Analyzing the Role of TNF-α and Autophagy in Regulation of TGF-β Induced Epithelial to Mesenchymal Transition in Cancer Cells

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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2018

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CERTIFICATE

This is to certify that the thesis entitled **"Analyzing the Role of TNF-α and Autophagy in Regulation of TGF-β Induced Epithelial to Mesenchymal Transition in Cancer Cells"** and submitted by **Ms. Subhra Dash** ID No**. 2013PHXF0410P** for award of Ph.D. of the institute embodies original work done by her under my supervision.

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Acknowledgement

First and foremost, I would like to express my deep and sincere gratitude to my Ph.D. research supervisor Prof. Rajdeep Chowdhury and co-supervisor Dr. Sudeshna Mukherjee, Department of Biological Sciences for providing me an excellent platform to pursue my passion. I am obliged to have got an opportunity to work under their skilled guidance, I am also grateful for the untiring attention they gave me from the initiation to the completion of my research work. Their supervision have improved my research and teaching aptitude and their never give up approach has always kept me motivated. I would also like to thank them, for encouraging me to attend international conferences and workshop for transcriptomic analysis which gave me a golden opportunity to interact with eminent scientists and researchers working in our field of interest, which will really be very helpful for my future.

This work would not have been possible without the infrastructural and administrative support from the institute for which I thank Prof. Souvik Bhattacharyya, Vice-Chancellor, BITS Pilani, Prof. A. K. Sarkar, Director, BITS Pilani, Pilani campus. I also thank Prof. Jitendra Panwar, Associate Dean, AGSRD and Prof. Shibashis Chowdhury BITS Pilani, Convener, Departmental Research Committee, Prof. Prabhat Nath Jha, HOD, Department of Biological sciences for their timely guidance and support regarding the academic formalities throughout the thesis work. I acknowledge DST and BITS, Pilani for helping me with the financial assistance. This research work would not have been the same without constructive evaluation of my DAC members, Prof. Uma S. Dubey, Department of Biological Sciences and Prof. Anil Bhanudas Gaikwad, Department of Pharmacy.

I would like to express my sincere gratitude to Prof. A.K Das and Dr. Symantak Majumder, Asst. Professor, Department of Biological Sciences for their constructive criticism and enthusiastic discussions which has always been a knowledge enhancing experience for me.

As a beginner, I received huge help and guidance from my lab mates Miss. Jyothi Nagraj, Mr. Abdul and seniors Dr. Zarna Pala, Dr. Gagandeep singh Saggu, Dr. Santosh Kumar Goru which is unforgettable. I would like to thank my friend Dr. Anuradha for

being a pillar of support during each and every hurdle I faced in completing this journey. My earnest thanks to my loving, enthusiastic, inspiring, mischievous and equally hardworking labmates and friends Ms. Leena and Mrs. Heena for being on their toes to help me in any situation, the most compatible labmates I can ever wish for. I would also like to thank Ms. Abhilasha, Ms. Ankita Dahiya, Ms. Ankita Sharma Mr. Sukalpa, Mr. Udit, Ms. Sukanya, Ms. Ifrah, Ms. Vishakha, Mr. Sumukh, Ms. Swetha, Ms. Swati not only for helping me with the smooth conductance of my experiments but also for keeping me motivated during all my failures. I also thank Ms. Neelam, Ms. Aastha, Mr. Manohar for their continuous support in the lab and coursework. I am blessed to have juniors yet counsellors like Mr. Lohitesh and Ms. Shivangi whose selfless help and motivation made this journey easier. I thank Mrs. Vandhana not only for being with me during standardization of initial experiments but also for her constant help during my coursework and qualifying examination.

I wish to thank the most cheerful juniors Ms. Nisha and Mr. Vajir for their untiring help and support both personally and professionally. Their presence have always provided a homely environment. I would also like to thank Ms. Shraddha, Mr. Sandeep, Ms. Tripti, Mr. Himanshu, Mr. Vikram, Mr. Vikas, Ms. Vishalakshi for making me laugh even in a difficult situation. Life at BITS would have been monotonous without you all. I wish to thank my friend, Mrs. Priyanka who has been a support since my very first day at Pilani. I would like to express my love to Sohom and Om for giving me a reason to smile with their acquitted queries and gestures wherever we meet. I would also, like to thank Mr. Debabrat for introducing me with BITS, Pilani.

This journey would not have been possible without the continuous encouragement from Prof. Suresh Arya (Sasha). I would like to extend my heartfelt thanks to all the faculty members and research scholars, department of Biological Sciences for extending their help and support during my research and teaching practices. I owe a special thanks to the non-teaching staff, especially Mukesh Ji for providing me with the pre-requisites for my experiments.

I wish to acknowledge Prof. Joyoti Basu, Bose Institute Kolkata and her extremely talented lab members Dr. Ranjeet, Dr. Manish, Dr. Sanjay and Mrs. Arijita for

introducing me with all the cell culture and molecular biology techniques and budding my interest in research during my post-graduate dissertation work.

I would like to acknowledge Genotypic, Bangalore for helping me with the RNA sequencing and data analysis. I am extremely thankful to Prof. Santosh Chauhan, ILS Bhubaneswar, for providing me with the siRNA for ATG5.

I am indebted to the countless blessings and sacrifices of my parents Mrs. Manju Dash, Mr. Surendra Nath Dash and my family members for having faith in my dreams which made me what I am today. I would like to thank my brother Mr. Santosh Dash for his care and guidance throughout my career, the innocent but scientific queries from my niece Sanvi has always been a stressbuster during this journey. I would like to thank my younger yet matured brother Lincon and sister Twinkle for their perpetual love and support in completing this journey.

SUBHRA

Abstract

Cancer typically develops in a chronic inflammatory setting causal to the release of a plethora of cytokines. In this regard, the progression of liver cancer, hepatocellular carcinoma (HCC) is almost always associated with persistent inflammation. Components of the inflammatory tumor microenvironment (TME) thus plays a critical role in HCC pathogenesis. As a consequence of the intricate crosstalk, HCC is often associated with complex carcinogenesis rendering hindrances to chemotherapy. Considering the importance of this crosstalk between secretary products from the tumor milieu with HCC cells, in this study, we selected two cytokines prevalent in HCC TME; TGF-β and TNF-α and investigated their effect on HCC progression, especially their implications on epithelial to mesenchymal transition (EMT) and on cellular homeostatic process- autophagy. EMT is a pre-requisite for cancer cells to disseminate; and autophagy, based on current evidences is considered a dual edged sword which can have both cancer promoting or inhibitory effects. We observed SMAD2 signalling dependent significant elevation of EMT markers upon TGF-β (transforming growth factor- β) exposure to HCC cells. Interestingly, simultaneous exposure to another cytokine, TNF-α significantly reduced TGF-β induced EMT by activating the expression of inhibitory SMAD7 and elevating intracellular ROS (reactive oxygen species) levels leading to cell death. TNF-α mediated antagonism of TGF-β induced effects was further validated in another cell type- human osteosarcoma cells as well. Importantly, TGF-β exposed cells undergoing EMT, showed induction of autophagy, which when inhibited pharmacologically using CQDP (chloroquine di-phosphate) or genetically (siATG5), drastically enhanced ROS and suppressed EMT. In contrary, quenching of ROS by NAC (N-acetyl cysteine), reduced autophagy and resulted in a significant elevation of EMT. TNF-α (tumor necrosis factor-α) was found to inhibit EMT by elevating ROS levels and reducing autophagy. We hence prove that regulation of ROS by autophagy is critical and acts as a pro-survival strategy that facilitates EMT in HCC cells. To gain further insights into global transcriptomic alterations associated with exposure to TGF-β, TNF-α or both, we performed RNA sequencing which further validated an induction of a subset of EMT and autophagy genes upon TGF-β exposure and their down-regulation at the transcriptomic level by TNF-α. Interestingly,

transcriptomic analysis showed an activation of non-canonical arm of TGF-β signalling in EMT undergoing cells. A significant increase in TAK1 (TGF-β activated kinase1) & p65 component of NF-κB signalling was observed. A pharmacological inhibition of p65, reduced ROS, autophagy, and showed a drastic increase in TGF-β induced EMT. An inhibition of p65 using JSH-23 resulted in an increased activation of SMAD2 signalling and vice versa, demarcating the existence of a reciprocal feedback loop between the two arms of TGF-β signalling. Here as well, TNF-α antagonized the non-canonical signalling arm. Through this study, we provide interesting mechanistic and molecular insights into the key connecting role of autophagy and ROS in EMT; we also provide cues to probable futuristic application of TNF-α agonists as EMT antagonists against HCC progression.

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List of Abbreviations

ATG- Autophagy related

BMPs- bone morphogenetic proteins

BME-UV1- Bovine mammary epithelial cell line

CQDP- Chloroquine di phosphate

CAFs- Cancer-associated fibroblasts

CDK- Cyclin dependent kinases

CMA- Chaperone mediated autophagy

DC- Dendritic cells

DCFDA- Dichlorofluorescin diacetate

ECM- Extracellular matrix

EGF- Epidermal growth factor

EMT- Epithelial to mesenchymal transition

ERK1 and ERK2- Extracellular signal regulated kinases 1 and 2

ELISA- Enzyme-linked immune-sorbent assay

FGF- Fibroblast growth factor

GDFs- Growth and differentiation factors

HOS- Human osteosarcoma

HNSCC- Head and neck squamous cell carcinoma

HIF-1α- Hypoxia inducible factor-1α

HCC- Hepatocellular carcinoma

HBV/HCV- Hepatitis B virus/Hepatitis C virus

IGF- Insulin like growth factor

IL- Interleukin

JNK- Jun N-terminal Kinase

LT- Liver transplantation

LAMP-2A- Lysosomal associated membrane protein 2A

MDC- Monodansylcadaverine

MMPs- Metalloproteinases

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide

NK cell- Natural killer cell

NASH- Non-alcoholic steatohepatitis

NAFLD - Non-alcoholic fatty liver disease

NAC- N-Acetyl-L-cysteine

NF-κB- Nuclear factor- κB

OLT- Orthotopic liver transplantation

OS- Osteosarcoma

PanIN- Pancreatic intra-epithelial neoplasia

PI- Propidium iodide

RFA- Radiofrequency ablation radio-embolization

ROS- Reactive oxygen species

SIRT- Selective internal radiation therapy

TGF-β2- Transforming Growth Factor-β2

TME- Tumor microenvironment

TIL- Tumor infiltrating lymphocytes

TH1/2- T helper 1/2

Tregs- T regulatory cells

TAFs- Tumor associated fibroblasts

TNF-α- Tumor necrosis factor α

TGF-β- Tumor growth factor β

TAM- Tumor associated macrophages

TCA- Tricarboxylic acid

TACE- Transarterial chemoembolization

CHAPTER- 1

General introduction

1. Cancer: a global epidemic

Our body comprises of millions of cells, each an autonomous living component. Usually normal cells in human body proliferate and divide for a stipulated time period post which they stop to grow and divide. Cancer arises when this regular phenomena goes out of control. To be more specific, it is characterized by the uncoordinated, uncontrolled and undesirable growth and proliferation of cells. According to the world health organisation, cancer is the second primary cause of death worldwide and is accountable for approximately 9.6 million deaths in the year 2018. Intake of tobacco being the most vital risk factor for causing cancer and is reported to be responsible for 22% (approximately) of cancer related deaths [1, 2].

Unlike normal cells the hallmark of cancer cells include abnormalities in biological processes like sustained proliferation, escaping growth suppressors, resistance to apoptosis, facilitating replicative immortality, induction of angiogenesis and metastasis. Above all genome instability is considered as a critical factor to accelerate cancer pathogenesis. Conceptual growth in preceding era has suggested two more emerging hallmarks of cancer cells which includes immune evasion and reconditioning energy metabolism. Adding on to the existing complexities the TME and inflammation have added another dimension to the cancer development making the treatments even more difficult [3, 4].

1.1 Inflammation a key regulator of cancer pathogenesis

Inflammation is recognized as the key step for cancer progression since the beginning of recorded medical knowledge. The first reported hypothesis, establishing an association between cancer and inflammation has been accredited to the German pathologist Rudolf Virchow way back in the mid- nineteenth century [5, 6]. Yet, a clear evidence of inflammation playing a critical role in tumorigenesis and its underlying molecular mechanism was not widely understood until the last decade.

However, accrued epidemiologic reports suggest that chronic inflammatory diseases are often associated with high risk of cancer development [7]. Most of the initial studies targeted the ROS and inducible nitric oxide synthase (iNOS) generated by leukocytes

dependent inflammation leading to cancer [8]. Now, it has been comprehended that inflammation mediated cancers can also be driven by other associated factors like inflammatory cells, cytokines, chemokines and matrix metalloproteases, which altogether develops the inflammatory microenvironment [8]. Chronic inflammation is evidently involved in remodelling the TME.

2. Tumor microenvironment in cancer

Tumorigenesis is an intricate and dynamic procedure, comprising of three stages: initiation, progression, and metastasis. The tumor microenvironment is composed of a heterogeneous population of cells composed of tumor cells, extracellular matrix (ECM) and nearby endogenous stromal cells recruited by the tumor which includes fibroblasts, myofibroblasts, pericytes, adipocytes, immune and inflammatory cells [9]. Interactions between these malignant and non-transformed cells in the milieu form the TME. These non-transformed cells present in the TME have tumor-promoting function at all the above mentioned stages of carcinogenesis [10]. This complex intercellular communication is facilitated by a network of growth factors, cytokines, chemokines and matrix remodelling enzymes which orchestrates intracellular and molecular processes like wound healing and inflammation [11-13]. Looking into the current information on tumor microenvironment it is clear that it has a strong influence on tumor initiation and progression (Fig 1.1). Hence, re-shaping of its atmosphere can offer unexpected therapeutic subsidies.

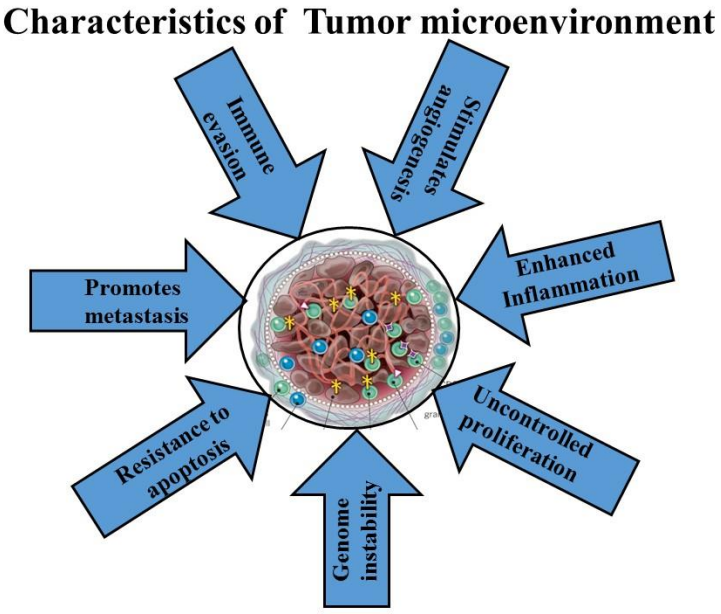


Figure 1.1. Schematic representation of salient features of TME *The tumor microenvironment in any type of cancer is wildly characterised by key features like promotion of metastasis, resistance to apoptosis, evading immune system, stimulating angiogenesis, enhancing inflammation, uncontrolled proliferation, genomic instability leading to poor prognosis hence treatment of cancer.*

2.1. Tumor microenvironment: a paradigm in hepatocellular carcinoma progression

Anatomically, the liver consists of five distinct parts which includes hepatocytes and hepatic lobule, vascular system, hepatic sinusoidal cells, biliary system and stroma [14]. Hepatocytes occupy approximately 60% of the total cells present in the liver, these are mostly parenchymal type cells which metabolize all the constituents that are absorbed by the portal vein from the gut [15, 16]. The non-parenchymal parts of the liver include stellate cells, Kupffer cells, endothelial cells and lymphocytes which are attributed to regulate the immune modulatory role of liver [16]. Upon hepatic injuries the liver undergo histological changes leading to fibrosis, which is characterized by unusual liver nodule development fenced by collagen fibrils secreted from stimulated hepatic stellate cells, during the process of fibrosis the parenchymal liver cells are irreversibly substituted by collagen enriched scar tissues [17]. In most of the acute injuries, liver can combat the damage by repairing the damaged tissue whereas, in chronic liver

injuries it fails to heal which can lead to HCC development [18]. Therefore, it's mandatory to understand the underlying mechanisms of hepatocarcinogenesis to find an effective HCC therapy tumor microenvironment therefore, have opened a new horizon in controlling HCC Pathogenesis.

2.2. Immune and inflammatory cells governing TME

The primary concept tells that immune surveillance has important roles in recognition and eradication of nascent cancer cells [12]. Conversely, unlike normal functions, inflammation associated immune cells would lead to various tissue pathologies like fibrosis, angiogenesis and neoplasia hence, these can be recognised as the initial crib of cancer progression [11, 19, 20]. To escape the immune scrutiny, cancer cells have a tendency to undergo numerous phenotypic alterations during the process of initiation and development, such as epithelial to mesenchymal transition [21]. These survivor cells, in due course would form primary solid tumor. Immune cells located inside a tumor milieu mostly include T lymphocytes, dendritic cells (DC), B lymphocytes, macrophages, polymorphonuclear leukocytes and natural killer (NK) cells [22].

2.2.1. NK cells are reported to induce antitumor cytotoxicity in vitro, due to its ability to downregulate the expression of HLA antigens and presence of MHC class I chain-related protein A and B (MICA and MICB) molecules though perforin or granzyme containing granules are conspicuously absent in tumor infiltrates and pre-cancerous abrasions [23, 24]. This paucity in tumor infiltrates may be an illustration of the evasion strategy preventing recruitment of NK-cell to the tumor site.

2.2.2. Tumor infiltrating lymphocytes (TIL) containing CD3+CD4+ and CD3+CD8+ T cells are major components of TME [7]. There are different types of T cells present within a TME which permeate the tumor areas, amongst these, CD8+ memory T cells are capable of destroying tumor cells [25]. CD4+ T helper 1 (TH1) cells are generally recognised by the production of interleukin-2 (IL-2). Other CD4+ cells include TH2 cells which produce IL-4, IL-5 and IL-13, this supports TH17 cells producing IL-17A, IL-17F, IL-21 and IL-22 that promote tumor growth [25].

2.2.3. T regulatory cells (Tregs) The tumor promoting CD4+ T cells are described as Tregs, which are generally characterized by the expression of FOXP3 and CD25 [26]. These cells exert immune suppressive function by producing TGF-β, IL-10 and hiding recognition and clearance of cancer cells by the immune system [27]. Hence, higher the number of Tregs in the TME, worse will be the prognosis [28, 29].

2.2.4. Tumor associated macrophages (TAMs) are a population of M2 polarized macrophage that resides inside the tumor milieu and promote cancer progression by regulating immune-suppression and pro-angiogenesis hence, migration and invasion [30]. Targeting of TAMs have shown enhanced antitumor abilities of chemotherapeutic drugs [31]. TAMs are re-programmed to obstruct lymphocyte function by releasing inhibitory cytokines like IL-10, ROS or prostaglandins [32-34]. Accumulating evidences suggest myeloid derived suppressor cells (CD34+CD33+CD13+CD15) differentiate into TAMs and dendritic cells and contribute to tumor progression by enhancing immune evasion mechanism, matrix remodelling followed by EMT [35].

2.2.5. Tumor-associated fibroblasts (TAFs) / cancer-associated fibroblasts (CAFs) in healthy cells, regulate extracellular matrix remodelling and wound healing. However, recent datas have indicated fibroblasts as the key players in regulation of tumorigenesis, constituting majority of stromal cells especially in pancreatic, breast and prostate cancers [36]. TAFs/CAFs are stimulated fibroblasts having many resemblances with typical fibroblasts involved inflammation and wound healing [37]. *Erez et. al.* have reported, TAFs/CAFs facilitates sustained inflammation via increased production of inflammatory cytokines, angiogenesis and macrophage recruitment to the tumor site hence, augmenting tumor growth [38]. TAFs/CAFs can also enhance the process of neoangiogenesis by secreting factors that stimulate pericytes and endothelial cells [10]. However, more investigation is required to understand the intricate mode of action of TAFs/CAFs due to many valid reasons like the origin of TAFs/CAFs, cellular heterogeneity inside the TME, sameness of TAFs/CAFs to normal fibroblasts in expression of similar bio-markers.

2.3. The paradox of cytokines in regulating HCC TME

Acute inflammation; a self-defence response used by cells against any alteration persuaded by xenobiotic agents, which actively participates in eliminating the infection and maintaining homeostasis of the affected tissues. However, prolonged inflammation elicits aberrant cellular events which might stimulate malignant transformation and carcinogenesis. A number of inflammatory mediators are known to induce metastasis via EMT, cytokines being the master regulators. Cytokines are lower molecular weight proteins which facilitate cellular communication. Stromal cells, like fibroblast cells and endothelial cells are known to synthesize these immune modulatory molecules which regulate crucial biological events like proliferation, differentiation, migration, immune cell activation and apoptosis [10].This thesis work is mainly focused on TGF-β and TNF-α; two pre-dominating cytokines present in TME. Apart from these, other cytokines involved in aggravating tumor growth and proliferation are listed below in the form of a table (table 1).

Table 1.1 List of pro-inflammatory cytokines predominating inside the TME and their functions

Pro-inflammatory cytokines	Role in TME
IFNγ	Induces proliferation of gastric carcinoma cells by upregulating Integrin-β3 mediated NF-κB Signalling.[39]
IL-6	Induces carcinogenesis by hypermethylating tumor suppressor genes and hypomethylating retro-transposon long interspersed nuclear element-1 (LINE-1), studied in oral squamous cell in vitro. [40]
IL-8	Colon cancer cells overexpressing IL-8 have shown high proliferation, migration and angiogenesis. Also, TNF- α mediated induction of IL-8 accelerates EMT. [41]

IL-10	IL-10 show pro-tumorigenic effect, by upregulating Bcl-2 and inducing apoptosis resistance activation. [42]
IL-1β	Polymorphisms in IL-1β and TNFα can have high risk of gastric carcinoma.[43]
IL-1α	Increases the expression of hypoxia inducing factor-1α, which in turn increases VEGF there by tumor progression and metastasis. [44]

2.3.1. TGF-β signalling and cancer

The TGF-β superfamily of cytokines consist of activins, nodal, inhibins, bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs). TGF-β is a small homodimeric signalling protein having 5 isoforms out of which three; TGF-β1, -β2, and -β3 are present in mammals [45]. These intracellular effector proteins have crucial roles in regulating diverse cellular machineries like immune suppression, senescence, EMT and extracellular matrix remodelling. Due to its involvement in a wide array of molecular events in cancer, the TGF-β signalling mechanism is being extensively explored now-a-days [46]. TGF-β primarily acts in a SMAD-dependent manner which initiates at the cell surface by dimerization of type I and type II receptor. Receptor I gets phosphorylated by the kinase domain of receptor II which recruits and phosphorylates SMADs. Once phosphorylated, SMAD2/3 uncouples from the receptor and associates with SMAD4 within the cytoplasm. This activated heterotrimeric complex then drifts to the nucleus and acts as a transcriptional regulator. Inhibitory SMAD 6/7 can oppose this pathway by stopping the translocation of this moiety into the nucleus or by degrading the receptors, resulting in the repression of a specific TGF-β-SMAD target gene (Fig 1.2) [47-50]. Alternatively, TGF-β mediated non-SMAD pathways also co-exist with the established SMAD pathway. Especially, MAPK pathways- p38 and JNK (Jun N-terminal Kinase) have been reported to be a down-stream targets of TGF-β inducing either programmed cell death or EMT [51-53]. Additionally, TGF-β can also signal through the activation of extracellular signal regulated kinases 1 and 2 (ERK1 and ERK2) leading to EMT induction. PI3K/Akt and mTOR pathway is also reported to be an indirect target of TGF-β in regulating EMT

and cytostasis [54]. TGF-β signalling is generally up-regulated in TME, providing a favourable niche for cell proliferation, differentiation, invasion, angiogenesis and interestingly controls apoptosis as well due to which its role as a tumor promoter or suppressor is still debatable [55]. These pleotropic role of TGF-β, is well explored in a panel of cancer types, including skin, colorectal, lung, prostate, breast, pancreatic, gliomas and liver [56]. In the initial stages of tumorigenesis, TGF-β is known to suppress tumor progression, by halting cell cycle as studied in epithelial, hematopoietic and endothelial cells. Which is modulated by downregulating cyclin dependent kinases (CDK) and c-myc while overexpressing CDK inhibitors, p15 and p21 [57]. Somatic mutations in TGF-β signalling components have anti-proliferative effects and it is also known to regulate cell cycle arrest by inhibiting estrogen receptor α-mediated proliferation [58, 59]. In breast cancer cells, overexpression of TGF-β receptor II re-established TGF-β sensitivity and regressed malignancy [60]. At later stages of carcinogenesis, TGF-β can stimulate the expression of c-myc by facilitating the nuclear translocation of nuclear factor of activated T cells and phosphorylation of SMAD3/4 which in turn will up-regulate MDM2 and destabilizes p53 [61, 62]. So it is clear from the above discussion that TGF-β can aviate its receptors to recruit and activate multiple intracellular pathway which controls many cellular processes out of which we have tried to address metastasis, autophagy, senescence, apoptosis and fibrogenesis. Therefore, selective targeting of the signalling pathway can render improved results compared to the currently used drug targets.

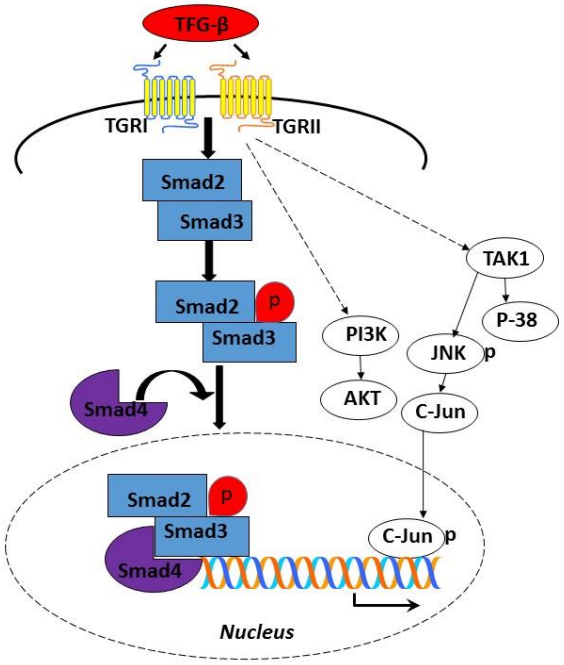


Figure 1.2. TGF-β mediated SMAD signalling pathway*. After TGF-β receptors dimerization upon ligand binding, SMAD2/3 gets phosphorylated by TβRI. This heterodimer forms a complex with SMAD4 which gets transported into the nucleus subsequently. Inside the nucleus, it binds with TGF-β dependent transcription factors and induces the transcription of TGF-β specific genes.*

2.3.2. TNF-α signalling in regulating tumorigenesis

TNF-α is known to regulate diverse cellular processes like cellular proliferation, differentiation and apoptosis. Inflammatory cells, involved in inflammation linked carcinogenesis secretes TNF-α which aggravates the process of metastasis by switching on the signalling cascade [63]. TNF-α mostly acts through two TNF super family receptors, TNF-α receptor I (TNF-R1, p55/p60) and TNF-α receptor II (TNF-R2, p75/p80). TNF-R1 is unanimously expressed in almost all cell types and has a significant role in NF-κB activation compared to TNF-R2. Different machineries of NF-κB trans-activation have been reported. As per the classical or the canonical pathway, there occurs a dimerization of either RelA or C with p50. This hetero-dimer lies in the cytoplasm in its inactive form by interacting with Iκ-B proteins [64]. Upon activation by any external stimuli Iκ-B gets phosphorylated in the serine residue by IKKβ subunit. Post which, Iκ-B protein undergoes proteasomal degradation followed

by nuclear translocation of NF-κB and it acts like a transcription factor regulating functions of TNF-α (Fig 1.3) [65, 66]. Existing evidences report TNF-α as one of the key mediators of cancer related inflammation. Malignant tumors can constitutively secrete TNF-α inside the tumour microenvironment leading to poor prognosis of the disease [67]. Additionally, TNF-α facilitates cancer development by controlling the proliferation of neoplastic cells, without inducing cell differentiation [68]. Nuclear factor-κB is considered to be critical for TNF-α mediated tumor initiation and progression, most importantly by its ability to accentuate the expression of other pro-inflammatory cytokines like IL-6, hinting towards its involvment in cancer associated inflammation. According to Hsu *et.al*., TNF-α driven activation of NF-κB facilitates neoplastic transformation in mouse epidermal JB6 cells [69]. Furthermore, inhibition of nuclear translocation of NF-κB has shown to inhibit TNF-α mediated cell proliferation [67, 70]. As discussed above, TNFα is equally vital in maintaining a normal homeostasis even though its anomalous production can lead to inflammation related cancers. Therefore, a clear understanding of the TNF-α pathway will not only help us to comprehend the overall modus operandi of TNFα, but it will also lead to identification of probable therapeutic targets that may be superior to currently available therapeutics.

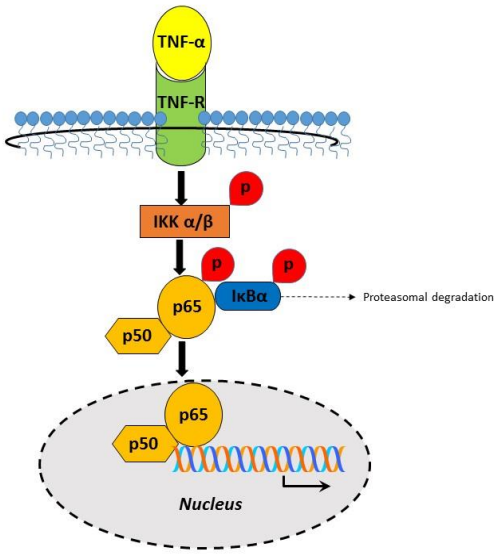


Figure 1.3. TNFα mediated p65 signalling pathway. *Binding of TNFα to its receptor (TNFR) leads to an activation of IKKβ in the IKK complexes, which leads to the phosphorylation of IκBα at serine S32 and S36 residues causing its poly-ubiquitination and proteasomal degradation. The heterodimeric complex of p65 and p50 now get translocated inside the nucleus, which acts as a transcriptional activator for many NF-κB specific genes.*

2.3.3. TGF-β and NF-κB cross talk

The concoction of cytokines released inside the tumour microenvironment play vital role in controlling cancer pathogenesis. Cytokines that are released by the cells counteract external infection and can inhibit tumor progression and metastasis. While, in turn cancer cells smartly regulate these host derived cytokines by promoting cellular proliferation and mitigating apoptosis. Therefore, a comprehensive understanding of the cytokines and tumour cell communication is required to improvise the existing cancer immunotherapies [71]. Here we are interested in deciphering the communion of TNF-α and TGF-β inside the tumor milieu (Fig 1.4). Accumulating evidences show that mouse fibroblast cell line upon simultaneous addition of TNF-α and TGF-β, blocked TGF-β induced nuclear translocation of SMAD2 in RelA wildtype cells [72]. It is well studied that SMAD7 stably interacts with TGF-β receptor I and prevents the binding, hence phosphorylation of SMAD2/SMAD3 by the receptor [73]. TNF-α introduction showed elevated SMAD7 levels in a good number of cell types including RelA positive fibroblasts, NIH-3T3, Mv1Lu and rat hepatic stellate cells (HSC). Additionally, TNF-α is observed to facilitate SMAD7 binding to TGF-β receptor complexes [72].

