

**TUMOR-STROMA COMMUNICATION THROUGH ACTIVATION OF AUTOPHAGY IN
FIBROBLAST CELLS, AN IMPORTANT COMPONENT OF STROMA**

by

HACER EZGİ KARAKAŞ SCHÜLLER

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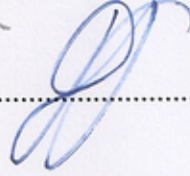
Sabanci University

August, 2017

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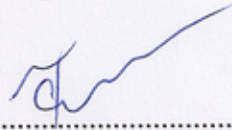
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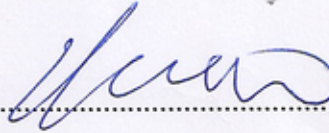
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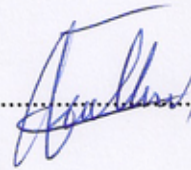
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HACER EZGİ KARAKAŞ SCHÜLLER

Molecular Biology, Genetics and Bioengineering, Ph.D. Thesis, August 2017

Thesis Advisor: Assoc. Prof. Devrim GÖZÜAÇIK

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ABSTRACT

Autophagy is an evolutionary conserved stress response mechanism. A bulk of cytoplasm including organelles or proteins is engulfed by a double membrane structure and later on degraded by fusion with the lysosome in order to provide building blocks for the need of the cell under stress conditions such as lack of nutrients or hypoxia. This mechanism has an important role in health as well as onset or the progress of different diseases including cancer. The communication between cancer and its surrounding cells, herein called microenvironment or stroma, is crucial for the initiation, progression and metastasis of the cancer cells. Basically, the tumor microenvironment provides a basis (soil) for the growth of cancer cells (seed). One of the major component of the stroma is fibroblast cells. They are known to be activated during tumorigenesis to support the survival and proliferation of cancer cells by reshaping the environment, and by providing growth factors and metabolites. During cancer cell-fibroblast cell communication, cancer cells cause induction of autophagy in fibroblast cells and they benefit from autophagy by using the secreted metabolites from fibroblast cells. However, it is not elucidated yet how cancer cells initiate this communication with surrounding cells to activate their autophagy, especially in fibroblasts. This information is important to prevent the support of fibroblast cells to cancer cells. In this thesis, the main aim is to mimic cancer-fibroblast communication outside of the body in order to define the key factors which initiate the crosstalk between cancer cells and fibroblasts through the modulation of autophagy. To create a sensitive system, microfluidic chips, or biochips, were used to monitor the crosstalk between single cancer cells with fibroblast cells through autophagy analysis in fibroblast cells. Moreover, a human growth factor and cytokine cDNA library was screened in order to cover potential communication signals broadly. According to the results, novel soluble paracrine factors were found to significantly induce autophagy in fibroblast cells. In order to create organized tissues outside of the body, 3D bioprinting of fibroblast cells was performed. During the preparation and bioprinting process, the importance of autophagy was tested using autophagy-competent (MEF WT) and autophagy deficient ((*Atg5*^{-/-}) MEF) cells. In summary, our research on microfluidic chip system shows the potential of this platform to analyze individual single cancer cells which can activate autophagy in fibroblasts, our research on novel growth factors and cytokines broaden our perspective in order to study the communication signal between cancer cell with fibroblasts in autophagy, and our research on 3D bioprinting shows the importance of autophagy during cell-cell communication in artificially created organized structures.

KANSER-MİKROÇEVRESİ İLETİŞİMİNİN MİKROÇEVRENİN ÖNEMLİ BİR UNSURU OLAN FİBROBLASTLARDAKİ OTOFAJİ DEĞİŞİMİ İLE İNCELENMESİ

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Anahtar Kelimeler: Otofaji, kanser, kanser mikroçevresi, bioçipler, hücre-hücre
iletişimi

ÖZET

Otofaji, evrimsel süreçte korunmuş stres yanıt mekanizmasıdır. Organelleri veya proteinleri içeren sitoplazmanın bir kısmı çift zarlı yapı ile çevrilir ve hücrenin besin eksikliği, hipoksi gibi durumlarında gereken yapı malzemelerini karşılamak için lizozomla birleşerek sindirilir. Bu mekanizma sağlıklı olduğu gibi kanser gibi hastalıkların başlangıcında ve ilerlemesinde de önemli bir rol oynamaktadır. Kanser ile çevresi, mikroçevresi veya stroma olarak da anılır, arasındaki iletişim kanserin başlangıcı, ilerlemesi ve metastazı için oldukça kritiktir. Temelde, tohum gibi davranan bir tümör için mikroçevresi onun büyümesini, gelişmesini sağlayacağı toprak görevi görmektedir. Bu mikroçevresinin başlıca unsurlarından birisi, fibroblast hücreleridir. Tümör oluşumu esnasında, kanserin büyümesini desteklemek için hücre dışı matriksini şekillendirdikleri, büyüme faktörleri ve metabolik ürünler sağladıkları ve tüm bunları yapabilmek için aktifleştikleri bilinmektedir. Kanser-fibroblast iletişiminde, kanser hücrelerinin fibroblastlarda otofaji aktivasyonuna neden oldukları ve onlardan salgılanan metabolik ürünleri kullanarak kendi büyümelerinde fayda sağladıkları bilinmektedir. Fakat, kanser hücrelerinin otofajiyi aktive etmek için çevreleriyle özellikle fibroblastlar ile olan iletişimi nasıl başlattıkları henüz anlaşılmış değildir. Bu bilgi, kanser hücrelerinin çevrelerindeki fibroblastlardan alacakları desteği önleyebilmek için oldukça önemlidir. Bu tezde, kanser ile fibroblast iletişimini fibroblastta otofaji aktivasyonuna neden olarak başlatan kritik faktörlerin tanımlanabilmesi için, kanser-fibroblast iletişiminin vücut dışında benzerinin yapılması hedeflenmiştir. Hassas bir sistem yaratılması ve fibroblastlardaki otofaji değişikliğinin kanser hücresi-fibroblast iletişimi ile eş-zamanlı görülebilmesi için mikroakışkan çip ya da bioçipler kullanılmıştır. Ayrıca, daha fazla faktörü kapsayabilmek için insandan alınan büyüme faktörü ve sitokin cDNA kütüphanelerinin taramaları yapılmıştır. Elde edilen sonuçlara göre, bazı özgün büyüme faktörleri ve sitokinler fibroblast hücrelerinde otofaji aktivasyonuna neden olmuşlardır. Bu tezde ayrıca, doku gibi organize yapıları vücut dışında oluşturabilmek için, fibroblast hücrelerinin 3-boyutlu bir şekilde yazımı gerçekleştirilmiştir. Hücrelerin hazırlanış ve yazım esnasında karşılaştıkları stres karşısında otofaji mekanizmalarını kullanmalarının önemini anlayabilmek adına otofaji yapabilen (MEF WT) ve otofaji yapamayan ((*Atg5^{-/-}*) MEF) hücreler kullanılmıştır. Özetle, mikroakışkan sistem ile yaptığımız çalışmalarımız kanser hücresinin fibroblast hücrelerindeki otofajinin aktive etmesini eş-zamanlı gösteren bir hücre dışı bir platform yaratmış, yeni etkilerini keşfettiğimiz faktörlerin kanser hücresi ile fibroblast arasındaki iletişimi daha geniş bakış açısı ile incelememize olanak sağlamış ve 3-boyutlu hücre yazım çalışmalarımız otofajinin oluşturulan organize yapılarda hücre-hücre iletişimindeki önemini analiz etmiştir.

“Only perseverance makes the impossible possible.”

*To my husband, Peter Schüller,
my family, Zübeyde Karakaş, Fevzi Karakaş, Umutcan Karakaş,
and my elder sister, Özge Karakaş*

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TABLE OF ABBREVIATIONS

3D	Three-Dimensional
ab	Antibody
ADP	Adenosine Diphosphate
AJCC	American Joint Committee on Cancer
AKT	AKT Serine/Threonine Kinase 1
AMBRA1	Activating Molecule in BECN1-Regulated Autophagy
Arg	Arginine
ATG	Autophagy -related gene
ATP	Adenosine Triphosphate
BCL2	B-Cell Leukemia 2 Protein
BCLXL/BCL2L1	Bcl-2-Like Protein 1
BECN1	Beclin 1, yeast Atg6 orthologue
BH	Benjamin Hochberg
BIF1	Endophilin B1
BNIP3L	BCL2/Adenovirus E1B 19-kD Protein-Interacting Protein 3-Like
B-Raf	B-Raf Proto-Oncogene
BRCA1	Breast Cancer Type 1 Susceptibility Protein
BSA	Bovine Serum Albumin
CAD	Computer Aided Design
CAF	Cancer-Associated/Carcinoma-Associated Fibroblast
Cav 1	Caveolin 1
Cdk2	Cyclin-Dependent Kinase 2
cDNA	complementary DNA
CMA	Chaperone-Mediated Autophagy
c-Myc	Myc Proto-Oncogene Protein
CNT	Control (condition)
CNTF	Ciliary Neurotrophic Factor
CQ	Chloroquine
CT	Computer Tomography
CT-1	Cardiotrophin-1
CTC	Circulating Tumor Cell
CXCL	Chemokine (C-X-C motif) Ligand
DAPK	Death-Associated Protein Kinase
DI	Deionized Water
DNA	Desoxyribonucleic Acid
DOI	Domain of Interest
DRP1	DAPk-related protein kinase-1
DTT	Dithiothreitol

E1	E1-like enzyme
E2	E2-like enzyme
E3	E3-like enzyme
EBSS	Earle's Balanced Salt Solution
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
ER	Endoplasmic Reticulum
FACS	Flourescence-Activated Cell Sorting (flow cytometry)
FAK	Focal Adhesion Kinase
FAP	Fibroblast-Activating Protein
FBS	Fetal Bovine Serum
FDGF	Fibroblast-Derived Growth Factor
FGF	Fibroblast Growth Factor
FIP200	FAK family kinase-Interacting Protein, 200-kD
FSP	Fibroblast-Specific Protein
GABA	Gamma-AminoButyric Acid
GABARAP	GABA-A Receptor-Associated Protein
GABARAPL1	GABA-A Receptor-Associated Protein-Like Protein 1
GABARAPL2/GATE16	GABA-A Receptor-Associated Protein-Like Protein 2
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GFP-LC3	Green Fluorescent Protein GFP-fused to MAP1LC3 protein
GFP-LC3 MEF	GFP-LC3 transgenic MEF cell
gp130	glycoprotein 130
GTP	Guanosine Triphosphate
HCC	Hepatocellular Carcinomas
HCl	Hydrogen Chloride
HEK	Human Embryonic Kidney
HeLa	Henrietta Lack's cervical cancer cells
HGF	Hepatocyte Growth Factor
HIF1 α	Hypoxia-Inducible Factor 1-alpha
HPLC	High Performance Liquid Chromatography
H-Ras	HRas proto-oncogene
HSC	Heat Shock Cognate
HUVEC	Human Umbilical Vein Endothelial Cell
hVPS34	Vacuolar Protein Sorting protein 34-VPS34-homolog

IDH3A	Isocitrate Dehydrogenase 3A
IGF-1	Insulin-like Growth Factor 1
IL	Interleukin
IL-6	Interleukin-6
iPSC	induced Pluripotent Stem Cell
JAK	Janus Kinase
kDa	kilodalton
KO	Knock Out
K-RAS	KRAS proto-oncogene
LAMP	Lysosome-Associated Membrane Protein
LC3	Microtubule-Associated Protein 1 Light Chain 3
LC-MS	Liquid Chromatography–Mass Spectrometry
LIF	Leukemia-Inhibitory Factor
LIFR	Leukemia-Inhibitory Factor Receptor
LIFR β	Leukemia-Inhibitory Factor Receptor-beta
LINE-1	Retrotransposon Long Interspersed Nuclear Element-1
Lys	Lysine
M.W.	Molecular Weight
MCL1	Myeloid Cell Leukemia Sequence 1 (BCL2-Related)
MEF	Mouse Embryonic Fibroblast
MMP	Matrix Metalloproteinase
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
MS	Mass Spectrometry
mTOR	mammalian Target of Rapamycin
mTORC1	mTOR Complex-1
NBR1	Neighbor of BRCA1 Gene 1
NDP52	Nuclear Domain 10 Protein 52
NF	Normal Fibroblast
NFKB	Nuclear Factor Kappa B
NOD.SCID	Charles River, NOD.CB17- Prkdc ^{scid} / NcrCrl, homozygote SCID mutation on the non-obese diabetic background mouse, Chain number: 394
NS	Not Significant
O/N	Overnight
Oct4	Octamer-Binding Transcription Factor 4
OPTN	Optineurin
OSM	Oncostatin M
p62/SQSTM1	Sequestosome 1
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline with Tween-20

PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PDGFR α	Platelet-Derived Growth Factor Receptor alpha
PDGFR β	Platelet-Derived Growth Factor Receptor beta
PDGF-BB	Platelet-Derived Growth Factor-BB
PDMS	Polydimethylsiloxane
PE	Phosphatidylethanolamine
PEO-PPO-PEO	Polyethylene Oxide and Polypropylene Oxide Trio Block Co-Polymer
PFA	Paraformaldehyde
PGF	Polypeptide Growth Factors
PI3K	Phosphatidylinositol 3-Kinase
PI3P	Phosphatidylinositol 3-Phosphates
PMSF	Phenylmethylsulfonyl Fluoride
Qbc-SNARE SNAP	Synaptosomal-Associated Protein
RAB22	Ras-Related Protein Rab-22
RAB24	Ras-Related Protein Rab-24
RAB7	Ras-Associated Protein 7
Rapa	Rapamycin
RASAL1	RAS Protein Activator Like 1
RFP	Red Fluorescent Protein
RIPA	Radioimmunoprecipitation Assay Buffer
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species
RT-PCR	Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
RUBICON	Run Domain- and Cysteine-Rich Domain-Containing BECN1-Interacting Protein
SD	Standard Deviation
SDF-1	Stromal Cell-Derived Factor 1
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
shGFP	short hairpin Green Fluorescent Protein
shTGF β -1	short hairpin Transforming Growth Factor-beta 1
shVEGFA	short hairpin Vascular Endothelial Growth Factor
SILAC	Stable Isotope Labeling in Cell Culture
SMAD	Sma- and Mad-Related Protein
SNARE	Soluble N-ethylmaleimide-Sensitive Factor Attachment Protein Receptor
STAT	Signal Transducer and Activator of Transcription

STL	Stereolithography
STV	Starvation (Condition)
Stx17	Syntaxin17
T1	Cancer Stage T1
T2	Cancer Stage T2
TGFβ	Transforming Growth Factor-beta
TGFβ1	Transforming Growth Factor-beta 1
TGFβ-RI	Type I TGFβ Receptor
TGFβ-RII	Type II TGFβ Receptor
ULK1/2	UNC51-Like Kinase 1
UPS	Ubiquitin Proteasome System
UVRAG	UV irradiation Resistance-Associated Gene
VAMP7	Vesicle-Associated Membrane Protein 7
V-ATPase	Vacuolar-type H ⁺ -ATPase
VEGF	Vascular Endothelial Growth Factor
VEGFA	Vascular Endothelial Growth Factor-A
VPS	Vacuolar Protein Sorting
w/v	Weight per Volume
WIPI	WD Repeat Domain, Phosphoinositide Interacting
WT	Wild Type
α-sma	alpha-Smooth Muscle Actin

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1. INTRODUCTION

In a multicellular organism, whether in healthy or diseased state, cell-cell communication and interaction are an important in deciding the next step on cell growth, differentiation, development, immune response, and death response. Cancer is not an exception. The communication between cancer cells and surrounding cells is also critical in the proliferation, progression and metastasis of cancer. While cancer cells grow more, invade the basal lamina or metastasize to distant locations, the communication between cancer cells and surrounding cells supports the decision of cancer cells and alters their characters for the benefit of cancer cells by changing their shapes, gene expressions, secretions as well as their metabolism. Since the growth and proliferation of cancer cells depends on the metabolism of the cancer cells, they recruit ready metabolites and growth factors/cytokines from their microenvironment. This support offers an intriguing possibility as an important target of cancer treatments.

Solid tumor microenvironments consist of different types of cells including fibroblast cells, mesenchymal stem cells, endothelial cells and different types of immune cells. Among these cell types, fibroblast cells are the major constituents of the tumor microenvironment. Recently, alteration in fibroblast catabolic process and reprogrammed metabolism have gained attention. In cells, there are two major catabolic processes for intracellular protein degradation and turnover: UPS (ubiquitin proteasome system), which degrades short-lived proteins by tagging them with single molecule or poly-ubiquitin chains and subsequently degrading them through proteasome system, and autophagy, which degrades long-lived proteins engulfed in lipid bilayer structure through fusion with lysosome. In addition to its role in protein homeostasis, autophagy degrades and recycles other macromolecules as well as organelles in either a selective or nonselective manner upon stress in order to support the needs of the cells until the stress is over. Depending on the duration and strength of the stress, autophagy can be considered as a stress response for survival or type II death response in the cells.

Autophagy is a basic cellular event conserved in all eukaryotes from yeast to man. Under normal conditions, it serves as an intracellular quality-control mechanism recycling long-lived or misfolded/aggregate-prone proteins and damaged organelles, such as mitochondria. Upon the stresses such as nutrient deprivation or hypoxia, autophagy is upregulated in order to generate building blocks that are necessary for cellular survival [10, 11]. Therefore, autophagy mainly serves as a stress-adaptation and survival mechanism [12]. Yet, there are increasing evidences which reports the role of autophagy on programmed cell death either indirectly through crosstalk mechanisms with apoptosis [13-15] or directly through autophagic cell death [16].

Impairments or abnormalities of autophagy have been reported in various diseases, including lysosomal storage diseases, neurodegenerative diseases, infections, and metabolic diseases including cancer [17]. The role of autophagy in cancer formation, growth, ischemia-resistance, metabolic changes, neo-vascularization, and even metastasis and tumor dormancy has been reported [18-20]. Moreover, autophagic capacity significantly affected responses of cancer cells to anticancer agents and radiation [21].

Recently, induced autophagy in stromal fibroblast cells has shown to provide ready metabolites and building blocks such as fatty acids, ketone bodies or amino acids to cancer cells [22, 23]. Although the reason for autophagy induction has been considered mainly as reactive oxygen species (ROS) created by cancer cells, the complex signaling network during tumor stroma points to the key role of secreted soluble factors from cancer cells to the environment which have been not elucidated yet. During this thesis, the main focus is to elucidate cancer-cell derived secreted factors which modulate the autophagy in fibroblast cells during their crosstalk with cancer cells [24]. Moreover, this thesis seeks to understand cell-cell communication during organized structures such as tissue formation using 3D bioprinters in the context of autophagy-competent or autophagy-deficient cells.

1.1. Basic mechanisms of autophagy

The ubiquitin-proteasome system and autophagy-lysosomal systems are the main catabolic mechanisms in mammalian cells. Proteosomal systems degrade mainly short-lived and soluble proteins following their regulated ubiquitinylation [25]. In contrast, lysosomal pathways degrade the engulfed cytosolic contents by its fusion with lysosome and by their further degradation by specific hydrolases. Here, ingested cytoplasmic targets

might be diverse including long-lived proteins; old and damaged organelles (e.g. mitochondria and peroxisomes); misfolded, mutant and/or aggregated proteins; and pathogens such as bacteria, viruses and parasites. So far, three subtypes of autophagy have been described in mammals: microautophagy, chaperone mediated autophagy (CMA), and macroautophagy [26].

Microautophagy proceeds through direct invagination of the vacuolar/lysosomal membrane and engulfment of nearby cytosolic materials [27]. In addition to cargo digestion, microautophagic vacuoles also contribute to the maintenance of organelle size and membrane composition [28].

Chaperone mediated autophagy (CMA) is an autophagic pathway allowing selective degradation of a subset of soluble proteins in lysosomes. During CMA, proteins containing the recognition and targeting motif KFERQ are selectively directed to lysosomes by a chaperone complex consisting of HSC70 (a heat shock cognate of the HSP70 family) and its co-chaperones. On lysosomal membranes, interaction with the lysosome-associated membrane protein LAMP2A allows translocation into the lumen of the organelle [29]. CMA plays a crucial role in the elimination of oxidized proteins and misfolded proteins, and it contributes to starvation responses [30].

Macroautophagy (refers to as simply “autophagy” for the remainder of this thesis) is the major and mostly studied cellular autophagy pathway. It involves the engulfment of cytosolic cargo by lipid bilayer structures called isolation membranes, which eventually close to form vesicles called “autophagosomes” or “autophagic vesicles”. The fusion of the outer membrane of autophagosomes with lysosomal membranes results in the delivery of autophagy cargos into lysosome lumen and the formation of autolysosomes. Hydrolases residing there degrade cargo contents to their building blocks, allowing their recycling back to the cytosol [31].

There are also selective and non-selective types of autophagy. For example, while starvation-induced autophagy is generally considered as a non-selective form of autophagy, the engulfment of organelles (mitophagy, pexophagy, ribophagy, ERphagy),

protein aggregates (aggrephagy), lipid droplets (lipophagy), and pathogenic invaders (xenophagy) occur in a selective manner [32-34]. Selective autophagy receptors include p62/SQSTM1 (sequestosome 1, shortly p62), NDP52 (nuclear domain 10 protein 52), NBR1 (a neighbor of BRCA1 gene 1), BNIP3L (BCL2/Adenovirus E1B 19-kD protein-interacting protein 3-like) and OPTN (optineurin) [35].

Autophagy can be induced by several factors such as starvation, hypoxia, or pathogens [36-39]. Autophagy proteins have initially been identified in yeast during genetic studies, and at least 35 of them have been functionally analyzed [40]. The basic autophagy system has shown to be preserved in mammals. Most of the autophagy-related (or ATG) genes play a role during autophagosome nucleation, expansion, vesicle closure, and finally fusion with lysosomes and degradation [41]. In the following parts, the major stages of autophagy will be explained and important molecular events involved in their regulation will be summarized.

1.1.1. Nucleation of Autophagosome

The mTORC1 protein kinase complex is the central nutrient sensing pathway in cells and also a key regulator of autophagy [42]. Under nutrient-rich conditions, mTOR kinase regulates the phosphorylation of autophagy proteins ULK1/2 (UNC51-like kinase 1, mammalian homolog of yeast Atg1) and ATG13, and blocks autophagy (Figure 1). Under stress conditions including hypoxia, deprivation of growth factor, or nutrient, mTOR kinase is inactivated, which results in the activation of ULK1/2. This event is followed by a direct phosphorylation of ATG13 and FIP200 (FAK family kinase-interacting protein, 200-kD) by ULK1/2 and the stimulation of autophagy. The active ULK1/2 complex (ULK1/2-ATG13-FIP200-ATG101) then translocates to autophagosome nucleation centers and triggers consecutive stages.

Another key autophagy complex is the phosphatidylinositol 3-kinase (PI3K) complex. In the mammalian system, this complex is composed of ATG14L (also known as ATG14 and Barkor), BECN1 (Beclin 1, yeast Atg6 orthologue), hVPS34 (vacuolar protein sorting protein 34-VPS34-homolog), and hVPS15 [43-46] (Figure 1). In the PI3K complex, hVPS34 is the kinase, and its activity and localization is controlled by other components of the complex. For example, ATG14L has been shown to recruit the complex onto the outer leaflet of the ER (endoplasmic reticulum) membranes. BECN1 is an important regulator of the hVPS34 activity. In fact, the PI3K complex activity is

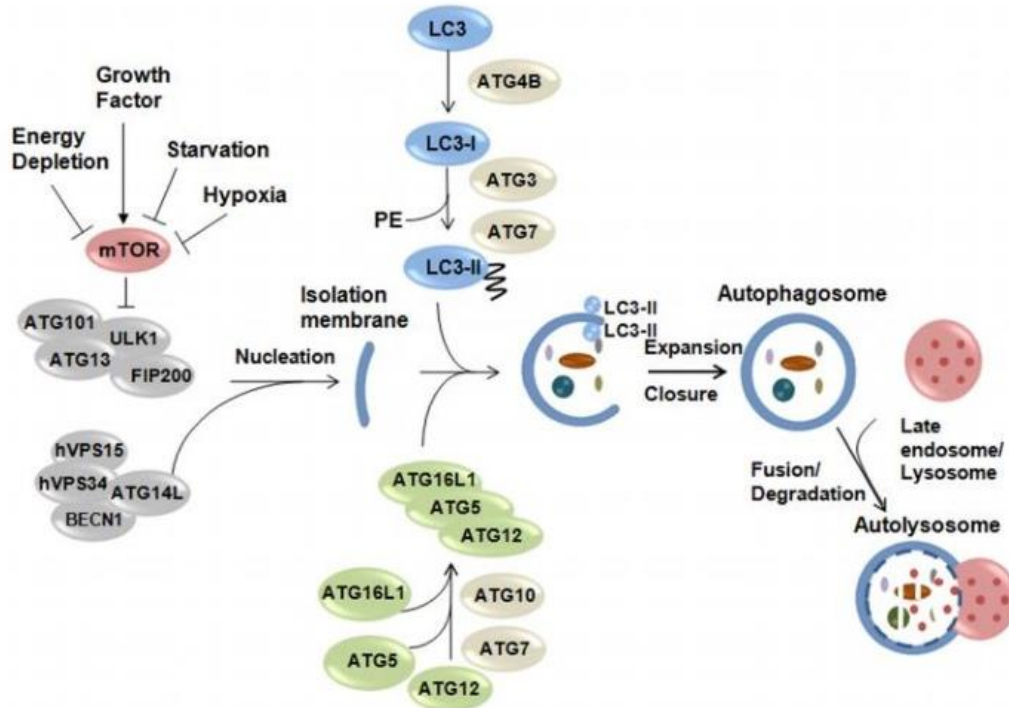


Figure 1. Steps in macroautophagy and important complexes [7].

responsible for the accumulation of autophagy-related lipid molecules, namely phosphatidylinositol 3-phosphates (PI3P) in autophagosome initiation/nucleation sites. PI3P islands that form on the outer membrane leaflet of the ER serve as landing pads for the recruitment of autophagy effectors containing phosphoinositide-binding domains, such as FYVE finger domains or PH domains (for example WIPI/Atg18 proteins). Additionally, AMBRA1 (activating molecule in BECN1-regulated autophagy), UVRAG (UV irradiation resistance-associated gene) and BIF1 (endophilin B1) have been shown to regulate the complex, whereas RUBICON (run domain- and cysteine-rich domain-containing BECN1-interacting protein) protein and anti-apoptotic BCL2 (B-cell leukemia 2) proteins including BCL2, BCLXL/BCL2L1 and MCL1 have been reported as inhibitors [47]. Activation of ULK1/2 was reported to be important for the localization of the PI3K complex to autophagic membranes, placing the ULK1/2 complex upstream to the PI3K [44]. Additionally, AMBRA1 has been shown to release a fraction of the PI3K complex from its association with dynein and to allow its localization to autophagosomal sites in a ULK1/2-dependent manner [41].

1.1.2. Expansion and Closure of Autophagosome

Expansion of autophagosome is mediated by two ubiquitylation-like systems: the Atg12-Atg5 conjugation system and the microtubule-associated protein 1 light chain 3 (Atg8 in yeast and MAP1LC3 or shortly LC3 in mammals) lipidation system (Figure 1) [48, 49]. In the first system, Atg12 is activated by Atg7 (an E1-like enzyme), then transferred to Atg10 (an E2-like enzyme). Finally, Atg12 is covalently conjugated to the Atg5 protein [49]. Subsequently, the Atg12-Atg5 conjugate interacts with Atg16 (ATG16L1 in mammalian) to form a higher molecular weight complex (Figure 1) [50]. In the yeast system, the Atg12-Atg5-Atg16 complex (ATG12-ATG5-ATG16L1 complex in mammals) is mainly localized to the surface of autophagosomes, and it dissociates from membranes following autophagic vesicle completion [49, 51].

In the second system, in order to expose a glycine residue at its C terminal, LC3 protein is cleaved by ATG4B proteins soon after its synthesis. This processed protein stays in the cytosolic free form called LC3-I. Subsequently, LC3-I is activated by ATG7, transferred to ATG3 (an E2-like enzyme), and covalently linked to a lipid molecule, namely phosphatidylethanolamine (PE). This lipid-conjugated LC3 form is called LC3-II, and it is localized both on isolation membranes and autophagosomes [52]. Hence, the demonstration of LC3 lipidation and LC3-I to LC3-II conversion, as well as the recruitment of cytosolic LC3 to autophagosomes are widely used as reliable markers of autophagic activity. The ATG12-ATG5-ATG16L1 complex is believed to act as an E3-like enzyme for LC3 lipidation, creating a direct link and interdependency between the two systems [53]. LC3 lipidation is necessary for the elongation and completion of autophagic vesicles. Moreover, LC3 proteins that are located in the inner membrane of autophagosomes serve as anchors for selective autophagy receptors, such as p62/SQSTM1, helping the recruitment of cargo proteins. GABARAP (GABA-A receptor-associated protein), GABARAPL1 (GABA-A receptor-associated protein-like protein 1), and GABARAPL2/GATE16 (GABA-A receptor-associated protein-like protein 2) are other homologs of LC3 in mammals, and the specific roles of these proteins and functional overlap between them are under investigation.

1.1.3. Fusion with lysosomes and degradation

In order to recycle cargo including long-lived proteins or damaged organelles, and to provide the cell with nutrients under stress conditions, autophagosomes fuse with late

endosomes or lysosomes and lead to the “digestion” of structural constituents into their building blocks (for example, protein macromolecules are digested into amino acid building blocks) (Figure 1). Vesicular structures that are formed by autophagosome-lysosome fusion are called “autolysosomes”. Several proteins play a role during fusion events, including lysosomal membrane proteins such as the V-ATPase complex and LAMPs (Lysosomal associated membrane protein) [54], and small GTPases such as RAB7 (Ras-associated protein 7), RAB22, and RAB24 [55-57]. Additionally, ATG binding proteins such as UVRAG and cytoskeletal components, especially microtubules that direct autophagosomes towards lysosomes, have been shown to be important for fusion [58]. SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins also play an important role [59]. Upon the induction of autophagy, Stx17 (Syntaxin17) is recruited to completed autophagosomes and interacts with cytosolic Qbc-SNARE SNAP (synaptosomal-associated protein)-29 and the SNARE proteins VAMP7 (vesicle-associated membrane protein 7) or VAMP8, which are located on lysosomal membranes. This process drives the fusion between the outer membrane of autophagosomes with lysosome membranes [60]. Lysosomal lumen that is packed with lysosomal hydrolytic enzymes, including cathepsin proteases and lipases, degrades both autophagosome inner membranes and cargos, a process that is followed by the recycling of digested constituents back to the cytosol.

1.2. Role of autophagy in cancer

Accumulating evidence in the literature indicates that abnormalities of autophagy play a role in cancer formation and development. Yet, the exact nature of this link between autophagy and cancer seems to be context-dependent. Figure 2 summarizes the role of autophagy in the early versus late stages of cancer. In early stages involving cancer formation, autophagy rather plays the role of tumor suppressor through the degradation

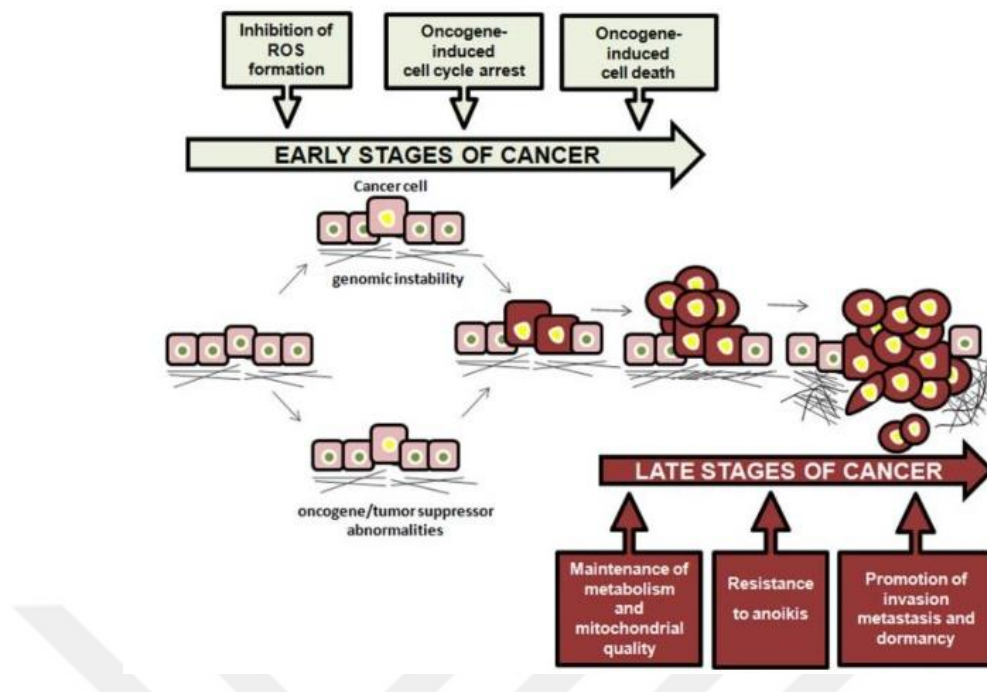


Figure 2. Role of autophagy in tumorigenesis [7].

of dysfunctional organelles such as mitochondria, limitation of ROS production, prevention of damaged DNA accumulation, and prevention of genomic instability [61]. However, at later stages and especially in some contexts (e.g. oncogenic K-RAS pathway activation), autophagy acts as a survival pathway supporting tumor growth under unfavorable metabolic conditions (scarce nutrient supply, hypoxia, and high energy/oxygen demand), in spite of abnormal and insufficient tumor vascularization [62]. Additionally, autophagy provides resistance to anoikis, a special type of apoptosis that cells activate following detachment from the basal lamina, a property that facilitates tumor cell evasion from primary sites, invasion, and spread [63]. Cells are constantly in an interaction with themselves or with their surroundings (Figure 3). These interactions are important for the decisions of the cells such as proliferation, differentiation or their death [4]. Thus, they induce stress response as they lose their cell-cell interaction and communication. Cancer cell dormancy and chemotherapy resistance have also been linked to the autophagic capacity of cells [20]. The link between cancer and autophagy is further supported by the fact that some autophagy-related proteins also function as tumor suppressors or oncogenes [13, 64]. In the following sections, relevant literature will be explained together with examples about the dual role of autophagy in cancer.

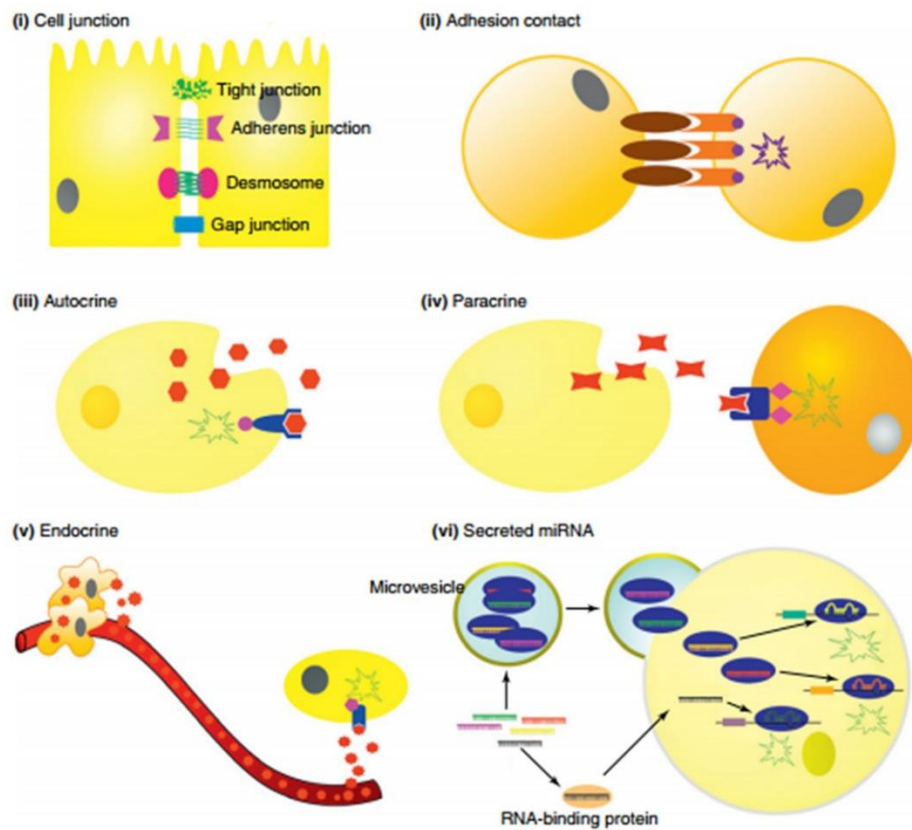


Figure 3. Cell-to-cell communication achieved in many ways in a cell/between cells [4].

1.2.1. Tumor suppressor role of autophagy

The manipulation of autophagy genes in mice has provided important clues as to the role of autophagy in cancer. For example, *Atg4C*-deficient mice not only have reduced starvation-related autophagy responses, but also an increased susceptibility to the development of chemical carcinogen-induced fibrosarcomas [65]. Mice with heterozygous *Atg5*-deletion, as well as mice with homozygous *Atg7*-deletion, developed benign liver adenomas [66]. Mice with a heterozygous disruption of *Beclin 1* gene (mouse orthologue of human *BECN1*) were generated by two independent laboratories [67, 68]. In both genetically modified mice, *Beclin 1* haploinsufficiency resulted in the formation of spontaneous tumors, including lung adenocarcinomas, hepatocellular carcinomas (HCC) and lymphomas. The tendency to develop HCC increased when these mice were crossed with liver cancer-prone mice [67]. Similarly, *Uvrag* or *Bif1* heterozygous deletion also enhanced cancer susceptibility in mice [69, 70]. These findings underline that tumor suppression is a shared property of different components of the autophagic pathway rather than being a phenotype unique to the disruption of an individual autophagy gene. In line

with these findings, mutations or deletions of several autophagy genes have been reported in human cancers (see below).

Accumulating data in the literature provide clues about how the suppression of autophagy may lead to cancer. It is now well-established that autophagy defective cells cannot recycle organelles, cytoplasmic long-lived proteins, and protein aggregates. As a result, these cells accumulate damaged mitochondria, and their cytoplasms are packed with deposits of p62 protein (and possibly other proteins). Hence, together with energy problems and metabolic stress, autophagy defective cells show a remarkable increase in reactive oxygen species levels, making them prone to DNA double strand breaks, gene amplifications, and chromosome rearrangements [61, 71]. These results have been confirmed both in 3D culture assays and *in vivo* in mice [61, 72]. Moreover, there is evidence that autophagy deficiency and p62 deregulation might be associated with the suppression of the canonical NF κ B pathway and accelerated oncogenesis through the non-canonical pathway [61]. Therefore, autophagy acts as a protection mechanism against proteotoxicity and genotoxic stress, and it helps maintain genome integrity and proper cellular signaling.

During cancer formation, cells generally inactivate apoptosis pathways and become less sensitive to apoptotic stimuli. In a context in which apoptosis pathways were blocked, autophagy incompetent cells were reported to be more prone to undergoing necrosis during metabolic stress, a cell death that results in the extracellular release of cellular contents and that triggers inflammatory responses. For example, the activation of an/the autophagy-suppressor AKT pathway or loss of a *Beclin 1* allele in combination with the overexpression of BCL2 made cells more susceptible to necrosis during metabolic stress and resulted in inflammatory cell infiltration to tissues *in vivo* [73]. In fact, necrosis and inflammation correlate with enhanced tumorigenic potential in solid tumors. Hence, the limitation of metabolic stress, prevention of necrotic cell death, and inflammation may also be factors contributing to the tumor suppressor function of autophagy *in vivo*.

Senescence is the status of cell cycle arrest with an active metabolism. Together with apoptosis induction, senescence is one of the two major anticancer mechanisms that are activated in normal cells in response to oncogene activation. In an interesting study, Young *et. al.* showed that autophagy was activated in human diploid fibroblast cells during oncogene-induced senescence [74]. Indeed, the expression of oncogenic Ras rapidly activated autophagy in this system. Autophagy was required for the establishment of senescence arrest since RNAi-mediated depletion of *Atg5* or *Atg7* facilitated the ability

of cells to escape from senescence and delayed senescence-associated cytokine production [74]. In line with these results, in an *in vivo* model, senescence marker positive regions of tumor tissues contained more LC3 positive puncta than proliferative regions, indicating that autophagic activity was higher in senescent cells [74]. These results indicate that oncogene-induced senescence may be another autophagy-related tumor suppressor mechanism that restricts normal cell growth and proliferation during oncogenic stress, preventing further genomic impairments.

In contrast with its role as a stress response mechanism for cell survival, under certain *in vitro* contexts and *in vivo* conditions, autophagy might lead to cell death [16]. Several tumor suppressor and death-related proteins, including DAPK, DRP1, ZIP and a p19ARF form, triggered a non-apoptotic and autophagy-dependent cell death [75-78]. In analogy with apoptosis, it was proposed that a defect in “autophagy-related cell death” or “autophagic cell death” could contribute to cancer formation [16]. For example, the introduction of oncogenic H-Ras protein to non-transformed cells was shown to activate a non-apoptotic cell death with autophagic characteristics [79]. In this way, autophagy was shown to block the clonogenic survival of transformed cells and prevented tumor formation. H-Ras-induced autophagic cell death was blocked by silencing the BH3-only protein NOXA or autophagy protein BECN1. BCL2 proteins that sequester BECN1 were also shown to block the effect [79].

1.2.2. Tumor promoting role of autophagy

Autophagy seems to mainly have a tumor suppressor role during the early stages of tumorigenesis. However, once tumor is established, autophagy might act as a mechanism supporting the survival of cancer cells. Cancer cells are exposed to various stress factors, including hypoxia, growth factor and nutrient deprivation, pH changes, and inappropriate extracellular matrix signals. To survive under these harsh and unfavorable conditions, cancer cells activate autophagy, allowing them to maintain cellular biosynthesis and ATP levels. Indeed, especially cancer cells in the interior parts of solid tumors, where the above-mentioned conditions are more dramatic, had elevated levels of autophagy compared with those at tumor margins [73]. In *in vivo* models of cancer, autophagy helps sustain tumor metabolism, maintain mitochondrial quality and mitochondria function, control reactive oxygen production, and allow nutrient recycling [80]. For example, autophagic activity was elevated in pancreas cancer cells and in tumor specimens, and the

suppression of autophagy resulted in tumor regression and extended lifespan in pancreas cancer xenografts and genetic mouse models [81]. In K-Ras-driven transgenic cancer models, autophagy supported tumor survival and growth by preserving mitochondrial energy production and metabolism [82]. Interestingly, in lung tumors developing on a mutant K-Ras background, the progression of tumors from adenomas to adenocarcinomas was blocked, and the knockout of the autophagy gene *Atg7* in this context led to the formation of benign oncocytoomas instead [80]. Similarly, autophagy-deficiency impaired tumor growth in B-Raf-driven lung tumors [83]. In a mammary-specific *Palb2* gene knock-out mouse model of breast cancer, autophagy impairment by allelic loss of *Beclin 1* delayed tumor development in a p53-dependent manner [84].

Moreover, autophagy has a role to promote tumor invasion. In epithelial cells transformed with oncogenic Ras, the depletion of autophagy genes resulted in a decrease in motility and invasion capacity in three dimensional cultures and reduced pulmonary metastases *in vivo* [85]. Autophagic activity was also important for the survival of dormant cancer cells *in vivo* [86].

All these findings underline the importance of autophagy in the maintenance of tumor metabolism and progression to malignancy, and even metastasis, in different biological scenarios.

1.3. Tumor and its microenvironment

Tumor is a composition of different cell types including cancer cells, reshaped extracellular matrix, various stromal cells such as immune cells, mesenchymal stem cells, adipocytes, endothelial cells and fibroblasts [5] (Figure 4). These neighbouring cells to cancer cells are called as tumor stroma (tumor microenvironment). Cell-cell communication is a key process occurring between cancer cells and surrounding cells. At 1889, Stephen Paget proposed that the growth of cancer cells (the “seeds”) depends on the microenvironment (the “soil”) which still keeps the validity today [87]. The communication between cancer cells and microenvironment can be done by signaling modulators such as growth factors, cytokines and chemokines that mainly affect (i) growth and proliferation of cancer cells, (ii) metastasis of cancer cells to tumor-distant tissues [88-90], (iii) transdifferentiation of the neighbouring cells, (iv) the composition and the stiffness of ECM (extracellular matrix), (v) the immune response, and (vi) formation of new blood vessels [91]. These signals will be explained in further sections.

Among the stromal cells, fibroblast cells (also called as cancer-associated fibroblasts, carcinoma-associated fibroblasts or shortly CAFs) represents the majority of the stromal cells especially in breast and prostate cancer. CAFs are activated fibroblasts that share similarities with fibroblasts stimulated by inflammatory conditions or activated during wound healing [92, 93]. Their role during tumorigenesis is mainly defined as tumor growth promoting. However, recent results obtained from *in vitro* co-cultures and

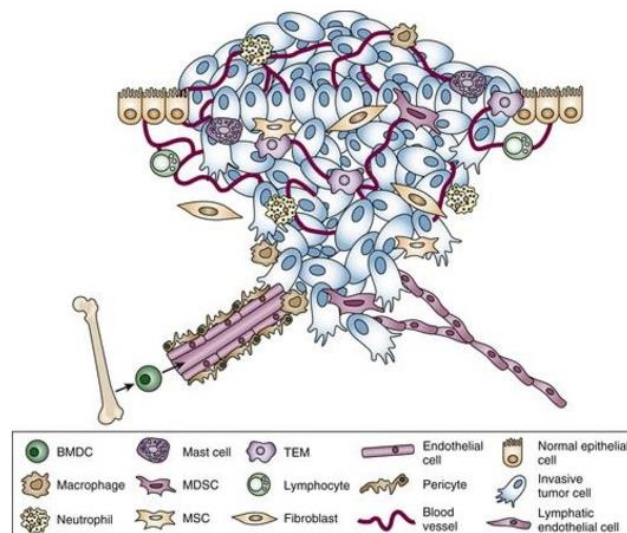


Figure 4. Tumor and its microenvironment components [5].

in vivo xenograft models claims tumor-inhibitory role of CAFs. It is important to note that this thesis will only cover the tumor growth promoting role of CAFs in tumor.

1.3.1. Fibroblasts

Fibroblasts were defined for the first time by Virchow around 1858 and later by Duval [94]. These cells were detected using classic anatomy techniques and microscopy. Basically, fibroblasts have spindle-shape morphology (Figure 5), they are non-epithelial, non-immune cells, but may originate from mesenchymal cells and a component of connective tissue [95]. These cells are the main producers of the extracellular matrix (ECM) components by producing fibrillar ECM-type I, type II and type V collagen and fibronectin- and by secreting basal membrane components- type IV collagen and laminin. And, formation of ECM is well-regulated and highly dynamic with its production by fibroblast cells and its degradation by fibroblast-derived matrix metalloproteinases (MMPs). In normal tissues, they have low level of metabolic and transcriptomic activity so that they are called in quiescent or resting state. Once they are “activated” by wound healing [96], acute and chronic inflammation, in tissue fibrosis [97-99] or stimulated by growth factors (such as transforming growth factor- β (TGF β), platelet-derived growth factor (PDGF) and interleukin-6 (IL-6) and others [94, 100], these “activated” fibroblasts secrete higher levels of ECM constituents, show higher proliferation rate, generate cytokines and chemokines, recruit immune cells [101-104].

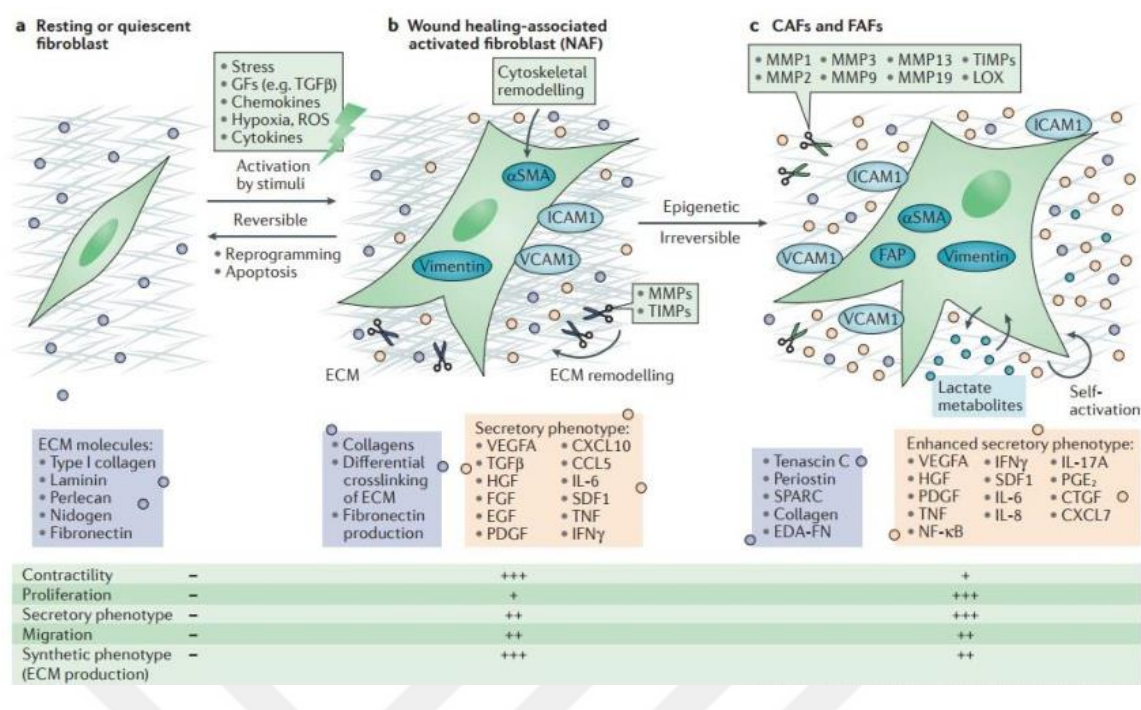


Figure 5. Comparisons between resting fibroblasts, wound healing-associated activated fibroblast, and carcinoma-associated fibroblasts [9].

1.3.1.1. Fibroblasts: in wound tissue

Injuries such as mechanical trauma, radiation-induced or extreme temperature-induced damage, toxins, pathogens or metabolic impairments cause a host response in order to restore tissue homeostasis [99, 103, 105-107]. In such a wound healing process, immune cells, mesenchymal stem cells and fibroblast cells are recruited in order to promote angiogenesis (new blood vessel formation) and reconstitution of ECM [99, 100, 108]. During the process, fibroblast cells are “activated” by increasing their proliferation, altering their phenotype and secretion profile. In order to increase their mobility and contract the wound region, alpha-smooth muscle actin (α -sma) production of these cells are upregulated. With the increased level of ECM proteins (such as type I, type III, type IV and type V collagens, laminins and fibronectin), growth factors, and chemotactic factors, fibroblast cells provide a scaffold to recruit inflammatory cells and vascular progenitor cells. During the wound healing, there is a dynamic communication between fibroblast cells and the injured epithelium. Although the direct source of “activated” fibroblast cells are still unknown, fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and transforming

growth factor- β (TGF β) are possible responsible secreted factors for the conversion of resting fibroblast to an activated fibroblast [109]. Activated fibroblasts are believed to originate from the local fibroblasts for tissue repair and their number dramatically decrease in recovered area. However, it is unclear whether the activated fibroblasts reverted to resting state and remained in the tissue, or whether the activated fibroblasts are eliminated and replaced by resting fibroblasts from surrounding normal tissue. Although the answer of this question is not clear yet, it is important to note that activated fibroblasts in wound share high similarities with educated fibroblasts in tumor.

1.3.1.2. Fibroblasts: in cancer tissue

The chronic tissue repair response also occurs in cancer. Stubborn accumulation of cancer cells in a tissue creates an ongoing tissue injury, causing a chronic wound healing response to the cancer cells. In this regard, tumors are considered as 'wounds that do not heal' [108] (Figure 5). Tumor results in a chronic host repair response which is called tumor stroma or tumor microenvironment. Fibroblasts are the main cellular component of tumor stroma. And, fibroblasts within tumors have an "activated" phenotype like fibroblasts in wound healing. CAFs are identified in tumor stroma by their high α -SMA expression and their cruciform or stellate shape. In the 1970s, it was proposed that cancer cells recruit activated fibroblast cells [110-114]. This recruitment is largely carried out by secreted factors from cancer cells and infiltrated immune cells. Similar to wound healing, TGF β is main factor for the recruitment of activated fibroblasts, for their proliferation and invasion in many cancer [115, 116]. Although there are evidence that activated fibroblasts is a host defense mechanism in order to prevent cancer progression and eliminate cancer [94, 117-119], it is a general view that cancer stroma is considered as promoting cancer initiation and progression [120-123].

In most organs, normal stroma contains a small number of resting fibroblasts embedded in ECM [120], however, tumor stroma generally accommodates with a large number of activated fibroblasts that usually express high amount of α -SMA or fibroblast activation protein (FAP) and increase deposition of ECM constituent (such as collagens, fibrin) [94, 124, 125]. Due to lack of nutrients and oxygen, the recruitment of new capillaries and recruitments of immune cells are mediated by many secreted molecules such as such as vascular endothelial growth factor A (VEGFA), PDGF, epidermal growth factor (EGF), IL-6 and IL-8. VEGFA is known for its ability to increase the permeability

of vessels by creating vascular leaks, leading to reactive perivascular areas in which immune cells, proliferative endothelial cells and activated fibroblasts are accumulated [126]. Enriched tumor stroma with ECM proteins (such as type I collagen and fibronectin) initiate tumor angiogenesis [126-128] (Table 1). While tumor grows, their stromal content also increases. In advanced stages of cancer, tumor stroma contains increased amounts of ECM proteins, including collagens, laminins, fibronectins, proteoglycans, periostin and tenascin C [129, 130]. In next section, cellular marker of carcinoma-associated fibroblasts will be explained.

Table 1. Quiescent versus activated fibroblasts.
(taken from [9])

Quiescent or resting fibroblasts	Activated fibroblasts
Morphologically bland (spindle shaped)	Morphologically active (cruciform or stellate shaped)
Metabolically indolent	Metabolically active
G0/G1 arrest or slow cycling self-renewal	Proliferative
Activated by growth factors FSP1, α 1 β 1 integrin	Further activated by growth factors α -SMA, PDGFR β , FAP
Non-migratory	Migratory
No ECM production	ECM production and synthetic phenotype
No active secretome	Active and dynamic secretome
Epigenetically stable	Epigenetically modified (e.g. <i>RASAL1</i> hypermethylation)
Precursor for activated fibroblasts	Precursor for iPSCs, chondrocytes, adipocytes, myocytes, and endothelial cells

α SMA, α -smooth muscle actin; ECM, extracellular matrix; iPSCs, induced pluripotent stem cells, PDGFR β , platelet-derived growth factor receptor- β ; *RASAL1*, RAS protein activator like 1.

1.3.1.3. Cellular marker of carcinoma-associated fibroblasts

In order to reveal differences between CAFs and resting fibroblasts in normal tissue, comparative gene expression profiling was performed. Upon their activation, CAFs produce various mesenchymal proteins such as FAP, α -SMA, vimentin and fibroblast-specific protein (FSP-1), also known as S100A4. In order to sustain their crosstalk with cancer cells and other stromal components, CAFs are the major source of secreted

cytokines, chemokines and growth factors including IL-6, CXCL8, CXCL12, VEGF (vascular endothelial growth factor), TGF- β , hepatocyte growth factor (HGF), EGF, FGF as well as receptors such as platelet-derived growth factor receptor alpha (PDGFR α) and platelet-derived growth factor receptor beta (PDGFR β). In addition to these factors, CAFs remodel the ECM by expressing various matrix-remodeling enzymes, matrix-components including tenascin C, type I collagen, fibronectin and MMP-1 (matrix-metalloproteinase 1) (Figure 5) [131, 132]. Markers such as α -SMA, vimentin, and PDGFR β have been used to identify CAFs from tissue and to analyze their role in tumor proliferation and malignancy [133-137].

1.3.1.4. Tumor-growth promoting role of CAFs

Over the last decade, several studies were conducted to understand the role of CAFs during multi-step process of tumorigenesis. Although there are conflicting studies whether CAFs can induce malignant transformation of normal epithelium (does not possess oncogenic mutations), there are strong evidence that CAFs have tumor growth promoting role from pre-malignant stage [92, 93, 131-138], to their dissemination to distant organs [139-141].

CAF-derived signals can directly promote cancer cell proliferation [142, 143], their migration and invasion [144-148], and their conversion to cancer stem cell phenotype [149-151]. Moreover, due to their role in reshaping ECM, CAFs help cancer cells to migrate and invade to the surrounding tissue [152, 153]. Additionally, in order to contribute the establishment of immunosuppressive, tumor-permissive environment, CAFs secrete several pro-inflammatory factors [154, 155] for the recruitment of immunosuppressive and tumor-promoting immune cells [156, 157]. In this thesis, CAFs and cancer metabolism will be explained.

1.3.1.5. Altered metabolism of CAFs

Due to their high proliferation rate, the cellular metabolism of CAFs is based on aerobic glycolysis [158]. During the activation of fibroblast cells, the metabolic changes may occur due to the presence of secreted factors such as TGF β , PDGF, and unfavorable environmental conditions such as hypoxia, reactive oxygen species (ROS) [159-161]. In order to preserve their altered proliferative state under intratumoral hypoxic condition;

metabolic adaptation, increased Warburg effect and an increased autophagy can be observed [159, 160, 162].

Metabolic adaptations in CAFs creates nutrient for other stromal cells and cancer cells. For example, Cav 1-knockout fibroblast cells increased ketone bodies, fatty acids, and glutamine levels which provides ready metabolites in order to support the mitochondrial respiration in cancer cells (Figure 6 and Figure 7) [160, 163]. Metabolic reprogramming of CAFs may be due to paracrine signaling from cancer cells or direct contact between CAFs and cancer cells [164]. According to global gene expression profiling studies of CAFs, isocitrate dehydrogenase 3A (IDH3A) gene is suppressed, HIF1 α (hypoxia-inducible factor 1-alpha) is stabilized and pro-glycolytic genes are induced [161]. Activation of p62 caused an impaired cellular redox and ROS-mediated increase in IL-6 production [165]. In one hand, the metabolic symbiosis of CAFs and cancer cells may dynamically evolve in response to oxygen, extracellular metabolite availability and chemokine or cytokine signaling. On the other hand, epithelial carcinogenesis may lead the metabolic reprogramming of CAFs in order to promote a reciprocal metabolic symbiosis between CAFs and cancer cells [166]. The impact of this symbiotic relationship on metastatic growth of cancer is largely unknown.

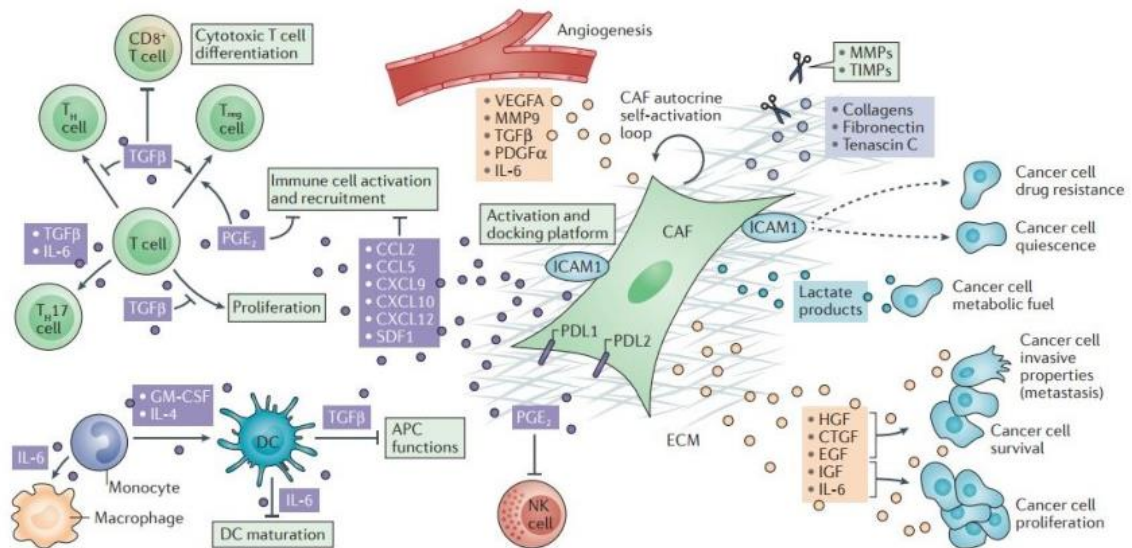


Figure 6. Complex communication between activated fibroblast cells with other components of tumor [9].

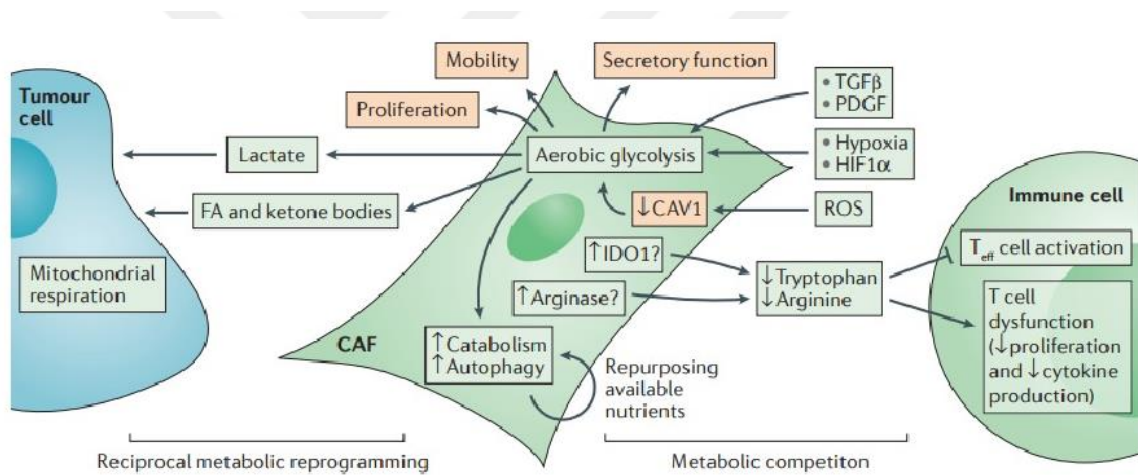


Figure 7. Autophagy induction in fibroblast cells and their metabolic reprogramming [9].

1.3.2. Soluble factors in tumor microenvironment

By Rudolf Virchow, the role of inflammation during the development of cancer was described circa 1863. According to his observations, inflammatory cells infiltrate tumors so that cancer may arise from inflammatory sites [167]. In addition to his observation, several cytokines and growth factors are detected in tumor microenvironment which are secreted by the stroma cells or by cancer cells.

Cytokines are low-molecular-weight proteins that mediate cell-to-cell communication. They are expressed by immune and stromal cells in order to regulate cell survival and death, immune cell activation, cell migration, proliferation, and differentiation. During chronic inflammation, like cancer, cytokines can induce cell transformation and malignancy, and activation state of the surrounding cells depending on the tumor microenvironment [168]. In addition to cytokines, tumor microenvironment contains number of polypeptide growth factors (PGF) and mediate their action through specific cell-surface receptors. Through binding of PGF to its cell-surface receptor, intracellular signal cascades initiated [169]. Both mesenchymal and epithelial cells contribute to the production of PGFs in stroma so that abnormal production or cellular response correspond to malignant transformation.

