem Cytochem. 2008;56(8):753-64.

50. Yoshida T, Akatsuka T, Imanaka-Yoshida K. Tenascin-C and integrins in cancer. Cell Adh Migr. 2015;9(1-2):96-104.

51. Sugimoto H, Mundel TM, Kieran MW, Kalluri R. Identification of fibroblast heterogeneity in the tumor microenvironment. Cancer Biol Ther. 2006;5(12):1640-6.

52. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, et al. Identification and characterization of a fibroblast marker: FSP1. J Cell Biol. 1995;130(2):393-405.

53. Xing F, Saidou J, Watabe K. Cancer associated fibroblasts (CAFs) in tumor microenvironment. Front Biosci (Landmark Ed). 2010;15:166-79.

54. Mitra AK, Zillhardt M, Hua Y, Tiwari P, Murmann AE, Peter ME, et al. MicroRNAs reprogram normal fibroblasts into cancer-associated fibroblasts in ovarian cancer. Cancer Discov. 2012;2(12):1100-8.

55. Kojima Y, Acar A, Eaton EN, Mellody KT, Scheel C, Ben-Porath I, et al. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. Proc Natl Acad Sci U S A. 2010;107(46):20009-14.

56. Toullec A, Gerald D, Despouy G, Bourachot B, Cardon M, Lefort S, et al. Oxidative stress promotes myofibroblast differentiation and tumour spreading. EMBO Mol Med. 2010;2(6):211-30.

57. Ishii G, Sangai T, Oda T, Aoyagi Y, Hasebe T, Kanomata N, et al. Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. Biochem Biophys Res Commun. 2003;309(1):232-40.

58. Quante M, Tu SP, Tomita H, Gonda T, Wang SS, Takashi S, et al. Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. Cancer Cell. 2011;19(2):257-72.

59. Weber CE, Kothari AN, Wai PY, Li NY, Driver J, Zapf MA, et al. Osteopontin mediates an MZF1-TGF-beta1-dependent transformation of mesenchymal stem cells into cancer-associated fibroblasts in breast cancer. Oncogene. 2015;34(37):4821-33.

60. McDonald LT, LaRue AC. Hematopoietic stem cell derived carcinoma-associated fibroblasts: a novel origin. Int J Clin Exp Pathol. 2012;5(9):863-73.

61. Greenburg G, Hay ED. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J Cell Biol. 1982;95(1):333-9.

62. Petersen OW, Lind Nielsen H, Gudjonsson T, Villadsen R, Ronnov-Jessen L, Bissell MJ. The plasticity of human breast carcinoma cells is more than epithelial to mesenchymal conversion. Breast Cancer Res. 2001;3(4):213-7.

63. Markwald RR, Fitzharris TP, Smith WN. Sturctural analysis of endocardial cytodifferentiation. Dev Biol. 1975;42(1):160-80.

64. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med. 2007;13(8):952-61.

65. Potenta S, Zeisberg E, Kalluri R. The role of endothelial-to-mesenchymal transition in cancer progression. Br J Cancer. 2008;99(9):1375-9.

66. Zeisberg EM, Potenta S, Xie L, Zeisberg M, Kalluri R. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. Cancer Res. 2007;67(21):10123-8.

67. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature. 2004;432(7015):332-7.

68. Ostman A, Augsten M. Cancer-associated fibroblasts and tumor growth--bystanders turning into key players. Curr Opin Genet Dev. 2009;19(1):67-73.

69. Kuperwasser C, Chavarria T, Wu M, Magrane G, Gray JW, Carey L, et al. Reconstruction of functionally normal and malignant human breast tissues in mice. Proc Natl Acad Sci U S A. 2004;101(14):4966-71.

70. Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science. 2004;303(5659):848-51.

71. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005;121(3):335-48.

72. Leigh S. The importance of breast cancer research from a patient's view: the voices and visions of advocates. Second International Symposium on the Molecular Biology of Breast Cancer: Breast Cancer Research; 2000.

73. Dimanche-Boitrel MT, Vakaet L, Jr., Pujuguet P, Chauffert B, Martin MS, Hammann A, et al. In vivo and in vitro invasiveness of a rat colon-cancer cell line maintaining E-cadherin expression: an enhancing role of tumor-associated myofibroblasts. Int J Cancer. 1994;56(4):512-21.

74. Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, Kuliopulos A. PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. Cell. 2005;120(3):303-13.

75. Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol. 1993;9:541-73.

76. Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, et al. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell. 1999;98(2):137-46.

77. Lochter A, Galosy S, Muschler J, Freedman N, Werb Z, Bissell MJ. Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. J Cell Biol. 1997;139(7):1861-72.

78. Karagiannis GS, Poutahidis T, Erdman SE, Kirsch R, Riddell RH, Diamandis EP. Cancer-associated fibroblasts drive the progression of metastasis through both paracrine and mechanical pressure on cancer tissue. Mol Cancer Res. 2012;10(11):1403-18.

79. De Wever O, Van Bockstal M, Mareel M, Hendrix A, Bracke M. Carcinoma-associated fibroblasts provide operational flexibility in metastasis. Semin Cancer Biol. 2014;25:33-46.

80. Dumont N, Liu B, Defilippis RA, Chang H, Rabban JT, Karnezis AN, et al. Breast fibroblasts modulate early dissemination, tumorigenesis, and metastasis through alteration of extracellular matrix characteristics. Neoplasia. 2013;15(3):249-62.

81. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. Cancer Cell. 2010;17(2):135-47.

82. Zhang XH, Jin X, Malladi S, Zou Y, Wen YH, Brogi E, et al. Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma. Cell. 2013;154(5):1060-73.

83. Verghese ET, Drury R, Green CA, Holliday DL, Lu X, Nash C, et al. MiR-26b is down-regulated in carcinoma-associated fibroblasts from ER-positive breast cancers leading to enhanced cell migration and invasion. J Pathol. 2013;231(3):388-99.

84. Bronisz A, Godlewski J, Wallace JA, Merchant AS, Nowicki MO, Mathsyaraja H, et al. Reprogramming of the tumour microenvironment by stromal PTEN-regulated miR-320. Nat Cell Biol. 2011;14(2):159-67.

85. Narra K, Mullins SR, Lee HO, Strzemkowski-Brun B, Magalong K, Christiansen VJ, et al. Phase II trial of single agent Val-boroPro (Talabostat) inhibiting Fibroblast Activation Protein in patients with metastatic colorectal cancer. Cancer Biol Ther. 2007;6(11):1691-9.

86. Wang Q, Qu C, Xie F, Chen L, Liu L, Liang X, et al. Curcumin suppresses epithelial-to-mesenchymal transition and metastasis of pancreatic cancer cells by inhibiting cancer-associated fibroblasts. Am J Cancer Res. 2017;7(1):125-33.

87. Ting H, Deep G, Kumar S, Jain AK, Agarwal C, Agarwal R. Beneficial effects of the naturally occurring flavonoid silibinin on the prostate cancer microenvironment: role of monocyte chemotactic protein-1 and immune cell recruitment. Carcinogenesis. 2016;37(6):589-99.

88. Yeung TL, Leung CS, Wong KK, Samimi G, Thompson MS, Liu J, et al. TGF-beta modulates ovarian cancer invasion by upregulating CAF-derived versican in the tumor microenvironment. Cancer Res. 2013;73(16):5016-28.

89. Lee J, Fassnacht M, Nair S, Boczkowski D, Gilboa E. Tumor immunotherapy targeting fibroblast activation protein, a product expressed in tumor-associated fibroblasts. Cancer Res. 2005;65(23):11156-63.

90. Xia Q, Zhang FF, Geng F, Liu CL, Xu P, Lu ZZ, et al. Anti-tumor effects of DNA vaccine targeting human fibroblast activation protein alpha by producing specific immune

responses and altering tumor microenvironment in the 4T1 murine breast cancer model. Cancer Immunol Immunother. 2016;65(5):613-24.

91. Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. Science. 2015;348(6230):74-80.

92. Hollingsworth RE, Jansen K. Turning the corner on therapeutic cancer vaccines. NPJ Vaccines. 2019;4:7.

93. Liao D, Luo Y, Markowitz D, Xiang R, Reisfeld RA. Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model. PLoS One. 2009;4(11):e7965.

94. Loeffler M, Kruger JA, Niethammer AG, Reisfeld RA. Targeting tumor-associated fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake. J Clin Invest. 2006;116(7):1955-62.

95. Kraman M, Bambrough PJ, Arnold JN, Roberts EW, Magiera L, Jones JO, et al. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. Science. 2010;330(6005):827-30.

