ANALYSIS OF THE CROSSTALK BETWEEN CANCER-ASSOCIATED FIBROBLASTS AND TUMOR CELLS IN HEPATOCELLULAR CARCINOMA

by

HATİCE ÇAKIR

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APPROVED BY:

Prof. Devrim GOZUACIK
(Thesis Supervisor)

Assoc. Prof. Ozlem KUTLU

Assoc. Prof. Devrim ÖZ ARSLAN

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ABSTRACT

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Hatice ÇAKIR

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Thesis Supervisor: Prof. Devrim GÖZÜAÇIK

Keywords: Tumor stroma, cancer associated fibroblast (CAF), hepatocellular carcinoma (HCC)

Most anticancer treatment approaches focus on malignant cells. However, tumors do not only comprise of malignant cells, but also contain many other cell types such as fibroblasts, mesenchymal cells, epithelial cells and non-cellular components. Hence, the progression of a tumor also depends on its crosstalk with neighboring cells called tumor stroma. Stroma can be activated by various stimuli and affects tumor progression, metastasis and drug resistance. Among stromal cells, fibroblast have attracted a significant amount of attention in the last decade, due to their unique functions. Naive fibroblasts are quiescent, but they are activated during physiological events, such as wound healing. On cancer, in response to interactions with tumor cells, fibroblasts (and some other cell types) undergo transdifferentiation and turn into cancer-associated fibroblasts (CAFs). CAFs are distinguished from other types of activated fibroblasts. CAFs were shown to support tumor growth, invasion, metastasis and even cancer resistance to treatment. In this study we isolated primary fibroblasts of normal liver and CAFs from hepatocellular carcinoma (HCC) tissues, and analyzed their effects on cancer behaviour. Co-culture experiments showed that, HCC-derived CAFs, but not normal liver fibroblasts, supported the growth of HepG2 and Huh7 tumor cell lines. Moreover, tumor promoting effects of CAFs did not require direct cell contact, indicating involvement of secreted factors. We identified a factor playing a key role in the observed cancer cell-CAF crosstalk. Secretion level of the factor was higher in conditioned media from CAFs that were co-cultured with cancer cells compared to those co-cultured with tissue fibroblasts. We demonstrated that the factor was predominantly secreted from CAFs, and cancer cells showed much lower expression levels. Importantly, proliferative effects of CAFs on cancer cells was reversed when a factor-specific neutralizating antibody was added to co-cultures. Hence, we defined a key regulator of CAF-induced tumor growth in HCC. The identified factor might be a potential target for new anticancer therapy approaches.

ÖZET

HEPATOSELÜLER KARSİNOMADA KANSER-BAĞLANTILI FİBROBLASTLAR İLE KANSER HÜCRELERİNİN KARŞILIKLI ETKİLEŞİMİNİN ANALİZİ

Hatice ÇAKIR

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Anahtar Kelimeler: Tümör stroması, kanser-bağlantılı fibroblast, hepatoselüler karsinoma

Günümüzdeki kanser terapilerinin büyük bir çoğunluğu yalnızca malign hücreleri hedeflemektedir. Oysa ki tümörler malign hücrelerin yanı sıra fibroblastlar, mezenkimal hücreler, epitel hücreler ve hücresel-olmayan içerikler olmak üzere birçok bileşenden oluşur. Tümörün gelişmesi, stroma olarak da bilinen komşu hücreler ile iletişimine bağlıdır. Stroma çeşitli uyaranlar ile aktifleştirilebilir ve aktifleşmiş stroma tümör gelişimini, metastazı ve ilaç direncini etkiler. Son yıllarda fibroblastlar benzersiz özelliklerinden dolayı stromal hücreler arasında en çok dikkat çeken hücrelerden biri olmuştur. Normal koşullarda fibroblastlar uyku halinde bulunurlar ve yaralanma gibi bazı fizyolojik durumlarda aktifleşirler. Fibroblastlar (ve bazı diğer tip hücreler) kanser hücreleriyle etkileşimleri sonucu transdiferansiyasyon geçirirler ve kanser-bağlantılı fibroblastlara (CAFlar) dönüşürler. Kanser-bağlantılı fibroblastlar birçok özellikleri ile diğer aktifleşmiş fibrolastlardan ayrılırlar. CAFların tümör gelişimini, invazyonu, metastazı ve hatta ilaç direncini etkilediği gösterilmiştir. Bu çalışmada hepatoselüler karsinoma hastalarının sağlıklı karaciğer dokularından primer fibroblast ve tümör dokularından CAFlar elde edilmiştir. Birlikte-kültür sonuçlarına göre, kanser-bağlantılı fibroblastların HepG2 ve Huh7 tümör hücre hatlarının büyümesini fibroblast birlikte-kültürüne göre anlamlı ölçüde artırdığı saptanmıştır. Dahası, bu büyümenin direkt hücre temasına gerek duymadığı bulunmuştur. Bu sonuç, büyümenin hücre dışına salınan sitokinler üzerinden gerçekleştiğine işaret etmektedir. Bu süreçte anahtar rol oynayan yeni bir faktör belirlenmiştir. Bu faktörün salınım düzeyi, CAF-birlikte kültür medyasında fibroblast birlikte-kültür medyasına göre daha yüksek bulunmuştur. Bu faktörün CAFlardan baskın bir şekilde salındığı ancak kanser hücrelerinde daha düşük düzeyde ifade edildiği gösterilmiştir. Önemli olarak, CAFların sebep olduğu büyüme, bu faktöre spesifik nötralize antikorun birlikte-kültür ortamına eklenmesi ile geri döndürülmüştür. Dolayısıyla bu çalışmada HCC'de, kanser-bağlantılı fibroblast tarafından indüklenen tümör büyümesinin anahtar düzenleyicisi olan yeni bir faktör tanımlanmıştır. Bu faktör, yeni kanser terapileri yaklaşımları için potansiyel bir hedef olabilir.

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To my mom.

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LIST OF ABBREVIATIONS

ADCC	Antibody-Dependent Cellular Cytotoxicity
ALKs	Activin Receptor-Like Kinases
BRAF	B-Raf Proto-oncogene
BMPs	Bone Morphogenic Proteins
CAF	Cancer-Associated Fibroblast
CXCL12	C-X-C Motif Chemokine Ligand 12
CYP	Cytochrome P450
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EGFs	Epidermal Growth Factors
EMT	Epithelial Mesenchymal Transition
ERK1/2	Extracellular Signal-Regulated Kinases 1/2
FAF	Fibrosis-Associated Fibroblast
FAP	Fibroblast Activation Protein
FBS	Fetal Bovine Serum
FGF2	Fibroblast Growth Factor
FSP1	Fibroblast-Specific Protein 1
GDF15	Growth Differentiation Factor 15
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HCL	Hairy Cell Leukemia
HDIL-2	High Dose Interleukin-2
HGF	Hepatocyte Growth Factor
HRP	Horseradish Peroxidase
IFN-A	Interferon-A
IL1R2	IL1 Receptor Type II

IL-6	Interleukin-6
INHBA	Inhibin Subunit Beta A
JNKs	c-Jun N-Terminal Kinases
LIF	Leukemia Inhibitory Factor
MAPK	p38 Mitogen Activated Protein Kinase
MM	Metastatic Melanoma
MMP	Matrix Metalloproteinases
MRCC	Metastatic Renal Cell Carcinoma
MSC	Mesenchymal Stem Cells
NAF	Normal Activated Fibroblasts
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween-20
PDGF	Platelet-Derived Growth Factors
PFA	Paraformaldehyde
PTEN	Phospatase And Tensin Homolog
SDF1 or CXCL12	Stromal Cell-Derived Factor 1
SHH	Sonic Hedgehog
SMAD	Mothers Against Decapentaplecgic Homolog
SRF	Serum Response Factor
SV40	Simian Virus 40
TCGA	The Cancer Genome Atlas
TGFβ	Transforming Growth Factor Beta
VEGFA	Vascular Endothelial Growth Factor A
VEGFR2	Vascular Endothelial Growth Factor 2
WHO	World Health Organization
α-SMA	Alpha-Smooth Muscle Actin

1. INTRODUCTION

Tumors do not only comprise of continuously dividing cancer cells, they also include many types of other cells. Tumor microenvironment consists of not only cellular components but also the non-cellular ones. One of the major component of tumor microenvironment is called as tumor stroma, which is a heterogeneous population that involves fibroblasts, mesenchymal cells and non-cellular components such as cytokines. Communication between cancer cells and tumor stroma is crucial for tumor progression, metastasis, invasion, and therapy resistance. Under normal conditions, stroma is not activated to maintain homeostasis and tissue structure. In recent years, fibroblasts are considered to be the major regulators of tumor stroma. They can be activated in order to contribute tumorigenesis. However, their function in the context of cancer is not fully understood yet.

1.1. Tumor and Tumor Microenvironment

Despite extensive research on advanced therapeutic options and improved molecular biology techniques, cancer remains a leading cause of death in worldwide. In cancer treatment, experimental and clinical outcomes are not overlapping. The most potential reasons behind this situation are that majority of anticancer therapies target only malignant cells, recurrence and lethal metastasis. In the last few decades, researchers have common opinions about the importance of tumor microenvironment in the progression and treatment of cancer.

According to a study conducted by Paget (1889) underlined the significance of tumor microenvironment and stroma with his “seed and soil” hypothesis, which gained attention only in the recent years. According to seed and soil hypothesis, tumors only metastasize to a secondary site, which has favorable condition. Tumor microenvironment involves many components including but not limited to epithelial cells,

immune system cells, soluble factors and stroma. There are other models describing the complexity of tumor microenvironment and its interactions called as optimal foraging theory, ecosystems networks (Amend & Pienta, 2015; Camacho & Pienta, 2012; De Groot, Roy, Brown, Pienta, & Amend, 2017). These models also propose that tumor microenvironment favors tumorigenesis from different perspectives. It has important effects on tumor initiation, progression, therapy resistance, metastasis, and relapse. Experimental studies conducted on tumor microenvironment ensured strong evidence to support these theories.

1.2. Tumor Stroma

The principal role of the tumor stroma is to maintain the structure of functional tissue and remodel it. Thus, interactions between non-cellular and cellular components of the stroma are significant to understand how they affect each other, and tumor behavior. Stroma comprises various components that are both cellular and noncellular. Cellular components of stroma include endothelial cells, pericytes, specialized connective tissue cells such as fibroblasts, osteoblasts, mesenchymal stromal cells, etc. On the other hand, noncellular components of stroma contain cytokines, collagens, growth factors, extracellular matrix (ECM) (Valkenburg et al., 2018). In this manner, it is easy to say that the communication between tumor microenvironment and tumor itself through secreted components of the stroma may and will affect the behavior of the tumor. Therefore, understanding the complex network of tumor stroma would serve better insights to interpret tumor behaviour.

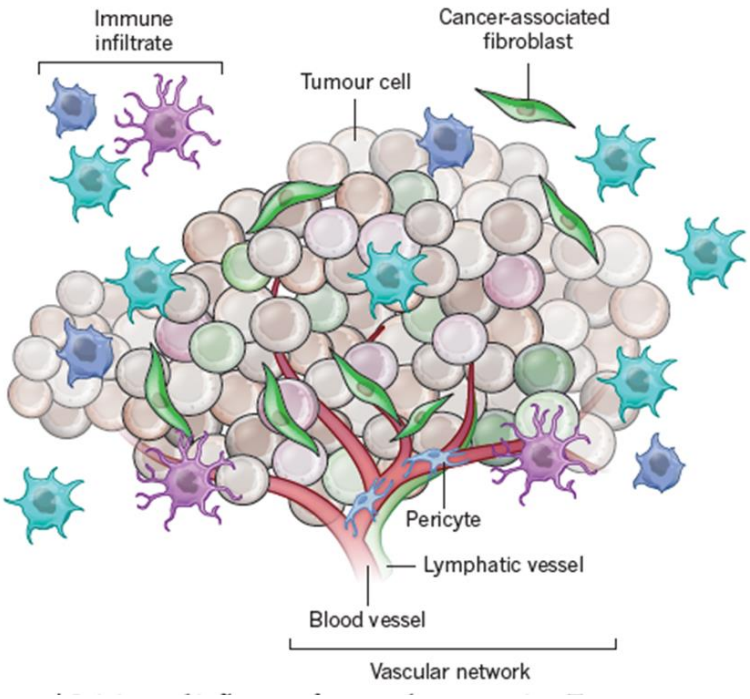


Figure 1.: Heterogeneity of tumor stroma (Junttila & Sauvage, 2013).

Construction and amount of stroma varies depending on cancer type. Particularly in pancreatic, liver, stomach cancers, it contributes to 60-90% of total tumor mass (Harold F Dvorak, 2016; Powell, Adegboyega, Mari, & Mifflin, 2005). From the general concept of cancer, stroma has severe effects on the hallmarks of cancer as a crucial component of the tumor microenvironment (Hanahan & Weinberg, 2011). Once cancer is initiated, stroma undergoes certain changes and it may become malignant stroma. During this event, normal stromal fibroblasts called “quiescent” are activated and transdifferentiated into cancer-associated fibroblasts (CAFs). Their shape, expression profile, secretion phenotype are changed (Valkenburg et al., 2018). During cancer progression, the ECM is also dramatically altered, becoming more rigid and favorable to invasion of cancer cells through ECM. Besides the physical effect on cancer cell invasion through structural changes, the ECM also facilitate genetic changes on cancer cells. For instance, increased stiffness of the ECM found to activate some factors including microRNAs which further downregulate tumor suppressor genes such as phospatase and tensin homolog (*PTEN*) and help cancer cells to gain more aggressive phenotype (Mouw et al., 2014). The differences between normal stroma and tumor stroma were shown in Figure 2.