In tumor microenvironment, tumor epithelial cells and stromal components communicate through the production of growth factors and cytokines [118]. For example, released platelet derived factor (PDGF) from cancer cells to fibroblast cells induce their transdifferentiation; the stromal cells reciprocate by releasing insulin-like growth factor 1 (IGF-1) which helps the growth and survival of cancer cells [170]. Thus, in general, these secreted factors mainly target increased proliferation rate, increased expression of proangiogenic factors, extracellular matrix-modifying enzymes to promote epithelial-mesenchymal transition (EMT), and facilitate other carcinogenesis programs such as genome instability, reprogramming of energy metabolism, and immune modulation [171]. Although these factors are numerous, in this thesis, TGF β , IL-6 and VEGF will be explained.

1.3.2.1. Transforming Growth Factor-beta (TGF β)

TGF is an immune-suppressing and anti-inflammatory cytokine. During physiological processes, its role in embryogenesis, cell proliferation and differentiation, apoptosis, adhesion and invasion is well documented [172]. In tumor microenvironment, the main sources of TGF β s are cancer and stromal cells, including immune cells and fibroblasts [173]. Due to elevated level in bone matrix, it is a common site for metastasis in many cancers, correlating with the tumor-promoting and invasive effects of this cytokine [174]. It has three isoforms: TGF β 1, TGF β 2, and TGF β 3. All TGF β s bind to Type II receptor (TGF β -RII), which induce type I TGF β - receptor (TGF β -RI) phosphorylation and subsequently the formation of SMAD 2/3 complex that translocates

into nucleus where they form complex with SMAD 4, for further binding to DNA, in order to induce gene expression [173].

In cancer, TGF β has dual role depending on the cell type and stage of tumorigenesis. In early stages, TGF β has tumor-suppressor role by inhibiting cell cycle progression and promoting apoptosis. In later stages, TGF β induces epithelial-mesenchymal transition (EMT) which increases invasion and metastasis of cancer cells [175]. During this process, TGF β shows a tumor suppressor effect through upregulation of p21 and downregulation of c-Myc [176]. The contribution of TGF β to tumorigenesis has been shown by conditional TGF β -RII knockout mice experiments in which highly proliferative epithelia (such as rectal and genital) developed spontaneous squamous cell carcinomas and furthermore showed accelerated carcinoma progression [177]. There are growing evidence that TGF β signaling involves in human cancer. Although increased TGF β 1 mRNA and protein levels have been observed in gastric carcinoma, non-small cell lung cancer, colorectal and prostate cancer [178], TGF β -receptor mutations or deletions have been associated with colorectal, prostate, breast, and bladder cancer in higher degree of invasion, and worse prognosis [179]. Due to serious side effects of systemic TGF β -inhibitors (specifically ligand traps, antisense oligonucleotides, receptor kinase inhibitors, and peptide aptamers) administration, further clinical trials are needed to be evaluated in terms of cancer types, stages and according to its effective dose [174].

1.3.2.2. Interleukin-6 (IL-6)

IL-6 is another pro-inflammatory cytokine with pro-tumorigenic effect. It has been claimed that IL-6 can be proposed as a malignancy predictor as a result of a comparison between the serum of healthy controls or patients with systemic cancer due to detecting an increased IL-6 levels in the serum of patients [180].

IL-6 binds to its receptor (IL-6R α) and coreceptor gp130 (glycoprotein 130), activates JAK/STAT signaling pathway of the Janus kinases (JAK) and signal transducers and activators of transcription (STATs) STAT1 and STAT3; as a result, it promotes proliferation and inhibits apoptosis [181] (Figure 8 and Figure 9). It was also shown that

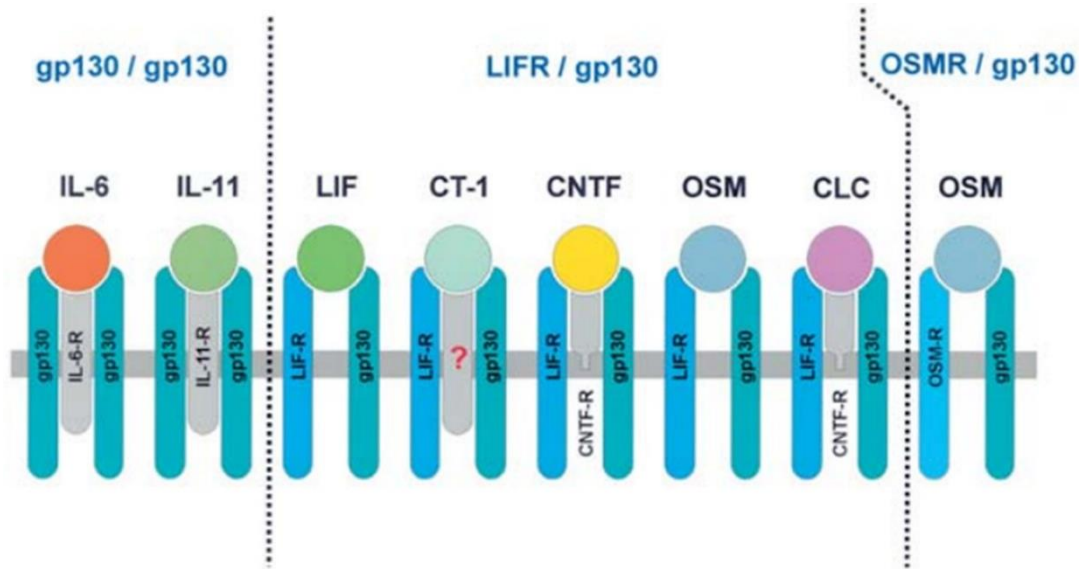


Figure 8. IL-6 family members and their receptors [6].

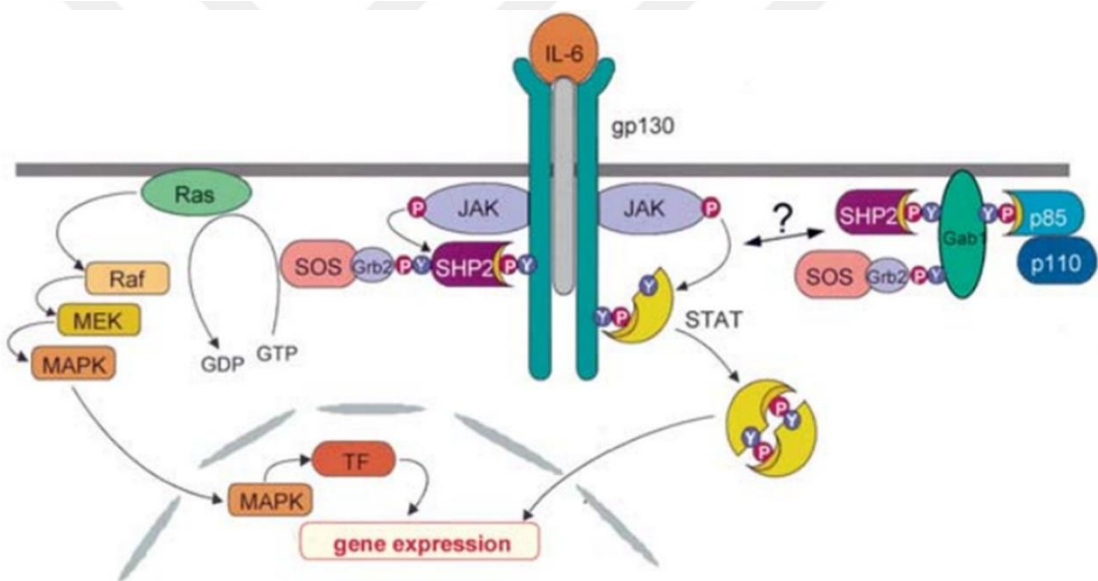


Figure 9. IL-6 signaling in the cell.

IL-6 can induce tumorigenesis by hypermethylation of tumor suppressor genes as well as by hypomethylation of retrotransposon long interspersed nuclear element-1 (LINE-1) in oral squamous cell cancer lines *in vitro* [182], a frequent event in various human cancers. Moreover, absence of IL-6 using deficient mouse model exhibits reduced tumorigenesis although the mouse is exposed to the carcinogen N-methyl-N-nitrosourea [183]. IL-6 facilitates tumorigenesis by taking an important role for the conversion of noncancer cells into tumor stem cells. In particular, IL-6 secretion by noncancer stem cells in low-attachment culture conditions upregulate Oct4 gene expression by activating the

IL-6R/JAK/STAT3 signaling pathway [184]. These findings have led researchers to propose IL-6 as a therapeutic target in cancer. Several phase I/II clinical trials are currently evaluating antibodies against IL-6 or IL-6R as therapeutic alternatives.

1.3.2.3. Cardiotrophin-1 (CT-1)

Cardiotrophin-1 is discovered by Pennica *et al.* in 1995 in an observation that conditioned medium from differentiated embryonic bodies induced hypertrophic response in neonatal cardiac myocytes. It is 21.5 kDa protein with 203 amino acids long. It shares 24% identity with leukemia inhibitory factor (LIF) and as a structure, CT-1 was considered as a new member of IL-6 family [185]. The coding regions of human and mouse CT-1 are contained on three separate exons which is around 6-7 kbp of genomic DNA. The similarity between these exons are 96%, 84 % and 81 % in exon 1,2, and 3 between human and mouse [186]. The human CT-1 gene is located on chromosome 16p11.1–16p11.2 [187]. CT-1 binds to the specific combination of glycoprotein 130 (gp130) and leukemia inhibitory factor (LIFR) together with third uncharacterized receptor (CT-1R α) [188]. Although IL-6 and CT-1 are the member of the same family, they have only 19.35% similarities in their protein sequence. Moreover, their receptors have structural differences (Figure 10). As a result, the activation of CT-1 in the system is unique and different from IL-6 activation. Signal transduction via binding to its receptor involves in three different major pathways: the JAK (Janus- activated kinase)-STAT (signal transducer and activator of transcription) axis, the Ras-Raf mitogen activated protein kinase (MAPK, MEK/ERK) signaling cascade and the Ras-Raf mitogen activated protein kinase (MAPK, MEK/ERK) signaling cascade, and the phosphatidylinositol 3-kinase-dependent (PI3K)/AKT (protein kinase B, PKB) pathway. CT-1 binds LIFR with low affinity and this binding stimulates its heterodimerization with the gp130 into a high affinity-binding complex [189]. gp130/LIFR activates JAK1, JAK2 and the tyrosine kinase (TYK2) associated with gp130, which phosphorylates tyrosine residues in the cytoplasmic domain of gp130. These phosphotyrosines subsequently serve as the docking sites to recruit STAT proteins, which are phosphorylated allowing STATs to translocate to the nucleus where they bind specific DNA sequences to modulate gene expression [190]. In the cells, CT-1 has cardioprotective role in cardiomyocyte survival [191]. It has an essential role in neural tissue development and protection of mature nervous system from damage [189, 192]. It protects liver against galactosamine liver damage [193]. It has



Figure 10. Similarity analysis between CT-1 vs IL-6 and also their receptors (modified from [8]).

a role in energy homeostasis and intermediate metabolism. It regulates body weight and increases whole-body oxygen consumption [194] (Figure 11).

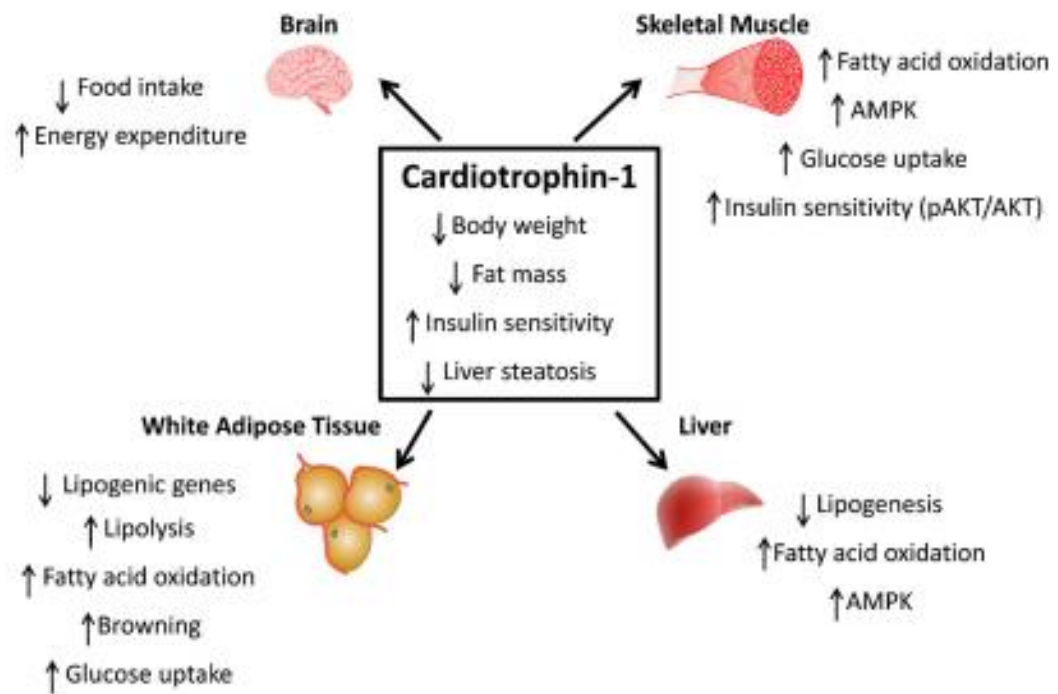


Figure 11. Role of Cardiostrophin-1 in different organs [2].

1.3.2.4. Vascular Endothelial Growth Factor (VEGF)

Tumor angiogenesis, a process of new vasculature formation, is appreciated to be an integral part of solid tumor development [195]. The supply of new blood vessels in tumors not only fosters autonomous tumor growth but also helps remove accumulated waste and ameliorate burdensome metabolism. In a fast-growing malignant tissue, tumor blood vessels are exposed to multiple growth factors and cytokines [196].

Tumor microenvironment contains several types of cells including fibroblasts/myofibroblasts, glial, epithelial, fat, immune, vascular, smooth muscle, and immune cells together with reshaped extracellular matrix (ECM) and extracellular molecules. Although none of these cells are malignant themselves, due to their environment and crosstalk with each other, and directly or indirectly with cancer cells, they gain an abnormal phenotype and altered function. Among all those cells, there are complex signaling networks both in tumor cells and its microenvironment which includes induction of inflammation of the area leading to recruitment of immune cells, proliferation of cancer cells due to growth factors, chemokines and cytokines released from the microenvironment, and suppression of cell death of cancer cells by altering the rates of apoptosis and proliferation rate through genetic mutations in tumor-suppressor genes. In addition to these changes, promoted angiogenesis and reshaped ECM induce blood vessel formation in order to support nutrient and oxygen for the growth of cancer cells [120, 197-199].

VEGF, from now on called as VEGFA, known also as vascular permeability factor (VPF), is a polypeptide and a member of platelet derived growth factor family. It is one of the most studied and growth factors secreted by cancer cells to the microenvironment. VEGF induces angiogenesis to provide oxygen and nutrient to cancer cells. It is produced and secreted by various tumor and normal cells as 34- to 43 kDa dimeric protein. There are several variants: VEGF₁₂₁, VEGF₁₄₅, VEGF₁₄₈, VEGF₁₆₅, VEGF₁₈₃, VEGF₁₈₉, VEGF₂₀₆. These isoforms are produced by an alternative splicing from a single VEGF gene which is located on chromosome 6p21.3 and contains eight exons [200]. Studies in the last decade have shown that the function of VEGFA is not only limited to angiogenesis and vascular permeability, it also affects immune cells which are present in the tumor microenvironment [201]. To increase the efficiency of targeted therapy for VEGFA, it is necessary to clarify the role of VEGFA on other stroma components.

1.3.3. Microfluidic chips (biochips) and their alternative usages

As understanding from their name, microfluidics is the combinational field of science and technology, process small amount of fluidics together with biological or chemical elements, reactions or analysis into various channels or pores with the dimensions of tens to hundreds of micrometers [202]. This integrated small size can bring several advantages including easy control, fast detection and flexibility [203]. In recent years, there is a rapid development for the biological analysis and other biological experiments such as polymerase chain reaction (PCR), protein analysis, biochemical reactions and drug treatments have been successfully transferred into chips [204, 205]. Cell culture is not an exception. The most common material for cell culture in microchip is polydimethylsiloxane (PDMS) which has several advantages due to air permeability, elasticity, biocompatibility, and its easy fabrication [206-208].

There are several advantages of microfluidic chips on cell applications [209-211]; (1) Depending on its design, single cells can be captured or manipulated; (2) It requires low cost and low consumption of the materials; (3) Cell movements can be controlled by adjusted fluidics which is not possible to achieve in traditional cell culture methods; (4) Together with controlled pressure of pumping system, complex body networks such as heartbeat or peristaltic movements can be achieved; (5) In a short time, multi-step operations can be achieved using high throughput design. It is also important to note that two different culture situations can be obtained: “static state” and “dynamic state”. In static state, a sufficient medium is supplied for the cells in a chip without any perfusion system [212]. In dynamic state, with the help of extra equipment such as micropump, the medium of the cells can be changed or cells can be separated [213]. With this precisely controlled environment, cells can be manipulated and body network can be simulated. These two states can be used for various purpose of cell culture experiments in chip. Microfluidic chip allows for cell-cell or cell-ECM interactions, their movement and drug delivery [214, 215]. Observations can be performed in single cell level [216-218]. Due to abundant and various cells work, interact and cooperate in the body, it is necessary to understand the mechanism of their action to discover new drugs in the diseased state. Thus, in microfluidic chip, numerous cells can be operated and detected at the same time.

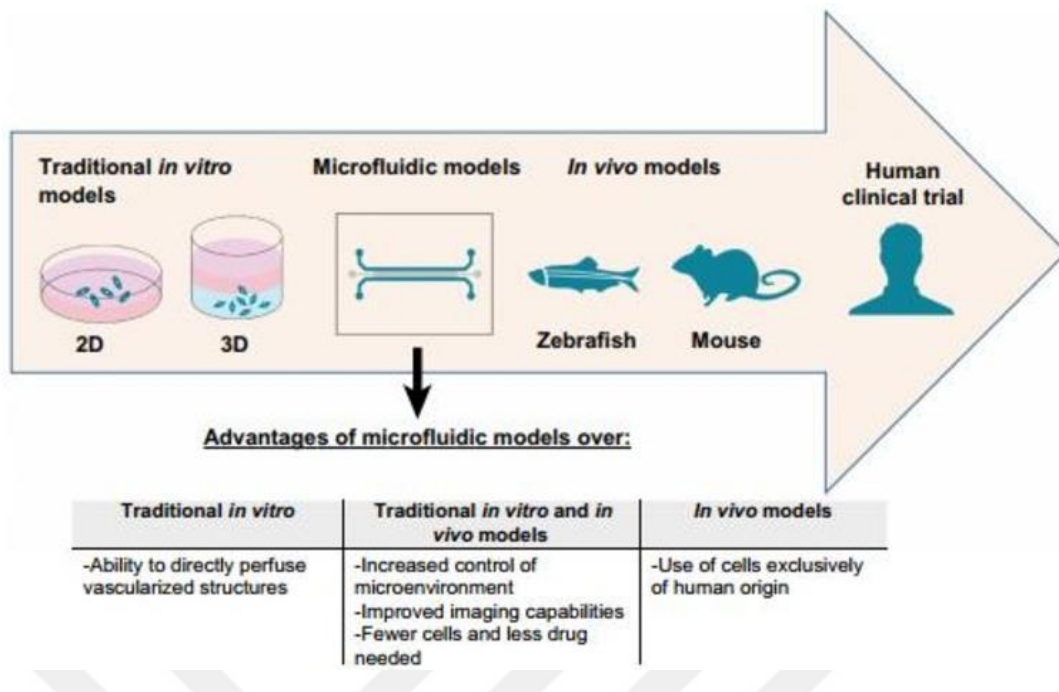


Figure 12. Comparison of microfluidic models with traditional *in vitro* and *in vivo* techniques [3].

1.3.3.1. Cancer Research using microfluidics

Tumor formation consists of different types of cells, with their dynamic communication and cell-cell interactions. Microfluidic co-culture systems were proposed in recent year to facilitate the understanding of tumor outside of the body (Figure 12). Fibroblasts or epithelial cells are the most common stromal cell types used in co-culture with cancer cells in order to understand tumor growth and migration [219, 220]. This type of co-culture chips allows for the observation of proliferation of the cancer cells as well as migration and invasion [221]. With low cost, dynamic analysis, and full automation, high throughput screening of anti-cancer drugs can be performed. Due to allowing co-culture systems and 3D cell culture, during tumorigenesis, the importance of secreted paracrine factors or cell-cell interaction can be tested (Figure 13). For example, Zheng et al. [222] presented cancer cell invasion progress. In that experiments, HeLa cells and human umbilical vein endothelial cells (HUVECs) were seeded into the chips separately, and them co-cultured with common medium. The results suggested that the crosstalk between these two cells is through soluble paracrine factors that has been achieved by varying the frequency of medium change on chip. In another study, Hsu *et al.* [223] showed that the migration of cancer cells is promoted by macrophages using microchannel chip design

2. MATERIALS AND METHODS

2.1. Cell Culture

Immortalized mouse embryonic fibroblast cells (MEF) were obtained following transfection with the SV40 whole genome of early passage primary GFP-LC3 (Green fluorescent protein GFP-fused to MAP1LC3 protein) transgenic MEF [78, 225] or primary MEF wild-type (WT), and primary (*Atg5*^{-/-}) MEF [78, 226]. MCF7-RFP and MDA-RFP breast cancer cells were derived from MCF7 (ATCC, USA) and MDA-MB-231 (ATCC, HTB-26) following lentiviral transduction. HEK 293T, MCF7, Huh7, PC3, and U2OS cells were cultured in full DMEM medium, which is DMEM (Sigma, D5671) supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine and 100 U/mL penicillin/streptomycin, at 37°C in a humidified 5% CO₂ incubator. GFP-LC3 transgenic MEF (shortly, GFP-LC3 MEF) and MDA-MB-231 (MDA) cells were cultured in full DMEM medium supplemented with 1xMEM non-essential amino acid solution (Gibco, 11140-035). MEF WT and (*Atg5*^{-/-}) MEF cells were cultured in MEF medium (full DMEM medium supplemented with 55 µM β-mercaptoethanol). For indicated experiments, low-FBS containing medium represents DMEM (Sigma, D5671) supplemented with 3% (v/v) fetal bovine serum (FBS, PAN, P30-3302), L-glutamine (Biological Industries, 03-020-1B) and 100 U/mL penicillin/streptomycin (Biological Industries, 03-031-1B).

2.2. Characterization of MEF WT and (*Atg5*^{-/-}) MEF Cells

2.2.1. Proliferation Assay for MEF WT and (*Atg5*^{-/-}) MEF Cells

MEF WT or (*Atg5*^{-/-}) MEF fibroblast cells were split as 1×10⁵ cells per well in 6 well plate. Their proliferations were tested at 12 h, 24 h, 36 h and 48 h. Attached cells were counted with Trypan Blue.

2.2.2. Attachment Assay for MEF WT and (*Atg5*^{-/-}) MEF Cells

2.2.2.1. Under normal conditions and in short time points, attachment kinetics of the fibroblast cells

MEF WT or (*Atg5*^{-/-}) MEF fibroblast cells were split as 4×10^5 cells per well in 6 well plate. Their attachments were analyzed at 30 min, 1 h, and 2 h. Attached cells were trypsinized and counted with Trypan Blue. Their cell pellets were used to analyze for autophagy markers using immunoblot technique.

2.2.2.2. Under normal conditions, and starvation treatment, attachment kinetics of the fibroblast cells in different time points

MEF WT or (*Atg5*^{-/-}) MEF fibroblast cells were split as 4×10^5 cells per well in 6 well plate in full DMEM medium or starvation (EBSS) medium. Their attachments were analyzed at 1 h, 2 h, 4 h, and 8 h. Attached cells were counted with Trypan Blue. Their cell pellets were used to analyze for autophagy markers using immunoblot technique.

2.2.3. Wound Healing Assay for MEF WT and (*Atg5*^{-/-}) MEF Cells

MEF WT or (*Atg5*^{-/-}) MEF fibroblast cells were split in 24 well plate in full DMEM medium (DMEM+ β -mercaptoethanol), in a medium (DMEM P/S) which does not contain any L-glutamine and FBS, or starvation (EBSS) medium. After scratches, t_i (initial) measurement was performed, and after 24 h, t_f (final) measurement was measured. Percentage of closure was calculated as:

$$[\text{Width}(t_i) - \text{Width}(t_f)] * 100 / \text{Width}(t_i)$$

Within each medium group, cells moved at the same speed and into the same direction, that means autophagy active or knock-out did not create any difference upon growth factor or nutrient deprivation. They were affected from starvation treatment in same pattern. Starvation and/or lack of important amino-acids and growth factors can cause obstacles during migration but this migration was independent from the autophagy activity in MEF cells within 24 h.

2.3. 3D Bioprinting Experiments

2.3.1. Decellularization of Animal Aorta

The protocol was modified from the reference article [227]. The lamb aorta was taken from Apikoglu Et. San. Ltd. (located at Orhanlı, provided by Ünsal Bey: +90535-555-2334). The aorta was transported on ice. The aorta was cleaned from adherent tissues and fat, dissected, and rinsed with deionized water (DI) and with PBS several times. Square samples (2 cm × 2 cm) were cut from the middle section of the aorta in order to minimize the changes of the mechanical properties along the longitudinal position of aorta. Three different types of decellularization protocols were applied: (i) In SDS treatment, the aorta sample was incubated in hypotonic 10 mM Tris Buffer (pH 8.0) with 0.1% EDTA for 1 h. Then, the sample was further incubated in 0.1% SDS with Tris Buffer for 72 h at room temperature and on orbital shaker. (ii) In Trypsin treatment, the aorta sample was incubated in 0.25% cell culture Trypsin for 72 h at room temperature on orbital shaker. (iii) In Triton-X 100 (Tert-actylphenyl polyacetylen) treatment, the aorta sample was incubated in 1% Triton X-100 with 0.2% EDTA in 10 mM Tris Buffer for 72 h at room temperature on orbital shaker.

2.3.2. 3D Bioprinting Optimization Experiments

In this part of the research, the goal was to prepare the cells to help their cell-cell contact after printing them in 3D structures and to analyze the role of autophagy during this process. In order to print fibroblast cells in defined positions for creating macrovascular structures using Organovo: NovoGen MMX Bioprinter™ (Organovo Holding, San Diego, CA), cells were prepared using different protocols: (i) Cells were prepared according to manufacturer instructions in separate cylindrical bioink formation, or (ii) in continuous bioprinting [228]. (i) For cylindrical bioink formation, immortalized MEF cells were cultured in 15 cm-diameter culture dishes. Cells were detached from the culture plate using 0.25% trypsin (Biological Industries) for 2-3 min. Following detachment, trypsin was neutralized using serum containing medium. Collected detached cells were centrifuged at $200 \times g$ and supernatant was discarded. The cell pellet was resuspended to obtain 10×10^6 cells / 20 ml medium incubated at 37 °C in 15 ml-conical tubes with rotation. Following pelleting, cells were resuspended in 1 ml medium and transferred into 1.5 ml Eppendorf tubes and centrifuged again ($1000 \times g$). Then, the cell pellets were drawn into capillary tubes. Following incubation, cells inside the capillary

tubes were extruded into cylindrical grooves on agarose gel (2% in PBS). Then, plates were covered with culture medium and put into the incubator until cylindrical bioinks are formed. Cylindrical bioink MEF cells were drawn back into capillary tubes and bioprinting was performed using the 3D bio-printer. (ii) For continuous bioprinting, cells were centrifuged at $200 \times g$. The pellet was re-suspended to have 20×10^6 cells / 6.5 ml and transferred into 15 ml-conical tubes. Following rotation at 37°C , cells were pelleted and transferred into Eppendorf tubes. Cell pellets in 1.5 ml Accepted Preprint 7 Eppendorf tubes (60×10^6 cells in total) were transferred into capillaries by continuous bioprinting.

In separate cylindrical bioink formation, mixing with collagen and/or longer incubations were tested (for detailed protocol, please see below).

Detailed bioprinting protocol: Fibroblast cells were trypsinized from 15 cm plates. Following detachment, trypsin was neutralized using serum containing medium. Collected detached cells were centrifuged at $300 \times g$ for 5 min and supernatant was discarded. (i) Cell pellets were mixed with 2 ml collagen or full medium (CNT), and incubated for 15 min in incubator. Cells were mixed every 3 min. After 15 min incubation, cells were centrifuged at $300 \times g$ for 5 min and 4 ml full DMEM medium is added. After collagen treatment, collagen was centrifuged and cell pellets were re-suspended either with Hoechst 33342 (1 $\mu\text{g/ml}$ final concentration) or Cell Mask Deep Membrane (5 $\mu\text{g/ml}$ final concentration). After 10 min incubation in 37°C incubator, cells are centrifuged and pre-printing process continued. Cells were rotated for 1 h at room temperature. Cells were centrifuged at $6000 \times \text{rpm}$ for 5 min. Supernatant of the cells were removed and cells were sucked into 500 μm capillary in order to keep the shape of the cells in cylinder. The capillaries were incubated in full DMEM medium in 50 ml tube for 20 min, 40 min, and 80 min in incubator. After the incubation period, cells were put into capillary plate (prepared with 2% Agarose) and incubated for 6 h or for 9 h. Cells were taken back into capillary and bioprinting process started. The results were tested: (i) in microscope to check their fusion efficiency, and (ii) in immunoblot technique for autophagy markers.

2.4. Preparation and Treatments of Conditioned Media

In order to analyze cancer cell-derived factors, conditioned media were obtained from different cancer cells (MCF7, MDA-MB-231, Huh7, PC3, and U2OS cells). As controls of the experiments, conditioned medium either from GFP-LC3 MEF cells or from MEF WT cells were used for microscope and for immunoblot analysis, respectively.

After 16 h of culturing the cells, their media were replaced with low-FBS containing medium, which is DMEM medium containing 3% FBS with L-glutamine, 100 U/mL penicillin and streptomycin. After 72 h, medium of the cells was collected and concentrated using Amicon Ultra-4 centrifugal filter units (Milipore-3kDa cut-off, UFC800396) with centrifugation at $4.000\times g$ for 40 min using a swinging bucket rotor. In order to avoid autophagy activation by impoverished media, conditioned media were concentrated and diluted with fresh culture media before adding onto fibroblasts. The concentrated media (approximately 300 μ l) were called as conditioned media and were diluted in 1 ml full DMEM plus 1xMEM non-essential amino acids in order to treat previously seeded GFP-LC3 MEFs (1×10^5 cells/well in 12-well plates). After 24 h of the treatments, cells were fixed with 4% paraformaldehyde and autophagy activation was evaluated under the fluorescence microscope with GFP-LC3 dot analysis. Moreover, the effect of concentrated media from indicated cancer cells on autophagy in fibroblast cells was tested using immunoblot techniques. Autophagy markers were analyzed in MEF WT cells using immunoblot technique in order to quantify autophagy induction after treatments with conditioned media.

2.5. Co-Culture of Cancer Cells with Fibroblast Cells

GFP-LC3 MEFs (1×10^5 cells/well in 12-well plate) were seeded on cover slides. After 16 h, breast cancer cells (MCF7-RFP or MDA-RFP) were prepared (1×10^4 cells/well) in 1 ml full DMEM medium and added onto previously seeded GFP-LC3 MEFs. After 24 h and 48 h of co-culturing cancer cells with fibroblast cells, all cells were fixed. CNT condition was autophagy analysis of the fibroblast cells without any cancer cells at 48 h. Autophagy activation in fibroblast cells was evaluated under fluorescence microscope with GFP-LC3 dot analysis.

2.6. Screening of cDNA Library for Cytokine and Growth Factors.

cDNA libraries were produced by Thermo Scientific (Catalog #: MHS4928; MHS4924) by cloning them into mammalian expression vector pCMV-SPORT6. Before the screening, individual clones provided by company in 96-well plate were transformed into bacteria. After single colony pick-up and growth, plasmids were isolated and stored separately. cDNA of 57 growth factors (11 pools) and 73 different cytokines (14 pools) were grouped in pools containing 5-6 different cytokines (Appendix C and Appendix D).

Among in total 130 different factors, first screening (i) was performed with randomly formed DNA cocktails, which contained 5 different plasmids. HEK 293T cells were transfected using calcium phosphate transfection method according to standard protocols [24] with these pools and after 8 h of post-transfection, low-FBS containing medium was added. After 72 h, the media of the transfected cells (around 5.5 ml) were collected and concentrated by using Amicon® Ultra- 4 centrifugal filter units (Millipore, Catalog #: UFC800396). This type of concentrated medium is called conditioned medium. Conditioned media (approximately 300 µl) of the pools were diluted in 1 ml full DMEM media and added on GFP-LC3 MEF cells which were cultured 16 h before the treatments. After 24 h, cells were fixed and autophagy activation was evaluated under 60X objective in upright fluorescence microscope (Olympus, BX60) with GFP-LC3 dot analysis. Based on the autophagy analyses in GFP-LC3 MEF cells which show more than two-fold autophagy induction, the highest pools were tested with their individual factors in a second screening (ii). The effects of individual factors from the autophagy-inducing pools were tested according to the effect of their conditioned medium on autophagy induction in fibroblast cells. Like our first screening, HEK 293T cells were transfected with the selected individual factors, their media were concentrated, and their concentrated mediums were added on GFP-LC3 MEF cells. After 24 h of treatments, the individual factors which induced autophagy more than two-fold in GFP-LC3 MEF cells were selected for third screening (iii). This screening is the final screening and HEK 293T cells were transfected with selected individual factors (For growth factors, twelve factors; for cytokines, eight factors were selected). Their media were collected, concentrated and analyzed on the autophagy of GFP-LC3 MEF as described for screenings (i) and (ii). The analyses of selected individual cDNA factors were repeated three times (n=3).

2.6.1. Conditioned Media Preparation for Candidate Factors

HEK 293T cells were transfected with TGFβ1, CT-1, VEGFA or pcDNA3 (CNT). After 8 h of post-transfection, their media were changed with low-FBS containing media. After 72 h of incubation, media of the transfected cells were collected and concentrated using an Amicon® Ultra-4 centrifugal filter units. Conditioned media were diluted in 1 ml full DMEM media and added on GFP-LC3 MEF which were cultured 16 h before the treatments. After 24 h, cells were fixed and autophagy activation was evaluated under upright fluorescence microscope (Olympus, BX60) with GFP-LC3 dot analysis.

2.6.2. Neutralization Antibody Treatment

Three different types of neutralization antibodies were used in this thesis: (i) In order to neutralize the effect of TGF β 1 in conditioned media from TGF β 1 overexpressing HEK 293T cells, conditioned media of these cells were mixed with 30 μ g/ml TGF β 1 neutralizing antibody (R&D, AB-100-NA) or control serum (rabbit serum: Sigma, R9133). Following rotation at 4 °C for 2 h, their volume is completed to 1 ml with the media (DMEM; 10% FBS; L-glutamine; 100 U/ml Pen/Strep.; 1X non-essential amino acids). GFP-LC3 MEFs were cultured in these mixed media for 24 h, fixed, and evaluated with GFP-LC3 dot analysis. (ii) In order to neutralize the effect of CT-1 in conditioned media from CT-1 overexpressing HEK 293T cells, conditioned media of these cells were mixed with 6 μ g/mL CT-1 neutralizing antibody (R&D, MAB2601) or with 6 μ g/mL control antibody (Monoclonal anti-Flag M2 antibody, Sigma, F3165) for 2 h at 4°C before diluting in fresh medium and adding onto fibroblast cells. After 24 h, cells were fixed and autophagy activation was evaluated under fluorescence microscope with GFP-LC3 dot analysis. (iii) In order to neutralize the effect of VEGFA in conditioned media from VEGFA overexpressing HEK 293T cells, conditioned media of these cells were mixed with 0.08 μ g/ml VEGFA neutralizing antibody (R&D, MAB293) or 0.08 μ g/mL control antibody (Monoclonal anti-Flag M2 antibody, Sigma, F3165). Following rotation at 4 °C for 2 h, their volume is completed to 1 ml with the media (DMEM; 10% FBS; L-glutamine; 100 U/ml Pen/Strep.; 1X non-essential aminoacids). GFP-LC3 MEFs were cultured in these mixed media for 24 h, fixed, and evaluated with GFP-LC3 dot analysis.

2.7. GFP-LC3 Dot Analysis

In order to detect autophagy activation by GFP-LC3 dot analysis, GFP-LC3 MEF cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, 15,812-7), mounted and inspected under 60 $\times$ magnification using an upright fluorescence microscope (Olympus, BX60, Japan). Basal autophagy threshold was determined as 20 GFP-LC3 dots per control-treated GFP-LC3 MEF cells. Dots in at least 150 cells per experimental point were counted and analyzed. Graphs were plotted as percentage of GFP-LC3 dots positive cells over total cell numbers.

2.8. Determination of Secreted Factors Using ELISA

2.8.1. Detection of Secreted TGFβ1 from Different Cancer Cells

TGFβ1 concentration in culture media was analyzed using ELISA kit (R&D, DB100B) according to manufacturer's recommendation. First of all, TGFβ1 concentration in media containing 1-10% FBS was measured in the absence of cells. Then, TGFβ1 concentration in the presence of 6.0×10^4 MEF or MDA cells in 200 μl of medium containing 3% FBS in 48-well plate was analyzed. 6.0×10^3 MDA cancer cells were seeded in co-cultures. After 6 h, cultured media were collected and analyzed. Average of two independent set of experiments were shown in Figure 37. In shTGFβ1 knock-down system, 1.0×10^5 cells were seeded in 2 ml of medium containing 3% FBS in 6-well plate. Cells were infected with shGFP/shTGFβ1 viruses together with 5 μg/ml polybrene. After 16 h, cells were washed and media were changed to 2 ml medium containing 3% FBS. After 72 h, media were collected and analyzed. Result of three independent experiments are shown in Figure 46B.

2.8.2. Detection of Secreted CT-1 from Different Cancer Cells

Serum samples were obtained from the supernatants of different cancer cells which were cultured in low-FBS containing media for 72 h. Secreted amount of CT-1 to the environment was tested using the CT-1 ELISA (Sigma, RAB0107) according to the instructions of the manufacturer.

2.8.3. Detection of Secreted VEGFA from Different Cancer Cells

Serum samples were obtained from the supernatants of different cancer cells which were cultured in low-FBS containing media for 72 h. Secreted amount of VEGFA to the environment was tested using the VEGFA ELISA (R&D, DVE00) according to the instructions of the manufacturer.

2.9. Recombinant Protein Treatment

2.9.1. Recombinant Protein of TGFβ1 Treatment on Fibroblast Cells

Recombinant protein of TGFβ1 was purchased from Sigma (Catalog #: H8541) and re-suspended in carrier, which is 5 mM HCl solution. For autophagy activation, several concentrations of recombinant TGFβ1 (1×10^{-4} , 1×10^{-3} , 1×10^{-2} , 1×10^{-1} , 1 and 10 ng/mL)

and several time points (30 min; 1 h, 2 h, 6 h, 12 h and 24 h) were tested under the microscope with GFP-LC3 dot analysis and with protein analysis for autophagy marker.

2.9.2. Recombinant Protein of CT-1 Treatment on Fibroblast Cells

Recombinant protein of CT-1 was purchased from Sigma (Catalog #: SRP4011) and re-suspended in carrier, which is 20 mM Tris solution (pH 8.0). For autophagy activation, several concentrations of recombinant CT-1 (5 and 25 ng/mL) and several time points (1 min; 5 min; 15 min; 30 min; 1 h) were tested under the microscope with GFP-LC3 dot analysis and with protein analysis for autophagy marker and for further downstream pathways.

2.10. Immunoblot Analysis and Antibodies

Protein extraction was performed with RIPA buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate) supplemented with complete protease inhibitor cocktail (Roche, Catalog #: 04-693-131-001) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, P7626). For phosphorylated proteins, protein extraction was performed with RIPA buffer supplemented with complete protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF; Sigma, P7626) and 100 nM okadaic acid, 1 μ M cyclosporine A, 1 mM NaF, 50 mM β -glycerophosphate. Cell extracts (30 μ g for LC3 shift analysis; 100 μ g for the phosphorylation of STAT3 analysis) were separated by SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Following blockage in 5% nonfat milk (or 3% BSA for phosphorylated-protein analysis), membranes were incubated in 3% BSA-PBST solutions containing primary antibodies (ab): anti-LC3B ab (Novus, NB100-2331, 1:1000), anti-SQSTM1/p62 ab (BD Transduct. Lab, 610832, 1:4000), anti-human CT-1 (R&D, MAB2601, 1:1000), anti-LIFR (Santa Cruz, sc-659, 1:1000), anti-STAT3 ab (CST, Catalog #: 9139, 1:1000), anti-P-STAT3 (Tyr705) ab (CST, Catalog #: 9145, 1:1000), anti-gp130 (Santa Cruz, sc-655, 1:1000), anti-LIFR (Santa Cruz, sc-659, 1:1000), anti-GFP (Roche, 11814460001, 1:1000), anti-VEGF (A-20) (Santa Cruz, sc-152, 1:250), anti-actin alpha-smooth muscle (Sigma, A2547, 1:1000) and anti- β -actin ab (Sigma-Aldrich, A5441, 1:10.000). Then, the appropriate secondary mouse or rabbit antibodies coupled to horseradish peroxidase (anti-mouse: Jackson ImmunoResearch Laboratories, 115035003; anti-rabbit: Jackson

Immunoresearch laboratories, 111035144, 1:10.000) were applied and protein bands were revealed with chemiluminescence. The band signals were quantified using ImageJ.

2.11. Retrovirus and Lentivirus Production and Infection

2.11.1. Retrovirus Production and Infection/ Created Stable Cell Lines

Retroviruses were produced by calcium phosphate transfection of 10 µg LMP-dsRed or LMP-GFP plasmid (kindly gifted by Dr. Batu Erman) together with 2.5 µg helper plasmid pCL-Eco (kindly gifted by Batu Erman) into Phoenix cells (2×10^6 cells per 10 cm plate) (kindly gifted by Dr. Batu Erman). After 16 h, the medium of the transfected cells was replaced with 5 ml full DMEM medium. Cell supernatants were harvested after 24 h and 32 h after transfection and used to infect cells. Collected medium was filtered with 0.45 µm filter. For stable cell line generation, MEF WT and (*Atg5*^{-/-}) MEF cells were seeded 1×10^6 cells per 10 cm plate. After 16 h, half of the filtered virus medium with 8 µg/ml polybrene (Sigma, H9268) was added onto seeded fibroblast cells. After 3 h of incubation, the other half of the filtered virus containing medium with 8 µg/ml polybrene was added onto infected fibroblast cells. After 3 h, the medium of the fibroblast cells was changed to full DMEM medium. After 24 h, the infected cells were selected with 0.8 µg/ml concentration of puromycin antibiotic (Sigma, P9620) containing medium for 4 weeks.

2.11.2. Lentivirus Production and Infection/ Created Stable Cell Lines

For stable cell line generation, lentiviruses were produced by transfection of pRSI9-U6-UbiC-TagRFP-2A-Puro plasmid (Addgene plasmid #: 28289) together with helper plasmids psPAX2 and pMD2.G (Addgene plasmid #: 12260 and #: 12259) into HEK 293T cells. Cell supernatants were harvested at 48 h and 72 h after transfection and used to infect cells. For stable cell line generation, MCF7 cells were infected at a 60% confluence for 24 h with lentiviral supernatants diluted 1:1 with full DMEM culture medium supplemented with 5 µg/ml polybrene. 24 h after infection, fresh medium was added. MCF7 cells stably expressing RFP (MCF7-RFP) and MDA-MB-231 cells stably expressing RFP (MDA-RFP) were obtained after selection of cells for 4 weeks with 1 µg/ml and 2 µg/ml concentrations of puromycin, respectively.

Moreover, the other lentiviruses were produced by transfection of shTGFβ1 (Sigma, SHCLNG- NM_000660 TGFβ1 MISSION shRNA, TRCN0000003320), shVEGFA

(Sigma, SHCLNG-NM_003376, TRCN0000003343), and shGFP (kindly gifted by Dr. Tamer Önder) with helper plasmids pCMV-VSVG (kindly gifted by Dr. Tamer Önder) and pCMV-dR8.2 (kindly gifted by Dr. Tamer Önder) into HEK 293T cells. Culture media were harvested at 48 h and 72 h after transfection, and used to infect cells or stored at -80 °C. To create stable cell lines, MDA cells were infected at 60% confluence for 24 h with lentiviral supernatants diluted 1:1 with fresh culture medium in the presence of 5 µg/ml polybrene (Sigma, H9268) or 5 µg/ml protamine sulfate (Sigma, P4505). 16 h later, low-FBS containing medium was added. After 72 h of incubation, media of the infected cells were collected and concentrated using an Amicon[®] Ultra-4 centrifugal filter units. Conditioned media of infected cells were diluted in 1 ml full DMEM media and added on GFP-LC3 MEF which were cultured 16 h before the treatments. After 24 h, cells were fixed and autophagy activation was evaluated under upright fluorescence microscope (Olympus, BX60) with GFP-LC3 dot analysis.

2.12. RNA Isolation and RT-PCR

Total RNA was extracted using TRIzol reagent (Sigma, T9424) according to the manufacturer's instructions. cDNA was reverse transcribed from total RNA (DNase treated) using M-MuLV reverse transcriptase (Fermentas, EP0351) and random hexamers (Invitrogen, 48190-011). PCR reaction was performed as follows: 94°C for 5 min, then 35 cycles of 94°C for 1 min, 50°C for 1 min. and 72°C for 1 min., followed by a single cycle of 72°C for 10 min.

Real-time RT-PCR for TGFβ1 mRNA quantification in shGFP/ shTGFβ1 infected MDA-RFP breast cancer cells, and for CT-1 mRNA quantification in different cancer cell lines were performed. Single step qRT-PCR reactions were performed using SYBR Green Quantitative RT-PCR kit (Roche, 04-913- 914-001) and a LightCycler 480 thermal cycler (Roche). Following an initial single cycle at 95°C for 10 min to activate the SYBR green, PCR was performed as follows: 40 cycles of 95°C for 15 sec and 60°C for 1 min and followed by the thermal denaturation protocol to generate the dissociation curves to verify amplification specificity (a single cycle of 95°C for 60 sec, 55°C for 60 sec, and 80 cycles of 55°C for 10 sec). Changes in mRNA levels were quantified using the 2-ΔΔCT method using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA as control. Primers used during the study were: TGFβ1 primers 5'-ACTGCAAGTGGACATCAACG- 3'; 5'-TGCGGAAGTCAATGTACAGC- 3', CT-1 primers 5'-

AGACGAGGTTTCGCCATGTTG 3'; 5'- CTCAGACGGAGTTACCATGCAGAC- 3', and GAPDH primers 5'-AGCCACATCGCTCAGACAC- 3'; 5'-GCCCAATACGACCAAATCC- 3'. Reactions were performed in duplicates and at least three independent experiments (n) were performed.

2.13. Microfluidic Chip (Biochip) Analysis

2.13.1. Biochip Production

2.13.1.1. Design and fabrication of the PDMS porous membrane

The PDMS porous membrane was fabricated by Junyoung Kim, Prof. Yoon-Kyoung Cho's Ph.D. student. According to Junyoung Kim's protocol, PDMS (Sylgard 184, Dow Corning, Midland, MI, USA) was injected into the gap between a surface-treated PDMS replica and glass Figure 32. The PDMS replica was fabricated using photolithography and soft lithography. A negative photoresist (SU-8-2025, Microchem, Newton, MA) was coated on a 6" silicon wafer at 1750 rpm for 30 s (JSPD, JD Tech) (Figure 32A), and a photoresist layer was patterned by photolithography (6 min soft baking at 95 °C, 170 mJ/cm² exposure energies, 6 min post exposure baking at 95 °C, and IPA & DI water rinse, MA-6, SussMicroTech) (Figure 32B). For facile release of the PDMS replica, the photoresist master on the silicon wafer was silanized by treatment with trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane (Sigma) in a vacuum desiccator (Bel-Art Scienceware) for 24 h. PDMS (base to curing agent ratio of 5:1) was cast onto the photoresist master and cured for 2 h at 65 °C (Figure 32C). The released PDMS replica (Figure 32D) and glass slide were treated by reactive ion etching (Labstar, TTL) with 50 sccm CHF₃, 150 W for 100 s and 50 sccm CHF₃, 15 W for 10 s, respectively (Figure 32E). The treated surfaces faced each other and were clamped together by magnets. Then, PDMS (5:1) was filled via capillary action (Figure 32F). After curing for 2 h at 65 °C, the PDMS membrane was released easily with 70% ethanol (Figure 32G). The membrane was 50 µm thick and consists of 800 holes with 30 µm diameter, separated by the center to center distance of 310 µm. The dimensions of the pores were verified by SEM measurement (SNE 4500M, SEC corp).

2.13.1.2. Design and fabrication of single cell screening platform.

According to Junyoung Kim's protocol, the membrane was first assembled with a 4 cm-thick PDMS reservoir with an opening window of 11 mm diameter, using oxygen plasma with 60 W plasma power and 50 sccm O₂ for 1.5 min (CUTE, Femto science) (Figure 32I). PDMS spin coating was employed to prepare the bottom substrate that faces the bottom side of the membrane where the fibroblasts are attached and cultured; the PDMS spin coating was conducted on cover glass at 2500 rpm for 5 min. The thickness of the coated PDMS layer was measured using a surface profiler (P6, KLA Tencor); the thickness was controlled to 8 μ m to prevent the cancer cells from escaping a hole and migrating to other holes. After polymerization at 65 °C for 2 h, the center part (11 $\times$ 11 mm) was cut and removed to make space for fibroblast culture media.

2.13.2. Biochip Optimizations

Biochip optimizations were performed using surface treatment, seeding fibroblast cells as monoculture, and trapping MDA-RFP cancer cells as single cells into the holes of the porous membrane.

2.13.2.1. Surface treatment of PDMS membrane with fibronectin and PEO-PPO-PEO solution

The device was thoroughly sterilized with 70% ethanol and UV for 1 h. After sterilization of the device, the bottom part of the membrane was coated with 10 μ g/ml fibronectin (Sigma, F1141) for 30 min at room temperature and washed with PBS. A 0.4% w/v PEO-PPO-PEO copolymer (Pluronic F-108, Sigma) was coated on the top part of the membrane for 30 min, after which the membrane was washed with PBS. In order to check the fibronectin coating status, 10 μ g/ml HiLyte-488 tagged fibronectin solution was coated on the bottom side of the PDMS membrane for 30 min, after which the membrane was washed with PBS. A fluorescence image test was conducted using a confocal microscope (Zeiss) to observe the fibronectin coating status. To check the effect of the PEO-PPO-PEO coating, the percentage of tumor cells remaining on the PDMS membrane was measured after five times of washing with media. Fluorescence images of RFP tumor cells were obtained using an IX-71 inverted microscope (Figure 33) (Olympus).

2.13.2.2. Monolayer of fibroblasts on the microfluidic device

GFP-LC3 MEFs were re-suspended at 6.6×10^4 cells in 200 μ l of the medium. Cells were seeded to the bottom side of the membrane and allowed to attach for 2 h in the incubator. Medium was removed and the surface was washed with DMEM. Then, the reservoir with fibroblasts was flipped to the original position and assembled with the substrate, which contained 200 μ l of a DMEM medium. The medium filling the reservoir could perfuse cells through membrane holes. After 14 h, fibroblasts covered the bottom part of the membrane. Seeded single tumor cells did not actively migrate through the holes during the experiments.

2.13.2.3. Single cell trapping experiments with MDA cells

When GFP-LC3 MEFs reached to the confluence, suspended tumor cells were loaded to the PDMS reservoir, and single cells were trapped in the holes via horizontal agitation (Neo Shaker, AS ONE). Then, the platform was kept in the incubator for 25 min allow in the trapped cells to settle. Non-trapped tumor cells were removed by washing five times with full DMEM medium. The trapping ratio was calculated as the number of holes with tumor cells divided by the total number of holes in the membrane. To determine the optimum conditions for single cell trapping, number of tumor cells input (5×10^3 - 2×10^4 cells) and the agitation velocity (0–200 rpm) were tested (Figure 34).

2.13.3. Single Cell Trapping- Retrieving Back and Further Genomic DNA Analysis

2.13.3.1. Live cell analyses

To study the kinetics of autophagy activation a confocal microscope (A1R, Nikon) equipped with a motorized X-Y stage and a live cell chamber maintaining CO₂ at 5% and the temperature at 37 °C was used. Images were taken using a Plan Apo λ 40x objective (NA 0.95). GFP-LC3 MEFs and MDA cells were captured at wavelengths 488.0 and 561.5 nm, respectively. Images (1,024 X 1,024 pixels) were automatically taken at 20 positions every 15 min during 6 h of interaction.

2.13.3.2. Retrieval of single tumor cells and single cell genomic DNA PCRs

Individual tumor cells were retrieved using a Kuiqpick micromanipulator (NeuroInDx). To withdraw a single tumor cell, a capillary with a diameter of 20 μ m was

positioned over membrane holes. Using optimized vacuum duration and power, the trapped single tumor cell was removed in a small volume of medium. Isolated cells were released into individual tubes and stored in lysis buffer for single cell gene analyses. After retrieval, genomic DNA was isolated using the REPLI-g Single Cell Kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. For PCR, following primers were used: Forward (5'-GACTATCATATGCTTACCGT-3') and reverse (5'-GTGGATGAATACTGCCATT-3') primers for the shTGFβ1 vector, and forward (5'-ATGGGTGTGAACCATGAGAA-3') and reverse primers (5'-GTGCTAAGCAGTTGGTGGTG-3') for GAPDH with reaction parameters 95 °C for 10 min (1 cycle); 95 °C for 45 s, 57 °C for 30 s, 72 °C for 90 s (for 35 cycles); and 72 °C for 3 min (1 cycle). PCR products were analyzed using a Labchip GX touch bioanalyzer (PerkinElmer).

2.14. Growth of Cancer Cells in Neutralization Antibody

Cancer cells (MCF7-RFP, MDA-RFP, PC3-GFP, U2OS-GFP) cancer cells were split in 12-well plate as 4×10^4 cells per well in 3% FBS containing medium. For each time point (24 h, 48 h, 72 h and 96 h), there are three different conditions: (i) TGFβ1 neutralization antibody (30 µg/ml)/ control serum (rabbit serum, 30 µg/ml), (ii) CT-1 neutralization antibody-added (6 µg/ml)/ control antibody (Flag ab)-added (6 µg/ml), or (iii) VEGFA neutralization antibody (0.08 µg/ml)/ control antibody (Flag ab)-added (0.08 µg/ml), no treatment. At indicated time points, cells were trypsinized and counted with Tryphan Blue (Sigma, T8154).

2.15. Quantification of Cancer Cell Growth in Co-Culture System

In order to analyze the importance of CT-1/VEGFA for cancer cell growth during cancer-fibroblast communication, CT-1/VEGFA neutralization antibody was used in co-culture experiments. MEF WT cells were seeded to have 1×10^5 cells/well (12 well plates were used). Indicated cancer cells were seeded after 16 h of fibroblasts seeding as 1:10 ratio of cancer:fibroblasts in low-FBS containing medium in the presence of CT-1 neutralization antibody (6 µg/ml) /VEGFA neutralization antibody (0.08 µg/ml) or in the presence of their control antibody (Flag antibody, 6 µg/ml or 0.08 µg/ml). In order to compare the importance of co-culture experiment, cancer cells alone were seeded as

1×10^4 cells/well. After 72 h or 96 h, experiments were terminated. For microscope and FACS experiments, stably fluorescence protein (RFP or GFP) expressing cancer cells were used. For Luciferase experiments, stably luciferase expressing cancer cells were used.

In order to analyze the importance of autophagy in fibroblast cells on the cancer cell growth during cancer-fibroblasts communication, MEF WT and (*Atg5*^{-/-}) MEF fibroblast cells were used in co-culture experiments. MEF WT or (*Atg5*^{-/-}) MEF fibroblast cells were seeded to have 1×10^5 cells/well. Indicated cancer cells were seeded after 16 h of fibroblasts seeding as 1:10 ratio of cancer: fibroblasts in low-FBS containing medium. In order to compare the importance of co-culture experiment, cancer cells alone were seeded as 1×10^4 cells/well. After 24 h, 48 h or 72 h, experiments were terminated. For microscope and FACS experiments, stably fluorescence protein (RFP or GFP) expressing cancer cells were used. For Luciferase experiments, stably luciferase expressing cancer cells were used.

2.15.1. Luciferase Assay for Quantification of Cancer Cell Growth in Co-Culture System

Fibroblast cells are seeded as 1×10^5 cells per plate in a 12-well plate. After 16 h, cancer cells were seeded as 1:10 cancer: fibroblasts ratio. After 72 h, luciferase assay was performed as following. The cells are washed with 1x PBS. 150 μ l Chris Buffer (50 mM Tris pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 10% Glycerol, 0.5% Nonidet P-40, $1 \times$ PI) is added and incubated for 5 min in order to lyze the cells. Then, cells were collected and centrifuged at $1000 \times g$ for 5 min. Luciferase assay was performed with 50 μ l of supernatant as duplicate wells. Luciferase reagent (1.07 mM (MgCO_3)₄XMg(OH)₂X5H₂O, 20 mM pH 7.8 Tricine, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 μ M coenzyme A, 530 μ M ATP and 470 μ M Luciferin) was added as three times of the supernatant (for 50 μ l of supernatant, 150 μ l of luciferase reagent was added). Mix was added in black 96 well plates, RE black bottom (Thermo Scientific, NC-95029840) in duplicates and the signal was measured with a luminometer (Thermo Scientific- Fluroskan Ascent FL).

2.15.2. Microscope Analysis for Quantification of Cancer Cell Growth in Co-Culture System

Co-cultured cells were fixed in 4% formaldehyde for 20 min, washed with PBS, mounted and inspected under $40 \times$ magnification using a BX60 fluorescence microscope

(Olympus, BX60). Random 20 different fields were counted and the graphs were plotted according to average cell number.

2.15.3. FACS Analysis for Quantification of Cancer Cell Growth in Co-Culture System

Cells were collected and centrifuged at 300×g for 5 min. Cells were washed 2 times with PBS and cell pellets were resuspended in cold PBS. Analysis was performed in BD FACS Canto.

2.16. Analysis of Metabolites in Fibroblast Cells upon CT-1 Treatment

2.16.1. Radioactivity Experiments

For radioactivity experiments, Valine-free medium was used. Radioactive valine was added as 0.2 µCi/ml (L-valine). Cells were split as 1×10^5 cells per well in 12 well plate in Valine free medium by adding 20 µl radioactive L-valine for 5 ml medium (1 ml for each well). After 16 h, old medium was removed, washed once with PBS and fresh medium with radioactive valine was added. The added medium had different conditions: (1) radioactive valine added medium; (2) radioactive valine added medium+ carrier (2.5 µl for 1 ml); (3) radioactive valine added medium+ 25 ng/ml CT-1 recombinant protein (2.5 µl from 10 ng/µl stock in 1 ml); (4) radioactive valine was added after 20 h and changed to STV (by treating the cells with EBSS)

After 24 h of the treatment, radioactive medium was changed to cold medium for 4 h. After 4 h, the media were collected into Eppendorf tubes (previously labeled). Caps were left open until finishing the experiment to prevent that closing and opening the cap distributes the radioactive material. The media were centrifuged at 2000 rpm for 1 min. In order to have equal medium, 250 µl were added from each condition. Medium was added onto UltraGold (1:10 diluted, 2- 2.5 ml, which was taken with single-use Pasteur pipette). In the machine (TRI-CARD 2900TR Liquid Scintillation Analyzer), **Protocol 12** was used. While transporting the samples, the rack of the device was used and the trashed of the samples including used gloves and other materials were collected in a separate container.

2.16.2. Ketone Body Analysis Assay

MEF WT or (*Atg5*^{-/-}) MEF fibroblast cells were split as 1×10^5 cells per well in 12 well plate. After 16 h, the media of the cells were changed to low-FBS containing media and these cells were treated with recombinant CT-1 protein (25 ng/ml) or carrier for 24 h. After 24 h, supernatants of the cells were collected and their β -hydroxybutyrate concentrations were measured according to manufacturer instructions (Biovision, K632-100).

2.17. LC-MS/MS Experiments for Target Analysis

2.17.1. Labeling Protein Lysates with SILAC (Stable Isotope Labeling in Cell Culture) Technique

LS-MS/MS experiments were performed by Seil Erbil, Ph.D. student of Dr. Devrim Gzaık. According to her protocol, both MEF WT and HEK 293T cells were cultured for 10 days in order to perform heavy labeling for CT-1 conditioned medium treatment, medium labeling for CNT conditioned medium treatment. Only MEF WT cells were cultured in light labeling for starvation treatment. SILAC medium contains DMEM (Thermo Fisher Scientific, 89985) including penicillin/streptomycin (100 unit/mL; PAN Biotech, 06-07100), L-glutamine (100 μ g/mL; PAN Biotech, P04-80100) and 10% FBS (Gibco, 26400-044). Depending on labeling types, different isotopes of amino acids were added. For heavy labeling, 42 μ g/mL L-arginine-13C6 15N4 hydrochloride (Arg10) (Sigma-Isotec, St. Louis, MO; 608033) and 40 μ g/mL L-lysine -13C6 15N2-hydrochloride (Lys8) (Sigma-Isotec, St. Louis, MO; 608041); for medium labeling, 42 μ g/mL L-arginine-13C6- hydrochloride (Arg6) (Cambridge Isotope laboratories Inc, Andover MA., CLM-2265) and 40 μ g/m L -lysine-4,4,5,5-*d*4 hydrochloride (Lys4) (Sigma-Isotec; 616192) and for light labeling, arginine (Arg0, Sigma, 11039) and lysine (Lys0, Sigma, L8662) were added into SILAC medium. After labeling, MEF WT cells were treated with conditioned medium of labeled HEK 293T cells in indicated mediums. After 24 h of conditioned medium treatment, from collected cells, protein lysates were obtained using RIPA solution.

2.17.2. Digest of Proteins with Trypsin Enzyme in the Gel

Obtained protein lysates were reduced with 10 mM DTT, alkalized with 55 mM iodoacetamide. Samples were loaded to NuPAGER gel to run gel electrophoresis and

stained with colloidal blue. In the gel, the regions, that contained proteins, are digested with Trypsin enzyme (Trypsin-sequencing grade, PROMEGA, V511C).

2.17.3. LC-MS/MS

After Trypsin treatment, peptide fragments were loaded into C18-filled columns and purified in order to sequence with HPLC-related mass spectrometry. Electrospray experiments were performed with Agilent 1200 nanoflow LC system (Agilent Technologies) connected nanoelectrospray ion source and LTQ Orbitrap XL mass spectrometry (Thermo Fisher Scientific). Chromatographic separation of peptides was performed in emitormicrocolon which is filled with reverse-phase mode ReproSil-Pur C18-AQ beads (Dr. Maisch) and packed with 15 cm silica (SilicaTipPicoTip; New Objective).

2.17.4. Proteomic Data Analysis

Raw chromatogram data, obtained from mass spectrometry, were analyzed with MaxQuant v.1.2.0.1.8 and Andromeda database. At the end of the analysis, Benjamin Hochberg statistic test was applied. According to the result of the analysis, proteins, which indicated differences depending on CT-1 conditioned medium treated samples versus negative control, or CT-1 conditioned medium samples versus starvation treated samples, were determined.

