MECHANISM OF TISSUE TRANSGLUTAMINASE UPREGULATION AND ITS ROLE IN OVARIAN CANCER METASTASIS

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DEDICATION

To my parents, my sisters, and my husband,

The wind under my wings.

And to my daughter,

The apple of my eye.

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ABSTRACT

Liyun Cao

MECHANISM OF TISSUE TRANSGLUTAMINASE UPREGULATION AND ITS ROLE IN OVARIAN CANCER METASTASIS

Ovarian cancer (OC) is a lethal disease due to metastasis and chemoresistance. Our laboratory previously reported that tissue transglutaminase (TG2) is overexpressed in OC and enhances OC peritoneal metastasis. TG2 is a multifunctional protein which catalyzes Ca^{2+}-dependent cross-linking of proteins. The purpose of this study was to explore the mechanism by which TG2 is upregulated in OC and its role in OC progression. We demonstrated that transforming growth factor (TGF)-β1 is secreted in the OC milieu and regulates the expression and function of TG2 primarily through the canonical Smad signaling pathway. Increased TG2 expression level correlates with a mesenchymal phenotype of OC cells, suggesting that TGF-β1 induced TG2 promotes epithelial-to-mesenchymal transition (EMT). TG2 induces EMT by negatively regulating E-cadherin expression. TG2 modulates E-cadherin transcriptional suppressor Zeb1 expression by activating NF-κB complex, which leads to increased cell invasiveness *in vitro* and tumor metastasis *in vivo*. The N-terminal fibronectin (FN) binding domain of TG2 (tTG 1-140), lacking both enzymatic and GTPase function, induced EMT in OC cells, suggesting the interaction with FN involved in EMT induction. A TGF-β receptor kinase inhibitor, SD-208, blocked TGF-β1 induced TG2 upregulation and EMT *in vitro* and tumor dissemination *in vivo*, which confirms the link between TGF-β1 and TG2 in EMT and tumor metastasis. TG2 expression was correlated with the number and size of self-renewing

spheroids, the percentage of CD44+CD117+ ovarian cancer stem cells (CSCs) and with the expression level of stem cell specific transcriptional factors Nanog, Oct3/4, and Sox2. These data suggest that TG2 is an important player in the homeostasis of ovarian CSCs, which are critical for OC peritoneal metastasis and chemoresistance. TG2 expression was also increased in CSCs isolated from human ovarian tumors, confirming the implication of TG2 in CSCs homeostasis. Further, we demonstrated that TG2 protects OC cells from cisplatin-induced apoptosis by regulating NF-κB activity. We proposed a model whereby TGF-β-inducible TG2 modulates EMT, metastasis, CSC homeostasis and chemoresistance in OC. These findings contribute to a better understanding of the mechanisms of OC metastasis modulated by TG2.

Daniela Matei, M.D., Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Akt	Protein kinase B
ALDH1	Aldehyde dehydrogenase 1
ALK	Activin receptor-like kinase
AMH	Anti-Mullerian hormone
AR	Adrenoceptor
ARID1A	AT rich interactive domain 1A
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
BMP	Bone morphogenetic proteins
BRCA1 and 2	Breast cancer susceptibility protein type 1 and type 2
CBP	CREB-binding protein
CDK	Cyclin-dependent protein kinase
CE	Cornified cell envelope
ChIP	Chromatin immunoprecipitation
CREB	Cyclic AMP-responsive element-binding protein
CSC	Cancer stem cell
DAG	1,2-diacylglycerol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition

EOC	Epithelial ovarian cancer
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FKBP12	The 12-kDa FK506-binding protein
FN	Fibronectin
FXIIIA	Factor XIII α subunit
GDFs	Growth differentiation factors
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GPCR	G-protein coupled receptor
G protein	GTP binding protein
GTP	Guanosine triphosphate
HA	Hyaluronic acid, hyaluronan
HSC	Hematopoietic stem cell
IC50	50% inhibitory concentration
IF	Immunofluorescence
IFN-γ	Interferon-gamma
IGFBP-3	Insulin-like growth factor-binding protein-3
IκBα	Inhibitor of kappa B alpha
ILK	Integrin-linked kinase
IP3	Inositol 1,4,5-trisphosphate

i.p.	Introperitoneal
JNK	c-Jun N-terminal kinase
LAP	Latency-associated propeptide
LLC	Large latent complex
LPA	Lysophosphatidic
LTBP	Latent TGF-β binding protein
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-Epithelial transition
MIS	Müllerian-inhibiting substance
MMP	Matrix metalloproteinase
MODY	Maturity onset diabetes of young
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NES	Nuclear export signal
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells
NLS	Neuclear localization signal
NOSE	Normal ovarian surface epithelial cells
OC	Ovarian cancer
OCICs	Ovarian cancer initiating cells
OPN	Osteopontin
PAI	Plasminogen activator inhibitor
PDI	Protein disulfide isomerase
PI3K	Phosphoinositide 3′ kinase

PIP2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PDGF	Platelet-derived growth factor
PMSF	Phenylmethylsulfonyl fluoride
qRT-PCR	Quantified RT-PCR
Rb	Retinoblastoma protein
RGD	Arginine-Glycine-Aspartic acid, Arg-Gly-Asp
RIPA	Radioimmunoprecipitation assay
RT-PCR	Reverse transcription-Polymerase chain reaction
SBE	Smad-binding element
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLC	Small latent complex
Smurf	Smad ubiquitination-related factor
TAK1	TGF-β-activated kinase 1
TBST	Tris Bufferred Saline containing Tween 20
TIMP-1	Tissue inhibitor of metalloproteinase-1
TG	Transglutaminase
TG2	Tissue transglutaminase
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
TSP	Thrombospondin
TUNEL assay	TdT (terminal deoxynucleotidyl transferase)-mediated

	deoxyuridine triphosphate nick-end labeling assay
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

CHAPTER 1: INTRODUCTION

1.1 Ovarian cancer (OC)

There are three types of cells in the ovary, each with distinct origin and functions: epithelial cells, which cover the ovary; germ cells, which develop into eggs (ova) that are released into the fallopian tubes every month; and stromal cells, which form the supporting or structural tissue holding the ovary together and produce the female hormones, estrogen and progesterone. Accordingly, ovarian tumors can arise from each of these components, giving rise to epithelial ovarian cancer (EOC, ~90%), germ cell tumors (~3%), and stromal cell tumors (~6%) (Chen et al 2003) (Figure 1).

EOC is a heterogeneous disease that has several histological subtypes with different origins and molecular profiles (Vaughan et al 2011). The most common histological subtype is papillary serous carcinoma that represents 50-60% of all cancers. Other subtypes include endometrioid (25%), clear cell (4%), and mucinous (4%) carcinoma (Farley et al 2008). High-grade serous ovarian cancers (HGS-OC) are derived from the surface of the ovary and/or the distal fallopian tube (Bowtell 2010). Endometrioid and clear cell ovarian cancers are derived from endometriosis, which is associated with retrograde menstruation from the endometrium (Wiegand et al 2010). Most invasive mucinous ovarian cancers are metastases to the ovary, often from the gastrointestinal tract, including the colon, appendix or stomach (Kelemen and Kobel 2011). These different histological subtypes have distinct molecular signaling pathways. Mutations in tumor protein p53 encoding gene TP53 occur in at least 96% serous

ovarian tumors and the breast cancer susceptibility protein type 1 and type 2 (BRCA1 and BRCA2) are mutated in 22% of HGS-OC samples. Clear cell ovarian cancers have few TP53 mutations, but are characterized by recurrent AT rich interactive domain 1A (ARID1A) and phosphoinositide-3-kinase catalytic alpha polypeptide (PIK3CA) mutations. Endometrioid ovarian cancers are associated with frequent β1-catenin encoding gene CTNNB1, ARID1A and PIK3CA mutations, and harbor a lower rate of TP53 mutations, as compared to serous cancers. Mucinous ovarian tumors are characterized by prevalent v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations (2011). As epithelial OC is the major form of OC, OC is used later on.

OC is the ninth most common cancer in women (not counting skin cancer) and the fifth cause of cancer death of women in United States. About 21,990 women were diagnosed with and 15,460 women died of OC in 2011 (www.cancer.gov). The lethality of OC is mainly due to the inability to detect the disease at an early stage, and the lack of effective therapies for advanced-stage disease. About 62% of ovarian cancer cases are diagnosed after the cancer has already metastasized (www.seer.cancer.gov), and advanced stage OC has only a 27% 5 year survival rate. Therefore, studies on understanding the mechanisms of OC metastasis may lead to potential targets that could be investigated for the development of more efficient treatment modalities for this disease.

Metastasis is a multistep process; the key steps being detachment of malignant cells from the primary tumor, migration to and proliferation at distant organs. OC differs from other hematogenously metastasizing tumors by

disseminating within the peritoneal cavity (Figure 2). To detach from the primary tumor, cancer cells undergo epithelial-mesenchymal transition (EMT) to lose their epithelial characteristics, including their apical-basal polarity and specialized cell-cell contacts, and acquire a migratory mesenchymal cell phenotype, which allows them to move away from their site of origin and spread to distant organs or tissues (Kalluri and Weinberg 2009). After dissociation from the original site, OC cells aggregate as multicellular spheroids and float in the peritoneal fluid (Allen et al 1987). The OC spheroids must overcome anoikis and hypoxic conditions in the peritoneal milieu, and attach to the mesothelium which covers all organs within the peritoneal cavity through several mechanisms, such as CD44 interaction with hyaluronic acid (Strobel et al 1997) (Lessan et al 1999), CA125/MUC16 interaction with mesothelin on mesothelial cells (Rump et al 2004), and β1 integrin interaction with fibronectin, laminin and collagen in the extracellular matrix (ECM) (Burleson et al 2004). After attachment, OC cells invade into the submesothelial basement membrane via proteolysis by matrix metalloproteinases (MMPs) and proliferate on abdominal organs such as the peritoneum, omentum, liver, stomach, colon, and diaphragm (Naora and Montell 2005).

Ascites is commonly found in ovarian cancer patients at advanced stage. Secretion of vascular endothelial growth factor (VEGF) by OC cells may increase vascular permeability and promote the ascites formation (Byrne et al 2003). Malignant ascites contains many growth factors, such as transforming growth factor-beta (TGF-β) (Abendstein et al 2000), platelet derived growth factor

(PDGF) (Matei et al 2006), vascular endothelial growth factor (VEGF) (Yabushita et al 2003), lysophosphatidic acid (LPA) (Xu et al 1995), cytokines, such as IL-6 (Offner et al 1995), and other extracellular matrix constituents. These factors can promote cancer cells' proliferation (Mills et al 1990), survival (Lane et al 2007), adhesion and invasion (Ahmed et al 2005). A proteomic analysis of 4 ascites samples from serous OC patients identified 80 overexpressed proteins that are involved in cell proliferation, differentiation, adhesion, migration, angiogenesis, proteolysis, and anti-apoptosis (Gortzak-Uzan et al 2008). Thus the ascites renders a favorable microenviroment to facilitate tumor growth and metastasis.

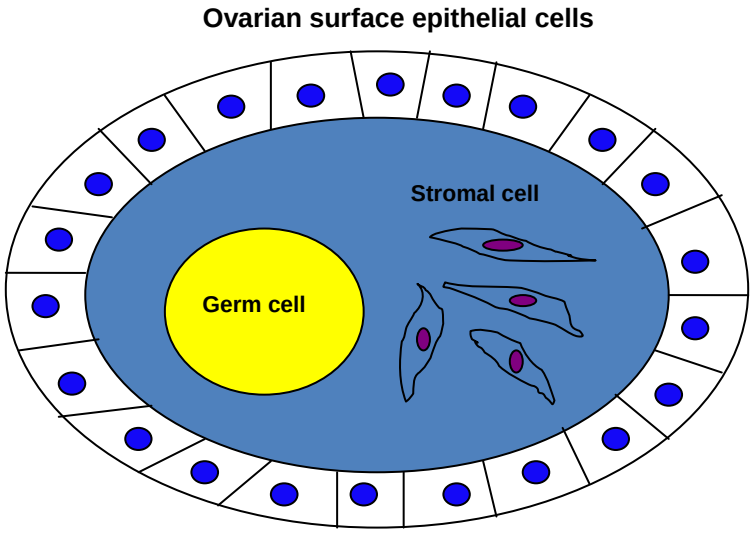


Figure 1: Three cell types in the ovary. There are three types of cells in ovary: surface epithelial cells that cover the ovary, germ cells that develop into eggs, and stromal cells that support the organ's structure and produce steroid hormones.

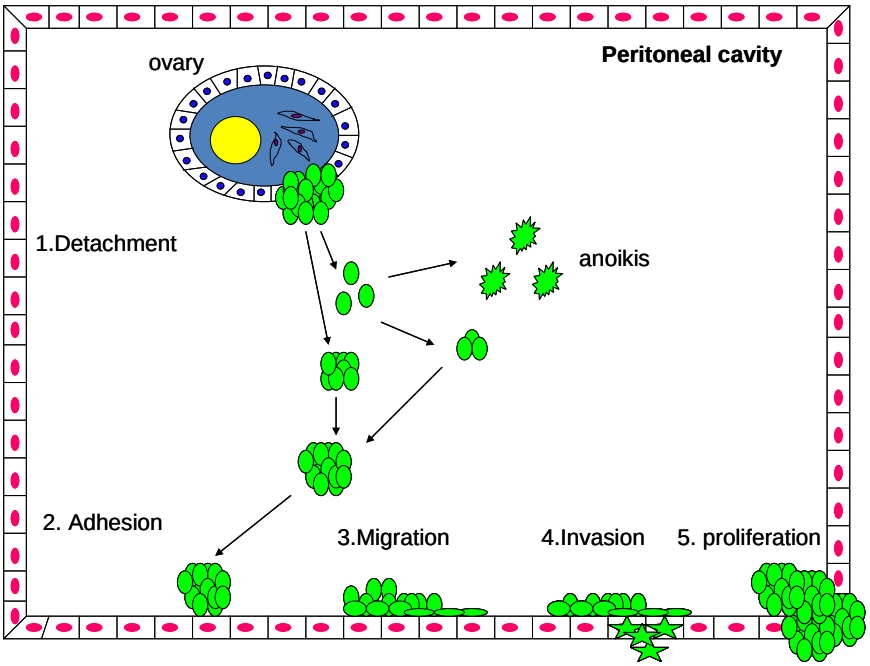


Figure 2: OC metastasis is a multistep process. 1. OC cells exfoliate from the primary site into the peritoneal cavity. Single cells undergo anoikis, while spheroids survive in the peritoneal cavity. 2. Spheroids adhere to the mesothelial lining in peritoneal cavity. 3. Spheroids dissociate and OC cells migrate on the mesothelial lining. 4. OC cells invade into the sub-mesothelial basement membrane. 5. OC cells proliferate at distant sites in the peritoneal cavity generating metastatic implants.

1.2 Tissue Tansglutaminase (TG2)

1.2.1 Transglutaminase family

Transglutaminase (TGase, EC 2.3.2.13) is a protein family consisting of 9 family members: a structural protein, band 4.2, which lacks catalytic activity and is expressed specifically in erythrocytes; and 8 enzymes, the factor XIII α subunit (FXIIIA) and TG 1-7.

So far, only 7 members of the family have been isolated and characterized at protein level (Table 1). They are: 1) FXIIIA, is essential for blood coagulation (Lorand 2001); 2) TG1, also called keratinocyte TGase, involves in keratinocyte differentiation (Rice et al 1992); 3) TG2, the tissue transglutaminase, is a multifunctional protein, details will be discussed later; 4) TG3, the epidermal/hair follicle TGase, that together with TG1 is involved in the assembly of the epidermal cornified cell envelope (CE), a structure formed beneath the plasma membrane in terminally differentiating stratified squamous epithelia, and is essential for skin maturation and integrity (Kalinin et al 2001); 5) TG4, the prostate specific TGase, involved in semen coagulation, is essential for sperm maturation (Porta et al 1986); 6) TG5, also called TGx, contributes to keratinocyte differentiation and CE assembly (Candi et al 2002); and 7) band 4.2, also called ATP-binding erythrocyte membrane protein band 4.2, is important in the regulation of erythrocyte shape and mechanical properties (Korsgren et al 1990).

The other 2 lesser studied TG family members, TG6 and TG7, also called TGy and TGz, were identified at DNA level (Grenard et al 2001). All TGase

encoding genes are closely related and have similar sequence and gene structure. The genes for FXIIIA (*F13A1*) and TG1 (*TGM1*) consist of 15 exons and 14 introns, whereas *TGM2-7* (genes for TG2-7) and *EPB42* (band 4.2 encoding gene) consist of 13 exons and 12 introns. These genes are scattered on different chromosomes: chromosome 3 for *TGM4*, 6 for *F13A1*, 14 for *TGM1*, 15 for *TGM5*, *TGM7*, and *EPB42*, and 20 for *TGM2, TGM3,* and *TGM6*. Given the high degree of similarity in gene structure, protein primary and three-dimensional structure, and their catalytic function, TGs genes are considered to have evolved from a common ancestral gene despite their distinct distribution in the human genome (Grenard et al 2001).

All TGase family members share 4 distinct domains: an N-terminal β-sandwich, a α/β catalytic core, and 2 C-terminal β-barrel domains (Yee et al 1994) (Liu et al 2002) (Figure 3). Similar to papain (EC 3.4.22.2) and the papain-like cysteine proteases, the catalytic core of TGase contains a Cys277-His335-Asp358 triad. Band 4.2 is catalytically inactive because it lacks this cysteine residue. TGase catalyzes three types of Ca^{2+}-dependent reactions: transamidation, esterification, and hydrolysis (Iismaa et al 2009). All three reactions involve 2 steps: acylation and deacylation. During the first step, a glutamine-containing protein or peptide acts as the acyl donor or amine acceptor substrate, which reacts with the Cys277 of the enzyme (acyl acceptor) to form a γ-glutamyl thioester intermediate, with ammonia released as a by-product. The acylation is followed either by a direct attack by water (hydrolysis/deamidation) or by binding of a second amine donor (transamidation) or an alcohol

(esterification). Transamidation results in either protein cross-linking, where a glutamine residue is cross linked to a lysine residue via a ε- (γ-glutamyl)-lysine isopeptide bond; or amine incorporation, where a primary amine is incorporated into a glutamine residue of the acceptor protein (Figure 4). The first TGase (which is designated TG2) was identified on the basis of its ability to incorporate amines into proteins (Sarkar et al 1957). Ca^{2+}-dependent amine incorporation remains the main method to detect TG activity.

Protein	Tissue distribution	Cellular localization	Gene	Gene map locus	Function
FXIIIA	platelets	Cytosol, extracellular	F13A1	6p24-25	blood coagulation
TG1	keratinocytes	Cytosol, membrane	TGM1	14q11.2	Keratinocyte differentiation
TG2	ubiquitous	Cytosol, nucleus, membrane, extracellular	TGM2	20q11-12	multiple
TG3	Squamous epithelium	cytosol	TGM3	20q11-12	Cell envelope (CE) formation
TG4	prostate	unknown	TGM4	3q21-22	Semen coagulation
TG5	ubiquitous	unknown	TGM5	15q15.2	CE formation, keratinocyte differentiation
TG6	unknown	unknown	TGM6	20q11	unknown
TG7	ubiquitous	unknown	TGM7	15q15.2	unknown
Band 4.2	cells of the erythroid lineage	membrane	EBP42	15q15.2	erythrocyte membrane integration

Table 1: Tansglutaminase family members. There are 9 members in the family: 8 enzymes including factor XIII α subunit (FXIIIA) and TG1-7, and non-enzymatic protein band 4.2. Each member has different tissue distribution, cellular localization and functions as shown in the table.

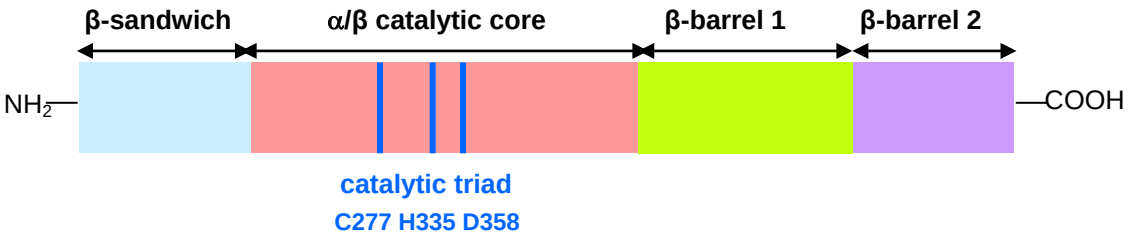


Figure 3: Schematics of TGase domains. TGases share 4 distinct domains: an N-terminal β-sandwich, a α/β catalytic core (containing the Cys^{277}-His^{335}-Asp^{358} catalytic triad), and 2 C-terminal β-barrel domains.

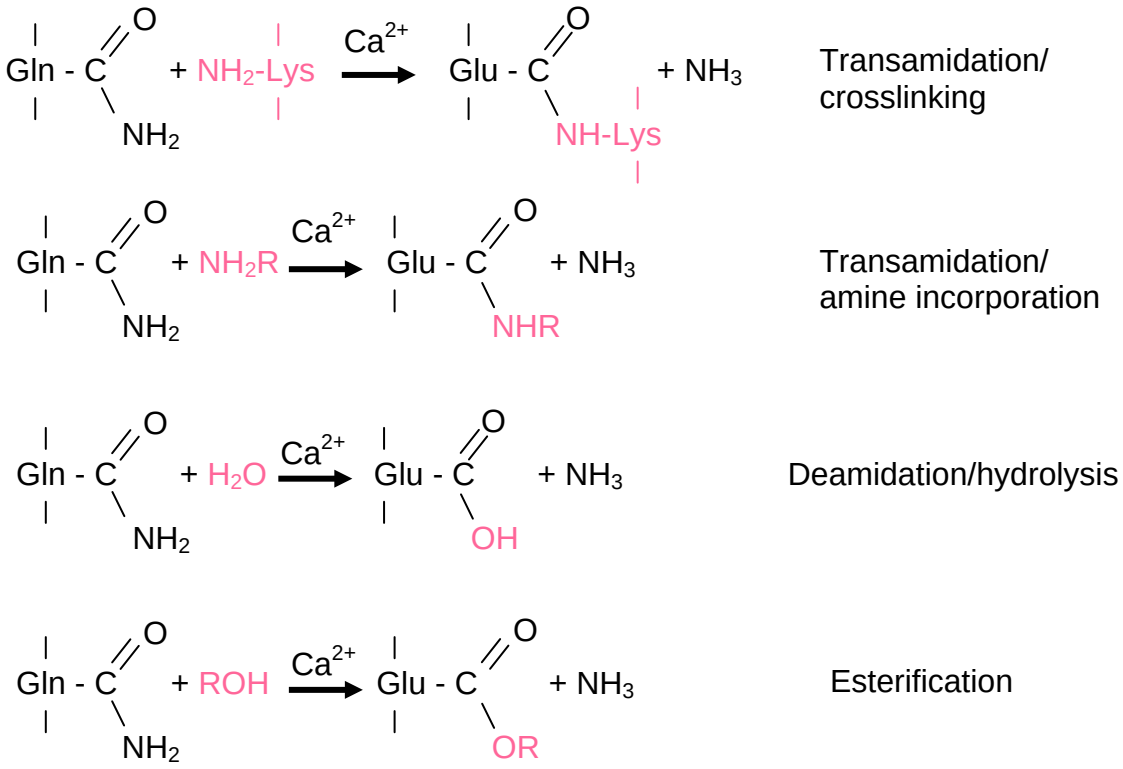


Figure 4: Catalytic activity of TGases. TGases catalyze 3 types of Ca^{2+}-dependent reactions: transamidation, hydrolysis, and esterification. In all these reactions, a glutamine-containing protein or peptide acts as the major substrate. It reacts with a lysine-containing protein or peptide to cause crosslinking of the 2 substrates. TG2 reacts with a polyamine to cause amine incorporation into the glutamine-containing protein or peptide. TG2 reacts with H_2O to cause deamidation. It reacts with an alcohol to cause esterification.

1.2.2 Tissue transglutaminase is a multifunctional protein

Tissue transglutaminase (TG2) is distinguished from other TGases by several unique characteristics: ubiquitous expression, widespread localization, ability to bind to and hydrolyze guanine nucleotides, and its non-enzymatic function involved in cell-matrix interaction (Figure 5). The ubiquitous distribution of TG2 is mainly due to its high expression on vascular endothelium and smooth muscle cells (Thomazy and Fesus 1989). TG2 is located mostly in the cytosol (~73%), and partly on the plasma membrane and in the ECM (~20%). A small proportion of TG2 (~7%) is localized in the nucleus (Bruce and Peters 1983). TG2 acts as a multifunctional protein due to its multiple functional domains and its functions vary depending on the protein's cellular localization and its regulators. Ca^{2+} and GTP are two important regulators that inversely regulate the protein's TGase and GTPase activity via an allosteric modulation of TG2 conformation (Di Venere et al 2000).

Folk et al demonstrated that Ca^{2+} induced a conformational change of purified guinea pig liver transglutaminase that promoted its enzymatic activity (Folk et al 1967). TG2 can bind 6 Ca^{2+}; 5 of the 6 Ca^{2+}-binding sites having been identified around the catalytic core (Kiraly et al 2009). However, the crystal structure of Ca^{2+}-bound TG2 is still unresolved. Pinkas et al reported a TG2 crystal structure with exposed active site using a pentapeptide Ac-P(DON)LPF-NH2, which acts as an irreversible TG2 inhibitor (Pinkas et al 2007). Based on the crystal structure of Ca^{2+}-bound FXIIIA (Fox et al 1999) and TG3 (Ahvazi et al 2002), it has been suggested that Ca^{2+} binding opens a channel to expose the active site of TG2, which then can exert its enzymatic activity.

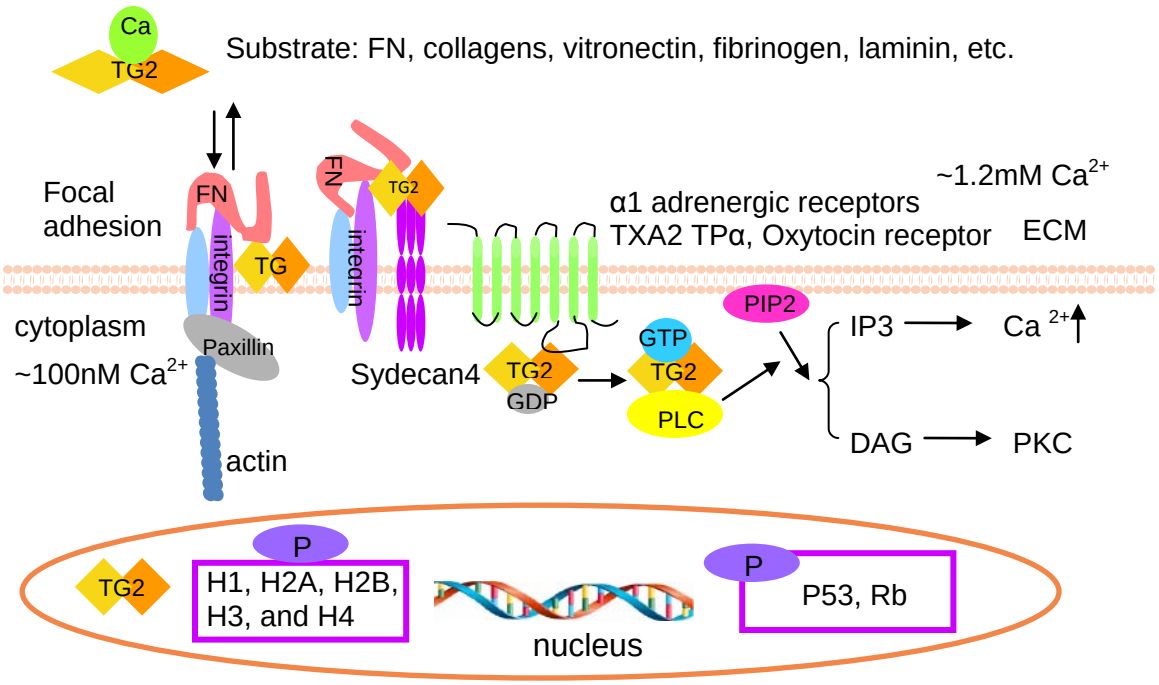


Figure 5: TG2 localization and corresponding functions. 1) TG2 is located mostly in the cytosol (70-80%), where the protein acts as GTPase (G_h). G_h stimulates phospholipase C (PLC)-mediated inositol phosphate (IP) production after coupling with α_1-adrenergic receptors, TXA2 TPα, and oxytocin receptor. 2) Approximately 7% of TG2 is located in the nucleus. Nuclear TG2 acts as a cross-linking enzyme, a G-protein (Singh et al 1995), and a protein kinase. As a protein kinase, TG2 phosphorylates p53, Rb, and the histones H1, H2A, H2B, H3, and H4. 3) Cell surface TG2 can form ternary complexes with integrins and fibronectin (FN) to facilitate cell adhesion and migration. 4) Extracellular TG2 has transamidating activity due to high extracellular Ca^{2+} concentrations. Many of the ECM components are substrates for TG2. Extracellular TG2 also interacts with syndecan 4 to form a FN-TG2-sydecan4 complex with or without integrins, and this complex is involved in the formation of focal adhesions and in the syndecan mediated signaling pathway.

Achyuthan et al found that guanosine 5'-triphosphate (GTP) could bind to guinea pig liver transglutaminase and inhibit its transamidation activity, a process which can be reversed by calcium. Guanosine diphosphate (GDP) binding also inhibited transglutaminase activity, but to a lesser extent than GTP. Guanosine monophosphate (GMP) didn't inhibit the enzyme activity (Achyuthan and Greenberg 1987). Later, purified guinea pig liver transglutaminase was reported to exert a GTPase activity to hydrolyze GTP with a Km of 4.4 μM (Lee et al 1989). By purifying the α_1-adrenergic agonist-receptor-G-protein ternary complex, Im et al found a 74-kD GTP binding protein (G-protein), named $G\alpha_h$ (Im and Graham 1990), which forms a heterodimer with a 50-kD subunit $G\beta_h$ (Im et al 1990), the inhibitory regulator of $G\alpha_h$ (Baek et al 1996a). By sequencing the newly found G-protein, $G\alpha_h$ was identified as TG2, exerting both transglutaminase activity and G-protein coupled receptor (GPCR) signal transduction function (Nakaoka et al 1994). Unlike the heterotrimeric G proteins and the Ras superfamily small GTPases, TG2 doesn't have a common GTP-binding motif NKXD, but contains a unique nucleotide binding pocket for the nucleotide (Iismaa et al 2000). The crystal structure of TG2 in complex with GDP revealed a unique GDP-binding site located between the catalytic core and β-barrel 1 domain. Several amino acids including Lys173 and Phe174 from the catalytic core, Arg478, Val479, Met483, Arg580, Leu582, and Tyr583 from the β-barrel 1 form a hydrophobic pocket to bind GDP. Arg580 seems to be indispensable for GDP binding, which forms two ion pairs with the α- and β-phosphates of the nucleotide (Liu et al 2002). Mutation of Arg580 abolished GTP-binding but didn't affect TGase activity

(Begg et al 2006b). This GDP-bound TG2 exhibits a closed conformation, where Cys277, the essential residue for TGase activity is blocked by 2 loops within the β-barrel 1 domain. Furthermore, Tyr516 forms a hydrogen bond with Cys277, which makes Cys277 inaccessible to the substrate. Thus, GTP/GDP binding induces a compact, catalytically inactive conformation of TG2. GTP binding to Arg580 is critical for TG2 to adopt the compact form while mutation of Arg580 destabilizes the compact form, indicating that Arg580 is an important allosteric site for TG2 conformational/functional regulation (Begg et al 2006b).

