The Role of Tissue Transglutaminase in the Progression of Colorectal Cancer

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Thesis Summary

There is a significant loss in the efficacy of the current therapeutic regime on advanced colorectal cancer, highlighting the need to understand the tumour progression process, and to identify potential prognostic and/or therapeutic targets. This study employs well characterised human primary and metastatic colorectal cancer cell lines (CRCs); RKO, SW480 SW620, and HCT116 to investigate the involvement of pro-inflammatory protein tissue transglutaminase (TG2) in tumour progression.

Cancer progression was assessed by evaluating Epithelial-Mesenchymal Transition (EMT), cancer cell invasion, drug resistance and the ability of CRCs to form spheroid containing cancer stem cells. TG2's expression was found to correlate with the advancement of the original tumour, markers of EMT, cell invasion, drug resistance, and cancer stemness. Manipulation of TG2 expression in the different CRCs by shRNA or TG2 transduction confirmed the relationship between TG2, EMT, tumour invasion and drug resistance.

TGFβ1 was shown to regulate TG2 expression and EMT in primary tumour cell lines by both canonical and non-canonical (RKO and SW480) signalling pathways. TGFβ1 also induced TG2 in the highly advanced HCT116 cells. However, the metastatic SW620 cell line was non-responsive to TGFβ1, but TG2 was associated with the increased presence of nuclear β-catenin in these cells, and TG2's inhibition/knockdown led to an increased interaction between β-catenin and ubiquitin as determined by co-immunoprecipitation. Interestingly, β-catenin and TG2 were found to be highly expressed in spheroids containing cancer stem-like cells only formed in the aggressive SW620 and HCT116 cell lines. These cancer stem cells were associated with heightened EMT, cellular invasion and angiogenic potential, which was attenuated following TG2 inhibition. TG2 may play a role in tumour progression in human CRCs through its involvement in EMT and the acquisition of cancer stem cell-like properties and may hold both prognostic and therapeutic potentials in colorectal cancer.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

Aβ	Amyloid beta- protein
AP-1	Activator protein 1
ATP	Adenosine 5'- triphosphate
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BSA	Bovine Serum Albumin
Ca_2^+	Free Calcium ions
$CaCl_2$	Calcium Chloride
CCl_4	Carbon tetrachloride
CD	Celiac disease
cDNA	Complementary deoxyribonucleic acid
c-MYC	V-myc avian myelocytomatosis viral oncogene homolog
CO_2	Carbon dioxide
CSC	Colon cancer stem cells
CRCs	Colorectal cancer cells
DAPI	4', 6-diamidino-2-phenylindole
DLK	Dual leucine zipper bearing kinase
DMEM	Dulbecco's modified Eagles' medium
DMSO	Dimethyl sulphoxide
DNA, cDNA, gDNA	Deoxyribonucleic acid, complementary deoxyribonucleic acid, genomic deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EGF	Epithelial growth factor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal regulated kinase
EV	Empty vector
FAK	Focal adhesion kinase
FBS	Foetal Bovine serum
FITC	Fluorescein isothiocyanate
FN	Fibronectin
FXIII	Factor XIII
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine 5'diphosphate
GTP	Guanosine 5'- triphosphate
GTPase	Guanosine 5'- triphosphatase
GSK-3	Glycogensynthase kinase-3
h	hour/ hours
HD	Huntington's disease
HNSCC	Head and neck squamous cell carcinoma
HRP	Horseradish peroxidase
HS	Heparan sulphate
IL-1	Interleukin -1
IL-6	Interleukin - 6
IκB	Inhibitor of -κB
JNK	c--Jun N-terminal kinases
kDa	Kilodalton
LEF	Lymphoid enhancer-binding factor
LTβP	Large latent TGFβ binding protein

M	Molar
MAPK	Mitogen activated protein kinase
MEK	MAPK kinase 1
MET	Mesenchymal-epithelial transition
Min	minute/ minutes
ml	Milliliters
MLH1	MutL homologue 1
MMR	Mismatch repair
mM	Millimolar
MMPs	metalloproteinase
MSI	Microsatellite Instability
MSS	Microsatellite stabile
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa - light chain enhancer of activated B cells
nM	Nanomolar
nm	Nanometer
NO	Nitric oxide
^{0}C	Degree Celcius
OCT 3/4	Octamer-3/4
OPD	*o*-phenylenediamine
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
pH	Negative log of hydrogen ion concentration
PI3K	Phosphoinositide 3- kinases
PKCα	Protein kinase C alpha
PKCδ	Protein kinase C delta
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
RA	Retinoic acid
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma
Rb	Retinoblastoma gene
RNA, mRNA	Ribonucleic acid, messenger ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
SMAD 2, 3	Mothers against decapentaplegic homolog 2, 3,
SDS	Sodium dodecyl sulphate
SDS- PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
shRNA	short hairpin ribonucleic acid
SOX2	Sex determining region Y (SRY)-related high mobility group (HMG) box 2
TEMED	N,N,N',N'-tetramethylene diamine
TBE	Tris/Borate/Ethylenediaminetetraacetic acid
TG	Transglutaminase
TG2	Tissue transglutaminase
TGase	Transglutaminases
TGFβ1	Transforming growth factor beta 1
TNFα	Tumour necrosis factor - alpha
Tris	Tris (Hydroxymethyl)-amino ethane
VEGF	Vascular Endothelia growth factor

VEGFR 1,2	Vascular Endothelia growth factor receptor 1,2
v/v	volume/volume
WNT	Wingless type
w/v	weight/ volume
x g	G- force
XTT	Sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate
ZEB	Zebularine
ZO-1	Zonal occludin
µ	Micro
µl	Microlitre
µM	Micromolar

Chapter 1
Introduction

1.1 INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the world and a major cause of morbidity and mortality. Although advances have been made in the treatment of CRC over the last decade with the introduction of new surgical techniques, radiotherapy, and chemotherapy, the overall survival rate of patients with CRC has not shown marked improvement (Liu et al., 2011). Survival of the disease is highly dependent upon the stage of disease at diagnosis and typically ranges from a 90% 5-year survival rate for cancers detected at the localized stage; 70% for regional; to 10% for people diagnosed with distant metastatic cancer (Leng et al., 2013). Metastasis plays a critical role in the poor prognosis, and more than one-third of patients with CRC will ultimately develop metastatic disease. The poor survival rate in metastatic cancer is partly due to increased aggressiveness of the cancer and reduced response rates to chemotherapy. Longley et al., (2003) reported a 10–15% response rate of advanced CRCs to 5fluorouracil (5FU) a single first-line treatment in advanced CRC.

The bulk of carcinoma cells generally exhibit predominantly epithelial characteristics. However, in order to invade, disseminate to distant tissues and subsequently form metastatic colonies, neoplastic epithelial cells must shift, at least transiently, into a more mesenchymal phenotype. Epithelial-Mesenchymal Transition (EMT) is a physiological process found in embryonic development, tissue remodelling, wound healing and fibrosis (Fan et al., 2012) however in neoplasms it is a critical step in the progression of tumour cells from the *in situ* carcinoma to distant metastases. Furthermore, recent reports also suggest that EMT results in the acquisition of other properties involved in carcinoma progressions, such as increased resistance to apoptosis, drug resistance, apoptosis and the acquisition of stem cell-like properties (Hanahan and Weinberg, 2011).

Tissue transglutaminase is a multifunctional enzyme involved in a number of cell functions including the post-translational modification of proteins, as a scaffold protein in cell adhesion and as a cell signalling protein (Griffin et al., 2002, Eckert et al., 2014). TG2 also acts as an important part of a pro-inflammatory response and has been associated with EMT in both fibrosis and cancer (Wang et al., 2017). TG2 is associated with various physiological and pathological conditions (Wang et al., 2012). Clinical studies have correlated TG2 expression in metastatic cancer and poor survival outcomes of ovarian, breast and colon cancer patients (Hwang et al., 2008, Oh et al., 2011). Studies also suggest

that TG2 mediates several aspects of cancer cell behaviour, including motility, invasion, growth, and survival (Verderio et al., 1999, Wang and Griffin, 2013, Kumar and Mehta, 2013). Although recent studies in different cancer cell types suggest a role for TG2 in EMT as highlighted by a possible cross-talk between TG2 and three critical pathways in EMT i.e. TGFβ1, WNT- β-catenin and NFκB (Mann et al., 2006, Cao et al., 2012), there still remains a number of conflicting reports on the importance of TG2 in cancer progression with respect to its pro and/or anti-cancer roles (Torricelli et al., 2013, Kotsakis et al., 2011, Fok and Mehta, 2007). This may in part be accounted for by the pleiotropic nature of TG2 owing to its multifunctional roles (Kotsakis et al., 2011).

Consequently, this study will attempt to understand the role of TG2 in human colorectal cancer by examining the correlation of TG2 expression with CRC disease stage. Using EMT as a threshold for progression in a myriad of dysregulated cellular processes preceding tumour metastasis and tumour drug resistance the importance of TG2 in facilitating EMT will be investigated and the mechanism(s) which TG2 and its partner proteins uses to facilitate EMT will be elucidated.

1.2 Cancer

Cancer is a pathological condition characterised by uncontrolled cell growth, metastasis, and invasion. It is the world's largest cause of non-communicable disease-related death after hypertension and heart-related pathologies (Chhabra et al., 2009). The aetiology of cancer as a disease can be associated with the need of incipient cancer cells to acquire the traits that enable them to become tumorigenic and ultimately malignant. In this regard Hanahan and Weinberg (2011) have described eight different capabilities or hallmarks of cancer cells which include: the ability of cancer cells to evade growth suppressors and avoid immune detection; possess replicative immortality, invasion and metastasis; ability to induce angiogenesis and possession of genome instability and resistance to cell death; deregulation of cellular energetics, sustaining proliferative signal and promoting inflammation.

1.2.1 Colorectal Cancer

In the United Kingdom, colorectal cancer accounts for 12% of all diagnosed cancers per year (37,514 people diagnosed/year. It is the third most common cancer affecting women (after breast and lung cancer) and men (after prostate and lung cancer). The incidence of colorectal cancer is strongly related to age, with nearly 85% of all cases arising in people aged 60 years or over (CRUK, 2017). Clinically colorectal cancer is classified using the Dukes' staging system, ranging from stage A to stage D with stage A being the less severe stage. The Dukes' system of classification is used to describe the extent of invasion or spread of a tumour, which is related to overall survival. For instance, colorectal cancer patients classified as stage D have a 3% survival chance compared with a Dukes' stage A classification with an 83% overall chance of survival; estimated over a five-year period (Campbell et al., 2001).

1.2.1.1 Aetiology of Colorectal Cancer

The aetiology of colorectal cancer can be varied and complex, including genetic, somatic, lifestyle and inherited mutations. Inflammation, diet type, hormones, gut flora and energy balance have been reported to play some significant role in the development of CRCs (Slattery and Fitzpatrick, 2009). There are broadly two molecular alterations that lead to increased function of oncogenes and loss of tumour suppressor genes. The first is the clonal selection of cells, which allows for the accumulation of a plethora of defects in a cancer cell. The conversion of cellular genes into oncogenic variants can result from specific point mutations or rearrangements that alter gene structure and function, and this describes the second major classification. In general majority of the mutations or inactivation of tumour suppressor genes are somatic and not hereditary in CRCs as often found in other cancer types. It is estimated that only 15-30% of CRCs have a hereditary component. Furthermore, the majority of hereditary CRCs are as a result of the hereditary nonpolyposis colorectal cancer (HNPCC) syndromes, and familial adenomatous polyposis (FAP) and other closely related variant syndromes (Fearon, 2011). Certain genes are frequently mutated in CRCs, and in a study by Muzny et al., (2012) covering 276 somatic genes APC, *TP53, KRAS, PIK3CA, FBXW7, SMAD4, TCF7L2*, and *NRAS*.; *CTNNB1, SMAD2, FAM123B* (also known as *WTX*), *SOX9, ACVR2A, APC, TGFBR2, MSH3, MSH6, SLC9A9,*

BRAF(V600E)and *TCF7L2* were the most frequently mutated genes. They further showed in 195 CRC tumours that there were recurrent alterations in the WNT, MAPK, PI3K, TGFβ1 and p53 pathways, and amongst these mutations, two signalling pathways stood out as being affected the most by mutations. The WNT signalling pathway was altered in 93% of all tumours assessed, similarly the TGFβ signalling pathway was characterised by mutations in the TGFβR1, TGFβR2, ACVR2A, ACVR1B, SMAD2, SMAD3 and SMAD4 in 27% of the non-hypermutated and 87% of the hypermutated tumours.

Tissue transglutaminase a multifunctional protein- enzyme has been reported to interact with both WNT and TGFβ signalling in pathologies such as carcinomas (Cao et al., 2012, Condello et al., 2013) and in fibrosis (Nyabam et al., 2016). However, little has been reported on the role of TG2 in these signalling pathways in colorectal cells.

1.2.1.2 Current Treatment for Colorectal Cancer

First line treatment for colorectal cancer is surgery followed by chemotherapy to try to kill off residual cancer cells (Goodnight et al., 2000). Surgery is curative in most patients, however cancers cells left from the primary site after surgery or patients with either circulating tumour cells or with metastatic lesions will require further chemotherapy. The most common chemotherapeutic treatment for advanced colorectal cancer has been 5-fluorouracil (5-FU) since its development in 1957 by Heidelberger (Jordan, 2016). The 5-FU molecule is bio-transformed within cells into an active metabolite that interferes and inhibits DNA synthesis and repair by forming a stable ternary complex with thymidylate synthase (TS) (deGramont et al., 1997). In a bid to increase the efficacy of chemotherapy other chemotherapeutic drugs have been developed that are 5-FU analogues (Goldberg and Gill, 2004). For instance, fluoropyrimidine (Capecitabine) is a prodrug of 5-FU. Fluoropyrimidine is converted to 5-FU by thymidine phosphorylase (TP) and/or uridine phosphorylase (UP), enzymes which are more active in tumour cell (Longley et al., 2003). This improves the specificity of Fluoropyrimidine as a 5-FU based drug in tumour cells as against normal cells.

Various combination therapies have been developed to further boost the efficacy of chemotherapy for instance fluoropyrimidine (Capecitabine) a prodrug of 5-FU has been used in combination with leucovorin (folinic acid) and irinotecan (also known as CPT-11, a topoisomerase I inhibitor) in a chemotherapeutic cocktail referred to as FOLFIRI [FOL from folinic acid, F from 5-FU and IRI from

irinotecan]; another combination strategy involves fluoropyrimidine and folinic acid combined with oxaliplatin (a platinum-based compound that forms DNA adducts) referred to as FOLFOX [OX from oxaliplatin] (Goldberg and Gill, 2004).

Toxicity of the various chemotherapeutic drugs mentioned can lead to serious side effects including, nausea, diarrhea, neurotoxicity, pulmonary toxicity and interstitial pneumonia are among the major limitations for patient treatment (Montagnani et al., 2011)

A number of new drugs that aim to improve efficacy of treatment while reducing side effects, include S-1, bevacizumab (Avastin), cetuximab (Erbitux), panitumumab, adriamycin (doxorubicin hydrochloride), mitomycin, 5'-deoxy-5-fluorouridine (5′-DFUR) and uracil-tegafur (UFT) which have been developed (Andre and Schmiegel, 2005), with response rates of CRCs to combination therapy now high. Subsequent retreatment following a relapse is however usually unsuccessful due to the development of resistance (Peters and Vangroeningen, 1991), and resistance to chemotherapeutic agents remains one of the major reasons for the death of colon cancer patients after surgery. Therefore, it is essential that new strategies are developed that can re-sensitise CRCs to 5-FU-based therapies or by the finding of new therapeutic targets which needs a better understanding of the mechanisms that cause drug resistance in CRCs. It is for this reason researcher are beginning to explore the potential role of tissue transglutaminase in tumour progression.

1.3 The Transglutaminase Family

Transglutaminases (TGs EC 2.3.2.13) are a family of Ca^{2+} dependent enzymes that are structurally and functionally related. These enzymes are involved in post-translational modification of proteins by catalysing protein-protein cross-linking, amine incorporation and site-specific deamidation (Mehta et al., 2010). The cross-linking activity of these enzymes is associated with an acyl-transfer reaction between the γ-carboxamide group of peptide-bound glutamine and the ε-amino group of peptide-bound lysine, resulting in a ε-(γ-glutamyl)lysine isopeptide bond. In general, TGs are widely distributed in nature and have been identified in micro-organisms, plants, invertebrates, and vertebrates. In mammals, eight TG isoenzymes including one TG-like protein have been identified at the genomic level. These

are keratinocyte transglutaminase (TG1), epidermal transglutaminase (TG3), prostate transglutaminase (TG4), trangutaminases 5,6, and 7; factor XIII, erythrocyte band 3.2, and TG2 (Brabletz, 2012).

1.3.1 Transglutaminase 1 (Keratinocyte transglutaminase)

Transglutaminase 1, (TG1) is a membrane-bound and soluble transglutaminase which is involved in the terminal differentiation of the cornified envelope in keratinocytes (Itoh et al., 2013), whereas an inactive zymogen of the enzyme exists and is activated only during the terminal differentiation of keratinocytes to form the cornified envelope. TG1 has also been found in cell-cell adheren junctions in lung, liver and kidney epithelial cells and has been reported to be localised with cell-cell adheren junction proteins such as E-Cadherin and zona occludens, (Hiiragi et al., 1999). Physiologically TG1 has been shown to be relevant in the keratinization process, as found in TG1 null mice which showed defective keratinization in epidermal development. Additionally, it was observed that there was no compensatory mechanism from other transglutaminases as the TG1 knockout in the mice led to fatality resulting from dehydration (Martinet, 2003).

1.3.2 Transglutaminase 3 (Epidermal Transglutaminase)

Transglutaminase 3 (TG3) has been found in the inner root sheath of the hair canal (Steinert et al.1999) and the distal end of the outer root sheath (Thibaut et al., 2009). TG3 performs protein crosslinking activity of trichohyalin and keratin intermediate filaments, which make up the structure of the hair follicle, giving the hair tensile strength and shape (Lee et al., 1996). Like TG1, TG3 exists as an inactive zymogen, which undergoes proteolytic activation into its active complex of 50/27 kDa transglutaminase. TG3 activity has been found to be predominately located in inner root sheath of the hair canal and hair shaft (Thibaut et al., 2009); thus suggesting a structural role for TG3 where it supports scaffolding.

Table 2.1 Transglutaminase location and Function

Transglutaminase	Function	Gene	Cellular localisation	Tissue distribution
TG1	Keratinocyte differentiation	*TGM1*	Cytosol, membrane	Epidermis
TG2	Multiple	*TGM2*	Cytosol, membrane, nucleus, extracellular matrix	Ubiquitous
TG3	Cell envelope formation	*TGM3*	cytosol	Squamous epithelium
TG4	Semen coagulation, protection of spermatozoa	*TGM4*	unknown	Prostate
TG5	Keratinocyte differentiation, Cell envelope formation	*TGM5*	unknown	Ubiquitous
TG6	Unknown	*TGM6*	Unknown	Unknown
TG7	Unknown	*TGM7*	Unknown	Ubiquitous
FXIIIA	Blood coagulation	*F13A1*	Cytosol, extracellular	platelets
Band 4.2	Erythrocyte membrane integration	*EBP42*	membrane	Cells of the erythroid lineage

1.3.3 Transglutaminase 4 (Prostate transglutaminase)

Transglutaminase 4 (TG4) is a 77kDa protein located in the prostate where it is stimulated by androgens (Dubbink et al., 1996). In rats, TG4 is a glycosylated protein, and plays a role in the formation of the copulatory track, by masking the antigenicity of male gamete preventing sperm cells from immune destruction after copulation. Similarly, TG4 in human is has been reported to be essential in male reproduction. However increased mRNA expression of TG4 has been associated with prostate cancer; and increased TG4 in sera has been identified as a male-specific autoantigen in male infertility associated with Autoimmune polyendocrine syndrome type 1 (APS1)(Landegren et al., 2015, Iismaa, 2016).

1.3.4 Transglutaminase 5

Transglutaminase 5 is a ubiquitous transglutaminase about 81kDa protein and has been associated with terminal differentiation of the cornified envelope and hair follicle, as an active enzyme (Candi et al., 2001). In keratinocytes, TG5 has been found to be deposited in the cytosol and localized perinuclearly

(Candi et al., 2001). TG5 has also been found to co-localise with vimentin which acts as an acyl donor in intracellular filament network of fibroblasts and thus may play a role in epithelial to mesenchymal transition (EMT) (Candi et al., 2001, Iismaa et al., 2009).

1.4 TISSUE TRANSGLUTAMINASE

Tissue transglutaminase (TG2) is the most characterized member of the transglutaminase family and is constitutively expressed in endothelial cells, smooth muscle cells and myofibroblasts as well as in a number of organ-specific cell types (Akimov et al. 2000) where its function is often associated with its location within the cell type. Although the protein is mainly localized in the cytosol, it can also be found extracellularly, bound to the cell surface, deposited to the matrix or intracellularly in the mitochondria and in the nucleus (Belkin 2011).

TG2 is a structurally and functionally complex protein, with both intracellular and extracellular functions including cross-linking, GTP-hydrolyzing activities (GTPase) (Johnson and Terkeltaub 2005), a protein disulphide isomerase (PDI) function and a serine/threonine protein kinase activity (Hasegawa et al., 2003). In general, TG2 play an essential role in biologic systems in maintaining tissue stabilization, repair and immediate defense against injury or infection. However aberrant expression and activity of TG2 in tissues contribute significantly to a host of disease processes, including neurodegenerative diseases, autoimmune diseases such as celiac disease, rheumatoid arthritis, tissue fibrosis and cancer (Kumar and Mehta 2013).

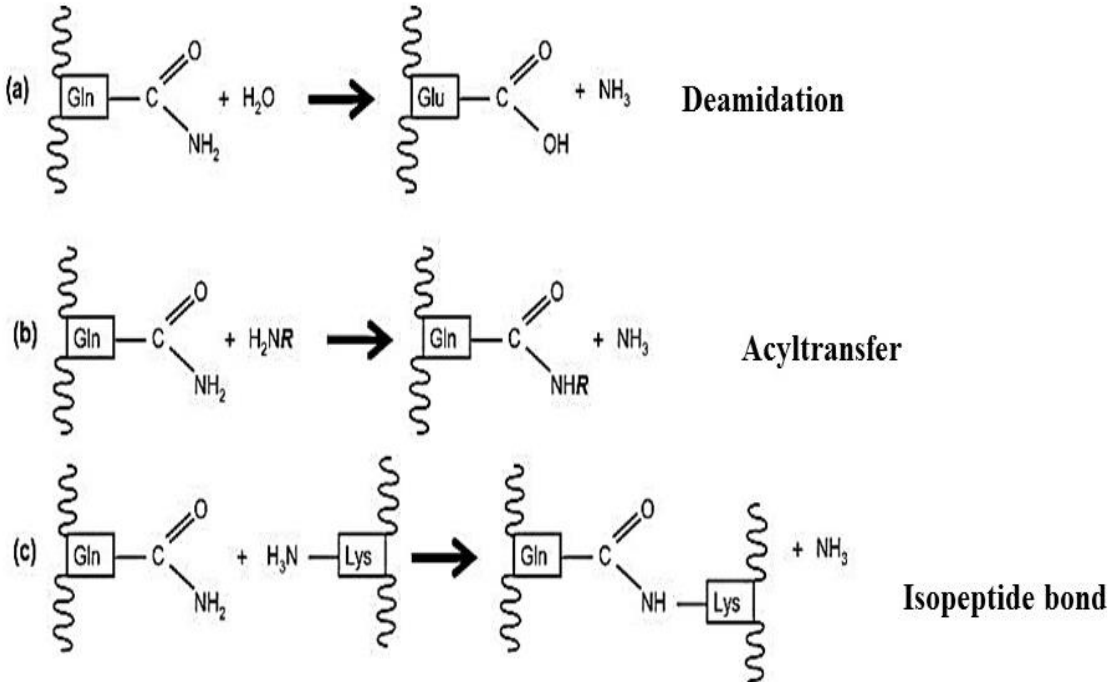


Figure 1.1. **The transamidating activity of Transglutaminase 2.** (**A**) Water can be used as an acyl acceptor in deamidating glutamine. (**B**) The acyl chain of an amino acid can be conjugated to the side chain amide of glutamine, thereby post modifying the glutamine structure. (**C**) An isopeptide bond is formed when the ε-group of a peptide bound lysine is the acyl-acceptor resulting crosslinking of two proteins (Modified from Nurminskaya and Belkin (2012).

1.4.1 Structure of TG2

The human TG2 is a 78 kDa monomeric protein consisting of 687 amino acids (Griffin et al., 2002). It is composed of four domains which comprises of an N-terminal b-sandwich domain, a catalytic core, and two C-terminally located b-barrels (**Figure 1.2**). These domains are made up of amino acids from 1–139, 140–454, 479–585, and 586–687, respectively existing in various secondary structures (Jang et al., 2014). Domains 1, 3, and 4 are folded in b-structures and domain 2 contains a α-helical secondary structure (Pinkas et al., 2007). The active site of the enzyme is located in the second domain which serves as the core catalytic domain and consists cysteine 277, histidine 335, and aspartate 358, arranged in a charge-relay system (Pinkas et al., 2007). The catalytic domain is hidden from contact with peptidylglutamine substrates due to overlayering of domains 3 and 4 in a resting or compact conformation thus conferring transamidating inhibitor roles to the third and fourth domains (Bergamini et al., 2011). During activation of the enzyme, the interaction between domain 2, domains 3 and 4 breaks down after the binding of Calcium ions, a cofactor in the transamidating activity. The catalytic activity of TG2 requires high calcium (>1 mM) and low GTP (<9 μM), essentially making intracellular TG2 a

cryptic enzyme under physiologic conditions due to the physiological low intracellular $Ca2^{+}$ ion concentration and high GTP concentration, and thus locking the enzyme in its role as a G-protein, and other non transamidating functions (Grenard et al., 2001).

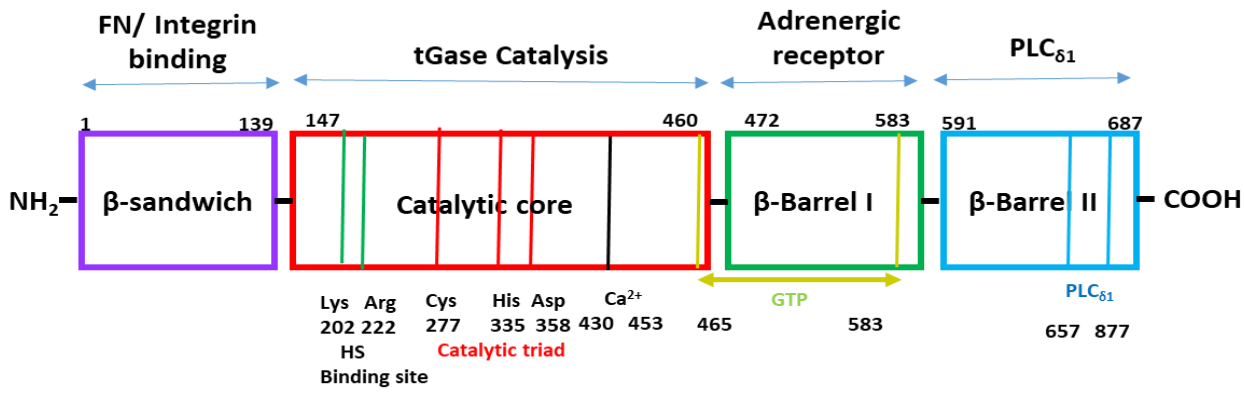


Figure 1.2 TG2 protein secondary Structure and Function
The TG2 protein consists of four distinct domains which perform different functions. The N-terminal β-sandwich domain modulates TG2 Fibronectin interaction. The β-sandwich thus functions as an anchor for TG2 in the ECM. The transglutaminase activity of TG2 is performed by the catalytic core domain which also holds the binding site for $Ca2^{+}$ a cofactor in the catalysis. The Barrel 1 domain contains a guanosine-tri/di-phosphate (GTP/GDP) -binding site which plays an important role in TG2-mediated signalling pathways. The C-terminal Barrel 2 domain under certain conditions (e.g., in GTP-bound form) can recruit and activate phospholipase C and contributes to the pro-inflammatory functions of TG2.

1.4.2 Regulation of TG2 Gene expression

TG2 is encoded by the gene *TGM2* in humans which is consists of 32.5Kb located in chromosome 20q11-12 (Gentile et al., 1991) and consists of 13 exons and 12 introns. The *TGM2* promoter is characterized by a retinoic acid (RA) response element (RRE) located 1.7 kb upstream from the initiation site, an interleukin-6 specific cis-regulatory element located within first the 4.0 kb of the promoter. Upstream of the open reading frame (ORF), classic TATAA and CAAT boxes are found along with sequences to bind transcription factors NF-kB and Sp-1, a TGFβ1 response element (GAGTTGGTGC) located 868 base pairs upstream, and two activator protein (AP)-2-like response elements located approximately 634 and 183 base pairs upstream from the transcription initiation site (Nadalutti et al., 2011). A 3.8 kb sequence 5' upstream of the initiation site is responsible for tissue-specific expression of the gene, and also contains the elements that convey the retinoid specificity in

the induction of the transcription of the TG2 gene (Bergamini et al., 2011). In general, the expression of TG2 is suggested to be controlled mainly at the transcription level (Griffin et al., 2002) and is regulated by an array of transcriptional inducers which act either directly or indirectly and have been identified to include inflammatory cytokines, such as tumour growth factor beta (TGFβ), tumour necrosis factor TNFα (Chiocca et al., 1988), interleukin 1 and 6 (IL-1 and IL-6)(Oh et al., 2011), interferons and several steroid hormones (chiefly glucocorticoids and progesterone); vitamin D (particularly in macrophages); and retinoids (**Figure 1.3**). It has been reported that the TG2 gene contains regulatory elements for several transcriptional factors, for example, two tandem RREs, which bind the retinoid receptors RAR and RXR. RA promotes the expression of TG2 via its interaction with its receptors to promote the formation of RAR/RXR heterodimers or RXR/RXR homodimers, which triggers the transcription of the gene (Mehta et al., 1996). With regards to TG2 expression by inflammatory cytokines, TNFα can induce the expression of TG2 either directly through a responsive element at the TG2 promoter or through activation of the NFkB system. TGFβ1 also induces the expression of TG2 via multiple pathways, in mouse it has been shown that TGFβ1 can directly induce TG2 via a response element on the TG2 gene promoter additionally TGFβ may also induce TG2 via TGFβ activated kinase 1 (TAK1) activation of NFkB (Cao et al., 2012).

Cacciamani et al., (2002) posits that DNA methylation alongside alterations in the chromatin structure may also play a role in TG2 expression. In this regard, the TG2 promoter region can be highly methylated at CpG islands, silencing the gene and thus making the promoter region a regulatory site for methylation and demethylation (Lu and Davies 1997). This was further shown by Ai et al., (2008) in breast cancer cells where hypermethylation at the CpG islands in the TG2 promoter resulted in the lack of expression of TG2. Similar observations were made in non-small cell lung cancer (Park et al., 2010) and in glioma (Dyer et al., 2011). Histone deacetylases may also down-regulateTG2 promoter activity by leaving the DNA bound to its histone proteins. Overall, TG2 is can be described as a stress response protein and is generally up-regulated under stress conditions.

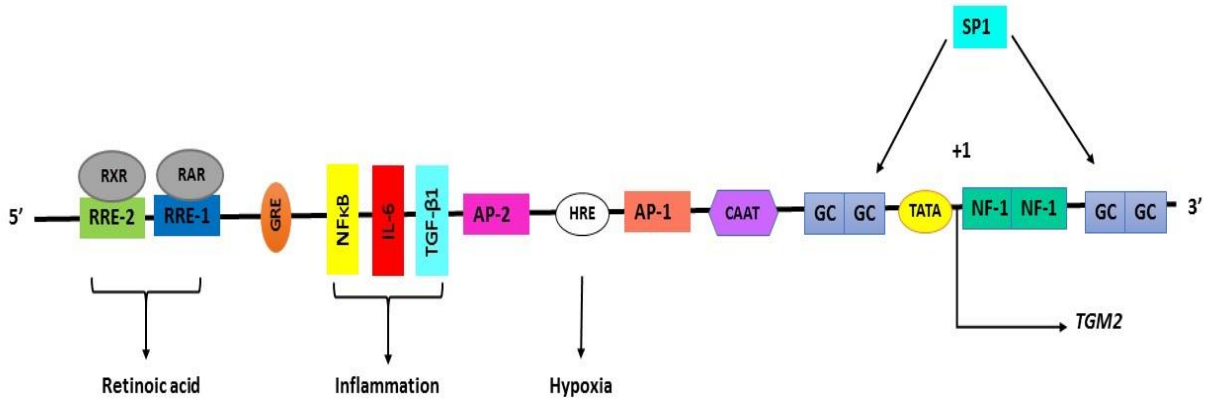


Figure 1.3 Regulation of TG2 gene expression (*TGM2*). Inflammatory and hypoxic elements regulate TG2 gene expression. Retinoic acid response and glucocorticoid response elements are located in the promoter region of *TGM2*. Retinoic acid response elements. Glucocorticoid response element (−1399 bp), NF-κB (−1338 bp), IL-6 response element (−1190 bp), AP-2 (−634 bp), HRE (−367 bp).

1.4.3 REGULATION OF TG2 ACTIVITY

Adding to the complexity of TG2 is its ability to exert opposing effects depending on its localization and predominant activity. However, three major regulators of TG2 activity are Ca^{2+}, GTP/GDP, and redox potential. Binding of Ca^{2+} ions to TG2 promotes its catalytically active 'open' or 'extended' conformation. Binding to GTP or GDP, in contrast, promotes the catalytically inactive 'closed' or 'compact' conformation (Agnihotri et al., 2013). Under physiological conditions, TG2 exists as a latent enzyme intracellularly as a result of the low Ca^{2+} levels and the inhibitory effect of GTP/GDP (Klöck and Khosla, 2012). The regulatory effect of GTP on TG2 activity has been well reported in vitro studies (Jang et al., 2014). Smethurst and Griffin (1996) confirmed in electropermeabilised islets of Langerhans that the binding of GTP to TG2 leads to conformational changes which results in reduced Ca^{2+} affinity and loss of activity. In the catalytically inactive or compact form, TG2 serves as a scaffold protein or a signal transduction GTP-binding protein and regulates signalling pathways by binding and altering the function or stability (or both) of certain effector proteins (Agnihotri et al., 2013).

In a similar fashion, nitric oxide (NO) is a potent inhibitor of TG2. NO is a powerful antioxidant and acts both by controlling activity of guanylate cyclase (which is crucial in several critical processes as,

e.g., vascular responsiveness and immunologic responses) and in a more direct way by promoting protein post-translational modification mainly through a Ca^{2+}-sensitive nitrosylation of multiple cysteine residues (Telci et al., 2009) but also at tyrosine residues. Nevertheless, under extreme conditions of cell stress or trauma after the disturbance or loss of Ca^{2+} homeostasis, TG2 may be activated to its catalytically active extended form and cause cross-linking of intracellular proteins, as is observed during apoptosis or necrosis (Agnihotri et al. 2013).

Figure 1.4 TG2 3D Structure regulates Function. 3D structure of TG2 showing the four domains. (A) Represents the structure of TG2 bound to GDP in the closed conformation, in which the active site of the catalytic domain is shielded from both Ca^{2+} and substrates. (B) Represents TG2 in the extended form, where Ca^{2+} binds and opens the catalytic triad for substrate binding and subsequent enzymatic reaction. Here an inhibitor is shown bound to the catalytic site of TG2. (Modified from Pinkas et al., 2007)

On the other hand, the extracellular milieu has a considerably lower concentration of GTP/GDP and abundance of free calcium, and thus it is safe to say that TG2 can be expected to be present in a catalytically active form. However, most extracellular TG2 is enzymatically inactive as a result of disulfide bonding between Cys230 and Cys370; and Cys370-Cys371. The state of the disulfide bond between these cysteine residues depends on the oxidizing conditions in the extracellular environment. The inter-strand disulfide bond between Cys230 and Cys370 facilitates the formation of the more stable Cys370-Cys371 disulfide bond that inactivates TG2. Reactivation of TG2 can be achieved either under

favourable redox potential or by protein cofactor thioredoxin 1. Once activated, TG2 can crosslink extracellular matrix (ECM) component proteins and stabilize the matrix for increased cell attachment, cell motility, and outside-in signalling cues resistance and a metastatic phenotype (Agnihotri et al., 2013). In addition, Magnesium ions (Mg^{2+}) which are also essential for the hydrolysis of both GTP and ATP. Mg^{2+}- ATP induces a conformational change in TG2 which inhibits GTPase but does not interfere with the cross-linking activity, while Mg^{2+}-GTP binding induces a different conformation which inhibits cross-linking activity without affecting the ATPase activity of TG2 (Lai et al. 1998). In general, the complexity of regulation of TG2 is brought about by changes in the properties of the enzyme in relation to its subcellular distribution (Piacentini et al., 2014).

1.4.4 Externalization of TG2

TG2 externalization does not follow the classical ER/Golgi pathway, this is due to the absence of a hydrophobic leader sequence (Verderio et al., 2004), and in the past, it was hypothesised that TG2 could not be secreted in healthy cells under physiological conditions except during stress or cellular damage when TG2 was thought to leak out of the cell into the ECM. However, Griffin and co-workers showed for the first time that increased expression of TG2 in Swiss embryonic fibroblast led to TG2 increase in the ECM and that this event was independent of external or stress-related stimuli (Verderio et al 1999). Till date, the secretion pathway of TG2 remains unclear, and several mechanisms have been identified involving integrins (Nurminskaya and Belkin, 2012) and syndecans (Wang et al., 2012). However, a consensus appears to involve the TG2 N terminal β-sandwich domain, and that a closed TG2 conformation is critical for externalisation (Badarau et al., 2015). More so, heparan sulphate (HS) binding site in TG2 may be required for the deposition of TG2 in the ECM via the shedding of syndecan-4, a member of the heparan sulphate proteoglycans, as mutations in the HS-binding of TG2 has been shown to limit TG2 matrix deposition a finding made in two independent studies (Lortat-Jacob et al., 2012; Wang et al., 2012).This is especially significant in fibrosis as it has been shown *in vivo* in renal fibrosis model, as sydecan 4 (Sdc4)-null mice subjected to either unilateral ureteric obstruction or aristolochic acid nephropathy (AAN) in order to mimic kidney fibrosis displayed significantly reduced extracellular TG2 and TG2 activity when compared to Sdc4 wt mice (Scarpellini et al., 2014). The use

of inhibitors or antibodies that target TG2-Sydecan 4 interaction may thus hold therapeutic value as the crosslinking activity of TG2 plays a role in the stabilization of the ECM components, such as FN and collagens, and has been reported to be important in the release of active TGFβ1 from the Latent TGFβ binding protein (LTBP) (Verderio et al., 1999).

1.5 PHYSIOLOGICAL ROLE OF TG2

TG2 plays an enigmatic enzyme owing to its multifunctional status, and it thus plays diverse roles in normal physiology, some of which are still unclear. Basically, TG2 is involved in protein transamidation and signalling activity, which alternatively predominate over each other ending in the enzyme location and milieu (Park et al., 2010). However, a growing body of evidence is suggestive of the role of TG2 in several biological and physiological processes including cell adhesion, proliferation, differentiation, apoptosis, wound healing and matrix stabilization (Agnihotri et al. 2013).

1.5.1 Intracellular Function of TG2

The transamidating reactions catalysed by TGs are potentially irreversible, and significantly alter the substrates by either affecting their solubility in the case of protein cross-linking, or altering surface charge by conversion of a neutral glutamine residue into a positively charged secondary amine following polyamidation or into a negatively charged glutamate residue; it is for this reason that TG2 is kept latent in the cell activated only during periods of cellular stress, imbalance in Ca^{2+} homeostasis or extreme insult (Bergamini et al. 2011). However, enzyme kinetic studies are beginning to shed light on a transient open TG2 conformation in equilibrium with a TG2 closed conformation (Keillor et al., 2015).

TG2 role in cellular apoptosis is still contradictory, Piacentini et al., (1991) reported that TG2 catalytic activity is increased in dying cells. Activated TG2 irreversibly crosslink cellular proteins resulting in the formation of a detergent-insoluble protein scaffold which modifies the cell's organization leading to ultra-structural changes characteristic of cells undergoing apoptosis. The insoluble TG2-catalyzed protein scaffold stabilizes the integrity of dying cells allowing for efficient phagocytosis and thus preventing the leakage of harmful intracellular components and the consequent inflammatory responses and scar formation in nearby tissue (Cho et al. 2010). Other studies also suggest that TG2 promotes

apoptosis by polymerising leucine zipper-bearing kinase (DLK) which subsequently increasing its kinase activity and activation of the JNK pathway in NIH-3T3 mouse fibroblasts and human breast epithelial cancer cells MDA-MB-231 (Robitaille et al., 2008). However previous findings suggest that increased expression of TG2 may prolong cell survival by preventing apoptosis in breast cancer cells (Herman et al.,2006).

Reports also indicate that TG2 may influence transcriptional or signalling events by acting as a scaffolding protein with several intracellular proteins involved in important physiological processes (Kumar et al. 2012). TG2 also exhibit a protein disulfide isomerase (PDI) function which is independent of Ca^{2+} or GTP. The TG2 PDI activity can mediate post-translational protein modifications. (Hasegawa et al., 2003). For instance, TG2 PDI has been reported to be crucial in maintaining the correct assembly of the mitochondrial ADP/ATP transporter adenine nucleotide translocator 1 (ANT1) (Herman et al., 2006). TG2 has also been shown to have intrinsic protein kinase activity, employing as phosphoryl donor. This suggests that TG2 may be involved in kinase activity with its ATP-binding site. This kinase activity targets serine and threonine residues in basic proteins such as histones, insulin-like growth factor binding protein-3 (IGFBP-3) and oncoprotein p53. In addition to these TG2 has many binding proteins such as PLCδ1 (Hwang et al., 1995; Kang et al., 2002), PKA anchor protein 13 (Lewis et al., 2005), Rac1 (Kim et al., 2010), and NFkB (Kumar and Mehta 2012) which are localized in the cytoplasm. There are numerous factors that might contribute to the regulation of intracellular TG2 asides from Ca^{2+}, for example, pH variations, endogenous nitric oxide, protein nitrosylation levels or monoamines (serotonin, histamine, dopamine, and norepinephrine, etc.) (Nadalutti et al., 2011).

1.5.2 Plasma membrane Functions of TG2

TG2 is located on the plasma membrane where the GTPase activity dominates. In the plasma membrane where the transaminating and cross-linking activity of TG2 remains inhibited, and the enzyme acts as an intracellular G-protein in response to endocrine-like factors and functions in intracellular signal transduction (Nakaoka et al., 1994). The plasma membrane role of TG2 may also include organization of the actin cytoskeleton, modification of extracellular ligands and receptors, control of growth and

metastatic spreading of tumour cells, terminal differentiation of specialized tissues, and modulation of local inflammatory responses (Kumar et al., 2014).

1.5.3 Extracellular Function of TG2

In keeping with some of the intracellular roles as a scaffolding protein not requiring transamidating activity, TG2 can also function in a similar fashion in the extracellular environment either at the cell surface where it may act as integrin co-receptor for the binding of Fibronectin (FN) via the 42-kDa gelatin fragment or following TG2's deposition into the (Extracellular matrix) ECM where it acts as a heterocomplex with FN in the binding of cell surface heparan sulphate proteoglycans (Bergamini et al., 2011)**.** TG2 is thought to be retained on the cell surface by its interaction with FN (Akimov et al., 2000) and syndecans (Wang et al., 2012). Furthermore, FN cross-linking mediated by TG2 offers FN protection from degradation. Aside from TG2's interaction with FN extracellularly, TG2 is also associated with integrin receptors in several different cell types via binding to the extracellular domains of the β1, β3 and β5 integrin subunits (Akimov et al. 2000). Based on the interaction between TG2 with FN or integrins, one can theorise that TG2 plays a role in the cell adhesion process. TG2's interaction with integrins may also be a stable non-covalent one independent of the transamidating activity of TG2 (Akimov et al. 2000). Although the binding sites for TG2 within integrins are still unknown, it has been demonstrated that integrin-TG2 complexes have a 1:1 stoichiometry and that TG2 on the cell surface is bound to integrin receptors in a FN-independent manner (Akimov et al. 2000). Additionally, TG2 can help stabilise the matrix by crosslinking collagen and by supporting the activation of TGFβ (Verderio et al., 1999). TG2 also exists in the ECM as a structural protein and may also function as an ECM protein in RGD-independent cell adhesion (Verderio et al., 2003). In this regard, TG2 bound to the FN matrix compensates for anoikis induced by RGD synthetic peptides in a protein kinase C (PKC α) and focal adhesion kinase (FAK) dependent mechanism. In addition, a recent study has shown that GTP-bound TG2, when present in the ECM, can induce hypertrophic differentiation of chondrocytes through a α5β1 integrin-dependent and FAK-associated cell adhesion process (Tanaka et al., 2007). Cell surface TG2 is highly sensitive to the proteolytic degradation mediated by membrane-type matrix metalloproteinases (MT-MMPs), thought to be primarily involved in ECM degradation (Kessenbrock

et al., 2010). It has been reported that proteolysis of TG2 by MT1-MMP specifically suppresses cell adhesion and migration to fibronectin (Belkin et al., 2011).

1.6 THE ROLE OF TG2 IN PATHOLOGICAL CONDITIONS

TG2 has been implicated in an array of pathological states: autoimmune disorders (including celiac disease), maturity-onset diabetes of the young (MODY) (Bernassola et al., 2002), neurodegenerative disorders (Bergamini et al., 2011)including Huntington's, Alzheimer's, and Parkinson's diseases, inflammation (Kumar and Mehta 2013), fibrosis (Nyabam et al., 2016) and cancer (Kumar et al., 2012).

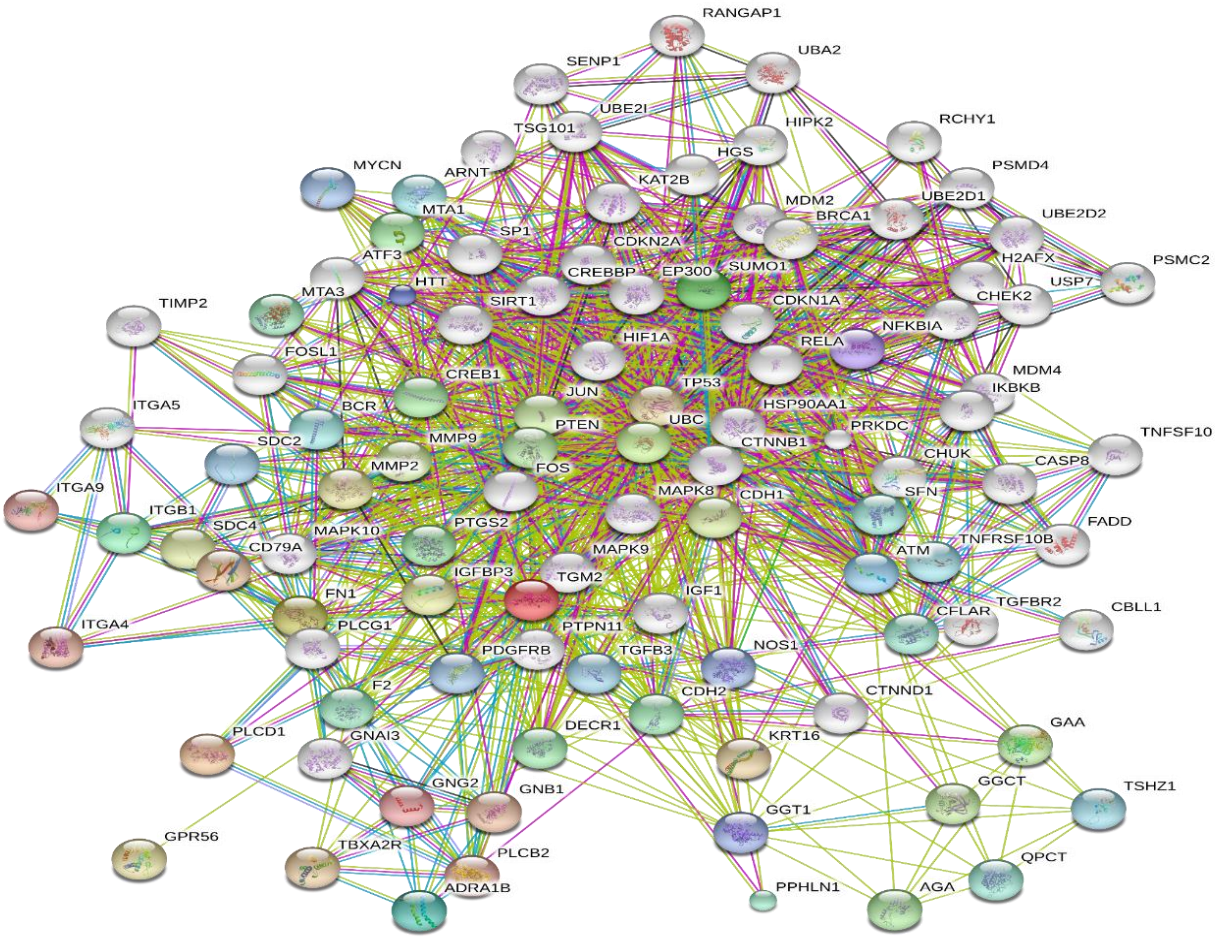


Figure 1.5 TG2 evolving cellular interactome. TG2 interaction with multiple cellular (inter and extracellular) partners (green stems experimental, blue stem predicted). TG2 is involved with multiple protein partners in affecting cellular processes. Interactions were modelled using STRING (*Search Tool for the Retrieval of Interacting Genes/Proteins*).

1.6.1 TG2 and Tumour Progression

The role of TG2 in cancer progression has been highly contested in the literature and can be found to have pleiotropic roles owing majorly to its multifunctional and ubiquitous nature (Kotsakis et al., 2011). It is suggested that in primary tumours, TG2 expression is usually reduced while increased TG2 expression is observed in chemoresistant and secondary metastatic tumours (Kotsakis and Griffin, 2007). In addition, it has been shown that TG2 influences several aspects of cancer cell behaviour, including motility, invasion, growth, and survival (Collighan and Griffin 2009). In performing this role, it is proposed that TG2 can make use of either its catalytic cross-linking, GTP binding or other protein function to mediates signal transduction via phospholipase C, focal adhesion kinase and PI3K activation. The extent to which TG2 may be relevant in cancer has been reported in many cancer cell types, including pancreatic carcinoma (Mehta and Han, 2011), breast carcinoma (Agnihotri et al., 2013), malignant melanoma (Yang et al., 2014), ovarian carcinoma (Cao et al., 2012), lung carcinoma (Park et al., 2010), Mesothelioma (Zonca et al., 2017) and glioblastoma. Furthermore increased TG2 expression in cancer cells has been linked to increased drug resistance, metastasis and poor patient survival (Chhabra et al., 2009).

Ai et al., (2008) have shown that that epigenetic silencing of TG2 expression may attenuate drug resistance, metastasis, and poor patient survival. However, Jones et al., (2006) have reported that TG2 may play an inhibitory role in tumours as injection of active TG2 into melanoma tumours inhibited tumour progression due to increased crosslinking of ECM proteins such as collagen and blocked the tumour growth. These observations suggest that TG2 may affect several aspects of tumour progression in a cell type-specific manner.

Hanahan and Weinberg, (2011) have described ten different capabilities or hallmarks of cancer cells to include the ability of cancer cells to evade growth suppressors, avoid immune detection, possess replicative immortality, invasion and metastasis, inducing angiogenesis possessing genome instability, resisting cell death, deregulating cellular energetics, sustaining proliferative signals and promoting inflammation (**Figure 1.6**). Depending on the cancer cell type, TG2 has been shown to facilitate some of these hallmarks.

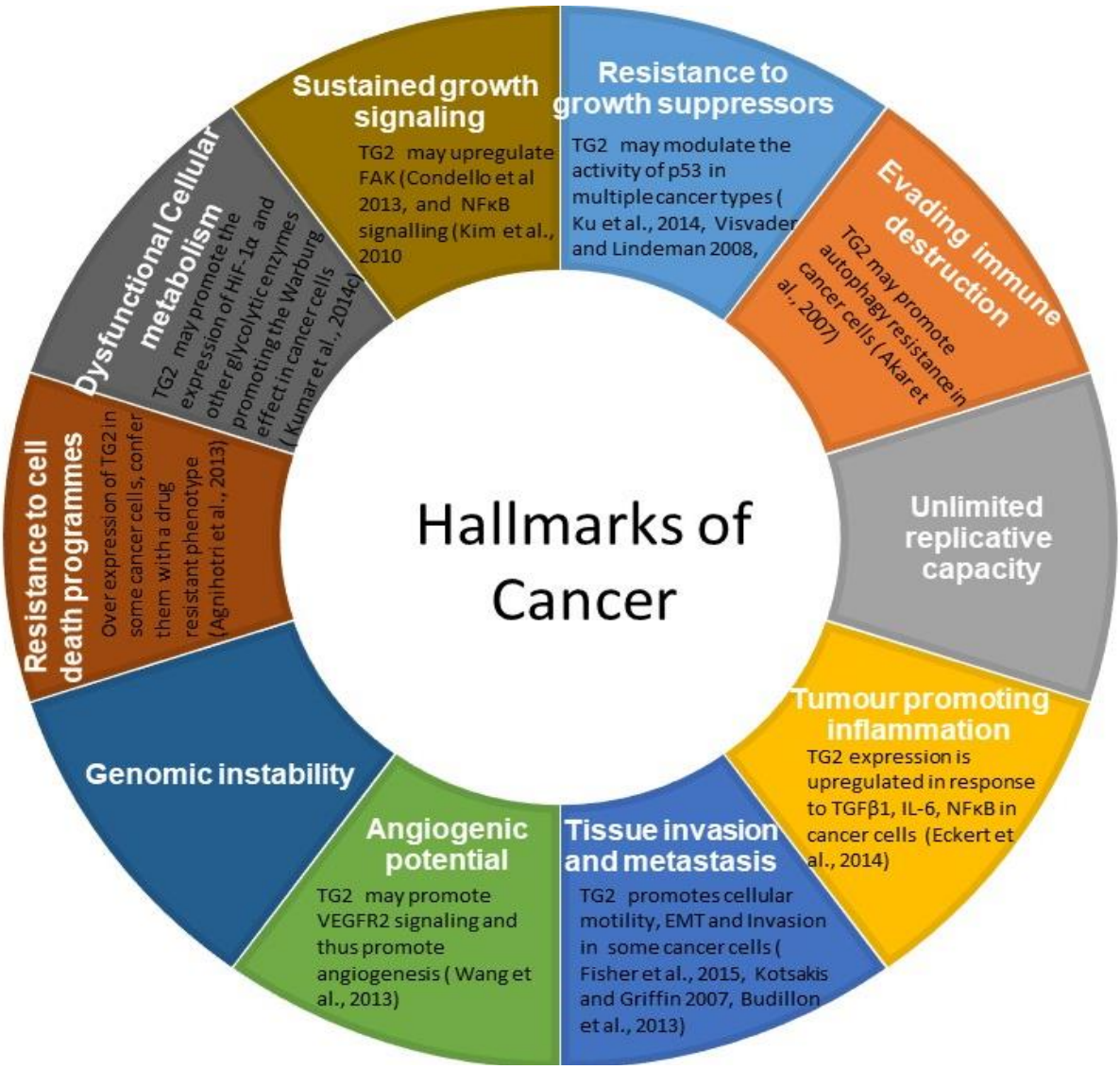


Figure 1.6 Hallmarks of Cancer. Cancer cells acquire the following traits to maintain their oncogenic status. These hallmarks are potential points for targeted therapy. A review of the literature suggests that TG2 may facilitate some of these traits in various tumour cells and may indicate that TG2 could hold therapeutic value in cancer cells

1.6.1.1 TG2 is involved in sustaining proliferative signals

TG2 can regulate cell signalling pathways that promote cell survival, cell motility, cell attachment, and invasive behaviour (Mehta and Han, 2011). Nuclear factor kappa B (NFκB) has been shown to be necessary for cellular proliferation in human breast cancer cells by promoting S-phase progression (Biswas et al., 2004). Verma and Mehta (2007) have shown that TG2 can form a direct complex with NFκB p50/p65 dimers in the cytoplasm and modify its affinity for IκBα. More so it has also been reported that IκBα the inhibitory component of NFκB is a good substrate for TG2 and that it can be crosslinked to form an insoluble cytosolic polymer that is unable to bind and sequester NFκB cytosol.

More recently has shown that TG2 can also associate with p65 in the nucleus and redirect it to non-canonical targets, including the TG2 gene itself, leading to the formation of a positive feedback loop in cancer (Kim et al. 2010). Furthermore, Li et al., (2011) demonstrated that TG2 mediates tumours necrosis factor-related apoptosis-inducing factor (TRAIL) resistance and cell migration through c-FLIP and MMP-9. More so, TG2 can interact with integrins, as TG2- mediated clustering of integrin potentiates outside-in signalling. Integrin-TG2 interaction modulates the integrin-dependent activation of FAK, (Ranganathan et al., 2007, Condello et al., 2013).

1.6.1.2 TG2 is employed by Tumour Cells to Evade Growth Suppressors

TG2 has been shown to play a role in the down-regulation of tumour suppressors in various cancer cells and via multiple mechanisms involving both intra and extracellular role of TG2. In renal carcinoma cells, it has been reported that TG2 mediated the expression of p53 a tumour suppressing protein. It was shown that TG2 directly cross-linked the DNA binding domain of p53, leading to p53 depletion *via* autophagy (Ku et al., 2014). Other reports also show that TG2 can bind to p53 irrespective of its mutation leading to ubiquitination and subsequent proteasomal degradation of p53 (Visvader and Lindeman, 2008). In neuroblastoma cell line SH-SY5Y, TG2 expression was associated with increased protection of cells from topoisomerase II inhibitor etoposide. This study further showed that the protective effect of TG2 was associated with the transamidation active form of the enzyme, because overexpression of the wild-type TG2, but not its transamidation inactive C277S form, resulted in a pronounced suppression of caspase-3 activity as well as p53 phosphorylation during etoposide-induced stress (Kumar et al., 2014b).

1.6.1.3 Tumour Promoting Inflammation

Chronic inflammation is an important driving force that provides a favourable platform for cancer's progression. NFκB is considered central to inflammation-induced tumours progression. As discussed earlier NFκB can facilitate TG2 expression non-canonically through the ataxia telangiectasia mutated (ATM) signalling or by binding to two independent consensus sites in the *TGM2* promoter region, resulting in a feedback loop as TG2 also facilitate activation of NFκB. In addition, TG2 in turn

complexes with NFκB, the TG2-NF-κB complexes binds to the *HIF-1α* promoter and results in its transcriptional regulation and expression. Increased expression of HIF-1α then modulates the expression of downstream genes that activate EMT, angiogenesis, and acquisition of stem cell-like traits (Agnihotri et al., 2013). In addition, other reports have shown that TG2 expression is up-regulated in the presence of other inflammatory signals such as TGFβ, IL-6, and TNFα all of which also induce EMT (Eckert et al., 2014).

1.6.1.4 Deregulating Cellular Energetics

Cancer cells survive by switching on to glycolysis for energy production this is in part due to the fact that tumour cells proliferate at a level where it is physiologically impossible for an extensive distribution of blood vessels. This condition is regarded as the Warburg effect. Kumar et al., (2014c) have shown that the genetic alterations leading from upregulation of TG2 involve the upregulation of various glycolytic enzymes. The major mechanism responsible for metabolic reprogramming is the upregulation of expression of hypoxia-inducible factor (HIF) 1α, a transcription factor that shifts energy production from mitochondrial to glycolytic sources in hypoxic regions of tumours.