2.18. Immunohistochemistry Analysis

Different cancer cells were cultured in low-FBS containing medium for 72 h. Cells were fixed with 4% paraformaldehyde and permeabilized in PBS with 0.1% BSA (Sigma, A4503) and 0.1% saponin (Sigma, 84510). Immunostaining was performed using anti-CT-1 antibody (R&D, MAB2601) followed by incubation with anti-mouse IgG Alexa Flour 568 (Invitrogen, A11004). Cover slides were mounted and inspected under 40× magnification using a Carl Zeiss LSM 710 confocal microscope (Zeiss, Germany). Human patient samples were stained by Yunus Akkoç Ph.D. student of Dr. Devrim Gözüaçık. According to his protocol, frozen tissues embedded in OCT (Tissue-Tek, VWR) and cut as 4-micron thickness sections by cryotome (Thermo Scientific Shandon Cryotome FSE). Frozen tissues were cut sections of the desired thickness, placed on poly-L-Lysine coated Fisher Superfrost slide and kept under -20 °C. Tissue sections then were

marked with hydrophobic pen and dehydrated by 1X PBS. Tissue sections were fixed with 4% paraformaldehyde and permeabilized in PBS with 0.1% BSA (Sigma, A4503) and 0.1% saponin (Sigma, 84510). Immunostaining was performed using anti-CT-1 antibody (R&D, MAB2601), anti- α -sma antibody (Sigma, A2547) followed by incubation with anti-mouse IgG Alexa Flour 488 (Invitrogen, 982425), anti-mouse IgG Alexa Flour 568 (Invitrogen, A11004), respectively. Cover slides were mounted and inspected under 20 $\times$ magnification using a Carl Zeiss LSM 710 confocal microscope.

2.19. Phalloidin Staining (for α -SMA Experiments)

MEF WT or (*Atg5*^{-/-}) MEF fibroblast cells were split as 4 $\times$ 10⁵ cells per well in 6 well plate. Cells were treated with CT-1 (5 ng/ml), TGF β 1 (0.01 ng/ml) or CT-1/TGF β 1 for 24 h, 48 h or 72 h. Cells were also treated with CT-1 (5 ng/ml), TGF β 1 (1 ng/ml) or CT-1/TGF β 1 for 3 days, 6 days or 9 days. At indicated time points, cover slides were fixed with 4% PFA for further Phalloidin staining (Invitrogen, A22283) and cell lysates were analyzed in protein level for α -sma, B-ACT and LC3 shift. Phalloidin staining was performed after fixation of the samples with 4% PFA. Then coverslips are placed in -20 °C with acetone for 5 min. Each sample was stained with 0.2-unit phalloidin for 20 min. After washing with PBS, cover slides were mounted and inspected under 20 $\times$ and 60 $\times$ magnification using a BX60 fluorescence microscope (Olympus, BX60, Japan).

2.20. *In vivo* Analysis and Patient Samples

2.20.1. Ethical Statements

Samples were collected under protocols approved by Samatya and Istanbul University Hospital (File number: 1223, Approved date: 12.08.2014), and written informed consent was obtained from all participants. For animal experiments, ethical permission was taken from Bogazici University Laboratory Animals Local Ethical Board (Approved date: 13.05.2015).

2.20.2. Chicken Chorioallantoic Membrane (CAM) Assay

The CAM assay was used to determine the effect of growth of cancer cell with fibroblasts in the presence of CT-1 neutralization antibody *in vivo*. Fertilized chicken eggs were incubated at 37.8 C in a humidified incubator and were prepared for cap opening on 4th day of incubation. Eggs were washed with 70% ethanol, and taped with transparent

tape. With the help of curled scissors, a round window (2 cm in diameter) was opened and covered with a tape. Silicone rings were placed on each membrane. On 6th day of incubation of cancer cells, fibroblast cells or cancer cells: fibroblast cells (1:10 ratio) were performed in 25 µl. The shell windows were covered again, and the eggs were incubated until 20th day.

2.20.3. Mouse Experiments (NOD.SCID Mouse Experiments)

1×10⁷ MCF7 cells were suspended in 100 µl growth factor reduced Matrigel (1:2 diluted growth factor reduced Matrigel, BD, Catalog #: 354230) and injected into the forth mammary fat pad of 6-8-week-old female NOD.SCID mice (Charles River, NOD.CB17-Prkdc^{scid}/NcrCrl, homozygote SCID mutation on the non-obese diabetic background mouse, Chain number: 394). On the day of injection, the mice received one estrogen pellet (17β-estradiol 0.36 mg/pellet, 60-day release; Innovative Research of America, Catalog #: SE-121). Tumor growth was followed by measuring the tumoral area twice a week with a caliper. The volume was calculated by the formula [(length×width²)/2]. 18 days after cancer cells injection, CT-1 neutralization antibody (CT-1 neutralization ab, 50 µg per animal) or flag antibody (CNT, 50 µg per animal) treatments were performed for each 3-4 day around of the tumor for 30 days. In second experimental setup, 3×10⁶ MCF7 were suspended in 100 µl growth factor reduced Matrigel (1:2 diluted growth factor reduced Matrigel, BD, Catalog #: 354230) and injected into the forth mammary fat pad of 6-8-week-old female NOD.SCID mice. 30 days after cancer cells injection, CT-1 neutralization antibody (CT-1 neutralization ab, 50 µg per animal) or flag antibody (CNT, 50 µg per animal) treatments were performed for each 3-4 day around of the tumor for 30 days. *In vivo* studies were terminated with CO₂ inhalation of the mice and followed with cervical dislocation. Tumor real dimensions and weight were taken, and graphs were plotted accordingly. Individual tumors and internal organs were fixed in 10% formalin and snap frozen in liquid nitrogen and then planned to be used for histological staining with hematoxylin and eosin.

2.20.4. Analysis of Tumor Samples from Breast Cancer Patient

Frozen tissue (present in liquid nitrogen) was broken into pieces, weighted, and washed in lysis buffer. After spinning at 13,000 rpm for 1 min at 4 °C (v:v), 2.5 µl/mg RIPA was added. Using a disposable tissue grinder, pieces were ground in a time with

approx. 40 strokes. Homogenization was performed on ice by spinning at 13,000 rpm for 15 min at 4 °C.

2.20.5. Primary Cell Isolation and Culture

Briefly, breast tissues diced in DMEM w/o FBS. Then for 250 µg tissue, 500 µl Liberase enzyme (Liberase TL Research Grade, Roche , 5401020001, 2.5 mg/ml stock) was added and tissues were incubated at 37°C degree for 90 min under rotation. Following three washes in DMEM w/o FBS, cells were cultured in full DMEM.

2.21. Statistical Analysis

Statistical analyses were performed using Student's two-tailed t-test. Data were presented as means of $\pm$ SD of ≥ 3 independent experiments. Values of $p < 0.05$ were considered as significant.

3. RESULTS

3.1. Aim and Summary of the Thesis

Autophagy is an evolutionary conserved stress response mechanism. Cell-cell communication is important for creating organized tissue formation and understanding disorganized cellular growth. For organized tissue formation, we aimed to understand the role of autophagy during creating macrovascular structures. For disorganized cellular growth, such as cancer, we aimed to understand the role of autophagy induction in the tumor microenvironment (stroma). In the literature, it has been shown that autophagy induction in fibroblast cells has a supportive role during tumor growth and proliferation. However, soluble factors secreted from cancer cells to initiate their communication with surrounding cells, and further, autophagy induction in their microenvironment is not clearly understood yet. Cytokines and growth factors are the abundant factors in such a microenvironment which mediate cell-cell communication in a paracrine manner. It is important to characterize their initiating role during cancer cell-stroma communication through autophagy activation in fibroblast cells. Therefore, the main motivations of the thesis are: (i) to analyze the role of autophagy in the context of bioprinted 3D cell systems for creating organized tissue like structures; (ii) to discover the autophagy initiating paracrine factors secreted from cancer cells to fibroblasts; (iii) a microfluidic chip for screening individual cancer cells via eavesdropping on autophagy-inducing crosstalk in the stroma niche: TGF β 1 as a model paracrine factor; (iv) to discover and analyze Cardiotrophin-1 (CT-1) as a mediator of tumor-fibroblast interaction; (v) to discover and analyze Vascular Endothelial Growth Factor A (VEGFA) as a mediator of tumor-fibroblast interaction. Therefore, the importance of autophagy during bioprinted 3D cell systems is analyzed with various aspects. Moreover, the discovery of novel roles for the soluble factors, namely cytokines and growth factors that controls autophagy in fibroblast cells through cancer cells-fibroblast cells communication is investigated by creating simplified tumor microenvironments.

3.2. Objectives of the Thesis

Fibroblast cells are the major components of the human body and tumor microenvironment. In order to create organized macrostructures, it is important whether the 3D bioprinting process creates a stress on the cells and whether presence of autophagy mediates faster cell-cell communication and thus creates more stable macrostructures. From tumor microenvironment point of view, the reason for the autophagy induction in fibroblast cells, which supports the growth and proliferation of cancer cells, is still unclear in cancer research. If the communication signals which initiate the communication between cancer cells and their microenvironment can be identified, those factors offer critical targets for cancer therapy. In this thesis, (i) the role of autophagy in the context of bioprinted 3D cell systems, and (ii) the role of critical soluble factors secreted from cancer cells to fibroblast cells are analyzed through the monitoring of autophagy in fibroblast cells.

3.2.1. Objective I – Analysis of the Role of Autophagy in the Context of Bioprinted 3D Cell Systems

Direct cell-cell communication is an important process for the decision of the cells during formation of organized structures. The role of autophagy in direct cell-cell communication was analyzed in printed 3D cell structures, and the advantage of autophagic response was investigated in 3D bioprinting process.

3.2.2. Objective II – Discovery of Novel Paracrine Factors Regulating Autophagy in Fibroblast Cells

Secreted factors from cancer cells to environment cause induction of autophagy in microenvironment. To cover a wide range of these soluble factors, 73 cytokines and 57 growth factors were purchased from the Thermo Scientific company. Their conditioned mediums were tested on autophagy activation in fibroblast cells. GFP-LC3 MEF cells modeled stroma fibroblasts in cancer and were used to quantify the autophagy response of the fibroblast cells. The cytokines and growth factors were prepared as pools, each of which contained 5-6 factors. After initial screening, the conditioned mediums of pools which induced autophagy in fibroblast cells equal or higher than two-fold were chosen to be analyzed as single factors. After the analysis of individual factors, the conditioned mediums of the factors which induced autophagy equal or higher than two-fold were chosen to be analyzed in three independent experiments. According to the results of the

screening, TGF β 1 (transforming growth factor β 1), VEGFA (vascular endothelial growth factor A), and CT-1 (cardiotrophin-1) were chosen for further analysis.

3.2.3. Objective III - A Microfluidic Chip for Screening Individual Cancer Cells via Eavesdropping on Autophagy-Inducing Crosstalk in the Stroma Niche: TGF β 1 as a Model Paracrine Factor

The role of TGF β 1 during tumor-fibroblast communication was studied in a microfluidic chip system which mimics a simplified tumor microenvironment. This biochip system allows single cancer cell-fibroblast cells communication through the monitoring of autophagy in fibroblast cells. Moreover, this biochip system has an open reservoir so that interested single cancer cells can be retrieved back from the biochip and their genomic DNAs can be further analyzed.

3.2.4. Objective IV – Discovery and Analysis of Cardiotrophin-1 (CT-1) as a Mediator of Tumor-Fibroblasts Interaction

CT-1 was analyzed regarding its effect on autophagy in fibroblast cells *in vitro*, its expression and secretion from different cancer cells, its expression in different stages of breast cancer (more specifically between T1 stages and T2 stages) and its role on the growth of cancer cells *in vitro and in vivo*. In addition to these analyses, its effect on the metabolism of fibroblast cells was analyzed.

3.2.5. Objective V - Discovery and Analysis of Vascular Endothelial Growth Factor A (VEGFA) as a Mediator of Tumor-Fibroblast Interaction

VEGFA was analyzed for its expression and secretion from different cancer cells. In order to further validate our screening results, this work sought to understand its effect on autophagy in fibroblast cells *in vitro* using different types of cancer cells including MDA, triple negative breast cancer cells.

3.3. RESULTS I - Analysis of the Role of Autophagy in the Context of Bioprinted 3D Cell Systems

In this part of the thesis, the role of autophagy during cell-cell communication *in vitro* and during 3D bioprinting will be analyzed. Cell-cell communication is important for organized structures in multicellular organisms. Moreover, dysregulation of the communication leads to several problems including diseases like cancer. In this chapter of the thesis, the results from creating organized structures during 3D bioprinting process were explained. 3D bioprinting was performed using two different cell types namely, MEF WT (autophagy-competent) and (*Atg5^{-/-}*) MEF (autophagy-deficient) cells. In order to understand the role of autophagy while creating organized structures, proliferation, attachment kinetics in short- and long- term, wound healing percentages *in vitro*, and bioprinting results of these cells were analyzed.

3.3.1. Proliferation Assay for MEF WT and (*Atg5^{-/-}*) MEF cells

To characterize MEF WT and (*Atg5^{-/-}*) MEF cells, proliferation assay was performed for 12 h, 24 h, 36 h and 48 h. According to the result of the assay, no significant difference was observed between autophagy-competent and autophagy-deficient fibroblast cells in terms of their growth and proliferation (Figure 14). Since the primary aim in 3D bioprinting is tissue formation, following up for longer growth and proliferation profiles of these cells may provide conclusive information.

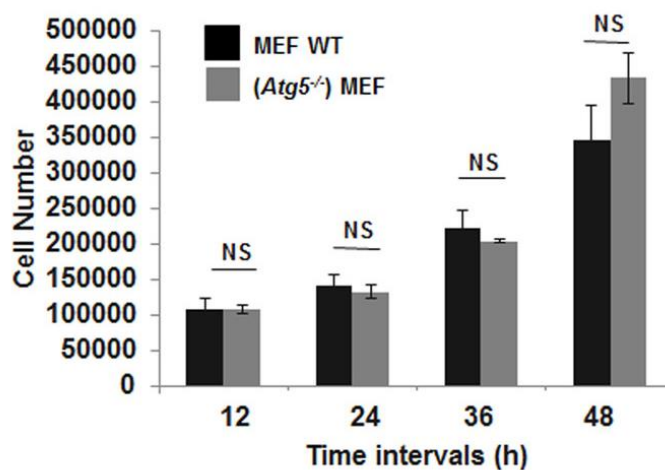


Figure 14. The proliferation assay of MEF WT and (*Atg5^{-/-}*) MEF cells for different time intervals.

The experiments were repeated three times and NS: Not significant.

3.3.2. Attachment Kinetics Assay for MEF WT and (*Atg5*^{-/-}) MEF Cells in Short-and Long-Time Points

In order to test attachment of the cells *in vitro*, attachment kinetics were performed for MEF WT and (*Atg5*^{-/-}) MEF cells for long-time points, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h. According to the result of the long-term attachment kinetics assay, there was significant difference between MEF WT and (*Atg5*^{-/-}) MEF at 1 h and 8 h (Figure 15A). Their detachment kinetics were also tested; however, no significant difference was observed (Figure 15B). In order to analyze the difference of their attachment profile at 1 h, shorter-time points (30 min, 1 h, and 2 h) were tested. However, no significant attachment difference was observed (Figure 15C). Autophagy markers were tested in immunoblot. Similar to microscope analysis, no significant changes in autophagy markers were observed (Figure 15D).

Next, attachment kinetics of MEF WT and (*Atg5*^{-/-}) MEF cells were tested for long-time points, 1 h, 2 h, 4 h, and 8 h in normal condition and under starvation. According to the results, no significant difference in their attachment was observed between these two cells (Figure 16).

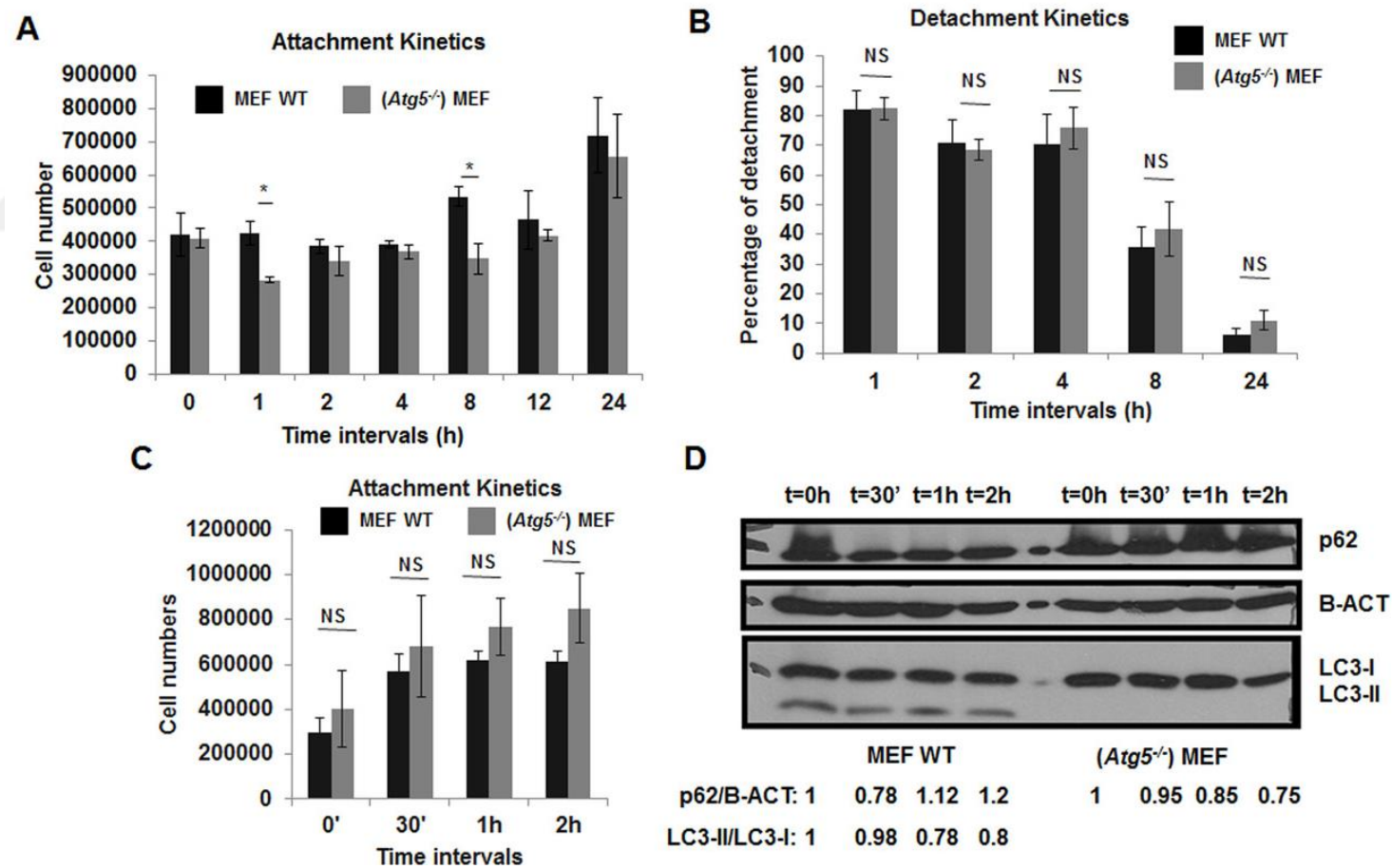


Figure 15. Attachment kinetics of MEF WT and (Atg5^{-/-}) MEF cells.

(A) Attachment kinetics of MEF WT and (Atg5^{-/-}) MEF cells for 24 h. The experiments were repeated three times and * $p < 0.05$. **(B)** Detachment kinetics of MEF WT and (Atg5^{-/-}) MEF cells in long-time intervals. The experiments were repeated three times and NS: Not significant. **(C)** Attached fibroblast cells were analyzed in shorter-time intervals, in 30 min, 1 h and 2 h. The experiments were repeated three times and NS: Not significant. There are no significant differences between their attachment. **(D)** Immunoblot analysis for autophagy markers.

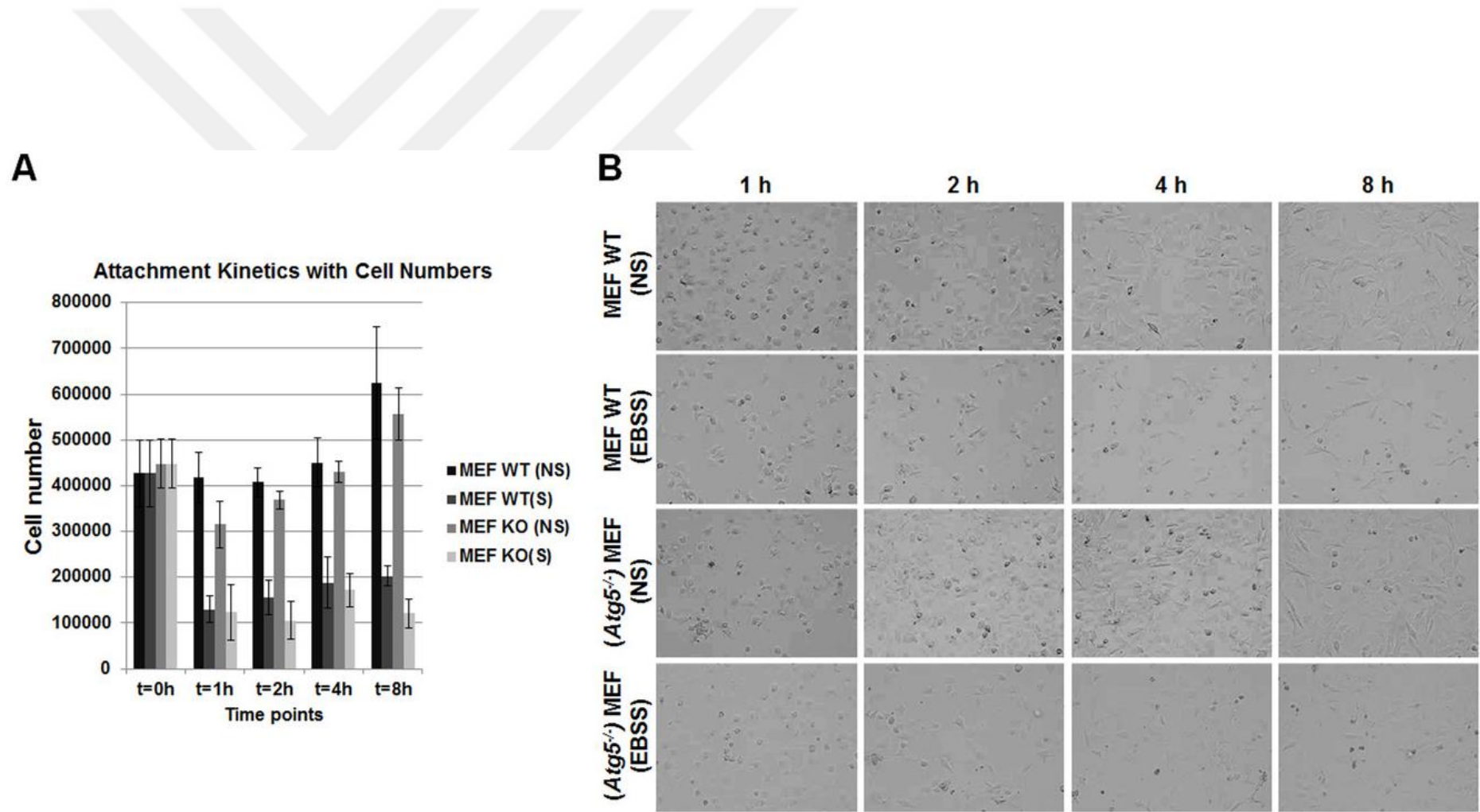


Figure 16. Attachment kinetics of MEF WT and (Atg5^{-/-}) MEF cells in long-time intervals in the presence of starvation (autophagy induction).

(A) Counting of attached cells percentage in 1 h, 2 h, 4 h and 8 h. **NS** represents non-starved condition and **S** represents starvation treatment. The experiments were repeated three times. There are no significant differences between their attachment. **(B)** Microscope analysis of the attachment of the cells.

3.3.3. Wound healing assay for MEF WT and (*Atg5*^{-/-}) MEF cells under different stress conditions

To understand whether autophagy has any role for the migration of MEF WT and (*Atg5*^{-/-}) MEF cells, wound healing assay was performed at different time points (4 h, 8 h, 12 h, and 24 h). According to the experiment, there was no significant differences between MEF WT and (*Atg5*^{-/-}) MEF cells at indicated time points (Figure 17A). Next, in order to test the importance of autophagy, the wound healing assay was tested under normal condition and under autophagy inducing conditions, namely (i) in normal medium, (ii) in no FBS containing medium and (iii) in starvation medium (EBSS) for 24 h. Although autophagy induction caused slower movements of both type of cells, there was no significant difference between autophagy-competent and autophagy-deficient fibroblast cells for each condition (Figure 17B). Since tissue formation was planned after 3D bioprinting, experiments with longer time points can be investigated for this experiment.

3.3.4. Decellularization of lamb aorta

As an alternative approach in scaffold-free tissue engineering, biological scaffolds can be created from decellularization of the body parts [229]. In order to apply this approach, a freshly taken lamb aorta was decellularized with different protocols (Figure 18). According to these protocols, to remove the cells from scaffold, sodium dodecyl sulfate, SDS, (anionic detergent, used to disrupt membranes totally), trypsin (serine

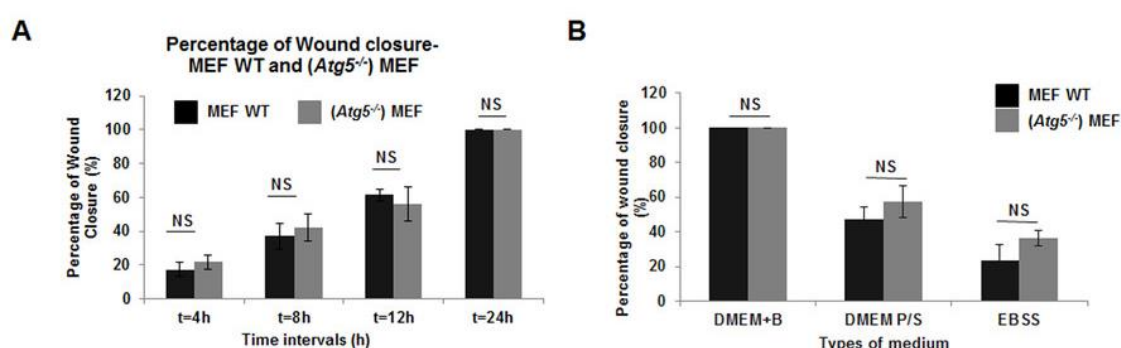


Figure 17. Wound closure of MEF WT and (*Atg5*^{-/-}) MEF cells in different conditions.

(A) Wound closure of MEF WT and (*Atg5*^{-/-}) MEF cells in different time intervals were tested (4 h, 8 h, 12 h and 24 h). The experiments were repeated three times and NS: Not-significant. (B) Wound closure of MEF WT and (*Atg5*^{-/-}) MEF cells in different conditions (in normal condition, in the absence of FBS in the medium, and under starvation) in 24 h. The experiments were repeated three times. NS: not significant.

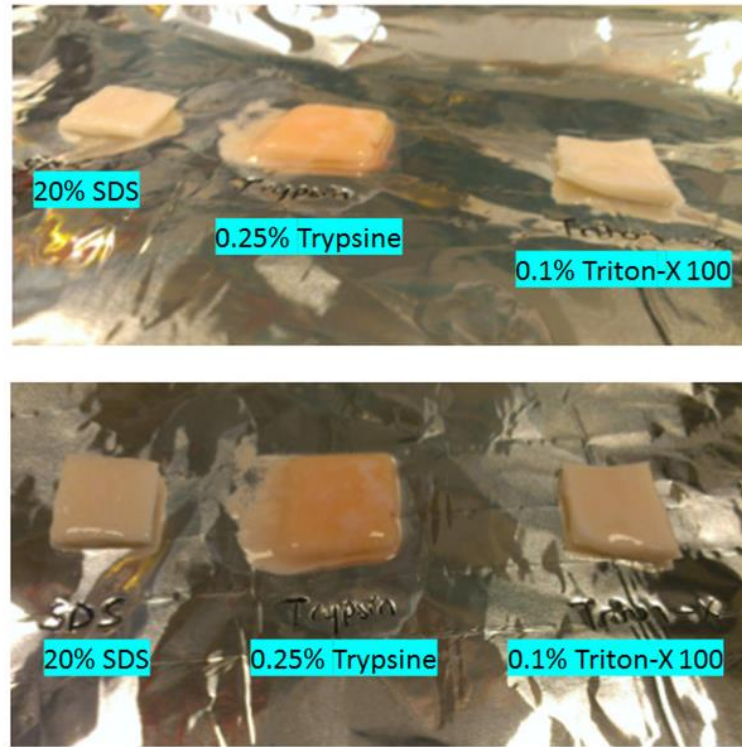


Figure 18. Decellularization of lamb aorta after 72 h of the treatments of 20% SDS, 0.25% Trypsin or 0.1% Triton X-100.

protease family member; cleaves peptides on the C-terminal side of Lysine and Arginine), Triton X-100 (non-ionic detergent, used to disrupt the cellular structure and over permeabilization of the cell membrane) were used. Although trypsin helped the most during decellularization, due to high contamination, experiments were terminated before cellularization process was completely achieved.

3.3.5. 3D bioprinting optimizations

In order to mimic tissue organization *in vitro* and minimize the adverse effects of biomaterials, 3D bioprinting of cells gained attention during last decade. In our experimental set-up, macrovascular cell organizations were aimed to be created and tested for their stress and cell death response (Figure 19).

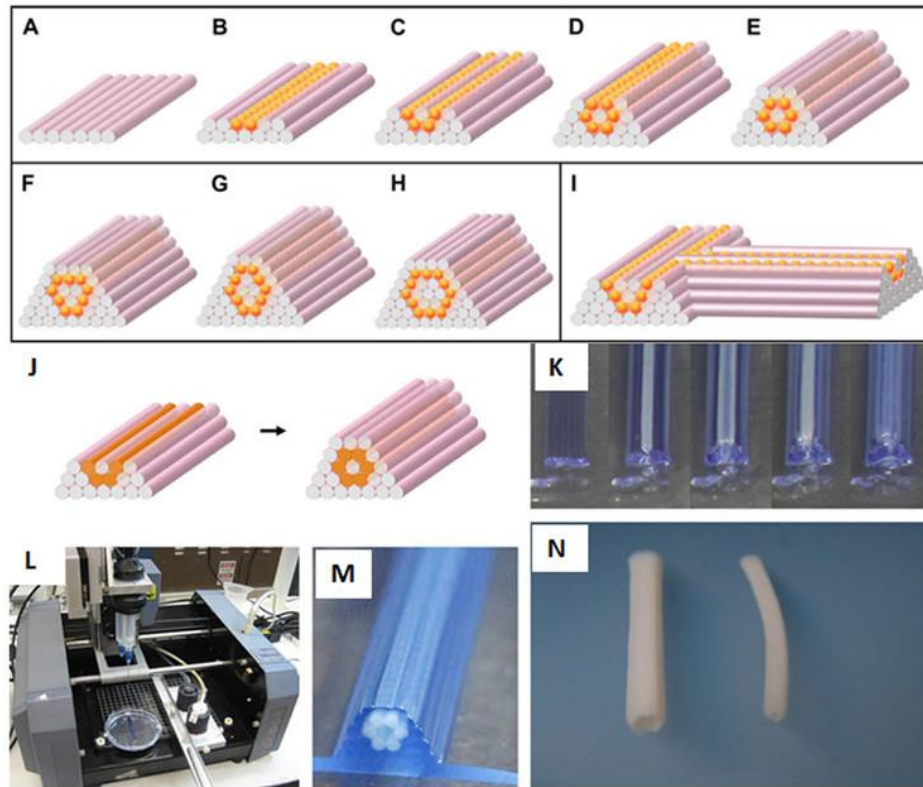


Figure 19. 3D bioprinting of macrovascular structure (pictures are taken from <http://organovo.com/>).

(A) Agarose based cylinders are printed (indicated in light purple color). (B) Cell cylinders are printed on agarose based cylinders (indicated in orange color). (D-H) 3D printing can precisely print the cells in indicated positions and shapes. (I) In order to create blood vessels, branches can be printed. (J-N) After printing the cells in a defined position and shape, by removing the agarose based structures, required shapes with desired dimensions can be obtained.

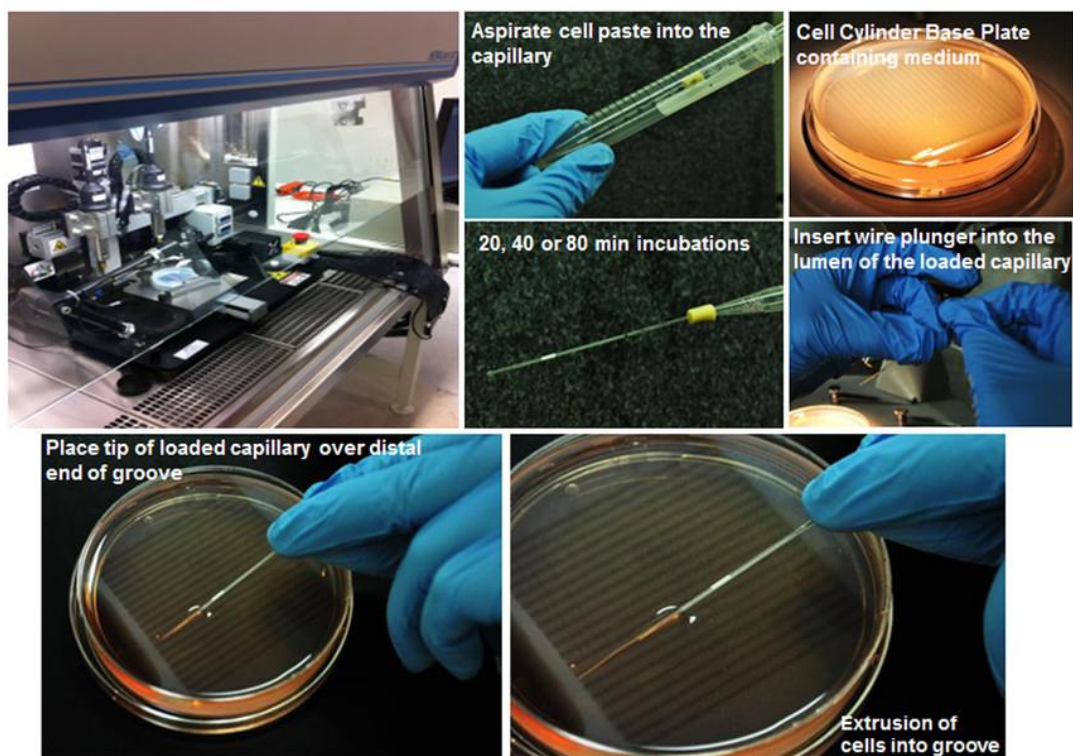


Figure 20. 3D bioprinting process for cell cylinders (pictures are taken from <http://organovo.com/>).

3D bioprinting can be performed using Organova 3D bioprinter. Cell cylinders were prepared accordingly: (i) Cell pellets were aspirated into capillary. (ii) 20 min, 40 min or 80 min incubation was performed in medium containing falcon tubes. (iii) Cell cylinder base plate was prepared using 2% Agarose with specific mold. (iv) Wire was inserted into the lumen of the loaded capillary. (v) Tip of the loaded capillary was placed over distal end of the groove. (vi) Cells were extruded into the groove.

This is achieved by a computer based-3D bioprinting device called Organova (Figure 20).

With computer-aided algorithms, bioprinting of self-supporting cell aggregates in 3D for fully biological and scaffold-free tissue engineering was performed. Before the actual printing process, cells are prepared for bioprinting with several steps, explained in detail in **Materials and Methods**. Due to several incubations and centrifugation steps (Figure 20), the role of autophagy during this preparatory process was unknown. Moreover, it was not elucidated yet whether the modulation of autophagy could help to obtain better results during and after bioprinting process.

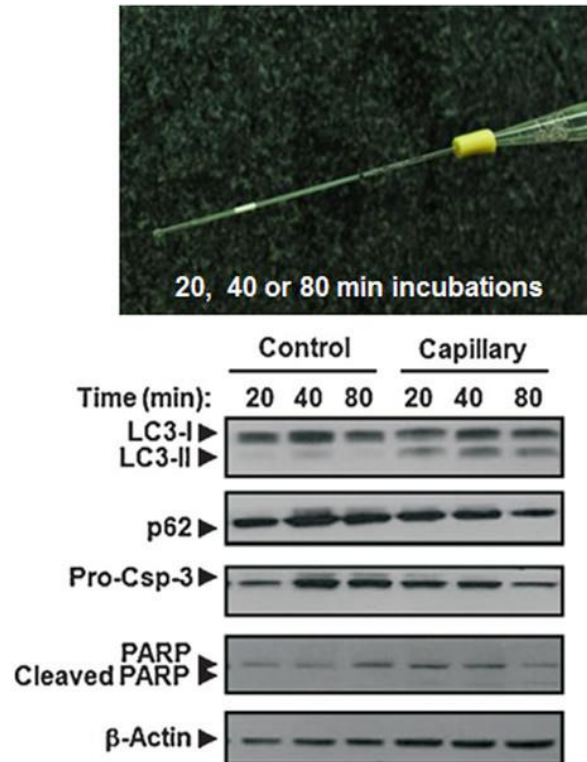


Figure 21. Autophagy and apoptosis markers analysis for MEF WT cells during bioprinting.

Different incubation times and autophagy and apoptosis analyses were performed (Figure 21). Compared to control cells, fibroblast cells which are incubated in a capillary showed an induced autophagy and an induced apoptosis depending on their incubation time in capillary (this was shown by Dr. Ster Irmak).

However, cell-cell connection was not optimal yet. Next, collagen addition during cell cylinder preparation was tested in order to increase cell-cell attachment and communication by increasing ECM. Moreover, the incubation times in cell cylinder base plate was increased to 9 h in order to help the cells to recover from the stress of capillary. Their autophagic response and strip size were analyzed (Figure 22).

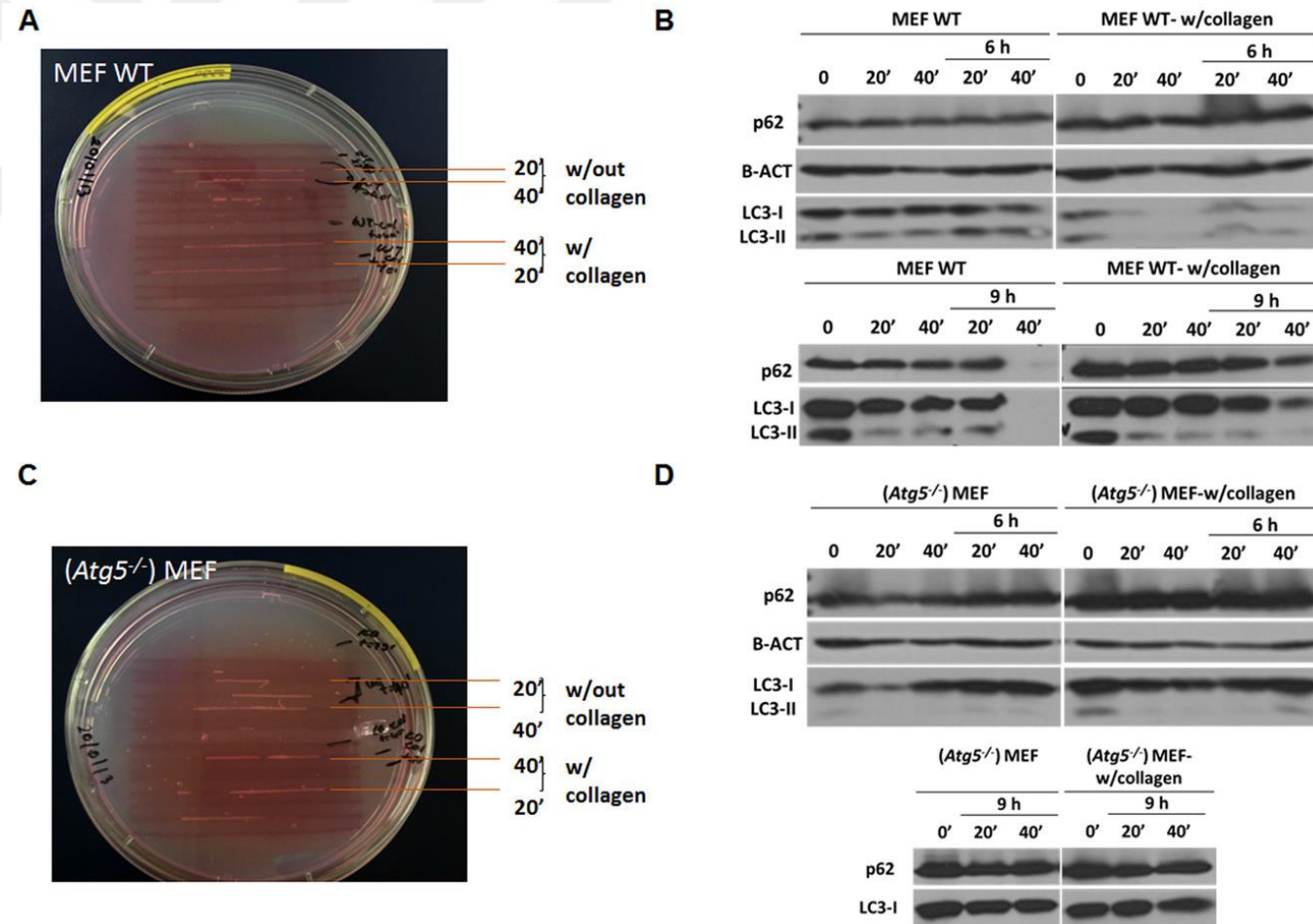


Figure 22. Autophagy analysis for addition of collagen and longer time intervals before bioprinting process.

(A) Cell cylinders with or without collagen mixture for MEF WT. **(B)** Autophagy marker analysis after 6 h and 9 h incubations in cell cylinder base plate for cell extracts from MEF WT cells. **(C)** Cell cylinders with or without collagen mixture for (Atg5^{-/-}) MEF cells. **(D)** Autophagy marker analysis after 6 h and 9 h incubation in cell cylinder base plate for cell extracts from (Atg5^{-/-}) MEF cells.

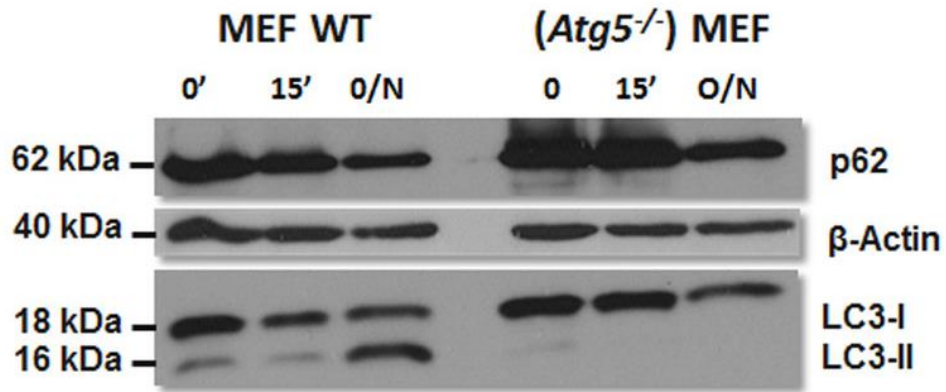


Figure 23. Autophagy markers were tested for 15 min and overnight (O/N) incubation for MEF WT and (*Atg5*^{-/-}) MEF.

Further optimization experiments such as longer and shorter incubation times were performed. It was observed that longer incubation time in the capillary caused significant autophagy induction in fibroblast cells (Figure 22). 15 min incubation in capillary was decided as optimum time point since cellular adhesion was already established and autophagy was not yet induced significantly (Figure 23).

Next, the prepared cell cylinders were used for 3D bioprinting. Basic principle of the 3D bioprinting was to print the cells in 3D with a computer-aided system using Organova bioprinter. According to modeled shape in the software, the arm of the device can print the cells in the decided position, precisely (Figure 19).

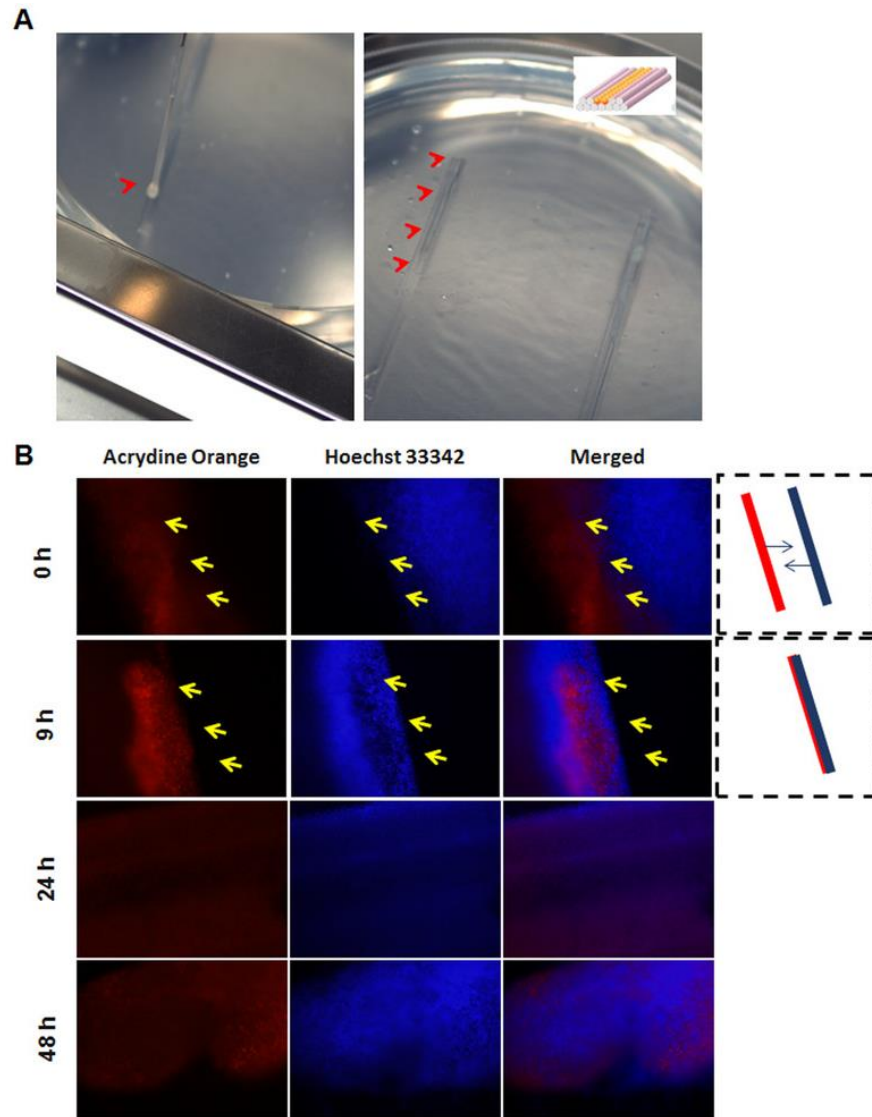


Figure 24. 3D bioprinting of the cells and following up to 48 h.

(A) 3D bioprinting of the stained cell cylinders with a computer aided program. **(B)** Printed cells were followed up to 48 h. The printed cell cylinders merged as early as 9 h.

3D-printed cells were observed for 48 h. In order to differentiate the cell cylinders under the microscope, one cell cylinder of MEF WT fibroblast cells was stained with Hoechst 33342 and one cell cylinder of MEF WT fibroblast cells was stained with Acridine orange. These two-printed cell cylinders could fuse with each other as early as 9 h (Figure 24). In later time points, completely fused structures were observed.

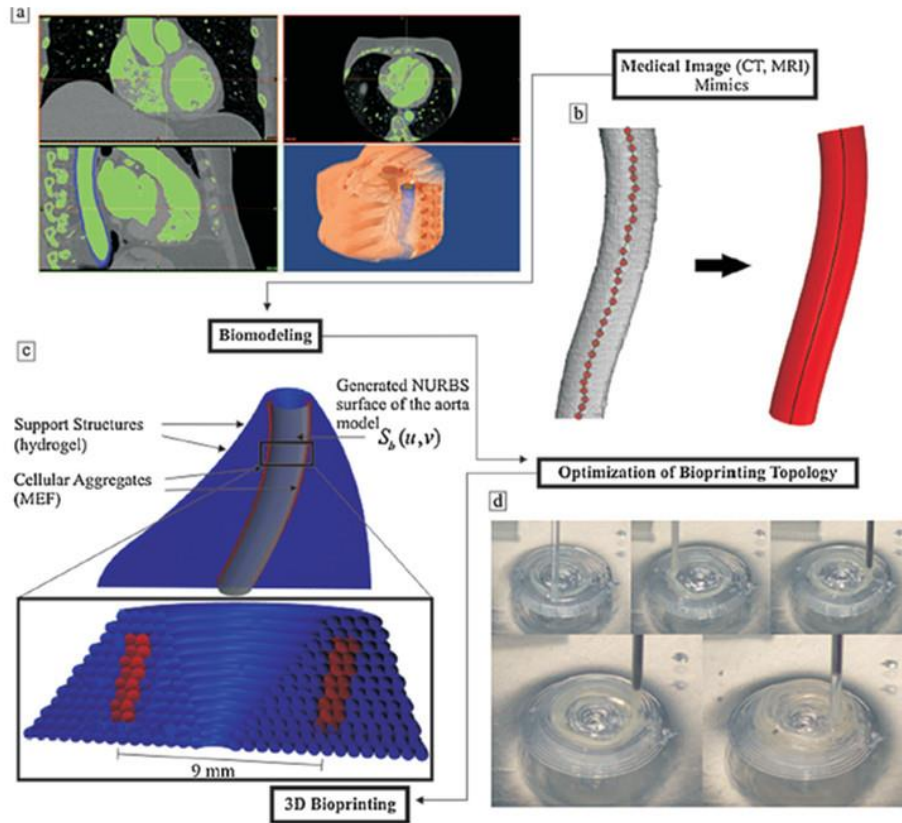


Figure 25 Modeling of human aorta for continuous 3D-bioprinting.

Geometric and topological information were obtained from computer tomography (CT) and magnetic resonance imaging (MRI).

Although mixing with collagen and longer time intervals could help to obtain longer cellular strips, these structures were not long enough for functional size of blood vessel formation. As a result, the bioprinting protocol was switched from printing cell strips to continuous bioprinting process (Figure 25) [228].

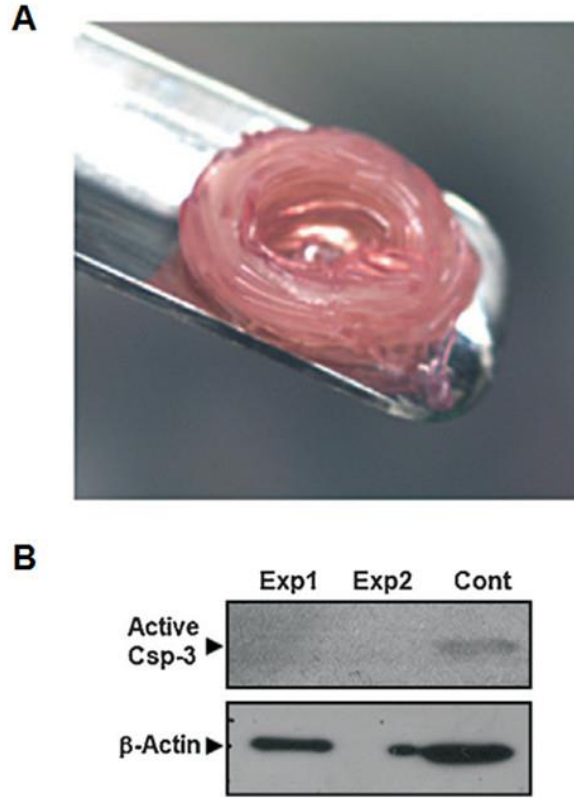


Figure 26. Continuous 3D-bioprinting of macrovascular structure and molecular analysis.

(A) Macrovascular structure was obtained with longer and scaffold-free using continuous 3D-bioprinting of fibroblast cells. **(B)** This process reduced apoptosis in the cells.

For continuous bioprinting, CT and MRI measurements of a real aorta were taken and used in Mimics software (Mimics, Medical Image Segmentation for Engineering on Anatomy) to representing the aorta in a stereolithography (STL) model. Modeling in Mimics was performed by Can Küçükgül, M.Sc. student of Dr Bahattin Koç. Using this model, longer and continuous cell strips with 3D-printing of the fibroblast cells were achieved with less apoptosis activity (Figure 26) As a result, due to creating high stress on the cells during the printing process and other limitations, autophagy analysis in the cells indicated that bioprinting process can be changed from cell cylinders to continuous bioprinting.

3.4. RESULTS II - Discovery of novel paracrine factors regulating autophagy in fibroblast cells

3.4.1. Cancer cells secrete soluble factors which induce autophagy in fibroblasts

Cell-cell communication can be achieved by direct contact and paracrine factors which are secreted to mediate the communication between cells in larger distance (Figure 3). In tumor microenvironment, the communication is mainly achieved by paracrine factors for recruiting immune cells, endothelial cells and activated fibroblast cells for their own benefits. Fibroblast cells are the major components of tumor microenvironment. Thus, in order to analyze tumor-stroma communication mediators which induce stress response in fibroblast cells, autophagy analysis for the treatment of fibroblast cells with the conditioned media of different cancer cells was performed. Technically, cancer cells were cultured in low-FBS medium for 72 h and concentrated with Amicon 3 kDa cut-off tubes. This type of medium is called conditioned medium. The conditioned media of indicated cancer cells were added onto GFP-LC3 MEFs, and conditioned media from GFP-LC3 MEFs were used as the control of the experiment. In fact, GFP-LC3 serves as a commonly used tool for autophagy detection. Under normal conditions, GFP-LC3 MEF cells show a diffuse cytoplasmic and nuclear localization. Upon the treatment of the cells with the conditioned medium of different cancer cells, GFP-LC3 dot formation appears in bigger and brighter dots reflecting autophagy induction through autophagic membrane formation, allowing the detection of autophagy activation in live cells under a fluorescent microscope (Figure 27A and B). All conditioned mediums from different cancer types, namely MCF7, MDA-MB-231, Huh7, and PC3, demonstrated the common feature of inducing autophagy in fibroblast cells. In order to further prove autophagy induction in fibroblast cells, the autophagy markers LC3 shift and p62 degradation were analyzed with immunoblot. LC3 is a cytosolic protein. Upon the induction of autophagy, LC3 protein is cleaved by ATG4B enzymes from its C-terminal to expose Gly¹²⁰ residue, and this form of the protein is called LC3-I. Further lipidation of LC3-I protein converts LC3-I to LC3-II, which is then recruited to the autophagosome membrane elongation sites [230, 231]. p62/SQSTM1 (called p62) protein selectively binds to cargo in order to target polyubiquitinated protein aggregates to the autophagic machinery. It can also recruit the autophagosomal protein LC3 and co-localizes with many types of polyubiquitinated protein aggregates. Degradation of p62 during autophagic process is another marker for

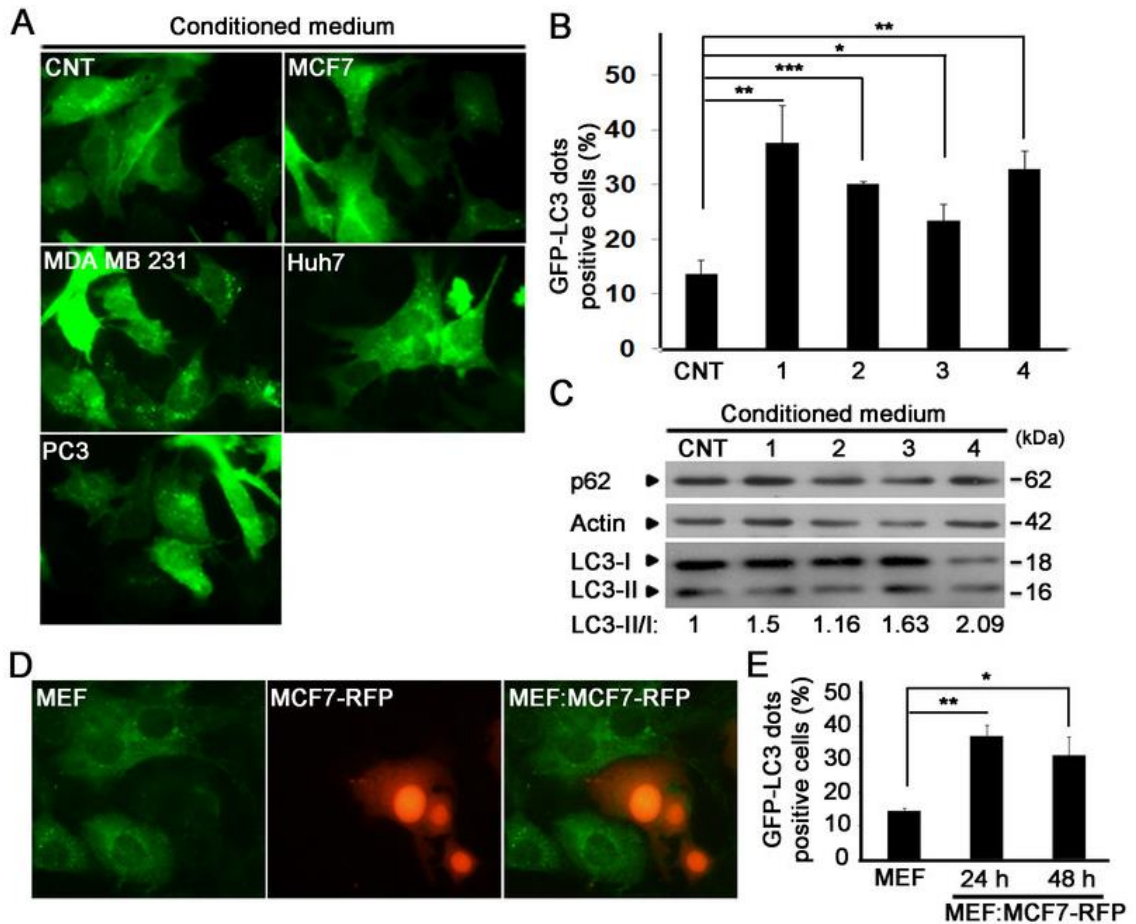


Figure 27. Effect of conditioned media of different cancer cells or co-cultured cancer cells on autophagy in fibroblast cells.

(A) Treatment of fibroblast cells with the conditioned media of MCF7 (1), MDA-MB-231 (2), Huh7 (3), and PC3 (4) cancer cells induce autophagy and increase GFP-LC3 dot formation in fibroblast cells. CNT was the conditioned medium of GFP-LC3 transgenic MEF cells. GFP-LC3 transgenic MEF cells were treated with the conditioned media for 24 h. GFP-LC3 dot counting was performed in fixed cells as described in the **Materials and Methods** section. (B) Quantitative analysis of the experiments in (A) (mean±SD of independent experiments, n=3, **<0.01, ***<0.001). (C) Immunoblots of autophagy analysis in MEFs that were grown in conditioned medium of cancer cells. Autophagy markers, p62 protein degradation and LC3 protein were analyzed using the immunoblot technique for the cell lysates, which were treated with the conditioned medium of cancer cells. CNT was the medium of MEF WT cells. Actin was used as the loading control. LC3-II/LC3-I protein band densitometric analyses were performed using Image J. (D) Co-culture of stably RFP-producing MCF7 (MCF7-RFP) breast cancer cells with GFP-LC3 MEFs (MEF) cells induce autophagy in fibroblast cells. MCF7-RFP cancer cells were cultured with fibroblast cells for 24 h and 48 h. CNT is regular medium of GFP-LC3 transgenic MEF cells. (E) Quantitative analysis of experiments in (D) (mean±SD of independent experiments, n=3, *p<0.05; **<0.01).

autophagy induction in cells [232, 233]. According to the immunoblot results, Huh7 and PC3 indicated a higher induction of autophagy based on their strong shift in LC3-I to

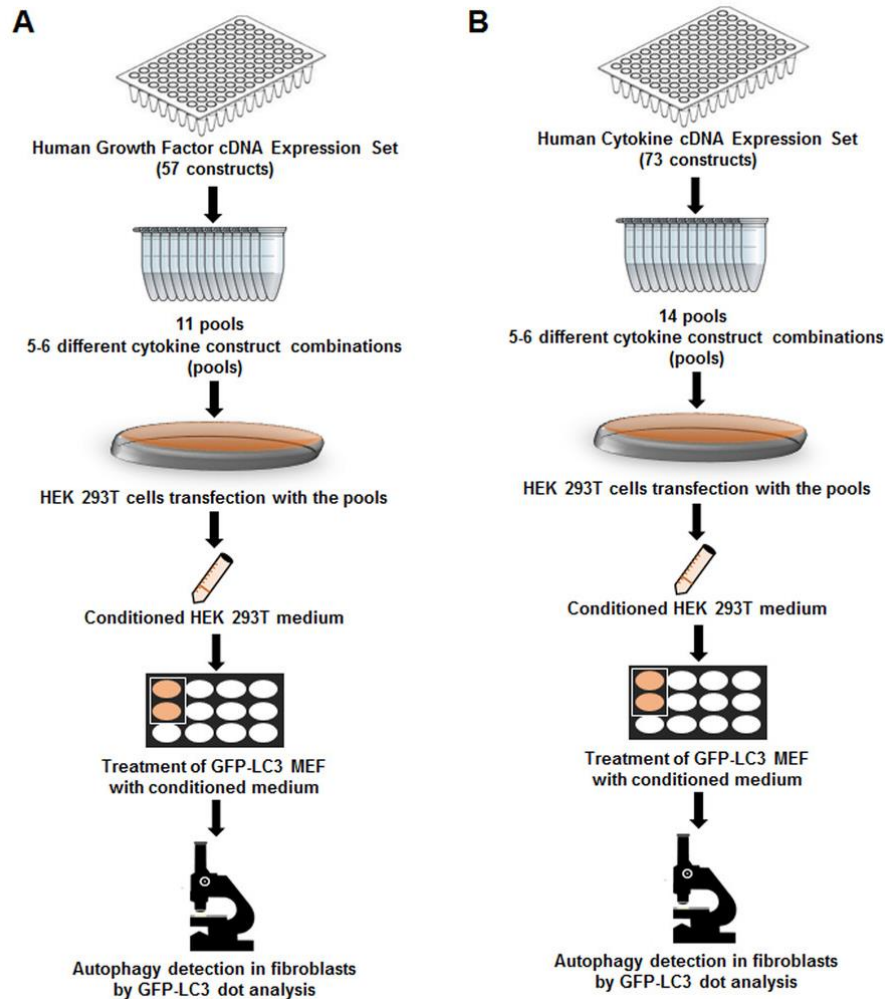


Figure 28. Preparation of the pools for growth factors and cytokines.

