Targeting Poor Prognostic CAF Markers in Gastric Cancer

by

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KOÇ
ÜNİVERSİTESİ

Targeting Poor Prognostic CAF Markers in Gastric Cancer

Koç University

Graduate School of Health Sciences

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and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
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Date: January 15th, 2024

Dedicated to Cemre Uçaryılmaz Metin

ABSTRACT

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Gastric cancer is the 5th most common cancer worldwide and the 4th leading cause of cancer deaths. Although highly cytotoxic chemotherapies are used in the clinic, curative strategies for advanced gastric cancer remain limited. Cancer-associated fibroblasts (CAFs) are the key elements of the tumor microenvironment that secrete various extracellular matrix proteins to increase tumor aggressiveness. Patients with CAF phenotype in their tumor microenvironment exhibit poor survival, prognosis, and chemoresistance. Recently through a bioinformatic study we identified poor prognostic markers associated with CAFs in gastric cancer, that decrease the patient survival significantly. Unraveling tumor-driving phenotypes associated with these markers of CAFs can show great promise in the treatment of gastric cancer. Therefore, in this study, we investigate the poor prognostic effects of CAFs using co-culture models of fibroblasts or patient-derived CAFs with gastric cancer cells.

ÖZETÇE

Mide Kanserinde Kötü Prognostik Kanserle İlişkili Fibroblastlar

Ece Özmen

Master of Science in Cellular and Molecular Medicine

15 Ocak 2024

Mide kanseri dünya çapında en sık görülen 5. kanser olup, kanserden ölümlerin 4. nedenidir. Klinikte yüksek oranda sitotoksik kemoterapiler kullanılmasına rağmen ileri evre mide kanserine yönelik tedavi edici stratejiler sınırlı kalmaktadır. Kanserle ilişkili fibroblastlar (KAF'lar), tümör agresifliğini arttırmak için çeşitli hücre dışı matris proteinlerini salgılayan tümör mikro ortamının temel unsurlarıdır. Tümör mikro-çevresinde KAF fenotipine sahip hastalar yüksek ölüm oranları, kötü hastalık seyri ve de kemoterapiye karşı direnç sergilemektedir. Yakın zamanda yaptığımız biyoinformatik çalışma ile mide kanserinde KAF'ların hastanın hayatta kalma oranını önemli ölçüde azaltan, kötü hastalık seyri gösteren belirteçler tespit ettik. Bu KAF belirteçleriyle ilişkili tümöre yol açan fenotiplerin çözülmesi, mide kanseri tedavisinde büyük umut vaat etmektedir. Bu nedenle, bu çalışmada, hastalardan türetilmiş kötü hastalık seyri gösteren belirteçlere sahip KAF'ların ya da fibroblast hücre hatlarının mide kanseri hücreleriyle birlikte kültür modellerini kullanarak kötü hastalık seyri fenotiplerini araştırmaktayız.

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ABBREVIATIONS

AJCC	American Joint Committee on Cancer
apCAF	Antigen-presenting CAF
RGD	Arginine-glycine-aspartate
ACRG	Asian Cancer Research Group
CAFs	Cancer-associated fibroblasts
CXCLs	Chemokine Ligands
DEG	Differentially Expressed Genes
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
EMT	Epithelial-Mesenchymal Transition
EndMT	Endothelial-Mesenchymal Transition
EGF	Epithelial Growth Factor
FBS	Fetal Bovine Serum
FN-1	Fibronectin-1
FAP	Fibroblast Activating Protein
FGF	Fibroblast Growth Factor
FSP-1	Fibroblast Specific Protein-1
GEJ	Gastro-oesophageal junction
HR	Hazard ratio
HGF	Hepatocyte Growth Factor
IC50	Half maximal inhibitory concentration
LOX	Lysyl Oxidases
MMP	Matrix metalloproteinases
miRNAs	MicroRNAs
myCAF	Myofibroblast CAF
PAGE	Polyacrylamide gel electrophoresis
PDAC	Pancreatic ductal adenocarcinoma
P/S	Penicillin/Streptomycin
PDGF	Platelet-derived Growth Factor
PDGFRα	PDGF Receptor α

PPI	Protein-Protein-Interaction
SPARC	Secreted Protein Acidic and Rich in Cysteine
scRNA-seq	Single cell RNA-sequencing
SDF-1	Stromal Cell Derived Factor-1
TIMPs	Tissue Inhibitors of MMPs
iCAF	Inflammatory CAF
IL	Interleukins
TGF-β	Transforming Growth Factor-β
TNF	Tumor Necrosis Factor
TS	Thymidylate Synthase
TME	Tumor microenvironment
WHO	World Health Organization
5-FU	5-Fluorouracil

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Chapter 1: **INTRODUCTION**

1.1.Gastric Cancer

1.1.1. Epidemiology and Classification of Gastric Cancer

Gastric cancer is one of the most prevalent cancer types. In 2020, with 1.1 million cases worldwide, it is estimated to be the 5th leading cancer type and 4th common cause of death (Sung et al., 2021) (Figure 1.1). Although most cases are recorded in Eastern Asia for both sexes and age groups, the overall incidence is higher in males, accounting for 66% of the cases (Morgan et al., 2022).

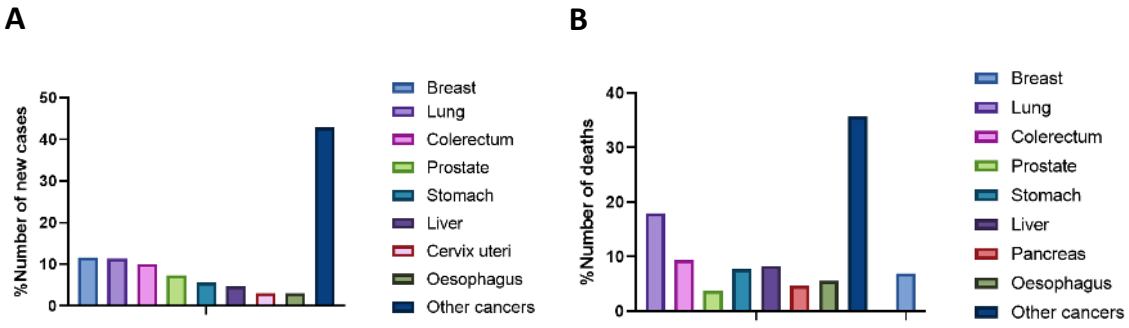


Figure 1.1 Epidemiology of gastric cancer in 2020 **A.** Number of cases. **B.** Number of deaths. (Sung et al., 2021).

There are anatomical, histopathological, and molecular classifications of gastric cancer. Anatomically, tumors located in non-cardia (gastric body, antrum, pylorus) and cardia are termed gastric adenocarcinoma. According to the Siewert Type classification, tumors are classified depending on the location relative to the gastro-oesophageal junction (GEJ) (Siewert et al., 2006) . However, the involvement of the GEJ is critical in the determination of the cancer type. For a better classification, the American Joint Committee on Cancer (AJCC) proposed a TNM classification that incorporates the epicenter of the tumor as a determinant of the cancer type. The AJCC classifies tumor epicenters that are distant to more than 5 cm to GEJ or within the 5 cm of GEJ but not extending to GEJ as gastric cancer (Edge and American Joint Committee on Cancer, 2010).

Laurén classification is the most widely used histopathological classification that enables to classify patients prognostically. Diffuse and intestinal are the two subtypes identified by the Laurén classification, although some cases exhibit a mix of these two types (Lauren, 1965). Diffuse type is characterized with gastric cancer cells that are poorly differentiated and lack cohesiveness (Cisło et al., 2018). Signet-ring cell carcinoma is characterized with diffuse subtype with high intracellular mucus deposition that pushes the nucleus to the cell periphery (Ma et al., 2016). Diffuse type is observed more commonly in females and at a younger age, has a worse prognosis compared to intestinal type, and arise due to genetic aberrations (Ma et al., 2016). Intestinal type is more common than diffuse type (54%), observed mostly in males, have a better prognosis, and occur due to environmental factors like *Helicobacter pylori* infection (Cisło et al., 2018). Intestinal gastric cancer cells show adhesion, have tubular and glandular appearance (Ma et al., 2016). The two histopathology is represented in Figure 1.2.

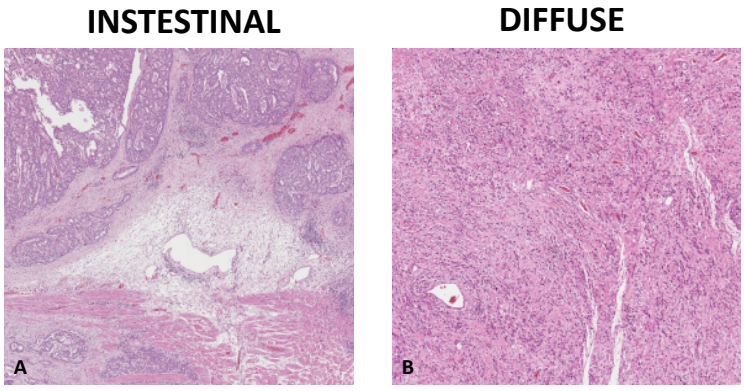


Figure 1.2 Laurén classification of gastric cancer. **A.** Intestinal type, glandular structures. Hematoxylin & Eosin staining, original magnification. **B.** Diffuse type, non-cohesive cancer cells. Hematoxylin & Eosin staining. (Dictionary - Pathology: Stomach cancer - The Human Protein Atlas, n.d.)

There are number of risk factors associated with gastric cancer; salty diet, alcohol consumption, smoking, *H. pylori* infection, genetic defects, age, and sex of the patient, are considered as major risk factors (Yusefi et al., 2018). Although, there is a decline in the

H.pylori caused gastric cancer, the incidence of younger patients is increasing which may be due to dietary choices (Morgan et al., 2022).

The Cancer Genome Atlas (TCGA) categorizes gastric cancer depending on the differences in molecular characteristics. The classification depends on high-throughput analysis of sequencing data and it identifies four subtypes of gastric cancer (Wang et al., 2019b). These are Eppstein-Bar Virus positive (8.8%), microsatellite-instable (MSI) (21.7%), genome stable (19.7%), and chromosomal instability tumors (49.8%) (Cancer Genome Atlas Research Network, 2014). The correlation between the molecular subtypes and clinical phenotypes is explained by the Asian Cancer Research Group (ACRG). According to the ACRG classification, diffuse type is associated with micro-satellite stable and epithelial-to-mesenchymal-transition (MSS/EMT) subtype, showing the worst prognosis and elevated recurrence rate (Cristescu et al., 2015).

Recent research has emphasized the identification of gene signatures and pathways to classify gastric cancer prognostically. One study further classified the diffuse and intestinal Lauren classifications into two molecular subtypes: core diffuse-type (COD) and intestinal-type-like (INT) gastric cancer. The study revealed that these subtypes are enriched in different gene clusters, therefore showed opposite characteristics. COD subtype was associated with worse clinical outcomes due to increased recurrence rates and a diminished survival rate (Kim et al., 2020). In contrast, INT subtype was linked with diminished levels of TGF-β and epithelial-mesenchymal transition (EMT) related genes, increased levels of DNA damage response genes, and better response to immune checkpoint inhibitors. Deciphering the variations in gene signatures of two molecular subtypes are necessary to develop treatments accordingly.

1.1.2. Treatment Approaches for Gastric Cancer

For the treatment of localized gastric cancer, radical surgery as gastrectomy or systematic lymphadenectomy is preferred for resectable tumors (Guan et al., 2023). Neoadjuvant or adjuvant chemotherapy is also employed with radical surgery (Joshi and Badgwell, 2021). Treatment of metastatic gastric cancer is comprised of varying cytotoxic agents like

fluoropyrimidines, platinums, taxens, anthracyclines like doxorubicin, and irinotecan (Joshi and Badgwell, 2021). Although treatment regimens for the first line treatment vary between countries, combination treatment of fluoropyrimidines and platinums is highly preferred, especially 5-Fluorouracil (5-FU) and cisplatin (Digklia and Wagner, 2016). The second-line treatment of gastric cancer aims to control the symptoms and increase the survival. For this purpose, application of taxanes and irinotecan as single therapy is widely accepted (Digklia and Wagner, 2016). Also, targeted therapies to HER-2 or VEGF is common to be used as a second line treatment (Joshi and Badgwell, 2021).

5-FU, is an analogue of uracil with an additional fluorine, damages DNA or RNA by incorporating itself (Longley et al., 2003). The main mechanism of action of 5-FU depends on inhibition of nucleotide synthesis enzyme Thymidylate Synthase (TS) functioning through forming a stable complex with nucleotide binding region of TS (Ma et al., 2018b). In contrast, paclitaxel exerts its effect by stabilizing the microtubules by binding irreversibly to the tubulin units, promotes the assembly of mitotic spindles and leads to malfunction (Jimenez et al., 2011). Therefore, it interferes with cell division and migration processes.

1.2. The Role of Cancer Stroma and Tumor Microenvironment in Gastric Cancer

Even though genetic aberrations like oncogene activation and inhibition of tumor suppressor genes are associated with development of malignancies, they are not the only contributors of this process. In fact, the surrounding cancer stroma has an immense impact on the initiation and development of carcinogenesis. Cancer cells are not independent clusters of cells, instead, they are part of a dynamic network comprised of a variety of cells and a supportive extracellular matrix (ECM). This composition of cellular and non-cellular elements is called the tumor microenvironment (TME), or tumor stroma as depicted in Figure 1.3 below. The TME is known to be a fertile soil for tumor initiation and progression. Paget suggested that cancer cells, as seeds, require a supportive environment for malignancies to develop (Langley and Fidler, 2011). Therefore, the composition of stroma highly matters for cancer progression and growth. Equilibrium state of the normal stroma is altered by the cancer cells. This

relationship between cancer cells and the stroma is reciprocal, together they trigger tumor initiation and progression. TME is highly influenced by cellular secretions and physical forces generated by cells, providing cancer cells a perfect environment to proliferate and spread. Cancer cells induce stromal and immune cells residing in the TME, to activate signaling cascades that drive tumor progression. These include the hallmarks of cancer, which are enhanced migratory and invasion capabilities, escaping from immune destruction, altered cellular metabolism, induction of angiogenesis, and tumor promoting inflammation (Wang et al., 2017).

Recent studies highlight the significance of TME in the progression of gastric cancer. Chronic inflammation and *H.pylori* infection highly potentiates the activation of surrounding epithelial gastric cells and induces them to activate tumorigenic pathways (Chung and Lim, 2014). Therefore, inflamed nature of the stroma becomes a fruitful environment for cancer cells and other stromal residents. Resistance to therapy, increased stemness properties, invasion and metastatic spread are induced by the TME, giving rise to aggressive and advanced stage gastric cancer (Chung and Lim, 2014; Monster et al., 2022). Cancer-associated fibroblasts (CAFs), endothelial cells, and various immune cells are known to promote tumorigenesis of gastric cancer (Oya et al., 2020). These cells are reprogrammed by gastric cancer cells, also stimulated by each other, and collectively worsen the prognosis of disease. Soluble factors like chemokines, cytokines, growth factors, and ECM modifying enzymes are effective in exerting such outcomes.

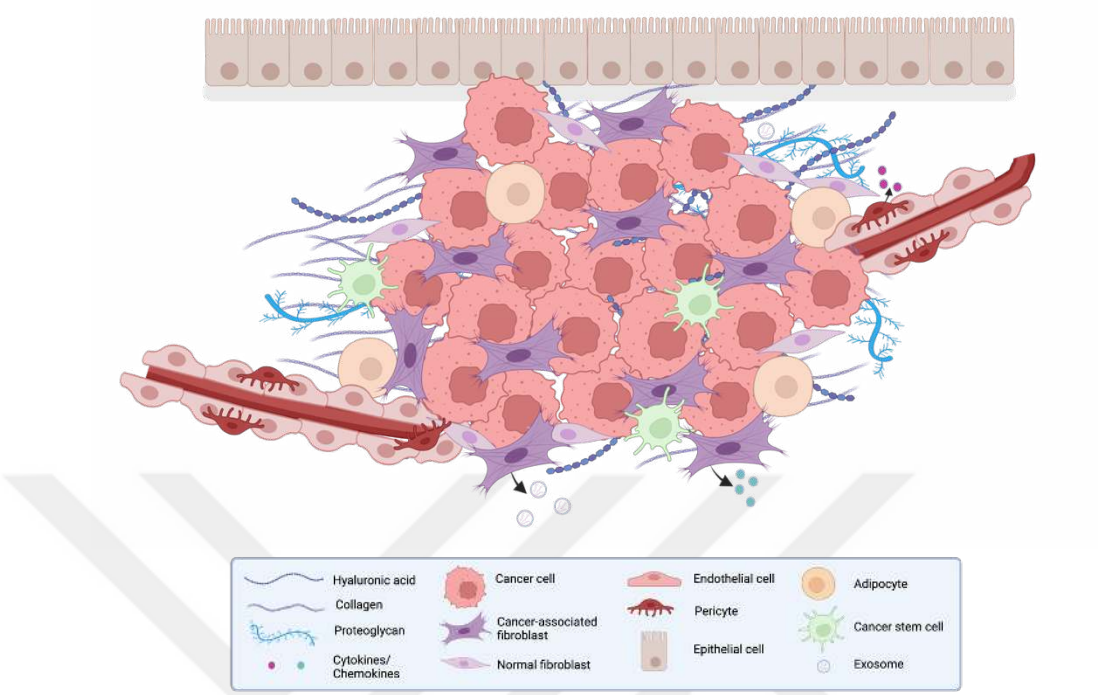


Figure 1.3 Complex nature of TME involves both cellular and non-cellular components. Cytokines, chemokines, and exosomes secreted by CAFs induce cancer cells and contribute to the tumorigenic phenotype.