96. Wen Y, Wang CT, Ma TT, Li ZY, Zhou LN, Mu B, et al. Immunotherapy targeting fibroblast activation protein inhibits tumor growth and increases survival in a murine colon cancer model. Cancer Sci. 2010;101(11):2325-32.

97. Cazet AS, Hui MN, Elsworth BL, Wu SZ, Roden D, Chan CL, et al. Targeting stromal remodeling and cancer stem cell plasticity overcomes chemoresistance in triple negative breast cancer. Nat Commun. 2018;9(1):2897.

98. Scott AM, Wiseman G, Welt S, Adjei A, Lee FT, Hopkins W, et al. A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer. Clin Cancer Res. 2003;9(5):1639-47.

99. Houthuijzen JM, Jonkers J. Cancer-associated fibroblasts as key regulators of the breast cancer tumor microenvironment. Cancer Metastasis Rev. 2018;37(4):577-97.

100. Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P, Becette V, et al. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. Nat Med. 2009;15(1):68-74.

101. Pickup MW, Mouw JK, Weaver VM. The extracellular matrix modulates the hallmarks of cancer. EMBO Rep. 2014;15(12):1243-53.

102. Shain KH, Dalton WS. Cell adhesion is a key determinant in de novo multidrug resistance (MDR): new targets for the prevention of acquired MDR. Mol Cancer Ther. 2001;1(1):69-78.

103. Soon PS, Kim E, Pon CK, Gill AJ, Moore K, Spillane AJ, et al. Breast cancer-associated fibroblasts induce epithelial-to-mesenchymal transition in breast cancer cells. Endocr Relat Cancer. 2013;20(1):1-12.

104. Yuan J, Liu M, Yang L, Tu G, Zhu Q, Chen M, et al. Acquisition of epithelial-mesenchymal transition phenotype in the tamoxifen-resistant breast cancer cell: a new role for G protein-coupled estrogen receptor in mediating tamoxifen resistance through cancer-associated fibroblast-derived fibronectin and beta1-integrin signaling pathway in tumor cells. Breast Cancer Res. 2015;17:69.

105. Amornsupak K, Insawang T, Thuwajit P, P OC, Eccles SA, Thuwajit C. Cancer-associated fibroblasts induce high mobility group box 1 and contribute to resistance to doxorubicin in breast cancer cells. BMC Cancer. 2014;14:955.

106. Huang J, Ni J, Liu K, Yu Y, Xie M, Kang R, et al. HMGB1 promotes drug resistance in osteosarcoma. Cancer Res. 2012;72(1):230-8.

107. Unsworth A, Anderson R, Britt K. Stromal fibroblasts and the immune microenvironment: partners in mammary gland biology and pathology? J Mammary Gland Biol Neoplasia. 2014;19(2):169-82.

108. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell. 2012;21(3):309-22.

109. Neuzillet C, Tijeras-Raballand A, Cohen R, Cros J, Faivre S, Raymond E, et al. Targeting the TGFbeta pathway for cancer therapy. Pharmacol Ther. 2015;147:22-31.

110. Ziani L, Chouaib S, Thiery J. Alteration of the Antitumor Immune Response by Cancer-Associated Fibroblasts. Front Immunol. 2018;9:414.

111. Takahashi H, Sakakura K, Kudo T, Toyoda M, Kaira K, Oyama T, et al. Cancer-associated fibroblasts promote an immunosuppressive microenvironment through the induction and accumulation of protumoral macrophages. Oncotarget. 2017;8(5):8633-47.

112. Fu Z, Zuo Y, Li D, Xu W, Li D, Chen H, et al. The crosstalk: Tumor-infiltrating lymphocytes rich in regulatory T cells suppressed cancer-associated fibroblasts. Acta Oncol. 2013;52(8):1760-70.

113. Tiainen S, Tumelius R, Rilla K, Hamalainen K, Tammi M, Tammi R, et al. High numbers of macrophages, especially M2-like (CD163-positive), correlate with hyaluronan accumulation and poor outcome in breast cancer. Histopathology. 2015;66(6):873-83.

114. Grossman JG, Nywening TM, Belt BA, Panni RZ, Krasnick BA, DeNardo DG, et al. Recruitment of CCR2(+) tumor associated macrophage to sites of liver metastasis confers a poor prognosis in human colorectal cancer. Oncoimmunology. 2018;7(9):e1470729.