1.6.1.5 Role of TG2 in angiogenesis during cancer progression

Angiogenesis is a very important stage in tumour development as tumour cells require a blood supply to grow and metastasize (Hillen and Griffioen, 2007, Zhang et al., 2001). The role of TG2 in angiogenesis is still unclear and very mixed (Fan et al., 2012). TG2 can support a stable ECM, which displays an anti-angiogenic property and can inhibit proliferation and spread of malignant cells mainly due to the collagen cross-linking caused by TG2 (Jones et al., 2006). Experiments have shown that over production of collagen due to TG2 stabilizes the ECM preventing blood vessel formation and may be a pathway undertaking by TG2 in exacerbating fibrosis (Haroon et al., 1999), it has been shown that administration of TG2 can stop angiogenesis without causing cell death. This is done by increasing the protein build up in the ECM (Jones et al., 2006). However, Griffin and co-workers, in their work identified that extracellular inhibition of TG2 activity using its specific inhibitors led to inhibition of angiogenesis in human umbilical vein endothelial cells via a reduction in VEGFR2 signalling (Wang et

al., 2013), and have shown that inhibition of TG2 activity blocked the vascular mimicry, where the vascular structures are derived from the tumour itself (Jones et al., 2013).

1.6.1.6 Activating Invasion and Metastasis

1.6.1.6. EMT, Cancer Invasiveness, Drug resistance and TG2

In normal physiology, Epithelial-Mesenchymal Transition (EMT) is a dynamic and essential process required in reprogramming epithelial cells during embryonic development. However persistent reactivation of EMT in adulthood is associated with various pathologic conditions including cancer and fibrosis (Kumar and Mehta 2013, Nyabam 2016). Recent studies have implicated EMT as the first necessary step in metastatic dissemination and tumour progression. Cells undergoing EMT acquire the ability to degrade the basement membrane as a result of increased activity of matrix metalloproteases (such as MMP2, MMP3, and MMP9); and these cells migrate through the ECM to populate different areas during cancer progression (Kumar and Mehta, 2013). Ilyas et al., (1997) suggests that EMT is essential for the development of drug-resistant phenotypes in cancer cells, and enables them to become invasive at the early stage of the disease.

EMT can be induced by a variety of different stimuli (growth factors / cytokines) including Hepatocyte Growth Factor (HGF), Transforming Growth Factor (TGFβ), Platelet-Derived Growth Factor Receptor (PDGFR), Wnt and Hedgehog (Christiansen and Rajasekaran, 2006). TGFβ is a major inducer of EMT, resulting in suppressed expression of various epithelial proteins such as E-cadherin and enhanced mesenchymal proteins such as fibronectin and vimentin. TGFβ operates as both a tumour suppressor and promoter by acting via Smad (e.g. Smad 2 and 4) or non-Smad (e.g. c-Myc, PI3-kinase and MAPK) pathways (Morris et al., 2010). Key targets of TGFβ signalling are the transcriptional repressors of E-cadherin; upregulation of Snail, Slug and Twist, Zeb-1 and Zeb-2. Wnt signalling is also a key regulator of EMT during cancer progression. The canonical βcatenin-dependent Wnt signalling pathway results in the nuclear accumulation of β-catenin. This causes transcriptional activation of target genes, involved in EMT, through the lymphoid enhancer factor 1 (LEF-1)/TCF transcription factors. These include vimentin and fibronectin, which are both markers of a mesenchymal morphology (Nawshad et al., 2007).

1.6.1.6.2 Key Alterations and Factors That Govern EMT

A number of key alterations occur during EMT, and several markers have been identified, that distinguish epithelial and mesenchymal cells (**Figure 1.7**). During EMT, the polarized epithelial cells, which normally interact with the basement membrane via its basal surface, undergo multiple biochemical changes that enable them to acquire a mesenchymal phenotype. Initially the perturbation of cellular junctions is required. This requires the loss of adherens junctions, loss of tight junctions, gap junctions and desmosomes (Voulgari and Pintzas, 2009). The 'cadherin switch' where epithelial cells downregulate E-cadherin and up-regulate N-cadherin is often associated with EMT. This results in the breakdown, or loosening, of cell-cell contacts and the cells adopt a more migratory phenotype due to the pro-migratory role of N-cadherin (Wheelock et al., 2008). Changes in the cytoskeleton is also common to promote the necessary structural machinery for cellular migration. Another key marker of EMT in this regard, is vimentin, an intermediate filament family member which is commonly used as a mesenchymal cell marker. In order to detach from the ECM and initiate migration or invasion during EMT, cells require matrix metalloproteinases- a set of enzymes that cleave structural proteins of the ECM enable degradation of the basement membrane. MMPs, specifically MMP2 and MMP9 are associated with mesenchymal cells and are upregulated in EMT. For example, MMP activities enable the degradation of the basement membrane and ECM to allow the more motile and invasive N-cadherin expressing mesenchymal-like colon cancer cells to infiltrate into the surrounding tissues and body circulatory systems (Wheelock et al., 2008, Curran and Murray, 2000). The transcriptional control of EMT is maintained by Zeb-1, Zeb-2, Twist1 Snail, and Slug which effectively turn on the EMT process (Agnihotri et al., 2013).

Figure 1.7 Cellular changes during EMT. Epithelial cells lose cell polarity, epithelial cell tight junctions with significant cytoskeletal reorganisation. The TGFβ, Wnt and Notch signalling process play a role in activating Snail, Slug and Zeb 1/2-transcription factors that regulate EMT. (Modified from Aroeira et al., 2007).

1.6.6.3.3 The Role of TG2 in EMT

TG2 as a proinflammatory protein has been shown in certain cancer and fibrotic models to induce promote EMT (Agnihotri et al., 2013, Eckert et al., 2014, Ku et al., 2014, Condello et al., 2013, Nyabam et al., 2016, Verderio et al., 2004), although, other studies also report that TG2 role in the ECM may perturb EMT (Kotsakis and Griffin 2007). In general TG2-mediated EMT in cancer cells have been reported to also confer metastasis , invasiveness, drug resistance, apoptotic resistance and a tumorigenic phenotype (Budillon et al., 2013). In breast cancer, Mehta and colleagues found that that Stable expression of TG2 in mammary epithelial cells resulted in the loss of epithelial markers (E- cadherin) and gain of mesenchymal markers (vimentin, fibronectin, N-cadherin, e.t.c). More so TG2-expression exhibited a significant increase in Snail1, Twist1, and Zeb1 transcription repressors which was

accompanied by increased invasiveness and the ability to form colonies in agarose (Agnihotri et al., 2013). Similar findings have been reported in the breast (Kumar et al., 2010a, Morin et al., 1997), Squamous (Fisher et al., 2015a), prostrate, and ovarian (Cao et al., 2008) cancer. The inhibitory role of TG2 in EMT in certain cell context has been shown with cell surface TG2 which modulates cell migration in an ECM dependent way and proteolytic degradation of cell surface TG2 by membrane-type matrix metalloproteinases (MT-MMPs) in gliomas and fibrosarcomas, resulting in increased cellular motility of tumour cells on FN (Kotsakis and Griffin 2007). Taken together TG2 may play an important role in EMT in cancer cells either by enhancing cell signalling processes that promote EMT in on the one hand or inhibiting cell migration by crosslinking the matrix.

1.6.1.6.4 TG2 enhances autophagy resistance in Tumour cells

Studies by Akar et al., (2007) have identified a potential new role of TG2 in tumour cells autophagy resistance. It was demonstrated that protein kinase C-delta (PKCδ) constitutively protected cells from autophagy by up-regulating TG2 expression in pancreatic cancer cells and significant autophagic cell death was observed following downregulation of TG2 by siRNA or by PKCδ inhibition.

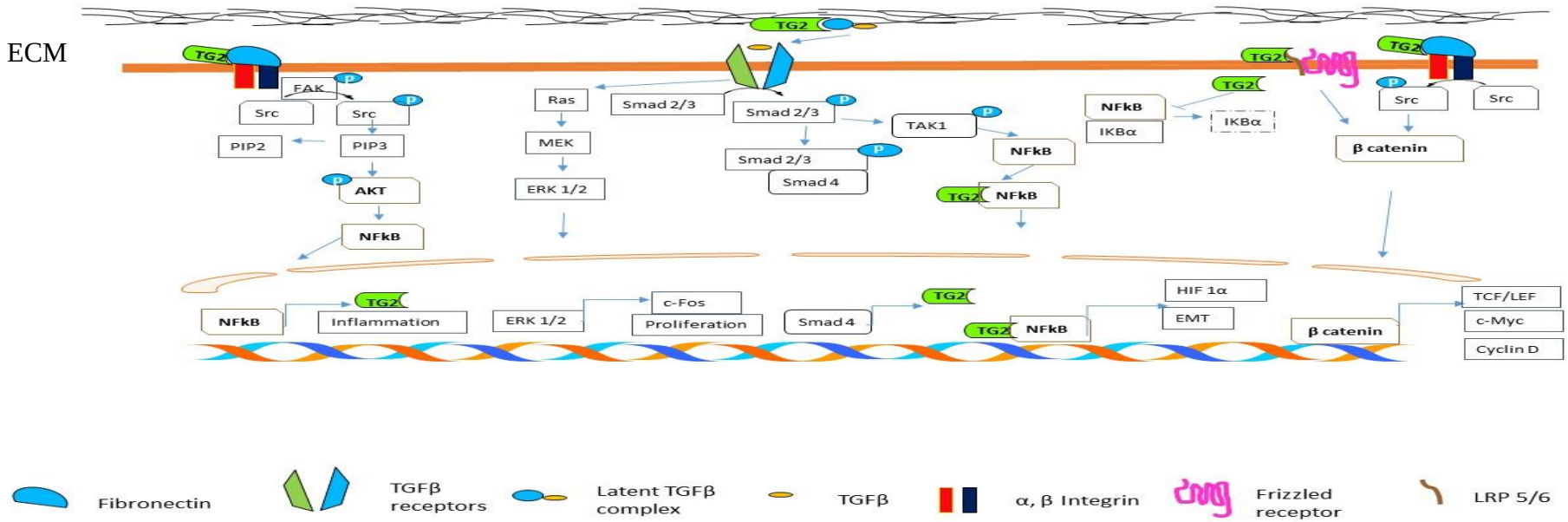


Figure 1.8. TG2 is involved in multiple cellular mechanisms that drive inflammation and EMT in fibrosis and cancer. TG2 interacts with fibronectin and induces integrin clustering, which leads to activation of Src, PIP3 and downstream activation of NFκB via phosphorylation of AKT. Extracellular TG2, via interaction with the ECM, can induce mechanical sheer stress that releases TGFβ from its latent TGFβ complex. Canonical TGFβ operates via Smad2/3 and activates TGFβ activated kinase 1 (TAK1), which in turn activates NFκB. Intracellular TG2 can bind NFκB, thus preventing its interaction with NFκB inhibitory unit (IKBα). Activated NFκB can translocate into the nucleus where it upregulates inflammation, EMT, or induces TG2 by binding to the *TGM2* promoter. Alternatively, intracellular TG2 can complex NFκB and TG2 facilitates the translocation of the complex into the nucleus to induce Hypoxia inducible factor 1 alpha (HIF 1α). Non-canonical TGFβ signalling activates extracellular kinase (ERK) which induces c Fos and proliferation. Canonical TGFβ signalling activates Smad4 which also induces TG2 expression by binding to the *TGM2* promoter site. Extracellular TG2 interaction with fibronectin and integrin activates Src, which inhibits proteasomal degradation of β-catenin. In addition, extracellular TG2 interaction with LRP5/6, a co-receptor with frizzled in canonical Wnt signalling facilitates β-catenin stability and nuclear translocation and β-catenin acts as a transcription factor for cell cycle regulators and EMT.

1.7 Cell Signalling aberrations in colorectal cancer

The pathogenesis of CRC varies and depends on the extent of genetic or epigenetic changes. These changes may follow multiple stages as theorized by Fearon and Vogelstein (Fearon and Vogelstein, 1990, Fearon, 2011), and the direct specific event within this sequence leads to CRC, by contributing to the "initiation" of neoplastic transformation of healthy epithelium and/or determining the "progression" towards more malignant stages of the illness (Meguerditchian and Bullard Dunn, 2013). The gastrointestinal tract is responsible for food digestion and absorption. These routine activities result in damage to the surface epithelium and replenishment of lost epithelium from abrasion is mediated by leucine-rich repeat containing G protein-coupled receptor (Lgr5) expressing intestinal stem cells (ISC) (van der Flier and Clevers, 2009). The ISCs proliferate from the crypt of the intestine and differentiate into various cell types, including enterocytes, Paneth cells, goblet cells, enteroendocrine cells, tuft cells, and M cells. The proliferation and differentiation of the ISCs are mediated by various signalling pathways along the crypt-villus axis, including Wnt, BMP, EGF, TGFβ and Notch pathways. Prominently the Wnt pathway is the primary driving force in the maintenance and proliferation of ISCs. This, therefore, suggests that any one of the signalling pathways above may play a role in the progression of CRCs (Novellasdemunt et al., 2015).

1.7.1 Pathogenic Classification of CRCs

On the basis of the pathogenesis of human CRCs, CRCs can be broadly classed into two groups based on their molecular profiles; non-hypermutated microsatellite stable (MSS) CRCs and hypermutated microsatellite instability (MSI) cancers. MSS CRCs constitute the clear majority, where loss of the tumor suppressor APC is the early event to initiate adenoma formation (Muzny et al., 2012). Subsequent cancer progression requires stepwise accumulation of other mutations, such as in *KRAS*, *PI3K*, *TGFβ*, *p53*, and/or *SMAD4*, which is known as the multistep somatic evolution model. On the other hand, around 10–15% of CRCs are caused by defective DNA mismatch repair (MMR) machinery that is often associated with *MLH1* hypermethylation (Vilar and Tabernero, 2013). These are characterized by the

presence of insertions or deletions of nucleotides in microsatellite repeat regions widespread across the genome, hence their name as MSI tumors (Vilar and Tabernero, 2013).

1.7.2 Cytokines and signalling pathways associated with colorectal cancer

Wnt, BMP, EGF, TGFβ, and Notch pathways are crucial for intestinal stem cell maintenance, and deregulation in any of these pathways may result in tumour initiation or progression (Novellasdemunt et al., 2015).

1.7.2.1 WNT Signalling Pathway

Wnt signalling is a key mediator of β-catenin signal transduction through processes involving phosphorylation and ubiquitin-mediated degradation. This regulation involves the cytoplasmic β-catenin destruction complex, consisting of AXIN, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). In the absence of the Wnt ligand, β-catenin is phosphorylated by CK1 and GSK3 in the complex, followed by recruitment of the E3 ligase βTrCP to the complex for ubiquitination and subsequent proteasomal degradation (**Figure. 1.9**).

Figure 1.9 Overview of the Wnt/β-catenin Pathway. Wnt plays a crucial a role as an agonist to the LRP5/6 and Frizzled transmembrane receptor activating Dishevel and inhibiting the APC-Axin death complex which targets β-catenin and recruits E3 ligase βTrCP for proteasomal degradation of the former. Wnt activation of Dishevel promotes β-catenin translocation to the nucleus from the cytoplasmic and membrane pool where in conjunction with its binding partner Lymphocyte enhancement factor/ T-cell factor (Lef-1/Tcf) act as transcription factors for cell cycle regulation, EMT and cell differentiation (Adapted from Sheikh et al., 2014).

However, with the engagement of Wnt to Frizzled (FZD) and low-density lipoprotein-related protein 5/6 (LRP5/6) receptors, the β-catenin destruction complex is recruited to the membrane, where βTrCP is dissociated from the complex. In the GIT Wnt signalling is crucial for stem cell maintenance and tissue homeostasis and its aberrant activation, loss of function of the β-catenin death complex, or mutation of β-catenin results in many human diseases including cancers and metabolic diseases (Novellasdemunt et al., 2015). Wnt signaling can proceed via a canonical β-catenin-dependent pathway and the noncanonical β-catenin-independent (planar cell polarity) pathway. However, it has been purported that the canonical pathway plays a role both in the physiology and pathology of the adult GIT, where it maintains stem cells in the crypts and when activated by mutation results in

gastrointestinal tract (GIT) related cancers including colorectal cancers including both MSS and MSI CRCs (Muzny et al., 2012). Inactivating mutations of tumour suppressor gene *APC* is the key mechanism for which the Wnt signalling pathway is activated in MSS CRCs. Whereas in MSI CRCs MMR defects result in high mutation rates in the entire genome known as the hypermutation phenotype. Consequently, there are frequent mutations observed in multiple oncogenes and tumour suppressor genes, including those associated with the APC/β-catenin/Tcf pathway (Novellasdemunt et al., 2015). Following the significant role of aberrant Wnt signalling on CRC various therapeutic molecules have been designed to target various aspects of the pathway and are now being used for human clinical studies (Bahrami et al., 2017).

Table 1.2 Anti-Wnt Therapeutics in human Phase 1 studies

Compound	Target Molecule (Action)	Cancer type	
LGK974 (Wnt 974)	O-acyl transferase Porcupine (Inhibitor)	Pancreatic adenocarcinoma, BRAF mutant Colorectal cancer, upstream wnt dependent tumours	(Liu et al., 2013)
Vanituctumab	Frizzled receptors (Inhibitory antibody)	Solid tumours	(Bahrami et al., 2017)
OMP-54 F28	Frizzled-8 receptor (decoy receptor)	Solid tumours	(Le et al., 2015)
PRI-724	Creb binding protein (CBP)-β-catenin interaction (CBP/ β-catenin antagonist)	Advanced solid tumours	(Nagaraj et al., 2015, Bahrami et al., 2017)

1.7.2.2 TGFβ1: A pleiotropic cytokine associated with EMT and tumour progression (canonical and non-canonical)

Tumour growth factor beta 1(TGFβ1) is a pleiotropic cytokine, associated with various pathological conditions including cancer and fibrosis (Nyabam et al., 2016, Cao et al., 2012), and TGFβ expression has been studied in a large panel of cancer types, including prostate, breast, lung, colorectal, pancreatic, liver, skin cancers, and gliomas (Neuzillet et al., 2015). The role of TGFβ1 in tumour progression is mixed with potential antitumor roles during the initiation process of a tumour, to a protumour role as the tumour progresses, designating TGFβ as a pleiotropic cytokine (Principe et al., 2014). In the early-

stage tumours, the TGFβ pathway promotes cell cycle arrest and apoptosis. However, at advanced stages, TGFβ1 promotes cancer cell motility, invasion, epithelial-to-mesenchymal transition (EMT), and cell stemness, promoting tumour progression and metastasis (Neuzillet et al., 2015).

1.7.2.2.1 TGFβ/SMAD signalling pathway

The TGFβ/SMAD signalling pathway consists of TGFβ receptor type I (TGFβRI) and type II type II (TGFβRII) and SMAD proteins (Munger and Sheppard, 2011)- **Figure 1.10**. TGFβ is secreted as part of an inactive complex of latent TGFβ bound non-covalently to its propeptide, latency associated protein (LAP) forming the small latent complex (SLC) (Verderio et al., 1999). In turn, the SLC is bound covalently to a large TGFβ binding protein (LTBP) to form the large latent complex (LLC) (Munger et al., 1997). TGFβ is released from LTBP by crosslinking and mechanical shear stress in the ECM. Free active TGFβ acts as a ligand canonically to TGFβRII which in turn complexes with TGFβRI, and then phosphorylates SMAD2, which binds to SMAD4. The SMAD2/4 complex translocates into the nucleus inducing the CDK inhibitors, p15 and p21, leading to growth arrest. However, mutations in the TGFβ receptors (Jonson et al., 2001, Biswas et al., 2008) and SMAD signalling proteins (Fleming et al., 2013), and/or a non SMAD dependent TGFβ signalling inhibits the antitumor role of TGFβ and cooperate with other genetic alterations (RAS-RAF-MAPK) to promote tumour initiation and progression (Morris et al., 2010). Mutations inactivating the *SMAD4* gene have been found in 16%–25% of colorectal cancer cases (Xu and Pasche, 2007), and alterations in the *SMAD2* gene has also been reported in 6% of cases (Takayama et al., 2006).

Figure 1.10 Smad-dependent and -independent transforming growth factor β (TGF β) pathways. TGFβ is sheared from the large latent complex (LLC) in the ECM, where it later binds to TGFβRI and TGFβRII. The kinase activity of TGFβ receptors are necessary for transducing canonical TGFβ signalling by phosphorylating Smads2/3. Activated receptor-associated Smads can form a heterotrimeric complex with Smad4, which interacts with other co-factors in the nucleus to regulate the expression of TGFβ target genes.In addition, downstream intracellular signalling may also be transduced via smad independent pathways including the MEK/Erk, the Rho-like GTPases, the phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt (PI3K/Akt) and the p38/mitogen-activated protein kinase (MAPK) pathways to regulate biological responses such as epithelial-to-mesenchymal transition (EMT), cell adhesion, migration and survival. (Adapted from Papageorgis and Stylianopoulos 2014).

TG2 has been reported to play a role in the release of TGFβ1 from the large latent complex (LLC) by crosslinking LLC via the latent TGFβ1 binding protein 1 (LTBP-1) to the extracellular matrix, thus facilitating the release of the small latency complex (SLC) comprising the inactive TGFβ1 bound to latency associated peptide (LAP) (Nunes et al., 1998, Telci et al., 2009).

Table 1.3 Anti- TGFβ1 signalling Therapeutics in human Phase 1 studies

Compound	Target Molecule (Action)	Treatment stage	
Fresolimumab (GC1008)	TGFβ1,2,3 (ligand Inhibitor)	Phase I/II Renal carcinoma, melanoma, mesothelioma, glioma, breast cancer	(Morris et al., 2014, Stevenson et al., 2013)
FANG™ Vaccine	TGFβ1,2 (Ligand inhibitor)	Melanoma, CRC, ovarian cancer	(Senzer et al., 2012)
Galunisertib	TGFβR1 (Receptor inhibitor)	Phase II in Pancreatic ductal adenocarcinoma, liver cancer, glioma, gliobastoma	(Rodón et al., 2015, Fujiwara et al., 2015, Brandes et al., 2016)
PF-03446962	TGFβR1 (Receptor inhibitor)	Phase I /II Liver cancer, mesothelioma, CRC	(Simonelli et al., 2016, Wheatley-Price et al., 2016)
IMC-TR1 (LY3022859)	TGFβRII (receptor inhibitor)	Phase I solid tumours	(Tolcher et al., 2017)

K-ras

Mutation in K-ras is common in various cancers particularly in gastroenterological cancers, including colorectal, pancreatic, and bile duct cancers. K-ras mutations may account for 15%–68% of sporadic colorectal adenomas and in 40%–65% of cancers (Takayama et al., 2001). Mutations in K-ras constitutively activates a variety of effector pathways, including RAF/MAPK, JNK, and phosphatidylinositol 3-kinase (PI3-K), leading to constitutive growth promotion (**Figure 1.11**). Other downstream target gene of K-ras injclude cyclin D1, DNA methyltransferase, and vascular endothelial growth factor (VEGF) genes (Downward, 2003).

The potential tumour promoting role of TGFβ in cancer cells has led to the development of therapeutic targets in the pathway, which have progressed to clinical trials (**Table 1.3**)

Figure 1.11 Colorectal cancer Progression and frequently mutated pathway. Left panel shows the progression of CRCs from normal epithelium to full blown carcinoma. Left panel frequently mutated molecules in Apoptotic, P53, PI3-Akt, TGFβ1 and Wnt signalling pathways in CRCs. Red typeset indicate frequently mutated genes (Oncogenes-β-catenin, K-Ras; Tumour suppressors-APC, DCC, TGFβRII, Smad2, Smad4, Bax p53) in CRCs, and grey highlight show pathways used by only microsatellite unstable CRCs(MSI). Mutations in tumour suppressors in the TGFβ1 pathway may regulate the pleiothropic nature of TGFβ1 and promote the pro-tumour role of TGFβ1 against its antitumour role. Mutations in APC and β-catenin also subvert the canonical Wnt signalling pathway to a more sinister one in CRCs (Kanehisa et al., 2017).

1.8 Colorectal cancer stem cells

An emerging concept in the biology of cancer cells is the cancer stem cells. These cells have self-renewing properties and ability to differentiate into diverse tumour population (Han et al., 2013). The emergence of cancer stem cells (CSC) was first established in the hematopoietic system and has now been progressively found in solid tumours such as breast (Kumar et al., 2010), ovarian (Cao et al., 2012), squamous (Fisher et al., 2015b), brain (Singh et al., 2003), hepatomas (Cao et al., 2011) and colorectal cancer (Fan et al., 2012). The origin of cancer stem cells is still debatable with one school of thought suggesting that cancer stem cells arise during developmental or tissue repair process either by cell fusion or horizontal gene transfer (Bu and Cao, 2012). These developmental or repair processes are often circumvented by tumour cells to enhance tumour survival and progression. On the other hand, it is postulated that gene mutations in normal stem cells, progenitor cells or differentiated cells may also drive the formation of CSCs (Ciurea et al., 2014).

In colorectal cancer, the CSC form the hierarchy of tumours and populate circulating tumour cells, possess metastatic, and drug resistant phenotypes (Willis et al., 2008). The current chemotherapeutic intervention in CRCs target hyperprolferative cells, but CSCs due to their stem cell phenotype are less proliferative and are usually not targeted. They may then acquire the necessary mutations that make them resistant to treatment, and once the bulk (non cancer stem cell population) of the tumour has been eradicated the reduced tumour mass triggers the potentially drug resistant CSCs to replicate, differentiate proliferate and build a highly drug resistant tumour (Hu and Fu 2012).

EMT a process in embryonic development and wound healing has been reported to play a role in driving colorectal cancer stem cells (Mani et al., 2008, Fan et al., 2012). It has been reported that EMT enhances the transcription of stem cell transcription factors such as Oct, Sox, and Nanog in cancer cells (Fan et al., 2012). Tissue transglutaminase has also been reported to play a role in the maintenance of a stem cell like phenotype in some cancers (Zonca et al., 2017). The consensus is that TG2 enables the formation of cancer stem cells by upregulating transcription factors associated with stem cells (Eckert et al., 2015).

1.8.1 Tumour spheroid culture as an *in vitro* method for cancer stem cell (CSC) enrichment

Despite significant advances in cancer research the isolation, identification and characterization of tumour initiating cells (cancer stem cells) remains incompletely established especially in colon cancer. In general, the isolation of cancer stem cells relies heavily on exploiting the properties or characteristics of cancer stem cells, and could involve isolation based on their functional or immunogenic properties (Islam et al., 2015). The antigenic approach exploits a variety of cell surface markers, including CD133, CD44, CD34, CD24, epithelial-specific antigen (EpCAM/ESA), CD166, CD29, Lgr5, CD49f and ALDH-1 which are expressed in cancer stem cells (Islam et al., 2015). Functional isolation of CSC uses various characteristics, including anchorage independent growth, chemoresistance, self-renewal, asymmetric division, and pluripotency. Over the last years spheroid cultures (SC), a form of functional isolation that relies on the anchorage-independent growth properties of stem cells have been used to enrich cancer stem cells in brain (Singh et al., 2003), breast (Agnihotri et al., 2013), and colon tissue (Todaro et al., 2007). It is well accepted that SC preserve more faithfully the characteristics of original tumours, including gene expression profiles, tumour heterogeneity and tumour morphology, compared to regular adherent cultures. Additionally, spheroids mirror the 3D cellular context and relevant pathophysiological gradients of *in vivo* tumours (Todaro et al., 2007).

1.9 Aims and Objectives

The aim of this study is to investigate the role(s) of TG2 in colorectal carcinoma progression and to identify its potential as a prognostic and therapeutic biomarker in the human disease. To fulfil this aim a pair of isogenic colorectal carcinoma cells, SW480 (primary colon carcinoma- Duke's B classification) and SW620 (lymph metastatic carcinoma Duke's C classification) have been employed, to investigate the role of TG2 in tumour progression. These two cell lines have been validated as an *in vitro* model of colon cancer progression from a primary tumour to metastatic disease and have retained relevant phenotypes despite long-term culture *in vitro* (Hewitt et al., 2000). In addition, a third colorectal cancer cell line RKO with a different monoclonal origin was assessed to gain a wider sense of TG2's role in human colon cancer progression. To fully delineate the potential role of TG2 in cancer stem cell formation a more aggressive colorectal cancer cell line, HCT116 with Duke's D classification was also studied.

To achieve the aim the following objectives were set:

Objective 1: Characterise CRCs for markers of disease severity to validate disease progression including epithelial to mesenchymal transition, invasion, and drug resistance.

Objective 2: By epigenetic manipulation of TG2, investigate the role of TG2 in epithelial to mesenchymal transition, invasion, and drug resistance. Following which the mechanism through which TG2 may influence these processes would be investigated.

Objective 3: Using an *in vitro* cancer stem cell enrichment culture assess the role of TG2 in colorectal cancer stem cell enrichment and behaviour vis-à-vis EMT, drug resistance, invasion and angiogenesis.

Chapter 2
Materials and Methods

Chapter 2

2.0 Materials and Methods

2.1 Materials

2.1.2 Chemicals

General chemicals used in this study where obtained from Sigma UK unless specified elsewhere.

Sigma Aldrich, UK

- ITS-Insulin, Transferrin, Sodium Selenite medium, 10X
- Diethyl pyrocarbonate (DEPC)
- SIGMAFAST® OPD tablets
- Fibronectin
- Dimethyl Sulfoxide (DMSO)
- Protein A and G Sepharose beads
- Ponceau S stain (P7170): 0.1 %(w/v) Ponceau S and 5%(w/v) acetic acid ready to use.

Melford (UK)

- Acrylamide (29:1)

Life Technologies Ltd (UK)

- Lipofectamine® 2000
- TRIzol ®

Qiagen (UK)

- RT^2 First strand Kit

Pierce (UK)

- Sulfo-NHS-LC-Biotin

Trevigen (UK)

- 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)

Vector Laboratories (UK)

- Vectashield hard set mounting medium with DAPI (4',6-diamidino-2- phenylindole)

Cell Signalling

- ERK inhibitor PD98059, CST, USA

R&D Systems

- human rTGFβ1
- TGF β1 1 neutralising antibody

Aston University (Prof Martin Griffin's Lab)

- R281:(N-benzyloxycarbonyl(CBZ)-L-phenylalanyl-6-dimethylsulfonium-5-oxo-Lnorleucine) (Jones et al., 2006)
- R283: (1,3-dimethyl-2-[(oxopropyl)thio] imidazolium. (Jones et al., 2006)
- R294: cell impermeable TG2 inhibitor (Griffin et al., 2008)
- 1-155: Peptido-mimetic cell-permeable TG2 inhibitor (Badarau et al., 2015)

2.1.3 Equipment

- Epifluorescent microscope Leica Microsystem (Milton Keynes, UK) MWG (USA)
- Primus 96 thermal cycler Stratagene (UK)
- GEL box (G:BOX F3) Syngene (UK)

2.1.4 Cell Lines

Human colorectal cancer cell lines RKO, SW480, SW620 and HCT116 were a kind gift from Dr Chris Tselepsis (University of Birmigham).

2.2 METHODS

2.2.1 Cell culture techniques

2.2.1.1 Culture conditions of cells

Human colorectal cancer cell lines RKO, SW480, SW620 and HCT116 were cultured in a humidified atmosphere at 37°C and 5% (v/v) CO_2. All the cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Lonza, UK) containing 10% (v/v) FBS (Gibco, UK), 1% (v/v) nonessential amino acids (Gibco, UK), unless otherwise indicated. For polyhema coated low attachment plates pre-coated with Poly-HEMA , SW620 and HCT 116 cells were cultured in serum free DMEM/F12 (1:1) (Lonza, UK) containing 2% (v/v) serum free supplement B27 (Invitrogen, UK), 20ng/ml EGF, 0.4% bovine serum albumin and 4 μg/ml insulin (Complete spheroid media).

2.2.1.2 Passaging of Adherent Cells

Cell lines were passaged to keep them at low density or to be seeded for experiments. For cell passaging the complete cell culture medium (DMEM) and 0.25% (w/v) trypsin in 2mM EDTA were pre-warmed prior to use. The adherent confluent (at least 90% confluency) cells were washed once with serum free DMEM to remove dead cells and debris. The cells were then treated with Trypsin for 5 min at 37^0C for the cells to detach. The cells were then collected in complete medium to inactivate the trypsin and centrifuged at 300g for 5 min. The supernatant was then discarded and the cell pellet was re-suspended in complete growth medium. Subsequently the cells were seeded into tissue culture flasks to obtain the desired confluency.

2.2.1.3 Cell counting using haemocytometer

Cells to be counted were collected as described in **Section 2.2.1.2** with 10μl of cell suspension from each cell line introduced into the haemocytometer. By means of an inverted microscope, the cells were counted in four separate chambers, neglecting the cells present on the top and right corner lines. The cell numbers obtained were multiplied by a factor of 10^4 to determine the cell number present per ml. The desired seeding density was achieved by diluting down the stock cell suspension.

2.2.1.4 Freezing and defrosting of cell lines

The cells were trypsinised and counted as described above in **Sections 2.2.2** and **2.2.3**

and subsequently re-suspended in freezing mixture which is composed of 10% (v/v) cell culture grade DMSO in heat-inactivated FBS. The cells were then distributed as 1ml aliquots into cryogenic vials and kept at -20ºC for 1 h. Subsequently, the cryogenic vials were kept in -80ºC for at least 24 h and stored in liquid nitrogen for a long-term storage.

2.2.2 Lentiviral Transduction

2.2.2.1 Lentivirus production of TG2 plasmids

To produce lentiviral particles, human embrayonic kidney cells- HEK293FT were transfected and used as viral packaging cells (Wang et al., 2013). HEK293FT cells were cultured in T75 flasks in DMEM medium supplemented with 2mM L-glutamine, 10% (v/v) heat-inactivated FBS, 1mM sodium pyruvate, 400µg/ml G418 (the antibiotic is omitted immediately after defrosting and prior to transfection) and 1x Nonessential amino acids (NEAA). For plasmid transfection, the HEK293FT cells were passaged as described in **Section 2.2.2** and allowed to reach 80% confluency, after which the medium was changed and then transfected with viral packaging mix containing packaging plasmid-pPax, viral envelop VSV-G, and the DNA of interest in a ratio a 9µg**:** 4.5 µg**:** 6µg with Lipofectamine® 2000. The transfection mixture was incubated at room temperature for 20 min to form the complex, and then added to the packaging cells drop by drop with gentle mixing. The transfected cells were incubated in cell culture conditions for 72 h within which the viral particles were released into the medium. After this incubation period, the infectious medium was carefully collected and centrifuged at 500×g for 10 min to remove the cell debris and viral aggregates. The supernatant was collected aseptically and stored for viral isolation and concentration.

Table 2.1 TG2 shRNA target sequences (Sigma Aldrich ,UK)

Taxon	Number (#)	Gene Symbol	Clone ID	Target Seq
Human	shRNA1	TGM2	TRCN0000000240	5' AGAAATACCGTGACTGCCTTA 3'
Human	shRNA2	TGM2	TRCN0000000241	5' ACAGCAACCTTCTCATCGAGT 3'
Human	shRNA3	TGM2	TRCN0000000243	5'TGAGAAATACCGTGACTGCCT 3'
Human	shRNA4	TGM2	TRCN0000000239	5'CCACCCACCATATTGTTTGAT 3'

2.2.2.2 Lenti-X viral concentration

Virus purification and concentration was performed using the Lenti-X virus concentrator. The clarified medium from **Section 2.2.2.1** was incubated with Lenti-X virus concentrator at the ratio of 3:1 (clarified media: concentrator), and gently inverted to mix. The mixture was incubated overnight at 4ºC. Following the incubation, the viral particles were pelleted at 1,500×g for 45 min at 4ºC. The supernatant was carefully discarded without disturbing the pellet. The viral pellet was then carefully re-suspended in DMEM complete cell culture medium with 1/10th of the original volume used, aliquoted into 200µl aliquots and stored at -80 ºC.

2.2.2.3 Lentiviral Titre value

Lentiviral titre was determined using the Lenti-X® Go-stix. To determine the titre, 20µl of the concentrated viral particle solution (**Section 2.2.2.2**) was added to the Lenti-X® Go-stix kit and 4 drops of Lenti- X® concentrator buffer was added. This was allowed to stand for 15 min. The presence of two visible bands (one for control and one for test) is used to qualitatively detect viruses, and the intensity of the test band used to predict viral density.

2.2.2.4. Viral transduction for epithelial CRCs in submerged culture

CRCs were seeded at 5 x 10^5 cells into 35 mm Petri dishes and allowed to reach 80% confluency. Prior to transduction, 700 µl of fresh complete cell culture media was added to the cells. 100 µl aliquot of Lentiviral particles with the desired DNA was then added to the wells drop by drop with gentle agitation to mix the viral particle and medium. Following 24 h incubation, second round of transduction was carried out. Cells were passaged two times before characterisation of TG2 expression and activity.

2.2.3 Preparation of whole cell lysate

Adherent CRC lines were seeded into 60mm cell culture Petri dishes and allowed to reach 80-90% confluency. After washing once with ice cold PBS pH7.4, the cells were lysed in cell lysis buffer [50 mM Tris-HCl, pH 7.4, with 1% (v/v) Nonidet, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1mM benzamidine, 1mM NaF, 1mM Na_3VO_4 and freshly added 0.1mM phenylmethylsulfonyl fluoride (PMSF) and 1% (v/v) protein inhibitor cocktail] and incubated for 30 minutes on ice. For spheroid cells, the spheroid suspension was collected and centrifuged at 300×g for 5 min at 4ºC. Following washing once with ice-cold PBS, pH7.4, the cell pellets were centrifuged at 300×g for 5 min at 4ºC and gently suspended in 100µl of lysis buffer and incubated on ice for 30 minutes. Cell lysates were then pre-cleared by centrifugation at 300×g for10 min at 4ºC. The samples were stored at -80ºC until use.

2.2.4 Protein Concentration

The protein content of cellular extracts was determined using the commercial kit from Bio-Rad based on the Lowry method (Lowry et al., 1951). Different concentrations of BSA solution (ranging from 0.0-1.0mg/ml) were used as standards. 5µl of BSA standards or whole cell lysates were added to a 96-well plate in triplicate. 25µl of Reagent A was added to the wells, followed by 200µl of Reagent B, and incubated for 15 min at room temperature. The absorbance was read at 750nm using a plate reader and the values were plotted as a standard graph to aid in determining the protein concentration of the cell samples.

2.2.5 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as introduced previously (Wang et al., 2012). Following the protein concentration assay (**Section 2.2.4**), the protein extracts that contained the desired amount of protein were dissolved in a 1:1 ratio with 2x Laemmli sample buffer (Sigma Aldrich) (Yoo et al., 2012) and denatured via boiling for 5 min and then stored at -80 ºC until needed in the SDS-PAGE. The SDS gels consisted of a stacking gel (containing 30% Acrylamide, Tris-HCL/SDS, pH 6.8, 10% ammonium persulphate and TEMED) and resolving gels that varied between 8-12% (w/v) polyacrylamide (containing 30% Acrylamide, Tris-HCL/SDS, pH 8.8, 10% ammonium persulphate and TEMED)

according to the molecular weights of the target proteins. The resolving and stacking gels were prepared as indicated in the **Table 2.3** below. The resolving gels were prepared using the BioRad mini gel system and isopropanol was poured onto the top of the gel to fill the gel system and provide the gel with a uniform flattened upper surface. The gels were then allowed to polymerise for 45 min to 1 h at room temperature. After polymerization of the resolving gel, the upper surface was washed thoroughly using distilled water. Excess water was removed from the gels, and the stacking gels were prepared (Table 2.4) and poured into the glass spacers and a 10-well comb was inserted into the stacking gel solution to form the sample wells.

Following polymerization of the stacking gel, the comb was carefully removed and the wells were washed with the SDS-PAGE running buffer containing 25mM Tris-HCl, 192mM Glycine and 0.1% (w/v) SDS, pH 8.3/8.4. Cell lysates containing denatured proteins in Laemmli buffer were loaded into the wells. Electrophoresis was initially carried out at 90V to allow the proteins to migrate through the stacking gel and then at 120V to aid protein migration through the resolving gel until the bromophenol blue tracking dye of the Laemmli buffer is seen to reach the bottom of the resolving gel.

Table 2.2 Composition of SDS-resolving Gel

	Acrylamide (v/v) Gel percentage		
	8%	10%	12%
30% (v/v) Acrylamide	6.0ml	7.5ml	9.0 ml
4x Tris HCl/SDS, pH8.8	7.5ml	7.5ml	7.5ml
dH2O	16.5ml	15.0ml	13.5ml
10% (w/v) Ammonium Persulphate	0.10ml	0.10ml	0.10ml
TEMED	0.020ml	0.020ml	0.020ml

Table 2.3 Composition of SDS-stacking gel

	Stacking Gel
30%(v/v) Acrylamide	0.65ml
4x Tris HCl/SDS, pH6.8	1.25ml
dH2O	3.05ml
10% Ammonium Persulphate	0.025ml
TEMED	0.005ml

2.2.6 Western Blotting of Proteins

The proteins previously resolved by SDS-PAGE were transferred onto nitrocellulose membranes using a Bio-Rad wet transfer system as per previously established protocols (Wang et al., 2012). The fibre pads and nitrocellulose sheets were cut to gel size and pre-soaked in ice cold transfer buffer [48.8mM Tris, 39mM Glycine and 20% (v/v) methanol]. The gel holder cassette was placed open and a sponge was placed on the black coloured surface of the cassette. The pre-soaked fibre pads were then placed on the sponge, on top of which the gel was placed. The soaked nitrocellulose paper was then laid on top of the gel, followed by the pre-soaked fibre pad and finally the sponge. A roller was used to remove any trapped air bubbles. The cassettes were then assembled and inserted into the apparatus used for blotting so that the membrane side faces the anode electrode. The apparatus was then filled with ice cold transfer buffer. The electro-transfer of the proteins in the gel was performed for 2 h at 200mA. The success of the transfer of proteins on to the nitrocellulose membrane was confirmed by the presence of high molecular weight markers on the membrane.

2.2.7 Immunodevelopment of Western blots

Following the transfer, the nitrocellulose membrane was blocked with blocking buffer [5% (w/v) non-fat milk in TBS-Tween, pH7.4] for 1h at room temperature and then probed with different primary antibodies (**Table 2.5**) overnight with shaking at 4^{o}C. The next day, the membrane was washed 4 times for 15 min each and then probed with the suitable secondary antibody diluted in blocking buffer (1:1000) for 2 h with shaking at room temperature. The membrane was then washed again with 1x TBS-Tween, 4 times for 15 min each. Immuno-detection of the blots was completed using the Amersham enhanced Chemiluminescence (ECL) system kit (G.E Healthcare) according to the manufacturers' protocol. Reagents A and B were mixed in a 1:1 ratio and 1ml of the mixture was added onto each blot. Following 1 min of incubation, the membranes were wrapped in cling film. The chemiluminescent signals were detected using a Gensys imaging system.

Tris buffered saline (10x)- 1.5M NaCl in 0.2M Tris base.

1x TBS-Tween (1x Tris-buffered saline, 0.1% Tween 20).

Table 2.4 List of primary antibodies used and their source

Primary Antibody	Source	Purpose
Twist	Santa Cruz Biotechnology Inc, UK	EMT transcription factor
Slug	Santa Cruz Biotechnology Inc, UK	EMT transcription factor
β -catenin	Santa Cruz Biotechnology Inc, UK	Signalling
p-Src(Y416)	Santa Cruz Biotechnology Inc, UK	Signalling
Src	Santa Cruz Biotechnology Inc, UK	Signalling
GAPDH	Santa Cruz Biotechnology Inc, UK	Housekeeping protein
Vimentin	Santa Cruz Biotechnology Inc, UK	Mesenchymal Marker
αSMA	Santa Cruz Biotechnology Inc, UK	Mesenchymal Marker
N cadherin	Santa Cruz Biotechnology Inc, UK	Mesenchymal Marker
TGFβ receptor 1	Santa Cruz Biotechnology Inc, UK	Signalling
TGFβ receptor II	Santa Cruz Biotechnology Inc, UK	Signalling
p-Smad 3	Santa Cruz Biotechnology Inc, UK	Signalling
Smad 2/3	Santa Cruz Biotechnology Inc, UK	Signalling
ERK1/2	Santa Cruz Biotechnology Inc, UK	Signalling
p-ERK (T202/Y204)	Santa Cruz Biotechnology Inc, UK	Signalling
C-Jun	Santa Cruz Biotechnology Inc, UK	Signalling
TGFβ1	Santa Cruz Biotechnology Inc, UK	Signalling
β1 Integrin	Santa Cruz Biotechnology Inc, UK	Signalling/Adhesion
β3 Integrin	Santa Cruz Biotechnology Inc, UK	Signalling/Adhesion
Nanog	Santa Cruz Biotechnology Inc, UK	Stem cell transcription factor
Sox	Santa Cruz Biotechnology Inc, UK	Stem cell transcription factor
Oct-4	Santa Cruz Biotechnology Inc, UK	Stem cell transcription factor
CD44	Santa Cruz Biotechnology Inc, UK	Stem cell Marker
HIF 1α	Santa Cruz Biotechnology Inc, UK	Transcription factor of angiogenesis
VEGF	Santa Cruz Biotechnology Inc, UK	Angiogenesis
ZO-1	life Technologies, UK	Epithelial Marker
TG2	Neomark Pierce UK	Cellular Protein
Fibronectin	Sigma Aldrich, UK	Mesenchymal Marker
S100A4	AbD Serotek	Tumour progression/ metastasis
LDH	Santa Cruz Biotechnology Inc, UK	Cytoplasmic Marker
Lamin A/C	Abcam , UK	Nuclear Marker

Table 2.5 List of secondary antibodies used and their source

Secondary Antibody	Source	Isotype
Swine anti-rabbit HRP conjugated	DAKO, DENMARK	Rabbit
Goat anti-mouse HRP conjugated	DAKO, DENMARK	Mouse

2.2.8 Stripping and re-probing of nitrocellulose membrane

To determine that equal amounts of protein was loaded onto each well during the SDS-PAGE gel were equal, or to re-probe for any other specific protein, the primary and secondary antibodies used to previously detect the protein of interest on the membrane was taken off from the blot using stripping buffer. The nitrocellulose membranes were immersed in stripping buffer pH 6.7 (65mM Tris HCL, 2% (w/v) SDS, 100mM 2-mercaptoethanol-freshly added) and incubated between 50-60 ^{0}C for 30min in an oven, with occasional shaking. The stripping buffer was then discarded and the blots were washed four times (15 min each) with 1x TBS-Tween, pH 7.4 at room temperature with gentle agitation. The membrane was then blocked in the blocking buffer for 1 hour at room temperature. The immune-development was later performed with relevant antibodies followed by immune detection as described in **Section 2.2.7** using the ECL system.

2.2.9 Detection of whole cell lysate TG2 activity via Biotin Cadaverine incorporation

The transglutaminase activity of TG2 was measured via biotin cadaverine incorporation on to immobilised N,N-dimethylcasein as described previously by (Jones et al., 2006). 50µl of 10mg/ml of casein in 50mM Tris-HCl, pH 8.0 was used to pre-coat the 96 well plates and left-over night at 4^{o}C. After washing the wells three times with 50mM Tris-HCl, pH 7.4, whole cell lysate protein (obtained as described in (**Section 2.2.3**) was diluted in 50mM Tris-HCl, pH 7.4 to a final concentration of 500µg/ml along with 0.1 mM biotin-cadaverine (Zedira Germany), 1mM DTT and 10mM $CaCl_2$. 400 ng/well of human recombinant TG2 (Zedira Germany) in 50mM Tris-HCl, pH 7.4 containing 1mM DTT, 0.1 mM biotin cadaverine, 10mM EDTA (negative control) or 10mM CaCl2 (positive control) was used as the control samples. 100µl of the mix above of sample and controls were added into 96well plates in triplicate and incubated for 2 h at 37 ^{o}C. After incubation, the sample and control solutions were discarded and the biotin cadaverine incorporated onto the casein layer was washed with 1xTBS-Tween, three times and then blocked with blocking buffer (heat inactivated 3% (w/v) bovine serum albumin in 50mM Tris-HCl pH 7.4) for 30 min at 37 ^{o}C. The biotin cadaverine incorporated into FN was incubated with Extravidin-peroxidase conjugate (Sigma-Aldrich, UK) diluted in blocking buffer (1:1000) for 1 h at 37^{o}C. The wells were again washed three times with 1xTBS-Tween, and once with

TBS and the reaction was developed by the addition of 100µl of developing buffer containing SIGMAFAST OPD (o-Phenylenediamine dihydrochloride) [Sigma, Uk] tablets in 20mls of distilled water to yield a ready to use buffered solution that contains OPD and urea hydrogen peroxide. The development of colour was terminated using 50µl 3N HCl and the absorbance was read at 490nm using a Spectrafluor plate reader.

2.2.10 Cell viability analysis using XTT Assay

The XTT assay was used to measure cell viability and proliferation of the colorectal cancer cell lines. XTT assay is based on the principle that the cleavage of the yellow tetrazolium salt to form an orange formazen dye only transpires in metabolically viable cells and was performed in accordance with the manufacturers' instructions (Trevigen, USA). The formazen dye formed can be directly quantified using a Spectrafluor plate reader. To test the viability of cells. 3000 cells were seeded into 96 well plates in 100µl with complete medium and left to incubate in a humidified atmosphere at 37 ^{0}C and 5% (v/v) CO_2. 1ml of XTT labelling agent was mixed with 20µl of electron coupling reagent, and 30µl of the resultant mixture were added into each well and incubated for 4h in a humidified atmosphere at 37^{0}C and 5% (v/v) CO_2. Following the incubation period, measurement of the orange formazen dye was performed by taking absorbance measure using a SpectraFluor plate reader at a wavelength of 490nm, and the reference wavelength used was 690nm. The background wavelength of 690nm was subtracted from the absorbance values obtained from 490nm.

2.2.11 Chemosensitivity Assay

Chemosensitivity assays were used to assess chemo resistance in CRCs. The assay was based on the traditional adherent monolayer cell culture system, and measuring cell viability using the XTT reagent (Trevigen USA). Cells were collected, counted and seeded as described in **Section 2.2.1.2-2.2.1.3**, and allowed to adhere for 4h before treatment with varying doses of 5 fluorouracil (5FU) and doxorubicin in 100µl of fresh complete cell culture media. After the duration of the treatment. Cell viability was measured as described in **Section 2.2.9** and the relative survival of cells treated with either 5 FU or doxorubicin was compared to that of the control vehicle (DMSO- Sigma, UK) treated cells.

2.2.12 Statistical analysis

Data were expressed as mean ± S.D. The data shown are derived from a representative experiment undertaken in triplicate. The differences between control and compound treated samples were determined using statistical software (GraphPad Instat software package). Comparisons among different groups were performed by student T-test or by an analysis of variance using ANOVA, where applicable. Significant differences between control and treatment groups were analysed by Bonferonni's Multiple Comparison Test. Statistical significant difference between data sets was defined in the text by $p < 0.05$ (two-sided).

Chapter 3

TG2 correlates with tumour progression and epithelial to mesenchymal transition *in vitro*

Chapter 3

3.0 TG2 correlates with tumour progression, and epithelial to mesenchymal transition i*n vitro*

3.1Introduction

Cancer metastasis describes the progression of cancer from the primary tumour microenvironment to its establishment at distant sites, by way of the lymphovascular system (Lugassy and Escande, 1997). In many cancer treatments, metastatic cancer implies a need for the use of a higher grade medical intervention, to identify, and treat the cancer. In spite of this, the 5-year survival rates for metastatic cancer patients remain very poor. Metastasis involves several stages, such as the activation of epithelial-mesenchymal transition (EMT) (**Figure 3.1.1**), during which cancer cells lose all cell-cell contact and substrate adhesion ; gain local invasion -enabling malignant cells to degrade the basal lamina, the special extracellular matrix that organises and separates epithelial tissues from the stroma ; intravasate, during which tumour cells pass through the walls of blood vessels and enter the bloodstream; the ability to survive in the bloodstream; extravasation, whereby tumour cells exit the bloodstream; and establishment of tumour cells in the tissues of the organ where metastasis will form by a reversal of EMT called Mesenchymal to Epithelial Transition (MET) (Arvelo et al., 2016).

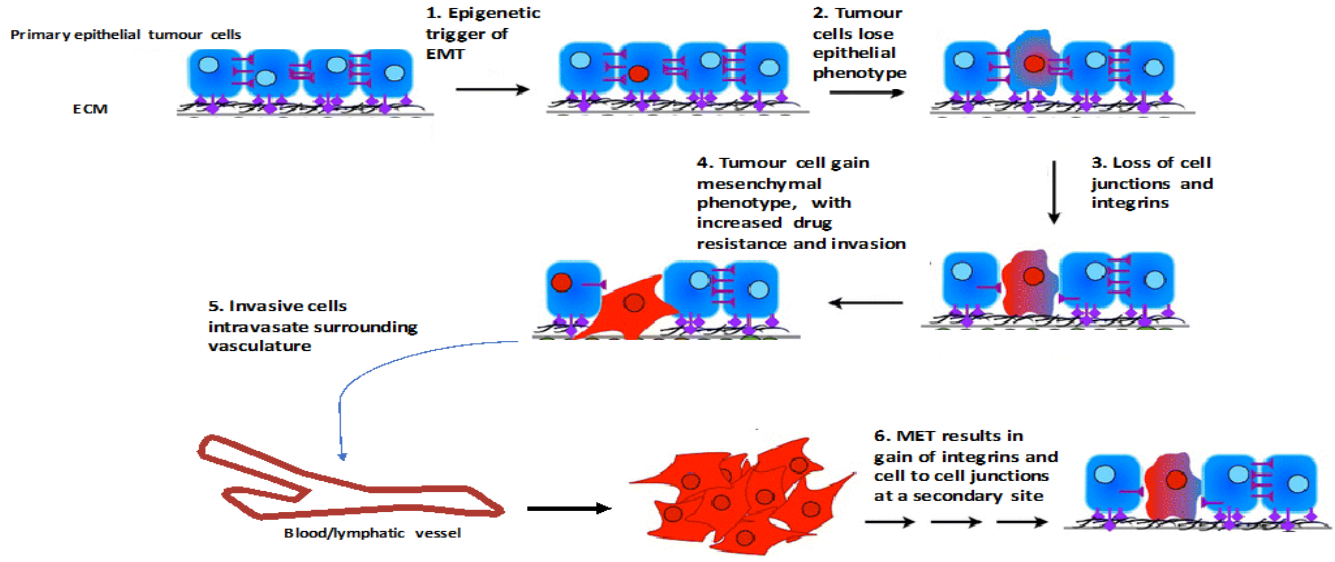


Figure 3.1.1 Epithelial to Mesenchymal Transition Promotes Tumour Progression. Epigenetic triggers in epithelial cells drive EMT, leading to loss of cell polarity, integrins and eventual gain of a mesenchymal phenotype which drives invasion, drug resistance and intravasation into the surrounding vasculature. Surviving circulating cells then revert to an epithelial phenotype via mesenchymal to epithelial transition (MET) at a secondary site to attach and seed a metastasis (Modified from Heerboth et al., 2015).

In colorectal cancer, the SW480 and SW620 cell lines have been validated as an *in vitro* model to study tumour progression (Hewitt et al., 2000). The SW480 cell line originated from a surgical specimen of a primary tumour of a moderately differentiated colon adenocarcinoma (grade 4, Duke class B). The SW620 cell line was established from a biopsy of a metastatic spread to the abdominal wall of the same patient (Leibovitz et al., 1976). Both of these cell lines represent an isogenic pair and have been used for a number of biochemical, immunological, and genetic studies on colon cancer (Kubens and Zänker, 1998, Zhao et al., 2007). Additionally, both SW480 and SW620 represent the microsatellite stable (MSS) subset of colorectal cancer based on molecular origins. The RKO cell line is a primary CRC cell line with a microsatellite instability (MSI) molecular basis, and it is for this reason that it may be generally regarded to be less aggressive or invasive than SW480 as it has been suggested that an MSI tumour is less invasive or aggressive compared to its corresponding MSS cancer (Rosty et al., 2014).

EMT has been identified as a crucial biological step for tumour metastasis and predicts a poorer 5-year survival in clinical colorectal cancer studies (Shioiri et al., 2006). This can be determined *in vitro* by assessing the expression of protein markers of EMT. Increased insensitivity to chemotherapy has been associated with metastasis, tumour progression (Agnihotri et al., 2013) and EMT (Kumar et al., 2010). In clinical studies, TG2 has been correlated with a poor 5-year survival and may play a role in EMT in cancer and fibrosis (Oh et al., 2011, Nyabam et al., 2016, Hwang et al., 2008). It has thus become important to identify potential biomarkers in colorectal cancer progression particularly as the genetic, especially the cytogenetic basis of colorectal cancer metastasis remains poorly understood. All the more so, as multiple studies in breast (Kumar et al., 2010), ovarian (Cao et al., 2008, Hwang et al., 2008), pancreatic (Mehta and Han, 2011) and lung cancer (Park et al., 2010) seem to be pointing to TG2 as a culprit in the progression of the disease by facilitating EMT and drug resistance. Therefore, a comparison between the primary (SW480-MSS), metastatic (SW620-MSS) human colorectal cancer cells may help identify oncogenic processes that are selectively upregulated in metastatic progression; and by studying all three cell lines the identity of common markers across the two broad molecular spectrums of CRCs-MSI or MSS may be identified. Additionally, since some clinical data suggests elevation of TG2 in patient tissue with poorer 5-year survival in CRCs, identifying if a similar trend is

obtainable in in vitro studies, and associating TG2 with processes that progress the disease may provide a clearer understanding of TG2's potential role in CRC and its potential as a prognostic marker or therapeutic target may then be established.

3.2 Aim and Objectives

The general aims of this chapter are: (i) to establish factors associated with tumour progression, and validate the current cell lines on these indices as showing a progression of the disease from primary to a metastatic tumour; and (ii) to determine if TG2 expression was related with progression of CRC in the current cell model.

Individual Objectives

1. Characterise RKO, SW480, and SW620 for markers of progression including invasion and drug resistance to conventional CRC chemotherapy
2. Identify Epithelial to Mesenchymal Transition as a cellular process in tumour progression in the current cell model by assessing both morphological and molecular markers of EMT
3. Determine if disease progression in the current CRC model correlates with the expression and activity of TG2.
4. Manipulate TG2 expression via lentiviral transduction and identify the role of altered TG2 expression on EMT, drug resistance, invasion and cell motility
5. Explore the potential of TG2 specific small molecule inhibitors in attenuating cancer progression in CRCs.

3.3METHODS

3.3.1 Determining Cell Morphology

To make observations on cell morphology, cells were collected as described in **Section 2.2.1.2-2.2.1.3**. 1.0 $X10^6$ cells were seeded (approx. 80% confluency) into 35mm Petri dishes and allowed to adhere for 16h, after which cellular morphology was assessed and images captured using a phase contrast microscope.