1.2.1. Cellular Components of the Tumor Microenvironment

The cellular composition of the TME is diverse. Immune cells including macrophages, tumor-associated macrophages, T, B, and Natural killer cells are present in the TME with altered functions that allows immune suppression. Apart from these, endothelial, mesenchymal, cancer stem cells, resident epithelial cells, adipocytes, pericytes, fibroblasts, and CAFs comprise the cellular component of TME (Bożyk et al., 2022). Although these cells have divergent roles in promoting tumorigenesis, they all employ paracrine signaling, cell to cell or cell to ECM contacts to induce tumorigenesis.

1.2.2. Non-cellular Components of the Tumor Microenvironment

Cancer stroma is distinct from the normal stroma. The transition to tumorigenic stroma is achieved by the altered secretory profiles of cells, reorganization, and increased production of ECM proteins. The non-cellular components of TME include cytokines, growth factors,

chemokines, metabolites, ECM proteins, exosomes, micro-RNAs (miRNAs) and matrix metalloproteinases (MMPs) (Patel et al., 2018). In normal tissues, ECM is in an equilibrium state of degradation and building up. In contrast, cancerous tissues as wounds that never heal, secrete inflammatory signals that alters the stromal content to allow tissue turnover (Flier et al., 1986). In fact, CAFs contribute to constant accumulation of ECM by producing non-cellular components of the TME (Wei et al., 2022a). In turn, stiff matrix causes activation of more fibroblasts and leads to production of complex ECM proteins even more (Ruiz-Zapata et al., 2020). Consequently, the TME serves as a deposit enriched with ECM proteins and proliferation stimulating factors for cancer and other residing cells.

ECM proteins constitute most of the non-cellular component of TME. They are mainly divided into two groups: proteoglycans and fibrous proteins (Frantz et al., 2010). Proteoglycans are defined as proteins that are linked to large polysaccharide chains called glycosaminoglycans (Yanagishita, 1993). Aggrecan, versican, neurocan, and brevican are known as ECM proteoglycans and bind to hyaluronan (Barkovskaya et al., 2020). They form hydrogels in the matrix due to their hydrophilic properties, therefore create a barrier against forces (Couchman and Pataki, 2012). In particular, versican is involved in angiogenesis, invasion, metastasis, and associated with poor clinical outcomes in different cancer types (Ricciardelli et al., 2009).

Fibrous proteins of ECM involve collagens, fibronectin, elastin, and laminin (Frantz et al., 2010). Collagens are the most common type of proteins located in ECM, there are approximately 28 subtypes of collagen (Sun, 2021). Although their common structure is comprised of triple helix of three polypeptide alpha chains, their molecular composition varies according to the subtypes (Gelse et al., 2003). COL1A1, COL1A2, COL3A1, and COL5A1 belong to fibril-forming collagen subtype and variations of COL4A belong to basement membrane collagen subtype (Gelse et al., 2003). High deposition of collagen is a characteristic feature of TME, these thickened collagens create a path for cancer cells to migrate and invade easily (Wyckoff et al., 2007). Laminin, another fibrous ECM protein, associates with type IV collagen to create a basis for basement membrane, which allows other proteins like secreted protein acidic and rich in cysteine (SPARC), tenascins or osteopontin to reside (Popova and Jücker, 2022). SPARC is also a critical protein in ECM, it is known

for regulating ECM turnover, cell adhesion, proliferation, and survival depending where its located in cell (Wong and Sukkar, 2017). Laminin anchoring is known to play role as a tumor suppressor, however, loss of laminin is characterized with poor prognosis due to increased cell proliferation and deregulated cell polarization (Akhavan et al., 2012). Lysyl oxidase (LOX) enzyme family catalyzes elastin and collagen crosslinking, therefore more robust fibers are obtained, which allows enhanced invasion of cancer cells (Levental et al., 2009). Fibronectins, as members of fibrous proteins, have many roles like interstitial ECM organization, regulating cell attachment and functions (Frantz et al., 2010). In fact, fibronectin is vital for providing cell to ECM communications since they are major proteins for integrin binding, which causes activation of further downstream pathways (Brücher and Jamall, 2014). For instance, fibronectin is required for the activation of latent Transforming Growth Factor-β (TGF-β) by binding to integrin αvβ6, which is an abundant growth factor in TME and known for its tumor promoting roles like inducing cell proliferation and chemoresistance (Fontana et al., 2005). A study revealed that expression of Fibronectin 1 (FN1), gene encoding for fibronectin, and SPARC are associated with poor prognosis in gastric cancer (Li et al., 2019)

1.3. Cancer-associated Fibroblasts are Vital in the Tumor Microenvironment

Recent evidence has shown that CAFs have an immense role on the progression of solid cancers. They regulate chemoresistance mechanisms, induce cancer stem cell generation, increase proliferation, invasion and metastasis in many type of solid cancers (Gandellini et al., 2015). A single cell sequencing study have revealed that CAFs are the second highest cell population after epithelial tumor cells at the primary site of pancreatic ductal adenocarcinoma (PDAC) tumors (Lin et al., 2020). In gastric cancer, stromal-enriched genes are found to be more powerful compared to immune-enriched genes in the progression of disease due to its association with poor prognosis (Li and Wang, 2021). Also, CAFs infiltration is associated with poor survival in TME of gastric cancer (Zeng et al., 2019). Among other solid cancers like bladder or kidney renal papillary cell carcinomas, gastric cancer is found to have a higher risk for the poor prognostic impact of CAFs (Ucaryilmaz Metin and Ozcan, 2022a). Therefore, deciphering the influence of CAFs on gastric cancer progression highly matters to prevent challenges faced in the treatment.

1.4. Cancer Associated Fibroblast Characteristics and Their Pro-tumorigenic Contribution

In normal tissue, fibroblasts are known to be in resting state and perform no significant metabolic activity unless they receive an activation signal, in response to inflammation and wound healing (Kalluri, 2016). The TME is abundant of these signals, stimulate the activation of fibroblasts and cause them to gain myofibroblastic CAF properties. However, CAFs not only derive from fibroblasts. Epithelial cells give rise to CAFs through EMT, similarly endothelial cells can differentiate into CAFs by Endothelial-Mesenchymal Transition (EndMT), bone marrow mesenchymal stem cells, cancer stem cells, adipocytes, and pericytes are also able to transdifferentiate into CAFs (Sun et al., 2022). Main CAF activating signals are bone morphogenetic protein, interleukins (IL) 1 and 6, TGF-β, Platelet-derived Growth Factor (PDGF), Tumor Necrosis Factor (TNF), Chemokine Ligands (CXCLs), miRNA containing exosomes, various other cytokines and chemokines (Wang et al., 2021). Activated fibroblasts are mainly characterized by the expression of α-smooth muscle actin protein, encoded by ACTA2 gene, Fibroblast Activating Protein (FAP), PDGF Receptor α (PDGFRα), vimentin, Fibroblast Specific Protein-1 (FSP-1) (Nurmik et al., 2020). In addition to these, there are cancer-type specific markers associated with activated CAFs (Han et al., 2020). For instance, in gastric cancer caveolin-1, a membrane protein that regulates cell survival and motility, is discovered to be linked with CAF activation (Shen et al., 2015). CAFs are known to promote carcinogenesis by promoting cell proliferation, inducing stemness in cancer cells, preventing cell death, stimulating migration and invasion, ECM remodeling, increasing angiogenesis, and desensitizing cancer cells to chemotherapy (Sun et al., 2022). Soluble factors like chemokines, cytokines, exosomes, are mediators of these events. However, not every activated CAF exhibit same tumor promoting phenotypes due to high cellular heterogeneity. Using state-of-the-art single cell RNA-sequencing (scRNA-seq) technique, subtypes of CAFs have been revealed. A study about PDAC cancer identified three different subtypes of CAFs as myofibroblast (myCAF), inflammatory (iCAF), and antigen-presenting CAFs (apCAF), each showing differences in their transcriptional activities (Hu et al., 2022). The study highlights that although iCAF population has a protective phenotype, myCAF is associated with poor prognosis (Hu et al., 2022). Whereas in gastric cancer, iCAF is associated with stemness-promoting characteristics due to high

expression of IL-6, CXCL1, and CXCL2 (Kim et al., 2022). Therefore, CAFs have a heterogeneous nature and employ different molecular functions to drive tumor progression.

1.4.1. The Secretome of Cancer-associated Fibroblasts

Although CAF secretions differ between subpopulations, they achieve to activate cancer promoting mechanisms and recruit other cell types through their secretome. The secretory profile of CAFs is particularly influenced by the cellular and non-cellular components of TME, as well as their location in the spatial pattern of TME (Linares et al., 2021). In response to the impact of TME elements, CAFs implement paracrine and autocrine signaling. TGF-β and Stromal Cell Derived Factor-1 (SDF-1), two mediators of fibroblast activation, are secreted by fibroblasts in autocrine manner. Thus, transdifferentiation of fibroblasts into CAFs is promoted, which leads to subsequent expression of these factors and further fibroblast activation (Kojima et al., 2010). Other than TGF-β, paracrine secretion of CAFs include PDGF, Fibroblast Growth Factor (FGF), Hepatocyte Growth Factor (HGF), PDGF, and Epithelial Growth Factor (EGF), each induces cancer cells to gain malignant phenotypes like chemoresistant, invasive, and proliferative properties (Fang et al., 2022). CAFs also secrete cytokines and chemokines like IL-6, CXCL-1, CXCL-2 to exert these effects (Kim et al., 2022). A wide range of CAF derived miRNAs encapsulated by extracellular vesicles has been identified to affect tumorigenesis as well (Fang et al., 2022). Apart from soluble signals received by CAFs, physical signals also potentiate secretory profile of CAFs to favor a stiffer ECM (Calvo et al., 2013). Collagens, secreted by CAFs constitute most of the ECM (Okumura et al., 2019; Sun, 2021). A recent study revealed that in solid tumors like PDAC, stomach, and breast cancer, expression of almost all fibrillar collagens upregulated and were associated with increased risk (Thorlacius-Ussing et al., 2024). The study also highlights that collagen expression in PDAC was attributed to fibroblasts which were further categorized into CAF subtypes. Although expression patterns of collagen vary among the iCAF and myCAF subtypes, both subtypes expressed COL1A1, COL3A1, and COL5A1 fibrillar collagens (Thorlacius-Ussing et al., 2024). Other than collagens, CAFs produce ECM proteins like fibronectin, laminin, and hyaluronan (Sapudom et al., 2020; Wei et al., 2022b).

Elevated levels of ECM proteins in TME enable cancer promoting signaling pathways and create a stiffer, hypoxic environment that drives tumor progression (Gilkes et al., 2014).

1.4.2. Contribution to Extracellular Matrix Remodeling

CAFs remodel the TME not only by producing complex ECM proteins, but also through secreting matrix modifying enzymes, which are known as Matrix Metalloproteinases (MMPs) and crosslinking Lysyl Oxidases (LOX) (Liu et al., 2019). Fibroblasts, which are activated upon cell-to-cell contact, induce proteolytic activity through the expression of MMPs (Sirén et al., 2006). MMPs induce pro-tumorigenic events like invasion and migration, angiogenesis (Jabłońska-Trypuć et al., 2016). LOX family catalyzes the crosslinking of collagen with elastin proteins, turning ECM into a stiffer and denser structure (Liu et al., 2019). In stiff matrices, abundant YAP/TAZ transcription factors induce CAFs to maintain their tumorigenic functions and activate positive feedback loop for YAP that further increases the matrix stiffness (Calvo et al., 2013). TGF-β, as an activator of YAP, also contributes to matrix stiffening through altered MMP and collagen production in fibroblasts (Feng et al., 2013).

1.4.3. Contribution to Migration and Invasion of Cancer Cells

Metastasis, start within the TME and if completed successfully, end up at distant tissues. Migration and invasion, two critical events of metastasis, are immensely influenced by the stromal members and especially by CAFs. Initially cells escape the tumor epicenter and migrate, by losing their cell-to-cell contacts and obtaining EMT characteristics like losing E-cadherin and gaining N-cadherin expression (van Zijl et al., 2011). CAFs are the main contributors to EMT in different cancer types, and they induce EMT by the activation of TGF-β/SMAD pathway (Chandra Jena et al., 2021; Szabo et al., 2023). Increased Fibroblast Growth Factor (FGF) secretion by fibroblasts is received by cancer cells through FGF-receptor and N-cadherin co-localization, which activates SRC downstream signaling, promotes integrin αvβ5 mediated migration and adhesion of colon cancer cells (Knuchel et al., 2015). In gastric cancer, CAF derived IL-11 stimulates migration as well by the activation of JAK/STAT3 and MAPK/ERK pathways (Wang et al., 2018). CXCL12 chemokine

secreted by CAFs, binds to CXCR4 and stabilizes integrin clustering with ECM proteins and activates FAK downstream signaling to promote invasion (Izumi et al., 2016). CAF derived MMPs and other ECM modifying enzymes like hyaluronan and proteoglycan link protein 1 (HAPLN1), are upregulated in CAFs, modify the dense structure of ECM and allow cancer cells to move along the TME (Liu et al., 2019; Zhang et al., 2022). Gastric CAFs induce an actin binding protein mediated contraction and invasion of cancer cells, which also influences MMP2 production (Yu et al., 2013). Therefore, in addition to enzymatic degradation of ECM, CAFs exert mechanical forces to mediate invasion of cancer cells (Yu et al., 2013; Yamaguchi et al., 2014; Zhang et al., 2022).

1.4.4. Contribution to Chemoresistance

In cancer, drug resistance either develops intrinsically or acquired by continuous exposure to the drug. Chemoresistance is associated with poor prognosis, recurrence of the disease, and decreased survival rates in different types of cancers (Kim et al., 2011; Zhang et al., 2021). CAFs facilitate the chemoresistance by upregulating expression of the drug transporter proteins, enhanced DNA repair mechanisms, inhibition of apoptosis, boosted survival signaling, and increased ECM stiffness (Jena et al., 2020). In gastric cancer, CAFs increase the cell survival and reduce apoptosis in response to 5-FU through secreting IL-6 (Ham et al., 2019). Resistance to cisplatin and doxorubicin is also mediated by CAFs by the secretion of IL-11 and subsequent inhibition of apoptosis in gastric cancer (Ma et al., 2018a). CAFs induce expression of the ATP binding cassette (ABCB1), also known as P-glycoprotein, through secreting IL-8 and Insulin-like Growth Factor 2 (IGF2) in gastric and non-small lung cancers respectively (Zhang et al., 2018; Zhai et al., 2019). In addition to these soluble factors, stiff ECM enriched with complex proteins contribute to drug resistance by preventing drug penetration, decreased nutrient supply, and inhibition of cytotoxic drug response (Henke et al., 2020). Elevated levels of collagen deposition and collagen crosslinker LOX, promote CAFs to secrete exosomes that induce drug resistance (Xiao et al., 2022). Hypoxic tumor epicenters, due to dense ECM architecture, is deficient in nutrients and oxygen (Ucaryilmaz Metin and Ozcan, 2022b). In hypoxic environments, CAFs further potentiate resistance to 5-FU by secreting Tissue Inhibitors of MMPs (TIMPs) (Jiang et al., 2023).