(A) Unbiased cDNA growth factor library screening was performed by treating fibroblast cells with a conditioned medium of overexpressed cDNA library pools in HEK 293T cells. Screening was based on human growth factor cDNA expression set which contains 57 different factors. These were grouped into 11 different pools in which each pool contained 5-6 different cDNA constructs. HEK 293T cells were transfected with these pools, and their mediums were switched to a low-FBS medium. After 72 h, mediums were concentrated by using Amicon tubes. The effect of growth factor pools on autophagy in fibroblast cells was checked by treating GFP-LC3 MEF cells with conditioned mediums of the pools. Quantification of autophagy in fibroblasts was performed by counting GFP-LC3 dots under the microscope. **(B)** Unbiased cDNA cytokine library screening was performed by treating fibroblast cells with a conditioned medium of overexpressed cDNA library pools in HEK 293T cells. Screening was based on human cytokine cDNA expression set which contains 73 different cytokines. These were grouped into 14 different pools in which each pool contained 5-6 different cDNA constructs. HEK 293T cells were transfected with these pools, and their mediums were switched to a low-FBS medium. After 72 h, mediums were concentrated by using Amicon tubes. The effect of cytokine pools on autophagy in fibroblast cells was checked by treating GFP-LC3 MEF cells with conditioned mediums of the pools. Quantification of autophagy in fibroblasts was performed by counting GFP-LC3 dots under the microscope.

LC3-II (Figure 27C). To further validate these results, GFP-LC3 MEFs were co-cultured

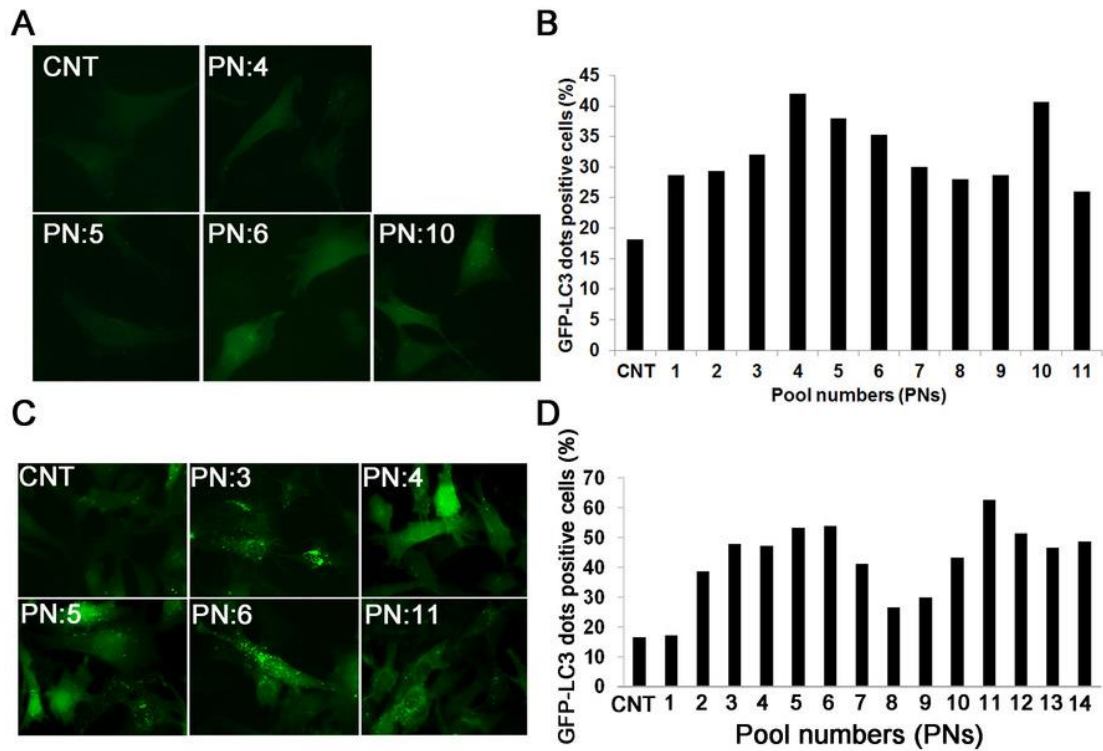


Figure 29. Autophagy inducing pools for growth factors and cytokines.

(A-B) The effect of growth factor pools on autophagy in fibroblast cells was checked by treating GFP-LC3 MEF cells with conditioned mediums of the pools. Quantification of autophagy in fibroblasts was performed by counting GFP-LC3 dots under the microscope. **(C-D)** The effect of cytokine pools on autophagy in fibroblast cells was checked by treating GFP-LC3 MEF cells with conditioned mediums of the pools. Quantification of autophagy in fibroblasts was performed by counting GFP-LC3 dots under the microscope.

with MCF7 breast cancer cells that stably express the red fluorescent protein RFP (shortly, MCF7-RFP) for 24 h and 48 h. MEFs that were in close proximity to cancer cells significantly activated autophagy under these co-culture conditions (Figure 27D and E). For further clarification of the key factors during tumor-fibroblast communication, an unbiased screening for the effect of various growth factors and cytokines was performed (see next section).

3.4.2. An unbiased screen to identify key factors which mediated cancer-fibroblast communication through modulation of autophagy

In order to identify key factors which have important roles during cancer cell-fibroblast communication, unbiased growth factor and cytokine screenings were performed on GFP-LC3 MEFs. 57 different growth factors were divided into 11

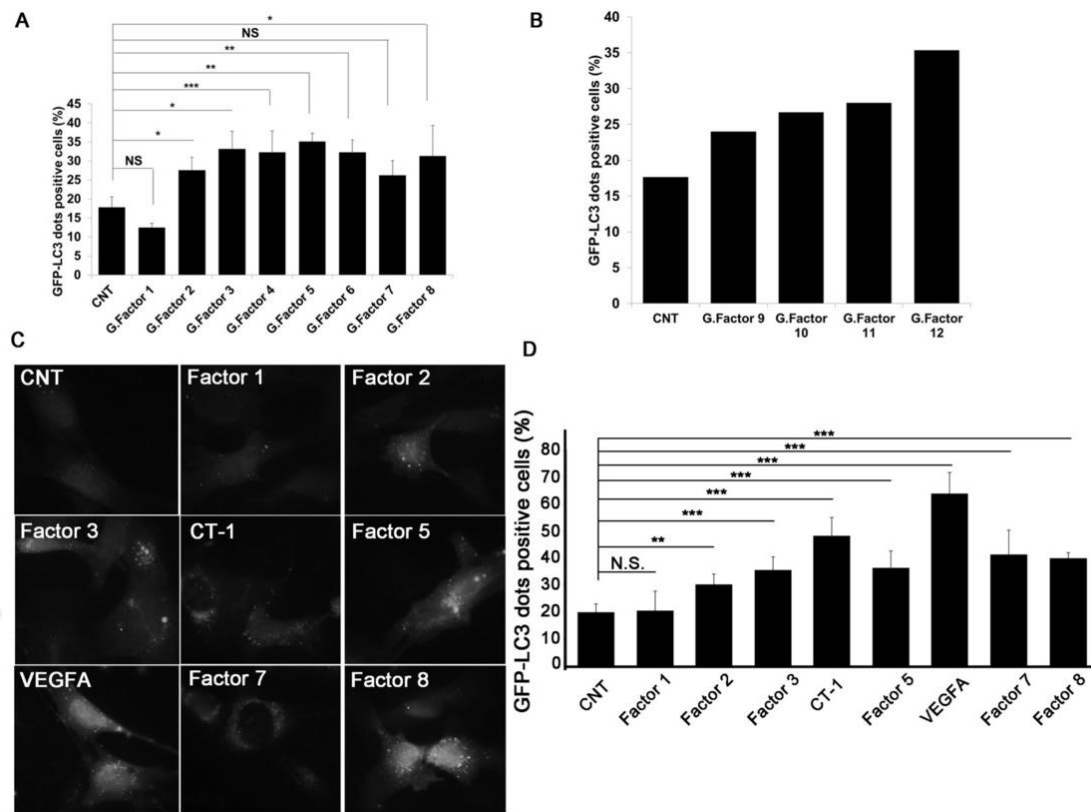


Figure 30. Screening for secreted factor which induces autophagy in fibroblast cells.

(A) Result of growth factor screening, (mean $\pm$ SD of independent experiments, n=3, NS: Not significant, * <0.05 , ** <0.01 , *** <0.001). **(B)** Result of growth factor screening, (mean $\pm$ SD of independent experiments, n=2). **(C)** Treatment of fibroblast cells with conditioned mediums of pools that highly induced GFP-LC3 dot formation in fibroblast cells. CNT was a/the conditioned medium of CNT (pcDNA3) plasmid overexpressing HEK 293T cells. Supernatants of transfected HEK 293T cells with the candidates were concentrated by using Amicon tubes, and GFP-LC3 MEF cells were cultured with the concentrated supernatants for 24 h. GFP-LC3 dot counting was performed in fixed cells. Among the 8 strongest cytokine candidates, CT-1 was selected to analyze its role on autophagy in fibroblast cells. **(D)** Quantitative analysis of the experiments in (C) (mean $\pm$ SD of independent experiments, n=3, ** <0.01 , *** <0.001). More details are given in section **Materials and Methods**.

different pools, each of which contains 5-6 different growth factors, and 73 different cytokines were divided into 14 different pools, each of which contains 5-6 different cytokines (Figure 28A and B) (Appendix C and D).

Growth factor and cytokine pools were overexpressed in HEK 293T cells, and the conditioned media of the pools were obtained by using Amicon 3kDa cut-off tubes. Treated GFP-LC3 MEFs were analyzed under a fluorescence microscope through the quantification of GFP-LC3 dot percentages. MEF cells which showed higher autophagy

activity according to their pool treatment were the possible candidates for a fine-grained screening (Figure 29A-D).

After the analysis of the pools, autophagy activation which was observed to be two-fold or more comparing to the control were converted to individual factors, and over-expression and conditioned medium preparation for individual factor analysis. At the end, twelve factors for growth factors and seven cytokines which significantly induced autophagy in MEFs were discovered (Figure 30A-D).

Among them, cardiotrophin-1 (CT-1) and vascular endothelial growth factor A (VEGFA) were identified as the most potent inducers of autophagy. Although CT-1 has been well studied about its proliferative roles, especially in cardiac myocytes though inhibiting apoptosis, there has been no study so far which shows the role of CT-1 during cancer cells and fibroblasts communication.

3.5. RESULTS III - A microfluidic chip for screening individual cancer cells via eavesdropping on autophagy-inducing crosstalk in the stroma niche: TGF β 1 as a model paracrine factor

3.5.1. Single-cell based microfluidic chip design for monitoring autophagy in tumor-stroma crosstalk

In a tumor microenvironment, cancer cells and surrounding cell are in dynamic interactions with each other (Figure 31A). Since the detailed mechanism of this crosstalk has not been elucidated yet, in order to study the crosstalk between a single tumor cell and stromal niche, a porous membrane-based single-cell trapping device was developed by our collaborator (Prof. Dr. Yoon-Kyoung Cho and her team) (Figure 31B). In this device, GFP-LC3 MEFs were modeled as cancer stroma fibroblasts. GFP-LC3 proteins were recruited to autophagic vesicles which transforms diffuse GFP signal into dot-like puncta formation. Hence, autophagy activation can be detected and quantified in live cells under a fluorescent microscope (Figure 31A and B). MDA-MB-231 triple-negative breast carcinoma cells, which stably express the red fluorescent protein (RFP) (in short, MDA cells) were used as cancer cells (Figure 31B).

The device platform was composed of a PDMS reservoir, a PDMS membrane, and a PDMS-coated thin glass (Figure 31B and C). The fabrication process of the microfluidic chip is explained in detail in Figure 32 and **Materials and Methods** section.

Our produced membrane has approximately 800 holes with diameter of 30 μm , which was optimized for a single cancer cell having 10-20 μm size, and the distance between adjacent holes was 280 μm (Figure 31D). After bonding the PDMS membrane with the PDMS reservoir, the top side of the membrane was covered with a cell repellent material, i.e., 0.4% (w/v) polyethylene oxide and polypropylene oxide triblock copolymer (PEO-PPO-PEO), while the bottom side of the membrane was coated with 10 $\mu\text{g}/\text{ml}$ of fibronectin (Figure 33). As a result, fibronectin coating provided evenly seeded fibroblasts on the bottom part of the membrane and PEO-PPO-PEO coating created hydrophobic force for tumor cells to be pushed to the hole of the membrane in order to have captured them as single cell in the holes (Figure 33D-F). In addition, we confirmed in independent control experiments that the fibronectin coating did not leak to the top side of the membrane through the holes (Figure 33B and C). Furthermore, we checked that coating with PEO-PPO-PEO copolymer prevented non-specific binding of cancer cells on the surface of the PDMS membrane (Figure 33D-F).

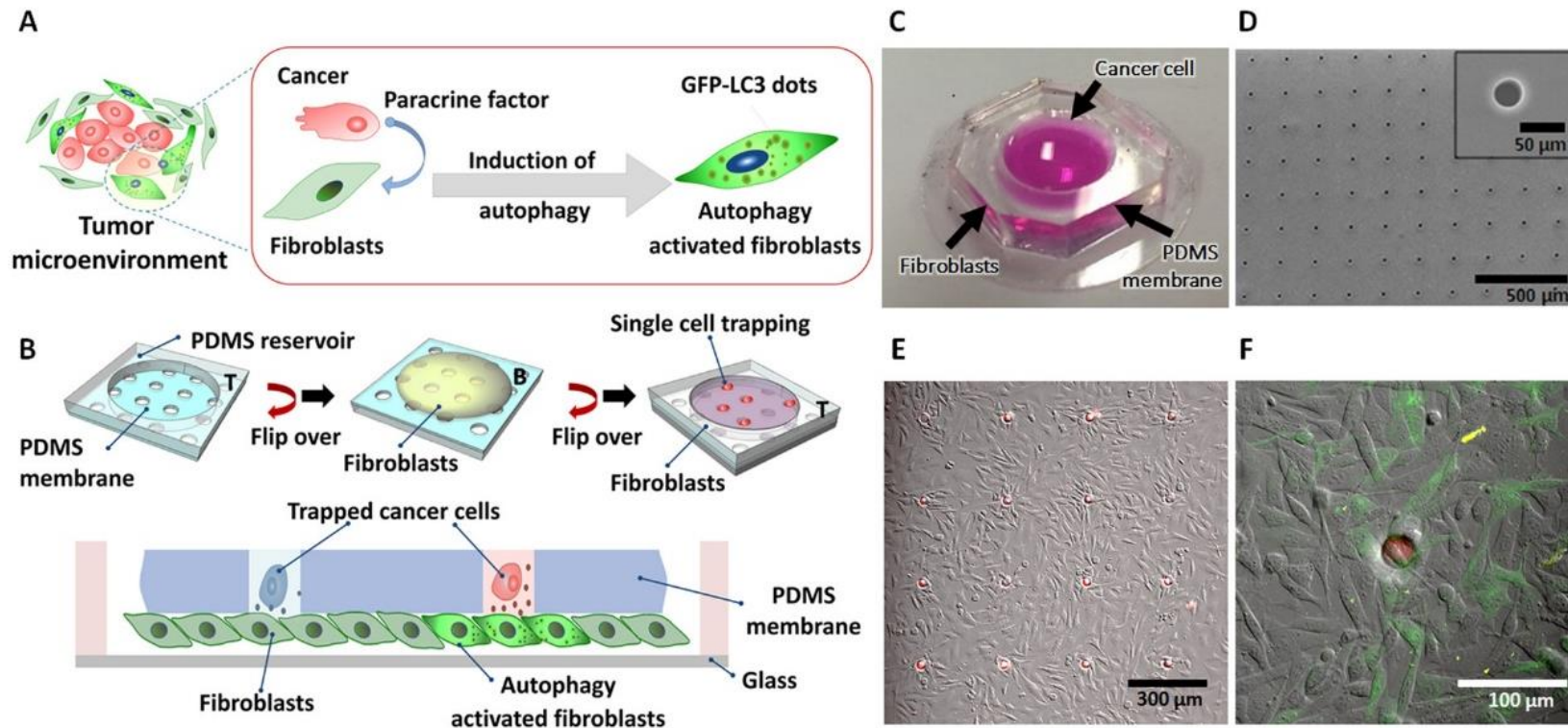


Figure 31. Experimental schemes and microfluidic devices for monitoring multicellular interaction between a single cancer cell and many fibroblasts in the neighbor.

(A) In a tumor microenvironment, cancer cells communicate with neighboring fibroblasts. Autophagy induction by paracrine factors can be detected using GFP-LC3 dots quantification. (B) The experimental set-up that allowed the study of the interaction between the single tumor cell and fibroblasts. Fibroblasts were first cultured on the bottom side (B) of the PDMS membrane for 2 h. The PDMS membrane was turned over and attached to a PDMS coated cover-glass. Then, a single tumor cell was trapped into the holes on top side (T) via gravitational forces and agitation. (C) A photograph of the microfabricated biochip system. (D) SEM image of the membrane. The pore diameter is 30 μm (Inlet. Scale bar, 50 μm), and the center-to-center distance between the pores is 310 μm (Scale bar: 500 μm). (E) A 10x microscopic image of fibroblasts neighboring individual MDA cells that were trapped in the hole array (Scale bar: 300 μm). (F) Visualization of autophagy activation in fibroblasts during communication with trapped single MDA cells (Scale bar: 100 μm).

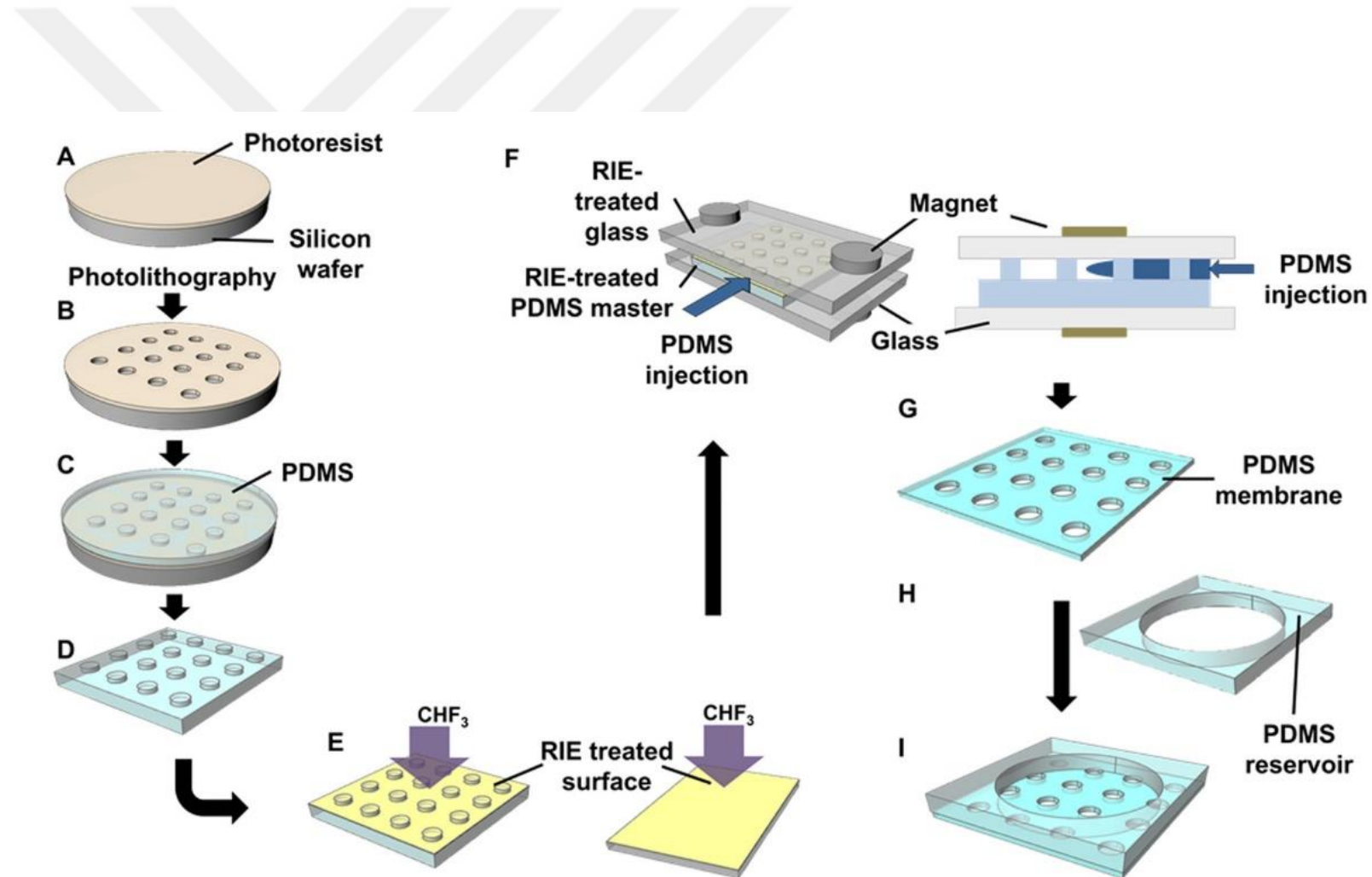


Figure 32. Fabrication procedure for the PDMS porous membrane.

(A) Deposit the photoresist on the silicon wafer. (B) Make a pattern using photolithography. (C) Add the pre-cured PDMS and cure. (D) Peel off the PDMS master. (E) Perform RIE treatment with CHF_3 . (F) Inject the pre-cured PDMS into the gap between the PDMS master and glass. (G) Release the PDMS membrane. (H) Fabricate an 11 mm-diameter PDMS reservoir. (I) Attach the PDMS membrane with the PDMS reservoir.

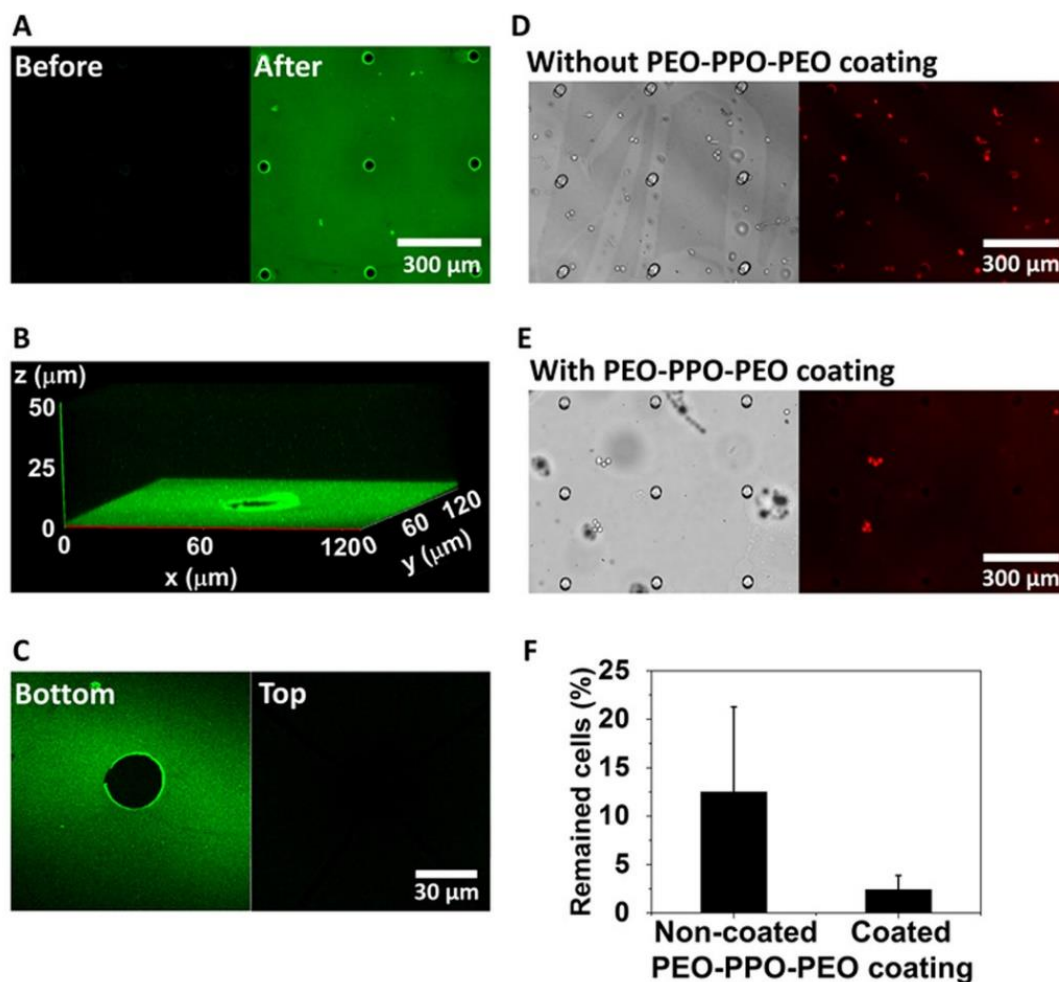


Figure 33. The effect of surface coating on the PDMS membrane.

(A) HiLyte488 fibronectin coating on the bottom side of the membrane. Before coating, the membrane did not show any fluorescent signal. The coated side of the membrane showed green fluorescence between and around the holes. (B) The fibronectin coating on the bottom side of the membrane did not affect the top surface, as is confirmed by the z-stack confocal image analysis of a hole. (C) The bottom side of the membrane showed the HiLyte488 fibronectin coating while the top surface did not. (D) Compared to the uncoated side, the coated side had a reduced number of cancer cells attached to the area between the holes. (E) Quantification of the experiments shown D (n=3)

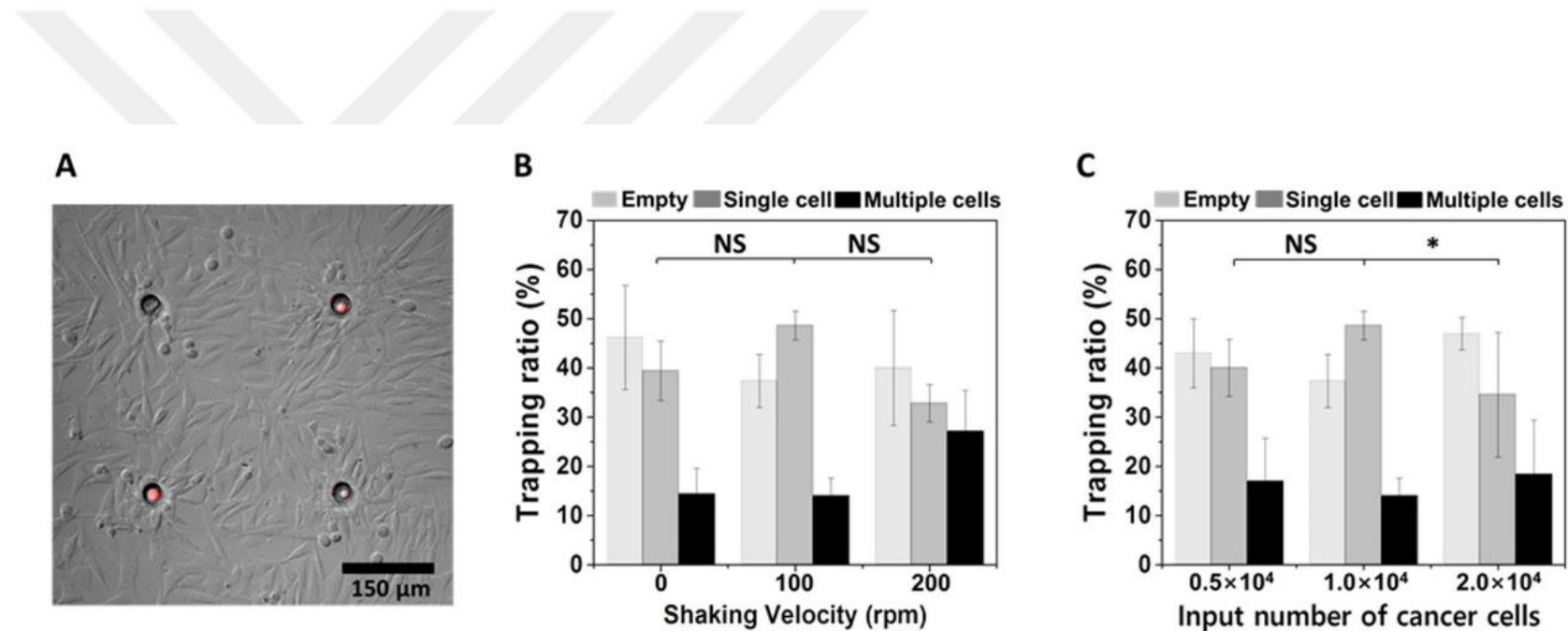


Figure 34. Optimization of trapping efficiency for MDA cells in the microfluidic chip system with a different number of cancer cells and orbital shaking velocities.

(A) Visualization of fibroblasts and trapped MDA cells in the microfluidic chip system under 20x. **(B)** Trapping efficiency of cancer cells with a different number of cancer cells and quantification of the experiment (mean $\pm$ SD of independent experiments, n=3). **(C)** Trapping efficiency of cancer cells with different orbital shaking velocities in the microfluidic chip system and quantification of the experiment (mean $\pm$ SD of independent experiments, *p < 0.05, n=3). Even though the Student t-test does not show statistically significant difference among different conditions which were tried, the shaking velocity of 100 rpm and 1×10^4 input number of cells provided relatively more reproducible data and used the condition in the follow-up experiments.

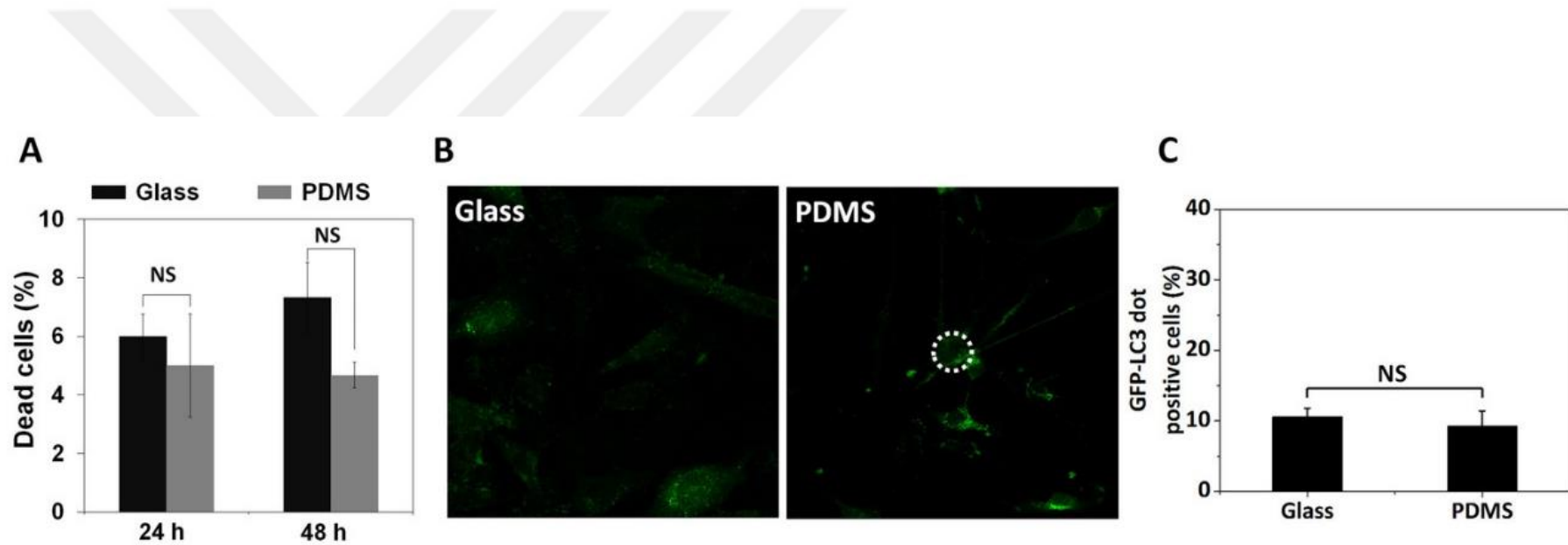


Figure 35. The effect of the microfluidic chip on biocompatibility and the basal level of autophagy in fibroblasts.

(A) In order to perform a live/death assay, PI staining was performed for the cells cultured in glass and on fibronectin-coated PDMS membrane. Cell viability was not affected by the system with time (mean $\pm$ SD of independent experiments, n=3, NS: Non-significant). **(B)** Compared to the control case when fibroblasts were seeded onto the glass slide, fibroblasts seeded into the microfluidic chip does not show elevated autophagy levels. **(C)** Quantification of the experiment (B) (mean $\pm$ SD of independent experiments, n=3, NS: Non-significant).

For the experimental set-up, fibroblasts were cultured on the fibronectin-coated bottom side of the PDMS membrane. Next, the membrane was flipped and the device was assembled. MDA cells were introduced to the top reservoir for single cell trapping in membrane holes. A different number of MDA cells and orbital agitation velocities were tested in order to obtain optimized single cell trapping ratio and exclusion of non-trapped cells from the top side of membranes (Figure 34). For the trapping experiments, the percentage of holes containing one and only one cell was determined in order to measure the performance of the single cell trapping ratio. Among the many conditions used, optimal single cell trapping ratio was obtained when 1×10^4 MDA cells were seeded, and under 100 rpm agitation for 5 min followed by 5 times washes (Figure 34B and C).

The viability test confirmed that the viability of fibroblasts was not affected (Figure 35A) and autophagy was not induced in GFP-LC3 fibroblasts by mere culturing on the platform (Figure 35B and C).

As shown in Figure 31E, under optimized conditions, individual cancer cells trapped in multiple arrays of holes in the membrane were in contact with the layer of fibroblasts cultured on the bottom side of the membrane. Due to the crosstalk between trapped single cancer cells and fibroblast cells, autophagy was induced in the fibroblasts (Figure 31F).

3.5.2. Autophagy activation by secreted paracrine factors in the tumor microenvironment

When MDA cells were co-cultured with MEFs on cover slides in a 1:10 ratio and GFP-LC3 dot formation ratios were quantified, autophagy activation was clearly observed in the MEFs that were in a close proximity of the MDA cells (Figure 36A). Furthermore, the fraction of autophagy positive fibroblasts, defined as cells with more than 20 GFP-LC3 dots, increased with time (Figure 36B). Detailed criteria for imaging and autophagic cell counting are provided in **Materials and Methods**.

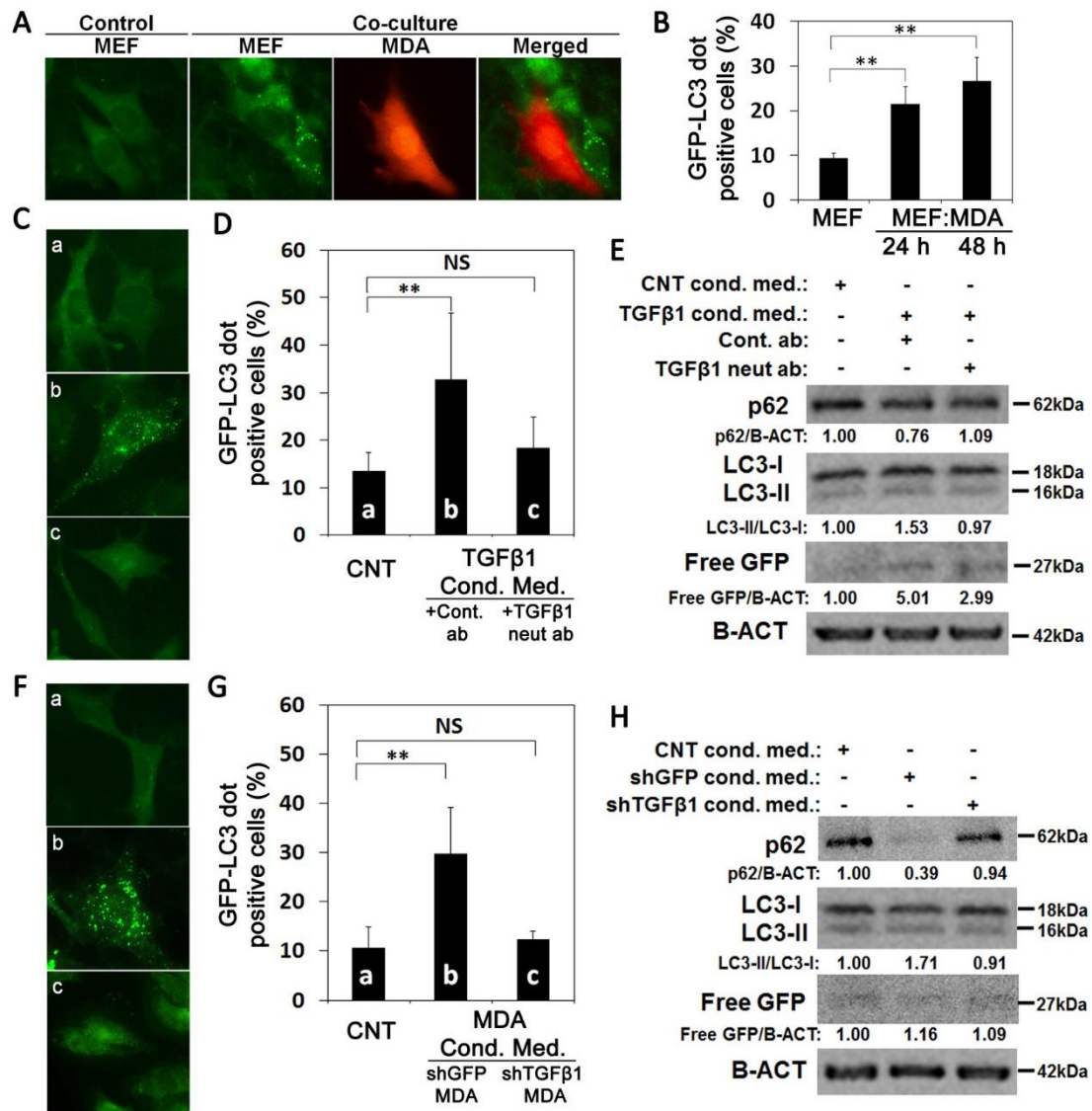


Figure 36. Effect of cancer cell co-culture with fibroblasts and conditioned media on fibroblast autophagy.

(A) Co-culture of MDA-MB-231 cells (MDA) with GFP-LC3 MEFs (MEF) induced autophagy in MEFs. (B) MEFs that were co-cultured with MDA cells for 24 h or 48 h showed significantly higher autophagy levels compared to MEFs alone (mean±SD of independent experiments, n=3, **p<0.01). (C and D) Effect of TGFβ1 neutralizing antibody (c, neut ab) or control antibody (b, cont. ab) on fibroblasts autophagy during incubation with TGFβ1 overexpressed HEK 293T cells conditioned media (cond. med.). pcDNA3 overexpressed HEK 293T cells conditioned medium was used as control (CNT) (a). The graph represents quantification of autophagy (GFP-LC3 dots) (mean±SD of independent experiments, n=4, NS: Non-significant, **p<0.01). (E) Immunoblots of the autophagy analysis in MEFs. p62, SQSTM protein. LC3 protein, MAP1LC3. Free GFP, GFP generation from the cleavage of GFP-LC3 protein in the autolysosomes. B-ACT was used as loading control. p62/B-ACT, endogenous LC3-II/LC3-I and free GFP/B-ACT protein band densitometric analyses were performed using Image J. (F and G) Effect of conditioned media (cond. med.) from control shGFP (b) or shTGFβ1 (c) infected MDA cells on fibroblast autophagy. Conditioned medium from MEF was used as control (a). The graph represents quantification of autophagy (GFP-LC3 dots) (mean±SD of independent experiments, n=4, NS: Non-significant, **p<0.01). (H) Immunoblot analysis of autophagy of cell extracts from MEFs that were grown in conditioned media from control shGFP or shTGFβ1 infected MDA cells. CNT, MEFs cultured in MEF conditioned medium.

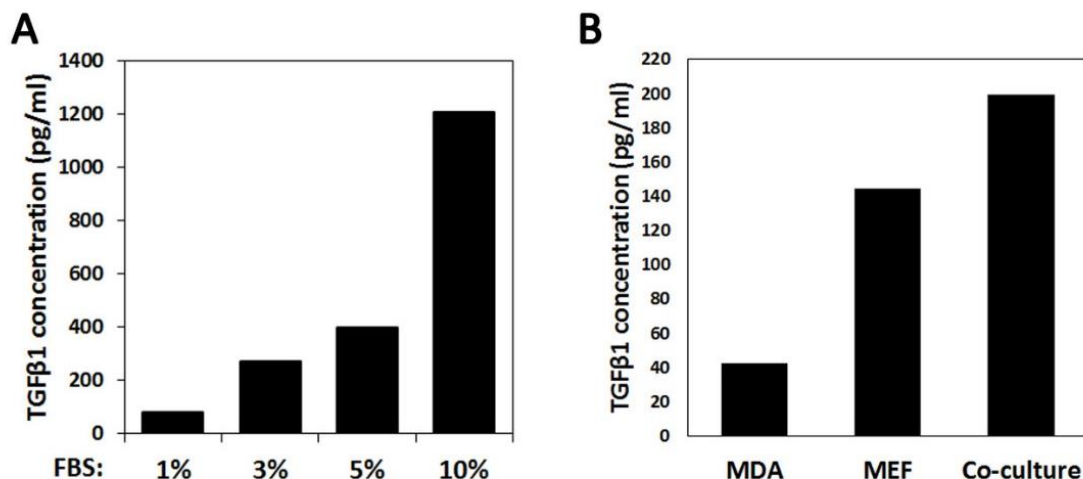


Figure 37. Analysis of TGFβ1 concentration.

(A) TGFβ1 concentration (pg/ml) was analyzed in different percentages (1-10%) FBS containing media (average of two independent experiments). **(B)** TGFβ1 concentration (pg/ml) in the media of MDA cancer cells, MEFs or co-cultures (Culture in 3% FBS containing medium for 6 h). (average of two independent experiments).

Among the many paracrine factors, TGFβ1 was proposed to play an important role in the formation of CAF from naive fibroblasts [94]. Moreover, TGFβ1 signals contribute toward myofibroblastic cell properties during fibrosis [234, 235] and CAF formation [159]. Studies that were published during the preparation of this thesis also indicated that TGFβ1 could induce autophagy in fibroblasts [236, 237]. Therefore, in proof-of-concept experiments, TGFβ1 was used as a model cytokine that could mediate cancer-fibroblast interaction.

First, TGFβ1 secretion in the system was confirmed using the ELISA method. While these tests confirmed that fibroblasts also secreted TGFβ1, a significant contribution of TGFβ1 was observed in cancer cell-fibroblast co-cultures (Figure 37). A 27.5% increase in TGFβ1 levels was observed compared to MEF-alone when MDA breast cancer cells were co-cultured with MEFs.

In order to confirm that TGFβ1 was indeed an inducer of autophagy, GFP-LC3 MEFs were cultured in recombinant TGFβ1 containing medium in the presence of a TGFβ1 neutralizing antibody or a control antibody. As shown in Figure 36C and D, while autophagy induction occurred in the GFP-LC3 MEFs that were incubated in cultured medium containing HEK 293T-derived recombinant TGFβ1 and control antibody, blockage of TGFβ1 using a specific neutralizing antibody prevented autophagy activation. Moreover, under similar conditions, classical molecular markers of autophagy

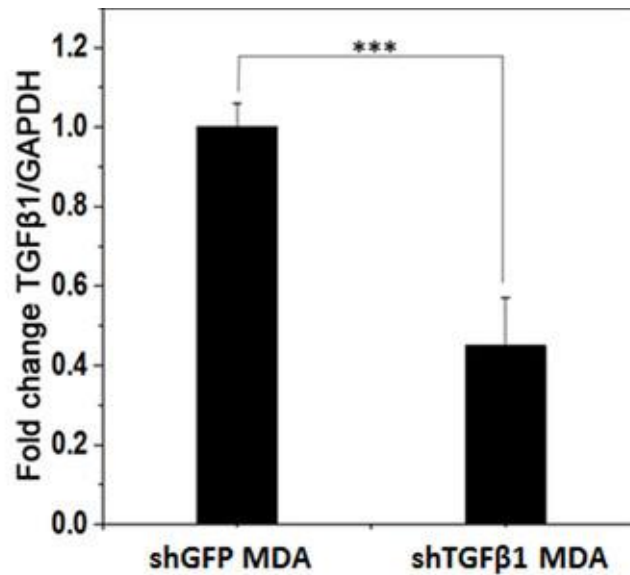


Figure 38. Analysis of TGFβ1 in MDA cells at mRNA level.

Compared to control case; MDA cells infected with control shRNA (shGFP), TGFβ1 levels are significantly reduced in shTGFβ1-infected MDA cells (mean±SD of independent experiments, n=3, ***p<0.001).

activation, namely endogenous LC3-I/LC3-II conversion, p62 protein degradation, as well as free GFP production from GFP-LC3 protein tests confirmed that autophagy activation was TGFβ1 dependent (Figure 36E).

To further prove that endogenous TGFβ1 that was secreted from MDA cells was the rate-limiting cytokine for the observed autophagy activating effects, media from MDA cell cultures that were infected either with lentiviruses containing a shRNA against GFP or TGFβ1 were collected and added onto the GFP-LC3 MEFs in culture. Moreover, TGFβ1 expression levels were significantly reduced in MDA cells that were infected with the shTGFβ1 virus compared to controls (Figure 38). Conditioned media from control GFP shRNA (shGFP) infected MDA cells could significantly stimulate autophagy in GFP-LC3 MEFs compared to the control cells incubated with MEF-derived media. Importantly, knockdown of the endogenous TGFβ1 in MDA cells using shRNA clearly prevented autophagy induction in fibroblasts (Figure 36F and G). By testing autophagy markers, endogenous LC3-I/LC3-II conversion, p62 protein degradation, as well as free GFP production tests, these results were also confirmed using immunoblot technique (Figure 36H). These experiments proved that TGFβ1 which is produced and secreted by the MDA cells is one of the key rate-limiting and important factors for the activation of autophagy in fibroblasts.

In order to analyze dose and time kinetics of the cytokine, GFP-LC3 dot formation assays were performed in the presence of purified recombinant TGF β 1 protein (Rec. TGF β 1). 1 ng/ml was determined as the minimum TGF β 1 concentration that was required to induce autophagy in GFP-LC3 MEFs at 2 h and 6 h incubation times (Figure 39A and B). Autophagy induction at concentrations under these conditions was confirmed using endogenous LC3-I/LC3-II conversion, p62 protein degradation, as well as free GFP production tests (Figure 39C). To determine the minimum concentration of TGF β 1 that was capable of inducing autophagy, GFP-LC3 MEF cells were incubated for 12 h or 24 h with decreasing concentrations of TGF β 1. The lowest autophagy-inducing dose of TGF β 1 was determined as 10⁻² ng/ml at 12 h and 24 h incubation time points (Figure 39D-F).

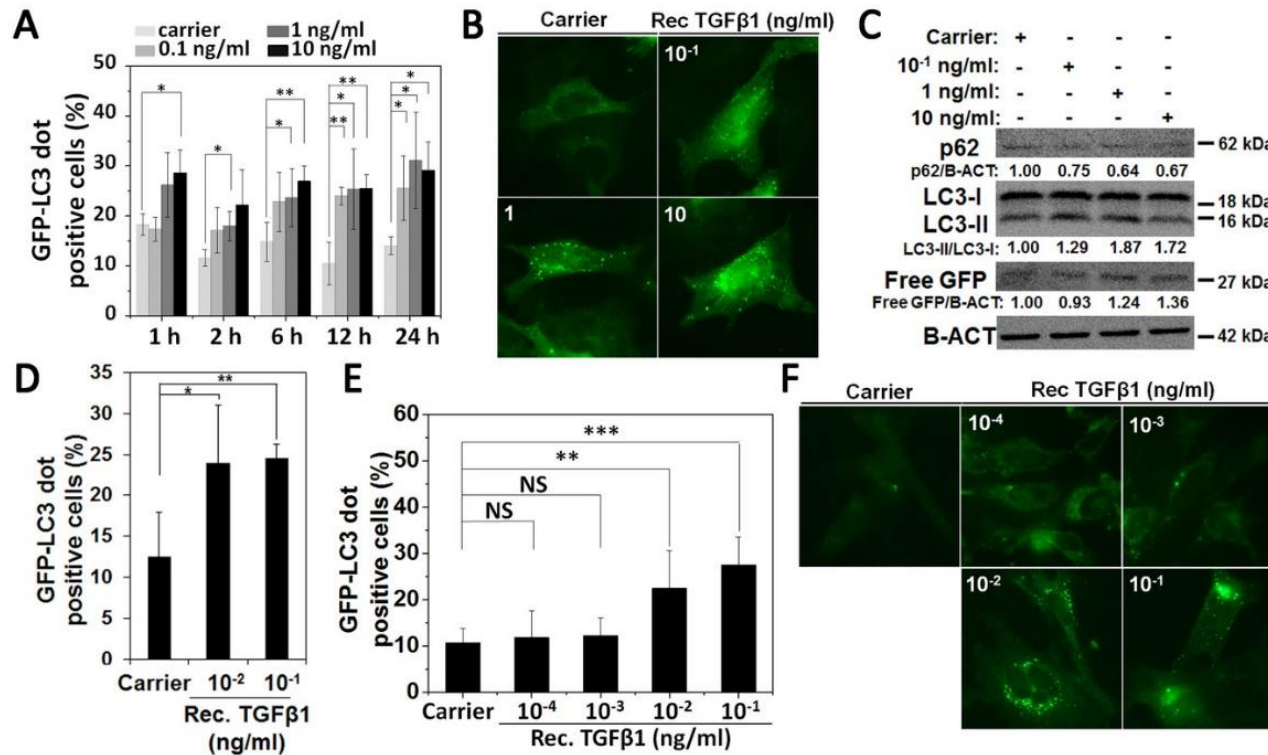


Figure 39. The effect of recombinant TGFβ1 on autophagy of fibroblast.

(A and B) Effect of recombinant TGFβ1 (Rec. TGFβ1) on autophagy of MEFs in a dose-dependent manner (10⁻¹, 1, 10 ng/ml Rec. TGFβ1). The graph represents quantification of autophagy (GFP-LC3 dots) after 1 h, 2 h, 6 h, 12 h and 24 h of incubation (mean±SD of independent experiments, n=4, *p<0.05, **p<0.01). **(C)** Immunoblot analysis for autophagy markers in MEF extracts following 24 h treatment with Rec. TGFβ1 (10⁻¹, 1 and 10 ng/ml) **(D and E)** Effect of decreasing doses of Rec. TGFβ1 on MEF autophagy. The graph represents quantification of autophagy (GFP-LC3 dots) after 12 h of incubation (mean±SD of independent experiments, n=3, *p<0.05, **p<0.01) **(D)** and 24 h (mean±SD of independent experiments, n=3; NS: Non-significant, **p <0.01; ***p <0.001) of incubation **(E and F)**.

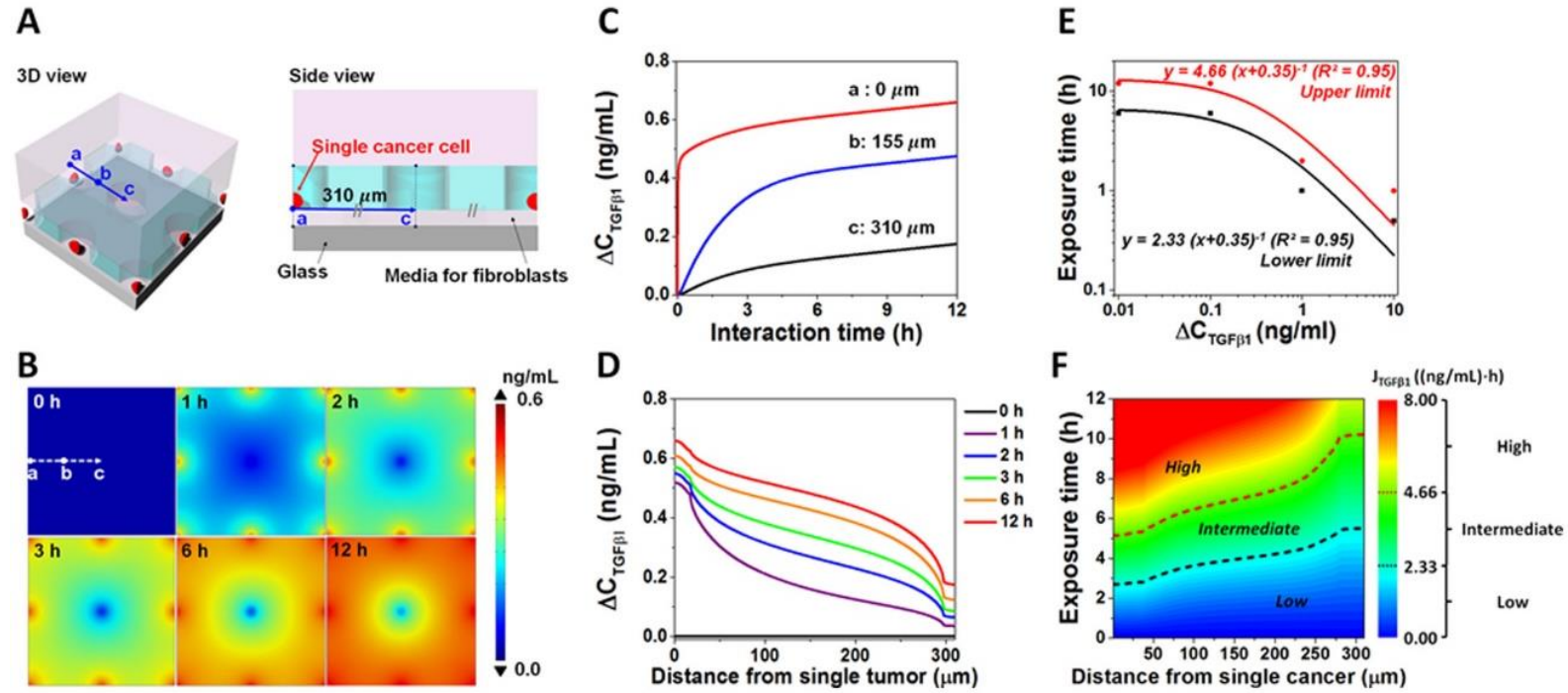


Figure 40. Numerical simulation of secreted TGF β ₁ diffusion in the chip system.

(A) Schematic diagram showing 3-dimensional and side views of DOI for a simulation scenario where an empty hole is situated near eight neighboring holes occupied by cancer cells. (B) Distributions of the concentration difference of TGF β ₁ ($\Delta C_{\text{TGF}\beta 1}$) in the fibroblast region with respect to time. (C) Time evolution of $\Delta C_{\text{TGF}\beta 1}$ at selected positions: (a) the center of the hole containing a single cancer cell, (b) the middle position between an empty hole and a tumor-occupied hole, and (c) the center of the empty hole. (D) Distribution of $\Delta C_{\text{TGF}\beta 1}$ along the path (a-c) from a cancer cell-occupied hole to an empty hole for various time. (E) Hyperbolic curve fitting (solid lines) to determine exposure condition. The minimum amount of TGF β ₁ exposure for highly probable autophagy activation was determined as the upper bound (red symbols) and the maximum amount of TGF β ₁ exposure that is unlikely to induce autophagy was determined as the lower bound (black symbols). (F) Contour plot of the TGF β ₁ exposure ($J_{\text{TGF}\beta 1}$) in (distance, exposure time)-space, where the numerical simulation was conducted. Based on $J_{\text{TGF}\beta 1} = \int_0^T (\Delta C_{\text{TGF}\beta 1} + b) dt$, the exposure condition space can be divided into high, intermediate, and low probable regimes for autophagy activation. The optimized exposure time, which is sufficiently short so as not to affect autophagy induction via TGF β ₁ diffusion from neighbor tumors and sufficiently long to induce autophagy activation at their own position, can be decided by the contour plot.

3.5.3. Numerical analysis of the diffusion of TGFβ1 secreted from a single tumor cell

In order to optimize the exposure time for autophagy in experiments, numerical simulations were conducted. The exposure time should be chosen to prevent cross-talk between holes of the membrane. Schematic descriptions of the simulation are shown in Figure 40A. A time-dependent diffusion equation for TGFβ1 was solved using COMSOL Multiphysics®. It was assumed that an empty hole is surrounded by the neighbor cancer cells. As the array of holes in the system was periodic, a single periodic unit (represented by the continuous line a-c in Figure 40A) was taken as the domain of interest (DOI) for simulation. And, the symmetric condition was applied to the boundaries.

The geometrical dimensions, including hole diameter, distance between neighboring holes, and space between the biochip membrane and glass slide, were identical to the real experimental platform. Concentration of TGFβ1 that was secreted from MDA cancer cells was around 250 pg/ml following 24 h of incubation ([238] and our observations). Using this value and time point, the secretion rate was calculated as $4.88 \times 10^{-14} \text{ mol/m}^2 \cdot \text{s}$ when the cancer cell size was assumed as 11 μm (measured by average diameters of trypsinized 100 MDA cells). The diffusion coefficient (D) of TGFβ1 in the reservoir was calculated as $6.4 \times 10^{-11} \text{ m}^2/\text{s}$, by using the equation of $D = 1.72 \times 10^{-8} (\text{M.W.})^{-0.552}$ and the molecular weight (M.W.) of TGFβ1 as 25 kDa. Furthermore, the media between the membrane and glass was assumed to be porous ($N_i = -D_{i,eff} \nabla C = -\frac{\varepsilon}{\tau} D \nabla C$, ε : porosity and τ : tortuosity) since it was packed with fibroblasts. We determined basal concentration (C_0) of TGFβ1 in the DMEM medium supplemented with various FBS concentrations (1~10 % (v/v)) using an ELISA assay (Figure 37A). When ELISA results were corrected according to cell numbers on chips (66,000 cells in 300 μl medium), TGFβ1 amount secreted from MEFs after 6 h of culture was calculated as 105.93 pg/ml. Basal concentration of TGFβ1 (C_0) was considered as the sum of TGFβ1 that comes from 3% FBS containing media (266.72 pg/ml) and MEFs in culture (105.93 pg/ml) (Figure 37). The corresponding molar density is $1.50 \times 10^{-8} \text{ mol/m}^3$.

TGFβ1 concentration difference profile ($\Delta C_{TGF\beta 1} = C - C_0$) around an empty hole (center) that is surrounded by eight cancer cell-containing hole is shown in Figure 40B. Figure 40C represents the time evolution of $\Delta C_{TGF\beta 1}$ at specified positions (a, b and c points) (Figure 40A). $\Delta C_{TGF\beta 1}$ near holes, that are occupied with a tumor cell (a), rises steeply at the beginning and then increases gradually to reach to approximately 0.66 ng/ml

at 12 h, meanwhile the concentration at the empty hole (c) increases slowly and does not reach 0.18 ng/ml even after 12 h. Figure 40D shows the distribution of $\Delta C_{TGF\beta 1}$ along the path (a-c distance in μm) in different time points.

According to our experimental results, a net 10^{-2} ng/ml TGF β 1 concentration was required to induce significant levels of MEF autophagy in our system at 12 h (Figure 39D and E); and the same concentration did not induce autophagy after 6 h incubation under similar conditions (Figure 41).

Taking these 6 h and 12 h data into consideration, concentration of TGF β 1 that was required for autophagy induction in MEFs was modeled as a function of time (Figure 40E and F). As shown in Figure 40E, the minimum amount of TGF β 1 exposure for highly probable autophagy activation was fitted as the upper bound (red symbols) and the maximum amount of TGF β 1 exposure that is unlikely to induce autophagy was fitted as the lower bound with a hyperbolic function ($T = A \cdot (\Delta C_{TGF\beta 1} + b)^{-1}$). The parameter of A corresponds to the accumulated amount of TGF β 1 in constant secretion condition and b is the fitting parameter. The contour plot of exposure amount for TGF β 1 ($J_{TGF\beta 1}$), which is divided into high, intermediate, and low probable regimes for autophagy activation, was drawn by integrating $\Delta C_{TGF\beta 1}$ with respect to exposure time ($J_{TGF\beta 1} = \int_0^T (\Delta C_{TGF\beta 1} + b) dt$) and the distance from single cancer cell (Figure 40F). Optimized exposure time was decided in the light of these results. 6 h exposure time was long enough to induce high to intermediate levels of autophagy in MEFs around a defined tumor cell (a to b: 0 to 155 μm). Yet, at this time point, autophagy in MEFs around neighboring

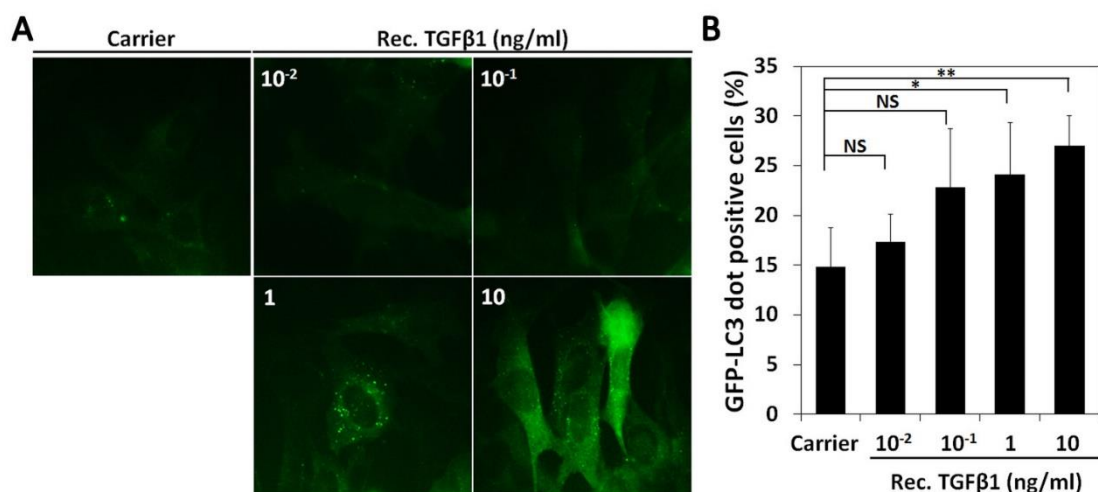


Figure 41. The effect of recombinant TGFβ1 on autophagy of fibroblast.

(A and B) Effect of decreasing doses of Rec. TGFβ1 on MEF autophagy (10⁻², 10⁻¹, 1 and 10 ng/ml Rec. TGFβ1). The graph represents quantification of autophagy (GFP-LC3 dots) after 6 h of incubation (mean±SD of independent experiments, n=4, NS: Non-significant, *p<0.05, **p<0.01)

holes was still at low levels (b to c: 155 to 310 μm). Therefore, 6 h time point was chosen and used in the biochip experiments.

3.5.4. Single cell-level monitoring of autophagy in fibroblasts interacting with a cancer cell

In the light of tumor–stroma autophagy model, it is crucial to confirm whether autophagy activation in response to tumor-secreted TGFβ1 in MEFs followed a gradient. In order to reduce heterogeneity in the system, using chloroquine (CQ, lysosomal pH modifier) or rapamycin (Rapa, an mTOR inhibitor and autophagy inducer), control vs TGFβ1 signal was tried to be improved. Unfortunately, there was no improvement for the enhancement of the signal (Figure 42).

Next, monoclonal of both MDA cancer cells and GFP-LC3 MEF cells were created. These monoclonal cells were similar to parental cells with respect to their TGFβ1 secretion, and autophagic response to TGFβ1 treatment and starvation (Figure 43).