Although TG2 has a different nucleotide binding mechanism from heterotrimeric GTPases, the signaling transduction cascade is similar. Upon binding of the agonist, the GPCR undergoes a conformational change which activates the $G\alpha_h$ by releasing GDP and binding to GTP. Once bound to GTP, $G\alpha_h$ dissociates from both the receptor and $G\beta_h$. GTP-bound $G\alpha_h$ then interacts with the effector which produces a second messenger to amplify the signal. Once GTP is cleaved to GDP and Pi by the GTPase function of $G\alpha_h$, GDP-bound $G\alpha_h$ dissociates with the effector and reassociates with $G\beta_h$, and thus completing an activation/deactivation cycle of GTPase (Im et al 1990). **Ligand:** $G\alpha_h$ was first found in a ternary form with an adrenergic agonist and a α_1-adrenoceptor (AR) (Im and Graham 1990). Catecholamines such as epinephrine and norepinephrine can bind to and activate α_1-adrenoceptor. **Receptor:** α_1-AR is a transmembrane glycoprotein and belongs to the GPCR superfamily with seven α-helical transmembrane-spanning domains connected by hydrophilic loops exposed to the intra- and extracellular environment. The intracellular loops bind and activate

the receptor-coupled G-proteins. They are classified into 3 subtypes: α1A, α1B, and α1D based on molecular structure, function, and signaling (Graham et al 1996). α1-AR activates phosphoinositide-specific phospholipase C (PI-PLC) via α subunit of Gq family (Wu et al 1992). TG2/Gh interacts with the third intracellular loop of α1B- and α1D- but not α1A-AR, and this interaction is not affected by transamidation inactive TG2 mutant C277S (Chen et al 1996). Multiple regions at the C-terminal β-barrel domains of TG2 including L547I561, R564−D581, and Q633−E646 interact with α1B-AR to mediate the signal to downstream effectors (Feng et al 1999). TG2/Gh also interacts with thromboxane A2 (TXA2) receptor TPα but not with TPβ to activate PLC (Vezza et al 1999), while thromboxane A2 (TXA2) is a prostaglandin derivative produced during arachidonic acid metabolism (Nakahata 2008). Except for α1-AR and TPα, TG2/Gh also couples with oxytocin receptor to activate PLC, while oxytocin is a neurohypophysial nonapeptide hormone (Baek et al 1996b). **Effector:** Phospholipase C (PLC) δ 1 was reported to be the effector of G_h (Feng et al 1996), which has an 8-amino acids recognition site located at the C-terminal β-barrel 2 domain (Leu665-Lys672) involved in interaction with PLC (Hwang et al 1995). PLC (EC 3.1.4.11) is a family of proteins including PLCβ, γ, δ, ε, ζ and η, which share a structure with an N-terminal pleckstrin homology (PH) domain, 4 EF hands, a catalytic TIM barrel, and a C-terminal C2 domain (Bunney and Katan 2011). The residues T721-L736 located in the C2 domain of PLCδ 1 are involved in the interaction with TG2 (Kang et al 2002). PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to two second messengers: 1,2-diacylglycerol (DAG), an

activator of protein kinase C (PKC) (EC 2.7.11.13), and inositol 1,4,5-trisphosphate (IP3), which binds to the IP3-sensitive Ca^{2+} channel at endoplasmic reticulum (ER) and triggers Ca^{2+} releasing to cytoplasm (Exton 1996). **Effects:** α1-ARs are important mediators of sympathetic nervous system responses, particularly arteriolar smooth muscle contraction and cardiac contraction, which are critical for cardiac function and blood pressure homeostasis (Graham 1990). TXA2 TPα is distributed on a variety of cells, including platelets, vascular smooth muscle cells, and macrophages. Upon activation, it exerts potent biological activities including platelet aggregation and secretion, vasoconstriction, and mitogenesis (Nakahata 2008). Oxytocin receptor is widely distributed in the central nervous system: the olfactory system, the basal ganglia, the limbic system, the thalamus, the hypothalamus, some cortical regions, the brainstem, and the spinal cord, where it involves in maternal, sexual, social, and stress-related behavior (Tribollet et al 1992).

Except for GTP, TG2 also binds and hydrolyzes adenosine triphosphate (ATP). ATP binding inhibits GTP hydrolysis but does not inhibit TGase activity (Lai et al 1998). ATP binds to the same pocket as GDP, but forms different hydrogen bonds and ions interaction with TG2. The four residues Arg476, Arg478, Val479 and Tyr583 are involved both in GDP and ATP binding to TG2. The residue Arg580, which is important for GTP/GDP binding, is not involved in ATP binding (Han et al 2010). By purifying the insulin-like growth factor-binding protein-3 (IGFBP-3) kinase in T47D breast cancer cells, Mishra et al found that TG2 on the cell membrane can act as kinase to phosphorylate IGFBP-3 and

IGFBP-5 at multiple serine residues in the central domain. This kinase activity is inhibited by calcium (Mishra and Murphy 2004). The authors later identified more substrates for TG2 as a kinase. They include P53 (Mishra and Murphy 2006), H1, H2A, H2B, H3, and H4 histones (Mishra et al 2006), and retinoblastoma protein (Rb) (Mishra et al 2007). Phosphorylation of P53 by TG2 alters the interaction between p53 and Mdm2, and phosphorylation of Rb by TG2 destabilizes the Rb/E2F1 complex. These results indicate that TG2 may act as a kinase to promote the function of some tumor suppressor proteins. However, all these studies were performed *in vitro*, and additional cell-based or *in vivo* analyses need to validate the kinase function of the protein.

Besides the functions mentioned above, Hasegawa et al reported a novel function of TG2 as a protein disulfide isomerase (PDI) by showing that TG2 converts the reduced/denatured inactive RNase A molecule to the native active enzyme, and this function is inhibited by bacitracin, a PDI inhibitor, but is not inhibited by calcium or GTP (Hasegawa et al 2003).

As a multifunctional protein, TG2 exerts different roles in different cellular compartments. Since Smethurst et al reported that at least 10μM Ca^{2+} is required to detect the transamidating activity of TG2 in a permeabilized cell system (Smethurst and Griffin 1996) and the intracellular Ca^{2+} concentration is relatively low (~100nM Ca^{2+}), it is believed that cytosolic TG2 acts as a GTPase, and not as a transamidating enzyme. However, the exact concentration of Ca^{2+} necessary for the enzyme's activation under physiological conditions is not clear. Basel level of transamidating TG2 activity was detected in Chinese-hamster

ovary cells (Fesus and Tarcsa 1989). Kiraly et al also described that enzymatic TG2 activity has been reported in many resting cells (Kiraly et al 2011), suggesting that under certain conditions intracellular TG2 can act as a TGase.

In contrast with cytosolic TG2, the extracellular matrix (ECM) TG2 exerts transamidating activity due to high extracellular Ca^{2+} concentrations (~1.2mM Ca^{2+}) (Turner and Lorand 1989). Since components of the ECM are TG2 enzymatic substrates: FN, collagens, vitronectin, fibrinogen, osteopontin, laminin, etc., extracellular TG2 is involved in the assembly, remodeling, and stabilization of ECM in various tissues (Aeschlimann and Thomazy 2000). Extracellular TG2 also interacts with syndecan 4 to form a FN-TG2-syndecan 4 complex with or without integrin. This complex is involved in the formation of focal adhesions and in syndecan mediated signaling (Wang et al 2010).

Cell surface TG2 forms ternary complexes with integrins and fibronectin (FN) in which the three proteins interact with each other, facilitating cell adhesion and migration (Turner and Lorand 1989). Interaction with integrins makes it possible for TG2 to be involved in integrin-dependent signal transduction, including in the focal adhesion kinase (FAK) and GTP-binding proteins Raf-1 and Rho regulated signaling pathways (Hang et al 2005). These interactions do not require Ca^{2+} and are independent of the transamidating and GTPase activity of the enzyme. Since most integrin ligands in the ECM are TG2 enzymatic substrates: FN, collagens, vitronectin, fibrinogen, osteopontin, laminin, etc., extracellular TG2 is involved in the assembly, remodeling and stabilization of ECM in various tissues (Aeschlimann and Thomazy 2000).

About 7% TG2 exists in the nucleus. The mechanism of nuclear translocation of TG2 is still under study. Importin-α3, a nuclear transporter protein, has been reported to be implicated in the active transport of TG2 into the nucleus (Peng et al 1999). TG2 has a putative nuclear localization signal (NLS) motif KQKRK between residues 598-602, which shares high homology with the NLS in the influenza virus NS1 non-structural protein (Greenspan et al 1988). TG2 also has a putative leucine-rich nuclear export signal (NES) motif LHMGLHKL at residues 657-664, which can be recognized by the nuclear exporter exportin1/Crm1p (Stade et al 1997). Therefore, the balance between the import and the export of TG2 regulates the amount of nuclear accumulation of TG2. TG2 acts as cross-linking enzyme (Lesort et al 1998), a G-protein (Singh et al 1995), and a protein kinase in the nucleus.

Therefore, TG2 acts as a multifunctional protein in different cellular compartments. Interestingly, TG2 knockout mice are viable and develop normally (De Laurenzi and Melino 2001) (Nanda et al 2001). To explain this normal phenotype, it has been speculated that the functions of TG2 may be compensated by the other members of the transglutaminase family. Further study of the TG2 null animals revealed impaired wound healing, defective phagocytosis, and maturity onset diabetes of young (MODY) (Bernassola et al 2002) (Szondy et al 2003). More detailed study of TG2 knockout mice is needed to fully understand the functions of this protein *in vivo*.

1.2.3 TG2 Involvement in Disease:

Given the complexity of its enzymatic and non-enzymatic functions, TG2 is involved in a number of inflammatory disorders, in wound healing, fibrosis, autoimmune diseases, neurodegenerative diseases, and cancer.

Our laboratory previously identified TG2 as an upregulated gene in OC cells compared with normal ovarian epithelial cells (Matei et al 2002). Overexpression of TG2 protein levels was also demonstrated in ovarian cancer cells, tumors and ascitic fluid (Satpathy et al 2007). Our group demonstrated that overexpressed TG2 facilitates OC intraperitoneal (i.p.) dissemination by enhancing cancer cell adhesion to the ECM and interaction with β1 integrin. Stable knockdown of TG2 in the OC cell line SKOV3 markedly affected cell adhesion, migration *in vitro* and i.p. dissemination in nude mice (Satpathy et al 2007). Recently, our laboratory reported that TG2 can upregulate matrix metalloproteinase-2 (MMP-2), which is an important mediator of ECM degradation and facilitates tumor cell invasion (Satpathy et al 2009). TG2 overexpression was also reported in other cancers such as breast cancer (Herman et al 2006), pancreatic cancer (Verma et al 2006), melanoma (Fok et al 2006), glioblastoma (Yuan et al 2007), and meningioma (Yuan et al 2008). The reported functions of TG2 in cancer were focused on metastasis and chemoresistance. However, the mechanisms by which TG2 is associated with cancer metastasis and chemoresistance are not fully understood. The function of TG2 in cancer is still an enigma since TG2 displays both pro- and anti-apoptotic effects in cells (Fesus and Szondy 2005).

TG2 has been linked to apoptosis, acting either as a promoter or as an antagonist, through mechanisms that are specific to the cellular context. Under physiological conditions, the intracellular enzymatic activity of TG2 is negatively regulated by low concentrations of Ca^{2+} and high level of guanosine triphosphate. However, in the late phases of apoptosis, when massive intracellular Ca^{2+} influx occurs, the enzymatic function of TG2 is activated leading to cross-linking of cytosolic proteins and finalization of the cell death process (Lorand and Graham 2003). In contrast, TG2 has an antiapoptotic role in malignant cells. For instance, in breast and pancreatic cancer cells, TG2 activates nuclear factor kappa B (NF-κB) by cross-linking the inhibitory subunit inhibitor of kappa B α (IκBα). This leads to its polymerization and displacement out of the complex with NF-κB (Park et al 2006) (Mann et al 2006). This process depends on TG2's enzymatic activity and leads to constitutive activation of NF-κB. In leukemia HL60 cells, TG2-mediated transamidation protects the retinoblastoma gene product from caspase-induced degradation and promotes cell survival (Boehm et al 2002). In colon cancer cells HCT116, TG2 suppresses apoptosis by protecting the cleavage and activation of caspase-3, through protein cross-linking (Yamaguchi and Wang 2006). Furthermore, TG2 is involved in anchoring epithelial cells to the extracellular matrix; this process leads to activation of 'outside-in' signaling that ultimately promotes cell survival (Akimov et al 2000). The interaction between TG2, fibronectin and integrins not only promotes cancer cell adhesion and migration, but also promotes cancer cell proliferation and survival by upregulating cell survival and anti-apoptotic signaling

pathways, such as focal adhesion kinase (FAK) and PI3K/Akt pathway (Verma et al 2008). These observations have direct applications for understanding the process of chemotherapy resistance and have incited an interest in developing TG2 inhibitors as anticancer therapy (Yuan et al 2005).

1.3 Transforming growth factor-beta (TGF-β)

Transforming growth factor-beta is a multifunctional polypeptide that signals through its serine-threonine kinase receptors and intracellular Smad effectors to regulate development, differentiation, and homeostasis.

Ligand: Transforming growth factors (TGFs) were first discovered as secreted polypeptides ranging from 7 to 25kDa in sarcoma virus-transformed fibroblasts, which can induce anchorage-independent growth of the fibroblasts in soft agar (de Larco and Todaro 1978). Further purification identified two distinct polypeptides, which were named TGF-α and TGF-β (Anzano et al 1983). TGF-α binds to the EGF receptor and competes with EGF to induce normal rat kidney cells' colony formation in soft agar. There are 3 isoforms in mammalian TGF-β family: TGF-β1, TGF-β2, and TGF-β3. As TGF-β1 is the prototype, TGF-β2 was purified from porcine platelets (Cheifetz et al 1987), and TGF-β3 was identified at the cDNA level (ten Dijke, 1988). TGF-βs 1–3 belong to the transforming growth factor beta superfamily which contains more than 30 polypeptide growth factors including TGF-βs (1–3), activins (A, B), inhibins (A, B), bone morphogenetic proteins (BMPs 1–20), growth differentiation factors (GDFs) including myostatin, nodal, leftys (1,2), and anti-Mullerian hormone (AMH) or Müllerian-inhibiting

substance (MIS). All family members share a similar structure: the disulfide-linked dimeric polypeptide.

The TGF-β superfamily ligands are secreted as latent form (Lawrence et al 1984) comprising a large dimeric N-terminal prodomain (latency-associated propeptide, LAP) and a C-terminal mature TGF-β (Gentry et al 1988), called small latent complex (SLC). The mature ligands are cleaved from the prodomain by furin-like enzymes during secretion (Dubois et al 1995). After cleavage, the propeptide LAP remains associated with the TGF-β dimer via non-covalent electrostatic interaction and inhibits TGF-β's binding to its receptors. Most cells secrete latent TGF-β as large latent complex (LLC), where the LAP associates with the latent TGF-β binding protein (LTBP) via a disulfide bond (Saharinen et al 1996). LTBPs are required for correct assembly and secretion of TGF-βs (Miyazono et al 1991). LTBPs belong to the LTBP/fibrillin family, including LTBP 1-4 and fibirillin 1-2, which are the main components of extracellular microfibrils. (Taipale et al 1996) (Ramirez and Pereira 1999). LTBPs are 125-240 KD proteins characterized by 14-20 copies of epidermal growth factor (EGF)-like repeats and 4 copies of eight cysteine (8-cys) repeats (Hyytiainen et al 2004). LTBPs bind to LAP via the third 8-cys repeat (Saharinen and Keski-Oja 2000), and localize the latent complex to the extracellular matrix (ECM) via the other three 8-cys repeats (Taipale et al 1994) (Unsold et al 2001). Thus the ECM bound latent complexes serve as a reservoir for TGF-β. Only after TGF-β is released from the latent complex, it binds to its cell surface receptors and initiates the intracellular signaling cascade. Release of active TGF-β requires two steps: first, to release

the TGF-β latent complex from ECM by cleavage of LTBP at its protease sensitive hinge region, which is between the N-terminal ECM binding domain and the C-terminal LAP binding domain. This can be achieved by many proteinases in the serine proteinase family, such as plasmin, chymase, and elastase (Taipale et al 1992) (Taipale et al 1995); second, to activate TGF-β by disruption of the non-covalent interaction between LAP and TGF-β. This can be achieved *in vitro* by physicochemical activation such as acid or base, heat and chaotropic agents treatment (Brown et al 1990). Under physiological situations, several mechanisms for TGF-β activation have been reported. A number of proteases including plasmin (Sato and Rifkin 1989) and MMP-2/9 (Yu and Stamenkovic 2000) can degrade LAP and thus release the active TGF-β. Thrombospondin (TSP) can release the active TGF-β by inducing a conformational change of LAP (Murphy-Ullrich and Poczatek 2000). Integrin $\alpha_v\beta_6$ can bind to the Arg-Gly-Asp (RGD) sequence in LAP, induce a conformational change, and activate TGF-β (Munger et al 1999). Normally, these two steps are finely tuned because they are critical for the regulation of TGF-β activity. For example, plasmin and MMP-2/9 not only can activate TGF-β, but also induce degradation of ECM, which is important in the process of TGF-β secretion and storage. Moreover, TGF-β induces the expression and activity of plasminogen activator inhibitor (PAI) (Laiho et al 1986) and tissue inhibitor of metalloproteinase-1 (TIMP-1) (Edwards et al 1987), thus a feed-back inhibitory loop is formed to modulate TGF-β activity. Deregulation of TGF-β activity will lead to pathological conditions such as fibrotic diseases, chronic inflammation and cancer.

Receptors: Active TGF-β signals through a heteromeric complex of type I and type II transmembrane serine-threonine kinase receptors. The ligand brings the receptors together in a heterotetrameric complex in which the type II receptors phosphorylate and activate the type I receptors. The receptor serine/threonine kinase family comprises 12 members: 7 type I receptors (Activin receptor-like kinases, ALKs1-7) and 5 type II receptors (TβR-II, ActR-II, ActR-IIB, BMPR-II, and AMHR-II). Both types of receptors consist of an N-terminal extracellular ligand binding domain, a transmembrane region, and a C-terminal intracellular serine/threonine kinase domain, whereas the type I receptors contain a specific glycine-serine-rich sequence, called GS region. This sequence is located upstream of the kinase domain in type I receptors and, once phosphorylated by the type II receptors, confers a conformational change that switches this region from a binding site for inhibitor the 12-kDa FK506-binding protein (FKBP12) to a binding site for its substrates, the Smad family of transcription factors. This switch results in full activation of the type I receptor kinases (Huse M, Massague J. 2001). For some ligand-receptor interactions, co-receptors, such as betaglycan, endoglin (Fonsatti et al 2001), and members of the EGF-Cripto, Frl-1, and Cryptic (EGF-CFC) family, are also required (Feng and Derynck 2005).

Intracellular signal mediators: Smad is a term derived from Sma in *Caenorhabditis elegans* and Mad (Mothers against decapentaplegic) in Drosophila (Derynck et al 1996). There are 8 Smad proteins encoded in mammalian genome, and they are divided into 3 subgroups based on structural

and functional criteria. Smad1, Smad2, Smad3, Smad5, and Smad8 act as substrates for TGF-β family of receptors, referred as receptor-regulated Smads, or R-Smads. Smad 1, 5, and 8 are substrates for BMP and AMH receptors, Smad 2 and 3 are substrates for TGF-β, activin, and Nodal receptors. Smad 4, also called Co-Smad, is a common partner for R-Smads. Smad 6 and 7 are inhibitory Smads (I-Smads) that interfere with Smad-receptor and Smad-Smad interactions. Smads are modular proteins with an N-terminal Mad-homology 1 (MH1) (conserved in all R-Smads and Smad4, lacked in I-Smads), a linker region and a C-terminal MH2 domain (conserved in all Smads). The MH1 domain participates in nuclear localization, DNA-binding, and protein–protein interactions. The MH1 domain binds to the sequence 5'-GTCT-3', or its complement 5'-AGAC-3', called the Smad-binding element (SBE), on target gene promoter region (Shi et al 1998). The linker domain recruits Smurf (Smad ubiquitination-related factor) ubiquitin ligases that regulate Smad and TGF-β receptor half-life. The MH2 domain is a major protein–protein interaction domain. It has a C-terminal conserved Ser-X-Ser motif, which can be phosphorylated by activated receptor serine-threonine kinase (Massague et al 2005).

TGF-β induced activation of the receptor complex leads to activation of Smad2 and Smad3 through direct phosphorylation by TGF-β RI. Phosphorylated Smad2 and Smad3 then form a trimeric protein complex with Smad4. Monomeric Smad proteins constantly shuttle in and out of the nucleus conferred by a lysine-rich nuclear localization signal (NLS) in MH1 domain (Xiao et al 2001), but only the receptor-activated R-Smad/Co-Smad complexes favors their nuclear

accumulation (Schmierer and Hill 2005). In the nucleus, the active R-Smad/Co-Smad complexes bind directly to cognate DNA sequence and associate with other transcription factors such as activating transcription factor 2 (ATF2) and cyclic AMP-responsive element-binding protein (CREB), co-activators CREB-binding protein (CBP) and p300 or co-repressors to activate or repress target gene transcription (Moustakas and Heldin 2009). Smad4 serves as co-activator for R-Smads by stabilizing the interaction between R-Smad, DNA and CBP/p300 (Derynck and Zhang 2003).

Besides Smad-mediated transcription, TGF-β can activate other pathways such as mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase (Erk), p38 and c-Jun N-terminal kinase (JNK), phosphoinositide 3′ kinase (PI3K), small GTPases of the Ras superfamily and NF-κB pathways . TGF-β activates TGF-β-activated kinase 1 (TAK1), which can activate p38 MAPK through MKK3, activate JNK through MKK4, and activate NF-κB through the Inhibitor of kappa B (IκB) kinase (Wang and Fu 2001) (Figure 6).

TGF-β in cancer: TGF-β exerts dual roles in cancer, as tumor suppressor at the early stages of tumorigenesis, through inhibition of epithelial cell proliferation, and as tumor promoter, at advanced stages, by enabling cancer cells to acquire the six hallmarks of cancer: cell proliferation, survival, immortality, angiogenesis, dissemination, and evading immunosurveillance (Tian et al 2011).

As a tumor suppressor, TGF-β inhibits cell proliferation by arresting cell cycle at the G1 phase via transcriptional regulation of target genes. TGF-β downregulates the expression of the growth-promoting transcription factors, c-

Myc (Chen et al 2002) and Id1 (Kang et al 2003), and upregulates the cyclin-dependent protein kinase (CDK) inhibitors, $p15^{INK4b}$, $p21^{CIP}$ and $p27^{kIP1}$ (Siegel and Massague 2003). While p15 inhibits the cyclin D/CDK4/6 complex, p21 inhibits cyclin B/CDK1, and p27 preferentially target and antagonize cyclin E/CDK2 complex. Taking all the events together, TGF-β works in concert to inhibit the progression of cells through the cell cycle.

As a tumor promoter, TGF-β activates PDGF, MAPK, NF-κB and PI3K/AKT pathways to promote cancer cell proliferation and survival (Arsura et al 2003) (Sanchez-Capelo 2005). TGF-β also acts as a potent inducer of angiogenesis, which is crucial for tumor growth, as blood vessels deliver nutrients and oxygen to the tumor cells. studies using transgenic KO mouse models of components of the TGF-β signaling pathway such as TβRI, TβRII, ALK5, ALK1, endoglin, and Smad5 revealed that TGF-β signaling is essential for vascular development, including proliferation and differentiation of endothelial cells, maintenance of the integrity of the vessel wall, recruitment of smooth muscle cells, as well as the deposition of extracellular matrix (Goumans et al 2009). TGF-β promotes tumor dissemination by inducing epithelial-mesenchymal transition (EMT), cell motility and invasion through both Smad-dependent and Smad-independent signaling pathways. TGF-β signals through Smad-mediated induction of Snail and Slug zinc-finger transcription factors to repress the E-cadherin gene (Takano et al 2007). TGF-β is also reported to downregulate E-cadherin expression via PI3K signaling pathway (Vogelmann et al 2005). TβRII-mediated phosphorylation of polarity protein Par6 promotes the dissolution of

tight junctions and disassembly of the cytoskeleton (Ozdamar et al 2005). TGF-β acts as an immunosuppressor by inhibition of B or T lymphocyte proliferation, differentiation and survival. During tumor progression, TGF-β suppresses anti-tumor immune response by regulating chemotaxis, activation, and survival of lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells, and granulocytes to evade immune surveillance (Li et al 2006). Overall, as a tumor promoter, TGF-β functions to promote cancer cell proliferation and survival by activating cell survival signaling pathways, to promote tumor metastasis by inducing motility, invasion and angiogenesis, to help tumor cells evade immunosurveillance by repressing anti-tumor immune response.

TGF-β in OC: In normal ovarian surface epithelial cells, TGF-β functions to inhibit epithelial proliferation (Berchuck et al 1992). However, this function is often lost in ovarian cancer, although the receptors and the downstream Smad signaling are intact including TGF beta 1, TGF beta RI, TGF beta RII, Smad2, -3, -4, and inhibitory Smad 6 and -7 (Yamada et al 1999) (Dunfield et al 2002). Moreover, the TGF-β ligand was found significantly increased in expression in both primary and recurrent OC compared with normal ovarian tissue (Henriksen et al 1995) (Bristow et al 1999). TGF-β1 and 2, but not 3, are secreted in ovarian cancer-associated ascites by both ovarian cancer cells and peritoneal mesothelial cells, and the secreted levels of TGF-β1 are higher than those of TGF-β2 (Abendstein et al 2000). It is possible that TGF-β plays a role in OC metastasis, as it significantly increases invasion of OC cells, but not of normal ovarian surface epithelial cells (NOSE) cells by increasing MMP-2 expression

and activity (Rodriguez et al 2001). In addition TGF-β1 induces EMT and MMP secretion in OC cells in a Smad-dependent signaling pathway (Do et al 2008).

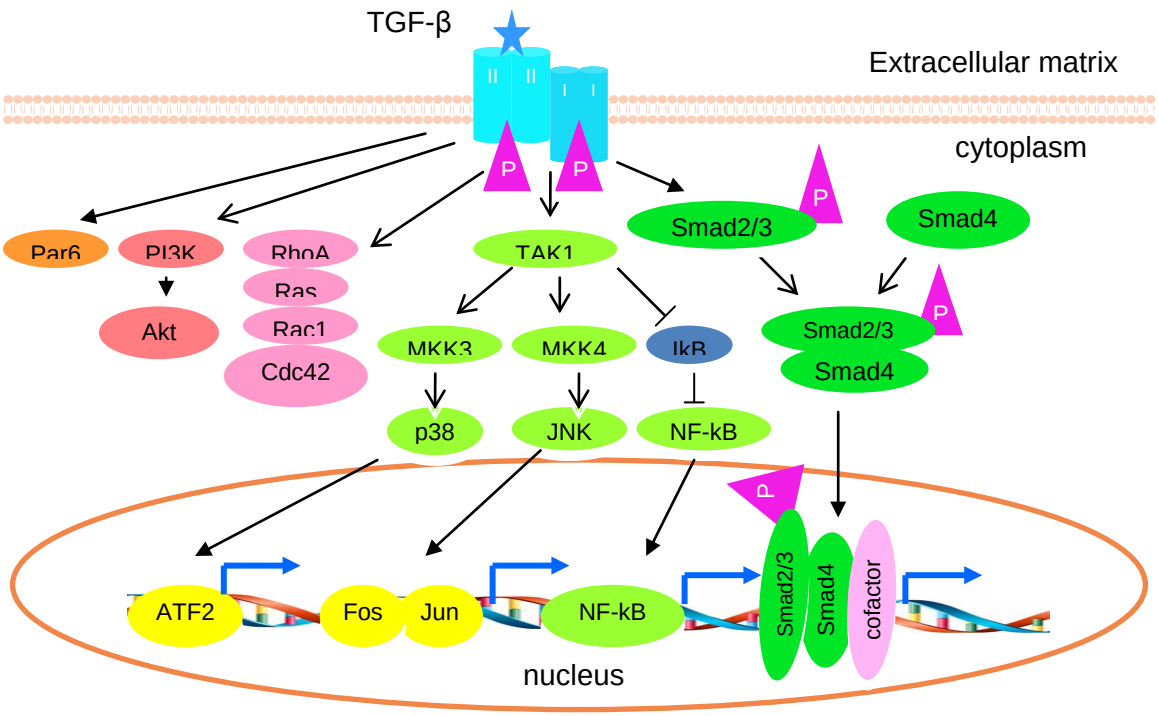


Figure 6: TGF-β signaling through Smad-dependent and Smad-independent pathways. TGF-β signals through a heteromeric complex of type I and type II transmembrane serine-threonine kinase receptors. In Smad-dependent pathway, TGF-β induced activation of the receptor complex leads to activation of Smad2 and Smad3 through direct C-terminal phosphorylation by TGFβ RI. Phosphorylated Smad2 and Smad3 then form trimers with Smad4, and translocate into the nucleus, where they associate and cooperate with other DNA binding transcription factors to activate or repress target gene transcription. In Smad-independent pathway, TGF-β activates TGF-β-activated kinase 1 (TAK1), which can activate p38 MAPK through MKK3, activate JNK through MKK4, and activate NF-κB through inhibition of IkB. TGF-β also can activate small GTPases, PI3K/Akt, and Par6 to regulate cell plasticity, motility and survival.

1.4 Epithelial-Mesenchymal Transition (EMT)

EMT is a biologic process by which adherent epithelial cells undergo morphological and molecular changes to gain a migratory mesenchymal cell phenotype. It is a critical step for many physiological and pathological conditions such as embryonic development, wound healing, fibrosis, tissue remodeling, and tumor progression. The reverse program, termed the mesenchymal-epithelial transition (MET), also occurs both during embryonic development and during several pathological processes (Boyer and Thiery 1993).

EMT is characterized by the breakdown of cell junctions and loss of cell polarity, rendering epithelial cells motile and invasive (Thiery 2002). In cancer, EMT causes cells to lose epithelial characteristics and acquire a mesenchymal phenotype, initiating invasion and metastasis. A variety of stimuli trigger EMT and many of these signals appear to converge on the same critical endpoint: the altered expression and/or function of cadherins.

Type I cadherins maintain stable cell-cell contacts through adherens junctions and desmosomes (Nagar et al 1996, Shapiro et al 1995). The extracellular immunoglobulin-like domains of E-cadherin bond neighboring cells through junctional complexes (Nagafuchi et al 1987, Patel and Gumbiner 1995). To preserve cellular shape and polarity, the intracellular domains of cadherins connect to the actin cytoskeleton through α-catenin and β-catenin (Cavey et al 2008). Downregulation of E-cadherin expression in cancer cells is a critical disruptor of epithelial homeostasis, leading to increased cell invasiveness and adoption of a mesenchymal behavior. A “cadherin switch” has been described,

whereby E-cadherin loss in cancer cells is accompanied by gain of N-cadherin (Kuphal and Bosserhoff 2006), which is a marker of mesenchymal differentiation (Kim et al 2000). The molecular mechanisms of this switch, leading to increased cell motility and invasion (Hazan et al 2000, Nieman et al 1999) are not known.

Loss of E-cadherin expression during cancer progression is regulated through genetic or epigenetic mechanisms. E-cadherin promoter hypermethylation leads to decreased expression level in metastatic breast and prostate cancer cells (Graff et al 1995, Tamura et al 2000). Genetic mechanisms regulating E-cadherin expression involve transcriptional repression at Ets sites or at palindromic E-boxes in the E-cadherin promoter (Hajra et al 2002). A number of transcriptional repressors are emerging as critical modulators of EMT, including the zinc-finger domain containing factors Snail, Slug, Zeb 1, Zeb 2 and the basic helix-loop-helix (HLH) factors *E47* and *Twist* (Julien et al 2007, Moreno-Bueno et al 2006, Olmeda et al 2007, Peinado et al 2007). Their function is partly regulated by oncogenic pathways activated in cancer, particularly by ras (Boyer et al 1997), Src (Rodier et al 1995), Akt (Grille et al 2003, Larue and Bellacosa 2005), GSK-3β(Zhou et al 2004), and NF-κB (Chua et al 2007). In addition, integrin signaling triggered by interaction of cancer cells with the extracellular milieu (ECM) leads to activation of the integrin-linked kinase (ILK) (Fuchs et al 2008, Li et al 2003) and modulation of the activity and balance between the small GTPases Rho and Rac (Zondag et al 2000). In turn, ILK, Rho and Rac regulate E-cadherin dependent cell adhesiveness and control the

assembly of the actin filaments required for cell shape and motility (Fuchs et al 2008, Zondag et al 2000).

1.5 Ovarian Cancer stem cells

Cells that have undergone EMT were reported to exhibit cancer stem cells (CSCs) phenotype (Mani et al 2008). CSCs, also called tumor-initiating cells, are cancer cells that possess characteristics associated with normal stem cells. They can undergo self-renewal (during cell division, one or both daughter cells retain the same biologic properties as the parent cell) to maintain tumor growth, and they also can differentiate into a heterogeneous population of cells that compose the tumor. CSCs are proposed to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors (Soltysova et al 2005). CSCs are chemoresistant (Gangemi et al 2009). CSCs can be identified and isolated using markers specific for normal progenitor or stem cells of the same organ (Visvader and Lindeman 2008). Several markers have been identified for isolation of cancer stem cells, including CD44/CD24 (Al-Hajj et al 2003), CD133 (Singh et al 2004) (O'Brien et al 2007), ATP-binding cassette B5 (ABCB5) (Schatton et al 2008), ALDH 1 (Ginestier et al 2007) as well as Hoechst 33342 exclusion (Szotek et al 2006) (Hadnagy et al 2006).

OC is a highly metastatic disease, and the metastasis process recapitulates the heterogeneity and morphology of the primary tumor. Moreover, although most patients with ovarian cancer achieve complete remission after surgical debulking and platinum-based chemotherapy, 70% of advanced stage ovarian cancer relapses. Even in stage I or II patients, the relapse rate is 20%–

25% (Ushijima 2010). Patients with recurrent tumors develop chemoresistance and ultimately succumb to the disease. This evidence strongly suggests the existence of ovarian cancer stem cells, which are responsible for metastasis, recurrence, and chemoresistance.

Ovarian cancer stem cells, also called ovarian cancer initiating cells (OCICs), were first identifided by Zhang et al (Zhang et al 2008). These CD44+CD117+ cells display several cancer stem cell characteristics: self-renewal as assayed by spheroid formation *in vitro*, ability of differentiation, tumorigenicity in serial transplantation in NOD/SCID mice, and chemoresistance. These cells express stem cell markers (Bmi-1, stem cell factor, Notch-1, Nanog, nestin, ABCG2, and Oct-4). As few as 100 of the CD44+CD117+ cells are sufficient to generate tumors in NOD/SCID mice.