It can be summarized as, TNF-α induced signalling is shown to activate SMAD7 transcription, which abrogates TGF-β dependent SMAD2 nuclear translocation and TGF-β type I receptor signalling. On the one hand, TGF-β is said to inhibit NF-κB pathway by activating the IκBα and retaining NF-κB in the cytoplasm as observed in hepatocytes and breast cancer cell lines [74, 75]. On contrary, in head and neck squamous cell carcinoma (HNSCC), TGF-β1 sequentially phosphorylates TAK1 and NF-κB pathway and promotes metastasis, depletion of TAK1 have shown to stop NF-κB activation and malignancies associated with it. Furthermore, it has also been studied

that TGF-β and NF-κB persuade SMAD7 expression which suppresses TGF-β induced phosphorylation of SMAD2 and TNF-α mediated NF-κB activation [76]. Therefore, functional cross-talk between TGF-β and NF-κB signalling in tumor microenvironment is still elusive and requires a more detailed exploration. From the above findings it can be said that, the crosstalk of TNF-α and TGF-β is under the governance of various downstream signalling molecules like SMAD2/3, p65 and TAK1. Hence, targeting these molecules can open up new therapeutic opportunities in controlling tumorigenesis.

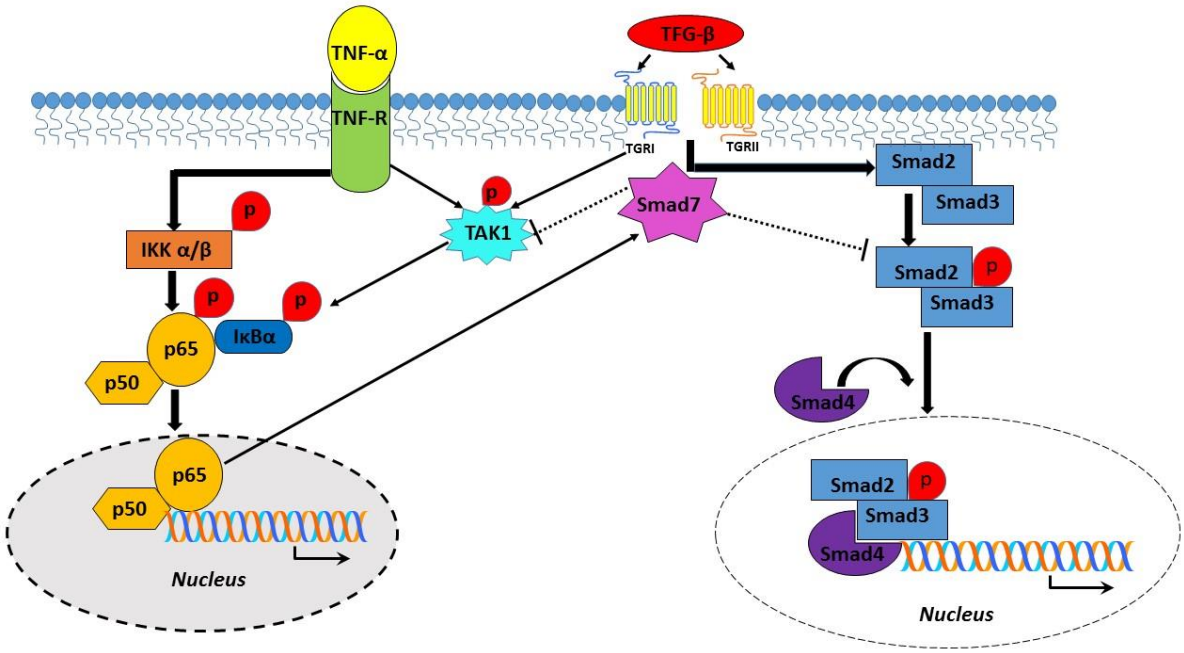


Figure 1.4. Illustration of TGF-β and TNFα signalling crosstalk. *TGF-β triggers NF-κB signalling by a sequential regulation of TAK1 and IKK kinases which leads to the phosphorylation of IκBα followed by nuclear translocation of p65. NF-κB signalling induces the expression of SMAD7, which in turn suppresses TGF-β induced SMAD2/3 phosphorylation and activation.*

3. Epithelial to mesenchymal transition

Mammalian system mainly comprises of two cell types epithelial cells and mesenchymal cells. The key characteristics of epithelial cells include tight interactions among the cells due to the presence of tight junction proteins, apicobasal alignment and lack of motility. Mesenchymal architecture differs in many ways from epithelial cells

like they have loose interactions among each other and hence, no apicobasal polarity and are motile having invasive properties. During the process of development, few cells switch from an epithelial to mesenchymal morphology by a firmly controlled cellular process known as EMT (Fig 1.5). EMT can be a reversible process, where depending on the environment cells can undergo the reciprocal mesenchymal to epithelial transition (MET) [77-79]

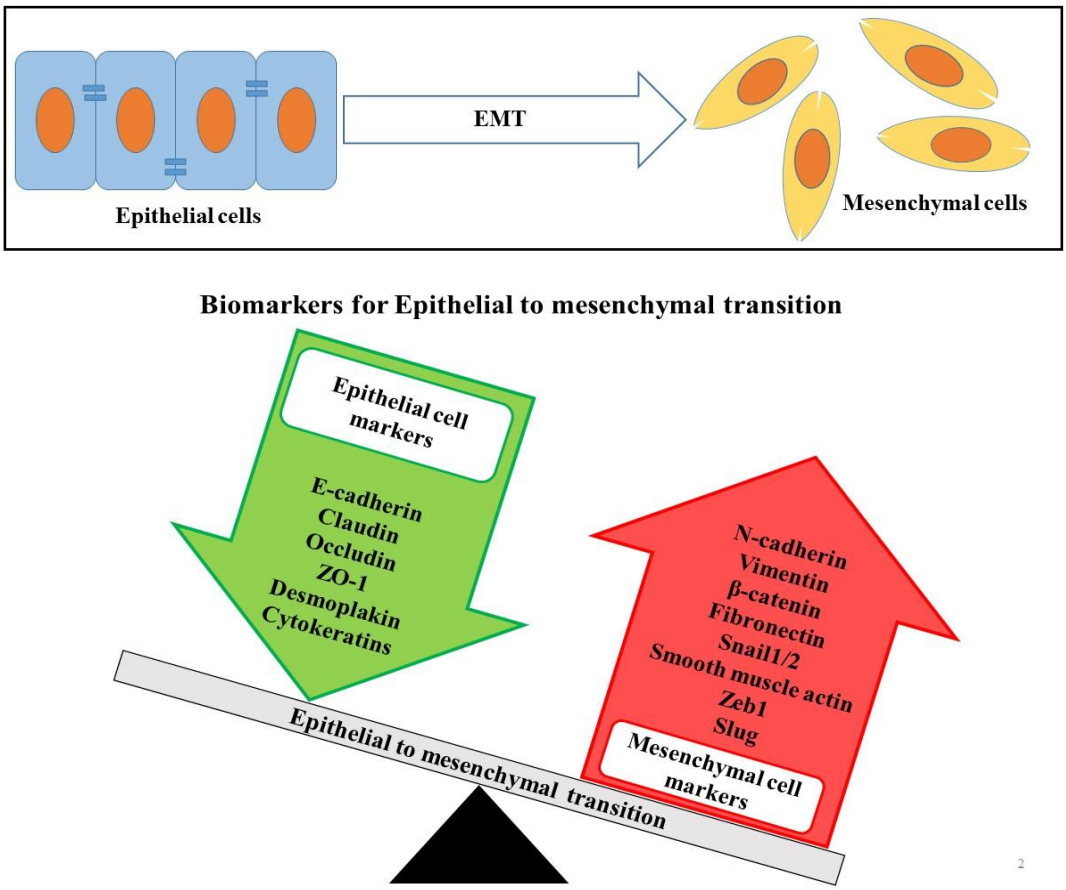


Figure 1.5. Image displaying crucial biomarkers involved in epithelial to mesenchymal transition. *During the process of EMT the key genes like N-cadherin, Vimentin, β-catenin, Fibronectin, Snail, Smooth muscle actin, Zeb-1, Slug gets turned on and downregulate the expression of epithelial markers like E-cadherin, Claudin, Occludin, ZO-1, Desmoplakin, cytokeratin.*

3.1. Epithelial to mesenchymal transition: an alliance towards cancer progression

Unlike normal cells the molecular and cellular events taking place during EMT in cancerous cells are stochastic, time independent or may be bypassed leading to the gain in invasive properties. Hence, EMT is considered to be the key step of metastasis, where epithelial type cells lose their polarity and acquire mobility; a key feature of mesenchymal type cell displayed by a diversity of cancer cells [80, 81].

During the process of metastasis cells localized inside the primary tumor snap off, inflowing the bloodstream or lymph nodes and settle down at a secondary location. In corroboration with existing reports metastasis is well recognized as a multistep process detachment being the first, in which cancer cells localized in the primary tumor gets detached from each other and gain motility triggered by EMT. Consequently, the cancerous cells can now invade neighbouring tissues entering the blood and lymphatic vessels which is a characteristic of the intravasation stage, where epithelial cells are not observed any more. From the existing population of cells few cells by the process of extravasation, invades to nearby secondary sites where they can proliferate and colonize forming micrometastases deposits which eventually can give rise to a secondary tumor [82, 83].

As discussed above, the interaction of tumour cells and the local microenvironment lead to the activation of the autocrine/paracrine exudation of cytokines, growth factors and extracellular matrix proteins leading to EMT [84]. Here we are elucidating few evidences supporting the above statement. Breast cancer associated fibroblasts have been reported to secrete chemokines which facilitates the proliferation and migration of cancerous cells [85]. A good number of growth factors like epidermal growth factor (EGF) and fibroblast growth factor (FGF) via their associated signalling cascades, Janus-activated kinase (JAK) pathway and ERK/MAPK respectively have been linked with EMT induction [86, 87]. Not only the MAPKs but also other signalling pathways like TGF-β, wnt, Notch, NF-κB and Hedgehog have also been observed to be critically associated with EMT induction [79, 88]. Additionally, mutations in signal transduction molecules like TGF-β receptors in different cancer types can also cause EMT [89]. Loss

of SMAD4; the downstream of TGF-β signalling is observed to inhibit the tumour suppressive role of TGF-β leading to cell transformation [90]. Genetic and epigenetic aberration in genes like E-cadherin and retinoblastoma (Rb) have been associated with the initiation of EMT [91, 92]. Also, co-culture of breast cancer cells and bone marrow-derived mesenchymal stem cells (MSCs) have shown a substantial upregulation of EMT markers like Twist, snail, N-cadherin and Vimentin and downregulation of E-cadherin [93]. Tumor associated macrophages (TAM) a crucial member of immune cells residing inside the tumor milieu as mentioned above, positively regulates EMT by the activation of TGF-β and β-catenin pathway [93, 94]. Another important component CAFs is reported to get activated by the secretion of metalloproteinases (MMPs) and IL-6 and induces EMT followed by metastasis in tumor cells [95]. Additionally, in ER+ breast cancer cells, IL-6 is observed to repress E-cadherin and induce EMT like phenotype leading to poor prognosis hence survival [95, 96]. Many leading factors, driving EMT are now documented some of which are reviewed in this thesis, still further research is required to unmask all the key players of this intricate process. This awareness will definitely have an enormous impact on identification of novel markers for proper control of cancer metastasis.

3.2. TGF-β and TNF-α cross talk in regulating EMT

EMT is a vital event during development by which epithelial cells change their morphology and acquire fibroblast-like properties and express reduced intercellular adhesion and gain motility. Hoarding evidences indicate a crucial role of EMT in providing cancer cells with invasive and metastatic properties [97]. Several biological agents are known to initiate EMT, where in cytokines present in tumor micro-environment being the most important ones. In adenocarcinoma cells, it has been seen that inhibition of IKKβ blocked TGF-β1 mediated EMT and the accentuating act of TNFα which is independent of SMAD 2/3 [98]. This indicates that the activity of TGF-β1 is not solely governed by the SMAD signalling pathway but can be modulated and regulated by other signalling pathways including those that can also be activated by TNFα such as ERK, NF-κB and p38 MAPK [99, 100]. Therefore, above results suggest a critical relation between inflammatory driven EMT and metastasis. These two cytokines are also reported to aggravate EMT in human colonic organoids, suggesting

a critical cross talk between the two under inflammatory background. TGF-β and TNF-α activates p38 MAPK pathway and promotes a rapid morphological transformation of the extremely organized colonic epithelium to isolated cells with a mesenchymal phenotype [101]. This suggests that TNF-α potentiates TGF–β mediated EMT via p38 MAPK activation contributing to tumor development. Another possible explanation for the rapid EMT induction on TNF-α stimulation is through ERK signalling which up-regulates TGF-β expression pointing towards synergy between TNF-α and TGF–β. Above statements highlight TGF-β functions not only in regulation of diverse cellular processes by activating SMAD and non-SMAD signalling but also in cross regulating the intracellular signalling induced by other pro-inflammatory cytokines present inside a tumor milieu.

4. Autophagy: a cellular homeostasis machinery

The word 'autophagy' means self-eating, first invented by Scientist named Christian de Duve, around 40 years ago. This invention was largely constructed on the observed degradation of intracellular structures like mitochondria and lysosomes in rat liver perfused with glucagon, a pancreatic hormone. Although the molecular mechanism involved was still illusive [102]. Recent findings have rediscovered autophagy, by unmasking the molecular markers and physiological conditions responsible for its initiation and progression at least in mammalian systems. As per reports, 32 different autophagy related genes (ATG) have been identified till date, through genetic screening in yeast, most of which are conserved in mammals, plants, slime moulds, worms and flies [103].

Autophagy can broadly be categorised into three types: **macro-autophagy**, where cytoplasmic cargos are delivered to the lysosome via an intermediary double membrane-bound vesicle, referred as autophagosome. This ultimately fuses with the lysosome and forms the autolysosome. In contrast **micro-autophagy** doesn't involve the autophagosome instead, delivers the cargos directly to the lysosome through invagination of the lysosomal membrane. Third one is the **chaperone mediated autophagy (CMA)**, where targeted proteins destined for degradation are translocated through the lysosomal membrane forming a complex with chaperone proteins (e.g. Hsc-

70) which then gets recognized by lysosomal associated membrane protein 2A (LAMP-2A), leading to their subsequent unfolding followed by degradation [104, 105].

4.1. Cellular machinery involved in autophagy

Autophagy starts with the formation of a lipid bilayer known as phagophore, contributed by the endoplasmic reticulum /or the trans-golgi and endosomes [106-108]. Which then elongates to engulf intracellular cargos of protein aggregates and cellular organelles, followed by sequestration inside a double membraned structure called as autophagosome [109]. The maturation of autophagosome then takes place by its fusion with the lysosome forming a complex called autolysosome which renders cargo degradation by the lysosomal proteases. The lysosomal permeases and other transporters helps in exporting back the amino acids and other by-products of degradation to the cytoplasm, where they are reused by the cells during stress conditions (Fig 1.6) [108]. Hence, autophagy is considered as a cellular recycling factory which facilitates ATP generation and help cells to combat intra-cellular damage by confiscating non-functional proteins.

Autophagy is known to regulate a diverse cellular machineries like, **maintenance of cellular homeostasis** by clearing off damaged cell organelles, misfolded proteins and protein aggregates [110]. In orchestration of stress responses, such as **nutrient starvation** by breaking down macro-molecules like carbohydrates, nuclei acids, proteins, triglycerides into their simpler forms for de-novo synthesis of biomolecules and ATP generation through tricarboxylic acid (TCA) cycle and other known metabolic procedures [111]. During **hypoxia,** autophagy mitigates the cellular stress triggered by reduced oxygen levels [112]. Hypoxia inducible factor-1α (HIF-1α), is reported to increase the levels of BCL-2 interacting protein 3 (BNIP3), which is a well-known

marker for inducing autophagy by disruption of the Bcl-2/BECN1 complex [113].

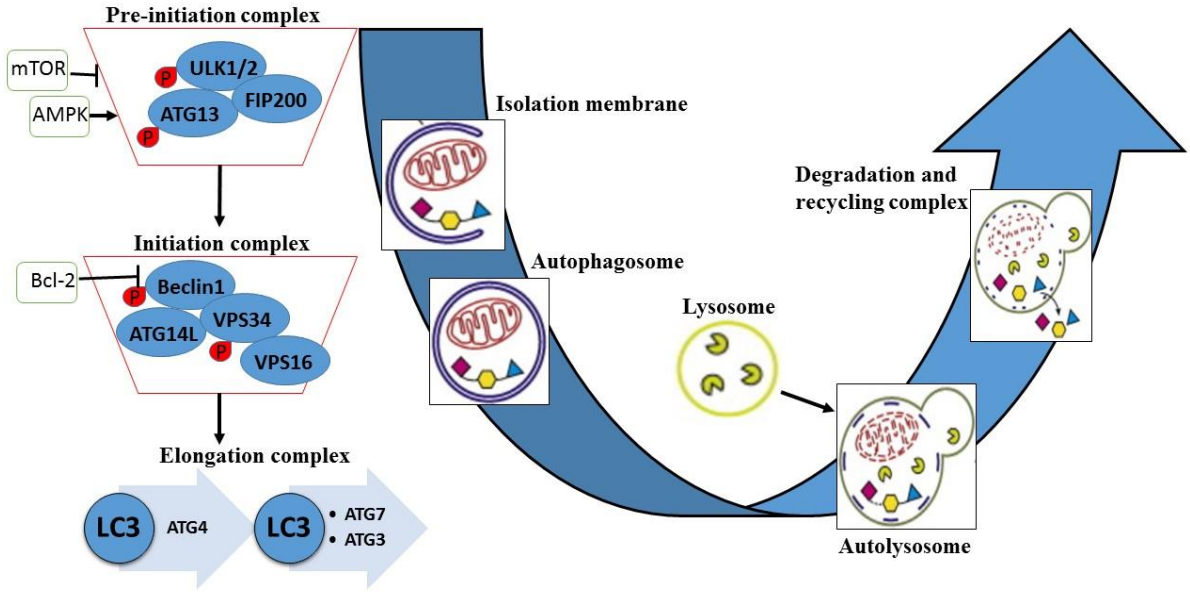


Figure 1.6. Schematic delineation of autophagic machinery. *Autophagy initiates by the formation of a sequestering/isolation membrane known as phagophore. Subsequent conjugation of LC3 to the sequestering membrane regulates the elongation of the phagophore complex. Post termination of elongation, sequestration complex gets closed forming a double-membrane structure called as autophagosome. Which is then delivered to fuse with the lysosome and forms the autolysosome degradation complex. Hydrolases and catalases destroys the cargo and nutrients generated are recycled. This autophagic machinery maintains homeostasis by sustaining cellular stress like nutrient starvation. However, autophagy can also lead to apoptosis.*

4.2. Autophagy and cancer: a question of cell fate

Defects in the above mentioned autophagic machinery have been linked with increased susceptibility to metabolic stress, genomic damage and most importantly tumorigenesis [114]. Autophagy is controversially discussed in context to cancer i.e. it has shown dual role in tumorigenesis as a tumor suppressor as well as a tumor promoter by regulating intracellular ROS levels (Fig1.7).

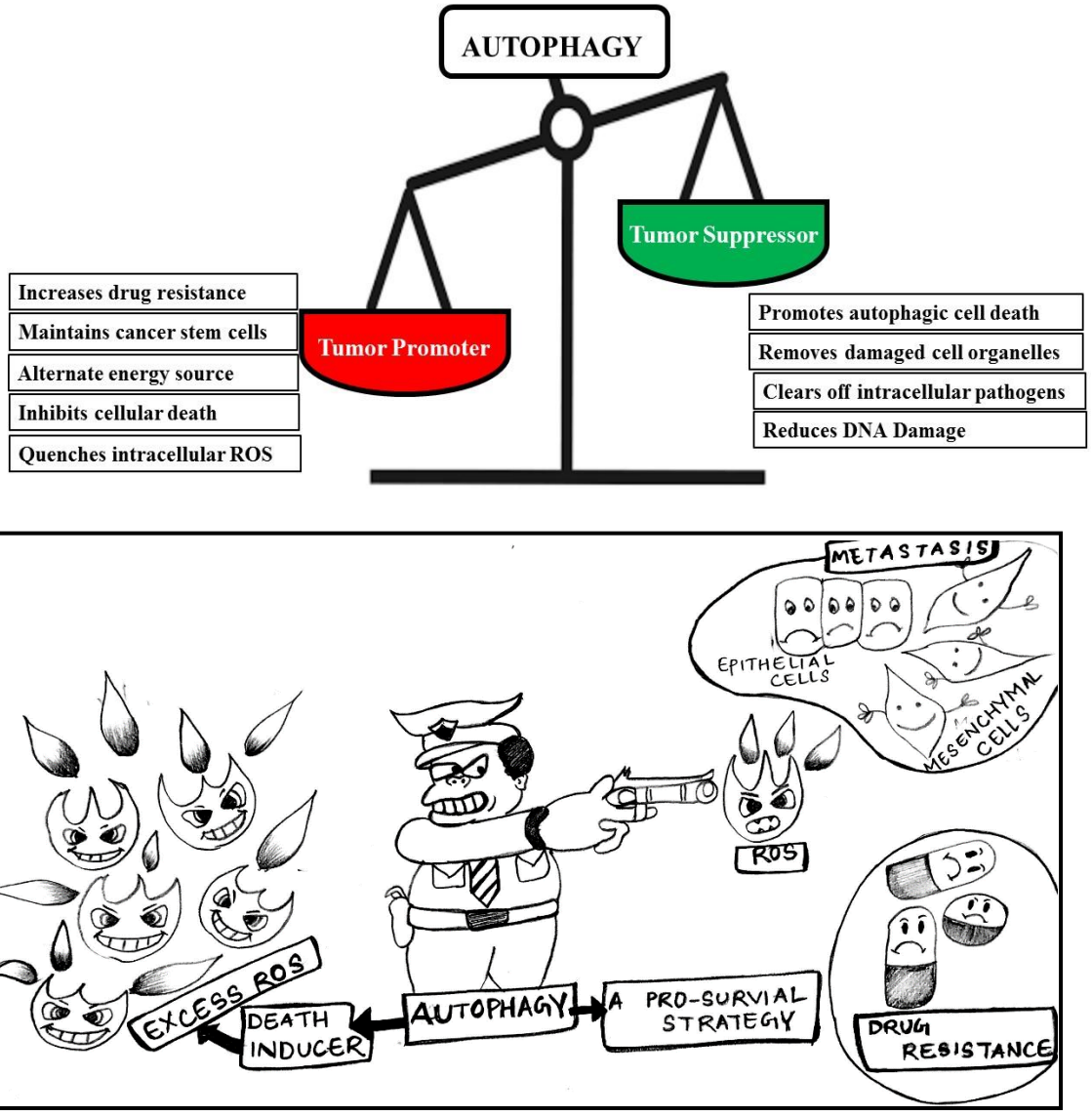


Figure 1.7. Diagrammatic representation of dual role of autophagy. *Autophagy acts as a double edged sword in context to cancer, it acts as a tumor promoter by facilitating drug resistance, maintaining stemness of cancer cells, providing alternate source of energy, inhibiting apoptosis and quenching intracellular ROS. On the other hand it suppresses tumor initiation and progression by promoting apoptosis, removing damaged cell organelles, clearing off intracellular pathogens and reducing DNA damage.*

4.2.1. Evidences of autophagy as a tumor suppressor

Autophagy is reported to be involved in controlling a wide range of inflammatory and degenerative diseases although there still lies a missing link in clear understanding of how autophagy regulates these diseases [115]. Arguably, till date cancer is the disease where autophagy is extensively studied. Qu et al and Yue et al have shown, becn1+/− mice are susceptible to liver and lung tumors also, the development of mammary hyperplasia is very high later in life [116, 117]. Most importantly, BECN1+/− cells showed significant reduction in the expression of p53; the tumor suppressor gene, signifying autophagy independent causes of becn1+/− genotype to develop spontaneous tumors when compared to other genetic defects in autophagy [118]. Mosaic deletion of autophagy gene, ATG5 or ATG7 in mice and rats respectively, is reported to initiate the development of benign hepatomas, suggesting autophagy deficiency can initiate liver tumor formation but confines advancement to malignant disease [119]. There are similar reports saying, ATG5 and ATG7 knockdown in mouse pancreas with Kras mutation stimulates pancreatic intra-epithelial neoplasia (PanIN) formation but also stops it's progression to malignant disease [120, 121]. Loss of autophagy in pancreas and liver is reported to cause oxidative stress, tissue damage followed by inflammation which are the major risk factors for promoting tumor initiation by producing cells susceptible to oncogenic transformations and mutations [122, 123]. Furthermore, accumulation of p62 and autophagic cargo in mice having autophagy deficient livers have shown increase in tumorigenesis [122]. Also, p62 accumulation can promote lung and mammary carcinogenesis persuaded due to autophagy deficiency [122, 124]. p62 binds to KEAP1; the inhibitor of NRF2 hence causing it's activation [125] and deficiency in p62 dampens lung, mammary and liver tumorigenesis induced by the loss of ATG7 [126]. Autophagy deficiency stimulates growth and proliferation of lung carcinogenesis by inducing NRF2 activation due to the accumulation of p62 [127]. Accumulating above observations highlighted the role of autophagy in controlling cancer progression.

4.2.2. Evidences underlining autophagy as a tumor promoter

Regulation of autophagy is evidently influenced by the complex crosstalk of tumor microenvironment and immune cells. Nutrient deprivation suppresses PI3K pathway and activates autophagy, which can be taken over by the cancerous cells as an alternate source of energy production for survival. *Degenhardt et.al.* were the first to observe the upregulation of autophagy in hypoxic areas of tumor, highlighting the role of autophagy in helping cancer progression by overpowering tumor mediated inflammation [107]. ATG7 deficiency in ras-driven lung carcinoma cells showed compromised fatty acid oxidation and mitochondrial respiration, suggesting the role of autophagy in promoting malignancy by maintaining mitochondrial quality control [128]. Human renal oncocytomas lacking Ras mutations are reported to develop pathogenic mutations in mitochondrial genome, defects in respiration and impaired autophagy [129]. Hence, autophagy is considered as a key mechanism to eliminate damaged mitochondria from the cells by mitophagy, getting accumulated in ATG7 deficient tumors.

Accumulating above observations suggest autophagy has context dependent roles inside the TME. As mentioned above in some circumstances, autophagy helps destroying cancer cells by inducting apoptosis, whereas in others it helps in proliferation and survival of cancer cells. Hence, autophagy can be used as a strategy to improve therapeutic efficacy of currently available anticancerous drugs.

4.3. Role of TGF- β and TNF-α in modulating autophagy

Autophagy is known as cellular degradative machinery, which clears off damaged and superfluous proteins and organelles from the body, which is further recycled and used as an alternate source of energy during metabolic stress to maintain cell homeostasis [130]. Autophagy and its involvement in cancer is a topic of debate due to its dual roles in cancer, both as a tumor suppressor by averting the gathering of damaged proteins and as a machinery to promote tumorigenesis [131]. To further investigate the above statement, we recently observed that, HCC cells upon exposure to TGF-β2 exhibited high autophagic flux. Interestingly, simultaneous addition of TGF-β2 and TNF-α inhibited TGF-β2-induced autophagy and EMT by elevating intracellular ROS and further promoting apoptosis [132]. Autophagy not only plays a crucial role in cancer

but also has significant functions in controlling non-cancerous processes. Studies suggest that autophagy is involved in regulation of TNF-α secretion by dendritic cells and macrophages, in particular, treatment of both murine and human cells with 3-MA (autophagy regulator) strongly inhibits TNF-α secretion [133, 134]. TGF-β plays a bi-directional role by either inducing or suppressing autophagy in a context dependent manner. TGF- β1 induces autophagy in the bovine mammary epithelial cell line (BME-UV1) and neonatal piglet gut epithelium [135, 136]. Moreover, TGF-β induced autophagy is reported to be suppressed by knockdown of SMAD2/3 or SMAD4, advocating that TGF-β induces autophagy, at least in part, via the SMAD pathway [137]. Additionally, inhibition of JNK or knockdown of death associated protein kinase also subdues TGF-β–induced autophagy [138], indicating the involvement of both SMAD dependent and SMAD-independent pathways in the process. Therefore, both TGF-β and TNF-α can be considered as key cytokines in regulating autophagy in tumorigenic as well as non-tumorigenic processes.

5. Apoptotic regulation of TGF-β and TNF-α crosstalk

Meticulous regulation of cell division and apoptosis is important for maintenance of normal cell development, and alteration in these processes can cause neoplastic transformation. During acute cellular stress, cells are frequently exposed to a variety of cytokines such as IL-1β, IFN-γ, TGF-β and TNF-α. These cytokines concomitantly trigger both pro-and anti-apoptotic pathways and create a redox imbalance. It is well reported that TGF-β has a multifunctional role ranging from modulating target gene activity to regulating cell growth and apoptosis [139-141]. For example, hepatic overexpression of TGF-β leads to apoptosis in transgenic mice and moreover its altered expression is linked to apoptosis in human and rodent hepatic neoplasia [142-144]. Interestingly, another cytokine TNF-α has shown to have cytolytic effects and is a potent inducer of NF-κB [145, 146]. Studies have shown that TNF-α induces apoptosis in neuronal cells by a pathway that involves formation of reactive oxygen species [147]. Not only individual role of TNF-α and TGF-β in supervising apoptosis is widely studied but their co-operation in aggravating cell death is reported as well. Previous studies indicate that the activation of both p38α as a pro-apoptotic effector and ERK1/2 as an anti-apoptotic protector via sTNFR1-induced autocrine TGF-β1 signalling loop helps

in maintaining a cellular balance in immune cells [148]. TNF-α mediated reverse signalling stimulates TNF receptor leading to increased TGF-β secretion from monocytes that could possibly activate p38α and ERK1/2 in an autocrine manner resulting into apoptotic induction. Although TGF-β and TNF-α induced apoptosis is well-documented in many different cell types, their antagonism and underlying molecular mechanism in regulating the same is still poorly understood. With this understanding we recently have tried to investigate the two cytokine's TGF-β and TNF-α cross regulation in apoptosis and interestingly found that, simultaneous introduction of TGF-β and TNF-α has shown an increased accumulation of intracellular ROS leading to apoptotic cell death in HCC cells [132].