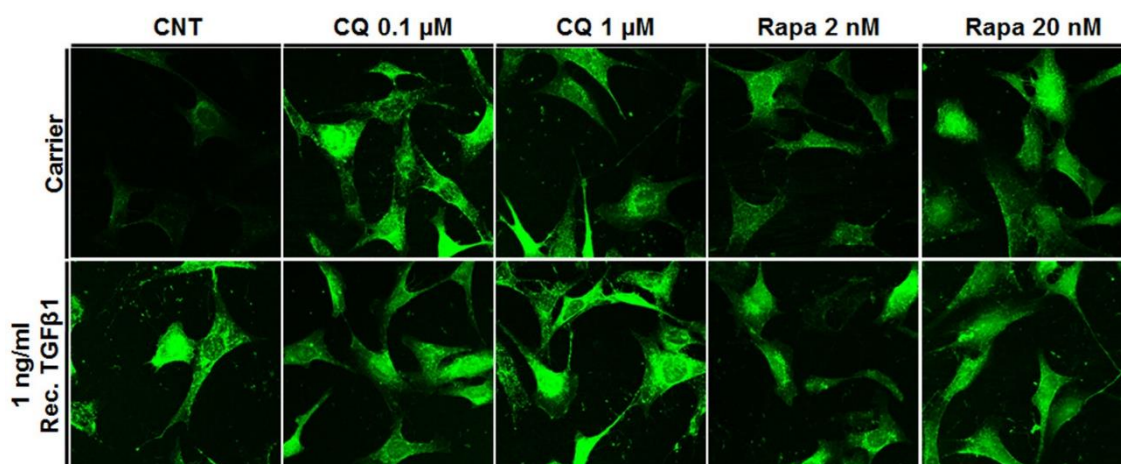


Figure 42. Autophagy analysis of MEFs in the presence or absence of chloroquine (CQ, 0.1 μ M or 1 μ M) or Rapamycin (Rapa, 2 nM or 20 nM) with or without recombinant TGF β 1 protein (1 ng/ml).

CNT, no addition of CQ or Rapa. Carrier, 4 mM HCl, 0.1% BSA.

In the biochip system, MEF monoclonal cells were cultured alone or with single MDA monoclonal cells captured in holes. Images of autophagy activation were taken from 3 different "region of interests" depending on the distance from the center of a hole. Cells that were found at a 50 μ m (S), 100 μ m (M), 155 μ m (L) radial distance from a hole were evaluated. Percentage of GFP-LC3 dot positive cells in these 3 regions were quantified in a cumulative manner (Figure 44).

According to the analysis, GFP-LC3 dot positivity in MEFs followed a clear gradient on biochips. MEFs that were closer to tumor cell-occupied holes had higher autophagic activity, compared to those that were farther away. However, lowest standard deviation levels and more homogenous autophagic response were obtained when autophagy activation in cells that were within 155 μ m (L) radial distance were quantified. As expected, MEFs around empty holes did not activate significant levels of autophagy. Therefore, autophagy activation in biochips followed a gradient and was not random. Activation in empty holes most probably reflected basal autophagic activity in the cells. In the light of these results, we decided to evaluate autophagy activation in MEFs at a 155 μ m radial distance from holes.

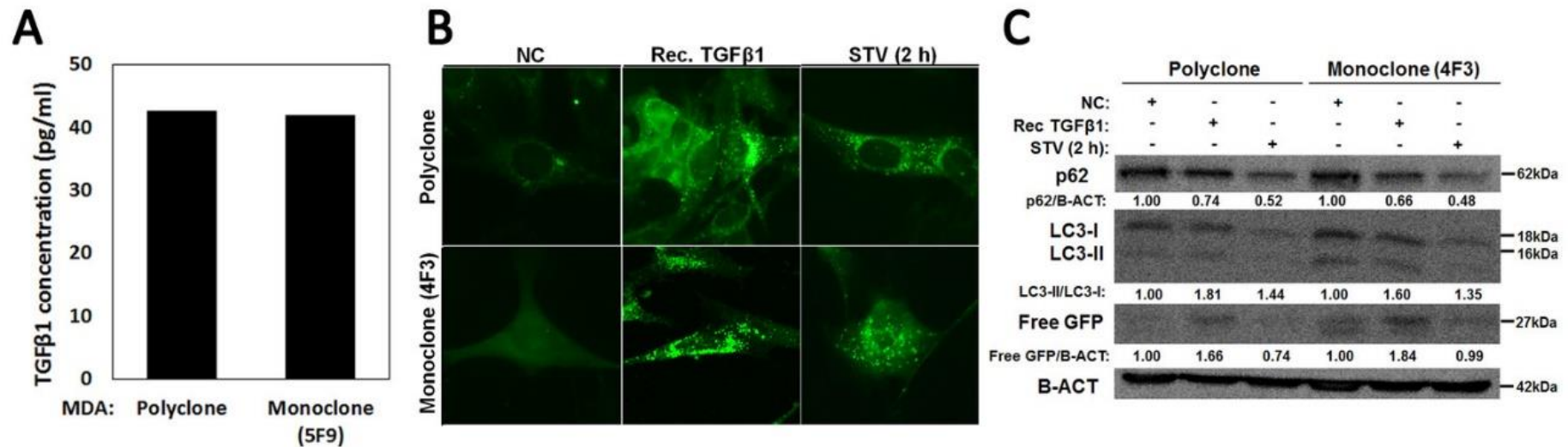


Figure 43. Characterization of MDA-RFP monoclonal cancer cells and GFP-LC3 MEF monoclonal cell.

(A) Analysis of TGFβ1 secretion from MDA poly or monoclonal (5F9 monoclonal was shown) (average of two independent experiments). Cells were cultured in low-FBS containing media for 6 h. Supernatant of the cells were analyzed using the ELISA assay. **(B)** Microscopy of GFP-LC3 dots in MEF poly or monoclonal (4F3 monoclonal was shown). Cells were treated with 1 ng/ml recombinant TGFβ1 for 6 h or starved for 2 h in order to induce autophagy. **(C)** Autophagy markers were analyzed in MEF poly or monoclonal (4F3) using immunoblotting technique. Cells were treated with 1 ng/ml recombinant TGFβ1 for 6 h or starved for 2 h in order to induce autophagy. B-ACT was used as loading control. Densitometric analysis of the bands was performed using Image J software.

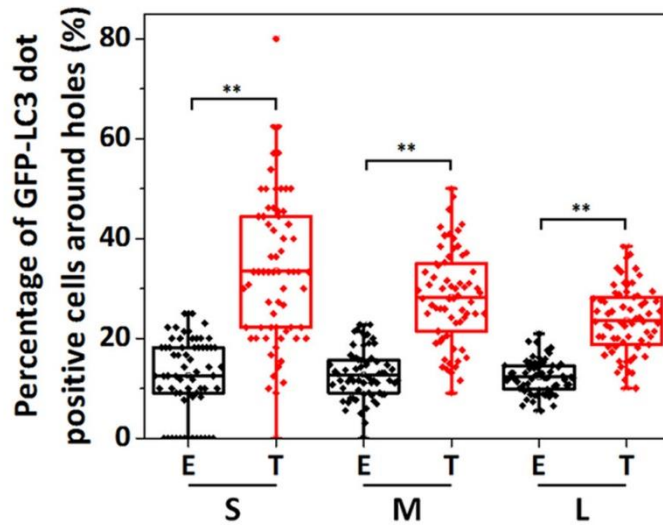


Figure 44. Autophagy analysis in biochips.

Autophagy in MEFs was analyzed in areas of 50 μm (S), 100 μm (M), 155 μm (L) diameter from the center of empty (E) or single cancer cell-captured (T) holes (mean $\pm$ SD of independent chip experiments, ** $p < 0.01$, $n=3$).

Next, time kinetics experiments of cells were performed in biochips. Autophagy activation in MEFs on biochips were analyzed at 1 h, 2 h, 3 h and 6 h. The most significant autophagy activation was obtained after 6 h of co-culture (Figure 45A and B). A live cell imaging video of real-time dynamics of autophagy activation in fibroblasts were also recorded (provided in enclosed DVD of this thesis, Movie 1).

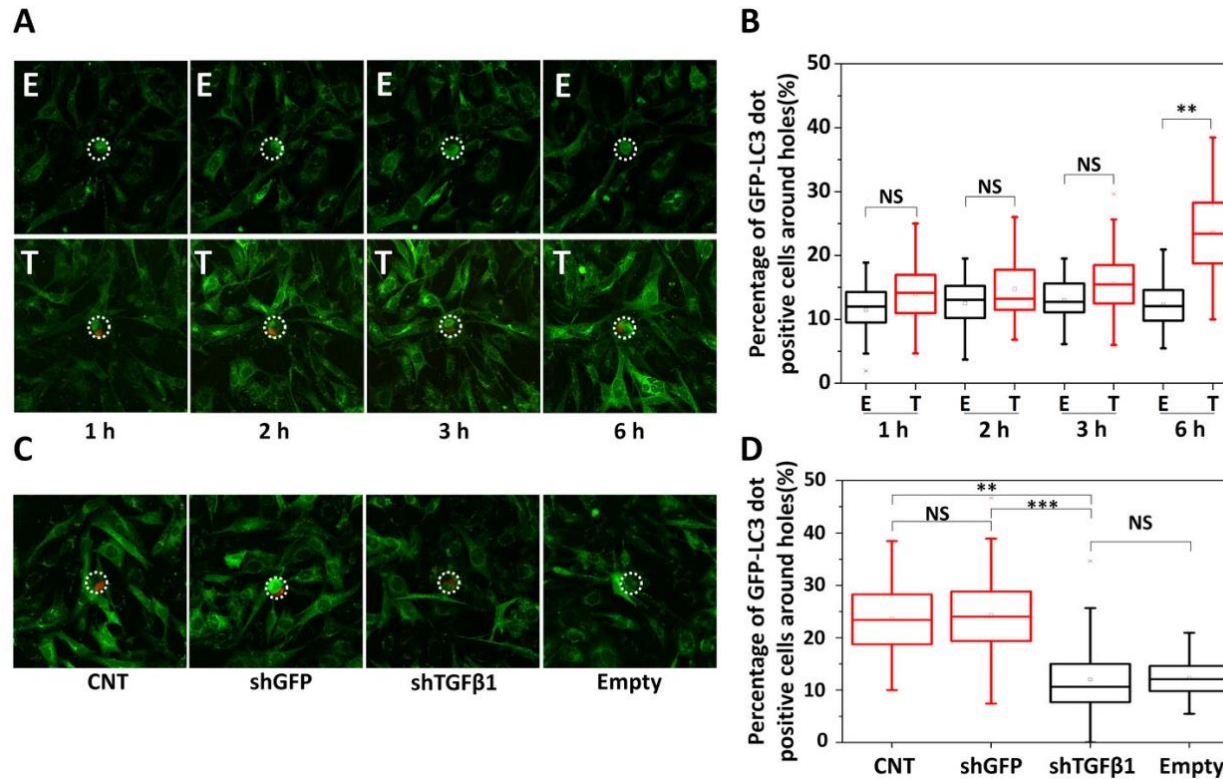


Figure 45. Single cell-level monitoring of autophagy in fibroblasts interacting with individual cancer cells.

(A) Live cell images of dynamics of autophagy activation in fibroblasts that are near an empty hole (E) or a hole with a trapped single tumor cell (T). (B) Box plot representation of GFP-LC3 dot quantification of fibroblasts near an empty hole (E) or a hole with a tumor cell (T) (1 h, 2 h, 3 h and 6 h co-culture). After 6 h of intercellular interactions, autophagy near holes with trapped tumor cells (Number of holes = 68) could well be differentiated from those near empty holes (Number of holes = 65). (C) Images of autophagy activation in MEFs near a hole with a wild-type MDA cell (CNT) (Number of holes = 68), shGFP infected MDA cell (shGFP) (Number of holes = 89), shTGFβ1 infected MDA cell (shTGFβ1) (Number of holes = 84) and empty holes (Number of holes = 65). (D) Quantification of autophagy activation in MEFs shown in C. MEFs near shTGFβ1 infected MDA cells (shTGFβ1) had lower autophagy levels (comparable to empty holes (Empty)) compared to those near wild-type (CNT) or shGFP infected MDA cells (shGFP). Images were analyzed by Imaris software (3 independent experiments, p-value was calculated by results of each set, NS: Non-significant, **p<0.01, ***p<0.001).

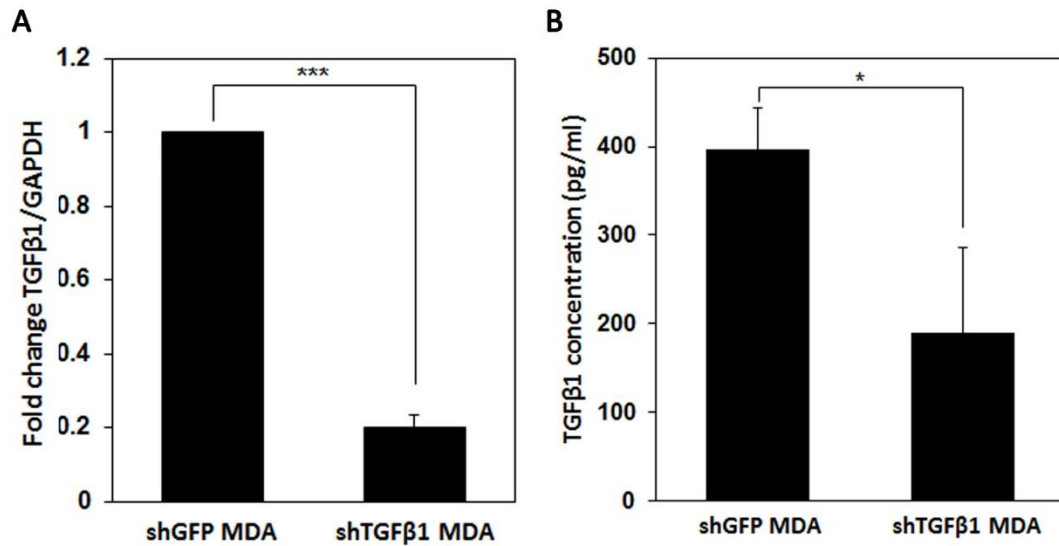


Figure 46. Analysis of TGFβ1 mRNA and secreted protein levels in with shGFP (control) or shTGFβ1 virus-infected MDA cells after 72 h.
(A) mRNA level of TGFβ1 was analyzed using qPCR (mean±SD of independent experiments, ***p < 0.001, n=3). **(B)** Secreted TGFβ1 concentration (pg/ml) in the media (mean±SD of independent experiments, *p < 0.05, n=3).

The role of TGFβ1 in cancer cell-stroma interaction was tested according to the following optimization of the autophagy detection system on biochips. MDA cells were infected with control shRNA (shGFP) or shTGFβ1 constructs and GFP-LC3 MEF autophagy induction levels were quantified. In line with our results in *in vitro* cell culture dishes, TGFβ1 knockdown in MDA cells (Figure 46) did significantly attenuate autophagy activation in MEFs on biochips (Figure 45C and D).

3.5.5. Facile retrieval of single tumor cells and downstream molecular analysis

In order to demonstrate that the proposed platform could identify and isolate autophagy-inducing single cancer cell clones, the following blind tests were performed

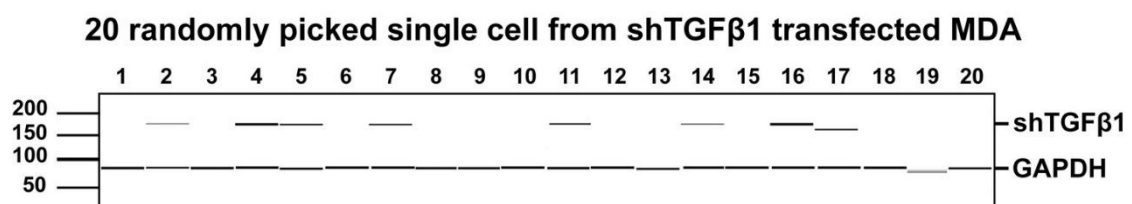


Figure 47. Analysis of MDA cells 72 h after infection with lentiviruses containing shTGFβ1.
 Only 8 out of 20 randomly selected MDA cancer cells contained shTGFβ1 sequence in their genome although the viral infection efficiency was near 80%.

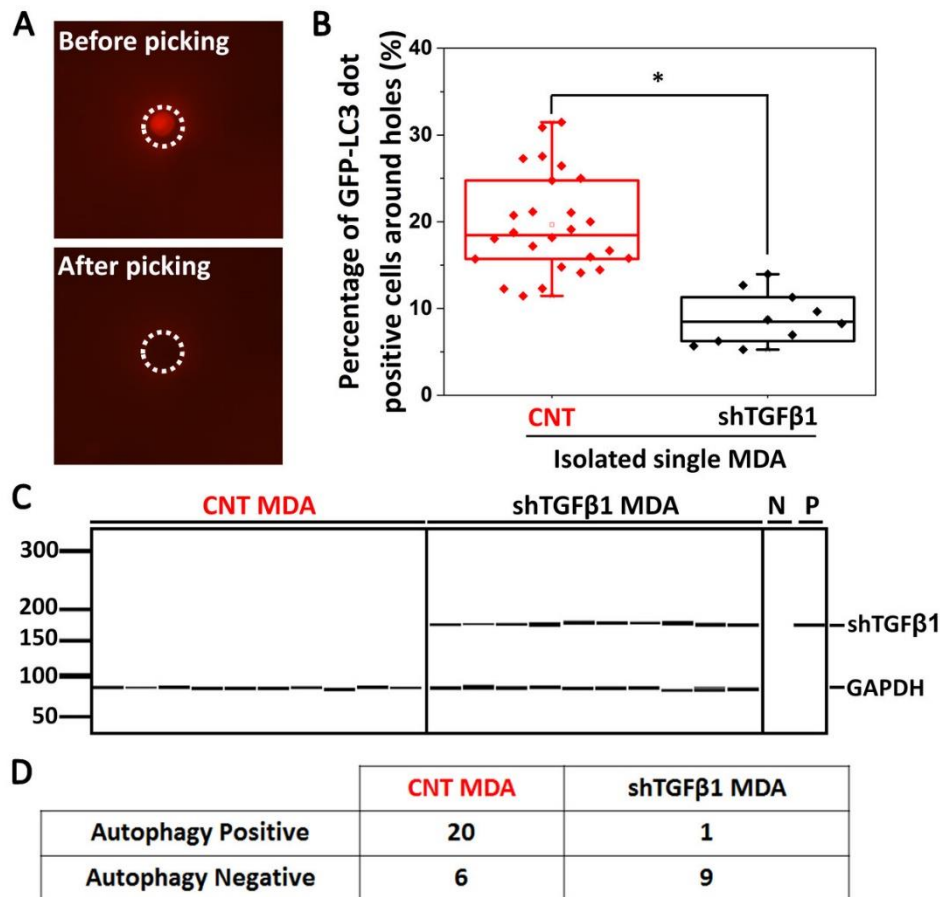


Figure 48. Retrieval and characterization of single tumor cells.

(A) Using a single cell-picking device, individual cancer cells were retrieved and used for further characterization. (B) Box plots depicting percentage of autophagic, GFP-LC3 dot positive, MEFs around (155 μ m diameter) single MDA cancer cell-containing randomly selected holes. Number of holes = 36. PCR analysis for the genomic DNA of single cancer cells was performed to confirm the presence or absence of the shTGF β 1 vector. Then, GFP-LC3 dot positivity was evaluated and results were depicted as shown in the graph. (C) Representative PCR results showing shTGF β 1 positivity or negativity of MDA cells that were analyzed in (B). 10 representative amplification results from wild-type MDA cells (CNT MDA) and shTGF β 1 MDA cells were shown. N represents negative control without template and P represents positive control using the shTGF β 1 vector (3 independent experiments, * p <0.05). (D) Summary of the results shown in (B) and (C).

by using a mixture of wild-type MDA cells and shTGF β 1 infected MDA cells. The single cell analysis of the MDA shTGF β 1 cells after viral infection was used to decide the ratio of cells in the mixture. As shown in Figure 47, only 8 out of 20 (40%) single MDA cells show the shTGF β 1 band although 80% infection efficiency was observed. Therefore, a mixture of wild type MDA and shTGF β 1 MDA cells with 1:4 ratio was used for blind test.

After 6 h of co-culture between cancer cells and fibroblasts on biochips, GFP-LC3 dot status of MEFs were documented. Regardless of their autophagy status, randomly

chosen single MDA tumor cells were collected from the chips using a microscope-based single-cell picking device (Figure 48A). Genomic DNA isolation from the picked cells was performed. Using primers that are specific for the shRNA vector, PCR amplification and identification of the TGF β 1 shRNA sequence in the vector were performed. PCR results confirmed that out of 36 randomly selected single cancer cell clones, 10 cells contained the shTGF β 1 sequence in their DNA. When blindly taken cell pictures were analyzed retrospectively, most MEF cells with less or no autophagy were around holes with MDA cells containing the shTGF β 1 sequence (Figure 48B and C).

The cut-off value was for the percentage of GFP-LC3 dot positive MEF cells near a hole was previously decided as 15% based on the capacity of the biochip system to differentiate the CNT MDA cells from shTGF β 1 containing MDA cells with a highest accuracy (82.23% in Figure 45D). In other words, in order to label a hole as autophagy positive, our criteria were decided that the hole should have around it (155 μ m radius area) more than 15% of the MEFs containing >20 GFP-LC3 dots per cell. Among 36 MDA cells which were randomly picked-up from 3 independent chips, pictures of MEF cells that were around 21 holes showed higher autophagy signals than the cut-off value, so they were considered as autophagy positive (Figure 48B and C). Among cancer cells retrieved from these 21 positive holes, 20 cells were indeed CNT cells and only one cell was shTGF β 1 positive. Remaining 15 holes were autophagy negative. 9 of 15 cells were indeed shTGF β 1 positive MDA cells (Figure 48B and C), whereas 6 cells were identified as CNT MDA cells. Figure 48D summarizes the results of these tests.

Therefore, the sensitivity (or, true positive rate) which is defined as the percentage of the holes, whose signal were higher than the cut-off value among total number of holes containing wild type tumor cells, was 77% (20/26). On the other hand, the specificity (or, true negative rate), which is defined as the percentage of the holes whose signal were lower than the cut-off value among total number of holes containing shTGF β 1 MDA cells, was 90% (9/10). As a result, we can differentiate CNT MDA and shTGF β 1 MDA cells based on their single-cell level capability of inducing autophagy in neighboring fibroblasts with 81% accuracy. These results suggest that our platform can be used in shRNA-based screens of cancer cells and allow a high-throughput study of the mechanisms of autophagy activation in fibroblasts in a tumor microenvironment.

3.6. RESULTS IV – The role of CT-1 during Tumor-Stroma Communication

According to the result of cytokine screening, cardiotrophin-1, CT-1, was one of the critical factor which is novel and caused autophagy induction in fibroblast cells (Figure 49). In this section of the thesis, the role of CT-1 in tumor-stroma communication will be analyzed *in vitro* and *in vivo*.

From *in vitro* perspective, its expression and secretion from different cancer cell types, its role as an autophagy inducer in fibroblasts, the role of its neutralization antibody on the cancer cell growth in mono- and co-culture systems with fibroblasts, its effect on α -sma production from cancer cells, and metabolic alterations in fibroblast cells which were treated with CT-1 will be analyzed, in detail.

From *in vivo* perspective, its role on cancer cells growth *in vivo*, and its expression in breast cancer patients who are in different stages of cancer will be examined.

3.6.1. CT-1 and its role during tumor-fibroblast communication *in vitro* analysis

3.6.1.1. CT-1 expression and secretion from different types of cancer cells

CT-1 is a member of interleukin-6 (IL-6) family which includes IL-6, leukemia-inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM) and IL-11 [189]. CT-1 was mainly studied in the heart where it was shown to have cardioprotective effects [239]. However, it is also expressed in several other tissues and organs, including liver, kidney, lung and skeletal muscle [185]. Several studies in literature involved CT-1 in a number of biological phenomena, including cell survival, proliferation, and tissue regeneration [2].

In order to confirm that CT-1 is expressed in cancer cells, its expression was checked in Huh7, MCF7 and PC3 cancer cell extracts and was detected clearly (Figure 50A). Furthermore, cancer cells were stained with CT-1 antibody and vesicle-like structures in these cells were observed (Figure 50B), and ELISA test revealed that CT-1 was secreted by all three cancer cell types at significantly higher levels than MEFs (Figure 50C). Moreover, CT-1 receptors gp130 and LIFR β were expressed in MEFs (Figure 50D).

All these data showed that cancer cells were a major source of CT-1 and cancer-secreted cytokine might affect MEF autophagy through its receptors on these cells.

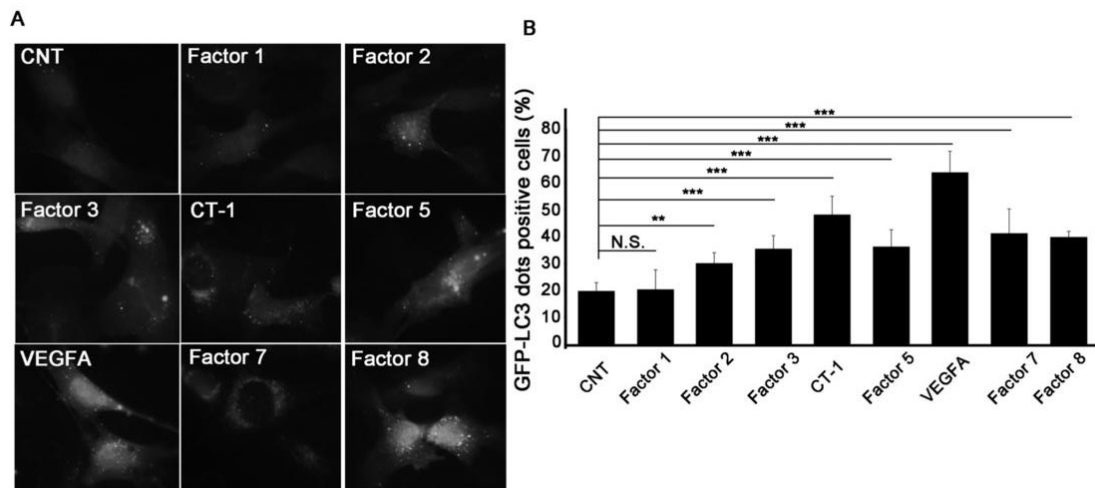


Figure 49. Cardiotrophin-1 (CT-1) is one of the critical factors which induce autophagy in fibroblast cells

(A) Microscope analysis of all candidate factors. (B) Quantifications of all candidate factors (mean±SD of independent experiments, n=3, **<0.01, ***<0.001).

3.6.1.2. Functional confirmation of CT-1 as an autophagy inducer in fibroblasts

To further confirm that CT-1 is indeed an autophagy inducer in fibroblasts, GFP-LC3 MEFs were treated with HEK 293T-derived recombinant CT-1 in the presence of a control antibody (CNT ab) or CT-1-specific neutralizing antibody (CT-1 Neut ab). Indeed, CT-1-stimulated autophagy in MEFs was inhibited significantly following incubation with the neutralizing antibody, confirming the cytokine was responsible for the observed effect (Figure 51A and B). A recombinant purified CT-1 protein could activate autophagy in a dose- and time-dependent manner (Figure 51C-F). Interestingly, treatment with 25 ng/ml CT-1 for 60 min could induce autophagy in MEFs that was comparable with levels obtained with Torin-1 (an mTOR inhibitor and a classical inducer of autophagy). A time-dependent and CT-1-mediated STAT3 phosphorylation was observed in MEFs, confirming that pathways that are downstream to CT-1 receptors were activated under these conditions (Figure 51G).

Therefore, all these data proved that CT-1 is a potent autophagy-inducer cytokine.

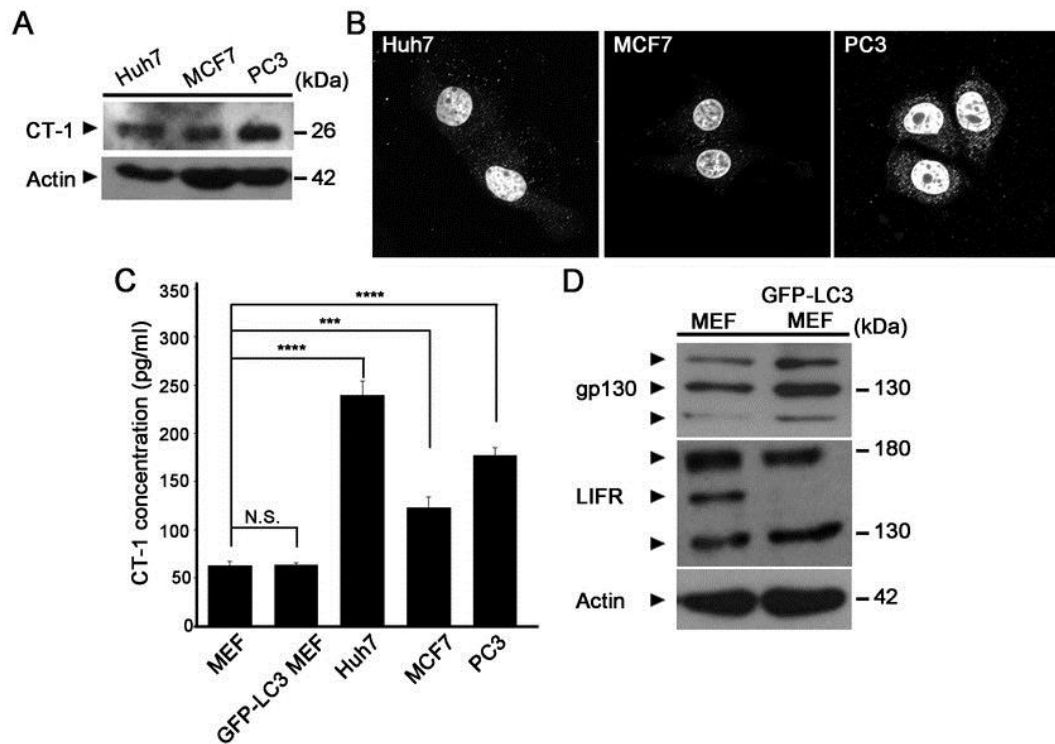


Figure 50. CT-1 was expressed and secreted from different cancer types. Moreover, its receptors, gp130 and LIFR, were detected in fibroblasts.

(A) Immunoblot analysis of CT-1 expression in different cancer types. **(B)** Immunostaining of CT-1 in different cancer types. **(C)** Both MEF and GFP-LC3 MEF cells have CT-1 receptors, namely gp130 and LIFR. **(C)** ELISA analysis of CT-1 secretion from different cancer types. Cancer cells were cultured in low-FBS for 72 h. Secreted CT-1 amounts were plotted according to standards (mean $\pm$ SD of independent experiments, n=3, ***p<0.001; ****p<0.0001).

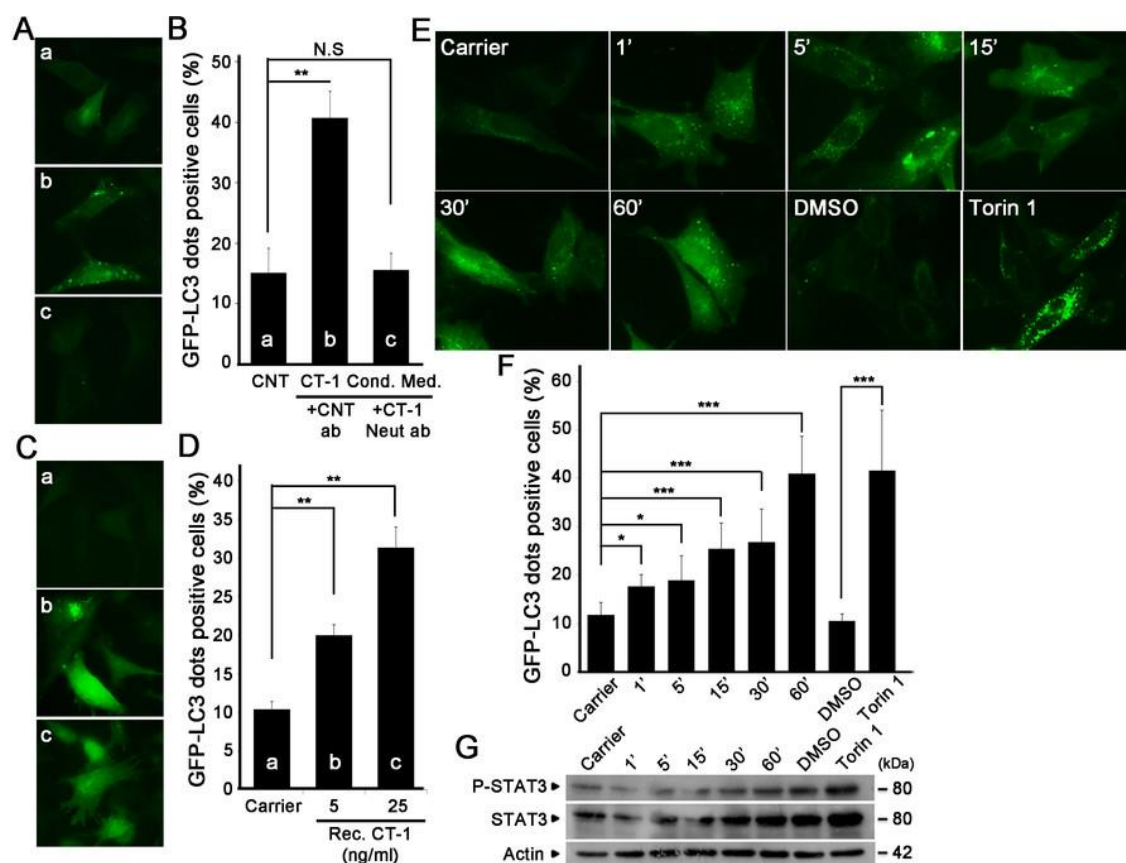


Figure 51. Effect of CT-1 conditioned medium or recombinant protein of CT-1 on autophagy in fibroblast cells.

The effect of CT-1 on autophagy in fibroblast cells is time- and dose-dependent. **(A)** Effect of CT-1 neutralizing antibody (c, CT-1 Neut ab) or control antibody (b, CNT ab) on fibroblasts autophagy during incubation with HEK 293T-derived CT-1 conditioned media (cond. med.). pcDNA3 overexpressed HEK 293T cells conditioned medium was used as control (CNT) (a). **(B)** Quantification of experiment (A) (mean±SD of independent experiments, n=3, *p<0.05, NS: not significant). **(C)** Effect of different concentrations of CT-1 recombinant protein on autophagy in fibroblast cells. **(D)** Quantification of experiment (C) (mean±SD of independent experiments, n=3, *p<0.05; **<0.01). **(E)** Effect of recombinant protein of CT-1 on autophagy in fibroblast cells depending on time. **(F)** Quantification of experiment (E) (mean±SD of independent experiments, n=4, *p<0.05; **<0.01). **(G)** STAT3 phosphorylation in fibroblast cells after treatment with CT-1 recombinant protein.

3.6.1.3. Effect of CT-1 neutralization antibody on cancer cells growth in mono- and co-culture systems

In order to understand whether cancer cell growth is CT-1 dependent or not, neutralization antibody of CT-1 was tested for the growth of cancer cells. According to the results, cancer cells, MCF7-RFP and MDA-RFP breast cancer cells, did not show any growth reduction in the presence of CT-1 neutralization antibody within low-FBS containing medium in time-dependent manner comparing to control antibody treated cancer cells growth (Figure 52A and Figure 53A).

Next, the growth of MCF7-RFP cancer cells was tested when these cancer cells were co-cultured with autophagy-competent fibroblast cells in the presence of CT-1 neutralization antibody. Initial tests were performed with low concentration of CT-1 neutralization antibody concentration (3 µg/ml) which was suggested by the company (R&D) to reduce the activity of CT-1. Although it was tried by three different Ph.D. students (by me, by Dr. Kumsal Ayşe Tekirdağ Koşar, previous Ph.D. student of Dr. Devrim Gözüa ık, and by Yunus Akko , Ph.D. student of Dr. Devrim Gözüa ık), different results were obtained. Technically, MCF7-RFP cancer cells were mono-cultured or co-cultured with fibroblast cells in the presence of neutralization antibody (it is added only fibroblast co-culture conditions in order to prevent crosstalk). After 72 h, cells are fixed and MCF7-RFP cancer cells were quantified under the microscope by counting randomly selected 20 different fields. According to all these three results, co-culturing MCF7-RFP cancer cells with autophagy-competent fibroblast cells were promoting the growth of cancer cells. However, only according to Yunus's result, the growth of cancer cells in co-culture system was significantly reduced upon the treatment of CT-1 neutralization antibody (Figure 52D). In my results and Kumsal's result, there was tendency; however, no significant reduction was observed (Figure 52B and C).

Next, the dose kinetics was performed for optimizing the concentration CT-1 neutralization antibody. According to the results, 6 µg/ml concentration of CT-1 neutralization antibody was decided as minimum concentration which can reduce the growth of cancer cells in co-culture system at 72 h (Figure 52E).

Then, the longer time points (24 h, 48 h, 72 h and 96 h) were tested for the growth of cancer cells in co-culture systems under the microscope. According to the microscope results, significant reduction of the cancer cell growth was observed at 72 h and 96 h when 6 µg/ml CT-1 neutralization antibody concentration was used while cancer cells were co-cultured with fibroblast cells (Figure 52F and G).

In order to confirm the results obtained with MEF cells, MCF7- Luc cancer cells were co-cultured with human breast carcinoma associated fibroblast cells (CAFs, isolated by Yunus Akkoç) and their growth were analyzed in the presence of control/CT-1 neutralization antibody at 72 h by Yunus Akkoç. MCF7-Luc stable cells which express luciferase in their genome were used. Luciferase assay, which is a simple and highly sensitive system. In this system, the light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. Firefly luciferase, a monomeric 61 kDa protein, catalyzes luciferin oxidation using ATP-Mg²⁺ as a co-substrate. In the conventional assay for luciferase, a flash of light is generated that decays rapidly after the enzyme and substrates are combined [240]. As a result, from technical point, the more cancer cells proliferate, the higher signals will be detected by machine. However, no significant difference was detected between the growth of MCF7-Luc cancer cells which were co-cultured with human CAFs in the presence of CT-1 neutralization antibody, and the growth of MCF7-Luc cancer cells which were co-cultured with human CAFs in the presence of control antibody at 72 h (Figure 52H). Longer time points can be tested after CT-1 neutralization antibody kinetics test.

The same experimental set-up was tested using MDA-RFP cancer cell growth in order to understand whether the observation is cell-type specific or not. Similar to MCF7-RFP cancer cell growth, mono-culture growth of MDA-RFP cancer cells did not show significant difference upon the treatment of CT-1 neutralization antibody (6 µg/ml was used) (Figure 53A). However, when MDA-RFP cancer cells were co-cultured with autophagy-competent fibroblast cells, no significant growth difference was observed between mono-cultured MDA-RFP cancer cells and co-cultured MDA-RFP cancer cells with fibroblast cells. Moreover, the treatment of CT-1 neutralization antibody did not change the growth of cancer cells (Figure 53B and C). MDA mCherry cells (bought from Cell. BioLabs) gave the same result with MDA-RFP cancer cells (Figure 53D).

Thus, co-culture growth pattern of MCF7-RFP (estrogen receptor (ER) +, progesterone receptor (PR) +, HER2-) cancer cells are different from MDA-RFP (triple negative) cancer cells. This observation may be relevant with the presence of ER and PR.

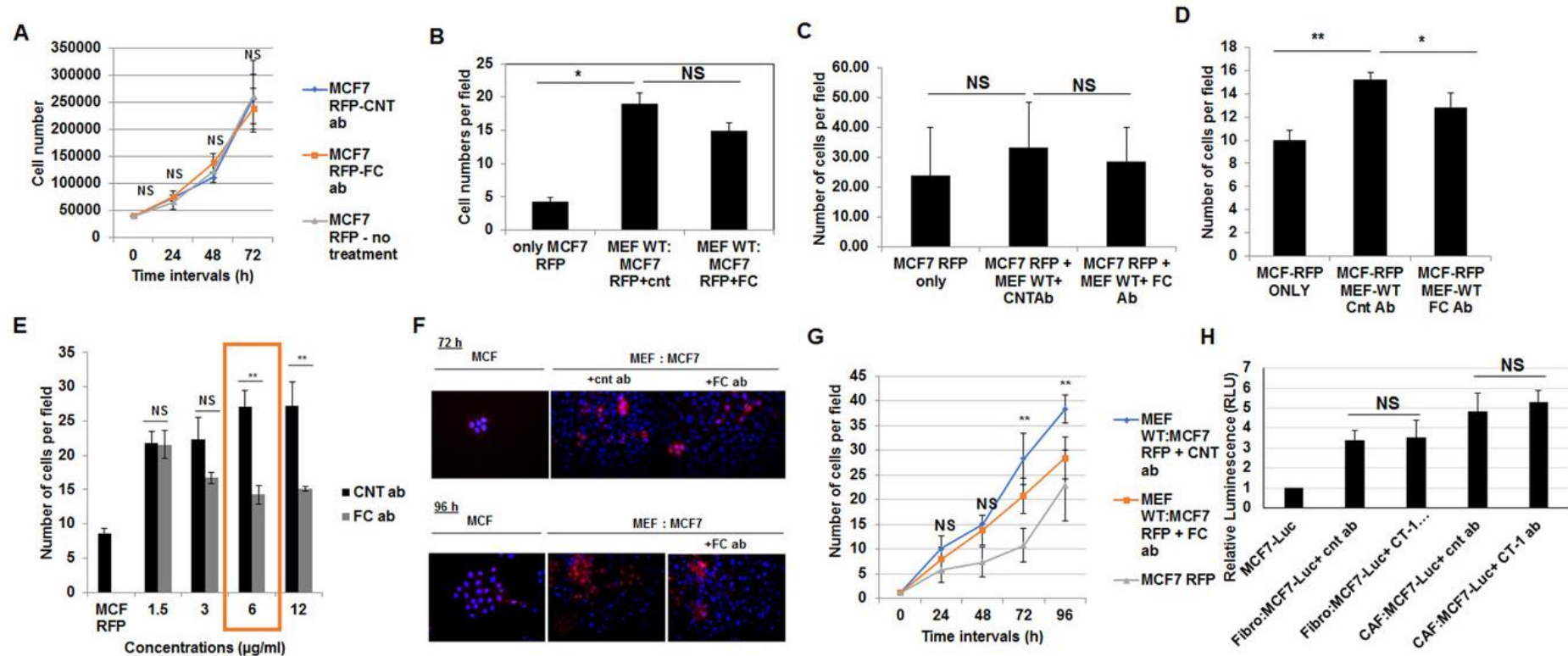


Figure 52. Growth of MCF7-RFP cancer cells in CT-1 neutralization antibody containing medium in the presence or absence of autophagy competent fibroblast (MEF WT) cells.

(A) Growth of MCF7 breast cancer cells in low-FBS medium in the presence of 6 µg/ml concentration of CT-1 (FC) neutralization antibody or control antibody for 24 h, 48 h and 72 h. (B) Growth analysis of MCF7-RFP cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 3 µg/ml concentration of CT-1 neutralization antibody or control antibody for 72 h under fluorescence microscope by counting 20 different fields (n=3, performed by Ezgi). (C) Growth analysis of MCF7-RFP cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 3 µg/ml concentration of CT-1 neutralization antibody or control antibody for 72 h under fluorescence microscope by counting 20 different fields (n=3, performed by Kumsal). (D) Growth analysis of MCF7-RFP cancer

cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 3 µg/ ml concentration of CT-1 neutralization antibody or control antibody for 72 h under fluorescence microscope by counting 20 different fields (n=4, performed by Yunus). **(E)** CT-1 neutralization antibody concentration test for the growth analysis of MCF7-RFP cancer cells when these cells were co-cultured with autophagy competent MEF cells in the presence of CT-1 neutralization antibody or control antibody for 72 h. **(F)** Microscope analysis for the growth of cancer cells when these cells were co-cultured with autophagy competent MEF cells in the presence of CT-1 neutralization antibody (6 µg/ml) or control antibody for 72 h. **(G)** Growth analysis of MCF7-RFP cancer cells when these cells were co-cultured with autophagy competent MEF cells in the presence of CT-1 neutralization antibody or control antibody for 24 h, 48 h, 72 h and 96 h. **(H)** Growth analysis of MCF7-Luc cancer cells when co-cultured with human fibroblasts or CAF cells in the presence of CT-1 neutralization antibody (6 µg/ml) or control antibody for 72 h by Luciferase assay (mean±SD of independent experiments, n=4, NS: not significant).

(Figure 54A and B). FACS experiments were performed for 72 h and 96 h of co-culture. According to the results of FACS analysis, MEF WT cells supported the growth of cancer cells more than (*Atg5^{-/-}*) MEF cells at 96 h (Figure 54C). To understand whether supportive feature of fibroblast cells in co-culture with cancer cells is cell type specific or not, the same experimental set-up was tested with MDA-RFP cancer cells. According to microscope analysis, MDA-RFP cancer cell line showed different growth pattern. These cancer cells grew independent from the presence of fibroblast cells. Autophagy deficient or autophagy competent fibroblast cells did not affect the growth of cancer cells during co-culture experiments at 24 h, 48 h and 72 h (Figure 54D and E).

As a result, the observation about MCF7 cells may be ER, PR dependent and also requires further confirmation with human CAF cells to highlight the importance of autophagy during co-culture system.

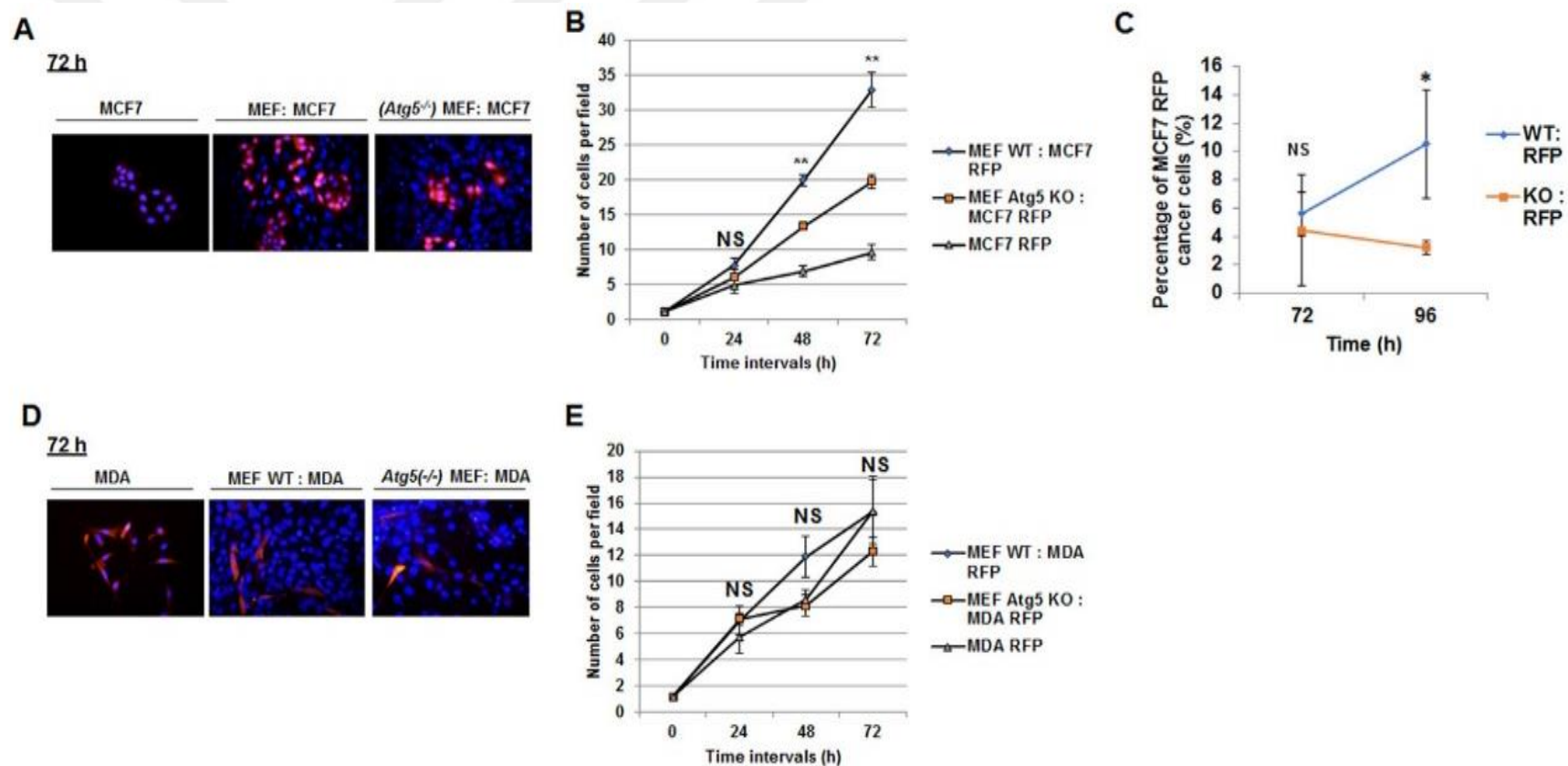


Figure 54. Growth analysis for MCF7-RFP or MDA-RFP cancer cells when these cells were co-cultured with autophagy competent MEF WT or with autophagy deficient (*Atg5^{-/-}*) MEF cells.

(A) Growth of MCF7-RFP cancer cells co-cultured with autophagy competent MEF WT or with autophagy deficient (*Atg5^{-/-}*) MEF cells. **(B)** Quantification of the experiment **(A)** (mean±SD of independent experiments, n=3, *p<0.05; **<0.01; NS: not significant). **(C)** FACS analysis for the growth of MCF7-RFP cancer cells co-cultured with autophagy competent MEF WT or with autophagy deficient (*Atg5^{-/-}*) MEF cells (mean±SD of independent experiments, n=3, *p<0.05; NS: not significant). **(D)** Growth of MDA-RFP cancer cells co-cultured with autophagy competent MEF WT or with autophagy deficient (*Atg5^{-/-}*) MEF cells. **(E)** Quantification of the experiment **(D)** (mean±SD of independent experiments, n=4, NS: not significant).

3.6.1.5. Production of α -sma protein in MEF *Atg5*^{-/-} cells, which is absent in normal conditions, upon CT-1 and TGF β 1 treatment

Carcinoma-associated fibroblast cells are one of the activated fibroblast cells in the stroma. They support growth and progression of the cancer cells with their pro-tumorigenic effects. These cells shared similarities with fibroblast cells which were activated by inflammatory response during wound healing [108]. Although their origin is controversial, transdifferentiating normal fibroblast cells is one of the formations of CAF cells [133]. Comparative gene expression analysis showed that there are several proteins which are expressed differently in their protein expression between normal fibroblasts (NFs) and CAFs. CAFs activates expression of fibroblast-specific protein (FSP-1), fibroblast-activating protein (FAP), vimentin, tenascin-C, and alpha-smooth muscle actin protein (α -sma) which are typical markers for activated fibroblast cells [94, 241].

When α -sma expression levels of MEF WT and (*Atg5*^{-/-}) MEF cells were analyzed, it was interesting to discover that (*Atg5*^{-/-}) MEF cells do not produce α -sma under normal conditions. In order to stimulate their α -sma expression, CT-1, TGF β 1 and combined treatments were performed.

First of all, high concentration of TGF β 1 (1 ng/ml) and low concentration of CT-1 (5 ng/ml, the lowest autophagy inducing dose in 24 h) with longer time points (3 days, 6 days and 9 days) were tested. According to our results, (*Atg5*^{-/-}) MEF cells started to express α -sma protein as early as 3 days (Figure 55). In order to detect the time points and conditions for the switch, α -sma expression in fibroblast cells was tested upon low concentration of TGF β 1 (10⁻² ng/ml, the lowest autophagy inducing dose in 24 h) and low concentration of CT-1 (5 ng/ml, the lowest autophagy inducing dose in 24 h) for 24 h, 48 h, and 72 h. As indicated, these concentrations of the treatments were decided according to minimum concentration which could induce autophagy in fibroblast cells. According to our results, α -sma protein expression was increased in MEF WT cells upon TGF β 1 treatment. CT-1 did not have any additional effect on α -sma expression. For (*Atg5*^{-/-}) MEF cells, after 72h, fibroblast cells start to produce α -sma protein upon TGF β 1 treatment (Figure 56).

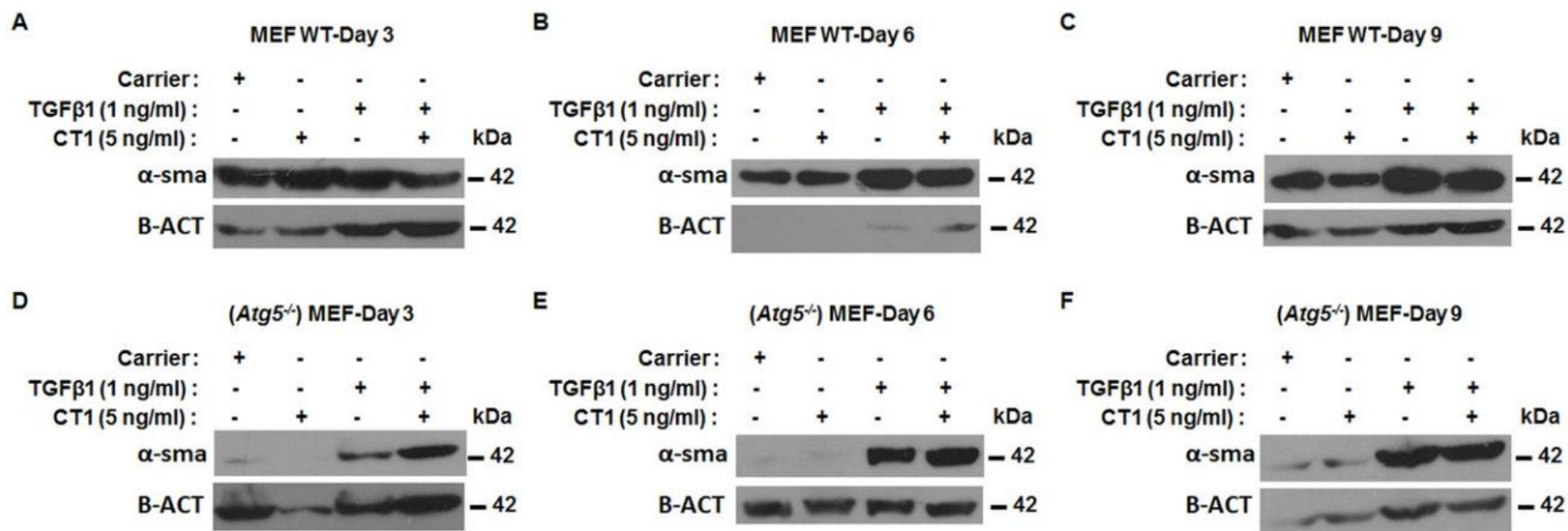
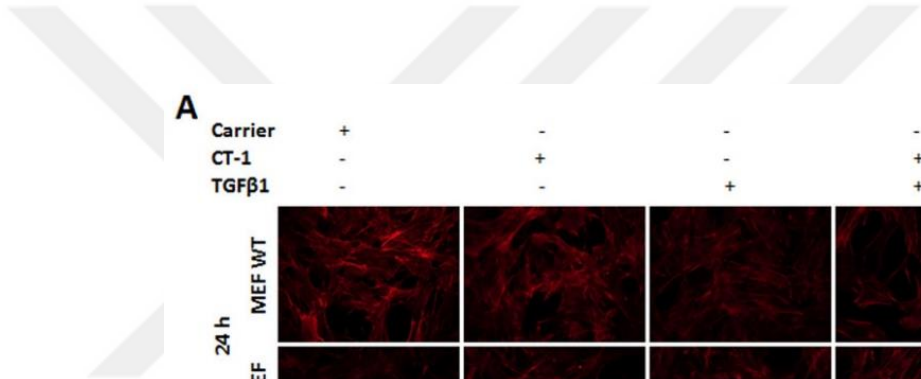


Figure 55. Analysis of α -sma expression upon CT-1, TGF β 1 (high concentration) or CT-1 and TGF β 1 treatment for longer time points. (A) Immunoblot analysis for the expression of α -sma in autophagy-competent MEF WT cells upon the treatment of CT-1 (5 ng/ml), TGF β 1 (1 ng/ml) or CT-1 (5ng/ml) and TGF β 1 (1 ng/ml) for 3 days. (B) Immunoblot analysis for the expression of α -sma in autophagy-competent MEF WT cells upon the treatment of CT-1 (5 ng/ml), TGF β 1 (1 ng/ml) or CT-1 (5ng/ml) and TGF β 1 (1 ng/ml) for 6 days. (C) Immunoblot analysis for the expression of α -sma in autophagy-competent MEF WT cells upon the treatment of CT-1 (5 ng/ml), TGF β 1 (1 ng/ml) or CT-1 (5ng/ml) and TGF β 1 (1 ng/ml) for 9 days. (D) Immunoblot analysis for the expression of α -sma in autophagy-deficient (*Atg5*^{-/-}) MEF cells upon the treatment of CT-1 (5 ng/ml), TGF β 1 (1 ng/ml) or CT-1 (5ng/ml) and TGF β 1 (1 ng/ml) for 3 days. (E) Immunoblot analysis for the expression of α -sma in autophagy-deficient (*Atg5*^{-/-}) MEF cells upon the treatment of CT-1 (5 ng/ml), TGF β 1 (1 ng/ml) or CT-1 (5ng/ml) and TGF β 1 (1 ng/ml) for 6 days. (F) Immunoblot analysis for the expression of α -sma in autophagy-deficient (*Atg5*^{-/-}) MEF cells upon the treatment of CT-1 (5 ng/ml), TGF β 1 (1 ng/ml) or CT-1 (5ng/ml) and TGF β 1 (1 ng/ml) for 9 days.



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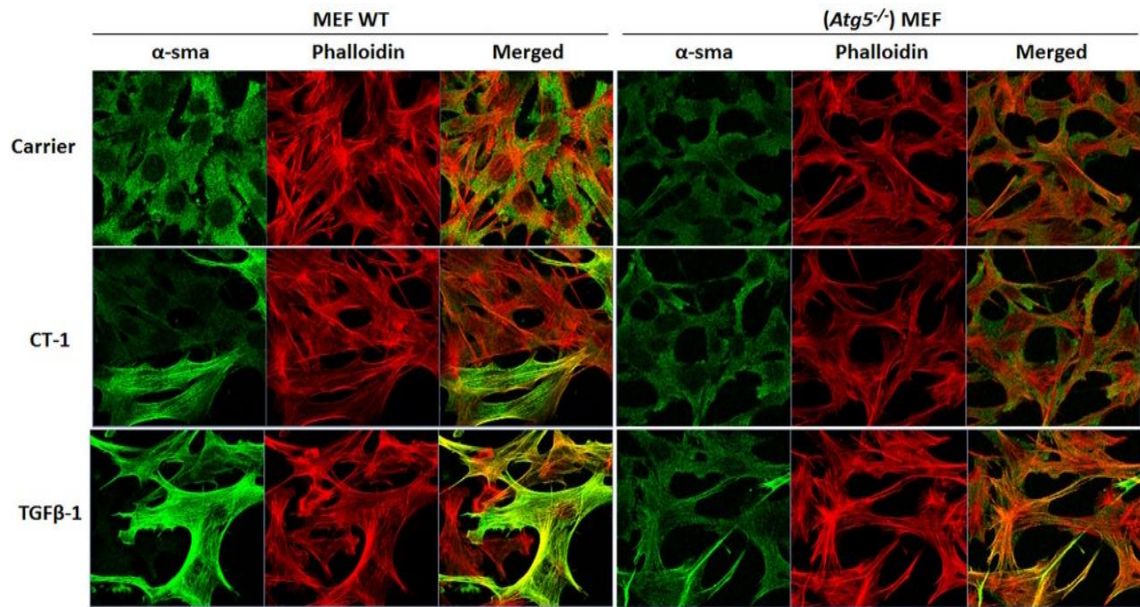


Figure 57. α -sma and phalloidin staining for MEF WT and (Atg5^{-/-}) MEF cells upon CT-1 (5 ng/ml), TGF β 1 (10⁻² ng/ml) or CT-1 and TGF β 1 for 48 h. α -sma staining was green and phalloidin staining was red (n=1).

The results were confirmed by Yunus staining MEF WT and (Atg5^{-/-}) MEF cells with α -sma (green) and phalloidin (red) at 48 h (Figure 57).

It has been shown that TGF β 1 is a major contributor for the fibrosis and myofibroblast transdifferentiation, but CT-1 has been identified as cytoprotective factor and an anti-fibrotic factor. In the study, it has been analyzed and shown the combined effects of CT-1 and TGF β 1 on proliferation of primary myofibroblast cells in serum free conditions and possible mechanisms for these changes specifically altered expression of cyclin E, Cdk2 and p27. It was also shown that contraction of cardiac myofibroblasts plated on the surface of prepared collagen matrices were increased upon TGF β 1 treatment. Thus, despite its proliferative effect of CT-1 on the cells, net collagen secretion per cell is diminished in the presence of CT-1 and thus the authors claimed that CT-1 effect may be to oppose the profibrotic signals of TGF β 1 and PDGG-BB. Moreover, they discussed that since CT-1 activates LIF/gp130 receptor dimer complex, although the most notable function of LIF is the prevention of stem cell differentiation, recent evidence suggests that LIF inhibits phenotypic switch of cardiac fibroblasts to myofibroblasts. LIF is associated with decreased matrix metalloproteinase function, which highlights its putative role on modulation of excessive ECM remodeling [242]. Thus, it is very likely that we cannot observe an increase in α -sma expression upon CT-1 treatment on fibroblast cells. On the other hand, it was shown that in human mammary fibroblast cells, SDF-1 and TGF β 1 are two-autocrine loops which initiates and maintain the differentiation of

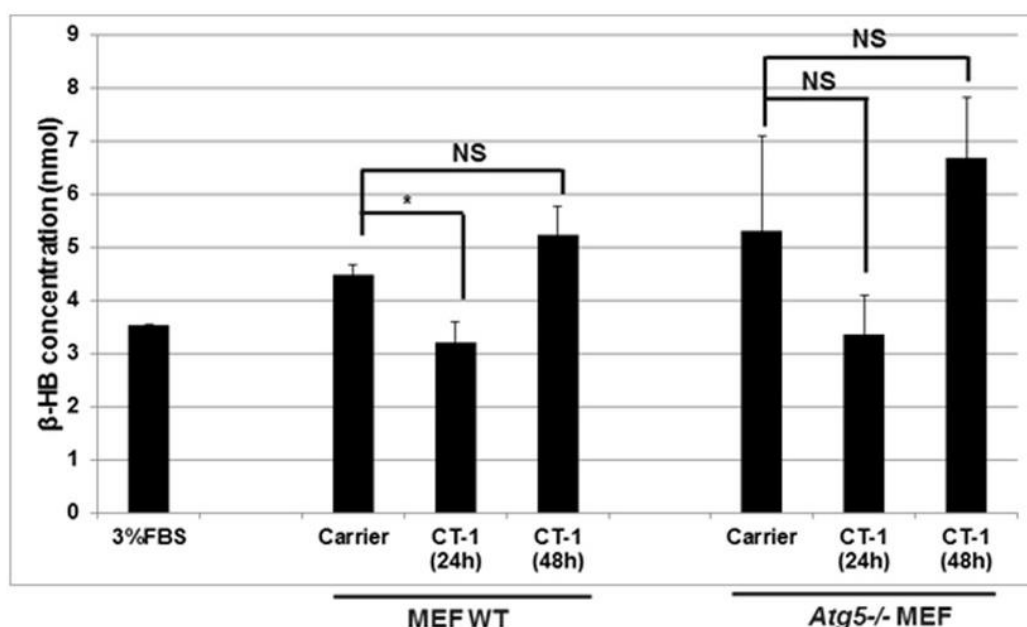


Figure 58. Ketone body release upon CT-1 treatment of MEF WT and (*Atg5^{-/-}*) MEF cells in 24 h.

(Mean $\pm$ SD of independent experiments, n=3, *p<0.05.)

fibroblasts to myofibroblasts and tumor-promoting phenotype [243]. This conversion might bring the novel role of Atg5 protein during NF to CAF transdifferentiation.