CD44 is a 80-250KD transmembrane glycoprotein family involved in cell proliferation, cell differentiation, cell migration, angiogenesis, presentation of cytokines, chemokines, and growth factors to the corresponding receptors, and docking of proteases at the cell membrane, as well as in signaling for cell survival (Ponta et al 2003). Epithelial CD44 (CD44E) is expressed on the normal epithelium of the skin, lung, stomach, intestine, esophagus, bladder, and uterine cervix, as well as on normal glandular epithelium, including that of the pancreas, bile ducts, sweat glands, prostate gland, mammary glands, salivary glands, and thyroid, and in tumors arising from these organs. Mesenchymal cells and all types of hematopoietic cells, including erythrocytes, T and B lymphocytes, natural killer (NK) cells, macrophages, Kupffer cells, interdigitating cells, follicular

dendritic cells, as well as granulocytes, preferentially express CD44s. Therefore, CD44s is known also as hematopoietic CD44 (CD44H). The major ligand of CD44 is hyaluronic acid (HA, hyaluronan), a linear polymer of repeating disaccharide units. It also can bind several other molecules such as collagen, fibronectin, fibrinogen, laminin, chondroitin sulfate, mucosal vascular addressin, serglycin/gp600, osteopontin (OPN), and the major histocompatibility complex class II invariant chain, as well as L-selectin and E-selectin. Normally CD44 does not bind to its ligand unless activated by cytokines such as IL-5, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), growth factors such as epidermal growth factor (EGF), oncostatin M, and transforming growth factor-β1 (TGF-β1) or phorbol ester. Interaction of HA fragments (<200 κDa) with cell surface CD44 activates cell cycling and proliferation via Ras-MAPK signaling pathway, cytoskeleton reorganization, and cell migration via Rho family of proteins, as well as maintenance of cell survival via PI3K and NF-κB signaling pathways. As all these functions may generate constitutive oncogenic signals leading to uncontrolled cell growth and invasive cell migration, CD44 is usually associated with malignant tumors. In ovarian cancer, SKOV3 cells demonstrate HA-dependent/CD44-specific HER2 and c-Src activation to promote cell growth and acquisition of the migratory phenotype (Naor et al 2002). CD44, along with other molecules such as CD24 and CD133, has been used to identify breast cancer stem cells (Al-Hajj et al 2003), pancreatic cancer stem cells (Li et al 2007), and prostate cancer (Collins et al 2005).

CD117 is a transmembrane tyrosine receptor kinase encoded by the c-kit proto-oncogene. The CD117 ligand is the stem cell factor, an important hematopoietic regulator. CD117+ ovarian cancer cells demonstrate the ability of self-renewal, differentiation, tumor initiation and chemoresistance (Luo et al 2011). CD117 combined with CD133 are used to identify CSCs in oral squamous cell carcinomas (Margaritescu et al 2011).

CD133 (Prominin-1), a 120 kDa glycosylated protein containing five transmembrane domains was identified initially by the AC133 monoclonal antibody, which recognizes a CD34+ hematopietic stem and progenitor cell population (Yin et al 1997). Unlike CD44 and CD117, the biological function of CD133 is unclear. Recent studies have shown that CD133 expression is not limited to primitive blood cells, but is detectable in non-hematopoietic tissues as well. CD133 alone has been used as a CSC marker for brain tumors (Singh et al 2004), liver cancer (Suetsugu et al 2006) (Yin et al 2007), colon cancer (O'Brien et al 2007) (Ricci-Vitiani et al 2007), lung cancer (Eramo et al 2008) and ovarian cancer (Curley et al 2009). CD133 together with ALDH1 have been used to identify HSC (Hess et al 2006), liver cancer stem cells (Ma et al 2008) and ovarian cancer stem cells (Silva et al 2011). In ovarian cancer, ALDH+/CD133+ cells showed increased proliferation *in vitro* and *in vivo* compared to ALDH+/CD133- cells, and as few as 11 ALDH+/CD133+ cells could initiate tumors in mice.

Aldehyde dehydrogenase (ALDH) catalyzes the irreversible oxidation of aldehydes to corresponding acids (Yoshida et al 1998). High ALDH expression

and activity is detected in stem and progenitor cells of various lineages including hematopoietic (Storms et al 1999) (Hess et al 2004), endothelial and mesenchymal (Gentry et al 2007), neural (Corti et al 2006), mammary (Ginestier et al 2007), colon (Huang et al 2009) and lung cancer (Jiang et al 2009). The ALDH+ population cells showed increased tumorigenesis and chemoresistance. It is believed that ALDH1 catalyzes the conversion of retinol (vitamin A) to retinoic acid and thus it can maintain hematopoietic stem cell (HSC) homeostasis (Douville et al 2009). However, retinoic acid acts to promote cell differentiation and only inhibition of ALDH and retinoid signaling can induce the self-renewal ability of HSC (Chute et al 2006). Penumatsa et al reported that ALDH1 expression is significantly reduced in malignant serous ovarian cancer compared to normal ovaries and benign tumors (Penumatsa et al 2010). Another study of 442 OC patients with long-term follow-up showed that high levels of ALDH1 expression were observed in 19% of the ovarian carcinoma samples, which correlated with endometrioid adenocarcinoma, early-stage disease, complete response to chemotherapy, low serum CA125 level and favorable survival (Chang et al 2009). Therefore, ALDH1 expression and function in the ovarian cancer may be different from breast, lung or colon cancer. Indeed, ALDH1 may serve as a marker to identify stem/progenitor cells in tissues where ALDH1 expression is limited (such as breast) or weak (such as colon). However, for the tissues with wide and strong expression of ALDH1 (such as liver and pancreas), ALDH1 may not be able to serve as a stem cell marker (Deng et al 2010).

Some other proteins have been reported as ovarian cancer stem cell markers, such as CD24 (Gao et al 2010), Lin28/Oct4 (Peng et al 2010), CD44/MyD88 (Alvero et al 2009), side population (Szotek et al 2006), and label retaining (Szotek et al 2008). Given the complexity of different histotypes and origins of ovarian cancer, and the heterogeneity of tumors, combination of several candidate markers may be necessary to yield the correct stem cell population.

1.6 Reseach Objective

Ovarian cancer is a highly metastatic disease. About 60% cases are diagnosed at advanced stage, when the tumor has already spread beyond the ovaries. Although most patients respond well to initial debulking surgery and chemotherapy, 70% patients with advanced disease suffer recurrence and chemoresistance and ultimately die of the disease. Thus, understanding the molecular mechanisms regulating ovarian cancer metastasis and chemoresistance may provide new targets for more efficient treatment of ovarian cancer.

Our laboratory has previously reported that TG2 is overexpressed in 80% of ovarian cancers but not in the normal ovarian surface epithelium, and overexpressed TG2 enhances ovarian cancer peritoneal dissemination. As a calcium-dependent enzyme which catalyzes intermolecular covalent cross-linking of proteins, the function of TG2 in cancer progression is unclear. The goal of this

study was to understand how TG2 is upregulated in ovarian cancer and how it contributes to ovarian cancer progression.

Preliminarily I found that TG2 expression level correlates with a mesenchymal morphology of ovarian cancer cells. This led to the general hypothesis that TG2 induces EMT, a critical process in tumor metastasis, to promote ovarian cancer progression. Three specific aims were proposed to test this hypothesis.

Aim 1: To identify the mechanism by which TG2 is overexpressed in ovarian cancer. In this specific aim, I tested whether TGF-β, the well-known EMT and metastasis inducer and a cytokine abundantly secreted in ovarian cancer ascites, increases TG2 expression in ovarian cancer. Further, I investigated the signaling pathways involved in this upregulation.

Aim 2: To determine the mechanism by which TG2 promotes ovarian cancer metastasis. In this specific aim, I tested whether TG2 can induce EMT by regulation of E-cadherin expression in ovarian cancer cells. As EMT is linked to CSCs, I also tested whether TG2 can promote ovarian cancer progression by modulating ovarian CSCs homeostasis.

Aim 3: To delineate the role of TG2 in ovarian cancer chemoresistance. In this specific aim, I tested whether TG2 protects ovarian cancer cells from apoptosis induced by cisplatin, the first line of chemotherapy for ovarian cancer patients. I further investigated the mechanism implicated in TG2 induced chemoresistance.

Overall, this study aimed to uncover the role of TG2 in ovarian cancer metastasis and chemoresistance, which are the major contributors to ovarian cancer related death. Therefore, this study will elucidate the progression of ovarian cancer and provide insight into TG2 implication in OC pathogenesis that can be ultimately targeted for treatment.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and reagents

Human TGF-β1 was from R&D Systems (Minneapolis, MN), TGF-β receptor I kinase inhibitor was from Calbiochem (La Jolla, CA) and TGF-β receptor I kinase inhibitor SD-208 was from Scios Inc. (Fremont, CA, USA). Cisplatin and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). TG2 enzyme inhibitor, KCC009, was provided by Alvine Pharmaceuticals (San Carlos, CA). The antibody against TG2 was from Neomarkers (Fremont, CA); antibodies against phospho-Smad2, phospho-TAK1, phospho-p38, phospho-JNK1/2, phospho-Akt, Smad2/3, TAK1, p38, JNK1/2, phospho-FAK, phospho-Ser^{473}Akt, N-cadherin, and E-cadherin were from Cell Signaling Technology (Danvers, MA); antibodies against TGF-β1 receptor I and II, Smad7 and Caspase-9 P35 were from Santa Cruz Biotechnology (Santa Cruz, CA); neutralizing TGF-β antibody (MAB1835) was from R&D (Minneapolis, MN); antibodies against cytochrome c and hILP/XIAP were from BD Bioscience (San Jose, CA); anti-GAPDH was from Biodesign International (Saco, ME) and anti-β-actin was from Sigma.

2.2 Human ovarian tumors and ascites specimens

Studies using human specimens were approved by the IU Institutional Review Board; all specimens being de-identified. Tumors were obtained at debulking surgery and the diagnosis of OC was verified. All tumors were serous

papillary. Ten samples of OC malignant ascites and three samples of inflammatory parapneumonic pleural fluid were obtained from the IU Tissue Bank.

2.3 Cell lines and primary cultures

Human OC cell lines SKOV3, Hey, OV90, and IGROV-1 were from the American Type Culture Collection (ATCC). The mesothelial cell line LP9 was from Dr. Y. Xu (Indiana University, IU). Normal ovarian surface epithelial (NOSE) cells were from Dr. R. Bigsby (IU) and were generated from normal human ovaries obtained during surgery from nonmalignant gynecological disorders. Pieces of an ovary were cultured as explants in medium containing 1:1 MCDB 105 (Sigma) and M199 (Cellgro, Herndon, VA) supplemented with 20% heat-inactivated fetal bovine serum (FBS, Cellgro) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Once cells grew out from the specimen, the tissue was transferred to another culture dish. After cells reached approximately 50% confluence, they were passaged and grown in a medium supplemented with 10% FBS (Wang et al 2008). The immortalized cell line C272/hTert/E7 was derived from CSOC (Cedars Sinai ovarian carcinoma, epithelial cells derived from EOC) culture by transducing human telomerase hTERT and the human papilloma virus E7 gene products (Gillan et al 2002). OVCA cells are primary cultures obtained from OC malignant ascites or from disaggregated human ovarian tumors. All cells were cultured in media containing 1:1 MCDB 105 and M199 supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C in a humidified 5% CO_2 atmosphere.

2.4 Cell proliferation

Cell proliferation was assayed by methylthiazolyldiphenyl-tetrazolium bromide (MTT) and BrdU assays. In brief, after treatment, cells were incubated with MTT (200 µl of 1 mg/ml in RPMI) for 4 h. The formazan crystals formed were solubilized in 200 µl acidified isopropanol (0.04 N HCl final) containing 1% Triton X-100 for 10 min, and the optical density was measured with an ELISA plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA) at 570 nm. In addition, an enzyme-linked immunosorbent assay colorimetric method based on measurement of BrdU incorporation into DNA (Roche Diagnostics, Penzberg, Germany) was used to assess cell proliferation. In brief, after cisplatin treatment, cells were incubated with BrdU (10 µM) for 3 h, and fixed. Cells were subsequently treated with a peroxidase-labeled anti-BrdU antibody for 90 min and the colorimetric reaction was developed with a tetramethyl-benzidine-based substrate. The reaction product was measured at 370 nm. All assays were performed in quadruplicate and were repeated twice in independent conditions. Data are presented as means ± SEM.

2.5 Chromatin immunoprecipitation (ChIP) assay

To detect the interaction between Smads and the TG2 promoter, ChIP assays were performed by using a kit from Millipore, Smad4 (Abcam) and phospho-Smad3 (Cell Signaling) antibodies. In brief, after crosslinking with formaldehyde, DNA from C272/hTert/E7 cells treated or not with TGF-β1 was immunoprecipitated with Smad4 or pSmad3 antibodies. Bound DNA was purified

and PCR amplified with primers flanking the -191 to -840 TG2 promoter regions. Ten putative SBEs were identified within this region by a bioinformatics tool PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo). As the exact SBE was not known, 4 pairs of primers, each encompassing 200bp domains were designed to cover this region (Table 2, Figure 7). PCR with primers covering the -269 to -479 promoter fragments yielded a positive signal, indicating that the Smad complex bound to this region. Chromatin immunoprecipitated with Smad4 or with pSmad3 antibody was amplified with primers designed for an upstream region (800-1000): TGC CAT GTG TTA GGA GCA CAA G (F), ATG CCT AGA ATC CCA GCG TTA GAG(R), as the negative control. Another negative control was DNA immunoprecipitated with IgG and subjected to PCR amplification. For real time quantitative PCR detection of bound DNA, SYBR-Green master mix was used on an ABI Prism 7900 platform, and percentage of input was calculated. For negative control, chromatin immunoprecipitated with normal mouse IgG was amplified with primers specific for TG2 promoter region. For positive control, chromatin immunoprecipitated with anti-RNA polymerase II was amplified with primers specific for human GAPDH.

Table2: Primers designed for ChIP assay.

Primer #	Regions covered	Primer sequence
1	1111-1312 (-688 to -889)	F: GGT GAG ATC GGA GGA TTA TCT G R: AGA CCT CCT AGA CAC CAA TG
2	1521-1731 (-269 to -479)	F: ATC TCC ACA GCC CTG TCT TG R: GCA GCA CCC TCA TAG AAA C
3	1390-1732 (-268 to -610)	F: GCT GTG TCA GGC TGT ATC TAT GTC R: ACG CAG CAC CCT CAT AGA AAC
4	1716-2034 (34 to -284)	F: CTA TGA GGG TGC TGC GTG TG R: GGC TGC GGT GAC TCT GAT AC

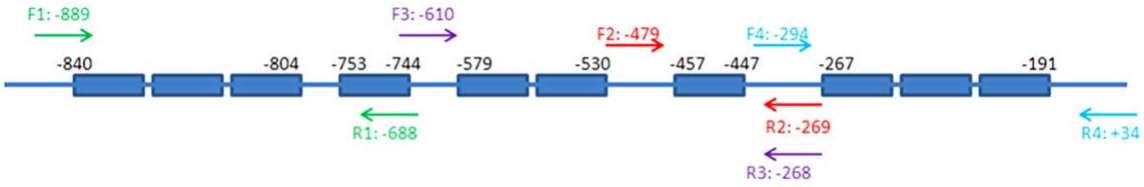


Figure 7: Schematics of primers designed for ChIP assay. Ten putative SBEs were identified within this region by a bioinformatics tool PROMO in the region from -191 to -840 of TG2 promoter. 4 pairs of primers, each encompassing 200bp domains were designed to cover this region.

2.6 Clonogenic assay

OC cells were seeded in six-well plates at a density of 100 cells per well. Triplicate wells were treated with 1, 2 and 5 μM cisplatin for 48 h after which the media was changed. Cells were allowed to grow for 10 days until visible colonies were formed. The colonies were fixed and stained with Hema 3 stain (Fisher) and counted. The number of colonies formed after treatment with cisplatin was expressed as a percentage of untreated controls (relative colony formation). Data are presented as means ± SDs.

2.7 Enzyme-linked immunosorbent assay (ELISA)

An ELISA assay (R&D) measured TGF-β1 concentrations in the conditioned media (CM) of OC or NOSE cells and in malignant OC ascites or pleural fluid from non-malignant conditions, following the manufacturer's protocol. In brief, 50 μL per well of standard, control, or activated sample were incubated with a monoclonal antibody specific for TGF-β1 coated on the polystyrene microplate for 2 hours at room temperature, followed by incubation with 100 μL polyclonal antibody against TGF-β1 conjugated to horseradish peroxidase for 2 hours at room temperature, then with 100 μL substrate (hydrogen peroxide with the chromogen tetramethylbenzidine) for 30 minutes at room temperature, with 4 times wash with was buffer between all steps. 100 μL stop solution (hydrochloric acid solution) was added before reading the plate at 450 nm within 30 minutes. TGF-β1 concentrations were calculated according to the standard curve.

2.8 Flow Cytometry

Single cell suspension in PBS containing 1% FBS (about 10^6cell/100uL) were incubated with FITC-conjugated anti-CD44 (BD Biosciences), APC-conjugated anti-CD117 (BD Biosciences), FITC- mouse IgG κ isotype control and APC-mouse IgG κ isotype control (BD Bioscience) at 4°C for 30-40 minutes in dark. Labeled cells were washed with PBS containing 1% FBS, fixed in PBS containing 1% paraformaldehyde, and detected on a BD FACS Calibure flow cytometer according to the manufacturer's procedure. Flow data were analyzed using BD CellQuest software. Ten thousand events were accumulated for each analysis. Three independent readings were obtained from separate experiments, and data were averaged for statistical analysis.

2.9 Fluorometric caspase-3 and -9 assays

Caspase-3 activity was measured in cell lysates by a fluorescence-based assay (Apo-ONE Homogeneous Caspase-3/7 Assay, Promega) following the manufacturer's protocol. Sequential cleavage and removal of the Asp-Glu-Val-Asp peptides from the profluorescent substrate (Z-Asp-Glu-Val-Asp-R100) by active caspase-3 releases the fluorescent rhodamine product, which can be measured by a SpectraMax M5 Microplate Reader, at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Recombinant active caspase-3 (Chemicon) was used as a positive control and denominator for analysis. Likewise, caspase-9 activity was measured in cell lysates using a fluorescence-based assay (BioVision, Mountain View, CA) according to the manufacturer's

protocol. The assay is based on detection of cleavage of substrate LEHD–7-amino-4-trifluoromethyl coumarin (AFC). LEHD–AFC emits blue light; upon cleavage of the substrate by active caspase-9, free AFC emits yellow–green fluorescence which can be quantified using a SpectraMax M5 Microplate Reader. Comparison of the fluorescence of AFC from an experimental sample with an uninduced control allows determination of the fold increase in caspase-9 activity. The fluorescent units for caspase-3 and -9 were normalized to the amount of protein measured for each sample using the Bradford method.

2.10 Gene reporter assay

A Dual-Luciferase Reporter Assay (Promega, Madison, WI) was performed to quantify TG2 and NF-κB-dependent promoter activities in SKOV3 and C272 hTert/E7 cells. In brief, cells were transiently co-transfected with the experimental reporter plasmid and control renilla plasmid at 10:1 ratio using DreamFect Gold transfection reagent (OZ Biosciences). Luminescence was measured with a TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA) and normalized to the internal control renilla. The experiments were performed in duplicate and repeated in independent conditions.

2.11 Immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA, pH 7.5 50mM Tris-HCl, pH 7.0 1mM EDTA, 150mM NaCl, 1% Triton X-100, 1% deoxycholic acid) containing protease inhibitors leupeptin (1 µg/mL), aprotinin (1

µg/mL), phenylmethylsulfonyl fluoride (PMSF, 400 µM), and sodium orthovanadate (Na_3VO_4, 1 mM). Cell lysates were sonicated briefly and subjected to centrifugation at 14,000 rpm for 15 min at 4°C to sediment particulate material. Protein concentration was measured by the Bradford method (BioRad). Equal amounts of protein were mixed with Laemmli sample buffer (BioRad) containing 5% 2-Mercaptoethanol (Sigma), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 1x Tris-glycine buffer (25 mM Tris base, 190 mM glycine) containing 0.1% SDS, and then electroblotted onto polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA) using 1x Tris-glycine buffer with 20% methanol. Proteins on the membrane were visualized with Ponceau Red (Sigma) to check if proteins were loaded equally or transferred to the membrane successfully. To prevent non-specific background binding of primary and/or secondary antibodies to the membrane, the membrane was blocked with 5% non-fat milk in 1xTris Bufferred Saline containing Tween 20 (TBST) for 1 hour at room temperature. The membranes were incubated with specific primary antibody overnight at 4°C with gentle rocking, then washed thrice with 1xTBST, 5 minutes per wash to remove unbound primary antibody. The membranes were then incubated with specific horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature, followed by thrice wash with 1xTBST. Antigen-antibody complexes were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, NJ).

2.12 Immunofluorescence assay

OC cells were plated on fibronectin-coated chamber slides (BD Biosciences) and allowed to adhere. 24 hours later, cells were washed with 1xPBS, then fixed in 4% paraformaldehyde for 10 minutes, washed with 1xPBS for 5 minutes, and permeabilized with 0.2% Triton X-100 for 5 minutes, washed again with 1xPBS for 5 minutes. After blocking at room temperature for 1 hour with 3% goat serum in 1xPBS, cells were incubated with primary antibodies, rabbit-anti–E-cadherin (1:200), Cy5-labeled anti-vimentin (Sigma, 1:200), and isotype-specific IgG (control) in blocking buffer at room temperature for 1 hour, washed thrice with 1xPBS, 10 minutes per wash. The cells were then incubated with Alexa Fluor 488–labeled anti-rabbit secondary antibody (1:1,000; Molecular Probes) in blocking buffer at room temperature for 1 hour, washed thrice with 1xPBS, 10 minutes per wash. Images were captured using a Zeiss LSM 510 confocal microscope system under UV excitation at 488 nm (Alexa Fluor 488), 630 nm (Cy5), and 360 nm [4, 6-diamidino-2-phenylindole (DAPI, Vectashield, Vector Laboratories)], and analyzed with Zeiss LSM software.

2.13 Immunohistochemistry

A tissue microarray from Pantomics (San Francisco, CA, USA) including 30 human epithelial ovarian tumors arrayed in duplicates was immunostained for pSMAD3 and TG2. In brief, the slides were heated at 56°C for 1 hour to melt the paraffin, then deparaffinized by consecutive washes with xylene, then rehydrated by graded washes with ethanol and water. 3% hydrogen peroxide was used to

quench the endogenous peroxidase activity. After antigen retrieval with 0.01M sodium citrate, the slides were blocked with 3% goat serum in TBS and incubated with primary antibody against TG2 (CUB 7402, NeoMarkers, 1:200, 1 hour at room temperature) and pSmad3 (Abcam, 1:50, 4°C overnight). The antigen-antibody complex formation was detected with the avidin/biotin system (LSAB2 kit, DAKO, Hamburg, Germany). Slides were stained with 3, 3′-diaminobenzidine and counterstained with hematoxylin. Negative controls were run in parallel, with omission of the primary antibody. Staining was graded from 0 (no staining) to 3+ (strong staining). Immunoreactivity was recorded only if noted in more than 15% to 20% of tumor cells. An H-score was calculated as a product combining both intensity and percentage of cells staining, using the following formula: H-score= (% at 0) * 0 + (% at 1+) * 1 + (% at 2+) * 2 + (% at 3+) * 3. Tumors were classified as positive or negative if H score was $\geq$ or < median score, respectively. The H-score of TG2 and pSmad3 staining were then used to calculate the correlation of TG2 and pSmad3 expression in human ovarian tumor sample by Fisher's exact test.

2.14 *In situ* TG2 activity assay

The assay was performed to determine the incorporation of 5-(biotinamido) pentylamine (BPA, Pierce) into proteins, as previously described (Zhang et al 1998). In brief, cells were treated with TGF-β1 or vehicle control for 48h, and incubated with 2 mM BPA for 1 hour, 5mM EDTA or 5mM $CaCl_2$ for 1 hour before harvesting. Cells were washed with cold PBS once and collected in

homogenizing buffer (pH 7.5 50mM Tris-HCl, 150mM NaCl, and 1mM EDTA) with protease inhibitors. Cell lysates were sonicated briefly on ice and protein concentration was measured using Bradford assay. Equal amounts of protein were run on 8% SDS gels (100v, 2 hours), and semi-dry transferred to nitrocellulose membrane (20v, 40 minutes). The membrane was blocked 2 hours at room temperature with 5% BSA, 0.05% SDS, 0.01% NP40 in BBS (100mM boric acid, 20mM sodium borate, 80mM NaCl), then incubated with HRP-conjugated streptavidin (1:200, Pierce) in 1% BSA, 0.05% SDS, 0.01% NP40 in BBS for 2 hours at room temperature. The blot was then washed thrice with1% BSA, 0.05% SDS, 0.01% NP40 in BBS, 10 minutes per wash, followed by a quick rinse with distilled water, then developed with ECL. TG2 activity is evidenced by BPA labeling of the cellular proteins on the blot, shown as the black bands on the blots.

2.15 Intraperitoneal ovarian xenograft model

3×10^6 SKOV3 cells stably transfected with AS-TG2 or pCDNA3.1 vector were injected i.p. in 7–8 weeks old female nude mice from Harlan (Indianapolis, IN). Treatment with SD208 (60mg/kg daily) or vehicle control began 1 week after i.p. implantation via gavage and was continued for 5 weeks. Each of the four experimental groups consisted of four animals. Six weeks after i.p. injection, mice were euthanized, peritoneal implants were counted. Tumors were measured bi-dimensionally if >5mm and tumor volume was calculated according to the formula $LxW^2/2$, where L is length and W is width. Acumulative tumor volume

was calculated by adding the volumes of dominant tumors for each animal. Large tumor masses were harvested and frozen in liquid nitrogen for future experiments. Student's *t* test was used to compare the volumes and numbers of peritoneal implants between any two animal groups. Experiments were approved by the IU Animal Care and Use Committee.

2.16 Isolation and detection of ovarian cancer stem cells

Fresh tumor samples were minced and digested in serum-free Dulbecco's modified Eagle's medium/F12 media (Invitrogen, Carlsbad, CA, USA) containing collagenase and hyaluronidase (Sigma-Aldrich). The resulting single cell suspension was filtered through a 40-μm cell strainer and red blood cells were removed with Histopaque-1077 (Sigma-Aldrich). Cells dissociated from tumors or OC cell lines were labeled with fluorescein isothiocyanate-conjugated anti-CD44 and antigen-presenting cell-conjugated anti-CD117 and sorted using a FACS-Aria flow cytometer (BD Biosciences, San Jose, CA, USA). Quantification of the CD44+/CD117+ population in tumor cells or OC cell lines was performed on FACSCalibur (BD Biosciences).

2.17 Migration Assay

A migration assay was done in a modified Boyden chamber method using 6.5-mm-diameter, 8.0-μm pore size polycarbonate membrane transwell inserts in a 24-well plate (Corning). To assess directional migration, the lower surfaces of the transwell membrane were coated with 50 μg/mL fibronectin or with 0.01%

type I collagen (Sigma). SKOV3 cells stably transfected with AS-TG2 or vector were serum starved for 18 h and then plated in the upper well at a concentration of 2×10^5 in 100 μL of serum-free medium. After 4 h of incubation at 37°C in a CO_2 incubator, the cells on the upper surface of the membrane were wiped off with a cotton swab. The cells on the lower surface of the transwell were stained with HEMA3 stain (Fisher) and counted at ×200 magnification. Cells were counted in five high-power fields (HPF) in duplicate experiments. Results are expressed as mean number of migrating cells ± SE. A similar experiment was done using serum-free conditioned medium from cells stably transfected with AS-TG2 or pcDNA3.1 as cell attractant in the lower chamber of the transwell.

2.18 Matrigel Invasion Assay

Invasion assay was done by using two-dimensional and three-dimensional cell cultures in a Matrigel matrix (BD-Biosciences). Briefly, 2.5×10^4 cells suspended in 50 μL of cell culture medium were seeded onto solidified Matrigel in 24-well plates as a monolayer (two-dimensional model) or mixed and embedded within the matrix (three-dimensional model). After incubation at 37°C for 24 to 48 hours, invasive cells adhered to the surface of the gel and spread to form networks (two-dimensional model) or degraded and invaded into the gel (three-dimensional). These were observed under an inverted microscope and photographed live. The experiments were done in duplicate and repeated in independent conditions.

2.19 Reverse transcription-Polymerase chain reaction (RT-PCR) and quantified RT-PCR (qRT-PCR)

RNA was extracted with RNA STAT-60 reagent (Tel-Test Inc., Friendswood, TX). In brief, cells were homogenized with RNA STAT-60 (1 ml per 5-10 x 10^6 cells) and RNA was extracted from the upper layer with 0.2 volume of chloroform, precipitated with 0.5 volume of isopropanol and washed with 75% ethanol. RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad). In brief, 1µg RNA was transcribed by 1 µL MMLV-derived reverse transcriptase in a 20µL reaction system at 42°C for 30 minutes, using a blend of oligo dT and random hexamer primers, followed by incubation at 85°C for 5 minutes to heat-inactivate the reverse transcriptase. The cDNA product was then used for semi-quantified PCR or qPCR.

The PCR reactions used Taq polymerase (Promega, Madison, WI) and the primers listed in Table 3. For qPCR, 1uL of the cDNA product was amplified in a 20uL reaction system with FastStart Universal Probe Master (ROX) (Roche) on an ABI Prism 7900 platform (Applied Biosystems), using specific primers (900nM) and probes (250nM) (Table 3). At the end of the PCR reaction, a melting curve was generated and the cycle threshold (Ct) was recorded for the target genes and for GAPDH. Relative expression of gene of interest was calculated as ΔCt, measured by subtracting the Ct of the reference gene from that of the control. Results are presented as mean ± SD of replicates. Each experiment was done in duplicates and repeated in independent conditions. Relative mRNA expression levels were normalized to GAPDH.

Table 3: Primers used for PCR reaction.

Target gene	Primers
Snail-1	F: ACC CCA CAT CCT TCT CAC TG
	R: TAC AAA AAC CCA CGC AGA CA
Slug	F: CTT TTT CTT GCC CTC ACT GC
	R: ACA GCA GCC AGA TTC CTC AT
Snail-3	F: AAA TCA ATG GTG CCT GCT CT
	R: GGC TGT CTT TGA GGG GTA CA
Zeb-1	F: TGC ACT GAG TGT GGA AAA GC
	R: CTT GCC ACA CTC TGT GCA TT
Zeb-2	F: CGC TTG ACA TCA CTG AAG GA
	R: CTT GCC ACA CTC TGT GCA TT
Twist-1	F: ACC ATC CTC ACA CCT CTG CAT TCT
	R:TGC AGG CCA GTT TGA TCC CAG TAT
Twist-2	F: AGC AAG AAG TCG AGC GAA GA
	R: CAG CTT GAG CGT CTG GAT CT
GAPDH	F: GAT TCC ACC CAT GGC AAA TTC C
	R: CAC GTT GGC AGT GGG GAC

Table 4: Primers and probes used for qPCR.

Target gene	Primers	Probe
TG2	F:AGG GTG ACA AGA GCG AGA TG	86
	R: TGG TCA TCC ACG ACT CCAC	
GAPDH	F: AGC ACA TCG CTC AGA CAC	60
	R: GCC CAA TAC GAC CAA ATC C	
Nanog	F: GAT GCC TCA CAC GGA GAC T	31
	R: TTT GCG ACA CTC TTC TCT GC	
Sox2	F: TGC TGC TCT TTA AGA CTA GGA C	35
	R: CCT GGG GCT CAA ACT TCT CT	
Oct3/4	F: CTT CGC AAG CCC TCA TTT C	60
	R: GAG AAG GCG AAA TCC GAA G	
Zeb-1	F: GCC AAC AGA CCA GAC AGT GTT	68
	R: TTT CTT GCC CTT CCT TTC TG	
Zeb-2	F: AAG CCA GGG ACA GAT CAG C	68
	R: GCC ACA CTC TGT GCA TTT GA	
Snail	F: GCT GCA GGA CTC TAA TCC AGA	11
	R: ATC TCC GGA GGT GGG ATG	
Slug	F: TGG TTG CTT CAA GGA CAC AT	7
	R: GTT GCA GTG AGG GCA AGA A	

2.20 Solid phase adhesion assay

Exponentially growing cells were detached from culture plates by trypsinization and labeled with calcein acetoxymethylester (calcein AM; 2 μM; Molecular Probes) for 20 min. After washing, cells were resuspended in serum-free medium. Equal numbers of cells (4×10^4 per well) were seeded into 96-well plates precoated with 5ug/mL fibronectin (Sigma) and blocked with bovine serum albumin (1% w/v). After 1 h of incubation at 37°C, the plate was immersed into PBS containing 1 mM $MgCl_2$ to remove non-adherent cells. The number of adherent cells was measured in a fluorescence plate reader (Applied Biosystems, Foster City, CA,) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All experiments were done in quadruplicate and repeated twice.

2.21. Spheroid culture

To induce formation of cellular spheroids, OC cells were seeded at a concentration of 1000 cells/mL in serum-free Dulbecco's modified Eagle's medium/F12 supplemented with 5 μg/mL insulin (Sigma), 20 ng/mL human recombinant epidermal growth factor (EGF; Invitrogen), 10 ng/mL basic fibroblast growth factor (bFGF; Invitrogen), and 0.4% bovine serum albumin (BSA; Sigma) in ultra-low attachment plates (Corning, Corning, NY) and allowed to form spheroids over 10–14 days. Spheroids were counted in 10 high-power fields (HPF) in duplicate experiments. Results are expressed as mean number of spheroids number ± SD.

