Autophagy: An Emerging Key Regulator of Epithelial-Mesenchymal Transition (EMT) in Non-Small Cell Lung Cancer (NSCLC) Cells

By

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Abstract

Lung cancer is considered as one of the most important causes of cancer-related death worldwide and Non-Small Cell Lung Cancer (NSCLC) accounts for 80% of all lung cancer cases. Autophagy is a cellular process responsible for the recycling of damaged organelles and protein aggregates. Transforming growth factor beta-1 ($TGF\beta_1$) is involved in Epithelial to Mesenchymal Transition (EMT) and autophagy induction in different cancer models and plays an important role in pathogenesis of NSCLC. It is not clear how autophagy can regulate EMT in NSCLC. In the present study, we have investigated the regulatory role of autophagy in EMT induction in NSCLC cells. we showed that $TGF\beta_1$ can simultaneously induce both autophagy and EMT. We observed that upon chemical inhibition of autophagy using Bafilomycin-A1 the expression of the mesenchymal marker vimentin is reduced. Also, using immune blotting and immunocytochemistry (ICC) we showed that mesenchymal marker (vimentin) was significantly downregulated upon $TGF\beta_1$ treatment in *Atg7* knockdown cells compared to corresponding scramble (negative control) cells (**, $P<0.01$; ***, $P<0.001$, respectively for A549 and H1975 cells) while E-cadherin was almost unchanged. Also, *Atg7* knockdown cells treated with $TGF\beta_1$ had less migration (mesenchymal function) compared to scramble counterparts. This study identified a crucial role of autophagy as a potential positive regulator mechanism in the induction of $TGF\beta_1$-induced EMT in NSCLC cells.

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DEDICATION

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LIST OF ABBREVIATIONS

α-SMA: alpha smooth muscle actin

ATGs: autophagy-related proteins

Baf-A1: Bafilomycin A1

BNIP3: BCL2/adenovirus E1B 19 kDa protein-interacting protein3

BSA: Bovine Serum Albumin

CMA: Chaperone-Mediated Autophagy

CQ: chloroquine

CSCs: Cancer stem cells

DAPI: 4',6' Diamidino-2 phenylindole Dia-1: Diaphanous-1

DMEM: Dulbecco's Modified Eagle's Medium

ECM: Extracellular Matrix

EGFR: Epidermal Growth Factor Receptor

EMT: Epithelial Mesenchymal Transition

ER: Endoplasmic Reticulum

ERK: Extracellular activated protein kinase

HIF-1α: Hypoxia Inducible Factor 1α

ICC: Immunocytochemistry

IHC: Immunohistochemistry

FBS: Fetal Bovine Serum

GBM: Glioblastoma

GFP: Green Fluorescent Protein

HCQ: Hydroxychloroquine

ITS: Insulin/Transferrin/Selenium

KD: KnockDown

kDa: Kilo Dalton

KO: KnockOut

LIR: LC3-interacting region

MAP1LC3: microtubule-associated protein 1 light chain 3 MAPK: Mitogen activated protein kinase

MET: Mesenchymal to Epithelial Transition

MMP: Matrix metalloproteinase

MOI: Multiplicity of

miR: microRNA

mTOR: mammalian target of rapamycin

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NSCLC: Non-Small Cell Lung Cancer

OIS: Oncogene-Induced Senescence

PAS: Phagophore Assembly Site

PBS: Phosphate Buffered Saline

PI3P: Phosphatidylinositol (PI) 3-phosphate

ROS: Reactive Oxygen Species

rpm: rotations per minute

RT: Room Temperature

sh: small hairpin

SMERs: small Molecule Enhancers of Rapamycin

TBS: Tris Buffered Saline

TEM: Transmission Electron Microscopy

$TGF\beta_1$: Transforming Growth Factor-Beta1

UBD: Ubiquitin-Binding Domain

ULK1: unc-51 like autophagy activating kinase 1

UPR: Unfolded Protein Response

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CHAPTER 1: INTRODUCTION

1.1 Autophagy Pathway

Autophagy function is evolutionarily conserved in yeast, plants and mammals as a basic stress-response and degradation mechanism. The role of autophagy has been widely investigated in humans, as it plays crucial roles in maintaining optimum conditions to maintain the best functions at the cellular and organismal level. These evidences support the idea that autophagy counteracts slow deleterious events which are associated with aging (1, 2). Autophagy plays effectual roles on several basal cellular mechanisms in different organs through its intracellular catabolism activities, influencing outcomes in disorders such as neurodegeneration, immunity and cancer (3-7). Historically, research on the field of autophagy was first started by the study which characterized the lysosome and this led to our current knowledge about regulatory and molecular aspects of autophagy (8). Autophagy can be defined as a catabolic process which degrades and recycles cytosolic materials. It is a highly regulated cellular process with three main types: Macroautophagy, Microautophagy and Chaperone-Mediated Autophagy (CMA) (Fig 1) (9). Macroautophagy includes the double membrane autophagosomes that engulf different cargos like organelles and cytoplasmic proteins. These autophagosomes sequester their cargo to lysosomes where it is degraded (10). Microautophagy results in the direct engulfment of substrates through the lysosomal or endosomal membrane invagination which then are degraded by lysosomal proteases (11). CMA acts in a very selective way and does not use membrane to engulf the cargo. This makes it different from macroautophagy and microautophagy. Proteins targeted by CMA contain a pentapetide motif containing

KFERQ sequence (Lys-Phe-Glu-Arg-Gln) that is detected by cytosolic heat shock cognate 70 kDa protein (hsc70). Then hsc70 together with the lysosomal-associated membrane protein 2A (LAMP2A) receptor helps cargo to be transferred into lysosomes through their membranes (12).

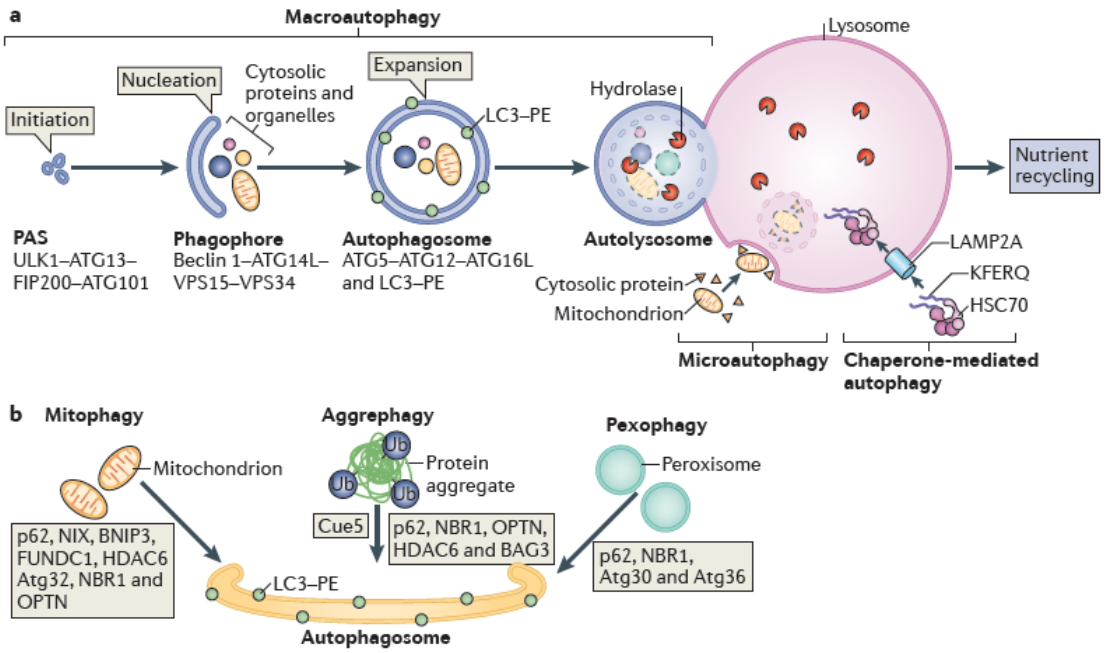


Figure1. Different autophagy pathways in mammals (9).

1.1.1 Macroautophagy

Macroautophagy (thereafter autophagy) was discovered in the late 1950s using morphological techniques (13). Being the degrading mechanism of the cell, macroautophagy contributes to the survival of cells under stressful conditions (14). Briefly**,** the cargo is sequestered into autophagosomes followed by their delivery to lysosomes for degradation (15). The autophagosomes is currently believed to be

originated from the Endoplasmic Reticulum (ER) under starvation and growth factor deprivation which is initiated by formation of phosphatidylinositol (PI) 3-phosphate (PI3P)-enriched membrane domains (omegasomes) which then expands to form the double-membrane autophagosome (16). Cytosolic entities are captured through a selective or nonselective process and enclosed during phagophore formation and expansion. The formed autophagosome fuses with the lysosome to form an autolysosome (autophagolysosome) where cargo is degraded and metabolic molecules are delivered to the cytoplasm (13).

1.1.2 The Generation of Autophagosomes

The first stage in macroautophagy is the sequestration of cytoplasmic components into the autophagosome. Formation of autophagosome can be ultrastructurally characterized and is triggered by different types of cellular stress such as amino acid starvation (13). Subsequently, autophagosome fuses with lysosome which degrades the sequestered cytoplasmic proteins and organelles (17). In Table 1 (13) autophagy-related proteins (ATGs) and their mammalian orthologues (18-20) have been briefly mentioned.

Table 1. List of various proteins in different stages of autophagy in yeast and mammalian. (13).

Protein Name	**Function**	**Yeast Homolog**
ULK1/2	Serine/threonine kinase	ATG1
ATG2a, b	Binds WIPI4	ATG2

ATG3	E2-like protein involved in conjugation of ATG8 proteins to PE	ATG3
ATG4A, B, C, and D	Activation of ATG8 proteins for conjugation to PE, and cleavage of ATG8–PE	ATG4
ATG5	Conjugated to ATG12 and acts as an E3-like protein in ATG8–PE conjugation	ATG5
BECLIN 1	Core component of Vps34 PI3P kinase complex	ATG6
ATG7	Acts as an E1-like protein in ATG12–5 and ATG8–PE conjugation cascades	ATG7
LC3A, B, C	Conjugated to PE	ATG8
GABARAP, GABARAPL1, GATE-16	Conjugated to PE	ATG8
ATG9	Multispanning membrane protein	ATG9
ATG10	Acts as an E2-like protein in ATG12–5 conjugation	ATG10
ATG12	Conjugated to ATG5 and acts as an E3-like protein in ATG8–PE conjugation	ATG12
ATG13	Core component of the ULK kinase complex	ATG13
ATG14L	Autophagy-specific subunit of Vps34 PI3P kinase complex	ATG14
ATG16L1	Forms a complex with ATG12–5 and acts as an E3-like protein in ATG8–PE conjugation	ATG16
ATG101	Core component of the ULK kinase complex	
FIP200	Core component of the ULK kinase complex	ATG17
WIPI1/2/3/4	WD propeller proteins binding PI3P WIPI2b binds ATG16L1	ATG18, ATG21
Non-ATG proteins		
AMBRA1	Regulator of Vps34 PI3P kinase complex	ND
DFCP-1	PI3P effector	ND
p150	Regulatory subunit of Vps34 PI3-kinase complex	VPS15
RUBICON	Negative regulator of Vps34 PI3-kinase complex	ND

UVRAG	Regulator of Vps34 PI3-kinase complex	ND
VPS34	PI3-kinase	VPS34

Autophagosome formation includes three steps: initiation, nucleation and expansion. The first step in formation of autophagosome is at the phagophore assembly site (PAS) (isolation membrane) where proteins of the unc-51 like autophagy activating kinase 1 (ULK1) complex combine in order to begin autophagosome formation (21). In the next stage (nucleation) activated ULK complex targets a class III PI3K complex to contribute to the production of a PI3K pool that is specific to autophagosomes (22). In the final stage, the autophagosome membrane recruits the *ATG12–ATG5–ATG16* complex where it facilitates microtubule-associated protein 1 light chain 3 (MAP1LC3; LC3) lipidation with phosphatidylethanolamine (PE). The isolation membrane expansion is dependent on LC3 (the mammalian homologue of yeast *ATG8).*Deacetylation of LC3 and cytosolic translocation is essential for its lipidation during starvation induced autophagy (23). The origin of autophagosome membranes in yeast is likely to be *de novo* (24, 25) however, in mammalian cells it has been a contentious subject. There are wide range of sources that can contribute to autophagosome formation (e.g.; ER–Golgi intermediate compartments, ER–mitochondria junctions, mitochondria, endosomes and the plasma membrane) however, evidence corroborate the notion that isolation membrane nucleation occurs at a distinct site and emanates from the ER (26). Altogether, these studies emphasize the intricate nature of autophagy initiation in mammals. Also, considering the diverse stimuli that can induce autophagy, a prominent research area is to understand the basics as to how these various membrane sources are used during autophagy (9).

1.1.3 Selective Sequestration of Deleterious Proteins and Damaged Organelles

Autophagy plays a crucial role in selective removal of the damaged organelles and unfolded proteins (27). It was believed that autophagy induced by growth factors deprivation acts in a non-selective manner however, the currently accepted theory is that autophagy sequesters its cargos (organelles, unwanted proteins and etc.) in a very selective mechanism (28). During selective autophagy, cargo is attracted through five well-known special receptors (p62 (SQSTM1), NBR1, NDP52, OPTN, and NIX) which recognize the degradation signals on cargo. Most of them have LC3-interacting region (LIR) and ubiquitin-binding domain (UBD) (29). This signal in mammals is usually ubiquitin which binds to the UBD of receptors (30). These receptors also have another domain mediating their binding to LC3 on the forming autophagosomes named the LIR (31). p62 (SQSTM1) is a cargo receptor which greatly contributes to the removal of protein aggregates a process called aggrephagy. This process is also dependent on the UBD and LIR2 (32). Furthermore, organelles are also targets of selective autophagy. As an example, mitophagy is involved in the process of damaged mitochondria degradation and recycling (33, 34). Recent studies have identified the presence of receptors involved in the mitophagy such as BCL2/adenovirus E1B 19 kDa protein-interacting protein3 (BNIP3) and *ATG32* in mammals and yeast, respectively (35-37). They regulate mitophagy via phosphorylation in some of their residues and they use LIR in order to sequester mitochondria (38).

1.1.4 Regulation of Autophagy

Autophagy is a highly regulated process and mammalian target of rapamycin (mTOR) complex I (mTORC I) is the most well-known and identified autophagy repressor (39). Therefore, inhibition of the mTORC I is the mechanism by which autophagy inducers like starvation and stress act. This facilitates the activation of ULK1 and autophagy-related 13 (*ATG13*) which trigger the autophagy induction (40, 41). AMP kinase (AMPK) is activated upon low ATP/AMP ratios and is one of the main inhibitors of mTORC I (42). It also activates autophagy through phosphorylating ULK1 (42-45). Autophagy can also be regulated based on the inducing stimulus which determines the autophagy functions in a selective or non-selective manner (46). Generally, changes in the metabolic processes of the cells cause the non-selective autophagy while the alterations in the homeostasis (such as damaged mitochondria, misfolded proteins, bacterial or viral infection) trigger selective autophagy (33, 47).

1.1.5 Crucial Role of Autophagy in Cell Quality Control

A basal level of autophagy acts as an intracellular quality control system in normal conditions by maintaining the cell from unwanted and damaged proteins and organelles (48, 49). Autophagy serves as an adaptive and cytoprotective response upon activation by various stimuli such as oxidative and genotoxic and nutritional factors (10, 50). This has been further proved by the observation that cells with non-functional autophagy (chemical or genetic intervention) do not have the necessary ability to adapt with the stressful conditions (49, 50). Therefore, due to its cytoprotective role in the cell, autophagy serves as a defensive mechanism against

different abnormalities like tumorigenesis where autophagy acts as an oncosuppressive. In line with this, it has been shown that oncoproteins cause autophagy inhibition while oncosuppressive proteins induce autophagy (51). Additionally, autophagy plays an important role in improving the immunity against cancer development so as to eliminate the cells susceptible to carcinogenesis (52). On the other hand, autophagy helps established cancer cells to sustain and more importantly autophagy acts in favor of the tumor metastasis and invasion in the stressful microenvironment of tumor lesions. This represents the oncogenic side (malicious face) of autophagy (53) and highlights the dual role of autophagy in cancer development (oncosuppressive and oncogenic) which will be discussed in more detail in next sections. Basal autophagy is also vital for the health and homeostasis of other cell types like neurons and muscle cells as it has been observed that autophagy dysfunction can lead to the formation of inclusion bodies because of damaged protein aggregation and result in the development of neurodegenerative and cardiac disorders (54). Importantly, when unfolded protein response (UPR) fails to remove the misfolded and aggregated proteins in the Endoplasmic Reticulum (ER) lumen, autophagy comes in to selectively eliminate these proteins (55). Also, autophagy contributes to the ER homeostasis through a process called reticolophagy where some areas of the ER and even part of the nucleus are targeted and sequestered by selective autophagy. Reticolophagy generally occurs under nitrogen-deprived conditions and *ATG39* and *ATG40* are required in the reticolophagy process (56). Another important function of autophagy is to spread the energy and nutrient material as the end products of autophagy in the cell. All in all, these observations show how autophagy can be a

determinant factor in controlling the cellular metabolic systems both in healthy and unhealthy cells such as cancer cells (9).

1.2 Lung Cancer

Despite the rapid advances in drug development and surgical procedures, lung cancer remains the leading cause of cancer related death in the United States (57) and worldwide (58). According to Canadian Cancer Society, it is estimated that about 29,000 Canadians were diagnosed with lung cancer in 2016. Lung cancer is classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (59). NSCLC is the most common type of lung cancer and usually spreads more slowly than other lung cancers. It accounts for 80% of all lung cancer cases and corresponds to a heterogeneous group and is subdivided to squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large cell carcinoma (57). Surgery is still considered to be one of the best treatments for resectable NSCLC (60) and the average survival after diagnosis is around 8 months and the overall 5-year survival rate is approximately 16% (61). Lung cancer is commonly diagnosed at the end stage of the disease and unfortunately only a small percentage of patients (about 25 percent) can use surgery (62). While chemotherapy is considered as a major treatment for lung cancer, it cannot eliminate the whole tumor cells which are a direct result of drug resistance. Patients with cancer show two main types of drug resistance: intrinsic resistance and acquired resistance (developed after treatment; however, they are first responsive to chemotherapeutic drugs) (58). The acquired resistance is a problematic issue even in NSCLC patients who respond to EGFR tyrosine kinase inhibitors (63). In fact, two

main reasons for the failure of lung cancer therapy are **metastasis** and drug resistance with the first one being also the main cause of death among patients with lung cancer (58). Thus, investigations to understand and dissect more the determining molecular mechanisms in the metastasis of lung cancer are of paramount importance in order to develop more effective therapeutic options.

1.3 Epithelial to Mesenchymal Transition (EMT)

Epithelial cells are characterized as polarized, well-differentiated, and cube-like with tight adhesion to the cells in their vicinity. During EMT and in response to stimuli, epithelial cells lose these properties and gain mesenchymal characteristics (invasive and spindle shape) and go through changes in their morphology (64) (Fig 2) (65).

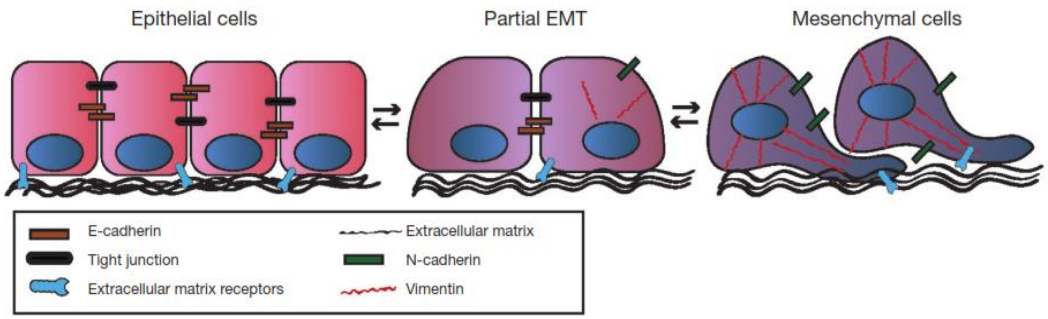


Figure 2. EMT induces a cellular phenotypic shift (65). EMT induces a cellular phenotypic shift. Epithelial cells are characterized by the presence of apico-basal polarity, intact cell-cell contacts, barrier function, and lack of movement. During the EMT process, cells with partial EMT appear characterized by the incipient presence of a front-rear polarity and have leaky cell-cell contacts. At the same time upregulation of the mesenchymal markers vimentin and N-cadherin begin. In addition, these partial EMT cells express lower amounts of E-cadherin than epithelial cells.

EMT process has remained conserved during evolution. The reverse process of EMT is Mesenchymal to Epithelial Transition (MET) during which cells convert from the

migratory and mesenchymal signature to the epithelial one (66). EMT plays a wide range of vital roles in different cellular functions both in normal and pathological conditions including wound healing, **metastasis** and chemoresistance of cancer cells, and embryonic development(67). One way to characterize the EMT is to look at the changes in the expression of different proteins where expression level of epithelial markers like E-cadherin and occludin is down regulated and that of mesenchymal markers like vimentin, N-cadherin, alpha smooth muscle actin (α-SMA), fibronectin and matrix metalloproteinases (MMP2, 3, 9) is upregulated. These gene expression changes have their effect on the morphology and adhesion properties of the cells (68). E-cadherin is the main component of tight junctions between cells and so has an important role in maintaining the cytoskeleton shape and contact between epithelial cells. E-cadherin loss is believed to help cancer cells to acquire invasive and metastatic features so it is considered as a strong marker of EMT (58). EMT has been categorized into three main types (69); type 1 and 2 play roles in normal cell functions with type 1 being involved in development of different tissues (70) and type 2 in wound healing and tissue repair (69, 71). Type 3 is the one with clinical significance which is identified as being involved in the metastasis and invasion of cancer cells (66, 72).

1.3.1 Transcriptional Regulation of EMT

EMT is a tightly regulated through different transcription factors including Snail, ZEB, and Twist which act as E-cadherin suppressors, thereby inducing EMT. These E-cadherin repressors are classified into two main groups based on their binding to the

E-cadherin promoter (66). Group 1 directly binds to the promoter and suppresses E-cadherin expression (Snail1, Snail2, ZEB1, and ZEB2) and group 2 does the same but by indirectly binding to the promoter (Twist). Snail proteins were the first ones identified to repress the E-cadherin expression and induce cells to undergo phenotypic and morphologic changes towards the EMT. Snail1 also increases the expression of proteins involved in the cell mesenchymal phenotype like N-cadherin (73), claudin (74) and fibronectin (75) and so contribute to the EMT in these cells.