3.3.2 Cell Invasion assay

Prior to the invasion assay cells were serum starved by seeding $1.0X10^6$ cells in 35mm Petri dish (approx. 80% confluency) in serum-free ITS (Insulin transferrin sodium selenite media supplement-Sigma Aldrich, UK) containing media for 16h. 3µM pore size inserts in an accompanying 24 well format plate (Corning plate) were coated with 33.3µl of 100µg/ml collagen IV in PBS. Following serum starvation Cell were collected, suspended (**Section 2.2.1.2-2.2.1.3**) and seeded at a density of $1.0X10^5$ per 100µl in serum-free media containing 1xITS. To the bottom chamber of the Transwell inserts of a 24 well plate, 600µl of 10% (v/v) serum in DMEM (complete media) was added. To the coated Transwell $1.0X10^5$ cells per 100µl of ITS media was added each insert placed in the wells of the plates. The plates were incubated for 24h in cell culture conditions. Following incubation, the Transwell inserts were removed from the plates and the media was discarded and a cotton-tipped applicator was used to remove any remaining cells that had not migrated from the top of the membrane without damaging it. Following this, the cells that were embedded in the Transwell insert were fixed with 650µl of 70% (v/v) ethanol added into the well of the 24-well plate for 10min. After which excess alcohol was discarded from the inserts and the Transwell membrane allowed to dry (typically 10-15 min). 620µl of 0.2% (v/v) crystal violet was added to the well of the 24-well plate and then the inserts introduced into the wells containing crystal violet and incubated at room temperature for 5-10 minutes, to stain the cells. Following staining the inserts were removed and excess crystal violet discarded, again the inserts were washed gently 3 times with PBS, pH 7.4 to remove the excess crystal violet, and the Transwell membrane was allowed to dry. The invasive cells **w**ere viewed underneath an inverted microscope and a cell count was made under x10 objective. Images of the cells were also captured.

3.3.3 Cell Adhesion Assay

To perform the cell adhesion assay adherent cells were collected and collected as described in **Section 2.2.1.2-2.2.1.3**. Cells were seeded $2.5x10^4$ in 100µl per well, and cell adhesion was observed every 20min for 2h. After which the cells were fixed with 3.7% (w/v) formaldehyde in PBS, pH 7.4 for 15 minutes at room temperature. Formaldehyde was discarded and the wells were gently washed 3 times with PBS, pH 7.4. After the wash cells were permeabilised with 0.1% (v/v) Triton in PBS, pH 7.4 for 15 min at room temperature, and then wash with PBS 3 times. The cytosol of the cells was then stained with May-Grunwald (Sigma, UK) for 15 min at room temperature, excess stain was removed and washed off with PBS 3 times. Giemsa (Sigma UK) was used for nuclear staining and was introduced into the wells and incubated for 20min at room temperature. Excess stain was removed and washed off with dH_2O, 3 times and the wells left to air dry. Numbers of cells adhered were counted under the X10 objective of a phase contrast microscope and Images from each well was then captured.

3.3.4 Cell motility as measured by cell scratch/wound assay

The cell scratch assay was used to measure cell motility. To evaluate the *in vitro* migratory profile of the CRC cells $2.5x\ 10^5$ cells were seeded into 24 well plates and allowed to adhere and settle for 16h in 0.5ml 10%(v/v) serum-containing, non-essential amino acid supplemented growth medium (complete growth medium) and maintained under cell culture conditions (**Section 2.2.1**). Following 16h incubation, a scratch was made on the monolayer across each well by using a 10µl pipette tip. The cell culture media was then changed to 1% (v/v) serum containing DMEM cell culture media supplemented with non-essential amino acids, and incubated for 2h in cell culture conditions, after which the cell culture media was changed to fresh 1% (v/v) serum containing DMEM cell culture media supplemented with non-essential amino acids. Images at time 0h was captured via phase contrast microscope and the cells were then incubated in cell culture conditions for 24 h after which images were taken using a phase contrast microscope and the percentage wound closure determined by ImageJ® software.

3.3.5 In situ TG2 Activity assay

TG2 incorporation of FITC-cadaverine into cells was detected using fluorescent microscopy. The CRCs (80,000 cells) were seeded into an 8-well chamber slide and incubated overnight. The next day, the cells were exposed to fresh complete medium containing 0.5mM FITC -cadaverine and incubated for 16 h (Nicholas, *et al.* 2003). Following the incubation, the medium was removed and the cells were washed gently three times with PBS, pH 7.4 and then fixed with pre-chilled methanol (-20^{0}C) and then incubated for 15 min at -20^{0}C. The cells were then washed three times with PBS, pH 7.4 and air dried. Nuclei were visualized by 4-,6-diamidino-2-phenylindole (DAPI) staining (Vectashield; Vector Laboratories, Burlingame, CA, USA) Fluorescent Imaging was performed using an inverted fluorescent microscope Leica DM1400B and the resulting images were analysed using Leica LAS AF Image browser software.

3.3.6 Immunofluorescence (IF) Staining

Immunofluorescent staining was performed as previously described by (Wang et al., 2013). CRCs were plated on chamber slides (BD Biosciences) 80,000 cells per chamber and allowed to adhere for 48h under cell culture conditions. After fixation in 4% (w/v) paraformaldehyde, cells were permeabilized using 0.2% (v/v) Triton X-100 in phosphate- buffered saline (PBS; 15 min) and blocked for 1 h with 3% (w/v) bovine serum albumin in PBS. Subsequently, cells were incubated for 1 h with the appropriate primary antibodies in blocking buffer at room temperature, followed by 1 h incubation with the FITC-conjugated anti-mouse secondary antibody (DAKO Denmark) or TRITC anti-rabbit secondary antibody (DAKO Denmark). Nuclei were visualized by 4=,6-diamidino-2-phenylindole (DAPI) staining (Vectashield; Vector Laboratories, Burlingame, CA, USA). Fluorescent Imaging was performed using an inverted fluorescent microscope Leica DM1400B and the resulting images analysed using Leica LAS AF Image browser software.

3.3.7 RNA Extraction and cDNA synthesis

TRIZOL Reagent (Invitrogen-Life Technologies) was used to isolate the total RNA from adherent cell monolayer as instructed in the manufacturers' protocol (Rio et al., 2010). Adherent CRCs at approximately 80-90% were washed once with ice-cold PBS. The cells were collected and lysed directly in 1 ml of TRIZOL reagent with the cell scraper. The cell solution in TRIZOL was vortexed thoroughly

and incubated for 1min. To the resulting cell lysate, 0.2 ml of chloroform was added and vortexed vigorously for 15 seconds after which the samples were incubated for 2 to 3 min at room temperature. The samples were then centrifuged at 12,000x g for 15 min using a refrigerated centrifuge (4^{o}C). Following centrifugation, the lysate separates into a colourless upper aqueous phase, an interphase and the red phenol-chloroform phase which is the lower phase. The cellular RNA is present in the aqueous phase. The upper aqueous phase was carefully collected in a fresh tube, and the RNA precipitated with the addition and incubation of 500µl of isopropyl alcohol at room temperature for 10min. The precipitated RNA was collected by centrifugation at 12,000x g for 10 min at 4^{o}C. The RNA pallet was washed twice with 1ml of 75 %(v/v) ethanol by vortexing and centrifugation at 7,500x g for 5 min at 4^{o}C. Each time the supernatant was discarded and the pellet retained. The final washed RNA pellet was then air dried for 15 min and then subsequently dissolved in DEPC-treated RNase free water by gentle pipetting. The dissolved RNA samples were analysed spectrophotometrically using a Nanophotometer (IMPLEN, Munchen, Germany) to determine the purity of the RNA (A260/A280) obtained as well as the concentration of RNA in the sample.

3.3.8 Reverse Transcription of RNA to cDNA

cDNA synthesis from RNA was performed Using the Qiagen® RT^{2} First Strand Kit according to the manufacturer's protocol. 5ng of RNA template from each cell line was added to the master mix of genomic elimination mixture (2µl of 5x gDNA elimination buffer) to get rid of any genomic contaminants. 10µl of RNase free water was added to the gDNA elimination buffer and gently mixed prior to incubation at 42^{o}C for 5 min. The tube was chilled on ice for 1 min and for each reaction tube a reverse transcription (RT) enzyme cocktail was made containing 4µl of 5x RT buffer mix, 1µl of a primer and external control mix, 2µl of a RT enzyme mix and RNAse free water to make up a total volume of 10µl per reaction. This was added to 10µl of the genomic DNA elimination mix and mixed gently pipetting up and down. The mixture was incubated for 15 min at 42^{o}C and heating the tube to 95^{o}C for 5 min stopped the reaction. To each mix of 20µl, 91µl of RNase free water was added and mixed. This mix containing the cDNA was then used for PCR.

3.3.9 Reverse transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed on cDNA obtained from the CRCs, the PCR reaction was performed using Taq DNA Polymerase kit from ThermoFisher according to the manufacturer's instructions. The solutions were initially kept on ice after being thawed completely. The reaction mix for the PCR was made up as follows;

Table 3.1 PCR Mix

Component	Volume	Final conc.
10X PCR buffer minus Mg	10 µl	1X
10 mM dNTP mixture	2 µl	0.2 mM each
50 mM $MgCl_2$	3 µl	1.5 nM
Primer mix (10 µM each)	5 µl	0.5 µM each
Template DNA	1–20 µl	n/a
Taq DNA Polymerase (5 U/µl)	0.2–0.5 µl	1.0–2.5 units
Autoclaved distilled water	to 100 µl	

Following the constitution of the PCR mix. A typical PCR thermal cycler program was set up as outlined below; using 30-35 cycles of PCR amplification. The PCR tubes were first incubated in a thermal cycler at 94°C for 3 minutes to completely denature the template. 30-35 cycles of denaturing at 94°C for 45 seconds, annealing 55°C for 30 seconds and extension for 72°C for 90 seconds was performed. After the completion of the PCR cycle, the tubes were incubated for an additional 10 min at 72°C and the reaction maintained at 4°C for subsequent loading or stored at -20°C for long storage. After the PCR reaction was performed, 10ng of the PCR products were mixed with the loading dye and run on a 1.5% agarose gel in TAE buffer and analysed by gel electrophoresis.

Table 3.2 Primer sequences for PCR

Primer (Sigma Aldrich, UK)	Forward	Reverse
N-Cadherin	5’-aacagcaacgacgggttagt-3’	5’-cagacacggttgcagttgac-3
Zeb 2	5’-caagcctctgtagatggtcc-3’	5’-atcgcgttcctccagttttct-3’
GAPDH	5’-tgcaccaccaacttgcttagc-3’	5’-ggcatggactgtggtcatgag-3’

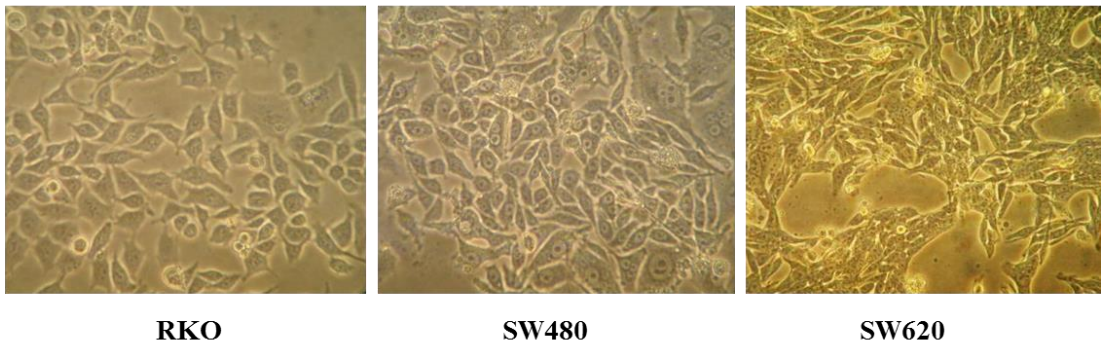
3.4 RESULTS

3.4.1 Characterisation of CRCs on basis of Invasion, Cell Motility, EMT, Drug resistance and TG2 expression and activity levels.

3.4.1.1 Cell morphology and cellular proliferation

SW480 and SW620 cells have different morphology in culture. Light microscopy with a phase contrast microscope reveals that most of the SW480 cells have a spreading, epithelial-type morphology when growing on tissue culture plastic (**Figure 3.4.1A**). In contrast, its metastatic counterpart SW620 displays an ovoid to elongated morphology. The RKO cells have a similar cellular morphology to SW480. **Figure 3.4.1A** suggest that the morphological characteristics of SW480 and RKO cells are indicative of predominately epithelial cell-like features compared to SW620 cells. **Figure 3.4.1B** shows the differences in the proliferative potential of the three CRCs. RKO and SW480 proliferate at similar rates over the 3-day (72h) period. However, SW620, the metastatic cell line, exhibits a significantly ($P<0.05$) lower proliferation rate compared to both RKO and SW480 after 24h as assessed by XTT cell viability assay.

A



B

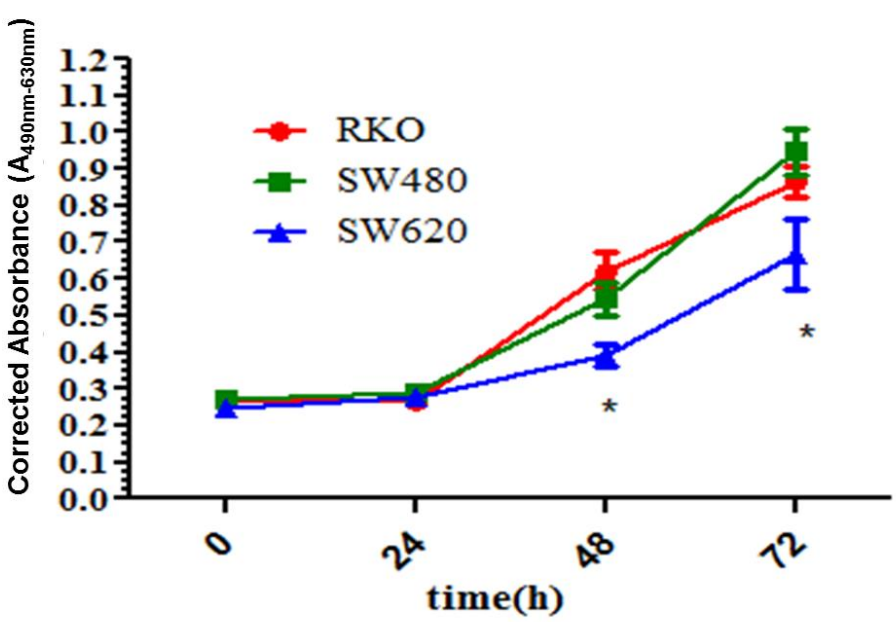


Figure 3.4.1. Physical characterisation of CRCs- RKO, SW480, and SW620. (**A**) Morphological differences in CRCs. Equal number of cells were seeded and allowed to reach ~80% confluency over 16h. Cell morphology was captured using an inverted microscope at 40x magnification. Images are representative (n=3). (**B**) Cell proliferation of RKO, SW480 and SW620 cells. 3000 cells were seeded in 96 well plates under cell culture conditions and cell proliferation determined by cell viability of cells using XTT as described in **Section 2.2.11** Absorbance at 630nm was subtracted from absorbance at 490nm to correct for smudges (* $P<0.05$, n=3, mean±S.D)

3.4.1.2 Invasive capacity amongst the three cell lines

The cell invasion assay was used to assess the invasive potential of CRCs and used to validate progression in the current CRC model. Tumour invasion describes an inherent characteristic of cancer cells to degrade and permeate the basement membrane. Collagen IV coated 3.0 μM inserts were used to mimic the basement membrane and used in assessing the invasive capacity of the three cell lines RKO SW480 and SW620. In **Figure 3.4.2** a count of cells that have invaded the matrix is shown and the metastatic cell line-SW620 displayed the highest potential for invasion and was significantly ($p<0.05$) higher than both primary cancer cell lines SW480 and RKO. More so, the SW480 cells displayed a significantly higher ($P<0.05$) invasive potential over the RKO cells.

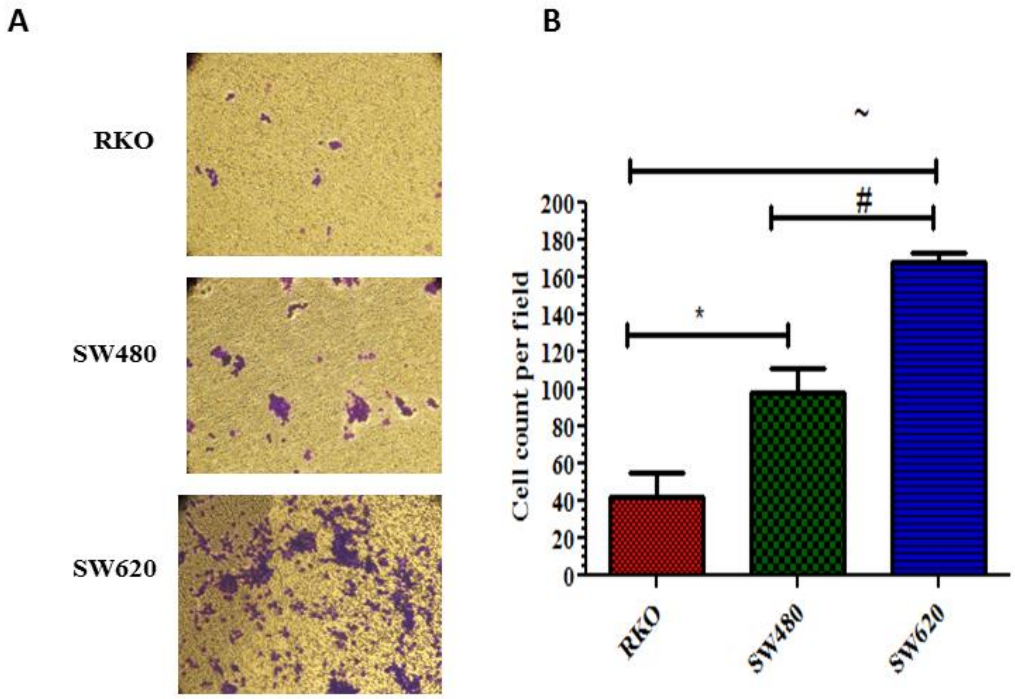


Figure 3.4.2 Characterisation on the invasive potential of CRCs. (**A**) Representative images of crystal violet stained cells that have invaded collagen IV and the Transwell inserts. Images were captured using phase contrast microscope with the 10X objective. (**B**) Cell count of stained cells after invasion assay as described in **Section 3.3.3.** Cells were counted in the 10X objective field from 10 different fields of view (n=3, mean±S.D). (* statistical ($p<0.05$) difference between RKO and SW480, statistical difference between SW480 and SW620, ~ statistical difference between SW620, SW480, and RKO.

3.4.1.3 Assays to determine capacity for adhesion and motility

Following the morphological changes observed among the cell lines, and the differences in invasion potential *in vitro* an *in vitro* assay for cell motility and adhesion were used to analyse factors that might underlie these differences in tumour cell invasiveness. **Figure 3.4.3A** shows representative images of the cell scratch assay over 24h. Primary cells SW480 exhibited significantly ($P<0.05$) increased wound closure compared to SW620. Similarly, RKO cells were also significantly more motile than SW620 cells. Although SW480 cells showed higher percentage wound closure than RKO cells, this difference was not significant. Following the observation of the cell scratch assay, a cell adhesion assay was performed (**Figures 3.4.3B and C**); RKO and SW480 cells displayed a progressive increase in attachment between 0.5 and 2 h on cell culture plates. At 2h, there were significantly ($P<0.05$) more SW480 and RKO cells than SW620 cells adherent on the cell culture plates. Additionally, RKO and SW480 showed significant increases in cell spreading when compared to SW620 cells (**Figure 3.4.3D**).

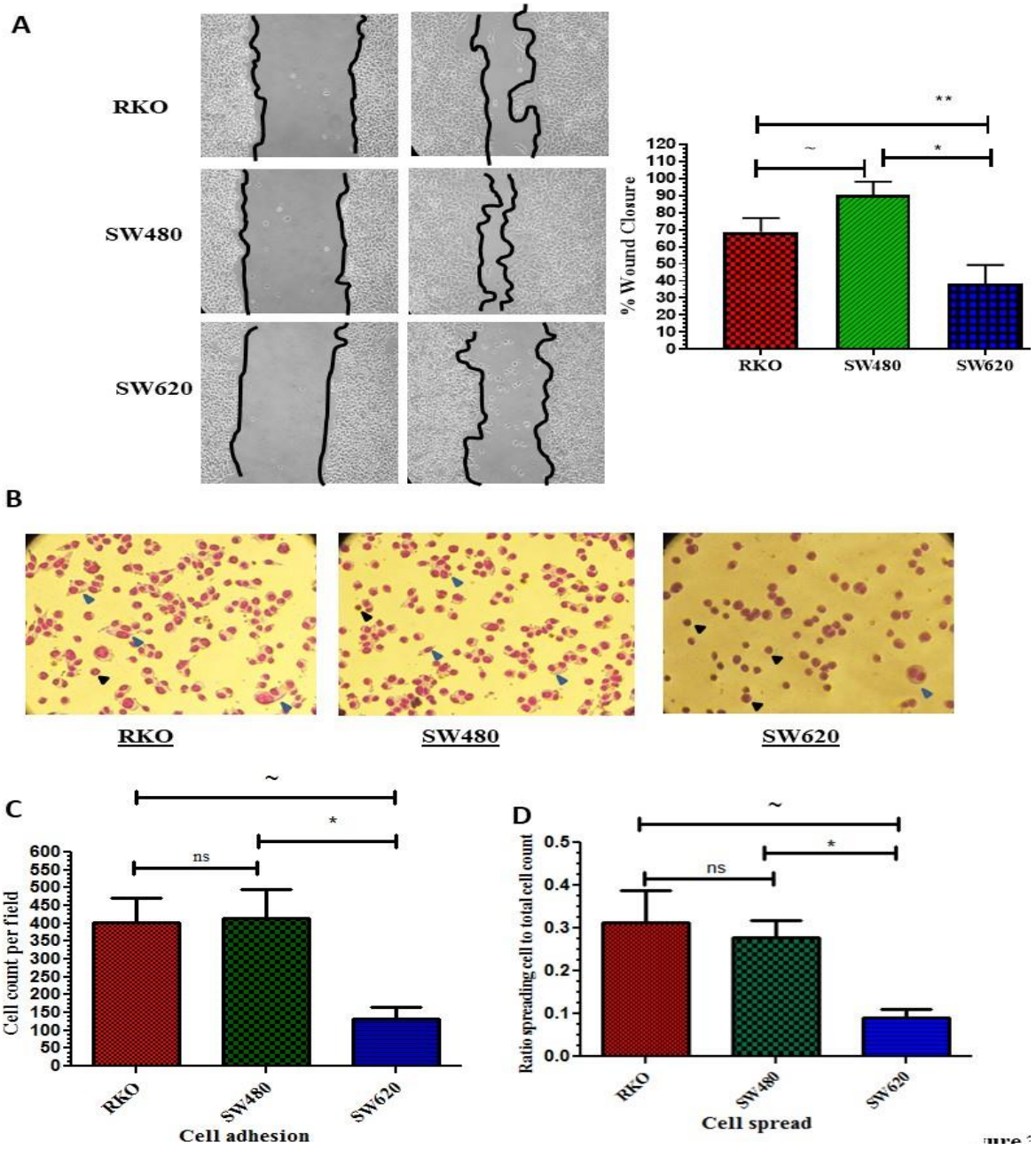


Figure 3.4.3 Metastatic cell line is distinct on the basis of cell motility and adhesion (**A**) Images of cell scratch or wound assay. Wound closure was monitored over 24h as described in **Section 3.3.5**. Representative images at the 20x objective. (* SW620 significantly different from SW480, **SW620 significantly different from RKO, ~RKO significantly different from SW480 $p<0.05$; n=3. mean±S.D (**B**) Representative images of cytoplasmic (light purple) and nuclear stained (dark purple) stained cells attaching to cell culture plate after 2h of seeding as described in **Section 3.3.4**. Blue arrows show attached cells with cell spreading (distinct cytoplasmic and nuclear stain). Black arrow shows non spreading attached cells. (**C**) Bar chart showing the number of adhered cells after 2h of equal cell seeding, cells were counted at the 10X objective (**D**) Ratio of spread cells to the total number of cell as viewed under the 10X objective (* SW620 significantly different from SW480, ~SW620 significantly different from RKO, $p<0.05$; n=3. n.s-not significant RKO vs SW480, mean±S.D).

3.4.1.4 Markers of Epithelial to mesenchymal Transition and CRC disease progression are upregulated in metastatic cell SW620.

EMT has been associated with disease progression, drug resistance and invasiveness in many cancer types and proven to be an authentic marker of tumour progression. In **Figure 3.4.4**, protein expression of EMT markers was assessed in RKO, SW480 and SW620 cells by Western blotting. Mesenchymal cell markers fibronectin (FN), vimentin, and N-cadherin had a significantly ($p<0.05$) higher expression in metastatic cell line SW620 compared to its isogenic primary cell line SW480 and RKO. Consistent with this observation epithelial cell marker ZO1 expression was significantly higher in the primary cell lines SW480 and RKO compared to SW620. To further establish the metastatic potential of SW620, proteins associated with metastasis and tumour progression; S100A4 and alpha smooth muscle actin (αSMA) were determined by western blotting in whole cell lysates of the CRCs. Only SW620 cells expressed significant and detectable amounts of the two proteins.

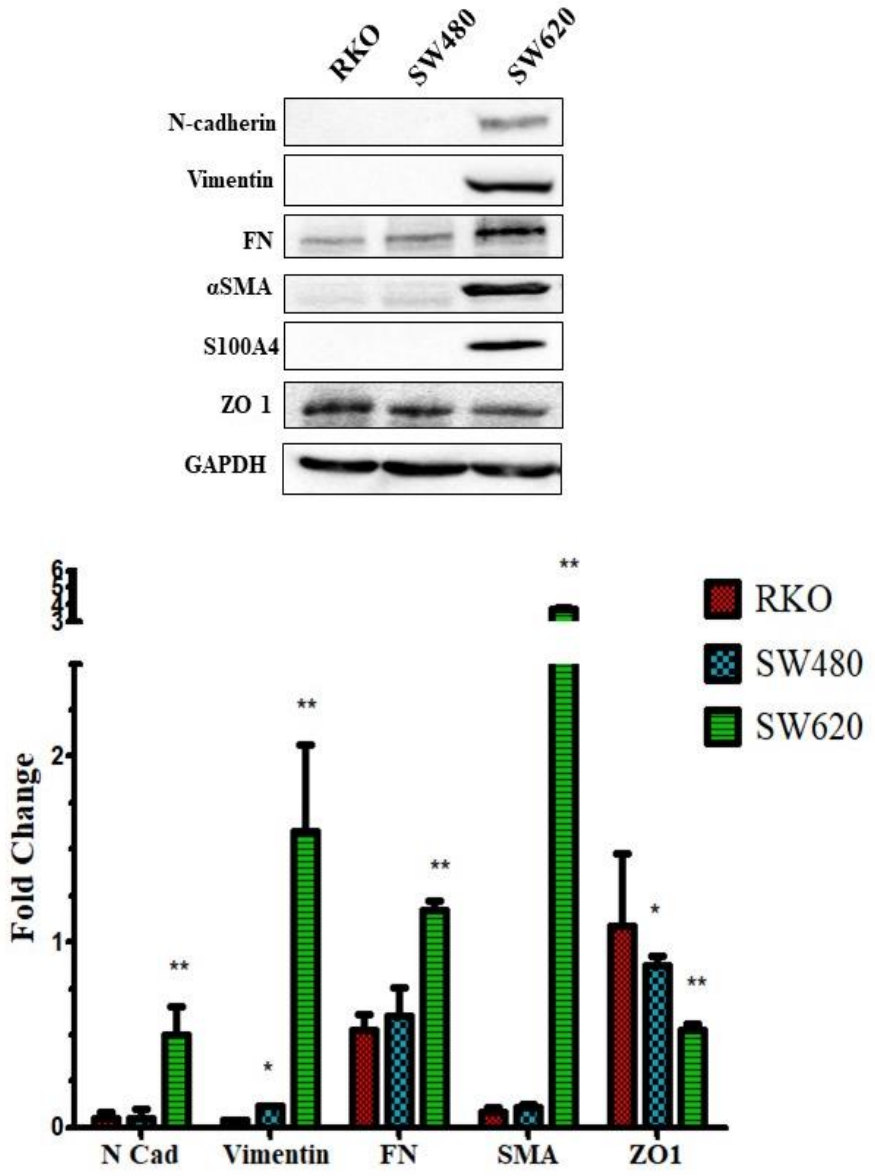


Figure 3.4.4. Characterisation of CRCs on EMT and markers of disease severity. Representative Western blots of whole cell lysates of CRCs were used for detection of epithelial marker ZO1, mesenchymal cell markers vimentin, fibronectin (FN), myofibroblast cell marker α smooth muscle actin (αSMA), and metastatic cell marker S100A4. GAPDH was used as a measure of equal protein loading. These markers were analysed by Western blotting as described in **section 2.2.6**. Blots are representative (n=3). Bar chart shows the densitometry ratios of protein expression of the EMT marker proteins against GAPDH. (n=3; mean±S.D) *-statistically significant from RKO, **-statistically significant from RKO and SW480

3.4.1.5 Metastatic cell line SW620 exhibit high drug resistance

Following the observation of the phenotypic and invasive differences between the metastatic cell line and the two primary cell lines, it was important to characterise the cells' response to the conventional CRC chemotherapy drug 5 Fluorouracil (5FU). **Figure 3.4.5** shows a dose-response assay with 5FU performed to determine the sensitivity of cells to 5FU. Growth inhibition was monitored in a dose-dependent manner in all three CRC cells, 5FU treatment resulted in different degrees of inhibition. The metastatic cell line SW620 displayed a significant ($p<0.05$) resistance to 5FU at doses (25-200μg/ml) during a 48-72h incubation when compared to primary cell lines RKO and SW480. Responses to 5FU by SW480 and RKO cells were similar. The drug-resistant of SW620 was also confirmed with doxorubicin with the metastatic cell line exhibiting significant resistance and higher viability to doxorubicin at (50-200 ng/ml) compared to the two primary cell lines SW480 and RKO during the 48-72h treatment period **(Figure 3.4.6**). No significant difference was found amongst the three cell line response to either 5Fu or doxorubicin within the first 24h of treatment. These findings, as well as other characterisation of the CRC's on cancer progression, confirm that SW620 metastatic cell line exhibits a progressive phenotype when compared to the two primary cancer cell lines.

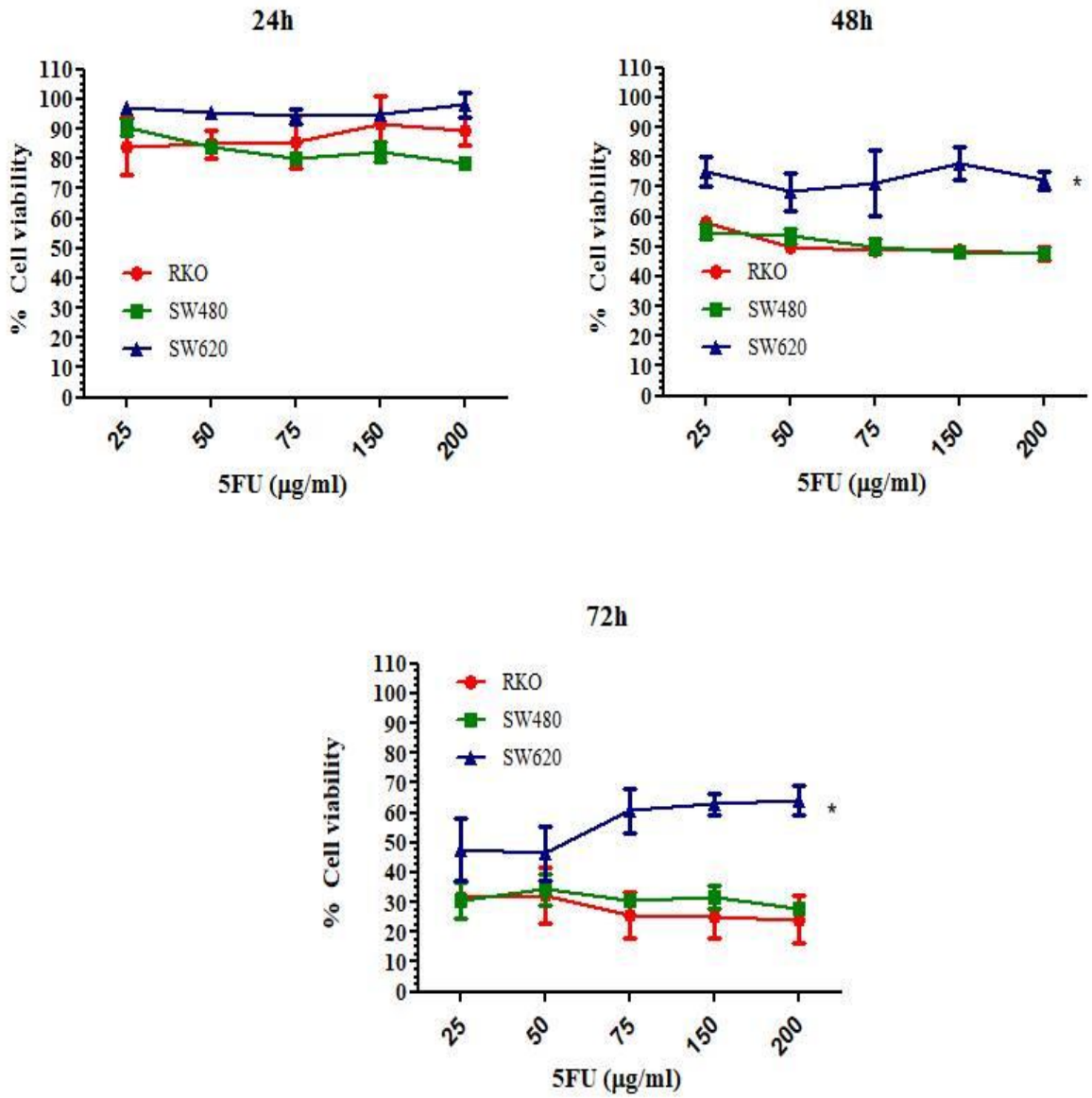


Figure 3.4.5. Drug Response of CRCs to 5-Fluorouracil (5FU). 3000 cells/well were seeded in 96 well plate and treated with 5FU (25-200µg/ml) 4h post seeding. DMSO was used as control vehicle for control cells. Cells were maintained under cell culture conditions and cell viability assessed by XTT at the different incubation periods. Cell viability of treated cells was measured as a percentage of control cells. (*SW620 significantly different from SW480 and RKO, $p<0.05$; n=3, mean±S.D)

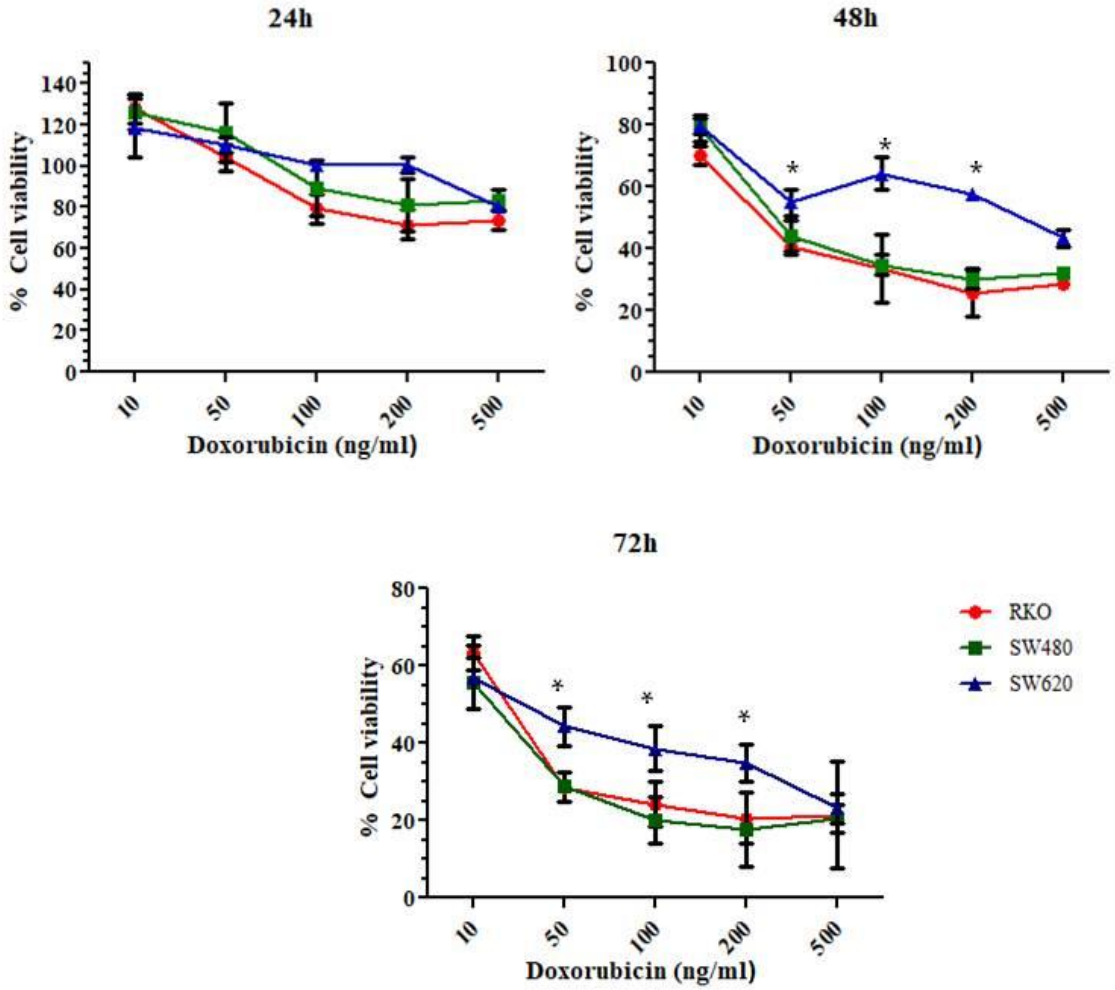


Figure 3.4.6. Drug Response of CRCs to doxorubicin. 3000 cells/well were seeded in 96 well plate and treated with doxorubicin (10-500ng/ml) 4h post seeding. DMSO was used as control vehicle for control cells. Cells were maintained under cell culture conditions and cell viability assessed by XTT at the different incubation periods. Cell viability of treated cells was measured as a percentage of control cells. (*SW620 significantly different from SW480 and RKO, $p<0.05$; n=3, mean±S.D).

3.4.1.6 TG2 is highly expressed in the Metastatic CRC disease model

After assessing the parameters associated with cancer progression including EMT, drug resistance and *in vitro* invasion and characterising the CRC cell lines for these parameters, it was now important to identify if TG2 was also associated with these characteristics. In **Figure 3.4.7A** TG2 protein epression was assesed via Western blotting and shows that TG2 is constitiutively and significantly ($p<0.05$) expressed in the metastatic cell line SW620 compared to the two primary cell lines SW480 and RKO, with SW620 cells expressing about ~12-folds higher TG2 compared to SW480 cells. SW480 expressed ~7-folds the level of TG2 detected in RKO cells. Since TG2 is an enzyme, whose activity can be easily detected. In **Figure 3.4.7B** TG2 crosslinking cataytic activity over a 2h period was assayed for in whole cell lysates of the three cell lines and confirmed the results of the Western blot, with SW620 cells exhibiting significantly higher TG2 activity in the whole cell lysates compared to SW480 and RKO. Although SW480 possesed higher TG2 activity than RKO, it was however not significant. Furthermore to validate this finding TG2 *in situ* incorporation of FITC-cadavarine was measured by fluorescent microscopy (**Figure 3.4.7C**) which confirmed findings observed using Western blotting and TG2 activity in whole cell lysates, with SW620 cells exhibiting higher fluorescent intensity than primary cells SW480 and RKO.

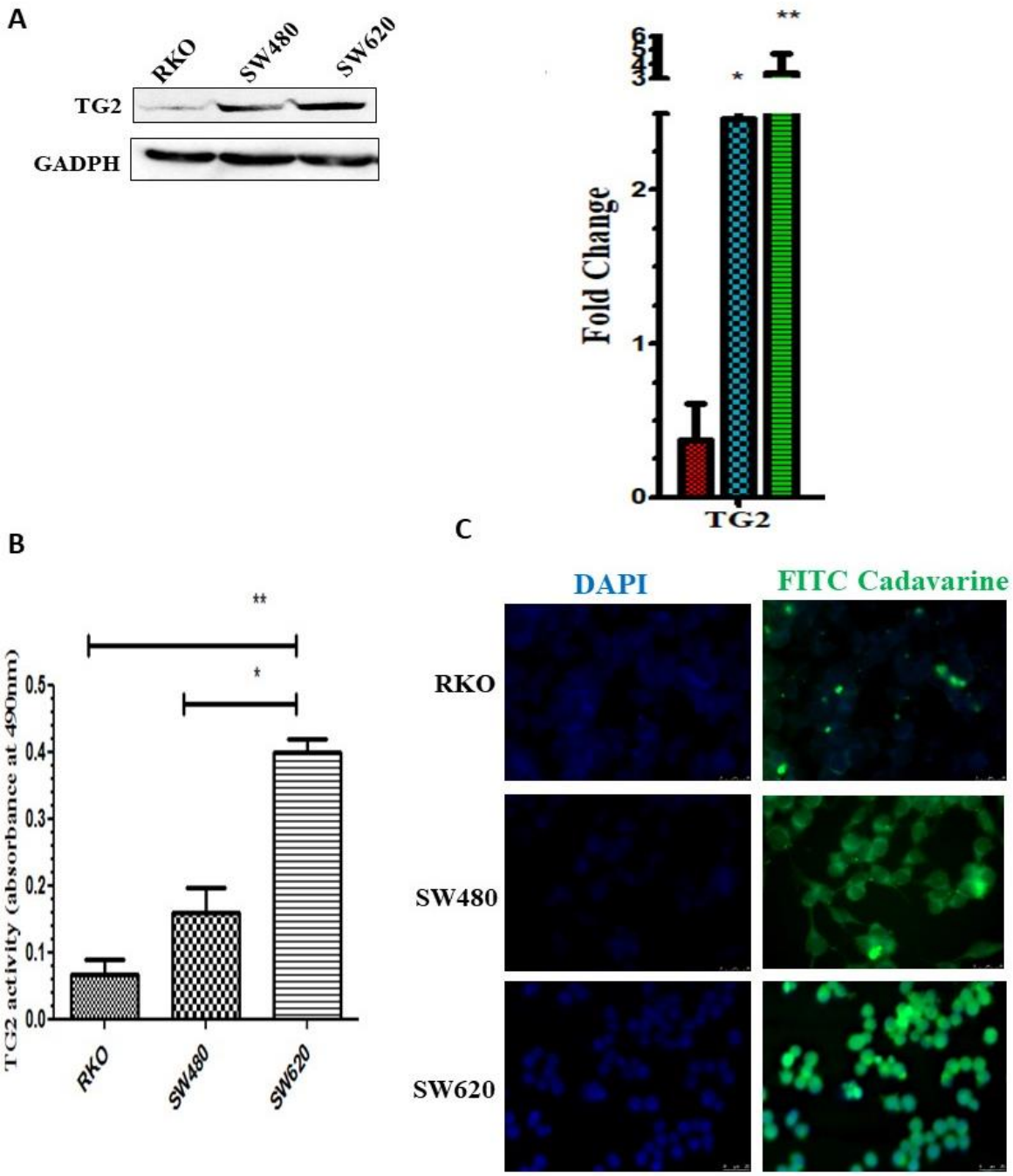


Figure 3.4.7 Characterisation of CRCs for TG2 expression level. (**A**)Whole cell expression of TG2. Whole cell lysates of CRCs were used for detection of cellular TG2. GAPDH was used as housekeeping protein and a measure of equal protein loading. Blots are representative, bar chart show densitometry ratios of protein expression normalised against GAPDH *-statistically significant from RKO, **-statistically significant from RKO and SW480) (**B**) TG2 whole cell lysate activity as measured by biotin cadavarine incorporation into casein coated wells (*SW620 significant from SW480, ** SW620 significant from SWRKO $p<0.05$; n=3, mean±S.D). (**C**) Fluorescent image of FITC-cadavarine incorporation *in situ* (**Section 3.3.5**). Fluorescent imaging was performed using an epifluorescent microscope and images are representative of two independent experiments.

3.4.2 TG2 expression and activity is associated with cellular invasion, EMT, cell motility and drug resistance

Next the involvement of TG2 in cell adhesion, cell motility, cell invasion and drug resistance was investigated. TG2 expression was silenced by a lentiviral construct containing shRNA for TG2 in the TG2 expressing SW620 and SW480 cells, conversely human TG2 was ectopically expressed by lentiviral construct in RKO cells. Additionally, TG2 was pharmacologically inhibited using TG2 site specific small molecule inhibitor 1-155 (1μM) to treat cells and determine the effect of TG2 inhibition on cellular activities. The toxicity of 1-155 and the different lentiviral treatments was investigated in the three CRCs by monitoring their effects on cell viability. Neither of these treatments affected the viability of the cells (**Figures 3.4.14** and **3.4.18C** respectively).

3.4.2.1 TG2 may be required for EMT in this CRC model

TG2 expression was silenced in SW480 and SW620 cells by transduction of cells with TG2 shRNA. The efficiency of the different shRNA constructs on TG2 expression and corresponding effect on the expression of EMT markers is shown in **Figure 3.4.8.** Following stably manipulating TG2 expression by stable ectopic expression in RKO, and silencing in SW480 and SW620 the effect TG2 expression on EMT was determined. In **Figure 3.4.9** increase in TG2 expression by viral trasduction in RKO cells led to significantly ($p<0.05$) increased expression of mesenchymal markers, including vimentin and FN, and a significant decrease in epithelial tight junction marker Zonal occludin 1 (ZO-1). The expression level of these EMT markers was significantly reversed once TG2 was downregulated by transduction of TG2 shRNA in SW480 and SW620 cells. TG2 downregulation by shRNA also restored levels of ZO-1 . More so by monitoring the mRNA levels of EMT markers by reverse transcriptase PCR, TG2 expression was associated with increased mRNA levels of N cadherin, and Zeb 2 an EMT transcription factor in SW620 and RKO cells with manipulated TG2 expression levels (**Figure 3.4.10A**). These changes in EMT markers with TG2 expression were validated in SW620 cells and RKO cells by immunofluorescence staining of Vimentin, Fibronectin and ZO-1 (Figure 3.4.10B).

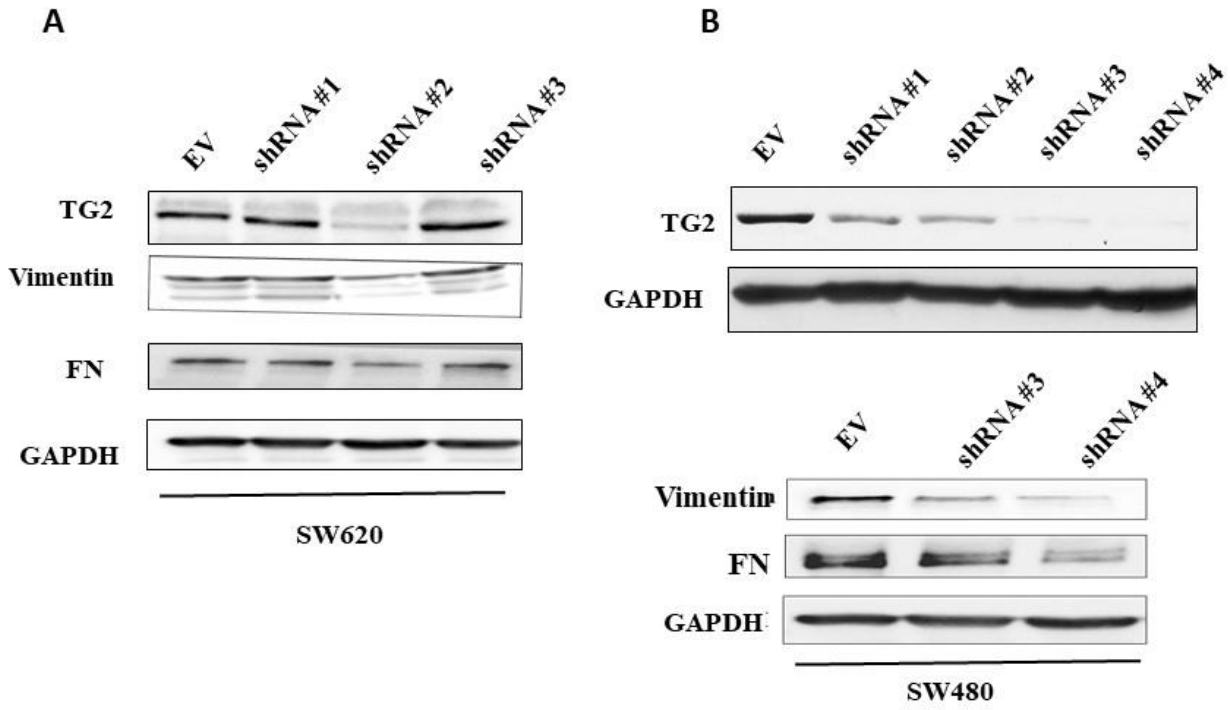


Figure 3.4.8 Transduction efficiency of TG2 shRNA in SW620 and SW480 cells. Representative Western blotting for TG2 and EMT markers expression following transduction with various lentiviral TG2 shRNA constructs. Blots are representative of 2 independent experiments. Ratios indicate mean relative densitometry ratios of protein expression of the proteins normalised to GAPDH.

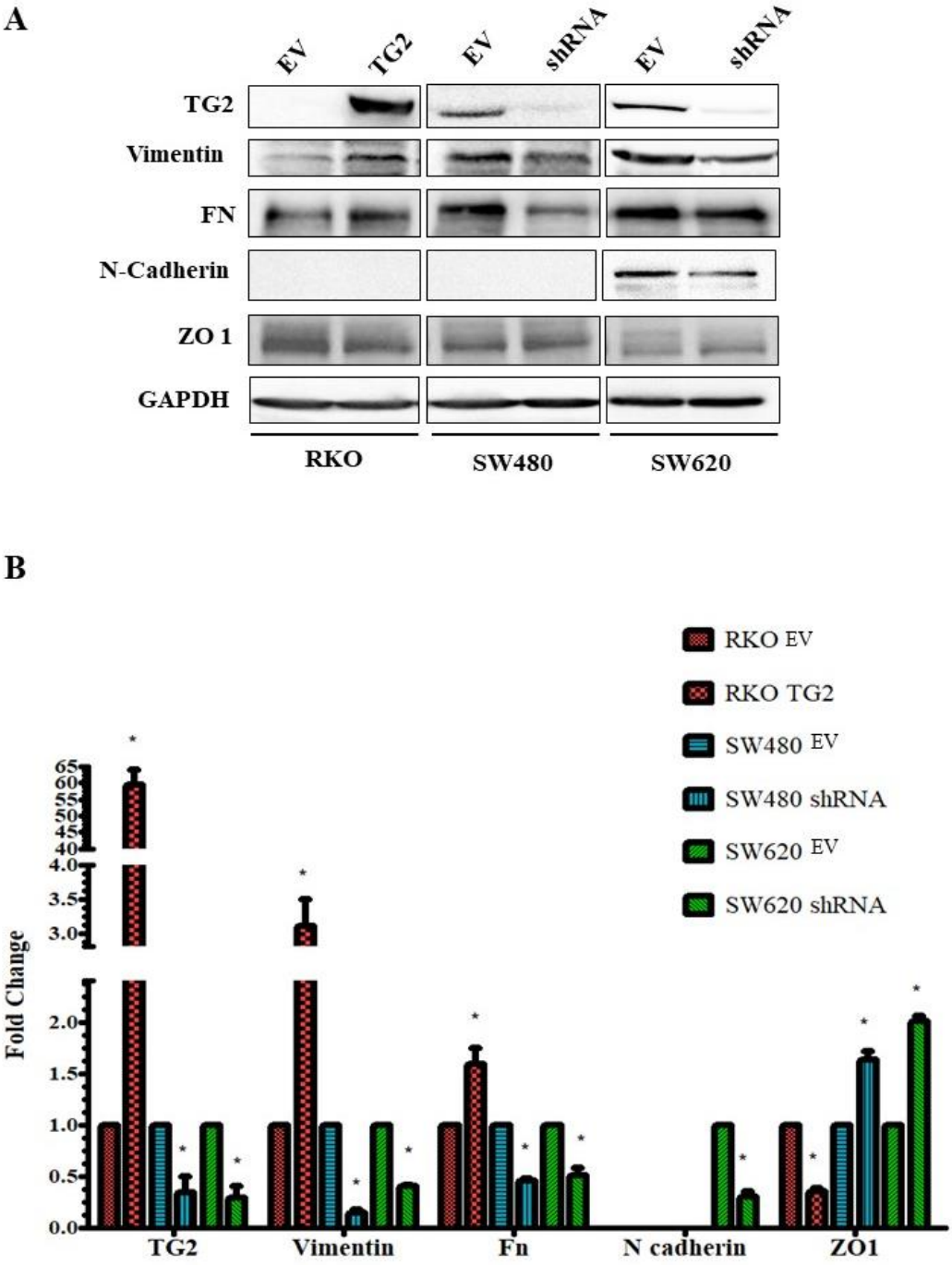


Figure 3.4.9 TG2 expression correlates with expression of EMT markers. (**A**) Representative Western blotting for TG2 and EMT markers in CRCs following TG2 expression increased by viral transduction (TG2) or reduced by transduction with TG2 shRNA (SW480shRNA and SW620shRNA) and their corresponding transduced empty vector (EV) controls. GAPDH was used as housekeeping protein and used to normalise protein loading. EMT markers were analysed by Western blotting. (B) Relative densitometry ratios of protein expression of the proteins against GAPDH. (n=3; mean±S.D) *-statistically significant from EV cells.

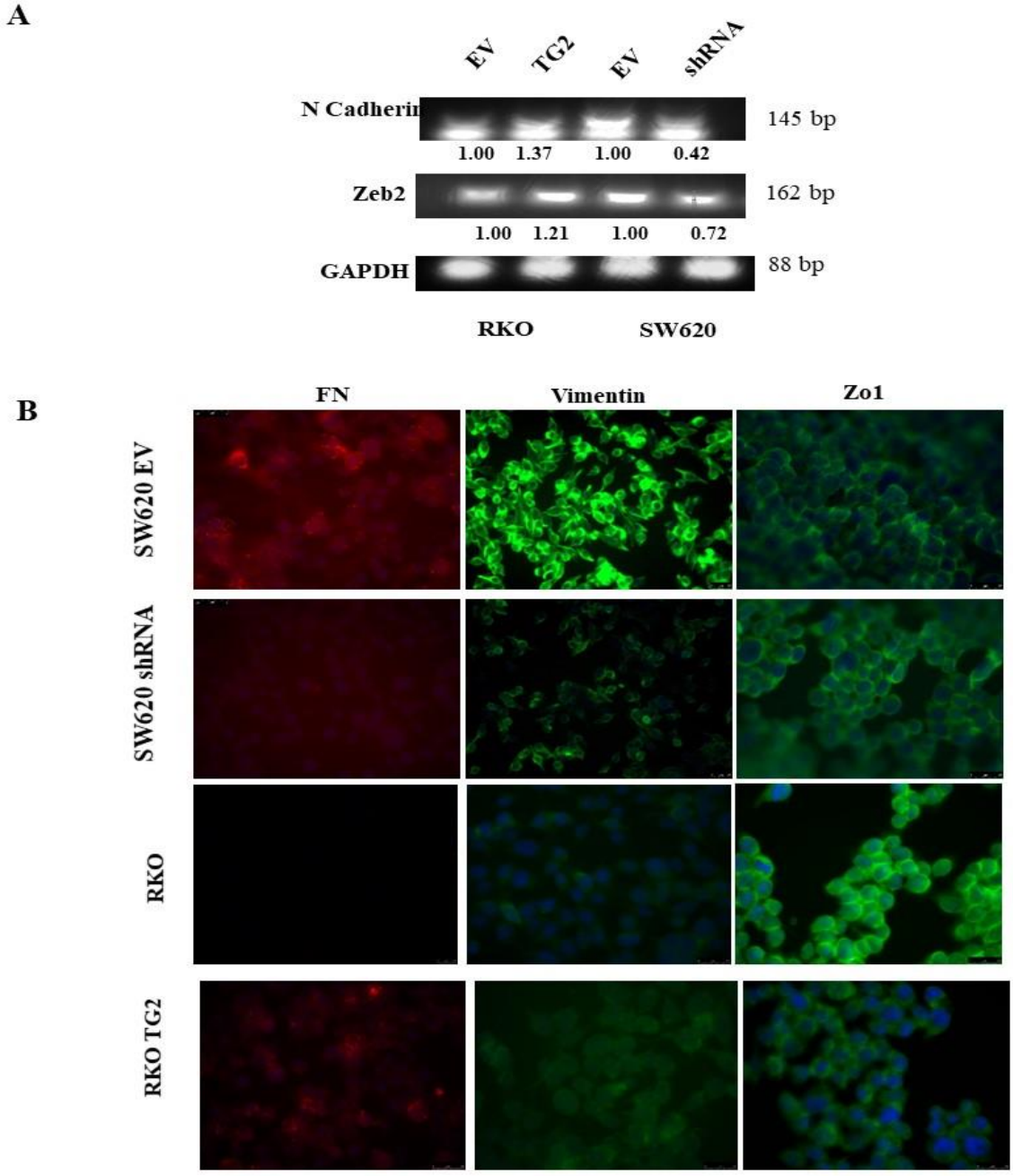


Figure 3.4.10 TG2 expression corresponds with EMT in CRCs. (**A**)Semi-quantitative RT-PCR for EMT marker N-cadherin and transcription factor Zeb2in TG2 manipulated RKO and SW620 cells, PCR blots are representative of 2 independent experiments performed in duplicate. GAPDH was used as housekeeping gene. Ratios indicate mean relative densitometry ratios of mRNA levels of the genes against GAPDH (**B**) Immunofluorescent detection of TG2 and EMT marker epitopes by fluorescence microscopy in TG2 manipulated cells and their corresponding controls (EV). Representative image (n=3).

3.4.2.2 TG2 expression induces a fibroblast like morphology in CRCs

Since TG2 expression altered the EMT potential of the cells, we next determined if TG2 expression would also alter the morphology of the cells. By manipulating TG2 expression in RKO, SW480 and SW620 the morphology of these cells (**Figure 3.4.11A)** altered with TG2 expression. The images show that increasing TG2 expression correlated with an elongated, fibroblast like appearance in cells ,while loss of TG2 expression coincided with a cuboidal, more epithelial cell like morphology**. Figure 3.4.11B**, shows that upon shRNA treatment, TG2 cellular catalytic activity is significantly ($p<0.05$) reduced by about 75% in SW620 cells and almost 80% in SW480 cells and conversely when TG2 is ectopically expressed in RKO cells TG2 cellular catalytic activity is significantly increased by over 800%, confirming the stable expression or knockdown of TG2 in these cells following TG2 gene manipulation.