6. Hepatocellular carcinoma

HCC is the most prevalent liver malignancy and is a primary cause of cancer associated death. Accumulating evidences suggest HCC as the fifth most common form of cancer worldwide and the third most common cause of cancer-related deaths [149]. Despite recent improvements in techniques used for prevention and treatment mortality continue to rise significantly. Orthotopic liver transplantation (OLT) is considered as an effective therapeutic option for treating both HCC and cirrhosis. Regrettably, most cases of HCC are detected at an advanced stage hence are not appropriate candidates for OLT. Major risk factors include liver cirrhosis, chronic hepatitis B infection and alcohol intake [150]. HCC occurrence is found to be more in males as compared to females [151] and the major risk factors for development of cirrhosis includes chronic infection with hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease, and non-alcoholic steatohepatitis (NASH). Additionally intake of aflatoxin contaminated food, diabetes, obesity, hemochromatosis and certain metabolic disorders can also lead to HCC [149, 152, 153].

6.1. Major risk factor for HCC

Major risk factors involved in HCC pathogenesis are described below (Fig 1.8)

6.1.1. Cirrhosis: Cirrhosis is the late stage of scarring of the liver instigated by chronic liver diseases and is considered as the key risk factor for HCC initiation, major

contributors being HBV and HCV infection. According to reports 50% of HCC cases worldwide are related with HBV infection and 25% with HCV infection [154]. Cirrhosis typically develops in a state of chronic liver diseases which can be characterized by reduced hepatocyte proliferation and an upsurge in the number of fibrous tissue, hinting towards an improper regenerative capacity of the liver. This provides a favourable niche for cancerous nodules development [155, 156]. Activation of stellate cells stimulate the production of cytokines, growth factors and reactive oxygen species which is a hallmark for liver cirrhosis and hence tumor formation [157].

6.1.2. Diabetes: Type 2 diabetes is now-a-days the most common metabolic disorder strongly associated with obesity and is characterized by many associated factors like hyper-glycemia, insulin resistance, and hyper-insulinemia. It has been linked with increased risk of several cancers with a substantial evidence on HCC initiation and progression [158, 159]. Due to the essential role of liver in maintaining glucose metabolism, diabetes mellitus can directly affect the liver. Additionally hyperinsulinemia is associated with a threefold increased chances of HCC development. Insulin like growth factor (IGF) and insulin receptor substrate1 have been reported to stimulate cellular proliferation and attenuate apoptosis and hence, regulate many cellular cascades controlling carcinogenesis [160, 161].

6.1.3. Non-alcoholic fatty liver disease (NAFLD), Obesity and non-alcoholic steatohepatitis (NASH): NAFLD is an ailment in which superfluous fat gets stored in the liver. NASH is considered as a type of NAFLD in which, along with the fat deposition in the liver, the individual has hepatitis infection and chronic liver inflammation. It arises in non-alcoholic individuals, although the hepatic histology resembles alcoholic hepatitis [162], with aberrant histology including inflammation, hepatic steatosis and fibrosis, NASH is the most severe form of NAFLD [163]. NAFLD is reflected as one of the crucial hepatic manifestation of obesity and related metabolic conditions which can also lead to HCC [164]. In non-obese conditions it is found to be associated with hyperinsulinemia and insulin resistance [165]. It has become one of the prevalent liver disorder arising almost universally among obese diabetic patients and in many industrialized countries including the United States [164, 166-168].

6.1.4. Other risk factors: In non-cirrhotic livers HCC can occur due to uptake of aflatoxin B1 containing food stuffs [169]. It is a mycotoxin produced by the Aspergillus fungus which cultivates readily on food when kept in warm and damp conditions [170]. After ingestion, it gets metabolized into its active AFB1-exo-8, 9-epoxide form which has a DNA binding affinity, including mutations of the p53 gene. Indeed, reports suggest 50% of HCC cases in southern Africa carry this mutation [171]. Alcoholic liver disease remains a major risk factor for HCC development which occurs due to high alcohol intake (>50–70 g/day) [172] this can act synergistically with HCV and HBV to aggravate liver cirrhosis [170].

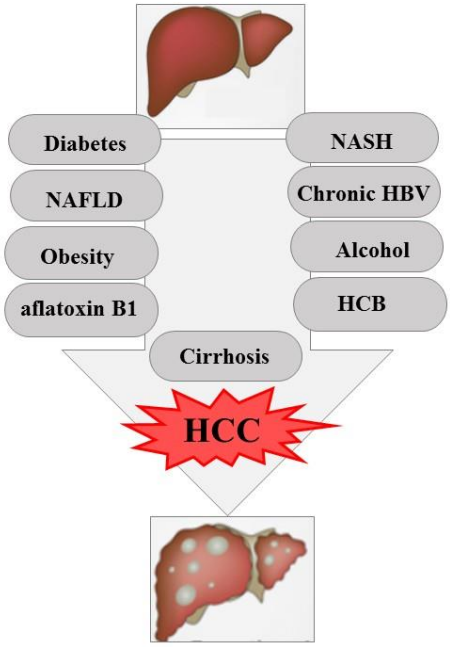


Figure 1.8. Crucial factors leading to HCC initiation and progression. *Major risk factors leading to HCC pathogenesis are chronic HBV/HCV infection, diabetes, NAFLD, obesity, aflatoxin B1, alcohol consumption, NASH which ultimately lead to cirrhosis followed by HCC.*

6.2. Screening and diagnosis

Early diagnosis is the key to get the appropriate treatment and cure for HCC. Unfortunately, maximum cases are diagnosed in their advanced stages hence, less than 20% of cirrhotic patients are screened for HCC [173].

Screening methods that are used currently includes liver ultrasound, Serum alpha fetoprotein (AFP), Ultrasound + alpha fetoprotein. As a result of uncontrolled hepatocyte proliferation, cirrhotic livers exhibit regenerative nodule. According to pathological studies, nodules < 1 cm are not HCC [174]. For lesions exceeding 1 cm, non-invasive diagnostic strategies are recommended followed by imaging methods like enhanced computed tomography and dynamic contrast-enhanced magnetic resonance imaging [175].

6.3. Available treatment options

Till date orthotopic liver transplantation (OLT) is considered as an effective treatment option for both underlying cirrhosis and HCC, limitation being detection in advance stages making the candidates unsuitable for OLT [176]. Patients diagnosed in their early stages have more chance of getting cured with different available treatment options although, tumor staging is essential for deciding a treatment option [2, 3]. A number of therapies have been used now-a-days with proven survival benefits mainly in the initial stages of HCC progression which includes, surgical resection, radiofrequency ablation (RFA), radio-embolization and transarterial chemoembolization (TACE) [177, 178].

6.3.1. Liver transplantation (LT) is considered to be a suitable treatment option for patients with decompensated liver cirrhosis. It is recommended for the patients whose tumor size is not bigger than 5 cm, or up to 3 lesions with each 3 cm or lesser. LT has shown 5 years overall success rate of 75% and a tumor relapse rate of less than 15% [178].

6.3.2. Surgical resection due to its high risk of hepatic decompensation, is considered as a treatment option for the patients with good liver function, single nodule and no underlying cirrhosis [179]. Patients who have undergone surgical resection have shown nearly 70% five year survival with a high risk of tumor recurrence due to the presence of microscopic vascular invasion in more than 30% of HCC patients [180]. Intrahepatic metastasis is one of the crucial reasons for early tumor recurrence i.e. within two years of surgery post that, the recurrence is mainly related to de-novo tumor development [181, 182].

6.3.3. Radiofrequency ablation (RFA) technique uses a radio wave to heat up a particular area of the nerve tissue to reduce pain. Local ablation with RFA is an appropriate treatment option for the patients having tumors < 2 cm in diameter which is can't be removed surgically. A group of study on RFA revealed that complete ablation of tumors smaller than 2 cm in diameter is possible in > 90% of cases having a recurrence rate <1% [183].

6.3.4. Transarterial chemo-embolization (TACE)

This technique is basically used to stop the flow of blood to a tumour and deliver chemotherapy drugs to the tumour, it is called chemoembolization. TACE is specific for HCC that blocks the hepatic artery to treat liver cancer. TACE is a standard treatment option for patients in their intermediate stage of HCC, having compensated liver function with bulky single nodule (< 5 cm) or multifocal HCC without extra hepatic spread [184].

6.3.5. Radio-embolization/selective internal radiation therapy (SIRT)

This is a type of radiation therapy mostly used as a therapeutic option for patients having intermediate stage HCC [185, 186]. In this technique, radioactive microspheres having size around 35 μm are delivered into the arteries to treat tumor nodules irrespective of their location, size and number. Now-a-days, the most prevalent radio-embolization therapy uses microspheres encoated with Y90 b-emitting isotope (SIR Sphere).

6.3.6. Chemotherapy

Currently, sorafenib is the sanctioned drug for HCC treatment which is an oral bi-aryl urea. It obstructs various cell surface and downstream kinases responsible for tumor development and progression. Cell surface tyrosine kinases targets of sorafenib comprise VEGFR-1, 2, 3, platelet derived growth factor receptor- (PDGFR-) β, FMS-like tyrosine kinase-3 and c-KIT. Additionally, sorafenib has been reported to inhibit Ras/MAPK pathway which helps in HCC progression [187]. Sorafenib is also used as a combinatorial therapy with other chemotherapeutic drugs like doxorubicin, octreotide, oxaliplatin, tegafur/uracil, cisplatin and gemcitabine with an aim to increase

the therapeutic efficacy [188], it has also been used in combination with AVE 1642, which is a human monoclonal antibody suppressing the insulin-like growth factor-1 receptor [189]. These drugs in combination with sorafenib have shown better results over its individual administration. Other clinically approved chemotherapeutic agents used for HCC treatment are Sunitinib, Linifanib, Brivanib, Tivantinib, Everolimus though they have associated side effects including fatigue, diarrhoea and hand-foot skin reaction [190].

7. Osteosarcoma

Osteosarcoma (OS) is one of the most prevalent form of bone tumor malignancies. This tumor starts from transformed mesenchymal cells and gives rise to the formation of underdeveloped bone. OS is reported to attack the longer bones of the body mainly the knee, other regions of the bone like pelvis, ulna, humerus, femur, radius and fibula can also get affected. It is stated as the eighth leading form of cancer affecting mostly children and adolescents in between the age group of 0-24 years [191, 192]. In last decades, a drastic improvement in survibility has been achieved with a significant reduction in mortality (1.3% approximately) [193]. The existing customary treatment for OS is radical surgery combined with chemotherapy [194, 195]. Widely used chemotherapeutic drugs for treating OS are cisplatin, doxorubicin and methotrexate individually or in combination. OS has a high propensity of pulmonary metastasis, lung being the most common; observed in around 30% of patients suffering from OS [196, 197]. Therefore, understanding the meticulous mechanisms of OS pathogenesis is essential to identify a novel therapeutic target to eliminate this disease [198].

CHAPTER- 2

Gaps in research and objectives

2.1. Gaps in research

A plethora of cytokines are produced in the HCC tumor milieu that invariably develops in a chronic inflammatory setting. Therefore, understanding of the roles of these pro-inflammatory cytokines is essential from both fundamental and therapeutic perspective for effective control of HCC, which is a very aggressive tumor and which is often diagnosed at an advanced stage of the disease. Multiple cytokines are predominantly prevalent in the HCC TME; of them, TGF-β, TNF-α, IL-6 and IL-1β are to name a few. Though the independent effects of these cytokines on tumor development is well studied; however, the molecular and functional crosstalk of these cytokines in context to epithelial to mesenchymal transition of HCC cells facilitating their migration and subsequent invasion are under explored. Recently, autophagy has emerged as a key cellular homeostatic process regulating cancer cell fate, especially under different stresses. We postulate EMT to be a stress associated event for cancer cells as well. However, what is the role of autophagy in HCC cells undergoing EMT, is controversially discussed over existing literature and deserves further research attention; furthermore, whether cytokines can modulate autophagy to regulate EMT is unknown. Hence, there is an existing gap in understanding the effect of different cytokines on EMT and autophagy and the interconnection, if any between each of these processes. To address the above lacunae for this study we selected two pro-inflammatory cytokines -TGF-β and TNF-α and framed the following objectives:

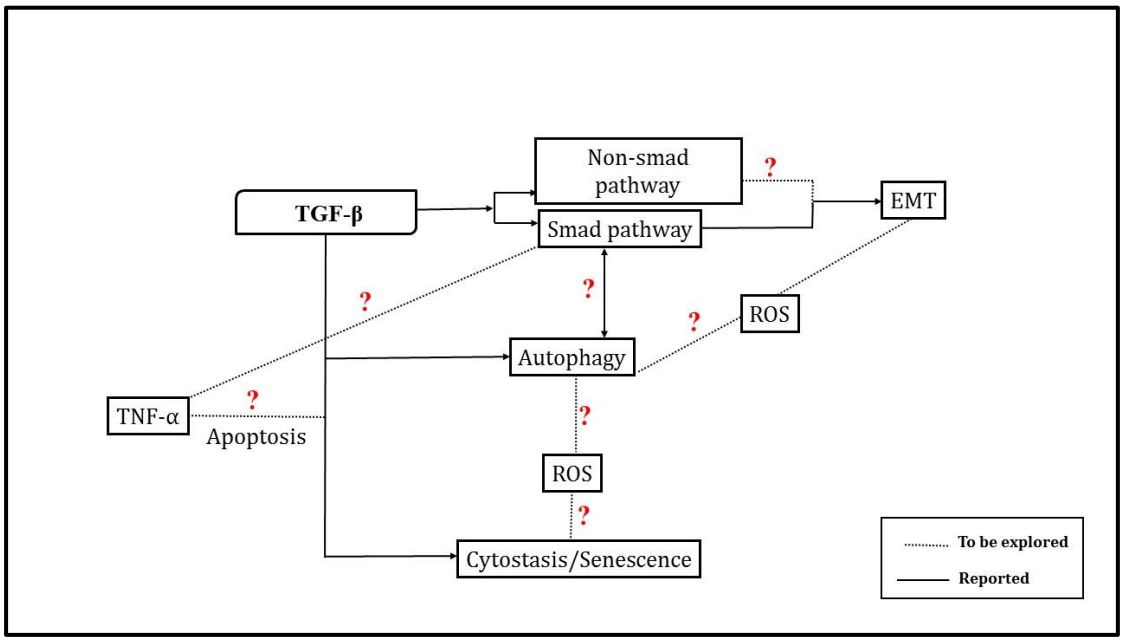


Figure 2.1. Schematic representation of gaps in existing research

2.2. Objectives

1. To understand the role of cancer associated pro-inflammatory cytokines and their crosstalk in regulating cell death, autophagy and EMT.

2. To understand alterations in global transcriptomic profile of HCC cells post exposure to pro-inflammatory cytokines.

3. To explore the intracellular signalling crosstalk post exposure to pro-inflammatory cytokines in HCC cells.

CHAPTER- 3

Materials and methods

3. Materials and methods

3.1. Chemicals

TGF-β2 (#SRP3170), TNF-α (# H8916), 2′,7′-dichlorofluorescin diacetate (DCFDA, # D6883), monodansylcadaverine (MDC, # D4008), chloroquine di phosphate (CQDP, #C6528), propidium iodide (PI; #P4864) were purchased from Sigma; N-Acetyl-L-cysteine (NAC, #47866) and 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT, #33611) were obtained from SRL. FITC conjugated Annexin-V (#A13199), Annexin-binding buffer (#1796344) were procured from Thermo Fisher Scientific and JC-1 was bought from Santacruz (#sc-364116A). SIS3 was procured from (Bio vision # 2227-1), JSH-23 was from sigma (#J4455). The siRNA for ATG5 was a gift from Prof. Santosh Chauhan, ILS Bhubaneswar (Dharmacon#M-004374-04) and lipofectamine 3000 was from bought from Invitrogen (#L3000-001).

3.2. Instruments

Major instruments used for conductance of the experiments described below are enlisted in the form of a table.

Table 3.1. List of major instruments used in the study.

Name of instruments	**Make**
Laminar airflow	MAC
Inverted Microscope	Olympus
Fluorescence Microscope	Olympus
Vertical gel electrophoresis unit	Bio-Rad
Semi-dry transfer apparatus	Bio-Rad
Real time PCR	Bio-Rad
Gradient Thermocycler	Bio-Rad

3.3. Methods

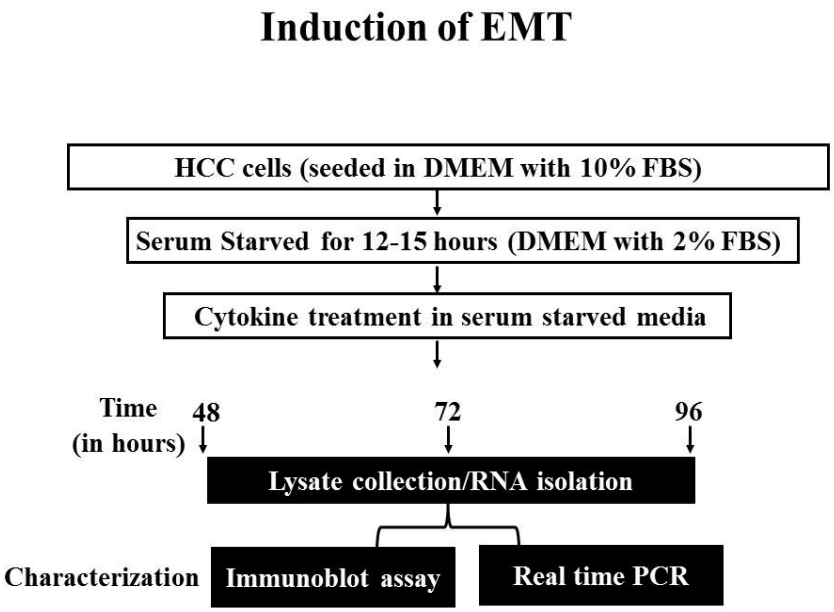


Figure 3.1. Flowchart depicting the process of EMT induction

3.3.1. Cell viability assay

Assessment of cell viability was performed by MTT assay. Briefly, cells growing in log phase were cultured in 96-well plates overnight. The following day, cells were washed with PBS and starved for 12h as mentioned above, post which cytokines at desired concentrations were added. Thereafter, MTT (20µl; stock concentration- 5mg/ml) was added. Cells were then incubated for 4h; post which formazan crystals were solubilized in DMSO and readings were obtained at 495nm with a differential filter at 630nm using an enzyme-linked immune-sorbent assay (ELISA) micro-plate reader (Start-fax 2100). Percentage of viable cells were calculated using the formula: viability (%) = (mean absorbance value of drug-treated cells) / (mean absorbance value of control) *100 [199].

3.3.2. RNA isolation and real-time PCR (RT-PCR)

Total RNA was isolated using TRIzol reagent (Sigma, #T9424). Complementary DNA (cDNA) was synthesized using GeneSure First Strand cDNA Synthesis kit (Genetix, #

PGK162-B) with oligodT, as per manufacturer's protocol. Templates were amplified using gene specific primers for Vimentin, N-cadherin and E-cadherin taking GAPDH as housekeeping control and detected using SYBR Green Supermix (Bio-Rad, #170-8882AP) in CFX Connect RT-PCR System (Bio-Rad). The primers used and their sequences are listed in the form of a table (table 4). The relative RNA expression was calculated using Livak method [200]. The melting temperature for the PCR reactions were 53°C for Vimentin and E-cadherin and 55°C for N-cadherin and P65 and the template was amplified for 30 cycles in each case.

Table 3.3. List of primers used for real time PCR.

Gene name	**Primer Sequence**
Vimentin	forward 5′- TCTACGAGGAGGAGATGCGG-3′ reverse 5'-GGTCAAGACGTGCCAGAGAC-3'
E-cadherin	forward 5'-TACACTGCCCAGGAGCCAGA-3' reverse 5'-TGGCACCAGTGTCCGGATTA-3'
p65	forward 5’- GGTCCACGGCGGACCGGT-3’ reverse 5’- GACCCCGAGAACGTGGTGCGC-3’
N-cadherin	forward 5'-CGAATGGATGAAAGACCCATCC-3' reverse 5'-GGAGCCACTGCCTTCATAGTCAA-3'
GAPDH	forward 5'-GCACCGTCAAGGCTGAGAAC-3' reverse 5'-TGGTGAAGACGCCAGTGGA-3'

3.3.3. Immunoblotting

Cells were lysed in modified RIPA buffer (Sigma-Aldrich) and protein content was measured using Bradford reagent (Thermo Scientific). The cellular protein lysates were run in denaturing polyacrylamide gels and thereafter transferred to PVDF membrane (Thermo Fisher Scientific, #88518) for blocking with 5% skimmed milk (HiMedia). The blots were then probed or re-probed with specific primary antibodies and detected using enhanced chemi-luminescence (ECL; Thermo Fisher Scientific, #3210) detection system following the manufacturer's protocol. The primary antibodies used were listed in the form of a table (table 3). The secondary antibodies were horseradish peroxide-

conjugated goat anti-rabbit IgG. Expression was quantitated using ImageJ and analyzed through Graph-pad Prism software [201].

Table 3.2. List of primary antibodies used for immunoblotting.

Antibodies	**Catalog number**
E-cadherin	CST, 24E10
N-cadherin	CST, D4R1H
Vimentin	CST, D21H3
β-catenin	CST, D10A8
ATG5	CST, D1G9
LC3-II	CST, D11
P62	BB-AB0130
Total SMAD2	CST, D43B4
Phosphorylated SMAD2	CST, 138D4
Total NF-κB p65	CST, D14E12
Phosphorylated NF-κB/p65	CST, 93H1
SMAD-7	Santacruz #sc-365846
β-actin	Santacruz #sc-69879
(Dilution used- CST-1:1000, Santacruz-1:2000)	

3.3.4 Determination of reactive oxygen species (ROS) levels

ROS levels were measured using 2, 7-dichlorofluorescein diacetate (H2DCF-DA) (Sigma) which measures intracellular generation of hydrogen peroxide, a procedure widely used for estimation of ROS. The H2DCF-DA passively enters the cell, where it reacts with ROS to form the highly fluorescent compound dichloro-fluorescein (DCF).

Briefly, cells were seeded at a density of 6000 cells / well in 96 well plates, allowed to grow till 50% confluency. The ROS scavenger, N-acetyl cysteine (NAC, 5mM) was added wherever mentioned, 1h prior to treatment with cytokine to inhibit ROS. Following exposure, cells were washed with PBS and then incubated in 100µl of working solution of H2DCF-DA (stock solution was diluted to yield a 10µM working solution) at 37°C for 30min. The fluorescence was measured at 485nm excitation and 530nm emission using a microplate reader (Fluoroskan Ascent) [202].

3.3.5. Determination of mitochondrial membrane potential

Flow cytometric analysis of mitochondrial membrane potential was done using the cyanine dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide). A shift in fluorescence intensity from red to green is an indication of mitochondrial depolarization. Huh7 cells were seeded at a density of 1×10^6 cells/plate, grown overnight and then treated with specific cytokine alone or in combination with CQDP (added 1h before cytokine addition). Cells were then harvested in 1ml of complete media. 5µg/ml of JC-1 was added to cell suspension and vortexed vigorously. After incubating for 20min at room temperature, samples were acquired using flow cytometer (Cytoflex, Beckmann Coulter) and analysis of acquired data was performed using CytExpert software [199].

3.3.6. Analysis of acidic vacuoles using monodansylcadaverine (MDC)

The compound MDC, a specific autophagolysosomal marker was used to analyze induction of autophagy [203-205]. For visualization of the autophagic vacuoles by fluorescence microscopy, cells were seeded on sterile cover slips and grown overnight. Following cytokine treatment, the cells were incubated for 10min at 37°C with 0.05mM MDC dissolved in PBS. The cover slips containing the cells were then washed with PBS and mounted with anti-fade mountant (containing DAPI). Intracellular MDC in the form of punctate dots were visualized using fluorescence microscopy. The images were taken at 100X under upright fluorescence microscope (Olympus, U-25ND25). For, fluorimetric measurement, cells after treatment were labelled with MDC for 10min, washed with PBS and collected in 10mM Tris-HCl (pH 8) containing 0.1% TritonX-100. Intracellular MDC was measured by fluorescence photometry (excitation

380nm and emission 525nm) in a microplate reader (Fluoroskan Ascent). An increase in MDC fluorescence upon treatment was expressed as fold change with respect to control.

3.3.7. Flow cytometric analysis of cell cycle

For DNA content analysis, cells were seeded in 6cm dishes at a density of 2×10^6 cells/plate and grown overnight. After 48h of treatment with cytokine, the cells were harvested, washed with PBS and centrifuged at 2000rpm for 5min at 4°C. The pellet was then re-suspended in 100µL of PBS and 900µL of ice cold 70% ethanol, used as a fixative. The fixed cells were incubated at 4°C overnight. The next day, cells were centrifuged and the pellet was re-suspended in 450µL PBS with 10µL of propidium iodide (PI; 2mg/ml) containing solution [206]. The samples were then incubated in dark for 10min followed by event acquisition using flow cytometer (Cytoflex, Beckmann Coulter) and analysis using CytExpert software.

3.3.8. Flow cytometric detection of apoptosis

During apoptosis phosphatidylserine (PS) which under normal physiological conditions remain on the cytoplasmic surface of the lipid bi-layer gets exposed, which can be detected by fluorochrome-tagged protein AnnexinV that binds to phosphatidylserine residues in the presence of calcium ions. Hence, for determination of apoptosis, cells were seeded in 6cm dishes at a density of 1×10^6 cells/dish. The following day, the cells were treated with specific cytokines and incubated for 48h. Thereafter, the cells were harvested, washed with PBS and re-suspended in 500µL of 1X binding buffer (Thermo Fisher Scientific). To detect both early and late apoptotic cells, 4µL of AnnexinV and 10µl of PI were added to the cells in binding buffer, followed by incubation in dark for 20min [206]. The samples were then acquired using flow cytometer (Cytoflex, Beckmann Coulter) and analysis of acquired data was performed using CytExpert software. Percentage of apoptotic cells was calculated by adding up percentage of cells in upper and lower right quadrant (UR and LR) is represented through bar diagram.

3.3.9. ATG5 knockdown, SMAD and p65 inhibition studies

To inhibit macroautophagy, cells were stably transfected with siATG5 (a kind gift from Prof. Santosh Chauhan, ILS, Bhubaneswar) a crucial regulator of macro-autophagy using lipofectamine3000. Studies were performed in Huh7 cells pre-incubated with 20nM siATG5 for 6 hours followed by exposure to TGF-β2 for 48h. For SMAD and P65 inhibition, Huh7 cells were treated with 2.5μM of SIS3 and 7.1 μM of JSH-23 respectively, one hour prior to TGF-β2 exposure for 48h.

3.3.10. Transcriptomic analysis through RNA sequencing

For transcriptomic sequencing total RNA was isolated and taken for fragmentation and priming. The fragmented and primed mRNA was further subjected to first strand synthesis followed by second strand synthesis. The double stranded cDNA was purified using HighPrep PCR magnetic beads (Magbio Genomics Inc, USA). The purified cDNA was end-repaired, adenylated and ligated to Illumina multiplex barcode adapters as per NEBNext® Ultra™ Directional RNA Library Prep Kit protocol. The adapters used in the study were Illumina Universal Adapter: 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC TTCCGATCT-3' and Index Adapter: 5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGTCTT CTGCTTG-3'. The adapter-ligated cDNA was purified using HighPrep beads and was subjected to Indexing-PCR to enrich. The final PCR product was purified with HighPrep beads. The sequencing library was initially quantified by Qubit fluorimeter (Thermo Fisher Scientific, USA) and its fragment size distribution was analyzed on Agilent Bio-analyzer. Finally, the sequencing library was quantified by qPCR using Kapa Library Quantification Kit (Kapa Biosy stems, USA). The quantified libraries were pooled in equimolar amounts to create a final multiplexed library pool for sequencing on Illumina sequencer for 150bp paired-end reads. The cleaned reads were aligned to the reference genome using Tophat2. Transcript quantification was done using Cufflinks. Differential expression analysis was performed using Cuffdiff. Transcripts with log2fold change of 1 and above were considered as up regulated and

those below -1 as down regulated. The raw reads were submitted to NCBI as BioProject PRJNA395629 (Transcriptomic analysis was done in Genotypic Bangalore). Expression of some of the key genes regulating EMT was validated by RT-PCR, the conditions and primer sequences of which are described before.

3.3.11 Senescence-associated β-Galactosidase activity

Cells were trypsinized and suspended in PBS. Galatosidase activity was thereafter measured following manufacturer's instruction (Thermo Fisher Scientific, # 75707). Briefly, M-PER reagent was added to the cell pellet. Cells were incubated for 10min in it followed by centrifugation at 13,000rpm for 15min. 50µl of cell extract was then transferred to a microplate well to which 50µl of β-gal assay reagent was added. Plate was incubated for 37^{o}C for 30min; reaction was stopped by addition of 100µl of stop solution and absorbance was measured at 405nm (Fluoroskan Ascent).

3.3.12 Statistical analysis

Tukey tests was used as a follow up to one way or two way ANOVA to compare every mean to a control mean and every mean with every other mean using Graph Pad Prism software version 5.0. The Bonferroni method was used to analyze multiple comparisons using Graph Pad Prism software version 5.0. The tests compute a confidence interval for the difference between the two means. Throughout the text the representative images are of experiments done in triplicates. Data represented in mean ± SEM (n=6). The symbols in parenthesis denotes the following: $^{*}p<0.001$ Vs Cntrl, #$p<0.001$ Vs TGF-β2, ns $P > 0.05$,*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

CHAPTER- 4

TNF-α attenuates TGF-β2 induced EMT by regulating ROS and autophagy in an epithelial type HCC cell

4.1. Introduction

Autophagy is an evolutionarily conserved lysosome-mediated degradation pathway by which cells self-digest selected cellular macromolecules and maintain homeostasis [207]. Autophagy also facilitates cellular survival under various stresses which may include, nutrient deprivation, growth factor depletion and hypoxia [208]. In recent years, autophagy has emerged as a focus of studies in cancer research, as increasing data indicate that autophagy functions as a central step allowing tumor cells to survive under drug/metabolic stress [209]. In contrary, a host of other studies also describe the role of autophagy in antagonizing cell survival and promoting cell death [210]. We assume that autophagy and cell death phenomenon like, apoptosis in cancer cells, might be linked to each other and occur in a cell type, stimulus and context-dependent manner, which needs to be further explored.

Interestingly, cancer develops in a complex milieu and almost invariably in an inflammatory setting. Under such circumstances, the cellular niche or tumor microenvironment (TME) can contribute substantially to the development, metastasis and therapy resistance of tumors. A plethora of cytokines, in the TME, play a crucial role in shaping tumor progression [211]. Many cytokines are also potent modulators of autophagy suggesting that autophagy-cytokine interaction has evolved as an important mechanism regulating pathogenesis of cancer. While, both archetypal cytokines, like, IFN-γ (Interferon) and TNF-α are known to induce autophagy, the classical cytokines like, IL-4 and IL-13 (Interleukins) are known to suppress it [212]. Additionally, recent studies have also shown that autophagy, apart from being regulated by a host of cytokines, can itself directly control secretion of a number of cytokines. Particularly, a perturbation of regular autophagic pathways has been associated with increased secretion of pro-inflammatory cytokines, like, IL-1α, IL-1β and IL-18 [213]. Activated inflammasome complexes are often engulfed by autophagosomes reducing IL-1β secretion and thus acting as an important homeostatic regulator [214]. Also, autophagy has been reported to positively regulate the transcription and secretion of IL-8 and IL-6 [212]. This establishes a strong connection between autophagy and cytokines and enforces the need to study further details of the interaction to facilitate better understanding of the disease.