1.3.2 EMT in Cancer Progression and Metastasis

It has been shown that EMT and the reverse process MET both are involved in the progression and invasion of different cancers and that its normal regulation is abrogated during cancer metastasis (76, 77). In order for cancer cells to metastasize, cancer cells in the primary tumor need to go through EMT and so acquire the capability to penetrate the blood vessels and invade the neighbor tissues (78). When cancer cells undergo EMT they can acquire different properties which are features of metastatic cells such as invasion, stemness, motility, drug resistance and anti-apoptosis characteristics and so they can detach from their primary sites and disseminate to other organs. This can significantly increase the cancer progression by giving the cells more invasiveness, tumorigenesis and unresponsiveness to different treatments (58, 79, 80). MET is thought to be crucial for the stabilization of cancer cells after they metastasized to new sites and acquired their epithelial features (81). It has been observed that the expression level of E-cadherin is upregulated in lymph nodes and other distant tissues compared to their primary sites suggesting the

conversion of metastatic cells to epithelial (EMT to MET) (82). Studies have further confirmed the important role of MET in tumor colonization through conversion and differentiation of metastatic cancer cells to epithelial cells in the metastatic tissue (an epithelial-like phenotype at the site of the metastasis formation) (83, 84).

1.3.3 EMT in Lung Cancer Progression, Prognosis and Drug Resistance

Different investigations have shown that E-cadherin downregulation and hypoxia inducible factor 1α (HIF-1α) upregulation are correlated with poor prognosis and inverse outcome in lung cancer. Pruklin and colleagues showed upregulated profile for EMT markers in NSCLC samples; however, the same markers were downregulated in the metastatic brain site and cells were more differentiated which suggested the conversion from EMT to MET (85). EMT is considered as a major determinant of the elevated invasive and metastatic features of lung cancer cells. It has been shown that EMT phenotype is related to a higher rate of drug resistance in lung cancer and in line with this it has been demonstrated that NSCLC cell lines with upregulated E-cadherin are more sensitive to EGFR inhibitors while NSCLC cells with lower expression level of E-cadherin and higher expression of fibronectin and vimentin are less sensitive to the same treatment (58, 86, 87). This fact points out the importance of EMT in determining the sensitivity to anti-cancer treatments in NSCLC (88). Also, two other studies have delineated that A549 cells acquire a more sensitive phenotype to cisplatin after suppression of Snail or Twist (89, 90). Therefore, one potential way to overcome the resistance of NSCLC to chemotherapy is to provide applicable approaches to reverse the EMT process. This can make lung cancer cells

sensitive to treatment regimens and improve the survival rate of patients with metastatic NSCLC (91).

1.3.4 TGFβ$_1$/SMAD Signaling Pathway and EMT in lung cancer

As mentioned earlier, EMT process is regulated through different transcriptional factors and signaling pathways which induce the downregulation of epithelial markers and up regulation of mesenchymal markers (58). Fig 3 shows a number of signaling pathways that are involved in EMT induction (86). TGFβ$_1$, a well-known cytokine is one of the main and well-studied EMT inducers. TGFβ$_1$ has three different ligands (TGFβ$_{1, 2}$ and 3) which are classified in the TGFβ$_1$ superfamily (92). During the activation of TGFβ$_1$ signaling pathway, it binds to its receptors where it forms a hetero-tetrameric complex of type I and II receptors. Receptor type II with its kinase activity phosphorylates the type I receptor resulting in the activation of receptor type I (93, 94). Next, activated form of receptor type I activates SMAD2 and 3 proteins through phosphorylation and they activate SMAD4, forming a trimeric protein complex. At this step, they can enter the nucleus and after joining with some other cofactors (major E-cadherin repressors) that increase their affinity to DNA they bind to the promoters of their target genes. Regulation of TGFβ$_1$ pathway has been shown to be tightly regulated via various adaptor and cofactor proteins. TGFβ$_1$ can induce a totally different range of responses which is dependent on the cell type and also context (95). All the proteins involved in the TGFβ$_1$ signaling activation are required for the TGFβ$_1$-induced EMT (SMADs and receptors) as their suppression has been shown to inhibit the EMT induced by TGFβ$_1$ (72).

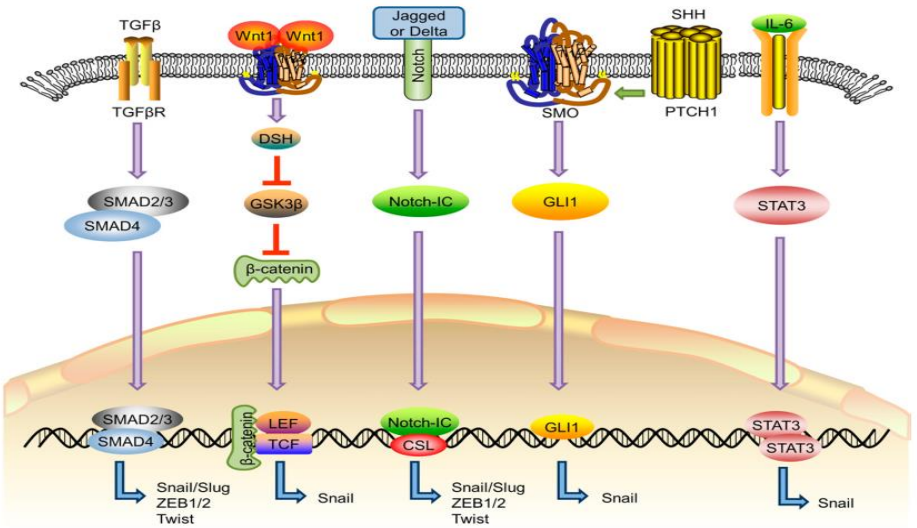


Figure 3. interaction of EMT process with other signaling pathways (86). Diverse signaling pathways associated with epithelial–mesenchymal transition (EMT). Transforming growth factor beta (TGFβ) signals activate SMAD2 and SMAD3 that complex with SMAD4. The trimeric SMAD complex enters the nucleus and leads to the transcription of EMT transcription factors (EMT-TFs). Activation of Wnt signaling inhibits the destruction complex containing glycogen synthase kinase 3 beta (GSK-3) through Disheveled (DSH), facilitating-catenin to enter the nucleus and activate the Snail transcription. Notch receptors can be activated by binding to Delta and Jagged ligands. After activation, Notch intracellular domain (Notch-IC) is released through a cascade of proteolytic cleavages and activates CSL transcription factor to express EMT-TFs. In Sonic Hedgehog (SHH) signaling, ligand binding to Patched 1 (PTCH1) receptors activates Smoothened (SMO) and Glioma (GLI) family transcription factors that induce Snail expression. Interleukin-6 (IL-6) can induce Snail expression by activating STAT3.

$TGF\beta_1$ signaling is involved in a wide range of cellular functions including cellular apoptosis, growth, secretion of cytokines and more importantly in the invasion and metastasis of cancer cells (66, 96). Additionally, $TGF\beta_1$ works with some other signaling pathways to induce EMT such as Hedgehog (97), Ras-MAPK (98), Notch (99), and Wnt (100). Fig 4 shows $TGF\beta_1$ association with signaling mechanisms which can regulate the EMT process (58). It has been well documented by different studies that $TGF\beta_1$ plays crucial roles in the progression and aggressiveness of lung

cancer especially induction of the EMT through regulating different proteins which determine the invasion of cancer cells such as Snail (101), E-cadherin (102), vimentin (103-105) and N-cadherin (105). As mentioned earlier, downstream proteins of TGFβ_1 pathway makes a complex with other cofactors before having the ability to regulate expression of target genes. These cofactors are mainly repressors of E-cadherin such as Twist, ZEB and Snail and therefore they can induce EMT. (106). Furthermore, TGFβ_1 can induce EMT (i.e. higher motility and invasion) in lung cancer by regulating the expression level of integrins (101, 107, 108). As metastasis is the main cause of death in lung cancer patients (109), it is vital to understand in depth the involvement of metabolic or other pathways in the molecular regulation of EMT.

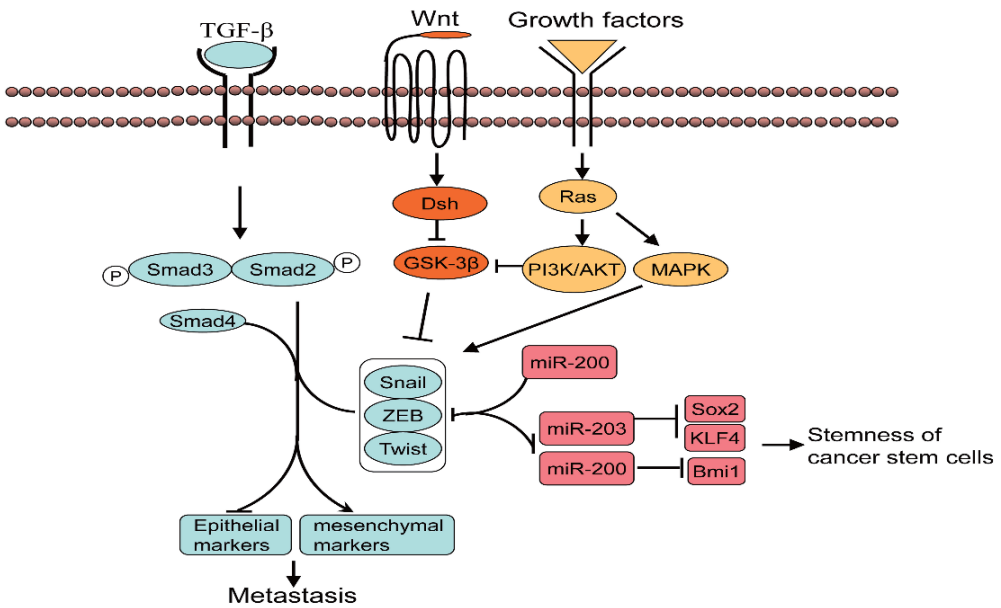


Figure 4. TGFß signaling, regulation and association with other pathways which can influence the EMT process (58). TGFß is a major inducer of EMT. It binds to the receptors leading to the phosphorylation of SMAD2 and SMAD3. Activated SMAD2 and SMAD3 form trimers with SMAD4, SMADs complex are then translocated into nucleus where they associate and cooperate with DNA binding transcriptional factors such as Snail, ZEB and Twist to regulate the expression of TGFß target genes, resulting in the downregulation of Epithelial markers and the upregulation of mesenchymal markers. TGFß also cooperates with

other signal factors such as Wnt and growth factors that act through receptor tyrosine kinase to regulate EMT. Several microRNAs have been indentified to regulate EMT. miR-200 suppresses EMT mainly through targeting ZEB factors and ZEB factors also regulate the expression of miR-200 and miR-203, linking the EMT and stem maintenance of cancer stem cells.

1.4 Dual Role of Autophagy in Cancer: A Double-Edged Sword

The complexity of the role of autophagy in cancer has been the focus of several investigations. Majority of these studies have observed that based on cancer type, stage and tumor microenvironment autophagy can have oncosuppressive or oncogenic role which is usually referred to as "a double-edge sword" (Fig 5) (110). Results from genetic studies have shown that autophagy has an onco-suppressive role in the early stages of cancer development and a survival role in established cancers with metastatic and invasive behavior. Also, the expression profile of autophagy proteins is used as markers for cancer prognosis. Therefore, the crucial role of autophagy in cancer development and metastasis together with its promising potential as a target in cancer therapeutics has attracted great deal of attention from scientific community (111-113).

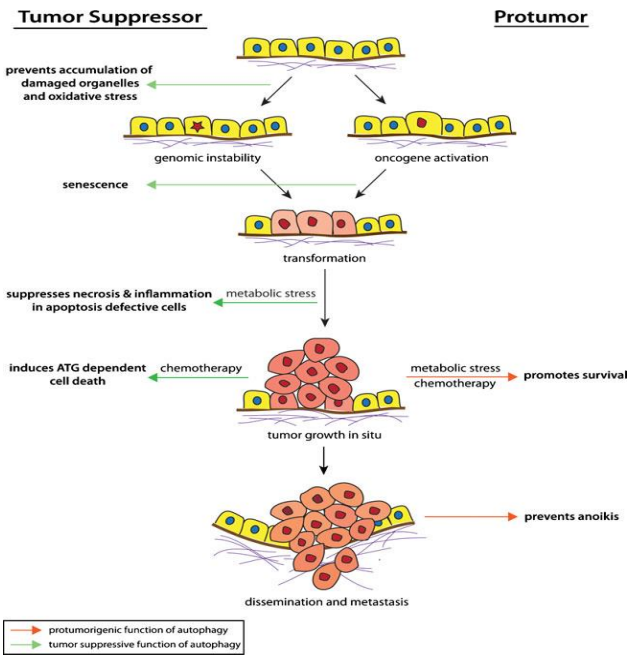


Figure 5. Double-Edged Sword characteristic of autophagy in cancer development (110). Tumor suppressive functions of autophagy include: (1) eliminating damaged organelles and mitigating oxidative stress, which prevents genome instability, and ultimately, malignant transformation; (2) promoting oncogene-induced senescence, a barrier to malignant transformation; and (3) preventing necrosis in apoptosis deficient cells in response to metabolic stress, which reduces pro-tumorigenic inflammation. Pro-tumorigenic roles for autophagy include: (1) promoting tumor cell survival in response to diverse micro-environmental and chemotherapy-induced stresses, and (2) protecting cells from anoikis, which may facilitate drug-resistance and metastasis.

1.4.1 Tumor Suppressive Functions of Autophagy

The generally accepted role of autophagy as a survival mechanism during stressful conditions seems to be in contrast to its tumor suppressive role. A study in 1999 on Beclin1 protein for the first time highlighted the role of autophagy as a tumor suppressor where the genetic mapping of Beclin1 showed its genetic location within a locus deleted in majority of human cancers (114). Interestingly, some oncogenes have appeared to inhibit autophagy through mTOR activation such as ERK, Ras (115), Akt (116) while tumor suppressors like PTEN (117), p53 (118, 119), and ARF (120, 121) induce autophagy activation. Accumulating evidence suggests that autophagy maintains genome integrity and plays a protective role during metabolic and genetic stresses like nutrient deprivation and hypoxia (110). There are different mechanisms by which autophagy exerts its protective mechanism against carcinogenesis which will be discussed in the following sections:

1.4.1.1 Maintaining Cellular Fitness

Apoptosis is considered as the first defensive mechanism against damaged cells. During cancer development apoptosis is usually abrogated and so autophagy comes into play to remove damaged organelles and misfolded proteins and maintain the

energy and fitness of the cells (122). It has been observed in the Bcl-2 (B-cell lymphoma 2) overexpressed mammary cells of mice that beclin1$^{+/-}$ cells are more susceptible to stresses while beclin1$^{+/+}$ cells are both resistant to metabolic stress and to carcinogenesis (122, 123). The reason for this observation is the fact that DNA breaks, damaged organelles and misfolded proteins are significantly increased when autophagy is compromised due to metabolic stress as opposed to in wild type cells (124). Moreover, in cells with defective autophagy the levels of reactive oxygen species (ROS) are increased leading to extensive damage to the DNA. Overall, autophagy acts as an onco-suppressive through removing ROS and limiting the genetic insults in the cell (125). Therefore, one might hypothesize that autophagy can be used by cancer cells to regulate DNA damage and increase genomic stability in cancer cells.

1.4.1.2 Inhibition of Necrotic Cell Death

Autophagy protects against development of tumors by inhibiting the necrosis in cells that are resistant to apoptosis. It has been shown that necrosis triggers the secretion of inflammatory cells and cytokines which contribute to the growth of tumor (126). Autophagy acts as a tumor suppressive mechanism via limiting necrosis of tumor cells and reducing the infiltration of inflammatory cells to the tumor.

1.4.1.3 Induction of Senescence

First barrier against the tumorigenesis is a state of cell cycle arrest known as the Oncogene-Induced Senescence (OIS). This process is mediated by some inflammatory

cytokines such as IL-6 and IL-8 (127). Young and colleagues showed that autophagy is also activated during the OIS (128) and proposed that autophagy provides the cells with basic building blocks for the synthesis of these cytokines which are essential for OIS induction. Therefore, autophagy has a key role in limiting genomic damage and cell growth by contributing to the OIS activation.

1.4.2 Tumor Promoting Functions of Autophagy

While autophagy is important in preventing the development of tumors, a low level of autophagy activation is thought to be essential for tumor survival as it is highly induced in cancer cells under stressful conditions (110). Therefore, it is strongly believed that autophagy is a vital mechanism for cancer cells to deal with the stressful tumor microenvironment and especially during metastasis where cancer cells are under much stress due to their elevated metabolism and high energy demand. There are three scenarios that autophagy improves cancer cell survival and progression and are being explained in the following sections.

1.4.2.1 Hypoxic Tumor Microenvironment

Low oxygen level or hypoxia is usually observed inside the tumor mass where the angiogenesis is limited. Hypoxia is related to a poor prognosis and increased cancer invasiveness and metastasis. Also, it has been shown that hypoxia is a driver of autophagy, for example hypoxia-inducible factor 1α (HIF-1α) is involved in invasion, chemoresistance and metastasis in tumors with hypoxia (129). A group of researchers showed that the deep and central part of the tumor mass has a higher level of

autophagy activity which provides optimum conditions for the survival of the cancer (126). More research is needed to address how autophagy regulate the cell fate of hypoxic tumor cells. Currently, autophagy in hypoxic area of tumors can be considered as a mechanism which provides optimum conditions for cancer cells survival in the stressful tumor microenvironment and meet their insatiable metabolic need (130).

1.4.2.2 Anoikis (Detachment Induced Cell Death)

Anoikis is the mechanism which is involved in cell death of detached cell and recent reports have showed that autophagy is a protecting mechanism against anoikis induced cell death (131). This observation is supported by the fact that autophagy is upregulated in detached cells and the knockdown of autophagy genes caused the clearance of these cells through activating anoikis (110). These evidences support the idea that autophagy in detached cells is a supporting mechanism against induced cell death and might be involved in cancer metastasis and invasion.

Tumor Dormancy

Small proportion of cancer cells are remained in the tumor mass after therapy which after some time can cause the cancer relapse. These cells are called dormant cells and are mainly resistant to regular treatments as they do not have a high rate of proliferation like cancer cells so they can avoid the effect of treatments which usually target high growing cells (132-134). It has been proposed that metastatic cancer cells go through dormancy and shut down their cell cycle as a mechanism to survive in the

stressful condition of the tumor microenvironment (132). Dormant cancer cells use autophagy for their survival in various ways (135). For instance, it is speculated that tumor migratory cells rely on autophagy for their survival and chemo-resistance (136)

1.5 Detecting Autophagy

Identification of better autophagy markers has attracted much attention as the critical role of autophagy in different diseases is unveiling (137). The most reliable and gold standard for autophagy detection is Transmission Electron Microscopy (TEM) which detects autophagosomes and autophagolysosomes and shows cellular ultrastructure in different stages of autophagy (138). TEM is costly and laborious and its routine clinical use for patient's samples is not possible. Thus, although the electron microscopy remains the most sensitive method for the detection of autophagy, using more quantitative and practical methods like cellular and biological methods are of more interest. One of the main hurdles in developing new methods for autophagy flux detection has been proposed as being the multi-step nature of autophagy mechanism where for example an increase in the number of autophagosomes means whether an elevated level of its formation or a reduction in its degradation. Therefore, autophagy detection by these methods can have few problems regarding analysis of data as autophagy is a dynamic cellular mechanism and these methods are static (110). Two very important autophagy markers are LC3 (Microtubule-Associated Protein1 Light Chain 3) and p62 that can be detected via different molecular and cellular techniques.

LC3: The Most Common Marker of Autophagy

There are three types of LC3 including LC3A, B, and C but only LC3B is associated with the levels of autophagy and so detecting the levels of LC3B-II is the most common and well-known method for autophagy detection and can be detected by different techniques such as immunoblotting, immunofluorescence, immunocytochemistry (ICC) and immunohistochemistry (IHC) (139). LC3B-II is formed upon phosphatidylethanolamine (PE) addition to LC3B-I and it is added to both inner and outer sides of the forming autophagosome upon activation of autophagy. Only after fusion with the lysosome LC3B-II is degraded and so its detection is a suitable indicator of autophagy flux. GFP-LC3 is another method to measure the number of autophagosomes and thus the animals or cells overexpressing GFP-LC3 are very suitable where localization of GFP-LC3 from cytosol to the autophagosomes membrane can be detected. Despite its high application there is one disadvantage to GFP-LC3 and that is the formation of aggregations which are like the localized GFP-LC3 puncta and so can result in false positive findings (140). Therefore, another good method for autophagy detection is detecting LC3-II using western blotting where the LC3-II moves faster than the LC3-I because of the hydrophobic PE in its structure. Researchers can now look into the different steps of the autophagy by inhibiting specific stages of the autophagy using autophagy inhibitors (141).

P62/SQSTM

P62 can be used together with LC3 as a great marker to interpret the autophagy flux. p62/SQSTM acts as a carrier protein to deliver cargos to the forming autophagosome through targeting the ubiquitin in aggregated proteins and also interacting with LC3 (142). p62/SQSTM is gradually degraded during autophagy and so its presence is indirectly associated with the activation level of autophagy (137, 143).

1.6 Targeting Autophagy Using Chemical Autophagy Inhibitors and Inducers

1.6.1 Autophagy Inducers

Studies have shown the potential effects of autophagy induction on different diseases. Therefore, the number of compounds that can induce autophagy is increasing rapidly (Table 2) which are discussed below:

Table 2. Autophagy Inducers: Mechanism of action, target and molecular characteristics.

Name	Mechanism	Target point	Solubility	References
Earle's balanced salt solution (EBSS)	Starvation inducer	Autophagy induction	Water-soluble	(144-146)
Brefeldin A	ER stressing inducer	Autophagy induction	Water-insoluble	(56, 147, 148)
Thapsigargin	ER stressing inducer	Autophagy induction	Water-insoluble	(56, 148, 149)
Tunicamycin	ER stressing inducer	Autophagy induction	Water-insoluble	(56, 147-149)
Rapamycin	mTOR inhibitor	mTOR-dependent signaling pathway	Water-insoluble	(150-152)
CCI-779	mTOR inhibitor	mTOR-dependent signaling pathway	Water-insoluble	(153-156)
RAD001	mTOR inhibitor	mTOR-dependent	Water-insoluble	(156-159)

		signaling pathway		
AP23576	mTOR inhibitor	mTOR-dependent signaling pathway	Water-insoluble	(156)
Small molecule enhancers rapamycin (SMER)	mTOR-independent activator	mTOR-independent signaling pathway	Water-insoluble	(160, 161)
Trehalose	mTOR-independent activator	mTOR-independent signaling pathway	Water-soluble	(162, 163)
Lithium chloride	IMPase inhibitor	mTOR-independent signaling pathway	Water-soluble	(164)
L-690,330	IMPase inhibitor	mTOR-independent signaling pathway	Water-soluble	(165)
Valproic acid sodium salt	IMPase inhibitor	mTOR-independent signaling pathway	Water-soluble	(166)
N-Acetyl-*D*-sphingosine (C2-ceramide)	Class I PI3K inhibitor	mTOR-dependent signaling pathway	Water-insoluble	(167)
Penitrem A	Ca^{2+} channel blocker	mTOR-independent signaling pathway	Water-insoluble	(168)
Calpastatin	Calpain inhibitor	mTOR-independent signaling pathway	Water-soluble	(169)

Starvation

Under physiological conditions, autophagy is mainly induced when the levels of amino acids, growth factors or nutrients are reduced. It has been observed that autophagy is induced upon incubation of cells in culture medium deprived from nutrients and necessary amino acids (144, 145). Also, large number of studies has shown the usefulness of physiological induction of autophagy through nutrient deprivation as an acceptable model for research purposes (170).