1.5. Poor Prognostic Markers of Gastric Cancer

Although CAFs are known to have a great impact on the progression of gastric cancer, more effort is required to address the significance of CAF related genes and their prognostic effect on the disease. Recently our lab group has identified a poor prognostic gene signature of CAFs in gastric cancer. The study highlights the importance of extracellular matrix components secreted by CAFs and holds a great promise for CAF targeted therapies. The functional annotation analysis of the Differentially Expressed Genes (DEG) between healthy adjacent and tumor tissues of gastric cancer has revealed ECM related pathways the most significant (Ucaryilmaz Metin and Ozcan, 2022a). Among DEGs, 8 upregulated genes overlapped with known CAF markers. Subsequent Protein-Protein-Interaction (PPI) analysis revealed COL1A1, COL1A2, COL3A1, COL5A1, FAP, FN1, and SPARC proteins to be the 6 highest interacted proteins with highest centrality among others (Ucaryilmaz Metin and Ozcan, 2022a). These 6 markers correlated with CAF infiltration, tumor stage, grade, and low overall survival (Ucaryilmaz Metin and Ozcan, 2022a). In addition, these markers are expressed more in mesenchymal phenotype rather than in epithelial, which is associated with CAF infiltration in gastric cancer (Ucaryilmaz Metin and Ozcan, 2022a). The study also highlights that although these markers are upregulated in different solid cancers, the poor prognostic effect of the markers is only specific to gastric cancer (Ucaryilmaz Metin and Ozcan, 2022a). Therefore, in this study we aimed to target these poor prognostic CAF markers and investigate the *in vitro* poor prognostic effects in gastric cancer.

Chapter 2: **METHODS**

2.1. Cell Culture and Other Reagents

In this study SNU484 and NUGC4 cell lines were used as gastric adenocarcinoma model. SNU484 was purchased from Korean Cell Line Bank. NUGC4 gastric cancer cell and BJ normal foreskin fibroblast cell line and MRC-5 normal lung fibroblast cell line were purchased from American Type Culture Collection. RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Biowest, France) and 1% Penicillin/Streptomycin (P/S) (Gibco, USA) was used for culturing gastric adenocarcinoma cell lines. Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 15% FBS and 1% P/S was used for culturing BJ fibroblast cell line and DMEM supplemented with 10% FBS and 1% P/S was used for culturing MRC-5 fibroblast cell line. All cell lines were incubated in 37°C, 5% CO_2 incubator. For co-culture experiments RPMI-1640 supplemented with 15% FBS and 1% P/S was used.

2.2. Co-culture Experiments

Investigation of the poor prognostic markers and the effect of fibroblasts on gastric cancer cells' survival was carried out integrating direct contact and no contact co-culture systems as seen in Figure 2.1 below. Experiments were carried out in three groups: monoculture and co-culture groups of gastric cancer and fibroblast cells. Direct co-culture groups were seeded in 1:1 ratio of gastric cancer and fibroblast cells.

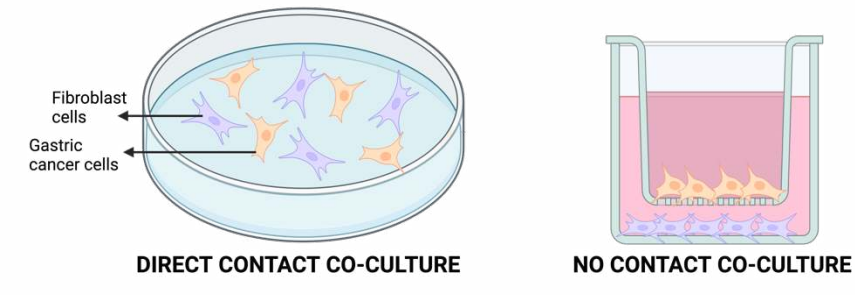


Figure 2.1 Co-culture systems used for investigating the poor prognostic markers and the effects of fibroblasts on gastric cancer cells' survival. Created by using Biorender.com.

2.2.1. qRT-PCR

Investigation of relative gene expression of CAF and poor prognostic markers was determined using qRT-PCR. Monoculture and co-culture of gastric cancer and fibroblast cells were used for RNA isolation. $1x10^6$ of cells were seed in 10 cm petri-dishes for mono and co-culture groups. RNA isolation was performed using RNA isolation kit following manufacturer's instructions (Macharey-Nagel, Germany). RNA concentrations were measured using Nanodrop.

1000 ng of total RNA was combined with 2.5 µl of 2 mM dNTPs (Thermo Fisher Scientific, USA) and 1µl of random hexamers (Invitrogen, USA). Total volume was completed to 16.5 µl with nuclease free water. The reaction was incubated in 65°C for 5 minutes. Then, 5 µl of 5x First Strand Buffer (Invitrogen, USA), 2 µl of 0.1M DTT (Invitrogen, USA), and 0.5 µl of RNAsin (Promega, USA) were added to each tube. Tubes were incubated for 10 minutes at room temperature and then 1 µl of MMLV-RT (Invitrogen, USA) enzyme was added to each tube. Tubes were incubated at 37°C for 1 hour, and then at 70°C for 15 minutes. After the incubation, 75 µl of nuclease free water was added. For qRT-PCR experiment 10 µl of 2x SYBR Green Master Mix (Qiagen, Germany), 1 µl of 5 µM reverse and forward primer mix, 2 µl of cDNA, and 7 µl of nuclease free water was mixed in a 96 well optical plate (Nest, China). GAPDH was used as housekeeping gene and to calculate relative expression levels of FAP, ACTA2, FN1, SPARC, COL1A1, COL1A2, COL3A1, and COL5A1 genes. For each gene of interest, experiment was performed in triplicates. The plate was sealed and centrifuged at 1300 rpm for 2 minutes. LightCycler 480 instrument (Roche, Switzerland) was used for qRT-PCR. The reaction took place in following conditions: pre-heating for 5 minutes at 95°C, amplification for 45 cycles of 10 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C, melting for 1 cycle of 10 seconds at 95°C and 65°C for 1 minute and cooling for 1 cycle of 30 seconds at 40°C. Relative gene expressions were calculated by using ΔΔCt method. Reverse and forward primers of genes of interest are provided in Table 2.1 below.

Table 2.1 Primer sequences used in the qRT-PCR experiment to amplify cDNAs

	Forward 5' > 3'	**Reverse 5'>3'**
FAP	TGA ACG AGT ATG TTT GCA GTG G	GGT CTT TGG ACA ATC CCA TGT
ACTA2	AAA AGA CAG CTA CGT GGG TGA	GCC ATG TTC TAT CGG GTA CTT C
SPARC	TGA GGT ATC TGT GGG AGC TAA TC	CCT TGC CGT GTT TGC AGT G
FN1	GGT GAC ACT TAT GAG CGT CCT AAA	AAC ATG TAA CCA CCA GTC TCA TGT G
COL1A1	GAG ACC TGC GTG TAC CCC ACT	GTC ATG CTC TCG CCG AAC CAG
COL1A2	GTG GCA GTG ATG GAA GTG TG	GCG TTA CCA ACA GCT CCA AT
COL3A1	GGA GCT GGC TAC TTC TCG C	GGG AAC ATC CTC CTT CAA CAG
COL5A1	CTG ACA AGA AGT CCG AAG GGG	CGT CCA CAT AGG AGA GCA GTT T
GAPDH	ACA ACT TTG GTA TCG TGG AAG G	GCC ATC ACG CCA CAG TTT C

2.2.2. Protein Isolation and Quantification

Investigation of relative protein expression of CAFs and poor prognostic markers was carried out using Western Blot. $1x10^6$ of cells were seeded in direct contact in 10 cm petri-dishes for mono and co-culture groups. After incubation for 5 days, the medium was discarded, and cells were washed with dPBS (Sigma, USA). Then, cells were scraped with 1 mL dPBS on ice and collected to an Eppendorf tube. Cells were centrifuged at 21.1G for 15 minutes, then the supernatant was discarded. Pellets were dissolved with 30 µl of protein isolation solution which was prepared by mixing 90 µl of RIPA (EcoTech, Turkey), 10 µl of cOmplete Protease Inhibitor Cocktail (Roche, Switzerland), and 1 µl PhosStop Phosptase Inhibitor Cocktail (Roche, Switzerland) solutions. Dissolved pellets were sonicated for 10 seconds with 30 second breaks for 3 cycles using Bioruptor Sonicator (Diagenode, Belgium). Protein quantification was performed with BCA Assay (Thermo Fisher, USA). Protein samples were prepared accordingly with nuclease free water and Laemmli Buffer to obtain 50 µg in 40 µl. Samples were denatured in 95°C heat block for 10 minutes and vortexed every 5 minutes before loading to the gel.

2.2.3. SDS-PAGE & Western Blot

10 µl of protein sample was loaded to each well of precast 4-15% gradient polyacrylamide gel (Bio-Rad, USA). After polyacrylamide gel electrophoresis (PAGE) with Tris-Glycine-SDS buffer, overnight wet western blot was performed at 4°C and 30 volts. PVDF membrane (Bio-Rad, USA) was used for blotting. Membrane used for blotting CO1A1 primary antibody was blocked with 5% BSA dissolved in TBS-T and for the rest of the membranes 7.5% skimmed milk powder dissolved in TBS-T was used for blocking. COL3A1 (Santa Cruz, USA), COL5A1 (Santa Cruz, USA), Beta-Actin (Abcam, USA), and FN1 (Abcam, USA) primary antibodies were prepared in 5% Skimmed Milk solution to 1:1000 and 1:5000 dilutions respectively. COL1A1 primary antibody was diluted in 1:1000 ratio using 5% BSA solution. Beta-Actin antibody was used as housekeeping protein. Anti-rabbit (Abcam, USA) and anti-mouse (Abcam, USA) horseradish peroxidase conjugated secondary antibodies, prepared in 2% skimmed milk solution, were used for signal detection. Chemiluminescent substrate Femto (Thermo Fisher, USA) and Odyssey Fc (Li-Cor, USA) imaging system was used for blot imaging and signal detection.

2.2.4. Establishment of GFP tagged SNU484 Cancer Cells

SNU484 cells were used for plasmid transfection. 200.000 cells/well were seeded to 6 well plates 24 hours before the transfection. Pgk-H2BeGFP plasmid (Figure 2.2) was thawed at room temperature. 1 mL of plasmid and 1 mL of Protamine Sulfate (Sigma, USA) was added to the well. After 24 hours, medium was discarded and replaced with fresh medium. The expression of GFP was checked under a fluorescent microscope. Cells were split into T25 flasks after reaching confluency.

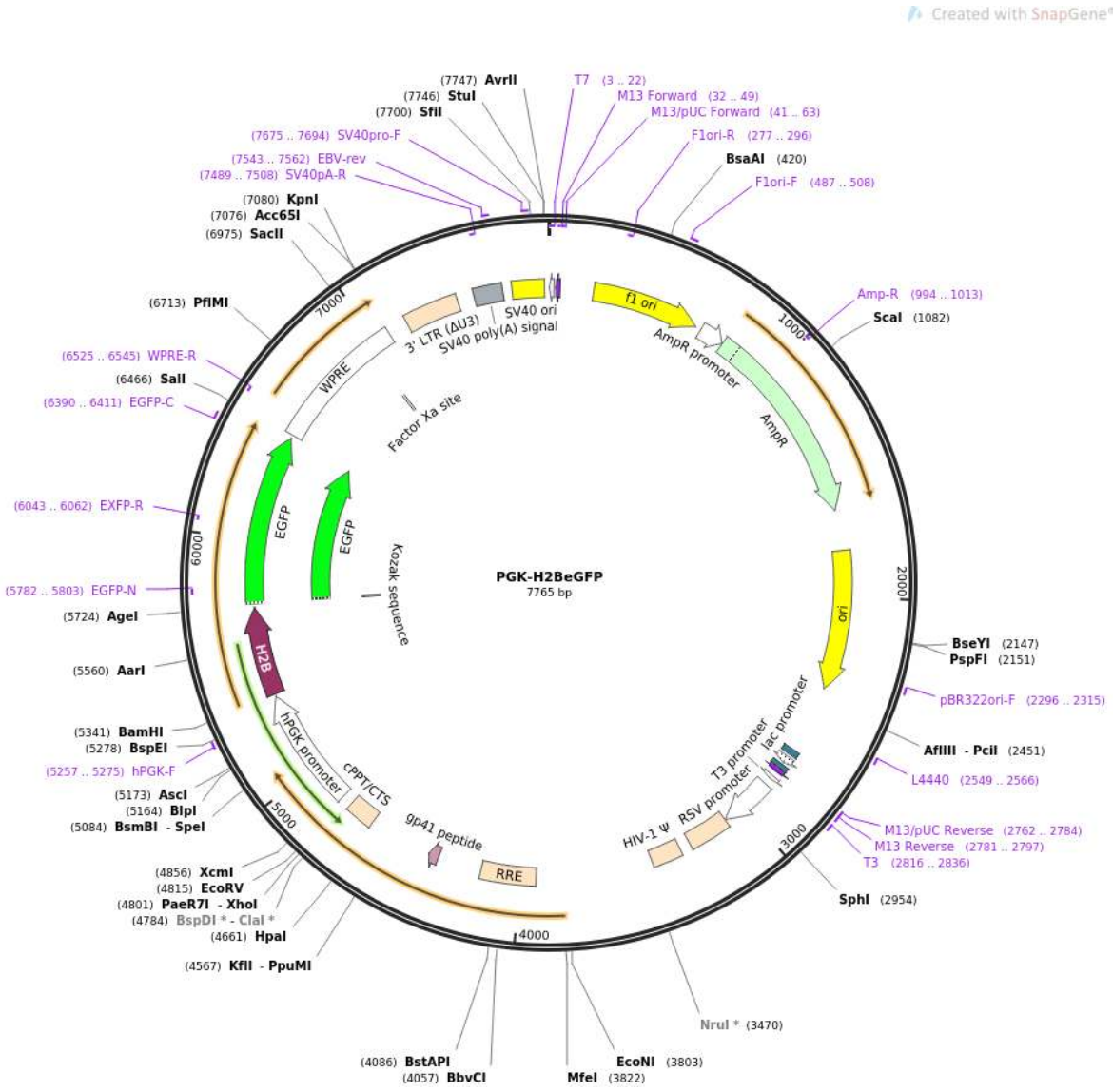


Figure 2.2 Plasmid map of Pgk-H2BeGFP.

2.2.5. Direct Contact Cell Proliferation Rate Assay

The cell proliferation rate of SNU484-GFP cells upon direct contact with BJ was investigated with this assay. Assay was performed with monoculture and co-culture groups of SNU484-GFP and BJ cells. 3500 cells in 100 µl was seeded per well of a 96-well plate. Co-culture group was seeded in 1:1 ratio for a total cell number of 3500 cells. After 24 hours, 50 µl of 1:1000 diluted Hoechst (Sigma, USA) was applied on cells for staining nucleus and

quantification of cell number in the well. Cell proliferation was observed for 5 days with 24-hour intervals using BioTek Cytation 5 Cell Imaging instrument (Agilent, USA). For cellular quantification, Gen 5 software (Agilent, USA) was used. DAPI channel was used for quantification of BJ cell number. For SNU484-GFP monoculture and co-culture groups, both DAPI and GFP channel was used. Doubling time was calculated by plotting a growth curve.

2.2.6. Paracrine-Cell Proliferation Rate Assay

The paracrine effect of BJ cells on cell proliferation rate of SNU484-GFP cells was investigated with this assay. The experimental design was illustrated below in Figure 2.3. Transwell inserts with 0.4 μm pore size for 6 well plates (Corning, USA) were used to culture cells together without contact. Assay was performed with monoculture and co-culture groups of SNU484-GFP and BJ cells. Insert with no cells and SNU484 only were used as control groups. Inserts with BJ monoculture and SNU484 and BJ co-culture were used to observe the paracrine effect. 110.000 of SNU484-GFP cells in 1 mL was seeded to each well of the 6 well plate. At the same time point, 50.000 cells were seeded to inserts. Co-culture group was seeded in 1:1 ratio for a total cell number of 50.000 cells. Cell proliferation was observed for 5 days with 24-hour intervals using BioTek Cytation 5 Cell Imaging instrument (Agilent, USA). Inserts were removed whilst imaging. GFP channel was used for quantification of cell number. Cell proliferation rate was calculated by plotting a growth curve.