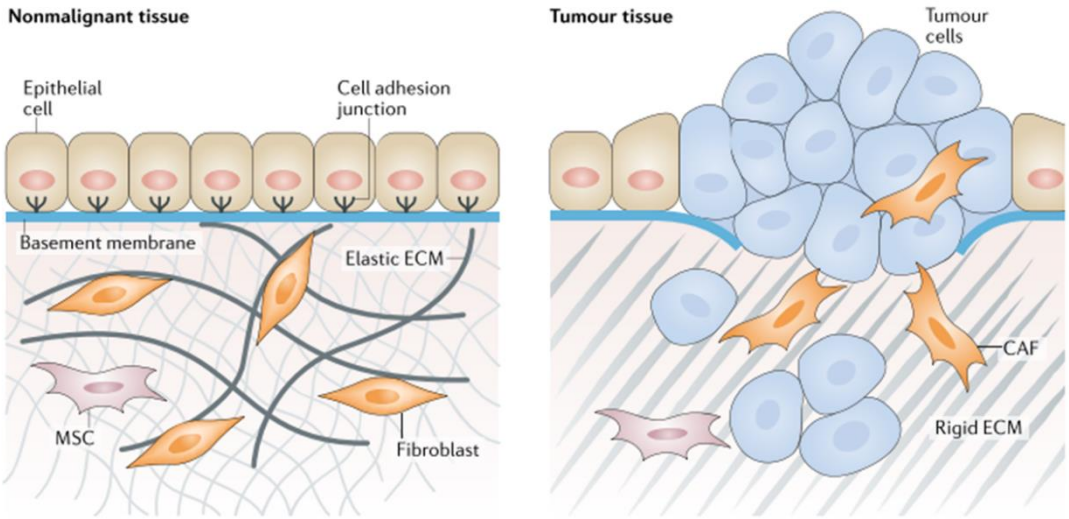


Figure 2.: Comparison of normal and tumor stroma (Valkenburg et al., 2018).

Another tumor-promoting effect of the malignant stroma is to support angiogenesis that is essential for tumor growth and survival. Endothelial cells, another cellular component of tumor microenvironment, are important for sustaining tumor growth by regulating angiogenesis. Angiogenesis is the formation of new vessels, neo-vascularization, from existing vascular organizations to supply cancer cells with oxygen and nutrients. It requires a variety of actions. Hypoxia is the main governer of angiogenesis in tumors. Angiogenesis begins with secretion of vascular endothelial growth factor A (VEGFA) from hypoxic cancer cells. Then, VEGFA interacts with VEGF receptor 2 (VEGFR2), which reside in surrounding endothelial cells. This interaction triggers the formation of motile endothelial cells, which are also called as tip cells (Potente, Gerhardt, & Carmeliet, 2011). Tip cells with the help of collagenases and matrix metalloproteinases (MMPs) degrade the ECM and this degredation leads to growth of new blood vessel offsets (Potente et al., 2011). Besides endothelial cells, another cellular components of stroma contribute to angiogenesis such as platelets through secreting fibroblast growth factor 2 (FGF2) and platelet-derived growth factors (PDGFs) (Palma, Biziato, & Petrova, 2017).

Briefly, surrounding stromal cells and cancer cells have continuous crosstalk. When stroma is activated, its key components like ECM and fibroblasts are changed. Malignant stroma may further promote tumor development, metastasis, and invasion.

1.3. Activation of Fibroblasts

Fibroblasts are classic spindle-shaped cells and have multiple distinct functions. Principally, they build up and degrade the ECM by releasing collagens, MMPs, and other fibrous molecules. The interaction among fibroblasts is driven by integrin signaling and it affects the distribution of collagens (ref). Similarly, collagen fibers affect the allocation of fibroblasts in ECM. They have also an impact on homeostasis and tissue maintenance through the expression of enzymes from cytochrome P450 (CYP) family that degrades potentially harmful molecules. Moreover, fibroblasts have an important role in inflammation by secreting cytokines and chemokines (Buckley et al., 2001; Smith, Smith, Blieden, & Phipps, 1997).

Under normal conditions, fibroblasts are at quiescent state, but they are able to migrate and secrete cytokines, ECM molecules, etc. Fibroblasts can be activated in different ways such as through regulation of growth factors (e.g. transforming growth factor-beta (TGFβ), PDGF, interleukin-6 (IL-6)) or upon certain pathological conditions, for instance, wound healing (Kalluri & Zeisberg, 2006; Öhlund, Elyada, & Tuveson, 2014). After activation, the ECM molecule production, remodeling properties, secretion phenotype of fibroblasts have changed. In acute wound healing, the response would become fast and temporary. Therefore, this process involves the recruitment of immune cells, activation of fibroblast, induction of angiogenesis and recovery of the ECM. A great part of the ECM and basement membrane such as collagens, fibronectin and laminins are created by activated fibroblasts or myofibroblasts (H F Dvorak, 1986). When the wound is healed, some of fibroblasts are eliminated by apoptosis, and a majority of the resting state “quiescent” is restored (Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002).

If the threatening factors become continious, for example, chronic diseases or extensive exposure of toxic materials, the wound losts its ability to heal. The tissue repair process becomes chronic and it is called as tissue fibrosis. Hence, tissue fibrosis can be defined as continuous wound healing activity by displaying endless repair functions. Epigenetic mechanisms are included in tissue fibrosis to control apoptosis, thus, fibrosis-associated fibroblasts (FAFs) may be included depending on the tissue (Figure 3) (Driskell et al., 2013; Dulauroy, Di Carlo, Langa, Eberl, & Peduto, 2012; Hamburg-Shields, Dinuoscio, Mullin, Lafayatis, & Atit, 2015; Rock et al., 2011).

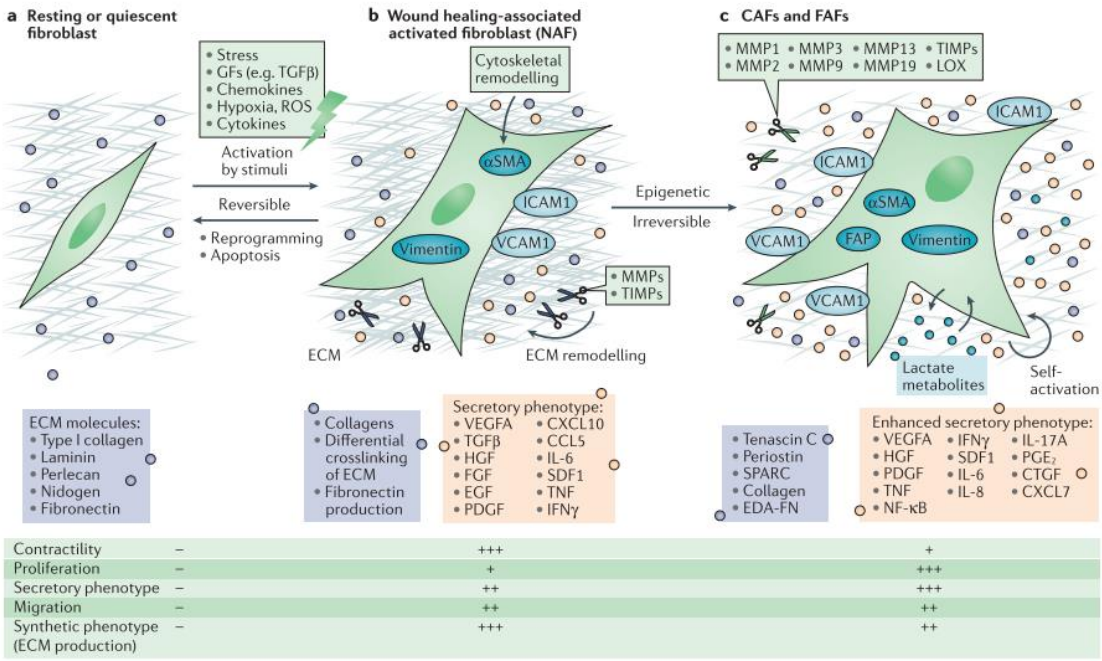


Figure 3.: Multi-step activation of fibroblasts (Kalluri, 2016).

Cancer is also an activator for fibroblasts and it is widely referred to as “wound that does not heal”. The role of myofibroblasts or CAFs in wound healing is well established, however, their functions in tumor progression are still under investigation due to their complexity and context-dependent behavior. They have both tumor-promoting and tumor-restraining effects (De Wever, Van Bockstal, Mareel, Hendrix, & Bracke, 2014; Öhlund et al., 2014). In acute or chronic damage, fibroblasts are recruited through secretion of TGFβ, PDGF and FGF2 from cancer cells (Elenbaas & Weinberg, 2001). In many types of cancer, enlisting of activated fibroblasts is dependent on TGFβ. Also, TGFβ induces the proliferation of neighboring CAFs. Of note, several studies were stated that fibroblast activation is a defense mechanism of host against tumor development (Dumont et al., 2013; Elenbaas & Weinberg, 2001; Ishii, Ochiai, & Neri, 2016; Kalluri & Zeisberg, 2006). Everything considered, it is still unknown the detailed mechanisms of tumor stroma accumulation affecting the tumorigenesis and awaits to be discovered.

Detailed association between wounding and cancer in mammals is not clarified yet, but some studies have indicated that presence of previous tissue fibrosis increases the risk of carcinoma outgrowth particularly in liver, pancreas and lung (Karampitsakos et al., 2017; J. Y. Li et al., 2014; Samet, 2000). The occurrence of hepatocellular carcinoma (HCC) was indirectly associated with previous existence of chronic damage including ROS accumulation, genomic instability, and liver fibrosis (Sangiovanni et al.,

2004; Wang et al., 2013).

Activated fibroblasts are considered to orchestrate the stromal activities and signaling pathways of cancer cells predominantly (Sappino, Skalli, Jackson, Schürch, & Gabbiani, 1988). Transdifferentiation of quiescent fibroblasts to CAFs is controlled by complex mechanisms and various signaling molecules including TGFβ. On the other hand, accumulating data demonstrates that this transition may be governed by epigenetic mechanisms (P. Li et al., 2015; Mrazek et al., 2014; Tampe & Zeisberg, 2014; Zeisberg & Zeisberg, 2013). In tumorigenesis, unlike wound healing CAFs are not eliminated by apoptosis, thus once activated follows irreversible cycle of activation through several factors. Therefore, CAFs are persistent players of malignant stroma.

1.4. From Fibroblasts to Cancer Associated Fibroblasts (CAFs)

The origin of CAFs is an arguable topic. Several studies have shown that CAFs might be originated from activated fibroblasts, mesenchymal stem cells (MSCs), adipocytes, and pericytes less probably. Ultimately, the consensus is that CAFs are originated from local resting fibroblasts (Sahai et al., 2020).

A variety of mechanisms has been proposed for the activation of CAFs (Figure 4). TGFβ is a well-understood molecule in terms of fibroblast activation. It may further enhance the activity of SMAD transcription factors and serum response factor (SRF). It also leads to expression of alpha-smooth muscle actin (α-SMA), which is an activated fibroblast marker, as well as an advance in the function of contractile cytoskeleton (Tomasek et al., 2002). Genomic and physiological stresses can also induce the activation of fibroblasts. For instance, double-stranded DNA breaks may enhance the production of IL-6 and Activin-A, a member of TGFβ superfamily (C. A. Fordyce et al., 2012; C. Fordyce et al., 2010). Changes in the structure of ECM can activate CAFs too. Crosstalk of cancer cells and fibroblasts can further promote CAF activation through the Notch signaling; however, it seems that it is dependent on cancer type. In breast cancer, CAFs were activated through Notch pathway, but in squamous cell carcinoma loss of Notch signaling was led to CAF activation (Procopio et al., 2015; Strell et al., 2019). Moreover, there are strong evidences for the management of activation by epigenetic alterations (Kalluri, 2016).

CAFs are usually identified by increased expression of α-SMA. There are other CAF markers such as vimentin, fibroblast activation protein (FAP) and fibroblast-specific

protein 1 (FSP1) (Kalluri, 2016).

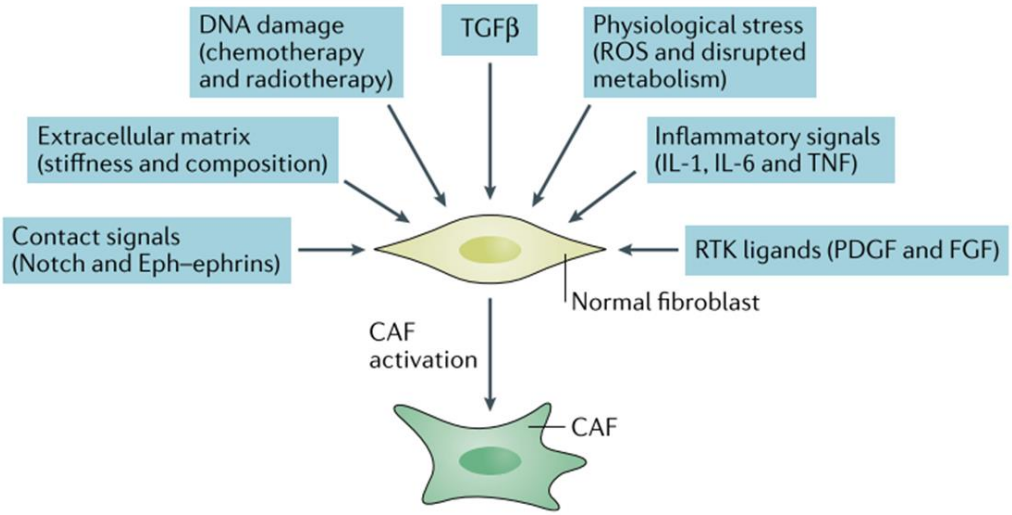


Figure 4.: Diverse mechanisms in the activation of fibroblasts (Sahai et al., 2020).