3.6.1.6. CT-1 and its role in modulating fibroblast metabolisms

3.6.1.6.1 Ketone bodies analysis

In a series of articles, Lisanti *et al.* suggested that nutrient catabolism through autophagic degradation enhanced energy source (ketone body and lactate) production in cancer stroma cells, and fueled tumor growth and metastasis (reviewed in [23]). Tumor-derived reactive oxygen species (ROS) were introduced as major signals that induce autophagy activation and energy source production in stroma cells. In order to confirm the literature, ketone body release from fibroblast cells upon CT-1 treatment was tested.

According to the result, upon 24 h of CT-1 treatment, ketone body concentration in the collected medium equals to the medium which did not contain cells. However, upon 48 h of CT-1 treatment in fibroblast cells caused an increase in ketone bodies concentration in the medium. However, this increase was not significant neither from MEF WT cells nor from (*Atg5^{-/-}*) MEF cells (Figure 58). It should be noted that the experiments were performed using mouse embryonic fibroblast cells. As a result, the

same mechanism may not be the explanation for the cells which have different origin than human.

3.6.1.6.2 Radioactivity analysis

The release of valine amino acids from fibroblast cells was checked upon the treatment of recombinant CT-1.

According to the results, no significant amino acid release was observed neither upon CT-1 treatment for 24 h, nor upon starvation (EBSS) for 4 h (Figure 59). Thus, similar to the ketone body release result, the result may be different from the literature due to having different origin (mouse embryonic fibroblast cells) and their support may come from a different mechanism.

3.6.1.6.3 LS/MS Analysis

In order to understand possible mechanisms that fibroblast cells supported the growth of cancer cells, changed proteins were analyzed upon the treatment of conditioned medium of CT-1 overexpressed HEK 293T medium on fibroblast cells, using LC-MS/MS analysis (this analysis was conducted by Seçil Erbil, Ph.D. student of Dr. Devrim Gözüaık, in Jern Dengjel's laboratory, Germany). First, HEK 293T cells were cultured

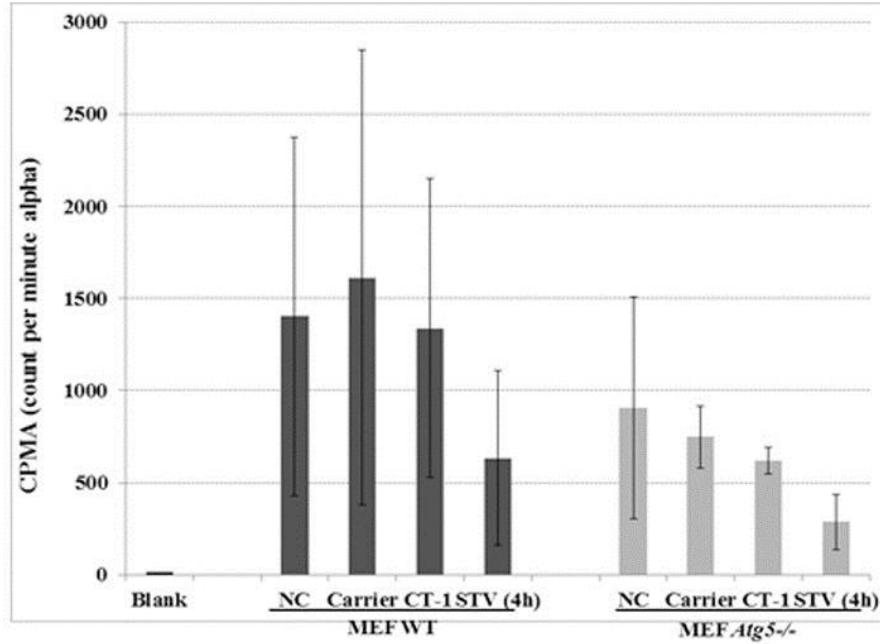


Figure 59. Valine amino acid release upon CT-1 treatment of MEF WT and (*Atg5^{-/-}*) MEF cells in 24 h.
(Mean±SD of independent experiments, n=3.)

in medium containing heavy (Arg10, Lys8) amino acids for CT-1 overexpression or in medium containing medium (Arg6, Lys4) amino acids for CNT conditioned medium for 10 days. Similarly, fibroblast cells were cultured in medium containing heavy (Arg10, Lys8), medium (Arg6, Lys4) or light (Arg0, Lys0) amino acids for 10 days. After labeling the cells, HEK 293T cells were transfected with CT-1 or CNT (pcDNA3) plasmid and after 8 h, medium was changed to low-FBS medium containing necessary Arg-Lys. After 72 h incubation, the medium of transfected HEK 293T cells was concentrated and added to the medium of fibroblast cells for 24 h incubation. For LC-MS/MS analysis, mixed cell lysates of treated fibroblast cells were run in gel electrophoresis. Separated proteins were digested with trypsin enzyme for further relative quantification and identification of the peptides (Figure 60A). According to the results, Pearson correlation coefficient of CT-1 conditioned medium over CNT conditioned medium treated fibroblast cells was 0.714156 (Figure 60B). The Pearson correlation coefficient is a measure of the strength of a linear association between two variables. It can take a range of values from +1 to -1. A value greater than 0 indicates a positive association, which means as the value of one variable increases, so does the value of the other variable [244]. Since the Pearson correlation coefficient between CT-1 conditioned medium over CNT conditioned medium treated fibroblast cells is 0.714156, it means there is a high correlation between these two

conditions. On the other hand, Pearson correlation coefficient of CT-1 conditioned medium over STV treated fibroblast cells (positive control) was 0.160965 (Figure 60C), which means there is a low correlation between these two conditions.

According to the result of LC-MS/MS, in total 49 proteins were changed upon CT-1 conditioned medium treatment. 32 of them were specific to CT-1 treatment. These proteins were grouped according to their functions. Moreover, 17 of these 49 proteins were common between CT-1 treated fibroblast cells and STV (starvation)-treated fibroblast cells (Figure 60D).



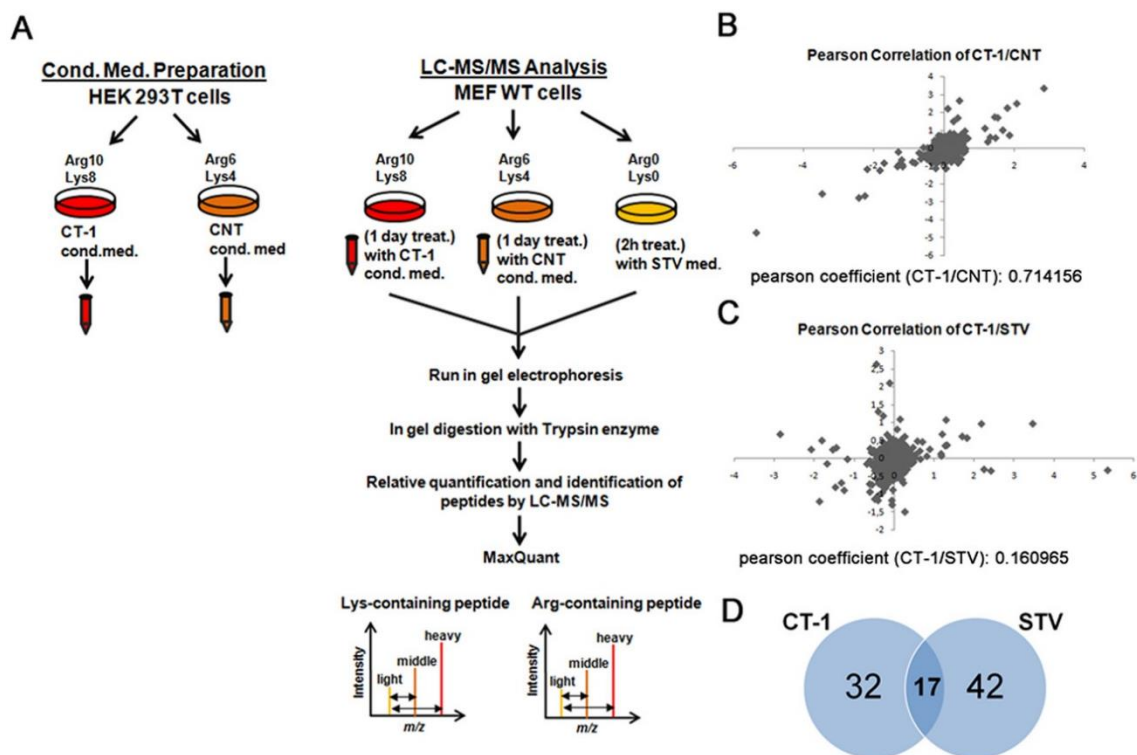


Figure 60. SILAC and LC-MS/MS analysis of the effect of CT-1 conditioned medium in fibroblast cells.

(A) SILAC labeling and experimental set-up was explained in scheme. In order to prepare conditioned medium of CT-1 or CNT, HEK 293T cells were cultured in heavy Arg and Lys or medium Arg and Lys for 10 days, respectively. After labeling HEK 293T cells with indicated conditions, cells were transfected with CT-1 or CNT plasmids and mediums were changed to low-FBS mediums labeled with above indicated amino acids. After preparation of conditioned mediums, previously labeled fibroblast cells were treated with indicated conditions for 24 h. As a positive control, 2 h of starvation was performed with HBSS medium. At the end of the conditioned medium or starvation treatment, cell lysates were run in gel electrophoresis. Digestion with trypsin enzyme in gel was performed, relative quantification was performed by LC-MS/MS, and identification of the peptides was performed with MaxQuant v.1.2.0.1.8 and Andromeda database (n=1). **(B)** Pearson coefficient calculation between CT-1 and CNT. **(C)** Pearson coefficient calculation between CT-1 and STV, which is positive control. **(D)** Venn diagram for the common and different protein numbers between CT-1 and STV treatments.

The role of the critical proteins will be discussed in detail in **Discussion**.

3.6.2. CT-1 and its role during tumor-fibroblast communication *in vivo* analysis

3.6.2.1. Cancer cells growth *in vivo*

3.6.2.1.1 CAM Assay

Eggs were kindly provided by Mudurnu Piliç. Fertilized eggs were cleaned and incubated in 37°C in humidified incubator. At Day 4, caps were opened and silicon rings were located (Figure 61). At Day 6, cancer cells (MCF7 alone or MDA-mCherry alone), fibroblast cells (MEF WT or (*Atg5*^{-/-}) MEF cells alone) or PBS (negative control) was tested. Experiments were terminated at Day 20 (Figure 62A). According to H&E staining results (Figure 62B), autophagy-competent fibroblast cells formed blood vessels. Since fibroblast cells were previously immortalized with SV40 plasmids, (*Atg5*^{-/-}) MEF cells showed atypical proliferation *in vivo*. MCF7 and MDA-mCherry cells formed dystrophic calcification. Unexpectedly, fungus hyphae were detected in the condition where MDA-mCherry cancer cell injected. Due to abnormal cell growth observed in MEF WT or (*Atg5*^{-/-}) MEF cells, it should be noted that even their growth may cause suppression the growth of cancer cells in co-culture system.

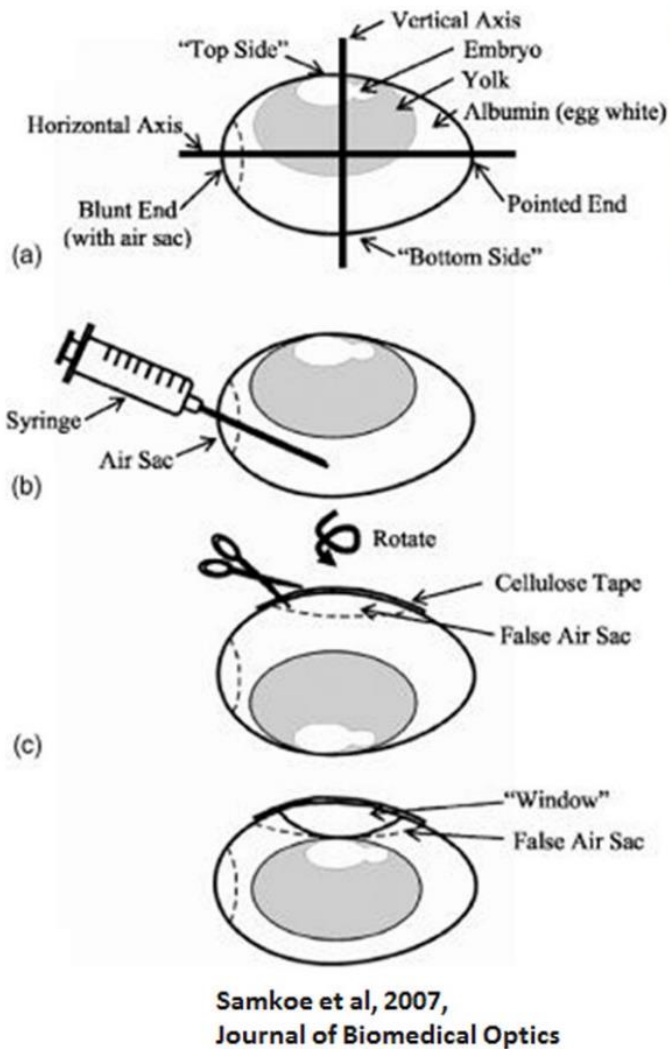
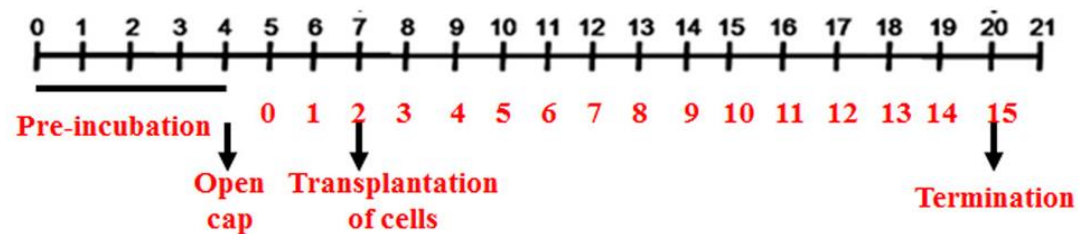


Figure 61. CAM Assay Preparation

(A) With syringe, part of albumin was taken. **(B)** This allows space on the top part of the fertilized to in order to see CAM. A cap was opened by scissors. **(C)** Silicon ring was added (Day 4).

A



B

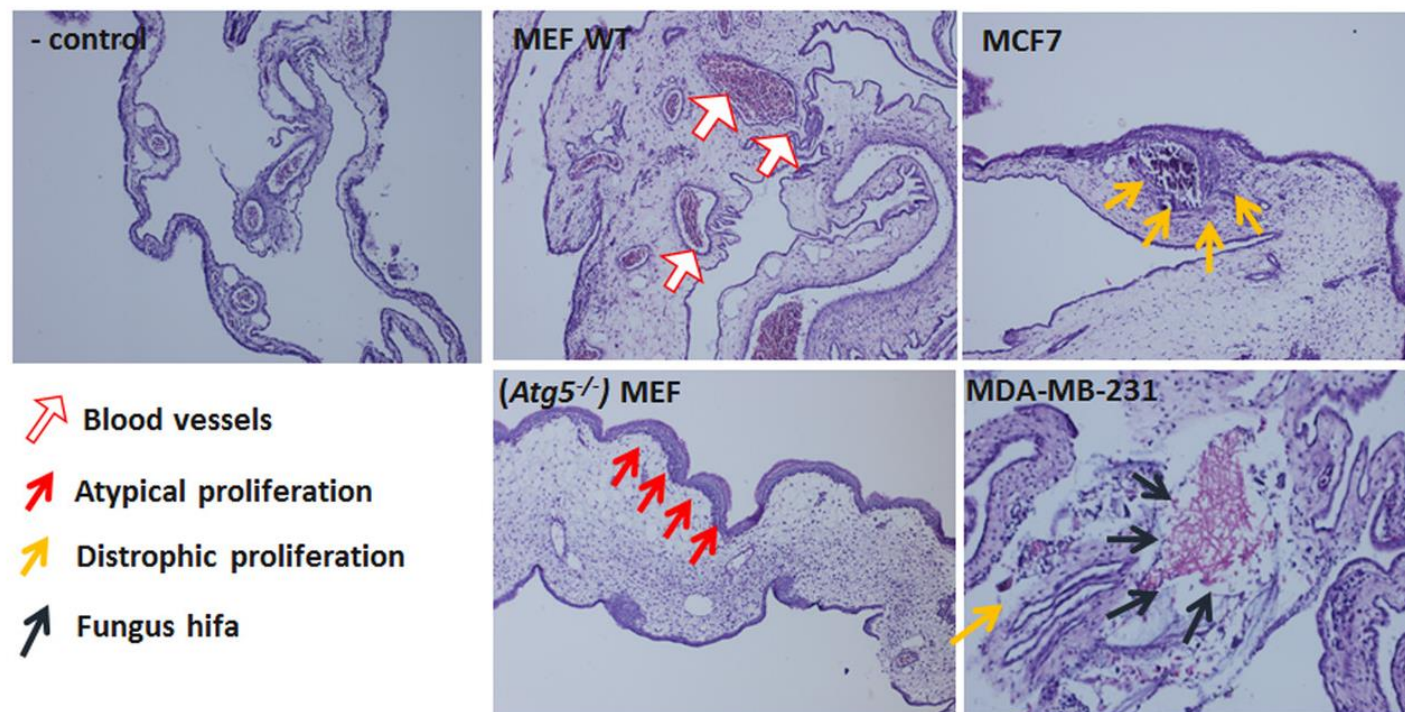


Figure 62. CAM Assay Protocol.

(A) Experimental setup for the CAM assay. **(B)** H&E staining for the conditions.

3.6.2.1.2 NOD.SCID mouse experiments

In order to analyze the absence of CT-1 during tumor growth and development, we performed CT-1 neutralization antibody after tumor formation in NOD.SCID mouse. The growth of tumor was targeted to be blocked using CT-1 neutralization antibody treatment after 18 days or 30 days of tumor injection. As described in timeline, injected MCF7 cancer cells into mammary pad of the mouse and tumor formations were followed for 30 days. After tumor formation, CT-1 neutralization antibody or control antibody were injected from different sites of the tumor each 3-4 day for 30 days so that secreted CT-1 from cancer cells can be blocked efficiently. 18 days after tumor injection, there was no significant growth reduction of tumor (Figure 63A-C). Next, with increased cancer cell numbers, 30 days after cancer cell injection, CT-1 neutralization antibody or control

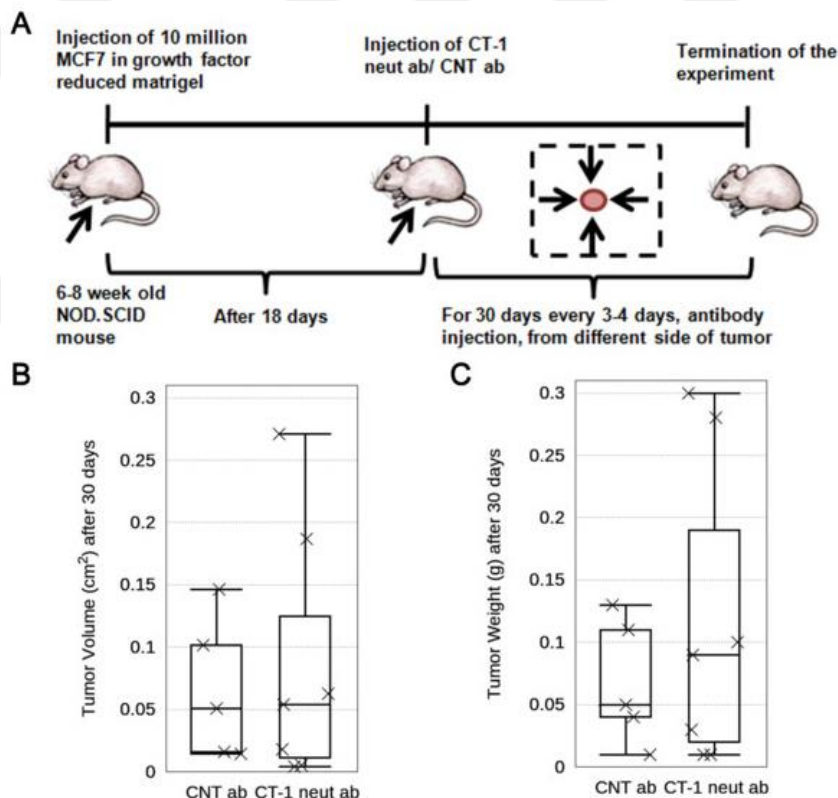


Figure 63. Tumor growth analysis *in vivo* in the presence of CT-1 neutralization antibody or control antibody starting after 18 days of tumor injection.

(A) Timetable for tumor and neutralization antibody/control antibody injection to NOD.SCID mouse. 10×10^6 MCF7 breast cancer cells were injected to the 4th mammary fat pad of the mouse. After 18 days of cancer cells injection, CT-1 neutralization antibody/control antibody injection was performed every 3-4 days for 30 days. At the end of the 30 days, experiments were terminated. **(B)** Distribution of the tumor volume for CT-1 neutralization antibody or control antibody injected tumor developing mouse **(C)** Distribution of the tumor weight for CT-1 neutralization antibody or control antibody injected tumor developing mouse.

antibody were injected from different sites of the tumor each 3-4 day for 30 days. Terminations of the experiments were done and tumor was taken as described in **Materials and Methods** part. Weight and volume of tumors were measured. According to tumor volume, there was no significant difference was observed between CT-1 neutralization antibody injected mouse and control antibody injected mouse. And according to tumor weight results, there was again no significant difference between CT-1 neutralization antibody injected mouse and control antibody injected mouse (Figure 64A-D).

No difference in the growth of cancer cells were observed upon the treatment of CT-1 neutralization antibody. Neutralization antibody concentration may not be enough to reduce the biological activity of the secreted CT-1 from tumor cells. Moreover, co-cultured MCF7-RFP cancer cells with human CAFs with control/CT-1 neutralization antibody treatment can be necessary to be tested.

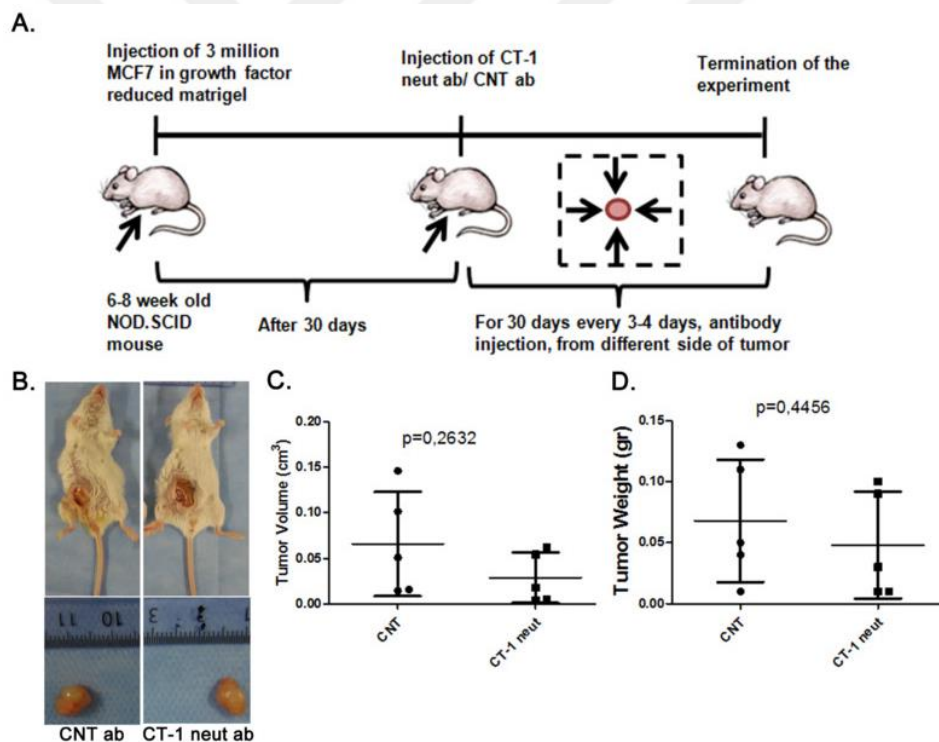


Figure 64. Tumor growth analysis *in vivo* in the presence of CT-1 neutralization antibody or control antibody starting after 30 days of tumor injection.

(A) Timetable for tumor and neutralization antibody/control antibody injection to NOD.SCID mouse. 1×10^7 MCF7 breast cancer cells were injected to the 4th mammary fat pad of the mouse. After 30 days of cancer cells injection, CT-1 neutralization antibody/control antibody injection was performed every 3-4 days for 30 days. At the end of the 30 days, experiments were terminated. **(B)** Representative images for the experiments. **(C)** Distribution of the tumor volume for CT-1 neutralization antibody or control antibody injected tumor developing mouse **(D)** Distribution of the tumor weight for CT-1 neutralization antibody or control antibody injected tumor developing mouse.

3.6.2.2. CT-1 and its role during tumor-fibroblast communication in patient samples

In vivo relevance of our observations in human breast cancer tissues was analyzed among T1 and T2 tumors. T1 defines tumors smaller than 2 cm in their larger dimension; while, tumors at T2 stage are bigger, their larger dimension being between 2-5 cm. Obtained paired tumor/non-tumor breast cancer tissues from patients with T1 or T2 diseases (American Joint Committee on Cancer (AJCC) classification) (Table 2 and Table 3) were checked for their levels of CT-1 protein expression. Strikingly, the results showed that a significant portion of tumors at T2 stage expressed higher levels of CT-1 protein compared to those in T1 stage (Figure 65). However, the expression difference between T1 and T2 was not related with ER or PR expression.

Besides to immunoblot analysis, selected tissues were stained using CT-1 specific antibody and α -sma antibody (analysis and staining were performed by Yunus Akkoç, Ph.D. student of Dr. Devrim Gözüaçık). α -sma is a marker of cancer-association stromal fibroblasts (CAFs). CT-1 was mainly expressed in the epithelial tumor cells while CAFs were devoid of it. When tumor tissues were stained with autophagy marker protein LC3, LC3 staining colocalized with α -sma staining and not with CT-1 staining was observed (Figure 66).

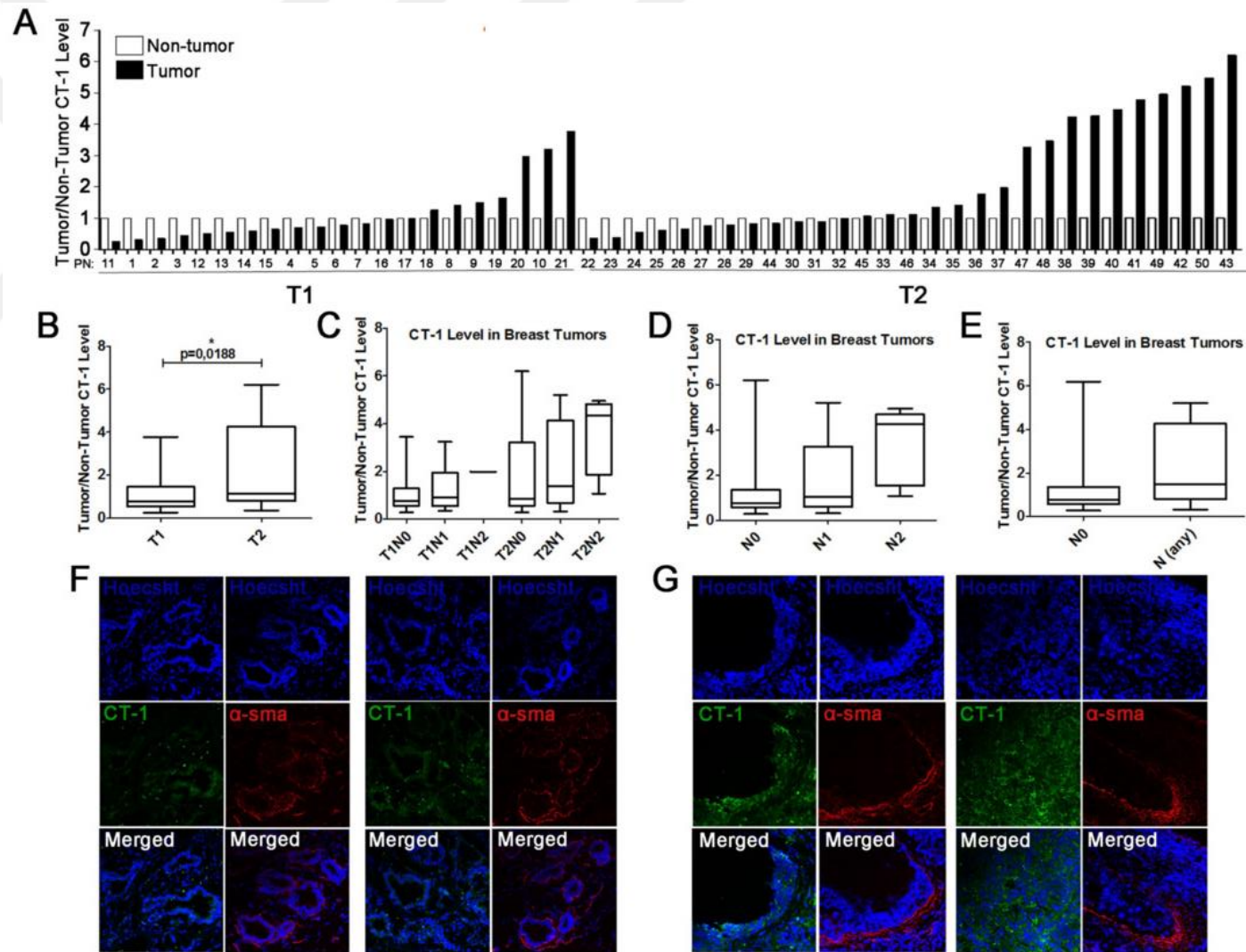


Figure 65. Tumor and non-tumor samples from breast cancer patients express CT-1 in stage-dependent manner.

(A) Immunoblot analysis of CT-1 expression in tumor and non-tumor tissue among T1 and T2 patients. (B) Quantification of tumor/non-tumor tissue CT-1 level according to T1/T2 classification ($*p<0.05$). (C) Quantification of tumor/non-tumor tissue CT-1 level according to TN stages. (D) Quantification of tumor/non-tumor tissue CT-1 level according to N stages. (E) Quantification of tumor/non-tumor tissue CT-1 level according to N0 vs N(any) stages. (F-G) Tumor sample from T1 (F) and T2 (G) staining using CT-1 (green) and α -sma (red).

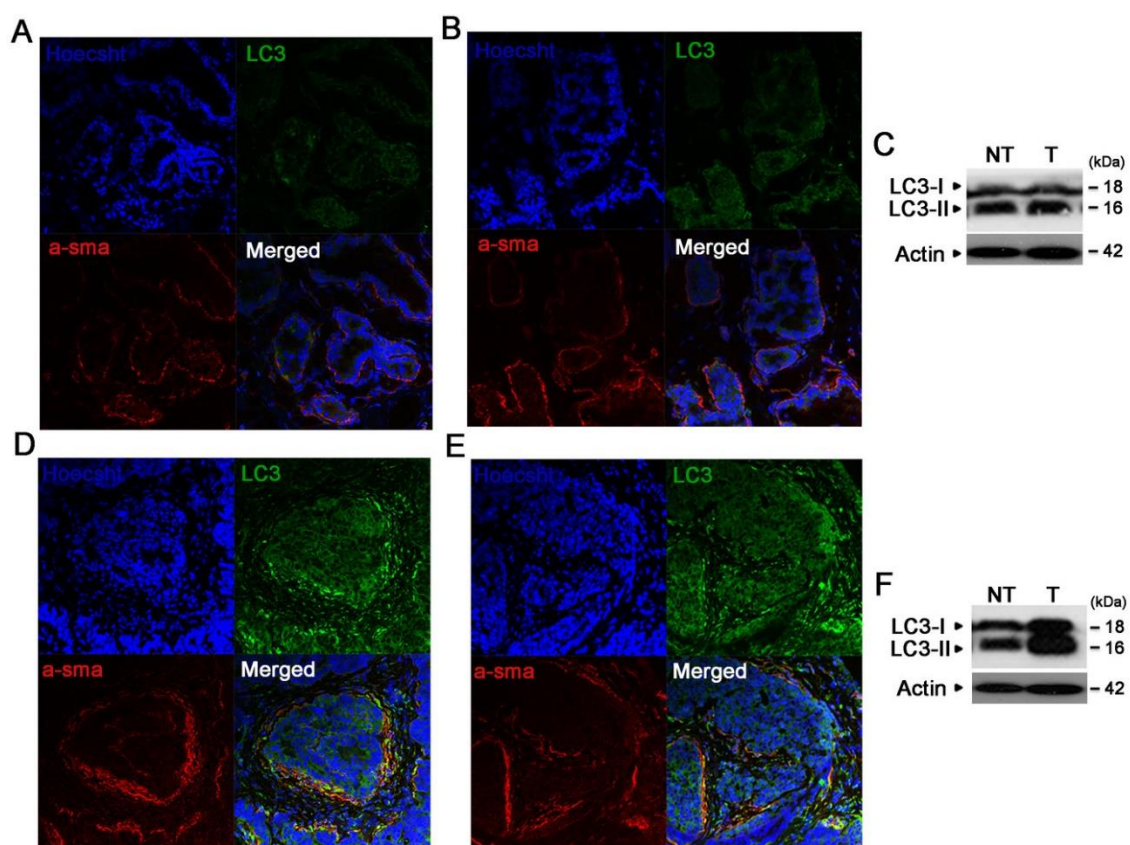


Figure 66. LC3 and α -sma staining of tumor and non-tumor samples from breast cancer patients.

(A-C) Staining and immunoblot analysis of α -sma and LC3 from tumor tissue samples which are smaller than 2 cm (sample from T1 patient). **(D-F)** Staining and immunoblot analysis of α -sma and LC3 from tumor tissue samples which are bigger than 2 cm (sample from T2 patient).

These data confirmed that CT-1 was mainly expressed in tumor tissue and stromal fibroblasts that are associated with CT-1 positive tissues showed higher level of autophagy. On the other hand, tissues with lower CT-1 expression level are associated with a lower CAF level and also lower autophagy (Table 2 and Table 3).

Table 2. Clinicopathological features of 50 breast cancer patients, in summary.

Parameters	No.
Age, (years mean±SD)	55,6±13,5
Female	50
AJCC Stage ^b , I/II/III/IV	8/36/6/0
T category, 1/2	21/29
N category, 0/1/2	25/18/7
M category, 0/1	50/0
Lymphovascular Invasion (LVI)(-/+)	22/22
ER Status (-/+)	5/27
PR Status (-/+)	8/25

Table 3. Detailed clinicopathological features of 50 breast cancer patients.

Age, years	CT-1			p value
	n	Low (%)	High (%)	
<=50	21	24	18	0.578
>50	29	28	30	
	50	52	48	
ER				
-	5	12.5	3.12	0.355
+	27	43.7	40.6	
	32	56.2	43.7	
PR				
-	8	18.2	6	0.416
+	25	39.4	36.4	
	33	57.6	42.4	
LVI				
-	22	29.5	20.4	0.547
+	22	22.7	27.7	
	44	52.2	48.1	

3.7. RESULTS V - Discovery and analysis of Vascular Endothelial Growth Factor A (VEGFA) as a mediator of tumor-fibroblast interaction

According to the cytokine screening, the conditioned medium of VEGFA (vascular endothelial growth factor A) caused the highest autophagy induction in fibroblast cells. In this chapter, the effect of VEGFA conditioned medium on autophagy in fibroblast cells, the expression and secretion of VEGFA from different cancer types, and the effect of VEGFA neutralizing antibody on cancer cell growth will be analyzed.

3.7.1. Conditioned medium of VEGFA overexpressed HEK 293T cells specifically induced on autophagy in fibroblast cells.

In order to validate the effect of VEGFA conditioned medium on autophagy, autophagy induction upon the treatment of fibroblast cells with HEK 293T-derived VEGFA conditioned medium was analyzed. HEK 293T cells were transfected with VEGFA or CNT (pcDNA3) plasmids in order to overexpress and release the factor to the low-FBS medium for 72 h. Then, the medium was collected and concentrated using Amicon 3 kDa cut-off tubes. Although under normal conditions, GFP-LC3 MEF cells showed a diffuse cytoplasmic and nuclear localization of GFP-LC3 dots, upon the treatment of these cells with the VEGFA conditioned medium, GFP-LC3 dot formation appeared in bigger and brighter dots reflecting the autophagy induction through autophagic membrane formation. In order to prove the specificity of autophagy induction, a neutralizing antibody which is specific to VEGFA was used. VEGFA neutralizing antibody specifically binds to VEGFA in order to block its binding to the receptor. This action mechanism reduces the biological effect of VEGFA floating around the cells. According to the result, VEGFA conditioned medium mixed with control antibody can induce autophagy in fibroblast cells; however, cells in VEGFA conditioned medium mixed with VEGFA neutralizing antibody have autophagy in basal level (Figure 67A and B). In order to confirm GFP-LC3 dot counting analysis, autophagy markers LC3 shift and

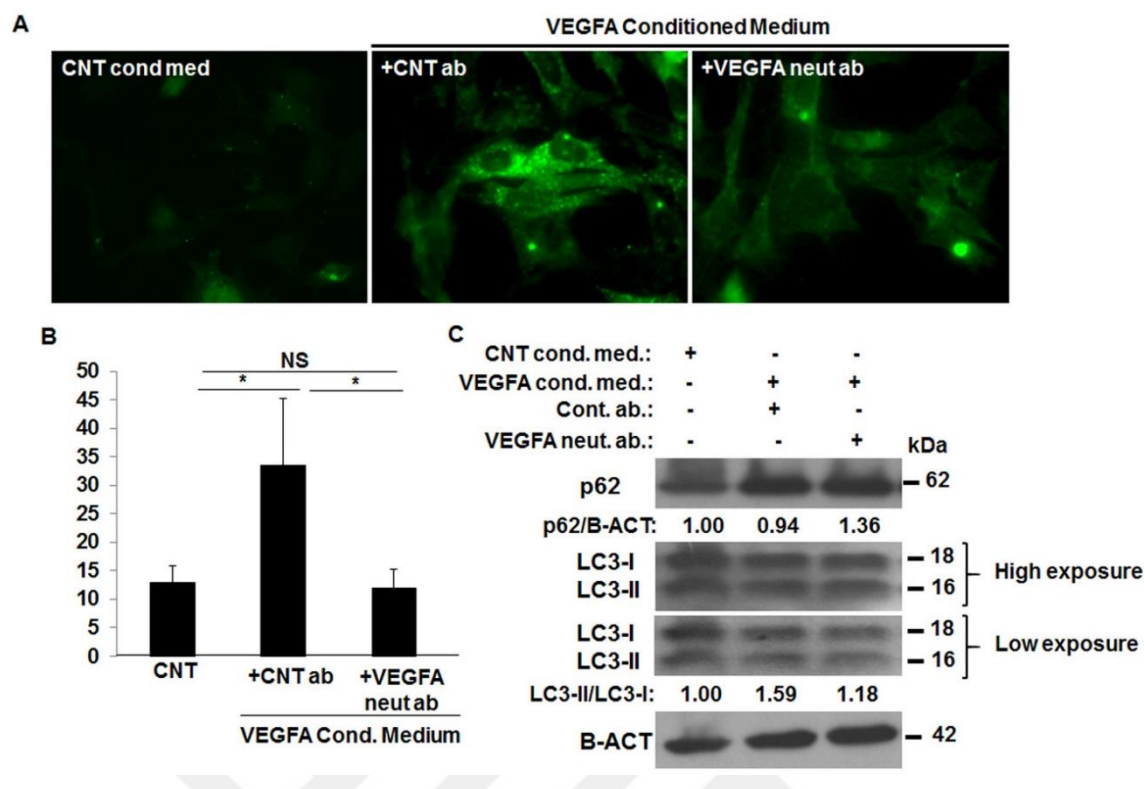


Figure 67. VEGFA conditioned medium induces autophagy in fibroblast cells; however, addition of VEGFA neutralization antibody to the conditioned medium reversed the effect to the basal level.

(A) The effect of condition medium of VEGFA overexpressed HEK 293T cells and that of conditioned medium mixed with VEGFA neutralization antibody on autophagy in fibroblast. **(B)** Quantitative analysis of the experiments in (A), (mean $\pm$ SD of independent experiments, n=3, *p<0.05; **<0.01, ***<0.001). **(C)** Immunoblot analysis: autophagy was induced in fibroblast cells upon VEGFA conditioned medium; however, autophagy occurred only at basal level upon treatment with VEGFA conditioned medium mixed with VEGFA neutralization antibody.

p62 degradation was analyzed using immunoblot technique. According to immunoblot results, fibroblast cells treated with VEGFA-conditioned medium showed higher LC3-I to LC3-II shift, means higher autophagy induction level (Figure 67C).

3.7.2. VEGFA expression and secretion by various cancer cell lines

VEGFA is a member of the family of platelet-derived growth factors. It is produced and secreted by various tumor and normal cells as 34- to 43 kDa dimeric protein. Its expression and secretion among different types of cancers were tested using immunoblot and ELISA methods. According to the results, all types of cancer cells, namely Huh7,

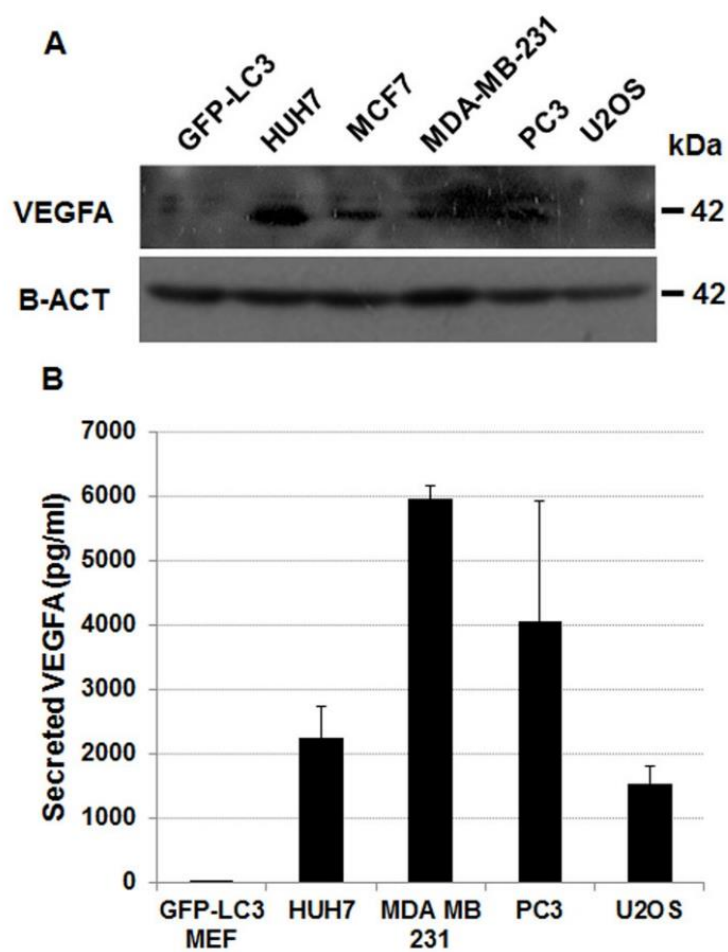


Figure 68. VEGFA was detected in different cancer cells in protein level and secreted from different cancer cells.

(A) The analysis of VEGFA in protein level was performed in different cancer cells using immunoblot technique. VEGFA protein was detected in all types of cancer cells, namely Huh7, MDA, PC3 and U2OS cancer cells, which confirms the obtained results from ELISA analysis. **(B)** Analysis of VEGFA secretion from fibroblasts and different cancer cells using ELISA (mean $\pm$ SD of independent experiments, n=3).

MDA, PC3 and U2OS cancer cells, expressed and secreted VEGFA, but not GFP-LC3 MEF cells (Figure 68A and B).

Among all cancer cells, MDA and PC3 cancer cells express and release VEGFA in the highest level. Based on experimental results, secretion of VEGFA level may have an initiating role for the crosstalk between cancer cells and fibroblast cells.

3.7.3. Neutralization of autocrine VEGFA does not affect the growth of cancer cells

In order to test whether the growth of cancer cells depends on VEGFA growth factor or not, the growth of different cancer cells, namely MDA-RFP, MCF7-RFP, PC3-GFP,

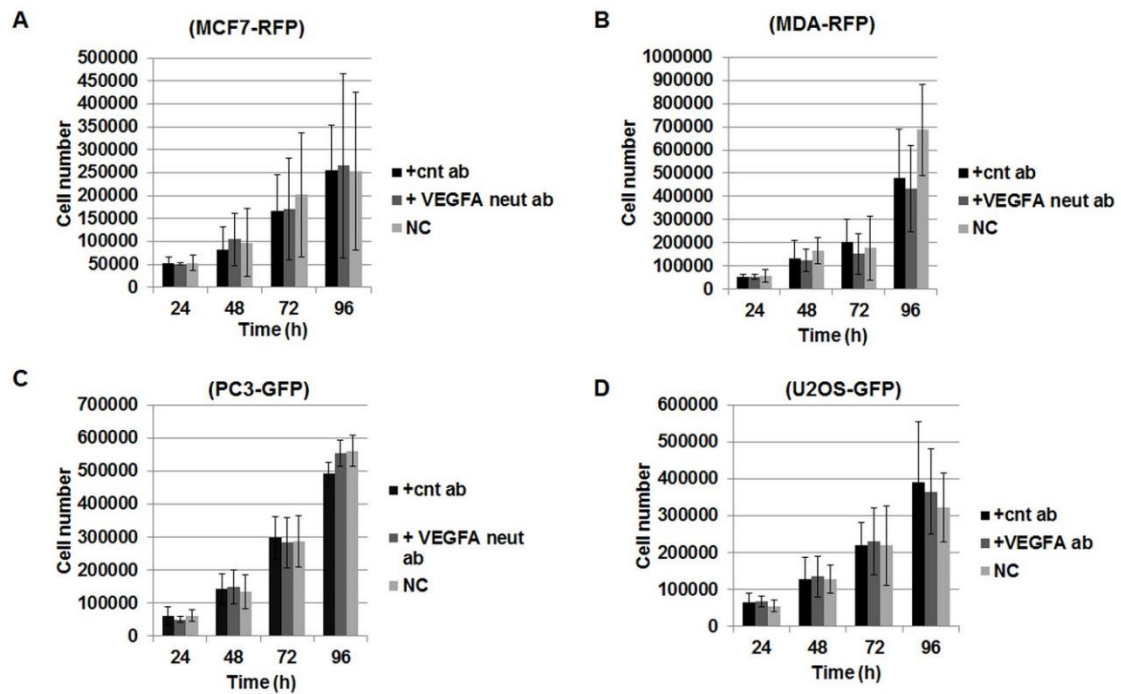


Figure 69. The growth of cancer cells does not change in the presence of VEGFA neutralization antibody. Cancer cells were cultured in the presence of VEGFA neutralization antibody.

(A) The growth of MDA-RFP breast cancer cells in VEGFA neutralization antibody (mean $\pm$ SD of independent experiments, n=3). **(B)** The growth of MCF7-RFP breast cancer cells in VEGFA neutralization antibody (mean $\pm$ SD of independent experiments, n=4). **(C)** The growth of PC3-GFP prostate cells in VEGFA neutralization antibody (mean $\pm$ SD of independent experiments, n=4). **(D)** The growth of U2OS-GFP osteocarcinoma cells in VEGFA neutralization antibody (mean $\pm$ SD of independent experiments, n=4).

and U2OS-GFP, were checked in low-FBS containing medium in the presence of VEGFA neutralizing antibody for 24 h, 48 h, 72 h and 96 h. The growth of none of these cancer cells was affected by the treatment of VEGFA neutralizing antibody (Figure 69).

As a conclusion, the growth of cancer cell is independent from their autocrine VEGFA secretion.

3.7.3.1. Effect of VEGFA neutralization antibody on cancer cells growth in co-culture systems

To test whether cancer cell growth is supported by fibroblast cells in the presence of cancer cell secreted VEGFA or not, the growth of cancer cells upon neutralization antibody of VEGFA treatment was analyzed in co-culture of cancer cells with fibroblast cells using three different techniques.

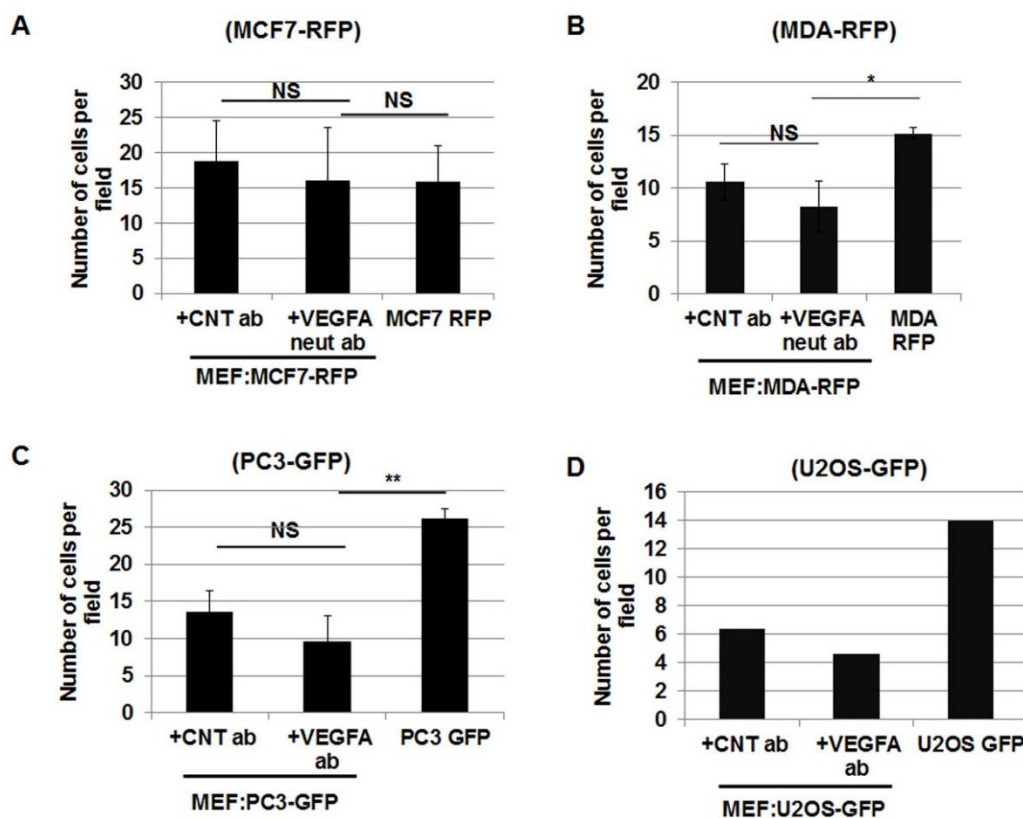


Figure 70. Growth analysis of MCF7-RFP cancer cells in VEGFA neutralization antibody containing medium in the presence or absence of autophagy competent fibroblast (MEF WT) cells under microscope.

(A) Growth analysis of MCF7-RFP cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g/ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h under fluorescence microscope by counting 20 different fields ($n=3$; NS: not significant). **(B)** Growth analysis of MDA-RFP cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g/ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h under fluorescence microscope by counting 20 different fields ($n=3$; $*p<0.05$, NS: not significant). **(C)** Growth analysis of PC3-GFP cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g/ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h under fluorescence microscope by counting 20 different fields ($n=3$; $**p<0.01$, NS: not significant). **(D)** Growth analysis of U2OS-GFP cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g/ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h under fluorescence microscope by counting 20 different fields ($n=2$).

3.7.3.1.1 Microscope analysis for the effect of VEGFA neutralization antibody on cancer cells growth in co culture systems

In microscope analysis, cancer (MCF7-RFP, MDA-RFP, PC3-GFP or U2OS-GFP) cells which are stably producing red or green fluorescence proteins were used. During the analysis for each condition randomly selected 20 different fields for the fluorescent cells

were counted under the microscope (Figure 70). According to the results, MCF7-RFP cells did not change their growth neither in the presence of fibroblast cells nor the presence of VEGFA antibody containing coculture system at 72 h. However, MDA-RFP cancer cells grew faster in the absence of fibroblast cells. The presence of VEGFA neutralization antibody in co-culture system did not affect the growth of cancer cells in their co-culture with fibroblast cells. Like MDA-RFP cancer cells, PC3-GFP and U2OS-GFP cancer cells grew faster in the absence of fibroblast cells. Also, the VEGFA neutralization antibody treatment did not affect the growth of cancer cell when the cancer cells were co-cultured with fibroblast cells.

3.7.3.1.2 FACS analysis for the effect of VEGFA neutralization antibody on cancer cells growth in co culture systems

In FACS analysis, like microscope analysis, cancer (MCF7-RFP, MDA-RFP, PC3-GFP or U2OS-GFP) cells which are stably producing red or green fluorescence proteins were used. During the FACS analysis, fluorescence-labelled cancer cells were detected by the machine (Figure 71). According to the results, the presence of VEGFA neutralization antibody in co-culture system did not affect the growth of MCF7-RFP

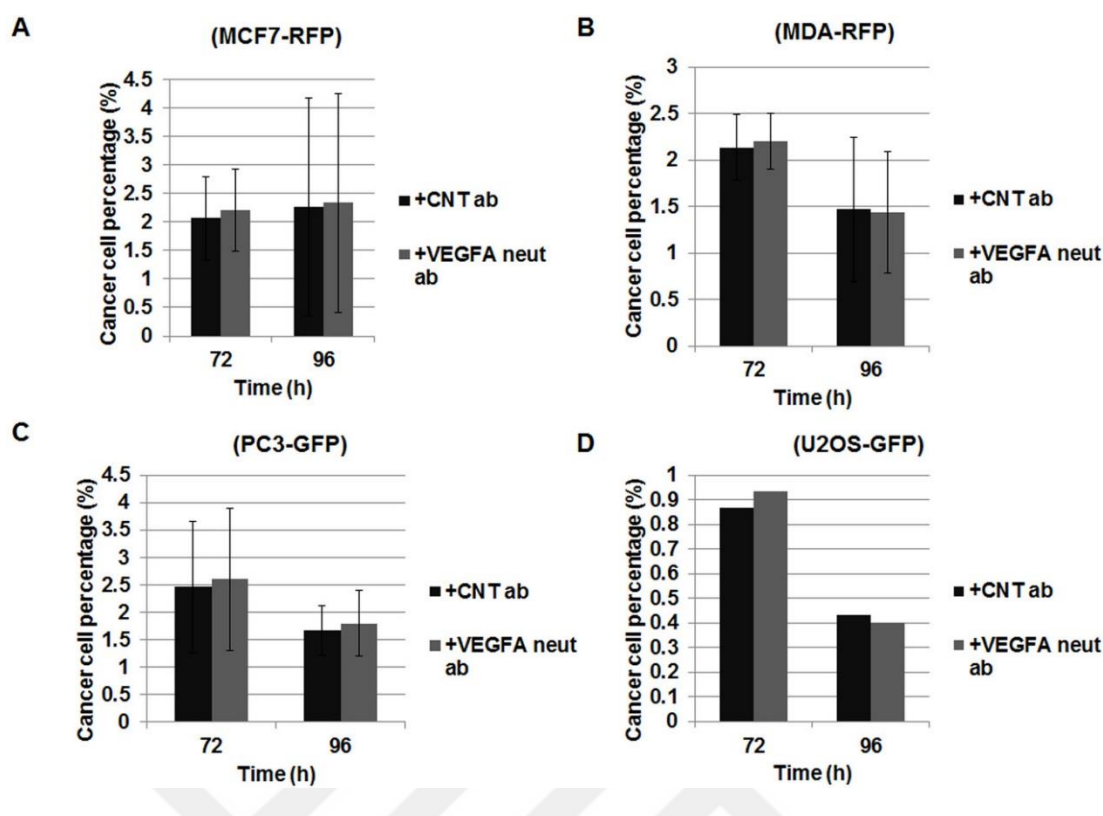


Figure 71. Growth analysis of MCF7-RFP cancer cells in VEGFA neutralization antibody containing medium in the presence or absence of autophagy competent fibroblast (MEF WT) cells using FACS.

(A) Growth analysis of MCF7-RFP cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g}/\text{ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h and 96 h (n=3). (B) Growth analysis of MDA-RFP cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g}/\text{ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h and 96 h (n=3). (C) Growth analysis of PC3-GFP cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g}/\text{ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h and 96 h (n=3). (D) Growth analysis of U2OS-GFP cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g}/\text{ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h and 96 h (n=2).

cancer cells in their co-culture with fibroblast cells. Similar results were obtained with MDA-RFP, PC3-GFP and U2OS-GFP cancer cells lines.

3.7.3.1.3 Luciferase analysis for the effect of VEGFA neutralization antibody on cancer cells growth in co culture systems

Third analysis method is Luciferase assay, which is explained in (page 125). For reminding, it will be repeated in here. In this system, the light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the

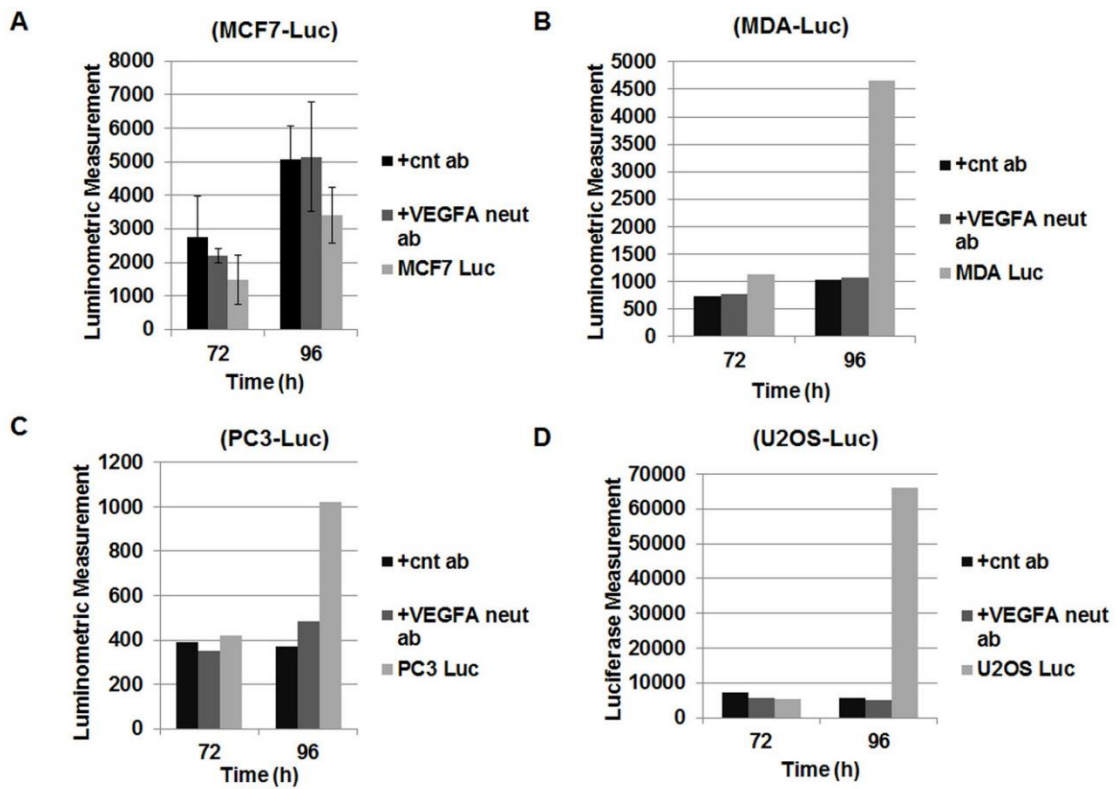


Figure 72. Growth analysis of MCF7-RFP cancer cells in VEGFA neutralization antibody containing medium in the presence or absence of autophagy competent fibroblast (MEF WT) cells using Luciferase Assay.

(A) Growth analysis of MCF7-Luc cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g}/\text{ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h and 96 h (n=3). **(B)** Growth analysis of MDA-Luc cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g}/\text{ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h and 96 h (n=2). **(C)** Growth analysis of PC3-Luc cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g}/\text{ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h and 96 h (n=2). **(D)** Growth analysis of U2OS-Luc cancer cells when these cells were co-cultured with autophagy competent fibroblast cells in the presence of 0.08 $\mu\text{g}/\text{ml}$ concentration of VEGFA neutralization antibody or control antibody for 72 h and 96 h (n=2).

product molecule oxyluciferin. Firefly luciferase, a monomeric 61 kDa protein, catalyzes luciferin oxidation using ATP-Mg^{2+} as a co-substrate. In the conventional assay for luciferase, a flash of light is generated that decays rapidly after the enzyme and substrates are combined [240]. During the performed experiment, cancer cells (MCF7-Luc, MDA-Luc, PC3-Luc and U2OS-Luc) which stably express luciferase construct in their genome were co-cultured with fibroblast cells in the presence or absence of VEGFA neutralization antibody. At 72h and 96 h, all cells were collected and lyzed using Chris Buffer. When luciferin is added, depending on the growth of cancer cells, difference in the intensity of

the light was detected (Figure 72). According to the results, no significant difference in co-culture system in the cancer cells growth between control antibody treated and VEGFA neutralization antibody treated samples were detected in 72 h and 96 h.

3.7.4. Secreted VEGFA from MDA cancer cells induces autophagy in fibroblast cells

To confirm the specificity of autophagy induction in fibroblast cells upon VEGFA treatment, VEGFA was knocked-down in MDA cancer cells using target specific shRNA. With the conditioned medium of shGFP (CNT) infected MDA cancer cells, fibroblast cells significantly induced autophagy significantly comparing to fibroblast cells under normal conditions (CNT). Upon shVEGFA infection in MDA cancer cells, fibroblast cells



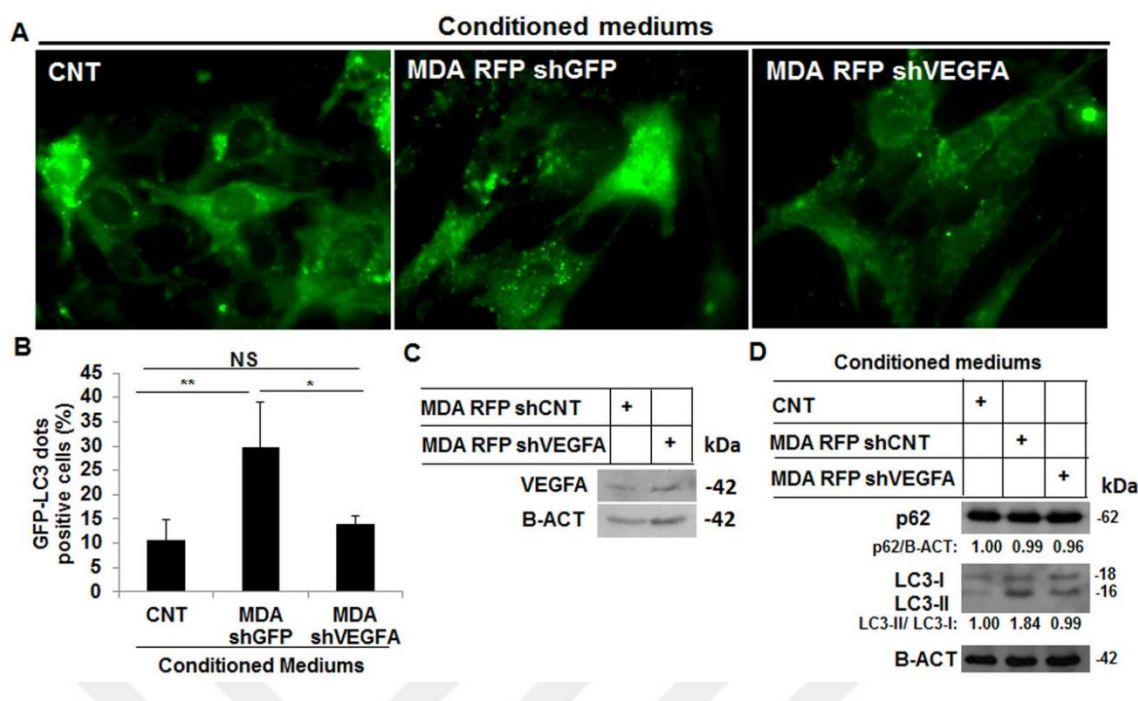


Figure 73. Knock down of VEGFA expression and secretion from cancer cells reduced autophagy in fibroblast cells to the basal level.