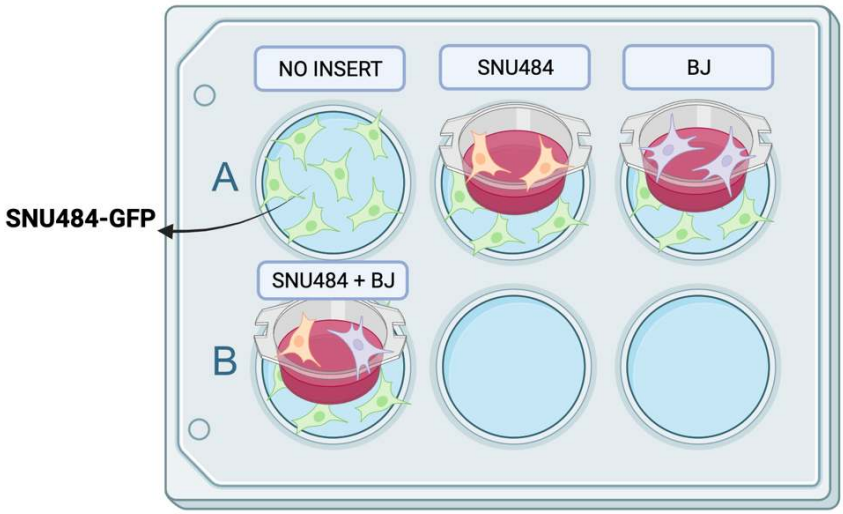


Figure 2.3 Experimental design of no contact co-culture model. Transwell inserts with with 0.4 µm pore size was used. Created by using Biorender.com.

2.2.7. Wound Healing Assay

150.000 cells/mL of SNU484-GFP were seeded to 24 well plates and left for 24 hours of incubation to become confluent. After the incubation, a wound was generated using a 200 µl pipette tip. Medium was discarded to remove detached cells and 1ml of 15% FBS RPMI was added. Before introducing other cell groups, wounds were imaged in GFP channel and with phase contrast setting on BioTek Cytation 5 Cell Imaging instrument (Agilent, USA). After imaging, 24 well plate transwell inserts with 0.4 µm pore size (Sarstedt, Germany) was used for no contact co-culture. SNU484-GFP cells were cultured with SNU484, BJ, and co-culture of SNU484 and BJ cell groups without direct contact. Inserts contained 20.000 cells/100 µL for each group. Co-culture group was seeded in 1:1 ratio. Wells without insert and SNU484 containing inserts were used as control groups. Experiment was carried out with three technical replicates as seen in Figure 2.4 below. Wound closure was examined every 12 hours for 1 day. ImageJ software was used to measure the wound areas. Wound closure percentage was calculated by using wound areas of 0 and 24 hours.

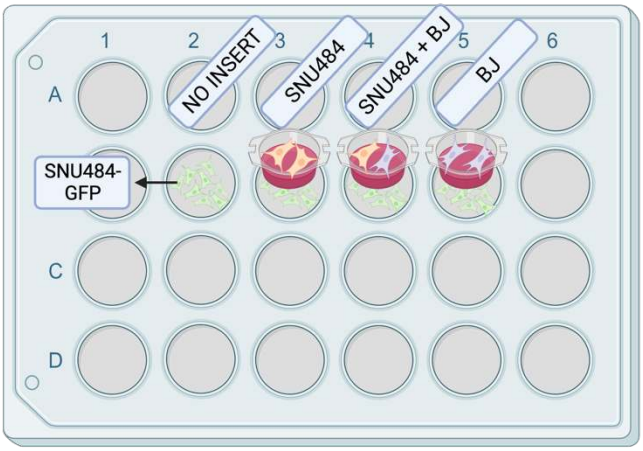


Figure 2.4 Experiment setup of wound healing assay. SNU484-GFP cells were seeded at the bottom of the plate. Insert containing SNU484, BJ monocultures and SNU484+BJ co-culture were placed after wound generation. Created by using Biorender.com.

2.2.8. Migration Assay

The experiment setup was represented in the Figure 2.5 below. 25.000 cells/mL of SNU484 monoculture, BJ monoculture, and 1:1 ratio of SNU484 and BJ co-culture were seeded to the wells of a 24 well plate. No cells were seeded to the first column. Well without cells and SNU484 monoculture containing well was used as control groups. Plate was left to incubate for cells to attach. After incubation, medium was discarded and 15% FBS containing complete medium was added. Then, 20.000 SNU484-GFP cells in 100 µL of serum free medium were seeded on top of 24 well plate transwell inserts with 8 µm pore size (Sarstedt, Germany). Plate was incubated for 20 hours to allow cell migration. After 20 hours, inserts were removed from the plate, medium was discarded, and top of the inserts were cleaned with damp cotton swabs to remove non-migrated cells. Cells on the insert were fixed with 99.9% pure ice-cold methanol (Merck, USA) for 15 minutes. Then methanol was removed, and inserts were left to dry for a few minutes. After drying, cells were stained with 1:1 diluted Crystal Violet (Merck, USA) for 15 minutes. Excess crystal violet was washed out with PBS (Sigma, USA) and then inserts were left for drying. After drying, inserts were imaged with BioTek Cytation 5 Cell Imaging instrument (Agilent, USA) in bright field and GFP channel, images were taken from 3 different regions of inserts.

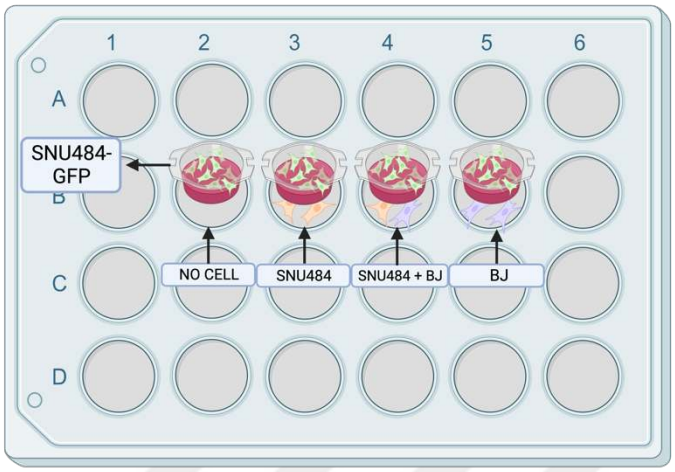


Figure 2.5 Experiment setup of migration assay. SNU484-GFP cells were seeded to inserts at top. No cells were seeded to the first column. SNU484 monoculture, SNU484 and BJ co-culture, BJ monoculture cells were seeded to the bottom of a 24 well plate. Created by using Biorender.com.

2.2.9. MTT Assay

MTT assay was used to determine the cell viability upon chemotherapy application. Drug response of 5-FU, paclitaxel, cisplatin, and doxorubicin was evaluated with this assay. Concentrations of the drugs were explained in Table 2.2. The experiment setup included three groups: monoculture of gastric cancer cells and fibroblasts, and co-culture of the two. 3500 cells in 100 µl was seeded per well of a 96 well plate, co-culture plates were seeded in 1:1 ratio. Cells were left for attachment for 24 hours and then treated with 100 µl of 9 different concentrations of the drugs. Treatment was carried out with 6 technical replicates in a single plate as demonstrated in Figure 2.6 below. Plates were incubated for 5 days and at the end of incubation, 50 µl of 3 mg/mL MTT (Sigma, USA) was added to each well. Plate was incubated in the 37°C, 5% CO_2 incubator for 4 hours for viable cells to reduce the MTT into formazan crystals. These were dissolved with 13.8% SDS (Bio-Rad, USA) solution containing 1% HCl and left for overnight shaking at room temperature and dark. Absorbance at 570 nm was measured after the incubation using BioTek Synergy plate reader (Agilent, USA). As background signal, absorbance values of no cell containing wells were subtracted

from treatment and control wells. Outlier values were removed, if there was any, by using GraphPad Prism9 software and applying ROUT method with 0.5% maximum false discovery rate. Percent cell viability was calculated for each concentration by dividing the treatment absorbance value to control and multiplying it with 100. For plotting the sigmoidal dose-response curves, percent viability values and corresponding drug concentrations in logarithmic scale were used. Four-parameter logistic regression was used to obtain IC50 values via GraphPad Prism9 software.

Table 2.2 Drugs and their concentrations used for MTT and live cell imaging experiments.

Drugs	**Highest Concentrations**
5-FU	100 μM
Paclitaxel	100 nM

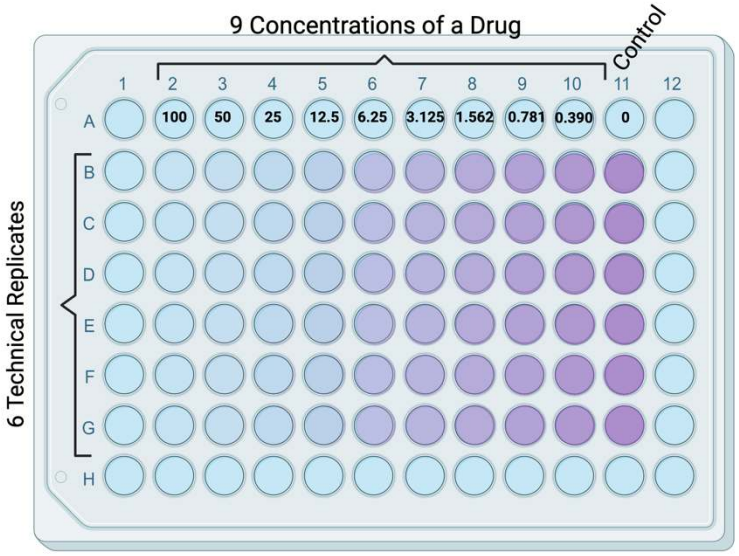


Figure 2.6 Experimental design of MTT and live cell imaging experiments. Created by using Biorender.com.

2.2.10. Live Cell Imaging for Cell Viability Measurement

Due to limitations of signal detection in MTT assay with co-culture group, we used live cell imaging for cell viability as an alternative method to MTT. We used SNU484-GFP tagged cells to quantify viable cells and investigate the drug response upon chemotherapy application. Seeding and treatment procedures were the same with MTT protocol as mentioned in the section 2.2.7. After 5 days of incubation, 50 µl of 1:1000 diluted Hoechst (Sigma, USA) was applied on cells for staining nucleus and quantification of cell number in the wells. Four different images were taken from a single well using BioTek Cytation 5 Cell Imaging instrument (Agilent, USA). Total cell number was calculated by quantifying nucleus stains either in DAPI or GFP channels with the cellular analysis feature of Gen 5 software (Agilent, USA). DAPI channel was used for quantification of BJ cell number. For SNU484-GFP monoculture and co-culture groups, both DAPI and GFP channel was used. Percent staining of GFP was calculated by dividing GFP signal to DAPI signal for true SNU484-GFP signal. Outlier values were removed, if there were any, by using GraphPad Prism9 software and applying ROUT method with 0.5% maximum false discovery rate. Percent cell viability was calculated for each concentration by dividing the treatment cell number to control and multiplying it with 100. For plotting the sigmoidal dose-response curves, percent viability values and corresponding drug concentrations in logarithmic scale were used. Four-parameter logistic regression was used to obtain IC50 values via GraphPad Prism9 software.

2.3.Primary Cell Culture

Tumor and parenchyma samples were collected from non-small cell lung adenocarcinoma patients with Koç University Institutional Review Board (2020.001.IRB2.001) ethics approval and patient consent.

2.3.1. Use of Cancer-associated Fibroblasts Derived from Lung Tumor Tissue

We used CAFs derived from lung tumor tissue provided by Assist. Prof. Ece Öztürk's group with the following protocols. Tissues were cut into 2-4 mm^3 pieces and washed. Tumor dissociation kit (Miltenyi Biotec, Germany) was used for isolating primary cells from lung tumor tissues, using manufacturer’s guidelines. Tissues were placed in gentleMACS C tubes with enzyme cocktail containing 100 µL Enzyme H, 50 µL Enzyme R, 12.5 µL Enzyme A

prepared in 2.2 mL complete DMEM/F12K. Tubes were placed in gentleMACS Octo Dissociator Miltenyi Biotec, Germany) and 37C_h_TDK_2 program was run. After digestion, tissues were filtered from pre-wet 70 µm cell strainers. Strainers were washed once more, and then flowthrough was centrifuged at 400G for 5 min, supernatant was discarded. Cells were washed with medium, centrifuged again, and supernatant was discarded. Pellet was dissolved in 1ml of medium and then 10 ml of red blood cell lysis solution (1X) was added, suspension was incubated at RT for 5 minutes. Suspension was centrifuged at 300G for 10 minutes, supernatant was discarded, and pellet was dissolved with DMEM/F12K and cells were incubated at 37°C, 5% CO_2 incubator. Anti-Fibroblast MicroBeads human (Miltenyi Biotec, Germany) kit was used for isolating fibroblasts from lung tumor cell suspensions using manufacturer's guidelines.

2.3.2. Use of Fibroblasts Derived from Lung Parenchyma

We used fibroblasts derived from lung parenchyma provided by Assist. Prof. Ece Öztürk's group with the following protocol. Tissues were cut into 2-4 mm^3 pieces and washed. Tissues were placed in gentleMACS C tubes with 3 mL of collagenase type II enzyme and 10 µl of Dnase I (2,000 U/mL) for 10 mL digestion. Tubes were placed in gentleMACS Octo Dissociator (Miltenyi Biotec, Germany) and 37C_m_NTDK program was run. Steps after digestion were applied as the same described in section 2.3.1. Anti-Fibroblast MicroBeads human (Miltenyi Biotec, Germany) kit was used for isolating fibroblasts from lung parenchyma cell suspensions using manufacturer's guidelines.

2.4.Statistical Analysis

For statistical analysis One-way Analysis of Variance (ANOVA) with Tukey post-hoc test setting was applied using GraphPad Prism 9 software. P values of <0.05 were considered statistically significant. The recommended significance settings of Prism's were applied as $p < 0.0001$ = extremely significant (****), $0.0001 < p < 0.001$ = extremely significant (***), $0.001 < p < 0.01$ = very significant (**), and $0.01 < p < 0.05$ = significant (*).

Chapter 3: **RESULTS**

3.1.Establishment of Co-culture Model

We established a co-culture model, comprised of normal fibroblasts and gastric cancer cells, to obtain CAFs. SNU484 and NUGC4 gastric cancer cells were paired with either BJ or MRC-5 fibroblast cells. After incubation of 5 days, the morphology of fibroblasts in co-culture groups have changed compared to monoculture. As seen in the Figure 3.1, mono-culture fibroblasts exhibited elongated, spindle like shape whereas co-cultured fibroblasts appeared to have a multi-polar cellular morphology. This cobble-stone like, polar cellular morphology is a characteristic feature of CAFs (Ma et al., 2018b; Jaeschke et al., 2020).

3.2.Investigation of Cancer-associated Fibroblast Markers

Normal fibroblasts can be activated by the secretions of cancer cells and become CAFs. The secretory profile of activated fibroblasts differs from normal fibroblasts; they are characterized by the increased expression of ACTA2 and FAP genes (Nurmik et al., 2020). Therefore, we investigated the gene expression levels of two CAF markers *FAP* and *ACTA2,* using qRT-PCR method. We observed that upon co-culture with BJ fibroblasts, there was no increase in any of the CAF markers compared to BJ monoculture group (Figure 3.2). There was a 9-fold increase of FAP gene expression in SNU484 and MRC-5 co-culture group. However, SNU484 monoculture group also showed a 6-fold higher FAP expression compared to MRC-5. In NUGC4 and MRC-5 co-culture group a slight increase in FAP and ACTA2 expression, but not significant, was observed compared to the gene expression in MRC-5 monoculture.