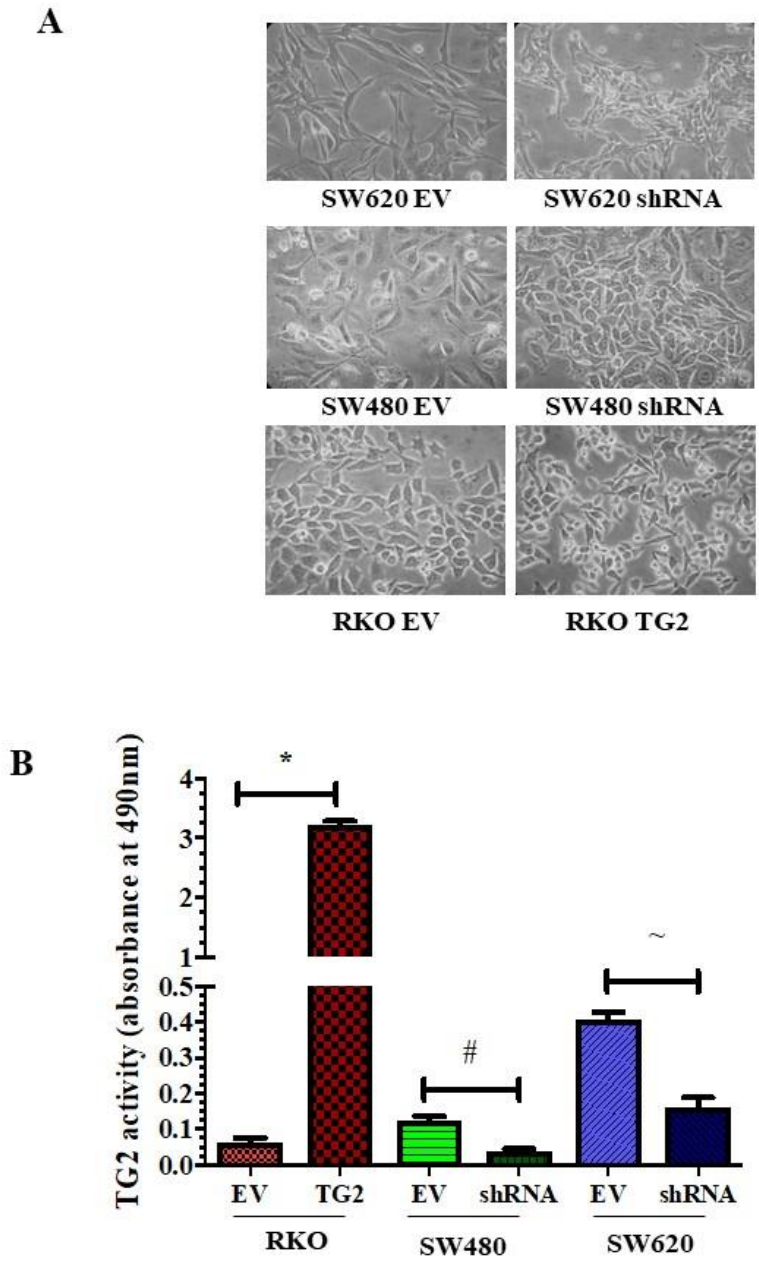


Figure 3.4.11 Lentiviral manipulation of TG2 expression affects cellular morphology and TG2 activity (**A**) Representative images of cell morphology of CRCs transduced with TG2 shRNA or wt TG2. Equal number of cells were seeded and 48h later images were taken at 40× objective magnification. (n=3) (**B**) TG2 whole cell lysate activity as measured by biotin cadavarine incorporation into casein coated wells (**Section 2.2.9**) (* statistical difference between RKO EV and RKO TG2, # statistical difference between SW480 EV and shRNA cells,~ statistical difference between SW620 EV and shRNA cells $p<0.05$; n=3, mean±S.D).

3.4.2.3 Empty vector transduction cells are comparable to the wt CRCs.

To eliminate any non-specific effect of the lentiviral construct on TG2 and cellular protein markers associated with EMT, a comparison of the effect of control plasmid (Empty vector-EV) on TG2 and EMT was carried out by Western blot and in **Figure 3.4.12** it can be seen that the empty vector containing cell which has plasmids that encode the Lentivirus showed comparable expression of TG2, and EMT markers- ZO1 and vimentin, as the control wt cells in the RKO, SW480 and SW620 cells. Additionally the figure also confirms the knockdown of TG2 by shRNA in SW480 and SW620 cells and the over 50 fold increase in TG2 expression in RKO cells transduced with the TG2 vector. Moreover, this confirms that the shRNA sequence selected to target TG2 expression (**Figure 3.4.8**) in SW620 and SW480, effectively and stably silenced TG2 in SW620 and SW480 cells respectively.

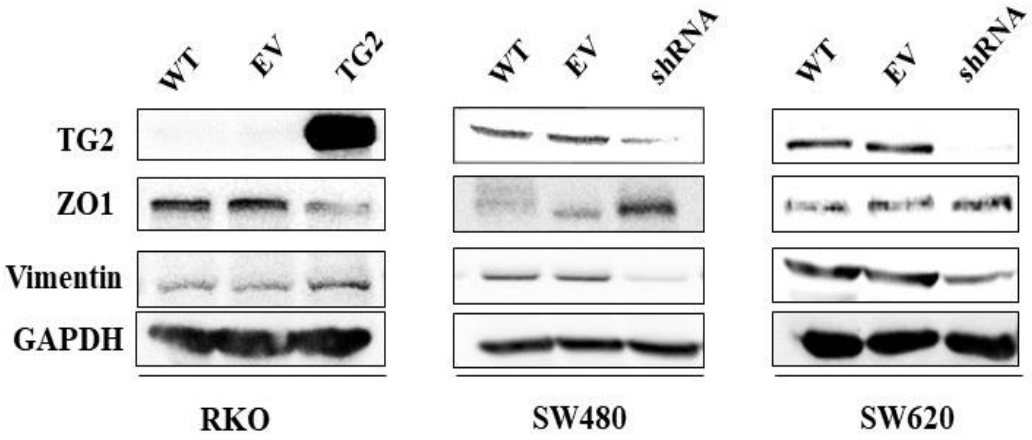


Figure 3.4.12 WT CRCs show comparable TG2 and EMT markers expression to control vector transduced cells. Representative Western blotting for EMT markers ZO1 and vimentin in whole cell lysates of wt cells, cells transduced with empty vector (EV, control cells) and cells transduced with lentiviral particles containing human TG2 (TG2) or shRNA targeting human TG2 (shRNA) in RKO, and SW480 and SW620 cells respectively(n=2).

3.4.2.3 TG2 expression correlates with expression of EMT transcription Factors.

After observing that TG2 expression correlates with the expression of EMT markers, and that TG2 expression resulted in a marked fibroblast like cellular morphology in these epithelial CRCs, it became necessary to determine the effect of TG2 expression on upstream regulation of EMT. **Figure 3.4.13** shows that knockdown of TG2 in the metastatic high TG2 expressing cell SW620 resulted in a corresponding significant ($p<0.05$) loss of approximately 60% in the expression of Slug and Twist. Slug and Twist are important transcription factors of EMT and upregulation of these proteins lead to

EMT in epithelial cells (Agnihotri et al., 2013). What this data suggests is that TG2's role in EMT is upstream of these two transcription factors in the EMT process.

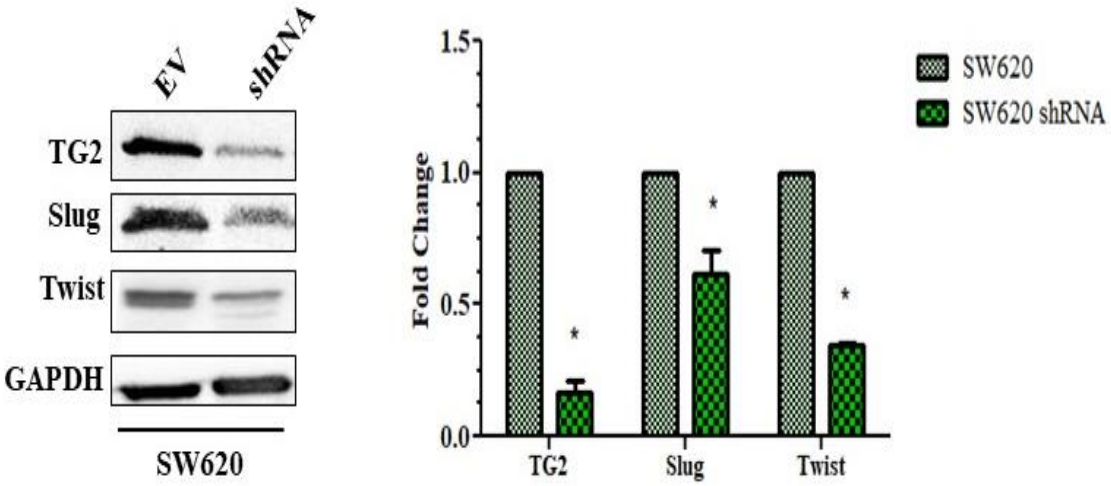


Figure 3.4.13 TG2 knockdown reduces cellular expression of EMT transcription factors in SW620 cells. Representative Western blot showing that TG2 expression correlates with increased expression of transcription factors of EMT (Slug and Twist) in SW620 (EV) control cells or SW620 cells transduced with TG2 shRNA (shRNA). Bar chart show relative densitometry ratios of protein expression normalised against GAPDH (n=3), *-statistically different from EV cells ($p<0.05$).

3.4.2.4 TG2 inhibitor 1-155 is a potent inhibitor of TG2 at small doeses

TG2 site specific cell permeable inhibitor 1-155 developed at Aston University was used to inhibit TG2 in RKO, SW480 and SW620 cells. 1-155 was chosen amongst an array of developed inhibitors due to its potency with an IC50 of 6nM (Badarau et al., 2015 , and a dose of 1μM which is specific for TG2 (Badarau et al., 2015). To establish that the 1μM concentration of TG2 inhibitor used did not pose any toxic effect on the CRCs- RKO, SW480 and SW620 cells, the cell viability of cells treated with 1-155 with a dose range of (0.1-2μM) was determined. (**Figure 3.4.14)** 1-155 at varying doses (0.1-2μM) did not affect cell viability after a 48h treatment, with cell viability in 1-155 treated cells showing a comparable levels tothe control vehicle (DMSO) treated cells.

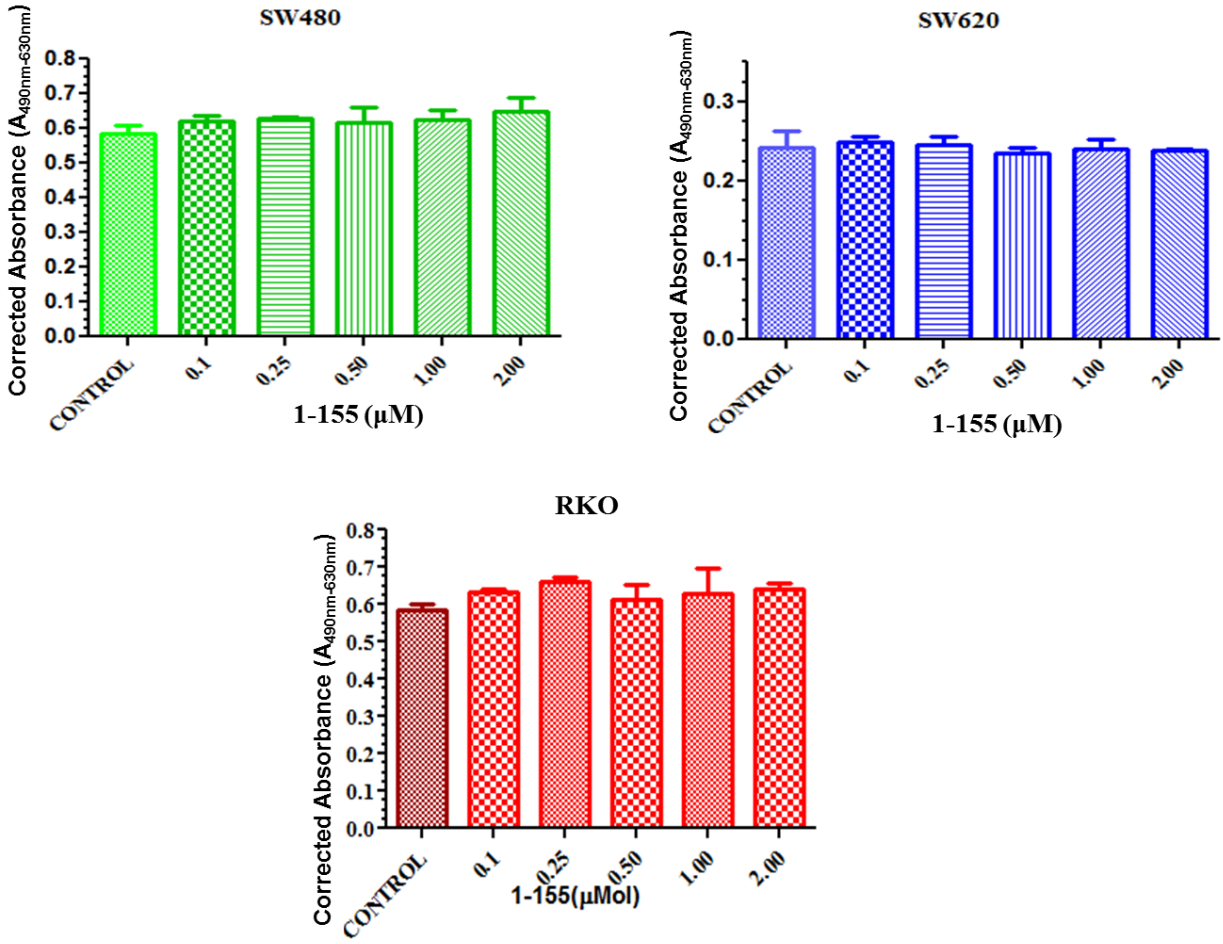


Figure 3.4.14: Treatment of CRCs with TG2 inhibitor 1-155 does not affect cell viability of CRCs. Effect of TG2 inhibition by 1-155 on cellular proliferation of CRCs over 48h incubation period. Cell proliferation was assessed via measuring cell viability with the XTT assay. Control cells received the control vehicle DMSO (n=2, mean±S.D). Corrected absorbance was obtained at (490-630)nm.

3.4.2.5 Cellular inhibition of TG2 attenuates EMT in CRCs

Figure **3.4.9** shows that TG2 expression correlates with expression of EMT markers, and following the establishment of the non toxic effects of 1-155 on cell viability of the CRCs, cells were treated with 1-155 (1µM) for 48h . **Figure 3.4.15 A and B** shows that treatment of SW480 and SW620 with the TG2 site specific inhibitor 1-155 (Badarau et al., 2015) significantly ($p<0.05$) reduced the expression of TG2 by about 50% in both SW620 and SW480 cells. Additionally inhibition of TG2 with 1-155 resulted in significantly reduced expression of EMT markers vimentin and fibronectin by about 30%

and 50% respectively when compared to the control cells. Additionally the expression of transcription factors Slug and Twist 1 in SW620 cells was significantly reduced compared to the detectable amounts shown in the control cells. 1-155 treatment also significantly enhanced expression of the epithelial tight junction protein ZO-1 by around 1.34 and 1.51 fold respectively in SW480 and SW620 cells when compared to control cells. Similarly in RKO cells transduced with TG2 (**Figure 3.4.15C**), treatment with TG2 inhbitor 1-155 significantly reduced expression of the EMT marker fibronectin (~70%) and upregulated ZO-1 (~1.97 fold) compared to control cells. Expression of EMT markers in the inhibited, TG2 transduced cells was similar to the amounts detected in the empty vector treated cells. However, expression of TG2 which is under the control of a viral promoter in these cells was not affected by TG2 inhibition.

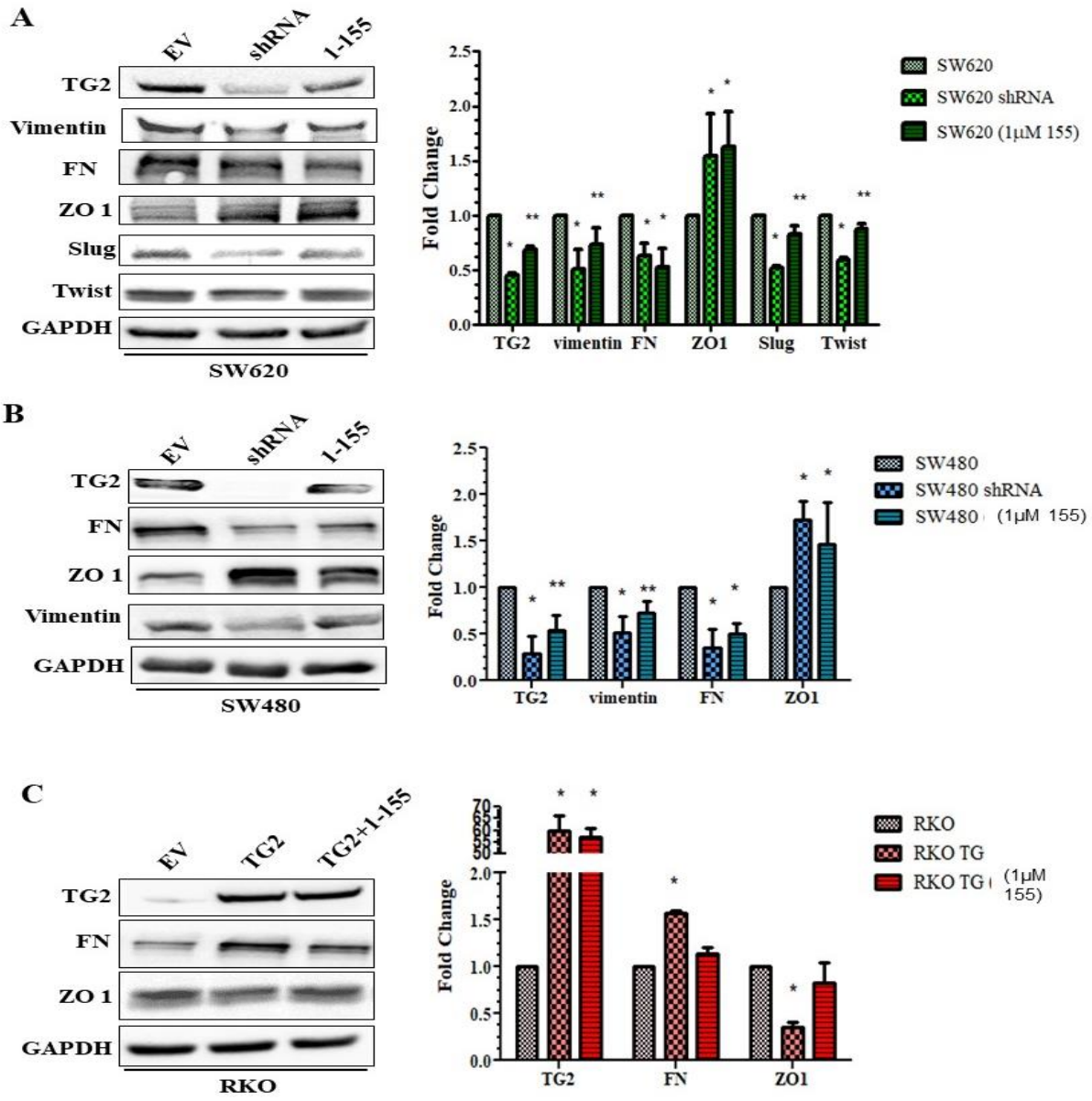


Figure 3.4.15 Cellular inhibition of TG2 attenuates EMT (**A**) Representative Western blot of whole cell lysates from SW620 empty vector control cells (EV) and SW620 cells transduced with TG2 shRNA or control EV cells treated with TG2 selective inhibitor 1-155 (1 μM). Control cells were treated with vehicle alone DMSO. (**B**) Representative Western blot of SW480 empty vector control cells and SW480 cells transduced with TG2 shRNA (SW480shRNA) or EV cells treated with TG2 selective inhibitor 1-155 (1 μM). Control EV cells were treated with vehicle alone DMSO. (**C**) Representative Western blot of whole cell lysates from RKO EV cells treated with the vehicle control DMSO and RKO cells transduced with TG2 (RKO TG2) or RKO-TG2 cells treated with TG2 selective inhibitor 1-155 (1 μM). All treatments were performed for 48h prior to cell lysis. Bar chart shows relative densitometry ratios of protein expression normalised against GAPDH (n=3, mean±S.D). *-Statistically different from EV cells, **-statistically different from EV and shRNA cells.

3.4.2.6 TG2 induces in-vitro cellular invasion in the SW480 and SW620

As shown in **Section 3.4.1.2**, the invasive potential of cells was significantly ($p<0.05$) increased in the SW620-metastatic cell line compared to the two primary cell lines SW480 and RKO. In **Figure 3.4.16** TG2 silencing in SW480 and SW620 resulted in a significantly reduced invasive capacity of the cells *in vitro*. However, increasing TG2 expression in RKO cells did not markedly increase the invasive potential of the cells. A similar trend was observed with the CRCs treated with the inhibitor 1-155 (1μM), as inhibiting TG2 in SW480 and SW620 significantly reduced the ability of the cells to degrade collagen IV and invade the Transwell inserts compared to the control cells however, TG2 inhibition led to comparable levels of invasion observed in the TG2 knockdown cells.

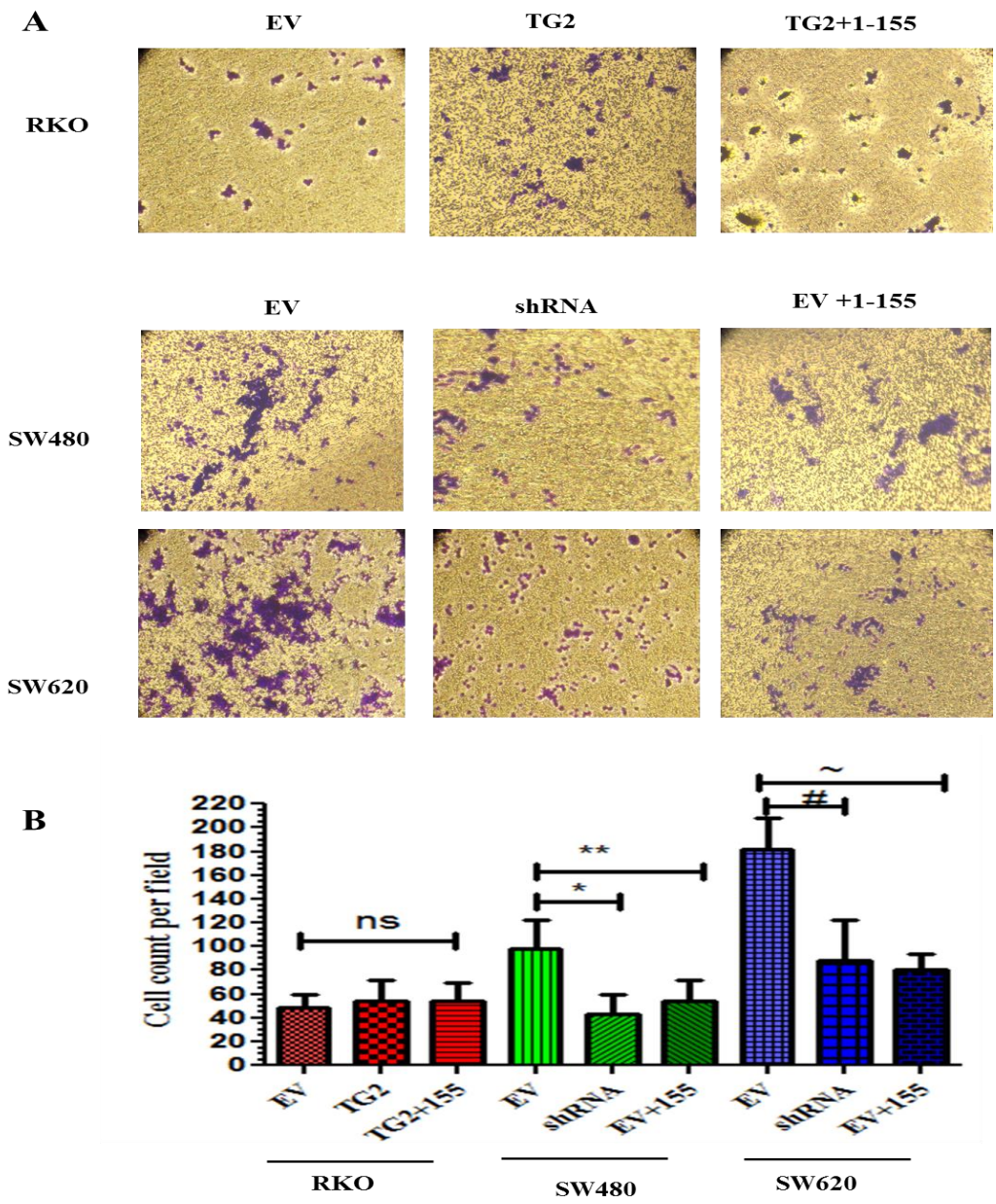


Figure 3.4.16 TG2 plays a role in increased cellular Invasion in CRCs. (**A**)Representative images of crystal violet stained cells that have invaded the collagen IV and the Transwell inserts. Images were captured using phase contrast microscope at the 10X objective. For 1-155 (1μM) treated cells, cells were pre-treated with 1-155 for 24h prior to seeding cells and were further treated with 1-155 (1μM) after cell seeding for the duration of the experiment. EV, shRNA and TG2 viral treated cells were treated with DMSO, the control vehicle. (**B**) Cell count of stained cells after invasion assay as described in **Section 3.3.2**. Cells were counted in the 10X objective field from 10 different field of each insert. (*- statistically difference between SW480 EV and SW480 shRNA, **-statistically difference between SW480-EV and1 -155 treated cells; # Statistical difference between SW620-EV and SW620 shRNA, ~statistical difference between SW620-EV and 1-155 treated cells. Statistical significance set as $p<0.05$, n=3, mean±S.D).

3.4.2.7 TG2 fosters *in vitro* cell motility in CRCs.

In **Section 3.4.1.3** RKO and SW480 cells displayed higher wound closure in a cell scratch assay, compared to SW620 cells. TG2 expression in the three CRCs showed a significant ($p<0.05$) effect on cell motility. In **Figure 3.4.17A** TG2 knockdown by shRNA in SW480 cells resulted in ~50% closure of the wound as against ~78 % wound closure in the control cells. More so TG2 inhibition by 1-155 (1μM) led to ~55% wound closure which was also significantly less relative to the control cells. The effect of TG2 inhibition and shRNA knockdown on cell motility in these cells were comparable although TG2 inhibited cells scored a higher wound closure percentage. In the SW620 cells a similar trend was observed with TG2 knockdown resulting in ~19% wound closure versus the ~38% wound closure observed in the control cells. TG2 inhibition further reduced cell motility to about 15% wound closure albeit non-significant when compared to the knockdown cells. As a proof of concept TG2 was ectopically expressed in low expressing TG2 celIs-RKO, resulting in a significantly increased cell motility with around 98 % wound closure in TG2 transduced cells compared to ~70% in the empty vector cells. When RKO cells made to ectopically express TG2 were treated with TG2 inhibitor 1-155 (1μM) cell motility was significantly less when compared to the control EV cells (**Figure 3.4.17C**). These data suggesst that TG2 expression was associated with significant increase in cell motility in all three CRCs.

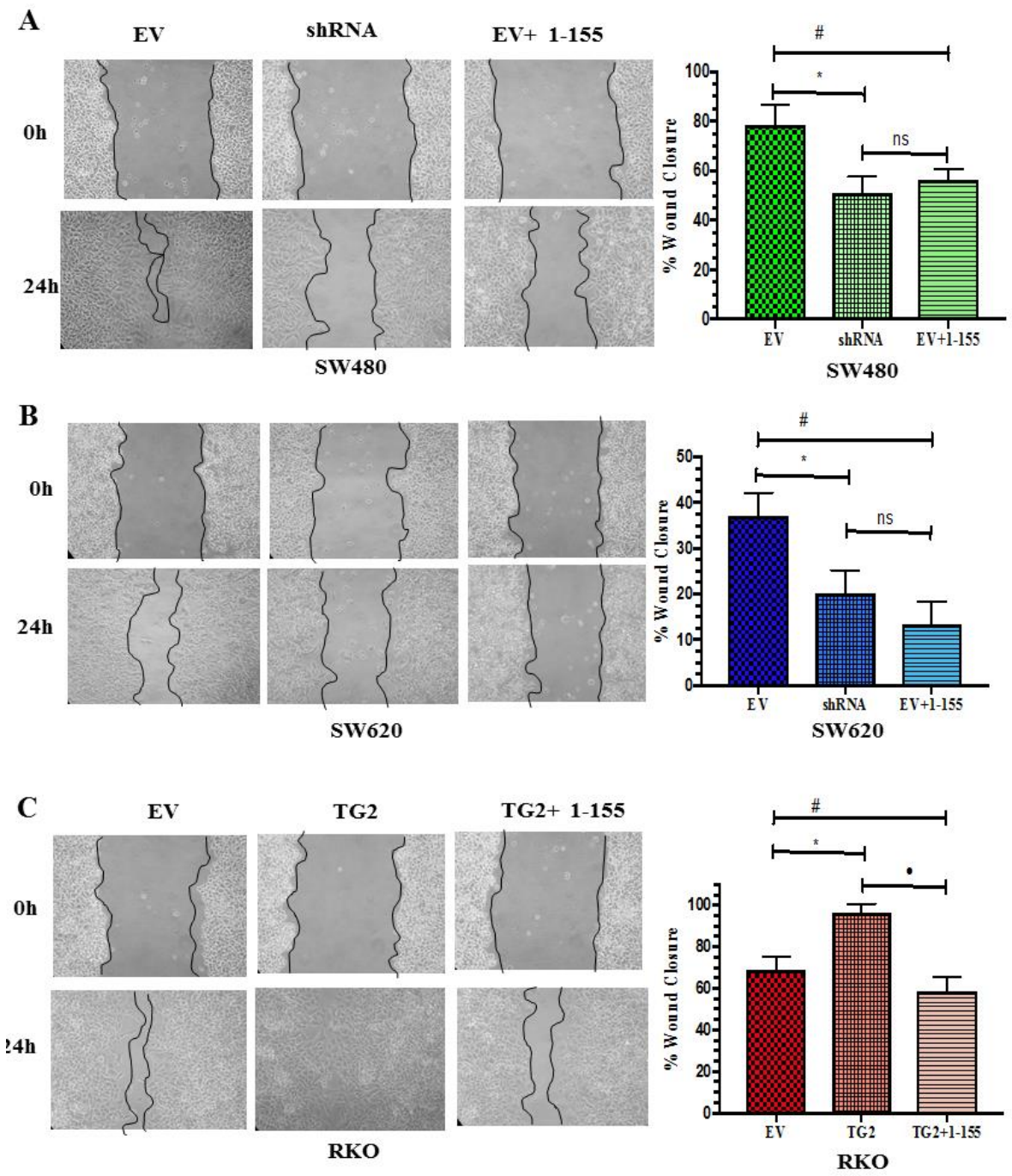


Figure 3.4.17 TG2 expression or activity impacts on cell motility of CRCs. Cell migration of cells assessed by monitoring wound closure was determined by a cell scratch assay monitored over 24h as described in (**Section 3.3.4**). Images are representative at 20x objective. For 1-155 (1μM) treated cells, cells were pre-treated with 1-155 for 24h prior to seeding cells and were further treated with 1-155 (1μM) after cell seeding for the duration of the experiment. EV, shRNA and TG2 viral treated cells were treated with DMSO, the control vehicle. (* ,# • indicates a statistical significant difference $p<0.05$, n=3; n.s not significant, mean±S.D).

3.4.2.8 TG2 may promote a drug resistance phenotype in CRCs

In **Section 3.4.1.5** metastatic cell line SW620 displayed significantly higher resistance to 5FU and doxorubicin- both chemotherapeutic treatment. Following that TG2 has played a role in EMT, cell motility and invasion, it was also important to assess the effect of TG2 manipulation on anticancer drug response. In **Figures 3.4.18 A and B**, TG2 knockdown in SW620 and SW480 significantly sensitised these cells to 5FU at varying doses (10-50)μg/ml. Following doxorubicin treatment, SW480 cells with TG2 shRNA were significantly sensitised to doxorubicin at lower doses (10-50 ng/ml). However, in SW620 cells, TG2 shRNA significantly sensitised the cells to doxorubicin at all doses. Conversely when TG2 was ectopically expressed in RKO cells drug response to both 5FU and doxorubicin was unchanged compared to the control cells (Figure 3.4.18 A and B**). Figure 3.4.18C** show that both lentiviral empty vector cells and lentiviral shRNA or TG2 target cells demonstrate similar cellular viability rates over 72h period, and the differences in viability following 5FU or Doxorubicin treatment is **Figures 3.4.18A and B** is attributable to TG2 knockdown effect.

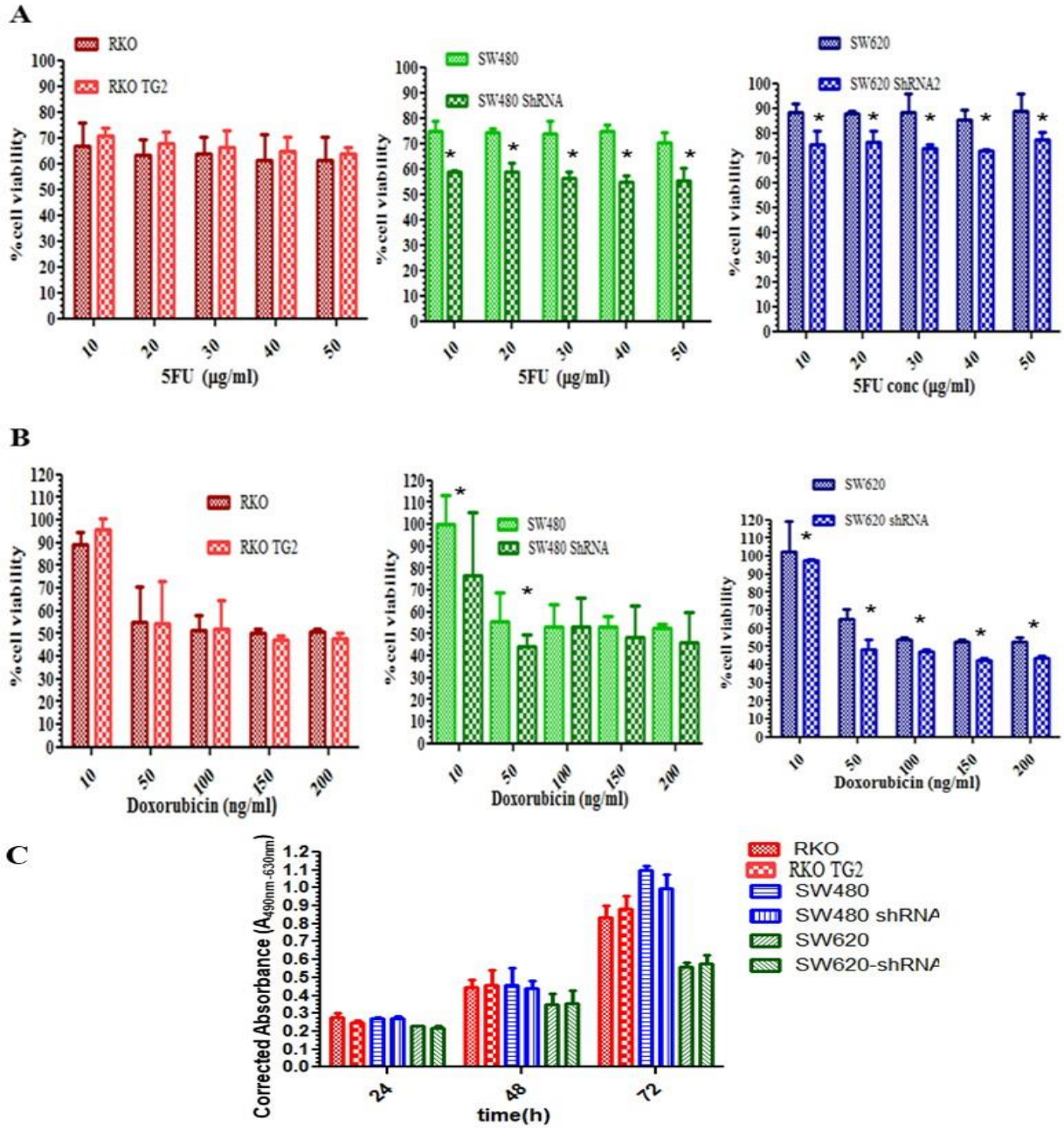


Figure 3.4.18 TG2 expression may modulate chemoresistance in CRCs. (**A**) Drug response of CRC with manipulated TG2 expression using shRNA or ectopic TG2 expression to 5FU treatment (10-50µg/ml) for 48h. (**B**)Drug response of CRCs with manipulated TG2 expression using shRNA or ectopic TG2 expression to doxorubicin treatment (10-200ng/ml) for 48h. 3000 cells/well were seeded in 96 well plate and treated with 5FU (25-200µg/ml) or doxorubicin (10-200ng/ml) 4h post seeding. DMSO was used as control vehicle for control cells. (**C)** Effect of shRNA, and TG2 viral particles on cell proliferation for 72h duration. Cells were maintained under cell culture conditions and cell viability assessed by XTT at the different incubation periods. Cell viability of treated cells was measured as a percentage of control cell cells. (*, $p<0.05$; n=3, mean±S.D). Corrected absorbance was obtained at (490-630)nm.

3.5 Discussion

Evaluating the molecular mechanisms and cellular processes involved in tumour progression is critical to understanding the disease and thus finding potential biomarkers or therapeutic targets. In view of this three phenotypic or biological processes have been highlighted. First is the ability of tumour cells to invade the biological membrane, and this serves to mimic the invasiveness of a tumour and its ability to intravasate into blood vessels as cancer progresses into a metastasis. By using a Boyden chamber Transwell insert with the pore size of 3µm and coated with collagen IV, the ability of cells to degrade collagen IV, and squeeze through the pores of the insert and migrate towards nutrient and growth factors present in the lower chamber was studied. The data clearly shows that the metastatic cell line possesses significantly higher invasive characteristics, compared to its isogenic primary tumour cell line SW480 and RKO cells. It is expected that metastatic SW620 cells and also higher graded cancer cells would demonstrate higher invasive potential. This is because as tumour cells progress they gain the necessary cellular machinery to invade and metastasise (Arvelo et al., 2016). Hewitt et al., (2002) reported increased *in vivo* invasive potential of SW620, compared to SW480 in immune compromised mice. Similarly, in a study performed by Young Sul et al., (1995), the invasive potential of colon metastatic tumour cell line Lovo was significantly higher than the primary cell line SW480. Epithelial to mesenchymal transition (EMT) is a cellular process that is purported to be used by cancer cells to progress from a primary tumour to a metastatic one (Kumar et al., 2010). This is because EMT promotes cell migration and invasion (Christiansen and Rajasekaran, 2006). The upregulation in the expression of mesenchymal cell markers by epithelial cells and loss of the typical cellular tight junction proteins indicates that an epithelial cell is undergoing EMT. It is for this reason that increased fibronectin which is required for extracellular matrix remodelling and cellular anchoring during cellular migration is increasingly expressed (Sung et al., 2015). More so vimentin, a type III intermediate filament, and a mesenchymal marker is also unregulated to enable reorganisation of the cellular cytoskeleton and architecture, to promote cell elongation, and the necessary tensile strength for lamellae contraction and relaxation as cells form lamellopodia and filopodia protrusions during migration and invasion (Mendez et al., 2010). Additionally, epithelial cells undergoing EMT may lose the cell-cell tight junction proteins

that anchor cells to each other and then to the matrix. One of such proteins is E-cadherin–a calcium regulated adherin transmembrane protein and zona occludin 1 (ZO 1) which serve as tight junction protein and aids adhesions of cells to one another and to the tissue. Such tight junction proteins are downregulated and replaced by N-cadherin which promotes fibroblastic phenotype in cancer cells, making them become more motile and invasive (Derycke and Bracke, 2004). In this current study, the metastatic cell line exhibited a mesenchymal like phenotype by significantly expressing markers of EMT, such as fibronectin, vimentin, and N-cadherin when compared to the two primary CRCs RKO and SW480. In addition, ZO1, an epithelial cell like protein, was significantly less expressed in SW620 when compared to SW480 and RKO, and would suggest that EMT is an upregulated cellular process during tumour progression in this cell model; and also fits with our finding on the invasive potential of SW620, and may explain the cellular changes in SW620 cells enabling migration through the pores of the Transwell inserts. More so the high vimentin expression in SW620 cells is consistent with the morphological differences observed between SW620 and the primary CRCs SW480 and RKO, with the former possessing a spindle like appearance which may be maintained by the inherently high expression of vimentin which maintains the cytoskeletal structure when compared to the cobble like morphology in SW480 and RKO with low vimentin expression. As part of characterising the three CRCs cells a adhesion and a scratch assay was performed. The results indicate that the primary cells possess significantly higher cellular adhesion on tissue culture plastic compared to the metastatic cell line and this phenotypic difference further suggests that RKO and SW480 have the adequate anchoring machinery to keep them within the ECM and tissues, further indicating an epithelial phenotype. Additionally, RKO and SW480 exhibited a higher wound closure capacity in a scratch assay, an indicator of a higher cell motility in these cells which is associated with increased attachment, since attachment is required for migration to occur an observation that has also been reported by Hewitt et al., (2000) who performed comparative phenotypic evaluation in SW480 andSW620 cells. More so, Kubens and Zanker (1998) also demonstrated that SW480 had increased cellular motility compared to SW620. The role of EMT in tumour progression has been widely studied in the literature and has been shown to be relevant in clinical studies where EMT is positively correlated with Duke's staging, tumour metastasis, and poorer 5-year survival rates in human colorectal cancer (Shioiri et al., 2006).

Drug resistance in cancer cells may also develop during the signalling processes of differentiation, which is essential for EMT (Housman et al., 2014). For example, the increased expression of transforming growth factor β (TGFβ) in colon cancer which is required for EMT, also serves as a survival signal for cancer cells against drugs (Bates and Mercurio, 2005).

A key characteristic of a progressed tumour is its ability to resist chemotherapy. Using 5FU a first line treatment for colorectal cancer we find in our current cell model that the metastatic cell line SW620 exhibited significant resistance to 5FU when compared to primary CRCs RKO and SW480 cells. A similar trend was observed using doxorubicin another chemotherapeutic agent. One possible mechanism for increased drug resistance in the SW620 cells may be due to the high inherent EMT occurring in the cells, another possible mechanistic characteristic could be the slower proliferation rate of the cells compared to the primary CRCs, as a lower proliferation rate may provide the cells with a more adaptive response. Some of the mechanism of drug resistance include increased xenobiotic efflux out of the cells (e.g., P-glycoprotein), mutation of the target of a particular toxin, and activation of alternate biological pathways instead of the one hit by a toxin (Hu and Fu 2012). Since the proliferation and doubling time of the SW620 cells is longer, it gives the cells more time to adapt to the xenobiotic in which case it can develop an effective efflux system, or activate an alternative biological pathway for survival. Consequences that may not be possible in a high dividing cellular population as the cells do not have sufficient time to adapt to the xenobiotic. Hewitt et al., (2000) also reported decreased susceptibility of SW620 cells to apoptosis-inducing factors such as TNF-α and serum deprivation compared to SW480 cells. The increased drug resistance of SW620 to 5FU compared to SW480 is similar to findings by (Bauer et al., 2012) who reported that the IC_{50} of SW620 was 2.7-folds higher than that for SW480.

By assessing in vitro cell invasion, EMT and drug resistance in RKO, SW480 and SW620 the current data suggest that the current cell line model exhibit a progression of CRC from a primary tumour to a metastatic one. This was also confirmed by detecting S100A4 (metastasin) (Boye and Maelandsmo, 2010, He et al., 2017) and αSMA- proteins associated with metastasis and tumour progression. And only in the metastatic cell line and not the two primary CRCs was metastatin and αSMA detected by Western blotting. This confirms reports that SW480 and SW620 provide and *in vitro* model for

studying cancer progression in CRCs. With this in hand, it was now important to identify the role of TG2 in the progression CRCs. The current data shows that TG2 expression increases with disease progression from primary to a lymph node metastatic tumour in microsatellite stable (MSS) CRC isogenic pair (SW480 and SW620). Similarly, TG2 expression increased between primary CRCs RKO and SW480. With the former hypothesised to be less invasive as indicated by the EMT markers and invasion assay studied. More so RKO possesses microsatellite instability (MSI) which is attributed with a less invasive phenotype compared to its corresponding MSS cancer (Rosty et al., 2014). These findings are similar to that reported by Miyoshi et al., (2010) who showed in clinical samples, using real time RT-PCR that TG2 expression was higher in CRC tissue compared to the corresponding normal tissue. Furthermore, they showed that TG2 expression correlated with tumour lymph node metastasis, lymphatic invasion, metastasis, and cancer disease staging. Clinical studies in various other cancer types also suggest that TG2 expression correlates with a poor 5-year survival rate (Hwang et al., 2008, Oh et al., 2011). In addition, a growing body of evidence suggests that EMT confers tumour cells with the ability to disseminate, invade surrounding tissues and eventually metastasise.

Having been able to establish the progression of CRCs in the current cell model, the role of TG2 in the cellular processes highlighted above was investigated. EMT is a major pathway that governs cell behaviour during cancer progression and is the initial step in tumour metastasis and has been linked to drug resistance (Cao et al., 2008, Mehta et al., 2010). Current data obtained suggest that TG2 may influence cancer progression in CRCs by facilitating EMT. Mesenchymal cell markers were up regulated in cells either constitutively expressing high levels of TG2 (SW620 cells) or ectopically expressing TG2 (RKO cells transduced with TG2) as confirmed by Western blotting and RT-PCR. While knockdown of TG2 using shRNA in SW480 and SW620 cells decreased the protein expression of these markers and concomitantly increased expression of the epithelial marker ZO-1 in SW620 and SW480 cells. Furthermore, pharmacological inhibition of TG2 by the highly specific TG2 irreversible, cell permeable peptidomimetic inhibitor 1-155 was also efficient in attenuating EMT. This finding proposes TG2 as a potential therapeutic target in CRC treatment using site specific TG2 small molecule inhibitors. The result also suggests that TG2 may play an upstream role in mediating EMT since TG2 expression correlates with the expression of the EMT transcription factors Slug and Twist, in SW620

cell and that inhibition of TG2 attenuated the expression of these transcription factors. Similar studies by (Kumar et al., 2010) also showed that in MCF10A and MCF12A mammary epithelial cancer cells, TG2 regulates the expression of EMT transcriptional regulators including Snail, Twist, Zeb1, and Zeb 2. TG2 also significantly affected the ability of cells to invade *in vitro*. Cancer invasion is an important element of the disease. In the isogenic pair SW480 and SW620 TG2 knockdown reduced the potential for invasion, similar reports have been made in breast cancer cells (Kumar et al., 2010). However, findings by Cellura et al., (2015) in the same isogenic pair SW480 and SW620 cells, suggests that TG2 correlates with reduced invasion. This disparity in findings could be due to the multifunctional and pleiotropic roles of TG2 as similar contradictory roles have been found in pancreatic ductal adenocarcinoma cells (Fok and Mehta 2007) prostate cancer cells-and in the human bladder carcinoma J82 cells (Yoo et al., 2012, Kotsakis and Griffin, 2007). This may also explain the non-significant impact of ectopic TG2 expression on invasion in the RKO cells, or as RKO appears to be significantly less progressive compared to SW480 and RKO, the necessary mutations or cellular changes pre-exquisite for TG2 induced alteration in invasion has not yet occurred. However, the effect of TG2 on cellular invasion in SW480 and SW620 cells fits with the earlier findings that show TG2 downregulation affects aspects of EMT such as reduced expression of vimentin, which plays a role in cell contraction necessary for invasion.

Cellular motility was also significantly increased in TG2 expressing cells. TG2 has been reported to be a binding partner or a modifier of many ECM proteins. Of relevance is TG2 interaction with integrin a cell adhesion molecule and the subsequent activation of focal adhesion kinase (Eckert et al., 2014), which supports cell attachment. During cell migration, the cell requires integrin interaction with FAK and the actin cytoskeleton to enable anchorage as the cell toggles through the ECM by a cycle of protruding the lamellipodia and filopodia, and this may explain the increased migration observed in TG2 expressing cells. In addition, TG2 mediated cellular motility in an *in vitro* cell scratch assay has been reported in breast cancer cell (Kumar et al., 2010, Wang and Griffin, 2013), epidermal squamous cancer cells (Fisher et al., 2015a). The increased cellular motility suggests an adoption of a mesenchymal-like cell and the progression of EMT.

EMT has also been reported to influence cancer response to chemotherapy, as EMT transcription factors have also been reported to regulate cell survivor genes, for instance ZEB2 an EMT transcription factor has been suggested to prevent ATM/ATR activation in response to a genotoxic stress in bladder cancer patients treated with radiotherapy (Sayan et al., 2009), furthermore Twist 1 has also been shown to trigger chemoresistance in an EMT-dependent manner through its ability to induce *AKT2* expression and to differently modulate the ratio between pro- and anti-apoptotic members of the BCL-2 family. SNAIL1 and SNAIL2 has been shown to interfere with p53-target gene activation in renal epithelial and hematopoietic precursor cells (Ansieau et al., 2014). In this regard, TG2 silencing in SW480 and SW620 cells sensitised these cells to chemotherapeutic drugs 5FU and doxorubicin. This agrees with reports shown in breast cancer (Kumar et al., 2010), ovarian cancer (Cao et al., 2008) renal cancer (Ku et al., 2014), lung cancer cells (Park et al., 2010) and very recently in colon cancer cells (Carbone et al., 2017) where TG2 enzymatic (Park et al., 2010, Ku et al., 2014) and non-enzymatic (Kumar et al., 2012, Carbone et al., 2017) functions have been associated with drug resistance in cancer cells. However, in the RKO cells over expressing TG2 did not increase resistance to 5FU and doxorubicin. 5 FU exerts its antitumor activity by creating thymidylate deficiency and creating imbalances in the nucleotide pool impairing DNA replication, transcription, and repair, subsequently leading to cell death (Wheeler et al., 2000). However, MSI tumours are resistant to 5FU because of the sporadic mutations in mismatch repair (MMR) genes that characterise MSIs. The loss of MMR proteins causes errors in repetitive DNA sequences, resulting from loss of scanning and recognizing errors during DNA replication (Gatalica et al., 2016). This loss of detection of mismatched and unpaired bases may be the primary source of resistance in RKO cells, which makes them tolerant to DNA damage, a cellular process that may supersede TG2's role in a possibly EMT induced drug resistance. Again, as observed in the invasion experiment, the insignificant effect of TG2 on the invasion capacity of the TG2 transduced RKO cells, and the nonsignificant effect of TG2 on doxorubicin resistance could be due to the need of further oncogenic mutations to enable TG2 and EMT mediate of drug resistance and invasion.

3.6 Conclusion

Conclusively by assessing certain hallmarks of cancer cells, we show that our current cell model shows a progression of colorectal cancer and that TG2 expression correlates with this progression. In addition, TG2 plays a significant role by inducing EMT, cell invasion, cell motility and drug resistance. With cellular TG2 inhibition ameliorating most of these hallmarks.

Chapter 4

TG2 Induces EMT via multiple cellular signalling mechanisms in colorectal cancer cells

4.0 TG2 Induces EMT via multiple cellular signalling mechanisms in colorectal cancer cells

4.1 INTRODUCTION

EMT confers cancer cells with the ability to detach and evade basement membranes, and adapt survival behaviour to evade immune responses from the host, circumvent stressful conditions such as hypoxia, nutrient factor deprivation and altered cell adhesion (Thorn et al., 2011). These processes are essential for migration and metastasis, and may help dedifferentiate cancer cells into cancer stem cells (Eckert et al., 2015). Furthermore, EMT may promote drug resistance in cancer cells, a role that may be performed by increased cellular expression of transcription factors of EMT such as Slug, Twist, Zeb, which facilitate the transcription of cell survival and anti-apoptotic proteins (Sayan et al., 2009, Ansieau et al., 2014). The transcription factors of EMT play a significant role in repressing the expression of cell tight junction proteins such as zona occluding 1 (ZO 1) and E-cadherin. On the other hand the EMT transcription factors induce expression of mesenchymal-like protein markers such as cell surface proteins (N cadherin), cytoskeletal proteins (α-smooth muscle actin, vimentin) and extracellular proteins (fibronectin, collagen) (Scanlon et al., 2013).

The loss of E cadherin and other cell tight junction proteins during EMT facilitate epithelial cells to lose their apical polarity. Mendez et al., (2010) also suggest that increased expression of intermediate filament protein vimentin also enable cell motility with an increase in contractile ability. Remodelling of ECM around the cell undergoing EMT enable cell anchoring during migration. In addition, MMPs are increasingly expressed during EMT to promote degradation of the basement membrane (Gialeli et al., 2011).

In colorectal cancer cells EMT has been associated with poorer survival outcomes (Shioiri et al., 2006). TGFβ1, NFκB and β-Catenin signalling pathways have been established as key mediators of EMT in physiology and pathologies (Novellasdemunt et al., 2015). The regulation of these signalling pathways is thus crucial for the maintenance of EMT. In colorectal cancer cells TGFβ1 and β-catenin signalling pathways are two commonly dysregulated pathways allowing for tumour progression, and facilitating EMT. The multifunctional enzyme Tissue transglutaminase (TG2) has been reported to mediate several aspects of cancer cell behaviour, including motility, invasion, growth, and survival (Wang and Griffin

2013; Kotsakis et al., 2011; Verderio et al., 1999) by a possible cross talk between TG2 and three critical pathways in EMT, i.e. TGFβ1, WNT, β-catenin and Nuclear factor kappa - light chain enhancer of activated B (NFκB) (Mann et al., 2006; Cao et al., 2012). The exact mechanism through which TG2 may induce EMT is varied (and may involve both extracellular and intracellular roles). In breast cancer cells it has been suggested that TG2 mediated NFκB nuclear translocation facilitates EMT, and via structural mutations of TG2 various studies suggest TG2's GTP binding function is essential for EMT, while other studies in breast cancer suggest that the fibronectin binding domain of TG2 may be required for driving EMT (Herman et al., 2006, Cao et al., 2008). TG2 activity has also been reported to be necessary for EMT in certain cells, cross-linking of the inhibitor of kappa B alpha (IκBα), results in dissociation of the NF-κB/IκBα complex, and the constitutive activation of NFκB, and its subsequent downstream target genes involved in promoting cell growth, survival and invasion (Chhabra et al., 2009). Wang and Griffin (2013) have shown that the extracellular crosslinking activity of TG2 is involved in the S100A4-related mammary cell migration via the syndecan-4/PKCα-dependent signalling.

In the previous chapter, TG2 expression was shown to correlate with EMT markers and also associate with the expression of transcription factors of EMT, suggesting a potential upstream role for TG2 in the regulation of EMT in colorectal cancer cells. This Chapter, therefore, explores the potential role of TG2 in mediating the cell signalling processes associated with EMT.

4.2 Aims and Objectives

The aim of this chapter is to explore the potential role of TG2 in mediating TGFβ1 and Wnt/β-catenin signalling, two signalling pathways that are often mutated in colorectal cancer, and that are known to drive EMT.

Objectives

1. Investigate the role of TGFβ1 in inducing EMT and TG2 in the current colorectal cancer cell model.
2. Explore the crosstalk between TG2 and TGFβ1 in colorectal cancer cells.
3. Investigate the role of TG2 in the Wnt/β-catenin signalling pathway.

4.3 METHODS

4.3.1 Dot Blotting for Proteins

Cells were collected and counted as described in **Section 2.2.1.2-2.2.1.3** and seeded $2.5X10^5$ in complete growth media for 4 h in 24well plates. After which the complete growth media was replaced with ITS (Sigma Aldrich) supplemented serum-free cell culture media, cells were incubated for 16-18h and the cell culture media was collected and transferred onto nitrocellulose membrane using a vacuum dot blotter. The dot blotted membrane was used for detection of proteins as described in **Section 2.2.8** by blocking the membrane in 5% w/v non-solid fat milk in Tris-buffered saline Tween (pH 7.4) and incubated with primary and HRP conjugated secondary antibody and detected by chemiluminescence.

4.3.2 Detection of secreted TGFβ1 by ELISA

Secreted TGFβ1 was measured by ELISA following the manufacturer's instructions (Novex™, Fisher, Hempstead, UK). Briefly, CRCs were seeded into 24-well plates and allowed to adhere for 4h in complete medium. Complete media was replaced with growth factor-free ITS replacement medium, and the cells incubated for 16 h and the culture medium then collected for analysis. Similarly, a cell-free ITS replacement medium incubated for 16h was collected and served as a cell medium control. Cell culture medium collected was precleared by centrifugation at 1000 X g for 10min, after which TGFβ1 was released from its latent form by addition of 50µl of extraction solution to 250µl of cell culture media followed by vortexing and incubation for 30min on ice. After this extraction step 250µl of diluent buffer was added to the TGFβ1 extraction mix to obtain a 2.2-fold dilution of the original culture medium volume. Standard TGFβ1 was prepared by reconstituting the TGFβ1 standard with diluent buffer, and serially diluted from 2000pg/ml to 0pg/ml. The diluent buffer was used as the zero reading. 200µL of standards, sample and control media (after extraction) were added to manufacturer pre-coated microtiter wells. To this 50µL of biotinylated anti-TGF-β1 (Biotin Conjugate) solution was added to each well and the side of the plate was tapped gently to mix the solutions thoroughly. A set of wells were kept empty to serve as chromogen blank(s). Plates were covered and incubated for 3 h at room temperature. After incubation, the contents of the plate were discarded and washed four times with wash buffer, and then aspirated. Following the wash procedure, 100µL Streptavidin-HRP working

solution was added to each well except the chromogen blank, the plate covered and incubated in the dark for 30min at room temperature. After incubation, the plate content was discarded and washed 4 times with the wash buffer and aspirated. 100µL of stabilized chromogen was added into each well including the set of empty wells which served as the chromogen blank. The plates were incubated for 30min in the dark after which 100µL of stop solution was added to each well including the chromogen blank. The absorbance of each well was obtained at 450 nm having first blanked the plate reader against a chromogen blank. The absorbance of the standard TGFβ1 is used to plot a standard curve and the concentration of samples determined by the standard curve and multiplied by the 2.2 dilution factor.

4.3.3 Cell Surface TG2 activity assay

2 x 10^4 cells were counted and resuspended in 400µl of medium containing 0.1mM biotin cadaverine as described by Jones et al., (2006). The cell mixture was added to a 96-well plate pre-coated with 50µl of 5µg/ml fibronectin and incubated at 37ºC for 2 h. The reaction was stopped by washing the well with 2mM EDTA in PBS, pH 7.4. 100µl of 0.1%(w/v) sodium deoxycholate in 2mM EDTA in PBS, pH 7.4 was added to each well and incubated on a slow shaker for 10 min at room temperature, after which the sodium deoxycholate solution was discarded and the wells washed three times with 50mM Tris-Cl, pH 7.4, followed by addition of blocking solution-heat inactivated 3% (w/v) Bovine Serum Albumin, BSA, in 50mM Tris-Cl, pH 7.4 at 37ºC for 30 min. The wells were washed and the immunoassay detecting the incorporation of biotin cadaverine by cell surface TG2 into fibronectin was determined using a 1:1000 dilution of Extravidin® peroxidase conjugate incubated at 37ºC for 1h. After washing the wells with 50mM Tris-Cl, pH 7.4, 100µl of OPD tablets (1:1) dissolved in 20ml of distilled water the reaction was stopped using 50µl of HCl (3M) and the absorbance determined at 490nm.