In this regard, the cytokine, TGF-β has been shown to induce autophagosome formation and enhanced expression of autophagic markers in multiple cancer types, like, human hepatoma, breast cancer cells, etc. [215]. However, similar to the dual role of autophagy, the role of TGF-β in cancers is controversially discussed. It is proposed to function both as a tumor suppressor and also a tumor promoter in a cellular and context dependent manner. At early stages of oncogenesis, it is known to serve as tumor suppressor, in contrary its role in facilitating EMT and metastasis of tumors in advanced cancers is well acclaimed [55]. Also, a recent study by Jiang *et al* in 2016 shows that sustained TGF-β treatment in mammary epithelial cells can result in induction of autophagy and reversal of EMT [216]. This endorses the contrasting role played by this cytokine purely in a context dependent fashion. Given that, TGF-β is a multifunctional cytokine with multidirectional role extending from inhibition of growth, induction of apoptosis, triggering of EMT, to senescence, all the more emphasizes the need for further investigating the molecular effects of this cytokine in various cancer cells. Also, whether TGF-β induced autophagy in cancer cells facilitates one or more of the diverse TGF-β mediated cellular functions remain to be explored.

Another important cytokine, with role in autophagy and other varied cellular processes is tumor necrosis factor α (TNF-α). It is reported to induce autophagy in various cancer cells, like, Ewing sarcoma cells [217], human breast cancer [218] and human T lymphoblastic leukaemia cells [219]. However, how TNF-α is connected to autophagy is not fully understood and actually differs across various cell types. TNF-α-induced autophagy has been found to be JNK-dependent in vascular smooth cells, ERK-mediated in human breast cancer cells and reactive oxygen species (ROS) induced in intestinal epithelial cells [217, 219].

All these extensive links, between cytokines like, TGF-β, TNF-α and autophagy has made this field an attractive area of future research. However, the role of TGF-β and TNF-α in autophagy and possible cross talk between the two cytokines in relation to autophagy needs to be further investigated. Also, whether the activation of autophagy in response to TGF-β enhances cancer cell killing or is a counter stress mechanism is still an open-ended question. Here in, our study shows that TGF-β2 treatment leads to a simultaneous induction of cytostasis and EMT like phenotype in Huh7 cells. The cells

undergoing EMT were found to utilize autophagy as a pro-survival strategy, as inhibition of later abrogated EMT-like features. Furthermore, we observed that simultaneous exposure of TNF-α with TGF-β2 antagonize its function and attenuate TGF-β2-induced SMAD signalling and EMT. Our study addresses the link between EMT, autophagy and functioning of two important cytokines with respect to their role in autophagy regulation, which can be of potential significance to the understanding of the complex cancerous milieu**.**

4.2. Results

4.2.1. TGF-β2 induces SMAD dependent EMT

TGF-β is a multi-functional cytokine that is known to be involved in tumor suppression, cancer invasion and also for its pro-fibrogenic role in almost all fibrotic diseases [220]. It can effectively orchestrate diverse cellular effects depending on the cell type and context. One of the primary established functions of TGF-β is to promote EMT of cancer cells [221]. EMT can be described as the process promoting metastasis where epithelial cells undergo trans-differentiation by shedding off their polarity and epithelial characteristics, which facilitate their migration into neighbouring tissues; and TGF-β is a well-known inducer of it. Taking this into consideration we were interested in exploring TGF-β2-mediated EMT induction in HCC cell type (Huh7) and the signalling associated with it. A distinct change in morphology, marked by extended cellular phenotype was observed in cells exposed to TGF-β2, when compared to untreated control (Figure 4.1A). During EMT, the down-regulation of E-cadherin is stabilized by the up-regulation of N-cadherin, which connects to the cytoskeleton through β-catenin resulting in a cadherin switch that controls cell adhesion [222]. Another crucial marker for EMT is Vimentin which facilitates invasion of cells into surrounding tissues [223]. In our study, the expression of these key genes was monitored post TGF-β2 exposure through real time PCR and immunoblot. A significant increase in transcript level of N-cadherin and Vimentin was observed along with a concurrent reduction of E-cadherin mRNA (Figure 4.1B). Immunoblot analysis further revealed a significant increase in Vimentin, N-cadherin and also β-catenin protein levels and a simultaneous decrease in E-cadherin protein levels in Huh7 cells upon 48h of exposure to TGF-β2 (Figure 4.1C).

Additionally, dose kinetics studies were also performed which showed similar trend in increased expression of N-cadherin and Vimentin at different doses of TGF-β2 with respect to untreated control; similarly, E-cadherin showed a significant decrease in protein levels at higher doses of TGF-β2 (Figure 4.1D). The altered regulation of the above markers signified an EMT like transition in the epithelial Huh7 cells after TGF-β2 administration. Investigation of the upstream signalling leading to EMT revealed that the canonical TGF-β signalling pathway was activated following TGF-β2 exposure. A high level of phospho-SMAD-2 protein was observed in cells undergoing EMT (Figure 4.1E). To further validate a SMAD-dependent effect, TGF-β induced SMAD signalling was inhibited with SIS3, an inhibitor of TGF-β and Activin signalling that acts through specific suppression of SMADs without affecting the MAPK/p38, ERK or PI3-kinase signalling pathways [224]. Importantly, a significant reduction in Vimentin protein levels was observed upon SMAD inhibition suggesting the role of SMAD signalling in TGF-β2-induced EMT (Figure 4.1F). Taken together, these observations suggest that TGF-β2 treatment induces EMT by activating SMAD signalling pathway in Huh7 cells.

Fig. 4.1A

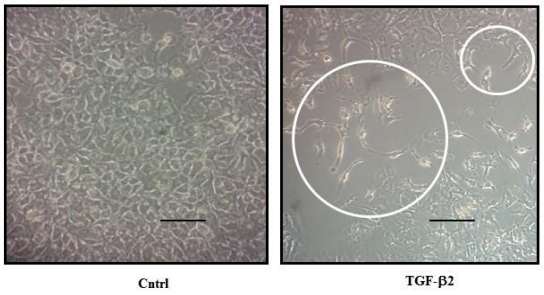


Fig. 4.1B

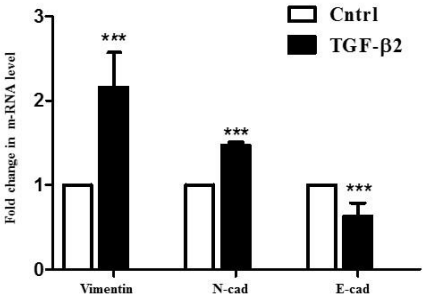
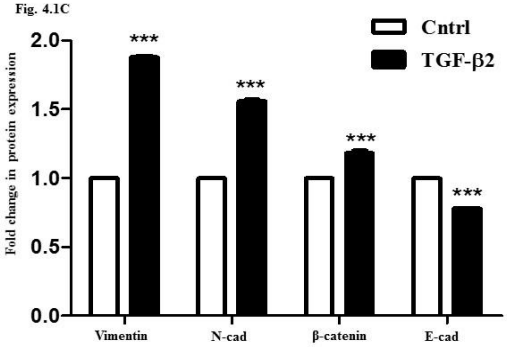
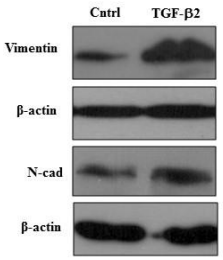


Fig. 4.1C





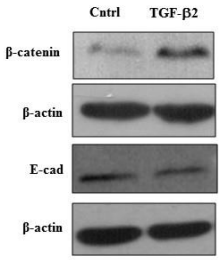


Fig. 4.1D

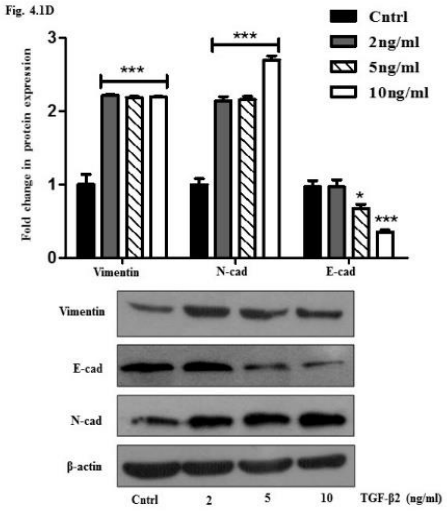


Fig. 4.1E

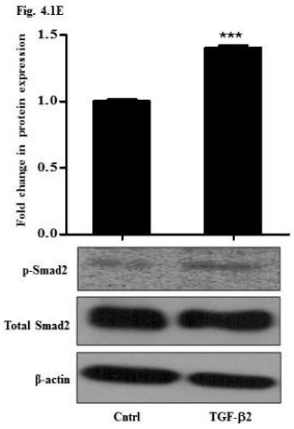


Fig. 4.1F

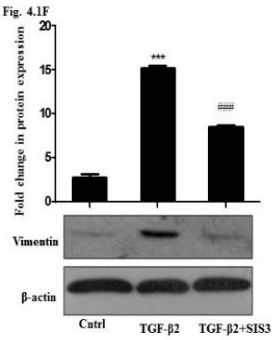


Figure 4.1. TGF-β2 induces EMT through SMAD signalling in Huh7 cells. *(A) Phase-contrast microscopic images of Huh7 cells post 48h of 5ng/ml TGF-β2 treatment; white circles indicate elongated cells. The scale bar represents 50μm, images taken at 40X. (B) Quantitative RT-PCR result showing expression of Vimentin, N-cadherin and E-cadherin after 48h of TGF-β2 (5ng/ml) treatment. (C) Immunoblot assay depicting expression levels of proteins like, Vimentin, N-cadherin, β-catenin and E-cadherin with and without addition of TGF-β2 (5ng/ml). (D) Immunoblot assay showing protein expression of Vimentin, N-cadherin and E-cadherin post TGF-β2 exposure for 48h at varied concentrations (2, 5, 10ng/ml). (E) Immunoblot assay showing an up-regulation of p-SMAD-2 after TGF-β2 treatment (5ng/ml) for 48h. (F) Immunoblot assay showing protein expression of Vimentin post p-SMAD-2 inhibition with SIS3 in TGF-β2 (5ng/ml; 48h) treated cells. SIS3 was added 1h before cytokine treatment. Expression in untreated control was taken as arbitrary unit "1". β-actin served as a loading control.*

4.2.2. TGF-β2 induced EMT is dependent on ROS levels

Reactive oxygen species (ROS)-induced signalling has often been linked to the diverse activities of TGF-β [225], [226]. TGF-β induced signalling can lead to redox unevenness through mitochondrial damage or by inhibiting cellular anti-oxidant activities. Recent evidences indicate that TGF-β and ROS can have opposing roles in pro- as well as anti-tumor effects [227],[228]. For these reasons, understanding the interplay between them is important for elucidating TGF-β –mediated effects in cancer progression. In this study, we observed that exposure of Huh7 cells to different doses of TGF-β2 led to a significant increase in intra-cellular ROS levels when compared to un-treated control as analyzed through H2DCF-DA fluorimetric assay (Figure 4.2A). Time kinetics analysis further showed significantly elevated levels of ROS after 48h and 72h of TGF-β2 (5ng/ml) treatment (Figure 4.2B). Interestingly, an increase in ROS was associated with TGF-β2-induced cytostatic effect. As compared to untreated control, significantly less number of viable cells was observed upon treatment with different doses of TGF-β2 for varied time points (Figure 4.2C and 4.2D). To further

verify that the reduced viable cell number is not due to cytotoxicity induced by TGF-β2, we performed apoptosis assay with AnnexinV/PI. TGF-β is reported to induce apoptosis in various cell types like, NIH3T3 and AML12 [229]. In our study, at the stipulated dose and time (5ng/ml; 48h), TGF-β2 failed to show an induction of apoptosis (Figure 4.2E). The conventionally used anti-cancer drug, cisplatin (35μM) was taken as a positive control. To further validate the cytostatic effect, cell cycle analysis was also performed using Propidium Iodide (PI) dye through flow cytometry after TGF-β2 exposure. A significant shift in number of cells in G1 phase of the cell cycle, indicative of arrest at G1/S juncture was observed establishing the cytostatic effect of TGF-β2 in the hepatocellular carcinoma cells studied (Figure 4.2F). An inhibition of ROS, with the widely used ROS quencher, NAC (N-acetyl cysteine) caused attenuation in ROS levels (Figure 4.2G) and significant reversal of cytostatic effect induced by TGF-β2 as analyzed by MTT assay (Figure 4.2H). An increased number of cells was also observed when Huh7 cells were exposed to both NAC and TGF-β2 when compared to only TGF-β2 treatment suggesting a rescue of the cytostatic effect upon quenching of TGF-β2-induced ROS (Figure 4.2I). This tempted us to investigate the role of ROS in TGF-β2-induced EMT. Interestingly a significant increase in EMT markers like N-cadherin and Vimentin was observed upon NAC-mediated quenching of TGF-β2-induced ROS (Figure 4.2J). These results suggest that generation of ROS was acting as a limiting factor attenuating EMT induction in Huh7 cells upon exposure to TGF-β2.

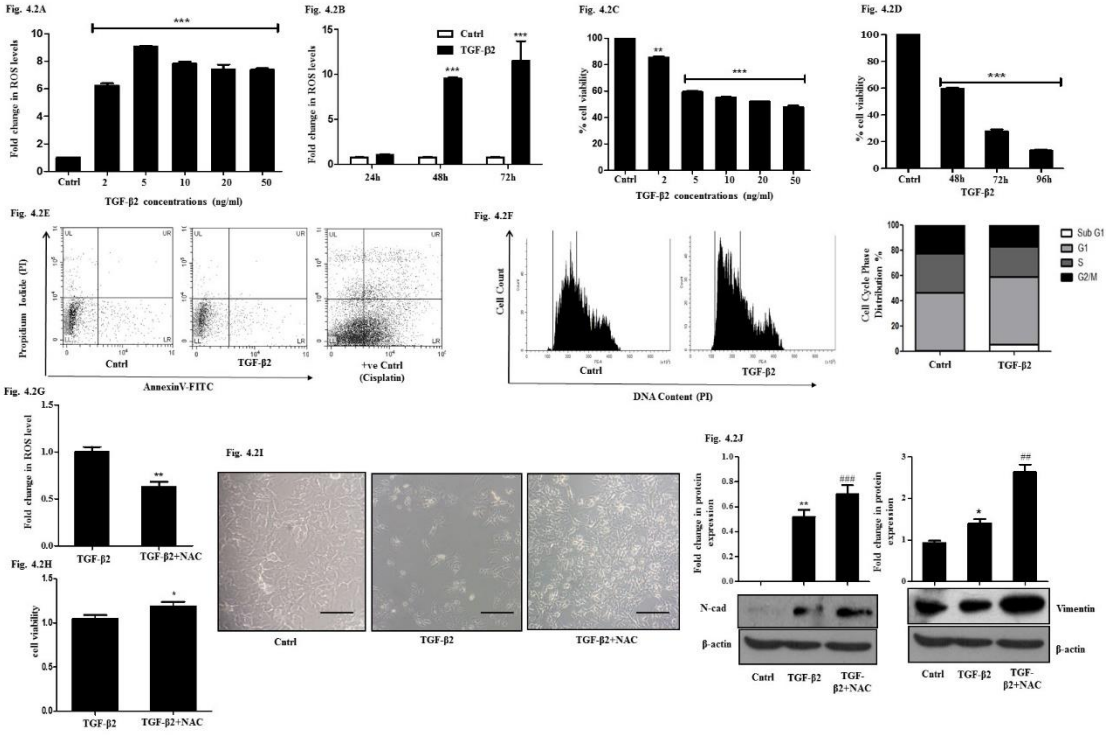


Figure 4.2. TGF-β2 induced EMT is ROS dependent. *(A) H2DCF-DA fluorimetric assay showing intra-cellular accumulation of ROS after treatment of Huh7 cells with TGF-β2 at the following concentrations (2, 5, 10, 20, 50 ng/ml; 48h). Untreated control was taken as arbitrary unit "1". (B) Bar graph showing time dependent increase in intracellular ROS accumulation in Huh7 cells after treatment with TGF-β2 (5ng/ml; 48h). (C) Viability of Huh7 cells measured through MTT assay upon exposure to varied doses of TGF-β2 for 48h. (D) Time dependent alterations in cell viability as analyzed through MTT assay upon treatment with TGF-β2 (5ng/ml). (E) Detection of apoptotic cells after TGF-β2-treatment (5ng/ml) for 48h, as analyzed through flow cytometry using Annexin V-FITC/Propidium Iodide (PI) staining. Cisplatin (35μm, 24h) treated cells were taken as positive control. (F) Cell cycle analysis of TGF-β2-exposed cells (48h) using PI dye by flow cytometry. A shift in G1 peak is marked by an arrow. Percentage of cells in each phase of cell cycle is represented by bar diagram. (G) Intracellular levels of ROS upon addition of NAC in TGF-β2 (5ng/ml) exposed Huh7 cells as analyzed through H2DCF-DA fluorimetric assay. (H) MTT assay showing percentage cell viability upon inhibition of ROS (5ng/ml) for 48h; 5mM of NAC was added 1h prior to TGF-β2 exposure (5ng/ml; 48h). (I) Phase-contrast microscopic*

images following TGF-β2 (5ng/ml, 48h) or TGF-β2 plus NAC treatment for 48h in Huh7 cells. The scale bar represents 50μm, images were taken at 40X. (J) Immunoblot analysis showing expression of N-cadherin and Vimentin upon quenching of ROS with NAC treatment.

4.2.3. TGF-β2 induced autophagy limits intra-cellular ROS levels

TGF-β has been previously reported to induce autophagy in various mammalian cancer cell types, extending from normal bovine mammary epithelial cells to mammary carcinoma cells in vitro [230]. Autophagy triggered, in turn can play a dual and paradoxical role either in tumor progression or suppression. It is known to mediate a cytoprotective phenomenon under nutrient deprivation [231], while, there are other evidences which document its role in promoting cell death [232]. The above observations prompted us to investigate whether autophagy is activated by TGF-β2 administration in Huh7 cells, and if so, what role it plays in such context. Exposure of Huh7 cells to TGF-β2 led to an increase in intracellular monodansylcadaverine (MDC) fluorescence indicative of autophagosome formation which was measured fluorometrically (Figure 4.3A). Microscopic analysis further revealed an increase in the number of MDC labelled spherical autophagic vacuoles distributed in the cellular cytoplasm, when compared to untreated control (Figure 4.3B). A significant increase in specific autophagic markers like, microtubule-associated protein light chain 3B-II (LC3B-II) and ATG5, indicative of enhanced autophagic activity was also observed by immunoblot analysis upon exposure to TGF-β2 (5ng/ml; 48h) (Figure 4.3C). Simultaneously, a decrease in p62, the turnover of which serves as a useful marker for induction of autophagy was obtained following exposure of Huh7 cells to TGF-β2 (Figure 4.3C). Since, the accumulation of autophagosomes is not always indicative of autophagy induction and may just represent either the increased generation of autophagosomes and/or a block in autophagosomal maturation, we hence checked for the difference in the amount of LC3B-II in the presence or absence of the lysosomal inhibitor, chloroquine di-phosphate (CQDP). The difference in the amount of LC3B-II in the presence and absence of CQDP represents the amount of LC3 that is delivered to lysosomes for degradation (i.e., autophagic flux) [228]. An increase in LC3B-II levels, indicative of autophagic flux, was observed in cells treated with CQDP plus TGF-β2

when compared to only CQDP-treated cells (Figure 4.3D). Considering the induction of autophagy and the context-dependent growth-inhibitory effect of TGF-β2, we were interested to investigate the role of autophagy. Interestingly, the inhibition of autophagy with CQDP caused a significant increase in intracellular ROS levels and also an associated shift in fluorescence of JC-1 dye indicative of a significant dip in mitochondrial membrane potential (Figure 4.3E and 4.3F). This was an important observation, given that we previously observed that ROS limits TGF-β2 induced EMT; however how, inhibition of autophagy and hence increased ROS effects EMT remained to be addressed.

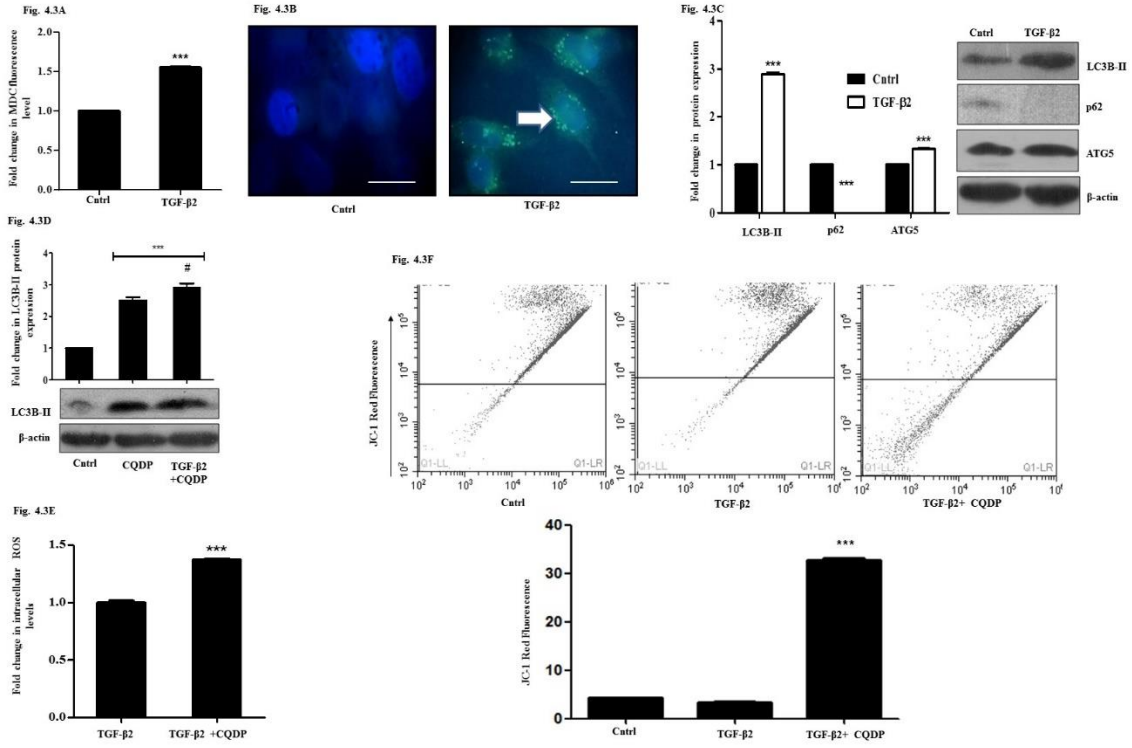


Figure 4.3. TGF-β2 exposure induces pro-survival autophagy in Huh7 cells. *(A) MDC fluorimetric assay showing autophagosome accumulation in Huh7 cells post 48h of TGF-β2 treatment (5ng/ml). (B) Fluorescent microscopy images of MDC fluorescence accumulation in TGF-β2-treated (5ng/ml, 48h) Huh7 cells. The scale bar represents 50μm, images were taken at 100X. (C) Immunoblot analysis showing expression of autophagic markers- LC3-BII, ATG5 and p62 in TGF-β2-treated (5ng/ml,*

48h) cells. Expression in untreated control was taken as arbitrary unit "1". β-actin served as loading control. (D) Determination of autophagic flux through immunoblot analysis of LC3B-II accumulation after TGF-β2 exposure (5ng/ml, 48h), in presence or absence of CQDP (10μM); the autophagy inhibitor was added 1h before addition of TGF-β2. The expression level of TGF-β2 was taken as arbitrary unit "1". (E) H2DCF-DA fluorimetric assay showing fold increase in ROS levels, upon inhibition of autophagy by CQDP (10μM) in TGF-β2-treated cells (5ng/ml, 48h). ROS level in only TGF-β2 treated cells was taken as arbitrary unit "1". (F) JC-1 fluorescence estimation by flow cytometry, showing a decrease in red fluorescence indicative of reduction of mitochondrial membrane potential after exposure to TGF-β2 (5ng/ml, 48h) and CQDP (10μM; added 1h before cytokine addition), compared to only TGF-β2.

4.2.4. TGF-β2 induced autophagy facilitates EMT by regulating ROS levels

Cancer cells are known to generate moderate to high levels of ROS to aid their need for enhanced proliferation, migration and metastasis; however, resistance to chemotherapeutic drugs can also be attributed to the higher intra-cellular levels of ROS in cancer cells. In contrary, increasing the level of ROS has often been utilized as a strategy to tip the balance of cancer cells from proliferation towards cell death. Under the above circumstances, the generation of enhanced intracellular ROS plays a pivotal role in initiation of cell death. In corroboration to above, in our study, inhibition of autophagy with CQDP resulted in an increased ROS and an associated cell death of Huh7 cells. Phase contrast images and cell viability analyzed through MTT assay provided evidences for the same (Figure 4.4A and 4.4B). To further validate the induction of cell death by autophagy inhibition, apoptosis was analyzed through AnnexinV/PI staining (Figure 4.4C). A significantly increased cell death was observed in cells treated with TGF-β2 and CQDP, compared to only TGF-β2. At 10μM concentration, for the studied time period, only CQDP treatment did not show a significant cell death. We speculate that TGF-β2 induced autophagy restricts intra-cellular ROS levels to go up substantially probably by clearing off damaged mitochondria; hence, an inhibition of lysosomal activity by CQDP results in a

significant boost in intra-cellular ROS levels triggering cell death. However further studies are required to confirm the hypothesis of mitophagy.

Interestingly, inhibition of autophagy with CQDP resulted in a significant decrease in EMT markers, as verified both at the RNA and protein levels (Figure 4.4D and 4.4E). Protein levels of Vimentin were significantly reduced upon inhibition of autophagic flux by CQDP (Figure 4.4E). Furthermore, p-SMAD2 levels were found to be significantly going down post CQDP treatment in TGF-β2 exposed Huh7 cells (Figure 4.4F). To further confirm that inhibition of autophagy has an effect on EMT, we transiently transfected Huh7 cells with siRNA against ATG5. In corroboration to above, knock down of ATG5 (Figure 4.4G) significantly reduced TGF-β2 induced Vimentin expression level (Figure 4.4H). The above results are conclusive of the fact that TGF-β2 induced autophagy facilitates EMT by suppressing and thus regulating intra-cellular ROS levels; with inhibition of autophagy ROS levels shoot up suppressing EMT as well as survival of Huh7 cells. To understand, what happens to autophagy levels if ROS is quenched, we checked for autophagic markers post NAC treatment. Interestingly, LC3B-II protein levels significantly went down upon addition of the ROS quencher (Figure 4.4I). This suggests that autophagy was instrumental in limiting ROS levels, and when intra-cellular ROS was quenched, autophagy was dispensable for EMT, as demonstrated in figure 2J. Although the role of TGF-β induced autophagy remains unclear in majority of cell types, and might be different in certain stages and aspects of tumor development, here we establish that autophagy supports TGF-β2 mediated cell survival and induction of EMT in Huh7 cells by controlling intra-cellular ROS.

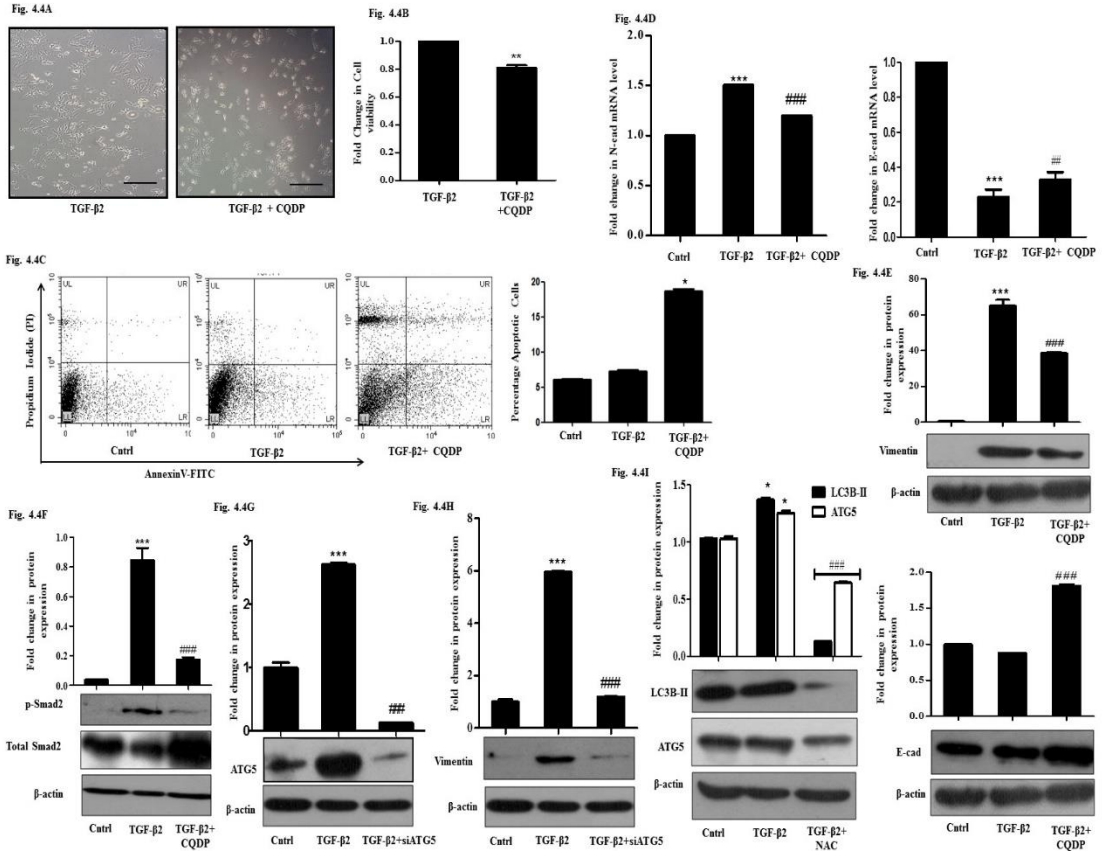


Figure 4.4. TGF-β2 induced autophagy suppresses ROS and promotes EMT. *(A) Phase contrast microscopic images showing increase in cell death upon inhibition of TGF-β2 (5ng/ml, 48h) induced autophagy with CQDP (10μM) addition. The scale bar represents 50μm, images were taken at 40X. (B) MTT assay showing percentage cell viability upon inhibition of TGF-β2 induced autophagy by CQDP addition prior to cytokine treatment. The percentage viability of cells after TGF-β2 treatment was taken as arbitrary unit "1". (C) Detection of apoptotic cells following addition of CQDP (10μM), 1h prior to TGF-β2 -treatment (5ng/ml, 48h) as analyzed through flow cytometry using Annexin V-FITC/Propidium Iodide (PI) staining. Percentage apoptotic cells are plotted in bar diagram. (D) Real time PCR analysis showing expression of N-cadherin and E-cadherin upon autophagy inhibition by CQDP (10μM) in TGF-β2-treated cells. (E) Immunoblot analysis showing Vimentin and E-cadherin protein expression upon autophagy inhibition by CQDP (10μM) in TGF-β2-treated cells. (F) Immunoblot analysis showing p-SMAD-2 protein expression upon inhibition of autophagy by CQDP (10μM) in TGF-β2-treated cells. (G) Immunoblot analysis*

showing knockdown of ATG5 in Huh7 cells transiently transfected with siATG5. (H) Immunoblot analysis showing protein expression of Vimentin upon inhibition of autophagy using siATG5. (I) Immunoblot analysis showing LC-3 and ATG5 protein expression upon inhibition of ROS by NAC. Expression in untreated control was taken as arbitrary unit "1". GAPDH expression was used as housekeeping control for RT-PCR and β-actin served as loading control for immunoblot.