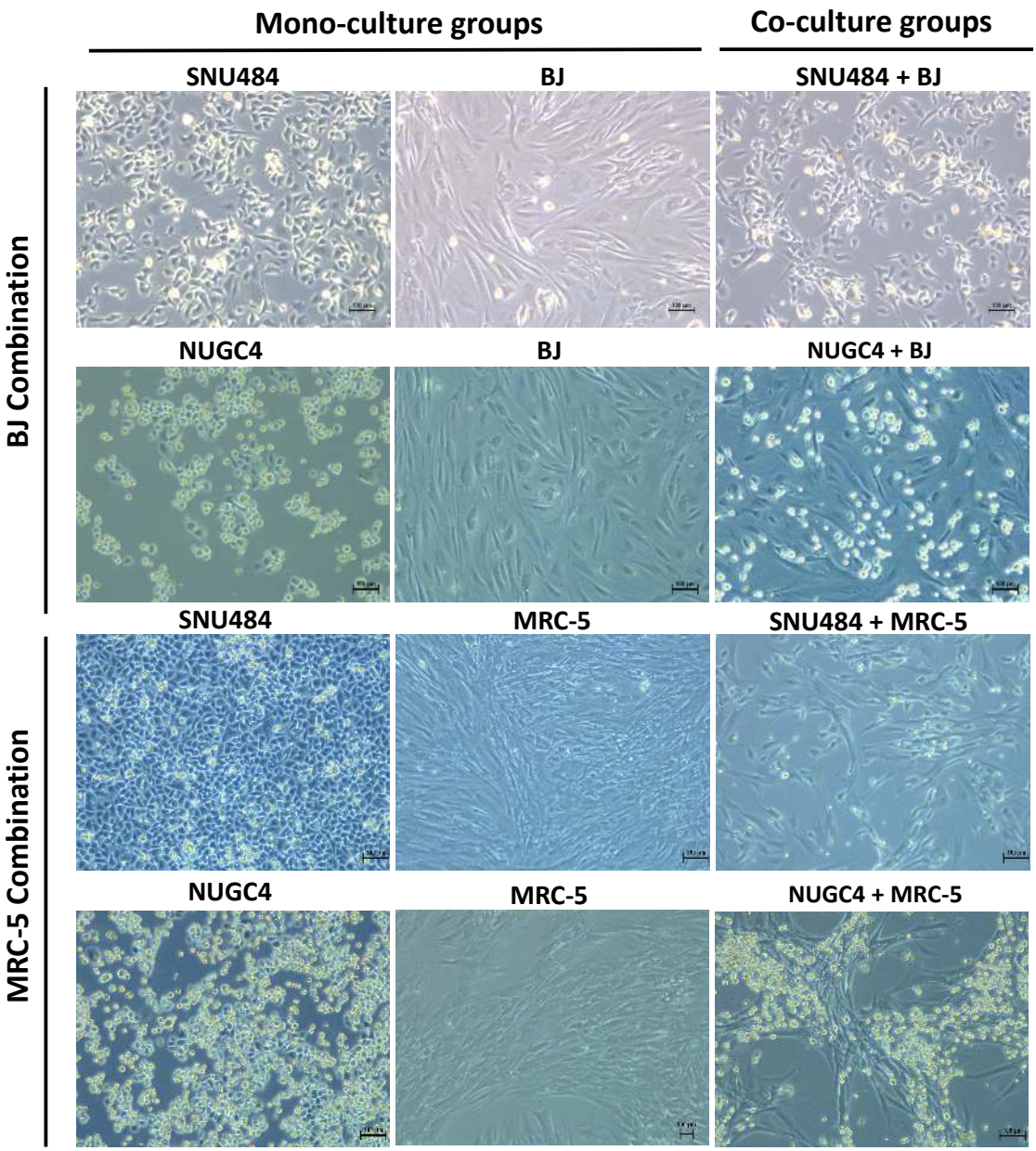


Figure 3.1 Bright field images of monoculture and co-culture groups of fibroblast and gastric cancer cell lines. BJ and MRC-5 fibroblast cell lines were co-cultured with SNU484 and NUGC4 gastric cancer cell lines. Characteristics of BJ and MRC-5 changed from spindle morphology into multi-polar appearance. Scale bar = 100 μm.

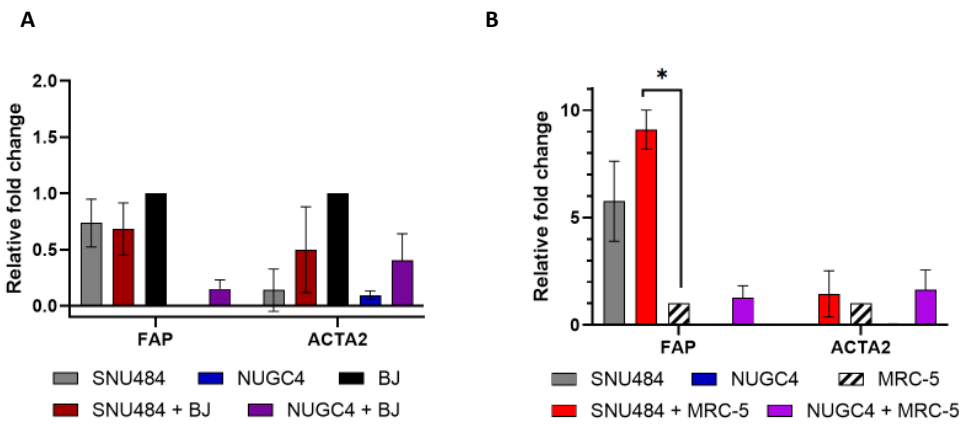


Figure 3.2 Relative fold change in FAP and ACTA2 mRNA expression levels. **A.** SNU484 and NUGC4 co-cultured with BJ fibroblast **B.** SNU484 and NUGC4 and co-cultured with MRC-5 fibroblast.

3.3.Investigation of Poor Prognostic Cancer-associated Fibroblast Markers

Previously we have identified a poor prognostic CAF gene signature through a bioinformatic study. The study revealed that FN1, SPARC, COL1A1, COL1A2, COL3A1, and COL5A1 genes were associated with poor prognostic gastric cancer (Ucaryilmaz Metin and Ozcan, 2022a). Therefore, we investigated the *in vitro* mRNA expression levels of poor prognostic CAF markers in mono and co-culture groups. The qRT-PCR results demonstrated a significant increase in the markers for SNU484 co-cultured with BJ, but not MRC-5 (Figure 3.3). No significant increase was detected for both combinations with NUGC4.

In SNU484 and BJ co-culture, *FN1, COL1A2,* and *COL5A1* mRNA expressions were greater compared to BJ monoculture as in Figure 3.3A. However, only *COL1A2* and *COL5A1* were found to be statistically significant with 2-fold and 2.5-fold increases respectively. The mRNA expressions of *SPARC, COL1A1,* and *COL3A1* markers were also increased in co-culture group compared to SNU484 monoculture, but not to BJ. For NUGC4 and BJ co-culture group, none of the markers were found to be increased compared to BJ. And no expression of these markers was detected in NUGC4 monoculture.

Changes in gene expression in MRC-5 co-culture group combinations were less prominent compared to BJ co-culture combinations, as can be seen in Figure 3.3B. SPARC was the only marker with increased expression in NUGC4 and MRC-5 co-culture, but no statistical significance was determined. However, in SNU484 and MRC-5 co-culture group, all markers were expressed higher than in MRC-5 monoculture.

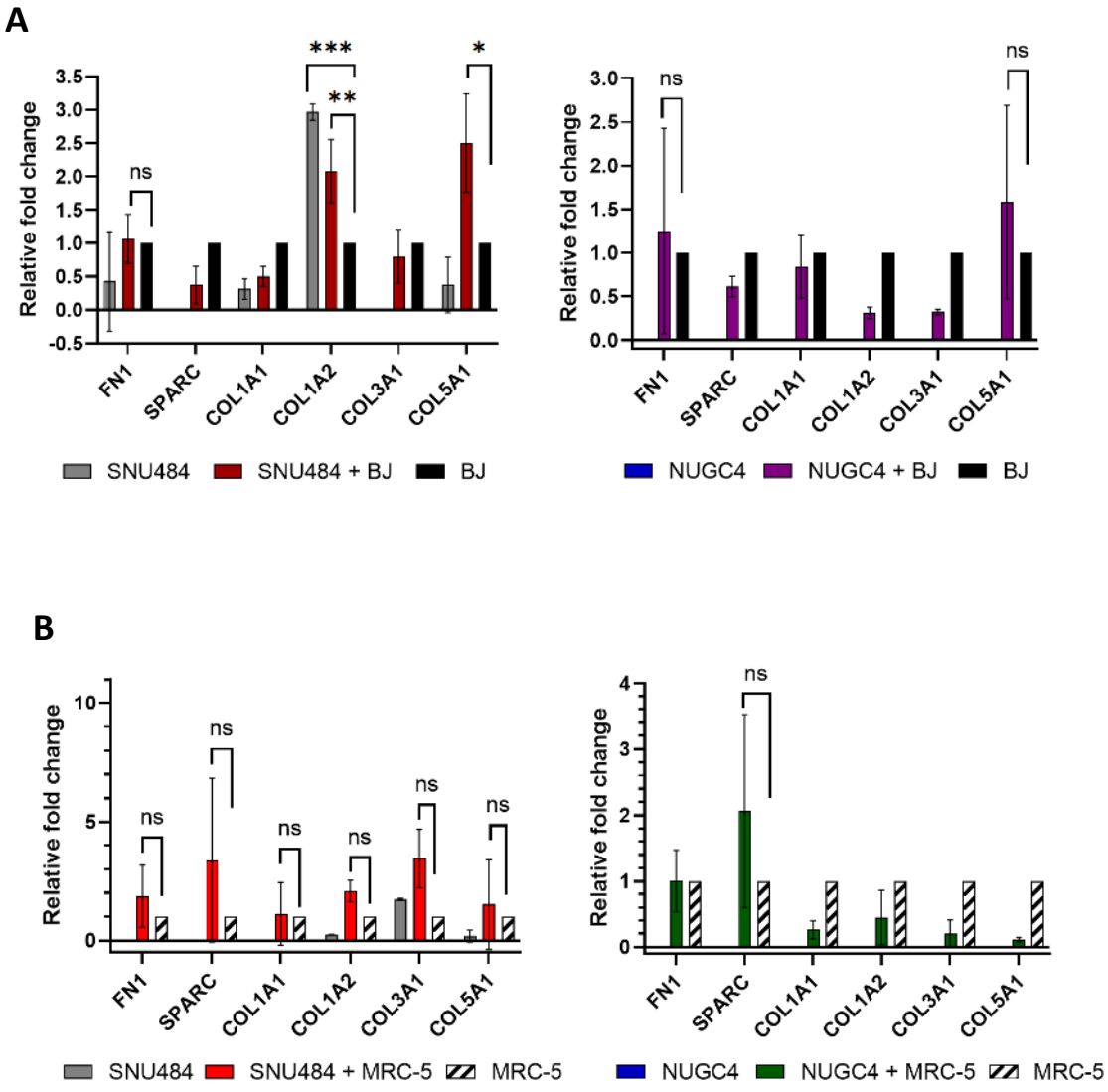


Figure 3.3 Relative fold change in expression levels of poor prognostic CAF marker genes. **A.** SNU484 and NUGC4 co-cultured with BJ fibroblast **B.** SNU484 and NUGC4 and co-cultured with MRC-5 fibroblast.

The expression of the most promising poor prognostic CAF markers was investigated further on protein level by western blot. Only BJ with SNU484 and NUGC4 combinations were investigated for protein expression since mRNA expressions of CAF markers were less significant in MRC-5 combinations. COL1A1 antibody was used for quantification of collagen type I alpha I chain and collagen type I and alpha II chain, COL3A1 antibody was used for collagen type III alpha chain I, COL5A1 antibody was used for collagen type V alpha chain I, and FN1 antibody was used for the quantification of fibronectin protein. Beta-actin antibody was used as a control housekeeping protein and for densiometric analysis. Protein expressions of each group were normalized over BJ.

Western blot analysis has revealed that COL1A1 was the only poor prognostic CAF marker with increased expression in SNU484 and BJ co-culture group (Figure 3.4). This result was compatible with the increased COL1A2 mRNA expression. As seen in the Figure 3.4., an increase in COL3A1 and COL5A1 markers was also recorded in the co-culture group compared to SNU484 mono-culture group, but not to BJ. No poor prognostic CAF marker expression was detected in SNU484 mono-culture group.

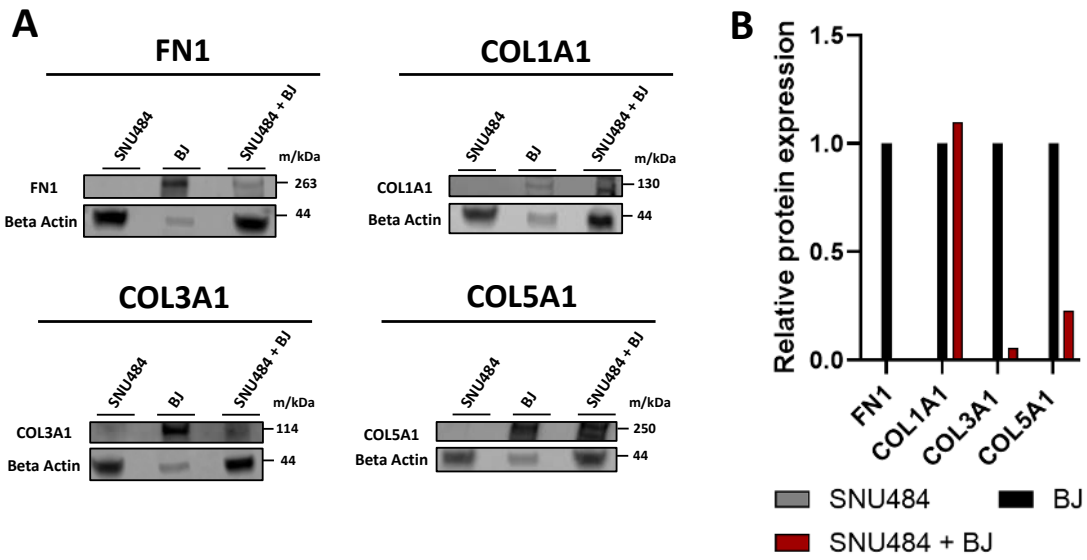


Figure 3.4 Protein expression levels of poor prognostic CAF markers in SNU484 and BJ combination. **A.** Western blots of FN1, COL1A1, COL3A1, and COL5A1 proteins. **B.**

Densiometric analysis of FN1, COL1A1, COL3A1, and COL5A1 expression using Beta-actin. Relative expression levels were calculated normalizing over BJ mono-culture group.

Western blot of NUGC4 and BJ combination group was also consistent with the qRT-PCR results. According to Figure 3.5, compared to BJ monoculture group, there was no increase in any of the poor prognostic CAF markers upon co-culture. Expression of these markers was not detected in NUGC4 monoculture group as well.

These qRT-PCR and western blot results indicated that SNU484 and BJ co-culture combination was the most prominent group for investigating poor prognostic effect of CAFs. Therefore, we conducted further experiments with this combination of cells.

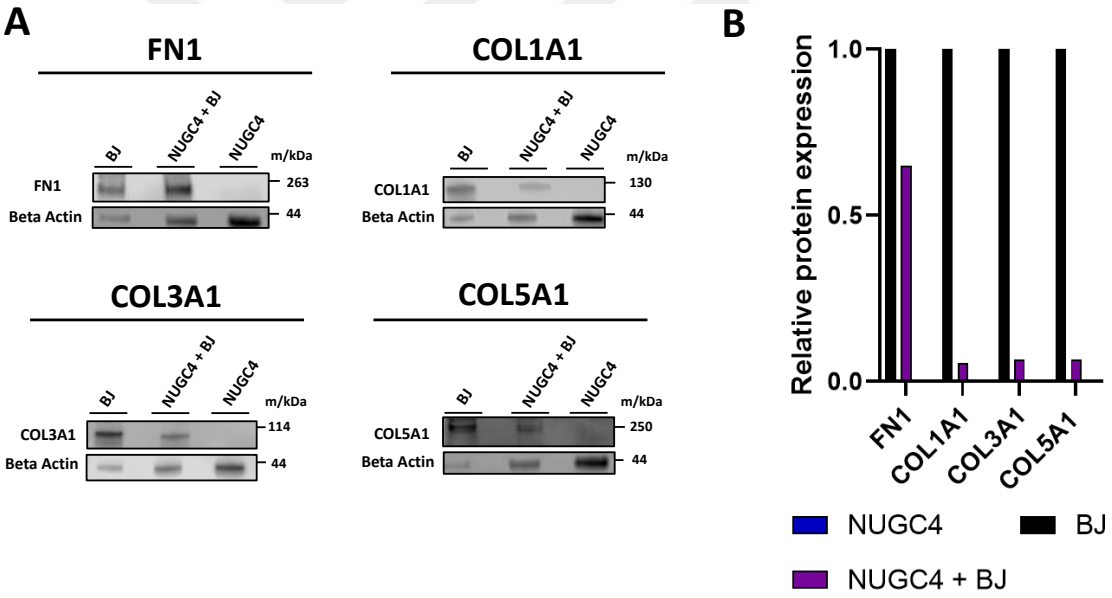
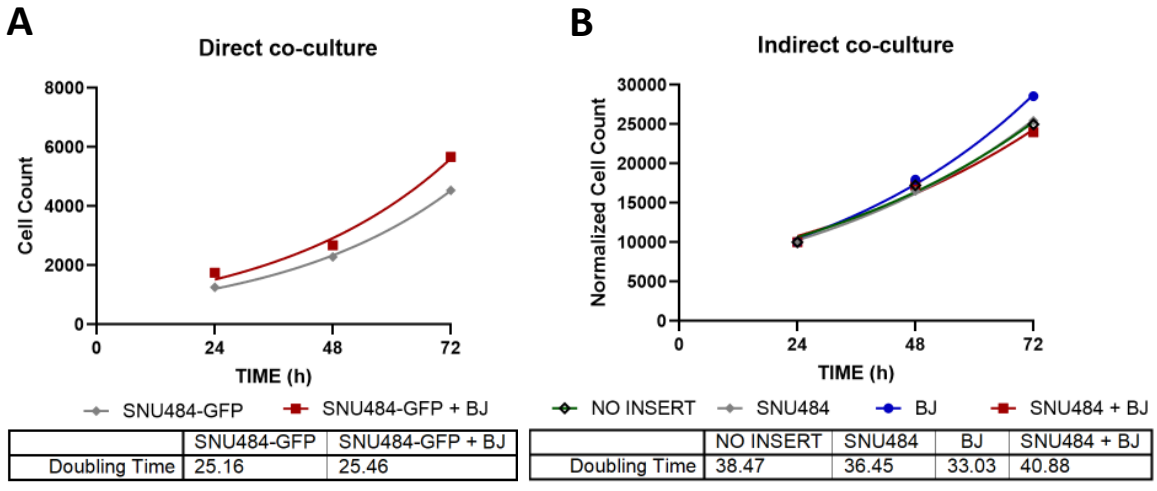


Figure 3.5 Protein expression levels of poor prognostic CAF markers in NUGC4 and BJ combination **A.** Western blots of FN1, COL1A1, COL3A1, and COL5A1 proteins. **B.** Densiometric analysis of FN1, COL1A1, COL3A1, and COL5A1 expression using Beta-actin. Relative expression levels were calculated normalizing over BJ mono-culture group.