2.22 Transfection and transduction

Stable knock-down of TG2 was achieved by transfection of an antisense construct (AS-TG2) or by transducing lentiviral particles containing shRNA targeting TG2 (Sigma). The AS-TG2 plasmid was a generous gift from Professor Janusz Tucholski, of University of Alabama. To generate SKOV3 AS-TG2 cells, an anti-sense construct (AS-TG2) cloned into pcDNA3.1 vector was transfected in SKOV3 cells using Fugene transfection reagent (Roche, Indianapolis, IN). As control, cells were transfected with pcDNA3.1 vector, carrying the G418 resistance gene. Stable transfected clones were established by selection with G418 (Sigma). Knock-down of TG2 in selected clones was demonstrated by Western Blot analysis. To generate IGROV-1 shTG2 cells, 3 packaging vectors (pMDL, pRSV, pMDG) and Sh RNA (scrambled Sh RNA control shc 002 (Sigma) and Sh-TG2 41, 43 (Sigma) were used to transfected packaging HEK293 cells with DreamFect (OZ Biosciences, Marseille, France). Virus were harvested from HEK293 cell supernatant and transduced into IGROV-1 cells with 8ug/mL polybrene (Sigma). Pooled stable transduced clones were established by selection with puromycin (Sigma). Knock-down of TG2 in selected clones was demonstrated by Western Blot analysis.

Stable overexpression of wild type TG2, TG2 fibronectin binding domain tTG 1-140, TG2 enzymatic mutant C277S, and TG2 GTPase mutant R580A in OV90 cells, an ovarian cancer cell line which doesn't express TG2, was achieved by transfection of pcDNA3.1 TG2 construct or by transduction of bicistronic retroviral vector pQCXIP (BD Biosciences, San Jose, CA). To transfect the

pcDNA3.1 TG2 construct, OV90 cells in the logarithmic phase of growth were transfected with wild-type TG2 cloned into the pcDNA3.1 vector using Fugene (Roche Applied Science). Stable transfected clones were established by selection with G418 (Sigma) and expression of TG2 was demonstrated by Western Blot analysis. For transduction of pQCXIP constructs, wild type TG2, tTG 1-140, TG2 C277S, and TG2 R580A were subcloned from pcDNA3.1 constructs to pQCXIP constructs, and transfected into packaging Amphophenix cells with DreamFect (OZ Biosciences). Virus was harvested and transduced into OV90 cells with 8ug/mL polybrene (Sigma). Pooled stable transduced clones were established by selection with puromycin (Sigma) and expression levels of TG2 and mutants were demonstrated by Western Blot analysis. The constructs pcDNA3.1 tTG10-140, pcDNA3.1 TG2 C277S, and pcDNA3.1 TG2 R580A were kind gifts from Dr. Belkin (Maryland University, MD) (Hang et al 2005), Dr. Cerione (Cornell University, NY), and Dr. Johnson (Rochester University, NY) (Ruan et al 2008) respectively.

To overexpress Zeb1 and reconstitute NF-κB and Akt activities in SKOV3 cells stably transfected with AS-TG2 or vector, full-length Zeb1 cDNA, constitutively active p65 subunit of NF-κB (p65NLS50) which has less affinity for IκBα by replacing the nuclear localization signal (NLS) of p65 with the NLS of p50, and a constitutively active form of Akt that lacks the pleckstrin homology domain were transferred by retroviral infection and pooled colonies were selected. These constructs cloned in pQCXIP were generous gifts from Dr. H. Nakshatri (Indiana University, IN).

Transient transfection of siRNAs targeting Smad2/3 (Cell Signaling), TAK-1 (Thermo Scientific Dharmacon, Lafayette, CO), p38 (Cell Signaling), JNK2, p65, Zeb1 or scrambled siRNA (Dharmacon) were performed by using DreamFect (OZ Biosciences).

2.23 TdT-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay

TdT (terminal deoxynucleotidyl transferase)-mediated deoxyuridine triphosphate nick-end labeling assay (Chemicon, Temecula, CA) was utilized to quantify cisplatin-induced apoptosis. In brief, after cisplatin treatment, cells were fixed in 1% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature and then in ethanol:acetic acid (2:1) for 5 min at −20°C. Fixed cells were blocked with equilibration buffer for 1 min at room temperature and treated with TdT enzyme and anti-digoxingenin conjugate. A mounting medium containing 4′,6 -diamidino-2-phenylindole (Vectorshield, Vector Laboratories, Burlingame, CA) was used. Cells were viewed by fluorescence microscope and counted at high power magnification (×400). Five fields were counted in each of the duplicated wells. Data are presented as means ± SEM.

2.24 Analysis of combined drug effects

Drug synergy was measured using the CalcuSyn software (Biosoft, Ferguson, MO). To perform these analyses, we used the data obtained from the growth inhibitory experiments, generated isobolograms and measured the

combination index (CI) based on the median-effect principle of Chou et al (Chou and Talalay 1984). The CI method is a mathematical representation that measures two-drug pharmacologic interaction. A CI of 1 indicates an additive effect between two agents, a CI <1 indicates synergism, whereas a CI >1 represents antagonism.

2.25 Statistical analysis

Comparisons between groups were performed using Student's *t*-test; Fisher's exact test was used to compare immunohistochemistry staining for TG2 and pSmad3. *P*-values <0.05 were considered significant. 50% inhibitory concentration (IC50) values for inhibition of cell proliferation by cisplatin and KCC009 were calculated as the concentration of drugs resulting in a 50% of reduction in viable cells compared with untreated controls by MTT or BrdU assays. The IC50 values and the combination effect of multiple drugs were determined using the Calcusyn software program for Windows (Version 1.2, 1996; Biosoft, Cambridge, UK).

CHAPTER 3: RESULTS

3.1 TGF-β1 induces TG2 overexpression in OC cells

3.1.1 TGF-β1 is secreted in OC microenviroment

First we determined if TGF-β is presented in the OC milieu. As TGF-β1 is reported to be the major isoform in OC and OC associated ascitic fluid (Abendstein et al 2000, Henriksen et al 1995), we focused our study on TGF-β1. To test whether TGF-β1 is secreted by OC cells, an ELISA assay was used to quantify TGF-β1 secretion in serum-free conditioned media from OC cell lines or primary OC cells (n=12) and in media conditioned by normal ovarian surface epithelial cells (NOSE) (n=3). Higher levels of TGF-β1 were detected in conditioned media from OC cells (1203 +/-271pg/mL) compared to NOSE (71+/-23pg/mL; p=0.002, Figure 8A), suggesting that TGF-β is secreted in higher amounts by malignant cancer cells as opposed to normal ovarian epithelial cells. As OC progression leads to accumulation of peritoneal fluid rich in secreted proteins, we also measured TGF-β1 secretion in OC associated ascites (n=10) compared with benign effusions (n=3). The cytokine was detected in the peritoneal OC fluid at higher levels (1719+/-275pg/ml) than in non-malignant effusions (387+/-341pg/mL; p=0.004, Figure 8B). This demonstrates that TGF-β1 is presented in OC microenviroment, and OC cells are a source of TGF-β1.

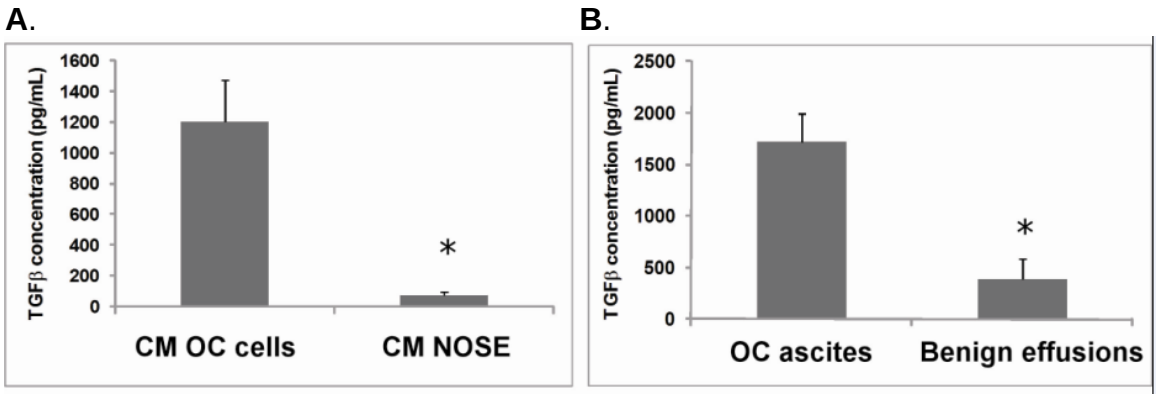


Figure 8: TGF-β1 is secreted in OC microenvironment. A. TGF-β1 measured by enzyme-linked immunosorbent assay (ELISA) in serum-free conditioned media (CM) of OC (*n*=12) vs. NOSE cells (*n*=3, *P*=0.002). B. TGF-β1 measured by ELISA in OC malignant ascites (*n*=10) vs. non-cancer pleural fluid (*n*=3, *P*=0.004).

3.1.2 TGF-β1 induces TG2 upregulation in OC cells

Previously our lab reported that TG2 is overexpressed in OC cells in association with a metastatic phenotype (Satpathy et al 2007, Satpathy et al 2009). As TGF-β1 exists in OC microenviroment and promotes OC metastasis (Do et al 2008), we tested whether TGF-β1 induces TG2 expression. Several OC cell lines and primary OC cells were serum starved for 16 to 24 hours and treated with TGF-β1 (5ng/mL) for 24 to 48 hours, and TG2 protein in these cells were detected by Western analysis. Increased TG2 expression was observed in primary OC cells (OVCA), immortalized C272/hTert/E7 cells, and ovarian cancer cell lines: SKOV3, Hey, and IGROV-1 cells treated with TGF-β1 for 24-48 hours (Figure 9A). In contrast, TGF-β1 did not augment TG2 expression in normal ovarian surface epithelial cells (NOSE), or in the non-transformed mesothelial cells LP9 derived from human peritoneum (Figure 9B). Dose-response experiments demonstrated that the effects of TGF-β1 on TG2 expression occur from concentrations from 0.5ng/mL to 5ng/mL which are physiologically relevant concentrations (Figure 10A). The effects of TGF-β1 on TG2 expression were more pronounced when cells were cultured on fibronectin mimicking the interaction with the ECM (Figure 10B), therefore subsequent experiments used FN-coated plates.

To elucidate why there is a difference in response to TGF-β1 between OC cell lines, non-transformed NOSE and LP9 cells, the signaling pathways downstream of TGF-β1 were analyzed. Cell surface TGF-β receptor I and II, intracellular signal transducers including regulatory Smad2/3 and inhibitory

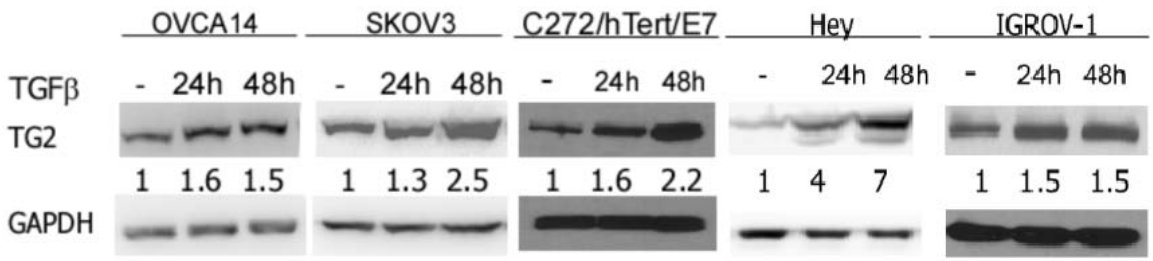
Smad7, and non-Smad pathway signal mediator TAK1 were detected by Western analysis in these cells. The results showed that OC cell lines and non-transformed NOSE and LP9 cells express TGF-β receptors I and II and TAK1 at similar levels. However, Smad2/3 expression levels were slightly lower in NOSE and LP9 cells compared to OC cells, while the inhibitory protein Smad7 was expressed in NOSE and LP9 cells, but not in all OC cell lines (Figure 11). These data reveal that the R-Smads may be more abundant in cancer cells, whereas the I-Smad is more abundant in normal cells.

The Western blot analysis showed that TG2 expression can be upregulated by TGF-β1 at protein level. To investigate whether TG2 was induced by TGF-β1 at transcriptional level, OC cell lines SKOV3 and C272/hTert/E7 were treated with TGF-β1. Total RNA was collected to measure TG2 mRNA level by qRT-PCR. Compared with control, the results showed that TGF-β1 induces TG2 mRNA expression in OC cells (Figure 12A, $P<0.05$), and this induction can be blocked by TGF-β neutralizing antibody (Figure 12B, $P<0.05$). Further, we tested whether TGF-β1 induces the TG2 promoter activity in OC cells. We transfected OC cells with a luciferase reporter gene downstream of the TG2 promoter, then treated the cells with TGF-β1, and collected the cell lysate to perform a luciferase assay. Compared with control, the results showed that TG2 promoter activity was enhanced by TGF-β1 (Figure 12C, $P<0.05$). All combined together, these data showed that TGF-β1 induces TG2 at transcriptional level.

To test whether secreted TGF-β alters TG2 expression, we evaluated the effects of CM from OC cells on TG2 expression. Significant increase in TG2

mRNA level was induced by media conditioned by OC cells (3-fold, Figure 13), comparable to the effects of recombinant TGF-β1. To demonstrate that the effects are caused by TGF-β1, we utilized a TGF-β receptor kinase inhibitor (TKI). Pre-treatment with TKI led to partial decrease in TG2 induction (p =0.04), suggesting the effects of the CM in part are caused by secreted TGF-β1. However the incomplete effect of the inhibitor supports that OC cells secrete factors other than TGF-β1 that regulate TG2. Such secreted factors may include LPA, VEGF or PDGF that are detectable in OC ascites (Xu et al 1995) (Yabushita et al 2003) (Matei et al 2006). Likewise, treatment of serum starved OC cells with the TGF-β receptor TKI diminished basal TG2 expression levels, suggesting that TGF-β1 is secreted by OC cells in an autocrine manner.

A.



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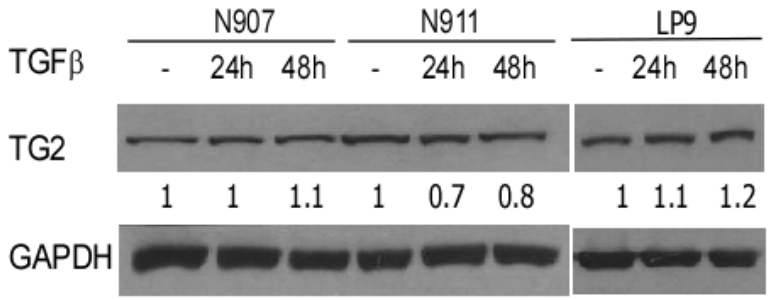
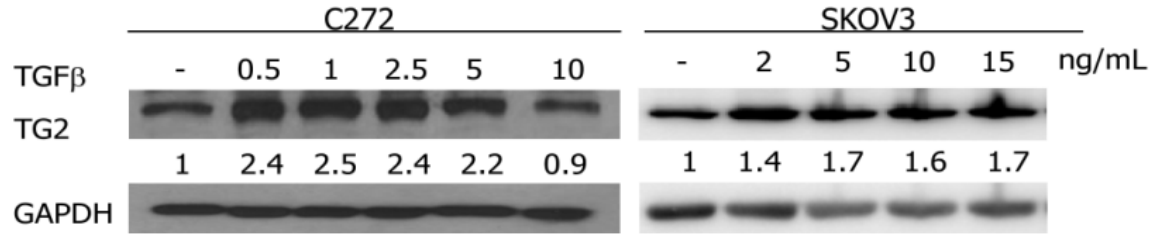


Figure 9: TGF-β1 induces TG2 upregulation in OC cells. A, Primary OC cell OVCA and OC cell lines SKOV3, C272/hTert/E7, Hey, and IGROV-1 were serum starved for 24 hours, then treated with 5ng/mL TGF-β1 for 24-48 hours. TG2 expression was detected by Western blotting. GAPDH was tested as loading control. B, Western blotting for TG2 and GAPDH in normal ovarian surface epithelial cells (NOSE) N907 and N911, non-transformed mesothelial cell LP9 treated with 5ng/mL TGF-β1 for 24-48 hours.

A.



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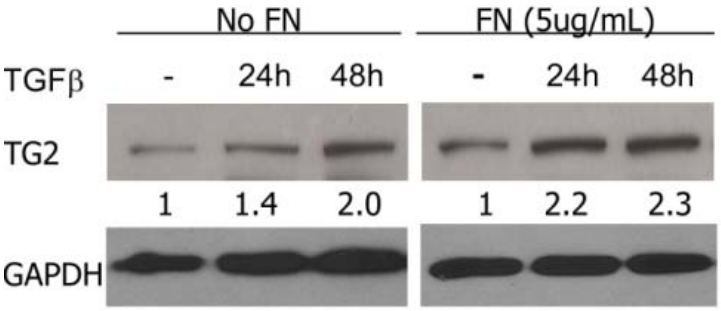


Figure 10: TG2 upregulation by TGF-β1 in OC cells at different conditions. A, To test the dose response to TGF-β1 in OC cells, C272hTert/E7 and SKOV3 cells were treated with TGF-β1 (0.5ng/mL-15ng/mL) for 48 hours. TG2 expression was detected by Western blotting. Densitometry quantifies the level of expression of TG2 relative to GAPDH. B, Western blotting for TG2 and GAPDH in SKOV3 cells plated on plastic (left panel) or on fibronectin (5ng/mL, right panel) coated plates.

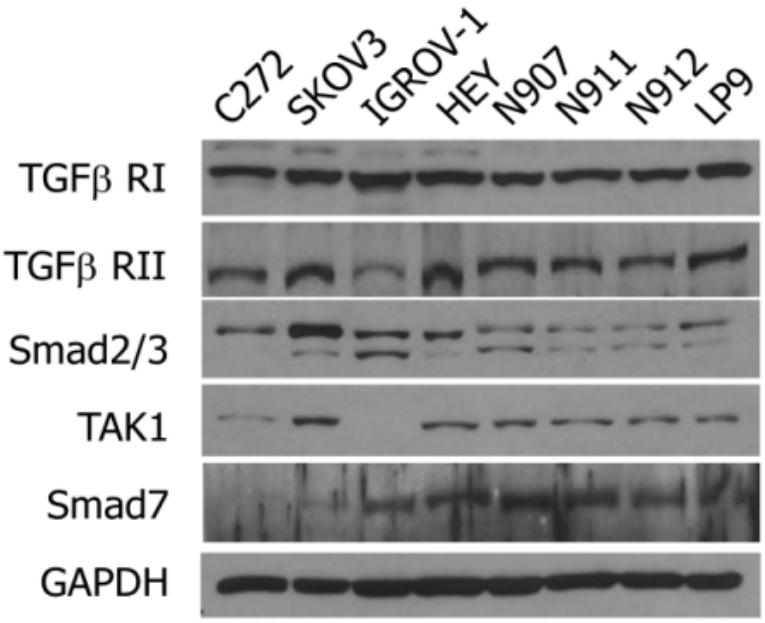
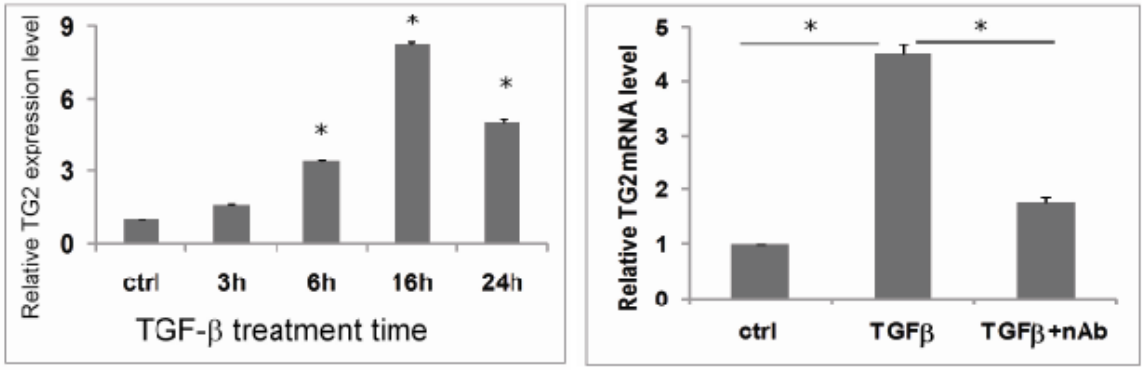


Figure 11: TGF-β signaling pathway in OC cells. Expression of TGF-β receptors I and II, TGF-β intracellular mediators Smad2/3, Smad7, and TAK1 were detected by Western blotting in OC cell lines C272, SKOV3, IGROV-1, and Hey, NOSE cells N907, N911, and N912, and mesothelail cell LP9.

A. **B.**



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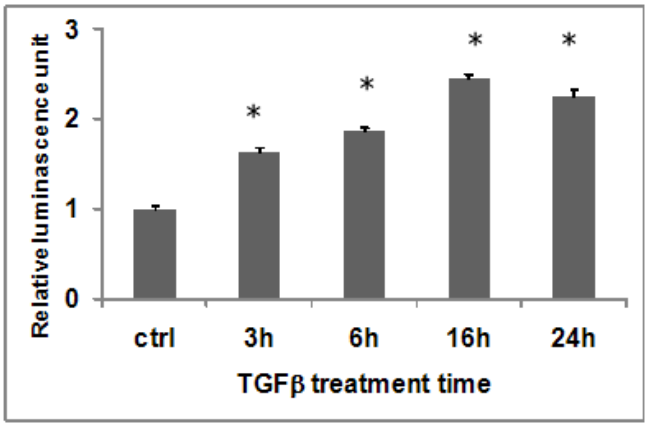


Figure 12: TGF-β1 induces TG2 upregulation in OC cells at transcriptional level. A, C272/hTert/E7 cells were treated with 5ng/ml TGF-β1 for 3–24 hours, TG2 mRNA level was measured by qRT–PCR. B, C272/hTert/E7 cells were pretreated with 5μg/mL TGF-β neutralizing antibody for 1 hour before treatment with 5ng/ml TGF-β1. Total RNA were collected after 16 hour treatment with TGF-β1. TG2 expression level was detected by qRT-PCR. C, C272/hTert/E7 cells treated with 5ng/ml TGF-β1 for 3–24hours. TG2 promoter activity was measured by luciferase reporter assay. *P<0.05. All these experiments were repeated in SKOV3 cells, and similar results were obtained.

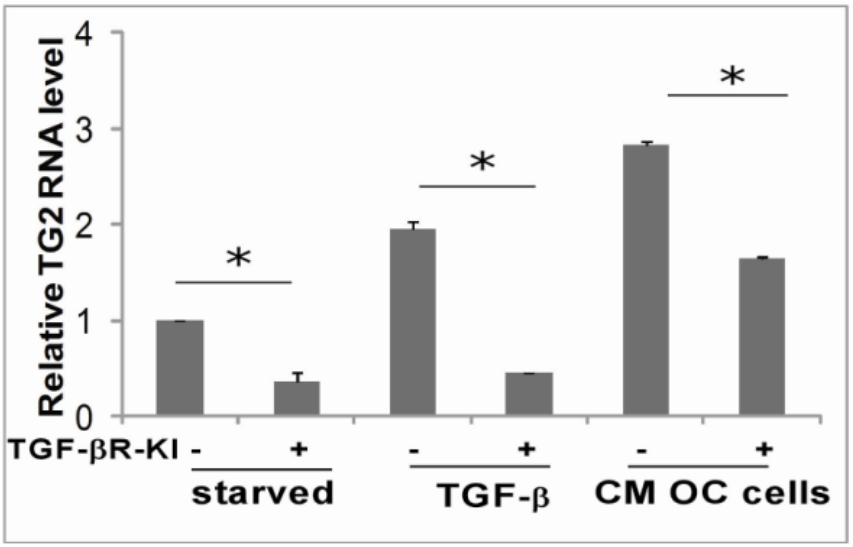
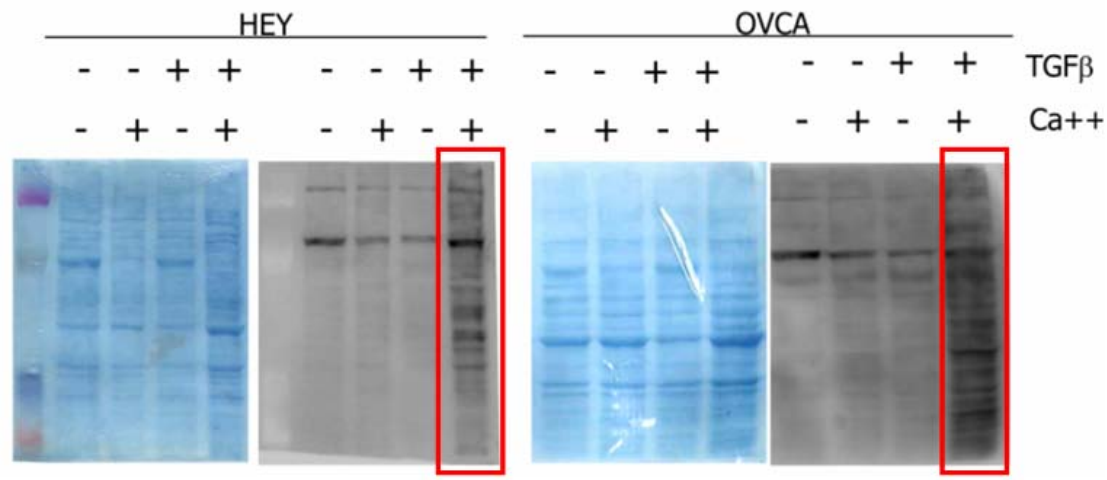


Figure 13: TGF-β1 is secreted in OC cells in an autocrine manner. C272/hTert/E7 cells were serum straved for 24 hours, treated with 1μM TGF-β receptor I kinase inhibitor or vehicle for 1 hour, and then treated with 5ng/mL TGF-β1 and conditioned media of C272/hTert/E7 cells for 16 hours. Total RNA were extracted and TG2 mRNA expression level was detected by qRT-PCR. *P<0.05.

3.1.3 TGF-β1 induces TG2 enzymatic activity in OC cells

As TG2 expression level is increased by TGF-β1, we investigated whether the enzymatic function of TG2 was also induced by TGF-β1. OC cell line Hey, primary OC cell OVCA, NOSE and LP9 cells were treated with TGF-β1, and TG2 enzymatic activity was measured by amine incorporation into total cellular proteins (Zhang et al 1998) (see materials and methods). Increased TG2 activity was observed in OC cells (Hey and OVCA#16) (Figure 14A), but not in non-transformed LP9 or NOSE# 1001 cells treated with TGF-β1 (Figure 14B). This increase in enzymatic function likely reflects TG2 expression induction by TGF-β1 in OC cells, but not in non-transformed cells. However, we cannot exclude a direct effect of TGF-β1 on the enzymatic activity of TG2.

A.



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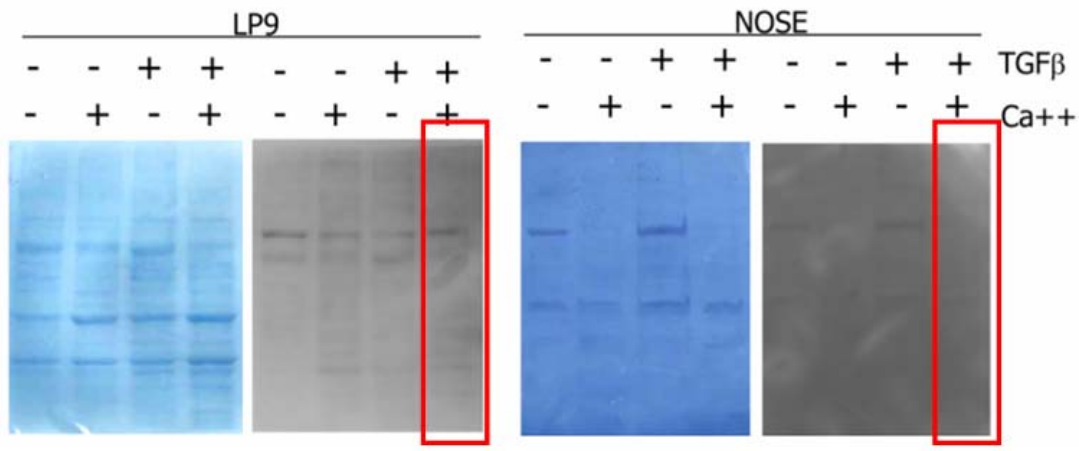


Figure 14: TGF-β1 induces TG2 enzymatic activity in OC cells. TGase assay were performed to detect TG2-catalyzed amine incorporation into proteins. Left panels: Napthol blue staining demonstrates equal loading of proteins per lane. Right panels: immunoblotting with HRP-conjugated streptavidin illustrates biotin-conjugated amine incorporation into proteins, as described in Materials and methods. A, Hey cells and primary ovarian cancer cells (OVCA#16), B, mesothelial cells (LP9) and normal ovarian epithelial cell NOSE#1001 were treated with TGF-β1, and incubated with BPA to detect TG2 activity in the presence or absence of Ca^{++}. Protein ladders formed when active TG2 catalyzes amine incorporation into proteins are framed in rectangles.

3.1.4. TGF-β1 induces TG2 in a Smad-dependent pathway

To understand how TG2 expression is regulated by TGF-β in OC cells, activation of TGF-β-mediated signaling was assessed by measuring the phosphorylation of Smad2/3, the canonical signal transducers of TGF-β. The results showed that Smad2/3 were activated within 15 minutes and remained activated for up 24 hours after TGF-β1 treatment in SKOV3 cells (Figure 15A). Activation of TGF-β signaling was also measured in non-transformed NOSE and mesothelial cell LP9 (Figure 15B), and the results showed that Smad2/3 were only modestly activated by TGF-β1 in these cells.

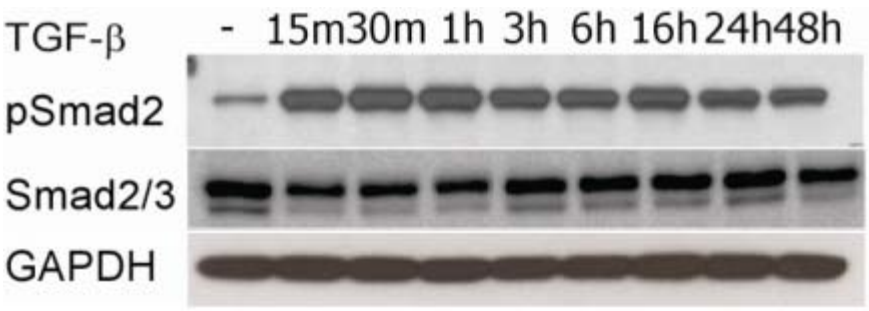
To investigate the effects of Smads on TG2 induction by TGF-β1, we utilized siRNA targeting Smad2/3 in OC cells. Knockdown of the Smad2/3 complex prevented the induction of TG2 by TGF-β1 both at protein (Figure 16A) and mRNA level (Figure 16B). Even the basal level of TG2 expression was decreased when Smad2/3 were knocked down in OC cells, suggesting that engagement of Smad is important to the regulation of TG2 expression by TGF-β.

Smads act as transcriptional regulators through direct binding to target gene promoter sequence (Zawel et al 1998) (Dennler et al 1998). To determine how Smads regulate TG2 expression in OC cells, a bioinformatic tool PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) was used to find out whether Smads directly regulate TG2 transcription. Several putative SBEs were identified in the -191 to -840 TG2 promoter region (Figure 17A). Binding of Smad4 and pSmad3 to the TG2 promoter after TGF-β stimulation was demonstrated by ChIP utilizing primers encompassing the -269 to -479 TG2 promoter fragment (Figure 17B & C), region where two SBEs were

predicted. These data suggest that TGF-β transcriptionally regulates TG2 expression mainly through canonical Smad2/3 signaling pathway.

Further, to consolidate the correlation between TG2 expression and Smad activation, we evaluated 30 human ovarian tumors on a tissue microarray by immunohistochemistry. H score was used to quantify the intensity and percentage of TG2 and pSmad3 staining in the tumor samples. Fisher's exact test was used to compare the relationship between TG2 and pSmad3 expression. The results showed a significant positive correlation between TG2 and pSmad3 staining in human ovarian cancer tissues (P=0.01, odds ratio=13.6, Figure 18 and Table 5), pointing to the clinical relevance of our findings.

A.



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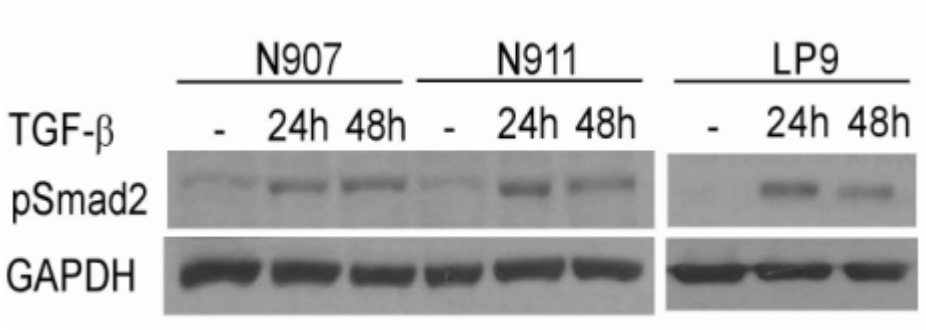
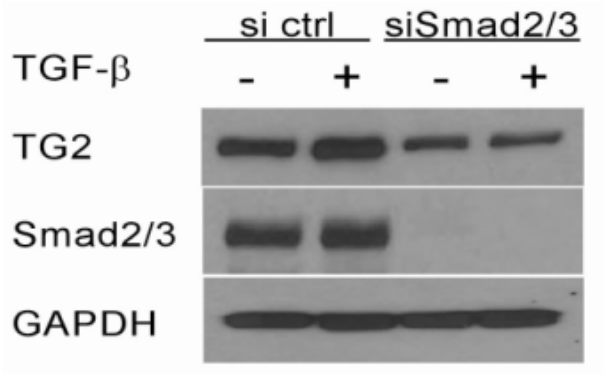


Figure 15: Smad2 is activated by TGF-β1 in OC cells. A, SKOV3 cells were serum starved for 24 hours, and then treated with TGF-β1 for 15 minutes to 48 hours. Smad2 activation was measured by Western blotting. B, NOSE cells (N907 and N911) and mesothelial cell LP9 were treated with TGF-β1 for 24 to 48 hours. Smad2 activation was measured by Western blotting.