Endoplasmic Reticulum Stress Inducers

Endoplasmic Reticulum (ER) stress is induced by the accumulation of misfolded proteins in the cell (171) and has been shown to induce autophagy through inhibiting of AKT/mTOR pathway (172) . Thapsigargin and Tunicamycin are well-known ER stress inducers which can also induce autophagy (56, 148, 149).

Rapamycin

Rapamycin or sirolimus has been mentioned in different studies as a very common inducer of autophagy (150, 152). It is an antifungal agent which is able to make a complex with mTOR and inhibits it with subsequent autophagy activation (151).

Small Molecule Enhancers of Rapamycin (SMERs)

Owing to the immunosuppressive side effects of rapamycin that preclude its use in therapy, a safer way of inducing autophagy urgently needs to be developed. These SMERs induce mammalian autophagy in an mTOR-independent manner, appearing to act either independently or downstream of the target of rapamycin. Three main types of SMERs exist including SMER 10, 18 and 28 (160, 173). They activate autophagy process in an independent mTOR mechanism (161).

1.6.2 Autophagy Inhibitors

There are different chemical inhibitors that can inhibit autophagy at a specific stage and they are now being used in many studies (Table 3). Despite their vast use in research studies, the results from autophagy inhibition using these inhibitors need to be analyzed very carefully as a large number of these inhibitors can have some non-

specific effects which can be managed via treatment time and different inhibitor doses.

Table 3. Autophagy Inhibitors: Mechanism of action, target and molecular characteristics.

Name	Mechanism	Target point	Solubility	References
3-Methyladenine	PI 3-kinase inhibitor	Autophagosome formation	Water-soluble	(174, 175)
Wortmaninn	PI 3-kinase inhibitor	Autophagosome formation	Water-insoluble	(176)
LY294002	PI 3-kinase inhibitor	Autophagosome formation	Water-insoluble	(176)
Cycloheximide	protein synthesis inhibitor	Autophagosome formation	Water-insoluble	(177)
Bafilomycin A1	Vacuolar-type H (+)-ATPase inhibitor	Autophagolysosome formation	Water-insoluble	(178)
Hydroxychloroquine	Lysosomal lumen alkalizer	Lysosome	Water-soluble	(179)
Lys05	Lysosomal lumen alkalizer	Lysosome	Water-soluble	(180)
Leupeptin	Acid protease inhibitor	Lysosome	Water-soluble	(181)
E64d	Acid protease inhibitor	Lysosome	Water-insoluble	(182)
Pepstatin A	Acid protease inhibitor	Lysosome	Water-insoluble	(183)

PI3K Inhibitors

PI3K inhibitors inhibit the formation of autophagosomes (174, 184). One chemical in this group is 3-methyladenine (3-MA) which is the first identified inhibitor of autophagy (185). 3-MA exerts inhibitory effect on autophagy through inhibiting the

PI3K class III (186). It has been observed that 3-MA can have two different effects on autophagy. It can induce autophagy when treated cells are rich in growth factors and nutrients and inhibit autophagy when the treated cells are under starvation condition. This unfavorable effect together with the fact that results from 3-MA treatments are not very clean compared to other autophagy inhibitors usually make scientists to use other inhibitors (187).

Vacuolar-type H (+)-ATPase Inhibitors

Vacuolar-type H-ATPases (V-ATPases) are multi-subunit enzymes in the membrane of many cell organelles including lysosomes where they acidify these organelles, so their function is very important for the proper function of organelles. Bafilomycin A1 (Baf-A1) is a well-known and commonly used compound which can specifically inhibit V-ATPase in the membrane of lysosome and interfere with its acidification and therefore disruption in the fusion of lysosomes with the autophagosomes (188, 189). Another less common inhibitor of V-ATPase is concanamycin A (190).

Lysosomal Lumen Alkalizers

This group of autophagy inhibitors impairs the lysosomes by alkalizing them. Popular chemicals like chloroquine (CQ) and hydroxychloroquine (HCQ) fall into this category and they have been used against malaria. More importantly, there are findings about the anti-cancer effects of CQ. It has been shown to rapidly kill serum-starved cancer cells (U251 glioma, B16 melanoma and L929 fibrosarcoma cells) in vitro (179).

Acid Protease Inhibitors

These inhibitors inhibit the lysosomal enzymes and so interfere with their function as digestive machines of the autophagosomes. Leupeptin is an inhibitor of lysosomal peptidases which inhibits the degradation of autophagosome cargos after fusion with lysosomes and so autophagolysosomes are accumulated (181). Pepstatin A, Cycloheximide and E64d act in the same fashion by inhibiting the degradation of autophagolysosomes and also sequestration of lysosomal proteases (183).

Genetic Intervention of Autophagy

Although autophagy inhibitors contribute to a great extent to unveiling the autophagy molecular basis and also discovering new therapeutic targets for different human disorders, most of these agents are not very specific and so a much more efficient and effective approach has proposed and that is genetic intervention in order to inhibit a specific step in autophagy pathway. For example, using specific siRNA sequences to knockdown *ATG* genes can provide scientists with more specific targets for investigations on autophagy (191). Also, many studies have shown the benefits of *ATG* knockdown cell and animal models in autophagy investigations. miRNAs are also useful in the genetic studies of autophagy. For instance, scientists have reported that miR-101 which is a well-known oncosuppressive and usually deregulated in patients with prostate cancer (192) is an autophagy inhibitor by targeting three novel targets, STMN1, RAB5A and ATG4D (193). miR-30a also targets Beclin1 and *ATG5* and downregulates their expression (194).

1.7 Autophagy, Tumor Cell Invasion and EMT

We discussed earlier that autophagy can have two different roles in cancer development. There is also couple of studies with intriguing findings that autophagy plays also a prominent role in the invasion and motility of metastatic cancer cells (195, 196) via providing the necessary building blocks and modulating the secretory system in these cells (197-199). Thus, induction or inhibition of autophagy can be harnessed as a therapeutic intervention against cancer depending on the type of the cancer, microenvironment and cancer stage which is discussed below. In some cancers, autophagy has a protective role and prevents the cancer metastasis and invasion where its inhibition causes an upregulation of mesenchymal markers and downregulation of epithelial markers. In a study on hepatocellular carcinoma, autophagy was reported to be involved in the degradation of EMT promoter Snail. Also, induction of autophagy was shown to drive cells toward the MET and prevention of EMT (200). The degradation of Twist and Snail upon activation of autophagy has been shown in breast cancer cells (201). In glioblastoma (GBM) cells autophagy induction could decrease the regulators of EMT, thereby reducing the invasiveness of these cells (202). On the other hand, in some other cancers autophagy is required for the progression and metastasis of cancer cells. Human cancer cells usually have a stressful microenvironment due to their high rate of protein synthesis and metabolism. Moreover, cancer cells need high energy in order to metastasize and invade other organs. Therefore, they induce autophagy to meet their energy needs for the metastasis process and also to survive the stressful condition (198, 203). The role of autophagy in these invasive and metastatic cells has been investigated using cellular and animal

models with genetically manipulated autophagy. Importantly, autophagy beside EMT has been involved with other common characteristics of metastasis like survival of cancer stem cells, providing a favorable microenvironment for metastasis of cancer cells and also hijacking the immune system (204-208). Interestingly, many of the stresses that can activate EMT can also induce autophagy like TGFβ_1 and hypoxia and especially stresses common to the migrating cancer cells such as growth factor and nutrient deprivation and basement detachment (50, 131, 199, 209-212). Several studies have pointed out a connection between metastasis and autophagy induction. It has been reported that elevated levels of LC3B-II punctate are associated with the low rate of survival and also high metastasis in breast cancer patients (213, 214). Also, it was shown that the intensity of LC3B staining is higher in metastatic tumors compared to the primary tumors (206, 207). It has been shown that autophagy is involved in the invasiveness and metastasis of hepatocellular carcinoma by contributing to TGFβ_1-induced EMT where TGFβ_1 pathway needs autophagy for its activation (199). ULK2 which is an inducer of autophagy (Beclin1 phosphorylation) has been reported to downregulate E-cadherin and induce EMT resulting in a more invasive form and metastasis (215). Autophagy is related to undifferentiated cellular types and essential for the metastasis of glioblastoma stem cells too (216). In a very recent study, Lum and colleagues found that the pharmacological inhibition of autophagy through Lys05 can improve the anti-tumor impact of sunitinib in ovarian carcinoma cells (217). Cancer stem cells (CSCs) contribute to the metastasis and invasiveness of cancer (218) and as autophagy and also EMT are involved in the state of CSCs so the relationship between these two are worth much research for development of new treatments against

cancer (205). Based on these findings, scientists have suggested that autophagy is required for dormant cells to survive in the secondary cancer sites for long time which later result in the relapse of the cancer (135). Autophagy might also contribute to the survival of deprived dormant cells by different ways such as production of ATP and amino acids (219) and induction of quiescence (220). Despite all these advancements, findings are totally different from each other about the association of EMT and autophagy in cancer. Also, the extent to which metastatic cancer cells rely on autophagy is yet to be investigated. Therefore, more studies are needed to find out if EMT and autophagy are connected in a direct or indirect manner.More importantly, it should be studied if cancer type, microenvironment, genetic factors or possibly other unknown factors play any role in different roles for autophagy in various cancers. .

1.8 Therapeutic Modulation of Autophagy in Cancer

Due to the remarkable role of autophagy in the development and metastasis of cancer, it can be considered as a promising target in developing novel anti-cancer therapeutics. Large number of research works have reported that the dual role of autophagy in cancer (death promoting and cytoprotective) can be used in cancer therapy strategies (110). In some cases, autophagy improves the effects of agents against cancers (221). Some other factors like sport and exercise can induce autophagy too and those who regularly do a kind of sport have a lower risk of cancer development (222). On the other hand, in some other cases it is used by tumor cells to survive the harsh microenvironment of the tumor and to help cancer cells to become resistant to therapies. In the first case, the aim is to induce autophagy and in the second case the

aim is to inhibit autophagy in cancer cells. It is worth mentioning that not always autophagy has a direct role in the disease progression and it can be just a passive mediator where its modulation does not have any influence on the survival or death of the cells (221).

1.8.1 Autophagy Inhibition as a Potential Therapy in Cancer

Identification of new autophagy inhibitors are now of high interest to the cancer research community as the inhibition of autophagy has been demonstrated to increase the efficacy of anti-cancer chemotherapies. Autophagy is usually highly activated in different types of human cancer cells upon anti-cancer treatments such as chemotherapy, radiation therapy, hormonal therapy and etc. For this reason, autophagy has been given the term “Achilles heel” in developing anti-cancer interventions. Many studies support the idea of targeting autophagy so as to increase the sensitivity of cancer cells to anti-cancer agents. Also, the efficacy of autophagy inhibition along with other anti-cancer treatments has been investigated with promising results in some cell and animal models. Findings from these studies show that autophagy inhibition can be regarded as an adjuvant therapy in cancer treatment strategies (126, 223-226). Two very commonly used chemicals used for the inhibition of autophagy are CQ and Baf-A1.

Chloroquine (CQ)

The anti-cancer effects of CQ were identified when it was found that it can decrease the risk of Burkitt's lymphoma in patients with malaria (227). The inhibitory role of CQ and its analog hydroxyl chloroquine (HCQ) has been the focus of some studies

and their potential role as adjuvant therapy in cancer therapies has been described. For example, autophagy inhibition by CQ could potentiate the anti-cancer effects of imantinib and suberoylanilide hydroxamic acid (SAHA) in chronic myelocytic leukemia (CML) cells (228). These findings together with the already clinical usage of CQ prompted some clinical trials to investigate more the efficiency of CQ in combination with anti-cancer therapies (110).

Bafilomycin A1

The V-ATPase is highly activated in cancer cells and therefore is a target for cancer therapy (229). Baf-A1 is a well-known autophagy inhibitor that can inhibit autophagy through inhibition of V-ATPase in the lysosomal membranes but also via mTOR activation, Beclin1-Bcl-2 binding, dissociation of Beclin1-Vps34 complex and so it can inhibit autophagy in early and late stages. Different studies have shown the effectiveness of Bafilomycin A1 against tumor growth and invasion. One very important study showed that the treatment of primary cells from patients with acute lymphoblastic leukemia (ALL) with low dose Bafilomycin A1 (1 nM) could specifically inhibit and cause cell death in cancer cells without influencing normal cells. Moreover, Baf-A1 has been demonstrated to reduce the metastasis and growth rate in cell lines of hepatocellular carcinoma and ovarian cancer (229). Regarding toxicity and safety concerns, the *in vivo* tests have shown that Bafilomycin A1 is a safe and potent chemical that can be used in cancer patients with caution (230).

1.8.2 Caveats of Autophagy Manipulation in Cancer Therapy

Notwithstanding the fact that autophagy inhibition can be a promising therapeutic strategy against cancer, there are some disadvantages to it. As autophagy plays a role in the cell quality control and also in tumor suppression, its inhibition might pose the risk of having secondary tumors or other disorders in patients (e.g.; neurodegenerative diseases and aging). Also, autophagy helps maintaining the hemostasis in organs such as heart, liver, brain, etc. Therefore, its inhibition in cancer patients could have undesirable effects on those organs (231). Moreover, it has been reported that because immune cells are dependent on autophagy so its inhibition can potentially reduce the immune responses against cancer cells (232). Furthermore, autophagy provides the ATP which after release from the cells can attract immune cells to the tumor area to attack the cancer cells (233).

1.9 Rationale of the Study

Lung cancer is highly deadly because it doesn't have symptoms until it is at an advanced stage. Novel ideas are needed to improve therapeutic strategies in this field. Metastasis represents the primary cause of death from lung cancer, and it is responsible for about 90% of all morbidity. A principal consideration within treatment of NSCLC metastasis is that about 40% of patients present with distant metastases, and thus new treatment options to prevent metastasis and tumor invasion are very important. Early investigations focusing on simplified model systems provided several essential insights into NSCLC metastasis mechanisms, but these model systems may not include the full range of signaling redundancy and compensatory mechanisms.

There is therefore a need for new strategies targeting NSCLC-derived metastases. The role of autophagy in EMT and lung cancer metastasis remains largely a mystery, especially how this process contributes to tumor invasion in lung cancer. Our study to some extent will pave the way to find the mechanism of autophagy involved in lung cancer metastasis and invasion and so could be targeted as a potential therapeutic target for lung cancer patients in future. In the long-term, we aim to help lung cancer patients by not only investigating the underlying mechanisms of autophagy, but also by investigating potential drug therapies that could lead to a new class of inhalers to target epithelial cells to decrease the EMT process.

1.10 Hypothesis and objectives

1.10.1 Hypothesis:

Pharmacologic inhibition or induction of autophagy and silencing *ATG7* gene modulates TGFβ_1-induced EMT in A549 and H1975 cells (Fig 6).

1.10.2 Objectives:

A: To investigate if inhibition of autophagy by Bafilomycin A1 can affect the EMT process in A549 and H1975 cells.

B: To determine if induction of autophagy by rapamycin can affect the EMT in A549 and H1975 cells.

C: To investigate if genetic intervention of autophagy using specific shRNA sequence against *ATG7* gene can affect the EMT process in A549 and H1975 cells.

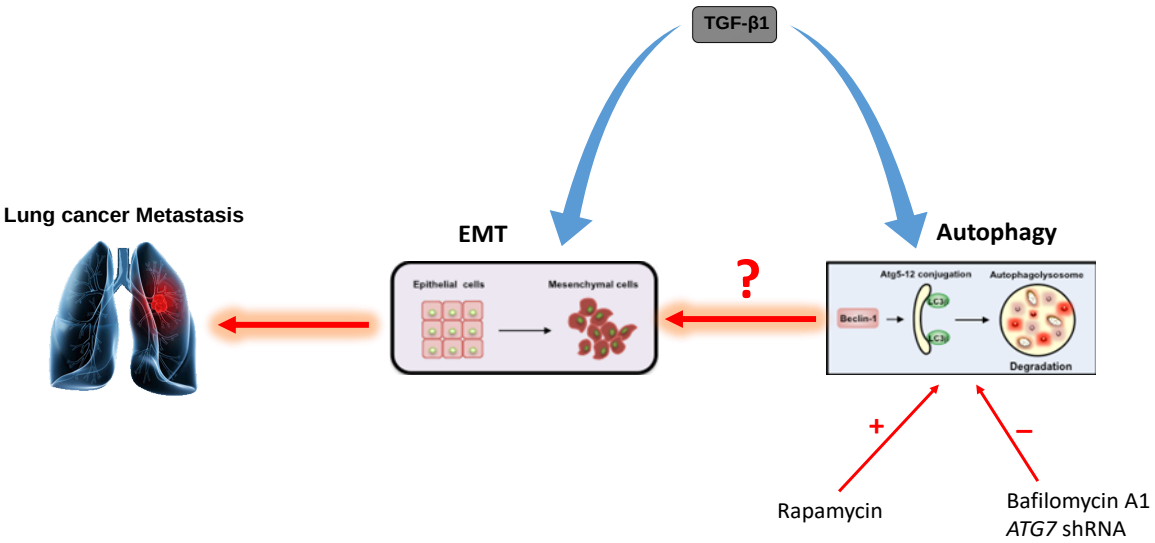


Figure 6. A concise overview of the rationale and hypothesis of the study focusing on the modulatory role of autophagy on TGFβ_1-induced EMT.

CHAPTER 2: Materials & Methods

2.1 Materials and Antibodies

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (CORNING; Cat #: 50-003-PB) with 10% Fetal Bovine Serum (FBS) (Gibco™; Cat #: 16000044). Insulin/Transferrin/Selenium (ITS) (1%) (Gibco™; Cat #:41400045) was used to starve the cells. Cells were maintained in a humidified incubator with 95% air and 5% CO_2 at 37˚C (standard cell culture incubator conditions). Tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (SIGMA; #M2128) was used for the cytotoxicity assay. Primary and secondary antibodies used in western blotting and immunofluorescence are listed in Tables 1-4. Autophagy inhibitor and inducer Bafilomycin A1 (Catalogue # B1793) and Rapamycin (Catalogue # R8781) were purchased from SIGMA-Aldrich Canada Co. Recombinant Human TGFβ_1 (rhTGFβ_1) was purchased from R&D Systems, Inc. (Catalogue #240-B) and a concentration of 5ng/ml used in experiments.

2.2. Cell lines Similarities and Differences:

Human non-small cell lung carcinoma (NSCLC) cell lines A549 (ATCC Number: CCL-185) and H1975 (ATCC Number: CRL-5908) were used as lung cancer cell lines. A549 is a hypotriploid human cell line with the modal chromosome number of 66, occurring in 24% of cells. Cells with 64 (22%), 65, and 67 chromosome counts also occurred at relatively high frequencies; the rate with higher ploidies was low at 0.4%. There were 6 markers present in single copies in all cells. They include

der(6)t(1;6) (q11;q27); ?del(6) (p23); del(11) (q21), del(2) (q11), M4 and M5. Most cells had two X and two Y chromosomes. However, one or both Y chromosomes were lost in 40% of 50 cells analyzed. Chromosomes N2 and N6 had single copies per cell; and N12 and N17 usually had 4 copies. Note: Cytogenetic information is based on initial seed stock at ATCC. Cytogenetic instability has been reported in the literature for some cell lines. H1975 cell line has mutations in different genes like CDKN2A, EGFR, EGFR, PIK3CA and TP53. Both A549 and H1975 cell lines comprise the majority population of cancer cells in NSCLC. The most important difference between A549 and H1975 is the mutated form of P53 in H1975 while it is intact (WT) in A549 (234-236).

Table 4: Primary antibodies used in western blotting

Primary Antibody	**Dilution**	**Source**	**Molecular size (KDa)**
Phospho-p44/42 MAPK (Erk1/2) Total p44/42 MAPK (Erk1/2)	1:1000	Cell signaling; #4370	42, 44 42, 44
Phospho-p38 MAPK Total-p38 MAPK	1:1000	Cell signaling; #4511	43
Phospho 46 and Phospho 54 - SAPK/JNK Total SAPK/JNK	1:1000	Cell signaling; #4668	46 54
Phospho-SMAD2 Total SMAD2	1:1000	Cell signaling; #8828	60
LC3B-I LC3B-II	1:1500	SIGMA; # L7543	18 16
ATG7	1:1000	Cell signaling; #8558	7

P62	1:1000	Cell signaling; #8025	62
Vimentin	1:1000	Cell signaling; #5741	57
E-cadherin	1:1000	Cell signaling; #3195	135
snail	1:1000	Cell signaling; #3879	29
slug	1:1000	Cell signaling; #9585	30
GAPDH	1:1500	Santa Cruz; sc-47724	37

Table 5: Secondary antibodies used in western blotting

Secondary Antibody-HRP conjugate	**Dilution**	**Source**
Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat	1:5000	SIGMA; # A6154
Anti-Mouse IgG (Fab specific)–Peroxidase antibody produced in goat	1:3000	SIGMA; # A-8924

Table 6: Primary antibodies used in immunofluorescence

Primary Antibody	**Dilution/ Conc.**	**Source**
Vimentin (Rabbit monoclonal)	1:200	Cell Signaling; #5741

Table 7: Secondary antibodies used in immunofluorescence

Secondary Antibody AlexaFluor (AF) conjugated	**Dilution**	**Source**
Donkey Anti Rabbit IgG–AF 647	1:500	JACKSON; #711-605-152

2.3. Production of Stable *ATG7* KnockDown (KD) NSCLC Cell lines

Human NSCLC cell lines A549 and H1975 were seeded at a density of $5x10^4$ cells/ well in 12-well plates and cultured in DMEM, 10% FBS for 24 hours. After reaching 40 percent confluent cells were treated with 10µg/ml polybrene (Santa Cruz; sc-134220) in DMEM medium (without FBS and) for 1h and then transfected with shRNA Lentiviral Particle for *ATG7* and scrambled control (a negative control strategy that has the same nucleotide composition, but not the same sequence, as the test shRNA) both carrying the coding gene for puromycin resistance (Santa Cruz; sc-41447-V, *APG7* shRNA (h) Lentiviral Particles). Cells were transfected at 3 and 6 multiplicity of infections (MOI) for 12 hrs, followed by replenishing the medium for recovery for 24h. After the recovery, cells that incorporated the shRNA plasmid were selected using Puromycin dihydrochloride (4µg/ml) (Santa Cruz; sc-108071) containing medium. Cells with activated the puromycin resistance gene survived the selection and clones were isolated. The *ATG7* status was later checked by Western blotting in shRNA transfected and scramble cells based on published original research papers (237-240).

2.4. Cytotoxicity Assay

A549 and H1975 cells grown to 30% confluence in DMEM media (high glucose) under 10% FBS media conditions. Cells were treated with a wide range of different concentrations of Bafilomycin A1 (0.1, 1, 2.5, 5 or 10 nM) and cell viability was assessed after 24 and 72 hrs using MTT assay. First, an amount of 20 µl of

tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added into each of the 96-well plate and incubated for 3 hrs. The medium in the wells were removed and 200ul of solvent control (DMSO) was added into each well and mixed well. The plate wrapped with a foil and then 570nm absorbance was read after 20 minutes incubation at RT (241). The assay is based on the reduction of the MTT to its insoluble formazan, which has a purple color. This reaction is facilitated by NAD(P)H-dependent cellular oxidoreductase enzymes which are active only in living cells with metabolic activity (242). Control cells for each time point were treated with DMSO.