3.4.Effect of Cancer-associated Fibroblasts on Gastric Cancer Cells' Proliferation Rate

CAFs are known to support cancer progression through release of growth factors and chemokines. This continuous supply of nutrients and stimuli help cancer cells to grow and proliferate. CAFs affect the proliferation and growth of cancer cells either by paracrine signaling or through direct contact. Therefore, we investigated the influence of poor prognostic CAFs on gastric cancer cells' proliferation rate.

We seeded cells together to obtain a direct contact co-culture model. Monoculture group was used as control to compare the proliferation rates. No contact co-culture system was generated by using transwell inserts. Four groups were established for no contact co-culture: SNU484-GFP without insert, with SNU484 insert, BJ insert, and SNU484+BJ insert. GFP tagged SNU484 cells were used to quantify cells by their GFP tagged nucleus. The cell number was quantified every 24 hours for 5 days to plot the growth curve. Log phase of the growth curve was used to derive the population doubling time.



	SNU484-GFP	SNU484-GFP + BJ
Doubling Time	25.16	25.46

	NO INSERT	SNU484	BJ	SNU484 + BJ
Doubling Time	38.47	36.45	33.03	40.88

Figure 3.6 Growth curves of SNU484-GFP and population doubling times. **A.** Monoculture SNU484-GFP and direct co-culture. Mono-culture group was used as control and only SNU484-GFP cells in the co-culture were quantified for doubling time calculation. **B.** No contact co-culture of SNU484-GFP cells via transwell inserts. No insert and insert with SNU484 groups were used as control. BJ and SNU484+BJ groups represent inserts with these

cells, indirectly co-cultured with SNU484-GFP cells on the bottom wells. Cell count was normalized to 10000.

Upon direct co-culture, there was no obvious difference in the proliferation rate of SNU484-GFP compared to monoculture. The doubling time of SNU484-GFP in monoculture was recorded to be 25.16 hours whereas in co-culture it was detected as 25.46 hours as in Figure 3.6A. Therefore, we concluded that direct contact of gastric cancer cells with CAFs do not enhance the proliferation rate of gastric cancer cells.

We also investigated the paracrine effect of CAFs on gastric cancer cells' proliferation rate by using transwell culture system (Figure 3.6B). When SNU484-GFP cells cultured alone, the doubling time was 38.47 hours. The presence of SNU484 cells in the environment did not contribute to facilitating the doubling time of SNU484-GFP remarkably. However, presence of BJ on the inserts greatly influenced the proliferation rate and lowered it to 33.03 hours. Even though two cell lines did not get in contact, BJ cells may have accelerated the proliferation rate in a paracrine manner. In contrast, insert containing the co-culture of SNU484 and BJ, increased the doubling time to 40.88 hours.

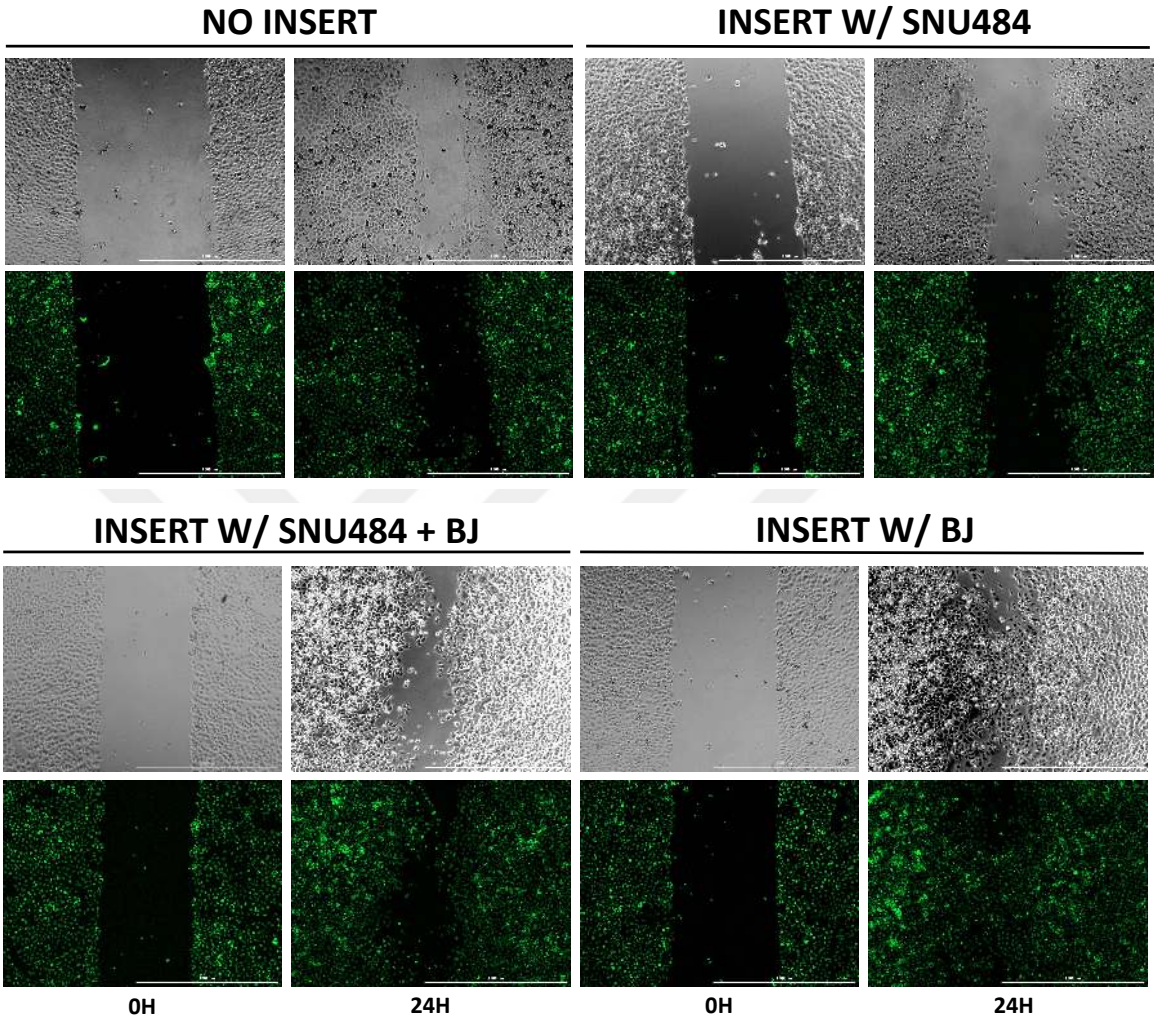
3.5.Effect of Cancer-associated Fibroblasts on Wound Healing Capabilities of Gastric Cancer Cells

Contribution of CAFs to wound healing capabilities of gastric cancer cells were examined via wound healing assay. For this purpose, no contact co-culture model was used. SNU484-GFP cells were paired with either SNU484, BJ monocultures or SNU484 + BJ co-culture containing inserts. Hence, paracrine effect of CAFs was observed on wound healing of gastric cancer cells. Wound closure percentage was calculated by using the wound areas at 0 and 24 hours.

No insert group was used as control. The wound closure percentage of SNU484-GFP cells without any effectors was observed as 35% in 24 hours Figure 3.7. When these cells were co-cultured with SNU484 cells a non-significant decrease in the wound closure percentage was observed. Although the paracrine effect of SNU484 + BJ co-culture led to an increase in the wound closure rate of SNU484-GFP cells, the statistical analysis revealed it to be non-significant compared to control group. However, paracrine effect of BJ resulted in an almost 2-fold significant increase in the wound closure rate.

These data suggested that presence of CAFs have an influence on wound healing capabilities of gastric cancer cells. Especially, paracrine effect of CAFs were observed to be effective in exerting such effect. Interestingly, paracrine signals relayed from the direct contact of SNU484+BJ attenuated the wound closure of SNU484-GFP cells, compared to only BJ paracrine signaling. Therefore, instead of cell-to-cell signaling, paracrine signaling seemed to be more effective in wound healing of gastric cancer cells.

A



B

TOP		SNU484	SNU484 + BJ	BJ
BOTTOM	SNU484-GFP	SNU484-GFP	SNU484-GFP	SNU484-GFP

C

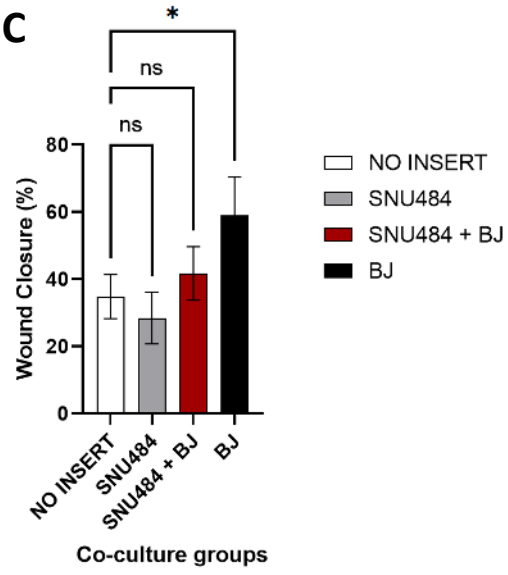


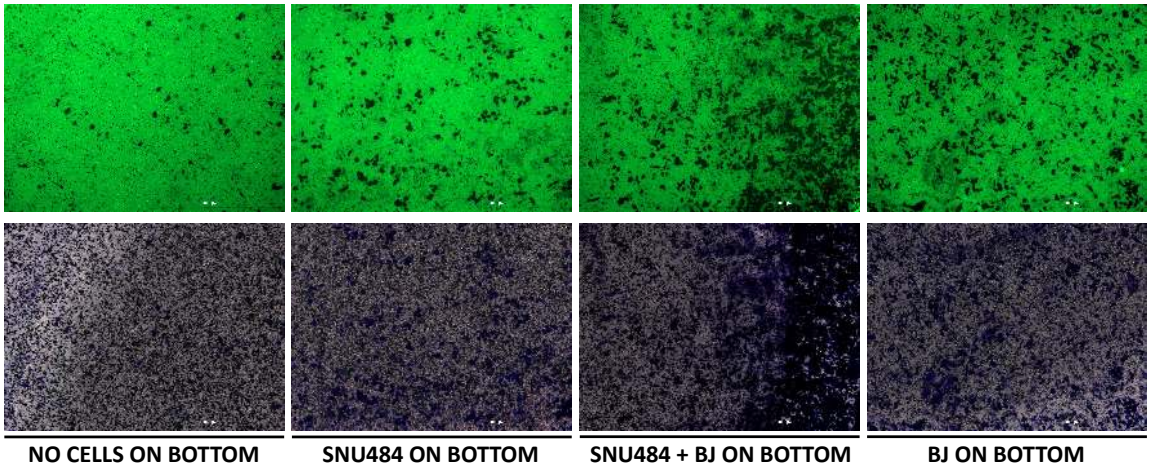
Figure 3.7 Wound healing capability of SNU484-GFP cells upon co-culture with inserts containing SNU484, BJ monocultures, SNU484 + BJ co-culture or without cells **A.** Images in phase contrast and GFP channel at 0 and 24 hours. Scale bar: 1000 μm. **B.** Representation of experiment setup. Bottom represents the bottom of the plate and top represents the inserts containing corresponding cell groups **C.** Wound closure percentages.

3.6. Effect of Cancer-associated Fibroblasts on Migration Capabilities of Gastric Cancer Cells

Migration capabilities of gastric cancer cells upon co-culture with fibroblasts were investigated. We utilized transwell inserts with 8 μm pores to observe migration of gastric cancer cells from the surface of the inserts through the pores. We used GFP tagged cells to observe migration. Hence, SNU484-GFP containing inserts were placed on top of the wells without any cells, SNU484 monoculture, SNU484 and BJ co-culture, or BJ monoculture as seen in Figure 3.8B. SNU484-GFP cells were seeded with serum-free medium and 15% FBS containing medium was present at the wells to promote migration to the bottom. Plate was incubated for 20 hours to allow migration of SNU484-GFP cells. Then, cells were fixed and stained with crystal violet to quantify the number of migrated cells.

SNU484-GFP cells without under the effect of other cell groups exhibited nearly no migration. SNU484 containing wells were not much different, number of migrated cells were low. However, monoculture of BJ cells positively influenced migration. Interestingly, highest number of migrated SNU484-GFP cells were recorded in bottom wells containing co-culture group of SNU484 and BJ. Therefore, with this data we concluded that fibroblasts induced migration capabilities of gastric cancer cells.

A



B

TOP	SNU484-GFP	SNU484-GFP	SNU484-GFP	SNU484-GFP
BOTTOM		SNU484	SNU484 + BJ	BJ

C

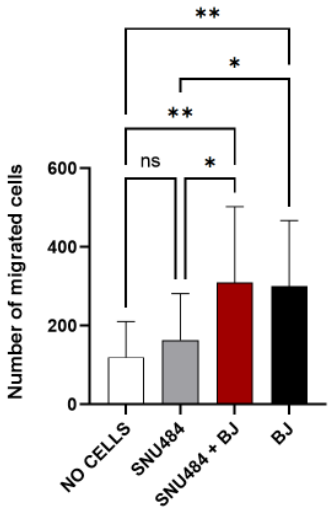


Figure 3.8 Migration capability of SNU484-GFP cells upon co-culture with SNU484, BJ monocultures, SNU484 + BJ co-culture or without cells **A.** Images in color bright field and GFP channel at 0 and 24 hours. Scale bar: 1000 μm. **B.** Representation of experiment setup. Top represents inserts containing SNU484-GFP. Bottom represents the bottom of the plate containing corresponding cell groups. **C.** Number of migrated cells.