When fibroblasts are activated and transdifferentiated to CAFs, they gain some features that distinguish them from normal fibroblasts (Table 1). For instance, CAFs are actively taking role in the production and remodeling of ECM by its secretory such as matrix-crosslink enzymes. Therefore, CAFs contribute to the increment of tumor stiffness more than resting stromal fibroblasts (Nguyen et al., 2019; Tang et al., 2016). Besides, CAF-mediated MMPs activation, CAFs also able to remodel ECM by physically forcing through leading fibroblasts which remodel the ECM and create a favorable spot for invasion (Gaggioli et al., 2007).

Table 1.: Comparison of normal fibroblasts and CAFs (Modified from Kalluri, 2016).

Normal (Resting) Fibroblasts	Cancer-Associated Fibroblasts (CAFs)
Spindle shaped	Cruciform or stellate shaped
Non-proliferative or slow self-renewal	Proliferative
Can be activated	Can be further activated
Activated by extrinsic factors	Capable of self-activation
FSP1$^+$, α1β1 integrin$^+$	αSMA$^+$, PDGFRβ$^+$, FAP$^+$, vimentin$^+$
No active secretome	Highly dynamic and active secretome
Epigenetically stabile	Epigenetically modified
Non-migratory	Migratory

CAFs become migratory, gain function of self-activation and display enhanced secretory phenotype including growth factors, chemokines and cytokines due to potentially epigenetic modifications and partly genetic alterations.

1.5. CAFs: Most Attracted Cells in Stroma

CAFs have attracted considerable attention in recent years due to their distinct functions, extraordinary secretions, effects on metastasis, autocrine growth signaling and capability of dominating tumor fate. Overwhelming knowledge supports that CAFs act as positive regulators in tumorigenesis. According to a variety of co-culture assays, CAFs promoted tumor development compared to normal activated fibroblasts (NAFs) (Orimo et al., 2005). Their tumor-promoting effect was shown for the first time in 1999. Simian virus 40 (SV40) transformed prostate epithelial cells were co-cultured with either CAFs or normal prostatic fibroblasts and injected into mice. As a result of co-culture, CAFs were dramatically enhanced tumor growth both *in vivo* and *in vitro* while normal prostatic fibroblast did not cause the same effect (Olumi et al., 1999). Additionally, their ability to trigger angiogenesis through stromal cell-derived factor 1 (SDF1 or CXCL12) secreted by CAFs partly drives pro-tumorigenic effects (Orimo et al., 2005). VEGF, produced by stromal cells, also induces angiogenesis. A number of exogenous signals, depending on cancer type, regulate the pro-tumorigenic and pro-invasive effects of CAFs. However, these regulation mechanisms are highly dynamic and complex. Therefore, describing their regulatory effects on CAFs in vivo using only a snapshot might be misleading because of the complexity of mechanisms. Most of the i*n vitro* studies of fibroblast activation were conducted on the widely used fibroblast activation ligands e.g. members of TGFβ superfamily, bone morphogenic proteins (BMPs), PDGFs, epidermal growth factors (EGFs), sonic hedgehog (SHH), and FGFs. Likewise, leukemia inhibitory factor (LIF) was involved in fibroblast activation and also in invasive properties of CAFs (Albrengues et al., 2014). In addition, hepatocyte growth factor (HGF) and growth differentiation factor 15 (GDF15) were demonstrated to enhance proliferation and invasion of cancer cells. However, more work is necessary to enlighten the dynamics of CAF-regulatory molecules and CAF-cancer cell interplay.

1.5.1. CAFs and Metastasis

Tumor metastasis is a multi-step process, which includes degradation and reorganization of ECM, invasion, migration of cancer cells followed by extravasation and blood circulation and then intravasation. Eventually residing on secondary site. CAFs are major drivers of secondary tumor development on metastatic site. Of note, tumors of S100A4 knock-out mice carrying deficiency of fibroblast differentiation, did not metastasize by demonstrating that S100A4 stromal fibroblasts play a key role in metastasis (Grum-Schwensen et al., 2005). Cytokines and growth factors secreted from CAFs on primary tumor site may enhance the invasive and proliferative behaviour of cancer cells through blood circulation (Bruzzese et al., 2014; Elkabets et al., 2011; Scherz-shouval et al., 2015). Moreover, several studies also documented that CAFs can be detected in blood circulation. Thus, rather than CAF-assisted cytokine secretion, CAFs can also metastasize to the secondary tumor site with tumor cells where help tumor cells to adapt their new environment (Chen & Song, 2019). During metastasis, cancer cells undergo epithelial mesenchymal transition (EMT) to increase their invasiveness. In breast cancer cells, CAFs induce EMT through paracrine TGFβ-SMAD pathway (Yu et al., 2014). Overall, CAFs govern metastatis by creating convenient niche by remodeling the ECM, and releasing effective factors to secondary sites.

1.5.2. Heterogeneousness and plasticity of CAFs

The insight we have reached today brought remarkable questions about CAFs; is there only a single type of CAF that performs all the functions in tumorigenesis or are there subspecial types of CAFs? If subspecialized types exist, is there a switch among types or functions? These questions are addressed by advanced techniques including array of function assays and single-cell analyses. Irresistible evidence indicates presence of supspecialization among CAFs (Croft et al., 2019; Pisco et al., 2018). Research on this field continues worldwide in a way to attract attention.

Mainly, CAFs display two different forms, either ECM producing contractile form or immunomodulating secretory phenotype. These are also called as myoCAFs and iCAFs due to their myofibroblast and immune response regulatory functions, respectively (Sahai et al., 2020). For instance in pancreatic cancer, CAFs nearby the cancer cells show myoCAF properties through α-SMA expression driven by TGFβ and a contractile behaviour in pancreatic cancer. However, distal CAFs are defined as iCAFs because IL-

6 expression was upregulated (Öhlund et al., 2017). It has been thought that classification and nomenclature of CAFs would be important for future research. Heterogeneity and plasticity of CAFs might be enlightened by mouse models.

1.6. Targeting CAFs to Improve Anticancer Therapies

As stated in previous sections, CAFs have diverse effects in tumorigenesis. They can increase the agresiveness of tumor, further promote tumor development, enhance stiffness of tumor, lead to therapy resistance. Therefore, including them on anticancer therapies is a promosing approach. Various patient studies reported that functions of CAFs strictly affect the clinical outputs. According to pre-clinical studies, targeting or deleting CAFs non-specifically may not be beneficial from patient aspects (Pentcheva-hoang et al., 2014). Hence, defining subtypes of CAFs and their communication networks is highly important to develop effective therapies. Currently, there are a few different CAF-targeting approaches, such as reprogramming CAFs, blocking extrinsic signals or inhibiting ECM components to improve clinical outcomes (Sahai et al., 2020). Of note, blocking the signals from CAFs could be beneficial. For instance, since CAFs are the major source of chemokines in most of the tumors, inhibiting C-X-C Motif Chemokine Ligand 12 (CXCL12) signaling may be considered as targeting CAFs (Feig et al., 2013). Likewise, inhibitors of B-Raf proto-oncogene can trigger the activation of stromal fibrloblasts and thus, can serve a compensative mechanism for activation of ERK-MAPK pathway in tumor cells (Hirata et al., 2015).

1.7. Cytokines

Cytokines are small soluble signaling molecules with their ability to initiate diverse pathways through membrane receptors. They are major players of immune responses. According to microenvironment, they may display pro-inflammatory or anti-inflammatory activity and pro-tumorigenic activity. (Seruga, Zhang, Bernstein, & Tannock, 2008). They are also involved in differentitation and activation of cells in autocrine and paracrine fashion. Cytokines are mainly released by immune cells, but endothelial cells, fibroblasts and most of other cell types can produce them as well (Steinke & Borish, 2005).

Cytokines may lead to tissue injury or autoimmune diseases by overactivation of host defense system (Holdsworth & Gan, 2015). Their effects depend on the type of cytokine, its down stream signaling, the dose of it and the cell surface receptor. Because of their effective roles, they were considered as great therapy targets. The first approved cytokine as a drug was interferon-α (IFN-α) in 1986 for use of hairy cell leukemia (HCL) treatment. It was followed by approval of high dose interleukin-2 (HDIL-2) in metastatic renal cell carcinoma (mRCC) in 1992 and for metastatic melanoma (MM) in 1998. However, cytokines have not meet the initial expectations as therapeutics yet (Conlon, Miljkovic, & Waldmann, 2019).

1.7.1. Cytokines in Cancer

In a healthy human being, inflammation is managed by various mechanism including cytokine signaling. IL10 and TGFβ are included in this process as anti-inflammatory cytokines (Seruga et al., 2008). Soluble receptors such as IL1 receptor type II (IL1R2) are as important as cytokines due to their neutralization role on cytokines. Also, neuronal activites and a number of hormones are involved in the response to inflammation (Elenkov, 2008; Pavlov & Tracey, 2005). A balance is maintained between pro- and anti-inflammatory mechanisms. Alterations in this balance result in inflammation. Perpetual inflammation causes chronic activation of immune system, which usually happens on patients with cancer. Deregulation of cytokines may lead to undesired outcomes in cancer (Figure 5).

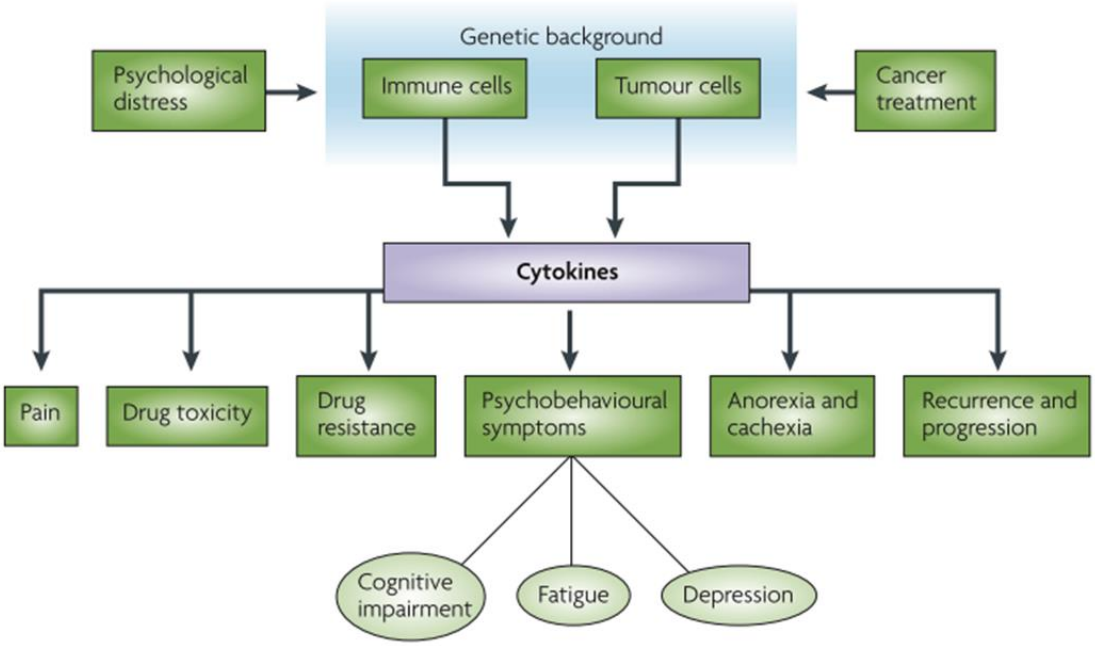


Figure 5.: A diagram for potential effects of cytokines in cancer (Seruga et al., 2008).

Cytokines indicate inflammation and undesired attack of non-malignant cells. Cytokines are present in tumor stroma abundantly and exhibit diverse roles such as pro-tumorigenic roles, inhibitory roles in tumorigenesis, and inducer roles for metastasis. A given set of cytokines and their roles in carcinogenesis were listed in table 2. Use of cytokines in cancer treatment at high doses seems promising; however, it potentially carries out another systemic impairment including neuropsychiatric disorders and hypotension. Thus, new approaches are developing. For instance, local management of related cytokines. For this purpose, plasmids or viruses may be used for the delivery of cytokine gene. A new approach is creating “superkines” which bind to selective receptors with high affinity to boost antitumor activities. Moreover, immunotherapy which is basically fortification of immune cells against cancer cells, have been attracted attention in recent years. Cytokines may be used as immunomodulators. Another clinical research documented a combination of cytokines and anticancer vaccines, anti-CTLA-4 or anti-PD-L1. They also aimed to extend the antibody-dependent cellular cytotoxicity (ADCC) caused by antibodies through injection of combined cytokines and monoclonal antibodies. Eventually, increased antitumor efficiency was targeted (Becker, Varkit, Gilliest, Furukawa, & Reisfeld, 1996; Carter, 2001; Levin et al., 2012; Schrama, Reisfeld, & Becker, 2006; Spangler, Moraga, Mendoza, & Garcia, 2015).