(A) Autophagy in fibroblast cells was significantly induced upon treatment with the conditioned medium of MDA-MB-231 cancer cells; however, they have basal level of autophagy upon the treatment with the conditioned medium of MDA-MB-231 cancer cells that have been infected with shVEGFA. **(B)** Quantitative analysis of the experiments in (A), GFP-LC3 dots positive cells upon conditioned mediums of MDA-MB-231 cancer cells under the microscope (mean $\pm$ SD of independent experiments, n=3, *p<0.05; **<0.01, ***<0.001). **(C)** Immunoblot analysis for VEGFA expression of shCNT infected and shVEGFA infected MDA-MB-231 cancer cells. **(D)** Immunoblot analysis for autophagy markers. Autophagy was induced in fibroblast cells after 24 h of treatment with the conditioned medium of MDA-MB-231 infected with shGFP; however, it was reduced to the basal level upon treatment with the conditioned medium of MDA-MB-231 infected with shVEGFA.

that were treated with the conditioned medium of these infected cells kept autophagy in basal level. According to the result, autophagy in fibroblast cells was increased due to secreted VEGFA from cancer cells (Figure 73A and B). Reduced VEGFA expression was confirmed using immunoblot technique (Figure 73C). Moreover, autophagy markers were analyzed under this condition. Immunoblot results confirmed the microscope analysis (Figure 73D).

4. CONCLUSION AND DISCUSSION

In our 3D bioprinting work, the goal was to create scaffold-free, well-defined positioned 3D cell printing in order to form macrovascular structures. Part of this thesis focused on the autophagy analysis during this cell printing process. Initially, the computer-aided designs and instructions for the Novogen MMX 3D Bioprinter was used. In the proposed bioprinting process, cells were exposed to several centrifuge and incubation steps before bioprinting process which significantly induced autophagy and increased apoptosis markers according to our analysis. Moreover, 3D bioprinter is equipped with 75 mm long capillaries for cell cylinders and 85 mm long capillaries for support material which may not be enough to create functional structures. Since, during the printing of each capillaries, the time passes, the printed cells without having medium expose to extreme stress under these conditions. Even though the conditions are sterile, long printing hours and extremely fragile structures make it difficult to obtain long and stable printed macrostructures. Due to all reasons explained in above, computer-aided algorithms were developed by our previous collaborator Dr. Bahattin Koç and his team. For this new design, the tool path design and 3D printing parameters were optimized for a well-formed 3D printing. Then, CT and MRI images were used to create the geometry of aorta which was later converted to CAD model. Using computational algorithms, a self-supporting structure was developed. As a result, support structure and MEF cells are successfully printed with the Self-Supporting methodology. A CAD software package, Rhino3D using Rhinoscript language, was used for the implementation of all methodologies.

Besides to 3D bioprinting studies, this thesis also showed the role of autophagy during tumor-stroma communication. Recently, it was shown in the literature that autophagy was induced in tumor stroma. It has also been shown that pancreatic stellate cells support tumor metabolism through alanine secretion upon autophagic induction [288]. Moreover, there are several studies which point out the released factors from tumor

microenvironment especially from fibroblast cells where it supports the growth and proliferation of the cancer cells [289-291]. However, it is still not fully elucidated how cancer cells educate the surrounding cells and what is the initial signaling which induces autophagy in surrounding cells, especially in fibroblast cells. In order to broadly analyze the initial signals from cancer cells, screening of cDNA library growth factor and cytokine library were performed. According to the results, from growth factor screening, TGF β 1 (transforming growth factor beta-1) was well-known factor due to playing an important role during transformation of fibroblast cells from normal fibroblast cells to carcinoma-associated fibroblast cells. From cytokine screening, CT-1 (cardiotrophin-1) and VEGFA (vascular endothelial growth factor-A) are the important factors which induce autophagy in fibroblast cells and potentially mediate crosstalk between cancer cells and fibroblast cells where it induces autophagy in fibroblast cells. In this thesis, their role will be discussed one by one.

In biochip work, a single cell-based screening platform was developed, in which the degree of autophagy activation induced by the intercellular communication between tumor cells and fibroblasts was used as criteria. In these chips, trapping of single tumor cells in the membrane holes of the microfluidic chip followed by co-culture with adjacent fibroblasts was possible. In proof-of-concept tests, the effect of TGF β 1 on paracrine autophagy activation in fibroblasts was shown in cell culture dishes and on microfluidic chips. Another group, Liu *et al.* confirmed autophagy-inducing effects of TGF β 1 on fibroblasts and in the formation of CAF phenotype in a tumor microenvironment [292].

This work is novel since it is the first study to demonstrate at a single cell level that autophagy in tumor stroma, and it can actually be used as a tool to screen for autophagy mediators in individual cancer cells [24]. Therefore, the technique has the potential to reveal key mediators secreted from tumor cells by performing a genome-wide analysis of target cancer cells after autophagy analysis in fibroblasts.

Importantly, the microfluidic platform was suitable for the retrieval of single tumor cells of interest, allowing genomic, transcriptomics and even metabolomics and determination of clonal backgrounds of individual cells. As such, the platform is suitable for large-scale unbiased omics screens. Future large-scale studies using this microfluidic chip platform might provide novel insights into tumor biology, and allow characterization of key molecular mediators of autophagy during tumor–stroma communication and other biological and pathological phenomena. Indeed, our platform has the potential to be applied to different cellular systems (e.g. tumor cell-macrophage, tumor cell-stem cell

couples etc.) and reveal further information on tumor-stroma interactions at a single cell level.

From cytokine screening, CT-1 (cardiotrophin-1) and VEGFA (vascular endothelial growth factor-A) were the critical factors and their roles analyzed in this thesis.

To enlighten the role of CT-1 during communication between cancer cells and fibroblasts, the expression of CT-1 among different cancer types in protein level and secretion to the environment was tested. CT-1 was expressed and secreted among different cancer cells, namely Huh7, MCF7, and PC3 cancer cells. It was shown that human and mouse CT-1 bind to the leukemia inhibitory factor receptor (LIFR) on both human and mouse cell lines indicating a lack of species specificity [187]. In order to confirm the binding of CT-1 to its receptors on MEF cells, LIFR and gp130 receptor proteins were analyzed in MEF cells, and shown that both receptor proteins were expressed in fibroblast cells. In the presence of the receptor, CT-1 protein can bind and induce the downstream pathways. Next, the specificity of autophagy induction of CT-1 in fibroblast cell was tested in the presence of neutralizing antibody. Since neutralizing antibody binds specifically to CT-1 protein, the binding of CT-1 to its receptor was reduced. As a result, autophagy activation in fibroblast cells was reduced to basal level which indicates the specific induction of CT-1 of autophagy in fibroblast cells. Autophagy in fibroblast cells was also induced in time- and dose- dependent manner of CT-1 treatment. During these treatments, induction of autophagy was analyzed with GFP-LC3 dot counting and phosphorylation of STAT3. According to microscope results, autophagy induction was active for 1 h. This result was correlated with phosphorylation of STAT3.

CT-1 neutralization antibody was tested during cancer cells-fibroblast co-culture systems. According to the results, only MCF7 breast cancer cells showed reduced growth when these cells were co-cultured with autophagy-competent fibroblast cells in the presence of CT-1 neutralization antibody at 72 h and 96 h. This system should be tested using human fibroblasts and CAF cells in later time points. It should be noted that CT-1 neutralization antibody does not change the growth of cancer cells (neither MCF7 nor MDA-MB-231 cancer cells), when they are mono-cultured.

Upon the treatment of fibroblast cells with CT-1 recombinant/conditioned medium, different metabolism tests were performed: (i) ketone bodies analysis, (ii) radioactivity for Valine amino acids release, and (iii) SILAC experiments followed by LC-MS/MS analysis.

According to the literature ketone body release was proposed one of the metabolic support which came from autophagy-induced fibroblast cells to cancer cells. However, according to the results, no significant ketone bodies release was detected. This may be explained due to having different origin of the fibroblast cells in our experiments. The support of MEF cells may come from another metabolic substance/secreted molecule. Next, release of valine amino acid was tested. However, no significant difference was observed upon CT-1 recombinant protein treatment on fibroblast cells. Last, SILAC experiments were performed with LC-MS/MS analysis. Since the Pearson correlation coefficient between CT-1 conditioned medium over CNT conditioned medium treated fibroblast cells is 0.714156, it has high correlation between these two conditions. Due to their higher correlation, the difference between these two conditions may come from autophagy induction. According to our results, which emphasize only the effect of CT-1 conditioned medium on fibroblast cells, the protein expressions of collagen fibrillogenesis [245], bone formation [246], DNA replication [247], base excision repair mechanisms [248], immune responses (including T cell activation, blood coagulation, antigen transport and host defense mechanism) [249-252, 254], biogenesis of sterol and cholesterol [259, 260, 262], gluconeogenesis [261] and endosomal trafficking related proteins [257] were reduced. However, translational initiation [255] and inhibition [256], intracellular trafficking [258], carbon metabolism [263], EMT [265], migration and metastasis [266], cancer progression [268], NF-kappaB [269] and especially iron uptake [270, 271] related proteins were significantly increased. Those pathways are critical to support the growth, proliferation and metastasis of cancer cells during tumorigenesis. Fibroblast cells treated with CT-1 conditioned medium show elevated levels for these pathways.

A comparison of the protein profile of conditioned medium of CT-1 treated MEF cells with that of MEF cells treated with STV shows, that in both conditions, MEF cells reduced their proteins which are related with glycogen synthesis, epithelial-mesenchymal transition (EMT), osteonecrosis, nuclear transport, vesicular trafficking and DNA repair comparing to CNT treated MEF cells protein profile [272-275, 277, 278]. Moreover, in both conditions, cancer progression (GLG1), tumor suppression (CUL2), intracellular trafficking (AP4E1), NF-kappaB (CRIP2 and PRKCB), cell proliferation (DIMT1), cell invasiveness and metastasis (FAT1) mechanisms related proteins indicated elevated level comparing to CNT treated MEF cells [281-287]. Interestingly, CT-1 conditioned medium treated MEF cells and STV treated MEF cells showed opposite profile for tumor growth,

distant metastasis and tumor progression [279, 280] where CT-1 conditioned medium treated fibroblast cells were promoted; whereas starvation treated fibroblast cells were suppressed.

Ferritin is a detoxification protein which stores iron. Ferritin expression is regulated by iron and by oxidative stress in order to maintain cellular iron homeostasis. Under normal conditions, ferritin shells are diffusely present in the cytoplasm. However, it can also be detected in mammalian mitochondria and nuclei, and in plant plastids. It consists of H- and L- ferritins and forms a hollow spherical complex of 12 or 24 subunits with 12 nm diameter. The ratio of H- and L- subunits may vary from tissue-to-tissue in order to adapt to cell needs. H-ferritin can translocate to the nuclei in some types of the cells to protect DNA from iron toxicity and oxidative stress, similar to mitochondrial ferritin [293, 294]. It was shown by Noboru Mizushima's group that ferritin which is small high-density particles with a diameter of 0.1 nm were clustered at the autophagosome formation site in FIP200- and ATG9A-deficient cells. With this result, they proposed that ferritin is a selective substrate of autophagy and may also have a role in autophagosome formation [295]. There are other studies which showed that ferritin can be degraded by autophagy [296-299], and iron depletion can induce autophagy [297]. Autophagy plays an important role for the delivery of ferritin to lysosome. However, according to their observation the total amount of ferritin was not increased in autophagy-deficient MEFs. [295]

According to the screening results, iron uptake related proteins, namely transferrin receptor protein 1, ferritin heavy chain and ferritin light chain 1/2 were significantly increased in CT-1 conditioned medium treated MEF cells comparing to control (CNT). Iron (Fe), is an important trace element. It plays an important role during oxygen metabolism, oxygen uptake, and electron transport in mitochondria, energy metabolism, muscle function, and hematopoiesis. Although iron is essential for physical functioning, free bivalent iron is quickly oxidized to its trivalent iron, therefore its free form produces free oxygen radicals, which may result in tissue damage [270]. Therefore, iron metabolism is strictly controlled to keep free iron levels as low as possible and to store enough iron for absorption and transportation. In patients with chronic diseases or cancer, regulation of iron is often dysregulated [300]. Cancer cells produce their own ferritin, which makes them partly independent of external iron supply. This can result in marked increase in serum ferritin levels and in certain cancers ferritin has been shown to closely correlate with tumor stage. Besides its role as indirect tumor marker, ferritin seems to

enhance tumor proliferation by stimulating angiogenesis and by suppressing the host's immune system [301]. Tomao et al. showed the role of iron as a direct carcinogen by describing a haemochromatosis patient who developed HCC without cirrhosis or any other cancer risk factor [302]. However, there are other examples in the literature, which shows the risk of developing HCC after iron removal in haemochromatosis patients. However, there are no studies which show dysregulation of iron metabolism in tumor microenvironment. Upregulation of ferritinophagy may aim to recycle iron in the system upon the stress [303]. Possible pathway analyses need to be investigated.

In order to test whether CT-1 could be used as a marker or not, CT-1 expression levels in non-tumor and tumor samples of the breast cancer patients were tested. According to the results, clinical correlation between CT-1 and breast cancer tumorigenesis, it was shown in this thesis that overexpression of CT-1 is more abundance in bigger sized tumors. Moreover, it was shown in this thesis that CT-1 is highly found in tumor foci in comparison with neighborhood which support our idea that CT-1 is a tumor secreted factor. Intriguingly, our results supported to *in vivo* relevance of the CT-1 induced autophagic activity in stromal cells with high level of LC3 staining in line with the cell culture experiments.

Treatment with chemotherapy which targets cancer cells creates genetically unstable cells and increases selection pressures and expansion of resistant clones [304]. On the contrary, stromal cells are not mutated [305] and turnover more slowly. From a therapeutic point of view, understanding the communication between cancer cells and fibroblast cells, and identifying critical factors which initiates this crosstalk facilitates the development of more effective cancer treatments. The general pattern of the candidate factor should be analyzed carefully in different types of cancer, in different stages with larger patient numbers. This may reveal the potential of this candidate as a disease marker.

In this thesis, the role of VEGFA on autophagy in fibroblast cells was also analyzed. Our analysis showed that VEGFA is commonly produced and secreted factor from different types of cancer cells. The blockage of secreted VEGFA either by using a specific antibody or by knocking down the gene in cancer cells caused the reduction of autophagy to the basal level in fibroblast cells (Figure 67). We also showed that growth of cancer cells alone does not change with the presence of neutralization antibody. Taken together, these results suggest that VEGFA has a potential to be one of the key factors in the tumor microenvironment, because it induces autophagy in another component of stroma, namely fibroblasts.

The tumor microenvironment can be categorized into two types of cells: (i) local cells before tumor development; and (ii) migratory cells from distal sides. Fibroblast cells and endothelial cells are the major groups of the first type; while immune/inflammatory cells, macrophages, neutrophils, other bone marrow-derived cells are the major groups in second type. During homeostasis, besides other roles in regulation of epithelial cell differentiation and inflammatory response on the tissue level, fibroblast cells are key regulators of wound repair by invading the wound, reshaping ECM to anchor other cells, recruiting immune cells to the site of the wound and facilitating healing of the wound via its contractile properties of collagen [104, 306]. While repairing the wounds, fibroblast cells are proliferating faster, producing larger amounts of ECM and expressing α -smooth muscle actin protein, and in that state of higher activity, they are called activated fibroblasts. During tumor progression, resident fibroblast cells obtain a similar character as has been described in wound healing, which promotes the growth and proliferation of neighboring cancer cells [112, 114, 118, 307]. It has been shown that mixing human breast carcinoma cells with CAFs in a mouse xenograft promotes larger and more angiogenic tumor formation due to binding of stromal-derived factor-1 (SDF-1) secreted by the CAFs to the CXCR4 receptor on tumor cells [308]. Besides their supportive role, CAFs deposit large amounts of ECM [309, 310]. Due to increased interstitial fluid pressure with accumulation of ECM in tumors, oxygen and nutrient diffusion can be reduced [311, 312]. As a result, CAFs could lead to increase in the expression of HIF1- α and VEGFA.

It was shown that autophagy is induced in fibroblast cells of tumor microenvironment to support the growth and proliferation of cancer cells metabolically. There are several factors such as hypoxia, high level of ROS, lack of growth factors and nutrient which can induce autophagy especially in CAFs [22, 23, 160, 313, 314]. Recently, the autophagic activities between tumor and stroma was proposed and called as ‘‘the autophagic tumor stroma model of cancer metabolism’’ [313-315]. In this model, it was proposed that by increasing ROS production and creating oxidative stress, cancer cells induce autophagy and mitophagy in CAFs. The induced autophagy in tumor stroma prevents cellular senescence and induces the secretion of high-energetic nutrients, such as ketone and lactate, which will be released to cancer cells in order to promote their growth during tumor growth and metastasis [316, 317]. Besides their own effect on autophagy and mitophagy induction in fibroblast cells, it is plausible that ROS produced by cancer cells induce VEGFA production. It was shown that exogenous ROS stimulate the induction of VEGF expression in different cell types, such as endothelial cells, smooth

muscle cells, and macrophages [318, 319]. Moreover, Xia et al. showed that elevated endogenous ROS level regulates hypoxia-inducible factor-1 (HIF-1) and VEGF expression in ovarian cancer [320]. Due to their close relationship, the reason of autophagy induction in fibroblast cells may also be due to secreted VEGFA depending on the elevated endogenous ROS level.

The growth of cancer cells when these cells are co-cultured with fibroblast cells in the presence of VEGFA neutralization antibody was tested. Similar to the growth of cancer cells in neutralization antibody, no significant growth change in the presence of neutralization of antibody together with fibroblast cells and cancer cells was observed. Although further analysis with different cancer types and patient samples are needed, it is plausible that VEGFA produced and secreted by cancer cells contributes to autophagy in fibroblast cells which makes the fibroblasts further support the metabolism of cancer cells.

4.1. Original Contributions

4.1.1. Original Contribution I - Analysis of the role of autophagy in the context of bioprinted 3D cell systems

This thesis analyzed the importance of autophagy activation during a 3D bioprinting processes. Autophagy-competent (MEF-WT) and autophagy-deficient cells ((*Atg5*^{-/-}) MEF) were characterized. Our experimental data showed that there was no significant difference between these two types of cells in terms of their proliferation, attachment and wound healing characteristics. However, autophagy analysis for cell-cell communication during 3D bioprinting processes creates realistic approaches and functional guidance for the printed scaffold-free macrostructures.

4.1.2. Original Contribution II - Discovery of novel paracrine factors regulating autophagy in fibroblast cells

This thesis showed that conditioned medium of different cancer cells causes induction of autophagy in fibroblast cells. This result points to the presence of secreted factors from cancer cells to their environment. According to our cytokine and growth factor screenings, TGFβ1, CT-1 and VEGFA are important secreted factors during cancer cells-fibroblast communication.

4.1.3. Original Contribution III - A microfluidic chip for screening individual cancer cells via eavesdropping on autophagy-inducing crosstalk in the stroma niche: TGFβ1 as a model paracrine factor

This thesis showed tumor-fibroblast communication and the role TGFβ-1 during this communication using a biochip system. With the created a simplified tumor microenvironment which contains different genomic types of cancer cells and captured these cancer cells as single cells in a close contact with fibroblast cells, and monitored autophagy in fibroblast cells using live cell microscope technique. Such monitoring of single-cell communication is a novel contribution and not possible with conventional cell-culture based methods.

4.1.4. Original Contribution IV - Discovery and analysis of Cardiotrophin-1 (CT-1) as a mediator of tumor-fibroblast interaction

This thesis showed the role of CT-1 during cancer cells-fibroblast communication through changes in autophagy in fibroblast cells for the first time in the literature.

According to the results, a significant portion of tumors at T2 stage (>2 cm) expressed higher levels of CT-1 protein compared to those in T1 stage (<2 cm) among breast cancer patients. In the line of our observations, there was higher autophagy activity in stroma in T2 stage breast cancer patient samples compared to T1 stage breast cancer patient samples. Moreover, we discovered that there was no α -sma expression in (*Atg5*^{-/-}) MEF until these cells were treated with TGF β 1 (low concentration) for at least 72 h. Further analysis needs to be performed.

4.1.5. Original Contribution V - Discovery and analysis of Vascular Endothelial Growth Factor A (VEGFA) as a mediator of tumor-fibroblast interaction

This thesis showed tumor-fibroblast communication and the role VEGFA during this communication. VEGFA is well studied in angiogenesis during tumorigenesis. However, our approach was the role of VEGFA on the induction of autophagy in fibroblast cells, for the first time in the literature.

4.2. Contributions to the Literature

The role of autophagy in microenvironment during tumor-stroma communication is a hot topic in cancer research. Since stromal autophagy is beneficial for cancer cells, instead of targeting cancer cells alone, targeting their support that is coming from microenvironment offers an increase in the efficiency of targeted therapy. This thesis explains the role of three factors which can be found in the tumor microenvironment by understanding their effect on autophagy in fibroblast cells, which are important components of the tumor microenvironment [24]. Moreover, in order to eliminate disadvantages of 2D cell culture method, we designed and created a 3D co-culture technique using a biochip system. Although it is not identical to the real tumor environment, with this method, we believe that the results will be closer to the real tumor-fibroblast communication, and monitoring this crosstalk is mimicked better than in traditional 2D cell culture systems. Besides our biochip work, the role of CT-1 and VEGFA on autophagy in fibroblast cells during tumor-fibroblast communication was analyzed from several further perspectives. We analyzed them *in vitro*, *in vivo* and also in breast cancer patient samples to gain a better understanding of their role on autophagy in fibroblasts and during cell-cell crosstalk. With our bioprinting work, we emphasize the importance of autophagy during 3D bioprinting. During the course of this thesis, several publications, review papers and a book chapter were accepted for publication in the

scientific literature and thereby increase the understanding of the scientific community about tumor-stroma and cell-cell communication (for detailed information see Appendix F- Publications).

In conclusion, cytokines and growth factors are important targets in tumor microenvironment since they are the communication signals between cancer cells and fibroblast cells. Using these signals, cancer cells can modulate the autophagy response of the surrounding fibroblasts cells in order to obtain metabolic help and support. To prevent their communication through the blockage of these signals, cancer cells can be weakened. Therefore, the combinational cancer therapeutics together with blockage of communication signals is a future direction for preventing cancer.

4.2.1. Publication List

JOURNAL PAPERS:

¹: co-author, * : corresponding author

- 1) **H.E. Karakas**¹, Y.Akkoc¹, S. Erbil, C. Kig, U. Zeybek, H. Akkız, J. Dengjel, and D. Gozuacik^{*}.
Cancer-derived paracrine factor mediates tumor-stroma interactions involving autophagy activation in fibroblasts.
Submitted to British Journal of Cancer.
- 2) **H.E. Karakas**¹, J. Kim¹, J. Park, J.M. Oh, Y. Choi, D. Gozuacik^{*}, and Y.K. Cho^{*}.
A microfluidic chip for screening individual cancer cells via eavesdropping on autophagy-inducing crosstalk in the stroma niche.
Scientific Reports 7, 2017, Article number: 2050, DOI: 10.1038/s41598-017-02172-7.
- 3) **H.E. Karakas** and D. Gozuacik^{*}.
Autophagy and Cancer.
Turkish Journal of Biology 38 (6), pages 720-739, 2014, DOI: 10.3906/biy-1408-16.
- 4) C. Kucukgul¹, S.B. Ozler¹, I. Inci, **H.E. Karakas**, S. Irmak, D. Gozuacik, A. Taralp, and B. Koc^{*}.
3D Bioprinting of Biomimetic Aortic Vascular Constructs with Self-Supporting Cells.
Biotechnology and Bioengineering 112 (4), pages 811-821, 2014, DOI: 10.1002/bit.25493.

BOOK CHAPTERS:

- 1) D. Gözüaık*, **H.E. Karakas**, Y. Akko, and B. Özata.
Otofaji ve kanser (Autophagy and cancer).
In book *Kanser Moleküler Biyolojisi (Cancer Molecular Biology)*, Yusuf Baran, editor, 2017, in press.

CONFERENCE PAPERS:

- 1) J. Kim, **H.E. Karakas**, Y. Choi, D. Gozuacik, and Y.K. Cho.
Monitoring Single Tumor Interactions With Fibroblasts In Autophagy Activation.
In: *8th International MMB Conference*, 2016, Seoul, Korea, April 20-22.
- 2) J. Kim, **H.E. Karakas**, Y. Choi, D. Gozuacik, and Y.K. Cho.
Kinetic Study of Autophagy Activation in Fibroblasts Interacted with Single Tumor Cell in Microfluidic Platform.
In: *Annual Autumn Meeting of the Korean BioChip Society*, 2015, Gangwon, Korea, November 18-20.
- 3) J. Kim, **H.E. Karakas**, D. Gozuacik, and Y.K. Cho.
Single Cell Analysis on the Role of TGF-β1 in Autophagy of Tumor Cell-Interacted Stroma Fibroblasts.
In: *19th International Conference on Miniaturized Systems for Chemistry and Life Sciences*, pages 358-360, 2015, Gyeongju, Korea, October 25-28.
- 4) J. Kim, Cedric Bathany, **H.E. Karakas**, D. Gozuacik, and Y.K. Cho.
Screening Chip for Autophagy of Fibroblast in Tumor cell Environment.
In: *18th International Conference on Miniaturized Systems for Chemistry and Life Sciences*, pages 452-454, 2014, San Antonio, USA, October 26-30.
- 5) C. Kucukgul, B. Ozler, **H.E. Karakas**, D. Gozuacik, and B. Koc.
3D hybrid bioprinting of macrovascular structures.
Procedia Engineering 59, pages 183-192, 2013, 3rd ECCOMAS Thematic Conference on Tissue Engineering (ICTE), Leiria, Portugal, June 06-08, 2013, DOI: 10.1016/j.proeng.2013.05.109.
- 6) B. Koc, C. Kucukgul, B. Ozler, **H.E. Karakas**, and D. Gozuacik.
3D hybrid bioprinting of macrovascular structures.
In: *IIE Annual Conference & Expo*, 2013, San Juan, Puerto Rico, May 18-20.

ABSTRACTS:

- 1) **H.E. Karakas**, J. Kim, J. Park, J.M. Oh, Y. Choi, D. Gozuacik, and Y.K. Cho.
Autophagy Analysis of Tumor-Fibroblasts Crosstalk on Biochips.

Turkish Journal of Molecular Biology & Biotechnology 02 (01/Supplement 1), pages 47-48, May 2017, Abstract no.: OP-3.

- 2) D. Gozuacik, S. Irmak, **H.E. Karakas**, and B. Koc.
Optimized 3D design and bioprinting of a blood vessel based on autophagy analyses.
Journal of Tissue Engineering and Regenerative Medicine 6, pages 377, September 2012.

ORAL PRESENTATIONS:

- 1) **H.E. Karakas**, J. Kim, J. Park, J.M. Oh, Y. Choi, D. Gozuacik, and Y.K. Cho.
Autophagy Analysis of Tumor-Fibroblasts Crosstalk on Biochips.
In: *6th International Molecular Medicine Congress*, 2017, Istanbul, Turkey, May 22-25.
- 2) **H.E. Karakas**, J. Kim, J. Park, J.M. Oh, Y. Choi, D. Gozuacik, and Y.K. Cho.
Functional cancer screens using biochip systems.
In: *4th International Gen-Arel Molecular Biology and Genetics Student Congress*, 2017, Istanbul, Turkey, May 5-6.
- 3) J. Kim, **H.E. Karakas**, Y. Choi, D. Gozuacik, and Y.K. Cho.
High throughput monitoring platform for heterogeneity of single tumor in autophagy of fibroblasts.
In: *Annual Winter Meeting of Global Ph.D fellowship conference*, 2015, Seoul, Korea, December 22.
- 4) **H.E. Karakas**, J. Kim, C. Bethany, Y.K. Cho, and D. Gozuacik.
Functional cancer screens using lab-on-a-chip systems.
In: *EMBO YIP*, 2014, Marseille, France, October 17-19.
- 5) O. Oral, D. Oz-Arslan, Z. Itah, **H.E. Karakas**, and D. Gozuacik.
Cleavage of Atg3 protein by caspase-8 regulates autophagy.
In: *Autophagy and Cancer Symposium*, 2014, Toulouse, France, October 15-18.
- 6) **H.E. Karakas**, J. Kim, C. Bethany, Y.K. Cho, and D. Gozuacik.
Functional cancer screens using lab-on-a-chip systems.
In: *MolBioTurk*, 2014, Istanbul, Turkey, April 8.
- 7) D. Gozuacik, **H.E. Karakas**, O. Oral, S. Irmak, and B. Koc.
Optimized 3D Design and Bioprinting of Cells Based on Autophagy Analyses.
In: *FEBS Special Meeting/Protein Quality Control in Health and Disease*, 2012, Kusadasi (Aydin), Turkey, November 14-16.
- 8) D. Gozuacik, **H.E. Karakas**, O. Oral, S. Irmak, and B. Koc.
Optimized 3D Design and Bioprinting of Cells Based on Autophagy Analyses.

In: *3rd TERMIS World Congress: "Tissue Engineering and Regenerative Medicine"*, 2012, Vienna, Austria, September 5-8.

POSTERS:

- 1) **H.E. Karakas**, J. Kim, J. Park, J.M. Oh, Y. Choi, D. Gozuacik, and Y.K. Cho.
A microfluidic chip for screening individual cancer cells via eavesdropping on autophagy-inducing crosstalk in the stroma niche.
In: *5th Heidelberg Forum for Young Life Scientists Conference*, 2017, Heidelberg, Germany, June 8-9.
- 2) J. Kim, **H.E. Karakas**, C. Bethany, D. Gozuacik, and Y.K. Cho.
Screening Chips for Autophagy of Fibroblast in Tumor Cell Environment.
In: *18th International Conference on Miniaturized Systems For Chemistry and Life Sciences*, 2014, San Antonio, Texas, October 26-30.
- 3) **H.E. Karakas**, B. Koc, and D. Gozuacik.
Analysis of the role of autophagy in tissue engineering studies.
In: *TERMIS-EU Conference*, 2013, Istanbul, Turkey, June 17-20.
- 4) D. Gozuacik, **H.E. Karakas**, O. Oral, S. Irmak, and B. Koc.
Optimized 3D Design and Bioprinting of a Blood Vessel based on Autophagy.
In: *3rd Stem Cell Symposium*, 2012, Istanbul, Turkey, November 30 - December 1.
- 5) D. Gozuacik, **H.E. Karakas**, O. Oral, S. Irmak, and B. Koc.
Optimized 3D Design and Bioprinting of a Blood Vessel based on Autophagy.
In: *1st International Congress of the Molecular Biology Association of Turkey*, 2012, Istanbul, Turkey, November 23-24.
- 6) D. Gozuacik, **H.E. Karakas**, O. Oral, S. Irmak, and B. Koc.
Optimized 3D Design and Bioprinting of Cells Based on Autophagy Analyses.
In: *FEBS Special Meeting/Protein Quality Control in Health and Disease*, 2012, Kusadasi (Aydin), Turkey, November 14-16.
- 7) D. Gozuacik, **H.E. Karakas**, O. Oral, S. Irmak, and B. Koc.
Optimized 3D Design and Bioprinting of Cells Based on Autophagy Analyses.
In: *3rd TERMIS World Congress: "Tissue Engineering and Regenerative Medicine"*, 2012, Vienna, Austria, September 5-8.

5. REFERENCES

1. Chaudhuri PK, Ebrahimi Warkiani M, Jing T, Kenry, Lim CT. Microfluidics for research and applications in oncology. *Analyst*. 2016 Jan 21;141(2):504-24.
2. Lopez-Yoldi M, Moreno-Aliaga MJ, Bustos M. Cardiotrophin-1: A multifaceted cytokine. *Cytokine Growth Factor Rev*. 2015 Oct;26(5):523-32.
3. Boussommier-Calleja A, Li R, Chen MB, Wong SC, Kamm RD. Microfluidics: A new tool for modeling cancer-immune interactions. *Trends Cancer*. 2016 Jan 01;2(1):6-19.
4. Chen X, Liang H, Zhang J, Zen K, Zhang CY. Secreted microRNAs: a new form of intercellular communication. *Trends Cell Biol*. 2012 Mar;22(3):125-32.
5. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer*. 2009 Apr;9(4):239-52.
6. Bauer S, Kerr BJ, Patterson PH. The neurotrophic cytokine family in development, plasticity, disease and injury. *Nat Rev Neurosci*. 2007 Mar;8(3):221-32.
7. Karakas HE, Gozuacik D. Autophagy and cancer. *Turk J Biol*. 2014;38(6):720-39.
8. Zabeau L, Lavens D, Peelman F, Eyckerman S, Vandekerckhove J, Tavernier J. The ins and outs of leptin receptor activation. *FEBS Lett*. 2003 Jul 03;546(1):45-50.
9. Kalluri R. The biology and function of fibroblasts in cancer. *Nat Rev Cancer*. 2016 Aug 23;16(9):582-98.
10. Kuma A, Mizushima N. Physiological role of autophagy as an intracellular recycling system: with an emphasis on nutrient metabolism. *Semin Cell Dev Biol*. 2010 Sep;21(7):683-90.
11. Rabinowitz JD, White E. Autophagy and metabolism. *Science*. 2010 Dec 3;330(6009):1344-8.
12. Su M, Mei Y, Sinha S. Role of the Crosstalk between Autophagy and Apoptosis in Cancer. *J Oncol*. 2013;2013:102735.
13. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene*. 2004 Apr 12;23(16):2891-906.
14. Oral O, Oz-Arslan D, Itah Z, Naghavi A, Deveci R, Karacali S, et al. Cleavage of Atg3 protein by caspase-8 regulates autophagy during receptor-activated cell death. *Apoptosis*. 2012 Aug;17(8):810-20.
15. Rubinstein AD, Kimchi A. Life in the balance - a mechanistic view of the crosstalk between autophagy and apoptosis. *J Cell Sci*. 2012 Nov 15;125(Pt 22):5259-68.
16. Gozuacik D, Kimchi A. Autophagy and cell death. *Curr Top Dev Biol*. 2007;78:217-45.
17. Schneider JL, Cuervo AM. Autophagy and human disease: emerging themes. *Curr Opin Genet Dev*. 2014 Jun 4;26C:16-23.
18. Guo JY, Xia B, White E. Autophagy-mediated tumor promotion. *Cell*. 2013 Dec 5;155(6):1216-9.
19. Murrow L, Debnath J. Autophagy as a stress-response and quality-control mechanism: implications for cell injury and human disease. *Annu Rev Pathol*. 2013 Jan 24;8:105-37.
20. Gewirtz DA. Autophagy, senescence and tumor dormancy in cancer therapy. *Autophagy*. 2009 Nov;5(8):1232-4.
21. Eberhart K, Oral O, Gozuacik D. Induction of autophagic cell death by anticancer agents. *Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, and Infection*. [Book Chapter]. 2013:179-202.
22. Martinez-Outschoorn UE, Lin Z, Whitaker-Menezes D, Howell A, Lisanti MP, Sotgia F. Ketone bodies and two-compartment tumor metabolism: stromal ketone production fuels mitochondrial biogenesis in epithelial cancer cells. *Cell Cycle*. 2012 Nov 1;11(21):3956-63.

23. Martinez-Outschoorn UE, Pavlides S, Howell A, Pestell RG, Tanowitz HB, Sotgia F, et al. Stromal-epithelial metabolic coupling in cancer: integrating autophagy and metabolism in the tumor microenvironment. *Int J Biochem Cell Biol.* 2011 Jul;43(7):1045-51.
24. Karakas HE, Kim J, Park J, Oh JM, Choi Y, Gozuacik D, et al. A microfluidic chip for screening individual cancer cells via eavesdropping on autophagy-inducing crosstalk in the stroma niche. *Sci Rep.* 2017 May 17;7(1):2050.
25. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem.* 1998;67:425-79.
26. Klionsky DJ, Schulman BA. Dynamic regulation of macroautophagy by distinctive ubiquitin-like proteins. *Nat Struct Mol Biol.* 2014 Apr 4;21(4):336-45.
27. Uttenweiler A, Schwarz H, Mayer A. Microautophagic vacuole invagination requires calmodulin in a Ca²⁺-independent function. *J Biol Chem.* 2005 Sep 30;280(39):33289-97.
28. Mijaljica D, Devenish RJ. A conference report from "Down Under": Talking autophagy at OzBio2010. *Autophagy.* 2011 Feb;7(2):252-4.
29. Massey AC, Zhang C, Cuervo AM. Chaperone-mediated autophagy in aging and disease. *Curr Top Dev Biol.* 2006;73:205-35.
30. Kaushik S, Cuervo AM. Chaperone-mediated autophagy: a unique way to enter the lysosome world. *Trends Cell Biol.* 2012 Aug;22(8):407-17.
31. Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat Rev Mol Cell Biol.* 2007 Nov;8(11):931-7.
32. Beau I, Esclatine A, Codogno P. Lost to translation: when autophagy targets mature ribosomes. *Trends Cell Biol.* 2008 Jul;18(7):311-4.
33. Kraft C, Reggiori F, Peter M. Selective types of autophagy in yeast. *Biochim Biophys Acta.* 2009 Sep;1793(9):1404-12.
34. van der Vaart A, Mari M, Reggiori F. A picky eater: exploring the mechanisms of selective autophagy in human pathologies. *Traffic.* 2008 Mar;9(3):281-9.
35. Shaid S, Brandts CH, Serve H, Dikic I. Ubiquitination and selective autophagy. *Cell Death Differ.* 2013 Jan;20(1):21-30.
36. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell.* 2008 Jan 11;132(1):27-42.
37. Mizushima N, Levine B. Autophagy in mammalian development and differentiation. *Nat Cell Biol.* 2010 Sep;12(9):823-30.
38. White E, Karp C, Strohecker AM, Guo Y, Mathew R. Role of autophagy in suppression of inflammation and cancer. *Curr Opin Cell Biol.* 2010 Apr;22(2):212-7.
39. Wong E, Cuervo AM. Integration of clearance mechanisms: the proteasome and autophagy. *Cold Spring Harb Perspect Biol.* 2010 Dec;2(12):a006734.
40. Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol.* 2009 Jul;10(7):458-67.
41. Mizushima N, Yoshimori T, Ohsumi Y. The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol.* 2011;27:107-32.
42. Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet.* 2004 Jun;36(6):585-95.
43. Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol Biol Cell.* 2008 Dec;19(12):5360-72.
44. Matsunaga K, Morita E, Saitoh T, Akira S, Ktistakis NT, Izumi T, et al. Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L. *J Cell Biol.* 2010 Aug 23;190(4):511-21.

45. Sun Q, Fan W, Chen K, Ding X, Chen S, Zhong Q. Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A*. 2008 Dec 9;105(49):19211-6.
46. Zhong Y, Wang QJ, Yue Z. Atg14L and Rubicon: yin and yang of Beclin 1-mediated autophagy control. *Autophagy*. 2009 Aug;5(6):890-1.
47. Funderburk SF, Wang QJ, Yue Z. The Beclin 1-VPS34 complex--at the crossroads of autophagy and beyond. *Trends Cell Biol*. 2010 Jun;20(6):355-62.
48. Kirisako T, Ichimura Y, Okada H, Kabeya Y, Mizushima N, Yoshimori T, et al. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J Cell Biol*. 2000 Oct 16;151(2):263-76.
49. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol*. 2001 Feb 19;152(4):657-68.
50. Fujita N, Itoh T, Omori H, Fukuda M, Noda T, Yoshimori T. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol Biol Cell*. 2008 May;19(5):2092-100.
51. Itakura E, Mizushima N. Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. *Autophagy*. 2010 Aug;6(6):764-76.
52. Satoo K, Noda NN, Kumeta H, Fujioka Y, Mizushima N, Ohsumi Y, et al. The structure of Atg4B-LC3 complex reveals the mechanism of LC3 processing and delipidation during autophagy. *EMBO J*. 2009 May 6;28(9):1341-50.
53. Otomo C, Metlagel Z, Takaesu G, Otomo T. Structure of the human ATG12~ATG5 conjugate required for LC3 lipidation in autophagy. *Nat Struct Mol Biol*. 2013 Jan;20(1):59-66.
54. Eskelinen EL, Tanaka Y, Saftig P. At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends Cell Biol*. 2003 Mar;13(3):137-45.
55. Bucci C, Thomsen P, Nicoziani P, McCarthy J, van Deurs B. Rab7: a key to lysosome biogenesis. *Mol Biol Cell*. 2000 Feb;11(2):467-80.
56. Kauppi M, Simonsen A, Bremnes B, Vieira A, Callaghan J, Stenmark H, et al. The small GTPase Rab22 interacts with EEA1 and controls endosomal membrane trafficking. *J Cell Sci*. 2002 Mar 1;115(Pt 5):899-911.
57. Munafo DB, Colombo MI. Induction of autophagy causes dramatic changes in the subcellular distribution of GFP-Rab24. *Traffic*. 2002 Jul;3(7):472-82.
58. Jahreiss L, Menzies FM, Rubinsztein DC. The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes. *Traffic*. 2008 Apr;9(4):574-87.
59. Fader CM, Sanchez DG, Mestre MB, Colombo MI. TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. *Biochim Biophys Acta*. 2009 Dec;1793(12):1901-16.
60. Itakura E, Kishi-Itakura C, Mizushima N. The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. *Cell*. 2012 Dec 7;151(6):1256-69.
61. Mathew R, Karantza-Wadsworth V, White E. Assessing metabolic stress and autophagy status in epithelial tumors. *Methods Enzymol*. 2009;453:53-81.
62. Levine B. Cell biology: autophagy and cancer. *Nature*. 2007 Apr 12;446(7137):745-7.
63. Fung C, Lock R, Gao S, Salas E, Debnath J. Induction of autophagy during extracellular matrix detachment promotes cell survival. *Mol Biol Cell*. 2008 Mar;19(3):797-806.

64. Morselli E, Galluzzi L, Kepp O, Vicencio JM, Criollo A, Maiuri MC, et al. Anti- and pro-tumor functions of autophagy. *Biochim Biophys Acta*. 2009 Sep;1793(9):1524-32.
65. Marino G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, Lopez-Otin C. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. *J Biol Chem*. 2007 Jun 22;282(25):18573-83.
66. Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, et al. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev*. 2011 Apr 15;25(8):795-800.
67. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A*. 2003 Dec 9;100(25):15077-82.
68. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest*. 2003 Dec;112(12):1809-20.
69. Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh BH, et al. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol*. 2006 Jul;8(7):688-99.
70. Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, et al. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol*. 2007 Oct;9(10):1142-51.
71. Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, et al. Autophagy suppresses tumor progression by limiting chromosomal instability. *Genes Dev*. 2007 Jun 1;21(11):1367-81.
72. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev*. 2007 Jul 1;21(13):1621-35.
73. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell*. 2006 Jul;10(1):51-64.
74. Young AR, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, et al. Autophagy mediates the mitotic senescence transition. *Genes Dev*. 2009 Apr 1;23(7):798-803.
75. Inbal B, Bialik S, Sabanay I, Shani G, Kimchi A. DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol*. 2002 Apr 29;157(3):455-68.
76. Shani G, Marash L, Gozuacik D, Bialik S, Teitelbaum L, Shohat G, et al. Death-associated protein kinase phosphorylates ZIP kinase, forming a unique kinase hierarchy to activate its cell death functions. *Mol Cell Biol*. 2004 Oct;24(19):8611-26.
77. Reef S, Zalckvar E, Shifman O, Bialik S, Sabanay H, Oren M, et al. A short mitochondrial form of p19ARF induces autophagy and caspase-independent cell death. *Mol Cell*. 2006 May 19;22(4):463-75.
78. Gozuacik D, Bialik S, Raveh T, Mitou G, Shohat G, Sabanay H, et al. DAP-kinase is a mediator of endoplasmic reticulum stress-induced caspase activation and autophagic cell death. *Cell Death Differ*. 2008 Dec;15(12):1875-86.
79. Elgendy M, Sheridan C, Brumatti G, Martin SJ. Oncogenic Ras-induced expression of Noxa and Beclin-1 promotes autophagic cell death and limits clonogenic survival. *Mol Cell*. 2011 Apr 8;42(1):23-35.
80. Guo JY, Karsli-Uzunbas G, Mathew R, Aisner SC, Kamphorst JJ, Strohecker AM, et al. Autophagy suppresses progression of K-ras-induced lung tumors to oncocytoomas and maintains lipid homeostasis. *Genes Dev*. 2013 Jul 1;27(13):1447-61.
81. Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, et al. Pancreatic cancers require autophagy for tumor growth. *Genes Dev*. 2011 Apr 1;25(7):717-29.

82. Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev.* 2011 Mar 1;25(5):460-70.
83. Strohecker AM, Guo JY, Karsli-Uzunbas G, Price SM, Chen GJ, Mathew R, et al. Autophagy sustains mitochondrial glutamine metabolism and growth of BrafV600E-driven lung tumors. *Cancer Discov.* 2013 Nov;3(11):1272-85.
84. Huo Y, Cai H, Teplova I, Bowman-Colin C, Chen G, Price S, et al. Autophagy opposes p53-mediated tumor barrier to facilitate tumorigenesis in a model of PALB2-associated hereditary breast cancer. *Cancer Discov.* 2013 Aug;3(8):894-907.
85. Lock R, Kenific CM, Leidal AM, Salas E, Debnath J. Autophagy-Dependent Production of Secreted Factors Facilitates Oncogenic RAS-Driven Invasion. *Cancer Discov.* 2014 Apr;4(4):466-79.
86. Lu Z, Luo RZ, Lu Y, Zhang X, Yu Q, Khare S, et al. The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. *J Clin Invest.* 2008 Dec;118(12):3917-29.
87. Paget S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev.* 1989 Aug;8(2):98-101.
88. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature.* 2005 Dec 08;438(7069):820-7.
89. McAllister SS, Gifford AM, Greiner AL, Kelleher SP, Saelzler MP, Ince TA, et al. Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell.* 2008 Jun 13;133(6):994-1005.
90. Gatenby RA, Gillies RJ. A microenvironmental model of carcinogenesis. *Nat Rev Cancer.* 2008 Jan;8(1):56-61.
91. Augsten M. Cancer-associated fibroblasts as another polarized cell type of the tumor microenvironment. *Front Oncol.* 2014;4:62.
92. Chang HY, Sneddon JB, Alizadeh AA, Sood R, West RB, Montgomery K, et al. Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. *PLoS Biol.* 2004 Feb;2(2):E7.
93. Cirri P, Chiarugi P. Cancer associated fibroblasts: the dark side of the coin. *Am J Cancer Res.* 2011;1(4):482-97.
94. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer.* 2006 May;6(5):392-401.
95. Tarin D, Croft CB. Ultrastructural features of wound healing in mouse skin. *J Anat.* 1969 Jul;105(Pt 1):189-90.
96. Gabbiani G, Ryan GB, Majne G. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia.* 1971 May 15;27(5):549-50.
97. Micallef L, Vedrenne N, Billet F, Coulomb B, Darby IA, Desmouliere A. The myofibroblast, multiple origins for major roles in normal and pathological tissue repair. *Fibrogenesis Tissue Repair.* 2012;5(Suppl 1):S5.
98. Desmouliere A, Darby IA, Gabbiani G. Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Lab Invest.* 2003 Dec;83(12):1689-707.
99. Darby IA, Laverdet B, Bonte F, Desmouliere A. Fibroblasts and myofibroblasts in wound healing. *Clin Cosmet Investig Dermatol.* 2014;7:301-11.
100. Ohlund D, Elyada E, Tuveson D. Fibroblast heterogeneity in the cancer wound. *J Exp Med.* 2014 Jul 28;211(8):1503-23.
101. Castor CW, Wilson SM, Heiss PR, Seidman JC. Activation of lung connective tissue cells in vitro. *Am Rev Respir Dis.* 1979 Jul;120(1):101-6.

102. Muller GA, Rodemann HP. Characterization of human renal fibroblasts in health and disease: I. Immunophenotyping of cultured tubular epithelial cells and fibroblasts derived from kidneys with histologically proven interstitial fibrosis. *Am J Kidney Dis.* 1991 Jun;17(6):680-3.
103. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol.* 2002 May;3(5):349-63.
104. Parsonage G, Filer AD, Haworth O, Nash GB, Rainger GE, Salmon M, et al. A stromal address code defined by fibroblasts. *Trends Immunol.* 2005 Mar;26(3):150-6.
105. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol.* 1999 Jul;277(1 Pt 1):C1-9.
106. Sriram G, Bigliardi PL, Bigliardi-Qi M. Fibroblast heterogeneity and its implications for engineering organotypic skin models in vitro. *Eur J Cell Biol.* 2015 Nov;94(11):483-512.
107. Driskell RR, Watt FM. Understanding fibroblast heterogeneity in the skin. *Trends Cell Biol.* 2015 Feb;25(2):92-9.
108. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med.* 1986 Dec 25;315(26):1650-9.
109. Zeisberg M, Strutz F, Muller GA. Role of fibroblast activation in inducing interstitial fibrosis. *J Nephrol.* 2000 Nov-Dec;13 Suppl 3:S111-20.
110. Ryan GB, Cliff WJ, Gabbiani G, Irle C, Statkov PR, Majno G. Myofibroblasts in an avascular fibrous tissue. *Lab Invest.* 1973 Aug;29(2):197-206.
111. Ryan GB, Cliff WJ, Gabbiani G, Irle C, Montandon D, Statkov PR, et al. Myofibroblasts in human granulation tissue. *Hum Pathol.* 1974 Jan;5(1):55-67.
112. Tsukada T, McNutt MA, Ross R, Gown AM. HHF35, a muscle actin-specific monoclonal antibody. II. Reactivity in normal, reactive, and neoplastic human tissues. *Am J Pathol.* 1987 May;127(2):389-402.
113. Schor SL, Schor AM, Grey AM, Rushton G. Foetal and cancer patient fibroblasts produce an autocrine migration-stimulating factor not made by normal adult cells. *J Cell Sci.* 1988 Jul;90 (Pt 3):391-9.
114. Durning P, Schor SL, Sellwood RA. Fibroblasts from patients with breast cancer show abnormal migratory behaviour in vitro. *Lancet.* 1984 Oct 20;2(8408):890-2.
115. Lohr M, Schmidt C, Ringel J, Kluth M, Muller P, Nizze H, et al. Transforming growth factor-beta1 induces desmoplasia in an experimental model of human pancreatic carcinoma. *Cancer Res.* 2001 Jan 15;61(2):550-5.
116. Aoyagi Y, Oda T, Kinoshita T, Nakahashi C, Hasebe T, Ohkohchi N, et al. Overexpression of TGF-beta by infiltrated granulocytes correlates with the expression of collagen mRNA in pancreatic cancer. *Br J Cancer.* 2004 Oct 04;91(7):1316-26.
117. Dumont N, Liu B, Defilippis RA, Chang H, Rabban JT, Karnezis AN, et al. Breast fibroblasts modulate early dissemination, tumorigenesis, and metastasis through alteration of extracellular matrix characteristics. *Neoplasia.* 2013 Mar;15(3):249-62.
118. Elenbaas B, Weinberg RA. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. *Exp Cell Res.* 2001 Mar 10;264(1):169-84.
119. Ishii G, Ochiai A, Neri S. Phenotypic and functional heterogeneity of cancer-associated fibroblast within the tumor microenvironment. *Adv Drug Deliv Rev.* 2016 Apr 01;99(Pt B):186-96.
120. Ronnov-Jessen L, Petersen OW, Bissell MJ. Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. *Physiol Rev.* 1996 Jan;76(1):69-125.

121. Bronzert DA, Pantazis P, Antoniadis HN, Kasid A, Davidson N, Dickson RB, et al. Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. *Proc Natl Acad Sci U S A*. 1987 Aug;84(16):5763-7.
122. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000 Jan 07;100(1):57-70.
123. Dvorak HF, Form DM, Manseau EJ, Smith BD. Pathogenesis of desmoplasia. I. Immunofluorescence identification and localization of some structural proteins of line 1 and line 10 guinea pig tumors and of healing wounds. *J Natl Cancer Inst*. 1984 Nov;73(5):1195-205.
124. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 2012 Mar 20;21(3):309-22.
125. Marsh T, Pietras K, McAllister SS. Fibroblasts as architects of cancer pathogenesis. *Biochim Biophys Acta*. 2013 Jul;1832(7):1070-8.
126. Brown LF, Guidi AJ, Schnitt SJ, Van De Water L, Iruela-Arispe ML, Yeo TK, et al. Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. *Clin Cancer Res*. 1999 May;5(5):1041-56.
127. Feng D, Nagy JA, Brekken RA, Pettersson A, Manseau EJ, Pyne K, et al. Ultrastructural localization of the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) receptor-2 (FLK-1, KDR) in normal mouse kidney and in the hyperpermeable vessels induced by VPF/VEGF-expressing tumors and adenoviral vectors. *J Histochem Cytochem*. 2000 Apr;48(4):545-56.
128. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*. 1989 Dec 08;246(4935):1306-9.
129. Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat Rev Cancer*. 2003 Jun;3(6):422-33.
130. Giussani M, Merlino G, Cappelletti V, Tagliabue E, Daidone MG. Tumor-extracellular matrix interactions: Identification of tools associated with breast cancer progression. *Semin Cancer Biol*. 2015 Dec;35:3-10.
131. Spaeth EL, Dembinski JL, Sasser AK, Watson K, Klopp A, Hall B, et al. Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PLoS One*. 2009;4(4):e4992.
132. Sugimoto H, Mundel TM, Kieran MW, Kalluri R. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol Ther*. 2006 Dec;5(12):1640-6.
133. Anderberg C, Li H, Fredriksson L, Andrae J, Betsholtz C, Li X, et al. Paracrine signaling by platelet-derived growth factor-CC promotes tumor growth by recruitment of cancer-associated fibroblasts. *Cancer Res*. 2009 Jan 01;69(1):369-78.
134. Feig C, Jones JO, Kraman M, Wells RJ, Deonaraine A, Chan DS, et al. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. *Proc Natl Acad Sci U S A*. 2013 Dec 10;110(50):20212-7.
135. Roberts EW, Deonaraine A, Jones JO, Denton AE, Feig C, Lyons SK, et al. Depletion of stromal cells expressing fibroblast activation protein-alpha from skeletal muscle and bone marrow results in cachexia and anemia. *J Exp Med*. 2013 Jun 03;210(6):1137-51.
136. Sharon Y, Alon L, Glanz S, Servais C, Erez N. Isolation of normal and cancer-associated fibroblasts from fresh tissues by Fluorescence Activated Cell Sorting (FACS). *J Vis Exp*. 2013 Jan 14(71):e4425.
137. Trimboli AJ, Cantemir-Stone CZ, Li F, Wallace JA, Merchant A, Creasap N, et al. Pten in stromal fibroblasts suppresses mammary epithelial tumours. *Nature*. 2009 Oct 22;461(7267):1084-91.
138. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res*. 1999 Oct 01;59(19):5002-11.

139. Calon A, Espinet E, Palomo-Ponce S, Tauriello DV, Iglesias M, Cespedes MV, et al. Dependency of colorectal cancer on a TGF-beta-driven program in stromal cells for metastasis initiation. *Cancer Cell*. 2012 Nov 13;22(5):571-84.
140. Zhang XH, Jin X, Malladi S, Zou Y, Wen YH, Brogi E, et al. Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma. *Cell*. 2013 Aug 29;154(5):1060-73.
141. Malanchi I, Santamaria-Martínez A, Susanto E, Peng H, Lehr H-A, Delaloye J-F, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature*. 2011;481(7379):85-9.
142. Jia CC, Wang TT, Liu W, Fu BS, Hua X, Wang GY, et al. Cancer-associated fibroblasts from hepatocellular carcinoma promote malignant cell proliferation by HGF secretion. *PLoS One*. 2013;8(5):e63243.
143. Subramaniam KS, Tham ST, Mohamed Z, Woo YL, Mat Adenan NA, Chung I. Cancer-associated fibroblasts promote proliferation of endometrial cancer cells. *PLoS One*. 2013;8(7):e68923.
144. Cai J, Tang H, Xu L, Wang X, Yang C, Ruan S, et al. Fibroblasts in omentum activated by tumor cells promote ovarian cancer growth, adhesion and invasiveness. *Carcinogenesis*. 2012 Jan;33(1):20-9.
145. Henriksson ML, Edin S, Dahlin AM, Oldenborg PA, Oberg A, Van Guelpen B, et al. Colorectal cancer cells activate adjacent fibroblasts resulting in FGF1/FGFR3 signaling and increased invasion. *Am J Pathol*. 2011 Mar;178(3):1387-94.
146. Owens P, Polikowsky H, Pickup MW, Gorska AE, Jovanovic B, Shaw AK, et al. Bone Morphogenetic Proteins stimulate mammary fibroblasts to promote mammary carcinoma cell invasion. *PLoS One*. 2013;8(6):e67533.
147. Pena C, Cespedes MV, Lindh MB, Kiflemariam S, Mezheyeuski A, Edqvist PH, et al. STC1 expression by cancer-associated fibroblasts drives metastasis of colorectal cancer. *Cancer Res*. 2013 Feb 15;73(4):1287-97.
148. Tyan SW, Hsu CH, Peng KL, Chen CC, Kuo WH, Lee EY, et al. Breast cancer cells induce stromal fibroblasts to secrete ADAMTS1 for cancer invasion through an epigenetic change. *PLoS One*. 2012;7(4):e35128.
149. Giannoni E, Bianchini F, Masieri L, Serni S, Torre E, Calorini L, et al. Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. *Cancer Res*. 2010 Sep 01;70(17):6945-56.
150. Luo Y, Lan L, Jiang Y-G, Zhao J-H, Li M-C, Wei N-B, et al. Epithelial-mesenchymal transition and migration of prostate cancer stem cells is driven by cancer-associated fibroblasts in an HIF-1 α / β -catenin-dependent pathway. *Molecules and Cells*. 2013;36(2):138-44.
151. Tsuyada A, Chow A, Wu J, Somlo G, Chu P, Loera S, et al. CCL2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. *Cancer Res*. 2012 Jun 01;72(11):2768-79.
152. Sanz-Moreno V, Gaggioli C, Yeo M, Albregues J, Wallberg F, Viros A, et al. ROCK and JAK1 signaling cooperate to control actomyosin contractility in tumor cells and stroma. *Cancer Cell*. 2011 Aug 16;20(2):229-45.
153. Scott RW, Hooper S, Crighton D, Li A, Konig I, Munro J, et al. LIM kinases are required for invasive path generation by tumor and tumor-associated stromal cells. *J Cell Biol*. 2010 Oct 04;191(1):169-85.
154. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. *Cancer Cell*. 2010 Feb 17;17(2):135-47.

155. Erez N, Glanz S, Raz Y, Avivi C, Barshack I. Cancer associated fibroblasts express pro-inflammatory factors in human breast and ovarian tumors. *Biochem Biophys Res Commun*. 2013 Aug 02;437(3):397-402.
156. Mace TA, Ameen Z, Collins A, Wojcik S, Mair M, Young GS, et al. Pancreatic cancer-associated stellate cells promote differentiation of myeloid-derived suppressor cells in a STAT3-dependent manner. *Cancer Res*. 2013 May 15;73(10):3007-18.
157. Comito G, Giannoni E, Segura CP, Barcellos-de-Souza P, Raspollini MR, Baroni G, et al. Cancer-associated fibroblasts and M2-polarized macrophages synergize during prostate carcinoma progression. *Oncogene*. 2014 May 08;33(19):2423-31.
158. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009 May 22;324(5930):1029-33.
159. Guido C, Whitaker-Menezes D, Capparelli C, Balliet R, Lin Z, Pestell RG, et al. Metabolic reprogramming of cancer-associated fibroblasts by TGF-beta drives tumor growth: connecting TGF-beta signaling with "Warburg-like" cancer metabolism and L-lactate production. *Cell Cycle*. 2012 Aug 15;11(16):3019-35.
160. Martinez-Outschoorn UE, Lisanti MP, Sotgia F. Catabolic cancer-associated fibroblasts transfer energy and biomass to anabolic cancer cells, fueling tumor growth. *Semin Cancer Biol*. 2014 Apr;25:47-60.
161. Zhang D, Wang Y, Shi Z, Liu J, Sun P, Hou X, et al. Metabolic reprogramming of cancer-associated fibroblasts by IDH3alpha downregulation. *Cell Rep*. 2015 Mar 03;10(8):1335-48.
162. Chaudhri VK, Salzler GG, Dick SA, Buckman MS, Sordella R, Karoly ED, et al. Metabolic alterations in lung cancer-associated fibroblasts correlated with increased glycolytic metabolism of the tumor. *Mol Cancer Res*. 2013 Jun;11(6):579-92.
163. Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, et al. The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. *Cell Cycle*. 2009 Dec;8(23):3984-4001.
164. Fiaschi T, Marini A, Giannoni E, Taddei ML, Gandellini P, De Donatis A, et al. Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay. *Cancer Res*. 2012 Oct 01;72(19):5130-40.
165. Valencia T, Kim JY, Abu-Baker S, Moscat-Pardos J, Ahn CS, Reina-Campos M, et al. Metabolic reprogramming of stromal fibroblasts through p62-mTORC1 signaling promotes inflammation and tumorigenesis. *Cancer Cell*. 2014 Jul 14;26(1):121-35.
166. Lisanti MP, Martinez-Outschoorn UE, Sotgia F. Oncogenes induce the cancer-associated fibroblast phenotype: metabolic symbiosis and "fibroblast addiction" are new therapeutic targets for drug discovery. *Cell Cycle*. 2013 Sep 01;12(17):2723-32.
167. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet*. 2001 Feb 17;357(9255):539-45.
168. Zamarron BF, Chen W. Dual roles of immune cells and their factors in cancer development and progression. *Int J Biol Sci*. 2011;7(5):651-8.
169. Hughes FJ, Turner W, Belibasakis G, Martuscelli G. Effects of growth factors and cytokines on osteoblast differentiation. *Periodontol 2000*. 2006;41:48-72.
170. Frasca F, Pandini G, Sciacca L, Pezzino V, Squatrito S, Belfiore A, et al. The role of insulin receptors and IGF-I receptors in cancer and other diseases. *Arch Physiol Biochem*. 2008 Feb;114(1):23-37.
171. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011 Mar 04;144(5):646-74.
172. Santibanez JF, Quintanilla M, Bernabeu C. TGF-beta/TGF-beta receptor system and its role in physiological and pathological conditions. *Clin Sci (Lond)*. 2011 Sep;121(6):233-51.
173. Massague J. TGFbeta in Cancer. *Cell*. 2008 Jul 25;134(2):215-30.