2.5. Treatment of A549 and H1975 cells and knockdown A549 and H1975 cells with Baf-A1, Rapamycin and TGFβ$_1$

A549 and H1975 cells were grown to 30% confluency in DMEM (high glucose) under 10% FBS media conditions and standard cell culture incubator conditions. Cells were then starved with insulin/transferrin/selenium (ITS) (1%) for 24 hrs and pretreated with Baf-A1 (1nM) and/or Rapamycin (500 μM) (2 hrs pretreatment). Then cells were co-treated with TGFβ$_1$ (5 ng/ml) and at various time points (12, 36 and 48 hrs) cell lysates were collected and proceeded for western blotting. Similarly, knock down of A549 and H1975 cells, grown to 30% confluency in DMEM medium (high glucose) with 10% FBS and puromycin (4μg/ml). Cells were then starved with ITS (1%) for 24 hrs followed by stimulation with TGFβ$_1$ (5 ng/ml) and studied at various time points (24 and 36 hrs).

2.6. Analysis of Cellular Morphology

To see the changes in the morphology of the cells after treatment with Baf-A1, Rapamycin and TGFβ_1 images were taken before collecting the cell lysates for western blotting. The phase contrast microscopy (Zeiss Axioverts 100) with Olympus DP10 CCD digital camera was used to capture images.

2.7. Western Blotting

Western blot analysis was used to detect markers of autophagy and EMT and SMAD phosphorylation in A549 and H1975 cells and *ATG7* KD cells. We examined markers of SMAD signaling, autophagy and EMT (Table 1) while GAPDH was used to normalize the results. We followed the procedures used in previous studies for protein assay, sample preparation and SDS-PAGE (240, 243, 244). Briefly, cells were washed and protein extracts were prepared in NP-40 lysis buffer (20 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 0.5 mM PMSF, 100µM β-glycerol 3-phosphate and 0.5% protease inhibitor cocktail) and samples were stored at -20˚C until used for western blotting. After centrifugation (13.000g for 10 min) supernatant protein content was determined by Lowry protein assay. Proteins were size fractionated by SDS-PAGE; samples were heated at 90˚C for 5 min and 15-20 µl of samples was loaded on a 10-15% polyacrylamide gels based on the molecular weight of the proteins, 10µl of precision plus protein were used as marker standard (Thermo Fischer Scientific, ON, Canada). After electrophoresis, separated proteins were transferred onto Immun-Blot PVDF Membranes (Bio-Rad; #1620177) under reducing conditions in transfer buffer (500nM glycine, 50mM Tris-HCl, and 20% methanol) for 2 hrs, RT at 100 volts.

Membranes were blocked with 5% non-fat dried milk and 1X Tris-buffered saline containing Tween (TBS/0.01% tween 20; TBST) at cold room overnight or RT for 1 h. After blocking, membranes were incubated at 4˚C overnight with appropriate primary antibodies (Table 1) in 1% milk in 1X TBST. After overnight incubation membranes were washed three times with 1X TBST for 20 min and incubated with appropriate horseradish peroxidase (HRP) coupled secondary antibodies (Table 2) for 2 hrs at RT. Membranes were washed again three times for 20 min and incubated with enhanced chemiluminescence (ECL) regents (Amersham-Pharmacia Biotech) for 2-3 min. The signals were visualized by autoradiography. Obtained protein bands were evaluated for changes in the autophagy, EMT and SMAD signaling pathway and to draw a conclusion and comparison on the role of autophagy inhibition and induction in EMT process in A549 and H1975 cells. The blots for both cells were quantified using dosimetry software Alpha Ease FC.

2.8. Fluorescence Live Cell Imaging using LC3-GFP

Previous works have used LC3-GFP in order to detect the autophagosomes (245). This method was used to confirm the autophagy and EMT in presence and absence of autophagy chemical inhibitor (Baf-A1), inducer (Rapamycin) and TGFβ_1. It involves the transfection of A549 and H1975 cells with LC3-GFP plasmid (Addgene; #24920) using the Effectene Transfection Reagent (QIAGEN; #301425). After transfection, the LC3-GFP plasmid expresses the mammalian LC3 fused to EGFP in the cells which can be detected as green under the immunofluorescent microscopy. A number of 30,000 cells/ml was counted and seeded on 6-well plates (2 ml of medium and cells)

and grown to 50% confluency by incubation for 24 hrs. The transfection of the cells was done with Effectene Transfection Reagent according to the manufacturer's protocol. After 18 hrs incubation transfected cells were checked under a fluorescent microscope (Olympus, Markham, ON, Canada) and after confirmation of the transfection their medium was replenished with ITS (1%) for 24 hrs and pretreated with Baf-A1 (0.1nM) or Rapamycin (500 µM) for 2 hrs. Then cells were co-treated with TGFβ_1 (5 ng/ml) and incubated. , lysosomes, mitochondria and DNA were stained after 36 hrs time point with Lysotracker Red (Molecular Probes™; LysoTracker® Red DND-99; L7528) at a concentration of 50 nM for 30 minutes. Also, a drop of DAPI (4', 6-diamidino-2-phenylindole) (ProLong® Gold Antifade Mountant with DAPI; Cat #: P36931) was simultaneously added for 30 minutes which stains the nucleus and also preserve the the fluorescent dyes from fading. After washing the cells with 1X PBS images were acquired using the fluorescent microscope. LC3 punctate localized with activated lysotracker red identified as autophagy cells and cells with autophagy inhibition were shown with accumulation of LC3 and inactivated lysosomes. These results validated the WB analysis results.

2.9. Immunocytochemistry (ICC)

To detect the changes of vimentin in A549 and H1975 cells and also in *ATG7* knockdown of these cells we used a combination of LC3-GFP and regular immunocytochemistry protocol according to previous studies (238, 246). After cells transfected with the LC3-GFP, they were cultured on coated slides (Fisherbrand™ Superfrost™ Plus Microscope Slides; Cat#: 12-550-15) (approximately 5000

cells/spot) in DMEM media (high glucose) with 10% FBS media conditions (using standard cell culture incubator conditions). Cells were starved with ITS (1%) for 24 hrs and pre-treated with indicated concentrations of Baf-A1 (0.1nM) or Rapamycin (500 µM) and then co-treated with TGFβ_1 (5 ng/ml) for 36 hrs. In the indicated time point, ICC was done with the following protocol. Cells were fixed with 3.7% formaldehyde for 20 mins at RT and washed 3 times for 10 min with 1X TBS. Then cells were permeabilized with 0.25% triton-X100 for 15 mins and blocked with 5% Normal Donkey Serum (NDS) (JACKSON; #017-000-121) for 1h at RT. Cells were then incubated overnight in 4˚C with the rabbit anti-Vimentin IgG primary antibody (Cell signaling; #5741) (1:200) and corresponding isotype control immunoglobulin (IgG's) diluted in 5% NDS to detect the changes in the EMT marker vimentin. Slides were washed 3 times for 10 min and incubated with appropriate fluorochrome-conjugated secondary antibodies (Alexa Fluor conjugated) for 1h at RT and washed 3 times for 10 min. Slides were then incubated with DAPI which stains the AT regions of the DNA, washed 3 times for 10 min and cover slipped in Antifade Mountant with DAPI and stored at -20˚C until imaged.

2.9.1 Image acquisition

Slides were imaged using the Axioimager Z1 microscope, AxiocamMR3 camera and a 63X/1.40 oil immersion DIC M27 lens (Carl Zeiss, Canada) and the automation tool on ZEN software (Carl Zeiss, Canada). The 3D nuclear optical sectioning was done by taking 80 stacks (Z stacks) along the X, Y and Z axis of the cell nucleus with a distance of 200nM between each Z stack and then rendering the sections using a

deconvolution algorithm in Axiovision 4.8 software (Carl Zeiss, Canada). The exposure time was set at 1000 milli seconds (ms) for the Alexa Flour 647 as this time did not show any background from IgG control.

2.10. Transmission Electron Microscopy (TEM)

Autophagy in presence and absence of Baf-A1 and TGFβ_1 was confirmed using TEM. We used the protocol for TEM based on the previous published research works (238, 246). Briefly, A549 and H1975 cells were cultured in 100 mm dishes (250,000 cells/dish) in DMEM media (high glucose) with 10% FBS in standard cell culture incubator conditions. Cells were starved with ITS (1%) for 24 hrs and then treated with Baf-A1 (1nM) and/or pre-treated with Baf-A1 and then co-treated with TGFβ_1 (5 ng/ml) for 36 hrs. TEM was performed on ultra-thin sections (100 nm on 200 mesh grids) 36 hrs after treatment and stained with uranyl acetate and counterstained with lead citrate. Autophagy induction was evaluated based on the autophagosome and autophagolysosome formation while autophagy inhibition was evaluated based on the accumulation of autophagosome in ultra-structure of the studied cells. Results from TEM showed the autophagy inhibition after treatment with Baf-A1 and TGFβ_1(5ng) in both A549 and H1975 cells and confirmed our imaging and western blotting findings.

2.11. Wound Healing Assay (Scratch Assay)

The migratory function of cells after treatment with Baf-A1, Rapamycin and TGFβ_1 was measured by wound healing assay per defined procedure (247). Culture inserts 2

Well (ibidi; #80209) were inserted in 12-well plates. A volume of 75µl of medium containing A549 or H1975 cells were added into each of the 2 small wells in culture inserts containing 1000 cells/well for A549 and 1500 cells/well for H1975. The surrounding area of the culture inserts in the plates were filled with medium to prevent evaporation. Plates were incubated for 24 hrs to grow to 30-40% confluency in DMEM media (high glucose and 10% FBS). Medium was gently withdrawn from the culture inserts and cells were starved for 24 hrs with medium containing 1% ITS followed by pretreatment with Baf-A1 (0.1nM) or Rapamycin (500 µM) for 2 hrs and then co-treatment with TGFβ_1 (5 ng/ml) for 48 hrs. Culture inserts were taken very carefully from the plates and cells were refreshed with medium (FBS 10%) to help cells start migration. Images were taken after time intervals of 24 and 48 hrs using phase contrast microscope. The same experiment was done for A549 and H1975 *ATG7* knockdown cells where both scramble and knockdown cells were starved for 24 hrs and then treated with TGFβ_1 (5 ng/ml) for 48 hrs. Culture inserts were gently taken and fresh medium (FBS 10%) was added to the wells. Images were taken after 24 and 48 hrs time intervals.

2.12. Statistical analysis

The statistical analyses were done using GraphPad Prism (Version 7; USA). The differences between the groups were calculated using one way ANOVA analysis (non-parametric, Brown–Forsythe test) and the confidence interval in each analysis was set at 95% so the comparisons having a $p<0.05$ value were considered significant.

CHAPTER 3: RESULTS

1. TGFβ$_1$ induces simultaneous Autophagy, EMT and SMAD signaling in A549 and H1975 Cells.

Our results showed that TGFβ$_1$ induces autophagy and EMT simultaneously. We treated A549 and H1975 cells with TGFβ$_1$ (5ng/ml) based on the protocol mentioned in Method and Material for 24, 48 and 72 hrs. We showed upregulation or downregulation of proteins involved in the activation of both autophagy and EMT. Western blot results showed downregulation of epithelial marker E-cadherin and upregulation of mesenchymal marker vimentin showing the induction of EMT in treated cells with TGFβ$_1$. Also, lipidation of LC3B-II and degradation of p62 showed the activation of autophagy. TGFβ$_1$ induced both EMT and autophagy in parallel with SMAD2 signaling pathway which is shown by the overexpression of phospho and total SMAD2 in both A549 and H195 cells (Figure7. A, B).

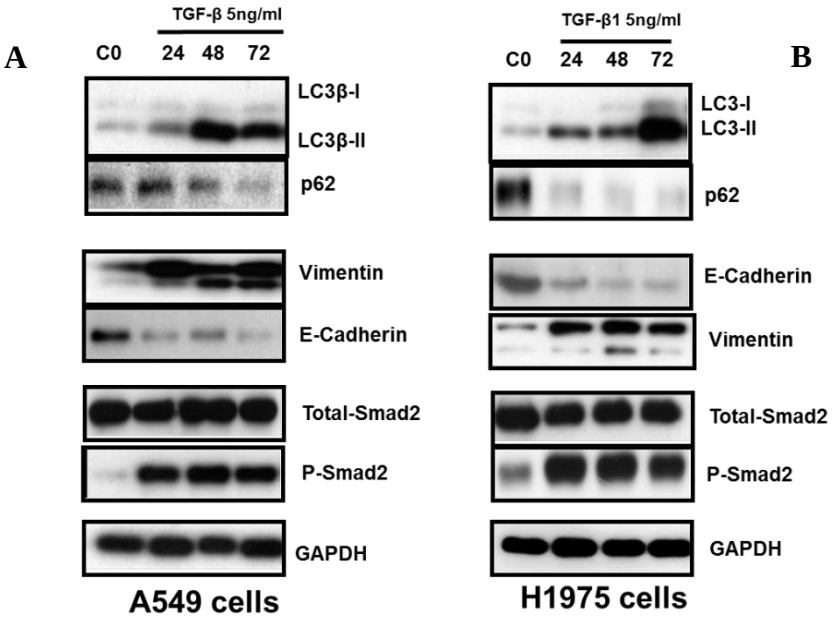
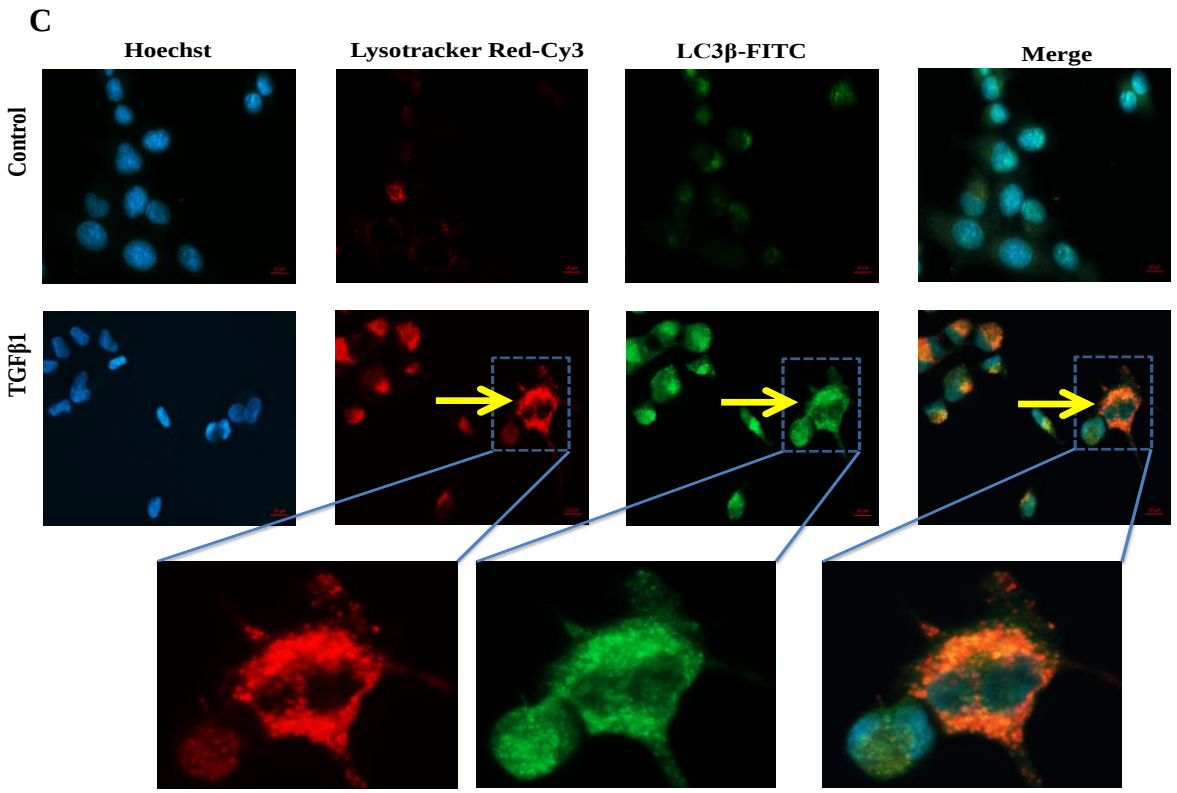


Figure 7. TGFβ$_1$ induces both autophagy and EMT in A549 cells. (A, B) Cells were treated with TGFβ$_1$ (5ng) and different proteins were assessed by western blotting after 24, 48 and 72 hrs TGFβ$_1$ treatment induces EMT (down regulation of E-cadherin and upregulation of vimentin) and also autophagy (Lipidation of LC3II and degradation of p62) in parallel with the activation of SMAD2 signaling pathway (upregulation of phospho-SMAD2 and SMAD2) in both A549 (A) and H1975 (B) cells. The protein loading was confirmed using GAPDH. The blots are representative of 3 different independent experiments.

Immunofluorescence results also showed LC3β-II puncta and lysosomal activation upon TGFβ$_1$ treatment for 48 hrs in both A549 and H1975 cells, highlighting the activation of autophagy in these cells (Figure 7. C, D).



D

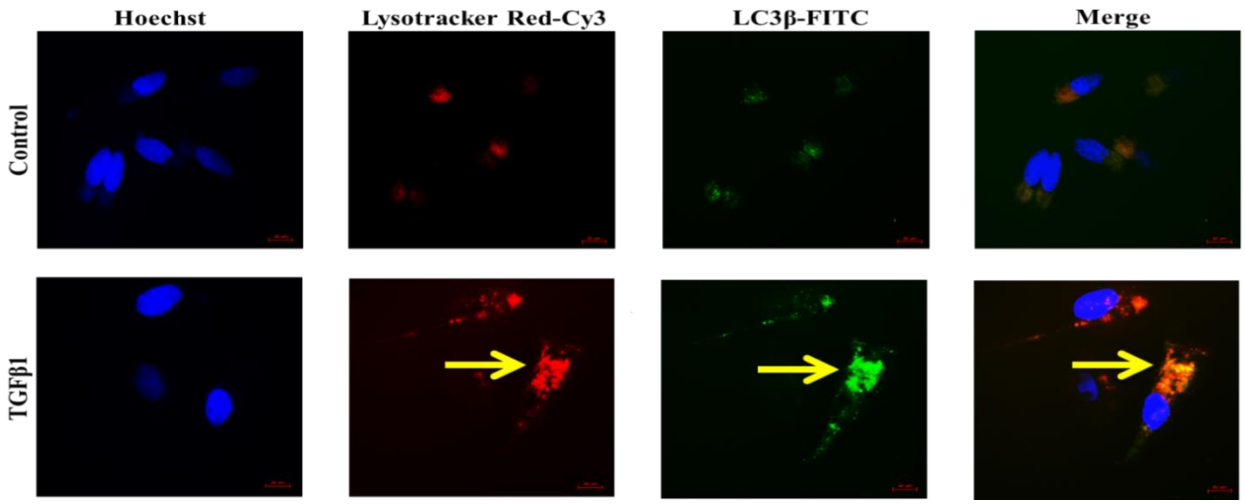


Figure 7. (C, D) TGFβ_1 induces LC3β-II punctuation and lysosomal activation after 48 hrs in A549 (C) and H1975 (D) cells. LC3B puncta (green) and lysosome activation (red) were analyzed with LC3-GFP transfecting plasmid and Lysotracker Red using Immunofluorescence. Representative immunofluorescent images of LC3β and activated lysosomes shown (scale bars 20μm). 10 fields were imaged for each condition with an average of 4 cells per field.

We further confirmed the autophagy activation through Transmission Electron Microscopy (TEM) in cells treated for 36 hrs with TGFβ_1. Results clearly confirmed autophagy activation (formation of the autophagolysosomes in A549 and H1975 cells after treatment with TGFβ_1. (Figure 7. E, F).

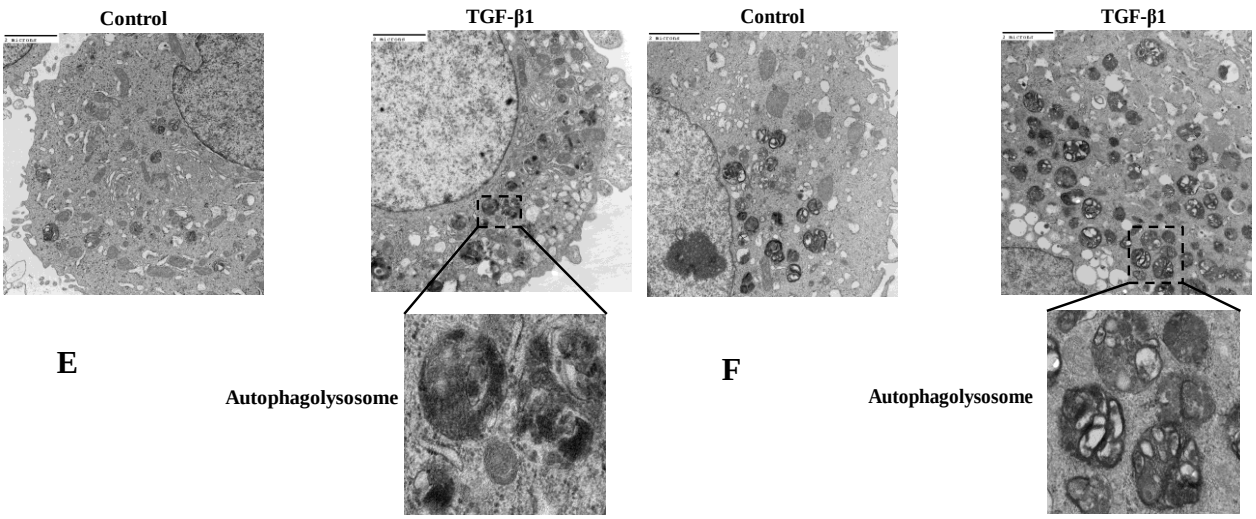


Figure 7. (E, F) Transmission Electron Microscopy (TEM) shows the activation of autophagy in A549 (E) (magnification 11600x) and H1975 (F) (magnification 20500x) cells after treatment with TGFβ$_1$ (5ng) for 36 hrs. Arrows show the forming autophagolysosomes containing the cargo.

Furthermore, TGFβ$_1$ treatment caused phenotypic changes in cells typical of mesenchymal cells (spindled shaped morphology). This shows that TGFβ$_1$ induced the EMT process in the treated A549 (G) and H1975 (H) cells after 24hrs of treatment which is a prerequisite for cancer cells migration (Figure 7. G, H).