Table 2.: A given set of cytokines and the roles in tumorigenesis

Cytokine	The role
TGFβ	Activation of CAFs, anti-inflammatory
IFN-α	Anti-angiogenic, promotes caspase-dependent apoptosis
IFN-γ	Activation of macrophages and antibody presentation
IL-10	Anti-inflammatory
GM-CSF	Stimulation of antigen presentation
IL-8	Induction of tumor growth
IL-12	Indirectly anti-angiogenetic
IL-2	T cell growth factor
IL-21	B cell differentiation
IL-15	Proliferation of activated T-cells

Presently, IL-2, IFN-α, IFN-β and granulocyte macrophage colony-stimulating factor (GM-CSF) are included in approved cytokines for therapy in clinical use. IL-12, IL-15, IL-7 and IL 21 are still under clinical investigation. A set of cytokines and their therapeutic effects from cancer aspect were shown in Table 3.

Table 3.: Cytokines which are either approved or under clinical research and the therapeutic roles

Cytokine	The therapeutic effect
IFN-α	Boost immune system
IFN-β	Potential treatment in triple-negative breast cancer
GM-CSF	Neutrophil recovery by stimulating antigen presentation
IL-12	Stimulation of effector T-cells
IL-2	Effective in cell growth and termination of Tcells
IL-21	Expansion of effector cells
IL-15	Enhancement of T cell activities

1.8. Activin A

Activin-A is a member of TGFβ superfamily. Two subunits, βA and βB, generate activins as homodimers or heterodimers. Inhibin subunit beta A (*INHBA*) encodes Activin-A (βAβA) (Vale et al., 2004). There are two other activin dimers known: Activin-B (βBβB) and Activin-AB (βAβB). Activin-A is the most studied activin dimer and its role in embryogenesis is well-established (Barton, Yang-Feng, Mason, Seeburg, & Francke, 1989; Ogawa, Funaba, Chen, & Tsujimoto, 2006; Sheen, Kim, Park, Park, & Nam, 2013). It modulates crucial processes including haematopoiesis, tissue repair and fibrosis. Nevertheless, the function of Activin-A in tumorigenesis is largely unknown.

Despite structural similarities of TGFβ and Activin-A, TGFβ is released as a precursor protein which needs activation and Activin-A is released as an active protein (Munger et al., 1997). Receptors of TGFβ comprise of type I and type II homodimers (Attisano et al., 1993). Type I receptors are widely called as activin receptor-like kinases (ALKs), and they carry highly conserved kinase domains which are required for phosphorylation and activation. Activin-A prefers ActRIB (ALK4) primarily (Loomans et al., 2014). Additionally, it has lower affinity to ActRIA (ALK2) and ActRIC (ALK7). Upon binding of ligand Activin-A, two type I and two type II receptors form heterotetramer. Type I receptors are phosphorylated by type II receptors and kinase activity of type I receptors was activated. Then, ALKs phosphorylate intracellular mothers against decapentaplecgic homolog 2/3 (SMAD2/3) signaling proteins which generate a complex with SMAD4, followed by translocation to the nucleus to further influence gene expression (Massague & Chen, 2000). Inhibitory of SMAD (I-SMAD) blocks the SMAD2/3 and SMAD4 binding and hereby transcriptional activity of them (Namwanje & Brown, 2016).

Moreover, Activin-A can trigger alternative non-canonical pathways such as extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNKs) and p38 mitogen activated protein kinase (MAPK) pathways. Thus it can affect cell differentiation and migration (Morianos, Papadopoulou, Semitekolou, & Xanthou, 2019). Also, Activin-A can activate canonical Wnt pathway by phosphorylated SMAD2 (Tsuchida et al., 2009). Activin-A signaling is presented in Figure 6.

Process of wound healing is described above. Activin-A is involved in wound healing process as well. Upon damage, Activin-A levels are rapidly increased in wounded area because of immune response. The function of Activin-A in wound healing is depending

on the concentration. Elevated levels of Activin-A leads to heal in a short-time period by linking to tissue fibrosis (Sulyok, Wankell, Alzheimer, & Werner, 2004).

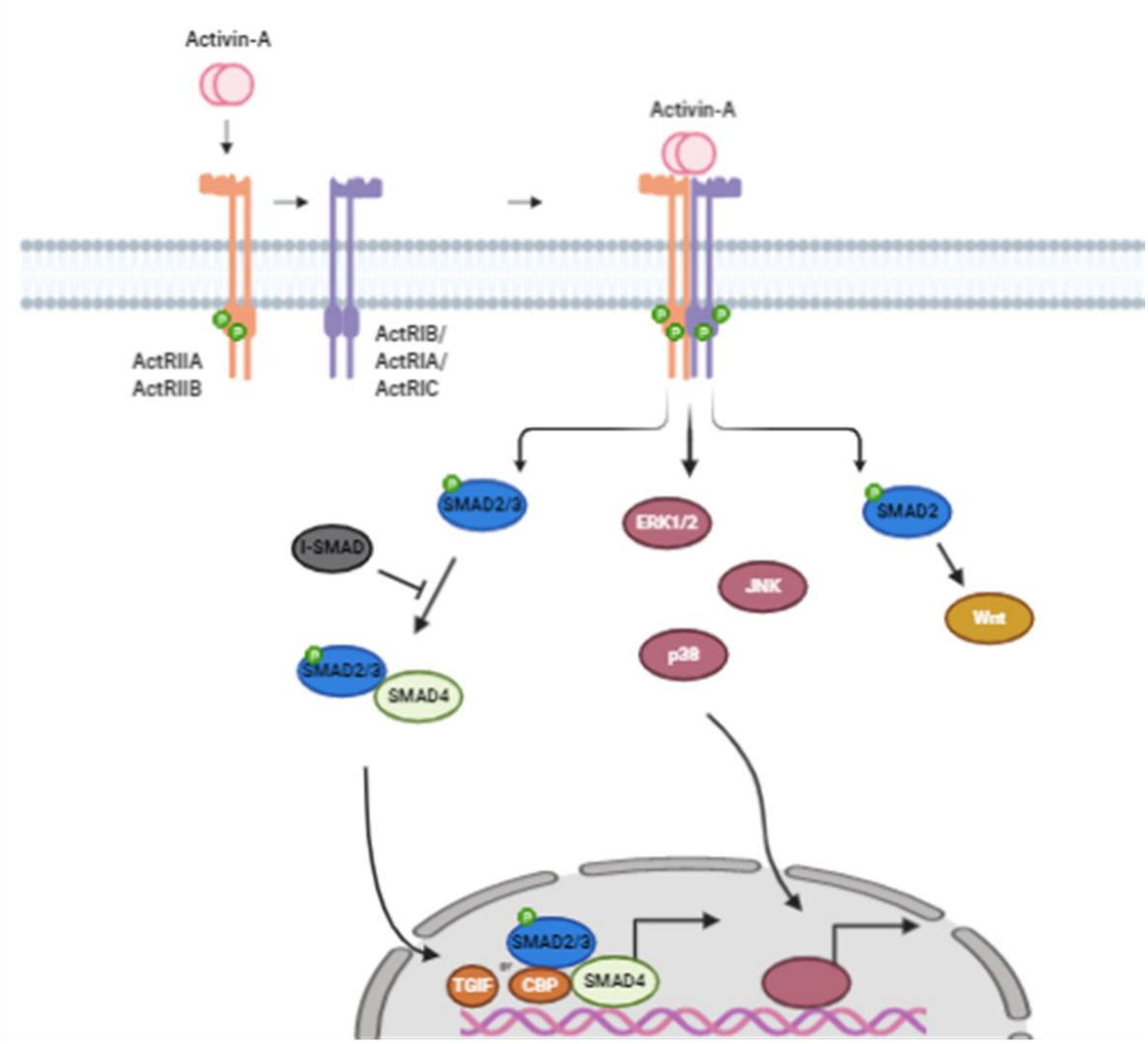


Figure 6.: Activin-A signaling. A) Canonical way of Activin-A signaling through SMAD proteins. B) Non-canonical way of Activin-A signaling.

The function of Activin-A in tumorigenesis was investigated in variety of cancer types, however, different functions were documented. Role of Activin-A depends on the type and stage of the cancer. It may enhance tumor growth, inhibit angiogenesis and promote immunosuppression.

1.9. Hepatocellular Carcinoma (HCC), HepG2 and Huh7 Cell Lines

Liver cancer comprises 4,7% of total cancer cases (7th most common cancer worldwide). It was 4th most common cause of cancer related deaths in 2018 (World Health Organization, WHO). Although liver cancer exhibits a little proportion of total cancer cases, it was one of the deadliest cancer among others. HCC is the most prevalent type of liver cancer. It generally develops following chronic liver damage for instance fibrosis and cirrhosis which are associated to activation of fibroblasts. Therefore stroma is important in the development of HCC. In this study HepG2 and Huh7 cell lines were used as HCC model. HepG2 cell line were generated from liver HCC of a 15 years old Caucasian male. Hepatitis B virus genome was not reported in HepG2 cell line (ATCC). Huh7 cell line was established from liver tumor of 57 years old Japanese male. Huh7 cell line was disposed to Hepatitis C virus infection.

2. MATERIALS AND METHODS

2.1. Ethical Statement

Patient samples were collected by Çukurova University. Written and informed consent form was obtained from all patients.

2.2. Cell Culture and Transfection

HEK293T, HepG2 and Huh7 cells were obtained from ATCC (USA). HepG2-LUC and Huh7-LUC cells (cells that are stably expressing luciferase gene) were previously generated by Dr. Yunus Akkoç in our laboratory. Fibroblasts and CAFs were isolated from fresh patient tissues and established in our lab by Dr. Yunus. All those mentioned cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, PAN-Biotech) supplemented with 10% (v/v) fetal bovine serum (FBS, PAN-Biotech), 100 U/ml Penicillin-Streptomycin (Biological Industries) and L-glutamine at 5% CO_2 humidified incubator at 37 °C. For luciferase stable Huh7 and HepG2 cells 10 µg/ml and 0,5 µg/ml HepG2 Blasticitidine was added, respectively, as a selection antibiotic. HEK293T cells were transiently transfected according to standard calcium-phosphate transfection protocol. DMEM that contains low-FBS containing medium (3% v/v), 100 U/ml Penicillin-Streptomycin (Biological Industries) and L-glutamine at 5% (referred as low-FBS containing medium hereafter) was used for co-culture and conditioned medium preparation purposes.

2.3. Western Blotting and Antibodies

Samples were boiled at 95 °C for 10 minutes and seperated on SDS-polyacrylamide gels. Gels were transferred to nitrocellulose membranes. Following the blockage with 5% non-fat milk in phosphate buffered saline-tween 20 (0,05%) (PBST), primary antibodies (ab): anti-Activin-A ab (1:1000, R&D, 130-10022-20), anti-FLAG M2 ab (1:1000, Sigma, F3165) were applied. After 3 times PBST washes, proper horseradish peroxidase (HRP)-coupled secondary anti-rabbit ab (Jackson Immunoresearch Laboratories, 111035144) or anti-mouse ab (Jackson Immunoresearch, 115035003) was applied onto membranes and signals from proteins were obtained with chemiluminescence. The intensity of bands was quantified using Image J.

2.4. Isolation of Primary HCC CAFs

Right after receiving fresh patient tissue samples in DMEM they were washed with PBS. Then, they were carefully chopped and treated with Liberase TL (Roche, 05401020001) for 2 hours at 37 °C under mild agitation. Samples in eppendorf tube were vortex in every 30 minutes to form a homogeneous mixture. At the end of the treatment, tubes were centrifuged at 0,1 G for 10 mins. Cell pellets were dissolved in fresh media and seeded onto 12-well plates. After first 4h, cells were washed with PBS to avoid the cross-contamination of other cell types. After overnight incubation wells were washed with PBS in order to remove any remaining tissue residues and finally single fibroblast or CAF culture was established.

2.5. Co-culture System and Conditioned Medium

Conditioned medium was obtained from fibroblast or CAF co-cultures and HEK293T cells as described below. In co-culture assays fibroblasts or CAFs seeded onto plates and attachment allowed for overnight. After the attachment of cells, they were washed with PBS and cancer cells were seeded onto fibroblasts or CAFs using low-FBS (3% v/v)

medium. Fibroblast or CAFs were co-cultured with cancer cells for 72h and conditioned medium was collected.

HEK293T cells were transfected with Flag-tagged plasmid inserted with *INHBA* gene encoding Activin-A (Sinobiological). After 8h post-transfection, culture medium was replaced with low-FBS containing (3% v/v) medium. Then, cells were incubated in 5% CO_2 humidified incubator at 37 °C. At the end of 72h, conditioned medium was collected and concentrated with Amicon Ultra-4 centrifugal filter units (Millipore-3kDa cut-off, UFC800324) via centrifugation at 4000 xg for 40 mins at +4 °C. Concentrated conditioned medium was aliquoted and kept at -80 °C for further use.

To provide a distance between CAFs and tumor cells we used transparent transwell inserts with 0,4 µm-pore-membrane (Millipore, MCHT12H48). Firstly, CAFs were seeded lower part of the plate as $5x10^4$ cell/well and incubated overnight. After adherence of CAFs, tumor cells were added upper part of the transwell insert as $5x10^3$ cell/insert.