A.



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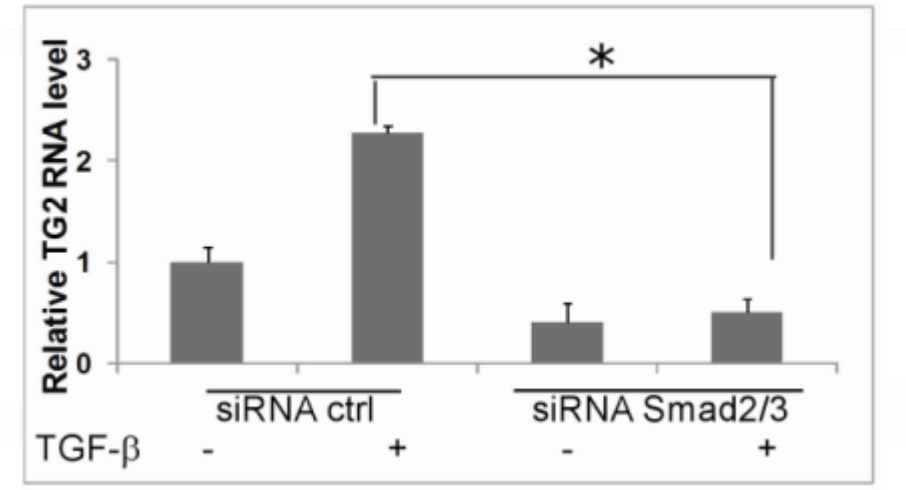
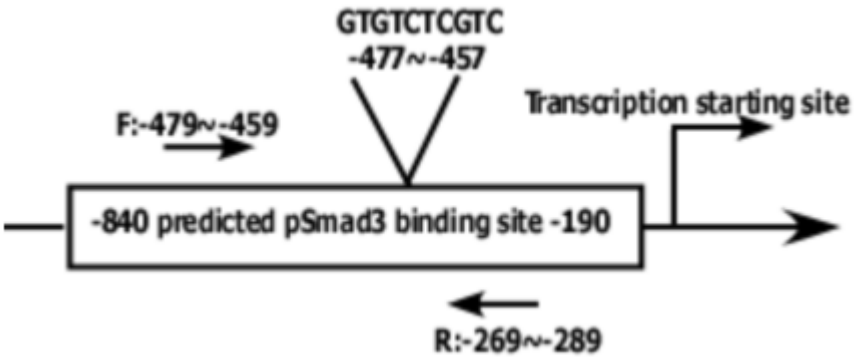


Figure 16: Knock down of Smad2/3 by siRNA blocked TG2 upregulation by TGF-β1. SKOV3 cells were transfected with Smad2/3 siRNA or control siRNA and treated with TGF-β1. TG2 expression level was detected by western blotting after 48 hour treatment (A) and qRT–PCR after 16 hour treatment (B). *P<0.05.

A.



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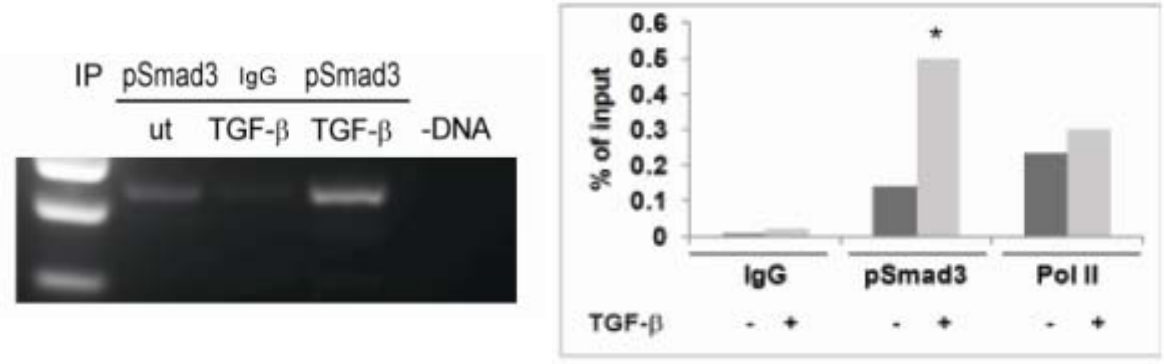

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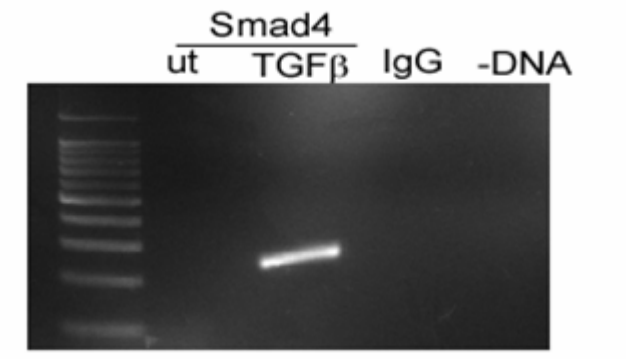


Figure 17: Smads bind to the TG2 promoter region.

Figure 17: Smads bind to the TG2 promoter region. A. Schematic Smads binding sites on TG2 promoter sequence. B & C. Chromatin immunoprecipitation (ChIP) assay demonstrates the binding of pSmad3 and Smad4 to the TG2 promoter. Cross-linked chromatin from C272/hTert/E7 cells treated with TGF-β1 or vehicle was immunoprecipitated with pSmad3 antibody and amplified by regular PCR (left) and quantitative PCR (right) with TG2 promoter-specific primers. C. Cross-linked chromatin from C272/hTert/E7 cells treated with TGF-β1 or vehicle was immunoprecipitated with Smad4 antibody and amplified by regular PCR with TG2 promoter-specific primers. For regular PCR, positive results are presented as PCR band about 210bp on agrose gel. For qPCR, results are presented as percentage of TG2 amplified from non-immunoprecipitated (input) chromatin. Chromatin immunoprecipitated with mouse immunoglobulin G (IgG) was amplified with primers for TG2 promoter as negative control. Chromatin immunoprecipitated with anti-RNA polymerase II was amplified with primers specific for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as positive control. *P<0.05

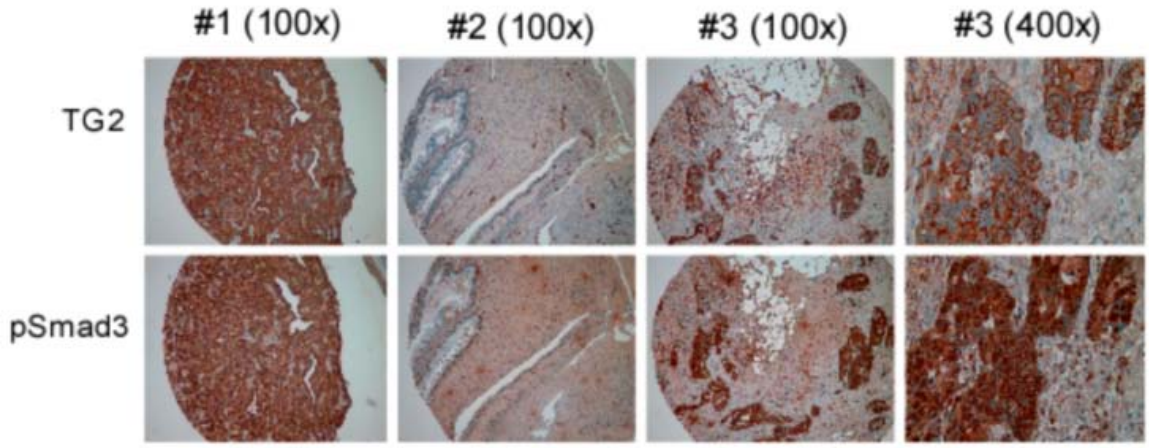


Figure 18: TG2 positively correlates with pSmad3 in human ovarian tumors. Representative immunohistochemistry (IHC) staining for TG2 and pSmad3 in human OC specimens are shown. Concordant positive staining in tumors #1 and #3 and concordant negative staining in tumor #2 are shown. Magnification (× 100–400) is indicated. Experiments performed in collaboration with Mrs. Bhadrani Chelladurai.

Table 5: Expression of TG2 and pSMAD3 in human ovarian tumors by histological type.

	Serous	**Endometrioid**	**Mucinous**	**Total**
number	10	12	8	30
TG2 positive	9	9	5	24
pSmad3 positive	10	8	6	24

3.1.5. TAK1 is involved in TG2 upregulation by TGF-β1

Except for canonical Smad signaling pathway, TAK1, another important TGF-β intracellular signaling mediator, was also investigated in the regulation of TG2 expression in OC cells. Similar to Smad2/3, TAK1 was activated within 15 minutes and remained activated for up 24 hours after TGF-β1 treatment in OC cells (Figure 19). Different than Smad2/3, TAK1 activation was not detected in non-transformed NOSE and LP9 cells (data not shown), suggesting that TAK1 is only activated in cancer cells.

Depending on the cellular context, after activation by TGF-β1, TAK1 may activate p38, the stress activated kinase JNK, or the NF-κB complex. Therefore, we measured the activation status of these TAK1 downstream signals. Phosphorylation of JNK was noted at late time-points (16-24 hours) after TGF-β1 treatment, while phosphorylation of p38 went down in response to TGF-β1. In contrast, the expression level of IKBα, the □inhibitory unit for the NF-kB complex was reduced within 1 hour after TGF-β1 treatment (Figure 19). This suggested that the NF-kB complex is a potential transducer of TGF-β-stimulated TAK1 activity in OC cells.

To find out if and which element in this pathway regulates TG2 expression, next we utilized siRNA targeting the main elements of the cascade (TAK1, JNK2, p38 and p65, the trans-activating subunit of the NF-κB complex). Knockdown of TAK1 blocked the induction of TG2 by TGF-β1 at both protein and mRNA level (Figure 20), suggesting that TAK1 signaling pathway is also important for the regulation of TG2 by TGF-β1.

However, knockdown of p38 and JNK2 did not prevent TG2's induction by TGF-β1, at neither protein nor mRNA levels (Figure 21A & B), suggesting that these pathways are not involved in TG2 upregulation by TGF-β1. Further, siRNA targeting JNK2 or p38 caused a small increase in the basal level of TG2 expression, suggesting these pathways exert an inhibitory effect on TG2 expression. In contrast, knockdown of p65, the other TAK1 target downstream of TGF-β (Figure 22A), blocked TGF-β1-induced TG2, supporting that the NF-κB complex, activated downstream of TAK1, regulates TG2 (Figure 22B). In addition, overexpression of p65 potently induced TG2 mRNA expression level (Figure 22C), demonstrating the additional role of this pathway in TG2's transcriptional regulation in OC cells. In conclusion, TAK1, via NF-κB, participates in TG2 expression regulation by TGF-β1 in OC cells.

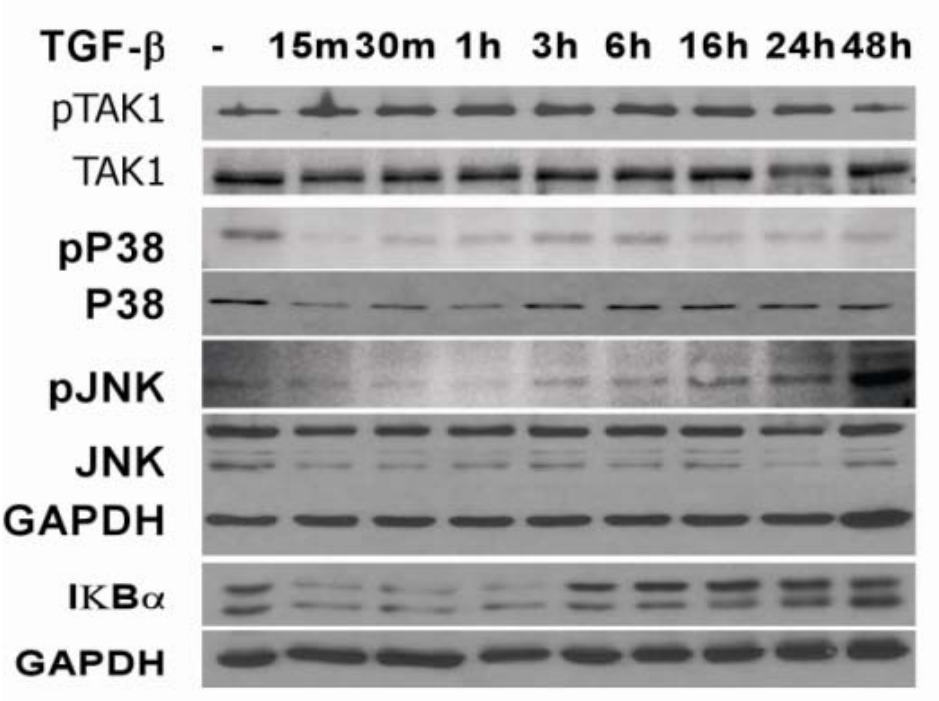
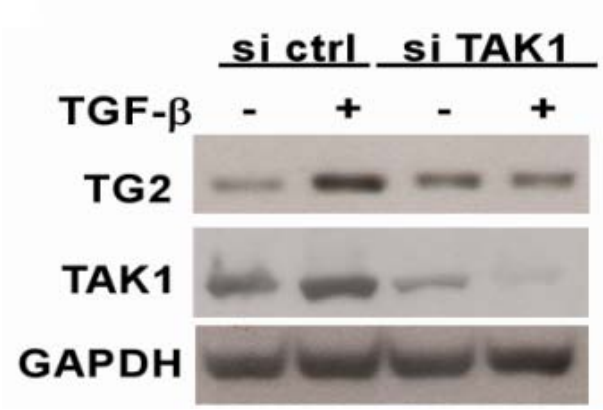


Figure 19: TAK1 and downstream signals in response to TGF-β1 in OC cells. SKOV3 cells were treated with TGF-β1 for 15 minutes to 48 hours. Phosphorylated and total TAK1, p38 and JNK, and IκBα were analyzed by Western blotting.

A.



B.

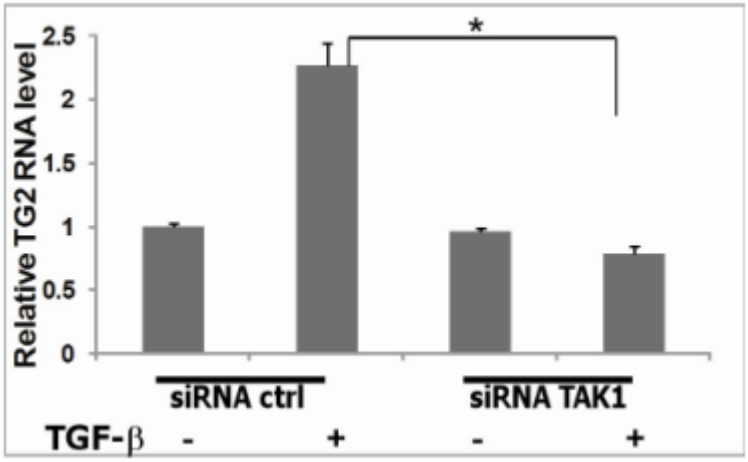
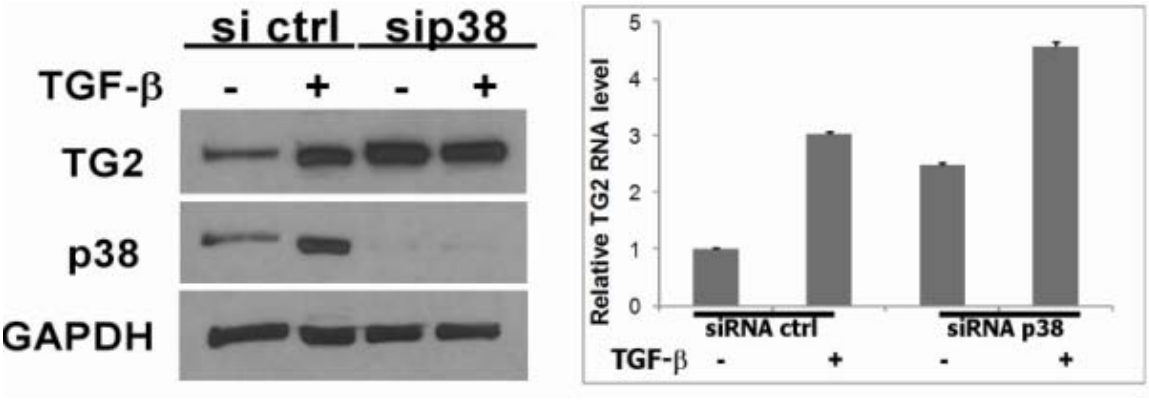


Figure 20: Knock down of TAK1 by siRNA blocked TG2 upregulation by TGF-β1. SKOV3 cells were transfected with TAK1 siRNA or control and treated with TGF-β1. TG2 expression level was detected by western blotting after 48 hour treatment (A) and qRT–PCR after 16 hour treatment (B). *P<0.05.

A.



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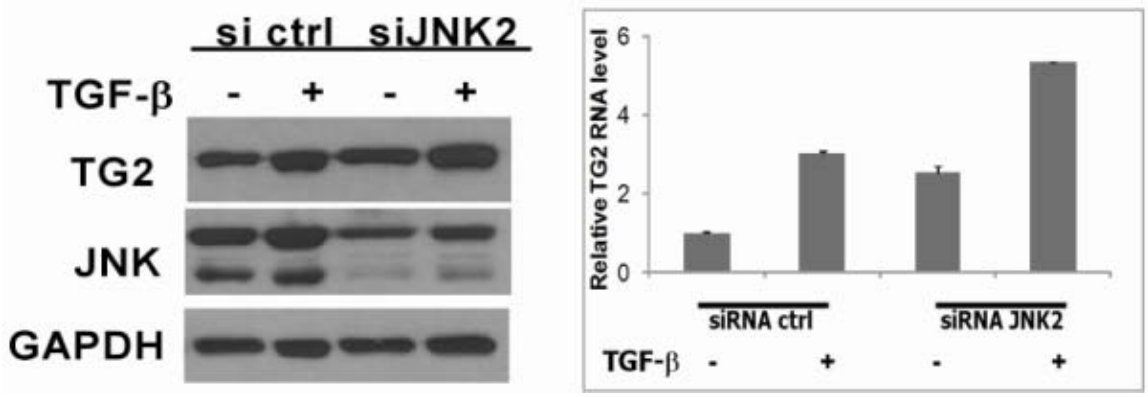
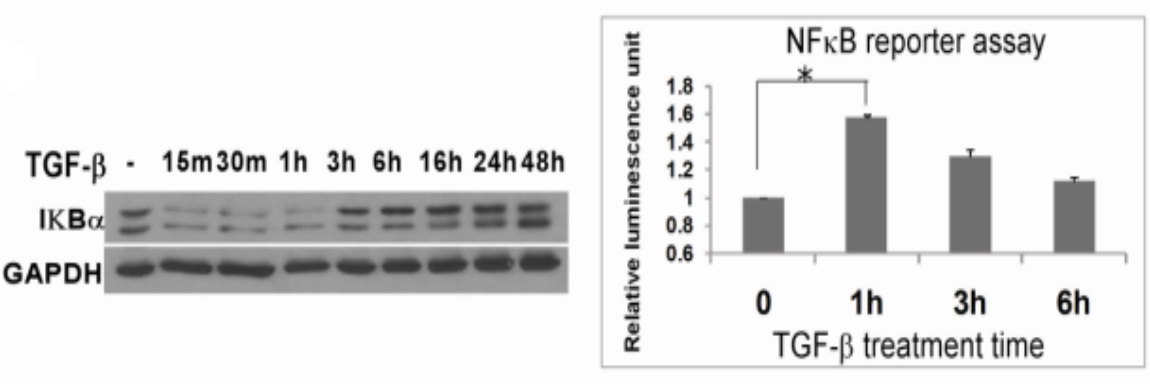


Figure 21: Effect of TAK1 downstream signals p38 and JNK on TG2 upregulation by TGF-β1. A, SKOV3 cells were transfected with p38 siRNA or control and treated with TGF-β1. TG2 expression level was detected by western blotting after 48 hours treatment with TGF-β1 (left) and qRT–PCR after 16 hours treatment with TGF-β1 (right). B, SKOV3 cells were transfected with JNK2 siRNA or control and treated with TGF-β1, and TG2 expression level was detected by western blotting after 48 hours treatment with TGF-β1 (left) and qRT–PCR after 16 hours treatment with TGF-β1 (right).

A.



B. **C.**

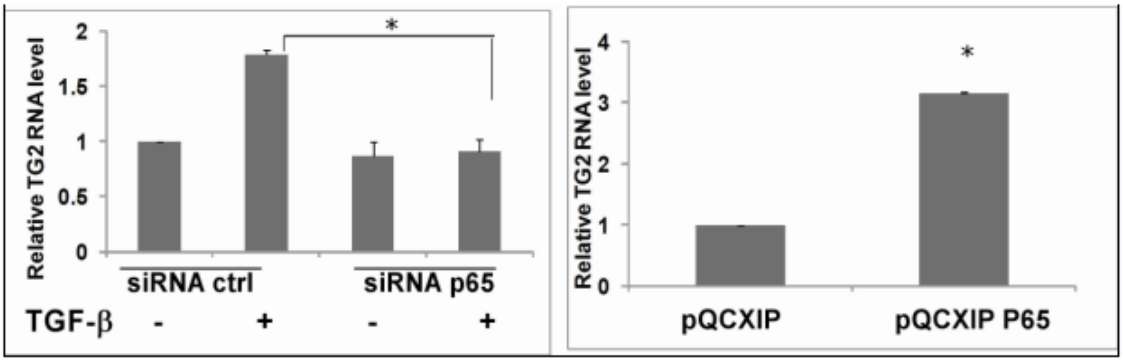


Figure 22: Effect of TAK1 downstream signal NF-κB on TG2 upregulation by TGF-β1. A, Western blotting for IκBα in SKOV3 cells treated with TGF-β1 (left). Gene reporter assay for NF-κB promoter activity in OC cells treated with TGF-β1 (right). B, SKOV3 cells were transfected with p65 siRNA or control and treated with TGF-β1. TG2 expression level was detected by qRT–PCR. C, SKOV3 cells were transduced with p65 or control vector (pQCXIP) and TG2 expression level was detected by qRT–PCR. *P<0.05.

3.2 TGF-β1 induced TG2 mediates Epithelial-Mesenchymal Transition and a cancer stem cell phenotype in OC cells

3.2.1 TGF-β1 induces EMT in OC cells

TGF-β1 is a known inducer of EMT in other epithelial cell models (Xu et al 2009), we therefore tested this function in OC cells. Our results showed that TGF-β1 treated SKOV3 cells underwent characteristic morphological changes of EMT, from a compact shape to an elongated, dispersed phenotype evident 24-72 hours after treatment (Figure 23A). The mesenchymal phenotype was confirmed by quantifying the expression of EMT markers, E-cadherin, N-cadherin, and of the transcriptional regulator Zeb1. A decrease in E-cadherin expression, associated with increased expression of N-cadherin, Zeb1, and TG2 were observed in TGF-β1 treated SKOV3 cells (Figure 23B). These data suggest that TGF-β1 can induce EMT in OC cells.

A. **B.**

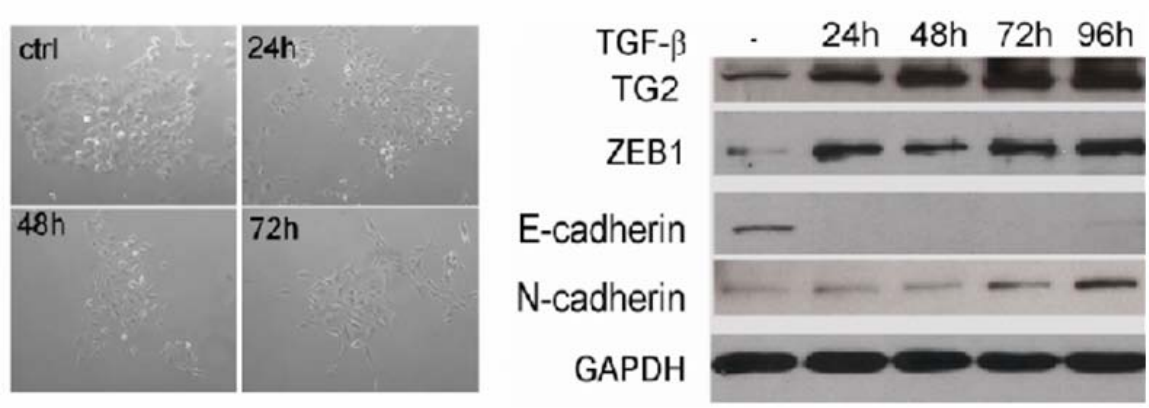


Figure 23: TGF-β1 induces EMT in OC cells. A, Morphological changes of SKOV3 cells treated with TGF-β1 for 24-72 hours (x100 magnification). B, SKOV3 cells were treated with TGF-β1 for 24-96 hours, epithelial marker E-cadherin, mesenchymal marker Zeb1 and N-cadherin, and TG2 expression level were detected by Western blotting.

3.2.2 TG2 induces EMT in OC cells

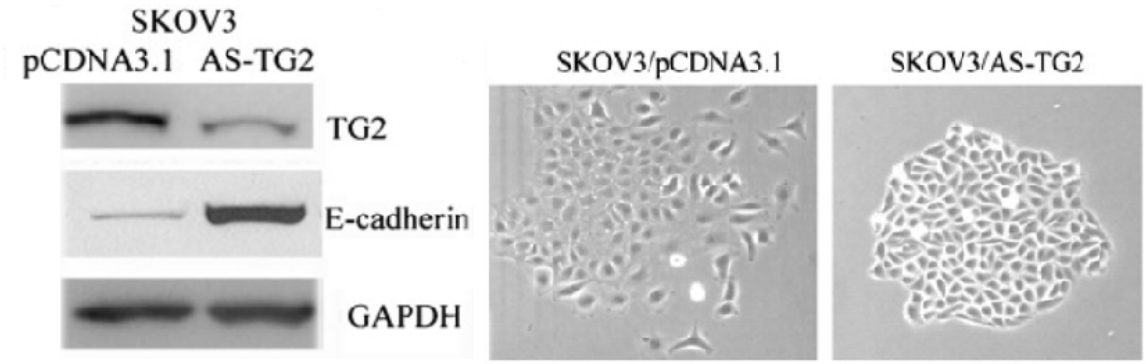
As TGF-β1 induces EMT in OC cells, and TG2 is upregulated by TGF-β1 in OC cells, we sought to determine whether TG2 plays a role in EMT. Two ovarian cancer cell lines were used, one expressing endogenous TG2 (SKOV3) and one lacking TG2 expression (OV90). To better dissect the role of TG2 in EMT, SKOV3 cells were stably transfected with an antisense TG2 construct (AS-TG2) or control vector (pcDNA3.1), and OV90 cells were stably transduced with full-length TG2 or control vector (pQCXIP). Decreased expression level of TG2 in AS-TG2 SKOV3 cells accompanied with increased expression level of epithelial marker E-cadherin was shown by Western blotting (Figure 24A, left). Control cells expressing endogenous TG2 displayed poorly organized adhesive junctions and loss of cell polarity and exhibited a fibroblast-like morphology. In contrast, cells stably transfected with AS-TG2 appeared compact, and cohesive (Figure 24A, right). A similar difference in phenotype was noted in OV90 cells with or without TG2 overexpression. Increased expression level of TG2 in TG2-transduced OV90 cells accompanied with decreased expression level of E-cadherin was shown by Western blotting (Figure 24B, left). While control cells are compact and organized in tight structures, TG2-transduced OV90 cells are elongated and dispersed (Figure 24B, right). These observations suggest that increased TG2 expression was associated with a mesenchymal morphology and decreased E-cadherin expression level.

Further, an immunofluorescent assay was done to test the expression of the epithelial marker, E-cadherin, and of the mesenchymal marker, vimentin, in

SKOV3 cells with and without TG2 expression. The results showed that SKOV3 control cells dimly expressed E-cadherin and strongly stained with a vimentin antibody. In contrast, E-cadherin expression was increased and vimentin staining was diminished in AS-TG2 cells (Figure 25). These observations suggest that increased TG2 expression was associated with loss of epithelial phenotype and gain of mesenchymal phenotype.

EMT renders cells more motile and invasive (Thiery 2002). We next examined whether the mesenchymal morphology observed in TG2-expressing cells is associated with increased invasiveness. For this, cells were plated in two-dimensional and three-dimensional cultures in matrigel matrix. SKOV3 control cells formed conspicuous networks in matrigel, whereas AS-TG2 cells remained as solitary clumps (Figure 26A). Likewise, OV90 cells transduced with TG2 invaded through the matrix as trabecullae, whereas OV90 control cells stayed in non-invading clumps (Figure 26B). These results show that TG2 promotes migration and invasion of ovarian cancer cells.

A.



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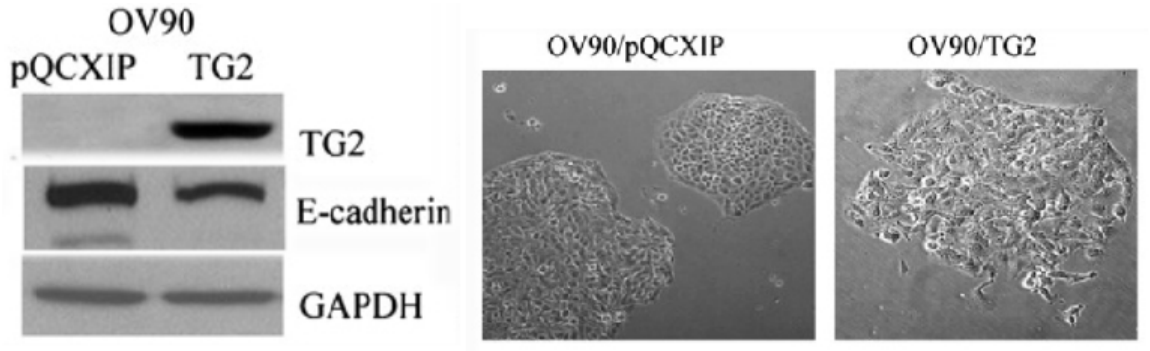


Figure 24: TG2 induces EMT in OC cells. A & B, Induction of EMT by TG2□ was assessed by EMT marker E-cadherin (left) and cell morphology (right, x100 magnification) in SKOV3 transfected with pcDNA3.1 vector and AS-TG2 cells (A) and OV90 transduced with pQCXIP vector and TG2 cells (B). Experiments performed in collaboration with Dr. Minghai Shao.

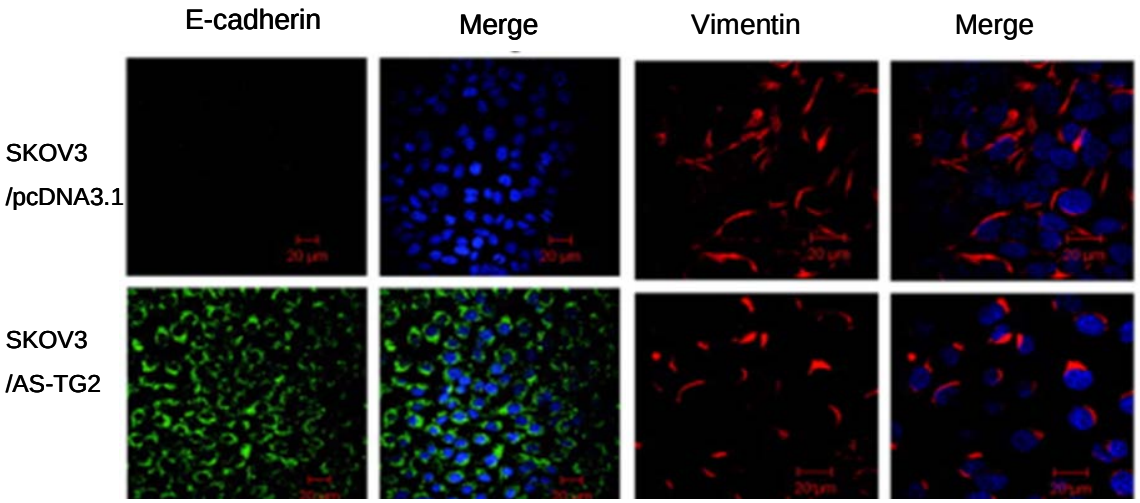


Figure 25: TG2 expressing SKOV3 pcDNA3.1 cells exhibit a mesenchymal phenotype. Immunofluorescent staining for epithelial marker E-cadherin (Green) and mesenchymal marker vimentin (red) in pcDNA3.1 and AS-TG2–transfected SKOV3 cells. Nuclei are visualized by DAPI staining (Blue).