4.3.4 Cytosolic and Nuclear Extraction of Proteins

Nuclear and cytosolic fractions were isolated using Thermo scientific ™ Subcellular Protein Fractionation Kit for Cultured Cells (Rockford IL, USA). Equal amounts of protein were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Briefly, cells were collected in ice-cold PBS using a cell scraper and cellular components sequentially extracted. The cell suspension in ice-cold PBS was centrifuged at 500 x g for 5 min, and the supernatant discarded. An aliquot of the packed

cell was retained and lysed using whole cell lysis buffer as described in Chapter 2, and this served as input. To 20µl of packed cell volume, 200µl of ice-cold cytoplasmic extraction buffer (CEB) containing freshly added protease inhibitor was added and incubated on ice for 10min. Following incubation, the cell extracts were centrifuged at 500 x g for 5 min at 4^0C. The supernatant was retained and contained the cytoplasmic contents. The pellet was washed 2 times with ice-cold PBS and further extracted by addition of 200µl of membrane extraction buffer (MEB) containing protease inhibitors, vortexed and incubated on ice for 10min. After this incubation, the cell extracts were centrifuged at 3000 x g for 10min at 4^0C. The supernatant contained the membranous fraction. The pellet was washed 2 times with ice-cold PBS and further extracted by addition of 100µl of ice-cold nuclear extraction buffer (NEB) containing protease inhibitors, vortexed for 15 seconds, and incubated at 4°C for 30 minutes with gentle mixing. Following incubation, the cell extracts were centrifuged at 5000 x g for 5 min at 4^0C. The supernatant was retained and served as the nuclear fraction. The cytosolic and nuclear extracts collected were then used for protein separation and Western blotting.

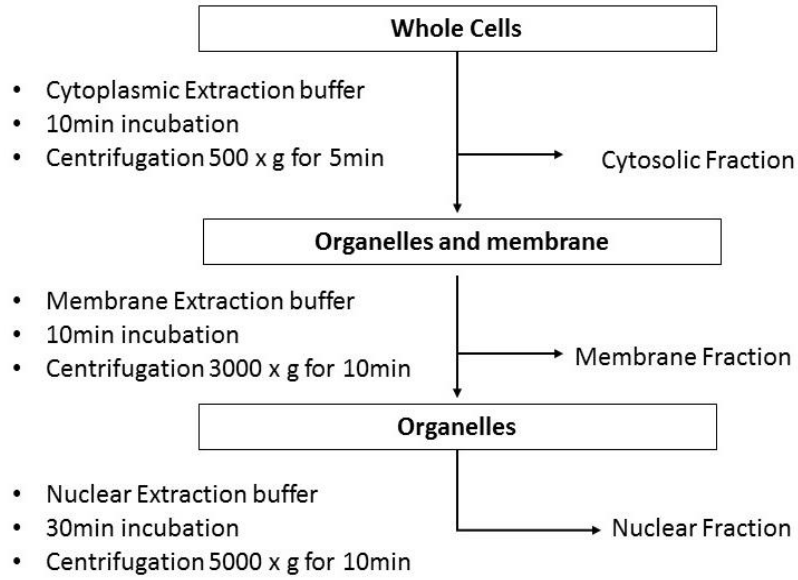


Figure 4.3.1 Flow chart of subcellular Fractionation. Whole cells are lysed and the subcellular components are extracted by exploiting varying buffers, incubation periods and centrifugation speeds.

4.3.5 Co-immunoprecipitation (co-IP)

Following appropriate treatment, CRC cells were lysed in the co-IP buffer [0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1% (v/v) protein inhibitor mixture] containing 500 µM TG2 inhibitor R283 as described previously (Kotsakis et al., 2011). Cell lysates (600 µg) were pre-cleared by incubating with 50 µl of non-specific protein A or G-Sepharose bead slurry (GE Healthcare, Buckinghamshire, UK) at 4°C for 90 min. The pre-cleared cell lysates were then incubation with 0.5 mg of primary antibody for 90 min at 4°C with gentle shaking. To the antibody-whole cell lysate mixture, 50 µl of Protein A or G Sepharose bead slurry were added and this slurry was further incubated overnight at 4°C. After this incubation, the Sepharose beads were collected by centrifugation at 300 x g for 5min at 4°C. The Sepharose beads were then washed twice with PBS, pH7.4, at 300 x g for 5min at 4°C. The immunocomplex on the beads was collected in 30 µl Laemmli buffer by boiling for 5min, centrifuging (300 x g for 5min) the slurry and retaining the supernatant for use in SDS-PAGE/ Western blotting analysis.

4.3.6 Measurement of biotin-X-cadaverine incorporation into cellular TG2 protein substrates

Cellular proteins acting as substrates for endogenous TG2-catalysed primary amine incorporation was investigated by incorporation of the cell permeable biotin-X-cadaverine**.** Prior to incubating the cells with 1 mM biotin-X-cadaverine (Life Technologies, Eugene, OR, USA) in complete growth medium overnight, the cells were treated with TG2 inhibitors 1-155 or R281 for 24h, control cells were treated with the treatment vehicle (DMSO). Following biotinylation of cellular proteins, 50µg of the whole cell extracts (**Section 2.2.3**) were then subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The biotinylated proteins were probed using ExtrAvidin HRP and visualized by enhanced chemiluminescence (ECL) (Almami et al., 2014).

4.4 RESULTS

4.4.1 TGFβ1 induces TG2 and EMT in CRCs via both canonical and non-canonical means

4.4.1.1TGFβ1 induces TG2 in Primary CRCs RKO and SW480

To assess the role of TGFβ1 in the CRCs, the cells were first characterised for signalling proteins associated with TGFβ1. In **Figure 4.4.1** Western blot analysis shows that RKO, SW480, and SW620 cells all express TGFβ1-receptor I and II and the downstream signalling molecules Smad2/3, with TGFβ1 receptor II being expressed over 2 fold higher in SW480 cells compared to RKO and SW620, although the expression of TGFβ1-receptor I and Smad 2/3 being relatively similar across the three cell lines. Several reports have shown that TGFβ1 induces TG2 (Wang et al., 2017) therefore in these three colorectal cells the effect of TGFβ1 on TG2 expression was determined by treatment with or without 2.5ng/ml TGFβ1, and with or without TGFβ1 neutralising antibody for 48h. In **Figure 4.4.2** TG2 expression was significantly increased by TGFβ1 treatment in the primary CRCs RKO and SW480 cells, with TGFβ1 inducing more than double the normal TG2 expression in RKO cells and also significantly increasing TG2 expression by over 60% in SW480 cells.

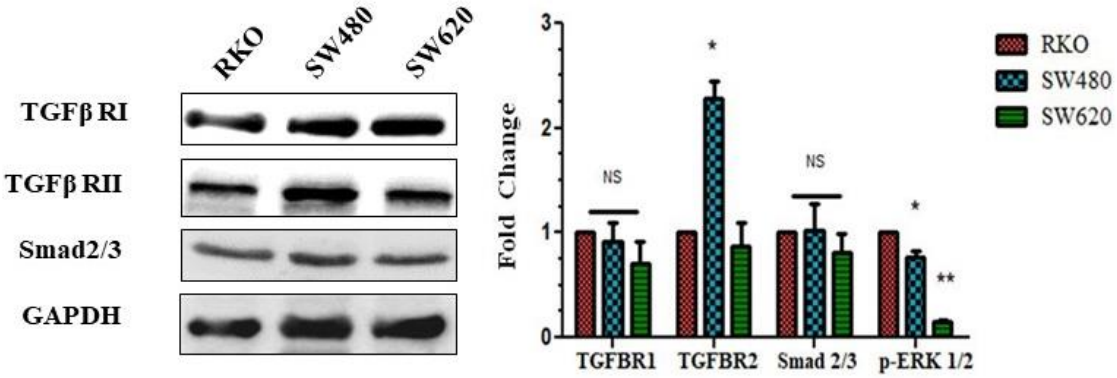


Figure 4.4.1 CRCs express elements of the TGFβ1signalling pathway. Representative Western blot showing the expression of TGFβ receptor (TGFβR) I and TGFβRII in RKO, SW480, and SW620 whole cell lysates. Bar chart shows the mean relative densitometry ratios of protein expression normalised against GAPDH (n=3; mean±S.D) *-statistically significant from RKO cells, **-statistically significant from RKO andSW480 cells. NS-not significant.

This was observed with a corresponding significant increase in the phosphorylation of Smad 3 by more than a 100% shown in both cell lines. The induction of TG2 by TGFβ1 treatment was also confirmed by treating the cells with TGFβ1 neutralising antibody which resulted in a significant reduction in TG2 expression in RKO and SW480 cells. However, neither TGFβ1 treatment nor TGFβ1 neutralizing antibody had any significant effect on TG2 expression in SW620 cells.

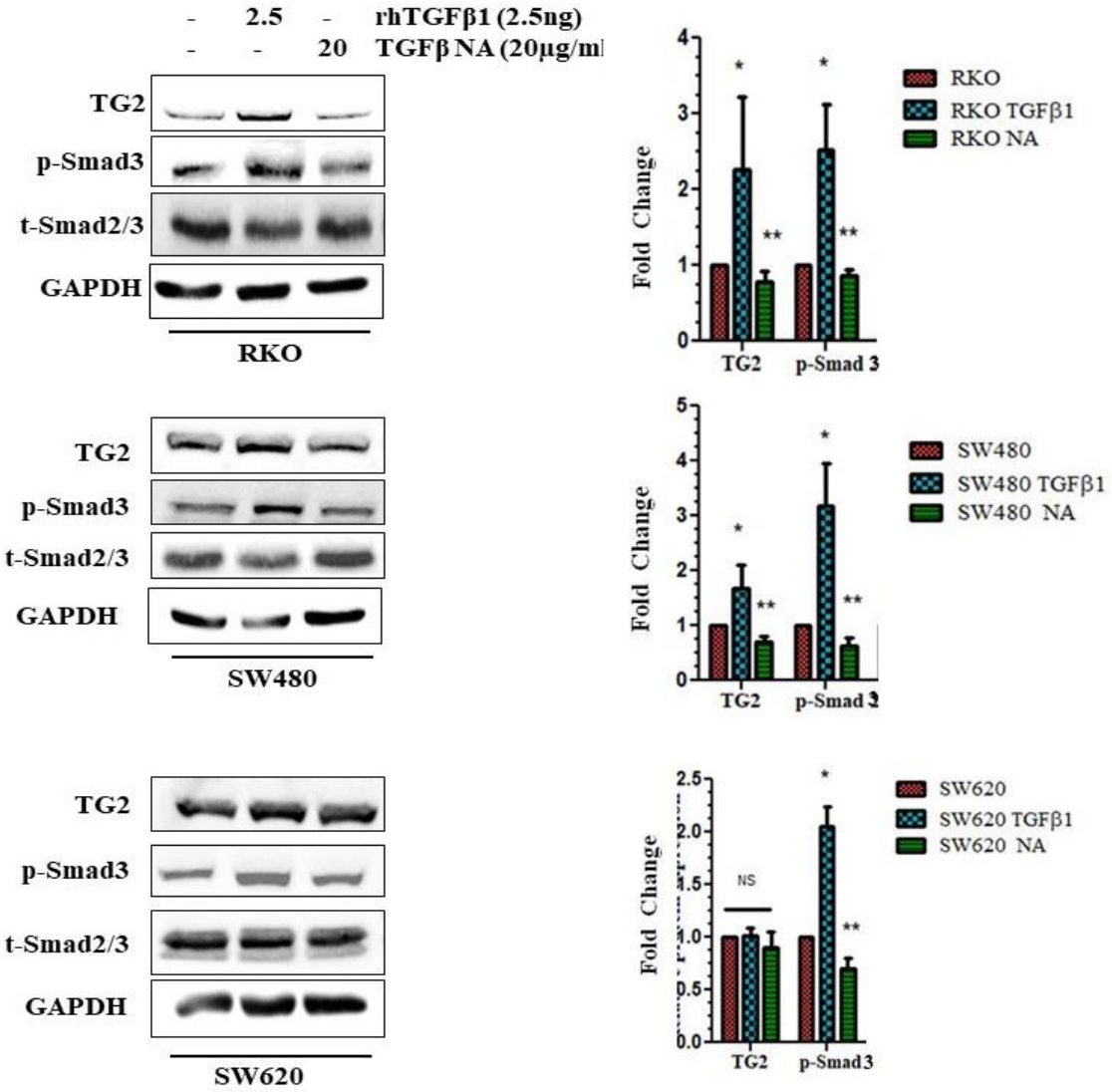


Figure 4.4.2 TGFβ1 induces TG2 expression in primary CRCs RKO and SW480. Representative Western blot of whole cell lysates of CRCs RKO, SW480, and SW620. Cells showing expression of TG2 and activated p-smad 3 against t-smad 2/3 following TGFβ1 treatment or treatment with TGFβ1 neutralising antibody. CRCs were treated with human recombinant TGFβ1 (2.5 ng/ml) and TGFβ1 neutralising antibody (NA) (20 µg/ml) for 48 h. Bar chart shows relative densitometry ratios of protein expression normalised against GAPDH or normalisation for p-smad 2 using total t-Smad 2/3 after normalising for protein loading. (n=3; mean±S.D) *-statistically significant from control cells, **-statistically significant from TGFβ1 (2.5ng/ml) cells, NS-not significant. NA- TGFβ1neutralising antibody (20µg/ml).

4.4.1.2 TGFβ1 induces TG2 in a dose-dependent fashion

Following increased TG2 expression in TGFβ1 treated primary cancer cells RKO and SW480, the extent to which TGFβ1 could induce TG2 expression was assessed by Western blotting following TGFβ1 dose treatment (0-10ng/ml) in CRCs SW480 and RKO (Figure **4.3**) Following treatment with TGFβ1 at varying doses in RKO cells, TG2 expression increases with increased TGFβ1 treatment from 2.5ng/ml to 10ng/ml. However in SW480 cells 1ng/ml TGFβ1 was sufficient to induce increased expression of TG2, and may be due to increased expression of TGFβ receptor II in these cells, suggesting that the SW480 cells may be more sensitive to TGFβ1 compared to RKO. **Figure 4.4.3** shows that TG2 expression is increasingly induced with increase in TGFβ1 concentration (1.0-5.0 ng/ml) over 48h in SW480 and RKO cells.

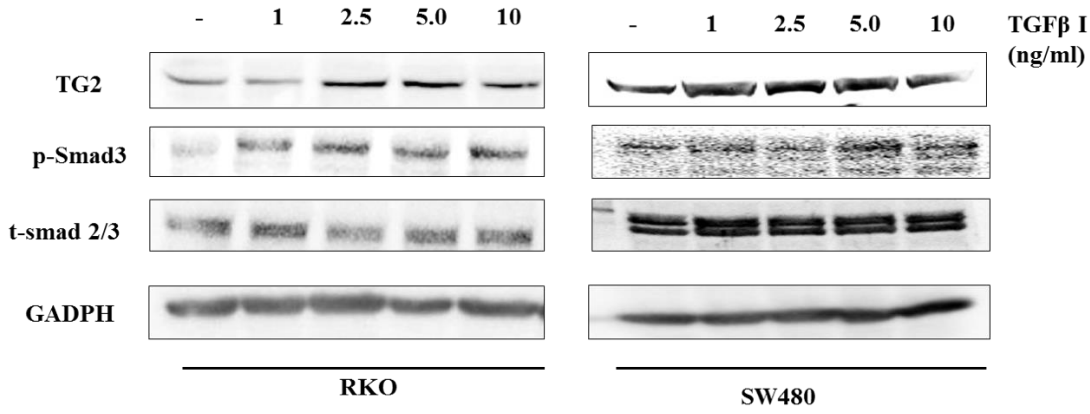


Figure 4.4.3 Increasing TGFβ1 concentration increase TG2 expression. Representative Western blots of whole cell lysates from CRCs RKO, SW480. Cells showing expression of TG2 and activated p-Smad against t-Smad following TGFβ1 treatment at varying concentrations (0-10)ng/ml for 48 h (n=2).

4.4.1.3 TGFβ1 induces EMT in CRCs following Smad activation

Multiple reports suggest that TGFβ1 is a potent inducer of EMT in cancer cells and given that TGFβ1 also induces TG2 in RKO and SW480 and that TG2 correlates with expression of EMT markers and a phenotype associated with EMT (**Figure 3.4.9**). It was, therefore, important to identify the role of TGFβ1 in EMT in these cells. When CRCs were treated with TGFβ1, EMT was induced in the RKO and SW480 cells with increased levels of vimentin and fibronectin and decreased ZO-1 (**Figure 4.4.4A**). In the RKO cells TGFβ1 significantly induced over 2 fold expression of vimentin and fibronectin with at least 50% reduction in the expression of tight junction epithelial cell marker ZO-1. Moreover, TGFβ1 neutralising antibody treatment reduced expression of these EMT markers. This similar trend was observed in SW480 cells with TGFβ1 treatment resulting in 16% increase in vimentin and a significant (~100%)increase in fibronectin compared to the untreated control, this effect was ablated by TGFβ1 neutralising antibody treatment which resulted in a significant reduction in the expression of EMT markers, with at least 20% reduction in vimentin expression and approximately 50% loss in expression of fibronectin. TGFβ1 neutralising antibody also resulted in 70% increase in the expression of epithelial tight junction marker ZO-1 in these cells. The effect of TGFβ1 on RKO and SW480 cells was also confirmed by the appearance of a fibroblast-like phenotype in TGFβ1 treated RKO and SW480 cells (**Figure 4.4.4B**). However in the SW620 cells neither TGFβ1nor its neutralising antibody had any significant impact on EMT in these cells.

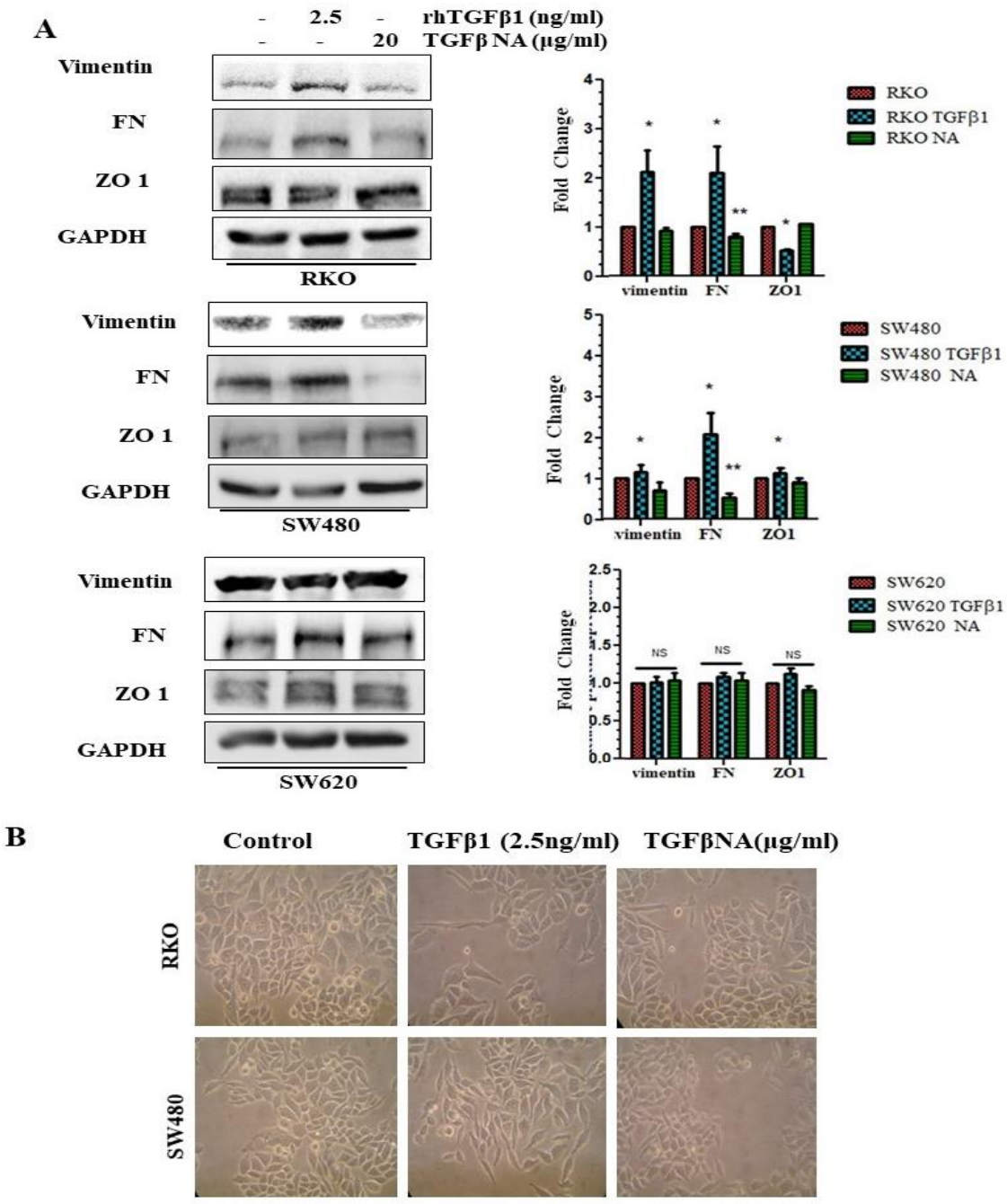


Figure 4.4.4 TGFβ1 induces EMT in primary CRCs RKO and SW480. (A) Representative Western blots of whole cell lysates of CRCs RKO, SW480, and SW620. Cells showing presence or absence of EMT markers following TGFβ1 treatment or treatment with TGFβ1 neutralising antibody. **(B)** Representative images of cell morphology of CRCs RKO and SW480 treated with rhTGFβ1 (2.5ng/ml) or TGFβ1 neutralising antibody PAN (20µg/ml). Equal numbers of cells were seeded and 48 h after treatment images were taken at x20 objective using a phase contrast microscope (n=3). Bar chart shows relative densitometry ratios of protein expression normalised against GAPDH. (n=3; mean±S.D) *-statistically significant from control cells, **-statistically significant from TGFβ1 (2.5ng/ml) cells, NS-not significant. NA- TGFβ1neutralising antibody (20µg/ml).

4.4.1.2 TGFβ1has no effect on EMT in SW480 cells transduced with TG2

Since TGFβ1 was not able to further induce expression of TG2 and EMT in SW620 cells, an attempt was made to ectopically express TG2 in SW480 to determine if further challenging SW480 cells with TG2 would increase EMT at a level similar to the high TG2 expressing SW620 cells. In **Figure 4.4.5A** transduction of TG2 in SW480 cells shows a slight but not significant impact on EMT compared to the control EV cells. Over a 40-fold increase in TG2 expression in SW480 cells by lentiviral transduction resulted in an approx. 1.2-fold increase in fibronectin when compared to EV cells. Furthermore treatment of the TG2 transduced SW480 with TGFβ1 at 2.5ng/ml or TGFβ1 neutralising antibody did not significantly affect the relative expression of EMT markers vimentin, fibronectin, and tight junction protein ZO-1, although the neutralising antibody increased Z0-1 expression by approx. 1.39-fold compared to the nontreated cells, this trend was also observed in the TGFβ1 treated cells with 1.10-fold increased expression of ZO-1 compared to untreated cells (**Figure 4.4.5B).**

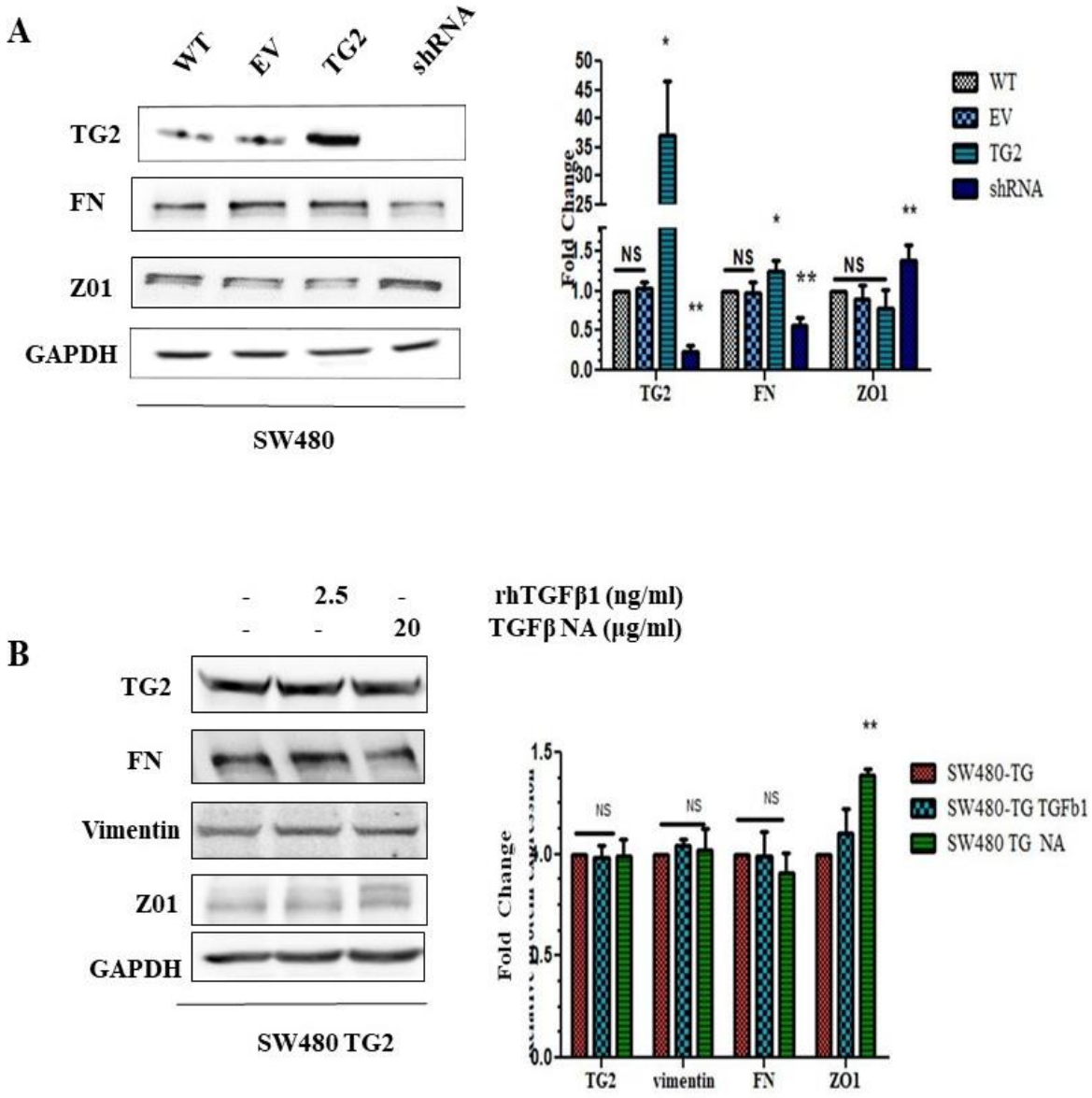


Figure 4.4.5. TGFβ1 does not significantly affect EMT in SW480 cells with ectopic TG2 expression. (A) Representative Western blot of TG2 and EMT markers (fibronectin and ZO1) in whole cell lysates from wt SW480 cells, SW480 cells transduced with empty vector (EV), human TG2 (TG2), and shRNA targeting TG2 (shRNA).(n=3; mean±S.D). *-statistically significant from wildtype cell (wt), **-statistically significant from TG2 transduced cells (**B)** Representative Western blot of whole cell lysates from SW480 cells transduced with TG2 (SW480 TG2) showing expression of TG2 and EMT markers (Fibronectin, vimentin, and ZO1) with or without treatment with TGFβ1 or TGFβ1 neutralising antibody. CRCs were treated with human recombinant TGFβ1 (2.5 ng/ml) and TGFβ neutralising antibody (NA) (20 µg/ml) for 48 h. (n=3; mean±S.D), **-statistically significant from TGFβ1 (2.5ng/ml) cells, NS-not significant. NA- TGFβ1neutralising antibody (20µg/ml). Bar charts shows the mean relative densitometry ratios of protein expression normalised against GAPDH.

4.4.2 TGFβ1 induces EMT in primary CRCs RKO and SW480 by non-canonical signalling involving extracellular signal-regulated kinase (ERK)

4.4.2.1 TGFβ1 activates extracellular signal kinase (ERK) in Primary CRCs

The data obtained for TGFβ1 with RKO and SW480 cells suggests a link between TGFβ1 signalling and TG2 expression. However, since SW480 is a Smad 4 null cell line but RKO expresses wild-type Smad 4 (Volmer et al., 2004; Chow et al., 2008), an attempt was made to identify a potential Smad 4 independent pathway in the SW480 cells which may also play a role in RKO cells is involved in inducing TG2 expression and EMT. One of such pathways is the mitogen-activated protein kinase (MAPK) pathway, where TGFβ1 noncanonically signals through its receptors which may possess intrinsic tyrosine kinase activity that phosphorylates ShcA an upstream regulator of ERK 1/2 (Lee et al., 2007). TGFβ1 induced EMT has been reported to follow the canonical pathway by activation of Smad signalling response or non-canonically via ERK 1/2 Lee et al., 2007). In **Figure 4.4.6A** CRCs-RKO, SW480 and SW620 were characterised for some members of the MAPK pathway including activated and total ERK 1/2 expression. **Figure 4.4.6A** shows that metastatic cell line SW620 exhibits significantly low basal expression of active ERK, with at least a 4-fold less level of detectable phosphorylated ERK 1/2 compared to SW480 and 5-fold less compared to RKO cells. In **Figure 4.4.6B** the ability of TGFβ1 to activate ERK 1/2 in RKO and SW480 cell lines was investigated, with ERK phosphorylation significantly increased above background between 30-120 min following TGFβ1stmulation. SW480 cells responded to TGFβ1 treatment after 30min with a 1.59-fold increase in activated ERK 1/2, which was kept constant for 120 min of TGFβ1 treatment. Although TGFβ1 stimulated ERK 1/2, the ERK 1/2 response to TGFβ1 treatment was delayed compared to the response in SW480 cells. At 30min the level of phosphorylated ERK 1/2 was similar to that of the untreated cell and not until 60min did levels of detectable phosphorylated ERK 1/2 significantly increase by 1.64 fold. This delayed ERK 1/2 response to TGFβ1 may again be due to the significant increased > 2-fold increase in expressed TGFβ receptor II observed in SW480 compared to RKO cells (**Figure 4.4.1**).

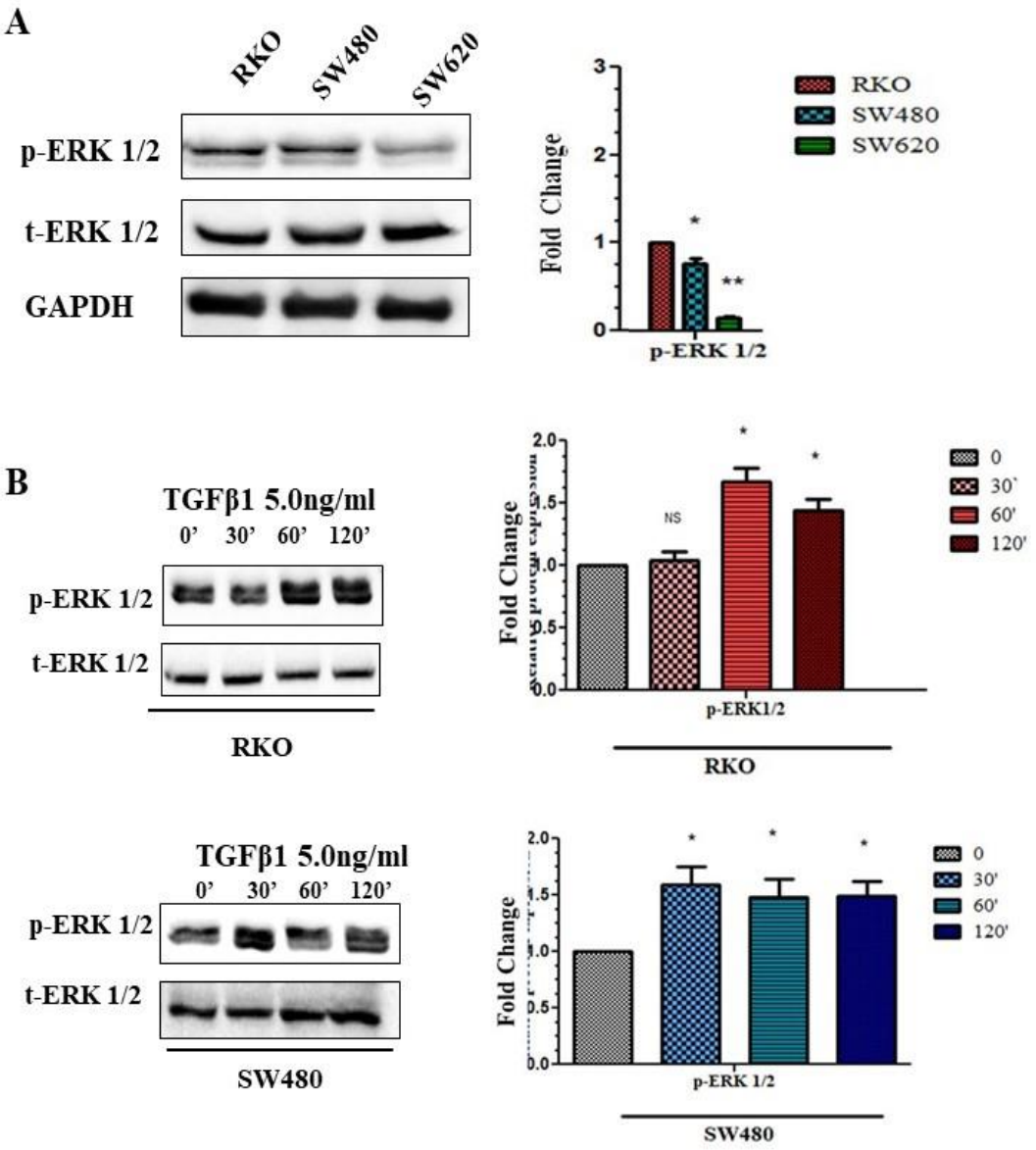


Figure 4.4.6 TGFβ1 activates ERK1/2 signalling in primary CRCs RKO and SW480. (A). Representative Western blots of whole cell lysates of CRCs showing reduced ERK1/2 activation in metastatic cell line SW620 compared to the two primary cancer cell lines RKO and SW480. (n=3; mean±S.D),Bar charts shows the normalized densitometry ratios. **-statistically significant from TGFβ1 (2.5ng/ml) treated cells, NS-not significant. **(B)** Representative Western blotting showing ERK 1/2 activation, after TGFβ1 (5.0 ng/ml) treatment over a time course of 2 h in wt RKO and SW480 cells. Bar chart shows the mean relative densitometry ratios of normalised protein expression of p-ERK 1/2 using total t-ERK 1/2 after normalising for protein loading. (n=3; mean±S.D), *-statistically significant from control cells, NS-not significant.

4.4.2.2 Extracellular signal-regulated kinase (ERK) may play a role in TGFβ1 induced TG2 expression

Following activation of ERK by TGFβ1 in RKO and SW480 cells it was now important to identify if ERK activation correlated with TGFβ1 induced TG2 expression in the primary CRCs RKO and SW480**.** To achieve this PD98059 a specific inhibitor of ERK 1/2 was used at a concentration of 10μM to inhibit ERK 1/2 in RKO and SW480 cells treated with or without 2.5ng/ml TGFβ1. In **Figure 4.4.7** inhibition of ERK1/2 with 10μM ERK1/2 inhibitor PD98059 over a period of 48h significantly reduced TG2 expression. Approximately 30% of detectable TG2 expression levels were lost following ERK 1/2 inhibition. The role of ERK1/2 on TGFβ1 induced TG2 was also investigated with ERK 1/2 inhibition in TGFβ1 treated cells accounting for a significant (<3.5 fold) loss in TG2 expression compared to the amount of TG2 detected in TGFβ1 only treated cells. Indeed, the levels of TG2 expressed in the cells treated with combined treatment of ERK 1/2 inhibitor and TGFβ1 was lower than the control untreated cells and similar to the PD98059 only treated cells. This suggests that TGFβ1 may also employ the mitogen-activated protein kinase (MAPK) pathway for induction of TG2 in both RKO and SW480 cells.

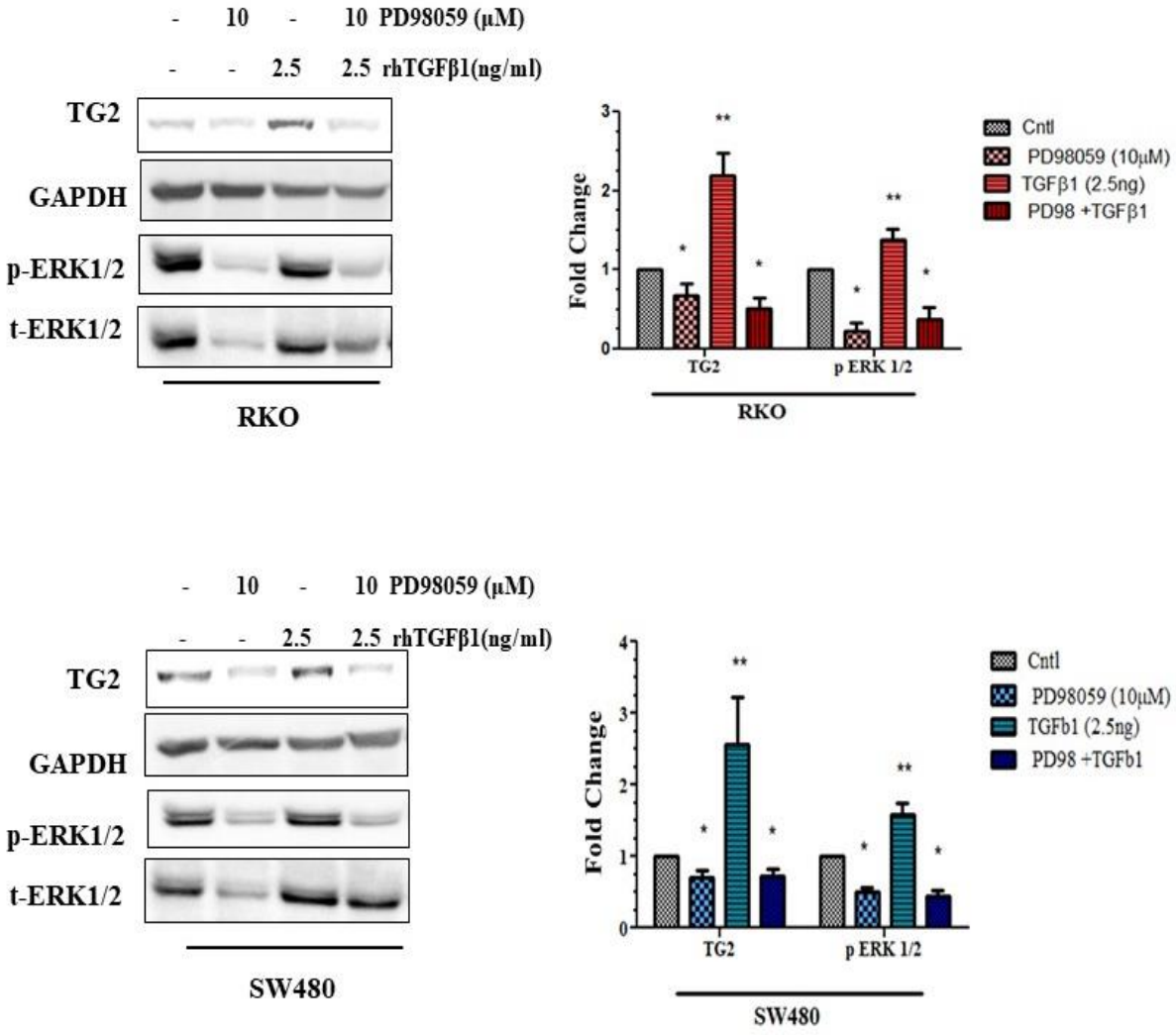


Figure 4.4.7 ERK1/2 plays a role in TGFβ1 **induced TG2.** Representative Western blotting of whole cell lysates of wt RKO and SW480 cells, showing expression of TG2 and ERK1/2 after treatment with TGFβ1 (2.5ng/ml) with or without ERK inhibitor PD98059 (10 μM) for 48 h. Ratios indicate mean. Bar chart shows relative densitometry ratios of protein expression normalised against GAPDH or normalisation for p-ERK 1/2 using total t-ERK 1/2 after normalising for protein loading. (n=3; mean±S.D) *-Statistically different ($p<0.05$) from control, **-statistically different from control and ERK inhibitor PD98059 treated cells.

4.4.3 TG2 and TGFβ1 contribute to a positive feedback loop

TGFβ1 has been reported and shown here to be an inducer of TG2. Many reports show that TGFβ1 may induce TG2 via multiple pathways involving NFκB and Smad dependent pathways (Cao et al., 2008). Some studies also suggest that TG2 may drive increased expression of TGFβ in fibrotic conditions (Telci et al., 2009) and in some cancerous conditions (Kotsakis et al., 2011). This suggests that TG2 and TGFβ1 may be involved in a feedback loop.

4.4.3.1 TG2 correlates with cellular TGFβ1 in CRCs

In CRCs, TGFβ1 signalling, an inducer of EMT, can be a major dysfunctional point during tumour progression (Lamouille et al., 2014). In the previous section and figures, it can be hypothesised that TGFβ1 induced EMT may involve TG2 since TG2 plays an important role in inducing EMT (**Figure 3.4.9**). It has also been suggested that TG2 may increase the abundance or release of TGFβ1 (Nyabam et al., 2016, Telci et al., 2009) In **Figure 4.4.8**A the amount of active TGFβ1 released into the cell culture medium was measured by ELISA and shows that TG2 transduced RKO cells released significantly higher amounts of TGFβ1 compared to EV RKO cells. In a similar fashion knockdown of TG2 by shRNA in SW480 cells also resulted in significantly less TGFβ1 in the cell culture media. Interestingly TGFβ1 levels in SW480 and SW620 EV (Empty Vector control) cells were similar and significantly higher than what was detected in RKO cells, which correlate with TG2's expression as shown in the previous chapter. However, TG2 knockdown did not significantly impact on the release of TGFβ1 into the cell culture media in TG2 shRNA treated SW620 cells. This observation was also confirmed by dot blotting of cell culture media from these cells **(Figure 4.4.8 B)**. Following the increase in released TGFβ1 in TG2 expressing primary CRCs, SW480 and RKO cells, an attempt was made to detect TGFβ1 expression in whole cell lysates of the CRCs. In **Figure 4.4.8C**, whole cell TGFβ1 expression is shown to be significantly increased by >50% in RKO cells transduced with TG2 compared to the EV cells and in a similar fashion TG2 knockdown by shRNA in SW480 cells led to a significant (approximately 50%) loss in TGFβ1 expression when compared to the EV cells. However, TG2 knockdown did not affect TGFβ1 expression in SW620 cells confirming previous data obtained using cell culture media.

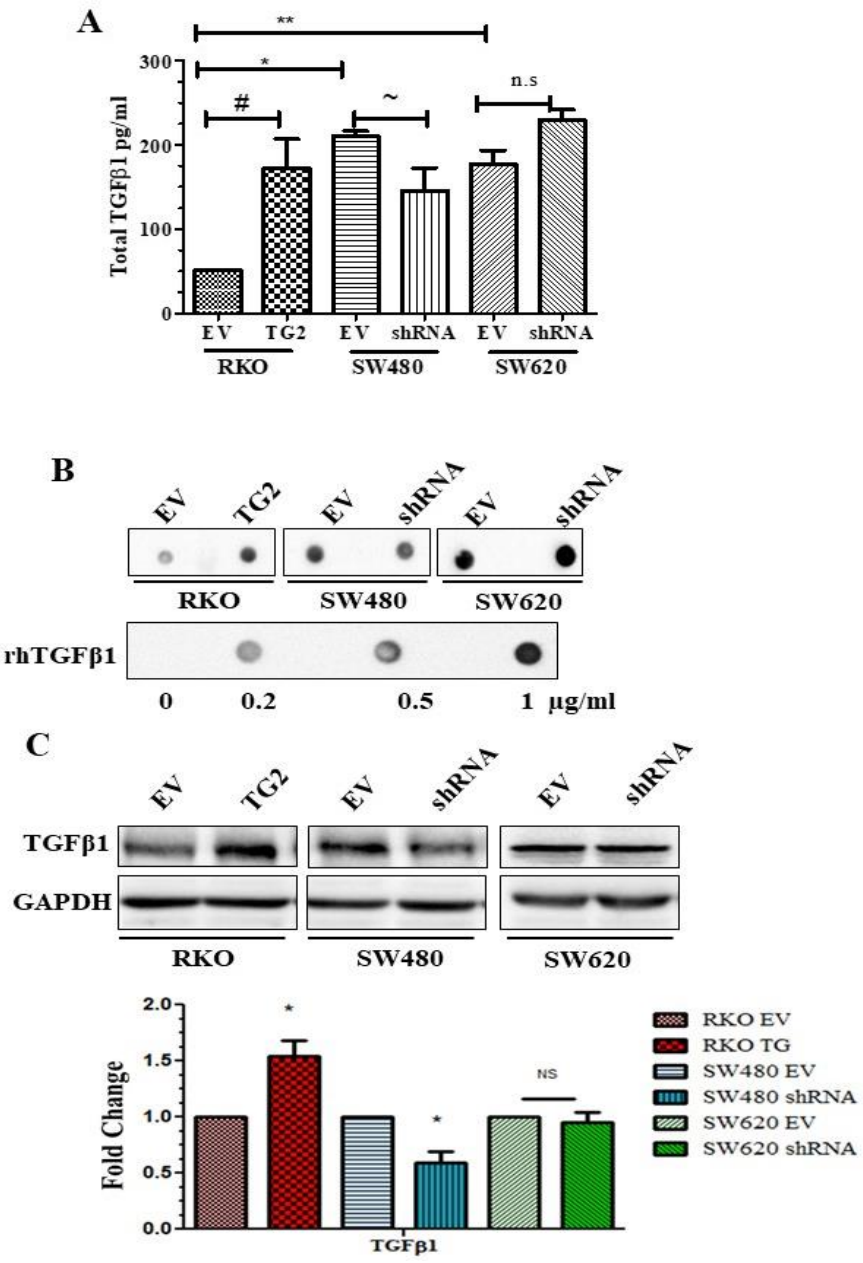


Figure 4.4.8 TG2 correlates with TGFβ1 expression and release into cell culture media. (A) TGFβ1 released into cell culture media of CRCs was determined by ELISA as described in the Materials and Methods. Data represent means ± S.D, from two experiments each performed in triplicate (*, **, #, ~, $p<0.05$). **(B)** Representative Dot blotting of cell culture media of CRCs both controls and cell transduced with TG2 (TG2) or shRNA (SW480shRNA and SW680shRNA) and recombinant human TGFβ1 probed with TGF β1 antibody (n=3). **(C)** Representative Western blots of whole cell lysates of CRCs showing expression of TGFβ1 in whole cell lysates of RKO control cells, RKO transduced with TG2 (RKOTG2), SW480 control cells or SW480 cells transduced with shRNA (shRNA), SW620 control cells or SW620 cells transduced with shRNA (shRNA). Graph chart shows relative densitometry ratios of protein expression normalised against GAPDH. (n=3) (n=3; mean±S.D), *-statistically significant from EV cells; NS-not significant.

4.4.3.2 TG2 inhibition reduces cellular and matrix-bound TGFβ1

Following the finding that TG2 expression correlates with TGFβ1 release and expression in primary CRCs RKO and SW480, an attempt was then made to investigate the effect of TG2 inhibition on cellular expression of TGFβ1. In **Figure 4.4.9**, treatment of RKO and SW480 cells with TG2 inhibitor 1-155 (1μM) significantly reduced cellular expression of TGFβ1 by approximately 50% compared to the control cells, in a similar fashion to the effect of TG2 knockdown on TGFβ1 expression. In addition, the amount of matrix-bound TGFβ1 was measured in RKO and SW480 cells, with or without TG2 inhibition (1μM 1-155) resulting in reduction in matrix-bound TGFβ1 in primary tumour CRCs RKO and SW480 (**Figure 4.4.9**)

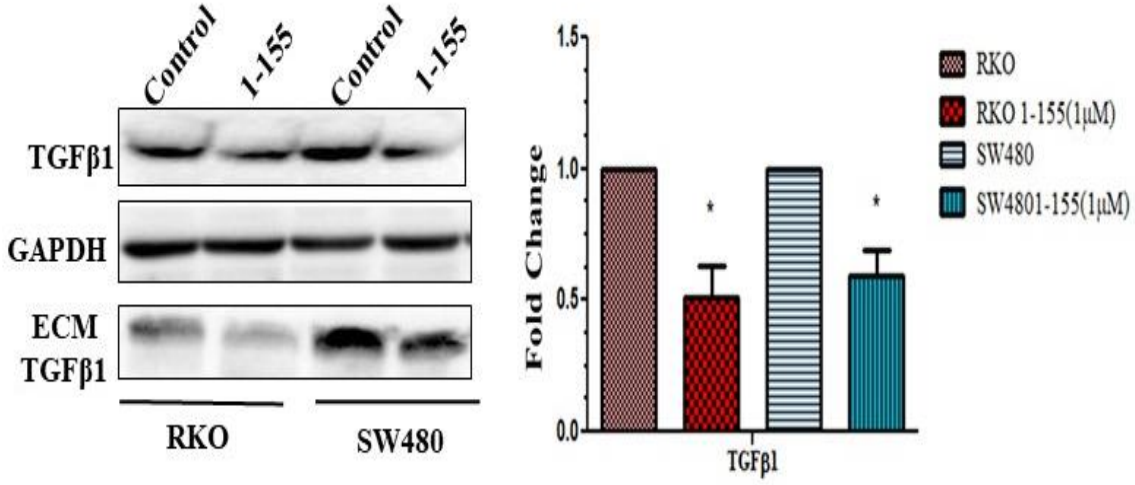


Figure 4.4.9 TG2 inhibition reduces cellular and matrix-bound TGFβ1
Representative Western blots of whole cell lysates from RKO and SW480 cells showing expression of TGFβ1 with or without treatment of cells with TG2 inhibitor 1-155 (1 μM) for 48 h. (n=3). Cell-matrix was collected following treatment of RKO and SW480 cells with or without TG2 inhibitor 1-155 (1 μM) for 72 h. Bar chart shows relative densitometry ratios of protein expression normalised against GAPDH. An equal number of cells were seeded for each experiment. (n=3; mean±S.D), *-statistically significant from control cells.

To demonstrate the activity of TG2 at the cell surface the cell surface activity of TG2 in RKO and SW480 cells was measured as described in **Section 4.3.3**. **Figure 4.4.10** shows that RKO cells transduced with TG2 possessed a significant increase in cell surface activity compared to the EV cells. Similarly TG2 knockdown by shRNA in SW480 cells resulted in a significant loss of TG2 cell surface activity when compared to the control empty vector (EV) cells. This may suggest increased that as well as increasing TGFβ1 expression, TG2 may also facilitate matrix-bound abundance of TGFβ in RKO and SW480 cells.

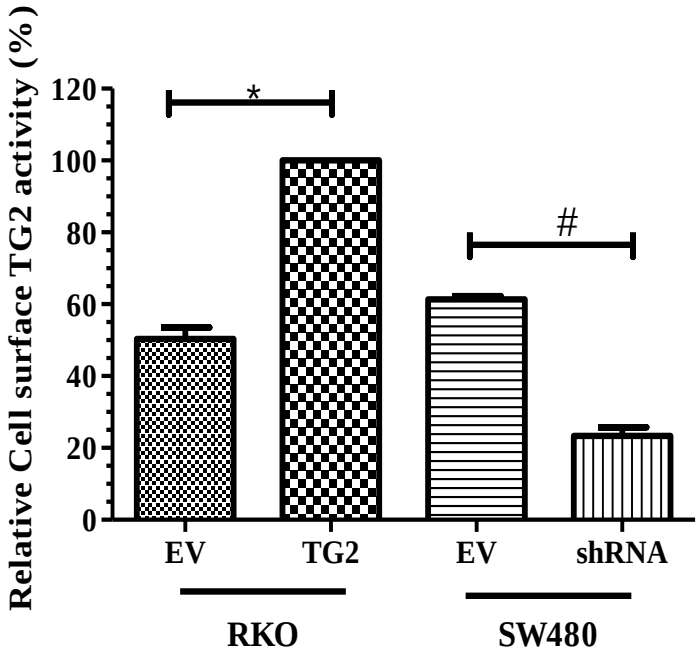


Figure 4.4.10 ***In vitro*** **Cell surface TG2 activity in primary CRCs.** Cell surface TG2 activity measured by incorporation of biotin cadaverine in RKO control cells, RKOTG2 cells, SW480 control cells and SW480shRNA cells measured as described in the Materials and Methods. Data are represented as mean ± S.D., (n=2), with each independent experiment performed in triplicate (*, **, #, ~, $p<0.05$).

4.4.3.3 Inhibition of Extracellular signal-regulated kinase (ERK) reduces c-JUN and cellular TGFβ1 expression

The expression of TGFβ1 may be upregulated by other inflammatory responses such as TNF α or NFκB (Telci et al., 2009). It has been suggested that TGFβ1 can be induced by C-Jun and C-Jun expression can be induced by ERK (Leppa et al., 1998). In **Figure 4.4.1**1, ERK inhibitor PD98059 significantly reduced the expression of C-Jun by 70% in RKO cells treated with the inhibitor. Furthermore, ERK inhibitor also reduced the expression of C-Jun by >30% in SW480 cells treated with the inhibitor. The ERK inhibitor-treated cells also exhibited a >45% decrease in TGFβ1 expression in both cell lines, indicating the involvement of C-Jun in ERK1/2-induced TGFβ1 expression in RKO and SW480 cells. This was confirmed by more than 70% inhibition of the phosphorylation of ERK 1/2 in RKO cells treated with the ERK inhibitor and more than 50% inhibition of phosphorylated ERK 1/2 in SW480 cells treated with ERK inhibitor.

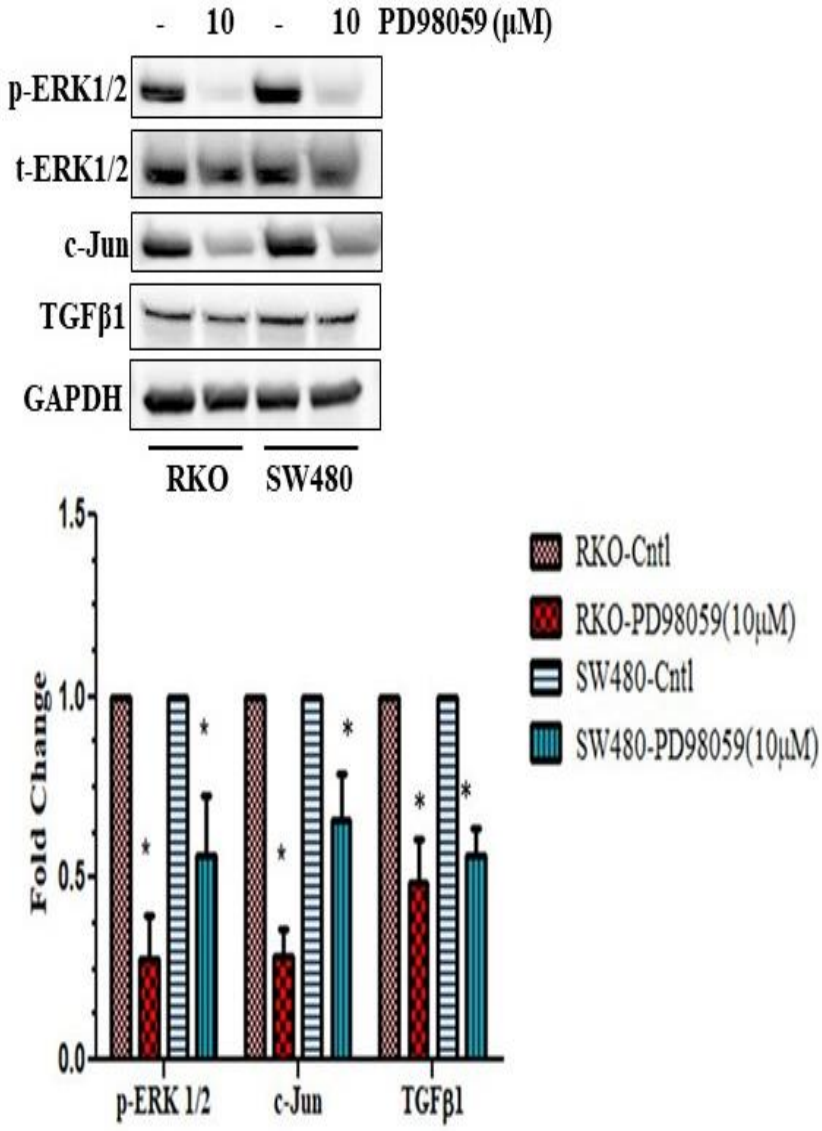


Figure 4.4.11 ERK1/2 activation correlates with cellular TGFβ1 expression. Representative Western blotting of whole cell lysates from RKO and SW480 cells showing expression of ERK1/2, C-Jun, and TGFβ1. p-ERK 1/2 was normalised against t-ERK 1/2. Cells were treated with or without treatment ERK 1/2 inhibitor PD98059 (10 μM) for 48 h. Bar chart shows relative densitometry ratios of protein expression normalised against GAPDH or normalisation for p-ERK 1/2 using total t-ERK 1/2 after normalising for protein loading. (n=3; mean±S.D), *-statistically significant from control (cntl) cells; NS-not significant.

4.4.3.4 TG2 induces activation of Extracellular signal-regulated kinase (ERK) may increase TGFβ1 expression

TG2 has been reported to induce ERK activation in human neuroblastoma cells (Singh et al., 2003b), human endometrial endothelial cells (Di Simone et al., 2013) and human embryonic kidney cells (Cho et al., 2010). Therefore, to determine if TG2 could activate ERK 1/2 and show if this correlated with C-Jun expression and TGFβ1, TG2 expression was manipulated in primary CRCs SW480 and RKO. In RKO cells ectopic expression of TG2 resulted in a significantly increased expression (1.37 fold) of detectable phosphorylated ERK (p-ERK 1/2) compared to the empty vector (EV) cells and this increase corresponded to a significant increase (1.3-fold) in C Jun and a significant increase (1.53-fold) in whole cell lysate TGFβ1. Similarly, TG2 knockdown in SW480 cells significantly reduced ERK 1/2 activation by about a third of the normal detectable phosphorylated ERK 1/2 in the EV cells, and lead to 30% loss in C Jun expression, followed by a 37% loss in whole cell lysate TGFβ1 expression in the SW480 cells with TG2 shRNA (**Figure 4.4.12A)**. However, TG2 expression did not significantly alter the expression of JNK another MAPK signalling protein associated with C-Jun expression and activation.

Since it was also observed that TG2 inhibition reduced whole cell TGFβ1 expression levels (**Figure 4.4.9**), the effect of TG2 inhibition on ERK 1/2 phosphorylation was determined on primary CRCs. In **Figure 4.4.12B** TG2 inhibition with the site-specific TG2 cell-permeable inhibitor 1-155 resulted in reduced expression of phosphorylated ERK in both SW480 and RKO cells.