2.6. Cytokine Array

To compare the factors that are secreted from fibroblasts and CAFs, they were co-cultured with cancer cells for 72h and conditioned medium was obtained. Conditioned medium was concentrated with Amicon Ultra-4 centrifugal filter units (Millipore-3kDa cut-off, UFC800324) at 4000 xg for 40 mins at +4 °C. Concentrated medium dissolved in 1 ml fresh DMEM before using. In order to detect difference among secreted factors we used Human Cytokine Array c4000 (RayBiotech, AAH-CYT-4000) and followed manufacturer's instructions. Intensity of dots was quantified with ImageJ.

2.7. Luciferase Reporter Assay

Luciferase stable HepG2 and Huh7 cells were used for measurement of cell proliferation. At the end of the experiment cells were washed with PBS and lysed with Chris buffer (50 mM Tris pH: 8,0, 200 mM NaCl, 0,1 mM EDTA, 10% glycerol, 0,5% Nonidet P-40, 1x PI, 1x PMSF) for 5 min at 37 °C. Then, cell lysates centrifuged for 10 mins at 1000 xg at +4 °C. Supernatant was taken to a new tube and kept on ice. Supernatant was mixed with homemade fresh luciferase reagent (1,07 mM $(MgCO_3)_4xMg(OH)_2x5H_2O$, 20 mM Tricine,

2,67 mM $MgSO_4$, 0,1 mM EDTA, 33,3 mM DTT, 270 µM Coenzyme A, 530 µM ATP and 470 µM Luciferin). Three fold of the supernatant was used from luciferase reagent, in other words 150 µL of luciferase reagent was mixed with 50 µL supernatant. Mixture in black 96-well plates (ThermoFisher Scientific, 9502867) was incubated for 5 mins at room temperature without light. Luciferase activity was detected using luminometer (ThermoFisher Scientific, Fluroskan Ascent FL).

2.8. Measurement of Cancer Cell Growth in CAF Co-culture System

$5x10^4$ or $3x10^4$CAF cells per well were seeded onto 12 well plates. 16h later, luciferase-stable HepG2 or Huh7 cells were seeded on CAFs as 1:10 cancer cell:CAF ratio. At the end of 72h, co-cultured cells were lysed with Chris buffer and their cell growth was measured with luciferase reporter assay as described above.

2.9. Neutralization Antibody Assays

To test whether Activin-A is the responsible for CAF-mediated tumor cell growth we used a neutralizing antibody against Activin-A. Neutralizing antibody was purchased from R&D (MAB3381) and reconstituted in sterile PBS. Neutralization antibody detects Activin-A and its precursor protein. After adherence of CAFs, cancer cells were seeded onto them and neutralizing antibody added into culture medium (low FBS, 3% v/v) as 1 µg/ml. They co-cultured for 72h, cells were collected and luciferase activity was measured as previously described.

2.10. Total RNA Isolation and qRT-PCR Analyses

Total RNA was extracted using TRI Reagent (Sigma-Aldrich, T9424) according to manufacturer's instructions. Total RNA was treated with DNase I (Thermo, EN0521) and reverse transcribed into cDNA using RevertAid reverse transcriptase enzyme (Thermo,

EP0442), random hexamers (Invitrogen, 481190011) and RiboLock RNase inhibitor (Thermo, EL0012). For qRT-PCR reaction, SYBR Green Master Product (Roche, 04887352001) and LightCycler 480 (Roche) RT-PCR machine were used. PCR was established as follows; an initial cycle of 95 °C 5 mins was done and followed by 45 cycles of 95 °C for 10 sec and 60 °C for 1 min 72 °C 10 sec. Then, single cycle of melting was done at 95 °C 5 mins and 55 °C 1 min. mRNA levels of related genes were quantified with $2^{-\Delta\Delta Ct}$ method and normalized to *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase) mRNA level. Primers that were used in this study are as follow, *INHBA:* fwd: 5' -TCGGAGATCACGTTTGC- 3' rev: 5' -TTGGGGACTTTTAGGAAGAGCC- 3', *ACVR1B* fwd: 5' -AGTGACAATTGAGGGGATGA rev: 5' -CATGCCATTTTTCTTCACCA- 3'. *GAPDH*: fwd: 5' -ATGGGTGTGAACCATGAGAA- 3' rev: 5' -GTGCTAAGCAGTTGGTGGTG- 3'

2.11. Analyses of Tissues from HCC Patients

For qRT-PCR analyses, patient tissues that are already in liquid nitrogen were pestled into small pieces and taken to eppendorf tubes. TRI Reagent (Sigma-Aldrich, T9424) was added onto samples and manufacturer's instructions were followed. Total RNA was reverse transcribed into cDNA as previously described. Same qRT-PCR template was used. mRNA levels were normalized to *GAPDH*. For each patient, healthy liver tissue that belongs to the same patient was used as control.

2.12. Immunofluorescence Analyses

Fibroblasts and CAFs were cultured on sterile glass coverslides and then fixed with ice-cold 4% paraformaldehyde (PFA) in PBS for 20 mins. After fixation, cells were permeabilized with 0,1% saponin and 0,1% BSA in PBS and treated with primary and secondary antibodies respectively. Anti-αSMA (Abcam, ab5694) and Anti-Vimentin (Abcam, ab92547) were used as primary antibodies. Then, proper secondary ab either Anti-rabbit Alexa Flour 488 (Invitrogen, A-11034) or Anti-mouse Alexa Flour Plus 488 (Invitrogen, A32723) were applied. Following antibody applications, cells were also

stained using Hoesct for nuclei staining and coverslides were mounted onto glass slides. Images were taken by Dr. Yunus Akkoç using Carl Zeiss LSM710 confocal microscope under 63x magnification.

2.13. Coomassie Blue Staining

We used coomassie blue staining to quantify the Activin-A protein in conditioned medium. For this purpose the gel was fixed for 30 mins with fixing solution (50% methanol, 10% glacial acetic acid, 40% distilled H_2O (dH_2O)). Fixation was followed by staining (0,1% Coomassie Brilliant Blue R-250, %50 methanol, 10% glacial acetic acid and dH_2O) for 20 mins. Later, destaining solution (40% methanol, 10% glacial acetic acid and 50% dH_2O) was applied onto the gel to remove residues. Solution was replenished a few times until the background of the gel is completely destained. The gel was washed with water overnight, if necessary. Gel images were obtained by Biorad imaging system and stained gels dried and kept for further analysis.

2.14. The Cancer Genome Atlas Program (TCGA) Analyses

INHBA expression levels were compared in Chen liver. *INHBA* copy numbers were compared in HCC and normal liver. Access date: 18.04.2020.

2.15. Statistical Analyses

GraphPad Prism 8 and MS Excel were used for analysis of data. Student's two- tailed t test was used for statistical analyses. Each data represent mean of 3 or more independent experiments. P value less than 0,05 was considered as significant. Results of statistical analyses indicated as mean ± SEM.

3. RESULTS

3.1. Characterization of CAFs Derived From HCC Patients

CAFs are important active components of tumor stroma. They distinguish from fibroblasts or NAFs with their unique features such as increased expression of α-SMA and Vimentin, formation of stress fibers. Besides, their promoting effects on tumor cell growth were demonstrated in the previous studies. Since, this study aimed to investigate tumor cells and CAFs crosstalk, we started with characterization of CAFs. CAFs were isolated from three different HCC patients. Right after receiving fresh tumor and healthy tissues, they were crumbled and treated with Liberase TL (Figure 7A). Then, in order to identify CAFs, we performed immunostaining using common CAF markers that are α-SMA and Vimentin. As seen in Figure 7B, both of α-SMA and Vimentin (green and red, respectively) protein levels were elevated in CAFs which are derived from tumor tissue compared to fibroblasts from normal liver tissue of the same patient. Also, confocal microscopy images show remarkable morphological changes between CAFs and fibroblasts, stress fibers were formed in CAFs (Figure 7B).

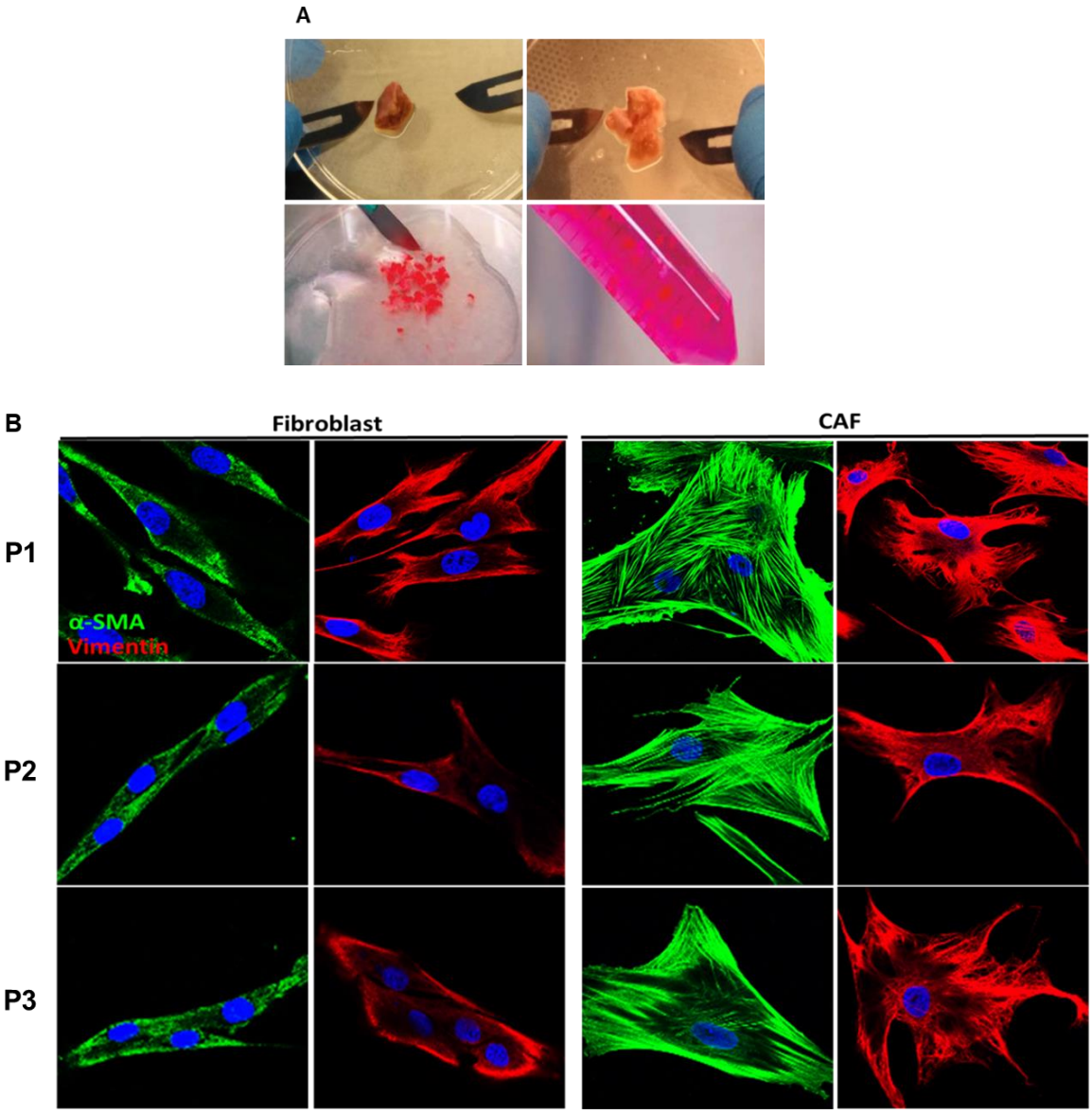


Figure 7.: Characterization of CAFs. A) Normal and tumor tissues of HCC patients were minced and treated with Liberase TL. B) CAFs and fibroblast derived from three different patients were stained with α-SMA (green), Vimentin (red) and Hoesct (blue). Images were obtained by confocal under 63x magnification. At least three different experiments were performed and representative images were chosen. In CAFs, protein levels of both α-SMA and Vimentin and formation of stress fibers were increased compared to fibroblasts.

3.2. Effects of fibroblasts and CAFs on HepG2 cells proliferation

A growing body of evidence claimed that CAFs are transdifferantiated form of fibroblasts. The certain role of CAFs in tumorigenesis is still under investigation. At the same time,

several studies demonstrated that CAFs have pro-tumorigenic effects and act as tumor-promoters. To expose the differential effects of fibroblasts and CAFs on tumor growth, we co-cultured them with HepG2 cells using low-FBS containing (3% v/v) medium for 72h in transwell. HepG2 cells without fibroblasts or CAFs were used as control. After 72h, cells were lysed, and their growth was measured with luciferase reporter assay. Consequently, CAFs isolated from two different patients were significantly increased HepG2 cell proliferation. Meanwhile, fibroblasts from same patients had no significant effect (Figure 8). Moreover, tumor promoting effect of CAFs did not require cell-to-cell attachment, indicating the involvement of cytokines.