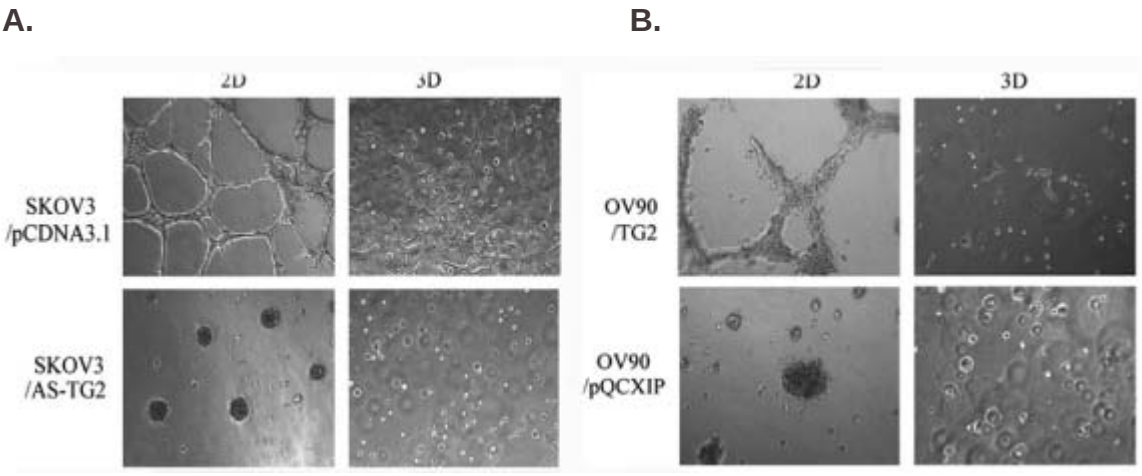


Figure 26: TG2 enhances OC cells migration and invasion. Migration on two-dimensional (2D) matrigel and invasion through three-dimensional (3D) matrigel were measured with SKOV3-pcDNA3.1 and AS-TG2 cells (A*;* ×100 magnification) or OV90-pQCXIP and TG2 cells (B; ×100 magnification).

3.2.3 TG2 negatively regulates E-cadherin at transcription level by modulating the transcription repressor Zeb1

Downregulation of E-cadherin expression is the central event in EMT (Zeisberg and Neilson 2009). We next investigated the mechanisms of E-cadherin repression by TG2. As transcriptional repression of E-cadherin is a major process involved in loss of E-cadherin expression in many carcinomas (Berx and van Roy 2009), we first analyzed whether TG2's effects on E-cadherin occur at the transcriptional level. For this, we measured E-cadherin mRNA in OC cells with and without TG2 expression. Reverse transcription-PCR (RT-PCR) showed that E-cadherin mRNA level was upregulated in AS-TG2 SKOV3 cells compared with control cells (Figure 27A, left). In contrast, E-cadherin mRNA level was downregulated in OV90 cells transduced with TG2 compared with vector-transduced cells (Figure 27A, right). We then tested E-cadherin promoter activity in AS-TG2 and control SKOV3 cells transfected with an E-cadherin-luciferase reporter construct (DiFeo et al 2006). The gene reporter assay showed that E-cadherin promoter activity was increased 2.6-fold by knocking down TG2 (Figure 27B; $P < 0.001$). These data show that TG2 levels are negatively correlated with E-cadherin transcription expression.

Several transcriptional repressors are reported as critical modulators of EMT, including Snail (Cano et al 2000), Slug (Bolos et al 2003), Zeb1 (Eger et al 2005), Zeb2 (Comijn et al 2001), and Twist (Yang et al 2004). To find out whether TG2 regulates E-cadherin expression through one or more of these repressors, semiquantitative RT-PCR and real-time PCR were used to test the

expression of these transcription factors. Reduced mRNA expression levels for Slug, Zeb1, and Zeb2, but not for Twist1, Twist2, Snail1, and Snail 3, were observed in AS-TG2 compared with control cells (Figure 28A, left). Real-time PCR showed that Zeb1, Zeb2, and Slug mRNA expression levels were decreased by 80% in AS-TG2–transfected cells compared with controls, but the Snail1 expression level was not significantly different between the two cell types (Figure 28B, top). Similar trends were observed in OV90 +/- TG2 cells. Slug, Zeb1, and Zeb2 mRNA levels were increased in OV90-TG2 compared with control cells. In addition, Twist1 and Snail 3 mRNA levels were slightly upregulated in TG2-overexpressing cells (Figure 28A, right). Real-time PCR confirmed a 75% increase in mRNA expression level for Zeb1 and Zeb2 in OV90-TG2 compared with control cells, but no significant difference for Slug and Snail1 (Figure 28B, bottom). These data suggest that in ovarian cancer cells, TG2 expression correlates with expression of E-cadherin transcriptional repressors, particularly with that of Zeb1 and Zeb2.

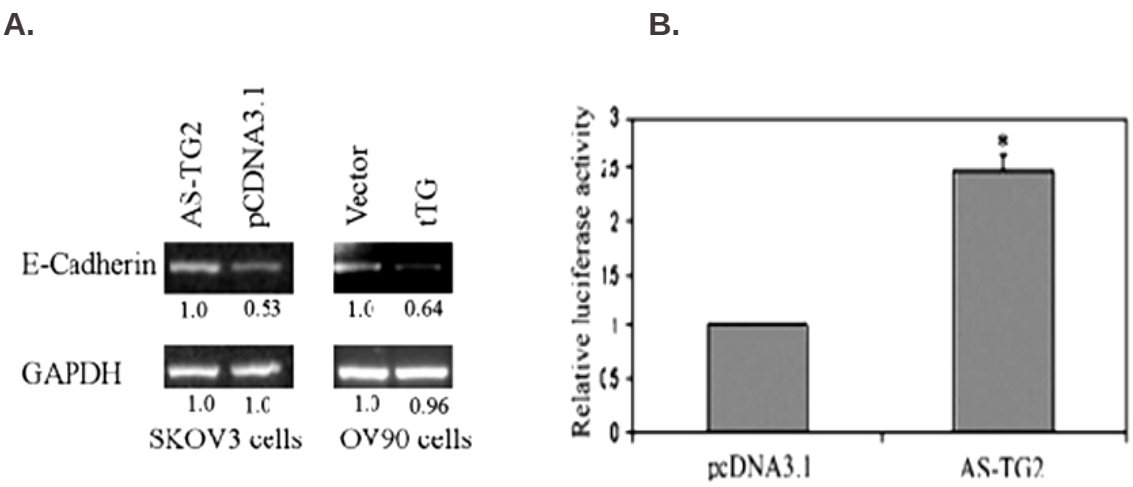


Figure 27: TG2 negatively regulates E-cadherin at transcriptional level. A, semiquantitative RT-PCR for E-cadherin and GAPDH in SKOV3 control and AS-TG2 cells (left) and in vector- and TG2-transduced OV90 cells (right). B, A gene reporter assay of E-cadherin promoter activity in AS-TG2 and pcDNA3.1 SKOV3 cells, conducted as described in Materials and Methods. Experiments performed in collaboration with Dr. Minghai Shao.

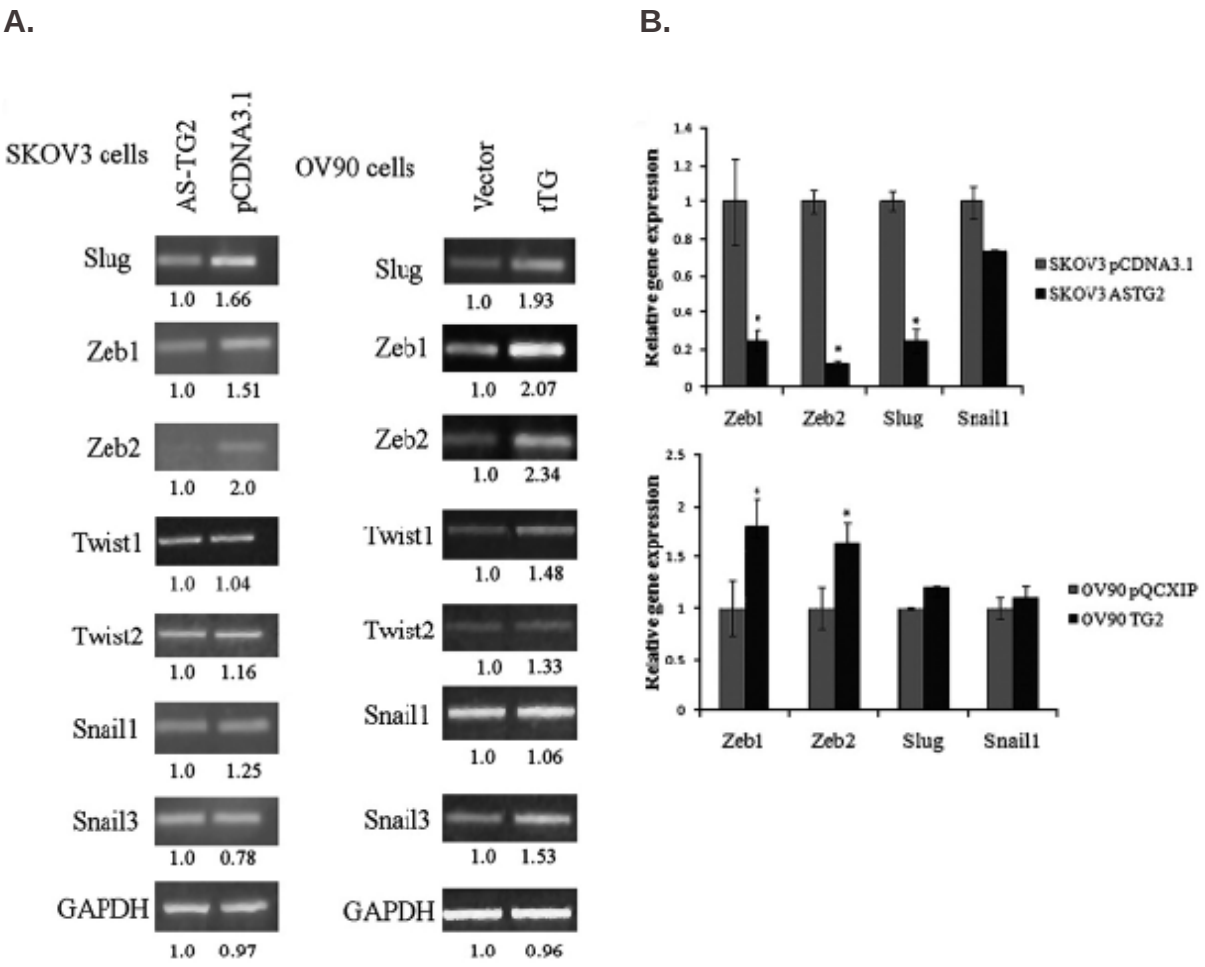


Figure 28: TG2 modulates expression of E-cadherin transcription repressors Zeb1 and Zeb2. A. semiquantitative RT-PCR for E-cadherin transcriptional repressors in pcDNA3.1 and AS-TG2 SKOV3 cells (left) and pQCXIP- and TG2-transduced OV90 cells (right). Densitometry quantifies the level of expression of E-cadherin repressors relative to GAPDH. B, quantitative PCR for Zeb1, Zeb2, Slug, and Snail1 in SKOV3 cells transfected with AS-TG2 or vector (top) and in OV90 cells transduced with TG2 or vector (bottom). *P<0.05. Experiments performed in collaboration with Dr. Minghai Shao.

Based on these results and on a recent report implicating Zeb1 in ovarian epithelial cell transformation (Hurt et al 2008), we focused on the effects of TG2 on Zeb1. Consistent with RT-PCR data, lower Zeb1 expression at the protein level was observed in AS-TG2 compared with control cells (Figure 29A). To test whether Zeb1 negatively regulates E-cadherin in ovarian cancer cells, we used siRNA targeting Zeb1. E-cadherin expression level was upregulated in SKOV3 cells transfected with Zeb1 siRNA compared with cells transfected with control siRNA (Figure 29B). These data suggest that TG2 is positively related with Zeb1 expression, and Zeb1 negatively regulates E-cadherin expression.

To investigate whether Zeb1 is needed for regulation of E-cadherin expression by TG2, we stably overexpressed Zeb1 in SKOV3 cells where TG2 was knocked down. The expression level of E-cadherin, which was markedly increased by TG2 knockdown compared with control cells (Figure 30A, lane 2 versus lane 1), was reduced significantly by overexpression of Zeb1 (Figure 30A, lane 3 versus lane 2). Consequently, overexpression of Zeb1 rescued the invasive phenotype in cells with decreased expression of TG2, as assessed by a two- or three-dimensional matrigel invasion assay (Figure 30B). These data suggest that Zeb1 plays a critical role in TG2-mediated regulation of E-cadherin expression and the invasive phenotype of ovarian cancer cells.

Given the previously described role of TG2 in activating the NF-κB complex and Akt (Mann et al 2006, Verma et al 2008), and the known functions of these pathways in modulating EMT (Chua et al 2007, Grille et al 2003), we investigated whether the effects of TG2 on Zeb1 and EMT are induced as a

consequence of Akt or NF-κB activation. To test this, we overexpressed the p65 subunit of NF-κB or a constitutively active form of Akt (CA-Akt) in ovarian cancer cells where TG2 was knocked down and compared the expression levels of Zeb1 and E-cadherin to those in cells expressing endogenous TG2. Overexpression of p65 in AS-TG2 cells was confirmed by Western blotting for IκBα (Figure 31A) and overexpression of CA-Akt was verified by Western blotting for Akt (Figure 31B). Zeb1 expression level was lower in AS-TG2 cells compared with control cells, and this corresponded to increased E-cadherin expression level (Figure 31C, lane 2 versus lane 1). Overexpression of p65 in AS-TG2 cells rescued Zeb1 expression level and repressed E-cadherin level (Figure 31C, lane 3 versus lane 2).

As assessed by a matrigel invasion assay, overexpression of p65 in AS-TG2 cells led to an invasive phenotype comparable to that of control cells (Figure 31D), suggesting that an important element in the process of TG2-induced EMT is the repressor Zeb1 induced by NF-κB. Overexpression of CA-Akt in AS-TG2 cells also repressed E-cadherin level (Figure 31C, lane 4 versus lane 2) and induced an invasive phenotype (Figure 31D). However, CA-Akt overexpression did not affect Zeb1 (Figure 31C, lane 4 versus lane 2), suggesting that Zeb1 is not a downstream target of Akt. Collectively, these data indicate that TG2 negatively regulates E-cadherin expression via inducing Zeb1 expression through activation of NF-κB, leading to EMT and increased cancer cell invasiveness.

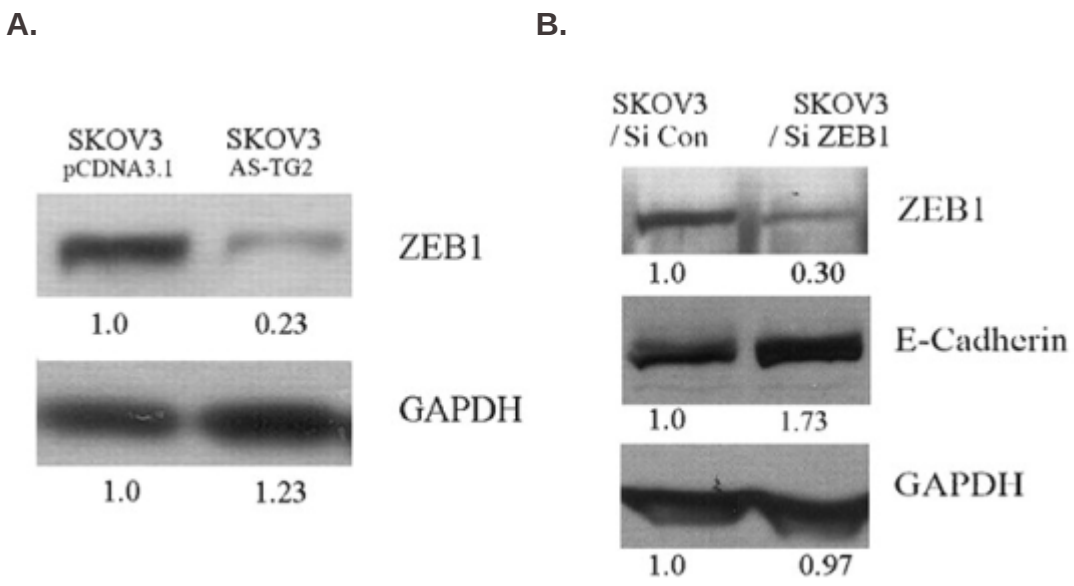
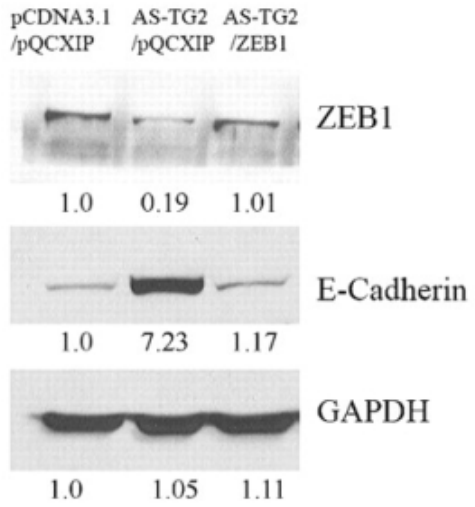


Figure 29: TG2 regulates E-cadherin expression by modulating Zeb1. A. Western blotting for Zeb1 in pcDNA3.1- and AS-TG2–transfected SKOV3 cells. B. SKOV3 cells were transfected with siRNA targeting Zeb1 or with control siRNA (right), expression of Zeb1 and E-cadherin were detected by Western blotting. Densitometry quantifies Zeb1 expression relative to GAPDH. Experiments performed in collaboration with Dr. Minghai Shao.

A.



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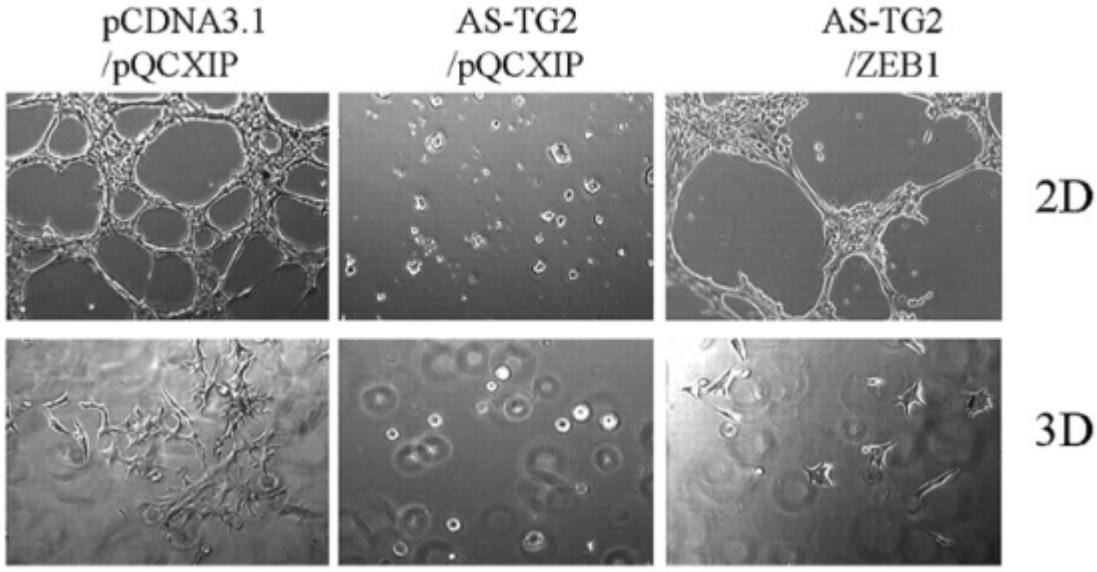


Figure 30: Zeb1 is the mediator of TG2 induced EMT. A, SKOV3 pcDNA3.1 and AS-TG2 cells were transduced with pQCXIP Zeb1 or control. Zeb1 and E-cadherin expression was detected by Western blotting. B, Migration and Invasion of SKOV3-pcDNA3.1 and AS-TG2 cells transduced with pQCXIP or Zeb1 (×100 magnification) were detected using Matrigel assay. Experiments performed in collaboration with Dr. Minghai Shao.

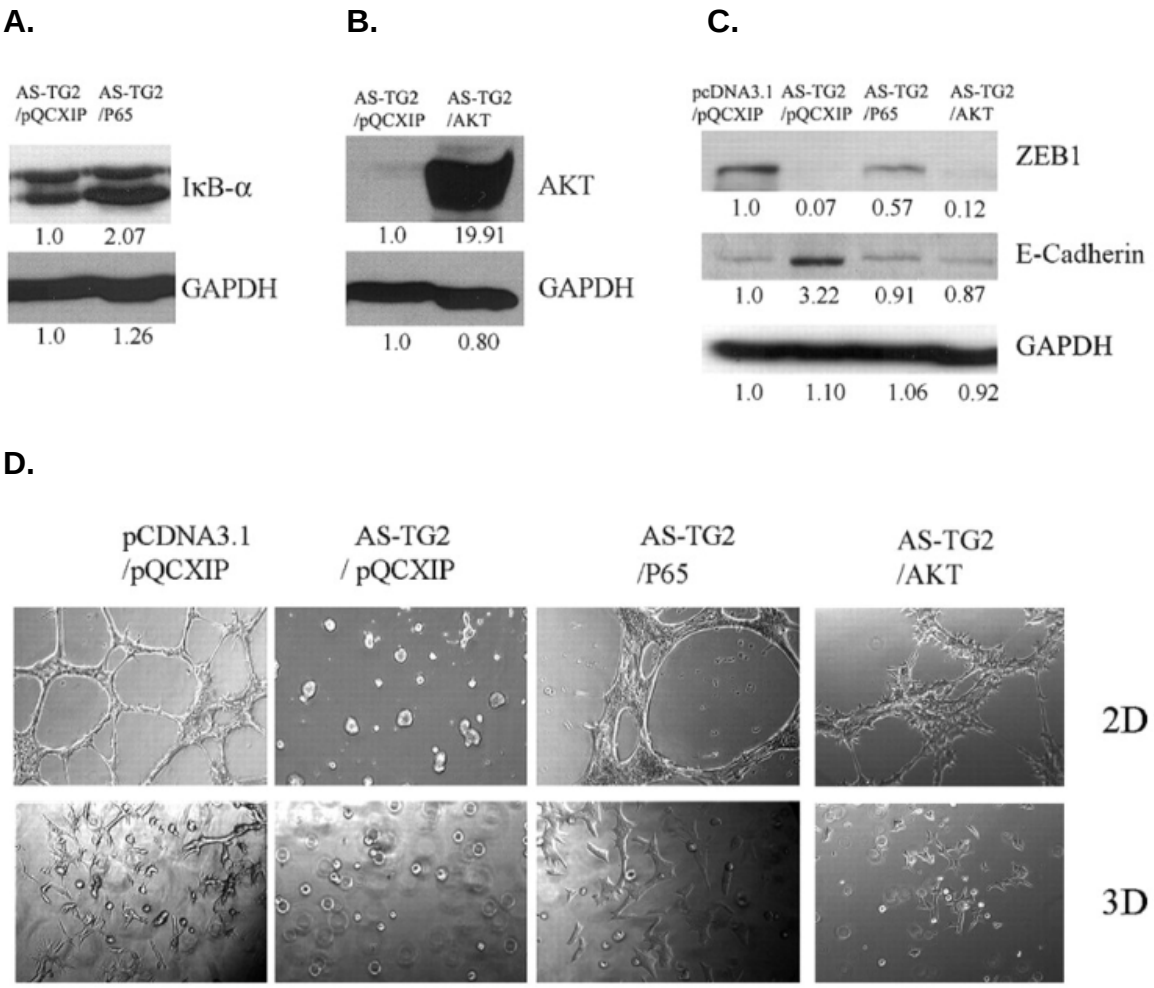


Figure 31: TG2 modulates Zeb1 expression by activation of p65. A. Western blotting for IκBα in AS-TG2 cells transduced with pQCXIP or p65. B. Western blotting for Akt in AS-TG2 cells transduced with pQCXIP or CA-Akt. C. Western blotting for Zeb1, E-cadherin, and GAPDH in SKOV3 pcDNA3.1 or AS-TG2 cells transduced with pQCXIP, p65, or CA-Akt. Densitometry quantifies the expression levels for Zeb1 and E-cadherin relative to GAPDH. D. Invasion through Matrigel of SKOV3-pcDNA3.1 and AS-TG2 cells transduced with pQCXIP, p65, or CA-Akt (×100 magnification). Experiments performed in collaboration with Dr. Minghai Shao.

3.2.4. N-terminal fibronectin binding domain of TG2 induces EMT in OC cells

TG2 is a multifunctional protein with several functional domains. To understand how TG2 participates in EMT induction, we used an OC cell model that does not express endogenously TG2 (OV90 cells) and stably transduced these cells by pQCXIP with wild-type TG2, a TG2 N-terminal fragment that contains the region binding FN (tTG1-140), a TG2 enzymatic mutant (C277S) and a TG2 GTPase mutant (R580A) (Figure 32). First we measured the expression of EMT markers in these engineered cells by Western blotting and qRT-PCR. Quantification of EMT markers at protein and mRNA levels demonstrated that both wild-type TG2 and tTG1-140 but not the TG2 enzymatic mutant C277S or TG2 GTPase mutant R580A induce downregulation of E-cadherin and upregulation of Zeb1 at both protein level (Figure 33A) and mRNA level (Figure 33B, $P<0.05$). Next, we measured the motility and invasiveness of these engineered cells. The results showed that both wild-type TG2 and tTG1-140 but not C277S or R580A induced cell invasion through matrigel consistent with a mesenchymal phenotype (Figure 33C). These data suggest that the FN-binding domain of TG2, lacking both enzymatic and GTPase function is sufficient to induce EMT. Interestingly, it has been recently shown that this fragment is essential for TG2's secretion into the ECM and interaction with the matrix (Chou et al 2011). Indeed, we measured cell adhesion to FN and observed that expression of both full length TG2 and tTG1-140 increases cell adhesion to FN, as previously reported (Akimov and Belkin 2001) (Hang et al 2005) (Figure 34,

P<0.05). These data suggest that TG2's role facilitating cell interaction with the ECM is implicated in EMT.

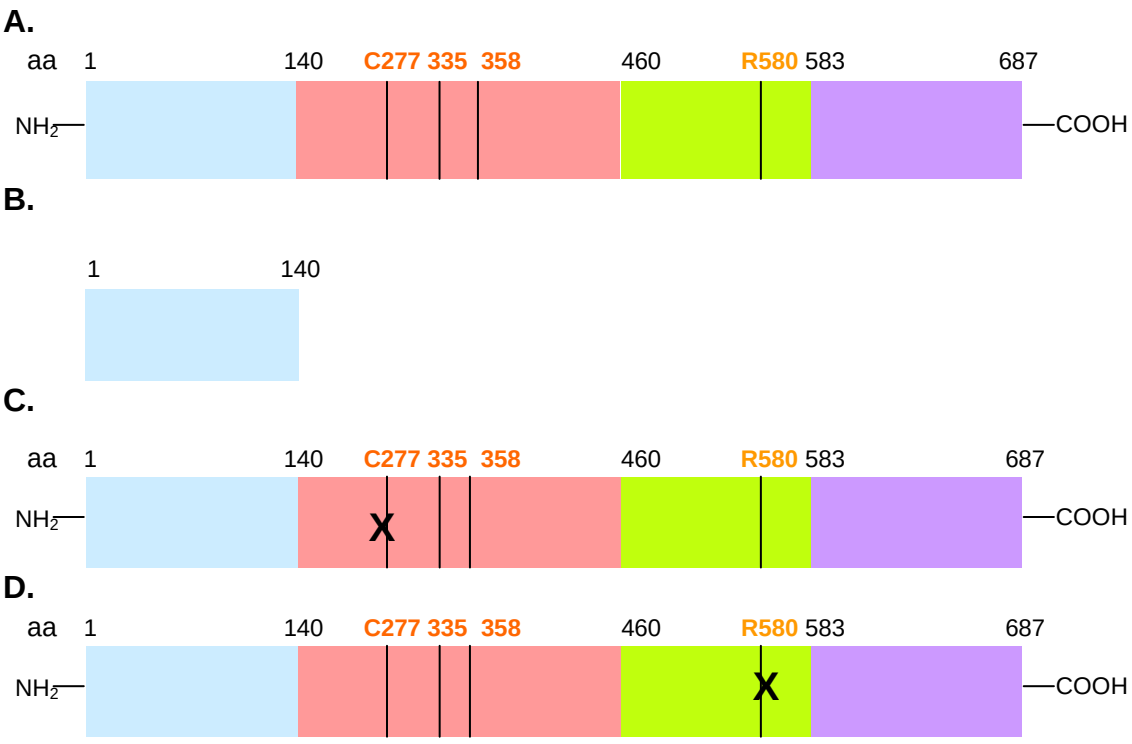
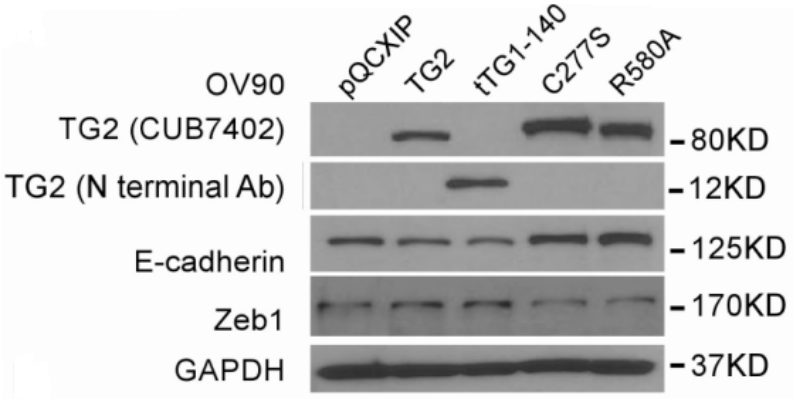
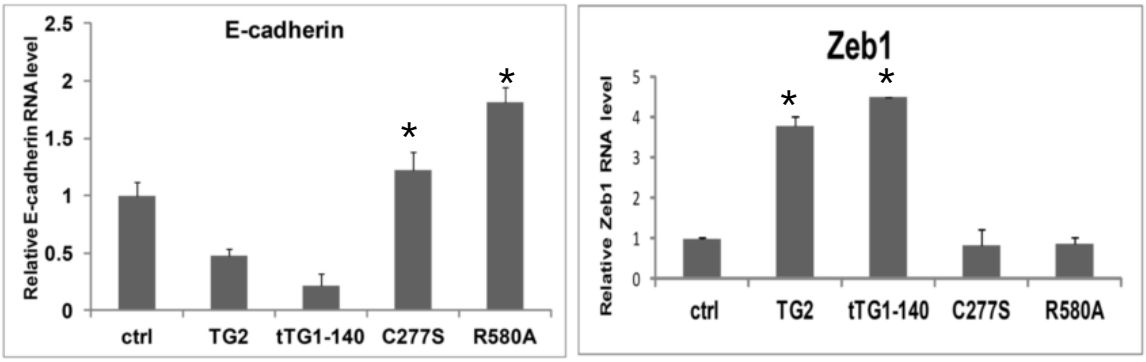


Figure 32: Schematics of TG2 constructs transduced into OV90 cells. A. wild-type TG2, B. a TG2 N-terminal fragment that contains the FN binding domain (tTG1-140), C. a TG2 enzymatic mutant (C277S) and D. a TG2 GTPase mutant (R580A).

A.



B.



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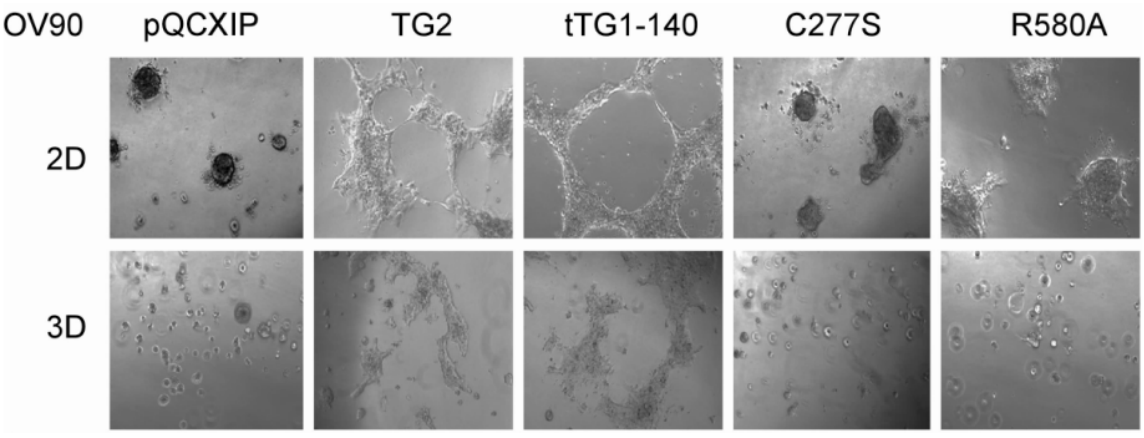


Figure 33: Wild-type TG2 and N-terminal fibronectin binding domain of TG2 induce EMT in OV90 cells.