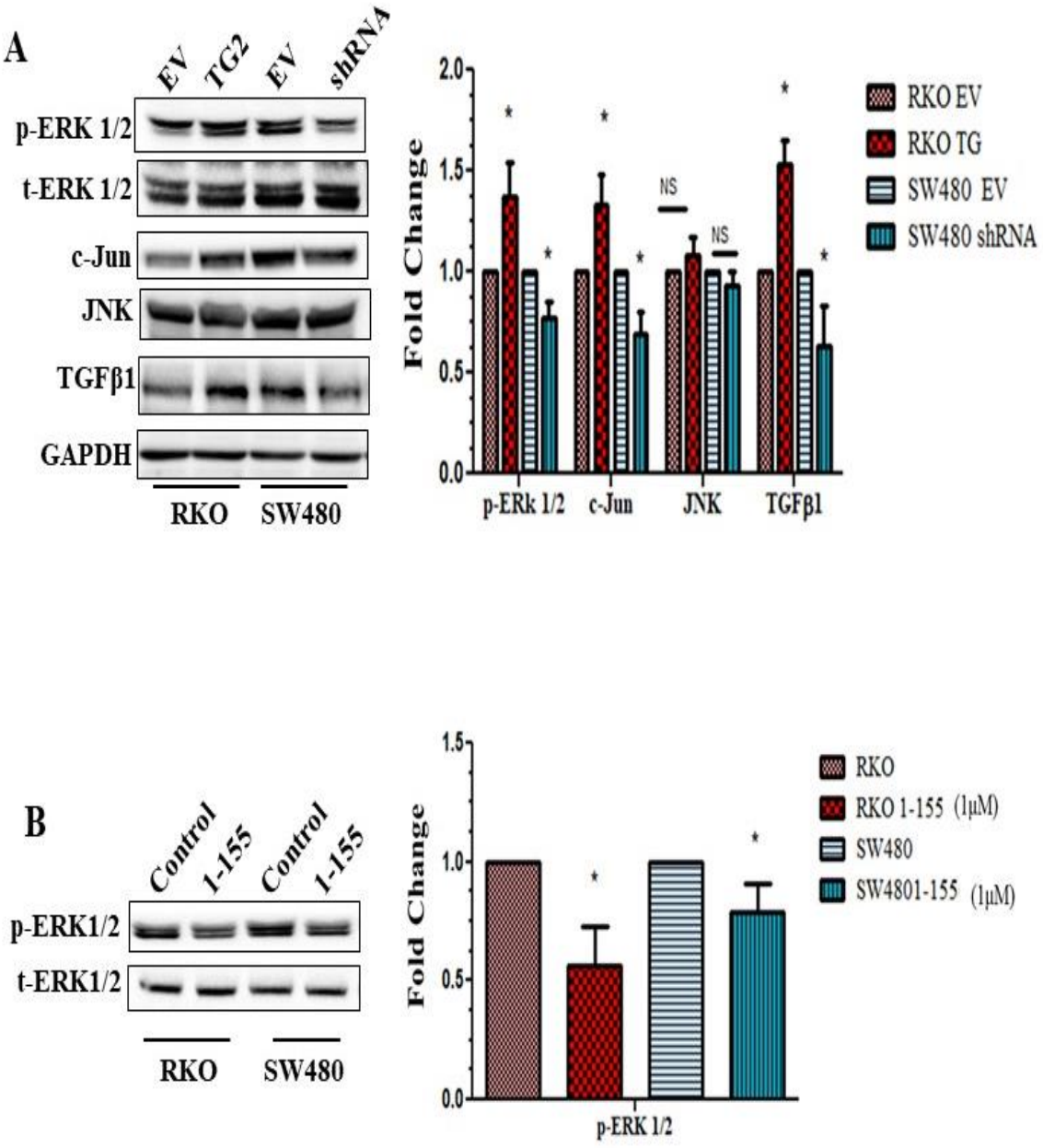


Figure 4.4.12 TG2 is associated with ERK1/2 activation and subsequent cellular TGFβ1 expression. (A) RepresentativeWestern blots of whole cell lysates of RKO and SW480 control cells and cells transduced with TG2 (RKO-TG) or shRNA (SW480shRNA) showing TG2 expression, ERK 1/2 activation, c-Jun, JNK and TGFβ 1 expression. (n=3; mean±S.D), *-statistically significant from control (cntl) cells; NS-not significant. **(B)** Western blots of whole cell lysates showing activation of ERK1/2 in RKO and SW480 cells with or without treatment with TG2 cell-permeable inhibitor 1-155 inhibitor (1 μM). (n=3; mean±S.D),*-statistically significant from EV cells; NS-not significant. Bar charts shows relative densitometry ratios of protein expression normalised against GAPDH or normalisation for p-ERK 1/2 using total t-ERK 1/2 after normalising for protein loading.

4.4.4 TG2 induces EMT via multiple mechanisms in the different colon cancer cell lines.

Since TGFβ1 does not impact on EMT in the metastatic SW620 cells despite TG2's ability to induce EMT in these cells (**Figure 4.4.4**), it was then important to investigate the potential involvement of TG2 on the Wnt/β-catenin signalling pathway an important deregulated pathway in cancer and EMT.

4.4.4.1 Characterisation of CRCs for Wnt/ β-catenin Signalling

In **Figure 4.13** CRCs RKO, SW480 and SW620 were characterised for members of the Wnt/ β-catenin signalling pathway by Western blotting. The expression of LRP 5 a co-receptor with LRP 6 and frizzled in the Wnt signalling pathway was significantly higher in metastatic SW620 compared to the primary CRCs RKO and SW480. This significantly higher expression of LRP 5 in the metastatic SW620 compared to its isogenic primary counterpart SW480, and primary cell line RKO was suggestive of a potential role of the Wnt signalling pathway in the SW620 cells. The detection of β-catenin a major transcription factor in the Wnt signalling pathway was then determined. β-catenin was significantly overexpressed in SW620 cells, with an almost 3-fold increase compared to the detected amount in isogenic pair SW480 (**Figure 4.4.13**), even though both have similar APC inactivating mutation (Ahmed et al., 2013). The level of cellular β-catenin in SW620 cells was significantly higher (>6 fold) compared to what was detected in RKO cells with wt APC (**Figure 4.4.13**).

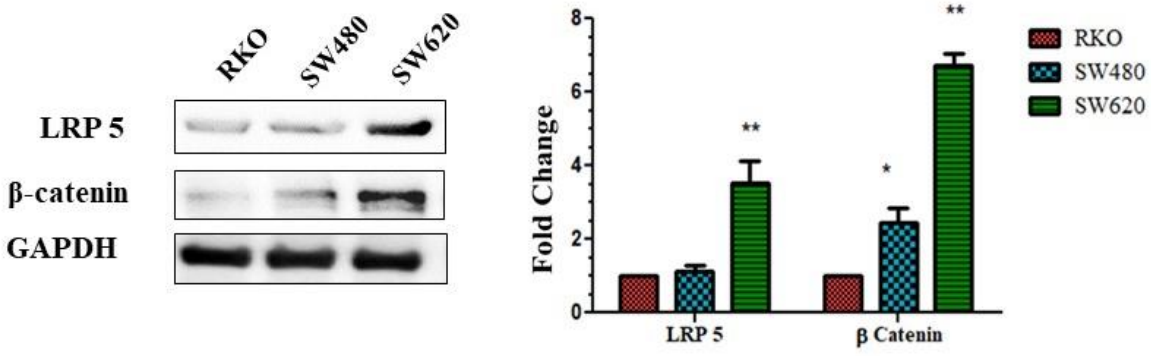


Figure 4.4.13 Western blots showing the characterisation of some of the elements of the β-catenin signalling pathway. Representative Western blots of whole cell lysates of RKO, SW480 and SW620 cells, showing expression of LRP5 (a receptor in the Wnt/β-catenin signalling pathway) and β-catenin. Bar chart showa mean relative densitometry ratios of protein expression normalised against GAPDH. (n=3; mean±S.D), *-Statistically different ($p<0.05$) from RKO cells, **-statistically different from RKO and SW480 cells.

4.4.4.2 TG2 increases β-catenin translocation into the nucleus by preventing β-catenin ubiquitination

Overexpression of β-catenin is associated with many cancers including colorectal carcinoma (Muzny et al., 2012), hepatocellular carcinoma (Tien et al., 2005) and ovarian carcinoma (Condello et al., 2015) amongst others. Following that β-catenin may also facilitate EMT in cancer cells (Nagaraj et al., 2015), and with the detection of high expression of β-catenin in SW620 cells compared to RKO and SW480 cells, it was important to investigate the potential role of TG2 in Wnt/β-catenin signalling in SW620 cells. Especially as TG2 has been shown to play an important role in the potentiation of Wnt signalling (Faverman et al., 2008). TG2 knockdown non-significantly reduced the expression of whole cell β-catenin as shown in **Figure 4.4.14**, however the effect of TG2 on the amount of β-catenin translocation into the nucleus, which is its most important role in inducing transcription factors of cell cycle regulators, EMT, and other cell survivor proteins were assessed. In **Figure 4.4.14**. TG2 expression facilitated increased expression of β-catenin in the nuclear fraction of SW620 cells, as knockdown of TG2 expression by shRNA led to a significant reduction in the translocation of β-catenin into the nucleus as detected by Western blotting. Additionally, some studies suggest that TG2 may translocate into the nucleus and act as a co-transcription factor to some EMT associated genes (Kumar and Mehta, 2012), however in the SW620 cells no detectable levels of TG2 was observed in the cell nucleus (**Figure 4.4.14**). The reduced abundance of β-catenin in the nucleus suggestive of a potential role of TG2 in β-catenin driven oncogenesis and tumour progression.

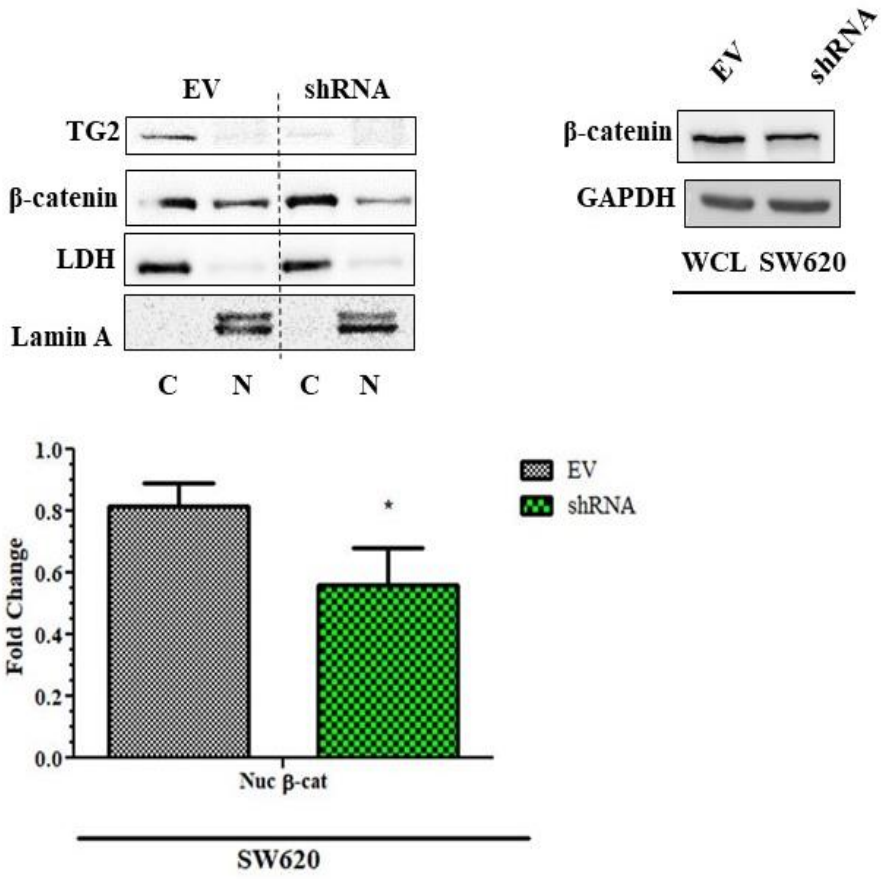


Figure 4.4.14 TG2 facilitates nuclear accumulation of β-catenin in metastatic SW620 cells
Representative Western blots of β-catenin presence in the nuclear (N) and cytoplasmic (C) cell extracts of SW620 control cells and SW620shRNA cells. LDH and Lamin A were used as cytoplasmic and nuclear protein markers respectively and the ratios obtained are normalise TG2 and β-catenin expression in the extracts to the respective subcellular markers. The N and C fractions were separated as described in **Section 4.3.4.** For the whole cell lysates (WCL) Bar chart indicate mean relative densitometry ratios of protein expression normalised to nuclear loading marker Lamin A. *-Statistically different ($p<0.05$) from EV cells.

4.4.4.3 TG2 interacts with cellular β-catenin

Following the role of TG2 in nuclear accumulation of β-catenin. It was important to investigate a mechanism in which TG2 promotes β-catenin nuclear translocation. By using co-IP, in **Figure 4.4.15A,** TG2 was co-immunoprecipitated from the whole cell lysate and probed for LRP-5 and β-catenin**.** This figure shows that amongst the TG2 pull-down proteins, β-catenin a major player in oncogenic progression and EMT was pulled down and detected. Additionally, LRP 5- a co-receptor with frizzled and LRP 6 in the propagation of Wnt signalling, was also detected amongst the TG2 pulled down protein. This suggests a form of interaction between TG2 and β-catenin, and TG2 and LRP 5. This also suggests that TG2 could play a role extracellularly with LRP 5, and intracellularly with β-catenin. Furthermore, the interaction between TG2 with LRP 5 and β-catenin, was found to be disrupted by the treatment of SW620 cells with TG2 specific cell-permeable inhibitor 1-155, and this is shown in **Figure 4.4.15A** by the reduced expression on LRP 5, and β-catenin in the inhibitor-treated cells compared to the untreated SW620 cells. To further validate the protein interaction between TG2 and β-catenin, in (**Figure 4.4.15B)** proteins interacting with β-catenin was pulled down by immunoprecipitating β-catenin from whole cell lysates, and again TG2 was detected amongst proteins associated with β-catenin and this interaction can be reduced by the addition of TG2 inhibitor 1-155 (**Figure 4.4.15B).**

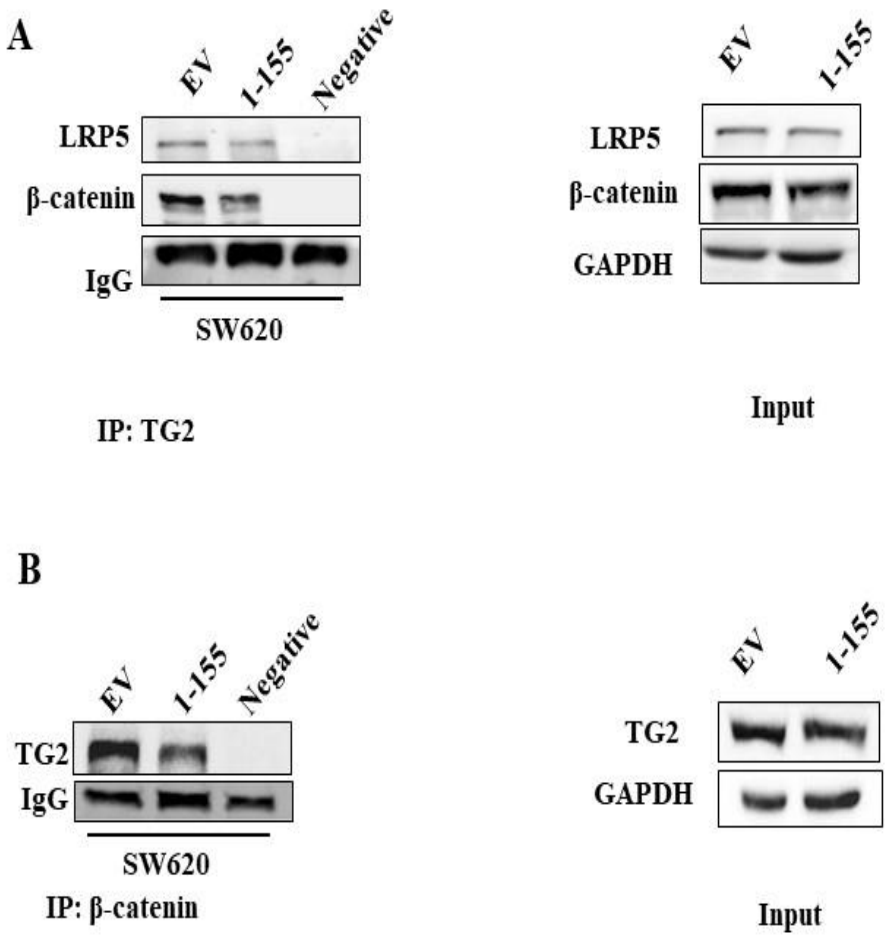


Figure 4.4.15 TG2 interacts with β-catenin in metastatic SW620 cells. (**A**)Representative Western blotting of LRP5 and β-catenin in wt SW620 and SW620 cells treated with TG2 selective inhibitor 1-155 (1 μM) following TG2 Co-IP from whole cell lysates. Co-IP was undertaken as described in **Section 4.3.5**. Input shows whole cell lysate level of LRP5 and β-catenin in the respective treated cells **(B)** Representative Western blotting of whole cell lysates for TG2 after β-catenin co-IP following treatment of cells with or without TG2 selective inhibitor 1-155 (1 μM). Input shows whole cell lysate level of TG2 in the respective treated cells. Control cells were treated with vehicle alone DMSO, and treatments were performed for 48 h (n=3).

4.4.4 4 TG2 interacts with cellular β-catenin and the consequent nuclear translocation of β-catenin is integrin and Src-independent in SW620 cells

As TG2 was seen to interact with LRP5 and β-catenin, and increase nuclear translocation of β-catenin, next the role of TG2 in Src-mediated β-catenin nuclear translocation, was investigated, as integrin-associated activation of Src has been reported to play a significant role in non-canonical β-catenin nuclear accumulation (Cao et al., 2008). In **Figure 4.4.16A** TG2 knockdown had no effect on LRP5 a co-receptor of Wnt Signalling. More so TG2 knockdown did not affect β1integrin expression. Importantly TG2 knockdown had no effect on the activation status of SRC in SW620 cells**.** This, however, was not the case with the isogenic primary cell SW480 and RKO cells. TG2 knockdown in SW480 cells significantly reduced the abundance of β1integrin found in these cells by almost 60%, concomitant with 70% loss in activated Src. The potential role of TG2 in Src activation in primary cells was further confirmed by overexpressing TG2 in RKO cells which led to a significant (1.41 fold) increase in β1integrin expression followed by 1.53 fold increase in activated Src compared to the detectable expressed levels in the EV cells (**Figure 4.4.16B**). This potentially suggests a β1integrin and Src-independent mechanism of TG2 induced nuclear accumulation of β-catenin in metastatic SW620 cells. To further establish this concept, Src was co-immunoprecipitated from cell lysates of SW620 treated with TG2 shRNA or TG2 inhibitor, and in **Figure 4.4.17** TG2 knockdown or inhibition did not alter the interaction between β-catenin and Src when compared to untreated cells, suggesting that TG2 plays a nonsignificant role in Integrin-Src-associated β-catenin nuclear accumulation in SW620 cells.

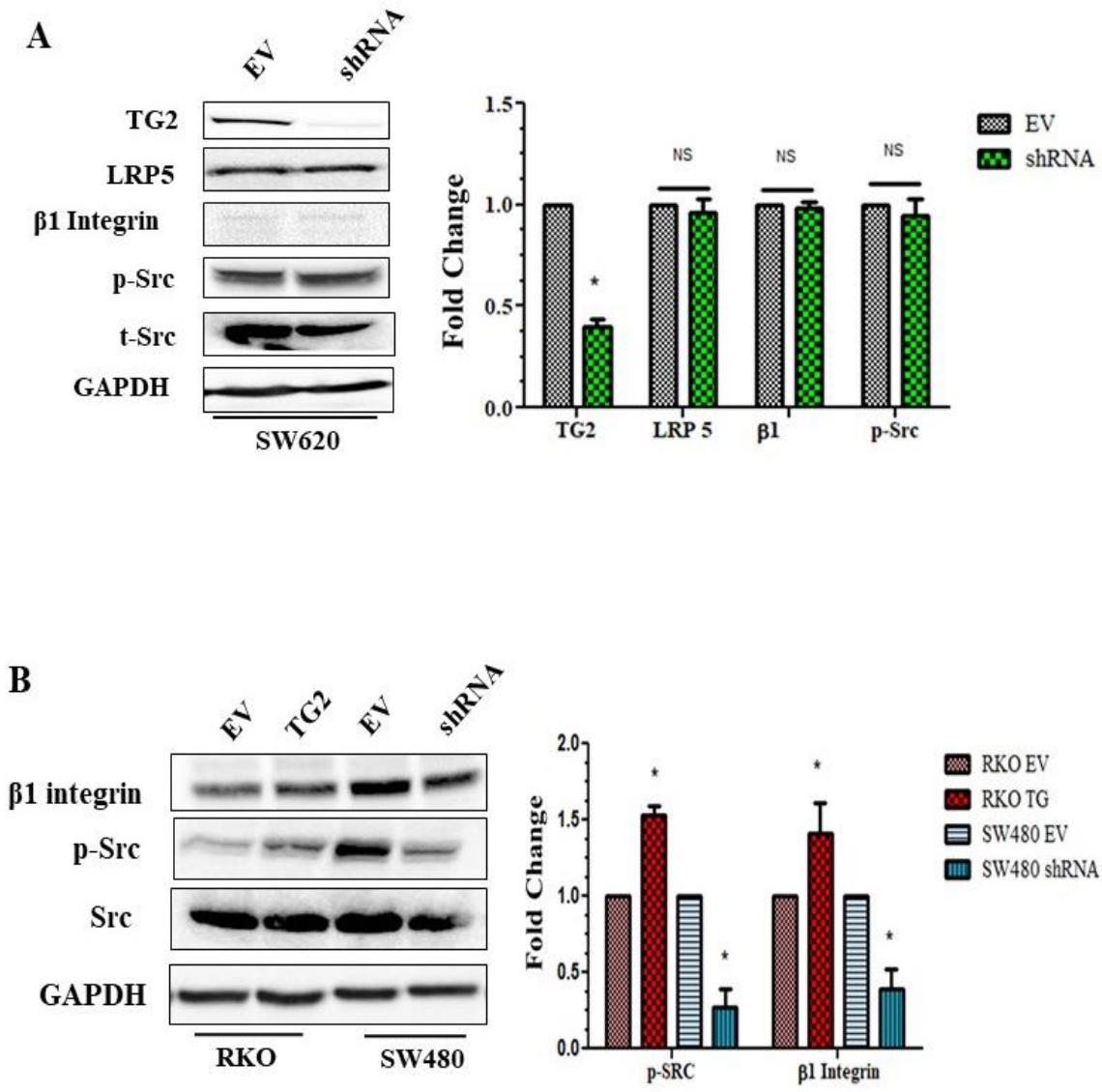


Figure 4.4.16 Expression of Src signalling molecules in CRCs with altered TG2 expression. (**A**) Src activation is independent of TG2 in SW620 cells. Representative Western blotting of whole cell lysates of SW620 cells with or without TG2 shRNA showing expression of β1 integrin, p-Src and total Src (n=3). *-Statistically different ($p<0.05$) from EV cell, ns-not significatnt. (**B**) Western blot of integrin and Src expression in RKO cells with empty vector (EV) or transduced with wt TG2 (TG2); and in SW480 cells with empty vector (EV) or with TG2 shRNA(shRNA). Ratios indicate mean relative densitometry ratios of protein expression normalised against GAPDH, p-Src was normalised against total Src (n=3). *-Statistically different ($p<0.05$) from EV cells. Bar chart show relative densitometry ratios of expressed proteins normalised to GAPDH and or Src, (n=3; mean±S.D).

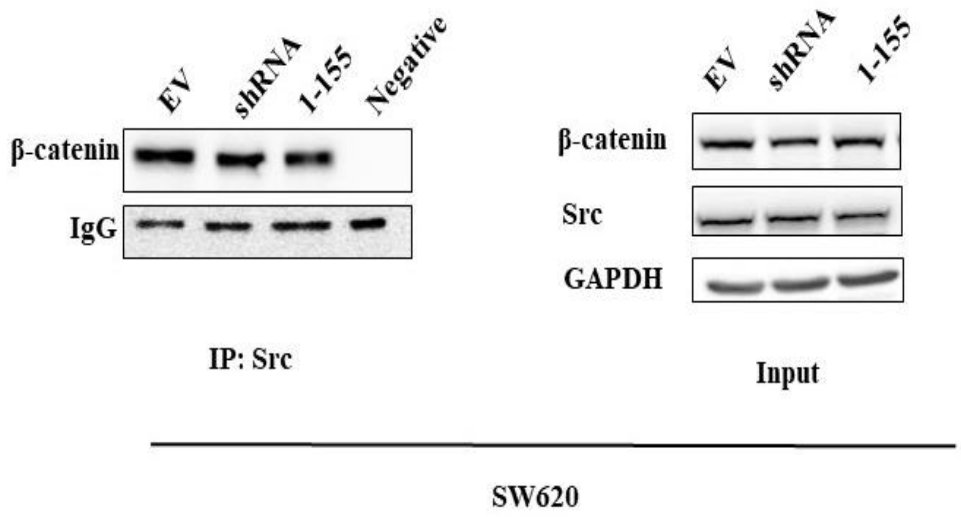


Figure 4.4.17 Src interacts with β-catenin in a TG2 independent manner in SW620 cells. Representative Western blots of β-catenin after Src co-immunoprecipitation from whole cell lysates of SW620 control cells and SW620shRNA cells or after treatment with TG2 inhibitor 1-155. Input shows whole cell lysate level of β-catenin and Src in the respective treated cells. Control cells were treated with vehicle alone DMSO, and treatments were performed for 48 h (n=3).

4.4.4.5 TG2 inhibition potentiates β-catenin ubiquitination

Following the finding that TG2 had no role to play in Src-mediated β-catenin activation in the metastatic SW620 cells, but played a role in inducing β-catenin accumulation, it was important to determine in what manner TG2 facilitated the nuclear accumulation of β-catenin. The more so as the functional APC mutation in SW620 cells facilitates unperturbed Wnt signalling by preventing the formation of the APC-axin death complex (Ilyas et al., 1997). **Figure 4.4.18** shows that the interaction between β-catenin and ubiquitin was found to be increased when cells were treated with TG2 inhibitor 1-155 when compared to control cells suggesting that TG2 is able to inhibit ubiquitination of β-catenin, downstream of Src in SW620 cells.

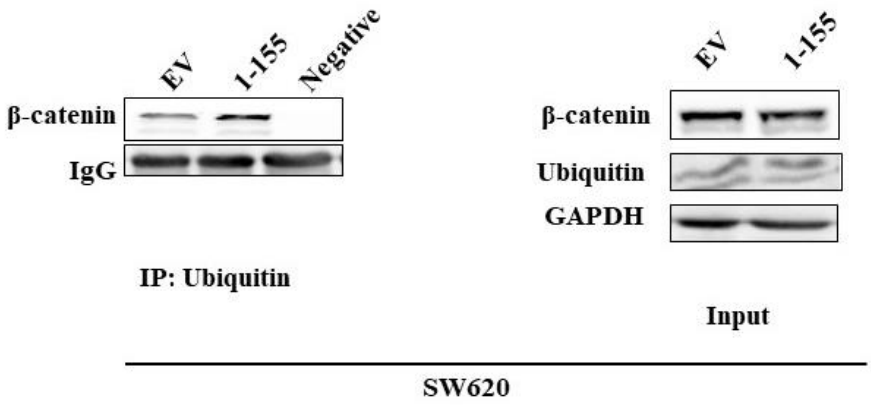


Figure 4.4.18 TG2 inhibition facilitates β-catenin interaction with ubiquitin in metastatic SW620 cells. Representative Western blots of whole cell lysates from wt SW620 cells after treatment with or without TG2 selective inhibitor 1-155 (1 μM) showing β-catenin after ubiquitin co-IP of whole cell lysates from wt SW620. Input shows whole cell lysate level of β-catenin and Ubiquitin in the respective treated cells. Control cells were treated with vehicle alone DMSO, and treatments were performed for 48 h (n=3).

4.4.5 Extracellular TG2 may be necessary for TG2 induced EMT in Primary CRCs.

An attempt was made to discriminate between extracellular and intracellular roles of TG2 induced EMT in CRCs, the effect of extracellular TG2 inhibitor R281 (Wang and Griffin 2013) was compared to cell-permeable TG2 inhibitor 1-155 inhibitor (Badarau et al., 2015). In **Figure 4.4.19**, RKO and SW480 cells treated with R281 had similar EMT attenuating effects as 1-155 with a reduction in fibronectin expression and a corresponding increase in ZO1 expression, suggesting the involvement of extracellular TG2 in EMT in these cells.

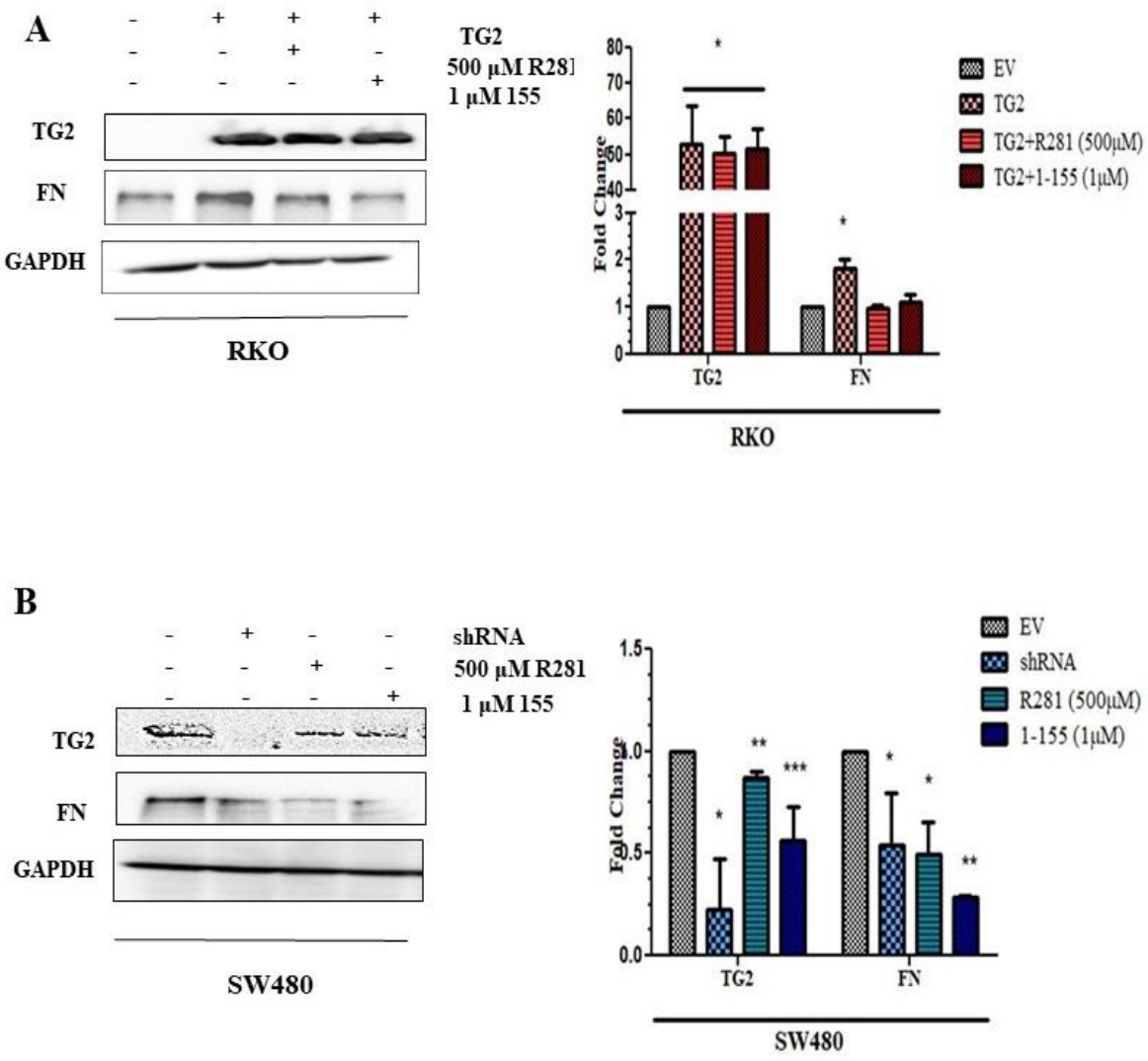


Figure 4.4.19 Extracellular TG2 may play a role in TG2 induced EMT in primary CRCs
(**A**) Representative Western blot of whole cell lysates of RKO and RKO TG2 transduced cells showing expression of TG2 and EMT markers (Fibronectin (FN); Zona Occludin 1 (ZO1) following treatment of the TG2 transduced cells with TG2 cell permeable (1-155) and impermeable inhibitor R281 (500 µM) (**B**) Western blotting of whole cell lysates of SW480 cells showing expression of TG2 and EMT marker Fibronectin (FN)after treatment with TG2 selective cell-permeable inhibitor 1-155 (1 µM) or impermeable inhibitor R281 (500 µM) . Control cells were treated with vehicle alone DMSO, and treatments were performed for 48 h. Bar charts show relative densitometry ratios of protein expression normalised against GAPDH. (n=3; mean±S.D), *-Statistically different ($p<0.05$) from control cells, **-statistically different from control and shRNA or TG2 transduced cells, ***-statistically different from R281 (extracellular inhibitor) treated cells.

4.4.6 Cytoplasmic TG2 may be essential for TG2 induced EMT in colorectal metastatic cell line SW620

Given the potential extracellular role of TG2 in primary CRCs, RKO and SW480, both extracellular and cell permeable TG2 inhibitors R281 and 1-155 respectively, were used to inhibit TG2 in SW620. **Figure 4.4.20A** shows that only the cell permeable inhibitor 1-155 was able to significantly affect EMT in a similar manner to the TG2 knockout cells, by reducing the expression of mesenchymal protein markers fibronectin and vimentin; and increasing the expression of ZO1 an epithelial marker. **Figure 4.4.20B** confirms the inhibitory ability of TG2 shRNA and TG2 inhibitors (cell permeable inhibitor 1-155 and cell impermeable inhibitor R281) on biotinylation of TG2 cellular substrates using biotin-X-cadavarine. The Western blot confirms the loss of TG2 activity in the shRNA treated cells and differential biotinylation of both cell permeable and cell impermeable TG2 inhibitors.

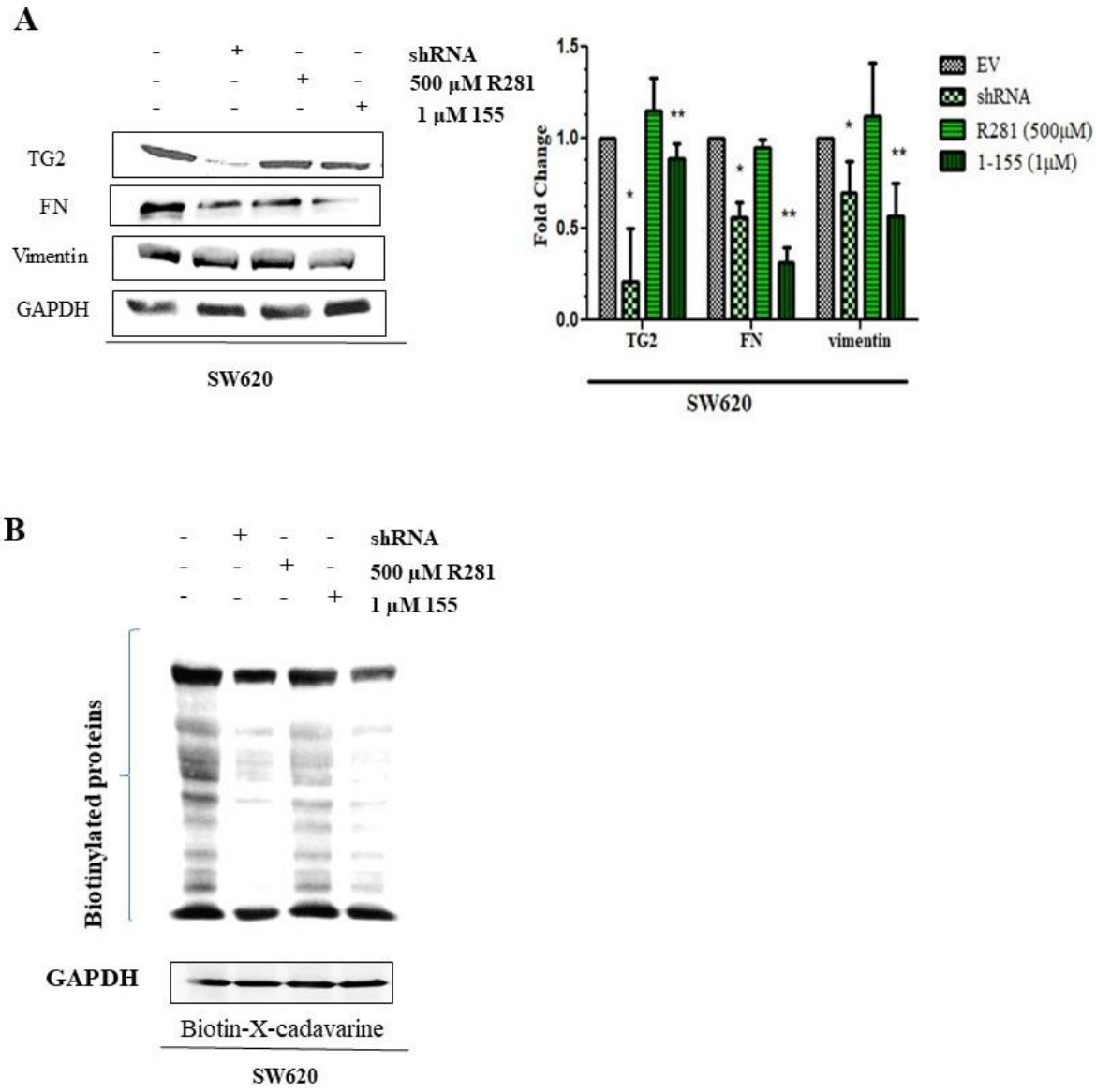


Figure 4.4.20 Inhibition of extracellular TG2 witg R281 does not affect EMT in metastatic SW620 cells. (A) Representative western blots of whole cell lysates showing expression of EMT markers after treatment with TG2 shRNA, or selective TG2 cell impermeable inhibitor R281 (500 µM) or TG2 selective cell-permeable inhibitor 1-155 (1 µM). Control cells were treated with vehicle alone DMSO, and treatments were performed for 48 hBar chart shows mean relative densitometry ratios of protein expression normalised against GAPDH (n=3, mean±SD). *-Statistically different ($p<0.05$) from control cells, **-statistically different from control, R281 and shRNA treated cells. (**B**) Representative western blot of biotin labelled proteins following treatment of SW620 cells with Biotin-X-cadaverine for 16h. SW620 cells were previously with (EV) or without shRNA, EV cells were treated with cell permeable (1-155) 1µM or cell impermeable (R281) 500µM for 48h prior to biotinylation (n=2).

4.5 DISCUSSION

In Chapter 3, the inhibition of TG2 activity, or silencing its expression reduced the expression of Slug and Twist in the metastatic SW620 cells. This proved that TG2 was acting upstream of these transcription factors, an observation that has been previously reported in MCF10A and MCF12A cells (Kumar et al., 2010). To investigate the cellular signalling pathways that may be associated with these TG2 related events involving the cellular expression of transcription factors of EMT, TGFβ1, and WNT/β-catenin signalling pathways were investigated.

Aberrations occurring in the TGFβ1 and WNT/β-catenin signalling pathways have both been implicated in EMT and tumour progression (Lamouille et al., 2014, Neuzillet et al., 2013), and both signalling pathways are commonly mutated in colorectal cancer (Novellasdemunt et al., 2015). It is well reported that TGFβ1 can specifically induce TG2 expression (Nyabam et al., 2016), an observation that has also been made in primary CRCs RKO and SW480 cells. Importantly, the specific effects of TGFβ1 on TG2 induction could be blocked by a TGFβ neutralizing antibody in these two cell lines. Additionally, results in **Figure 4.4.4** demonstrate that TGFβ1 can induce EMT in RKO and SW480, suggesting that TG2 could be a downstream target of TGFβ1 and is recruited by TGFβ1 to induce EMT as it was found out in SW480 cells transduced with TG2, where further treatment with TGFβ1 did not induce further EMT transition compared to control treated TG2 transduced cells (**Figure 4.4.5**). This may explain the findings in the metastatic SW620 where TG2 expression is much higher, and TGFβ1 does not impact on EMT. This suggests a more intrinsic mechanism for TG2 that is independent of growth factors or the ECM. It also suggests that the interplay between TG2 and TGFβ1 may be necessary to acquire a metastatic phenotype, but further cellular mutations are required for metastasis to take place, and an alternative path for TG2 induced EMT is observed in the SW620 cells. While the current data suggest that TGFβ1 may induce TG2 and EMT canonically via increased activation of Smad 2/3; ERK activation and Smad signalling have also been reported to be both necessary for TGFβ-induced epithelial-mesenchymal transition (Davies et al., 2005).

The mechanism by which TGFβ1 induces TG2 in cells may be varied and cell-specific. TGFβ1 was reported to upregulate TG2 in ovarian cancer by upregulating the activity of NFκB (Cao et al., 2012) and via the ERK and PI3/AKT pathway in diseased pulmonary fibroblast (Olsen et al., 2014).

Furthermore, genomic studies on TGFβ1-regulated gene expression profiles also suggest that in the human lung cancer cell line A549, TGFβ1 induces TG2 expression by a pathway that is partially dependent on the ERK signalling pathway (Ranganathan et al., 2007). Hence, in this study the data suggests that ERK1/2 a downstream signalling molecule in the Ras/MAPK kinase pathway is rapidly activated in response to TGFβ1 in both primary CRCs RKO and SW480 (**Figure 4.4.6**), and that blockage of TGFβ1 binding to the receptor, and /or inhibition of ERK 1/2 is sufficient to mitigate TGFβ1 induced expression of TG2 in SW480 and RKO cells, suggesting that ERK 1/2 may be necessary for the TGFβ1 induced TG2 expression in these cells.

More so, the current dataset also demonstrates that TG2 was associated with increased expression and levels of TGFβ1 in the whole cell lysates and culture medium respectively. This agrees with previous studies in murine colon carcinoma that showed increased levels of TGFβ1 in the medium of TG2 transfected clones, which was inhibited by the inclusion of site-directed TG2 inhibitors in the culture medium (Kotsakis et al., 2011). It has been suggested by others that the MAP kinase, ERK1/2 signalling pathway may result in activation of Activator protein-1 (AP-1), a transcription factor which is critical for auto-induction of TGFβ1 (de Caestecker et al., 2000) by mediating the expression of C-Jun. Here it is shown that TG2 increases the activation of ERK1/2 which can be reversed by silencing TG2 or by selective TG2 inhibition using 1-155. Again the mechanism through which TG2 may activate ERK 1/2 may be varied, and may involve TG2 and integrin interactions on the one hand, as it has been shown that TG2-Fibronectin interaction with α5β1 integrin, may potentiate integrin clustering (Telci et al., 2008) or that TG2 may participate as a co-receptor with β1 or β3 integrins (Akimov et al., 2000), with both forms of integrin interaction facilitating the activation of Focal Adhesion Kinase (FAK) which in turn activates other downstream signalling molecules including ERK 1/2 (Cho et al., 2010). On the other hand, TG2 via its cell surface activity may increase the abundance of active TGFβ1 by facilitating the release of TGFβ1 from its latent complex (Verderio et al., 1999). Alternatively, TG2 may also increase matrix-bound TGFβ1, as it has been suggested by Griffin and colleagues that active TGFβ1 crosslinked into the matrix might be necessary to enhance and stabilise the signalling potential of TGFβ1 (Nyabam et al., 2016), this will result in a corresponding activation of ERK 1/2. The former

may explain the more than 4-fold reduced activated ERK 1/2 detection in SW620 cells compared to RKO and SW480, as these cells express very little if any β1 integrin, therefore abating any TG2-integrin-associated cell response including ERK activation.

It is, therefore, plausible to suggest that TG2 induced activation of ERK1/2 can induce c-Jun and in turn recruit transcription factor AP-1 for increased expression of TGFβ1. This is suggested by the increased expression of c-Jun in ERK1/2 activated SW480 and TG2 transduced RKO cells. Taken together these effects suggest a loop and mechanism, in which TGFβ1 directly, or indirectly induces TG2 via ERK1/2, and TG2 via mediating activation of ERK1/2 recruits the necessary machinery for increased TGFβ1 expression and secretion, thus sustaining EMT (**Figure 4.5.1**). The involvement of TG2 in upregulating TGFβ1 expression could be validated in the SW620 cells where loss of β1 integrin expression prevents TG2 from playing a role in ERK activation, and thus provides a proof of concept wherein TG2 silencing in these cells have no effect on TGFβ1 cellular expression or release into the extracellular milieu.

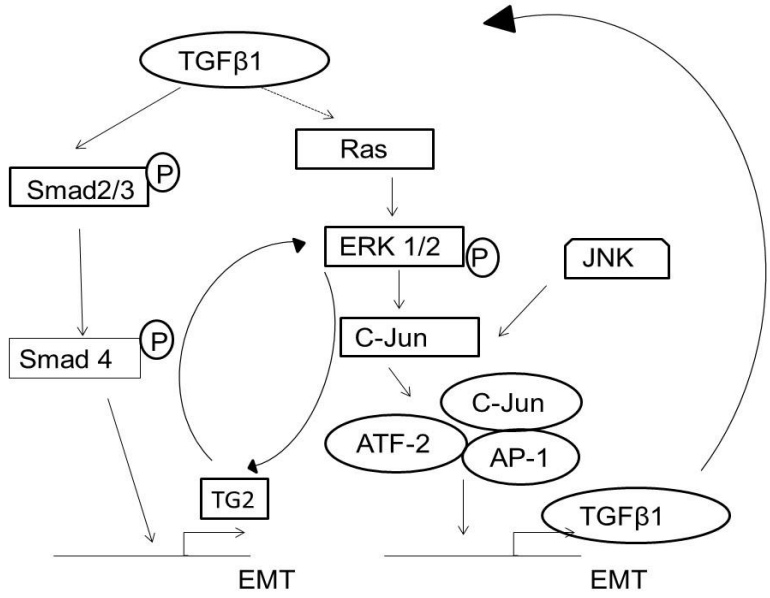


Figure 4.5.1 Proposed mechanism of TG2 induced expression of TGFβ1 and TGFβ1 regulation of TG2. TGFβ 1 induces ERK activation in a non-canonical fashion via Grb and SOS activation of Ras, Activated ERK induces the expression of TGFβ1transcription factor c-Jun and TG2. c-Jun is activated by JNK, activated c-Jun translocates into the nucleus where it associates with other TGFβ 1 transcription factors, to form activated protein 1(Ap-1)- an autoinducer of TGFβ1, this along ATF-2 induces TGFβ1 expression. In an auto-regulatory loop, TGFβ1 may induce TG2 via Smad 4, or by activating ERK. TG2 may then autoregulates itself by activating ERK which then induces TGFβ1expression and secretion.

Since TGFβ1 had no impact on EMT in the metastatic cell line SW620, alternative pathways that might be used by TG2 in driving EMT in SW620 cells were investigated. β-catenin is a known transcriptional regulator with oncogenic activity attributed to either activating mutation or interactions with mutated β-catenin interacting proteins (Morin et al., 1997), or by canonical activation of Wnt signalling. In carcinomas, β-catenin degradation is often blocked by either loss-of-function mutations of *Adenomatous Polyposis Coli (APC)* or by β-catenin mutations that render β-catenin stable (Condello et al., 2013). In SW620 cells, β-catenin is overexpressed and stabilised due to mutations in the *APC* gene (Ilyas 1997). The current data show that TG2 is able to interact with LRP 5, a Wnt co-receptor, which could lead to β-catenin stabilisation, and nuclear accumulation (**Figure 4.4.14**). This interaction can be reduced by the TG2-selective inhibitor 1-155 which can also lock TG2 into its inactive open conformation and prevent its externalisation (Badarau et al., 2015). More importantly, TG2 may also be able to stabilise β-catenin intracellularly since SW620 cells already possess loss or mutation in APC leading to loss of function of the glycogen synthase kinase-APC-Axin death complex, which induces proteasomal degradation of β-catenin (Ilyas et al., 1997). The results show that TG2 interacts with β-catenin an interaction which is reduced upon TG2 inhibition by 1-155, thus allowing for increased β-catenin association with ubiquitin. The results further suggest that TG2 stabilisation of β-catenin and its subsequent nuclear accumulation is independent of Src in SW620 cells since neither knockdown nor inhibition of TG2 resulted in reduced Src activation or reduced interaction between Src and β-catenin. However, in RKO and SW480 cells, TG2 did induce increased phosphorylation of Src (**Figure 4.4.16**). In ovarian carcinomas, TG2 recruits Src via β1-integrin and FN interaction for the stabilisation of β-catenin (Condello et al., 2013). Interestingly, in the SW620 cells, β1-integrin was poorly expressed. Therefore, Src in our cell model may have undergone some form of mutation that has left it constitutively activated with the subsequent loss of β1-integrin, or the TG2-induced activation of Src is tumour specific. The proposed mechanism of TG2 in β-catenin nuclear accumulation is shown in **Figure 4.5.2**.

While TG2 may play both extracellular and intracellular roles in WNT signalling (Condello et al., 2013; Myneni et al., 2015) upon further examination of the cellular location within which TG2 may

influence EMT in SW620 cells, only the cell permeable inhibitor (1-155) and not the cell impermeable inhibitor (R281) contributed to the reversal of EMT in a manner comparable to that found in TG2 knockdown cells. This suggests that intracellular TG2 may be key to the EMT process in the SW620 cells. The absence or low expression of integrins in these cells may diminish an extracellular role for TG2, as TG2-fibronectin-integrin interaction has been reported to be essential for cell adhesion, migration and invasion in epidermoid carcinoma cell line A-431 (Chen et al., 2010). This, however, may still be true for the primary cells RKO and SW480, as extracellular inhibition of TG2 resulted in a reduction in the EMT potential in this cells.

Figure 4.5.2. The proposed mechanism of TG2 regulation of β-catenin. Schematic showing extracellular TG2 interaction with LRP5in the propagation of Wnt signalling. Cytosolic TG2 interaction with β-catenin inhibits ubiquitination of β-catenin allowing for cytosolic accumulation and nuclear translocation of β-catenin where it interacts with lef-2/TCf transcription factors to regulate cell renewal, EMT and cell cycle progression.

4.6 Conclusion

This chapter shows that TGFβ1 may regulate TG2 expression via both canonical and non-canonical pathways, and thus potentiate EMT, and that TG2 may also regulate TGFβ1 expression, activation, and matrix abundance leading to a positive feed back loop involving both TG2 and TGFβ1 in which ERK1/2 activation may play a central role. Additionally, based on the different molecular signatures between SW480 and SW620 cells TG2-β1integrin association may be crucial in discriminating between potential extracellular and intracellular roles of TG2 in EMT and tumour progression, where the loss of β1 integrin may potentiate a dominant intracellular role for TG2. With this role in the SW620 associated with stabilisation of intracellular β-catenin and increased nuclear presence of β-catenin, which is responsible for activation of EMT and invasive genes such as *Fibronectin* (Gradl et al., 1999) and *S100A4* (Stein et al., 2006) and other genes associated with cancer stemness like *c-myc* (Hadjimichael et al., 2015), *CD44* (Brabletz et al., 2001), *Sox 9* (Blache et al., 2004) and angiogenesis such as *VEGF* (Zhang et al., 2001). Importantly, TG2 site-specific inhibitor (1-155) is able to perturb the TG2-TGFβ1 feedback loop and increase β-catenin association with ubiquitin, therefore antagonising the EMT process and the associated tumour progression.

Chapter 5

TG2 is upregulated in colorectal tumour spheroids characterised with stem-like phenotype

Chapter 5

5.0 TG2 is upregulated in colorectal tumour spheroids characterised with stem-like phenotype.

5.1 INTRODUCTION

There is a growing body of evidence showing that the formation and seeding of circulating tumour cells is highly dependent on a subpopulation of tumour cells with self-renewal potential and ability to differentiate into diverse tumour population, this subpopulation of cells is called the cancer stem cells (Han et al., 2013), and can repopulate a tumour leading to a relapse, or seed a metastasis (Polyak and Hahn, 2006). In colorectal cancer, patient survival depends on the stage of the disease, with metastasis accounting for poor patient prognosis. Cancer researchers have become aware of the existence of colorectal cancer stem cells (CSCs) with innate ability to propagate a metastasis and form highly drug-resistant phenotypes (O'Brien et al., 2007). These cells are considered to have the potential to acquire the many mutations that drive tumourigenesis (Wilson et al., 2011). Wei et al., (2012) have shown that cancer stem cells can sustain carcinogenesis, angiogenesis, metastasis, and recurrence of colorectal cancer after remission.

In cancer, many of the embryonic and wound healing processes are subverted for pathological gains, one of which is epithelial to mesenchymal transition (EMT) a process which enables epithelial cells to gain a mesenchymal phenotype (Kalluri and Weinberg, 2009). Various studies are now providing evidence that the EMT process is important in the development and acquisition of a cancer stem cell phenotype (Mani et al., 2008, Fan et al., 2012). Transglutaminase 2, has been reported to regulate EMT in various cancerous and fibrotic conditions (Fishers et al., 2015a, Nyabam et al., 2016) and in chapter 3 and 4 of this thesis TG2 has been shown to play a significant role in the induction of EMT in CRCs. In recent times TG2 expression has also been reported to be increased in certain cancer stem cell-like enriched populations including ovarian (Cao et al., 2012), breast (Kumar et al., 2011), squamous carcinomas (Fisher et al., 2015b) and mesotheliomas (Zonca et al., 2017). In addition, β-catenin, an oncogenic protein shown to be upregulated in colorectal cancer (Huang et al., 2017), gastric (Pasche et al., 2002) and ovarian cancer (Condello et al., 2015) is thought to be important in the formation of cancer stem cells by upregulating transcription factors associated with stem cells (Kiyonari et al., 2010).

For example, β-catenin is known to play an important role in the maintenance of intestinal stem cells (Novellasdemunt et al., 2015). In the previous chapters of this thesis, TG2 was shown to potentiate nuclear accumulation of β-catenin in colorectal cancer cells. In this chapter, an attempt is made at the enrichment of a cancer stem cell population *in vitro* using four different colorectal cancer cell lines. These include RKO, isogenic primary and metastatic pair (SW480 and SW620) and HCT116 cell- an MSI Duke's category D colorectal cancer cell line (Ahmed et al., 2013). These cells have been used to characterise and identify if TG2, EMT, and β-catenin have a role to play in colorectal cancer stem cells and to also establish the interaction of these players in the induction and maintenance of the colorectal cancer stem cells.

5.2 AIM AND OBJECTIVES

In the previous chapters, the work was directed to characterising colorectal cancer cells on disease severity using TG2, EMT, invasion and drug resistance. In this chapter, the focus is directed towards undertaking colorectal cancer stem cell enrichment from four different colorectal cancer cell lines RKO, SW480, SW620 and HCT116, and determining the role of TG2 in the acquisition of the cancer stem like cell phenotype in these colorectal cancer cells *in vitro*.

Objectives

1. Undertake cancer stem cell enrichment *in vitro* using the current set of colorectal cancer cell lines
2. Isolate cells with a cancer stem cell phenotype, and characterise them for markers of stemness
3. Characterise cells from (2) for EMT and β-catenin which are reported to be inducers of cancer stemness
4. Determine the role of TG2 in cancer stemness and its consequent role in cell invasion and angiogenesis.

5.3 METHODS

5.3.1 Tumour Spheroid Formation Assay on Poly-HEMA coated Plates

Cells in culture were collected and counted as described in **section 2.2.1.2-2.2.1.3**. The cells were suspended in spheroid medium and plated at 40,000 cells per 9.5 cm^2 plates coated with 1.2% (w/v) poly-HEMA in 95% ethanol coated dishes (Antonyak et al., 2011). The tumour sphere culture medium consisted of serum free DMEM/F12 (1:1) (Lonza, UK) containing 2% serum free supplement B27 (Invitrogen, UK), 20 ng/ml EGF, 0.4% bovine serum albumin and 4 μg/ml insulin. Tumour spheres were also cultures in humidified atmosphere at 37ºC and 5% (v/v) CO_2 and allowed 10 days for sphere formation.

5.3.2 Passaging of Tumour Sphere cells

Tumour spheroids cultured in suspension were collected by centrifugation at 300 ×g for 5 min, washed with sterile Phosphate buffered Saline (PBS), pH 7.4, and then the cells within the spheroid dispersed into single cells by treatment with pre-warmed 0.25% (w/v) trypsin in 2mM EDTA for 5 min at 37ºC. Complete cell culture medium was used to neutralise trypsin and cells were centrifuged for 300×g for 5 min. The supernatant was discarded and the pallet was suspended in complete spheroid media, counted and subsequently seeded into poly-HEMA-coated dishes to obtain the desired confluency.

5.3.3 Poly-HEMA Coating of cell culture plates

20mg/ml poly-HEMA solution was prepared via dissolving 1 g of Poly-HEMA (Sigma-Aldrich, UK) in 50 ml 95% (v/v) ethanol at 37ºC. Following the dissolution of poly-HEMA, the solution was clarified by centrifugation at 2,000×g for 20 min.). The desired volume of poly-HEMA (as listed in **Table 2.1**) was used to coat the tissue culture plates or Petri dishes at room temperature. Ethanol was allowed to evaporate gradually, after coating the cell culture vessel. The coated vessels were then UV-sterilized and rinsed with sterile PBS, pH 7.4, prior to seeding the cells (Kuroda et al., 2013).

Table 5.1 Required amount of poly-HEMA solution for dish coating.

Dish size	Volume of poly-HEMA solution (per dish or per well)
90 mm (diameter)	3.2 ml
60 mm (diameter)	1.3 ml
35 mm (diameter)	500 µl
12 well	200 µl
24 well	100 µl
48 well	70 µl
96 well	25 µl

5.3.4 Immunophenotyping by Flow Cytometry

Adherent monolayer cells and cells derived from tumour spheres were collected as described in **Sections 2.22-2.2.5** by trypsinisation and suspended in PBS containing 3%w/v bovine serum albumin (BSA) (Sigma Aldrich). The cells were then initially blocked with 10% (100mg/ml) normal human immunoglobulin (Ig) (Grifols, Cambridge, UK) for 30 min at 4 °C, after which cells were collected by centrifugation at 500 x g for 5min at 4 °C, and then incubated with CD44 phycoerythrin (PE) conjugated mouse monoclonal anti-human antibodies (Immunotools, Friesoythe, Germany) (1:100) diluted with 3% w/v BSA in PBS for 1h at 4^0C. Non-specific fluorescence was determined by incubating cells with isotype-matched control phycoerythrin-conjugated antibodies IgG2a (Immunotools). Cells were then washed 4 times by centrifugation at 500 x g with 3% w/v BSA in PBS. Immunoreactivity for each CD 44 was assessed by flow cytometry using a Beckman Coulter FC500 flow cytometer and data were analysed using Flowing Software 2.5.1 analysis Software.

5.3.5 Dot Blotting for Proteins

Cells were collected and counted as described in **Section 2.2.1.2-2.2.1.3** and seeded $2.5X10^5$ in complete growth media for 4 h in 24well plates. After which the complete growth media was replaced with ITS (Sigma Aldrich) supplemented serum-free cell culture media, cells were incubated for 16-18h and the cell culture media was collected and transferred onto nitrocellulose membrane using a vacuum dot blotter. The dot blotted membrane was used for detection of proteins as described in **Section 2.2.8**

by blocking the membrane in 5% w/v non-solid fat milk in Tris-buffered saline tween (pH 7.4) and incubated with primary and HRP conjugated secondary antibody and detected by chemiluminescence.