4.2.5. TGF-β2 induced EMT is antagonized by TNF-α

Few studies have previously addressed the antagonistic activities exerted by pro-inflammatory cytokines and TGF-β. The opposing roles exerted by pro-inflammatory cytokines against TGF-β are known to play an essential role in context to maintaining tissue homeostasis and extracellular matrix deposition [233]. We therefore wondered whether the functionally opposing nature of these cytokines can represent a useful paradigm in the study of complex cellular signals regulating HCC pathogenesis. In this context, we explored the effect of TNF-α administration on TGF-β2 induced effects described above. TNF-α, generally released by activated macrophages, is a key player modulating inflammatory responses in the HCC tumor microenvironment [234]. Our findings reflect that Huh7 cells when exposed to TGF-β2 and TNF-α simultaneously, showed a significant reduction of TGF-β2 induced EMT features. A significant attenuation in mRNA expression of the EMT markers like, Vimentin and N-cadherin in TGF-β2 and TNF-α-treated samples was observed through RT-PCR (Figure 4.5A). Furthermore, immunoblot analysis also revealed a marked reduction in Vimentin, N-cadherin and β-catenin protein levels upon simultaneous exposure to TGF-β2 and TNF-α compared to only TGF-β2 treatment (Figure 4.5B). At the same time, E-cadherin expression substantially recovered in cells treated with TGF-β2 and TNF-α, when compared to only TGF-β2-treated cells (Figure 4.5B). Similar results were obtained at different time points studied where TNF-α antagonized TGF-β2 induced effects (Figure 4.5C and 4.5D). Immunoblot analysis showed a significant reduction in Vimentin and N-cadherin protein levels analyzed after 72h and 96h of TGF-β2 plus TNF-α exposure (Figure 4.5C and 4.5D). Moreover, a significant down-regulation of N-cadherin and Vimentin along with an up-regulation of E-cadherin in TGF-β2 and TNF-α-treated

samples, was observed, even after serum rescue emphasizing the fact that TNF-α-mediated antagonistic effects are serum independent (Figure 4.5E).

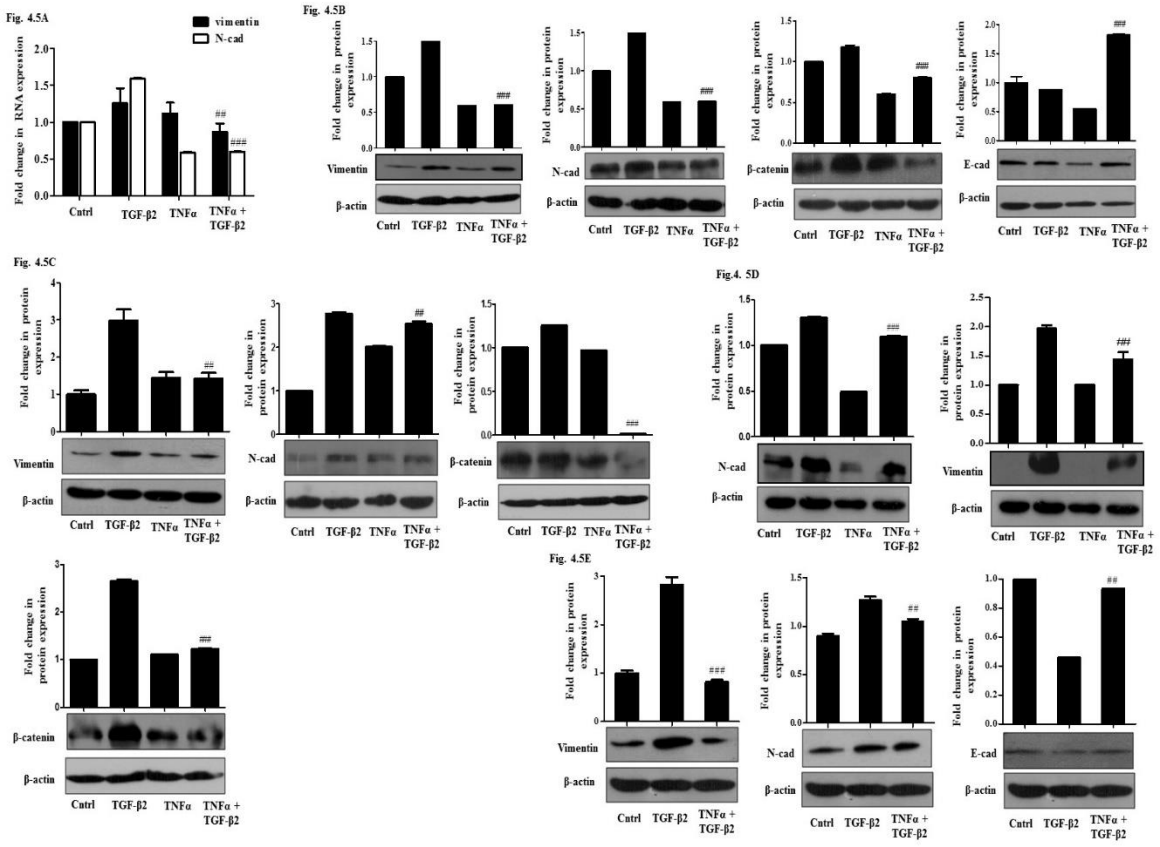


Figure 4.5. TNF-α suppresses TGF-β2 induced EMT. *(A) Real time PCR results showing expression of Vimentin and E-cadherin mRNA levels, upon simultaneous exposure of Huh7 cells to TGF-β2 (5ng/ml) and TNF-α (20ng/ml) for 48h. (B) Immunoblot analysis showing protein expression of Vimentin, N-cadherin, β-catenin and E-cadherin upon simultaneous exposure of TGF-β2 (5ng/ml) and TNF-α (20ng/ml) for 48h in Huh7 cells. (C) Immunoblot analysis showing protein expression of Vimentin, N-cadherin and β-catenin upon 72h of simultaneous exposure to TGF-β2 (5ng/ml) and TNF-α (20ng/ml) in Huh7 cells. (D) Immunoblot analysis showing protein expression of N-cadherin and β-catenin upon 96h of exposure to TGF-β2 (5ng/ml) and TNF-α (20ng/ml). (E) Immunoblot analysis showing protein expression of Vimentin, N-cadherin and E-cadherin upon exposure to TGF-β2 (5ng/ml) and TNF-α (20ng/ml) following rescue of serum with 10% FBS. Expression in untreated control was taken as*

arbitrary unit "1". GAPDH expression was used as housekeeping control for RT-PCR and β-actin served as loading control for immunoblot.

4.2.6. TNF-α inhibits TGF-β2 induced autophagy and SMAD signalling

More recently, existing studies have shown that several signalling pathways, activated in response to various stimuli, can lead to an increased expression of inhibitory SMAD-7, which, in turn, prevents TGF-β induced signalling [235]. Alternatively, specific cytokines have also been shown to directly interfere with SMAD-2/3 functioning resulting in similar effects. Therefore, we checked for SMAD-7 expression and activation of SMAD-2, upon exposure to TGF-β2 and TNF-α. Interestingly, we observed a striking increase in SMAD-7 levels and a stark decrease in phospho-protein levels of SMAD-2 upon simultaneous exposure of both the cytokines (Figure 4.6A). Additionally, an attenuation in protein expression of TGF-β2 induced autophagic markers- LC3B-II and ATG5 was also observed in TGF-β2 and TNF-α-treated samples suggesting that a reduction of EMT like features could probably be attributed to inhibition of autophagy by TNF-α (Figure 4.6B). Similar results depicting a significant reduction of LC3B-II were obtained when cells were exposed to TGF-β2 for prolonged time periods like, 72h and 96h as well (Figure 4.6C and 4.6D). LC3B-II levels were found to go down significantly even after serum rescue suggesting TNF-α-mediated autophagy inhibition is a serum independent effect (Figure 4.6E). Interestingly, a significant elevation in ROS levels was also observed when Huh7 cells were exposed to both TGF-β2 and TNF-α (Figure 4.6F). To explore the effect of increased ROS on fate of cells, we analyzed viability of cells upon exposure to TGF-β2 and TNF-α. This resulted in an increased cytotoxicity of Huh7 cells when compared to any of the cytokine exposure independently (Figure 4.6G). Collectively our results suggest that TNF-α can abrogate TGF-β induced metastatic spreading and proliferation of HCC cells by targeting autophagy signalling and regulating intra-cellular ROS levels.

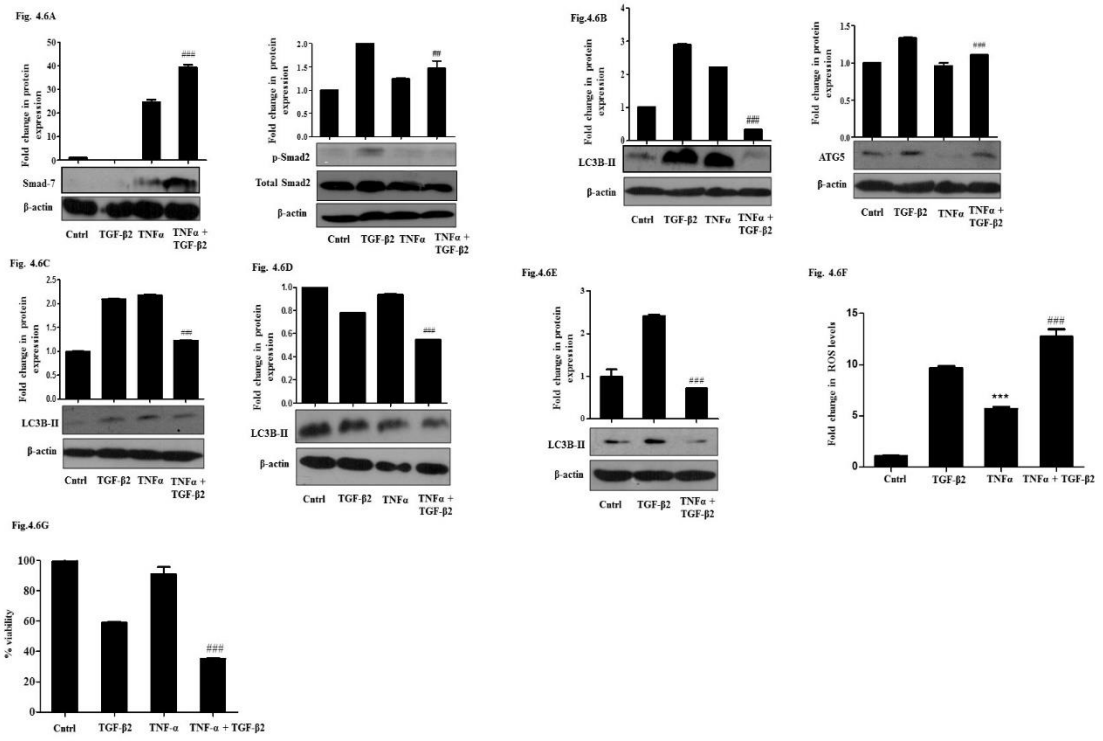


Figure 4.6. TNF-α antagonizes TGF-β2 induced signalling and autophagy. *(A) Immunoblot analysis showing SMAD7 and p-SMAD-2 protein expression in Huh7 cells exposed to TNF-α (20ng/ml, 48h) along with TGF-β2 (5ng/ml) for 48h. (B) Immunoblot analysis of autophagic markers- LC3B-II and ATG5 in the presence or absence of TNF-α (20ng/ml, 48h) along with TGF-β2 (5ng/ml). (C) Immunoblot analysis of LC3B-II post 72h of TNF-α (20ng/ml) and TGF-β2 (5ng/ml) treatment. (D) Immunoblot analysis of LC3B-II post 96h of TNF-α (20ng/ml) and TGF-β2 (5ng/ml) treatment. (E) Immunoblot analysis of LC3B-II upon simultaneous exposure to TGF-β2 (5ng/ml) and TNF-α (20ng/ml) for 48h when serum was rescued with 10% FBS. (F) H2DCF-DA fluorimetric analysis showing elevated ROS levels upon simultaneous exposure to TGF-β2 (5ng/ml) and TNF-α (20ng/ml) for 48h. (G) MTT assay showing percentage cell viability upon TGF-β2 (5ng/ml) and TNF-α (20ng/ml) treatment for 48h, when compared to independent cytokine treatment or untreated cell.*

4.3. Discussion and conclusion

HCC is one of the most prevalent cancers worldwide with a relative 5-year survival rate as low as 15% [223]. This authenticates the need for more studies related to the understanding of the disease. The tumor microenvironment is known to play a critical role in the complex etiology of HCC where an intricate interplay exists between the hepatocytes and surrounding cells [224]. Cytokines like, TGF-β and TNF-α are secreted in the HCC tumor milieu, which alongside other cytokines, growth factors and tumor infiltrating leukocytes invariably create a chronic inflamed state contributing significantly to the progression of the disease [224] . Hence, in this study we investigated the effect of the above cytokines in HCC cells and their functional co-relation with respect to disease progression. TGF-β, as a cytokine has a plethora of biological functions. TGF-β signalling is known to play a tumor suppressive function, attenuating cell growth and inducing apoptosis. On the contrary, TGF-β is one of the most powerful activator of EMT during tumor progression. TGF-β in late stages of cancers induces EMT, promoting cellular migration and anoikis resistance [225]. This complicates the biological understanding of the spectrum of its functional diversity encouraging more studies elucidating its role. Interestingly, autophagy seems to be amongst the wide plethora of cellular processes that is under the control of TGF-β. It is known to activate autophagy in both normal and cancer cells [226]. It has been observed that cancer cells exposed to TGF-β can result in a strong activation of autophagy marked by an increased expression of pro-autophagic genes. This effect is primarily facilitated by SMAD signalling. Functional exploration of TGF-β induced autophagy supports its role primarily in induction of apoptosis. Additional supportive evidences also emphasize on the fact that TGF-β induced autophagy inhibits metastatic progression of cancer cells [227]. However, TGF-β can probably trigger both pro-tumorigenic and anti-tumorigenic signals and the choice may be completely dependent on the cellular context and the stage of tumor progression [225]. Interestingly, in our results, we observed that TGF-β2 treatment led to simultaneous induction of cytostasis and EMT like phenotype in Huh7 cells. The cells utilized autophagy as a pro-survival strategy, as inhibition of it by CQDP or siRNA-mediated ablation of ATG5, abrogated EMT-like features. Interestingly, Yang et al in 2006 suggested that TGF-β, being a

pleiotropic molecule can not only induce EMT, but can also simultaneously induce other cellular processes like, apoptosis in mouse hepatocyte cells [228]. This endorses the fact that TGF-β can concurrently regulate multiple biological functions. In corroboration to above, we observed that pro-survival autophagy was required for the Huh7 cells to promote EMT features. Our results are in corroboration to Pang et al (2016) who showed that TGF-β can induce both autophagy and EMT in mouse tubular epithelial cells and inhibition of autophagy reduced TGF-β induced EMT [229]. We speculate that, during metastatic spreading, a massive reorganization of cellular interaction properties and loss of the adhesion, can render cancer cells without an effective anchorage thus inducing apoptosis. Under these circumstances, autophagy can come to the rescue and induce resistance to cell death. In our study, pre-treatment of Huh7 cells with CQDP or siRNA against ATG5, resulted in down-regulation TGF-β induced metastatic features, enhanced cytotoxicity and apoptotic cell death thus proving that autophagy was acting as a survival response facilitating EMT. Similar pro-survival role of autophagy has been observed before but it is completely context and cell type dependent [211]. Since, recent studies suggest that mitochondrial ROS are essential for TGF-β mediated gene expression [230], we explored ROS levels upon TGF-β exposure as well. Significantly increased ROS levels were observed in cells exposed to TGF-β. Interestingly, quenching of ROS by NAC resulted in an increased expression of EMT markers. In contrary, inhibition of autophagy resulted in a significant enhancement in ROS levels suggesting that TGF-β induced autophagy limits ROS facilitating EMT. We assume that this increase in ROS levels, upon autophagy inhibition, was the trigger to tip the balance of Huh7 cells from survival towards cell death. Previous report suggests that the synthesis of type-I collagen is regulated by cytokines especially, TGF-β [231], and the matrix-remodelling function of the pro-inflammatory cytokine, TNF-α is opposite to TGF-β [232]. We hence explored the effect of TNF-α addition on TGF-β induced effects in Huh7 cells. Interestingly, we observed that simultaneous exposure of TNF-α with TGF-β antagonized the function of later and attenuated TGF-β induced SMAD signalling, EMT and autophagy by regulating intracellular ROS levels (Figure 4.7).

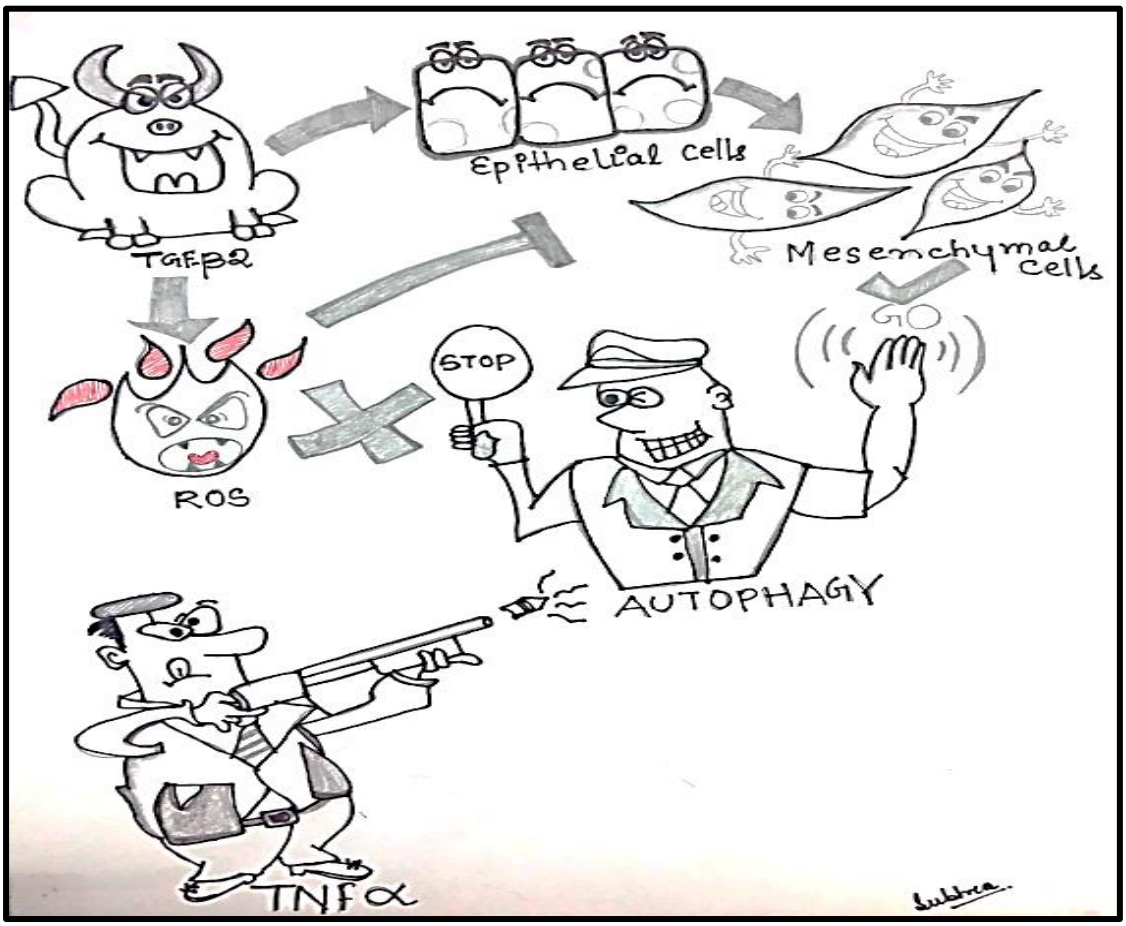


Figure 4.7. A schematic representation of the effect of TGF-β2 and TNF-α on Huh7 cells.

CHAPTER- 5

TGF-β2 mediated increased expression of mesenchymal markers in osteosarcoma cells is suppressed by TNF-α through regulation of autophagy

5.1. Introduction

The mixture of cytokines that is produced in the TME has an important role in cancer pathogenesis [71]. Infection, inflammation and immunity trigger the release of cytokines that can function to inhibit tumour progression. Though, it is completely context-dependent, in early stages of tumour formation cytokines might provide signals regulating cancer-cell growth, whereas later in tumour development, cytokine derived cues can modulate cancer-cell invasion and metastasis. Therefore, it becomes important to understand cytokine-tumour cell crosstalk that may open new horizons in area of cancer research and immunotherapy.

OS occurs eighth in general incidence among childhood cancers and has a confidence interval of about 95% with low survival rate of 30-75% making it the most common primary bone malignancy [191, 192, 236]. It is well known that one of the hallmarks of cancer is TME, rich in plethora of cytokines that contribute to tumour progression [10, 237]. However, their effects on OS pathogenesis have not been fully explored. The major cytokines present in the inflammatory TME of OS include, TNF-α, interferon-gamma (IFN-γ), interleukin-6 (IL-6), IL-8, IL-1 and TGF-β [238, 239]. These cytokines together activate osteoclastogenesis that leads to bone de-regulation, followed by bone resorption. During the process, factors that are trapped in the bone matrix, such as TGF-β, get released in the bone microenvironment [240]. TGF-β is well known for its ability to inhibit cell proliferation at early stages of tumour progression and stimulate invasiveness and more mesenchymal like features at later stages of tumour growth [241]. Because of its diverse effects on tumour growth and development, we intended to explore its role in OS progression. Similar to TGF-β, the pro-inflammatory cytokine, TNF- α has also been shown to have multi-dimensional effects. It is an acute response cytokine produced by cancer or stromal cells and is often associated with tumour development. It can activate NF-κB signalling and is also central to interactions between tumour cells and macrophages leading to increased invasiveness [242]. However, its role in migration of a mesenchymal type cell needs further understanding. Therefore, crosstalk between these cytokines regulating migratory properties of OS cells can open up new hope and possibilities in targeting OS pathogenesis.

Interestingly**,** while the role of apoptosis in tumour suppression is well known, the importance of other forms of cell death, like autophagy, is only recently becoming realized [107]. Autophagy being a highly conserved homoeostatic mechanism [212], has emerged as a critical pathway in both tumour development and therapy; although it's precise function remains a conundrum [243]. Current scenario is that autophagy has a dual role. On one hand, it can function as a tumour suppressor by degrading organelles, proteins and regulating ROS while, on the other hand it can act as a key survival mechanism in tumour development. Therefore, determining the contextual role of autophagy in OS becomes very critical [119].

Various environmental stimuli, cellular stresses and pathogen derived molecules, including cytokines promotes autophagosome formation. Particularly, cytokines like TNF-α, IFN-γ, interleukins and TGF-β all have been shown to promote autophagy independently, while some cytokines like IL-4 and IL-13 are inhibitory [212]. Recent studies suggest that TGF-β regulates autophagy in turn regulating critical aspects of kidney fibrosis in renal cells [138]. Additionally, another cytokine, TNF-α has also been shown to induce apoptosis and autophagy of cancer cells in a totally context dependent manner. High TNF-α level has shown to promote tumour progression while, death receptors of TNF family trigger apoptosis. This suggests that TNF-α can mediate balance between cell survival and death. This framework evokes the idea of targeting OS by regulating TGF-β and TNF-α-mediated action determining cell fate.

All these observations and links between cytokines, like TGF-β, TNF-α and autophagy have convincingly triggered our interest in exploring this area for future research. Herein, our study shows that TGF-β2 treatment in HOS cell line induced increased expression of mesenchymal markers like, Vimentin and N-cadherin at both RNA and protein levels. Inhibition of autophagy caused reduction in TGF-β2 induced SMAD signalling and mesenchymal traits. Furthermore, TNF-α antagonized TGF-β2 induced response by suppressing autophagy. Our study provides critical information on the role of cytokines in the pathogenesis of OS which can be useful for future therapeutic strategies targeting the disease.

5.2. Results

5.2.1. TGF-β2 mediated increase in mesenchymal markers is independent of ROS and senescence

One of the key features of OS is their ability to deregulate bone remodelling by disturbing the balance between bone resorption and formation. This imbalance inside the bone micro-environment by tumour cells persuades the release of numerous cytokines and growth factors entrapped in the bone matrix, such as TGF-β, which in turn can promote tumour progression [240]. TGF-β is reported to be one of the influential factors driving bone metastasis of breast cancer by regulating angiogenic factor- connective tissue derived growth factor and osteolytic factor interleukin 11 (IL-11) which are direct targets of TGF-β [244]. Keeping this in mind, we were interested in checking the role of TGF-β in HOS cells. As expected, Vimentin a key gene regulating mesenchymal phenotype is found to be significantly going up when HOS cells were exposed to TGF-β2 for 72h and 96h, compared to untreated control (Figure 5.1A). Furthermore, immunoblot analysis was also done to investigate the change in protein levels and a substantial elevation in mesenchymal protein markers like Vimentin and N-cadherin was seen (Figure 5.1B and 5.1C). TGF-β being a multifunctional cytokine is well reported to induce ROS dependent senescence in other cancer cell types, like liver cancer [245, 246]. To explore similar effects in OS, HOS cells were exposed to TGF-β2 at various concentrations and H2DCF-DA fluorimetric assay was performed to measure ROS. No significant accumulation of intracellular ROS was observed when compared to untreated cells, checked with and without addition of ROS scavenger NAC (Figure 5.1D). Additionally to assess the ROS mediated cytostatic effect of TGF-β2 in HOS cells a time and dose kinetic (5, 10, 20ng/ml) assay was also performed for 24h, 48h, 72h and 96h interestingly unlike an epithelial type cell type (Huh7), TGF-β2 failed to induce cytostasis in HOS cells, as investigated through MTT assay [132] (Figure 5.1E). Further, TGF-β2 mediated senescence in HOS cells was studied using β-galactosidase fluorimetric assay; TGF-β2 failed to induce senescence in HOS cells at 48h (Figure 5.1F). However, in Huh7 where ROS levels were found to be significantly going up, TGF-β2-mediated induction of

senescence alongside EMT [227, 247] (Figure 5.1G). This suggests that similar doses of the same cytokine TGF-β2 can mediate its effects through different intra-cellular mechanisms in HOS and Huh7 cells [20]. Taken together, these observations suggest that TGF-β2 can elevate mesenchymal markers in HOS cells in a ROS and senescence independent manner.

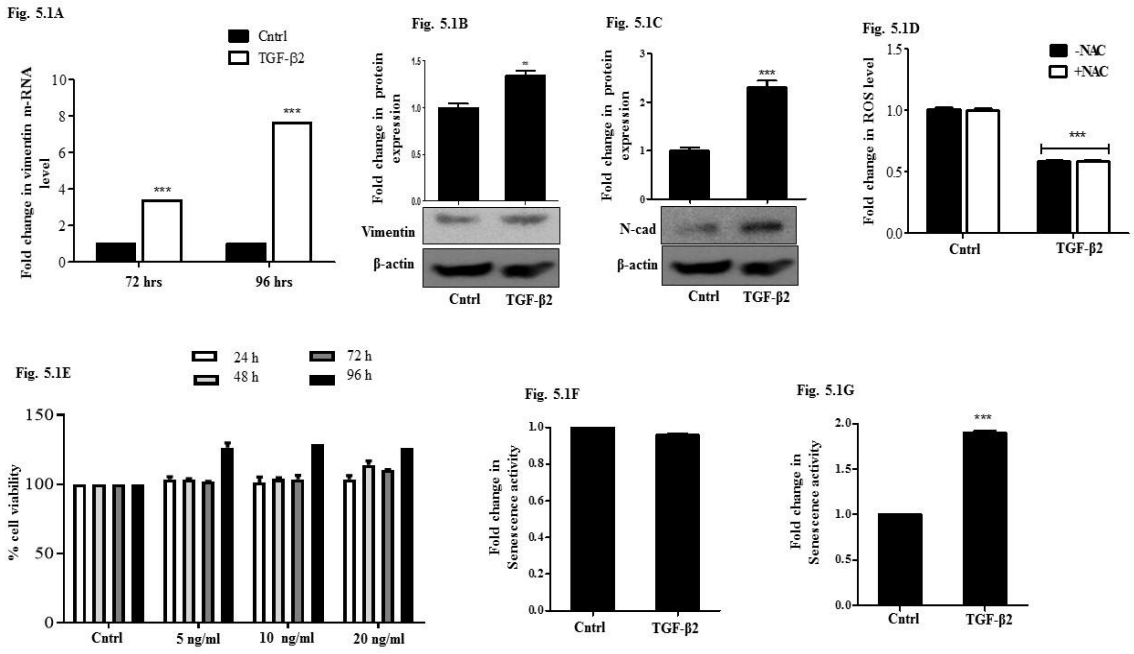


Figure 5.1. TGF-β2 mediated increase in mesenchymal markers is independent of ROS and senescence in HOS cells *(A) Real-time PCR showing expression of Vimentin after 72h and 96h of TGF-β2 (5ng/ml) exposure. (B) Immunoblot assay showing protein expression of Vimentin post 5ng/ml of TGF-β2 exposure. (C) Immunoblot assay showing protein expression of N-cadherin post 5ng/ml of TGF-β2 exposure. (D) Bar diagram showing H2DCF-DA fluorimetric assay with and without TGF-β2 and NAC treatment. (E) MTT assay showing percentage cell viability upon TGF-β2 treatment at varied concentrations for 48h, 72h and 96h. (F) Bar diagram representing β-galactosidase senescence assay done in HOS cells after TGF-β2 exposure for 48h. (G) Bar diagram representing β-galactosidase senescence assay done in Huh7 cells after TGF-β2 exposure for 48h. β-actin served as loading control for immunoblots and GAPDH as housekeeping control for RT-PCR.*

5.2.2. Autophagy is a regulator of TGF-β2 induced effects in OS cells

TGF-β family is known to regulate several fundamental traits of cellular behaviour. There is a more coherent understanding of the cytostatic and apoptotic effects induced by TGF-β in different cell types. However, molecular mechanisms governing TGF-β induced cellular responses like, autophagy are poorly understood. TGF-β is known to induce autophagy in various cancer cells like, human hepatocellular carcinoma [132] and MDA-MB-231 cells [215], whereas the link between TGF-β and autophagy in OS cells is least explored. In our study, TGF-β2 (5ng/ml for 48h) induced a substantial increase in autophagic markers, LC3B-II, and simultaneously, a significant reduction in p62 protein level, as analyzed through immunoblot (Figure 5.2A and 5.2B). Furthermore, connection between autophagy and mesenchymal features was investigated. Interestingly, inhibition of autophagy with CQ caused a significant reduction in protein levels of mesenchymal marker N-cadherin and interestingly, a gain of epithelial marker E-cadherin, suggesting a probable differentiation effect (Figure 5.2C and 5.2D).