115. Mazur A, Holthoff E, Vadali S, Kelly T, Post SR. Cleavage of Type I Collagen by Fibroblast Activation Protein-alpha Enhances Class A Scavenger Receptor Mediated Macrophage Adhesion. PLoS One. 2016;11(3):e0150287.

116. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454(7203):436-44.

117. Skalniak L, Mizgalska D, Zarebski A, Wyrzykowska P, Koj A, Jura J. Regulatory feedback loop between NF-kappaB and MCP-1-induced protein 1 RNase. FEBS J. 2009;276(20):5892-905.

118. Liang J, Saad Y, Lei T, Wang J, Qi D, Yang Q, et al. MCP-induced protein 1 deubiquitinates TRAF proteins and negatively regulates JNK and NF-kappaB signaling. J Exp Med. 2010;207(13):2959-73.

119. Tanaka H, Arima Y, Kamimura D, Tanaka Y, Takahashi N, Uehata T, et al. Phosphorylation-dependent Regnase-1 release from endoplasmic reticulum is critical in IL-17 response. J Exp Med. 2019;216(6):1431-49.

120. Jura J, Wegrzyn P, Korostynski M, Guzik K, Oczko-Wojciechowska M, Jarzab M, et al. Identification of interleukin-1 and interleukin-6-responsive genes in human monocyte-derived macrophages using microarrays. Biochim Biophys Acta. 2008;1779(6-7):383-9.

121. Liang J, Wang J, Azfer A, Song W, Tromp G, Kolattukudy PE, et al. A novel CCCH-zinc finger protein family regulates proinflammatory activation of macrophages. J Biol Chem. 2008;283(10):6337-46.

122. Akira S. Regnase-1, a ribonuclease involved in the regulation of immune responses. Cold Spring Harb Symp Quant Biol. 2013;78:51-60.

123. Xu J, Peng W, Sun Y, Wang X, Xu Y, Li X, et al. Structural study of MCPIP1 N-terminal conserved domain reveals a PIN-like RNase. Nucleic Acids Res. 2012;40(14):6957-65.

124. Yokogawa M, Tsushima T, Noda NN, Kumeta H, Enokizono Y, Yamashita K, et al. Structural basis for the regulation of enzymatic activity of Regnase-1 by domain-domain interactions. Sci Rep. 2016;6:22324.

125. Xu J, Fu S, Peng W, Rao Z. MCP-1-induced protein-1, an immune regulator. Protein Cell. 2012;3(12):903-10.

126. Mizgalska D, Wegrzyn P, Murzyn K, Kasza A, Koj A, Jura J, et al. Interleukin-1-inducible MCPIP protein has structural and functional properties of RNase and participates in degradation of IL-1beta mRNA. FEBS J. 2009;276(24):7386-99.

127. Mino T, Murakawa Y, Fukao A, Vandenbon A, Wessels HH, Ori D, et al. Regnase-1 and Roquin Regulate a Common Element in Inflammatory mRNAs by Spatiotemporally Distinct Mechanisms. Cell. 2015;161(5):1058-73.

128. Conne B, Stutz A, Vassalli JD. The 3' untranslated region of messenger RNA: A molecular 'hotspot' for pathology? Nat Med. 2000;6(6):637-41.

129. Mayr C. Evolution and Biological Roles of Alternative 3'UTRs. Trends Cell Biol. 2016;26(3):227-37.

130. Schwerk J, Savan R. Translating the Untranslated Region. J Immunol. 2015;195(7):2963-71.

131. Vlasova-St Louis I, Bohjanen PR. Feedback Regulation of Kinase Signaling Pathways by AREs and GREs. Cells. 2016;5(1).

132. Vrotsos EG, Kolattukudy PE, Sugaya K. MCP-1 involvement in glial differentiation of neuroprogenitor cells through APP signaling. Brain Res Bull. 2009;79(2):97-103.

133. Niu J, Shi Y, Xue J, Miao R, Huang S, Wang T, et al. USP10 inhibits genotoxic NF-kappaB activation by MCPIP1-facilitated deubiquitination of NEMO. EMBO J. 2013;32(24):3206-19.