5.5.6 Matrigel endothelial cord formation assay

Endothelial cord formation was measured by a Matrigel endothelial cord formation assay as described by Wang et al., (2013). Phenol red-free basement membrane Matrigel (BD, Oxford, UK) with reduced grown factors was defrosted at 4^0C and used to coat 96 well plates, 30 µl/well. Plates were incubated at 37 ^{0}C for 30 min to allow the basement membrane to form a thin layer of gel. Human Umbilical Vein Endothelial Cells HUVECs (Promocell, Heidelberg, Germany) were cultured in endothelial growth medium (EGM) containing endothelial cell basal medium, 2% v/v FBS, hydrocortisone, bFGF, VEGF, hEGF, ascorbic acid, R3-IGF, heparin, and GA-100 (Lonza, Verviers, Belgium) under normal cell culture conditions. 1.5 X10^5 cells were seeded (**Section 2.2.1.2.-2.2.1.3**) onto Matrigel-coated wells and treated with either conditioned media from spheroid cells or the spheroid forming media with or without rhVEGF as positive and negative controls.

5.3.7 Soft Agar Colony Formation

Soft agar assay was performed for spheroid cells as according to (Kato et al., 2012). Each well of a 24-well plate was loaded with 1mL 0.5% w/v agar in DMEM: F12 supplemented with 10% (v/v) FBS as base. After polymerization of the base agar, 4000 cells were mixed in 0.75 mL 0.375% (w/v) agar in DMEM supplemented with 10% (v/v) FBS were added. The soft agar cultures were maintained in a humidified incubator at 37°C and 5% (v/v) CO_2 for 2 weeks before colonies photographed.

5.4 RESULTS

5.4.1 Colorectal cancer stem cell phenotype correlates with the aggressive nature of the cancer

The *in vitro* spheroid enrichment culture has been established as a way of enriching cancer cells exhibiting a stem cell phenotype. This involves culturing adherent cells in non-adherent condition in a serum-deprived medium (Fisher et al., 2015a). By performing the spheroid enrichment assay in **Figure 5.4.1A** only metastatic SW620 cells (Duke's C cancer disease stage) and high diseased stage HCT116 (Duke's Stage D) were able to form compact spheroids in culture. Surviving single cells in suspension first aggregate, and then form compact anchor-independent spheres. This was not, however, the case with the RKO and SW480 (Duke's B Stage) as surviving cells formed large loose aggregates which became sediments on the bottom of the plates, losing their adherence independence and spheroid compaction. Given these differences in spheroid formation, the clonogenic ability of RKO, SW480, SW620, and HCT116 on soft agar was assessed in Figure **5.4.1B**. Metastatic SW620 and Duke's D category HCT116 cells readily formed colonies on soft agar compared to primary cells SW480 and RKO confirming the aggressiveness of these cells.

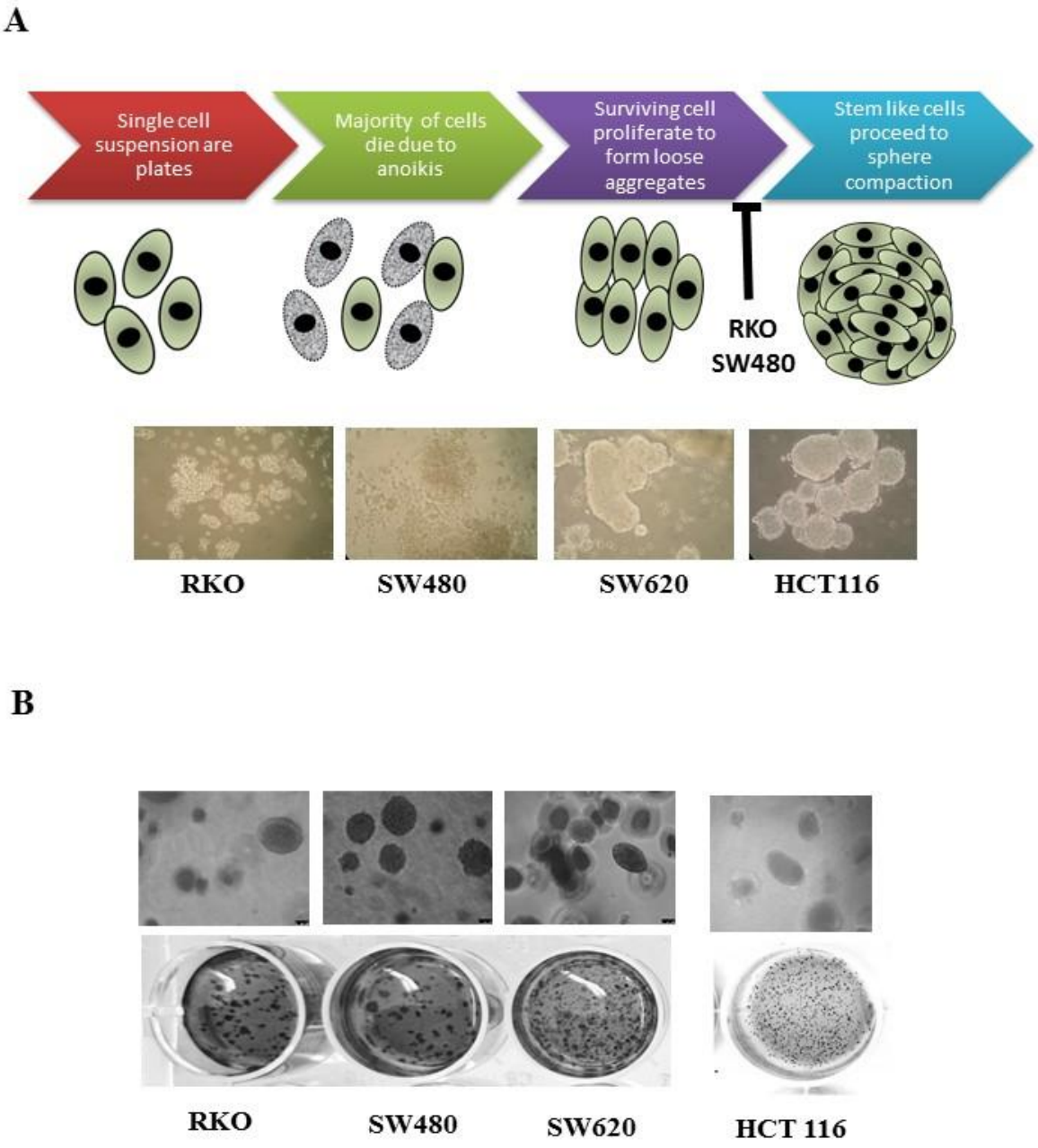


Figure 5.4.1Aggressive cancer cell lines form spheroids in nonadherent cell culture. (**A**)Spheroid formation *in vitro*, primary cells SW480 and RKO form loose aggregates, while metastatic SW620 and more aggressive HCT116 cells proceed to form compact spheroids. Representative images were taken after 12days of culture. (**B**) Soft Agar colony formation assay for CRCs. Colonies were allowed to grow for 14 days, and then stained with crystal violet. Representative image of colony formation in soft agar (top panel) at 10X objective (n=3)

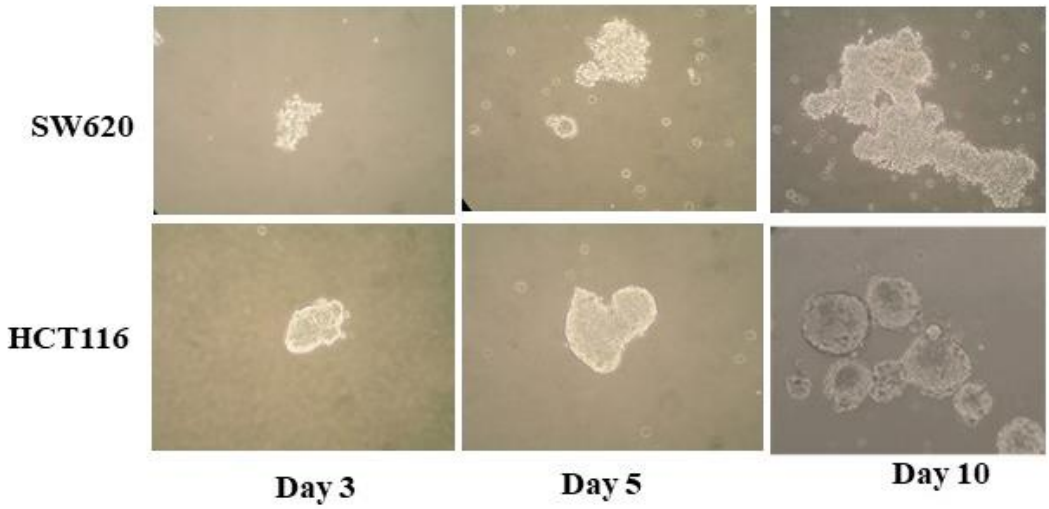


Figure 5.4.2 *In vitro* Spheroid Formation.Stages of spheroid formation in SW620 and HCT116 cells allowed to grow for 10days. Compaction of spheroids is observed from 5 days. Spheroids formation was captured at 20X objective (n=3).

5.4.2 CD44 and transcription marker of stem cells is upregulated in Spheroid cells

A time course of the culture of HCT116 and SW620 spheroids is shown in **Figure 5.4.2** and shows surviving cells proliferate, aggregate and eventually form compact spheroids after 10 days. Spheroids were next characterised for the presence of stem cell markers. In **Figure 5.4.3** whole cell lysates of cells derived from the spheroid culture and the traditional monolayer, adherent cell culture was collected and used for the detection of stem cell markers. CD44 a cell surface protein marker for colorectal stem was found to be expressed significantly (>2 folds) over what was detected in the corresponding SW620 and HCT116 monolayer cells (SW620-M and HCT116-M). Furthermore, the expression of the stem cell transcription factors Sox 2, Nanog, and Oct3/4 was determined by Western blotting in both monolayer and spheroid cells. In the SW620 cell line, the cells derived from the spheroids (SW620-S) expressed significantly ($p<0.05$) higher amounts of detectable Sox 2 (2.53 folds) Nanog (1.93 folds) and Oct 3/4 (1.92 fold) compared to the parental monolayer cell (SW620M). This trend was also observed in the HCT116 cells, with spheroid cells (HCT116-S) expressing significantly higher stem cell transcription factor, SOX (1.49 folds) Nanog (1.50 folds) and Oct 3/4 (2.70 folds) compared to the monolayer cells. In **Figure 5.4.4** the expression of CD44 on the cell surface was measured by flow cytometry on cells derived from spheroid and monolayer cultured cells in both HCT116 and SW480

cells. The flow cytometry data shows that the cells obtained from the spheroids significantly expressed increased CD44 when compared to their respective monolayer cells both SW620 and HCT116 cells, confirming the findings by Western blotting (**Figure 5.4.3**).

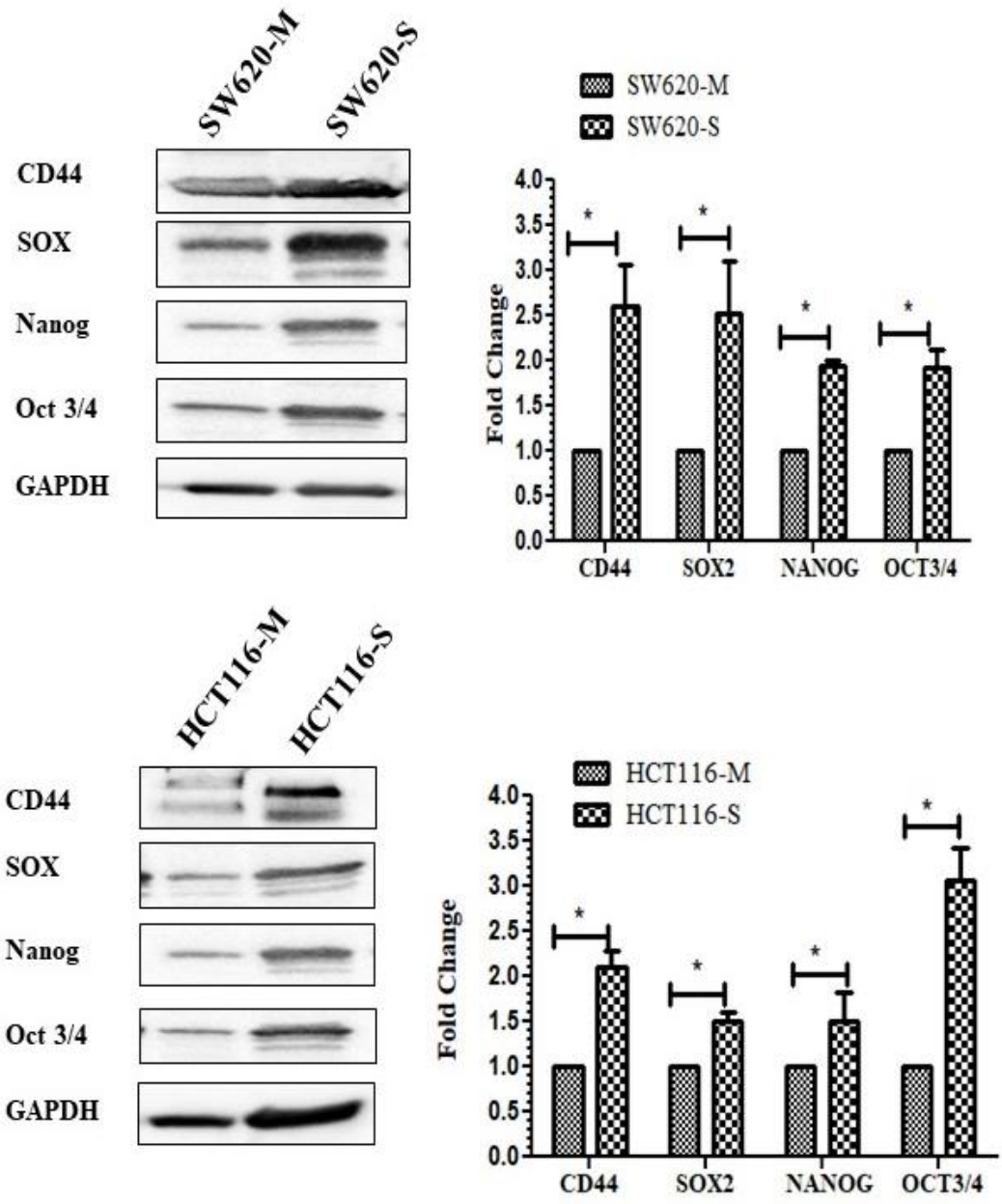


Figure 5.4.3 Spheroid cells display stem-like properties. Representative Western blot of whole cell lysate proteins containing cell surface protein associated with GIT stem cells CD44, and transcription markers associated with stem cells Sox 2, Nanog, and Oct 3/4. Bar chart shows mean relative densitometry ratios of protein expression normalised against GAPDH (mean±SD, n=3). *-Statistically ($p<0.05$) different from monolayer cells.

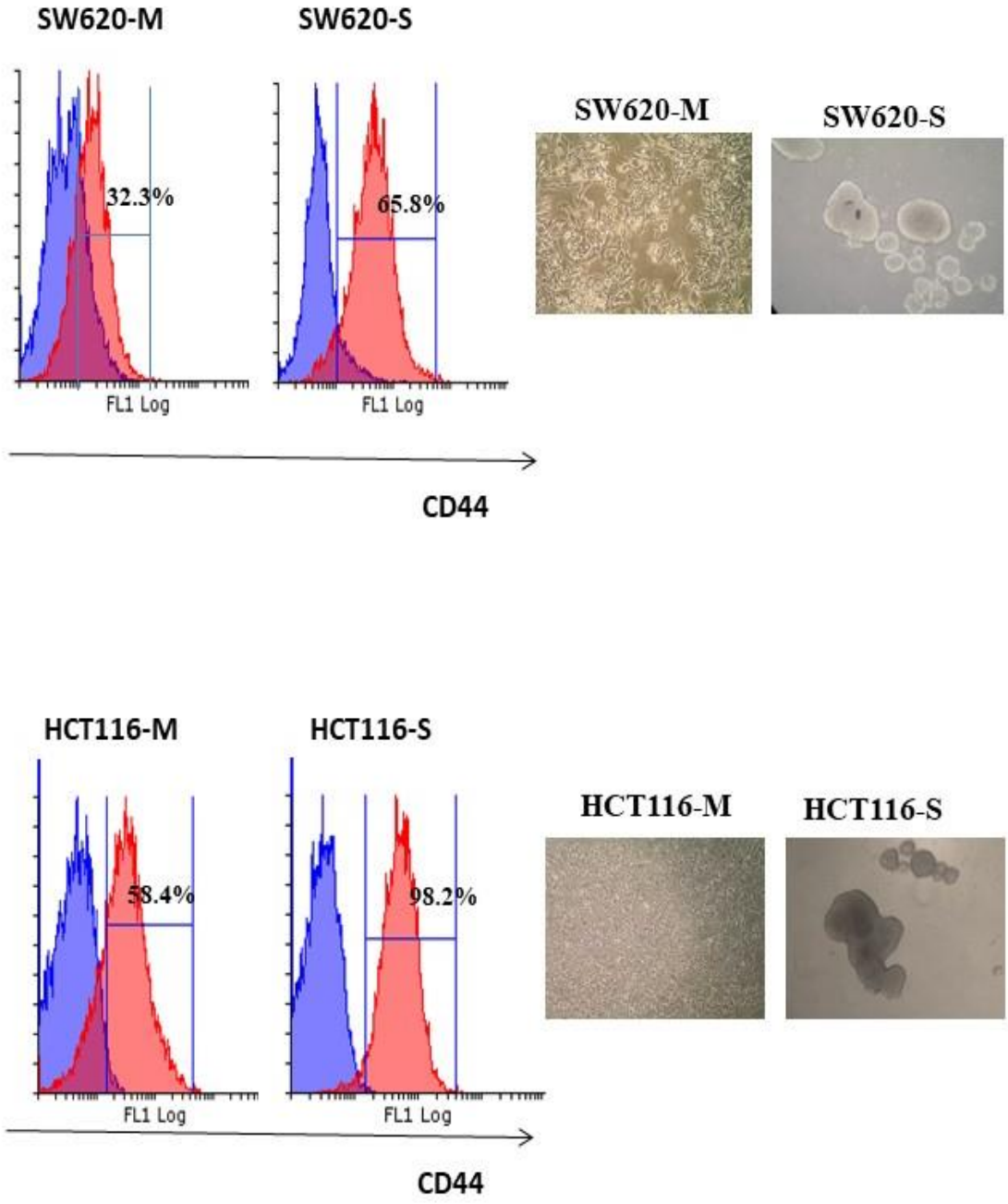


Figure 5.4.4 Flow cytometry detection of CD44 in monolayer and spheroid cells. Flow cytometry analysis of $CD44^{+}$ sub-populations of SW620 and HCT116 monolayer and spheroids. Cells were stained with an anti-human CD44-phycoerythrin (PE) antibodies. Isotype-matched human antibodies served as control. Isotype represented in blue and CD44 positive cells represented by the red histogram. (n=3). Representative images of HCT 116 monolayer (M), HCT 116 Spheres (S), SW620 monolayer and SW620 spheroid cells. Phase-contrast microscope images at 20X objective (right panel) (n=3).

5.4.3 TG2 and EMT is upregulated in colorectal cancer stem cells

Following the characterisation and validation of the spheroid cells as possessing stem cell characters, the spheroid cells were characterised for TG2 expression in the whole cell lysates by Western blotting. **Figure 5.4.5** indicates that TG2 expression is significantly expressed in spheroid cells, SW620-S and HCT116-S compared SW620 and HCT116 monolayer cells. In addition, epithelial to mesenchymal transition which has been reported to be a driver of a stem cell phenotype was next characterised in the spheroids. EMT marker vimentin showed significantly higher expression in the spheroids of both HCT 116 and SW620 compared to their respective monolayer counterparts. Additionally, to confirm the increase of the EMT process in the spheroids, the expression of transcription factors that regulate EMT were determined by Western blotting and show that transcription factors that induce EMT such as Slug and Twist were significantly expressed. In contrast, the expression of epithelial marker ZO1 which was significantly reduced in the spheroids compared to the monolayer cells.

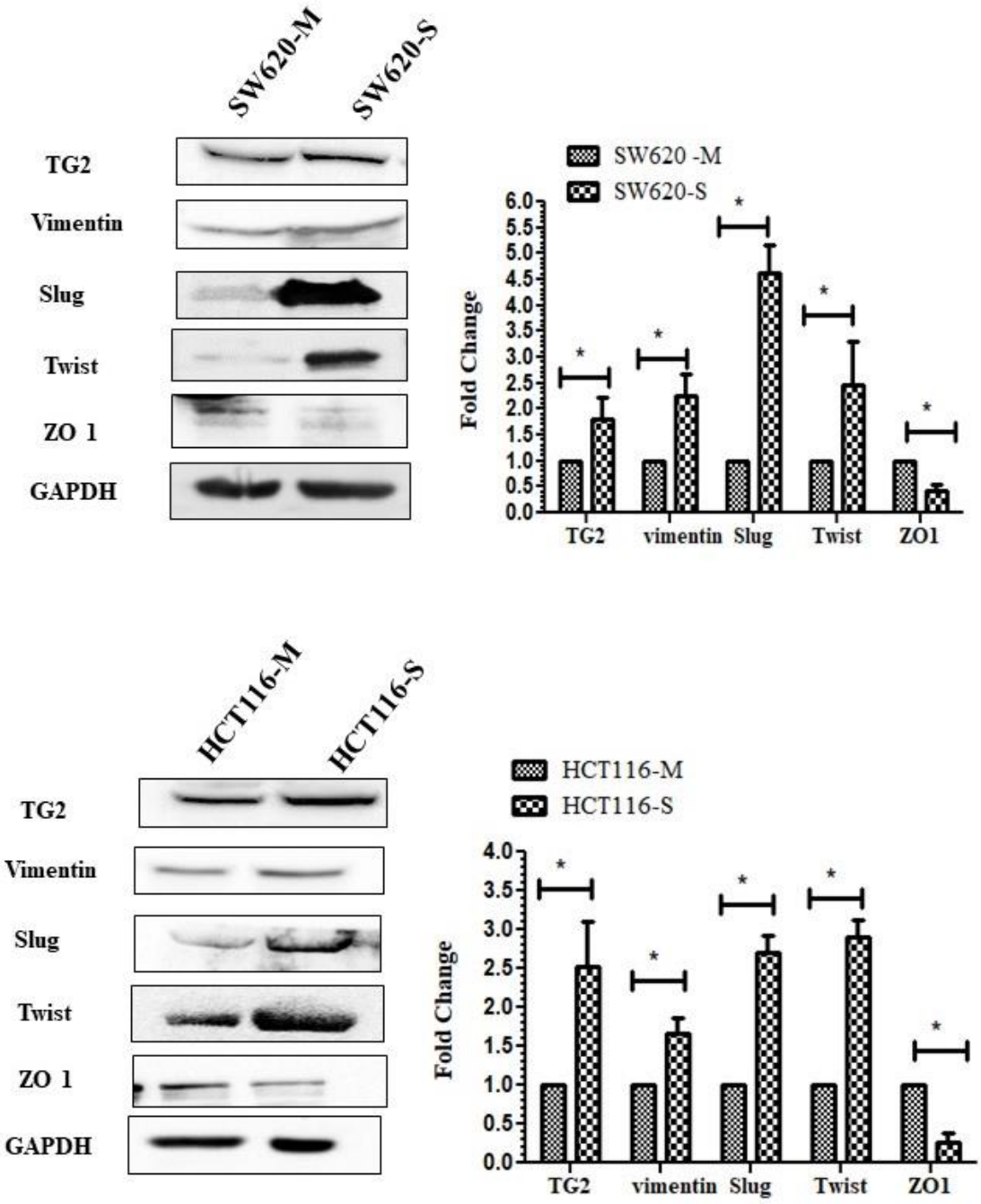


Figure 5.4.5 TG2 and EMT is upregulated in Spheroid cells with stem cell-like properties. Representative Western blot of whole cell lysates of parental adherent monolayer cells and spheroid cells from SW620 and HCT116 cells. Expression of TG2 and vimentin and transcription markers of EMT are increased in spheroid containing stem-like cells. Expression of tight junction protein ZO-1 is reduced in spheroid. Bar chart show mean relative densitometry ratios of protein expression normalised against GAPDH (n=3, mean±SD). *-Statistically ($p<0.05$) different from monolayer cells.

In **Figure 5.4.6** an attempt was made to delineate the role of TG2 on EMT in HCT116 cells. The role of TG2 in EMT in SW620 cells have been established (Chapters 3 and 4 of this thesis) by TG2 knockdown or pharmacological inhibition. This was also confirmed in HCT116 cells, and in **Figure 5.4.6** shRNA specific for human TG2 was used to knockdown TG2 expression in HCT116 which led to a concomitant significant reduction in the expression of EMT marker vimentin, and a significant reduction in the expression of EMT transcription factor slug. In addition, ZO1 a tight junction protein and an epithelial marker were seen to be significantly increased following TG2 knockdown by shRNA. A similar trend was observed following treatment of HCT116 cells with TG2 selective inhibitor 1-155 at a dose of 1μM, for 48h.

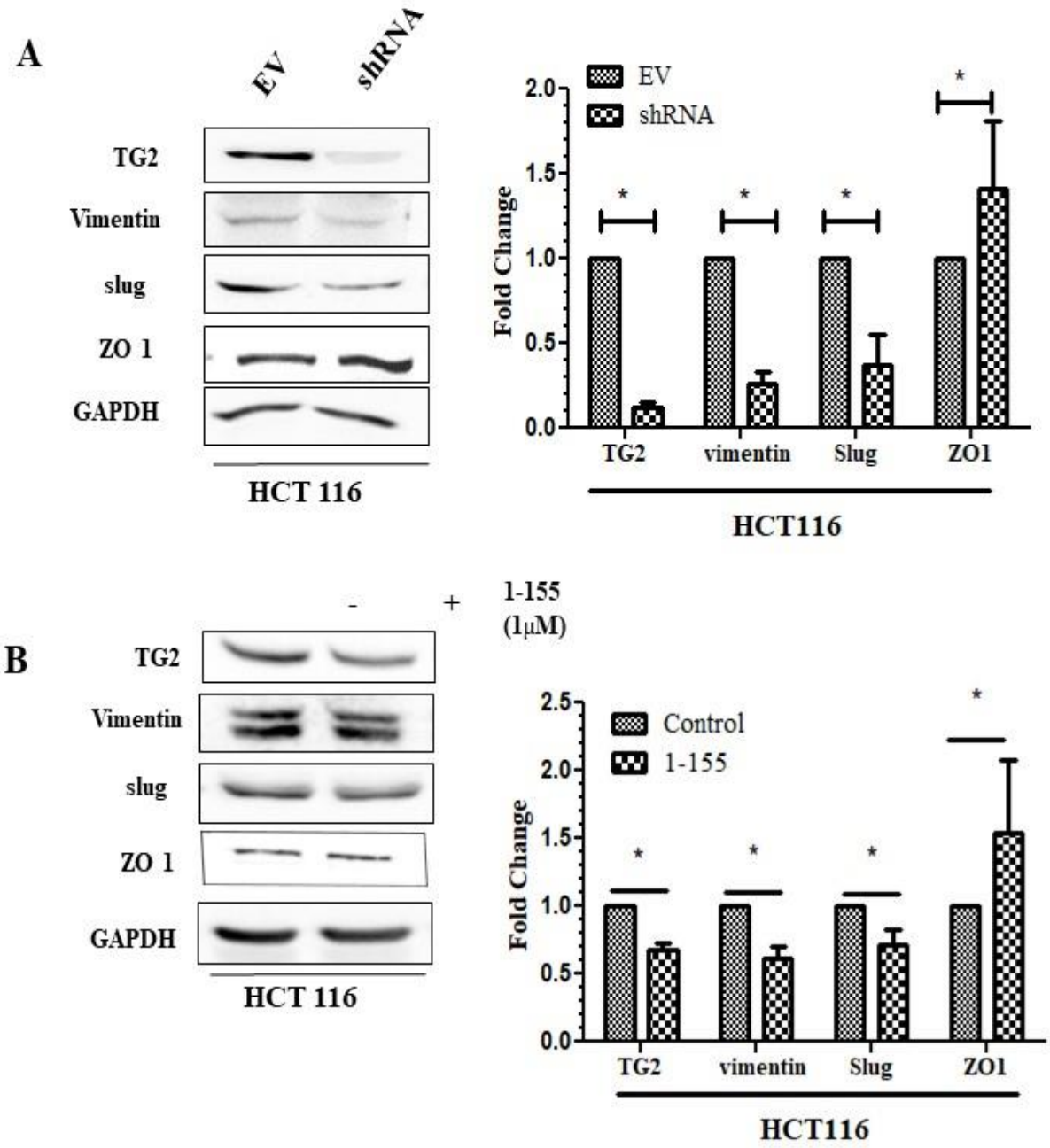


Figure 5.4.6 TG2 knockdown or inhibition attenuates EMT in HCT116 cells. (**A**) Representative Western blot of TG2, mesenchymal marker vimentin, transcription factors of EMT slug, and expression of tight junction protein ZO in whole cell lysates of TG2 knockdown cells and (**B)** cells treated with TG2 cell-permeable inhibitor 1-155, 1μM for 48h. Bar chart shows mean relative densitometry ratios of protein expression normalised against GAPDH (n=3, mean±SD)). *-Statistically ($P<0.05$) different from EV or control cells.

In **Figure 5.4.7A** the effect of TG2 EV (empty vector) transduction on the expression of TG2 and EMT marker vimentin was, determined, with the empty vector transduction having no effect on TG2 and EMT expression. Additionally, the effect of TG2 site-specific inhibitor 1-155 (0.1-2μM) was determined in HCT116 cells. In **Figure 5.4.5B**, TG2 inhibitor did not significantly affect cell viability over a 48h treatment period.

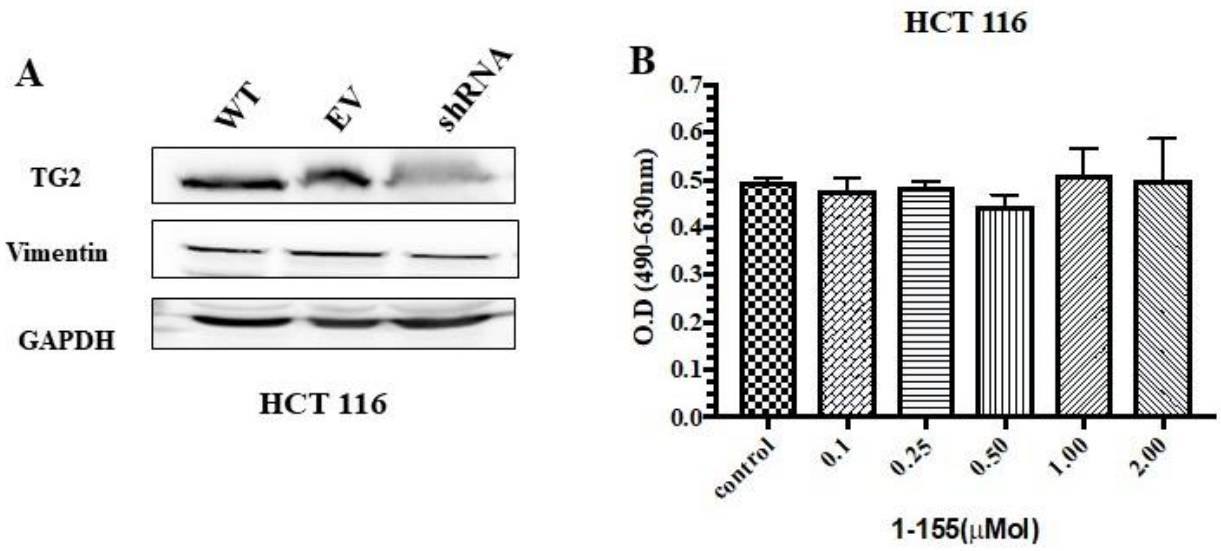


Figure 5.4.7 Empty Vector transduction has comparable TG2 and EMT profile to wild-type cells. (**A**) Western blot detection of TG2 and vimentin in empty vector, TG2 shRNA, and wild-type HCT116 cells (n=2). (**B**) TG2 inhibition does not affect alter cell viability in HCT116 cells as measured by XTT over a 48h treatment period (n=3, mean±S.D).

5.4.4 Colorectal stem-like cells display an invasive phenotype *in vitro*

The cancer stem cells subtype is suggested to be the more aggressive type of cancer (O'Brien et al., 2007, Fan et al., 2012, Fisher et al., 2015a), This hypothesis was tested and cells from spheroid and monolayer cells were collected and used for a Transwell invasion assay. In the SW620 cell line, spheroid cells were able to significantly invade collagen IV-coated inserts in Transwell migration chambers compared to their parental monolayer counterparts, with approximately 50% more migrated cells counted for in the spheroid cells. Since cell invasion in these cells correlated with TG2 expression in SW620 cells (**Section 3.4.2.6**), and TG2 expression is also upregulated in SW620 spheroid, the

spheroid cells were then treated with TG2 site-specific inhibitor 1-155 (1μM) and this treatment significantly impeded cellular invasion when compared to the control cells. In addition, the reduction in cell invasion of the spheroid cells treated with TG2 inhibitor was also significantly less when compared to the invasive cell count of the monolayer cells (**Figure 5.4.8**).

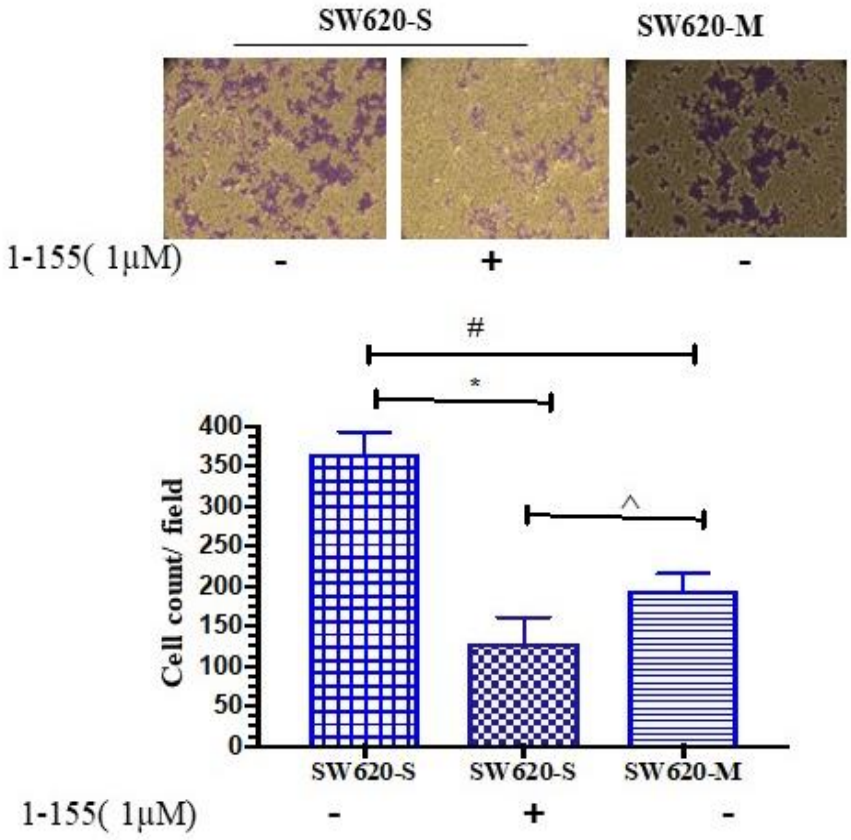


Figure 5.4.8 TG2 inhibition reduces the invasiveness of colorectal cancer spheroids with stem cell properties in SW620 cells. Representative images of crystal violet stained cells that have invaded collagen IV and the Transwell inserts. Images were captured using phase contrast microscope with the 10X objective. For 1-155 (1μM) treated cells, cells were pre-treated with 1-155 for 24h prior to seeding cells and were further treated with 1-155 (1μM) after cell seeding for the duration of the experiment. Cells were counted in the 10X objective field from 10 different fields of each insert. DMSO, the control vehicle was used to treat spheroid and monolayers SW620 cells. (n=3, mean±S.D, * significant different from 1-155 treated spheroid cells, # significantly different ($P<0.05$).from monolayer cells, ^ monolayer cells significantly different from1-155 treated spheroid cells).

A similar cellular invasion trend was observed in the HCT116 cells, with the spheroid cells scoring significantly higher cellular invasion compared to the monolayer cells, and spheroid cell treated with TG2 site-specific inhibitor 1-155 (1μM) (**Figure 5.4.9**). Again, it was observed that TG2 inhibition

resulted in a further reduction of cellular invasion in the spheroid cells compared to the monolayers, although this difference was not significant.

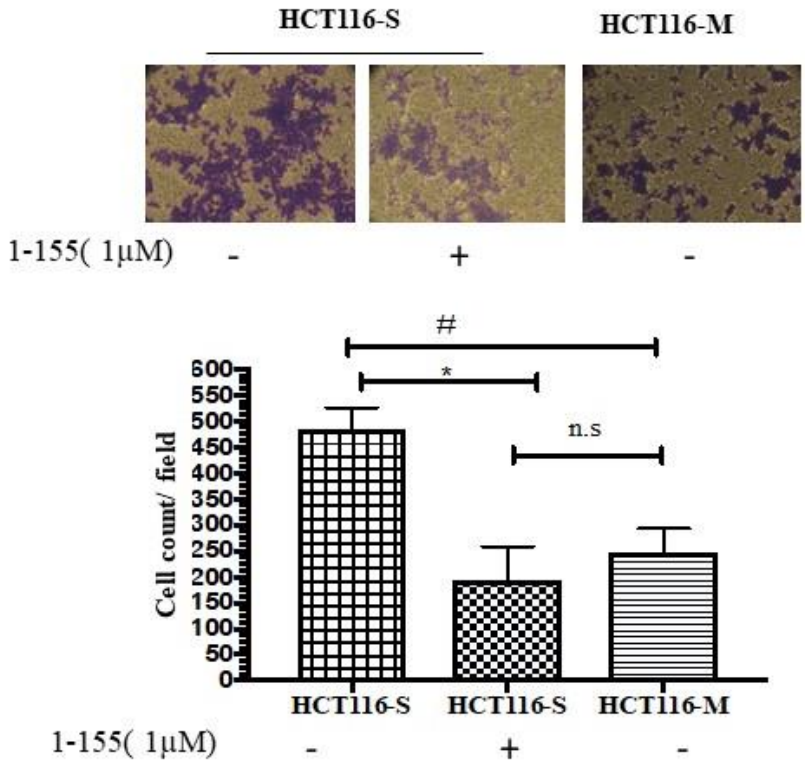


Figure 5.4.9 TG2 inhibition reduces the invasiveness of colorectal cancer spheroids with stem cell properties in HCT116 cells. Representative images of crystal violet stained cells that have invaded collagen IV and the transwell inserts. Images were captured using phase contrast microscope with the 10X objective. For 1-155 (1μM) treated cells, cells were pre-treated with 1-155 for 24h prior to seeding cells and were further treated with 1-155 (1μM) after cell seeding for the duration of the experiment. DMSO, the control vehicle was used to treat HCT116 spheroid and monolayer cells. (n=3, mean±S.D * significantly different from 1-155 treated spheroid cells, # significantly ($P<0.05$) different from monolayer cells, N.S-not significant $P>0.05$).

5.4.5 TG2 plays a role in the formation of spheroids

Since the spheroid cells are associated with EMT and TG2 has been shown to play a role in the induction of EMT, the functional role of TG2 in the formation of spheroids in SW620 and HCT116 cells was investigated. Here the involvement of TG2 in spheroid formation was investigated by knocking down or inhibiting TG2. Cells treated with TG2 shRNA or treated with TG2 inhibitor 1-155 (1μM) were allowed to form spheroids *in vitro*, and after 10days of spheroid formation, XTT was used to measure

the cell viability of the cells. The cell viability of cells treated with TG2 inhibitor or TG2 shRNA was comparable and not significant, but cell viabilities were significantly lower in these cells than in the control untreated cells, and this observation was seen in both SW620 and HCT116 cell lines **(Figure 5.4.10)**.

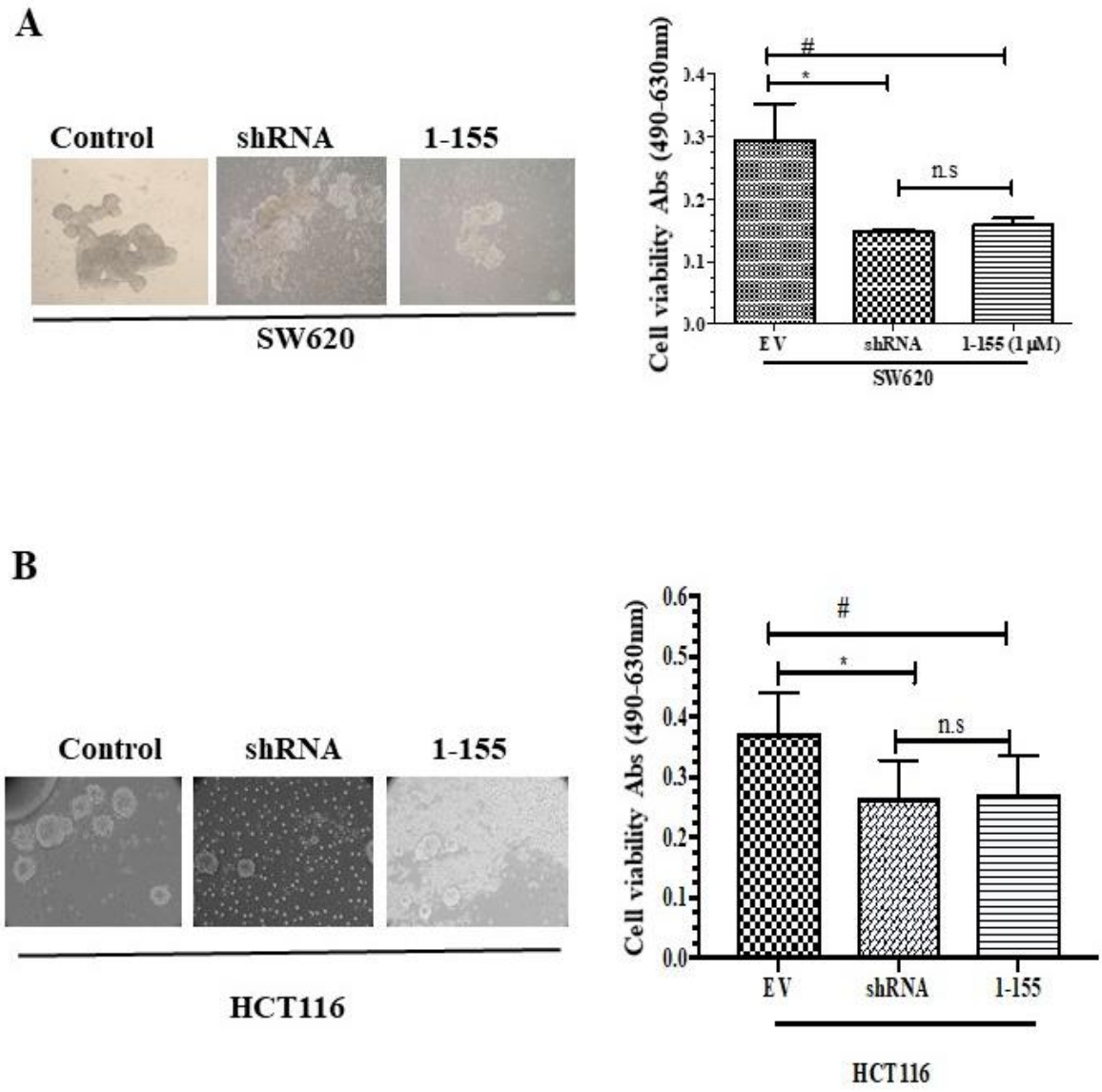


Figure 5.4.10 TG**2 induces a colorectal stem cell-like property in SW620 and HCT116 cells.** TG2 knockdown(shRNA) or TG2 inhibition(by 1-155) 1µM reduces the enrichment of spheroids with stem cell-like phenotype in (A) SW620 cells and (B) HCT116 cells For the treatment group CRCs were seeded in spheroid forming media contain 1µM TG2 selective inhibitor 1-155 for 10 days, then spheroid images were captured and cell viability was performed using XTT. (n=3, mean±S.D * EV-control significantly ($P<0.05$) different from shRNA, EV-control significant different from 1-155 treated cells. N.S-not significant $P>0.05$).

5.4.6 Stem cell regulator β-catenin is upregulated in colorectal cancer stem cells

β-catenin is a regulator of stem cell maintenance and differentiation (Miki et al., 2011) and is upregulated in cancer stem cells (Lee et al., 2014). In **Figure 5.4.11** β-catenin expression in SW620 cells was significant and over 3 folds higher in the spheroid cells compared to the monolayer cells. Additionally, the expression of cyclin D1 was observed to be significantly increased by 2 fold in the spheroid cells compared to the monolayer cells in SW620 cells. The increased expression of cyclin D1 may confirm not just increased cellular β-catenin in the spheroid cells but also a potential increase in nuclear activity of β-catenin. This pattern of observation was also confirmed in the HCT116 cells with β-catenin significantly expressed (>2 folds) in the spheroid-derived cells compared to the monolayer-derived cells, with a corresponding significant increase (in excess of a 2-fold increase) in cyclin D1 expression the spheroid cells compared to the monolayer cells. This confirmed the association between spheroid cells, β-catenin, and increased β-catenin nuclear activity by measuring cyclin D1 expression.

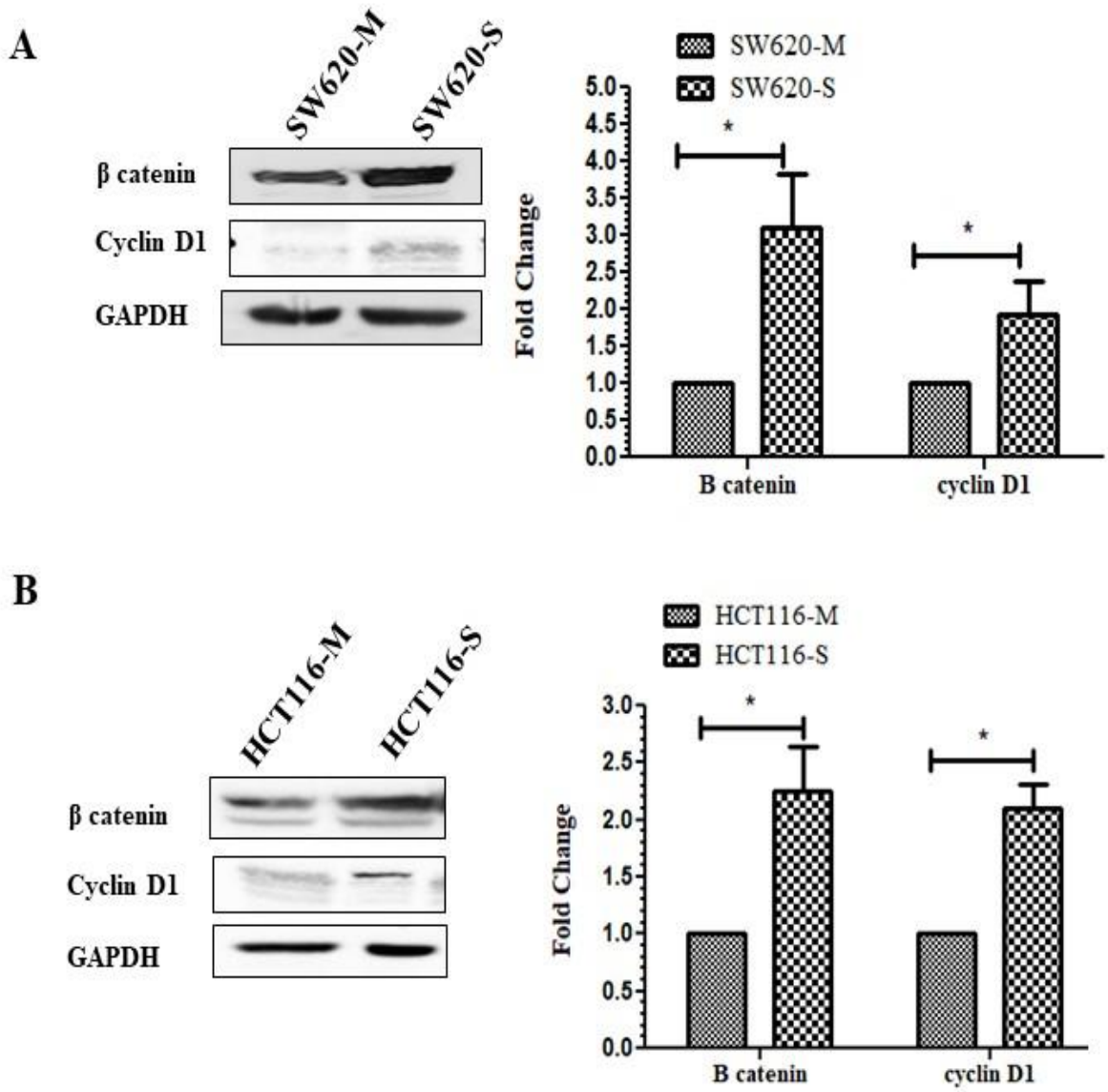


Figure 5.4.11 Intestinal stem cell regulator β-catenin is overexpressed in Spheroid derived cells. Representative Western blot of monolayer and spheroid cells from (**A**) SW620 and (**B**) HCT116 detecting the presence β-catenin and cyclin D1 expression in the whole cell lysate. Bar charts show mean relative densitometry ratios of protein expression of the proteins against GAPDH (n=3, mean±SD). *-Statistically ($P<0.05$) different from monolayer cells.

5.4.7 TG2 may regulate β-catenin expression in colorectal cancer stem cells

In Chapter 4 of this thesis, it was shown that TG2 was able to interact with β-catenin, increasing nuclear translocation of β-catenin and that inhibition of TG2 increased the interaction between β-catenin and ubiquitin in SW620 cells. This alongside the significantly increased expression of TG2 and β-catenin in spheroids derived from SW620, and the effect of TG2 knockdown or inhibition may suggest TG2 regulation of β-catenin in spheroid cells derived in SW620 cells. For the HCT116 cells and in **Figure 5.4.12A** TGF β1 at 2.5ng/ml significantly increased TG2 expression by 1.74 folds, with a corresponding 2.19-fold increase in β-catenin, and 1.59-fold increase in activated ERK 1/2 (p-ERK) in HCT 116 cells. This effect of TGF β1 was found to be abrogated by ERK1/2 inhibition using 10μM ERK inhibitor PD98059. ERK 1/2 inhibition in the presence of TGF β1 led to about 50% loss in TG2 expression compared to control cells, and 70% loss compared to the TGF β1 treated cells. This corresponded to a significant 20% loss in β-catenin expression compared to the control untreated cells and to about 60% loss compared to the TGF β1 treated cells. Indicating a potential role of ERK 1/2 in β-catenin expression. Furthermore, following that TG2 inhibition also reduced spheroid formation (**Figure 5.4.10**), TG2 inhibition in HCT116 cells led to a significant 20% loss in the β-catenin expression a consequence of a significant 50 % loss in ERK 1/2 activation. This may suggest that TG2 mediated ERK 1/2 activation in the HCT116 cells may play a role in regulating β-catenin expression (**Figure 5.4.12B**).

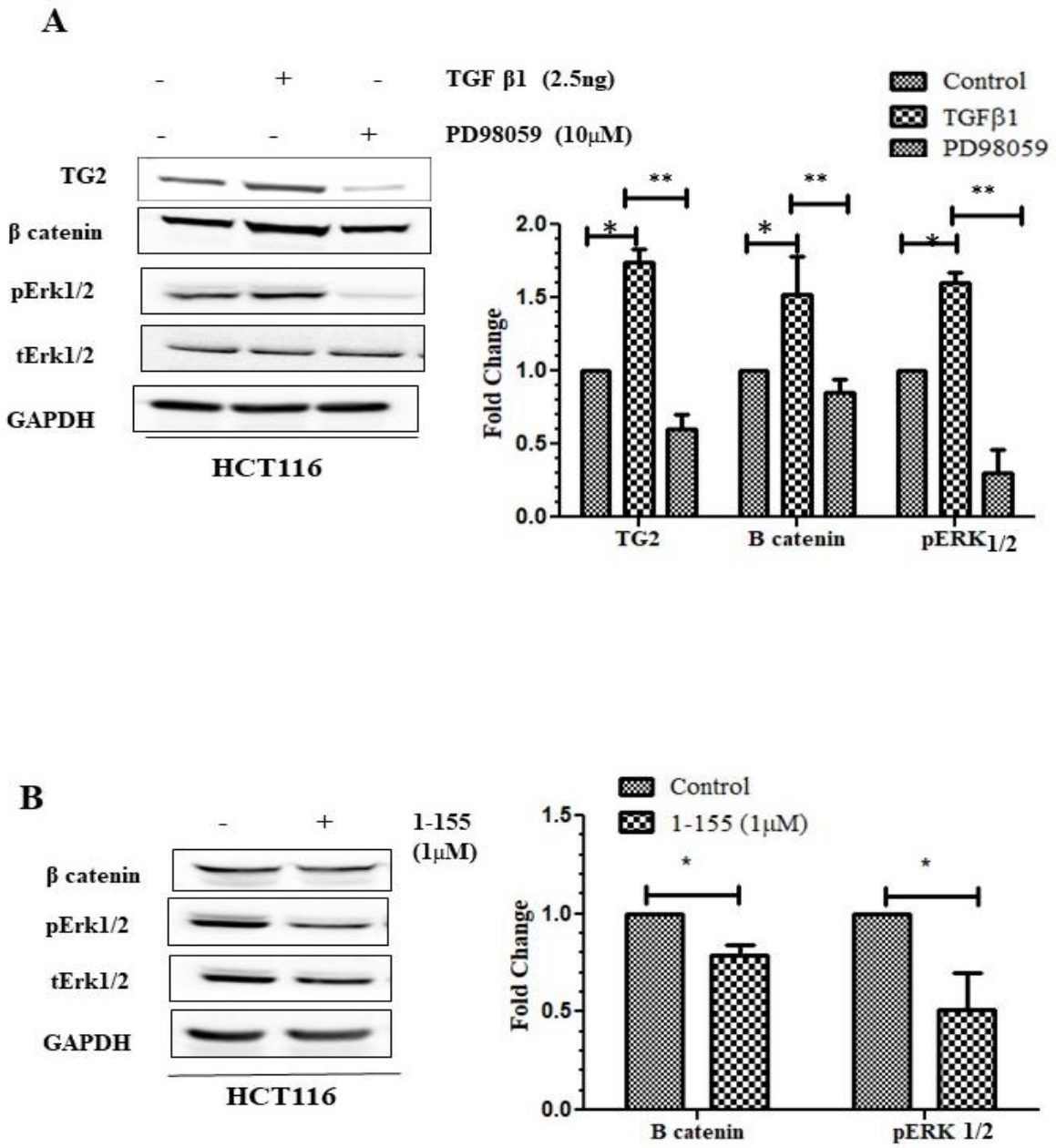


Figure 5.4.12 TG2 governs intestinal stem cell regulator β-catenin (A) ERK activation increases β-catenin expression in HCT116 cells. Western blot of TG2, β-catenin and p-ERK 1/2 in whole cell lysates of HCT116 cells treated with human recombinant TGFβ1 (2.5ng/ml) and ERK 1/2 inhibitor PD98059 (10μM) for 48h. Bar chart show relative densitometry ratios of protein expression normalised against GAPDH, p-ERK 1/2 normalised against t-ERK 1/2 (n=3, mean±S.D).*- Statistically ($P<0.05$) different from control cells, **-statistically different from both control and TGFβ1 treated cells (**B**) TG2 activates ERK and β-catenin expression. Western blot of TG2, β-catenin and ERK activation in whole cell lysates of HCT116 monolayer cells following treatment with TG2 inhibitor 1-155 (1μM) for 48h. Bar chart shows mean relative densitometry ratios of protein expression normalised against GAPDH, p-ERK 1/2 normalised against t-ERK (n=3, mean±S.D). *-Statistically ($P<0.05$) different from control cells.

5.4.8 ERK 1/2 inhibition repeals Spheroid formation in HCT116 cells

Since TG2 inhibition led to reduced ERK 1/2 activation and β-Catenin expression in the HCT116 cells. The HCT116 cells were treated with TGF β1 or ERK 1/2 inhibitor to determine their effect on spheroid formation via determining cell viability. In **Figure 5.4.13** TGFβ1 increased spheroid measured by cell viability, albeit not significantly when compared to the control cells. ERK1/2 inhibition with 10μM PD98059 significantly reduced the spheroid forming potential of HCT116 cells as determined by XTT, when compared to the control or TGF β1 treated cells**.**

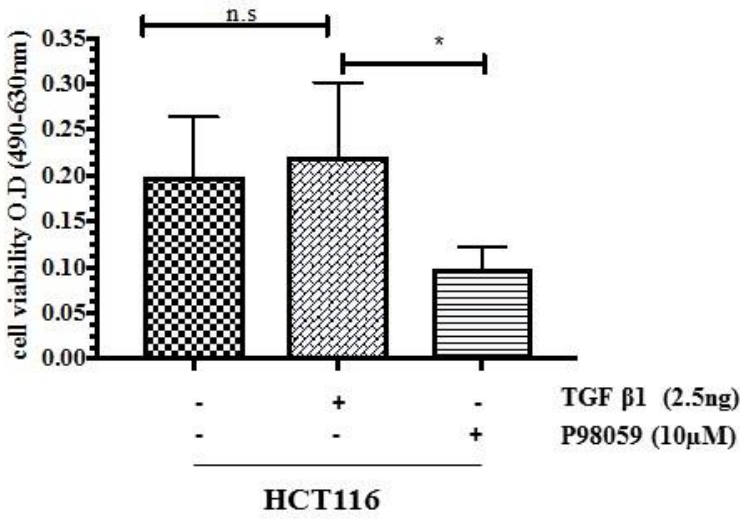


Figure 5.4.13 ERK 1/2 inhibition reduces spheroid formation. ERK inhibition reduces spheroid forming potential of HCT116 cells. HCT116 cells were seeded in spheroid forming media containing PD98059 (10μM) or TGFβ1 (2.5ng/ml) for 10days and cell viability was performed using XTT. Control cells were treated with DMSO. (n=2, mean±S.D * significantly different ($P<0.05$) from PD98059 treated cells. N.S-not significant $P>0.05$).

5.4.9 Tumour Spheroids containing cancer stem cells promote increased angiogenesis

Tumours originating from cancer stem cells are more vascular compared to tumours from monolayer cells (Wei et al., 2012). Therefore, the effect of conditioned media from spheroid cells on HUVEC cord formation when cultured on Matrigel was determined. In **Figure 5.4.14** HUVEC cells seeded on Matrigel were treated with rhVEGF (10ng/ml) or conditioned media from the SW620 spheroids grown over a 24h period. The conditioned media treated HUVEC's at 6h started aligning to form cords and by 24h endothelial cords were observed in both rhVEGF and conditioned media treated HUVEC cells. Following this, the spheroid containing stem-like cells were characterised for angiogenic factors. In **Figure 5.4.15** both SW620 and HCT116 spheroid cells displayed a significant cellular expression of VEGF (3.99 and 1.99-fold increase for SW620 and HCT116 respectively), HIF 1α (1.45 and 1.52-fold increase for SW620 and HCT116 respectively) compared to their respective monolayer cells.