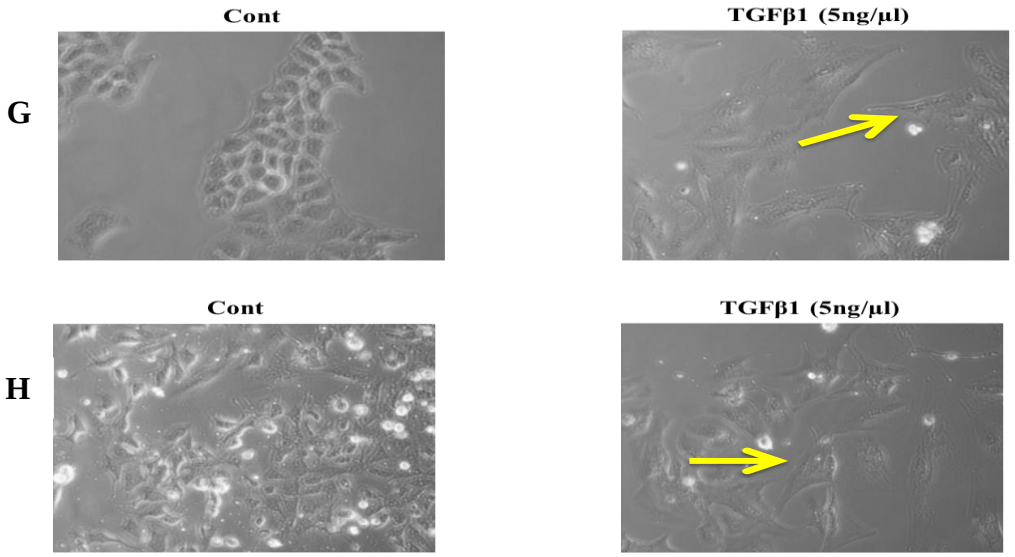


Figure 7. (G, H) TGFβ$_1$ induced the A549 (G) and H1975 (H) cells to go through the pheno-conversion and acquire a mesenchymal phenotype (yellow arrows). Images were taken using phase contrast microscope after 24 hrs of treatment with TGFβ1. (magnification 20x).

2. Chemical Inhibition of autophagy (Baf-A1) abrogates TGFβ$_1$-induced EMT in A549 and H1975 cell lines.

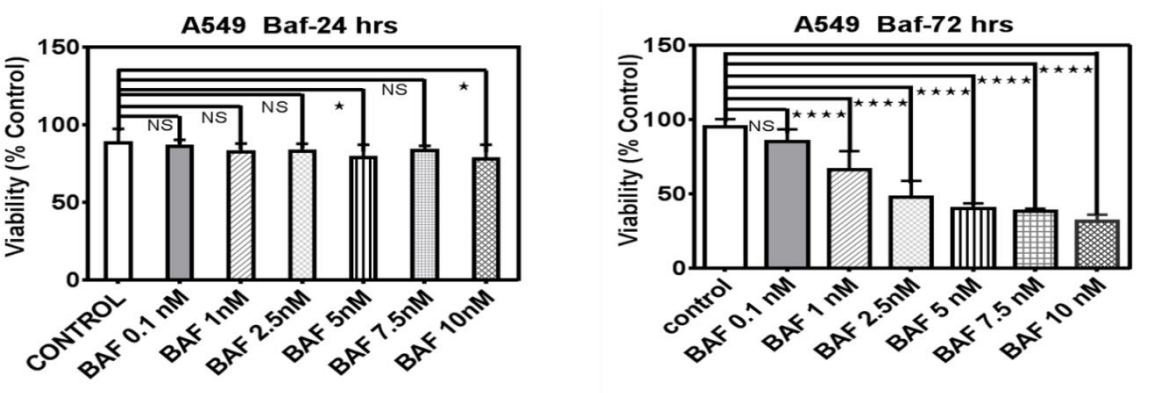
We have found in our experiments that autophagy and EMT are both induced by TGFβ$_1$. In next step, we wanted to investigate how EMT is possibly regulated by autophagy and to answer the question if autophagy might regulate the EMT process.

We first manipulated autophagy using chemical inhibition of autophagy and later confirmed the results through silencing of *ATG7* gene in A549 and H1975 cells.

2.1. Chemical inhibition of autophagy (Baf-A1) abrogates TGFβ$_1$-induced EMT in A549 and H1975 cell lines.

We did cytotoxicity assay (MTT assay) in order to find the optimal dose of Baf-A1 which can inhibit autophagy with the lowest toxicity. We treated the cells with a wide range of Bafilomycin A1 concentrations (0.1-10 nM) for 24 and 72 hrs. Our results showed that Baf-A1 (1 nM) has low toxicity on the time point we investigate while inhibiting autophagy flux in both A549 and H1975 cells (Figure 8. A, B, C).

A



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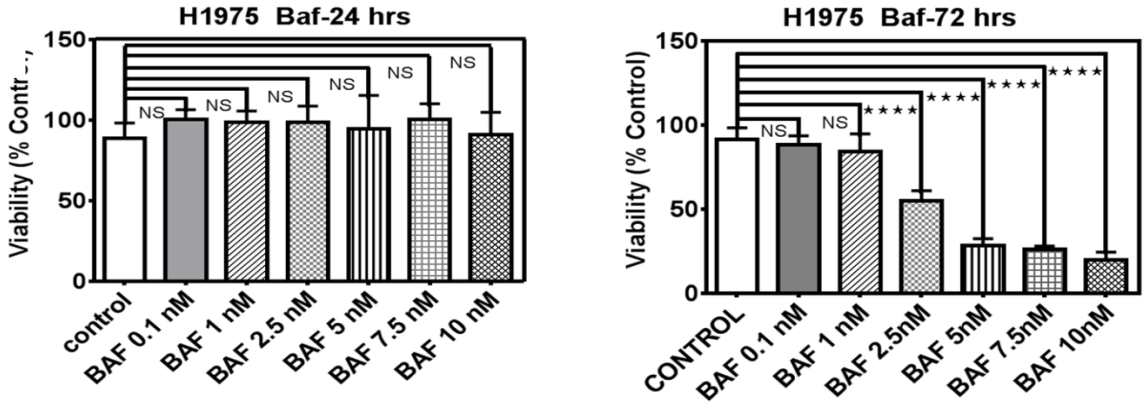


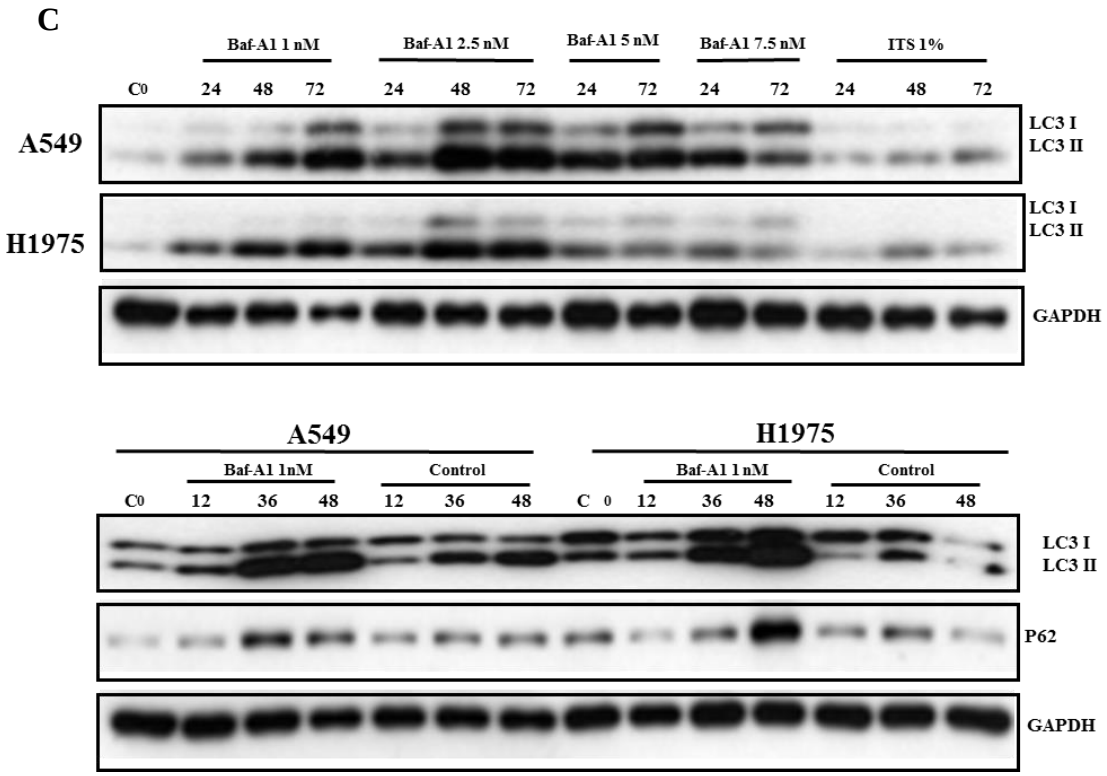
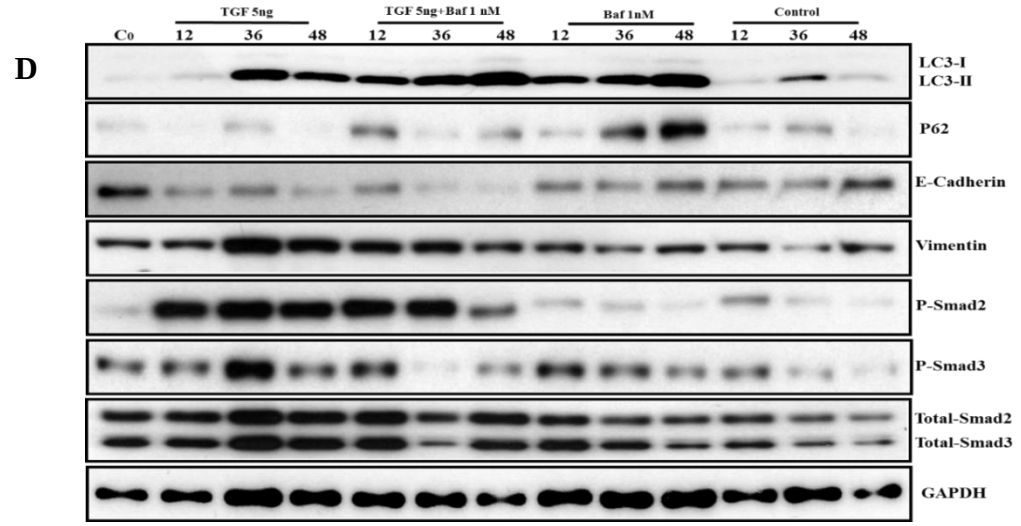
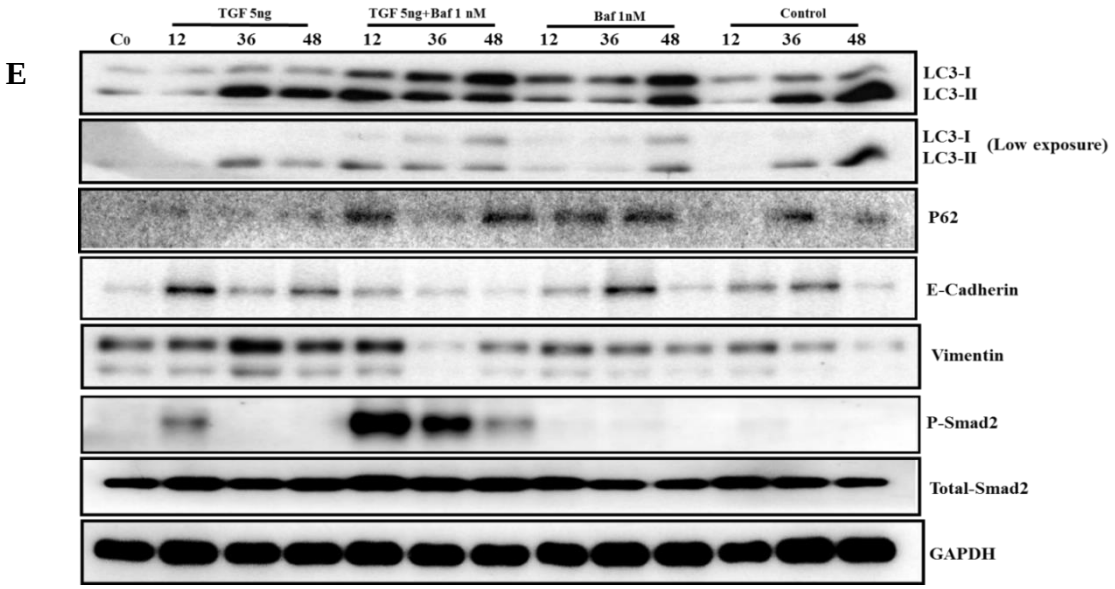


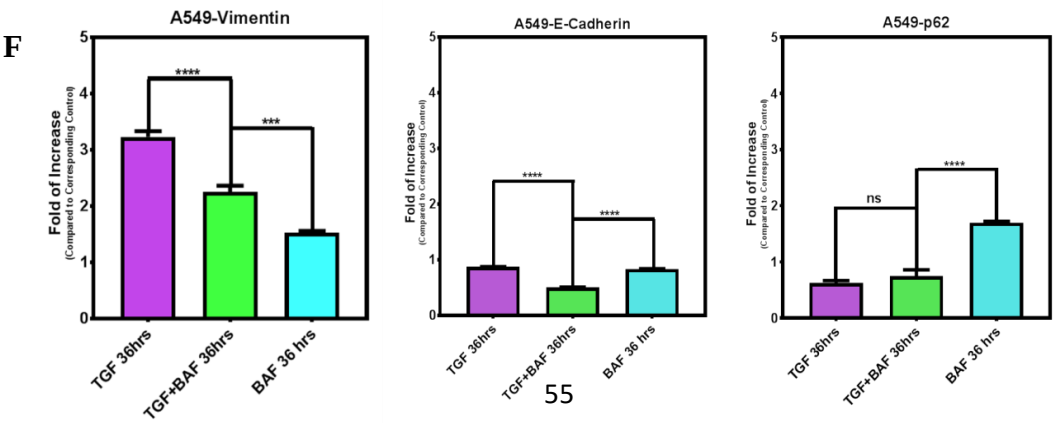
Figure 8. Treatment of cells with Bafilomycin A1 (1nM) or shRNA targeting *ATG7* gene can inhibit autophagy which in turn can prevent the EMT induction in both cells. (A, B, C) Cytotoxicity assay for Bafilomycin A1 in A549 (A) and H1975 (B) cells. A549 and H1975 cells were treated with a wide range of different concentrations of Bafilomycin A1 (0.1, 1, 2.5, 5 or 10 nM) and cell viability was assessed after 24 and 72hrs using MTT assay. Control cells for each time point were treated with the solvent control (DMSO). Results are expressed as percentage of corresponding time point control and represent the means +- SD of 15 replicates in three independent experiments (**, P<0.01; ***, P<0.001). (C) cells were treated with different concentration of Bafilomycin A1 (1-10nM) to see the inhibition of autophagy in different time points. Cell lysates were collected and using western blotting the abundance level of LC3-II were analyzed for both cell lines. Also, we showed that Bafilomycin-A1 with concentration of 1nM can inhibit autophagy for both cell lines using autophagy markers LC3-II and p62. GAPDH was also measured as control for loading.

Cells were treated with Baf-A1 (1nM) followed by co-treatment with TGFβ_1 (5ng/ml) for 12, 36 and 48 hrs. Autophagy was inhibited by Baf-A1 which was shown by the lipidation of LC3B-II and reduced degradation of p62. More importantly, we found that inhibition of autophagy causes a reduction in the mesenchymal marker (vimentin)

and upregulation of epithelial marker (E-Cadherin) in both A549 and H1975 cells (Figure 8. D-G).







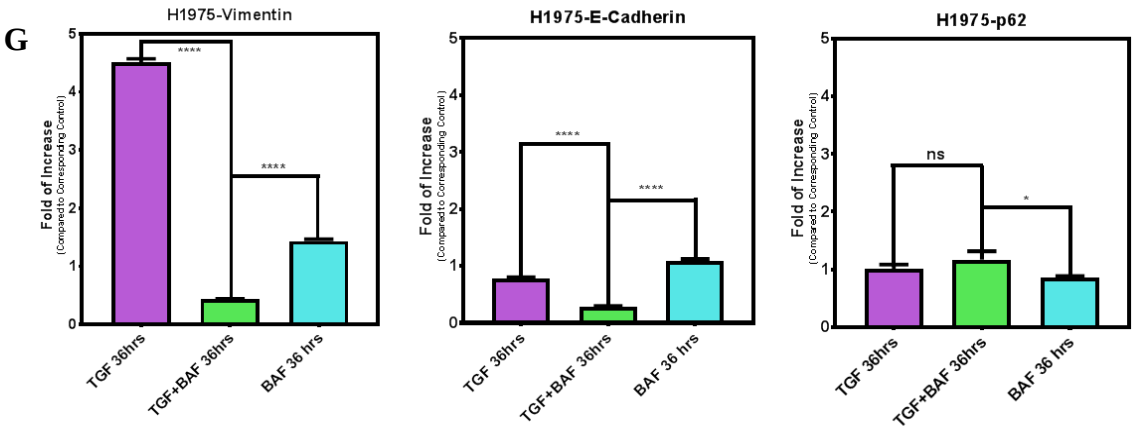
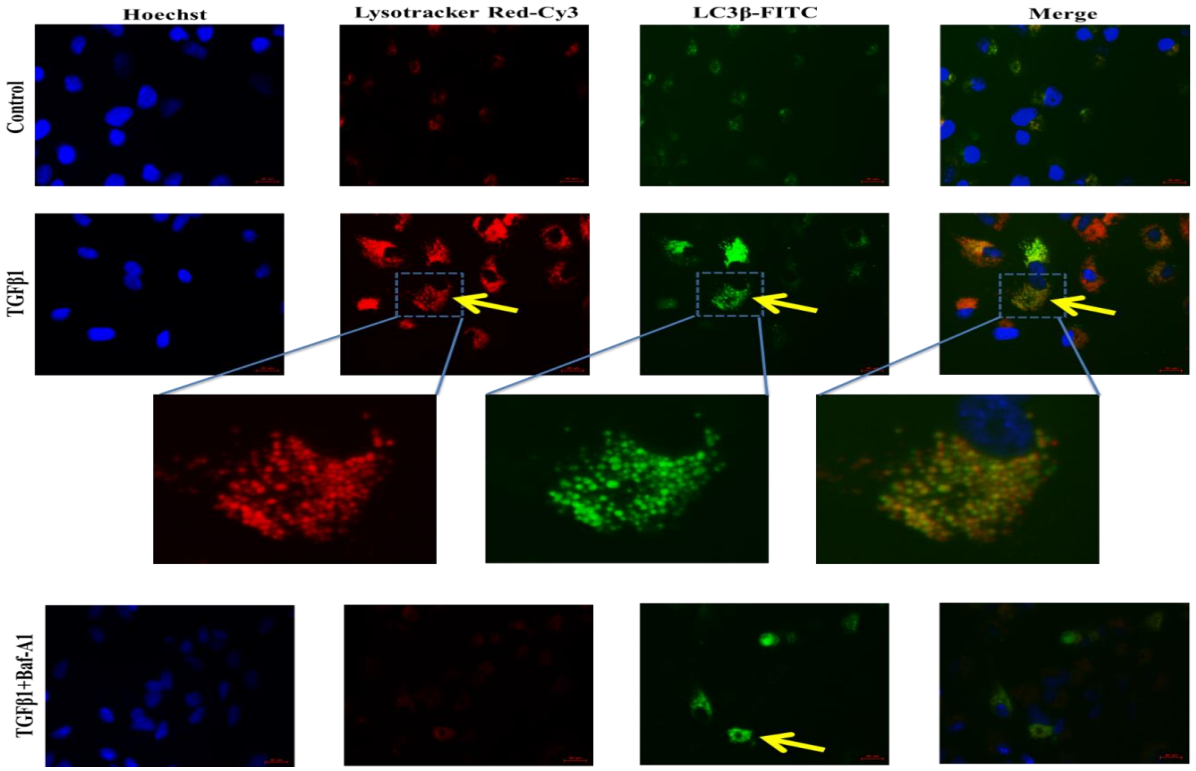


Figure 8. (D-G) A549 (D) and H1975 (E) cells were treated with Bafilomycin A1 (1nM) and co-treated with TGFβ_1 (5ng) for 12, 36 and 48 hrs. Cell lysates were collected and using western blotting the abundance level of EMT and autophagy markers were analyzed for both cell lines. GAPDH was also measured as control for loading. (F, G) The blots for vimentin, E-Cadherin and p62 were quantified for both cells compared to their correspondent controls using densitometry software Alpha Ease FC and graphs were prepared by GraphPad Prism using one way ANOVA (non-parametric, Brown–Forsythe test). P-value ≤ 0.05 and lower were considered as statistically significant.

These promising findings highlight the possible regulation of EMT by autophagy. Results from immunofluorescence imaging showed that LC3β-II puncta is increased and we had lysosomal activation confirming again the autophagy activation after treatment with TGFβ_1. However, autophagy flux is inhibited in treated cells with Baf-A1+TGFβ_1 shown by accumulation of LC3B-II puncta and inactivation of lysosomes (Figure 8. H, I).

H


Hoechst
Lysotracker Red-Cy3
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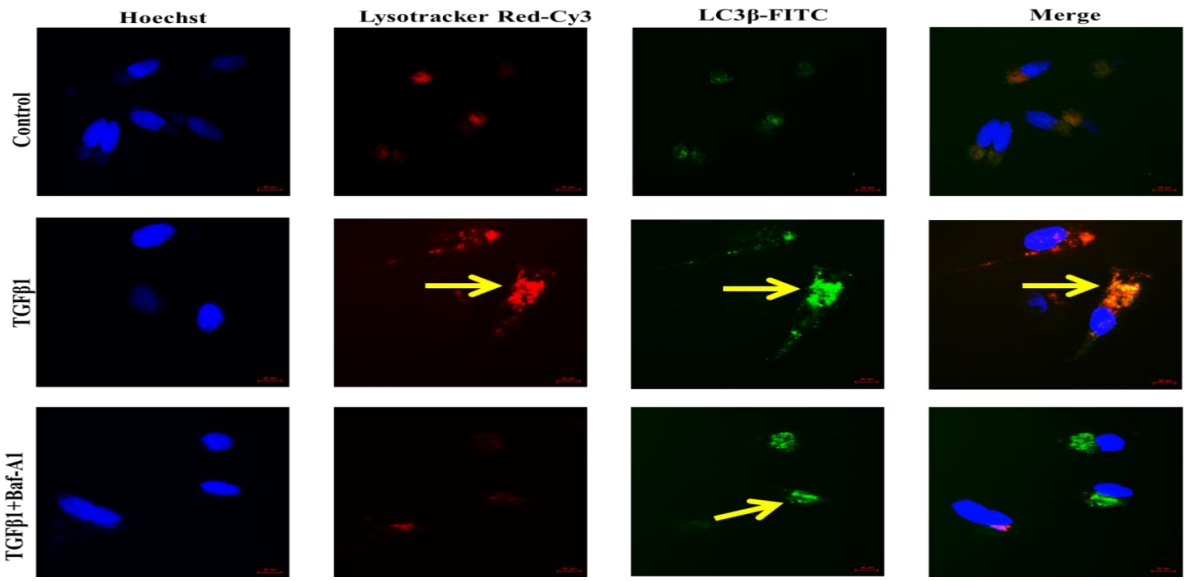
Control
TGFβ1
TGFβ1+Baf-A1

I


Hoechst
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Control
TGFβ1
TGFβ1+Baf-A1

Figure 8. (H, I) immunofluorescent images showed that Baf-A1 inhibited the lysosomal activation upon co-treatment with TGFβ_1 in both A549 (H) and H1975 (I) cells after 48 hrs. In TGFβ_1 treated samples, as expected, we had LC3β-II puncta and lysosomal activation after 48hrs. Representative immunofluorescent images of LC3B-II and activated lysosomes shown (scale bars 20μm).