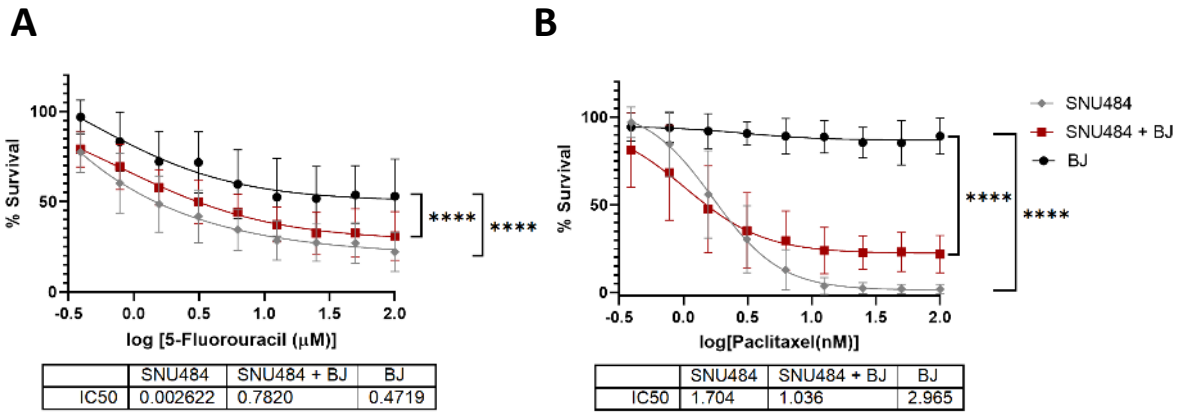
3.7.Effect of Cancer-associated Fibroblasts on Drug Response in Co-culture

3.7.1. Cell Viability of Total Cell Population Upon Treatment

The proliferation rate assays have revealed that upon co-culture, CAFs shorten the doubling time of gastric cancer cells in paracrine manner. Since CAFs boosted gastric cancer cells' proliferative capabilities, we investigated whether if CAF relayed signaling could potentiate other tumorigenic effects of CAFs on gastric cancer cells. Therefore, we assessed their effect on cellular viability of gastric cancer cells upon chemotherapeutic application. MTT method was utilized to observe response to 5-FU and Paclitaxel.

The dose-response curves of 5-FU showed that SNU484 and BJ co-culture group had a higher half maximal inhibitory concentration (IC50) of 0.7820 μM compared to SNU484 monoculture which had 0.0026 μM. The co-culture group was less sensitive to the drug, presence of BJ caused a slight shift of the curve to the right. The IC50 value of BJ monoculture group was recorded as 0.47 μM as in Figure 3.9A. In contrast, the co-culture group was more sensitized to paclitaxel compared to SNU484 monoculture. In Figure 3.9B, IC50 values were recorded as 1.704, 1.036 and 2.965 nM for SNU484, co-culture group and BJ respectively. BJ monoculture almost showed no response to paclitaxel treatment.

Although MTT experiment results enabled us to do a comparative analysis between monoculture and co-culture groups, it remained short to differentiate the signal of each cell in the co-culture group. Thus, we investigated the drug response with live cell imaging as an alternative method. For this purpose, we established a GFP tagged SNU484 cell line which allowed us to examine its drug response in the co-culture, rather than obtaining a pooled response of BJ and SNU484 cells together.



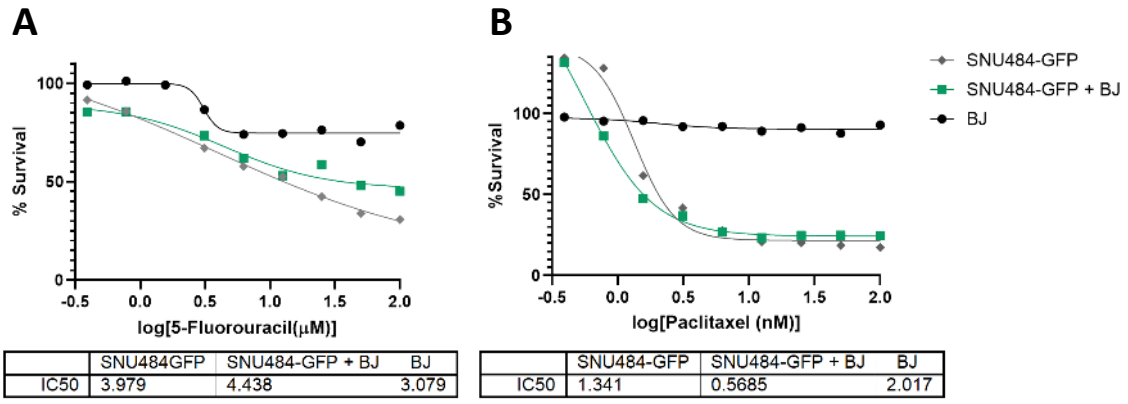
	SNU484	SNU484 + BJ	BJ
IC50	0.002622	0.7820	0.4719

	SNU484	SNU484 + BJ	BJ
IC50	1.704	1.036	2.965

Figure 3.9 Dose-response curves of SNU484, BJ monoculture and SNU484 + BJ co-culture groups obtained via MTT assay. **A.** Response to 5-Fluorouracil **B.** Response to Paclitaxel.

3.7.2. Viability of Gastric Cancer Cells in Co-Culture Conditions upon Treatment

Drug response was calculated by counting the number of viable cells. Nucleus labeling of SNU484 with GFP and nucleus staining of BJ by Hoechst enabled to quantify cells. The results were found to be similar with MTT assay as seen in Figure 3.10. SNU484 cells showed higher IC50 value in BJ co-culture group compared to SNU484 monoculture for 5-FU treatment, a slight shift to the right was recorded. However, no such effect was seen in paclitaxel treatment. Results were coherent with MTT assay. Collectively, these results indicated that CAFs decrease the 5-FU response of SNU484 gastric cancer cells in co-culture.



	SNU484GFP	SNU484-GFP + BJ	BJ
IC50	3.979	4.438	3.079

	SNU484-GFP	SNU484-GFP + BJ	BJ
IC50	1.341	0.5685	2.017

Figure 3.10 Dose-response curves of SNU484-GFP, BJ monoculture and SNU484-GFP + BJ co-culture groups obtained via live cell imaging. **A.** Response to 5-Fluorouracil **B.** Response to Paclitaxel.

3.8.Primary Fibroblasts and Their Influence on Gastric Cancer

We asked whether if primary fibroblasts could have poor prognostic effect on gastric cancer cells. Therefore, we utilized fibroblasts that were isolated from primary lung parenchyma and tumor lung cells.

3.8.1. Investigation of Cancer-associated Fibroblast Markers

We investigated whether if the fibroblasts isolated from parenchyma and tumor tissues express the two CAF markers; *FAP and ACTA2*. Expression levels were compared with BJ fibroblast cell line. According to Figure 3.11, neither of the lung parenchyma nor the tumor lung fibroblasts showed expression for *FAP* gene. In contrast, *ACTA2* gene expression was elevated in lung parenchyma fibroblast cells, but not in tumor lung fibroblasts.

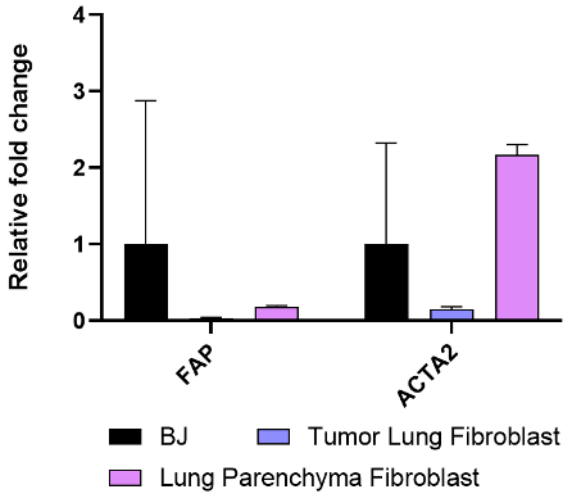


Figure 3.11 Relative fold change in *FAP* and *ACTA2* mRNA expression levels in BJ, Lung Parenchyma and Tumor Lung monocultures. BJ monoculture was used as control group and to calculate relative fold change.

3.8.2. Investigation of Poor Prognostic Cancer-associated Fibroblast Markers in Primary Fibroblasts

We investigated the poor prognostic CAF markers that we have previously identified through a bioinformatic study in monoculture primary fibroblasts. As in Figure 3.12, poor prognostic CAF markers were expressed more in lung parenchyma fibroblast monocultures compared to tumor fibroblasts. The rank of highest expressed genes in lung parenchyma fibroblasts were recorded as: *FN1* followed by *SPARC*, *COL1A1, COL5A1, COL3A1,* and *COL1A2*. However, the results were unstable with high error bars were calculated. In tumor lung fibroblast, only *FN1* was detected to be higher than BJ monoculture.

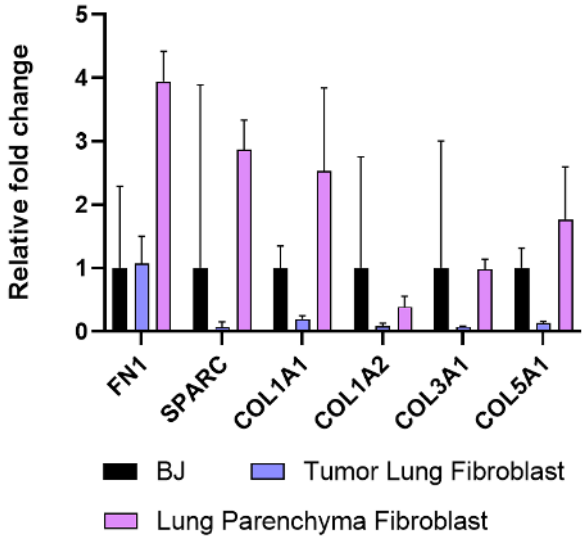


Figure 3.12 Relative fold change in expression levels of poor prognostic CAF marker genes in BJ, Tumor Lung, and Lung Parenchyma Fibroblast monocultures. BJ monoculture was used as control group and to calculate relative fold change.

Expression levels of the poor prognostic CAF markers in lung parenchyma and tumor lung fibroblasts were further investigated on protein level by western blot. According to the densiometric analysis in Figure 3.13, no expression of the poor prognostic CAF markers was observed in tumor lung fibroblasts, except FN1 showed a slight increase. In contrast, expression of these markers increased in lung parenchyma fibroblasts. These data were consistent with the qRT-PCR results.

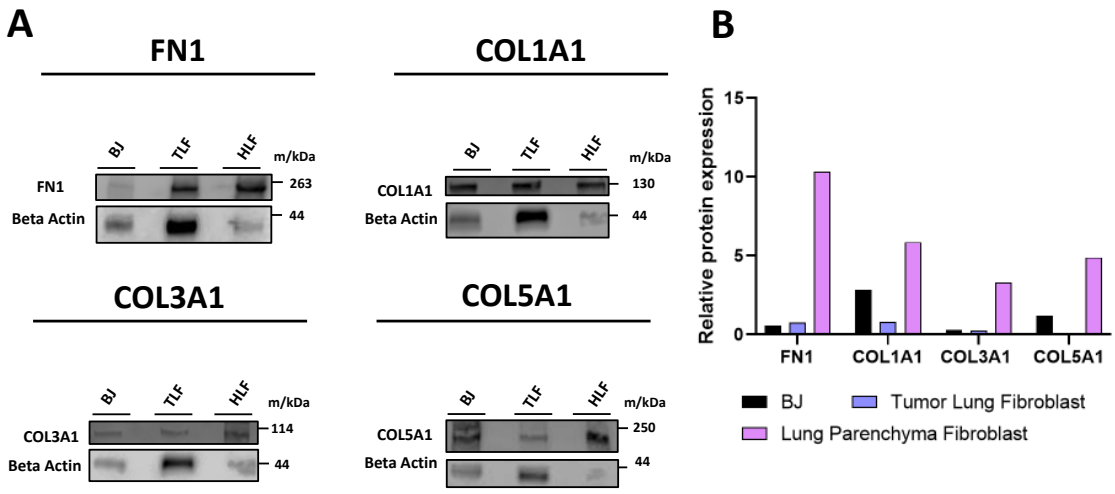


Figure 3.13 Protein expression levels of poor prognostic CAF markers in BJ, Tumor and Lung Parenchyma Fibroblast monocultures. **A.** Western blots of FN1, COL1A1, COL3A1, and COL5A1 proteins. **B.** Densiometric analysis of FN1, COL1A1, COL3A1, and COL5A1 expression using Beta-actin.

3.8.3. Investigation of Drug Response of Gastric Cancer Cells upon Co-culture with Primary Fibroblasts

Lung parenchyma fibroblasts exhibited increase in the poor prognostic markers. Therefore, we checked the response of SNU484-GFP gastric cancer cells to 5-FU, upon co-culture with tumor lung and parenchyma fibroblasts. Although tumor lung fibroblasts did not show increase in these markers, we investigate the drug response anyways. To observe the drug response of gastric cancer cells, we utilized live cell imaging method.

According to the cell viability results, none of the primary fibroblasts caused a decrease in the response to 5-FU (Figure 3.14). Tumor lung and parenchyma fibroblasts sensitized SNU484-GFP cells to 5-FU. Especially, lung parenchyma fibroblast was observed to decrease the maximal inhibition of SNU484-GFP cells in co-culture group compared to monoculture SNU484-GFP. Monocultures of these primary fibroblasts almost did not show response to the cytotoxic effect of 5-FU. Therefore, we concluded that primary fibroblasts caused gastric cancer cells to respond better to 5-FU.

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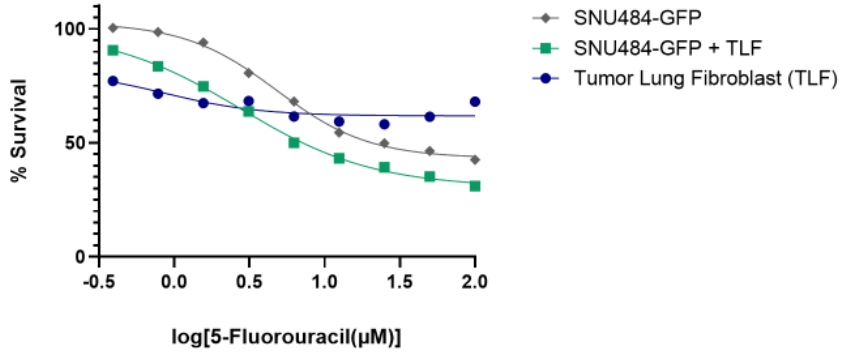
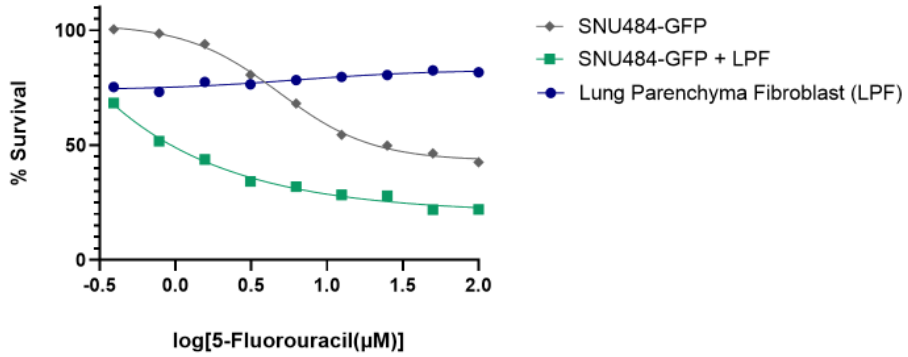


	SNU484-GFP	SNU484-GFP + TLF	TLF
IC50	4.814	2.746	0.8674

B



	SNU484-GFP	SNU484-GFP + LPF	LPF
IC50	4.814	3.624e-007	6.271

Figure 3.14 Dose-response curves of SNU484-GFP to 100 μM 5-FU treatment. Results were obtained via live cell imaging. **A.** SNU484-GFP co-cultured with tumor lung fibroblasts. **B.** SNU484-GFP co-cultured with lung parenchyma fibroblasts.

Chapter 4: **DISCUSSION**

Cancer initiation and progression highly depends on the surrounding stroma. Cancer cells disrupt the tissue homeostasis of the normal stroma by inducing the resident cells (Bremnes et al., 2011). This altered reactive stroma is abundant of different stromal and immune cells, which support cancer cells to proliferate and gain an aggressive phenotype. These cells secrete growth factors, inflammatory cytokines, chemokines, and complex ECM proteins to provide cell to cell communications and facilitate tumorigenesis. TME preserves these cellular and non-cellular components and serves as a deposit for cancer cells. Thus, cancer cells enhance angiogenesis, become resistant to chemotherapy, promote metastasis, and escape from immune destruction. In many different cancer types, various studies has emphasized the prognostic significance of TME (Zeng et al., 2019; Ma et al., 2020; Liu et al., 2021). Therefore, deciphering the characteristics of TME is vital for developing new treatment strategies as well as overcoming the current challenges faced in cancer treatment.

CAFs are one of the most common stromal cell types in the TME. They are known to exhibit pro-tumorigenic and anti-tumorigenic phenotypes depending on the stage of the tumor, as well as the frequency of cancer derived secretions (Wang et al., 2021). Although there may be a portion of anti-tumorigenic CAFs, cancer cells maintain to reprogram these by secreting microRNA (miRNA) containing extracellular vesicles or other soluble factors (Yan et al., 2015). Tumor promoting CAFs are particularly important for cancer progression. Through their secretions they induce not only cancer cells but also other stromal and immune cells. Also, they are known to integrate autocrine signaling to further activate molecular events that drive malignancy (Unger et al., 2017). Thus, they restrain the efficiency of chemotherapeutics, exert physical forces to help cancer cells invade and enable cancer cells to gain stemness properties.