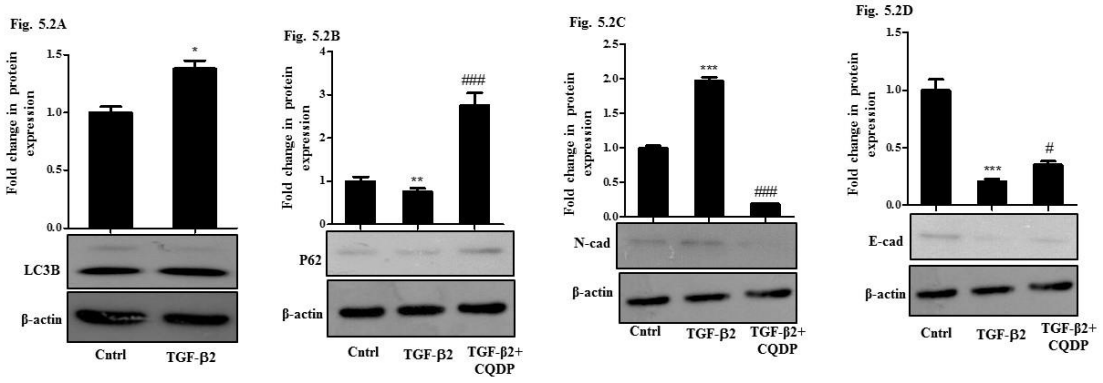


Figure 5.2. TGF-β2 mediated effects are regulated by autophagy in HOS cells *(A) Immunoblot analysis showing expression of autophagic marker LC3B-II in TGF-β2 treated (5ng/ml, 48h) HOS cells. Expression in untreated control was taken as arbitrary unit "1". (B) Immunoblot analysis showing expression of autophagic marker p62 in TGF-β2 treated (5ng/ml, 48h) and TGF-β2 plus CQ treated HOS cells. (C) Immunoblot analysis showing expression of mesenchymal marker N-cadherin in TGF-β2 treated (5ng/ml, 48h) and TGF-β2 plus CQ treated HOS cells. (D) Immunoblot analysis*

showing expression of epithelial marker E-cadherin in TGF-β2 treated (5ng/ml, 48h) and TGF-β2 plus CQ treated HOS cells.

5.2.3. TNF-α administration reverses TGF-β2 induced effects in OS cells

A reciprocal collaboration between tumour cells and the adjacent cells promote the commencement, advancement, metastasis and chemo-resistance of tumours [248, 249]. Till date the crosstalk between micro-environment and cytokines is relatively less explored and hence a thorough understanding is urgently required to address the irresponsiveness of different cancers to immunotherapy [250]. Taking this into consideration, we were interested in studying the crosstalk between TGF-β and the pro-inflammatory cytokine TNF-α in OS cells. HOS cells were treated with TGF-β2 (5ng/ml) / TNF-α (20ng/ml) or a combination of both for different time points. According to existing reports, co-treatment of TGF-β and TNFα in A549 cells accentuated the process of EMT by regulating IKKβ, while, in our previous study we observed a mutually antagonistic effect [98, 132]. This ambiguity prompted us to analyze the effects in OS cells. Interestingly in HOS cells a significant down-regulation in Vimentin was observed both at 72 and 96h, as evaluated through real time PCR (Figure 5.3A). At the same time immunoblot analysis also revealed a marked reduction in N-cadherin and Vimentin protein expression levels on simultaneous treatment of TGF-β2 and TNF-α (Figure 5.3B and 5.3C). This urged us to investigate any alteration in TGF-β2 induced intracellular signalling taking place in OS cells post TNF-α addition. Interestingly, a significant down-regulation in phospho-SMAD2 was observed, as analyzed through immunoblot (Figure 5.3D). Moreover, attenuation in TGF-β2 induced autophagic marker LC3B-II was also observed in TGF-β2 and TNF-α-treated samples. It is thus implied that TNF-α mediates decrease in mesenchymal features through down-regulation of autophagy (Figure 5.3E). Furthermore, simultaneous treatment of TGF-β2 and TNF-α showed an increase in cell death in HOS cells, compared to un-treated control and TGF-β2 exposure alone (Figure 5.3F). Taken together these observations provide clues on how to control TGF-β induced

dissemination of HOS cells by an antagonistically functioning cytokine through regulation of autophagy.

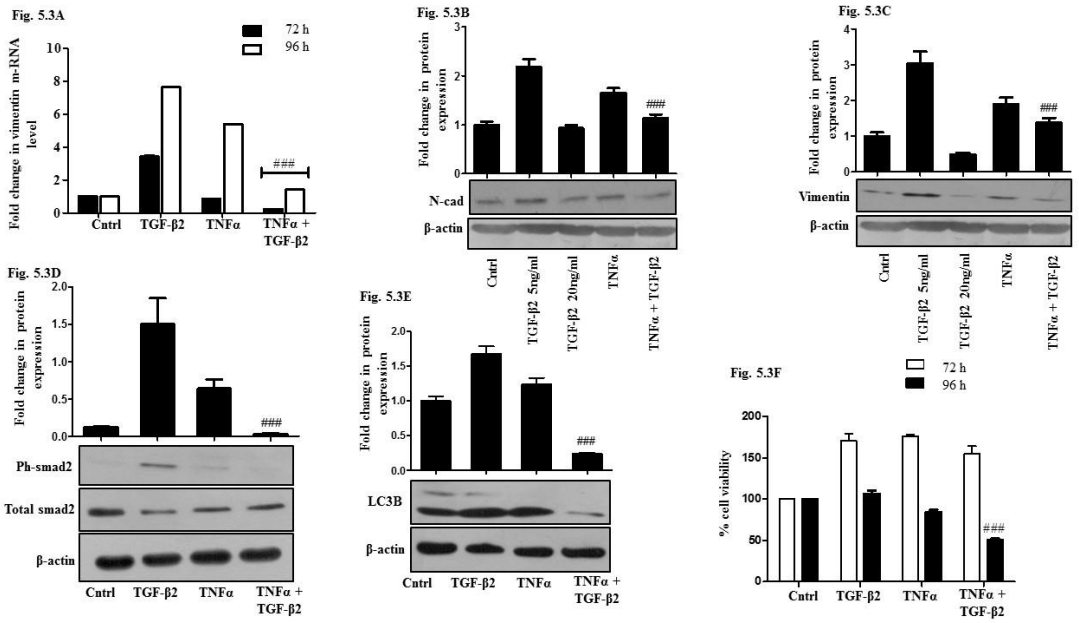


Figure 5. 3. TNF-α administration reverses TGF-β2 induced effects in HOS cells *(A) Real-time PCR showing expression of Vimentin upon simultaneous exposure of HOS cells to TGF- β2 (5ng/ml) and TNF-α (20ng/ml) or a combination of both for 72h and 96h. (B) Immunoblot analysis showing protein expression of N-cadherin upon simultaneous exposure of TGF-β2 (5ng/ml) and TNF-α (20ng/ml) for 48h in HOS cells. (C) Immunoblot analysis showing protein expression of Vimentin upon simultaneous exposure of TGF-β2 (5ng/ml) and TNF-α (20ng/ml) for 48h in HOS cells. (D) Immunoblot analysis showing phospho-SMAD2 expression upon simultaneous exposure of HOS cells to TGF-β2 (5ng/ml) and TNF-α (20ng/ml). (E) Immunoblot analysis showing expression of LC3B-II upon simultaneous exposure to TGF-β2 and TNF-α in HOS cells. (F) MTT assay showing percentage cell viability upon TGF-β2 (5ng/ml) and TNF-α (20ng/ml) treatment for 72h, when compared to independent cytokine treatment or untreated cells.*

5.3 Discussion and conclusion

OS is the third most frequent cause of cancer among adolescents and represents over 56% of all the bone tumours [243, 244]. Compounding the problem, the molecular

mechanism underlying OS pathogenesis is poorly understood. The microenvironment of bone is very unique in providing a support for cancers to flourish. Many growth factors and cytokines such as TGF-β and TNF-α along with other growth factors are embedded in the mineralized bone matrix which are released and activated upon tumour-induced osteoclastic bone resorption [245]. This categorizes the above cytokines as crucial factors which are responsible for prompting the feed-forward malicious cycle of tumour growth in bone. Therefore, in this study we have focused on the role and interplay of the above cytokines and subsequently investigated the potential use of targeting them, leading to a promising strategy to treat OS cells. TGF-β acts as a homeostatic perpetuator in tumours by managing the proliferation and inter-communication within the microenvironment comprising cell types of different cellular origin and function [246]. Previous studies further suggest that in later stages of tumour development TGF-β can aggravate metastasis leading to cellular migration and anoikis resistance through increase in expression of mesenchymal cytoskeleton markers like, fibronectin and Vimentin [132, 235, 247]. Toshiyuki et al 2005, showed that TGF-β is high in culture supernatants of resorbing bones that promotes the production of PTH-rP, a cytokine playing a central role in bone cell migration [248]. This shows the diversified function of TGF-β and the predominant role played by it in bone micro-environment, encouraging more studies elucidating its role. Interestingly, some emerging data advocates that the microenvironment of tumours undergo an AST, which leads to the activation of the TGF-β pathway facilitated by SMAD signalling in fibroblasts [249]. Interestingly, in our results, we observed that TGF-β2 treatment led to induction of enhanced mesenchymal phenotype in HOS cells though cytostasis or senescence was not associated with it, as was observed in our previous study on epithelial type Huh7 cells [132]. HOS cells employed autophagy as a migration protagonist as treatment with CQ partly abrogated mesenchymal features induced by TGF-β2. Therefore, our study indicates that autophagy is a requisite for the HOS cells to promote enhanced mesenchymal features. Similar role of autophagy, in migration has been observed before but it is completely context and cell type dependent [211].

Our previous work suggests that, TGF-β2-induces autophagy facilitating EMT by regulating ROS levels in Huh7 cells [132]. Strikingly, in the mesenchymal type cell,

TGF-β2 failed to induce ROS. One of the reasons for the differential effect in HOS cells could be a pre-dominantly higher pre-disposition of OS cells to TGF-β in OS microenvironment [250]. However, this opens up a new area for research in deciphering the mechanism underlying the regulation of TGF-β mediated effects in mesenchymal type cells.

Previous studies suggest that, TNF-α antagonizes the effects of TGF-β2-induced by down-regulating autophagy and inhibiting EMT in HCC cells [132, 232]. Also, TGF-β mediated inhibition of cytotoxic T cell development is reported to be suppressed by TNF-α production in mixed Lymphocyte Cultures [251]. This strong opposing effect of TNF-α on TGF-β induced effect in epithelial cell type drew our interest in investigating the same on mesenchymal cell type as well. Our results corroborates previous findings where TNF-α inhibits TGF-β induced SMAD signalling and its downstream effects [20]. A significant decrease in mesenchymal cell type specific markers was observed on simultaneous exposure of TNF-α and TGF-β.

Several reports till date have shown that cancer cells in the bone microenvironment activates osteoclastic bone resorption, which further results in the secretion of active TGF-β [252, 253]. TGF-β then bestows the tumour cells with enhanced mesenchymal like properties. The net result is tumour progression as well as osteolytic bone metastasis. Therefore, blockade of TGF-β signalling or neutralization of TGF-β induced effects can be an effective way to decrease mesenchymal phenotype. In this regard, several strategies have been employed so far but, the use of antagonistically functioning cytokines to reduce TGF-β induced effects in OS cells has not been explored. In this regard, our study provides a novel and promising alternative for the treatment of OS.

CHAPTER- 6

Transcriptomic analysis depicting TNF-α mediated antagonism of TGF-β2 induced effects

6.1. Introduction

With recent advancement in research the demand for high throughput sequencing using RNA-Seq has gained interest [251]. RNA-Seq has a wide range of application including the identification of nucleotide variations, methylation patterns, differentially regulated gene, signalling pathways involved in pathogenesis [252]. Additionally, RNA-Seq has several advantages over microarrays mainly the accuracy and reproducibility of data makes the experiment less tedious by reducing the number of technical replicates. Besides, this also allows the identification and quantification of novel transcripts and isoforms [253, 254]. RNA-Seq mainly adopts five simple steps which start with the fragmentation of RNA and conversion into complementary DNA sequences (cDNA). Second step includes the mapping of these small fragments to the reference genome. Followed by the estimation of expression levels for each gene and normalization of mapped data using available software's to identify differentially expressed genes (DEGs). Finally, the authenticity of the data generated is evaluated from a biological perspective [255, 256].

As mentioned in previous chapters, TGF-β is considered as a pleotropic cytokine due to its involvement in various patho-physiological processes, till date three functional isoforms of TGF-β are identified in mammals [257]. The dual role of TGF-β in cancer; as a tumor suppressor in initial stages of carcinogenesis and a tumor promoter during metastasis has made it a topic of debate [258]. However, a clear understanding of the responsible factors is still missing which makes this study more attractive [259]. The transcriptomic profiling of TGF-β signalling in context to EMT is well studied and is found to significantly high in breast cancer cell, bone, brain and skin tumors, whereas endometrium and colorectal cancers showed lesser expression [260]. Existing literature have also shown, the global transcriptomic profile of TGF-β intervened gene regulation in normal and transformed HPL1D and A549 cells highlighting the non-canonical regulation of TGF-β [259].Furthermore, when lung adenocarcinoma cell lines (A459 and H358) were exposed to TGF-β, 137 genes involved in EMT were found to be up-regulated and 32 downregulated common to both cell lines. These findings help in validation of TGF-β as an EMT inducer providing novel insights in understanding cancer pathogenesis [261].

Gathering above evidences suggest, the role of TGF-β in EMT is well explored using micro-array and RNA-Seq techniques. Whereas, its role in regulation of another critical pathway "autophagy" and its crosstalk with other cytokines is least explored. Therefore, here we are presenting a comprehensive review of two antagonistically acting cytokines; TGF-β2 and TNF-α and their role in regulation of cellular events like EMT and autophagy using RNA-Seq as a tool.

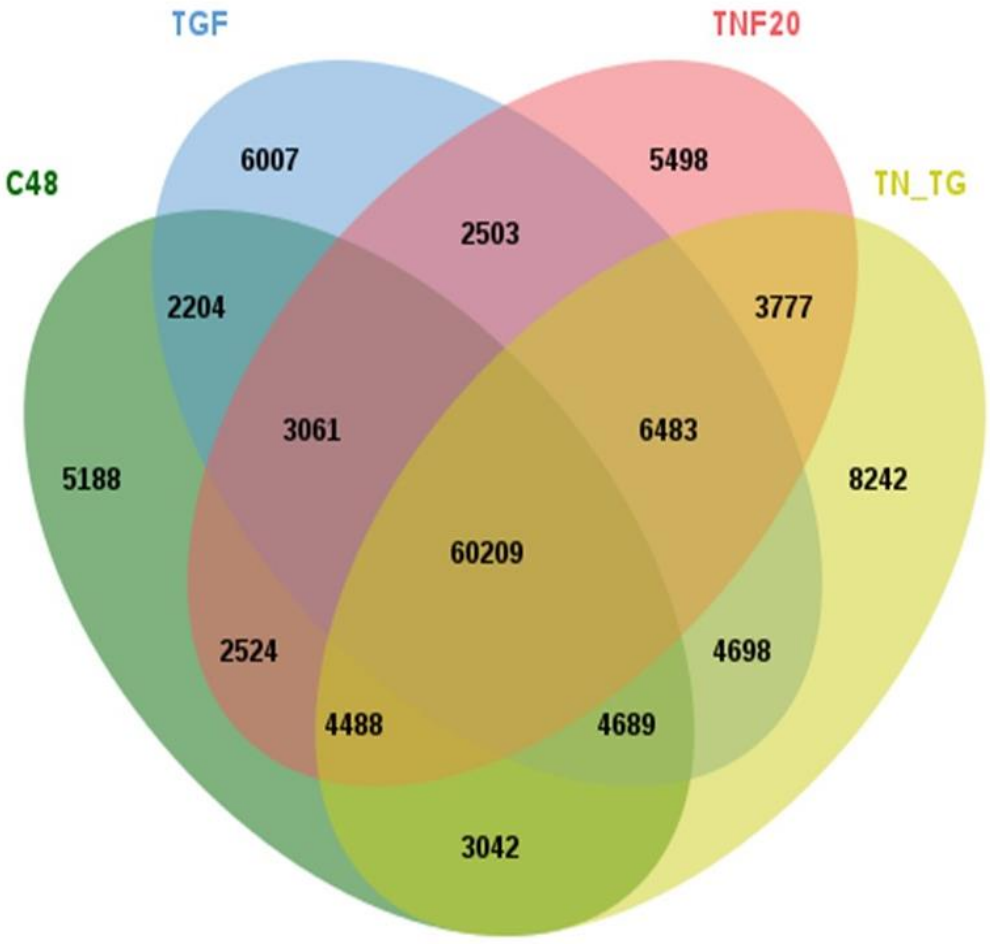
6.2. Results

6.2.1. Transcriptomic analysis depicting TNF-α-mediated antagonism of TGF-β2-induced effects

Illumina paired end reads (150*2) were generated for untreated control, TGF-β2, TNF-α and TGF-β2 plus TNF-α exposed Huh7 cells and reference based transcriptome analysis was carried out. An average of 91.7% of the reads were aligned to the reference genome. Approximately, 89K transcripts covering ~22K genes were found to be expressed across all the samples. On an average, approximately 32% of the transcripts were differentially regulated in cytokine-treated samples of which 6.2K transcripts were found to be uniquely expressed and 6029 transcripts were found to be common across all the samples, which is represented in the form of a Venn diagram (Figure 6.1A). Top 40 de-regulated transcripts in TGF-β2 compared to control, and in TGF-β2 plus TNF-α compared to TGF-β2-exposed samples are represented in the form of a Heatmap (Figure 6.1B and 6.1C). The key genes regulating EMT, like, N-cadherin, Vimentin and β-catenin were found to be significantly up-regulated in TGF-β2-treated samples when compared to control with a log2fold change value of 2.28, 1.10281 and 1.15 respectively. Substantiating our previously observed results, the expression of N-cadherin, Vimentin and β-catenin were either neutrally regulated or significantly down-regulated in TGF-β2 plus TNF-α treated samples (N-cadherin-0.092569, Vim-0.174, β-catenin-0.98). Also, in corroboration to results described earlier, E-cadherin and SMAD7 were found to be drastically going up with log2fold change values of 2.14 and 6.4 respectively following TGF-β2 plus TNF-α exposure in comparison to only TGF-β2-treated samples. To have a holistic idea on transcriptomic alterations associated with intracellular signalling, pathway enrichment analysis was performed. Amongst the top

pathways that were severely de-regulated post exposure to cytokines, we observed that pathways related to autophagy signalling, lysosome and protein degradation machinery were the ones that were pre-dominantly altered (Figure 6.1D). This is suggestive of the fact that there is a significant alteration of intra-cellular material or protein turn-over post exposure to cytokines which might determine cellular fate. Importantly, in accordance to earlier results, a significant down-regulation of autophagic genes was observed in TGF-β2 plus TNF-α-treated samples when compared to only TGF-β2-exposed cells. The log2fold change in some of the key autophagy-related genes in response to cytokines is represented in the form of a graph (Figure 6.1E). Two key genes involved in autophagy which were found to be significantly down-regulated in TGF-β2 plus TNF-α-treated samples included, ATG16L1 and WIPI2. ATG16L1 along with other ATGs are some of the final ATGs recruited that determines the site of LC3-II formation; LC3 family of proteins are required for phagophore expansion, closure, and cargo recruitment. Whereas, WIPI2 acts immediately upstream of ATG16L1 and is responsible for ATG12–5-16L1 recruitment to omegasomes (autophagosomes arise from endoplasmic reticulum-derived omegasomes), resulting in LC3 lipidation and subsequent autophagy. Furthermore, KEGG autophagy pathway graph rendered by Pathview also shows an up-regulation of genes (in red) (Figure 6.2A) involved in autophagic pathway in TGF-β2-treated samples compared to control; however, the same genes did not show a significant up-regulation in TGF-β2 plus TNF-α-treated samples compared to only TGF-β2 (Figure 6.2B). Overall, the transcriptomic analysis authenticates and emphasizes on the up-regulation of autophagy supporting TGF-β2-induced EMT and attenuation of the same upon addition of TNF-α in Huh7 cells; we also provide valuable information on critical genes that are key to regulation of autophagy and EMT upon cytokine exposure.

Fig. 6.1A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C48
TN_TG
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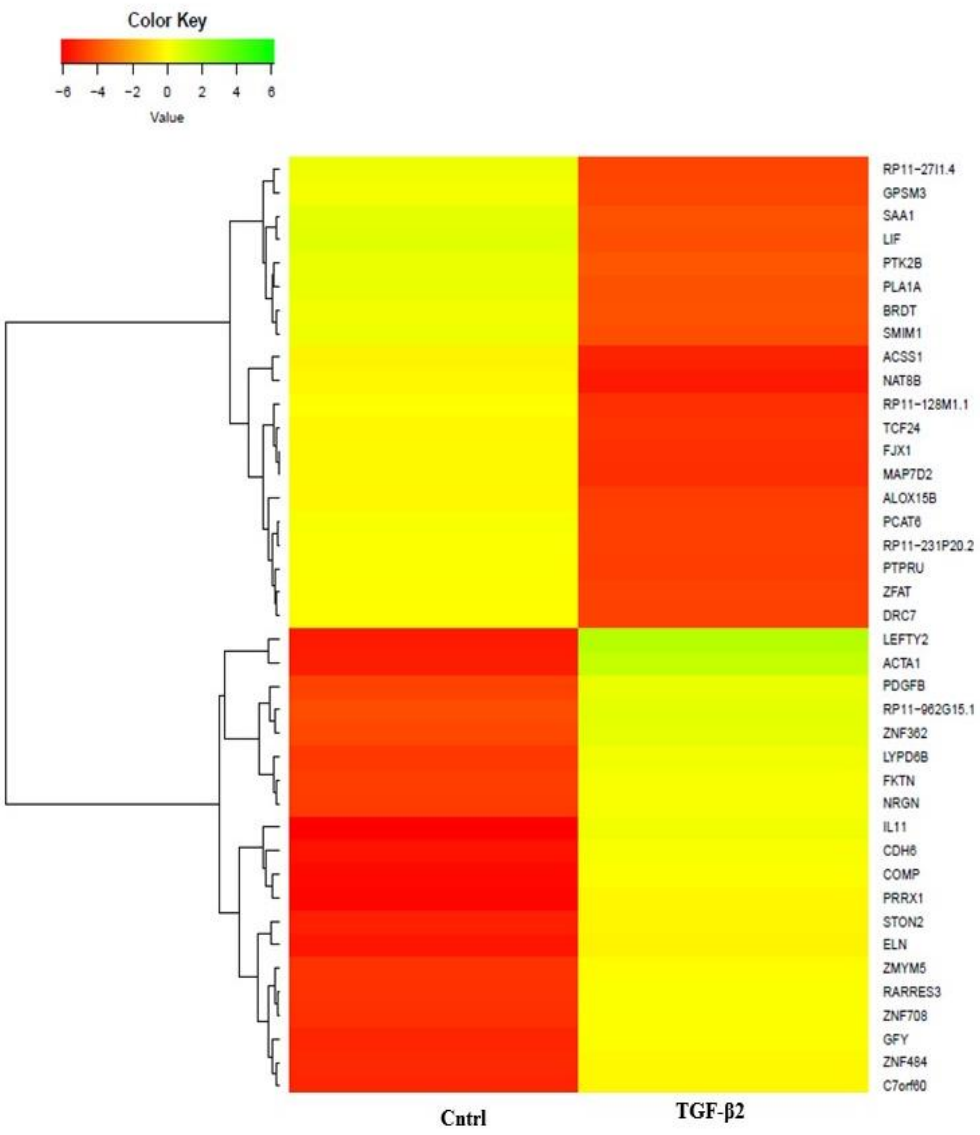
Fig. 6.1B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MAP7D2
ALOX15B
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PTPRU
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DRC7
LEFTY2
ACTA1
PDGFB
RP11−962G15.1
ZNF362
LYPD6B
FKTN
NRGN
IL11
CDH6
COMP
PRRX1
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RARRES3
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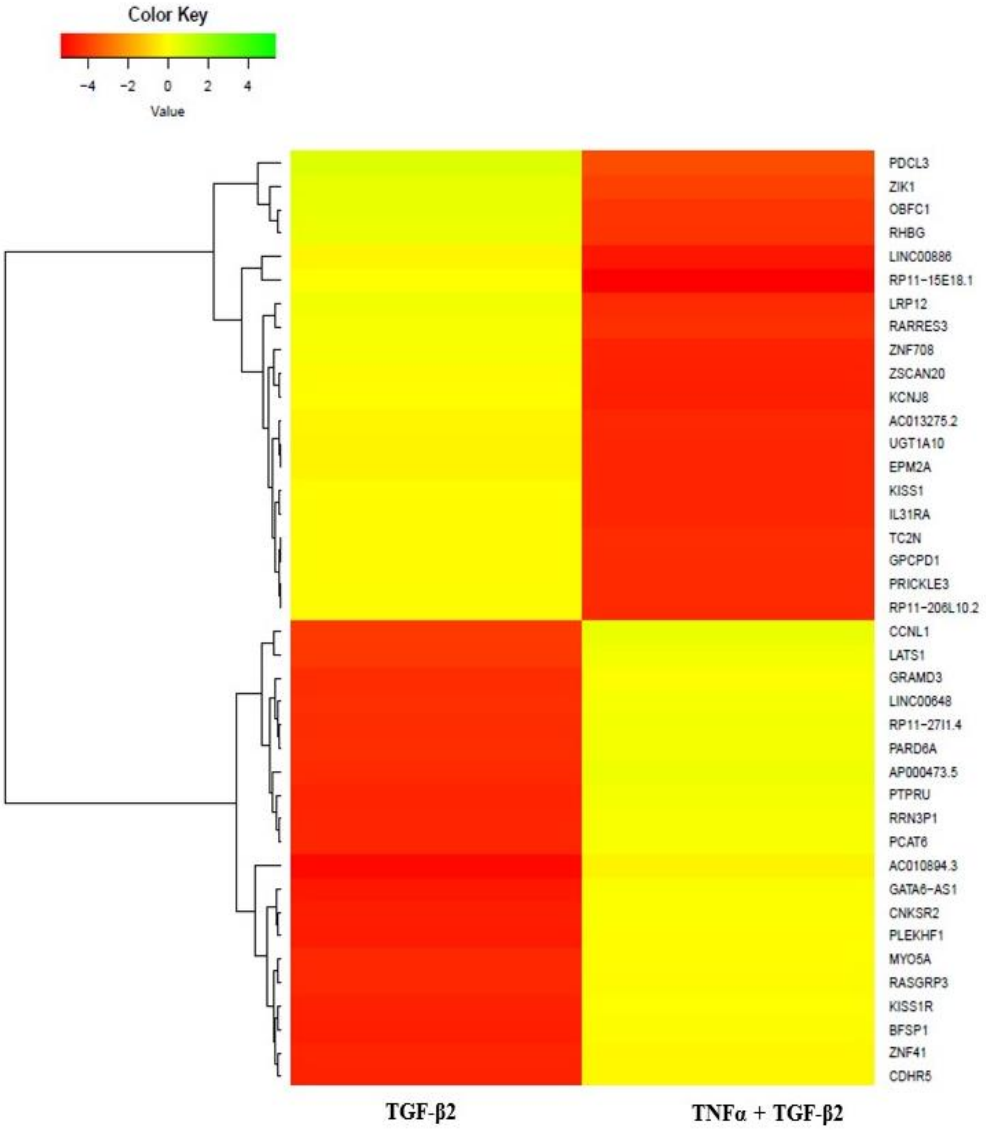
GFY
ZNF484
C7orf60
Cntrl
TGF-β2

Fig. 6.1C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UGT1A10
EPM2A
KISS1
IL31RA
TC2N
GPCPD1
PRICKLE3
RP11−206L10.2
CCNL1
LATS1
GRAMD3
LINC00648
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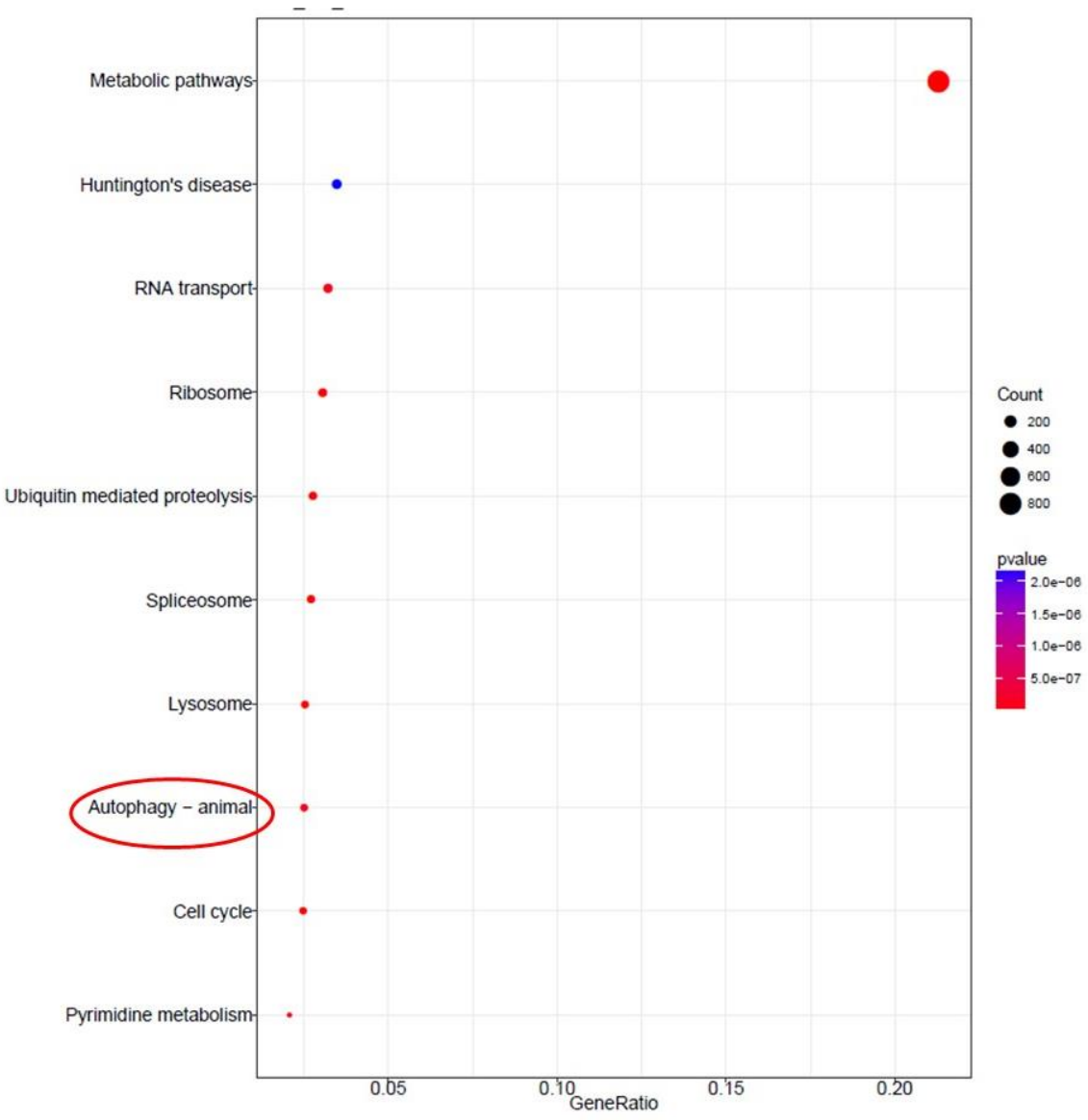
PARD6A
AP000473.5
PTPRU
RRN3P1
PCAT6
AC010894.3
GATA6−AS1
CNKSR2
PLEKHF1
MYO5A
RASGRP3
KISS1R
BFSP1
ZNF41
CDHR5
TGF-β2
TNFα + TGF-β2