TEM images also confirmed the autophagy inhibition in treated cells with Baf-A1 where there are many accumulated autophagosomes in the treated cells after 36 hrs. Interestingly, we observed huge vacuole-like autophagosomes in A549 and H1975 cells treated with Baf-A1+TGFβ1 (Figure 8. J, K).

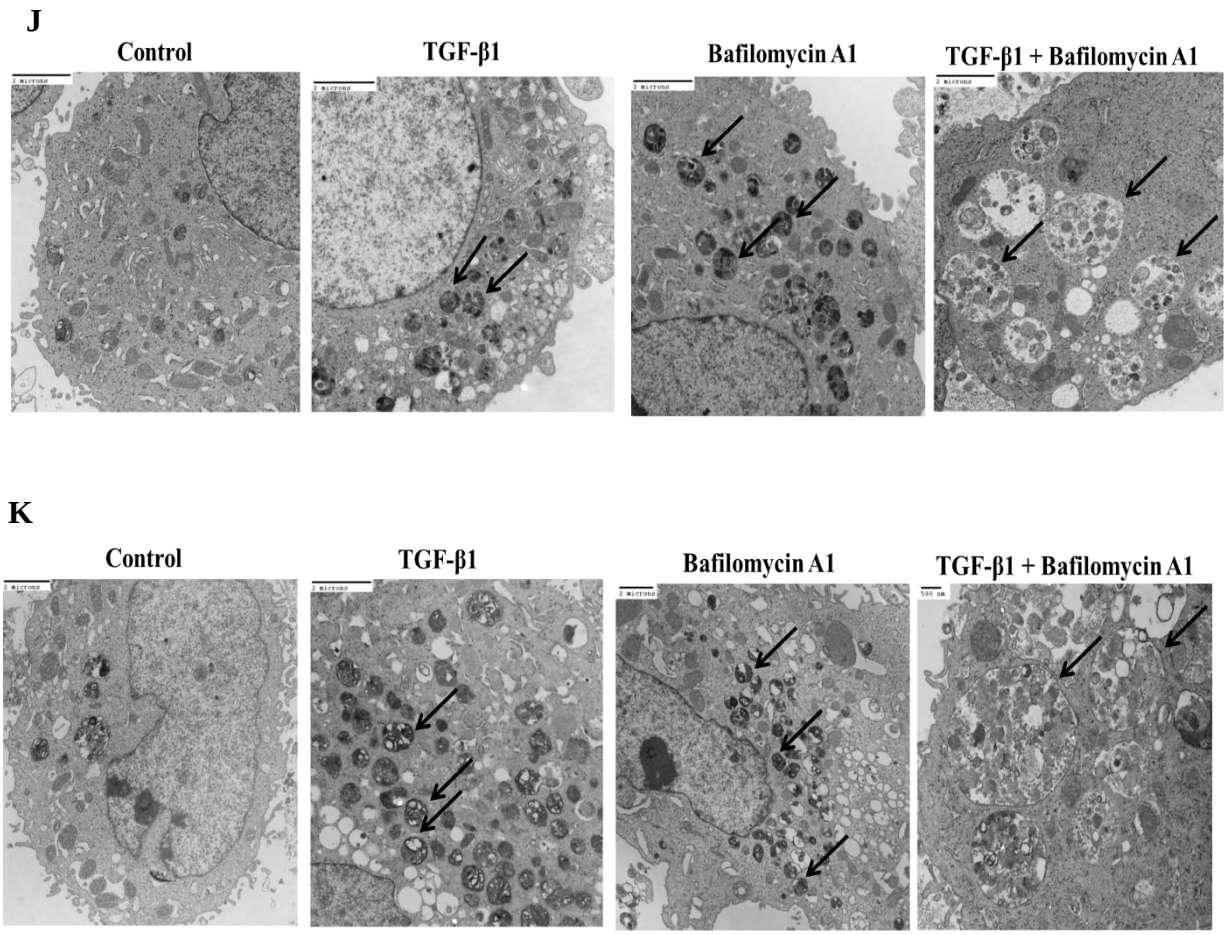


Figure 8. (J, K) Ultrastructure of A549 (J) (magnification 20500x) and H1975 (K) (magnification 27700x) cells treated with Bafilomycin A1 & Bafilomycin A1+TGFβ1. Transmission Electron Microscopy (TEM) shows the inhibition of autophagy in both cells after treatment with Bafilomycin A1 (1nM) and TGFβ$_1$ (5ng) for 36 hrs. Arrows in the left image show the accumulated autophagosomes and in the right image show huge vacuole-like autophagosomes. Interestingly, treatment with Bafilomycin A1+TGFβ1 induced a peculiar morphology of huge and vacuole-like autophagosomes in both cells.

We assume that these large double membrane vacuoles are the result of merging many autophagosomes in the cells treated with Baf-A1+TGFβ$_1$ after 36 hrs. We also showed the morphological changes in the A549 and H1975 cells upon treatment with Baf-A1+TGFβ$_1$. TGFβ$_1$ caused the cells to go through phenotype conversion from epithelial to mesenchymal. However, in cells with Baf-A1+TGFβ$_1$ treatment, Baf-A1 caused cells to re-convert from their mesenchymal form to their original epithelial phenotype after 24hrs of treatment. This shows that Baf-A1 can in fact abrogate the TGFβ$_1$ induced EMT (which is a prerequisite for cancer cells mesenchymal phenotype conversion and migration) in A549 and H1975 cells via inhibition of autophagy (Figure 8. L, M).

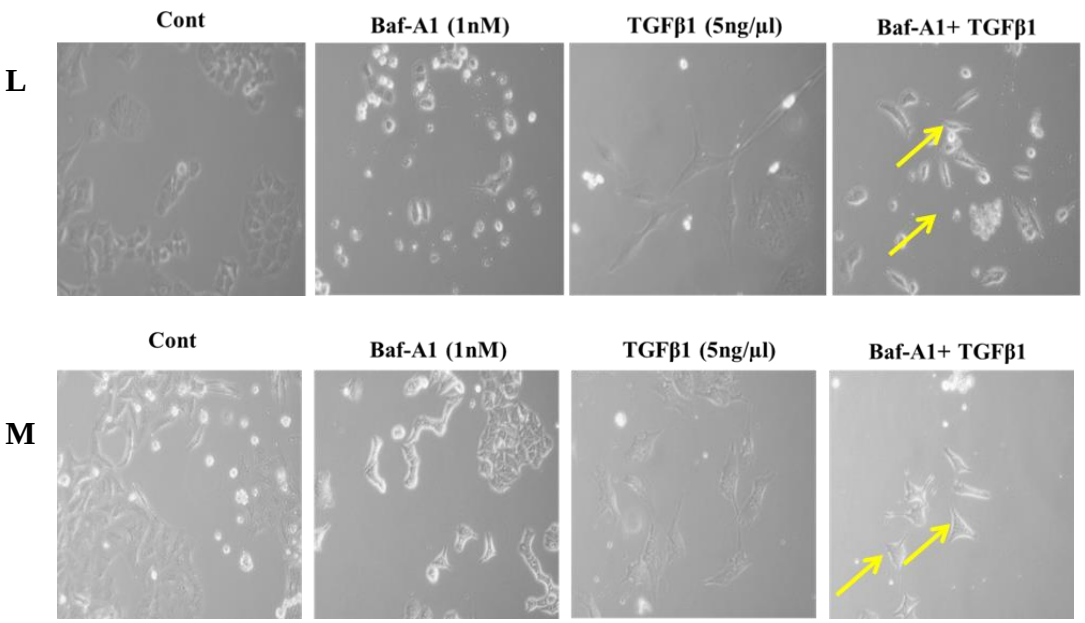


Figure 8. (L, M) As opposed to TGFβ$_1$ effect on pheno-conversion of A549 (L) and H1975 (M) cells, Baf-A1 causes the cells to acquire their epithelial phenotype after 24hrs treatment with Bafilomycin A1+TGFβ1 (yellow arrows). Images were taken using phase contrast microscope after 24hrs of treatment with Bafilomycin A1+TGFβ1. (magnification 20x).

2.2. Targeted knockdown of *ATG7* gene modulates TGFβ$_1$-induced EMT in A549 and H1975 cells.

We asked the question if knockingdown of an autophagy gene will have the same results as the chemical inhibition of autophagy. In order to address this, we selected *ATG7* gene as the target gene for autophagy knockdown. *ATG7* encodes the E1-like activating enzyme which is essential in the formation of autophagosome. More importantly, it activates *ATG12* for its conjugation with *ATG5* and also activation of the *ATG8* (LC3B) family proteins for their conjugation with phosphatidylethanolamine (PE) (248). By using lentiviral particles carrying the shRNA against *ATG7* gene, we generated stable *ATG7* knockdown clones in A549 and H1975 cells. A549 and H1975 cells were transfected with lentivirus at different MOI (3 and 6) containing shRNA plasmid for *ATG7* in order to identify the most efficient one to knockdown the *ATG7* gene, and non-silencing shRNA plasmid (scramble). *ATG7* expression was evaluated with Western blot using an anti-*ATG7* antibody after selection of *ATG7* KD clones using puromycin (4µg/ml) medium. The Western blot analysis displayed knockdown of the *ATG7* protein in clones transfected with shRNA against *ATG7* whereas scramble clones expressing non-silencing shRNA did not show any effect on *ATG7* protein expression. The knockdown was much more

efficient with the MOI 6 in both A549 and H1975 cells and it was about 80-90 percent knockdown in samples with shRNA against *ATG7* compared to that of scramble samples (Figure 8. N).

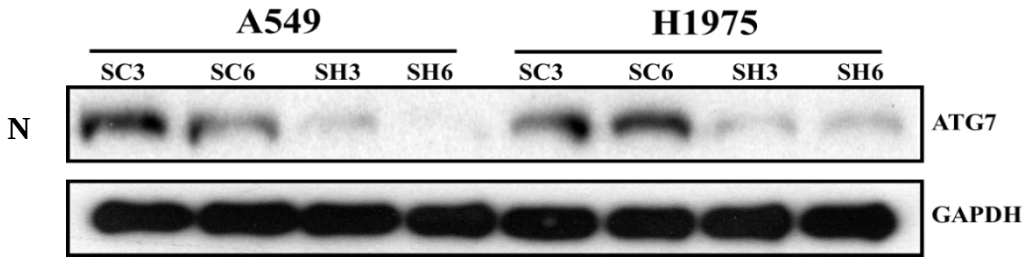
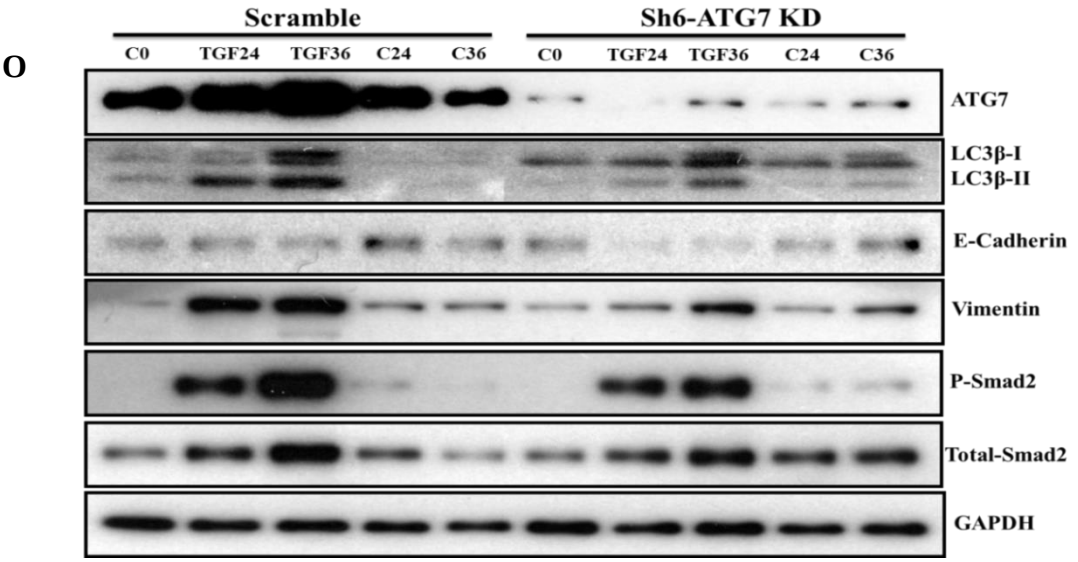
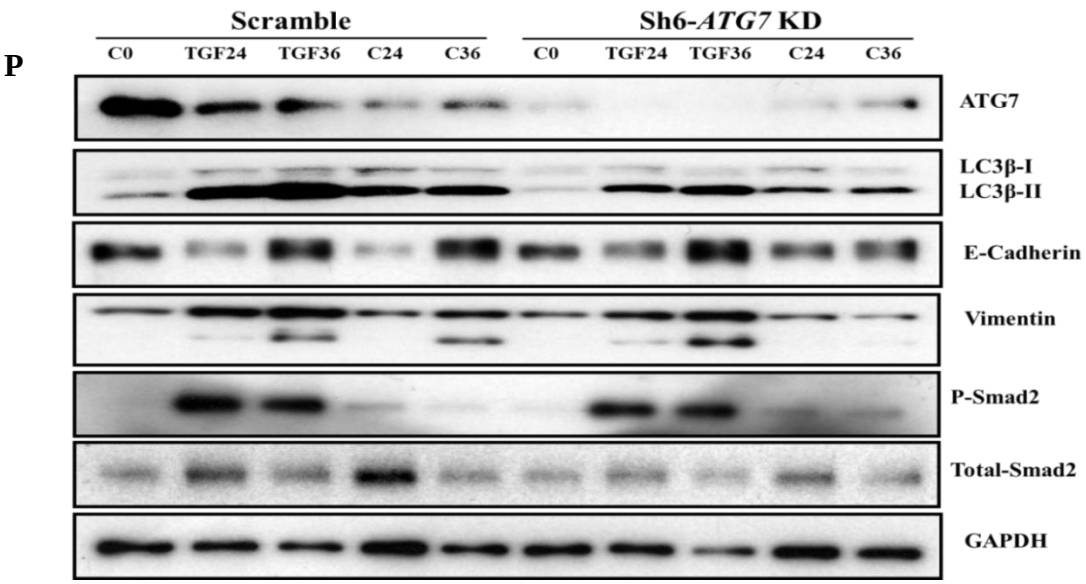


Figure 8. (N) *ATG7* gene was knockeddown in both cell lines using lentivirus carrying the specific siRNA against the target gene. Different MOIs (3 and 6) of lentiviral particles were used in order to identify the most efficient one to knockdown the *ATG7* gene. Scramble siRNA (without the specific sequence for *ATG7* gene) was used as control.

ATG7 KD A549 and H1975 cells were treated with TGFβ_1 (5ng/ml) for 24 and 48 hrs according to the protocol described in Method and Material section. Western blotting was done on cell lysates and showed changes in the markers of EMT, autophagy. As it is shown in Figure 8. O, P: WB, TGFβ_1 induces autophagy in scramble cells while it did not in the *ATG7* KD cells as it was shown by the lipidation of LC3B-II and also downregulation of *ATG7*. These findings show that autophagy is inhibited in *ATG7* KD cells. Additionally, we could observe that in scramble cells treated with TGFβ_1 the vimentin expression was upregulated in both cells while in *ATG7* KD cells it was down regulated (Figure 8. O-Q). In contrast to this, E-Cadherin did not change significantly as an epithelial marker in either scramble or *ATG7* KD A549 and H1975 cells upon TGFβ_1 treatment. These results again highlight that autophagy inhibition can abrogate the induction of EMT. Also, we further detected the activation of

phosphorylated SMAD2 and total SMAD2 in parallel with our results in the *ATG7* KD A549 and H1975 cells treated with TGFβ_1 (Figure 8. O, P).





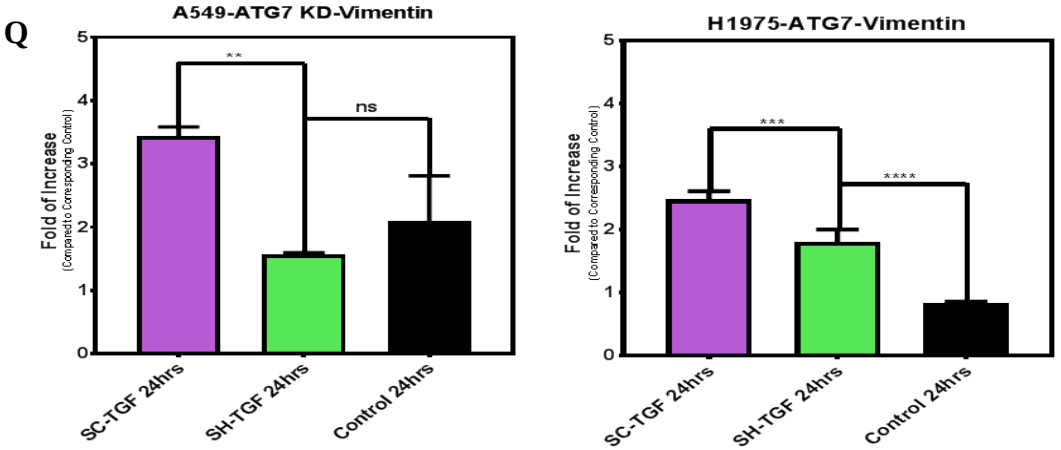
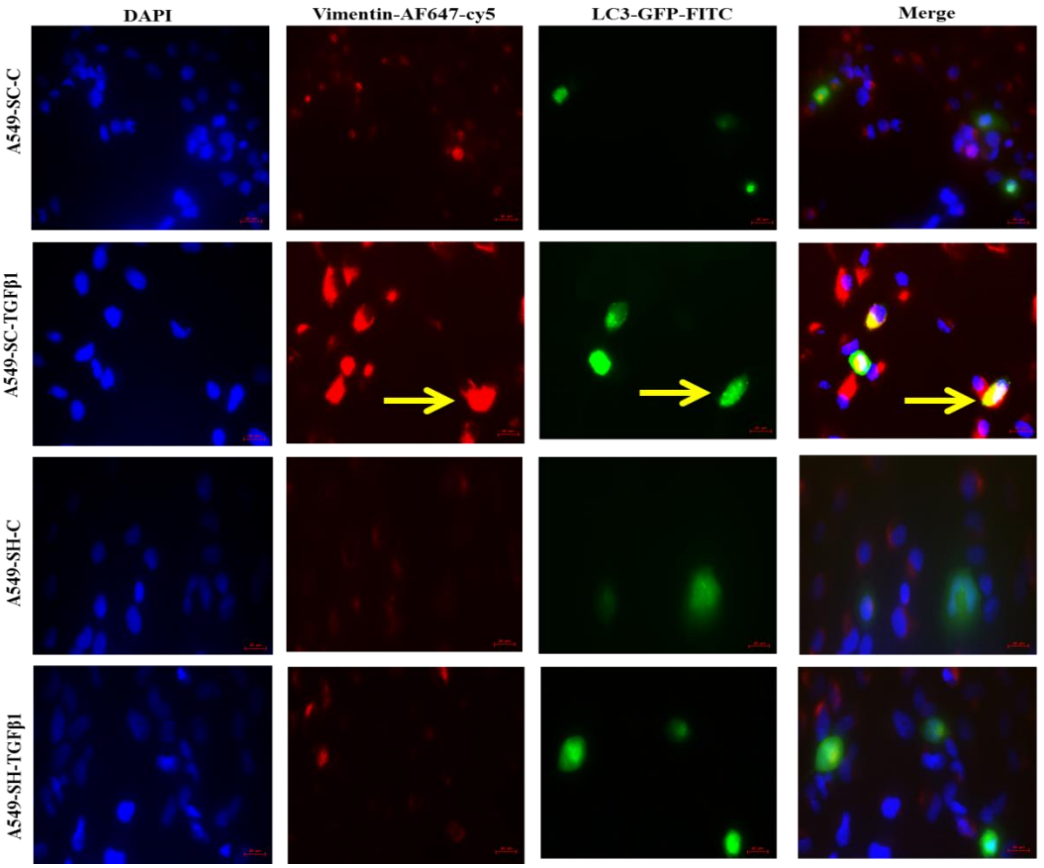


Figure 8. (O-Q) A549 (O) and H1975 (P) cells (*ATG7* KD) were treated with TGFβ_1 for 24 and 36 hrs. The abundance of EMT, SMAD signaling and autophagy markers were evaluated using western blotting. Figures show the representative western blotting bands. GAPDH was used as the loading control. (Q) The blots for vimentin were quantified for both cells compared to their correspondent controls using densitometry software Alpha Ease FC and graphs were prepared by GraphPad Prism using one way ANOVA (non-parametric, Brown–Forsythe test). P-value ≤ 0.05 and lower were considered as statistically significant.

Furthermore, our immunofluorescence results showed that the vimentin is downregulated and the LC3B-II puncta is decreased (since *ATG7* is upstream to LC3 in the autophagy pathway) in *ATG7* KD A549 and H1975 cells compared to the scramble counterparts (Figure 8. R, S).

R


DAPI
Vimentin-AF647-cy5
LC3-GFP-FITC
Merge
A549-SC-C
A549-SC-TGFβ1
A549-SH-C
A549-SH-TGFβ1

S

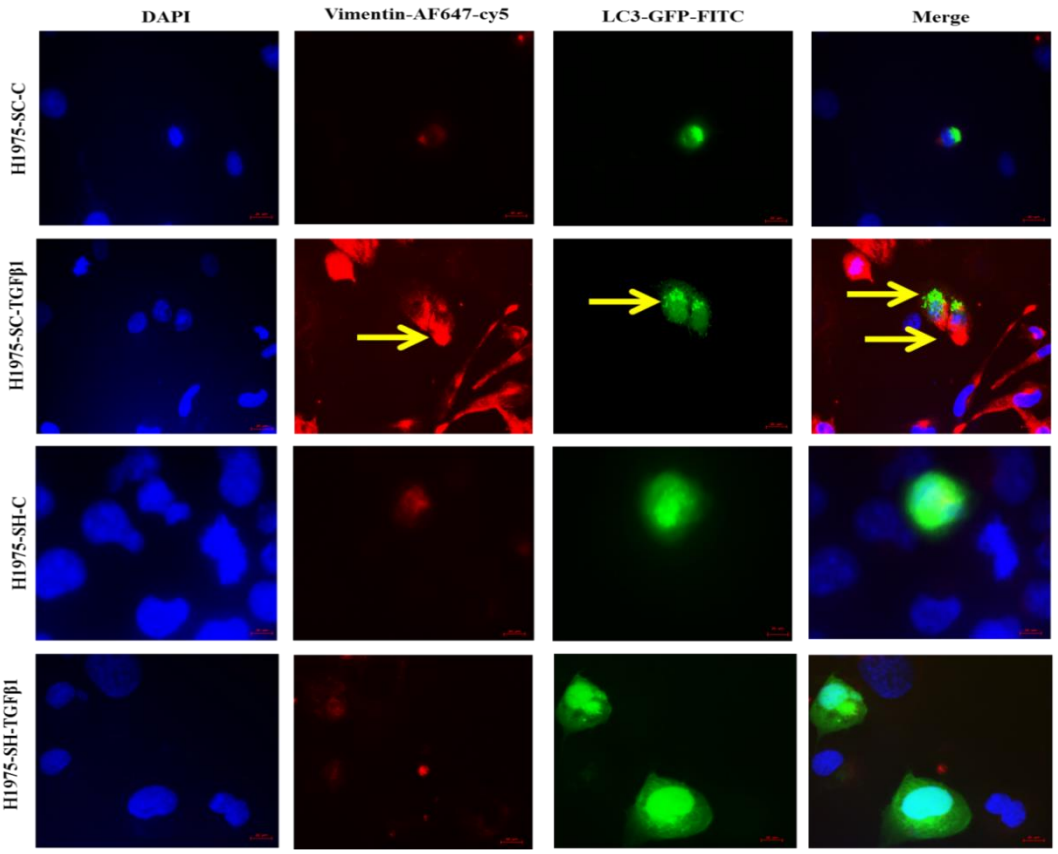

DAPI
Vimentin-AF647-cy5
LC3-GFP-FITC
Merge
H1975-SC-C
H1975-SC-TGFβ1
H1975-SH-C
H1975-SH-TGFβ1

Figure 8. (R, S) ICC results showed that the *ATG7* KD cells have a lower signal for vimentin and less level of LC3B-II puncta as compared to their scramble counterparts after treatment of both *ATG7* KD A549 (R) and H1975 (S) cells with TGFβ_1 (5ng/µl) for 48hrs. yellow arrows show the LC3 punctuate and highly expressed vimentin in scramble cells treated with TGFβ_1. Representative immunofluorescent images of LC3B-II and activated lysosomes shown (scale bars 20µm).