Gastric cancer is the fifth most prevalent cancer type and fourth in cancer related deaths (Sung et al., 2021). Among various risk factors associated with gastric cancer, the TME is a major contributor to the disease progression. Inflamed nature of gastric metaplasia, provides a rich environment for recruitment of surrounding stromal and immune cells (Chung and Lim, 2014). Accumulating evidence has revealed that stromal and immune cell related genes

are correlated with poor prognosis in gastric cancer (Wang et al., 2019a; Wei et al., 2020). Especially, fibroblasts are considered as the main infiltrating cell types in gastric cancer with elevated levels of risk-associated transcription factors, cancer related pathways, and associated with dismal prognosis (Liu et al., 2020). Our lab group has identified a poor prognostic CAF gene signature in gastric cancer, which associated the expression of these markers with low survival rates and advanced tumor stages (Ucaryilmaz Metin and Ozcan, 2022a). Therefore, focusing on the TME elements requires great attention to develop new alternative treatment strategies.

In this thesis study, we aimed to generate a co-culture model to observe the poor prognostic effect of CAFs on gastric cancer cells. Fibroblasts can be activated by cancer cells, become CAFs, and facilitate tumor progression. We investigated how CAFs drive malignancy in gastric cancer by utilizing the poor prognostic gene signature that has been identified by our lab group. Although it is known that CAFs augment the tumorigenesis of gastric cancer, there is limited information about how poor prognostic CAFs exert these effects. According to the bioinformatic study, the gene signature was found to be correlated with the CAF infiltration rates, aggressive mesenchymal type gastric cancer, low survival rate and tumor stages (Ucaryilmaz Metin and Ozcan, 2022a). Hence, we focused on depicting tumor driving outcomes of poor prognostic CAFs in gastric cancer.

To obtain CAFs we utilized direct and no contact co-culture systems comprised of gastric cancer cells and fibroblasts. Direct contact co-culture system was used to demonstrate cell-to-cell and cell to ECM contact. In TME, cells are linked to each other and to the ECM by cadherin and integrin anchoring junctions respectively (Brücher and Jamall, 2014). Therefore, signal relaying is carried out by the interactions of receptor of a cell with another cell and/or ECM molecules. Cadherin anchoring junctions are known to affect functions of many growth factor receptor tyrosine kinases like EGFR, VEGFR, TGF-β, and FGFR (Lin et al., 2023). For instance, FGF signaling is maintained only in the presence of N-cadherin, which allows to activate downstream signaling that induces EMT, migration and invasion (Suyama et al., 2002). Whereas, integrin anchoring junctions mainly interact with ECM proteins like fibronectin, laminin, as well as growth factors like TGF-β (Brücher and Jamall,

2014; Khan and Marshall, 2016). Among arginine-glycine-aspartate (RGD) binding integrins, vast majority of αv family is known to regulate TGF-β binding and activation of latent TGF-β (Khan and Marshall, 2016). Fibronectin secretion is also known to contribute to TGF-β activation through binding to integrin αvβ6 (Fontana et al., 2005). TGF-β and FGF signaling being the major contributors to fibroblast activation (Fang et al., 2022), interact with anchoring junctions to exert their effects. In addition to direct contact, cells communicate through paracrine signaling in TME. Cancer cells induce fibroblast activation in paracrine manner by secreting growth factors and/or exosomes (Wu et al., 2013; Yang et al., 2020). Therefore, by integrating direct and no contact co-culture systems we aimed to potentiate activation of fibroblasts and observe their tumor driving phenotypes.

Direct co-culture of gastric cancer cells and fibroblasts caused morphological changes in fibroblasts. Monoculture fibroblasts exhibited spindle-like, elongated cellular morphology whereas fibroblasts in the co-culture groups showed stellate-like, polar morphology cells. This myofibroblastic, polar appearance of cells are characterized with CAF morphology (Sung et al., 2011; Kalluri, 2016; Ma et al., 2018b). Therefore, we emphasized that fibroblasts may have been activated by the gastric cancer cells and transitioned into CAFs. To further validate fibroblast activation, we checked for the expression of CAF markers. FAP and α-SMA (ACTA2) expressions are commonly used for CAF identification, but expression of FSP-1 and PDGFRα/β is also associated with gastric CAFs (Zhang et al., 2020; Hong et al., 2023). We investigated FAP and ACTA2 gene expression levels with qRT-PCR method for CAF activation in co-culture groups. The most significant increase in FAP gene expression was recorded in SNU484 and MRC-5 co-culture group. ACTA2 expression was also found be increased in this group. Expression of the CAF markers in SNU484 and BJ co-culture group were not prominent as much as in MRC-5 group. But no such increase was recorded for NUGC4 group co-cultured with BJ or MRC-5. We concluded that co-culture combinations with NUGC4 did not give rise to activation of fibroblasts molecularly. In contrast, SNU484 combinations caused activation of fibroblasts. Although CAF markers were recorded to be high in co-culture groups, SNU484 itself also showed high expression levels for these markers. Therefore, the expression levels in co-culture groups both gastric cancer cells and fibroblasts contributed to gene expression. To observe true activation levels

of fibroblasts, expression levels could have been observed only in fibroblasts. This could have been detected via using impermeable transwell inserts containing gastric cancer cells or conditioned medium instead of using direct co-culture method. This will be addressed in our future studies.

Upon investigation of CAF activation in co-culture groups, we investigated expression of the poor prognostic CAF markers using qRT-PCR and western blot methods. qRT-PCR results have revealed that co-culture of CAFs with SNU484 caused an increase in the poor prognostic CAF markers, whereas NUGC4 combinations did not. This suggested that since NUGC4 cells did not cause activation of fibroblasts, they did not potentiate the expression of these poor prognostic markers as well. Among SNU484 combinations, BJ co-culture group led to elevated levels of COL1A2 and COL5A1 expressions, the latter being more significant. This finding was particularly interesting since the bioinformatic study of our group also highlighted the significance of the hazard ratios for CAF infiltration levels and expression of these markers. The study depicts hazard ratio (HR) for CAF infiltration rate as 5.240. High COL1A2 expression increases the HR further to 5.631, and high COL5A1 expression increases it to 8.584, and COL5A1 with COL1A2 expressions together increases the HR to 7.701 (Ucaryilmaz Metin and Ozcan, 2022a). These results implied that activated BJ fibroblasts have exerted its poor prognostic effects by the expression of these two markers. On the other hand, COL3A1 expression was found to be increased in SNU484 and MRC5 co-culture group. However, it was not more robust than the significant markers detected in BJ combination.

Next, we further examined the expression of the poor prognostic CAF markers on protein level. Results were coherent with the qRT-PCR results, NUGC4 and BJ co-culture group showed no expression for the markers. In SNU484 and BJ co-culture group, increased mRNA levels of COL1 were detected in protein level as well with COL1 antibody. However, COL5A1 protein levels were not elevated as much as in gene level. These data suggested that SNU484 and BJ co-culture could be a promising model to observe tumor driving roles of CAFs in gastric cancer. Hence, we used SNU484 and BJ co-culture group in further experiments.

CAFs promote malignancy in many types of cancers, through different mechanisms. Increasing the proliferation rate of cancer cells is one of the tumorigenic features of CAFs. In colorectal, endometrial and prostate cancers conditioned medium obtained from CAFs have caused increase in the cell proliferation rates of these cancer cells (Subramaniam et al., 2013; Jahangiri et al., 2019; Sun et al., 2019). Therefore, we investigated whether if no contact or direct contact of CAFs would facilitate the cell proliferation rate of gastric cancer cells. According to results of the assay, direct contact of CAFs did not increase the cell proliferation rate, whereas paracrine effect of monoculture CAFs showed opposite results. Otomo and colleagues have shown that in lung cancer, lung fibroblasts facilitate the cell proliferation of lung cancer cells in a direct co-culture system through p53 downregulation and Tetraspanin 12 derepression, but such effect was not recorded in no contact co-culture system (Otomo et al., 2014). Therefore, contact dependent effect of CAFs on cell proliferation could be specific to cancer types or require a better representation of TME. In head and neck squamous carcinoma, co-culture of CAFs and cancer cells as 3D spheroids, showed increased proliferation rates (Magan et al., 2020). Thus, using 3D models could be more promising for observing contact dependent proliferation rate due to their resemblance to TME in terms of high ECM protein production, cell to cell and cell to ECM interactions. However, the paracrine effect of CAF monocultures resulted in lowering the doubling time of gastric cancer cells. Therefore, we deduced that secretions of CAFs could have exerted such effects. Although we did not investigate the effector further, it is known that CAF derived paracrine cytokine secretions or exosomes containing miRNA are involved in increasing the cell proliferation in different cancer types (Richards et al., 2017; Yang et al., 2021).

Cancer, also referred as wounds that never heal, promotes constant inflammation and ECM deposition to drive its progression (Flier et al., 1986). CAFs are activated in response to the inflammatory environment and actively produce ECM proteins, and reorganize the ECM to allow cancer cells to proliferate (Yang et al., 2023). Therefore, we expected to observe poor prognostic CAFs to boost wound healing capabilities of gastric cancer cells. For this purpose, we investigated the paracrine effect of CAFs. CAFs exhibited a significant contribution to wound healing only when they were monocultured in inserts.

It is also known that CAFs induce migration and invasion capabilities of cancer cells to potentiate metastatic cascade (Yang et al., 2023). We utilized transwell inserts with cell permeable pores to observe migration of gastric cancer cells in the presence of CAFs. We also created a serum gradient to facilitate the migration. Without the effect of any cells, migration was not induced by serum gradient. Paracrine effect of SNU484 cells did not affect migration of SNU484-GFP cells either. In contrast, SNU484 and BJ co-culture increased the migration significantly. Also, the paracrine effect of BJ monoculture significantly increased the migration of SNU484-GFP cells in top inserts. Similar to wound healing results, paracrine signaling of CAFs inferred to be critical in promoting migration of SNU484-GFP cells.

Another tumor driving phenotype of CAFs is increasing the survival of cancer cells in response to chemotherapeutics (Jena et al., 2020). Consequently, we investigated whether the poor prognostic CAFs induce chemoresistance in gastric cancer. For this purpose, we tested 5-FU and paclitaxel chemotherapeutic agents that are conventionally used in the first- and second-line treatment of gastric cancer respectively.

5-FU, as a pyrimidine analog, inhibits TS that leads to cytotoxicity due to integration of fluoronucleotides into DNA and RNA (Longley et al., 2003). According to the results of MTT assay, BJ monoculture showed the least response to 5-FU, followed by SNU484 and BJ co-culture group. Highest response was recorded in SNU484 mono-culture group, meaning that CAFs decreased the anti-cancer effect of 5-FU and caused a slight shift of the curve to the right. Similar results were recorded in a study investigating the effect of primary gastric fibroblasts on gastric cancer cells' drug response to 100 ng/mL of 5-FU (Ma et al., 2018b). However, another research pointed out that effect of BJ fibroblasts caused an increase in the anti-cancer effect of 10 μM 5-FU on colon cancer cells whereas colon fibroblasts decreased the response to 5-FU (Koh et al., 2019). Therefore, in our study using gastric fibroblasts could have been more promising to observe CAF induced chemo resistance. Also, a study highlights that 5-FU treatment inhibits not basal COL1A1 but TGF-β induced COL1A2 synthesis in foreskin fibroblasts (Wendling et al., 2003). In our co-culture model, CAFs exhibit elevated levels of COL1A2, which have been associated with poor prognosis. Although, we did not investigate TGF- β levels in co-culture, it is likely for it to be present in the environment since it is a major contributor to CAF mediated mechanisms. If 5-FU

caused COL1A2 inhibition in our co-culture model as well, the CAFs may not have exhibited their poor prognostic effect robustly.

Paclitaxel binds to microtubules and prevents cell cycle progression due to stabilizing tubulin proteins (Jimenez et al., 2011). MTT cell viability assay revealed that BJ monoculture almost did not show response to paclitaxel treatment. Thus, we inferred fibroblasts that are not under the effect of cancer cells might have been in resting state and not affected by paclitaxel treatment. However in renal fibrosis, low dose of paclitaxel leads to inhibition of fibroblast activation by downregulating fibronectin, α-SMA, collagen I expression through deregulating microtubule and STAT3 association (Zhang et al., 2015). In SNU484 + BJ co-culture group we observed that presence of fibroblasts increased the response to chemotherapy. Although, Bartling and colleagues have demonstrated a protective role of fibroblasts on lung cancer cells upon paclitaxel treatment by regulating apoptosis related protein kinases (Bartling et al., 2008). Consequently, more effort is required to understand the mediators of decreased cell survival effect of CAFs in response to paclitaxel.

We suspected that the experiment setup of MTT assay might have been insufficient to observe the SNU484 cells' response to treatment in the co-culture group since the signal obtained from co-culture plates belong to both type of cells. Thus, we generated SNU484 cells with GFP tagged nucleus, which allowed to measure its cellular viability in co-culture. However, the results were coherent with MTT assay for both 5-FU and paclitaxel treatments. We validated the effect of CAFs on gastric cancer cells and concluded that poor prognostic CAFs induce a slight resistance to 5-FU.

Although CAFs are known to have cancer promoting phenotypes, not all induce cancer cells in the same extent. In gastric cancer, different CAF subtypes have different tumor promoting roles, and even some subtypes do not induce tumorigenesis (Kim et al., 2022). Therefore, we asked whether primary fibroblasts obtained from tumor tissue could have a robust poor prognostic effect compared to fibroblast cell line. We used primary fibroblasts derived from lung parenchyma and tumor tissue to observe this effect. Although the magnetic bead separation allowed to differentially select the fibroblasts in the heterogenous cell suspension, we further checked for the gene expression of FAP and ACTA2 CAF markers. According to

the qRT-PCR results, only expression of ACTA2 gene was increased in lung parenchyma fibroblasts. However, in tumor lung fibroblasts none of the markers were expressed. Even though there are cancer specific CAF markers in the literature, FAP and ACTA2 are the most observed CAF markers (Han et al., 2020). Since tumor lung fibroblasts did not exhibit any of these markers, we suspected that they were not classified as CAFs. Also, they did not express any of the poor prognostic CAF markers except FN1. In contrast, lung parenchyma fibroblasts showed an increase almost in all the markers.

Although we observed that lung parenchyma fibroblasts expressed poor prognostic CAF markers, we did not observe the poor prognostic effect. Cell viability experiments revealed that primary fibroblasts did not induce gastric cancer cells to obtain chemoresistant phenotype. Instead, they induced gastric cancer cells to be sensitive to the drug. Therefore, we inferred that these CAFs may have different phenotypes as recent studies suggest (Wong and Sukkar, 2017; Kim et al., 2022). Thus, other poor prognostic phenotypes of these CAFs can be investigated like their contributions to wound healing abilities or increasing cell survival of cancer cells. Moreover, other cellular or non-cellular components of the TME may be necessary to induce a poor prognostic phenotype for CAFs. In our future studies, we aim to address this.

In summary, we tested different co-culture models to observe how poor prognostic CAFs exert their effect on gastric cancer. We observed that COL1A2 and COL5A1 expressing CAFs illustrates a malignant phenotype in gastric cancer. Especially, we pointed out the differences between paracrine signaling and direct contact of CAF and gastric cancer cells. This study has identified findings about the poor prognostic effects of CAFs as boosting the cell proliferation rate, decreasing the anti-cancer effects of 5-FU, increasing the wound healing and migration capabilities in gastric cancer. Associating these cancer promoting features of CAFs greatly matters to overcome handicaps faced in treatment of gastric cancer. To our knowledge, there is limited evidence on *in vitro* investigation of poor prognostic CAFs in gastric cancer. Therefore, identifying CAF markers and its relevance in disease progression could be promising for new treatment strategies. As our lab group has identified in the bioinformatic study, these CAF markers can be robust targets to eradicate the poor prognostic

phenotype. Agents like Halofuginone and collagenase that are known for inhibition of fibroblast maturation and collagen degradation respectively, could be repurposed to target these markers (Ucaryilmaz Metin and Ozcan, 2022a). Therefore, as future prospective of this study, combination of 5-FU and these agents could bring a new insight in the treatment of poor prognostic gastric cancer. This will be addressed in our future studies.

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