Fig. 6.1D


Metabolic pathways
Huntington's disease
RNA transport
Ribosome
Ubiquitin mediated proteolysis
Spliceosome
Lysosome
Autophagy – animal
Cell cycle
Pyrimidine metabolism
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0.10
0.15
0.20
GeneRatio
Count
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800
pvalue
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1.0e−06
5.0e−07

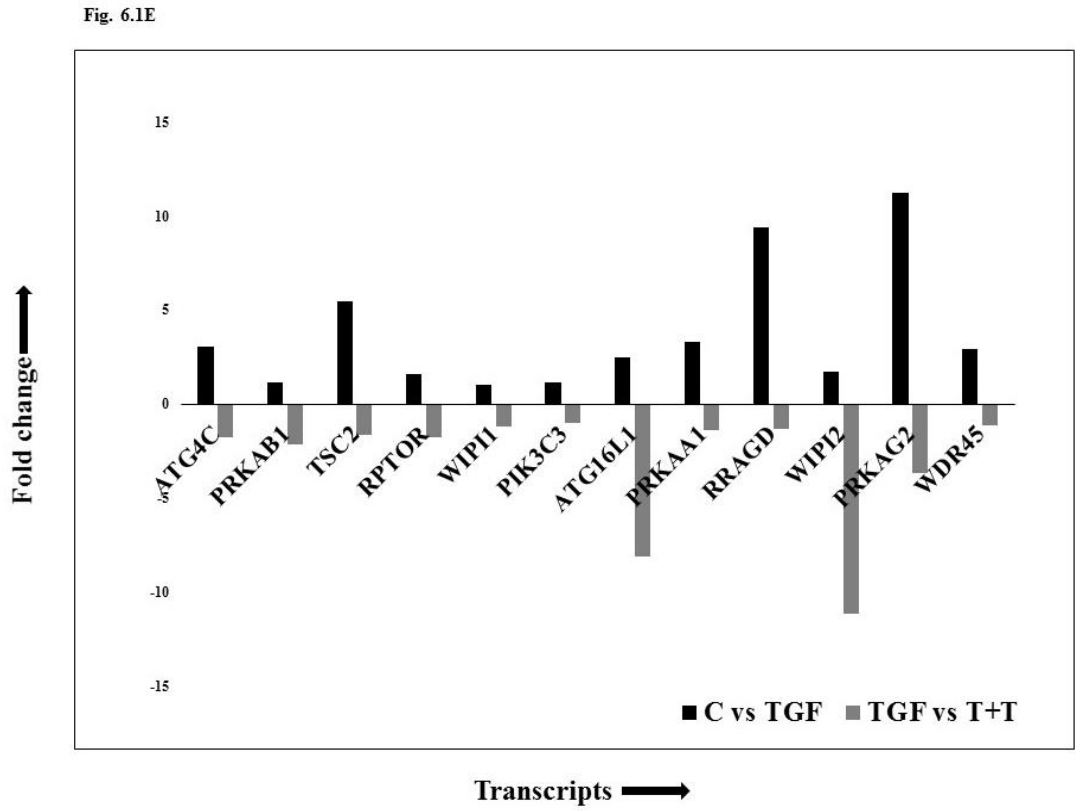
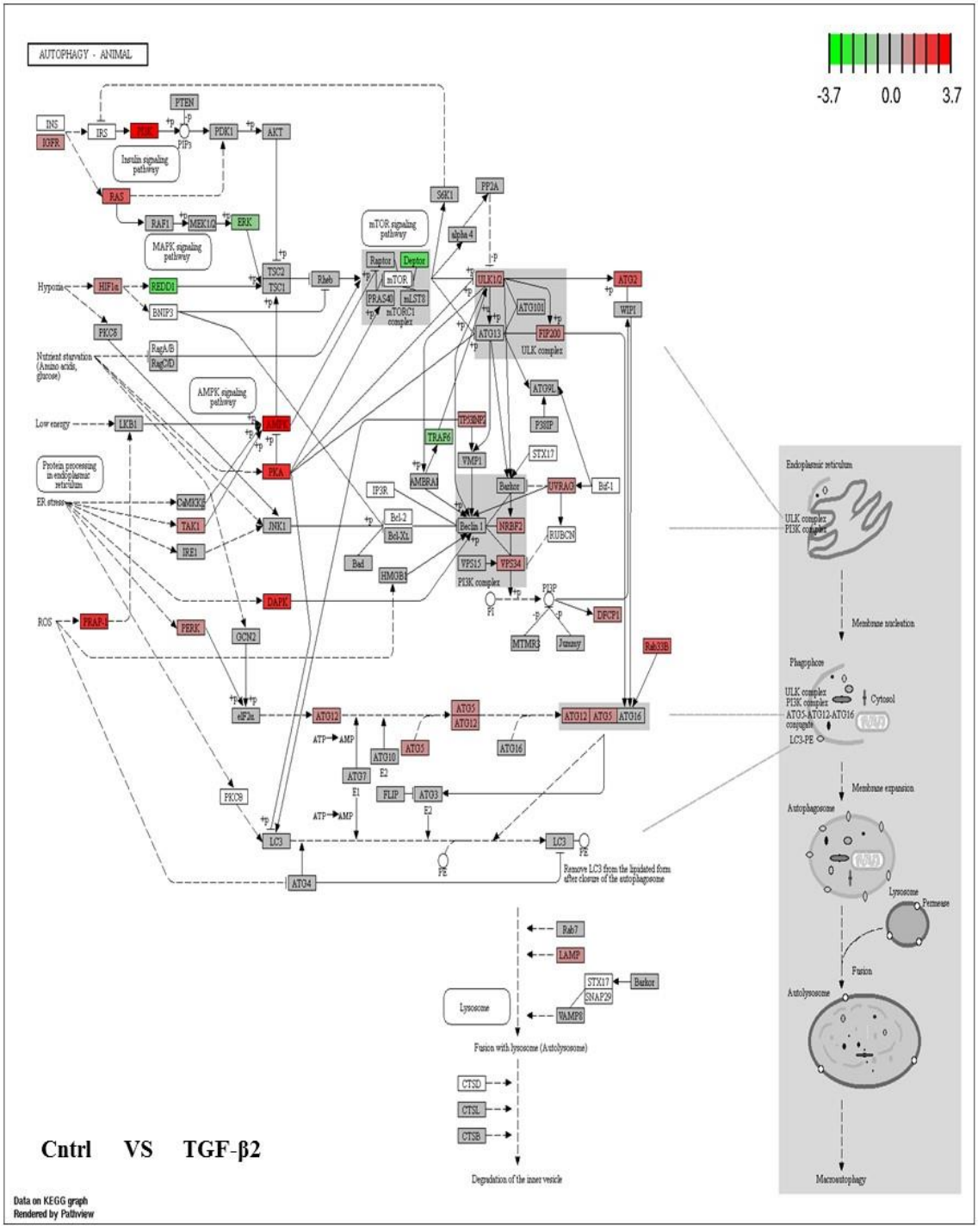


Figure 6.1. Transcriptomic analysis showing TNF-α-mediated antagonism of TGF-β2 induced effects. *(A) Venn diagram showing total number of transcripts and common transcripts across four samples sequenced- untreated control (C48), TGF-β2 (5ng/ml; 48h), TNF-α (20ng/ml; 48h) and TGF-β2 (5ng/ml; 48h) plus TNF-α (20ng/ml; 48h). (B) Heatmap demonstrating top 40 de-regulated transcripts in TGF-β2 (5ng/ml; 48h) treated samples when compared to untreated control; (C) and in TGF-β2 (5ng/ml; 48h) plus TNF-α (20ng/ml; 48h) treated samples when compared to only TGF-β2 segregated based on their log2 fold change values. (D) Pathway enrichment analysis showing the top 10 pathways getting de-regulated post TGF-β2 treatment. (E) Graph showing up or down regulation of transcripts involved in autophagy, upon exposure to TGF-β2 (5ng/ml; 48h) in comparison to untreated control; and in TGF-β2 (5ng/ml; 48h) plus TNF-α (20ng/ml; 48h) treated samples when compared to only TGF-β2.*

Fig. 6.2A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3.7
Cntrl VS TGF-β2
Data on KEGG graph
Rendered by Pathview

Fig. 6.2B

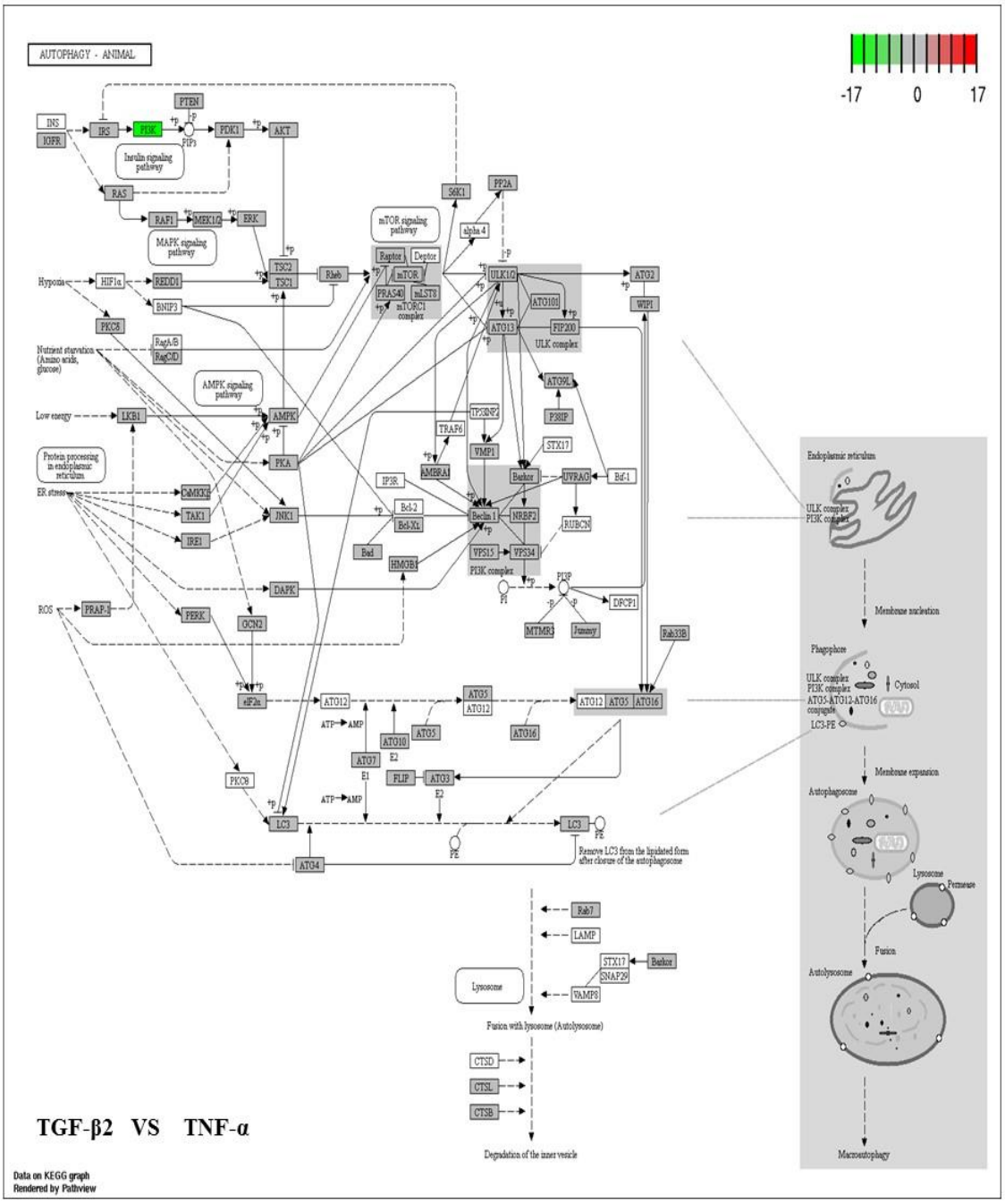
AUTOPHAGY - ANIMAL
-17
0
17
TGF-β2 VS TNF-α
Data on KEGG graph
Rendered by Pathview

Figure 6.2. KEGG pathway graph rendered by pathview showing major genes involved in TGFβ-2 induced autophagy. *(A) KEGG pathway showing autophagy genes up or down regulated in TGF-β2 (5ng/ml; 48h) treated samples with respect to control, (B) and in TGF-β2 (5ng/ml; 48h) plus TNF-α (20ng/ml; 48h) treated samples compared to only TGF-β2 (5ng/ml; 48h).*

6.3. Discussion and conclusion

In the present study, HCC cells exposed to TGF-β2 and TNF-α individually and in combination were sent for RNA-seq to investigate the molecular mechanisms involved in HCC pathogenesis. Up and downregulated differentially expressed genes were screened between/across the samples. In addition, the functions of individual genes were collected from KEGG pathway and OMIM. Top 40 significantly up/down regulated transcripts were taken to generate a heatmap. Based on the cellular and biological functions pathway enrichment analysis was done and top 10 deregulated pathways were represented using pathview software. In corroboration to our previous findings autophagy was found to be one of the top 10 pathways getting significantly up-regulated post TGF-β2 exposure. Interestingly simultaneous exposure to TNF-α considerably reduced autophagy. In conclusion, this transcriptomic analysis has enriched the pool of existing information about HCC TME and metastasis.

CHAPTER- 7

TGF-β2 induced p65 activation is inhibited by TNF-α: a crosstalk in context to EMT and autophagy

7.1. Introduction

As discussed in previous chapters, HCC is the sixth most predominant malignant tumor and the third leading cause of cancer associated mortality indicating to its poor prognosis [262]. It is refractory to most of the currently available anti-cancer therapies and frequently arises in response to chronic infection resulting into cirrhosis and persistent inflammation due to cytokine infiltration [263]. Various signalling pathways are associated with this cytokine mediated inflammatory response in HCC development. SMAD dependent classical TGF-β signalling, a regulator of either cell survival or differentiation and/or metastasis is one of the important pathways that is activated during hepatic damage induced inflammation and has been studied quite extensively in liver carcinogenesis [132]. TGF-β modulates numerous cellular phenotypes including growth arrest in epithelial cells and proliferation in fibroblasts. Although the SMAD pathway is fundamental for the majority of these responses, recent evidence indicates that non-SMAD pathways may also have a critical role [76]. However, the non-canonical or non-SMAD dependent TGF-β pathway in regulating EMT in HCC is not fully elucidated. Interestingly, the two modes of TGF-β signalling do not always act in isolation but may engage in extensive crosstalk, which needs further exploration. The understanding of this pathway could be attractive for future treatment of HCC pathogenesis.

Cytokine mediated inflammation leads to dysregulation of several regulatory pathways that are implicated to modulate metastasis, ROS levels, apoptosis and autophagy in tumor cells. The MAPK, NF-κB, TGF-β2 and PI3K/ATK pathways are some of the most commonly dysregulated pathways [264]. Among these, TGF-β signalling mechanism is extensively explored due to its involvement in a wide array of molecular events in cancer. As discussed above, TGF-β mediated activation of non-SMAD pathway also co-exists with the established SMAD pathway. More recently, many targets, including Rho GTPases (Cdc42 and Rac1), p38, ERK, JNK, TAK, NF-κB, PI3K, PAK2, Akt, and c-Abl, have emerged as downstream targets to be activated by TGF-β receptors independent of the SMAD proteins [265]. For example, MAPK pathways-p38 and JNK have been reported to be a down-stream target of TGF-β

inducing either programmed cell death or EMT [266]. Importantly, both arms of TGF-β signalling cross-talk and regulate each other's activities and functions. For instance, studies reveal that the NF-κB signalling pathway can also be regulated in a non-SMAD manner playing an essential role in regulating EMT through the transcriptional up-regulation of the EMT inducer Snail [267]. In contrary, NF-κB signalling pathway and the transcription factors that it activates has also emerged as key regulators of the apoptotic response. Thus, NF-κB can either promote cancer pathogenesis or it can promote programmed cell death in response to certain death-inducing signals [268]. Furthermore, reports have shown that ROS has the potential of bidirectional regulation of NF-κB in various cancer cell types [269]. Not only EMT, ROS and apoptosis, p65 subunit of NF-κB is reported to regulate autophagy as well, by regulating BECN1 expression leading to apoptotic cell death [270]. Though, the NF-κB signalling has diversified functions yet being part of the non-canonical arm of TGF-β its precise role in modulating TGF-β induced effects in correlation to HCC pathogenesis is least explored which could serve in better understanding of HCC pathology.

Among all of the aforementioned non-Smad signals stimulated by TGF-β, p65 (NF-κB/ REL A) is critical because it is the downstream component identified necessary for the EMT upregulation in HCC cells on global transcriptomic analysis in our previous study [132]. Additionally, the role of cross-talk between components of the TGF-β and NF-κB pathways playing in altered activation of these pathways has not been established. Few reports show that TGF-β and TGF-β-activated kinase 1 (TAK1) are predominantly expressed in a subset of HNSCC tumors with nuclear activation of NF-κB family member RELA (p65) [271]. Though, the extent of inter-communication between the two modes of TGF-β action in HCC pathogenesis is still not addressed which could serve as an effective therapeutic target in HCC pathogenesis.

Another pro-inflammatory cytokine, TNF-α has shown to be involved in many physiologic and pathologic processes. In addition, accumulating evidence has suggested its role in promoting tumor metastasis, although the mechanism is not clarified [272]. Furthermore, TNF-α along with TGF-β have shown to regulate MMP-9 expression/activation in the cells of human skin suggesting their role in tissue remodelling. Much to our interest, carcinogenic exposure affects TNF-α, which serves

to activate NF-κB and enable its many roles like metastasis, angiogenesis etc. in the cell [273]. Additionally, it has been shown that the p65 and p50 subunits of NF-κB are constitutively active and are overexpressed in breast cancer, resulting in further transcription of anti-apoptotic genes [274]. Surprisingly, not much has been looked upon how p65 one of the downstream targets of TGF-β non-classical pathway acts in presence of TNF-α in TME. Here, we examined the hypothesis that TGF-β activation of p65 activation is inhibited by TNF-α and their crosstalk in context to EMT and autophagy. We further validated the above finding post pharmacological inhibition of p65 using, JSH-23 to inhibit p65-mediated TGF-β signalling and explored the malignant phenotype in HCC. As TNF-α can induce SMAD7, we examined the potential role of p65 and SMAD7 in the cross-talk between TNF-α and TGF-β pathway, and suppression of TGF-β induced signalling and gene expression. Our findings support a model whereby TGF-β induced p65 enhances metastasis, whereas TNF-α can attenuates non-canonical TGF-β signalling, thereby suppressing the malignant phenotype of HCC.

7.2. Results

7.2.1. TGFβ-2 exposed HCC cells showed upregulation of p65 pathway and TNF-α antagonizes it

In previous chapters we have observed that TGF-β is an effective inducer of mesenchymal features in HCC as well as OS cells. This increase in mesenchymal feature was found to be SMAD dependent; the canonical arm of TGF-β. However, there are several non-canonical targets of TGF-β through which it can induce EMT and are yet to be unmasked. To get a global gene expression profile of HCC cells upon TGF-β and TNF-α exposure, transcriptomic analysis was done using RNA sequencing. While exploring the transcriptomic data, interestingly we have found that the NF-κB pathway components- RELA/p65 and RELB/p50 significantly went up upon TGF-β treatment in Huh7 cells for 48h. Additionally, downregulation of IKBα; an inhibitor of p65 nuclear translocation was observed in the same sample. Interestingly, simultaneous exposure to TGF-β and TNF-α substantially downregulated the RELA/p65 and RELB/p50 components, while upregulating IKBα. Added to the stated observations,

MAP3K7/TAK1 was found to be highly expressed in TGF-β exposed HCC cells which significantly went down when TNFα was introduced. As discussed in chapter-1, TGF-β is known to promote EMT by phosphorylating TAK1 and NF-κB. TAK1 can therefore be considered as a connecting link between TGF-β and NF-κB in regulating EMT in HCC cells (Fig 7.1A). Wet lab validation of RELA/p65 component was further carried out using real time PCR and a significant upregulation in transcript level of p65 was obtained while TNFα significantly reduced p65 levels transcriptionally (Fig 7.1B). Besides, immunoblot analysis was also carried out to check the change in protein levels of p65 post 48h of TGF-β and TNFα treatment individually and in combination; and similar results were obtained (Fig 7.1C). In addition, dose kinetics immunoblot experiments were also performed for 72h and 96h and a drastic decrease in p65 protein levels were observed upon simultaneous introduction to TGF-β2 and TNF-α compared to only TGF-β2 treatment (Fig 7.1D).

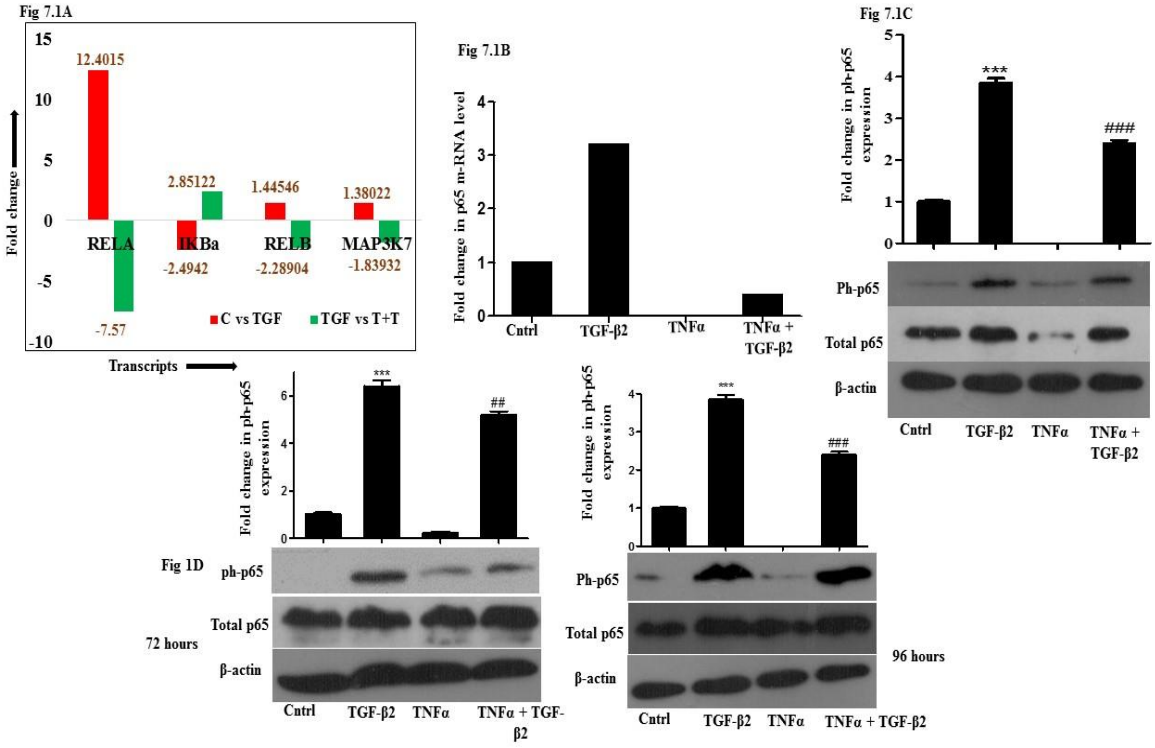

Figure 7.1. TGF-β2 induces p65 activation and TNF-α antagonizes it. *(A) Graph showing transcriptomic data of Huh7 cells post 48h of TGF-β2 (5ng/ml) and TNF-α (20ng/ml) treatment; Red bar indicates the log2fold values signifying the upregulation of p65 pathway components upon TGF-β2 addition. Green bars indicate the*

downregulation of p65 pathway components upon addition of TNF-α to TGF-β2 treated HCC cells. (B) Quantitative RT-PCR result showing expression of p65 after 48h of TGF-β2 (5ng/ml) treatment. (C) Immunoblot assay depicting expression levels of p65 protein post 48h of TGF-β2 (5ng/ml) and TNFα (20ng/ml) exposure, individually and in combination. (D) Immunoblot assay depicting expression levels of p65 protein post 72h and 96h of TGF-β2 (5ng/ml) and TNFα (20ng/ml) treatment. Expression in untreated control was taken as arbitrary unit "1". β-actin served as a loading control.

7.2.2. Pharmacological inhibition of p65 using JSH-23 quenches ROS and enhances EMT upon TGF-β2 treatment

In continuation to the above findings p65 was inhibited using a pharmacological inhibitor JSH-23. This inhibitor is reported to inhibit the nuclear translocation of p65, however it has also shown to alleviate oxidative stress by quenching intracellular ROS [275]. JSH-23 was added to Huh7 cells at a concentration of 7.1μM for 48h and the inhibition of p65 was confirmed through immunoblot analysis (Fig 7.2A). We earlier observed that introduction of Huh7 cells to TGF-β (5ng/ml) led to a substantial upsurge of intra-cellular ROS when compared to the un-treated control. Interestingly, as expected simultaneous exposure of TGF-β (5ng/ml) and JSH-23 significantly reduced intracellular ROS levels as analyzed using H2DCF-DA fluorimetric assay (Fig 7.2B). As discussed in chapter 4, an escalation in TGF-β2-induced ROS was associated with cytostasis and a simultaneous exposure to NAC, a ROS quencher rescued the cytostatic effect. While investigating the role of ROS in regulation of EMT we observed that, inhibition of ROS using NAC drastically increased the expression of mesenchymal markers. Similar results were obtained when JSH-23 was introduced in TGF-β treated Huh7 cells for 48h. An upregulation of N-cadherin and Vimentin was observed as analyzed using immunoblot probably suggesting the role of ROS in attenuating TGF-β2 induced EMT (Fig 7.2C). Additionally, immunoblot analysis was also performed for 72h and an increase in N-cadherin and Vimentin was again observed in TGF-β2 and JSH-23 treated sample compared to TGF-β2 alone (Fig 7.2D). This suggested us that p65 might not be indispensable for EMT induction under the current scenario, at least in presence of JSH-23 which could enhance EMT by controlling ROS levels. We

further checked the expression of p65 post inhibition of ROS by NAC. Fascinatingly, quenching of intracellular ROS using NAC in TGF-β2 treated Huh7 cells significantly elevated p65 levels, however when ROS levels were controlled by JSH-23 the role of p65 in terms of EMT seemed redundant (Fig 7.2E).

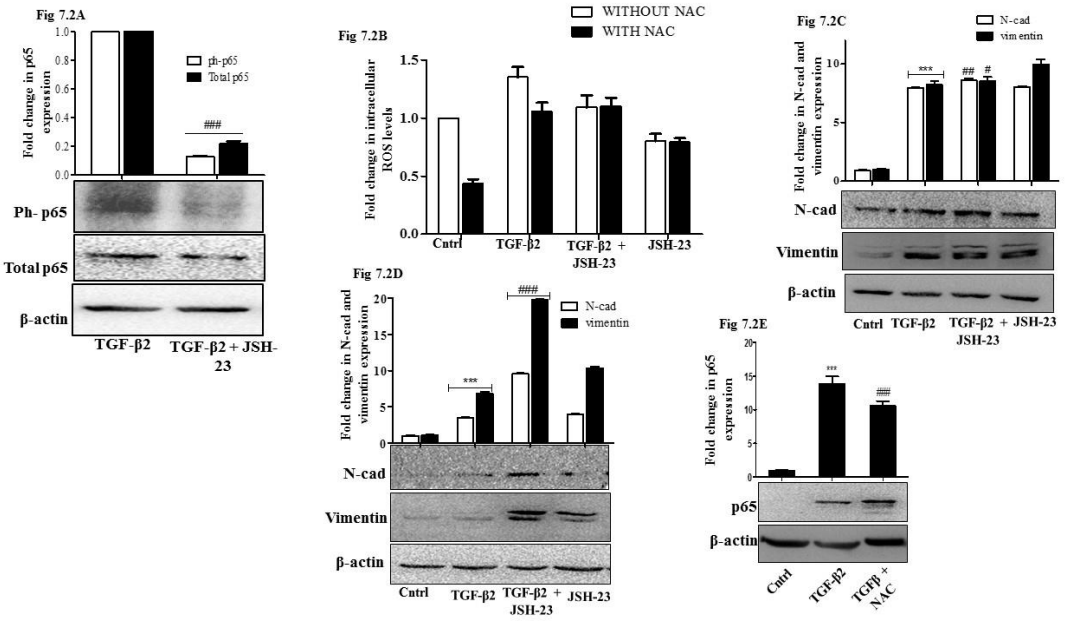

Figure 7.2. Analysis of EMT post p65 inhibition using JSH-23 in TGF-β2 and TNF-α treated cells. *(A) Immunoblot assay showing p65 expression post 48h of TGF-β2 (5ng/ml) with and without JSH-23 addition. (B) Bar diagram showing H2DCF-DA fluorimetric assay with and without TGF-β2 and NAC treatment post inhibition of p65 using JSH-23. (C) Immunoblot assay showing protein expression of N-cadherin and Vimentin post 48h of TGF-β2 exposure with and without JSH-23 addition. (D) Immunoblot assay showing protein expression of N-cadherin and Vimentin post 72h of TGF-β2 exposure with and without JSH-23 addition. (E)Immunoblot assay showing p65 expression post 48h of TGF-β2 (5ng/ml) with and without addition of NAC. Expression in untreated control was taken as arbitrary unit "1". β-actin served as a loading control.*

7.2.3. TGFβ-2 induced pro-survival autophagy is attenuated upon pharmacological inhibition of p65

TGF-β is well reported to activate autophagy in a wide range of cancer [230]. Autophagy as discussed previously can act as a double edged sword it can either promote tumor progression or suppression [231, 232]. In this study we have already shown that, TGF-β2 induced pro-survival autophagy by limiting ROS and facilitating SMAD driven EMT. Hence, we were interested in checking the role of autophagy post inhibition of TGF-β (by JSH-23) induced p65 activation. Huh7 cells treated with TGF-β2 led to an increase in autophagic marker LC3B-II and ATG5. However, pharmacological inhibition of p65 significantly reduced these autophagy markers (Fig 7.3A and 7.3B). This observation putatively suggests that when JSH-23 quenches intracellular ROS, the role or function of autophagy in limiting ROS becomes redundant; hence a decline in autophagy levels with JSH-23 introduction. It has also been well documented that, p65 has consensus sites in the promoter region of beclin 1 and hence can positively regulate canonical autophagy in many human cell lines [276]. That means there can be crosstalk between autophagy and p65. Interestingly, we observed that, inhibition of TGF-β induced pro-survival autophagy using CQDP significantly reduced p65 levels, as checked through immunoblot analysis (Fig 7.3C). This observation further suggests that autophagy and p65 co-operates in promoting survival of cancer cells undergoing EMT upon TGF-β treatment.