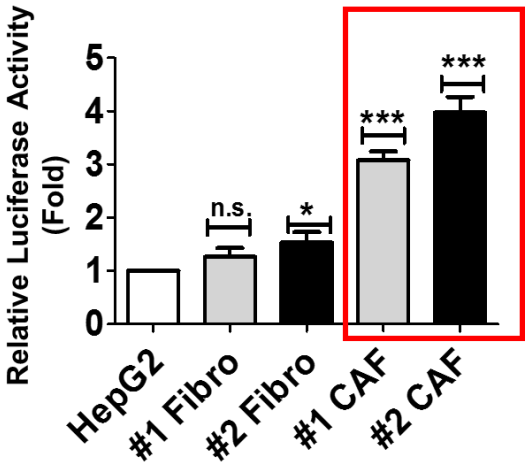


Figure 8.: Effects of fibroblasts and CAFs on HepG2 cell proliferation. Relative luciferase activity was measured after 72h co-culture or only HepG2 cells culture. Mean ± SEM of 3 independent experiments. *P<0,05, ***P<0,001, n.s.: Non-significant.

3.2.1. Screening of secreted factors in fibroblast and CAF co-cultures using Human Cytokine Array

Cytokines are small and secreted factors that may affect cell growth *in vitro* and *in vivo*. We thought that cytokines might be the reason of this tumor-promoting effect of CAFs. In order to examine differentially released cytokines in fibroblast and CAF co-cultures, we collected and analyzed co-culture mediums using Raybiotech Human Cytokine Array (Figure 9). As a result, Activin-A was upregulated in CAF co-culture compared to fibroblast co-culture (Figure 10A and 10B).

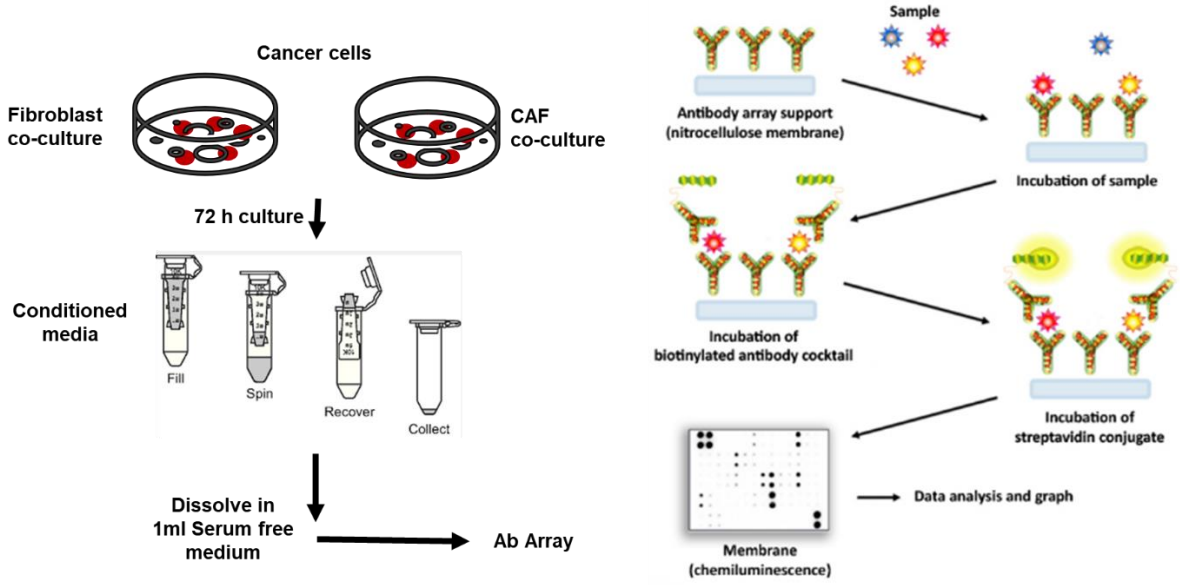


Figure 9.: Schematic representation of conditioned medium collection and principle of Human Cytokine Array

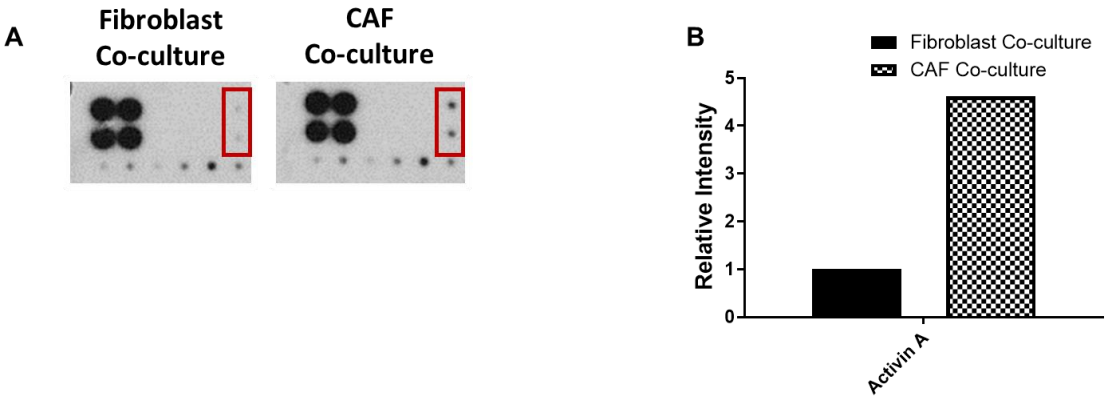


Figure 10.: Analysis of differential secreted cytokines in fibroblast and CAF co-cultures with Human Cytokine Array. A) Representative image of cytokine array result. B) Quantification of cytokine array. Two independent experiments.

3.3. Production and Quantification of Recombinant Activin-A

To test the growth promoting effect of Activin-A on HCC model cell lines, we produced it recombinantly. First, HEK293T cells were transfected with *INHBA* plasmids and produced flag tagged proteins. Since Activin-A is a secreted cytokine, it must present in the medium. Then, 72h later post-transfection, we collected and concentrated the culture medium. Following concentration, medium was run on the gel and anti-Activin-A and

anti-flag antibodies were applied to control the presence of Activin-A. Low-FBS containing medium was used as control (Figure 11A). Following production, we further wanted to quantify Activin-A in the conditioned medium and performed coomassie blue staining. Unfortunately, Activin-A band was not detected after staining (Figure 11B).

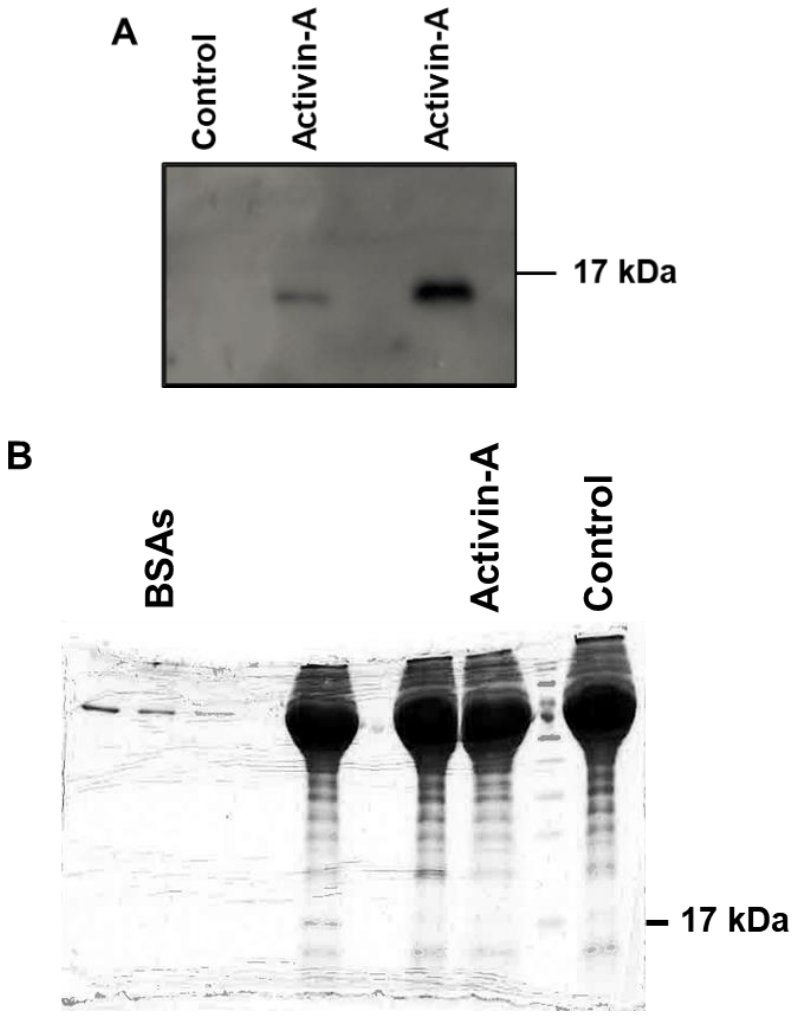


Figure 11.: Recombinant Activin-A obtained from conditioned medium. A) Activin-A was produced succesfully. B) Activin-A band was not detected on coomassie blue staining. Activin-A lane is indicated.

3.4. Effect of recombinant Activin-A on proliferation of HCC cells

To examine the effect of Activin-A on tumor growth *in vitro,* recombinant Activin-A was

applied to HepG2 and Huh7 cells which are stably expressing luciferase. After 72h treatment, relative luciferase activity was measured and compared to HepG2 or Huh7 alone conditions. Low-FBS DMEM applied to HepG2 or Huh7 cells are used as a control medium. As a result, Activin-A was significantly enhanced proliferation of HepG2 cells compared to control. Activin-A treated Huh7 cells showed comparable increase of growth (Figure 12A and 12B).

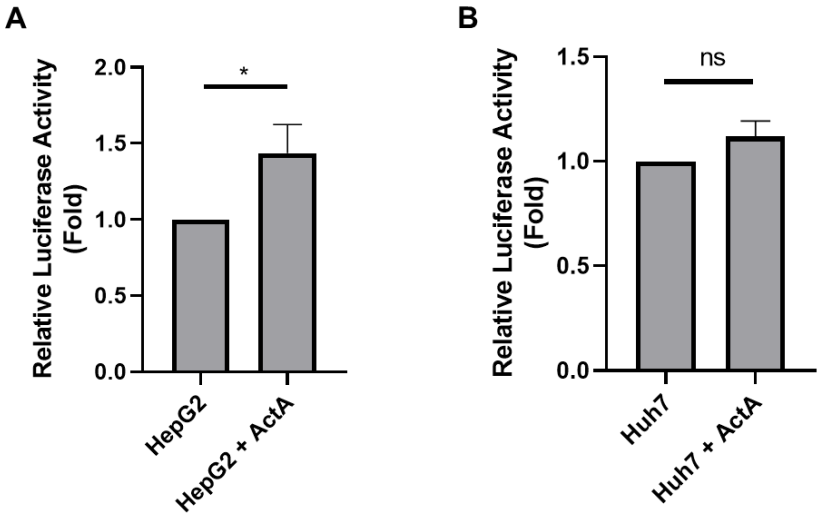


Figure 12.: Recombinant Activin-A was enhanced tumor growth. A) Relative luciferase activity of HepG2 cells 72h after treatment of Activin-A. 6 independent experiments. *P<0,05. B) Relative luciferase activity of Huh7 cells. Mean ± SEM of 5 independent experiments. ns: Non-significant. ActA: Activin-A, HEK293T-produced.

3.5. Co-culture of CAFs and HepG2 or Huh7 Cells and Neutralization Antibody Treatment

To obtain effects of CAFs on HCC growth, we co-cultured CAFs with either luciferase stable HepG2 or Huh7 cells using low-FBS medium containing (Figure 13A). In addition, since we speculate that Activin-A is the responsible cytokine of CAFs' tumor promoting feature, we used a neutralization antibody to suppress activity of Activin-A. After 72h, HepG2 and Huh7 cells in different conditions were collected and their proliferation was compared. Cells were lysed with Chris buffer. Cells lysates were used in luciferase reporter assay. Consequently, CAFs isolated from three different patient were significantly increased proliferation of HepG2 cells. Moreover, Activin-A neutralization

antibody significantly reversed growth-promoting effects of CAFs (Figure 13B). In addition, Activin-A neutralization antibody was reduced proliferation of HepG2 cells without CAFs (Figure 13C). Similar results were obtained with Huh7 cells. CAFs which were isolated from patient 1 and 2 significantly increased Huh7 cell proliferation and Activin-A neutralization antibody was prevented the tumor promoting effects of CAFs (Figure 13D). However, we could not repeat the experiments with patient 3 on Huh7 cells due to lack of primary tissue. Taken all together, CAFs induce cell proliferation in HepG2 and Huh7 cell lines and this event probably occurs through Activin-A. In other words, these results indicate that Activin-A is a major player in CAF-induced HCC cell proliferation.