134. Niu J, Wang K, Graham S, Azfer A, Kolattukudy PE. MCP-1-induced protein attenuates endotoxin-induced myocardial dysfunction by suppressing cardiac NF-small ka,

CyrillicB activation via inhibition of Ismall ka, CyrillicB kinase activation. J Mol Cell Cardiol. 2011;51(2):177-86.

135. Mao R, Yang R, Chen X, Harhaj EW, Wang X, Fan Y. Regnase-1, a rapid response ribonuclease regulating inflammation and stress responses. Cell Mol Immunol. 2017;14(5):412-22.

136. Miekus K, Kotlinowski J, Lichawska-Cieslar A, Rys J, Jura J. Activity of MCPIP1 RNase in tumor associated processes. J Exp Clin Cancer Res. 2019;38(1):421.

137. Zhou L, Azfer A, Niu J, Graham S, Choudhury M, Adamski FM, et al. Monocyte chemoattractant protein-1 induces a novel transcription factor that causes cardiac myocyte apoptosis and ventricular dysfunction. Circ Res. 2006;98(9):1177-85.

138. Qi D, Huang S, Miao R, She ZG, Quinn T, Chang Y, et al. Monocyte chemotactic protein-induced protein 1 (MCPIP1) suppresses stress granule formation and determines apoptosis under stress. J Biol Chem. 2011;286(48):41692-700.

139. Ligeza J, Marona P, Gach N, Lipert B, Miekus K, Wilk W, et al. MCPIP1 contributes to clear cell renal cell carcinomas development. Angiogenesis. 2017;20(3):325-40.

140. Lu W, Ning H, Gu L, Peng H, Wang Q, Hou R, et al. MCPIP1 Selectively Destabilizes Transcripts Associated with an Antiapoptotic Gene Expression Program in Breast Cancer Cells That Can Elicit Complete Tumor Regression. Cancer Res. 2016;76(6):1429-40.

141. Ono M. Molecular links between tumor angiogenesis and inflammation: inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy. Cancer Sci. 2008;99(8):1501-6.

142. Roy A, Kolattukudy PE. Monocyte chemotactic protein-induced protein (MCPIP) promotes inflammatory angiogenesis via sequential induction of oxidative stress, endoplasmic reticulum stress and autophagy. Cell Signal. 2012;24(11):2123-31.

143. Sciences SL. Cell Culture Slide II SPL Life Sciences SPL Life Sciences 2021 [Available from: http://www.spllifesciences.com/en/m21.php?cate=1&idx=358.

144. Jacobs JP, Jones CM, Baille JP. Characteristics of a human diploid cell designated MRC-5. Nature. 1970;227(5254):168-70.

145. Fozzatti L, Alamino VA, Park S, Giusiano L, Volpini X, Zhao L, et al. Interplay of fibroblasts with anaplastic tumor cells promotes follicular thyroid cancer progression. Sci Rep. 2019;9(1):8028.

146. Vembadi A, Menachery A, Qasaimeh MA. Cell Cytometry: Review and Perspective on Biotechnological Advances. Front Bioeng Biotechnol. 2019;7:147.

147. Scientific T. Mr. Frosty Freezing Container Thermo Scientific: Thermo Scientific; 2021 [Available from: https://www.thermofisher.com/order/catalog/product/5100-0001#/5100-0001.

148. Industries B. EZ-PCR Mycoplasma Detection Kit Biological Industries: Biological Industries; 2021 [Available from: https://www.bioind.com/worldwide/media/wysiwyg/product/mycoplasma/EZ-PCR-Mycoplasma-Test-Kit-IFU.pdf.

149. Laboratories B-R. Trans-Blot Turbo RTA Mini 0.2 µm PVDF Transfer Kit Bio-Rad Laboratories: Bio-Rad Laboratories; 2021 [Available from: https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10016505D.pdf.

150. Teicher BA, Herman TS, Holden SA, Wang YY, Pfeffer MR, Crawford JW, et al. Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. Science. 1990;247(4949 Pt 1):1457-61.

151. Jain RK. Vascular and interstitial barriers to delivery of therapeutic agents in tumors. Cancer Metastasis Rev. 1990;9(3):253-66.

152. Li XY, Hu SQ, Xiao L. The cancer-associated fibroblasts and drug resistance. Eur Rev Med Pharmacol Sci. 2015;19(11):2112-9.