174. Connolly EC, Freimuth J, Akhurst RJ. Complexities of TGF-beta targeted cancer therapy. *Int J Biol Sci.* 2012;8(7):964-78.
175. Morrison CD, Parvani JG, Schiemann WP. The relevance of the TGF-beta Paradox to EMT-MET programs. *Cancer Lett.* 2013 Nov 28;341(1):30-40.
176. Malliri A, Yeudall WA, Nikolic M, Crouch DH, Parkinson EK, Ozanne B. Sensitivity to transforming growth factor beta 1-induced growth arrest is common in human squamous cell carcinoma cell lines: c-MYC down-regulation and p21waf1 induction are important early events. *Cell Growth Differ.* 1996 Oct;7(10):1291-304.
177. Guasch G, Schober M, Pasolli HA, Conn EB, Polak L, Fuchs E. Loss of TGFbeta signaling destabilizes homeostasis and promotes squamous cell carcinomas in stratified epithelia. *Cancer Cell.* 2007 Oct;12(4):313-27.
178. Bierie B, Moses HL. TGF-beta and cancer. *Cytokine Growth Factor Rev.* 2006 Feb-Apr;17(1-2):29-40.
179. Levy L, Hill CS. Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev.* 2006 Feb-Apr;17(1-2):41-58.
180. Heikkila K, Ebrahim S, Lawlor DA. Systematic review of the association between circulating interleukin-6 (IL-6) and cancer. *Eur J Cancer.* 2008 May;44(7):937-45.
181. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. *Eur J Cancer.* 2005 Nov;41(16):2502-12.
182. Gasche JA, Hoffmann J, Boland CR, Goel A. Interleukin-6 promotes tumorigenesis by altering DNA methylation in oral cancer cells. *Int J Cancer.* 2011 Sep 01;129(5):1053-63.
183. Kinoshita H, Hirata Y, Nakagawa H, Sakamoto K, Hayakawa Y, Takahashi R, et al. Interleukin-6 mediates epithelial-stromal interactions and promotes gastric tumorigenesis. *PLoS One.* 2013;8(4):e60914.
184. Kim SY, Kang JW, Song X, Kim BK, Yoo YD, Kwon YT, et al. Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. *Cell Signal.* 2013 Apr;25(4):961-9.
185. Pennica D, King KL, Shaw KJ, Luis E, Rullamas J, Luoh SM, et al. Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. *Proc Natl Acad Sci U S A.* 1995 Feb 14;92(4):1142-6.
186. Funamoto M, Hishinuma S, Fujio Y, Matsuda Y, Kunisada K, Oh H, et al. Isolation and characterization of the murine cardiotrophin-1 gene: expression and norepinephrine-induced transcriptional activation. *J Mol Cell Cardiol.* 2000 Jul;32(7):1275-84.
187. Pennica D, Swanson TA, Shaw KJ, Kuang WJ, Gray CL, Beatty BG, et al. Human cardiotrophin-1: protein and gene structure, biological and binding activities, and chromosomal localization. *Cytokine.* 1996 Mar;8(3):183-9.
188. Robledo O, Fourcin M, Chevalier S, Guillet C, Auguste P, Pouplard-Barthelaix A, et al. Signaling of the cardiotrophin-1 receptor. Evidence for a third receptor component. *J Biol Chem.* 1997 Feb 21;272(8):4855-63.
189. Pennica D, Shaw KJ, Swanson TA, Moore MW, Shelton DL, Zioncheck KA, et al. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J Biol Chem.* 1995 May 05;270(18):10915-22.
190. Kurdi M, Booz GW. Jak inhibition, but not Stat1 knockdown, blocks the synergistic effect of IFN-gamma on Fas-induced apoptosis of A549 human non-small cell lung cancer cells. *J Interferon Cytokine Res.* 2007 Jan;27(1):23-31.
191. Hirota H, Chen J, Betz UA, Rajewsky K, Gu Y, Ross J, Jr., et al. Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress. *Cell.* 1999 Apr 16;97(2):189-98.
192. Barnabe-Heider F, Wasylnka JA, Fernandes KJ, Porsche C, Sendtner M, Kaplan DR, et al. Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. *Neuron.* 2005 Oct 20;48(2):253-65.

193. Ho DW, Yang ZF, Lau CK, Tam KH, To JY, Poon RT, et al. Therapeutic potential of cardiotrophin 1 in fulminant hepatic failure: dual roles in antiapoptosis and cell repair. *Arch Surg*. 2006 Nov;141(11):1077-84; discussion 84.
194. Moreno-Aliaga MJ, Perez-Echarri N, Marcos-Gomez B, Larequi E, Gil-Bea FJ, Viollet B, et al. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab*. 2011 Aug 03;14(2):242-53.
195. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature*. 2000 Sep 14;407(6801):249-57.
196. Weis SM, Lindquist JN, Barnes LA, Lutu-Fuga KM, Cui J, Wood MR, et al. Cooperation between VEGF and beta3 integrin during cardiac vascular development. *Blood*. 2007 Mar 01;109(5):1962-70.
197. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002 Dec 19-26;420(6917):860-7.
198. Park CC, Bissell MJ, Barcellos-Hoff MH. The influence of the microenvironment on the malignant phenotype. *Mol Med Today*. 2000 Aug;6(8):324-9.
199. Tlsty TD, Hein PW. Know thy neighbor: stromal cells can contribute oncogenic signals. *Curr Opin Genet Dev*. 2001 Feb;11(1):54-9.
200. Turley H, Scott PA, Watts VM, Bicknell R, Harris AL, Gatter KC. Expression of VEGF in routinely fixed material using a new monoclonal antibody VG1. *J Pathol*. 1998 Nov;186(3):313-8.
201. Senger DR. Vascular endothelial growth factor: much more than an angiogenesis factor. *Mol Biol Cell*. 2010 Feb 01;21(3):377-9.
202. Whitesides GM. The origins and the future of microfluidics. *Nature*. 2006 Jul 27;442(7101):368-73.
203. Haeberle S, Zengerle R. Microfluidic platforms for lab-on-a-chip applications. *Lab Chip*. 2007 Sep;7(9):1094-110.
204. Ma S, Loufakis DN, Cao Z, Chang Y, Achenie LE, Lu C. Diffusion-based microfluidic PCR for "one-pot" analysis of cells. *Lab Chip*. 2014 Aug 21;14(16):2905-9.
205. Jebrail MJ, Renzi RF, Sinha A, Van De Vreugde J, Gondhalekar C, Ambriz C, et al. A solvent replenishment solution for managing evaporation of biochemical reactions in air-matrix digital microfluidics devices. *Lab Chip*. 2015 Jan 07;15(1):151-8.
206. Sia SK, Whitesides GM. Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies. *Electrophoresis*. 2003 Nov;24(21):3563-76.
207. Giulitti S, Magrofuoco E, Prevedello L, Elvassore N. Optimal periodic perfusion strategy for robust long-term microfluidic cell culture. *Lab Chip*. 2013 Nov 21;13(22):4430-41.
208. Zhang Q, Liu T, Qin J. A microfluidic-based device for study of transendothelial invasion of tumor aggregates in realtime. *Lab Chip*. 2012 Aug 21;12(16):2837-42.
209. El-Ali J, Sorger PK, Jensen KF. Cells on chips. *Nature*. 2006 Jul 27;442(7101):403-11.
210. Mehling M, Tay S. Microfluidic cell culture. *Curr Opin Biotechnol*. 2014 Feb;25:95-102.
211. Xiong B, Ren K, Shu Y, Chen Y, Shen B, Wu H. Recent developments in microfluidics for cell studies. *Adv Mater*. 2014 Aug 20;26(31):5525-32.
212. Torisawa YS, Mosadegh B, Luker GD, Morell M, O'Shea KS, Takayama S. Microfluidic hydrodynamic cellular patterning for systematic formation of co-culture spheroids. *Integr Biol (Camb)*. 2009 Dec;1(11-12):649-54.
213. Skafte-Pedersen P, Hemmingsen M, Sabourin D, Blaga FS, Bruus H, Dufva M. A self-contained, programmable microfluidic cell culture system with real-time microscopy access. *Biomed Microdevices*. 2012 Apr;14(2):385-99.
214. Wang DY, Wu SC, Lin SP, Hsiao SH, Chung TW, Huang YY. Evaluation of transdifferentiation from mesenchymal stem cells to neuron-like cells using microfluidic patterned co-culture system. *Biomed Microdevices*. 2011 Jun;13(3):517-26.

215. Wei CW, Cheng JY, Young TH. Elucidating in vitro cell-cell interaction using a microfluidic coculture system. *Biomed Microdevices*. 2006 Mar;8(1):65-71.
216. Kobel S, Valero A, Latt J, Renaud P, Lutolf M. Optimization of microfluidic single cell trapping for long-term on-chip culture. *Lab Chip*. 2010 Apr 07;10(7):857-63.
217. Mazutis L, Gilbert J, Ung WL, Weitz DA, Griffiths AD, Heyman JA. Single-cell analysis and sorting using droplet-based microfluidics. *Nat Protoc*. 2013 May;8(5):870-91.
218. Yin H, Marshall D. Microfluidics for single cell analysis. *Curr Opin Biotechnol*. 2012 Feb;23(1):110-9.
219. Liu T, Lin B, Qin J. Carcinoma-associated fibroblasts promoted tumor spheroid invasion on a microfluidic 3D co-culture device. *Lab Chip*. 2010 Jul 07;10(13):1671-7.
220. Ma H, Liu T, Qin J, Lin B. Characterization of the interaction between fibroblasts and tumor cells on a microfluidic co-culture device. *Electrophoresis*. 2010 May;31(10):1599-605.
221. Hockemeyer K, Janetopoulos C, Terekhov A, Hofmeister W, Vilgelm A, Costa L, et al. Engineered three-dimensional microfluidic device for interrogating cell-cell interactions in the tumor microenvironment. *Biomicrofluidics*. 2014 Jul;8(4):044105.
222. Zheng C, Zhao L, Chen G, Zhou Y, Pang Y, Huang Y. Quantitative study of the dynamic tumor-endothelial cell interactions through an integrated microfluidic coculture system. *Anal Chem*. 2012 Feb 21;84(4):2088-93.
223. Hsu TH, Kao YL, Lin WL, Xiao JL, Kuo PL, Wu CW, et al. The migration speed of cancer cells influenced by macrophages and myofibroblasts co-cultured in a microfluidic chip. *Integr Biol (Camb)*. 2012 Feb;4(2):177-82.
224. Li P, Stratton ZS, Dao M, Ritz J, Huang TJ. Probing circulating tumor cells in microfluidics. *Lab Chip*. 2013 Feb 21;13(4):602-9.
225. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, et al. The role of autophagy during the early neonatal starvation period. *Nature*. 2004 Dec 23;432(7020):1032-6.
226. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*. 2006 Jun 15;441(7095):885-9.
227. Zou Y, Zhang Y. Mechanical evaluation of decellularized porcine thoracic aorta. *J Surg Res*. 2012 Jun 15;175(2):359-68.
228. Kucukgul C, Ozler SB, Inci I, Karakas E, Irmak S, Gozuacik D, et al. 3D bioprinting of biomimetic aortic vascular constructs with self-supporting cells. *Biotechnol Bioeng*. 2015 Apr;112(4):811-21.
229. Hjelle JT, Carlson EC, Brendel K, Meezan E. Biosynthesis of basement membrane matrix by isolated rat renal glomeruli. *Kidney Int*. 1979 Jan;15(1):20-32.
230. Tanida I, Ueno T, Kominami E. Human light chain 3/MAP1LC3B is cleaved at its carboxyl-terminal Met121 to expose Gly120 for lipidation and targeting to autophagosomal membranes. *J Biol Chem*. 2004 Nov 12;279(46):47704-10.
231. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J*. 2000 Nov 01;19(21):5720-8.
232. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol*. 2005 Nov 21;171(4):603-14.
233. Larsen KB, Lamark T, Overvatn A, Harneshaug I, Johansen T, Bjorkoy G. A reporter cell system to monitor autophagy based on p62/SQSTM1. *Autophagy*. 2010 Aug;6(6):784-93.
234. Shimoda M, Mellody KT, Orimo A. Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression. *Semin Cell Dev Biol*. 2010 Feb;21(1):19-25.

235. Bernard M, Dieude M, Yang B, Hamelin K, Underwood K, Hebert MJ. Autophagy fosters myofibroblast differentiation through MTORC2 activation and downstream upregulation of CTGF. *Autophagy*. 2014;10(12):2193-207.
236. Ghavami S, Cunnington RH, Gupta S, Yeganeh B, Filomeno KL, Freed DH, et al. Autophagy is a regulator of TGF-beta1-induced fibrogenesis in primary human atrial myofibroblasts. *Cell Death Dis*. 2015 Mar 19;6:e1696.
237. Frassanito MA, De Veirman K, Desantis V, Di Marzo L, Vergara D, Ruggieri S, et al. Halting pro-survival autophagy by TGFbeta inhibition in bone marrow fibroblasts overcomes bortezomib resistance in multiple myeloma patients. *Leukemia*. 2016 Mar;30(3):640-8.
238. Martinez-Carpio PA, Mur C, Rosel P, Navarro MA. Constitutive and regulated secretion of epidermal growth factor and transforming growth factor-beta1 in MDA-MB-231 breast cancer cell line in 11-day cultures. *Cell Signal*. 1999 Oct;11(10):753-7.
239. Sheng Z, Knowlton K, Chen J, Hoshijima M, Brown JH, Chien KR. Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase-dependent pathway. Divergence from downstream CT-1 signals for myocardial cell hypertrophy. *J Biol Chem*. 1997 Feb 28;272(9):5783-91.
240. Smale ST. Luciferase assay. *Cold Spring Harb Protoc*. 2010 May;2010(5):pdb prot5421.
241. Utispan K, Thuwajit P, Abiko Y, Charngkaew K, Paupairoj A, Chau-in S, et al. Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker. *Mol Cancer*. 2010 Jan 24;9:13.
242. Drobic V, Cunnington RH, Bedosky KM, Raizman JE, Elimban VV, Rattan SG, et al. Differential and combined effects of cardiotrophin-1 and TGF-beta1 on cardiac myofibroblast proliferation and contraction. *Am J Physiol Heart Circ Physiol*. 2007 Aug;293(2):H1053-64.
243. Kojima Y, Acar A, Eaton EN, Mellody KT, Scheel C, Ben-Porath I, et al. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. *Proc Natl Acad Sci U S A*. 2010 Nov 16;107(46):20009-14.
244. Gayen AK. The frequency distribution of the product-moment correlation coefficient in random samples of any size drawn from non-normal universes. *Biometrika*. 1951 Jun;38(1-2):219-47.
245. Sun M, Chen S, Adams SM, Florer JB, Liu H, Kao WW, et al. Collagen V is a dominant regulator of collagen fibrillogenesis: dysfunctional regulation of structure and function in a corneal-stroma-specific Col5a1-null mouse model. *J Cell Sci*. 2011 Dec 01;124(Pt 23):4096-105.
246. Izu Y, Sun M, Zwolanek D, Veit G, Williams V, Cha B, et al. Type XII collagen regulates osteoblast polarity and communication during bone formation. *J Cell Biol*. 2011 Jun 13;193(6):1115-30.
247. Yoshida MM, Ting L, Gygi SP, Azuma Y. SUMOylation of DNA topoisomerase IIalpha regulates histone H3 kinase Haspin and H3 phosphorylation in mitosis. *J Cell Biol*. 2016 Jun 20;213(6):665-78.
248. Gossmann TI, Ziegler M. Sequence divergence and diversity suggests ongoing functional diversification of vertebrate NAD metabolism. *DNA Repair (Amst)*. 2014 Nov;23:39-48.
249. Chong LC, Twa DD, Mottok A, Ben-Neriah S, Woolcock BW, Zhao Y, et al. Comprehensive characterization of programmed death ligand structural rearrangements in B-cell non-Hodgkin lymphomas. *Blood*. 2016 Sep 01;128(9):1206-13.
250. Molinas FC, Giavedoni E, Frigerio MJ, Calello MA, Barcat JA, Weissenbacher MC. Alteration of blood coagulation and complement system in neotropical primates infected with Junin virus. *J Med Virol*. 1983;12(4):281-92.

251. Wynne JW, Woon AP, Dudek NL, Croft NP, Ng JH, Baker ML, et al. Characterization of the Antigen Processing Machinery and Endogenous Peptide Presentation of a Bat MHC Class I Molecule. *J Immunol*. 2016 Jun 01;196(11):4468-76.
252. Wang B, Ekblom R, Strand TM, Portela-Bens S, Hoglund J. Sequencing of the core MHC region of black grouse (*Tetrao tetrix*) and comparative genomics of the galliform MHC. *BMC Genomics*. 2012 Oct 15;13:553.
253. Zhang YS, Xiong GF, Peng JP. Effects of TGF beta-1 on mouse embryo implantation and expression of H2-D1 and H2-DM. *Front Biosci (Elite Ed)*. 2010 Jan 01;2:351-60.
254. Kim BH, Shenoy AR, Kumar P, Das R, Tiwari S, MacMicking JD. A family of IFN-gamma-inducible 65-kD GTPases protects against bacterial infection. *Science*. 2011 May 06;332(6030):717-21.
255. Lee SG, Song K. Identification and characterization of a bidirectional promoter from the intergenic region between the human DDX13 and RD genes. *Mol Cells*. 2000 Feb 29;10(1):47-53.
256. Hiraishi H, Oatman J, Haller SL, Blunk L, McGivern B, Morris J, et al. Essential role of eIF5-mimic protein in animal development is linked to control of ATF4 expression. *Nucleic Acids Res*. 2014;42(16):10321-30.
257. Donaldson JG, Johnson DL, Dutta D. Rab and Arf G proteins in endosomal trafficking and cell surface homeostasis. *Small GTPases*. 2016 Oct;7(4):247-51.
258. Schill NJ, Hedman AC, Choi S, Anderson RA. Isoform 5 of PIPKIgamma regulates the endosomal trafficking and degradation of E-cadherin. *J Cell Sci*. 2014 May 15;127(Pt 10):2189-203.
259. Robertson KA, Hsieh WY, Forster T, Blanc M, Lu H, Crick PJ, et al. An Interferon Regulated MicroRNA Provides Broad Cell-Intrinsic Antiviral Immunity through Multihit Host-Directed Targeting of the Sterol Pathway. *PLoS Biol*. 2016 Mar;14(3):e1002364.
260. Ghosh S, Mukherjee S, Basu A. Chandipura virus perturbs cholesterol homeostasis leading to neuronal apoptosis. *J Neurochem*. 2015 Oct;135(2):368-80.
261. Ye G, Liu J, Liu Y, Chen X, Liao SF, Huang D, et al. Feeding glycerol-enriched yeast culture improves lactation performance, energy status, and hepatic gluconeogenic enzyme expression of dairy cows during the transition period. *J Anim Sci*. 2016 Jun;94(6):2441-50.
262. Lu M, Hu XH, Li Q, Xiong Y, Hu GJ, Xu JJ, et al. A specific cholesterol metabolic pathway is established in a subset of HCCs for tumor growth. *J Mol Cell Biol*. 2013 Dec;5(6):404-15.
263. Ding F, Li HH, Li J, Myers RM, Francke U. Neonatal maternal deprivation response and developmental changes in gene expression revealed by hypothalamic gene expression profiling in mice. *PLoS One*. 2010 Feb 24;5(2):e9402.
264. Chandrakesan P, Panneerselvam J, Qu D, Weygant N, May R, Bronze MS, et al. Regulatory Roles of Dclk1 in Epithelial Mesenchymal Transition and Cancer Stem Cells. *J Carcinog Mutagen*. 2016 Apr;7(2).
265. Wu N, Wei J, Wang Y, Yan J, Qin Y, Tong D, et al. Ribosomal L22-like1 (RPL22L1) Promotes Ovarian Cancer Metastasis by Inducing Epithelial-to-Mesenchymal Transition. *PLoS One*. 2015;10(11):e0143659.
266. MacDonald G, Nalvarte I, Smirnova T, Vecchi M, Aceto N, Dolemeyer A, et al. Memo is a copper-dependent redox protein with an essential role in migration and metastasis. *Sci Signal*. 2014 Jun 10;7(329):ra56.
267. Abid A, Ajaz S, Khan AR, Zehra F, Hasan AS, Sultan G, et al. Analysis of the glutathione S-transferase genes polymorphisms in the risk and prognosis of renal cell carcinomas. Case-control and meta-analysis. *Urol Oncol*. 2016 Sep;34(9):419 e1- e12.
268. Caselli A, Paoli P, Santi A, Mugnaioni C, Toti A, Camici G, et al. Low molecular weight protein tyrosine phosphatase: Multifaceted functions of an evolutionarily conserved enzyme. *Biochim Biophys Acta*. 2016 Oct;1864(10):1339-55.

269. Fenner BJ, Scannell M, Prehn JH. Identification of polyubiquitin binding proteins involved in NF-kappaB signaling using protein arrays. *Biochim Biophys Acta*. 2009 Jul;1794(7):1010-6.
270. Ponka P, Lok CN. The transferrin receptor: role in health and disease. *Int J Biochem Cell Biol*. 1999 Oct;31(10):1111-37.
271. Jahshan A, Esteves-Villanueva JO, Martic-Milne S. Evaluation of ferritin and transferrin binding to tau protein. *J Inorg Biochem*. 2016 Sep;162:127-34.
272. Irgens HU, Fjeld K, Johansson BB, Ringdal M, Immervoll H, Leh S, et al. Glycogenin-2 is dispensable for liver glycogen synthesis and glucagon-stimulated glucose release. *J Clin Endocrinol Metab*. 2015 May;100(5):E767-75.
273. Lee WJ, Kim SC, Yoon JH, Yoon SJ, Lim J, Kim YS, et al. Meta-Analysis of Tumor Stem-Like Breast Cancer Cells Using Gene Set and Network Analysis. *PLoS One*. 2016;11(2):e0148818.
274. Kim TH, Bae SC, Lee SH, Kim SY, Baek SH. Association of Complement Receptor 2 Gene Polymorphisms with Susceptibility to Osteonecrosis of the Femoral Head in Systemic Lupus Erythematosus. *Biomed Res Int*. 2016;2016:9208035.
275. Plafker KS, Plafker SM. The ubiquitin-conjugating enzyme UBE2E3 and its import receptor importin-11 regulate the localization and activity of the antioxidant transcription factor NRF2. *Mol Biol Cell*. 2015 Jan 15;26(2):327-38.
276. Ma L, Piirainen S, Kuleshkaya N, Rauvala H, Tian L. Association of brain immune genes with social behavior of inbred mouse strains. *J Neuroinflammation*. 2015 Apr 18;12:75.
277. Dirk BS, Jacob RA, Johnson AL, Pawlak EN, Cavanagh PC, Van Nynatten L, et al. Viral bimolecular fluorescence complementation: a novel tool to study intracellular vesicular trafficking pathways. *PLoS One*. 2015;10(4):e0125619.
278. Majka J, Chung BY, Burgers PM. Requirement for ATP by the DNA damage checkpoint clamp loader. *J Biol Chem*. 2004 May 14;279(20):20921-6.
279. Wang M, Zhao XP, Xu Z, Yan TL, Song Y, Song K, et al. EphA2 silencing promotes growth, migration, and metastasis in salivary adenoid cystic carcinoma: in vitro and in vivo study. *Am J Transl Res*. 2016;8(3):1518-29.
280. Basakran NS. CD44 as a potential diagnostic tumor marker. *Saudi Med J*. 2015 Mar;36(3):273-9.
281. Morisaki T, Yashiro M, Kakehashi A, Inagaki A, Kinoshita H, Fukuoka T, et al. Comparative proteomics analysis of gastric cancer stem cells. *PLoS One*. 2014;9(11):e110736.
282. Maeda Y, Suzuki T, Pan X, Chen G, Pan S, Bartman T, et al. CUL2 is required for the activity of hypoxia-inducible factor and vasculogenesis. *J Biol Chem*. 2008 Jun 06;283(23):16084-92.
283. Raza MH, Mattera R, Morell R, Sainz E, Rahn R, Gutierrez J, et al. Association between Rare Variants in AP4E1, a Component of Intracellular Trafficking, and Persistent Stuttering. *Am J Hum Genet*. 2015 Nov 05;97(5):715-25.
284. Cheung AK, Ko JM, Lung HL, Chan KW, Stanbridge EJ, Zabarovsky E, et al. Cysteine-rich intestinal protein 2 (CRIP2) acts as a repressor of NF-kappaB-mediated proangiogenic cytokine transcription to suppress tumorigenesis and angiogenesis. *Proc Natl Acad Sci U S A*. 2011 May 17;108(20):8390-5.
285. Kiga K, Mimuro H, Suzuki M, Shinozaki-Ushiku A, Kobayashi T, Sanada T, et al. Epigenetic silencing of miR-210 increases the proliferation of gastric epithelium during chronic *Helicobacter pylori* infection. *Nat Commun*. 2014 Sep 04;5:4497.
286. Antonell A, Llado A, Sanchez-Valle R, Sanfeliu C, Casserras T, Rami L, et al. Altered Blood Gene Expression of Tumor-Related Genes (PRKCB, BECN1, and CDKN2A) in Alzheimer's Disease. *Mol Neurobiol*. 2016 Nov;53(9):5902-11.

287. Pileri P, Campagnoli S, Grandi A, Parri M, De Camilli E, Song C, et al. FAT1: a potential target for monoclonal antibody therapy in colon cancer. *Br J Cancer*. 2016 Jun 28;115(1):40-51.
288. Sousa CM, Biancur DE, Wang X, Halbrook CJ, Sherman MH, Zhang L, et al. Pancreatic stellate cells support tumour metabolism through autophagic alanine secretion. *Nature*. 2016 Aug 25;536(7617):479-83.
289. Librizzi M, Tobiasch E, Luparello C. The conditioned medium from osteo-differentiating human mesenchymal stem cells affects the viability of triple negative MDA-MB231 breast cancer cells. *Cell Biochem Funct*. 2016 Jan;34(1):7-15.
290. Yu Y, Xiao CH, Tan LD, Wang QS, Li XQ, Feng YM. Cancer-associated fibroblasts induce epithelial-mesenchymal transition of breast cancer cells through paracrine TGF-beta signalling. *Br J Cancer*. 2014 Feb 04;110(3):724-32.
291. Soon PS, Kim E, Pon CK, Gill AJ, Moore K, Spillane AJ, et al. Breast cancer-associated fibroblasts induce epithelial-to-mesenchymal transition in breast cancer cells. *Endocr Relat Cancer*. 2013 Feb;20(1):1-12.
292. Liu FL, Mo EP, Yang L, Du J, Wang HS, Zhang H, et al. Autophagy is involved in TGF-beta1-induced protective mechanisms and formation of cancer-associated fibroblasts phenotype in tumor microenvironment. *Oncotarget*. 2016 Jan 26;7(4):4122-41.
293. Arosio P, Ingrassia R, Cavadini P. Ferritins: a family of molecules for iron storage, antioxidation and more. *Biochim Biophys Acta*. 2009 Jul;1790(7):589-99.
294. Watt RK. The many faces of the octahedral ferritin protein. *Biometals*. 2011 Jun;24(3):489-500.
295. Kishi-Itakura C, Koyama-Honda I, Itakura E, Mizushima N. Ultrastructural analysis of autophagosome organization using mammalian autophagy-deficient cells. *J Cell Sci*. 2014 Sep 15;127(Pt 18):4089-102.
296. Asano T, Komatsu M, Yamaguchi-Iwai Y, Ishikawa F, Mizushima N, Iwai K. Distinct mechanisms of ferritin delivery to lysosomes in iron-depleted and iron-replete cells. *Mol Cell Biol*. 2011 May;31(10):2040-52.
297. De Domenico I, Ward DM, Kaplan J. Specific iron chelators determine the route of ferritin degradation. *Blood*. 2009 Nov 12;114(20):4546-51.
298. Kidane TZ, Sauble E, Linder MC. Release of iron from ferritin requires lysosomal activity. *Am J Physiol Cell Physiol*. 2006 Sep;291(3):C445-55.
299. Ollinger K, Roberg K. Nutrient deprivation of cultured rat hepatocytes increases the desferrioxamine-available iron pool and augments the sensitivity to hydrogen peroxide. *J Biol Chem*. 1997 Sep 19;272(38):23707-11.
300. Ludwig H, Muldur E, Endler G, Hubl W. Prevalence of iron deficiency across different tumors and its association with poor performance status, disease status and anemia. *Ann Oncol*. 2013 Jul;24(7):1886-92.
301. Alkhateeb AA, Connor JR. The significance of ferritin in cancer: anti-oxidation, inflammation and tumorigenesis. *Biochim Biophys Acta*. 2013 Dec;1836(2):245-54.
302. Tomao S, Romiti A, Mozzicafreddo A, Raffaele M, Zullo A, Antonaci A. Onset of hepatocellular carcinoma in a non-cirrhotic patient affected with haemochromatosis. *Oncol Rep*. 1998 May-Jun;5(3):723-5.
303. Deugnier YM, Guyader D, Crantock L, Lopez JM, Turlin B, Yaouanq J, et al. Primary liver cancer in genetic hemochromatosis: a clinical, pathological, and pathogenetic study of 54 cases. *Gastroenterology*. 1993 Jan;104(1):228-34.
304. Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature*. 2013 Sep 19;501(7467):328-37.
305. Allinen M, Beroukhi R, Cai L, Brennan C, Lahti-Domenici J, Huang H, et al. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell*. 2004 Jul;6(1):17-32.

306. Tomasek JJ, McRae J, Owens GK, Haaksma CJ. Regulation of alpha-smooth muscle actin expression in granulation tissue myofibroblasts is dependent on the intronic CArG element and the transforming growth factor-beta1 control element. *Am J Pathol.* 2005 May;166(5):1343-51.
307. Schor SL, Schor AM, Rushton G. Fibroblasts from cancer patients display a mixture of both foetal and adult-like phenotypic characteristics. *J Cell Sci.* 1988 Jul;90 (Pt 3):401-7.
308. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell.* 2005 May 06;121(3):335-48.
309. Chiquet-Ehrismann R, Mackie EJ, Pearson CA, Sakakura T. Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell.* 1986 Oct 10;47(1):131-9.
310. Inaguma Y, Kusakabe M, Mackie EJ, Pearson CA, Chiquet-Ehrismann R, Sakakura T. Epithelial induction of stromal tenascin in the mouse mammary gland: from embryogenesis to carcinogenesis. *Dev Biol.* 1988 Aug;128(2):245-55.
311. Netti PA, Berk DA, Swartz MA, Grodzinsky AJ, Jain RK. Role of extracellular matrix assembly in interstitial transport in solid tumors. *Cancer Res.* 2000 May 01;60(9):2497-503.
312. Brown EB, Boucher Y, Nasser S, Jain RK. Measurement of macromolecular diffusion coefficients in human tumors. *Microvasc Res.* 2004 May;67(3):231-6.
313. Sotgia F, Martinez-Outschoorn UE, Howell A, Pestell RG, Pavlides S, Lisanti MP. Caveolin-1 and cancer metabolism in the tumor microenvironment: markers, models, and mechanisms. *Annu Rev Pathol.* 2012;7:423-67.
314. Pavlides S, Vera I, Gandara R, Sneddon S, Pestell RG, Mercier I, et al. Warburg meets autophagy: cancer-associated fibroblasts accelerate tumor growth and metastasis via oxidative stress, mitophagy, and aerobic glycolysis. *Antioxid Redox Signal.* 2012 Jun 01;16(11):1264-84.
315. Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, et al. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell.* 2006 Jul 14;126(1):107-20.
316. Castello-Cros R, Bonuccelli G, Molchansky A, Capozza F, Witkiewicz AK, Birbe RC, et al. Matrix remodeling stimulates stromal autophagy, "fueling" cancer cell mitochondrial metabolism and metastasis. *Cell Cycle.* 2011 Jun 15;10(12):2021-34.
317. Iozzo RV. Autophagic tumor stroma: a biofuel for cancer growth. *Cell Cycle.* 2011 Oct 01;10(19):3231-2.
318. Ushio-Fukai M, Tang Y, Fukai T, Dikalov SI, Ma Y, Fujimoto M, et al. Novel role of gp91(phox)-containing NAD(P)H oxidase in vascular endothelial growth factor-induced signaling and angiogenesis. *Circ Res.* 2002 Dec 13;91(12):1160-7.
319. Ruef J, Hu ZY, Yin LY, Wu Y, Hanson SR, Kelly AB, et al. Induction of vascular endothelial growth factor in balloon-injured baboon arteries. A novel role for reactive oxygen species in atherosclerosis. *Circ Res.* 1997 Jul;81(1):24-33.
320. Xia C, Meng Q, Liu LZ, Rojanasakul Y, Wang XR, Jiang BH. Reactive oxygen species regulate angiogenesis and tumor growth through vascular endothelial growth factor. *Cancer Res.* 2007 Nov 15;67(22):10823-30.

APPENDIX A: CHEMICALS

Material	Company	Catalog number
Acrylamide 30%	Sigma	A3699
Agarose	Roche	11388991001
Albumin (BSA)	Sigma	A4503
Albumin, Bovine Serum (BSA)	Sigma	A3059
Ampicillin	Roche	10835269001
Anthrone	Sigma	319899
Anthrone	Fluka	52445
anti-actin alpha-smooth muscle	Sigma	A2547
anti-GFP	Roche	11814460001
anti-gp130	CST	sc-655
anti-LC3	Novus	NB100-2331
anti-LIFR	Santa Cruz	sc-659
anti-mouse coupled to horseradish peroxidase	Jackson Immuno-research Laboratories	115035003
anti-mouse IgG Alexa Flour 488	Invitrogen	982425
anti-mouse IgG Alexa Flour 568	Invitrogen	A11004
anti-P-STAT3 (Tyr705)	CST	9145
anti-rabbit coupled to horseradish peroxidase	Jackson Immuno-research Laboratories	111035144
anti-SQSTM1/p62	BD transduct. Lab.	610832
anti-STAT3	CST	9139
anti-VEGFA (A-20)	Santa Cruz	sc-152
anti- β -actin	Sigma	A5441
Arginine (Arg0)	Sigma	11039
Blasticidine	Sigma	15205
B-mercaptoethanol	Sigma	M6250
Bradford Reagent	Sigma	B6916
Chloroform	Sigma	C2432
Chloroquine (CQ)	Sigma	6628
Coumaric	Sigma	C9008
CT-1 ELISA kit	Sigma	RAB0107
CT-1 neutralizing antibody	R&D	MAB2601
CT-1 recombinant protein	Sigma	SRP4011
D(+) glucose	Calbiochem	346351
Developer and Fixer	Hacettepe Kimya	
DMEM	Sigma	D5671
DMEM (for SILAC analysis)	Thermo Scientific	89985
DMSO (Dimethyl Sulfoxide)	Applichem	A1584

EBSS	Biological Industries	02-100-1A
Ethidium Bromide	Applichem	A1151
FBS	Hyclone	SH3025601
FBS (for SILAC analysis)	Gibco	26400-044
Fibronectin	Sigma	F1141
Formaldehyde solution 37%	Sigma	F1635
Free Valine Medium		
Glycerol	Applichem	A4453
Growth factor reduced Matrigel	BD transduct. Lab.	354230
Hepes	Sigma	54457
Hexadimethrine bromide (polybrene)	Sigma	H9268
Hoechst 33342 trihydrochloride trihydrate	Invitrogen	H3570
Human Full-Length cDNA (pCMV) Set-Cytokine	Thermo Scientific	MHS4928
Human Full-Length cDNA (pCMV) Set-Growth Factor	Thermo Scientific	MHS4924
Isopropanol for Analysis	Merck	1.09634
Kanamycin	Promega	A1493
L -lysine-4,4,5,5- <i>d</i> 4 hydrochloride (Lys4)	Sigma	616192
L-arginine-13C6 15N4 hydrochloride (Arg10)	Sigma	608033
L-arginine-13C6- hydrochloride (Arg6)	Cambridge Isotope lab Inc, Andover MA.	CLM-2265
LB Agar	Sigma	L2897
LB Broth	Sigma	L302214
L-glutamine	Biological Industries	03-020-1B
L-glutamine (for SILAC analysis)	PAN Biotech	P04-8010
L-lysine -13C6 15N2-hydrochloride (Lys8)	Sigma	608041
Luminol	Fluka	9253
Lysine (Lys0)	Sigma	L8662
M-MuLV reverse transcriptase	Fermantas	EP0351
Monoclonal anti-Flag antibody	Sigma	F3165
Non fat milk powder	Applichem	A0830
Nonidet P40	Applichem	A1694.0250
Paraformaldehyde	Sigma	15,812-7
PDMS	Dow Corning	Sylgard 184
Penicillin/ Streptomycin	Biological Industries	03-031-1B
Penicillin/ Streptomycin (for SILAC analysis)	PAN Biotech	06-07100
PEO-PPO-PEO copolymer	Sigma	Pluronic F-108
Phalloidin	Invitrogen	A22283
Phenol Red, sodium salt, A.C.S Reagent	Sigma	114537-5G
picoLucent Luminol	Gbioscience	195P-A

picoLucent peroxide solution	Gbioscience	193P-A
PMSF (Phenylmethanesulfonyl fluoride)	Sigma	P7626
Potassium Chloride KCl	Sigma	P9333
Propidium Iodide	Invitrogen	P3566
Protamine sulfate	Sigma	P4505
Protease Inhibitory Cocktail	Roche	04-693-131-001
Protease Inhibitory Cocktail	Sigma	P8340
Puromycin	Sigma	P9620
Rabbit serum	Sigma	R9133
Random hexamers	Invitrogen	48190-011
Rapamycin	Calbiochem	553210
REPLI-g Single Cell Kit	Qiagen	150343
Rnase A	Roche	10109169001
Rnase Away	Sigma	83931
Saponin	Sigma	84510
Sodium Azide NaN ₃	Riedel de Haen	13412
Sodium Chloride	Sigma	53014
Sodium hydroxyde (NaOH)	Merck	1.064.625.000
SU-8	Microchem	2025
SYBR Green Quantitative RT-PCR kit	Roche	04-913-914-001
TEMED	Sigma	T7024
TGFβ1 ELISA kit	R&D	DB100B
TGFβ1 neutralizing antibody	R&D	AB-100-NA
TGFβ1 recombinant protein	Sigma	H8541
TRI reagent	Sigma	T9424
Trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane	Sigma	
Trizma base	Sigma	T1503
Trizol	Invitrogen	15596-026
Trizol	Sigma	T9424
Tryphan Blue	Sigma	T8154
Trypsin enzyme (Trypsin-sequencing grade)	PROMEGA	V511C
Tween 20	Sigma	P5927
VEGFA ELISA kit	R&D	DVE00
VEGFA neutralizing antibody	R&D	MAB293
β-hydroxybutyrate	Biovision	K632-100
17β-estradiol 0.36 mg/pellet, 60-day release estrogen pellet	Innovative Research of America	SE-121
1X non essential aminoacids	Gibco	11140-035

APPENDIX B: EQUIPMENT

Equipment	Company	Number	Country
Agilent 1200 nanoflow LC system	Agilent Technologies		USA
Balance	Sartorius	BP211D	GERMANY
C25HC Incubator Shaker	New Brunswick Scientific		USA
Centrifuge	Eppendorf	5415C	GERMANY
Centrifuge	Eppendorf	5415R	GERMANY
Confocal Microscope	Zeiss	LSM 710	GERMANY
Confocal microscope	Nikon	A1R	USA
Deepfreeze: -70o C	Kendro Lab. Prod.	Heraeus Hfu486 Basic	GERMANY
Distilled Water: Millipore	Elix-S		FRANCE
Electrophoresis:	Biogen Inc.		USA
Gel Documentation	Biorad		UK
GFL		Shaker 3011	USA
Heating Magnetic Stirrer			ITALY
Heraeus Multifuge 3L	Hitachi		GERMANY
Horizontal agitation	Neo Shaker, AS ONE		MonotaRO Thailand
Ice Machine	Scotsman Inc.	AF20	USA
Incubator	Memmert	Model 300	GERMANY
Inverted microscope	Olympus	IX-71	JAPAN
Kuiqpick micromanipulator	NeuroInDx		USA
Labchip GX touch bioanalyzer	PerkinElmer		Houston, TX
Laminar Flow	Kendro Lab. Prod., Heraeus	HeraSafe HS12	GERMANY
LightCycler 480 Thermo cycler	Roche		SWITZERLAND
LTQ Orbitrap XL mass spectrometry	Thermo Scientific		Massachusetts, ABD
Luminometer	Thermo Scientific	Fluoroskan Ascent FL	Massachusetts, ABD
Magnetic Stirrer	VELP Scientifica		ARE
Microliter Pipette	Gilson	Pipetman	FRANCE
Microwave Oven	Bosch		TURKEY
Millipore,	MilliQ Academic,		FRANCE
New Brunswick Sci	Innova™ 4330		USA
O ₂ plasma	CUTE, Femto science		Gyeonggi-Do, Korea
Organovo: NovoGen MMX Bioprinter™	Organovo Holding		San Diego, CA

pH meter	WTW	pH540 GLP MultiCal®	GERMANY
Power Supply	Biorad	PowerPac 300	USA
Reactive ion etching	Labstar, TTL		
Refrigerator: +4 °C	Bosch		TURKEY
SEM	SEC corp	SNE 4500M	Gyeonggi-do, Korea
Shaker	Forma Scientific	Orbital Shaker 4520	USA
Sorvall RC5C Plus	USA Hitachi, Sorvall Discovery 100 SE		USA
Spectrophotometer	Schimadzu		JAPAN
Surface profiler	KLA Tencor	P6	USA
Thermocycler	Eppendorf	Mastercycler Gradient	GERMANY
TRI-CARD 2900TR Liquid Scintillation Analyzer	PerkinElmer		Houston, TX
Upright microscope	Olympus	BX60	JAPAN
UV-Transilluminator 2000	Biorad		USA
Vacuum desiccator	Bel-Art Scienceware		USA
Water bath	Huber	Polystat cc1	GERMANY

APPENDIX C: GROWTH FACTOR POOLS

Pool #	Position in Plate		Factor Name	Gender	Origin	Gene ID
Pool-1	a	1	S100A6	Unknown	anaplastic oligodendroglioma with 1p/19q loss	NCI_CGAP_Brn67
	b	1	BMP7	Unknown	glioblastoma with EGFR amplification	NCI_CGAP_Brn64
	c	1	IL1A	Unknown	large cell carcinoma	NIH_MGC_68
	d	1	IL1B	Unknown	large cell carcinoma	NIH_MGC_68
	e	1	NOV	Unknown	melanotic melanoma	NIH_MGC_72
Pool-2	f	1	FGF19	Unknown	adenocarcinoma	NIH_MGC_65
	h	1	CGB, CGB7	Female	adenocarcinoma	NIH_MGC_66
	b	2	ESM1	Unknown	large cell carcinoma	NIH_MGC_68
	c	2	IL6	Unknown	large cell carcinoma	NIH_MGC_68
	d	2	HDGFRP3	Female	leiomyosarcoma	NIH_MGC_71
Pool-3	e	2	BMP4	Unknown	duodenal adenocarcinoma, cell line	NIH_MGC_88
	g	2	IL11	Male	adenocarcinoma, cell line	NIH_MGC_91
	a	3	GRN	Female	carcinoma	NIH_MGC_12
	b	3	FGF21	Unknown	large cell carcinoma	NIH_MGC_68
	c	3	FGF13	Unknown	melanotic melanoma	NIH_MGC_72
Pool-4	d	3	TGFB1	Unknown	duodenal adenocarcinoma, cell line	NIH_MGC_88
	e	3	VEGFC	Unknown	Skin carcinoma	NCI_CGAP_Skn4
	f	3	ILIF5	Unknown	choriocarcinoma	NIH_MGC_10
	g	3	TRIP6	Female	carcinoma	NIH_MGC_12
	h	3	LEFTY1	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
Pool-5	b	4	FIGF	Female	Lung, spleen (pooled)	NIH_MGC_122
	c	4	EDN2	Both	Colon,kidney,stomach (pooled)	NIH_MGC_116
	d	4	BDNF	Male	Brain	NIH_MGC_114
	e	4	NDP	Unknown	Brain,lung,testis (pooled)	NIH_MGC_115
	f	4	PDGFB	Unknown	Brain,lung,testis (pooled)	NIH_MGC_115

Pool-6	g	1	CXCL1	Female	adenocarcinoma	NIH_MGC_66
	a	2	TYMP	Unknown	large cell carcinoma	NIH_MGC_68
	f	2	CLCF1	Unknown	hypernephroma, cell line	NIH_MGC_89
	h	2	TGFB3	Male	adenocarcinoma, cell line	NIH_MGC_91
	a	4	BMP5	Female	Lung, spleen (pooled)	NIH_MGC_122
Pool-7	g	4	PPBP	Unknown	leukocyte	NIH_MGC_118
	h	4	GDF10	Both	Brain	NIH_MGC_121
	a	5	GDF5	Both	Brain	NIH_MGC_121
	b	5	OSGIN1	Unknown	leukocyte	NIH_MGC_118
	c	5	TFF1	Both	Colon,kidney,stomach (pooled)	NIH_MGC_116
	d	5	GMFG	Both	Colon,kidney,stomach (pooled)	NIH_MGC_116
Pool-8	e	5	NGF	Unknown	retinoblastoma	NIH_MGC_67
	f	5	FGF1	Unknown	adenocarcinoma, cell line	NIH_MGC_90
	g	5	LEFTY2	Female	ovary	NIH_MGC_125
	h	5	SPEM1	Male	medulla	NIH_MGC_119
	a	6	CXCL12	Male	hippocampus	NIH_MGC_124
Pool-9	b	6	TYMP	Unknown	Brain,lung,testis (pooled)	NIH_MGC_115
	c	6	RABEP1	Male	embryonal carcinoma, cell line	NIH_MGC_92
	d	6	CECR1	Unknown	Brain,lung,testis (pooled)	NIH_MGC_115
	e	6	IL7	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
	f	6	RABEP2	Female	mammary adenocarcinoma, cell line	NIH_MGC_87
Pool-10	g	6	VEGFC	Unknown	transitional cell papilloma, cell line	NIH_MGC_93
	h	6	GKN1	Both	Colon,kidney,stomach (pooled)	NIH_MGC_116
	a	7	HGF	Both	Brain	NIH_MGC_121
	b	7	VEGFA	Unknown	large cell carcinoma	NIH_MGC_68
	c	7	VGF	Male	dorsal root ganglia	Lupski dorsal root ganglion

Pool-11	d	7	CNTF	Unknown		NIH_MGC_181
	e	7	GDF1	Male	sympathetic trunk	Lupski sympathetic trunk
	f	7	CTGF	Male	dorsal root ganglia	Lupski dorsal root ganglion
	g	7	CSF2	Unknown	large cell carcinoma, undifferentiated	NIH_MGC_69
	h	7	BMP8B	Male	embryonal carcinoma, cell line	NIH_MGC_92
	a	8	WISP3	Unknown	Transitional cell papilloma, cell line	NIH_MGC_93

APPENDIX D: CYTOKINE POOLS

Pool #	Position in Plate		Factor Name	Gender	Origin	Gene ID
Pool-1	a	1	BRE	Female	carcinoma	NIH_MGC_12
	a	2	CXCL1	Female	adenocarcinoma	NIH_MGC_66
	a	3	YARS	Unknown	osteosarcoma, cell line	NIH_MGC_86
	a	4	LTA	Unknown	lymphoma, cell line	NIH_MGC_85
	a	5	LEFTY1	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
	a	1_2	KITLG	Unknown	mixed organs, full-length reagent, FLP	FLPRX0022
Pool-2	a	6	CMTM6	Female	adenocarcinoma	NIH_MGC_66
	a	7	FAM3C	Unknown	duodenal adenocarcinoma, cell line	NIH_MGC_88
	a	8	VEGFC	Unknown	transitional cell papilloma, cell line	NIH_MGC_93
	a	9	NAMPT	Unknown	melanotic melanoma	NIH_MGC_72
	b	1	CCL13	Unknown	glioblastoma with EGFR amplification	NCI_CGAP_Brn64
	b	1_2	AREG	Unknown	Colon	COLNNOF03
Pool-3	b	2	FAM3D	Unknown	adenocarcinoma	NIH_MGC_65
	b	3	BMP4	Unknown	duodenal adenocarcinoma, cell line	NIH_MGC_88
	b	4	RHOQ	Male	embryonal carcinoma, cell line	NIH_MGC_92
	b	5	CCL3L1	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
	b	6	PPBP	Unknown	leukocyte	NIH_MGC_118
Pool-4	b	7	TYMP	Unknown	Brain,lung,testis (pooled)	NIH_MGC_115
	b	8	CTF1	Female	Lung, spleen (pooled)	NIH_MGC_122
	b	9	CSF2	Unknown	large cell carcinoma, undifferentiated	NIH_MGC_69
	c	1	BMP7	Unknown	glioblastoma with EGFR amplification	NCI_CGAP_Brn64
	c	2	IL6	Unknown	large cell carcinoma	NIH_MGC_68

Pool-5	c	3	CLCF1	Unknown	hypernephroma, cell line	NIH_MGC_89
	c	4	VEGFC	Unknown	Skin carcinoma	NCI_CGAP_Skn4
	c	5	CCL22	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
	c	6	GDF10	Both	Brain	NIH_MGC_121
	c	7	CXCL16	Female	Lung, spleen (pooled)	NIH_MGC_122
Pool-6	c	8	VEGFA	Unknown	large cell carcinoma	NIH_MGC_68
	c	9	BMP8B	Male	embryonal carcinoma, cell line	NIH_MGC_92
	d	1	CCL5	Unknown	glioblastoma with EGFR amplification	NCI_CGAP_Brn64
	d	2	IL8	Unknown	large cell carcinoma	NIH_MGC_68
	d	3	CXCL6	Unknown	adenocarcinoma, cell line	NIH_MGC_90
Pool-7	d	4	IL36RN	Unknown	choriocarcinoma	NIH_MGC_10
	d	5	CCL21	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
	d	6	GDF5	Both	Brain	NIH_MGC_121
	d	7	CCL14	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
	d	8	PXMP2	Unknown	retinoblastoma	NIH_MGC_67
	d	9	FLT3LG	Male	Brain	NIH_MGC_114
Pool-8	e	1	IL24	Unknown	large cell carcinoma	NIH_MGC_68
	e	2	TYMP	Unknown	large cell carcinoma	NIH_MGC_68
	e	3	IL11	Male	adenocarcinoma, cell line	NIH_MGC_91
	e	4	TRIP6	Female	carcinoma	NIH_MGC_12
	e	5	CCL19	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
Pool-9	e	6	TNFSF10	Unknown	lymphoma, cell line	NIH_MGC_85
	e	7	IL7	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
	e	8	CNTF	Unknown		NIH_MGC_181
	e	9	CTF1	Unknown	Brain, lung, testis (pooled)	NIH_MGC_115
	f	1	IL1A	Unknown	large cell carcinoma	NIH_MGC_68

Pool-10	f	2	EBI3	Unknown	epithelioid carcinoma	NIH_MGC_70
	f	3	GRN	Female	carcinoma	NIH_MGC_12
	f	4	BMP5	Female	Lung, spleen (pooled)	NIH_MGC_122
	f	5	IL16	Unknown	Brain, lung, testis (pooled)	NIH_MGC_115
	f	6	LEFTY2	Female	ovary	NIH_MGC_125
Pool-11	g	3	IL15	Unknown	adenocarcinoma	NIH_MGC_65
	g	4	FIGF	Female	Lung, spleen (pooled)	NIH_MGC_122
	g	5	SCGB3A1	Unknown	Brain, lung, testis (pooled)	NIH_MGC_115
	g	6	CXCL12	Male	hippocampus	NIH_MGC_124
	g	7	EBI3	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
Pool-12	f	7	CMTM3	Both	Brain	NIH_MGC_121
	f	8	GH1	Unknown		NIH_MGC_179
	f	9	CCL14	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
	g	1	IL1B	Unknown	large cell carcinoma	NIH_MGC_68
	g	2	CMTM5	Unknown	anaplastic oligodendroglioma with 1p/19q loss	NCI_CGAP_Brn67
Pool-13	g	8	CERS1	Male	sympathetic trunk	Lupski sympathetic trunk
	g	9	GLMN	Unknown	choriocarcinoma	NIH_MGC_10
	h	1	CCL2	Unknown	epithelioid carcinoma	NIH_MGC_70
	h	2	SECTM1	Unknown	adenocarcinoma, cell line	NIH_MGC_90
	h	3	SIVA1	Female	adenocarcinoma	NIH_MGC_66
Pool-14	h	4	CCL4	Male	Pancreas, Spleen (pooled)	NIH_MGC_120
	h	5	PDGFB	Unknown	Brain, lung, testis (pooled)	NIH_MGC_115
	h	6	CMTM3	Both	Colon, kidney, stomach (pooled)	NIH_MGC_116
	h	7	MIF	Unknown	melanotic melanoma	NIH_MGC_72
	h	8	CXCL11	Unknown	Brain, lung, testis (pooled)	NIH_MGC_115

APPENDIX E: CT-1 TISSUE SAMPLE PATIENT LIST

Istanbul University Patients			
T1 (Catalog no/wb)	FOLD CHANGE	PN (Article number)	Stage (T/N)
1	0,36	1	T1N1
39	0,40	2	T1N0
9	0,54	3	T1N0
13	0,75	4	T1N0
3	0,77	5	T1N0
8	0,81	6	T1N1
38	0,85	7	T1N0
2	1,35	8	T1N0
48	1,50	9	T1N1
50	3,29	10	T1N0
T1 (Catalog no/wb)	FOLD CHANGE	PN (Article number)	Stage (T/N)
51	0,28	22	T2N0
17	0,32	23	T2N1
31	0,37	24	T2N0
41	0,48	25	T2N0
15	0,56	26	T2N0
36	0,57	27	T2N0
5	0,58	28	T2N1
12	0,58	29	T2N0
11	0,62	30	T2N1
49	0,85	31	T2N0
37	0,95	32	T2N1
6	1,12	33	T2N1
10	1,25	34	T2N0
4	1,39	35	T2N0
43	1,50	36	T2N?
34	1,66	37	T2N1
32	3,80	38	T2N0
42	4,26	39	T2N2
35	4,39	40	T2N1
16	4,42	41	T2N2
33	5,21	42	T2N1
46	6,20	43	T2N0

Cukurova University Patients			
T1 (Catalog no/wb)	FOLD CHANGE	PN (Article number)	Stage (T/N)
20	0,29	11	T1N0
21	0,58	12	T1N0
5	0,61	13	T1N1
15	0,63	14	T1N0
8	0,69	15	T1N0
14	0,97	16	T1N0
3	0,99	17	T1N1
17	1,26	18	T1N0
22	1,99	19	T1N2
16	3,24	20	T1N1
4	3,44	21	T1N0
T1 (Catalog no/wb)	FOLD CHANGE	PN (Article number)	Stage (T/N)
2	0,59	44	T2N0
9	1,07	45	T2N2
11	1,13	46	T2N0
13	3,21	47	T2N0
10	3,37	48	T2N1
7	4,95	49	T2N2
18	5,47	50	T2N0

APPENDIX F: PUBLICATIONS



Turkish Journal of Biology

<http://journals.tubitak.gov.tr/biology/>

Review Article

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Autophagy and cancer

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Abstract: Autophagy is an evolutionary conserved intracellular degradation and stress response mechanism that is mainly responsible for the breakdown and recycling of cytoplasmic materials, including long-lived proteins, protein aggregates, and damaged organelles. In this way, autophagy provides the cell with building blocks and allows the maintenance of homeostasis under stress conditions such as growth factor deficiency, nutrient deprivation, hypoxia, and toxins. Consequently, abnormalities of autophagy contribute to a number of pathologies ranging from neurodegenerative diseases to cancer. Autophagy was reported to have a dual role in cancer. Depending on cancer stage, autophagy seems to act as tumor suppressor or as a mechanism supporting tumor growth and spread. In this review, we provide a summary of the relevant literature and discuss the role of autophagy in cancer formation and chemotherapy responses.

Key words: Autophagy, stress, cancer, tumor, oncogenes, tumor suppressors, metastasis, cell death, chemotherapy

1. Introduction

Autophagy is a basic cellular event conserved in all eukaryotes from yeast to man. It serves as an intracellular quality-control mechanism recycling long-lived or misfolded/aggregate-prone proteins and damaged organelles, such as mitochondria. Moreover, under stress conditions, autophagy is upregulated in order to generate building blocks that are necessary for cellular survival (Kuma and Mizushima, 2010; Rabinowitz and White, 2010). Therefore, autophagy mainly serves as a stress-adaptation and survival mechanism (Su et al., 2013). However, under certain conditions, autophagy was reported to contribute to programmed cell death either indirectly through crosstalk mechanisms with apoptosis (Gozuacik and Kimchi, 2004; Oral et al., 2012; Rubinstein and Kimchi, 2012) or directly through autophagic cell death (Gozuacik and Kimchi, 2007).

Abnormalities of autophagy were reported in various diseases, including neurodegenerative diseases, lysosomal storage diseases, infections, metabolic diseases, and ischemia/reperfusion injury (Schneider and Cuervo, 2014). Cancer is no exception. The roles of autophagy in cancer formation, growth, ischemia resistance, metabolic changes, neovascularization, and even metastasis and tumor dormancy were reported (Gewirtz, 2009; Guo et al., 2013b; Murrow and Debnath, 2013). Moreover, autophagic capacity was shown to significantly affect responses of

cancer cells to anticancer agents and radiation (Eberhart et al., 2013). In this review we will mainly focus on the role of autophagy in cancer formation and cancer therapy.

2. Basic mechanisms of autophagy

There are 2 major catabolic mechanisms in mammalian cells: the ubiquitin-proteasome system and the autophagy-lysosomal system. Proteasomes degrade mainly short-lived and soluble proteins following their regulated ubiquitinylation (Hershko and Ciechanover, 1998). In contrast, lysosomal pathways rely on the delivery of cytosolic contents into the lumen of the organelle, an event that is followed by their degradation by specific hydrolases. Here, ingested cytoplasmic targets might be various. The list includes long-lived proteins, old and damaged organelles (e.g., mitochondria and peroxisomes); misfolded, mutant, and/or aggregated proteins; and pathogens such as bacteria, viruses, and parasites. So far, 3 subtypes of autophagy have been described in mammals: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy (Klionsky and Schulman, 2014).

Microautophagy proceeds through direct invagination of the vacuolar/lysosomal membrane and engulfment of nearby cytosolic materials (Uttenweiler et al., 2005). In addition to cargo digestion, microautophagic vacuoles also contribute to the maintenance of organelle size and membrane composition (Mijaljica and Devenish, 2011).

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3D Bioprinting of Biomimetic Aortic Vascular Constructs With Self-Supporting Cells

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ABSTRACT: Cardiovascular diseases are the leading cause of deaths throughout the world. Vascular diseases are mostly treated with autografts and blood vessel transplantations. However, traditional grafting methods have several problems including lack of suitable harvest sites, additional surgical costs for harvesting procedure, pain, infection, lack of donors, and even no substitutes at all. Recently, tissue engineering and regenerative medicine approaches are used to regenerate damaged or diseased tissues. Most of the tissue engineering investigations have been based on the cell seeding into scaffolds by providing a suitable environment for cell attachment, proliferation, and differentiation. Because of the challenges such as difficulties in seeding cells spatially, rejection, and inflammation of biomaterials used, the recent tissue engineering studies focus on scaffold-free techniques. In this paper, the development of novel computer aided algorithms and methods are developed for 3D bioprinting of scaffold-free biomimetic macrovascular structures. Computer model mimicking a real human aorta is generated using imaging techniques and the proposed computational algorithms. An optimized three-dimensional bioprinting path planning are developed with the proposed self-supported model. Mouse embryonic fibroblast (MEF) cell aggregates and support structures (hydrogels) are 3D bioprinted layer-by-layer according to the proposed self-supported method to form an aortic tissue construct.

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KEYWORDS: 3D bioprinting; scaffold-free tissue engineering; tissue engineering of macrovascular structures; biomimetic modeling

Introduction

Cardiovascular diseases are the primary reasons of deaths and they rank among the top ten leading causes of morbidity and mortality (Nemeno-Guanzon et al., 2012). Autografts and blood vessel transplantation are found to be the most effective treatments for vascular diseases. However, autografts have limitations such as lack of autografts in donor site or the patient's deficient health condition. Recently, tissue engineering and regenerative medicine research have been developed for regeneration of damaged or diseased vascular tissues.

In earlier tissue engineering strategies, the cells are seeded into synthetic, biological or composite scaffolds. Especially, synthetic-biologic hybrid scaffold with their biochemical and mechanical properties mimicking the native ECM are strong 3D cell culture platforms for cell physiology and tissue printing studies (Tibbitt and Anseth 2009). However, fabrication of a controlled porous structure with the desired internal architecture might be challenging with traditional scaffold fabrication techniques. Despite these difficulties, several researchers have developed methods for design and bioprinting of functionally gradient porous scaffolds with controllable variational pore size and heterogeneous porous architecture (Khoda et al., 2011; Khoda et al., 2013).

Recently, there are a few studies focusing on vascular and valve tissue engineering using 3D printing techniques. In order to mimic complex 3D anatomy and heterogeneity of aortic valve, root wall and tri-leaflets are 3D printed with poly-ethylene-glycol-diacrylate (PEG-DA) hydrogels. Porcine aortic valve interstitial cells (PAVIC) seeded scaffolds maintained near 100% viability over 21 days (Hockaday et al., 2012). Another study demonstrates that

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A microfluidic chip for screening individual cancer cells via eavesdropping on autophagy-inducing crosstalk in the stroma niche

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Autophagy is a cellular homeostatic mechanism where proteins and organelles are digested and recycled to provide an alternative source of building blocks and energy to cells. The role of autophagy in cancer microenvironment is still poorly understood. Here, we present a microfluidic system allowing monitoring of the crosstalk between single cells. We used this system to study how tumor cells induced autophagy in the stromal niche. Firstly, we could confirm that transforming growth factor $\beta 1$ (TGF $\beta 1$) secreted from breast tumor cells is a paracrine mediator of tumor-stroma interaction leading to the activation of autophagy in the stroma component fibroblasts. Through proof of concept experiments using TGF $\beta 1$ as a model factor, we could demonstrate real time monitoring of autophagy induction in fibroblasts by single tumor cells. Retrieval of individual tumor cells from the microfluidic system and their subsequent genomic analysis was possible, allowing us to determine the nature of the factor mediating tumor-stroma interactions. Therefore, our microfluidic platform might be used as a promising tool for quantitative investigation of tumor-stroma interactions, especially for and high-throughput screening of paracrine factors that are secreted from heterogeneous tumor cell populations.

Interactions between cancer cells and the neighboring stroma play a critical role in tumorigenesis, and an in-depth understanding of intercellular communication is of great significance for the development of novel therapeutic strategies^{1–3}. Heterogeneity of tumor cells is evident, and its profound impact in clinical applications is highly recognized⁴. However, conventional tools used to study cell-to-cell interactions only deliver averaged information from a population of cells and fail to provide information on the distribution of responses reflecting the heterogeneity of individual cells.

Microfluidic devices have emerged as useful tools for single-cell analysis^{5–7}. Phenotype heterogeneity⁸, paracrine secretion⁹, and DNA repair capacities with different genetic backgrounds¹⁰ are among the cellular properties that have been analyzed using single-cell based systems. Cell-to-cell interactions may also be studied at a single-cell level. For example, using single-cell pairing techniques, effects of cell-to-cell interaction on migration and proliferation patterns¹¹ and contact-dependent organoid formation¹² have been analyzed. In addition, the heterogeneous dynamics of CD8 T-cells during their interaction with lymphocytes have been investigated¹³. However, to the best of our knowledge, single-cell-based techniques have been rarely used for studying the interactions of tumor cells with tissues surrounding them, i.e., the stroma. Furthermore, the retrieval of individual

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