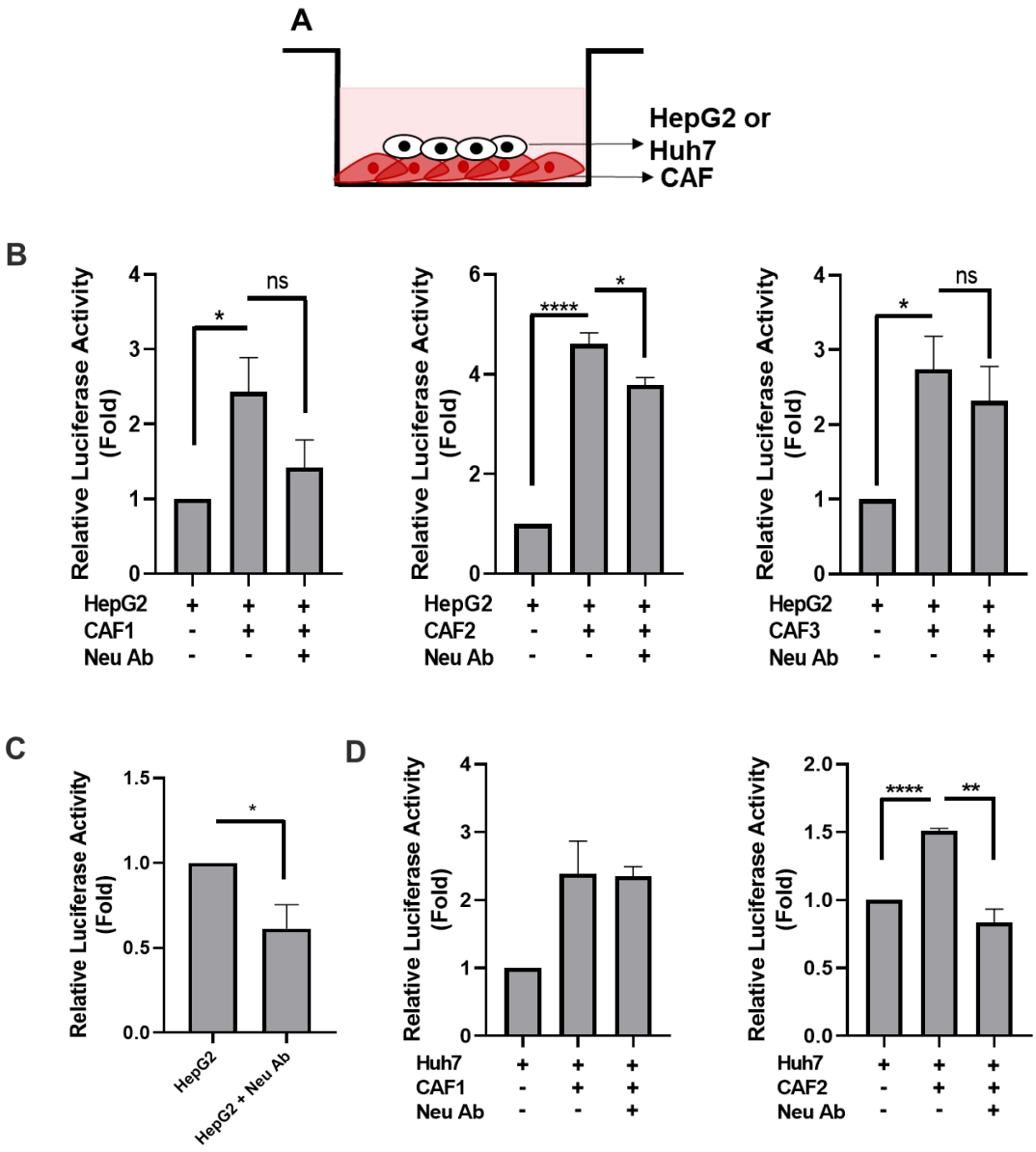


Figure 13.: CAF co-culture of HepG2 and Huh7 cells. A) Schematic view of co-culture conditions. B) Relative luciferase activity of HepG2 cells under different conditions. CAF1, CAF2, CAF3 were isolated from different patients. Data represent mean ± SEM of 3 independent experiments. C) Relative luciferase activity of HepG2 cells. Data represent mean ± SEM of 6 independent experiments. D) Relative luciferase activity of Huh7 cells. CAF1 and CAF2 cells were the same as previous data. Mean ± SEM of 2 and 3 independent experiments, respectively. *P<0,05, **P<0,01, ****P<0,0001, ns: Non-significant Neu Ab: Activin-A neutralization antibody.

3.6. Western Blot and qRT-PCR Analyses of CAF Co-culture in Transwell

After we demonstrated that CAFs were able promote HCC cell proliferation, we changed co-culture conditions and provided a distance between CAFs and tumor cells using Transwell with 0,4 µm-pore membrane. CAFs were seeded lower part of the plate and HepG2 cells were added to upper part of the transwell insert. The schematic representation of transwell plate was given in Figure 14A. 72h later, co-culture mediums were collected and concentrated as previously described. This conditioned medium was analysed with western blot using anti-Activin-A antibody. As a result, Activin-A secretion was the highest in CAF co-culture medium (Figure 14B). Afterwards, we further wanted to investigate which cell type predominantly releases Activin-A. For this purpose, total RNA was isolated from 72h co-cultured HepG2 alone, CAF alone and CAF-HepG2 co-culture (CAF and HepG2 cells separately) conditions. mRNA expression of *INHBA* was 2,8 fold higher in CAF co-culture compared to CAF alone culture (Figure 14C). Interestingly, *INHBA* expression was reduced in HepG2 cells when they are co-cultured with CAFs compared to HepG2 alone culture. This result points out that Activin-A is predominantly secreted from CAFs in the presence of tumor cells.

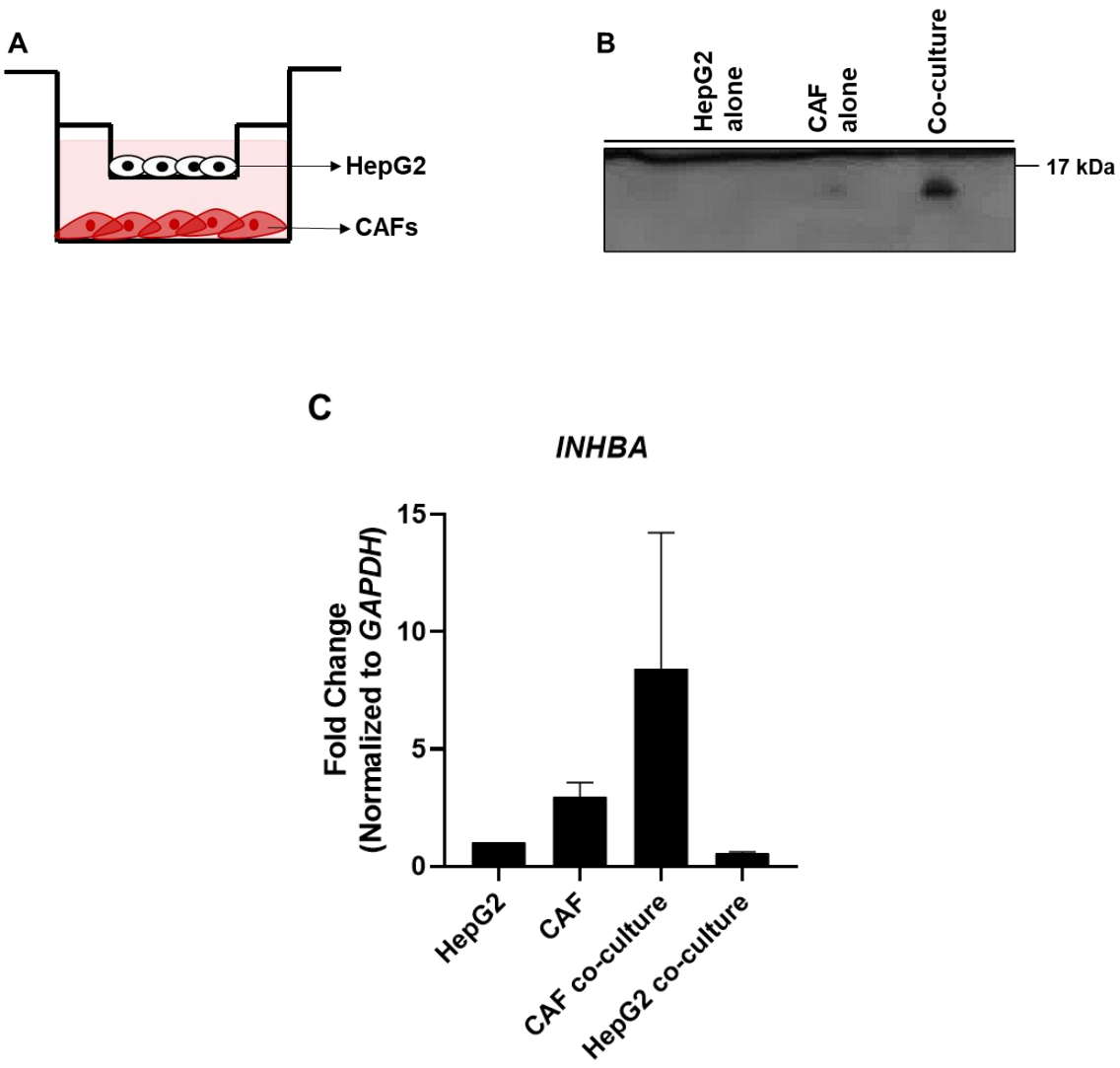


Figure 14.: Western blot and qRT-PCR analyses of CAF co-culture in transwell. A) Schematic representation of transwell plate. B) Western blot result of conditioned mediums. 3 independent experiments. C) qRT-PCR analysis of *INHBA* from HepG2 alone, CAF alone and CAF co-culture conditions. Data represent means ± SEM of 2 independent experiments.

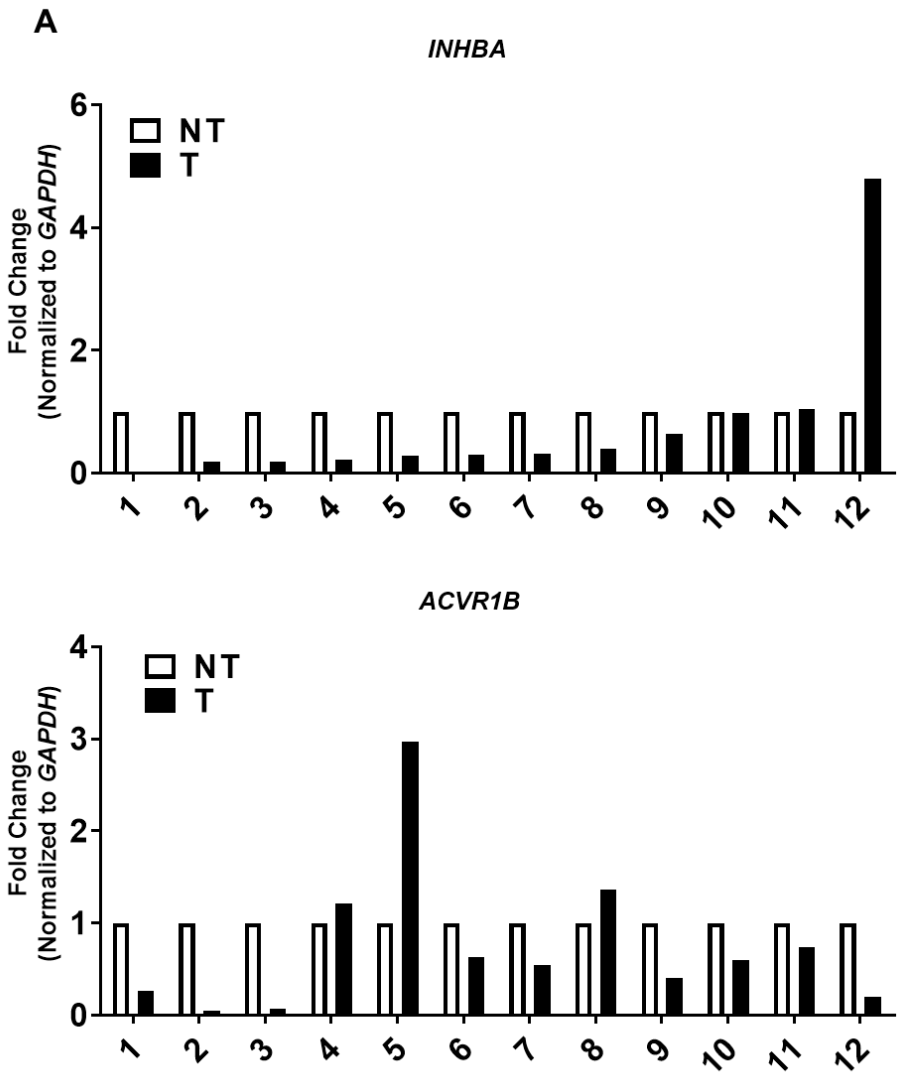
3.7. qRT-PCR Analyses From Frozen Patient Tissues

To examine how the expressions of *INHBA* and its receptor *ACVR1B* were changed in HCC tumors, we performed a qRT-PCR analysis. Total RNA was isolated from frozen tumor and healthy tissues of 12 HCC patients. The gene expression levels of tumor samples were compared to normal tissue counterparts for each individual. Expression levels of *INHBA* and *ACVR1B* were altered in tumor samples (Figure 15A). We further

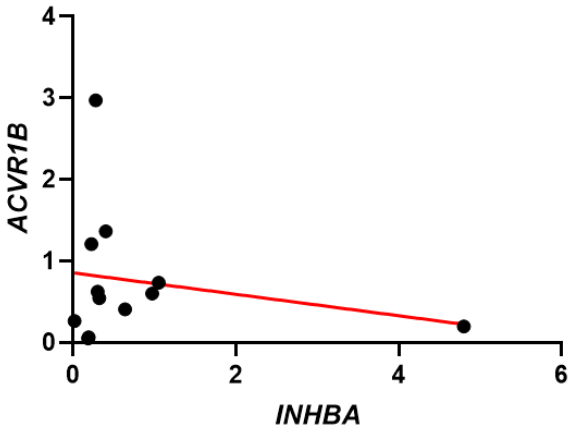
analyzed this data to find a correlation between *INHBA* and its receptor. We were expecting a positive correlation but instead, we found a weak negative correlation (Figure 15B). Nevertheless, data set should be expanded to certain results.