153. Silzle T, Kreutz M, Dobler MA, Brockhoff G, Knuechel R, Kunz-Schughart LA. Tumor-associated fibroblasts recruit blood monocytes into tumor tissue. Eur J Immunol. 2003;33(5):1311-20.

154. Allaoui R, Bergenfelz C, Mohlin S, Hagerling C, Salari K, Werb Z, et al. Cancer-associated fibroblast-secreted CXCL16 attracts monocytes to promote stroma activation in triple-negative breast cancers. Nat Commun. 2016;7:13050.

155. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature. 2011;475(7355):222-5.

156. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009;29(6):313-26.

157. Cochran BH, Reffel AC, Stiles CD. Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell. 1983;33(3):939-47.

158. Brown Z, Strieter RM, Neild GH, Thompson RC, Kunkel SL, Westwick J. IL-1 receptor antagonist inhibits monocyte chemotactic peptide 1 generation by human mesangial cells. Kidney Int. 1992;42(1):95-101.

159. Yoshimura T, Yuhki N, Moore SK, Appella E, Lerman MI, Leonard EJ. Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. FEBS Lett. 1989;244(2):487-93.

160. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol. 2009;86(5):1065-73.

161. Zhang R, Qi F, Zhao F, Li G, Shao S, Zhang X, et al. Cancer-associated fibroblasts enhance tumor-associated macrophages enrichment and suppress NK cells function in colorectal cancer. Cell Death Dis. 2019;10(4):273.

162. Proost P, Struyf S, Couvreur M, Lenaerts JP, Conings R, Menten P, et al. Posttranslational modifications affect the activity of the human monocyte chemotactic proteins MCP-1 and MCP-2: identification of MCP-2(6-76) as a natural chemokine inhibitor. J Immunol. 1998;160(8):4034-41.

163. Ji WT, Chen HR, Lin CH, Lee JW, Lee CC. Monocyte chemotactic protein 1 (MCP-1) modulates pro-survival signaling to promote progression of head and neck squamous cell carcinoma. PLoS One. 2014;9(2):e88952.

164. Jura J, Skalniak L, Koj A. Monocyte chemotactic protein-1-induced protein-1 (MCPIP1) is a novel multifunctional modulator of inflammatory reactions. Biochim Biophys Acta. 2012;1823(10):1905-13.

165. Uehata T, Iwasaki H, Vandenbon A, Matsushita K, Hernandez-Cuellar E, Kuniyoshi K, et al. Malt1-induced cleavage of regnase-1 in CD4(+) helper T cells regulates immune activation. Cell. 2013;153(5):1036-49.

166. Rosenbaum M, Gewies A, Pechloff K, Heuser C, Engleitner T, Gehring T, et al. Bcl10-controlled Malt1 paracaspase activity is key for the immune suppressive function of regulatory T cells. Nat Commun. 2019;10(1):2352.

167. Wei J, Long L, Zheng W, Dhungana Y, Lim SA, Guy C, et al. Targeting REGNASE-1 programs long-lived effector T cells for cancer therapy. Nature. 2019;576(7787):471-6.

168. Lee CH, Hung PF, Lu SC, Chung HL, Chiang SL, Wu CT, et al. MCP-1/MCPIP-1 Signaling Modulates the Effects of IL-1beta in Renal Cell Carcinoma through ER Stress-Mediated Apoptosis. Int J Mol Sci. 2019;20(23).

T.C.
HACETTEPE ÜNİVERSİTESİ
Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu

Sayı : 16969557-1829
Konu : ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 01 EKİM 2019 SALI
Toplantı No : 2019/23
Proje No : GO 19/955 (Değerlendirme Tarihi: 01.10.2019)
Karar No : 2019/23-22

Üniversitemiz Kanser Enstitüsü Temel Onkoloji Anabilim Dalı öğretim üyelerinden Dr. Öğr. Üyesi Gürcan GÜNAYDIN'ın sorumlu araştırmacı olduğu, Prof. Dr. Ali KONAN, Doç. Dr. Ahmet Bülent DOĞRUL, Doç. Dr. Kemal KÖSEMEHMETOĞLU, Uzm. Bio. M. Emre GEDİK ile birlikte çalışacakları ve Göksu SARIOĞLU'nun yüksek lisans tezi olan, GO 19/955 kayıt numaralı, ***"Tümör Mikroçevresinin Şekillendirilmesinde Regnase-1'in Rolü"*** başlıklı proje önerisi araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş olup, 02 Ekim 2019–02 Ekim 2020 tarihleri arasında geçerli olmak üzere etik açıdan **uygun bulunmuştur.** Çalışma tamamlandığında sonuçlarını içeren bir rapor örneğinin Etik Kurulumuza gönderilmesi gerekmektedir.