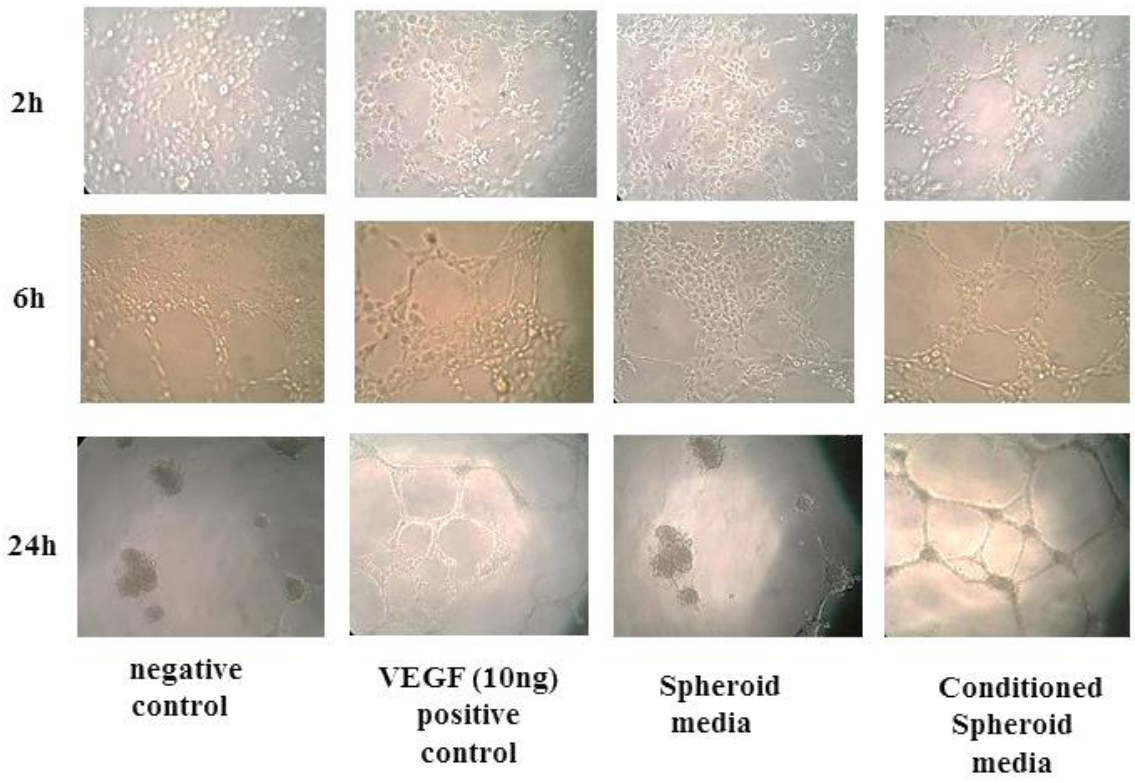


Figure 5.4.14 Spheroid containing colorectal cancer stem-like cells induce angiogenesis *in vitro.* HUVECs cells-3000 cells per well was seeded 96-well plates containing Matrigel and induced to form tubule-like structures (cords). HUVEC cells were treated with 10ng rhVEGF which served as positive control, sphere media which served as blank for the sphere condition media, and condition media from spheroid cells. Cells were treated for 24 h and phase contrast images at the 10X objective were taken at 2, 6, and 24h periods. Images are representative of n=2 experiments.

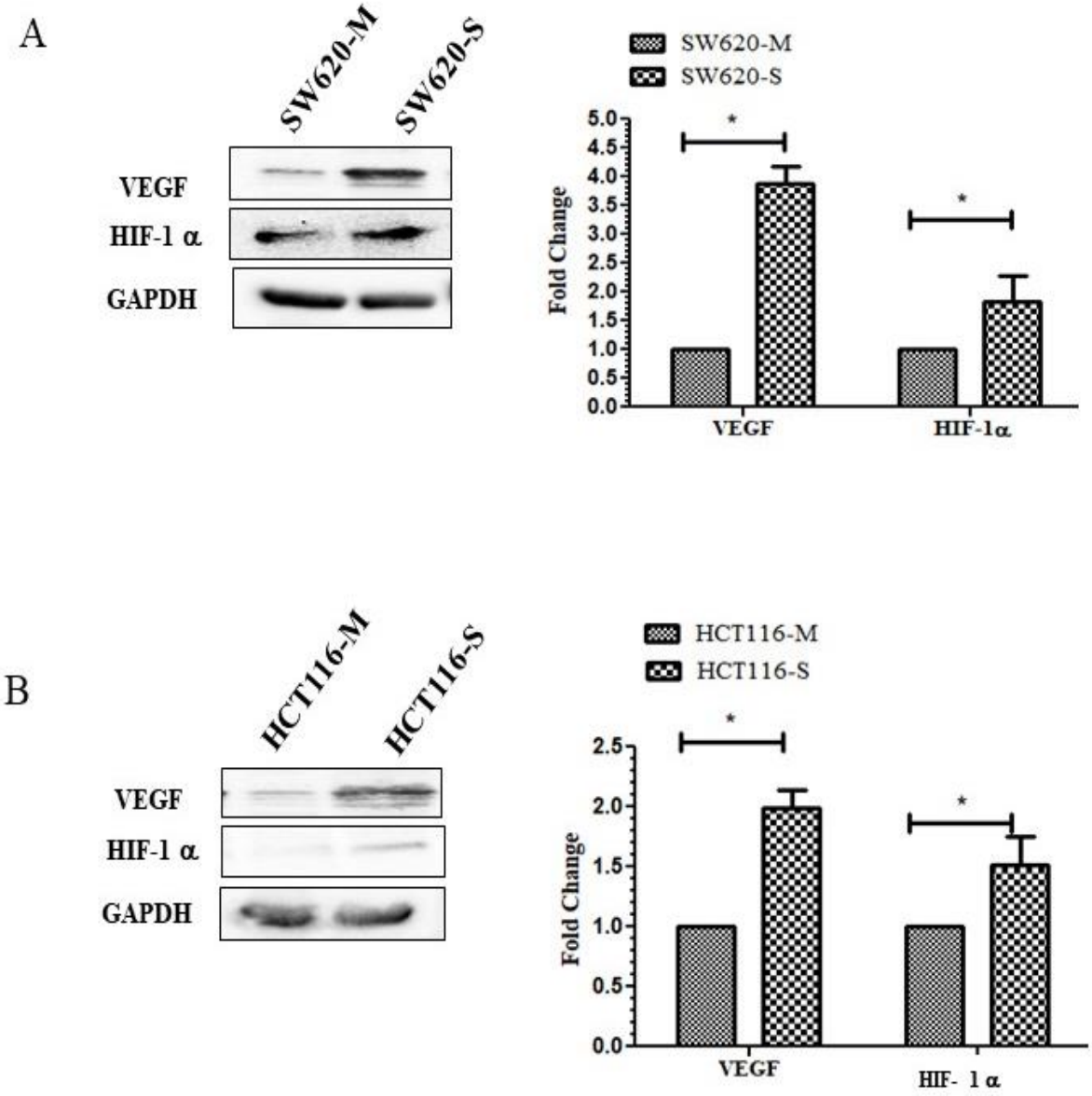


Figure 5.4.15 Angiogenic factors are overexpressed in colorectal cancer spheroids containing stem cells. Western blot of monolayer and spheroid cells from (**A**) SW620 and (**B**)HCT116 detecting the presence VEGF and HIF 1α expression in the whole cell lysate. Bar chart shows mean relative densitometry ratios of protein expression of the proteins against GAPDH (n=3, mean±SD). *- Statistically ($P<0.05$) different from monolayer cells.

5.4.10 TG2 induced β-catenin expression increases VEGF release into culture medium inducing angiogenesis in endothelial cells

Next the role of TG2 on VEGF release into conditioned media by spheroids was examined and in **Figure 5.4.16** VEGF was detected by dot blot and more VEGF was detected in spheroid conditioned media compared to their monolayer counterparts in both SW620 and HCT116 cells, additionally TG2 inhibition resulted in reduced release of VEGF in spheroid media (**Figure 5.4.16A)**. It has been reported that β-catenin plays a role in VEGF transcription (Easwaran et al., 2003), so here whole cell lysates of spheroid cells treated with TG2 inhibitor showed a reduction in the expression of β-catenin in the spheroid cells by almost 50% in the SW620 spheroids, and approx. 65% in the HCT116 spheroids, mirroring the detection of VEGF in the condition media. (**Figure 5.4.16B**).

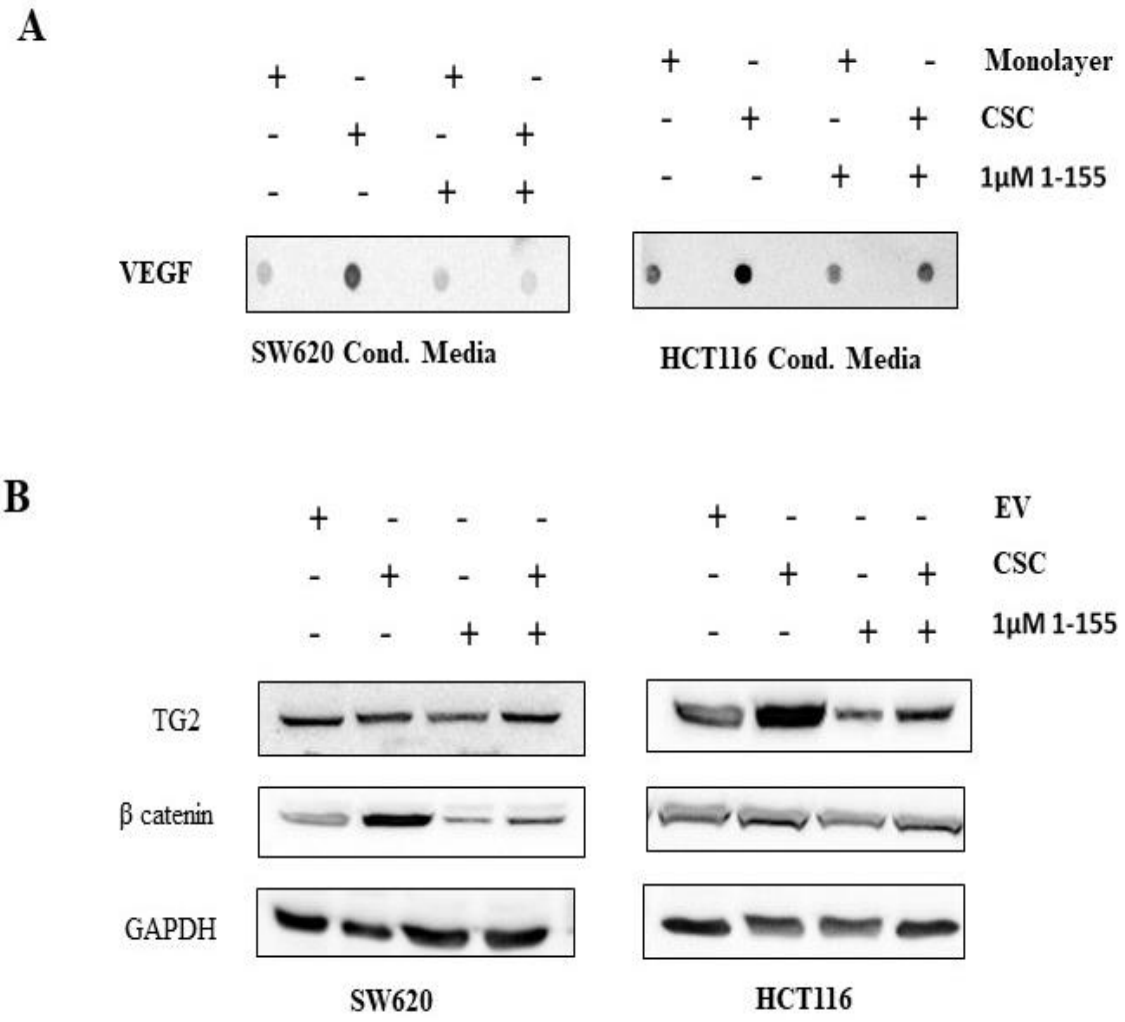


Figure 5.4.16 TG2 inhibition reduces the release of VEGF from CRC spheroids. (**A**) Representative Dot blot of VEGF in condition media of SW620 and HCT 116 cells monolayer and spheroid cells. (n=3) (**B**) Representative Western blotting of TG2 and β-catenin from whole cell lysate of HCT116 and SW620 monolayer and spheroid cells following treatment with TG2 inhibitor 1-155 (1μM) for 48h, (n=2).

To further establish TG2's role in VEGF release into cell culture media, monolayer cells of SW620 and HCT116 cells transduced with TG2 shRNA were used and in **Figure 5.4.17A.** TG2 knockdown reduced the release of VEGF by cells into the cell culture medium, a similar trend was also observed in TG2 inhibitor-treated cells. Next, the role of TG2 on cellular expression of VEGF was determined and **Figure 5.4.17B** shows that TG2 knockdown or inhibition significantly reduced cellular expression of VEGF.

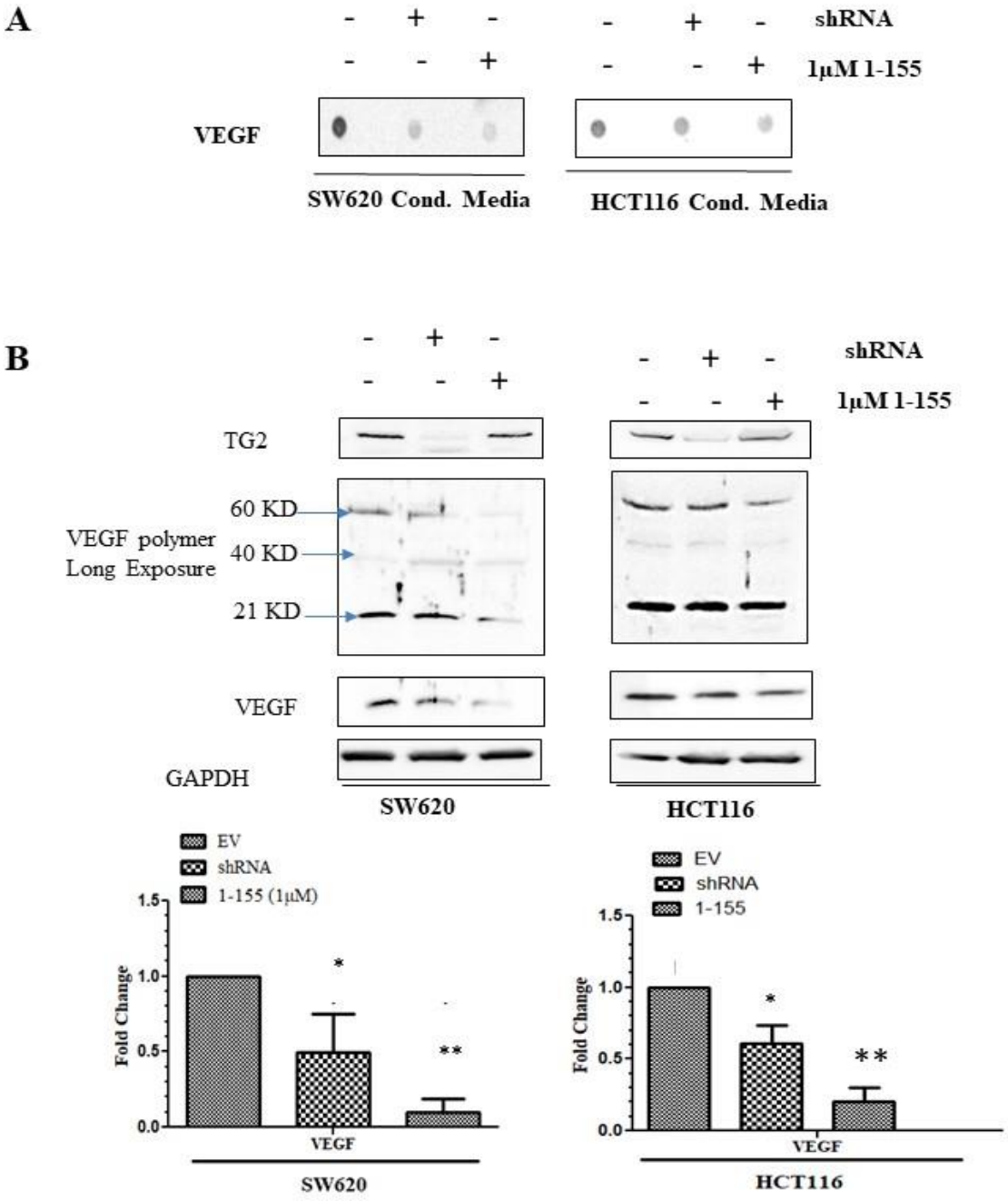


Figure 5.4.17 TG2 knockdown or Inhibition reduces VEGF expression in CRCs. (A) Representative Dot blot of VEGF in conditioned media of monolayer cells (n=3) (B)Representative Western blot of whole cell lysates of monolayer cells from SW620 and HCT116 detecting the presence VEGF (n=3). Bar charts show mean relative densitometry ratios of protein expression of VEGF monomer normalised against GAPDH. *-Statistically ($P<0.05$) different from EV, **-statistically significant from EV and shRNA.

5.5 DISCUSSION

The *in vitro* spheroid formation culture has been widely shown and used as a means to isolate, enrich, maintain or expand potential CSC subpopulations from various types of cancers (Fisher et al., 2015a, Cao et al., 2011, Kumar et al., 2012, Fan et al., 2012), and these CSC population showed stem cell-like properties and expressed CSC markers. Here we characterised the spheroids obtained for stem cell marker CD44 and show that the expression of CD44 was upregulated in the spheroids of SW620 and HCT116 as assessed by both flow cytometry and Western blotting, suggesting that the *in vitro* spheroid formation enriches colorectal cancer cells with stem cell characters. More so, the transcription factors of stemness including Oct 3/4, Nanog, and Sox2 were highly expressed in the spheroid cells of SW620 and HCT116 compared to the parental monolayer cells. These findings are in concert with those reported in ovarian (Cao et al., 2012), breast (Kumar et al., 2010) and squamous carcinomas (Fisher et al., 2015b).

Of the four colorectal cancer cell lines used in the spheroid-cancer stem cell enrichment culture, only high TG2 expressing SW620 and HCT116 cells were able to form compact spheroids. This interestingly also coincides with the grade of the CRC cell line, SW620 being a line with Duke's C classification and HCT116 a more aggressive primary tumour with, D classification. This observation is in concert with other studies that have also reported the inability of the lower grade primary cell lines to form compact spheroids in culture (Leng et al., 2013).

Various studies are now providing evidence for a role of EMT in the acquisition of cancer stem cell phenotype in colon cancer cells (Fan et al., 2012, Han et al., 2013). It has been proposed that while EMT is necessary for cells to dissociate, evade and disseminate (metastasise), the last metastatic step involves the growth of micrometastases into macroscopic metastases, and may require cells with self-renewal capacity and proliferative potential (Polyak and Weinberg, 2009). However, since EMT is transient, circulating tumour cells upon reaching a metastatic site can be re-epithelialised a process which will then enhance proliferation and differentiation. Results in **Figure 5.4.3** suggests that the EMT process is heightened in spheroid of SW620 and HCT116 cells with stem cell-like phenotype. Fan et al., (2012) showed that transcription factors of EMT (Snail, Twist) confer a cancer stem cell phenotype by inducing the expression of pluripotency maintaining transcription factors associated with

stem cells vis-à-vis Oct 4, Sox2, Nanog, Bmi in colorectal cancer cell lines HT29 and HCT116. In this current study, the spheroid cells exhibited increased expression of EMT transcription factors Slug and Twist in both SW620 and HCT116 spheroid cells compared to monolayer cells. Elsewhere in this thesis, TG2 has been shown to induce EMT in CRCs (RKO, SW480, and SW620) and regulate the expression of both Slug and Twist in SW620 cells. This was further established in HCT116 cells where TG2 knockdown or inhibition reduced EMT (**Figure 5.4.6)**.

Interestingly, the current data also show that TG2 expression is also increased in cancer stem cells. As shown in Chapter 4, and here with the HCT116 cells, TG2 may regulate EMT via multiple mechanisms, it is thus plausible to say that increased TG2 and EMT facilitate the stem cell formation in these CRCs. This was substantiated by the reduced spheroid forming potential of these cells when treated with shRNA specific for human TG2, or when treated with a TG2 specific inhibitor (1-155); an effect that has been previously reported in squamous cancer (Fisher et al., 2015b).

β-catenin is an oncoprotein whose dysregulation is associated with colorectal cancers (Novellasdemunt et al., 2015), and plays an important role in the maintenance of intestinal stem cells. Nuclear β-catenin in embryonic stem cells, depending on its binding partner can maintain pluripotency or play a role in lineage decision/commitment process (Miki et al., 2011).

β-catenin has been shown to up-regulate pluripotency factors e.g. Oct4, Nanog, and Sox2 (Kiyonari et al., 2010). So, it is not surprising that β-catenin is sufficiently upregulated in the spheroid cells enriched with cancer stem cells. Very recent studies report increased β-catenin expression in SW620 stem cell populations (Huang et al., 2017). In Chapter 4 TG2 influenced β-catenin accumulation in the nuclei of SW620 cells and in SW620 spheroids both TG2 and β-catenin is upregulated compared to monolayer cells, which may further describe a role of TG2 in maintaining a cancer stem cell phenotype by facilitating β-catenin translocation into the nucleus. Indeed, a very recent study showed that the self-renewal capacity of cancer stem cells in colon and head and neck cancer was blocked by perturbation of the interaction between β-catenin and the transcription factor TCF4 in the nucleus (Fang et al., 2016). To further pursue this line of thought cyclin D1 a protein that is transcribed following β-catenin nuclear activity (Shtutman et al., 1999) was measured. Through live imaging and measurement of YFP tagged

mRNA cyclin D1, and GFP tagged β-catenin, Kafri et al., (2016) was able to show that cells with nuclear accumulation of β-catenin had a 3-fold increase in cyclin D1 mRNA. Results in this Chapter show that cyclin D1 expression is higher in spheroid cells compared to monolayer cells, this alongside the heightened expression of β-catenin in the spheroid cells is suggestive of increased nuclear transcriptional activity of β-catenin.

Additionally, the data in this chapter provide further evidence on a potential TG2 regulated stabilisation of β-catenin which may involve the MAPK pathway in the HCT116 cells. Indeed, it has been suggested that ERK1/2 may activate the Wnt/β-catenin signaling (Lemieux et al., 2015). In addition, Horst et al., (2012) showed that ERK 1/2 activation resulted in nuclear accumulation of β-catenin in clinical human colorectal cancer cells, and concluded that MAPK signalling contributes significantly to determining the impact of WNT activity on stemness phenotypes in CRC cells. Here, ERK 1/2 inhibition perturbed spheroid formation *in vitro*, and TG2 mediated ERK 1/2 phosphorylation correlated with increased β-catenin expression. Taken together this suggests that TG2 driven β-catenin nuclear accumulation may be necessary for maintaining EMT and cancer stem cells. Others have reported that TG2 may induce activation of NFkB and HIf-1α to drive EMT, cancer stemness and prevent apoptosis in certain breast cancer cell types (Kumar et al., 2011).

Wei et al., (2012) have reported that cancer stem cells maintain angiogenesis, and there are considerable reports of β-catenin regulation of angiogenesis. According to (Zhang et al., 2001), there are seven β-catenin/Tcf binding sites on the vascular endothelial growth factor (VEGF-A) gene promoter. In mice heterozygous for the multiple intestinal neoplasia gene (Min/) there was increased redistribution of VEGF in proximity to those cells expressing nuclear β-catenin with a corresponding increase in vessel density (Easwaran et al., 2003). Zhang et al., (2001) further showed that in HeLa cells transfected with constructively active β-catenin, there was an up-regulated VEGF promoter activity by about 4.5-fold. In **Figure 5.4.16** spheroid cells containing cancer stem cells that sufficiently expressing β-catenin, demonstrated increased release of VEGF into the cell culture medium compared to monolayer cells in both SW620 and HCT116 cells, and the condition media from these spheroid cells mediated endothelial cord formation in HUVEC cells seeded on Matrigel. Furthermore HIF-1α a

transcription factor for angiogenesis was elevated in spheroid cells derived from SW620 and HCT 116 cells, and in these spheroid cells inhibition of TG2 by site-specific TG2 inhibitor 1-155 reduced the expression of VEGF and β-catenin. In addition, TG2 inhibition resulted in a significant reduction in VEGF polymer as detected by western blotting, suggesting that TG2 catalytic activity may play a role in the polymerisation of VEGF. More so Wang et al., (2013) have shown that TG2 may increase the bioavailability of matrix-bound VEGF-A in HUVEC cells. Taken together an important role forTG2 in the crosstalk between colorectal cancer stem cells and endothelial cells can be proposed in which TG2 may induce nuclear β-catenin accumulation or HIF 1α resulting in the up-regulation of VEGF, and the presence of extracellular TG2 may further increase the availability of matrix bound VEGFA, which stimulates endothelial cell migration and proliferation. This neovascularisation of CSCs in tumours potentiate tumour growth and or metastasis by favouring extravasation.

5.6 Conclusion

This Chapter shows that only the aggressive cancer cell lines showed the potential of being populated with cancer stem cell, and these cancer stem cells possessed a highly invasive potential. TG2 expression is upregulated in colorectal cancer stem cells, and TG2 may maintain a cancer stem cell phenotype by inducing EMT or by fostering accumulation of β-catenin. More so TG2 may play a role in the expression and release of VEGF necessary for sustaining angiogenesis. Inhibition of TG2 reduces EMT, cancer stem cell formation which proposes TG2 as a potential therapeutic target in CRC progression.

Chapter 6

Discussion and Future Work

6.0 DISCUSSION AND FUTURE WORK

6.1 DISCUSSION

Cancer accounts for the second largest non-infectious disease in the world following heart-related conditions. The survival of cancer patients is highly dependent on the stage of the cancer at diagnosis with a metastatic cancer presenting a poorer diagnostic outcome. In colorectal cancer which is the third largest cancer type (Liu et al., 2011), 5-year survival rates drop from nearly 90% for a primary diagnosed cancer to about 10% in a metastatic one (Leng et al., 2013). This significant drop in the efficacy of the current therapeutic approach calls for the development and identification of biomedical markers that associate with disease progression to enhance easier detection and opens up an opportunity to try to understand cellular processes that are associated with tumour progression, and that may result in poorer clinical therapeutic outcomes.

Epithelial to mesenchymal transition (EMT), a physiological process in wound healing and embryogenesis, is being recognised as a mediator in tumour cell behaviour and metastasis (Agnihotri et al., 2013). Multiple studies have shown that the inception of EMT in cancer cells results in upregulation of cell survival signalling pathways (Bates and Mercurio, 2005), and recent clinical evidence now points to the correlation between EMT and poorer five-year survival in colorectal cancer cells (Mima et al., 2013). In addition, EMT has been shown to play a significant role in the acquisition of a metastatic potential *in vitro*, *in vivo* and in clinical studies, in prostate cancer Hori et al., (2017).

Son and Moon (2010) suggest that EMT can facilitate metastasis by promoting cellular invasion which is critical for dissemination. This was shown by highlighting the role of EMT in inducing the expression of matrix metalloproteinases (MMPs), responsible for proteolytic degradation of the ECM. Other studies have also shown that the transcription factor of EMT, Twist, induces the formation of invadopodia which are actin-based protrusions that recruit various proteases such as membrane-tethered proteases (MT-MMPs), ADAMs, and MMPs, etc. to cell–matrix contact points to degrade ECM (Murphy and Courtneidge 2011). Similar roles for Twist in the development of invadopodia has been reported in mammary epithelial cells (Eckert et al., 2011). Following cell invasion and intravasation into the vasculature, it has been reported that EMT facilitates the formation of circulatory tumour cells

(CTCs). Rhim et al., (2012) reported that the majority of CTCs in a mouse model of pancreatic cancer were presented with a mesenchymal phenotype and expressed the EMT associated transcription factor Zeb2, suggesting that the EMT program in these cells was active. These findings corroborate with the studies performed by Tsai et al. (2012) who also showed increased production of CTCs in a squamous cell carcinoma mouse tumour model in response to Twist1 expression.

In addition to the role of EMT in sustaining CTCs for metastasis, it has also been reported that EMT significantly confers a drug resistance phenotype on cancer cells (Kumar et al., 2012). This has been substantiated by reports from Sayan et al., (2009) who showed that Zeb 2 promotes drug resistance in bladder cancer cells by upregulating the expression of cell survival mechanisms.

Multiple studies also suggest that EMT drives the acquisition of stem-like properties in cancer cells (Eckert et al., 2015). Importantly, Fisher et al., (2015a) suggested that the correlation between EMT and the cancer stem cell (CSC) phenotype, maybe due to the process of de-differentiation, a status that cancer cells adopt in order to evade death signals and seed neoplasms. Fan et al., (2012) reported that knockdown of EMT transcription factor Snail and Twist resulted in perturbation in the expression of CSC transcription factors and the ability of cancer cells to form cancer stem cells in colorectal cancer cells. Taken together this makes EMT a crucial point in the progression of CRCs by fostering invasion, drug resistance, metastasis and cancer stem cell formation.

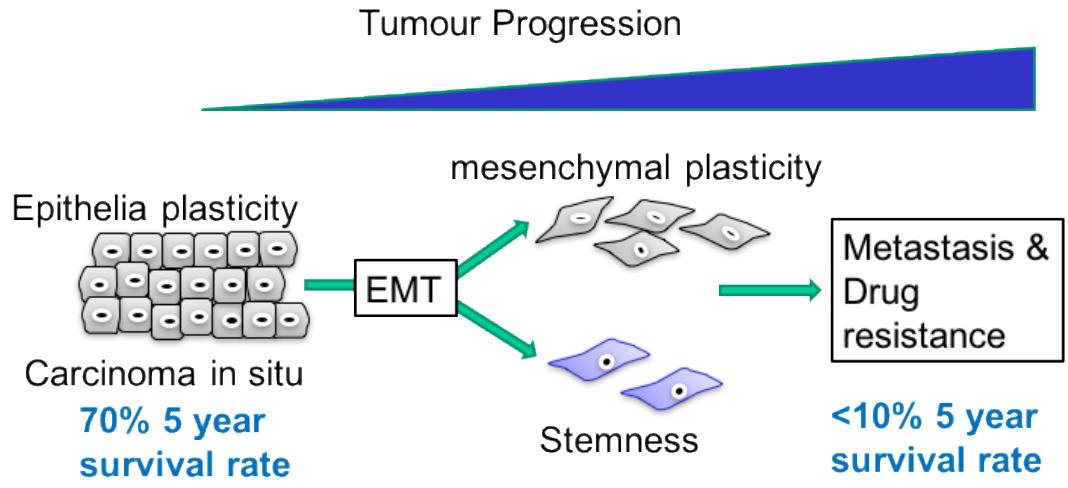


Figure 6.1 Epithelial to Mesenchymal Transition plays an essential role in Tumour Progression. As epithelial tumours progress, epithelial carcinomas acquire a mesenchymal phenotype which drives invasion, metastasis, drug resistance and contribute to the maintenance of cancer stem cells.

Tissue transglutaminase has been extensively reported as an enzyme that can act as a protein crosslinker, amino transferase, and as a protein disulphide isomerase (Wang and Griffin 2013), and is often described as a pro-fibrotic protein, but in recent years the increase in research and method development, is highlighting more protein regulation/cell signalling properties for TG2 (Agnihotri et al., 2013). The TG2 interactome is growing with evidence of TG2 involvement in cellular architecture, cell motility and migration, cellular apoptosis, protein degradation (Wang and Griffin 2012), and binding partners of nuclear transcription factors (Agnihotri et al., 2013). In cancer cells, the role of TG2 is varied with some studies identifying TG2 as a potential antitumor protein (Yoo et al., 2012; Kotsakis and Griffin, 2007) while others suggest a more tumour protective role (Kumar et al., 2010, Fisher et al., 2015). The general consensus is that the cellular location of TG2 may be relevant in predicting its tumour associated role(s) (Agnihotri et al., 2013). The GTP/GDP function of TG2 has been reported to be associated with cancer cell survival and EMT in breast cancer cells (Kumar et al., 2012), in addition, TG2- fibronectin-Integrin association has been reported to activate Src in ovarian cancer cells (Condello et al., 2008). New roles have also been reported for TG2 as a co-transcription factor with NFκB in HIF-1α transcription (Kumar and Mehta 2012). The literature also suggests that TG2 induces EMT via multiple mechanisms in cancer and fibrosis (Nyabam et al., 2016, Fisher et al., 2015b, Kumar and Mehta 2012, Cao et al., 2008), and additionally, TG2 has been reported to clinically correlate with poor disease outcome in ovarian cancer (Hwang et al., 2008), breast cancer (Oh et al., 2011) and colon cancer (Miyoshi et al., 2010). Based on the crucial role of EMT in tumour progression and the potential of TG2 in regulating EMT it was important to determine the interplay between TG2 and EMT in the progression of colorectal cancer cells.

To achieve this colorectal cancer cell lines which have been validated as a progressive model of the disease were employed. In a bid to study the cellular mechanisms that promote tumour progression and metastasis many researchers have attempted to study SW480 and SW620 as a validated *in vitro* model for tumour progression in CRCs, as these cells represent an isogenic pair collected form a patient as the cancer progressed (Hewitt et al., 2000). Gene spectrum analysis confirms that the genetic signature of both cell lines differs significantly with genes differentially up-regulated in SW620 consistent with the

more aggressive cancer phenotype (Futschik et al., 2002). Furthermore, RKO cells which are microsatellite instability (MSI) are thought to be less aggressive compared to its corresponding staged microsatellite stable (MSS) tumour (Rosty et al., 2014).

The characterisation of RKO, SW480 and SW620, indicated distinct morphological and cellular difference between these cells. The primary CRCs RKO and SW480 display increased cellular adhesion and migration, characteristics that are often dependent on interaction of integrins, focal adhesion kinases and Rho GTPases (Parsons et al., 2010). Therefore, it was not surprising to observe increased expression of integrins in the primary cells compared to the metastatic SW620 cell line. Indeed, while SW620 possesses reduced cellular adhesion and migration, these cells showed increased potential for invasion as shown in **Figure 3.4.15**. The invasive potential of SW620 cells is incumbent on it being a metastatic cell, following the development of the necessary cellular machinery to degrade the basement membrane and intravasate blood vessels. A key mediator in the progression from primary to metastatic tumour is EMT, with the metastatic cell line displaying a predominantly mesenchymal phenotype compared to the epithelial phenotype of the primary CRCs SW480 and RKO. In addition to the predominantly mesenchymal phenotype associated with SW620 cells, protein S100A4 (Metastatin) was also found to be expressed but only in the SW620 cells confirming the metastatic potential of these cells. In colorectal cancer metastasis is often associated with drug resistance and a poor five-year survival. In **Figure 3.4.6** drug responses of the cancer cell lines RKO, SW480 and SW620 was assessed using both first line CRC chemotherapeutic therapy 5FU, and doxorubicin. Again, the metastatic cell line SW620 displayed a significantly higher resistance to chemotherapy. Furthermore, in an *in vitro* spheroid assay to enrich cancer stem-like cells, only the metastatic cell line SW620 was able to form compact spheroids with stem-like cells. The observations made from this study confirms the initial hypothesis on EMT, with the presence of EMT associating with a metastatic, more invasive, drug resistant and a cancer stem like cell phenotype.

Following the establishment of the potential role of EMT in the progression of colorectal cancer disease using these cell lines. It became important to identify the role of TG2 in these cells, given that TG2 has been found to play a role in EMT in fibrosis and in certain cancer types, and that clinical evidence by two independent groups associate TG2 with a poor five-year survival in colorectal cancer (Miyoshi, et

al., 2010), and increased metastatic potential (Fernandez-Acenero et al., 2016). Characterisation of the three cell lines RKO, SW480 and SW620 for TG2, showed that the metastatic SW620 expressed significantly higher TG2 compared to the other two primary cell lines (SW480 and RKO). These observations in the human CRC cell line are suggestive of a potential role for TG2 in the progression of the disease, and this further corroborates clinical findings and indeed, proposes that TG2 may hold prognostic value in colorectal cancer.

On the assumption that EMT is associated with the progression of cancer by inducing invasiveness, drug resistance, and cancer stem cell formation. The role of TG2 in facilitating EMT was investigated, especially as TG2 expression in the current cell model is associated with EMT. By manipulating the expression of TG2 through transduction with lentiviral particles TG2 expression was found to significantly associate with EMT markers, and also the upstream transcription factors that regulates EMT. It was not surprising that TG2 expression also correlated with the invasiveness of cells, with TG2 significantly fostering invasion, migration and drug resistance. This confirms that the induction of EMT is crucial for these processes and that blocking EMT may significantly perturb tumour progression. The ability of TG2 to affect EMT, cell invasion and migration has been reported in breast (Wang and Griffin 2013, Kumar et al., 2010), and squamous (Fisher et al., 2015a) cancer cells. Furthermore, the potential for TG2 as a therapeutic target was investigated in these cells, using the TG2 selective permeable inhibitor 1-155 (Badarau et al., 2015). TG2 inhbition was found to attenuate EMT, which then impacted on cell migration, and invasion. This corroborates findings made in squamous cell carcinoma (Fisher et al., 2015b)

TGFβ1 and β-catenin signalling were investigated, most especially as these signalling pathways are sites of multiple mutations in human colorectal cancers and associate with EMT and tumour progression (Novellasdemunt et al., 2015). Primary cells RKO and SW480 cells displayed a significant EMT response to TGFβ1 treatment with accompanying activation of Smad 2/3 and ERK1/2 activation. Inhibition of either TGFβ1 by its inactivating antibody or ERK inhibition resulted in reduced EMT in these cells. TGFβ1's ability to induce EMT in epithelial cells have been widely reported where Smad 2/3 interaction with smad 4 act as transcription factors in the transcription of Slug, Snail and Twist

which control the EMT process. TGFβ1 can also induce EMT by activation of the master inflammatory protein NFKB through the TGFβ1 activation Kinase 1 (TAK1) (Cao et al., 2012). More importantly TGFβ1 ability to induce EMT may be hinged on TG2 in cancer cells. TGFβ1 is a potent inducer of TG2 in epithelial (Cao et al., 2008) and Fibroblast cells (Nyabam et al., 2016); and Matei and colleagues have shown that TGFβ1 induced EMT in ovarian cancer cells is dependent on TG2 (Cao et al., 2012). Also important is the finding that TG2 can upregulate TGFβ1 expression and ECM abundance in CRCs. This was previously reported in a murine model of colorectal cancer (Kotsakis et al., 2011). In this current study TG2 induced activation of ERK facilitates c-JUN transcription and potentiates AP-1 transcription of TGFβ1. Previous studies in Swiss 3T3 fibroblast also show that TG2 overexpression induces TGFβ1 (Telci et al., 2009). This thus proposes a positive feedback loop in which TGFβ1 activates TG2 canonically or non-canonically via Smad and MAPK kinase respectively and activated TG2 via possible integrin co-receptor function (Akimov et al., 2000) activates ERK1/2 which in turn, turns on the necessary machinery for TGFβ1 expression, and continued maintenance of EMT (**Figure 4.5.1)**. The ability of TG2 to activate ERK has been recently reported in Gastric cancers *in vivo*, where TG2 promoted gastric cancer cell proliferation, migration, invasion, as well as tumorigenesis and peritoneal metastasis by activating ERK 1/2 (Wang et al., 2016).

It is also worth noting that in this study, TG2 inhibition significantly reduced the expression of matrix bound TGFβ1, as was seen in the RKO and SW480 cells. It has been proposed elsewhere that TG2 ability to facilitate the incorporation of other growth factors such as VEGF to the matrix probably by way of crosslinking VEGF to the ECM, plays an important role in increasing the half-life of VEGF (Wang et al., 2013). In other studies, TG2 induced formation of VEGF polymers render VEGF resistant to neutralising antibodies such as Bevacizumab which act as therapeutic treatment in cancer (Feng et al., 2017).

This however, was not the case with the metastatic cell line SW620, which according to biochemical and phenotypical measures appeared to be independent of adhesion related signalling, as observed with significantly reduced adhesion and cell motility in a wound closure assay compared to the primary CRCs. In addition, these cells exhibited significantly reduced expression of integrins compared to its primary counterpart SW480. Following this observation, TG2 was found to regulate β-catenin nuclear

translocation, in a Src independent way. Previous studies in ovarian carcinoma suggested that TG2 promoted β-catenin nuclear activity by activating Src via TG2 interaction with β integrin (Cao et al., 2008). Other studies have also shown that extracellular TG2 interacts with LRP 5/6 in potentiating Wnt signalling (Deasey et al., 2013). However, the very low-level expression of β integrins in SW620 cells, may explain the null effect of TG2 knockdown on Src activation, especially as TG2 expression correlated with increased activation of Src in the β integrin expressing RKO and SW480 cells, or it may be that Src has been left constitutively activated following a mutation in SW620. However, these proposes an alternate intrinsic role for TG2 in the cytoplasm, where TG2 facilitates β-catenin nuclear translocation by limiting cytosolic β-catenin interaction with ubiquitin. TG2 inhibition or knockdown in SW620 cells facilitated increased interaction between βcatenin and ubiquitin, indicating increased proteasomal degradation of β-catenin. Indeed, this was proven by the fact that TG2 extracellular inhibition did not have any effect of EMT in SW620 cells unlike the primary RKO and SW480 cells. Additionally, in an attempt to further prompt the primary CRC SW480 to a more invasive phenotype by ectopic expression of TG2 in these cells, failed to significantly push EMT beyond the status of the control vector treated cells, more so TGFβ1 treatment of the TG2 transduced SW480 cells did not have any significant impact on EMT, similar to the observation in the high TG2 expressing SW620 cells. This presented a conceptual dilemma, in which TGFβ1 had no effect on TG2 transduced SW480 cells-which may indicate TGFβ1 influence on EMT is subject to TG2, but again TG2 overexpression did not significantly advance the EMT status of the cells unlike the role TG2 played in the SW620 cells. This may be explained by the assumption of tumour clonal selection whereby only cells with a particular subset of mutation are able to assume metastasis. Therefore, TGFβ1 and TG2 may drive EMT in the SW480 cells, however, further oncogenic mutations maybe necessary to facilitate further involvement of TG2 in EMT especially in the TG2 transduced SW480, and possibly even metastasis, as evident by the differential expression of proteins in both cell lines.

The aggressiveness of the CRCs RKO, SW480 and SW620 was further tested by the ability of these cells to form spheroids *in vitro*. *In vitro* spheroid formation has been established as a method to enrich cancer cells with stem like phenotype (Eckert et al., 2015), and also as a way to select cells that are

resistant to anoikis. Of all three cell lines only the metastatic SW620 cells formed compact spheroids. This observation again coincides with SW620 exhibiting a predominantly mesenchymal phenotype. Analyses of the cell lysates from the *in vitro* spheroids and the monolayer cells further established that the spheroid cells expressed significantly higher EMT markers compared to the monolayer cells and this was further confirmed using another aggressive but primary cell line HCT116. HCT116 is a Duke's class D colorectal carcinoma, which is the highest disease staging under this classification. Importantly TG2 was also found to be upregulated in these spheroid cells compared to the monolayer cells in both SW620 and HCT116 cells. The spheroid cells from these two cell lines were confirmed to be enriched with stem cell marker CD44 and transcription factors of stem cells, Oct 3/4, Nanog, and Sox 2. Suggesting that TG2 and EMT are upregulated in colorectal cancer stem cells. Indeed, TG2 knockdown or site-specific inhibition, significantly reduced the potential of SW620 and HCT116 to form spheroids in culture, an observation that has also been reported in squamous (Fisher et al., 2015b), breast (Kumar et al., 2010) and ovarian cancer cells (Cao et al., 2008). Since EMT has been shown to be a critical process for the acquisition of cancer stem like properties (Fan et al., 2012) and in this current study EMT correlated with spheroid formation amongst RKO, SW480 and SW620, and that EMT is upregulated in the spheroid containing stem like cells compared to the monolayer cells, it is plausible to say that EMT may mediate the formation of cancer stem cells. Additionally, since TG2 has been shown to drive EMT in this cancer cell type and in multiple cancers including breast (Kumar et al., 2010), ovarian (Cao et al., 2008) and squamous (Fisher et al., 2015b). TG2 induced EMT may drive the cancer stem cell phenotype, which is lost upon TG2 inhibition or knockdown. This was further established by the ability of TG2 inhibition to significantly reduce the invasive potential of SW620 and HCT116 spheroids, with cellular invasion being characteristic of cancer stem cells. However, following that TG2 viral transduction did not further induce EMT in SW480 cells, other critical mutations or oncogenes may need to be activated which may then recruit TG2 to further facilitate EMT and its associated pathological processes.

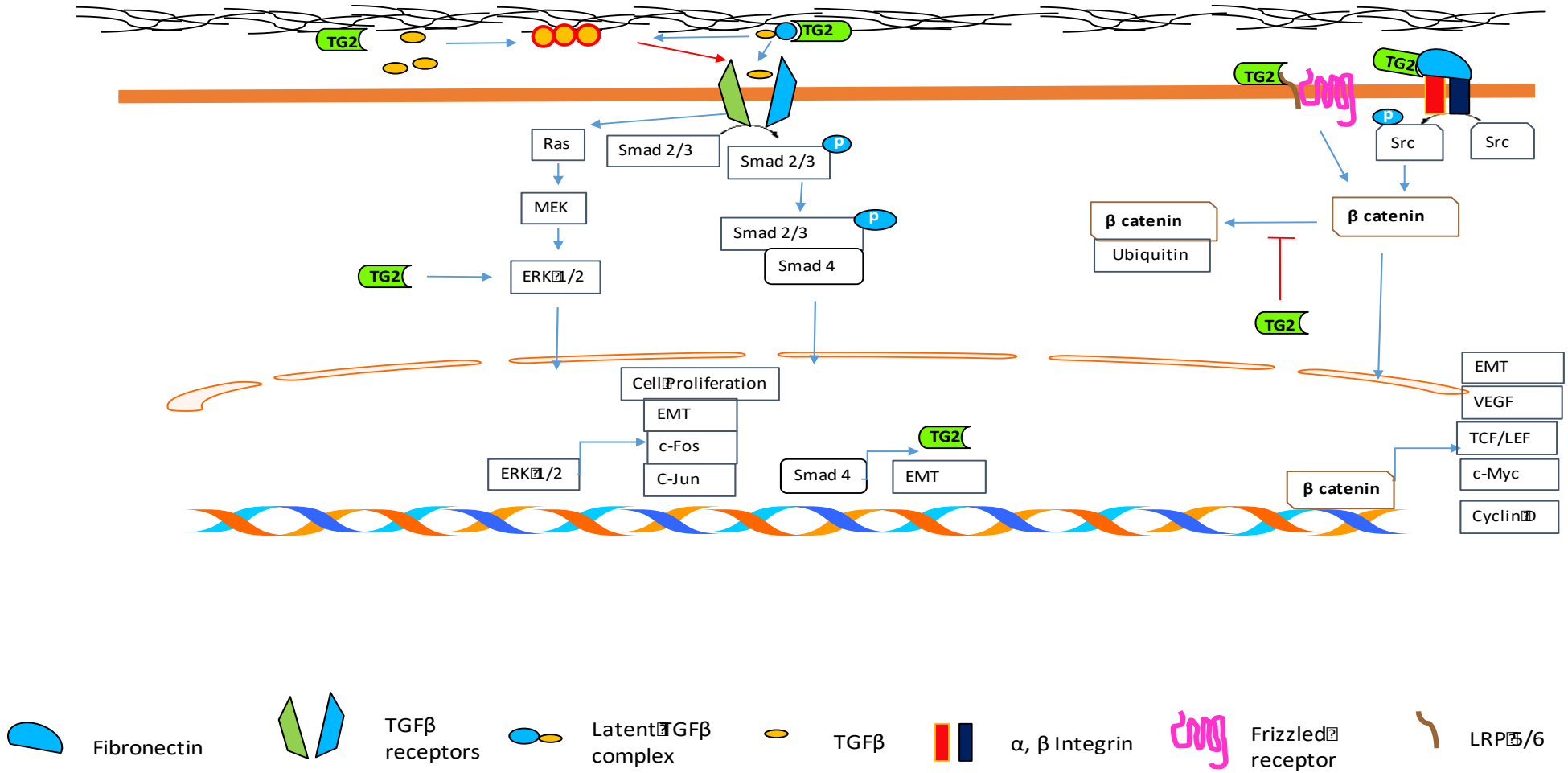


Figure 6.2. Proposed mechanism of TG2 regulation of EMT and stem cell formation in colorectal cancer cells. Extracellular TG2, via interaction with the ECM, can induce mechanical sheer stress that releases TGFβ1 from its latent TGFβ complex. Canonical TGFβ1 operates via Smad2/3 and Smad 4 in the induction of TG2 and EMT. Extracellular TG2 may also incorporate TGFβ1 into the matrix increasing its half-life and signalling. Non canonical TGFβ1 signalling activates extracellular kinase (ERK) which induces, TG2. TG2 may also activate ERK, inducing C-jun, C-fos and TGFβ1 thus driving a vicious auto-regulatory cycle involving TG2 and TGFβ1. Extracellular TG2 interaction with fibronectin and integrin activates Src, which inhibits proteosomal degradation of β-catenin. In addition, extracellular TG2 interaction with LRP5/6, a co receptor with frizzled in the canonical Wnt signalling facilitates β-catenin stability and nuclear translocation and β-catenin acts as a transcription factor for cell cycle regulators and EMT. More so, Intracellular TG2 may stabilise β-catenin independent of Src, by inhibiting the association of β-catenin with ubiquitin

Multiple reports have shown *in vivo* that spheroid cells form more aggressive, and larger tumours (Kumar et al., 2010, Adhikary et al., 2013). In addition, these tumours appear to be highly vascularised compared to tumourss formed from traditional monolayer cells (Leng et al., 2013; Adhikary et al., 2013). Tumour vascularisation plays an important role in tumour growth and dissemination, and may explain the significantly increased tumour size and metastatic potential of the CSC tumours relative to their parental cell lines. The current data in this study shows that spheroids containing cancer stem cells express HIF 1α, a transcription factor of angiogenesis, and vascular endothelial growth factor (VEGF), furthermore the condition media from both monolayer and spheroid cells reveal that VEGF is highly released in the spheroid cells compared to the monolayer cells, and these condition media induces endothelial cord formation on HUVECs on Matrigel further substantiating that cancer stem cells have expedited angiogenesis. The ability of spheroid cells to promote angiogenesis was shown to be reduced by inhibiting TG2 in this study. This maybe potentially due to inhibiting TG2 ability to drive HIF 1α on the one hand, or by inhibiting TG2 ability to promote nuclear β-catenin accumulation. Mehta and colleagues have shown that TG2 can act as a co-transcription factor with NFκB for the transcription of HIF 1α (Kumar et al., 2010). Moreover, β-catenin has also been reported to possess binding sites in the promoter region of the VEGF gene (Easwaran et al., 2003; Zhang et al., 2001). Taken together TG2 may be relevant in promoting the aggressiveness of cancer stem cells by promoting EMT and driving angiogenesis in multiple ways.

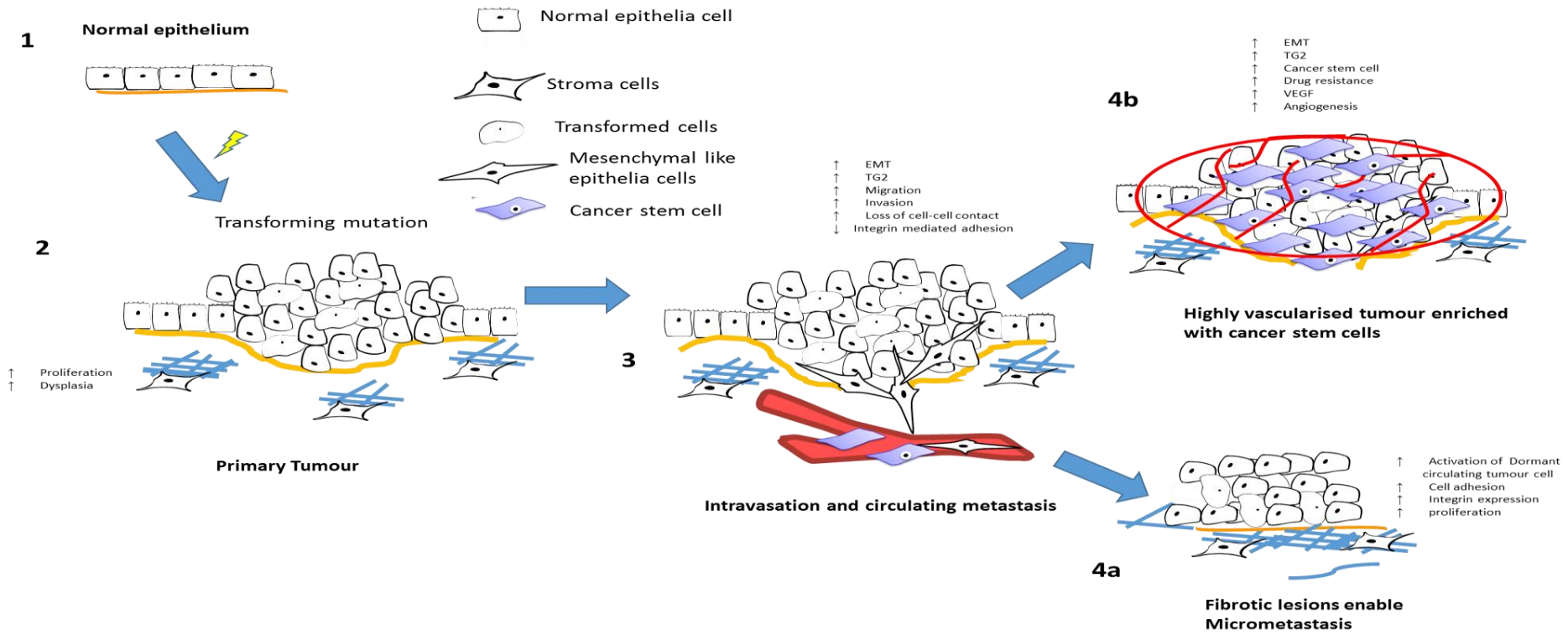


Figure 6.3 TG2 promotes EMT, drug resistance, cancer stem cell formation, angiogenesis and tumour Progression in colorectal cancer cells
The presence of a transforming mutation on normal epithelium (1) can result in pre-neoplastic cells, which aid cell survival and cellular proliferation.(2) Increased cell survival and uncontrolled proliferation a consequence of oncogenic mutations drive cells into a dysplastic state. (3) Further mutations in the primary tumour potentiate EMT mediated by cytokines and TG2. Cell to cell contact is reduced with increasing loss of cell adhesion compounded by the capacity to produce basement degrading enzymes, leading to intravasation and dissemination of tumour cells. (4a) TG2 and EMT drive circulating tumour cells to remain dormant in circulation having lost cell adhesion mechanism, they are unable to proliferate. However, upon reaching a suitable site with remodelled ECM that support adhesion and growth factors, circulating tumour cells can then be seeded, re-express integrins and proliferate via mesenchymal to epithelial transition (MET) to form micrometastasis or (4b) TG2 sustains EMT and a cancer stem cell phenotype in tumours, the increasing number of cancer stem cell enables the tumour to become more aggressive, invasive and drug resistance. TG2 may also drive angiogenesis by regulating VEGF to nourish tumours and consequently aid tumour intravasation.

Summarily, in colorectal cancer cells TG2 correlates with disease progression as observed in the validated *in vitro* human cell model for colorectal cancer progression, and as benchmarked by EMT, drug resistance, *in vitro* invasion and tumour sphere formation. Additionally, it can be proposed that the significant role of TG2 in facilitating EMT via multiple cellular mechanisms, may be responsible for metastasis, drug resistance, invasion and cancer stem formation. Inhibiting TG2 by small molecule inhibitors attenuate EMT and consequently most processes associated with EMT in tumour progression. These studies as well as those found in clinical studies in colorectal cancer thus propose TG2 as a potential biomarker for disease progression and a pharmacological target in colorectal cancer.

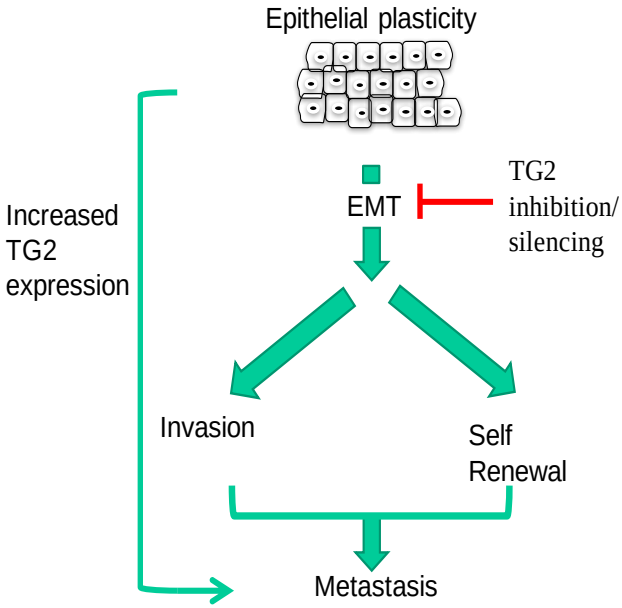


Figure 6.4. TG2 as a potential Target in regulating colorectal cancer progression. TG2 inhibition or knockdown reduces the capacity of epithelial colorectal cancer cells to undergo EMT. EMT favours invasion, drug resistance, cancer cell self-renewal and metastasis. With a significant gap in prognostic outcomes between primary and metastatic tumours. TG2 may be a target for diagnosis and may hold a potential for treatment, using small molecule inhibitors.

6.2 Future Work

To make confirmatory comments and to further understand some processes carried out in this study, further investigation can be undertaken as follows:

- There is a lot of debate as to if the functional unit or the conformation of TG2 is responsible for driving EMT in certain epithelial cell context. Although the general consensus is that inhibitors alter both conformation and function, it will be important to discriminate between function and conformation as a disposition towards EMT, using TG2 structural and functional mutants.
- To further establish the relationship between TG2 and β-catenin in CRCs- especially SW620 cells, it would be important to knockout β-catenin since the Wnt Signalling pathway cannot be perturbed owing to the APC mutation downstream of GSK-3β, and determine if sustained TG2 expression still promotes EMT in β-catenin deficient cell.
- Perform a DNA microarray of oncogenic proteins/ signalling pathways in TG2 expressing cells versus TG2 knockdown cells to determine which oncogenes are upregulated or down regulated and map them with processes associated with tumour progression.
- Since TG2 inhibition appears to attenuate EMT, invasion and stem cell formation *in vitro,* it will be important to introduce TG2 inhibitors in an *in vivo* mice system with human CRCs, especially the metastatic SW620 to determine if TG2 inhibition slows down metastasis.
- In addition to determining the role of TG2 in slowing down tumour progression and metastasis *in vivo,* it will also be important to inoculate immune compromised mice with colorectal cancer stem cells and dose these mice with TG2 inhibitors to determine if TG2 *in vivo* inhibition attenuates the invasiveness of colorectal cancer stem cells in one part, and if TG2 inhibition perturbs tumour angiogenesis, to confirm the *in vitro* findings.

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