These findings from *ATG7* KD cells are consistent with those from chemical inhibition of autophagy with Baf-A1 in both cell lines and point out the regulatory role that autophagy inhibition has on the prevention of EMT induction.

3. Autophagy induction with Rapamycin favors the regulation of TGFβ_1-induced EMT.

To answer the question if autophagy can regulate the EMT process, in next step we chemically induced autophagy in A549 and H1975 cells using the mTOR inhibitor Rapamycin. First, we evaluated the efficiency of rapamycin in inducing autophagy by treating both A549 and H1975 cells with two different concentrations of rapamycin (250 and 750nM). As it is shown in Figure 9. A rapamycin was able to effectively induce autophagy in both concentrations. This is shown by the degradation of p62 and lipidation of both LC3B-I and LC3B-II. Therefore, we chose the 500nM as our target concentration for our other experiments. Also, we starved both cells for 48hrs and evaluate if it causes the induction of autophagy. As shown in the Figure 9. A, starvation of cells caused the induction of autophagy in both A549 and H1975 cells as evidenced by the degradation of p62.

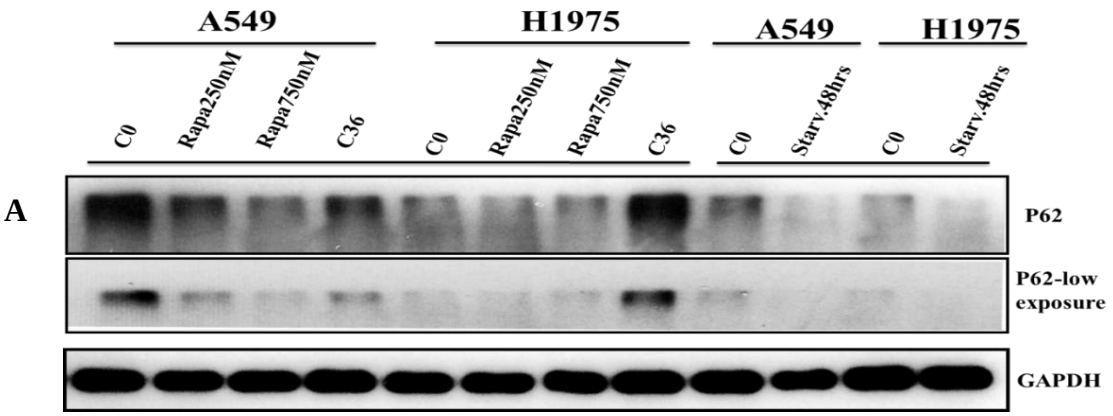


Figure 9. Treatment of cells with Rapamycin (500 nM) can induce autophagy which in turn enhances the EMT induction in both cells. (A) Rapamycin induces autophagy in A549 and H195 cells. A549 and H1975 cells were treated with Rapamycin (250, 750nM) and the autophagy markers were analyzed using western blotting to detect LC3 II lipidation and p62 degradation. Also, after both cells reached a confluency of about 40 percent they were starved for 48hrs (DMEM without FBS). Starvation of A549 and H1975 cells induced autophagy activation. GAPDH was used as loading control.

We treated the cells with rapamycin (500nM) followed by co-treatment with TGFβ_1 (5ng/ml) for 12, 36 and 48 hrs. The results showed the activation of autophagy shown by lipidation of both LC3B-I and LC3B-II, upregulation of *ATG7* and also increased degradation of p62. Autophagy was highly activated in treated cells with Rapamycin+TGFβ_1 compared to the TGFβ_1 alone and control samples. Also, the mesenchymal markers vimentin and slug were upregulated more in Rapamycin+TGFβ_1 compared to the TGFβ_1 alone and control samples. For E-Cadherin we observed the same results as the Baf-A1 where there was no change in E-Cadherin upon treatment with rapamycin in both A549 and H1975 cells (Figure 9. B, C).

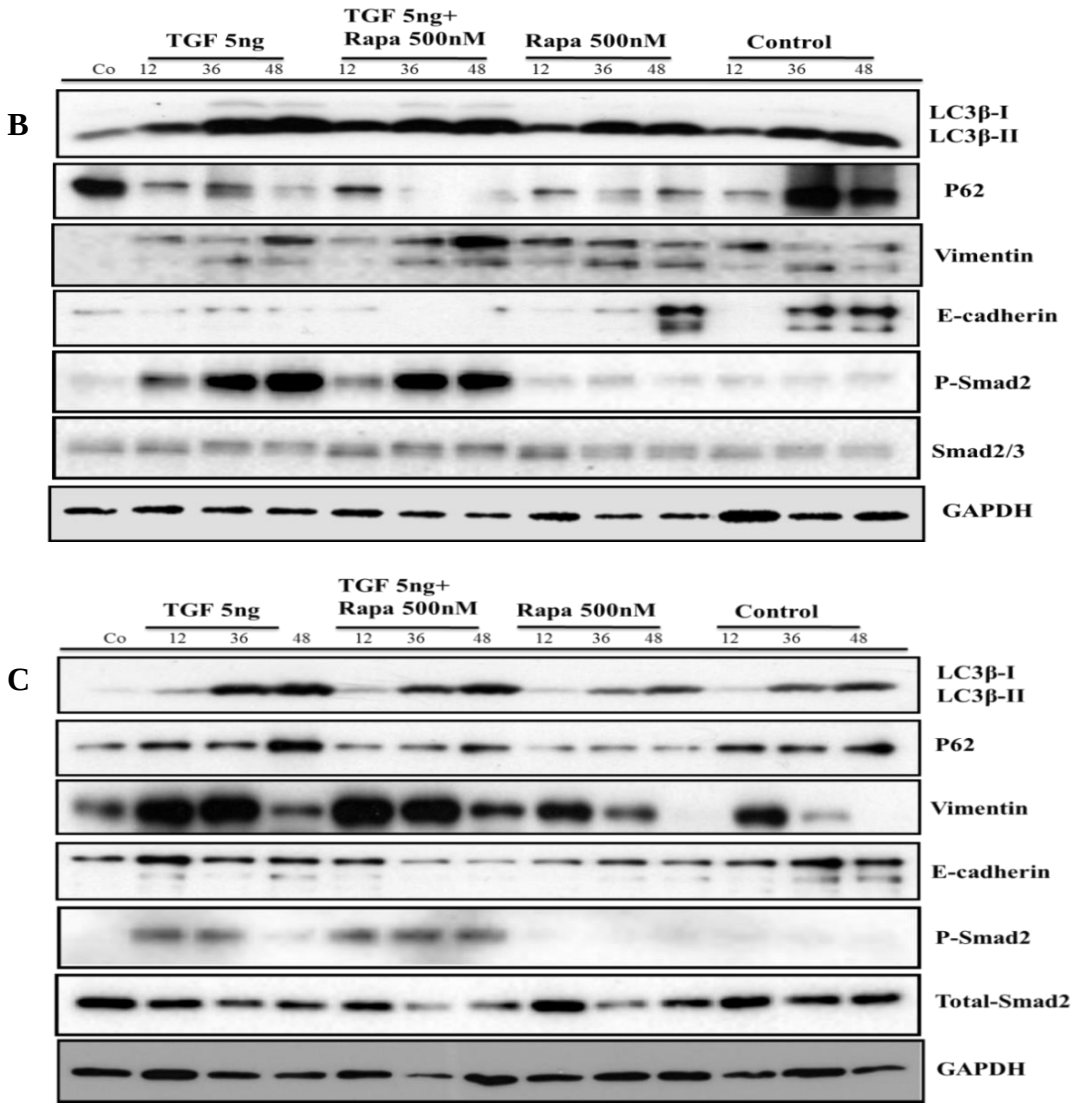


Figure 9. (B, C) Autophagy induction by co-treatment with Rapamycin (500nM) and TGFβ_1 (5ng) directs A549 (B) and H1975 (C) cells towards EMT as opposed to Bafilomycin A1 and TGFβ_1. A549 cells were treated with rapamycin, TGFβ_1 and/or both and the change in the abundance of SMAD signaling, EMT and autophagy markers were evaluated after 12, 36 and 48hrs time points. GAPDH was used as the loading control.

Immunofluorescence imaging results further confirmed the western blotting results. We showed that upon treatment of A549 and H1975 cells with Rapamycin+TGFβ_1 for 48hrs Rapamycin activates autophagy and we have a higher lysosomal activation and

LC3 punctuate compared to TGFβ$_1$ alone (Figure 9. D, E). Also, again we showed that TGFβ$_1$ induces autophagy activation in both cell lines.

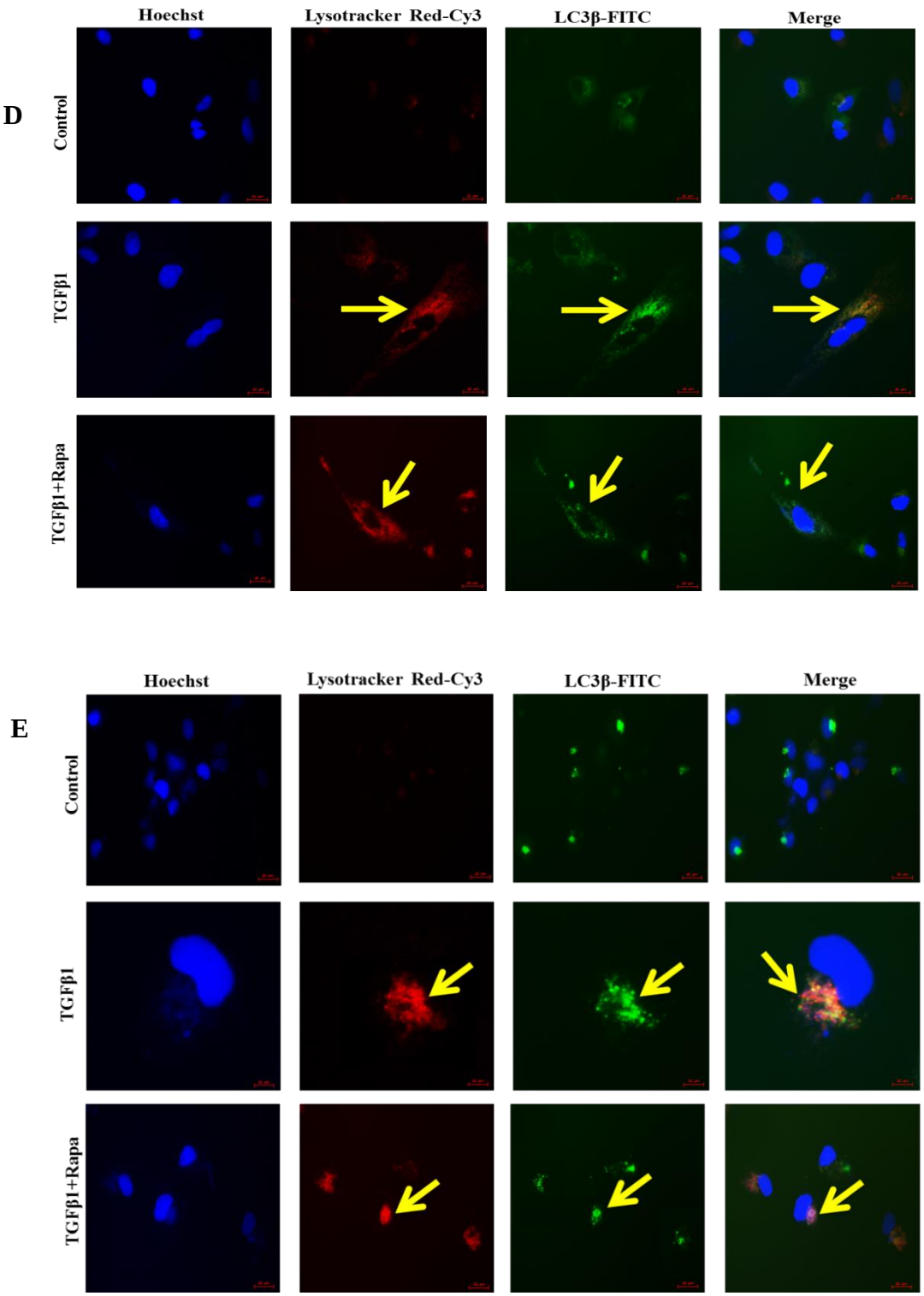


Figure 9. (D, E) immunofluorescence and ICC images showed that Rapamycin caused an increase in the lysosomal activation upon co-treatment with TGFβ_1 in both A549 (D) and H1975 (E) cells after 48 hrs. Representative immunofluorescent images of LC3B-II and activated lysosomes shown (scale bars 20μm).

Finally, we showed the changes in the morphology of A549 and H1975 cells upon treatment with Rapamycin (500nM)+TGFβ_1 (5ng). TGFβ_1 caused the cells to go through phenotype conversion from epithelial to mesenchymal. However, in cells with Rapamycin+ TGFβ_1 treatment, Rapamycin induced a higher level of autophagy activation, thereby EMT activation in these cells. Therefore, they acquired an even more mesenchymal phenotype compared to the control and cells treated only with TGFβ_1. This shows that Rapamycin could enhance the TGFβ_1 induced EMT in both A549 and H1975 cells through activation of autophagy in these cells (Figure 9. F, G).

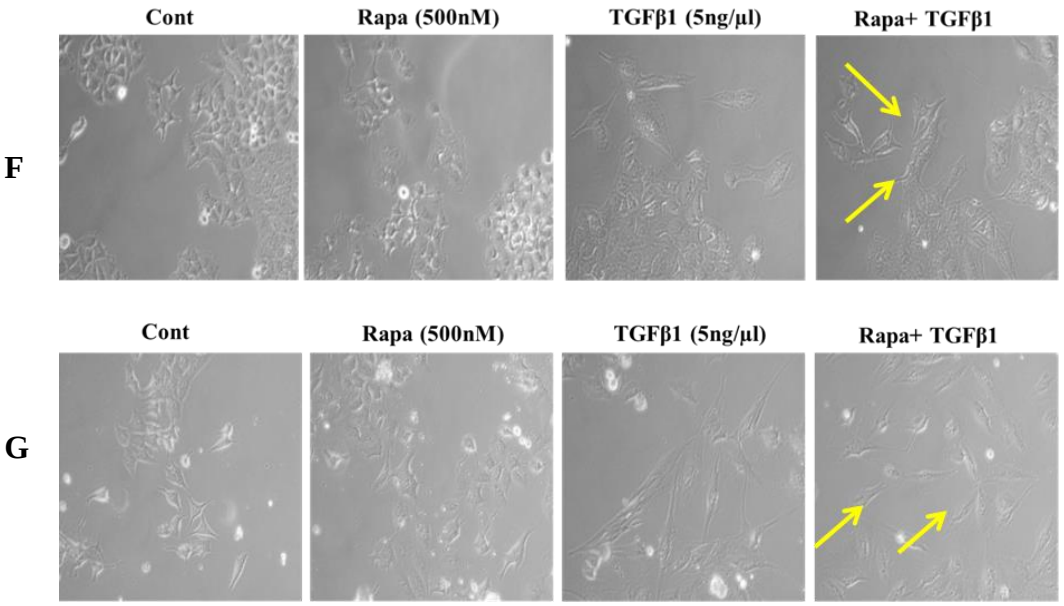
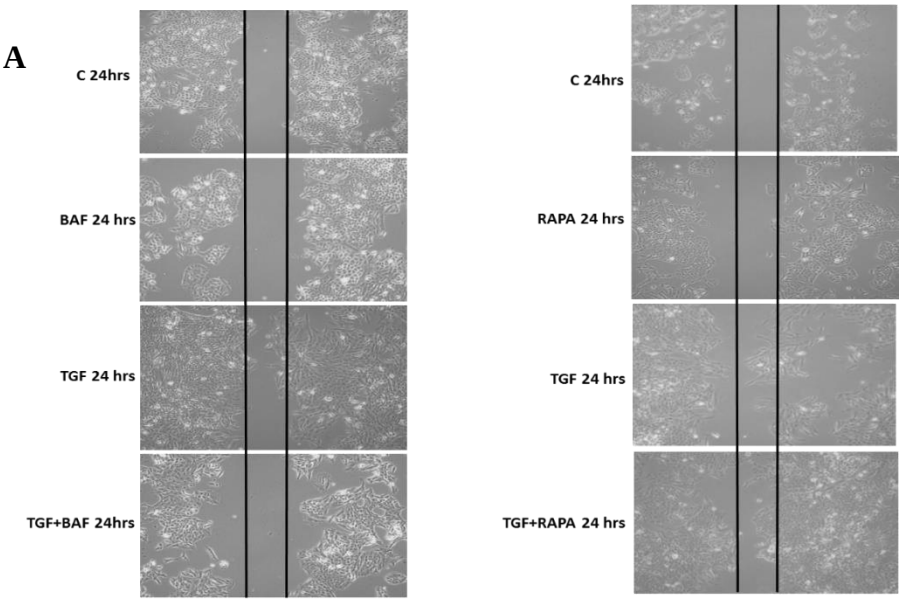


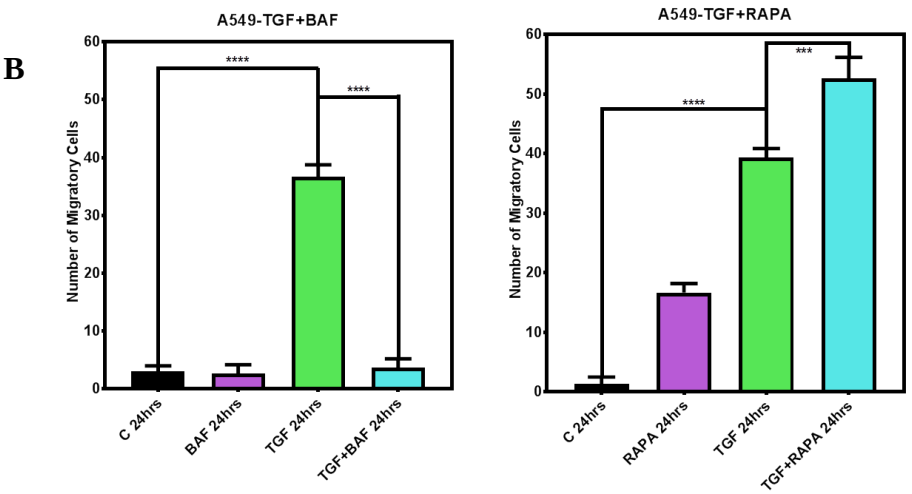
Figure 9. (F, G) Rapamycin induced A549 (F) and H1975 (G) cells towards acquiring a more mesenchymal phenotype as compared to TGFβ_1 alone and control cells after 24 hrs treatment with Rapamycin+TGFβ1. Images were taken using phase contrast microscope after 24 hrs of treatment with Rapamycin+TGFβ1. (magnification 20x).

4. Wound Healing Assay (Scratch Assay): Treatment with TGFβ_1 and/or TGFβ_1&Bafilomycin A1/Rapamycin changes the migratory behavior of A549 and H1975 cells.

In order to further confirm our results from western blotting and immunofluorescence we performed two functional assays, namely Wound Healing Assay (Scratch Assay) and contractile assay. As explained in the Method and Material section we used specific culture inserts for this experiment. After treatment of the A549 and H1975 cells with Baf-A1 (0.1nM) and/or Rapamycin (250nM) and TGFβ_1 (5ng/µl) for 48 hrs fresh medium with FBS 10% was added to cells and images were taken after 24 and 48 hrs. As it is shown in the Figure 10. A-D both cells migrated into the scratch area after treatment with TGFβ_1 while cells were inhibited from migrating in the Baf-A1+ TGFβ_1 treatments. This shows that the Baf-A1 inhibition of autophagy affects negatively the ability of cells to migrate. To put it another way, autophagy inhibition abrogates the cells from undergoing EMT which is required for the cells so they convert into a mesenchymal phenotype and also changes in their molecular signature and gain mesenchymal characteristics before they can start migrating. In contrast to these results, A549 and H1975 cells treated with rapamycin could migrate even more well into the scratch area compared to the cells treated only with TGFβ_1 or control

treatment. This highlights the same scenario as we explained above; the activation of autophagy through rapamycin increases the EMT induction in both cells which results in the acquirement of a mesenchymal phenotype with higher ability for migration. Therefore, cells treated with rapamycin+ TGFβ_1 could migrate more into the scratch area as compared with TGFβ_1 treatment and control treatment (Figure 10. A-D).