1.Prof. Dr. Ayşe Lale DOĞAN	(Başkan)	İZİNLİ 9. Doç. Dr. Fatma Visal OKUR
2. Prof. Dr. Sevda F. MÜFTÜOĞLU		İZİNLİ 10. Doç. Dr. Can Ebru KURT
3. Prof. Dr. M. Yıldırım SARI		İZİNLİ 11. Doç. Dr. H. Hüsrev TURNAGÖL
4. Prof. Dr. Necdet SAĞLAM		12. Dr. Öğr. Üyesi Özay GÖKÖZ
İZİNLİ 5. Prof. Dr. Mintaze Kerem GÜNEL		13. Dr. Öğr. Üyesi Müge DEMİR
6. Prof. Dr. Oya Nuran EMİROĞLU		14. Öğr. Gör. Dr. Meltem ŞENGELEN
7. Prof. Dr. M. Özgür UYANIK		15. Av. Meltem ONURLU
8. Doç. Dr. Gözde GİRGİN		

Hacettepe Üniversitesi Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu
06100 Sıhhiye-Ankara
Telefon: 0 (312) 305 1082 • Faks: 0 (312) 310 0580 • E-posta: goetik@hacettepe.edu.tr
Ayrıntılı Bilgi için:

T.C.
HACETTEPE ÜNİVERSİTESİ
Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu

Sayı : 16969557-1149
Konu :

26.05.2021

Doç. Dr. Gürcan GÜNAYDIN
Kanser Enstitüsü
Temel Onkoloji Anabilim Dalı
Öğretim Üyesi

Sayın Doç. Dr. GÜNAYDIN,

Kurulumuzun 01.10.2019 tarihli toplantısında GO 19/955 kayıt numarası ile onaylanmış olan ve ***"Tümör Mikroçevresinin Şekillendirilmesinde Regnase-1'in Rolü"*** başlıklı projeniz için vermiş olduğunuz 25.05.2021 tarihli protokol revizyonu ve süre uzatma dilekçeniz Kurulumuzun 26.05.2021 tarihli toplantısında değerlendirilmiş ve **uygun bulunmuştur**. Çalışmanın yeni sonlanım tarihi 27 Mayıs 2022 olarak belirlenmiş ve kayıtlarımıza eklenmiştir. Çalışma tamamlandığında sonuçlarını içeren bir rapor örneğinin Etik Kurulumuza gönderilmesi gerekmektedir.

Bilgilerinize rica ederim.

Prof. Dr. Ayşe Lale DOĞAN
Başkan

EK :
Toplantı Katılım Tutanağı

turnitin

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	Goksu Sarioglu
Assignment title:	Thesis
Submission title:	GOKSU SARIOGLU THESIS_20.07.2021
File name:	GOKSU_SARIOGLU_THESIS_20.07.2021.docx
File size:	4.98M
Page count:	86
Word count:	17,416
Character count:	100,848
Submission date:	20-Jul-2021 07:49PM (UTC+0300)
Submission ID:	1622017295

REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

THE ROLE OF REGNASE-1 PROTEIN IN SCULPTING OF TUMOR MICROENVIRONMENT

GOKSU SARIOGLU

TUMOR BIOLOGY AND IMMUNOLOGY PROGRAM
MASTER THESIS

ANKARA
2021

GOKSU SARIOGLU THESIS_20.07.2021

ORIGINALITY REPORT

20%	15%	16%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.nature.com Internet Source	2%
2	www.mdpi.com Internet Source	1%
3	www.openaccess.hacettepe.edu.tr:8080 Internet Source	1%
4	link.springer.com Internet Source	1%
5	m.cafe.daum.net Internet Source	1%
6	www.ncbi.nlm.nih.gov Internet Source	1%
7	"17th International Congress of Immunology, 19–23 October 2019, Beijing, China", European Journal of Immunology, 2019 Publication	1%
8	jeccr.biomedcentral.com Internet Source	1%

CURRICULUM VITAE