3.8. TCGA Analyses

Expression levels and copy numbers of *INHBA* were examined in TCGA database. In consensus with our results, *INHBA* expression and copy number were increased in HCC. (Figure 16A-B).


A
INHBA
NT
T
Fold Change
(Normalized to GAPDH)
0
2
4
6
1
2
3
4
5
6
7
8
9
10
11
12
ACVR1B
NT
T
Fold Change
(Normalized to GAPDH)
0
1
2
3
4
1
2
3
4
5
6
7
8
9
10
11
12

B



Pearson r	
r	-0.2119
95% confidence interval	-0.7006 to 0.4121
R squared	0.04490
P value	
P (two-tailed)	0.5085
P value summary	ns
Significant? (alpha = 0.05)	No

Figure 15.: qRT-PCR analysis of tumor and normal tissue samples of HCC patients. A)Expression levels of *INHBA* and *ACVR1B*, respectively. Each tumor sample was compared to its normal tissue sample counterpart. B) Correlation analysis of *INHBA* and *ACVR1B*.

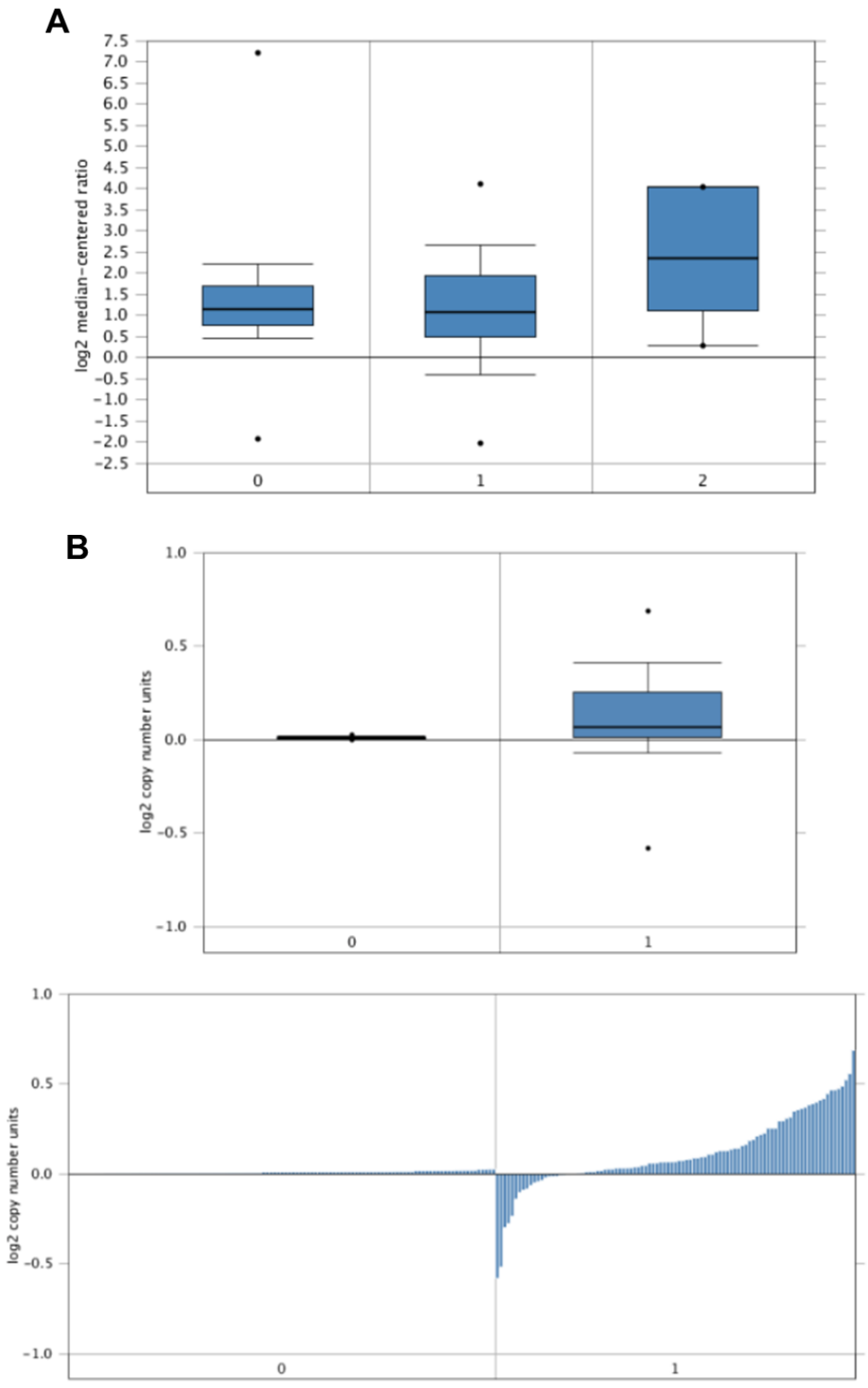


Figure 16.: TCGA Analyses. A) Expression levels of *INHBA* in Chen liver. 0: No value (85), 1: HCC (100), 2: Liver cancer precursor (7). B) Copy number of *INHBA* in TCGA liver. HCC vs. normal liver. 0: No value (115), 1: HCC (97).

4. DISCUSSION

Today, cancer still remains as a leading cause of annual deaths worlwide. The underlying reason is probably relapse of cancer and targeting only malignant cells in anticancer therapies. Despite the importance of tumor microenvironment highlighted by Paget (1889) over a hundred years ago, the researches have draw attention fairly only in recent years. Overwhelming majority of anticancer therapies are mainly focused on malignant cells. However, tumors are not composed of cancer cells solely, but comprise of many different cell types. Therefore, understanding mechanisms of stromal activation, which is an important part of tumor microenvironment, the crosstalk between cancer cells and neighboring stromal cells, and considering tumor microenvironment as therapy target as well as cancer cells, are essential for better clinical outcomes.

Various cell types including but not limited to immune cells, epithelial cells, pericytes, platelets and fibroblasts are involved in tumor stroma. Numerous studies documented that tumor stroma is able to promote tumorigenesis. A few studies also reported that stroma has tumor-restraining effects through TGFβ (Bhowmick et al., 2004). Overall, tumor stroma may potentially have a tumor-suppressive activity at the very beginning of carcinogenesis, but in secondary sites or advanced stages of cancer, stroma becomes acidic and further enhances tumorigenesis, stiffness, invasion and metastasis. These effects mostly occur through signaling between cancer cells and stromal cells.

Among heterogeneous population of stromal cells, fibroblasts are thought to drive the stroma predominantly. Thus, exact mechanisms involved in activation of fibroblasts and tumorigenesis are emerging. Fibroblasts are activated by different stimuli. CAFs are irreversibly transdifferentiated from activated fibroblasts and distinguished from NAFs with certain properties. It has been reported that abundance of CAFs in tumor stroma makes tumor more aggressive and metastatic. They are also capable to increase proliferation of cancer cells robustly. Contributions of CAFs to tumor development from various aspects make them potential therapy targets in recent years.

Here, we investigated the crosstalk between CAFs and HCC cells. For this

purpose, we collected normal and tumor tissue samples from HCC patients. Firstly, fibroblasts from normal tissue and CAFs from tumor tissue were isolated. Then, we co-cultured fibroblast and CAFs with HepG2 cells to observe their effect on tumor growth. After 72h of co-culture, luciferase activity was measured. We obtained a significant incerase in proliferation of HepG2 cells in CAF co-culture. Additionally, we repeated these experiments using transwell inserts and showed that tumor-promoting effect of CAFs does not require direct cell contact. Afterwards, we collected conditioned medium from CAF and fibroblast co-cultures and performed an unbiased screening using Human Cytokine Array. As a result, secreted Activin-A was upregulated in CAF co-cultures. To further analyse the role of Activin-A on tumor growth, it was recombinantly produced. We tried to quantify the amount of Activin-A in conditioned medium, however, we were not successful. Recombinant Activin-A was applied to luciferase stable HepG2 and Huh7 cells. HepG2 cells showed significant increase, while Huh7 cells showed comparable levels of proliferation upon Activin-A treatment. These results indicate that exogenous Activin-A is able to enhance growth of HCC cell lines model.

Tumor-promoting behaviour of CAFs was demonstrated previously in different cancer types (Olumi et al., 1999; Orimo et al., 2005). In this study, we checked this behaviour using CAFs isolated from 3 different HCC patients. We observed that all CAFs were able to promote HepG2 and Huh7 cell growth. Moreover, the significant level of increase varies from patient to patient. Furthermore, we wanted to target the communication between CAFs and HCC cells and for this reason a neutralization antibody for Activin-A was used in co-culture experiments. Consequently, Activin-A neutralization antibody was significantly reversed the growth-promoting effects of CAFs. This result suggests that, Activin-A is the major regulator of CAF-induced tumor growth in HCC. Blocking the effects of Activin-A with neutralization antibody may be a promising way to inhibit cancer proliferation; notwithstanding, side effects may rise due to extensive expression of *INHBA* in most of human tissues.

Under normal conditions Activin-A regulates regeneration and embryogenesis. Deregulation of Activin-A was reported in several cancer types such as testicular cancer, endometrial carcinoma, and HCC (Loomans et al., 2014). Another study was documented that, consistent with our results, Activin-A secreted by myofibroblasts enhanced tumor growth in oral squamous cell carcinoma (Sobral et al., 2011).

After we showed that CAFs enhance HCC tumor growth and Activin-A is a major player, we wanted to investigate which cell type predominantly release Activin-A. First, we co-cultured CAFs and HepG2 cells in transwell system for 72h. Then, we collected conditioned medium from HepG2 alone, CAF alone and CAF co-culture conditions and analysed them with western blotting. Activin-A secretion was higher in CAF co-culture

compared to both HepG2 and CAF alone conditions. More than that, total RNA was isolated from HepG2 cells and CAFs in transwell, independently. Our qRT-PCR results revealed that mRNA expression of *INHBA* was highest in CAFs, co-cultured with HepG2 cells. This result indicates that, CAFs are the main source of Activin-A secretion under co-culture conditions. A very recent study reported that cancer cell-derived Activin-A enhances carcinogenesis through activating *mDia2* using Smad2 signaling in squamous cell carcinoma. Besides, they proposed a model that describes a loop between cancer cells and fibroblasts mediated by Activin-A secretion (Cangkrama et al., 2020). Our results were indicated that Activin-A was predominantly secreted from CAFs, whereas Cangkrama et al. claimed that Activin-A was a secreted factor from cancer cells. This conflict might be context-dependent behaviour of Activin-A.

In addition, expression levels of *INHBA* and its receptor *ACVR1B* was evaluated using normal and tumor tissues of 12 different HCC patients. *INHBA* expression was impaired in all patients. We were expecting a positive correlation between *INHBA* and *ACVR1B*, however, statistical analyses uncovered a weak negative correlation. Although *ACVR1B* was reported as one of the most frequent receptor of Activin-A, analyzing of the correlation between *INHBA* and other receptors, namely *ACVR1* or *ACVR2A* may reveal a different correlation. In addition, patient set should be precisely expanded.

Despite the gaps in the literature, it has been clarified that stroma have crucial roles at different stages of cancer. Considering tumor stroma as a new approach for cancer therapy will lead to improve clinical outcomes. Therefore, understanding interplay between stromal cells and cancer cells will provide us better insight to develop new treatments. CAFs and cytokines are key components of the interplay with their autocrine and paracrine fashion. Thus, CAFs, cytokines and their crosstalk are potential new therapy targets.

Here, we proposed Activin-A as a major regulator of CAF-induced tumor growth in HCC. Our results revealed that Activin-A may be a potential target for treatment of cancer.

Future research will surely enlighten the downstream pathway of Activin-A in HCC. Also, Activin-A might be targeted via genetic engineering. Since we presented the release of Activin-A by CAFs, experiments may be conducted to knock-out *INHBA* or its receptor.

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PUBLICATIONS

1. **Hatice Cakir**[*], Yunus Akkoc[*], Hikmet Akkiz, Devrim Gozuacik, 2020**, Activin-A is the key component of CAF and tumor cell crosstalk.**
2. Sinem Demirbag-Sarıkaya**, Hatice Cakir**, Devrim Gozuacik, Yunus Akkoc, 2020, **Crosstalk between autophagy and DNA repair systems.**

PRESENTATIONS & AWARDS

1. Poster presentation, **Hatice Çakır**, Hikmet Akkız, Yunus Akkoç, Ümit Karaoğullarından, Tuğsan Ballı, Abdullah Ülkü, Figen Doran, Devrim Gözüaçık, **The role of cytokines in hepatocellular carcinoma-stroma interactions.** 3rd HCC Conference, Adana, Turkey, 2019.
2. Oral presentation, **Hatice Çakır**, Yunus Akkoç, Hikmet Akkız, Ümit Karaoğullarından, Figen Doran, Oğuz Üsküdar, Sedef Kuran, Devrim Gözüaçık, **The Discovery of a New Cytokine Secreted From CAFs and Controls HCC Cell Growth.** 4th HCC Conference, Adana, Turkey, 2020.
3. Oral presentations first place winner, 4th HCC Conference, Adana, Turkey, 2020.