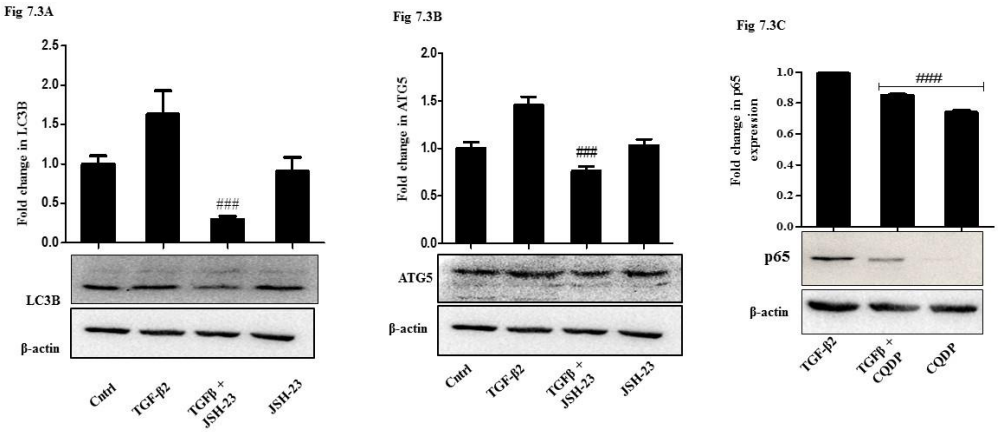

Figure7.3. p65 inhibition suppresses TGF-β2-induced autophagy. *(A) Immunoblot analysis showing protein expression of LC3B post simultaneous exposure of TGF-β2 (5ng/ml) and JSH-23 for 48h in Huh7 cells. (B) Immunoblot analysis showing protein expression of ATG5 post simultaneous exposure of TGF-β2 (5ng/ml) and JSH-23 for 48h in Huh7 cells. (C) Immunoblot analysis showing protein expression of p65 post inhibition of autophagy using CQDP for 48h in Huh7 cells.*

7.2.4. Increase in apoptotic cell death is observed post inhibition of p65

The p65 component of NF-κB pathway is well studied to promote proliferation of cancer cells. In oral squamous cell carcinoma inhibition of p65 was previously shown to suppress cellular proliferation [277]. Also, in colon cancer, overexpression of p65 is studied to promote cell proliferation and motility [278]. However, the role of TGF-β2 induced p65 activation in context to HCC is least explored. We observed that, simultaneous exposure of TGF-β2 and JSH-23 showed increased cytotoxicity when compared to TGF-β2 alone as analyzed after 48h through bright field imaging and MTT assay (Fig 7.4A and 7.4B). Additionally, cell death was confirmed through AnnexinV/PI staining through flow cytometry and similar results were obtained (Fig 7.4C). An increase in both apoptotic and necrotic cell death was also observed. Immunoblot analysis further showed an upregulation of cleaved PARP upon inhibition of p65 in TGF-β2 exposed HCC cells (Fig 7.4D). These observations suggest that TGF-β2-induced p65 activation might help the cells in survival as they undergo EMT.

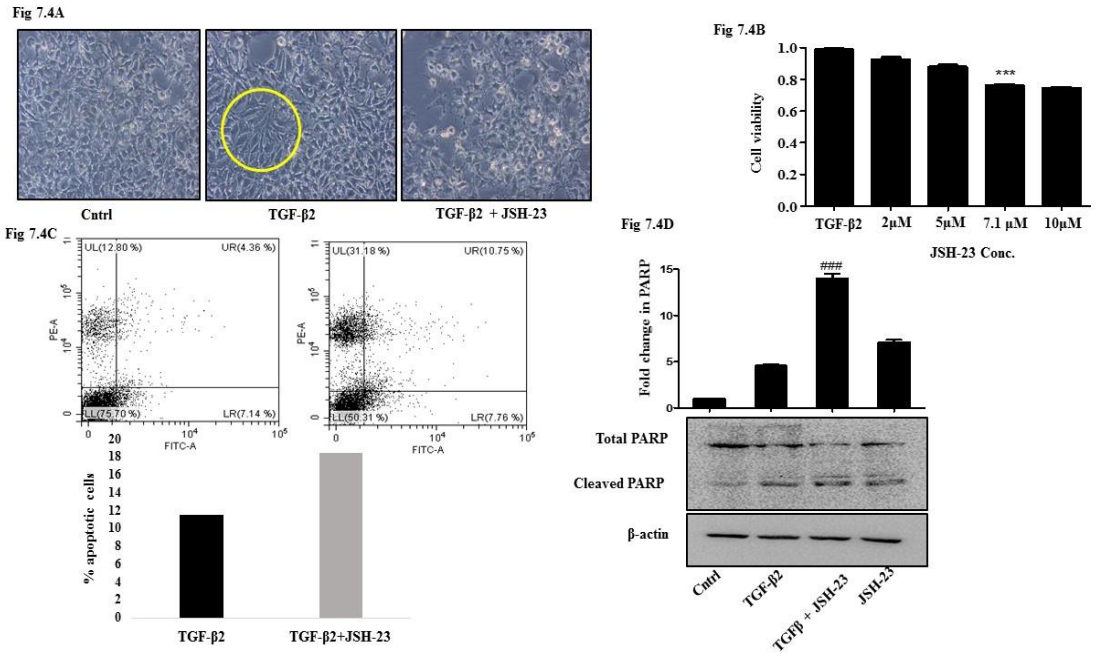

Figure 7.4. p65 inhibition by JSH-23 induces cell death in TGF-β2 treated HCC cells. *(A) Phase contrast image of Huh7 cells post TGF-β2 (5ng/ml) treatment with and without JSH-23 treatment. (B) Graph showing cell viability of TGF-β2 (5ng/ml) treated Huh7 cells exposed to different doses of JSH-23 for 48h, analyzed using MTT assay.(C) Flow cytometry plots and histogram depicting Annexin V/PI results analyzed after 48h of exposure. (D) Immunoblot analysis showing protein expression of total/cleaved PARP post simultaneous exposure of TGF-β2 (5ng/ml) and JSH-23 for 48h in Huh7 cells, alongside respective controls.*

7.2.5. Pharmacological inhibition of p65 up-regulates SMAD to induce EMT in HCC cells

TGF-β regulates a multitude of signalling pathways other than SMADs to control varied cellular functions- p65 being one. Lack of research regarding the TGF-β induced p65 activation and its signalling crosstalk with canonical arm, urged us to investigate the same. We observed that simultaneous treatment of TGF-β and p65 inhibitor- JSH-23 in Huh7 cells up-regulated SMAD2 as analyzed using immunoblot analysis (Fig 7.5A); the effect was more pronounced in 72h (Fig 7.5B). This observation led us to check the co-existence and further crosstalk of SMAD and non-SMAD pathways in TGF-β exposed HCC cells undergoing EMT. We observed that inhibition of SMAD signalling

by SIS3 (discussed earlier), in turn, significantly elevated p65 activation, as observed through immunoblot assay (Fig 7.5C). From the above observation it can be inferred that, TGF-β exposure stimulates the canonical as well as non-canonical signalling arm parallelly to maintain EMT and survival of HCC cells and there is existence of feedback loop between the two arms [279].

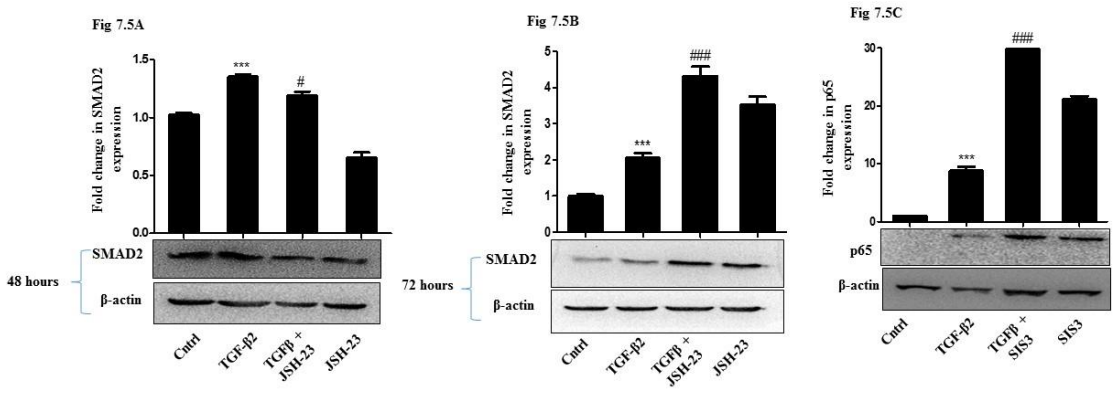

Figure 7.5. Pharmacological inhibition of p65 induces cellular proliferation through SMADs. *(A) Immunoblot analysis showing protein expression of SMAD post simultaneous exposure of TGF-β2 (5ng/ml) and JSH-23 for 48h in Huh7 cells. (B) Immunoblot analysis showing protein expression of SMAD post simultaneous exposure of TGF-β2 (5ng/ml) and JSH-23 for 72h in Huh7 cells. (C) Immunoblot analysis showing protein expression of p65 post TGF-β2 (5ng/ml) exposure with and without SMAD inhibition for 48h in Huh7 cells.*

7.3. Discussion and conclusion

In the present study, we provide evidence for a cross-talk between TGF-β canonical and non-canonical mode of signalling pathway in context to EMT, autophagy, cell survival and how TNF-α antagonizes this effect. Transcriptomic analysis data provides evidence showing increased expression of p65/RELA component of NF-κB pathway upon TGF-β exposure while TNF-α rendering an opposing effect on the same in HCC cells. We further demonstrate that TGF-β treatment results in increased expression of

TAK1, a p65 upstream element; whereas down-regulates IKBα. We assume that TAK1 is a key node in cross talk between TGF-β and p65 pathway regulating cell survival and EMT. Further studies, showed that TNF-α counters this TGF-β mediated activation of p65 non-canonical signalling.

Activation and nuclear translocation of p65 is one of the critical steps alongside IκB phosphorylation in NF-κB pathway. We inhibited the activation and subsequent nuclear translocation of p65 upon TGF-β exposure with a synthetic compound 4-methyl-N-(3-phenyl-propyl)-benzene-1,2-diamine (JSH-23) [280]. Previous reports have shown that, NF-κB inhibition partially reverses functional, behavioural and biochemical deficits with JSH-23 treatment in diabetic neuropathy [281]. Interestingly, opposite to our anticipation, the attenuation of p65 with JSH-23 led to a significant increase in mesenchymal markers in HCC cells. We then explored existent reports describing the functions of JSH-23. It is a well-established anti-oxidant and is known to decrease the levels of intracellular ROS. Our results are in corroboration to what we observed earlier with NAC; where NAC, a ROS quencher administration, in presence of TGF-β was found to enhance the EMT inducing effect of the cytokine.

Autophagy has been recognized as a critical response of normal and malignant cells to environmental changes in a context dependent manner. Reports show that TGFβ induces autophagy in a manner dependent on both SMAD and non-SMAD pathways. Additionally, TGF-β induced accumulation of autophagosomes and the conversion of microtubule-associated protein LC3II has been shown to promote the degradation rate of long-lived proteins [282]. These observations suggest that autophagy contributes to the varied effects of TGF-β. Here, in this study we observed that post pharmacological inhibition of TGF-β induced p65 activation with JSH-23, there was a substantial decrease in expression of autophagic markers. Since autophagy is known to control cellular redox homeostasis, we assume that a decrease in autophagy can be attributed to quenching of ROS with JSH-23, making autophagy dependent functions redundant. Therefore, we postulate from this study that autophagy promotes cell survival by regulating ROS levels and when ROS levels are decreased by an anti-oxidant autophagy levels go down. The activation of p65 by TGF-β, while its down-regulation with JSH-23, accompanied with increasing levels of EMT markers suggests that p65 might have

an alternate cellular function in EMT undergoing cells and might not directly contribute towards EMT of HCC cells.

Several studies have earlier provided indirect evidence for pro- and anti-apoptotic role of p65 signalling in different cell types. However, the role of p65 as downstream non-canonical arm of TGF-β in HCC cells is not understood. We observed that simultaneous exposure of TGF-β2 and JSH-23 showed significant cell death. From these observations, we speculate that TGF-β induced p65 activation might help HCC cells in survival rather than in EMT, and hence an inhibition of p65 though promotes EMT due to reduced ROS but also promotes cell death due to in-activation of p65. This proposition and the series of event need to be further validated by genetic knock down of p65, which is currently been conducted in our laboratory but has been excluded from the current thesis. In our study, we further observed that there is crosstalk between the SMAD and non-SMAD pathways, in TGF-β exposed HCC cells undergoing EMT. An inhibition of SMAD signalling by SIS3 significantly elevated p65, while, inhibition of p65 arm significantly increased SMAD levels. Based on the observations, we speculate that the canonical arm is more stringently required for EMT; suppression of mesenchymal markers was observed with inhibition of canonical signalling. While the non-canonical arm contributes towards HCC cell survival under TGF-β exposure as cells undergo EMT, however, whether there is a switch in functionality under inhibition of each arm needs to be confirmed by further experiments. In this context, autophagy serves as a bystander controlling intra-cellular ROS at decreased levels; an increase of which hinders EMT. Understandably, when ROS levels drop, due to use of ROS quenchers, autophagy levels also go down. Our study highlights an interesting aspect of TGF-β induced signalling where each arm promotes linked but independent functions. HCC progression can be better controlled if therapeutic approaches are appropriately undertaken to suppress both arms of TGF-β signalling.

CHAPTER- 8

Conclusions, limitations and future perspective

8.1. Thesis conclusion

Several pharmacological agents have till date been tested as a treatment modality to attenuate the effect of TGF-β, specifically, with respect to collagen deposition in fibrotic diseases. One approach has been to reduce TGF-β gene expression, either by suppressing transcription or by altering RNA stability; or by the use of antioxidants such as α-tocopherol; or through administration of anti-TGF-β anti-serum. However, the use of antagonistically functioning cytokines to reduce TGF- β-induced effects in cancer cells has not been explored. In this study, we provide concrete evidences to probable futuristic use of antagonistically functioning cytokines like, TNF-α or introduction of TNF-α agonists as alternatives to existing therapy limiting HCC progression. While investigating the cross-talk of two pre-dominant cytokines TGF-β2 and TNF-α, we found that simultaneous introduction of TNF-α with TGF-β antagonized TGF-β induced SMAD dependent EMT in HCC cells. Hence we propose TNF-α as a novel and promising alternative for the treatment of advanced HCC. However, further studies especially in *in vivo* models are required to conclusively claim the above antagonistic function of TNF-α.

From this study, we further provide evidences for autophagy, a genetically regulated and finely orchestrated cellular process, for its role in sustenance of TGF-β induced EMT in HCC cells. An up-regulation of autophagy both at genetic and protein level was observed in TGF-β exposed cells showing enhanced mesenchymal properties. Inhibition of autophagy resulted in a significant down-regulation in the expression of mesenchymal markers and induced apoptosis. This suggested that autophagy acted as a pro-survival strategy facilitating TGF-β induced EMT. However, we were inquisitive to know the precise role of autophagy in the current context to EMT induction. In this regard, we observed that TGF-β2 exposure resulted in cytostasis and accumulation of intracellular ROS in HCC cells. Quenching of ROS increased cell viability with a significant reduction in autophagy levels. In contrary, suppression of autophagy significantly enhanced ROS levels suggesting the role of TGF-β induced autophagy in limiting ROS and thus facilitating EMT. Interestingly, TNF-α was not only found to antagonize TGF-β induced SMAD2 signalling by activating inhibitory SMAD7 but also resulted in reduction of autophagy genes both at transcriptional and protein level.

Therefore, we propose that TGF-β induced EMT in HCC cells can either be antagonized by TNF-α or inhibited by suppression of autophagy.

The antagonistic role of TNF-α on TGF-β induced EMT was further validated by RNA sequencing. Apart from the EMT-specific genes like N-cadherin, Vimentin and others, which were significantly down-regulated, the autophagy genes e.g., Atg16L1 and WIPI2 also showed reduced expression upon TNF-α treatment. Atg16 and WIPI2 are required for autophagosome maturation and lipidation. Both the genes significantly went down in TGF-β plus TNF-α-treated samples, indicating that TNF-α might inhibit autophagy by suppressing the final steps of autophagosome maturation and lipidation.

We further explored the role of non-canonical signalling arm in TGF-β induced EMT. Transcriptomic analysis showed that RELA/P65 component of the NF-κB signalling pathway is highly up-regulated in TGF-β-treated samples and TNF-α inhibits this pathway as well. We were therefore interested to investigate the role of p65 in the given circumstances. Interestingly, pharmacological inhibition of p65 with JSH-23 resulted in an enhanced TGF-β induced EMT. We assume that JSH-23 apart from being a p65 inhibitor is also a potent anti-oxidant which facilitated EMT by quenching of ROS. An up-regulation of SMAD2/4 canonical arm was also observed upon JSH-23 exposure in presence of TGF-β. Alternatively, a suppression of SMAD2/4 signalling showed an increase in p65 activation highlighting an existent feedback mechanism. It is apparent from the existing observations that p65 might not play a direct role in EMT, but might have a role in survival of cells undergoing EMT as an increased cell death was observed in cells treated with JSH-23 and TGF-β.

In summary, we postulate that a neutralization of TGF-β induced effects by regulating autophagy can be an effective strategy to decrease EMT in HCC cells. Further we provide evidences that antagonistically functioning cytokines like TNF-α can potentially reduce TGF-β induced effects in HCC cells. Our study provides a basic understanding on the molecular interplay of cytokines in HCC cells and also provides promising alternatives for the treatment of HCC (Fig 8.1).

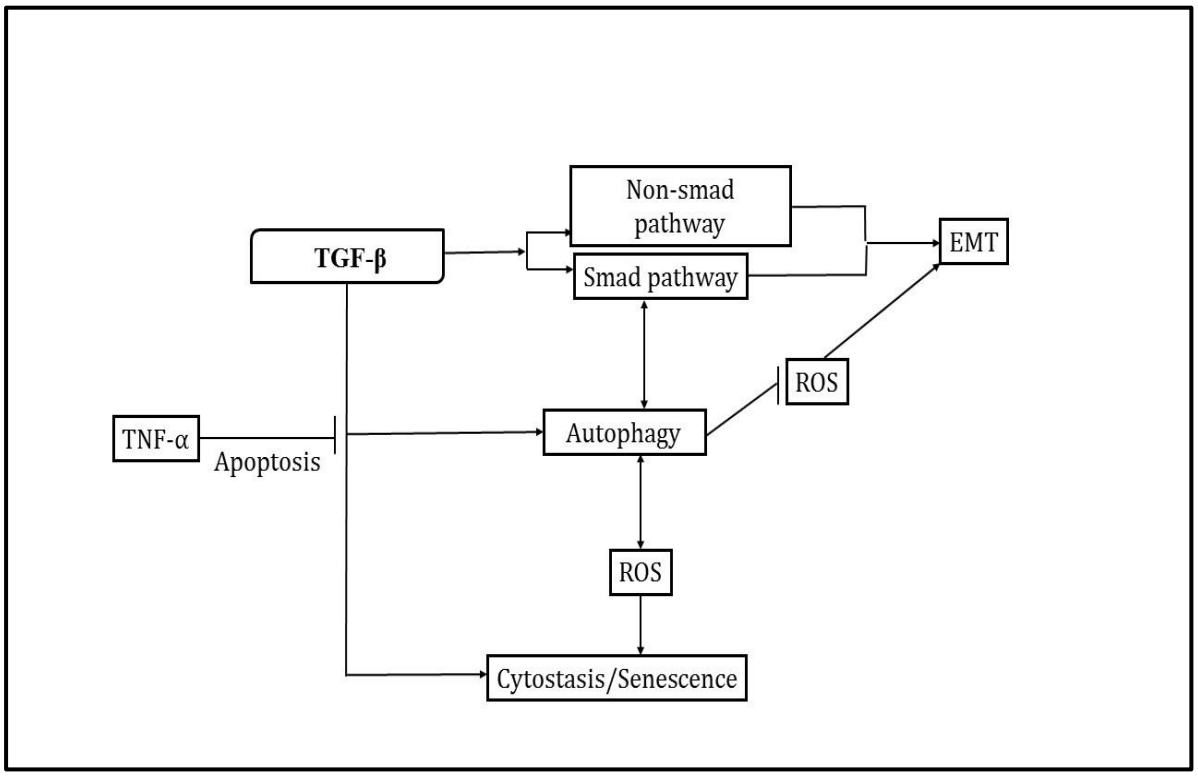

Figure 8.1. Schematic diagram highlighting major findings from the thesis.

8.2. Limitations and future perspective

- Multiple cytokines are present in the tumor microenvironment of HCC. This study highlights the functional interplay between two predominant cytokines found in HCC tumor milieu. However, practically the outcome in terms of HCC pathogenesis is the crosstalk between all the cytokines present in a given context at a specific time, which needs to be mimicked through future appropriate studies. Nevertheless, our study provides crucial hints to the probable molecular effects and crosstalk of the two most important cytokines in HCC.
- The existing study is mainly focused on macro-autophagy and its role in context to EMT. However, further experiments could be carried out to assess the effect of selective autophagy like mitophagy, ER-phagy in regulating TGF-β induced EMT.
- The non-canonical axis of TGF-β is constituted by numerous other imperative components other than p65 which could play important role in regulation of EMT and autophagy. Hence, the role of these non-SMAD molecules need to be investigated for a better understanding of TGF-β induced signalling and EMT.
- Further, *in vivo* studies could be designed to better understand the functional crosstalk of TGF-β, TNF-α and autophagy in context to EMT.
- Existing transcriptomic data have shown the up-regulation of long non-coding RNAs (lncRNAs) (e.g. MALAT1, LINC00662) in TGF-β exposed HCC cells. Involvement of these lncRNAs in EMT and autophagy could be studied, which will add another dimension to this study by clarifying the epigenetic regulation of TGF-β mediated EMT in HCC cells.

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APPENDIX

A.1. List of publications

From Thesis

i. **Subhra Dash**, Prasad M Sarashetti, Balaji Rajashekar, Rajdeep Chowdhury* and Sudeshna Mukherjee*. TGF-β2-induced EMT is dampened by inhibition of autophagy and TNF-α treatment. Oncotarget 9.5 (2018): 6433. (**Published**)

ii. **Subhra Dash**, Shivangi Mishra, Rajdeep Chowdhury and Sudeshna Mukherjee* TGF-β2-mediated induction of mesenchymal markers is suppressed by TNF-α through regulation of autophagy in human osteosarcoma cells. (**Communicated**)

iii. **Subhra Dash**, Rajdeep Chowdhury* and Sudeshna Mukherjee* TGF-β2-induced p65 activation is inhibited by TNF-α: a crosstalk in context to EMT and autophagy. (**In pipeline**)

iv. **Subhra Dash**, Shivangi Mishra, Sudeshna Mukherjee and Rajdeep Chowdhury* Exploring the extensive crosstalk between the antagonistic cytokines-TGF-β and TNF-α in regulating cancer pathogenesis. **(In pipeline)**

v. **Subhra Dash**, K. Lohitesh, and Sudeshna Mukherjee. BOOK CHAPTER: Rediscovering Cancer From Mechanism to Therapy, Chapter no.7: Cytoplasmic Signalling Circuitry: An Important Trait of Cancer, Apple Academic Press, 2018. (**Book Chapter**)

Other Publications

i. Mukherjee S, **Dash S**, Lohitesh K and Chowdhury R*. The dynamic role of autophagy and MAPK signalling in determining cell fate under cisplatin stress. PloS One. 2017, 12(6): e0179203.

ii. Fageria L, Pareek V, Dilip R, Bhargava A, Pasha S, Laskar I, Saini H, **Dash S**, Chowdhury R*, Panwar, J* (*co-corresponding authors). Biosynthesized protein-capped silver nanoparticles induce ROS-dependent pro-apoptotic signals and pro-survival autophagy in cancer cells. ACS Omega, 2017, 2 (4), pp 1489–1504.

iii. Alam P, **Dash S**, Climent C, Kaur G, Choudhury AR, Casanova D, Alemany P, Chowdhury R*, Laskar IR*. Aggregation Induced Emission' Active Iridium (III)

Complexes with Applications in Mitochondrial Staining. RSC Advances, 2017, 7, 5642-48.

iv. Raghuram HS, Pradeep S, **Dash S**, Chowdhury R and Mazumder S. Chitosan encapsulated ZnS:M (M: Fe3+ or Mn2+) quantum dots for fluorescent labeling of sulphate reducing bacteria. Bulletin of Materials Science; April 2016:1-9.

v. Shukla P, Sharma A, Chowdhury R, **Dash S** and Pallavi B. Fast microwave assisted synthesis of pyrazolopyran derivatives as new anticancer agents. Current Microwave Chemistry; 2015, 3:1-6.

vi. Pasha S, Alam P, **Dash S**, Kaur G, Banerjee D, Chowdhury R, Rath N, Choudhury RA and Laskar IR. Rare Observation of 'Aggregation Induced Emission' in Cyclometalated Platinum (II) Complexes and their Biological Activities. RSC Advances; 2014, 4:50549-50553.

A.2. List of conferences

Presented poster in the following conferences:

i. TGF-β2-induced EMT is dampened by inhibition of autophagy and TNF-α treatment.

Subhra Dash, Prasad M. Sarashetti, Balaji Rajashekar, Rajdeep Chowdhury and Sudeshna Mukherjee. Keystone Symposia meeting on Selective Autophagy, Westin Miyako, **Kyoto, Japan**. 22/04/2018 - 26/04/2018.

ii. Autophagy: a pro-survival strategy for cells undergoing Epithelial to Mesenchymal Transition upon TGF-β treatment and analysis of cytokines antagonizing this effect.

Subhra Dash, Rajdeep Chowdhury* and Sudeshna Mukherjee*The EMBO event on Autophagy: Cellular mechanism(s) and significance in health and disease, **Bhubaneswar, Odisha**, India. 11/12/2017 – 13/12/2017.

iii. Autophagy: a pro-survival strategy for cells undergoing EMT upon TGFβ-2 treatment.

Subhra Dash, Rajdeep Chowdhury and Sudeshna Mukherjee. International conference on challenges in drug discovery and delivery (ICCD3), held at **BITS-Pilani, Rajasthan,** India. 2/03/2017-4/03/2017.

iv. The Effect of Pro-inflammatory Cytokines on Epithelial to Mesenchymal Transition in Hepatocellular Carcinoma Cells.

Subhra Dash, Leena Fageria, Vandhana Cheziyan, N Sarda, Sudeshna Mukherjee and Rajdeep Chowdhury R. Global Cancer Summit, Indian Institute of Sciences, J.N.TATA Auditorium Bengaluru, **Bengaluru, Karnataka**, India. 18/11/2015 – 20/11/2015

A.3. Biographies

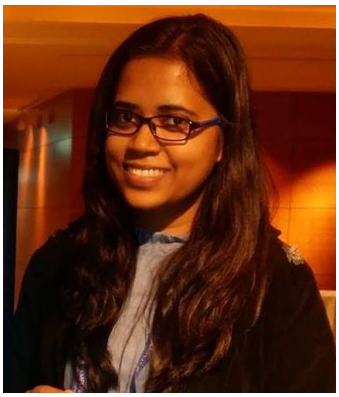
Brief biography of the supervisor

Dr. Rajdeep Chowdhury is currently working as Associate Professor, Department of Biological Sciences, BITS Pilani, Pilani Campus, Rajasthan. He earned his Bachelors and Masters degrees from Calcutta University. In 2003 he joined Indian Institute of Chemical Biology, Molecular & Human Genetics Department, Kolkata for his PhD (CSIR-NET). He made significant contributions in the field of Cancer Biology and Toxicology with special emphasis on arsenic-induced carcinogenesis and its remediation during his PhD. He was awarded DBT Post Doctoral Research Fellowship in 2008. In 2009, he joined Massachusetts Institute of Technology (MIT), USA, Department of Bioengineering, as a postdoctoral researcher. At MIT he studied the myriad set of genetic events following Nitric Oxide (NO) exposure; his project extended from understanding the effects of NO-induced post translational modifications to its cancer promoting effect and also its role in cell death mechanisms like, Autophagy. In Oct 2012 he joined BITS-Pilani as an Assistant Professor in Dept of Bio-Sciences. He has expertise in the field of cancer biology and is currently investigating causes to cancer drug resistance, with special emphasis on autophagy. He has received research grants from various government funding authorities like (i) Department of Science and Technology (DST) under fast track scheme of young scientist (ii) Science and Engineering Research Board (SERB) under Extra-Mural Research Funding (iii) University Grants Commission (UGC) under Minor Research Project and (iv) Department of Biotechnology under Pilot Project and (v) projects from SERB as co-PI as well. The findings from his works have been published in more than 25 reputed international scientific journals. He currently has a Google scholar h-index of 13 and total citations of more than 900. At present he is guiding four Ph.D. students and guided more than four M. Tech Bio Sciences students for the fulfilment of their dissertation.

Brief biography of the co-supervisor

Dr. Sudeshna Mukherjee Chowdhury is currently working as Assistant Professor, Department of Biological sciences, BITS Pilani, Pilani campus, Rajasthan. She did her PhD from Chittaranjan National Cancer Institute (CNCI, Kolkata) under Jadavpur University. Her graduate research was primarily focused on elucidating the molecular mechanisms to carcinogenesis with special emphasis on cell cycle dys-regulation and chromosomal aberrations on embryonic fibroblasts. After my graduation she moved on to USA where she did her post-doctoral research from Dept. of Biochemistry, Tufts School of Medicine, Boston, USA. The research was focussed on Polyoma Middle T Antigen mediated signalling in cancer. She joined BITS-Pilani on July, 2013 a visiting faculty with DST-SERB Young Scientist Grant. Post completion of that project she got a regular position as an Assistant Professor. Her expertise is in the field of cancer biology specifically epithelial to mesenchymal transition. She is interested in studying role of cytokines in tumor microenvironment and their cross-talk in EMT and cancer progression. She has received projects from DST-SERB core research grant and start up grants from BITS-Pilani as well. Findings from her works have been published in reputed international scientific journals. At present she is guiding 2 Ph.D. students.

Brief biography of the candidate



Ms. Subhra Dash has graduated in Biotechnology from Utkal University, Bhubaneswar, India in 2011 and has completed her Masters in Biotechnology from Kalinga Institute of Industrial Technology, Bhubaneswar, India in 2013. In the same year, she has joined under Prof. Rajdeep Chowdhury, BITS-Pilani, Pilani Campus to pursue her doctoral research work. Her area of interest is cancer biology and autophagy. She has presented poster in four international conferences and has received travel award from DBT and CSIR for presenting her research work in Keystone symposia meeting on Selective Autophagy, held in Kyoto, Japan. She has published 7 research articles and 1 book chapter in peer-reviewed journals. Along with research, she was also involved in teaching and has taken courses for first degree and higher degree students in BITS Pilani, Pilani Campus.