Figure 33: Wild-type TG2 and N-terminal fibronectin binding domain of TG2 induce EMT in OV90 cells. A, TG2, E-cadherin and Zeb1 expression levels were measured by western blotting in OV90-pQCXIP, wild-type (wt)-TG2, tTG1-140, TG2 enzymatic mutant C277S and TG2 GTPase mutant R580A transduced cells. B, E-cadherin and Zeb1 mRNA expression levels were measured by quantitative reverse transcriptase (qRT)–PCR in OV90-pQCXIP, wt-TG2, tTG1-140, C277S, and R580A transduced cells. *P<0.05. C, Invasion through matrigel using two-dimensional (2D) and three-dimensional (3D) cultures of OV90-pQCXIP, wt-TG2, tTG1-140, C277S, and R580A transduced cells (×100 magnification).

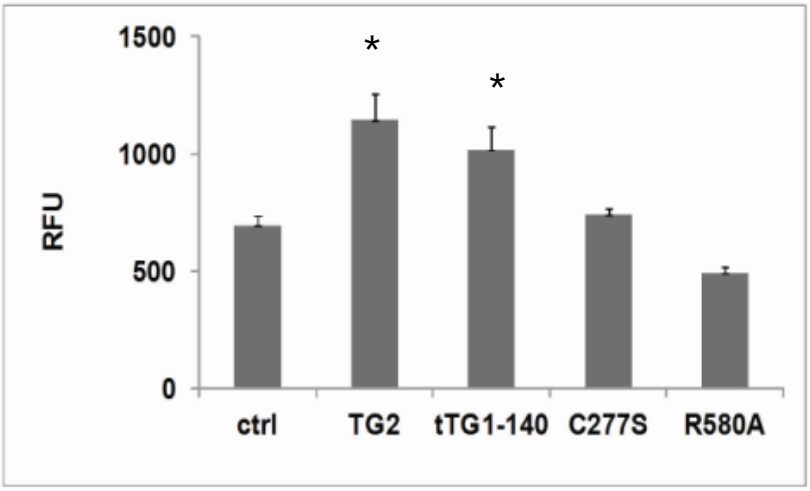


Figure 34: Wild-type TG2 and N-terminal fibronectin binding domain of TG2 promote OV90 cells adhere to FN. Adhesion to FN was measured by solid phase assay using OV90-pQCXIP, wt-TG2, tTG1-140, C277S, and R580A transduced cells. *P<0.05.

3.2.5. TGF-β1 induces an ovarian cancer stem cell phenotype

An important step in the process of OC dissemination is the formation of cellular spheroids. These allow cancer cells to float in the abdominal cavity as multi-cellular aggregates, protecting them from environmental stresses and facilitating invasion into the mesothelium (Allen et al 1987, Sodek et al 2009). Spheroid formation is also recognized as a cancer stem cell feature (Dontu et al 2003). As TGF-β1 can induce EMT, and EMT is associated with cancer stem cell phenotype (Mani et al 2008), we investigated whether TGF-β1 enhanced OC spheroid formation by inducing TG2. Increased size and number of spheroids were induced by TGF-β1 in IGROV1 (Figure 35, $P<0.05$, left) and SKOV3 cells (not shown). TGF-β1 also increased the size of spheroids formed by IGROV1 cells and by cells disaggregated from human ovarian tumors (Figure 35, right).

Prior studies indicated that cancer cells with stem cell phenotype aggregate as spheroids when cultured under low adherence conditions in the absence of differentiating factors (Al-Hajj et al 2004, Zhang et al 2008). Further, cancer cells cultured as spheroids are enriched in cells with stem phenotype (Dontu et al 2003). We therefore quantified the percentage of cells harboring the OCIC phenotype within spheroids and tested the effects of TGF-β1 on this cell population. We used CD44 and CD117 as the cell surface markers, based on the demonstration that cells double staining for these markers display the functional characteristics of stem cells, including the ability to differentiate, to generate tumors in mice and to be chemoresistant (Zhang et al 2008). We observed that as compared to monolayer cultures, IGROV1 spheroids were

enriched in CD117+ (35% vs. 3%) and CD44+/CD117+ (2.5% vs. 1%) cell population (Figure 36A). TGF-β1 □treatment of IGROV1 cells enriched the CD44+ (36% vs. 23%) and CD44+/CD117+ cell subpopulations (2.5% vs. 1.4%, Figure 36B). These data show that TGF-β1 induces spheroid formation in OC cells and increases the CD44+/CD117+ population.

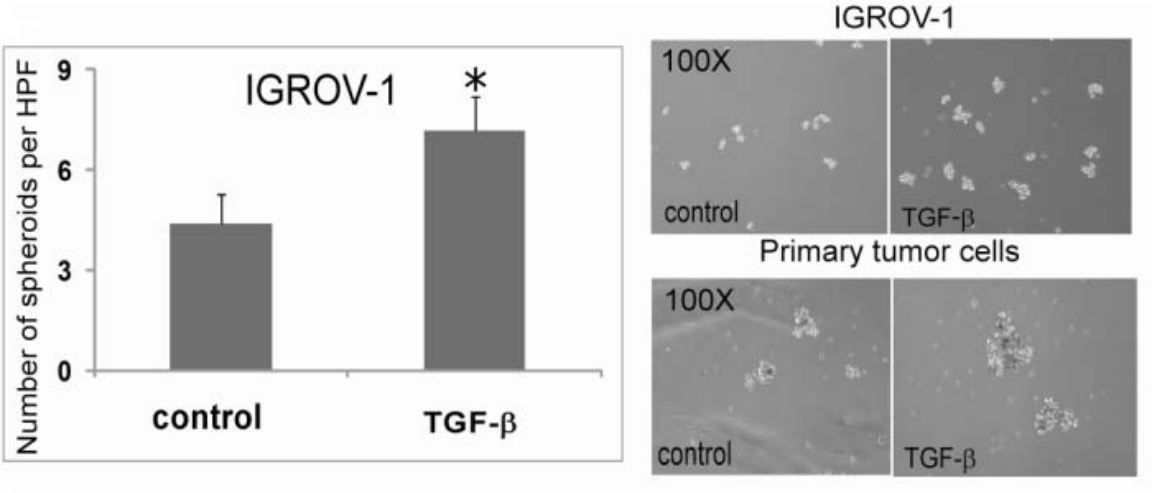
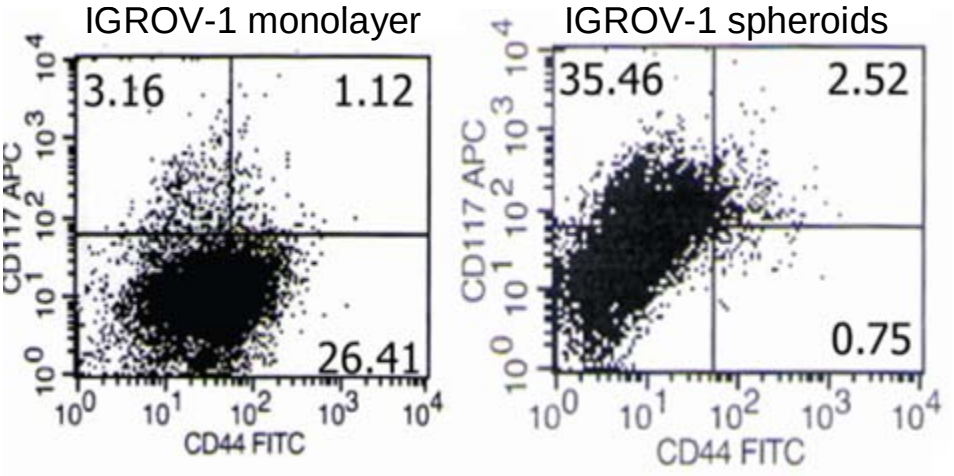


Figure 35: TGF-β1 induces spheroid formation of OC cells. *S*pheroid formation induced by TGF-β1 in IGROV-1 cells. Columns correspond to mean number of spheroids ± SEM (left). *P<0.05. Morphology of spheroids formed by IGROV-1 cells and primary OC cells treated with TGF-β1 (right, x100 magnification).

A.



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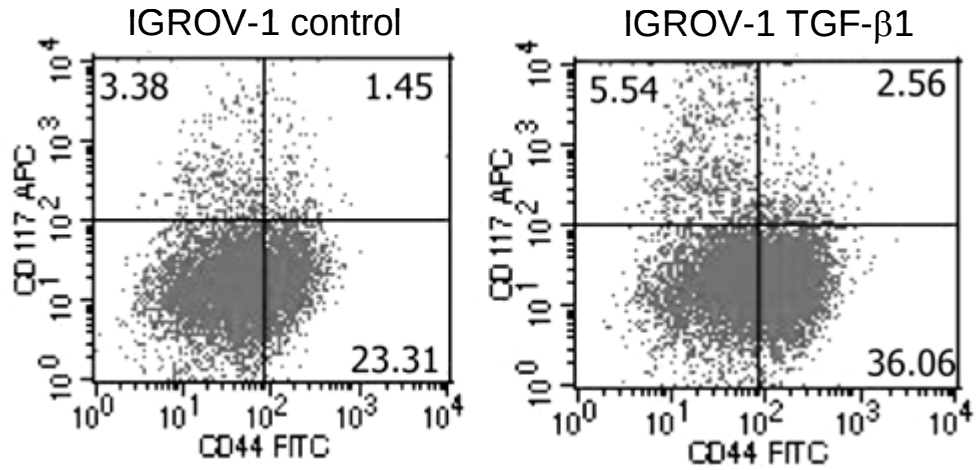


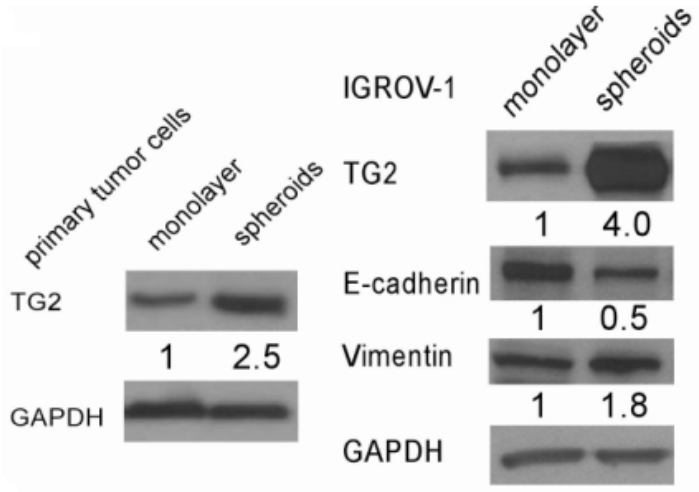
Figure 36: TGF-β1 induces an ovarian cancer stem cell phenotype. A. Flow cytometry quantifies CD44+/CD117+ population in IGROV-1 cells cultured as monolayer or spheroids. B. Flow cytometry quantifies CD44+/CD117+ population in IGROV-1 cells treated with and without TGF-β1 for 24 hours.

3.2.6. TG2 is upregulated in ovarian cancer stem cells

In summary, we have shown that TGF-β1 induces EMT and a stem cell phenotype in OC cells, which are crucial for OC metastasis. We also demonstrated that TGF-β1 upregulates TG2, which induces EMT in OC cells (data shown above) and promotes OC metastasis (Satpathy et al 2007). Next we investigated the relationship between TG2 expression level and OC stem cells. For this, we measured TG2 expression in OC spheroids and in CD44+/CD117+ OC cells. We found that cells growing as spheroids expressed higher TG2 levels and displayed mesenchymal characteristics (increased vimentin, decreased E-cadherin expression) compared to cells cultured as mono-layer (Figure 37A, left panel- primary OC cells, right panel- IGROV-1 cells).

To investigate relevance to human OC, we isolated CD44+/CD117+ cells from human tumors and measured TG2 mRNA expression levels in double staining cells, un-fragmented tumor, ascites, and double negative cells. Our results showed that TG2 expression level was increased in CD44+/CD117+ cells compared to whole tumor or cells isolated from ascites (Figure 37B, $P<0.05$) or to CD44-/CD117- cells dissociated and sorted from ovarian tumors (n=3, Figure 37C, $P<0.05$). These data suggest that TG2 expression level is increased in OC cells grown as spheroids and in the CD44+/CD117+ cell population.

A.



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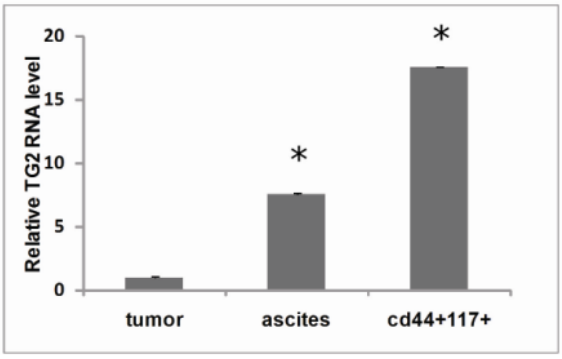

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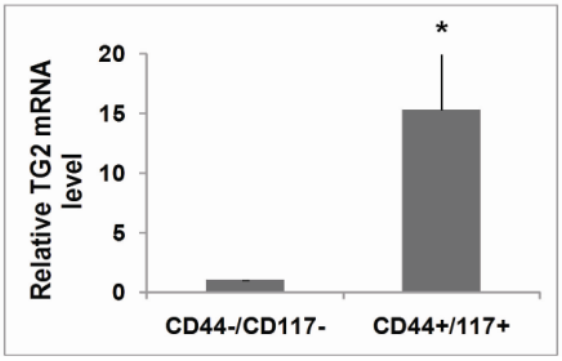


Figure 37: TG2 is upregulated in ovarian cancer stem cells. A, Western blotting for TG2, E-cadherin and vimentin in IGROV-1 (right) and primary OC cells (left) cultured as monolayer or spheroids. B, TG2 *mRNA* expression levels were measured by qRT-PCR in CD44+/CD117+cells from human ovarian tumor and compared with bulk tumor and cells isolated from ascites. C, Average TG2 expression levels measured by qRT-PCR in CD44+/CD117+ cells isolated from human ovarian tumors compared with CD44-/CD117- (n=3). *P<0.05.

3.2.7. TG2 induces an ovarian cancer stem cell phenotype

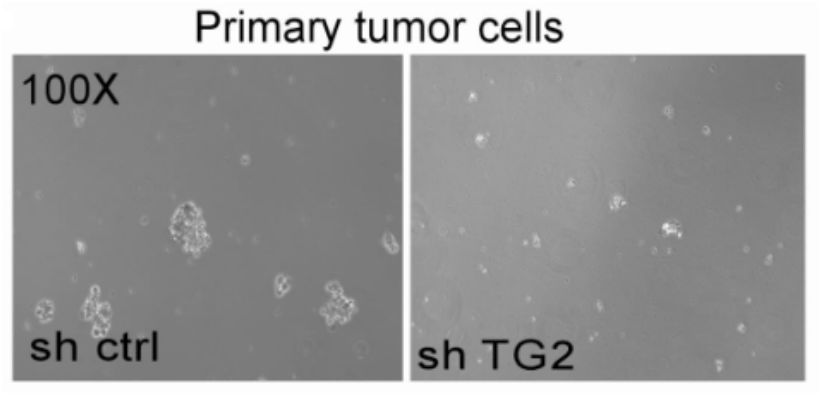
To test whether TG2 plays a role in spheroid formation, TG2 was knocked down by transducing in primary cancer cells dissociated from tumors (n=3) with shRNA targeting TG2 followed by sorting based on expression of stem cells markers (CD44+/CD117+), and by stable transfection of AS-TG2 in SKOV3 cells. We then measured spheroid formation by OC cells under stem cell condition. Our results showed that spheroid formation was blocked when TG2 is knocked down in both SKOV3 cells and human OC stem cells (Figure 38).

We next investigated whether TG2 alters the abundance of OCIC cell population by quantifying the percentage of CD44+/CD117+ cells in SKOV3 cells +/- TG2. Significantly fewer CD44+/CD117+ cells were identified in SKOV3-AS-TG2 (0.7%) compared to control cells (7.8%; Figure 39), suggesting that TG2 is important to this phenotype.

To further support the link between TG2 and cancer stem cells, the expression levels of stem cell specific transcription factors Nanog, Oct3/4, and Sox-2, were measured in cells +/- TG2. Increased levels of Nanog and Sox-2 were noted in SKOV3-control and OV90-TG2 cells compared to SKOV3-ASTG2 and OV90-vector, respectively (Figure 40A). Oct 3/4 expression level was increased in OV90-TG2 compared to control cells, but was not different in SKOV3 +/-TG2. Likewise, the Western blot results showed that STAT3, a transcription factor which is important for the maintenance of stem cell self-renewal and pluripotency, was activated in TG2 expressing SKOV3 pcDNA3.1 and OV90 tTG cells (Figure 40B). Furthermore, Western blot and IF results showed that β-catenin, the main effector of Wnt signaling pathway is induced in

TG2 expressing SKOV3 pcDNA3.1 cells compared with SKOV3 AS-TG2 cells (Figure 41). These data suggest that TG2 may promote OC stem cell phenotype by increasing the expression of stem cell specific transcription factors. Collectively these data support the concept that TG2 expression level correlates with an ovarian cancer stem cell phenotype.

A.



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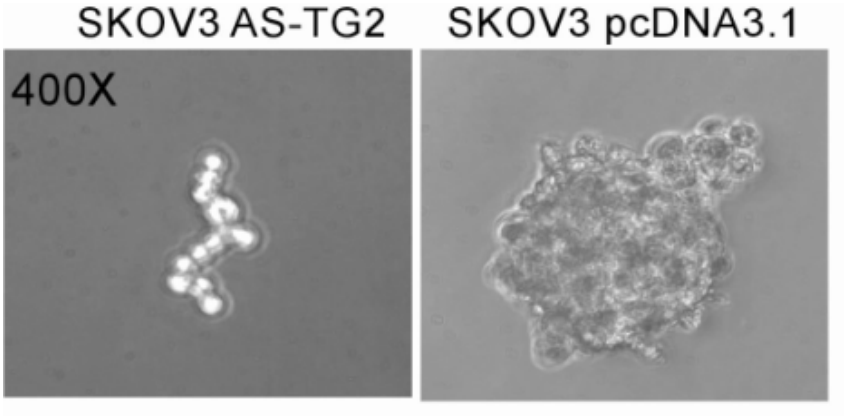
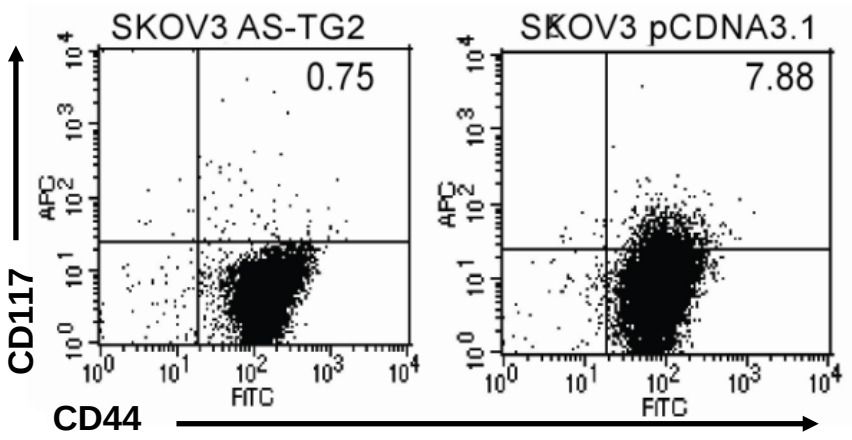


Figure 38: TG2 promotes spheroid formation of OC cells. Morphology of spheroids formed by CD44+/CD117+ cells from human OC transduced with short hairpin RNA (shRNA) targeting TG2 or control (A, × 100) or by SKOV3 AS-TG2 and pcDNA3.1 cells (B, × 400).

A.



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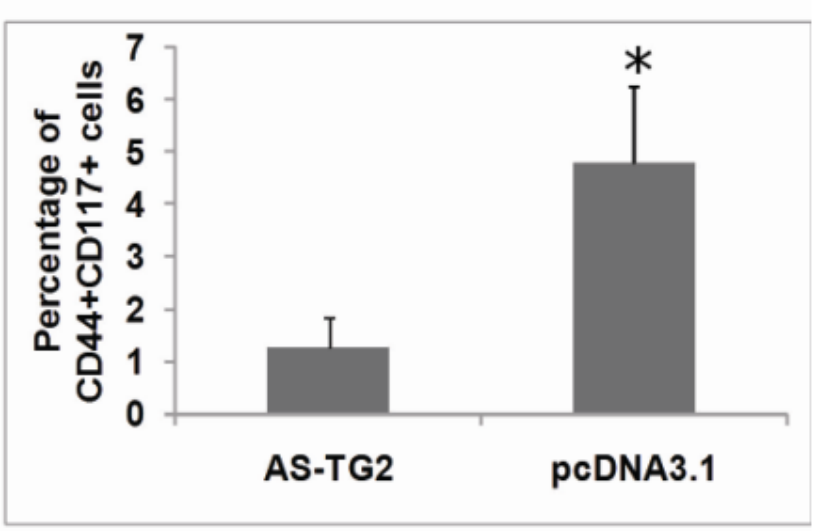
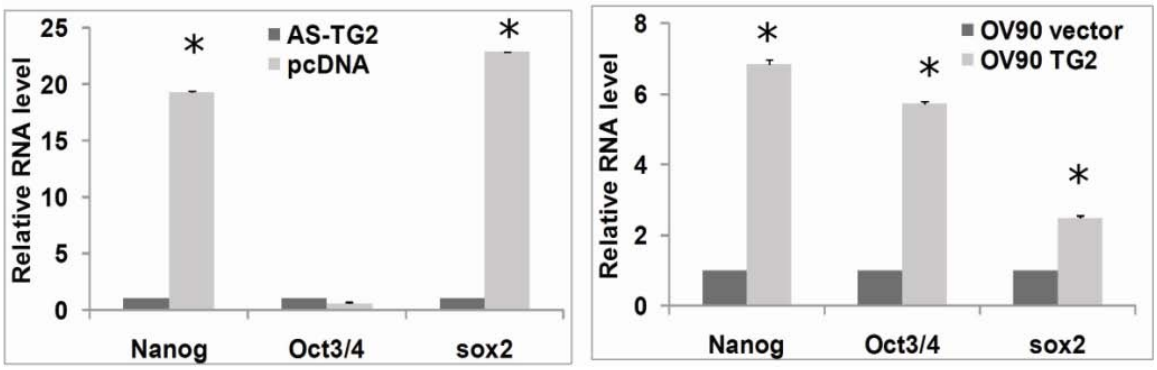


Figure 39: TG2 enriches CD44+CD117+ population. Flow cytometry quantifies CD44+/CD117+ cells in SKOV3 cells transfected with AS-TG2 or vector. Representative (A) and averages of four replicates (B) are shown. *P<0.05.

A.



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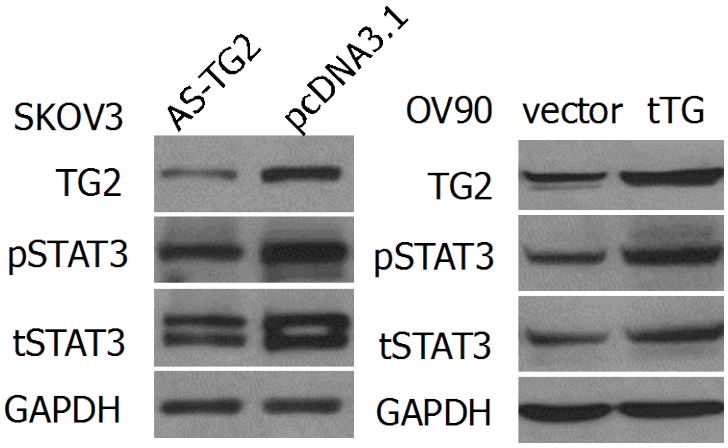


Figure 40: Stem cell specific transcription factors are upregulated in TG2 expressing cells. A. Quantitative PCR measured expression levels for Nanog, Oct3/4 and Sox 2 in SKOV3 AS-TG2 or control cells (left) and in OV90-TG2 or control cells (right). *P<0.05. B. TG2, phosphorylated STAT3, and total STAT3 were measured by Western blot in SKOV3 AS-TG2 or control cells (left) and in OV90 TG2 or control cells (right).

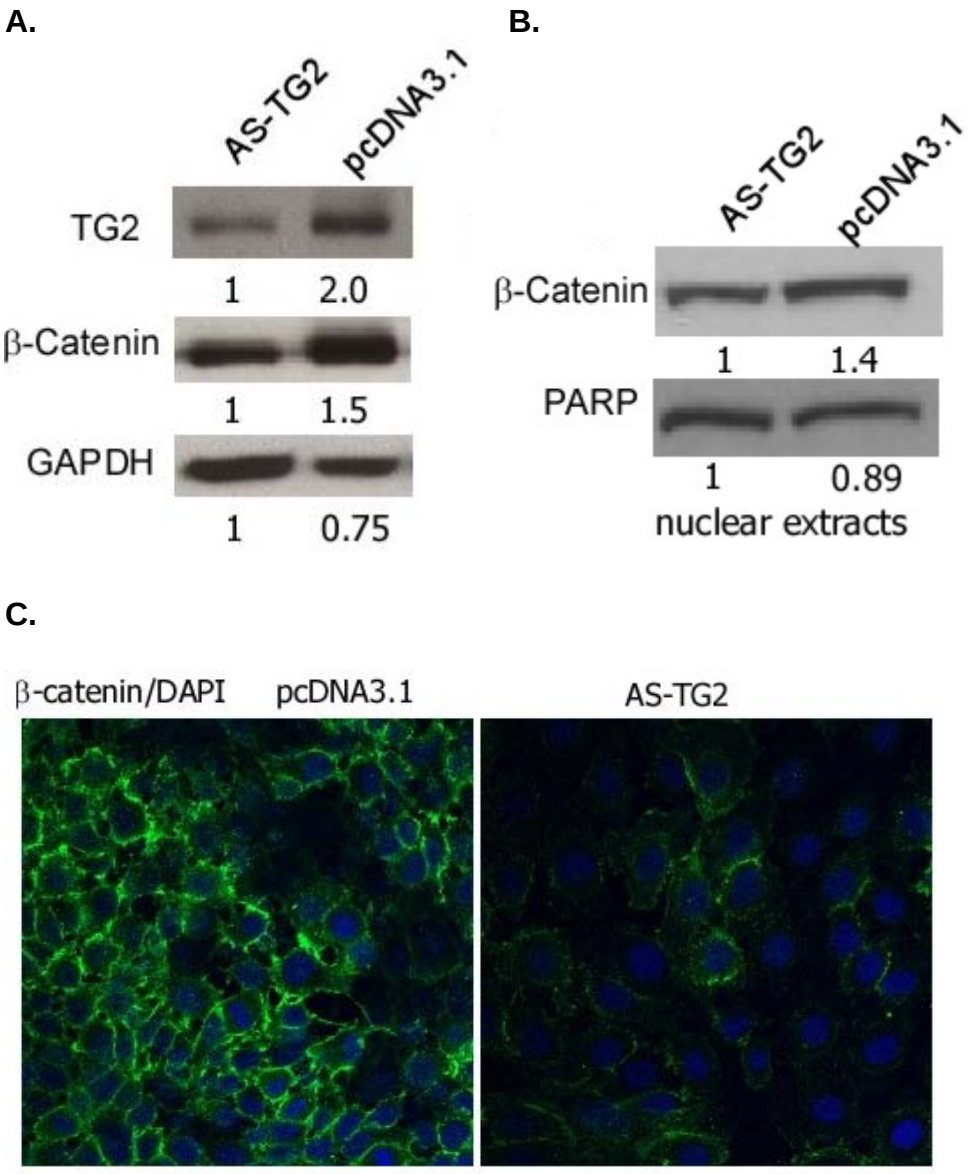


Figure 41: β-catenin is upregulated in TG2 expressing SKOV3 pcDNA3.1 cells compared with SKOV3 AS-TG2 cells. A. TG2 and β-catenin were detected by Western blot in SKOV3 pcDNA3.1 and AS-TG2 cells. B. β-catenin was detected by Western blot in nuclear extracts of SKOV3 pcDNA3.1 and AS-TG2 cells. C. β-catenin was detected by IF in SKOV3 pcDNA3.1 and AS-TG2 cells.

3.2.8. TG2 is required for TGF-β1 induced EMT, cancer stem cell phenotype and OC metastasis

To test the significance of TG2 to TGF-β1 induced EMT, stem cell phenotype and metastasis, we utilized SKOV3 cells stably transfected with an antisense construct (AS-TG2) or the empty vector pcDNA3.1. Induction of EMT by TGF-β1, as assessed by cell morphology (Figure 42A) or quantification of EMT markers (Figure 42B), was prevented in SKOV3-ASTG2 compared with control cells. This suggests that TG2 is an important downstream mediator for TGF-β induced EMT.

Next, we investigated whether TG2 is relevant to TGF-β1 induced OC stem cell phenotype. SKOV3 pcDNA3.1 and AS-TG2 cells were treated with TGF-β1, and CD44+/CD117+ OC stem cell population were measured by flow cytometry. Our results showed that TGF-β1 induced three-fold increase in the number of CD44+/CD117+ in SKOV3 pCDNA3.1 cells, but not in SKOV3 AS-TG2 cells (Figure 43). These data suggest that TG2 is needed for TGF-β1 induced cancer stem cell phenotype.

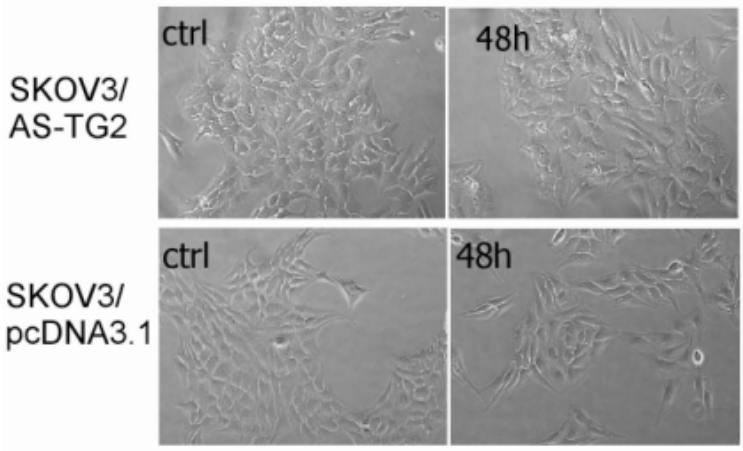
Then we used the TGF-β receptor I serine threonine kinase inhibitor, SD208 (Mohammad et al 2011) to block the TGF-β signaling pathway in SKOV3 pcDNA3.1 and AS-TG2 cells. TG2 induction and EMT markers were detected by Western blotting. Our results showed that SD208 blocked TGF-β-induced Smad 2/3 activation and TG2 induction in both SKOV3-ASTG2 and control cells (Figure 44). This confirms that TG2 is upregulated by TGF-β through Smad signaling pathway, and that this upregulation can be blocked by the TGF-β receptor

inhibitor. Treatment with SD208 also increased E-cadherin expression in SKOV3-ASTG2 cells, to a greater degree than in control cells. This suggests that the TGF-β receptor inhibitor can block TGF-β induced EMT in OC cells, and cells without TG2 expression are more sensitive to the TGF-β inhibitor treatment, further supporting the concept that TG2 is important in TGF-β induced EMT.

Next, we tested the effect of this inhibitor *in vivo*, in an intraperitoneal (i.p.) xenograft model in nude mice. We observed a significant difference between the number of peritoneal implants derived from control vs. AS-TG2 cells (142 vs. 383, P=0.006, Figure 45A), consistent with our previous observations (Satpathy et al 2007) and supporting TG2's role in regulating OC peritoneal dissemination. We also found that SD-208 blocked almost completely the formation of peritoneal metastases derived from SKOV3 AS-TG2 cells (16 vs. 142, P=0.006, Figure 45A), as compared with metastases derived from SKOV3 pCDNA3.1 cells (278 vs. 383, P=0.08, Figure 45A). These data suggest that SD208 not only inhibits EMT *in vitro*, but also blocks tumor metastasis *in vivo*. These data are also consistent with the *in vitro* finding that TG2 knockdown cells are more sensitive to the inhibitory effect of SD208, which confirms that TG2 is required for OC metastasis. To further confirm that TG2 is a TGF-β downstream effector required for OC metastasis, TG2 expression level was detected by Western blotting in tumor samples from SD208 treated and untreated SKOV3 pCDNA3.1 and AS-TG2 i.p. mice. Our results showed that TG2 expression level in AS-TG2 derived tumors treated with SD-208 was almost undetectable, whereas TG2 expression level was only modestly decreased in control tumors from animals treated with

SD208 (Figure 45B). Collectively, these data suggest that TG2 is required for TGF-β induced EMT, cancer stem cell phenotype *in vitro* and tumor metastasis *in vivo*.

A.



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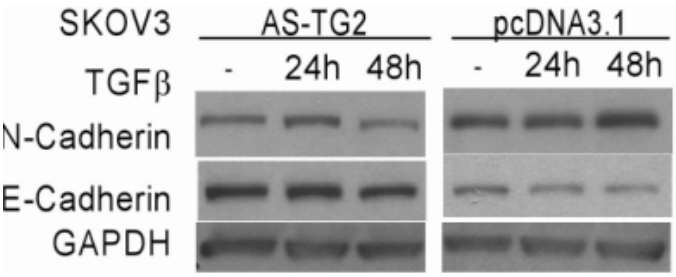


Figure 42: TG2 is required for TGF-β1 induced EMT in OC cells. A. Cellular morphological change in SKOV3 pcDNA3.1 and AS-TG2 cells after TGF-β1 treatment (x100 magnification). B. EMT markers E-cadherin and N-cadherin were detected by Western blot in SKOV3 pcDNA3.1 and AS-TG2 cells after TGF-β1 treatment.