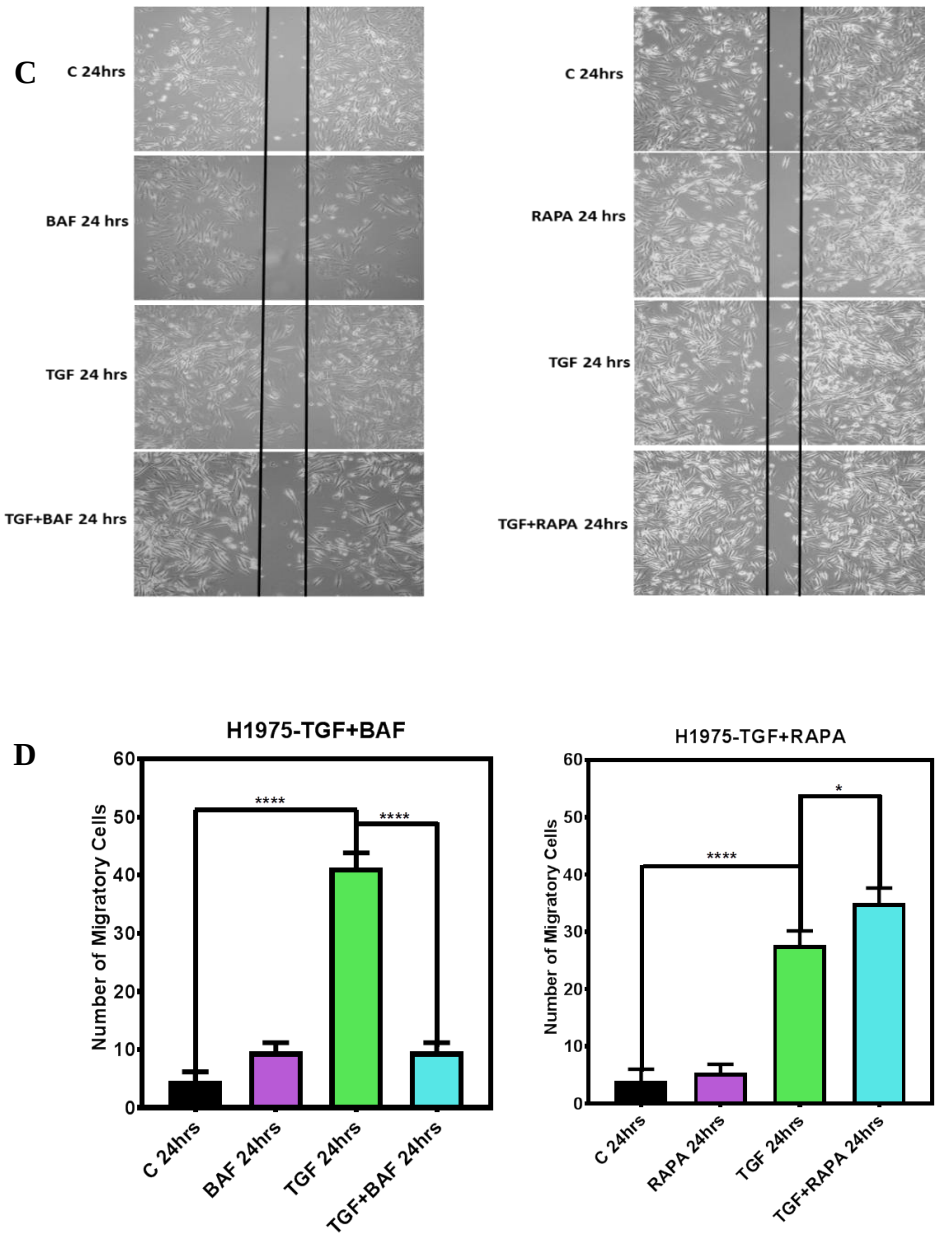
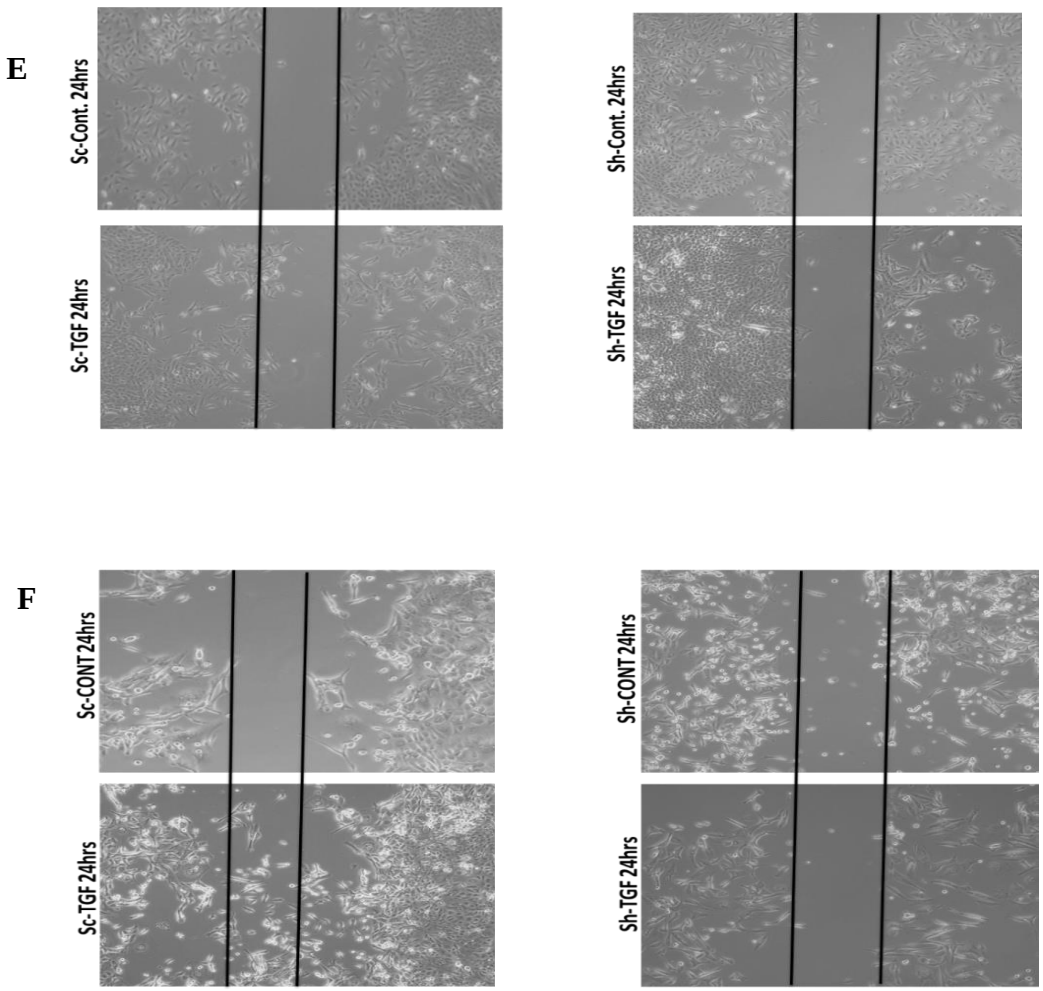


Figure 10. Treatment of cells with Bafilomycin A1 (1nM) decreases the migration of cells while Rapamycin (500nM) increases their migration. (A, C) Cells were grown in the culture inserts and were starved for 24 hrs. cells pretreated with Baf-A1 (1nM) for 2hrs and the treated with TGFβ_1 (5ng/µl) for 48 hrs. media was then refreshed with fresh medium (FBS 10%) and images were taken after time intervals of 24 and 48 hrs using phase contrast microscope. Images are representative of three different experiments for 24 hrs time point. (A: A549 cells, C: H1975 cells). (B, D) The number of migrated cells into the scratch area was counted and quantified for A549 (B) and H1975 (D) cells and compared to their correspondent controls and graphs were prepared by GraphPad Prism using one way ANOVA (non-parametric, Brown–Forsythe test). P-value $\leq$ 0.05 and lower were considered as statistically significant.

Also, we did the same experiment for the *ATG7* KD A549 and H1975 cells in order to find out if the results would be the same as Baf-A1 or not. Interestingly, after treatment of the cells with TGFβ_1 we observed the exact pattern of movement in the *ATG7* KD cells with that of cells treated with Baf-A1. *ATG7* KD cells did not migrate into the scratch area after TGFβ_1 treatment for 24 and 48 hrs. However, scramble cells could migrate much more after TGFβ_1 treatment (Figure 10. E-G). These results again confirm our aforementioned results in above sections in terms of the key role that autophagy inhibition can have on the prevention of A549 and H1975 cells to undergo EMT.



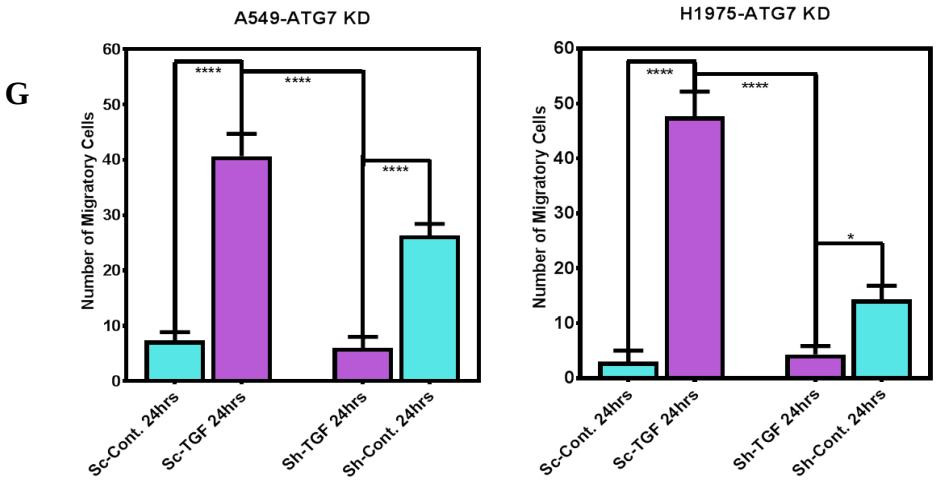


Figure 10. (E-G) Cells grown in the culture inserts and were starved for 24 hrs followed by TGFβ_1 (5ng/ml) treatment for 48 hrs. Culture media was then refreshed with fresh medium (FBS 10%) and images were taken after time intervals of 24 and 48 hrs using phase contrast microscope. Images are representative of three different experiments for 24 hrs time point. (E: *ATG7* KD A549 cells, F: *ATG7* KD H1975 cells). (G) The number of migratory cells into the scratch area was counted and quantified for both scramble and *ATG7* KD cells treated with TGFβ_1 (5ng/ml) and compared to their correspondent controls. Graphs were prepared by GraphPad Prism using one way ANOVA (non-parametric, Brown–Forsythe test). P-value $\leq$ 0.05 and lower were considered as statistically significant.

CHAPTER 4: DISCUSSION

We have shown in our study that TGFβ_1 could induce both autophagy and EMT in both NSCLC cell lines (A549 and H1975). We have also shown that chemical inhibition of autophagy using Bafilomycin A1 and gene silencing of autophagy (*ATG7* KD) can decrease the level of EMT induction in both A549 and H1975 cell lines. On the other hand, and in line with these results, induction of autophagy using autophagy inducer Rapamycin could increase EMT induction in both A549 and H1975 cell lines. Based on our immuno blot results, we have found that vimentin as a mesenchymal marker is downregulated significantly after autophagy is inhibited using Bafilomycin A1 or in *ATG7* KD cells. However, we could not see a statistically significant change in the expression of E-Cadherin as an epithelial marker. In contrast to autophagy inhibition results, autophagy induction by Rapamycin could increase the vimentin expression while having no effect on the E-Cadherin expression. Immunocytochemistry analysis showed that autophagy is activated in both A549 and H1975 cells upon treatment with TGFβ_1. In cells treated with Baf-A1+TGFβ_1 the level of vimentin expression is reduced while it was significantly increased in cells treated with Rapamycin+TGFβ_1 as compared to time match control or TGFβ_1 treated samples. Also, TEM analysis of cells showed activation of autophagy (formation of autophagolysosomes) upon TGFβ_1 treatment where there was an accumulation of activated autophagolysosomes. In contrast to this, we were able to observe an accumulation of autophagosomes in cells treated with Baf-A1 and Baf-A1+TGFβ_1 showing the inhibition of autophagy. Finally, wound healing scratch assay also confirmed our results that autophagy inhibition prevents the EMT where A549 and

H1975 cells treated with Baf-A1+TGFβ$_1$ impeded the ability of cells to migrate compared to TGFβ$_1$ treated cells. In contrast, cells migrated to a higher extent in treated cells with Rapamycin+TGFβ$_1$ as compared to the TGFβ$_1$ treated cells. Both sets of results are in line together and show that autophagy inhibition limits the EMT induction in A549 and H1975 cells resulting in less migration as opposed to when autophagy is induced by Rapamycin where it contributes to the induction of EMT and higher ability to migrate. **Our findings reported here are consistent with each other and highlight the possible modulatory role of autophagy in the regulation of EMT in NSCLC cells.**

It is hypothesized that autophagy preserve the integrity of the genome and prevents initiation of carcinogenesis and tumor formation. On the other hand, when tumor is formed and established, autophagy improves the health of cancer cells and promotes their survival by adapting them to the existing stress in the tumor microenvironment (200, 202, 203). Several studies have pointed out a connection between EMT/metastasis and autophagy. It has been shown that autophagy induction causes downregulation of mesenchymal markers (vimentin, snail and αSMA) and conversion of hepatocellular carcinoma cells towards MET (200). They observed that EMT inducer Snail is degraded through autophagy. Also, the degradation of two main EMT inducers Twist and Snail has been shown in breast cancer cells upon activation of autophagy (201). It has also been observed that autophagy induction reduces the expression of mesenchymal markers and therefore inhibition of EMT and less invasiveness in glioblastoma cancer cells (195, 202). On the other hand and in contrast to aforementioned studies, in some different contexts autophagy is required

for the progression and metastasis of cancer cells by contributing to the EMT. In this scenario, when cancer cells become very motile and on the verge of metastasis they induce autophagy to help them with the invasion and metastasis process. This is because metastasis and basically the pheno-conversion into mesenchymal phenotype is an energy demanding process. Autophagy provides cancer cells with this required energy and also help them survive in the stressful tumor microenvironment and during the metastatic spreading (198, 203). The punctua form of LC3B in imaging results shows the activation level of autophagy. It has been reported that elevated levels of LC3B-II punctate are associated with the low rate of survival and high rate of metastasis (and EMT) in breast cancer patients (213, 214). Also, the intensity of LC3B staining has been shown to be higher in metastatic tumors compared to the primary tumors (207, 208). These findings show the direct association between autophagy activation and the EMT induction. ULK2 which is an inducer of autophagy (Beclin1 phosphorylation) has been reported to downregulate E-cadherin and induce EMT in A549 lung cancer cells resulting in metastasis and a more invasive form (215). One very interesting study was to elucidate the role of autophagy in aggressiveness of hepatocellular carcinoma (HCC). They showed that autophagy is involved in the invasiveness and metastasis of HCC by contributing to TGFβ_1-induced EMT in hepatocellular carcinoma cells (199). Our data in this study correlates with findings of this study where we showed that autophagy helps A549 and H1975 cells to undergo EMT providing them with the required phenotypic and molecular characteristics for metastasis and invasion of lung cancer cells. In a very recent study, Lum and colleagues found that the pharmacological inhibition of autophagy through

Lys05 can improve the anti-tumor impact of sunitinib in ovarian carcinoma cells (217). Lys05 is a dimeric chloroquine which more efficiently blocks autophagy compared with HCQ by accumulating more in the lysosome. Moreover, autophagy is related to undifferentiated cellular types and essential for the metastasis of glioblastoma stem cells too (216). Importantly, cancer stem cells (CSCs) contribute to the metastasis and invasiveness of cancer (218) and as autophagy and also EMT are involved in the state of CSCs so the relationship between these two are worth much research for development of new treatments against cancer (205). Cancer cells require an uninterrupted nutritional supply for maintaining their proliferative needs and this high demand in concurrence with inadequate supply of blood and nutrition induces stress in these cells. These cells utilize autophagy to avoid cell death and overcome nutritional deficiency. Autophagy allows the cancer stem cells to generate ATP and other essential biochemical building blocks necessary under such adverse conditions. Based on these findings, scientists have suggested that autophagy is required for dormant cells to survive in the secondary cancer sites for long time which later result in the relapse of cancer (135). All of these findings highlight the crucial role of autophagy as a supportive and contributing factor to the cancer metastasis through induction of EMT in cancer cells. It is also worth mentioning that manipulation of autophagy, inhibition or induction, does not always follow the same pattern of effects on EMT. As mentioned in above examples, autophagy inhibition or induction can have a positive or negative impact on the EMT dependent on the cancer type, stage, tumor microenvironment and etc. The results from our study pinpoint a regulatory role for autophagy inhibition in the prevention of EMT.

Cellular stimuli like TGFβ_1 can activate EMT and can also induce autophagy (199, 210-212). Not only we have confirmed this previously shown role of TGFβ_1 as an inducer of both autophagy and EMT but we also found that TGFβ_1 acts in parallel with SMAD signaling pathway shown by activation of phospho SMAD2 and that autophagy does not affect the SMAD2 phosphorylation in A549 and H1975 cells. Therefore, based on our findings and those of previous studies on the role of TGFβ_1 in induction of autophagy and EMT we aimed to investigate if manipulation of autophagy can abrogate the EMT process in NSCLC A549 and H1975 cells.

Autophagy is highly activated in a variety of malignant tumor cells induced by different inducers such as starvation, growth factor withdrawal and hypoxia (2). However, there is a lack of knowledge as to the exact and direct role of autophagy in the invasion and metastasis of lung cancer. Autophagy has been involved with common characteristics of metastasis like survival of cancer stem cells, providing a favorable microenvironment for the metastasis of cancer cells and also evading from the immune system (206, 207). Moreover, autophagy might contribute to the survival of deprived dormant cancer cells by different ways such as production of ATP and amino acids (219) and induction of quiescence (220). In this study, inhibition of autophagy was achieved by *ATG7* KD and chemical inhibition using Baf-A1. Baf-A1 or *ATG7* KD was enough for autophagy inhibition in A549 and H1975 cell lines which resulted in statistically significant reduction in mesenchymal markers of TGFβ_1-induced EMT in these cells. Although in this study we provided findings to show that autophagy inhibition abrogates the EMT process in NSCLC cells the question of how autophagy contributes to this fundamental mechanism for metastatic

lung cancer cells remains to be answered. Autophagy has been defined as a defensive mechanism against stresses in the tumor microenvironment. It can increase the survival of the cancer cells and keep them in a healthy condition through eliminating the damaged organelles and aggregated or misfolded proteins harmful to the cancer cells (110). This way cancer cells can restrict the production of ROS which help maintain their genome stability and therefore their survival (197). However, as discussed, from a molecular perspective direct role of autophagy in EMT induction and metastasis of cancer cells is yet to be elucidated.

Our findings mentioned here show the crucial role that autophagy plays in the TGFβ_1-induced EMT. Targeting autophagy can be considered as the Achilles heel of cancer for developing new treatment options against it.

Conclusion and Future Prospects

Despite all the studies, findings are totally different from each other about the association of EMT and autophagy in cancer. This apparent paradox can perhaps be reconciled by the differential effects of autophagy in different stages of tumorigenesis. It may serve both pro- and anti-metastatic functions depending on the contextual demands placed on tumor cells throughout the metastatic process (and EMT). There is no consensus as to what factors determine a role for autophagy in EMT but as stated earlier tumor microenvironment, tumor stage and type of cancer play a role in it. Also, the extent that metastatic cancer cells rely on autophagy is yet to be proved. Therefore, more research is needed to answer these questions and to find out if EMT and autophagy are connected in a direct or indirect manner and more importantly if these

different roles for autophagy in various cancers are as a result of cancer type, microenvironment, genetic factors or possibly other unknown factors. As our understanding of the biological roles of autophagy is increasing, at the same time its involvement in cancer development and metastasis is increasing too. One of the main concerns for the future of autophagy targeting as a strategy for cancer treatment is to develop more specific modulators especially for use in clinical settings. Although our current knowledge to surely target autophagy in cancer patients is still limited and the translation of bench results to the patients bedside is a long process we can count on autophagy as a promising candidate for the development of novel treatment strategies against cancer. There are couple of clinical trials in phase I and II currently under investigation using the lysosome inhibitors chloroquine or hydroxychloroquine in combination with chemotherapy for the treatment of a range of haematological and solid tumors (249, 250). A phase III trial in glioblastoma patients treated with radiation and carmustine with or without daily CQ found a median overall survival of 24 and 11 months in CQ- and placebo-treated patients, respectively (251). A phase I/II trial of HCQ with temozolomide and radiation for glioblastoma patients was launched through the American Brain Tumor Consortium, and included pharmacodynamic (PD) and pharmacokinetic (PK) analyses. PD evidence of HCQ dose –dependent autophagy inhibition was observed using a novel electron microscopy assay on serial blood mononuclear cells (252). Currently there are more than 20 trials involving HCQ accruing cancer patients (prostate, lung, pancreas, breast, etc.) nationwide and many of them have evidence of preliminary antitumor activity (249). . One challenging issue, as mentioned earlier, is that autophagy may either act against or in favor of the cancer

progression and that the stage specific role of autophagy in development of cancer is not well known. Therefore, extensive analysis of the particular role of autophagy must be taken into account in each specific type of cancer. For this reason, researchers would greatly benefit to work on the mouse models in which autophagy can specifically be modulated. This allows for the appropriate modulation of autophagy with a favorable outcome in a specific type of cancer. There have been developed different types of mouse animals for autophagy studies like Atg5 Tg mice which express Atg5 ubiquitously, Atg7 f/f which are knockout for the Atg7 where they develop different tumors, transgenic reporter mice GFP-LC3 expressing the fluorescent autophagosome marker, GFP-LC3 (253).

It is worth noting there are studies that contradict with our findings here. One of which reported that induction of autophagy by nutrient deprivation or mTOR pathway inhibition (rapamycin) is able to reduce the invasiveness and migration of GBM cancer cells (downregulation of SNAIL and SLUG, two of the major transcription factors of the EMT process). While autophagy inhibition through silencing ATG5, ATG7 or BCN1 resulted in increased cell motility and invasiveness (202). Another study has shown that autophagy is activated upon interaction of DEDD with PI3KC3 which reduced the EMT in human breast cancer cells (201). Another group showed that lysosome inhibition using a V-ATPase inhibitor or its siRNA could significantly reduces the epithelial markers (P-cadherin and ZO-1) and increases the mesenchymal markers (FSP-1 and α-SMA) in podocytes and therefore contributing to the EMT in these cells. They found that the enhanced EMT associated with lysosome dysfunction may be related to accumulation of p62 and associated reduction of p62

phosphorylation (254). Gugnumi and colleagues (255) did a study based on a thyroid cancer patients library and found that Cadherin-6 (CDH6) directly interacts with GABARAP, BNIP3 and BNIP3L, and that through these interactions CDH6 impedes autophagy and promotes re-organization of mitochondrial network. Analysis suggests that the interaction with the autophagic machinery may be a common feature of many cadherin family members. The analysis of CDH6 expression in a cohort of human Papillary thyroid carcinomas (PTCs) also showed that CDH6 expression marks specifically EMT cells. and it is strongly associated with metastatic behavior and worse outcome of PTCs.

In this study, we showed the important regulatory role of autophagy modulation in EMT prevention in NSCLC cells. More importantly based on our results, we speculate that inhibition of autophagy in NSCLC A549 and H1975 cells is a promising candidate in order to develop novel therapeutic strategies to limit EMT and invasiveness of NSCLC cancer cells. However, the mechanism by which autophagy contributes to EMT are clear gaps in our study that requires further research. Development of novel biomarkers to detect and analyze autophagy in tissues of human tumors can also be very helpful to improve new autophagy-based treatments against cancer. Also, investigations on the MAPK and SMAD signaling might explain the mechanistic pathways by which autophagy contributes EMT and might answer at least partly to these questions. More studies using in vivo models appears to be crucial in order to shed some light on the exact functions of autophagy in metastasis process and hopefully to come up with a scientific rationale as to when and how we should manipulate autophagy to prevent the EMT and metastasis in cancer cells.

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