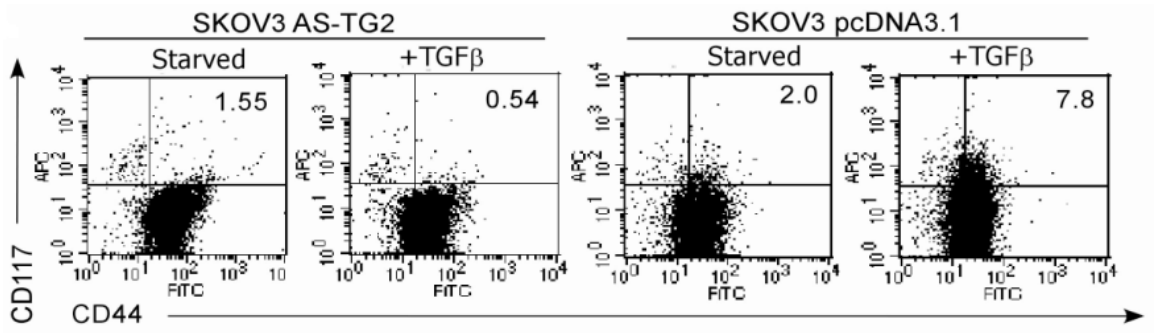


Figure 43: TG2 is required for TGF-β1 induced ovarian cancer stem cell phenotype. Flow cytometry quantifies CD44+/CD117+ population in SKOV3 AS-TG2 and pcDNA3.1 cells treated with TGF-β1 for 24 hours.

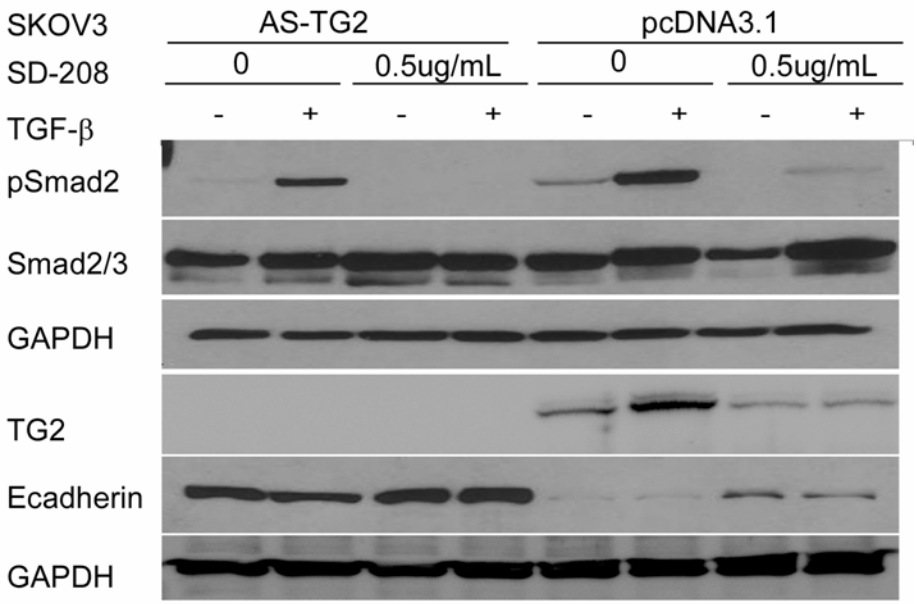
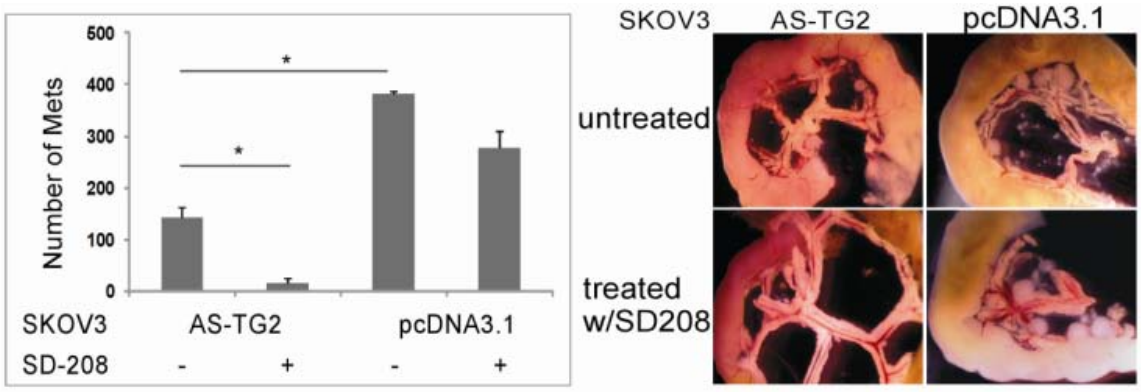


Figure 44: TGF-β receptor I kinase inhibitor, SD208, blocked TG2 upregulation and EMT in OC cells. SKOV3 AS-TG2 and control cells were pre-incubated with SD208 (0.5 μg/ml) or vehicle for 1h before treatment with TGF-β1 (5 ng/ml). Activation of Smad3 and total Smad3 were detected by western blotting after treatment with TGF-β1 for 30 minutes. E-cadherin and TG2 expression level were detected by western blotting after treatment with TGF-β1 for 48 hours.

A.



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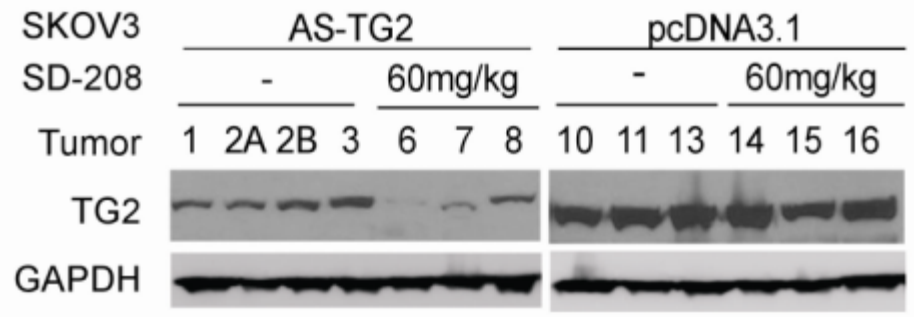


Figure 45: TGF-β receptor I kinase inhibitor, SD208, blocked OC metastasis *in vivo*. A. Left, average number of peritoneal implants ± SD in mice injected with SKOV3 pcDNA3.1 or AS-TG2 cells and treated with SD208 or control (*n*=4 per group). **P*<0.05. Right, representative pictures of peritoneal implants on mesentery adjacent to the bowel in the four experimental groups. B. Western blotting measured TG2 expression level in xenograft tumors from the four experimental groups.

3.3 TG2 induces chemoresistance in OC cells

3.3.1 TG2 mediates response to cisplatin in OC cells

One important property of cancer stem cells is chemoresistance. Cisplatin, a DNA cross-linking agent, is the first line chemotherapy for OC (Armstrong et al 2006). Given TG2's role in maintenance of stem cell homeostasis in OC cells, we next investigated whether TG2 plays a role in response to cisplatin. For this, SKOV3 cells stably transfected with AS-TG2 and control vector (pcDNA3.1) were treated with varying concentrations of cisplatin for 24 h. To avoid potential clonal bias, the effects of cisplatin were measured in two different clones expressing low TG2 levels (clone G and M, Figure 46A). Knockdown of TG2 sensitized cells to cisplatin, with a 2-fold decrease in the inhibitory concentration (IC50), from 5 μM in control cells to 2.5 μM in AS-TG2 cells (Figure 46B). Results were confirmed by BrdU assay (data not shown).

To mimic the interaction with the extracellular matrix, cells were plated on fibronectin and treated with cisplatin. Similar results were obtained, the IC50 being decreased from >5 μM in controls to 2.5 μM in AS-TG2 cells and data were verified by BrdU assay (Figure 46C). This was accompanied by a corresponding increase in apoptotic or necrotic cells, as counted by Trypan blue exclusion, with >10-fold increase in the number of dead cells after exposure to cisplatin in AS-TG2 cells versus controls (Figure 46D).

We next assessed the effects of cisplatin on the clonogenic potential of SKOV3 cells transfected with vector or with AS-TG2. Clonogenic assay, also called colony formation assay, is an *in vitro* assay to test cell survival based on

the ability of a single cell to grow into a colony. It is used to determine the effects of cytotoxic agents on tumor cell proliferation (Franken et al 2006). Our results showed that colony formation by cells with low TG2 expression was suppressed more significantly by low concentrations of cisplatin (1 and 2 μM) compared with control cells (Figure 47). Along with the results of the proliferation assays, these observations suggest that OC cells in which TG2 was knocked down are more sensitive to cisplatin treatment.

Next, we tested whether stable overexpression of TG2 in OC cells decreased sensitivity to cisplatin. For this, an ovarian cancer cell line with low endogenous expression of TG2 (OV90) was transfected with human full-length TG2. A stably transfected clone was identified by immunoblotting after selection with G418 (Figure 48A). OV90 cells with and without TG2 expression were treated with various concentrations of cisplatin, and MTT assay was performed to detect the effect of cisplatin on these cells. Our results showed that stable expression of TG2 increased resistance to cisplatin compared with cells transfected with empty vector (pcDNA3.1), with a 2-fold increase in IC50 from 5 μM in control cells to 10 μM in cells overexpressing TG2 (Figure 48B). These data suggest that TG2 induces ovarian cancer cells to become resistant to cisplatin treatment. Together with the effects of TG2 knockdown, these results indicate that TG2 is implicated in ovarian cancer cells' response to cisplatin.

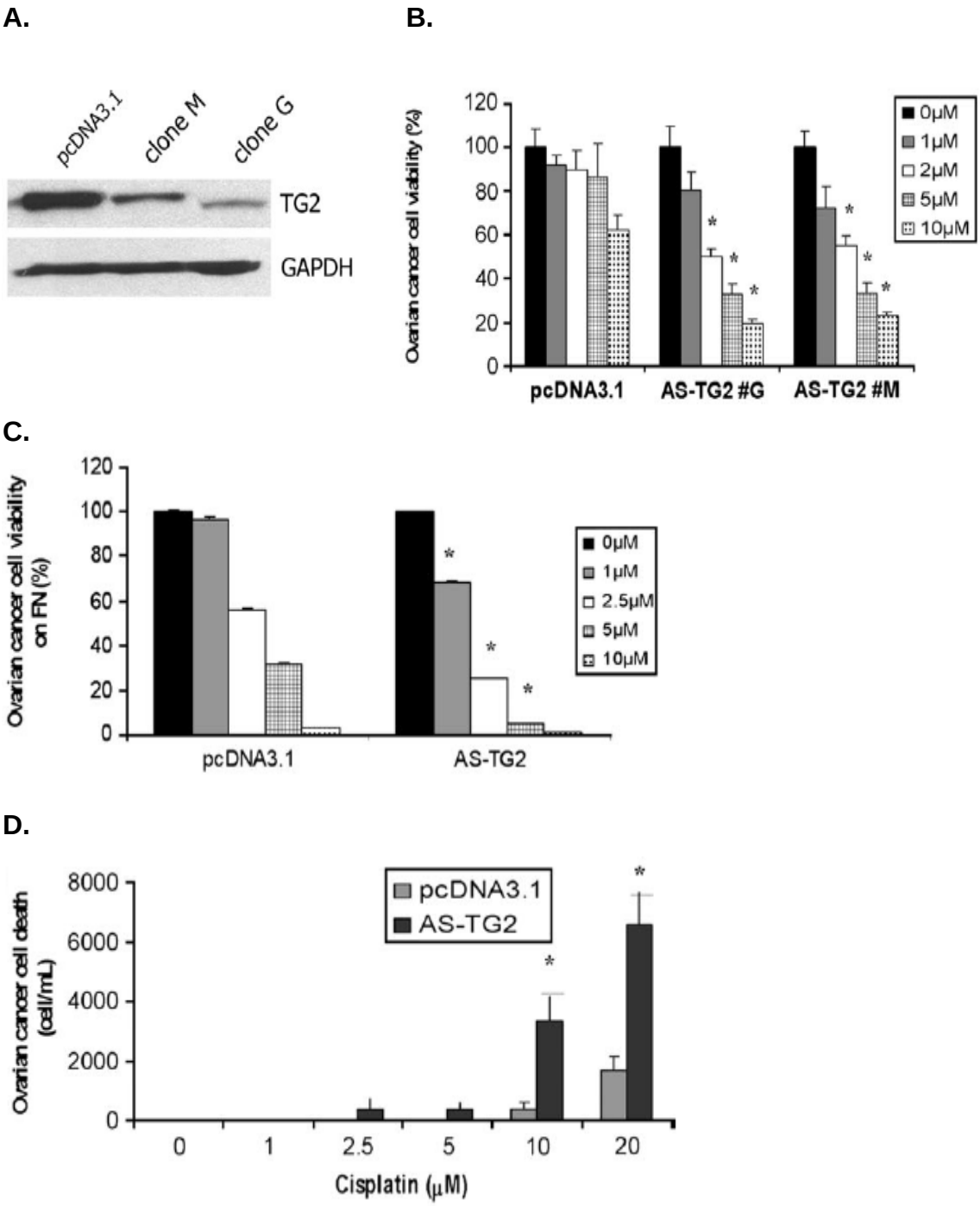


Figure 46: TG2 knockdown in SKOV3 OC cells enhances sensitivity to cisplatin.

Figure 46: TG2 knockdown in SKOV3 OC cells enhances sensitivity to cisplatin. A. TG2 was knocked down using stable transfection with an antisense (AS-TG2) construct cloned into pcDNA3.1. Western blot demonstrates TG2 levels in SKOV3 cells stably transfected with pcDNA3.1 vector (control) and with AS-TG2 (clones #G and #M). B. Effects of cisplatin on cell proliferation were assessed by MTT assay. SKOV3 cells transfected with pcDNA3.1 (control) and two AS-TG2 stable clones (#G and #M) were treated with different concentrations of cisplatin for 48 h. C. Effects of cisplatin on cell proliferation in SKOV3 cells transfected with pcDNA3.1 (control) and AS-TG2 cells being plated on the matrix protein, fibronectin (FN). D. Number of floating cells after 48 h treatment with cisplatin in AS-TG2 versus control cells. *$P < 0.05$.

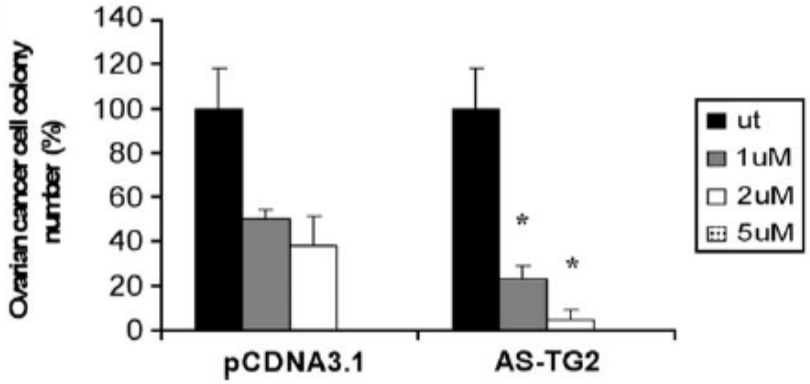
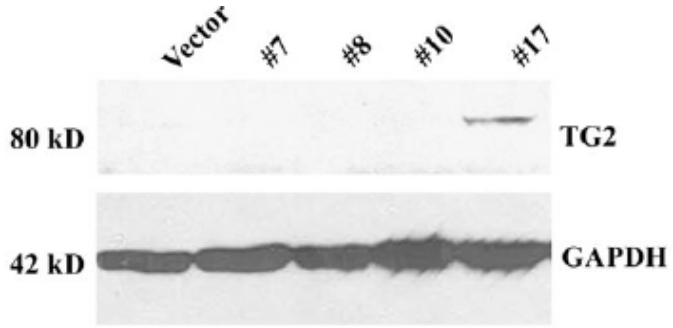


Figure 47: Knock down of TG2 in SKOV3 cells induces response to cisplatin. Clonogenic assay assessed colony formation after 48 h exposure to cisplatin in SKOV3 cells transfected with AS-TG2 or pcDNA3.1. Data represent averages of triplicate experiments ± SEM. *P< 0.05.

A.



B.

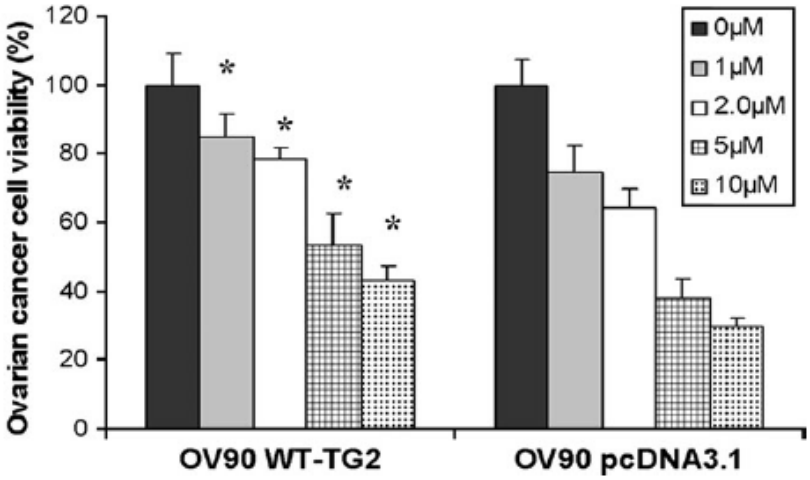


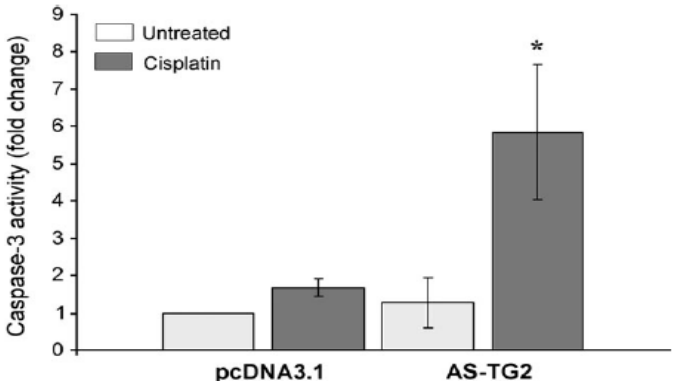
Figure 48: Overexpression of TG2 in OC cells induces resistance to cisplatin. A. Wild-type (WT) TG2 was transfected in OV90 ovarian cells. Stable transfection were selected based on resistance to G418. Western blot shows levels of TG2 in several clones (clone #17 is positive) and in vector-transfected cells (controls). B. OV90 cells transfected with pcDNA3.1 (control) and WT-TG2 were treated with different concentrations of cisplatin for 48 hours, and the effects of cisplatin were measured by MTT assay. Data represent averages of triplicate experiments ± SEM. *$P < 0.05$.

3.3.2 TG2 protects against apoptosis induced by cisplatin in EOC cells

Cisplatin induces apoptosis in EOC cells by activating the mitochondrial machinery (Henkels and Turchi 1997). Having noticed a remarkable increased number of detached cells in SKOV3 AS-TG2 cells exposed to cisplatin compared with controls (Figure 44D), we measured apoptosis induced by cisplatin. Apoptosis was first determined by measuring the activity of caspase-3 in OC cells after cisplatin treatment in SKOV3 pcDNA3.1 and AS-TG2 cells versus untreated cells as controls. We observed increased activation of caspase-3 in SKOV3 AS-TG2 cells treated with cisplatin compared with untreated control and with cisplatin-treated SKOV3 pcDNA3.1 cells (Figure 49A).

Next, we measured apoptosis in SKOV3 pcDNA3.1 and AS-TG2 cells via TdT-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. Our results showed that the percentage of apoptotic cells after exposure to cisplatin was 5-fold higher in cells stably transfected with AS-TG2 compared with control. Cisplatin increased the percentage of TdT-positive cells from 1% in controls to 9% in AS-TG2 cells (Figure 49B). These results suggest that apoptosis induced by cisplatin is enhanced in cells with decreased TG2 expression. We also measured cleavage of pro-caspase-9, degradation of the X-linked inhibitor of apoptosis protein (XIAP) and cytoplasmic release of cytochrome c after cisplatin treatment and found them to be more pronounced in AS-TG2 cells compared with controls after cisplatin treatment (Figure 49C), suggesting that TG2 exerts an anti-apoptotic role in EOC cells exposed to cisplatin.

A.



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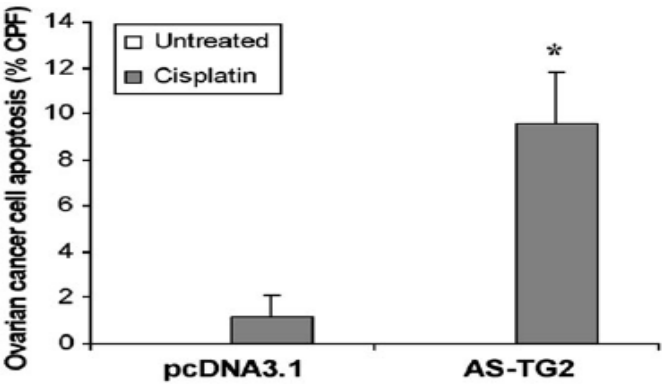


C.

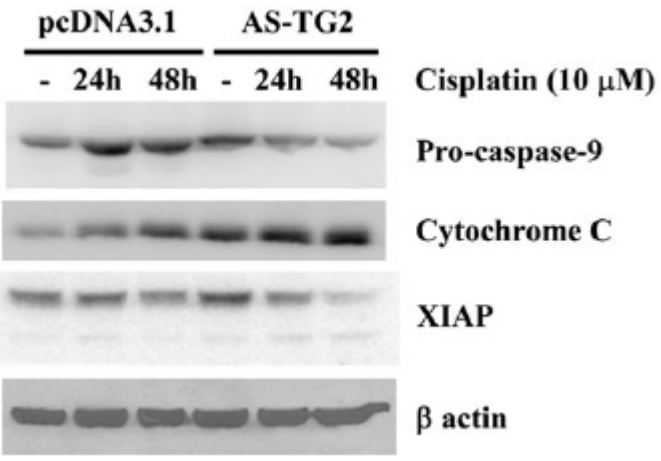


Figure 49: TG2 protects OC cells against cisplatin-induced apoptosis.

Figure 49: TG2 protects OC cells against cisplatin-induced apoptosis. A. Activation of caspase-3 was measured by a fluorometric assay. SKOV3 cells stably transfected with pcDNA3.1 and AS-TG2 were treated with 10 μM cisplatin for 24 h. Data represent averages of two independent experiments performed in duplicate ± SEM. (B) Percentage of apoptotic cells after treatment with cisplatin was assessed by TdT-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. SKOV3 cells stably transfected with AS-TG2 or vector were treated with 10 μM cisplatin for 24 h. Percentage of TUNEL-positive cells was quantified by counting of 10 microscopic fields (x200) per specimen. Data represent average of duplicate experiments ± SEM. All counts were obtained in duplicate. The number of TdT-positive cells in wells not treated with cisplatin was close to zero. C. Cisplatininduced apoptosis was measured by western blotting using antibodies for pro-caspase-9, cytochrome c and XIAP. β-Actin was used as a control. SKOV3 cells stably transfected with pcDNA3.1 and AS-TG2 were treated with cisplatin (10 μM) for 24 and 48 h. *P< 0.05. Experiments performed in collaboration with Dr. Daniela Petrusca.

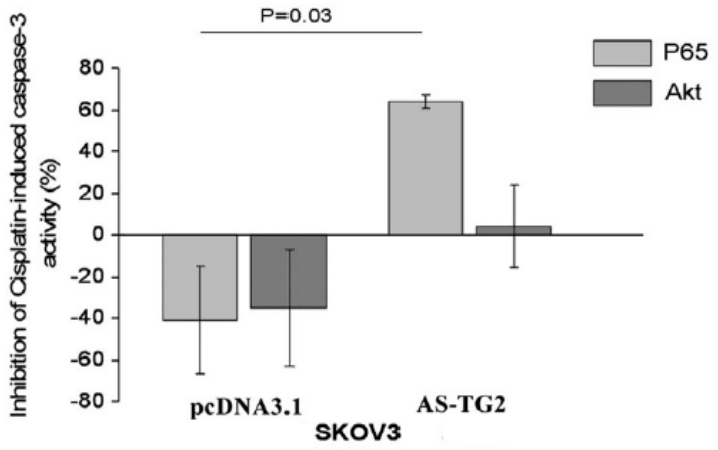
3.3.3 TG2 protects EOC cells from cisplatin-induced apoptosis through activation of NF-κB signaling pathway

To understand the mechanism by which TG2 exerts its anti-apoptotic role OC cells, we examined the effects of Akt and NF-κB, two survival pathways commonly involved in development of drug resistance in EOC. For this, we overexpressed a constitutively active form of Akt (CA-Akt) and the p65 subunit of NF-κB in SKOV3 AS-TG2 and pcDNA3.1 cells and examined cisplatin-induced apoptosis in these cells. There was no statistically significant difference between cisplatin-induced caspase-3 activity in SKOV3 pcDNA3.1 cells transduced with empty vector and cells transduced with p65 (Figure 50A). Similarly, Akt overexpression failed to inhibit cisplatin-induced apoptosis in these SKOV3 pcDNA3.1 cells. In contrast, cisplatin-induced caspase-3 activation was rescued by overexpressing constitutively active p65, but not by CA-Akt in AS-TG2 cells (Figure 50A), suggesting that the anti-apoptotic effect of TG2 was exerted mainly through activation of NF-κB.

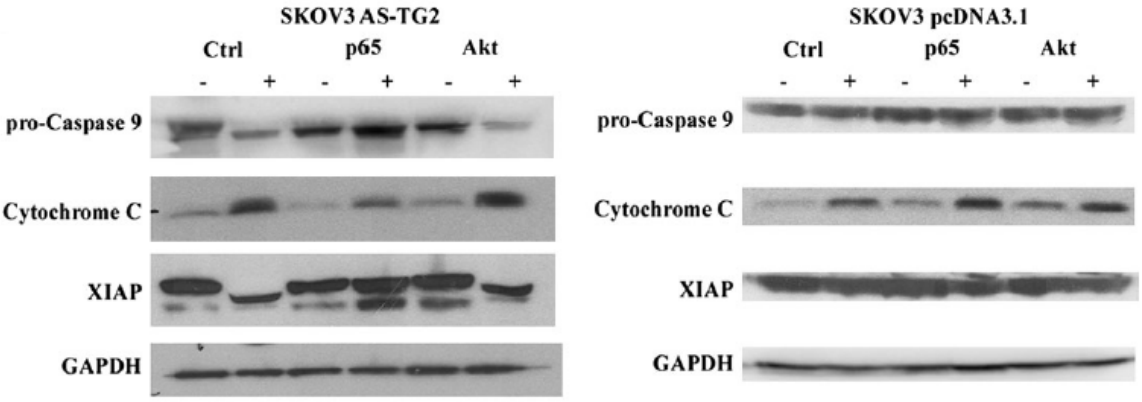
Knowing that cisplatin-induced apoptosis proceeds primarily through activation of the mitochondrial pathway, we examined the effects of cisplatin on the mediators of this intrinsic apoptotic pathway. We found that cisplatin-induced activation of caspase-9 in AS-TG2 cells was significantly inhibited by overexpression of p65, but to a lesser extent by CA-Akt compared with vector-transfected cells (Figure 50B). These data support NF-κB as the major mediator in TG2 induced anti-apoptotic effect.

Next we investigated by western blot the level of expression of other markers of apoptosis induced by cisplatin (pro-caspase-9, cytochrome *c* and XIAP) in SKOV3 AS-TG2 and pcDNA3.1 cells transduced with vector, p65 or CA-Akt. Overexpression of constitutively active p65 subunit prevented cleavage of pro-caspase-9 and XIAP in AS-TG2 cells treated with cisplatin (Figure 50C, left panel). Partial cleavage of XIAP after exposure to cisplatin was still detected in AS-TG2 cells transduced with p65, however, to lesser degree than in AS-TG2 cells transduced with empty vector. Cisplatin-induced cytochrome c release was also decreased by overexpression of p65 in AS-TG2 cells. These findings support the previous observations that p65 rescues AS-TG2 ovarian cancer cells from cisplatin-induced apoptosis. In contrast, CA-Akt did not affect cisplatin-induced cleavage of pro-caspase-9 or XIAP and release of cytochrome c in AS-TG2 cells, suggesting that Akt does not play a major role in TG2-mediated ovarian cancer cells' resistance to cisplatin. Interestingly, in control cells, expressing intact levels of TG2, cleavage of pro-caspase-9 or XIAP was not observed after 48h exposure to cisplatin, showing a cisplatin-resistant phenotype. Overexpression of CA-Akt or p65 did not significantly alter basal or cisplatin-induced pro-caspase-9 or XIAP levels in control cells. Collectively, these data suggest that TG2 induces cisplatin resistance via NF-κB signaling pathway.

A.



B.



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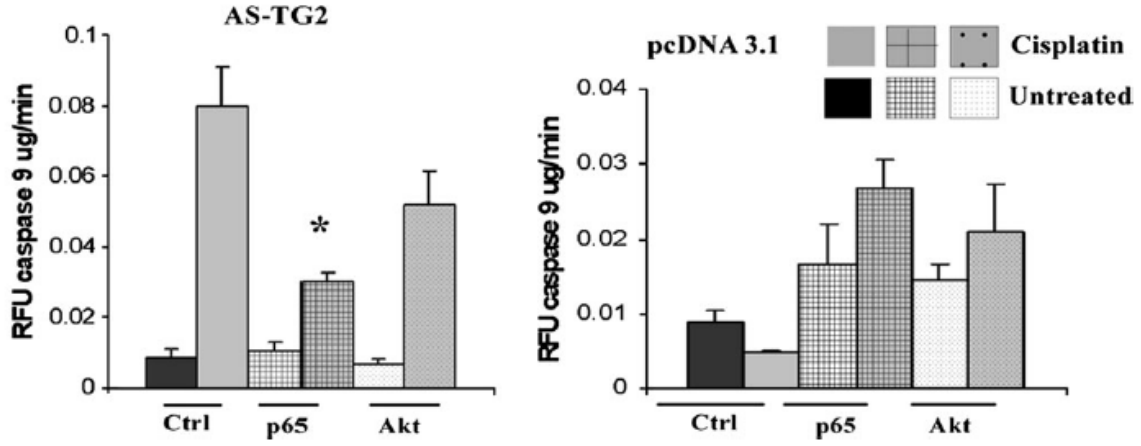

Figure 50: Reconstitution of NF-κB, but not of Akt, restores resistance to cisplatin in AS-TG2 cells.

Figure 50: Reconstitution of NF-κB, but not of Akt, restores resistance to cisplatin in AS-TG2 cells. SKOV3 cells transfected with AS-TG2 or pcDNA3.1 were transduced with constitutively active p65, CA-Akt or vector (pQCXIP) and treated with cisplatin. A. Caspase-3 activity was measured by fluorometric assay at 24 h after cisplatin treatment. The results are expressed as inhibitory potency (mean % inhibition ± SEM; n=2) of constitutively active p65 or CA-Akt compared with empty vector on the caspase-3 activity induced by cisplatin treatment. Note the potent inhibitory effect of p65 overexpression on the cisplatin-induced caspase-3 activity in cells transfected with AS-TG2. All other treatments failed to significantly inhibit (or further activate) cisplatin-induced caspase-3 in either control vector- or ASTG2-expressing cells. B. Apoptosis was assessed by western blotting of cytochrome c, pro-caspase-9 and XIAP after 48 hours of cisplatin treatment. β-Actin was used as a control. C. Caspase-9 activation was measured using a fluorometric assay in pcDNA3.1 or AS-TG2 cells transduced with constitutively active p65, CA-Akt and vector control (pQCXIP) and treated with cisplatin for 24 h. Data are expressed as mean fold increase versus control caspase-9 activity (measured as fluorescent units per minute per milligram protein) ± SEM (n = 3). Note that only p65-overexpressing AS-TG2 cells failed to activate caspase-9 in response to cisplatin treatment. *P< 0.05. Experiments performed in collaboration with Dr. Daniela Petrusca.

3.3.4 TG2 regulates NF-κB activity in EOC cells

Having noticed that p65 rescued cisplatin-induced apoptosis in EOC cells to greater extent than CA-Akt, we next examined the function of the Akt and NF-κB pathways in EOC cells in the presence or absence of functional TG2. For this, we compared levels of phosphorylated Akt in SKOV3 cells stably transfected with AS-TG2 or pcDNA3.1 in basal conditions. We found decreased activation of Akt in AS-TG2 cells compared with controls as measured by decreased phosphorylation at Ser^{473}. This occurred in parallel to decreased phosphorylation of the focal adhesion kinase (FAK) in AS-TG2 cells compared with control cells (Figure 51A).

To see if this phenomenon depends on TG2 enzymatic activity or not, we used a chemical inhibitor of TG2 KCC009 to test its effect on Akt, FAK and NF-κB activity. Interestingly, inhibition of TG2 enzymatic activity by the TG2 inhibitor KCC009 did not affect activation of Akt, suggesting that the effects of TG2 on this survival pathway may be independent of its enzymatic function (Figure 51B). However, FAK phosphorylation was modestly reduced after treatment with KCC009, suggesting that TG2 enzymatic activity may be needed for focal adhesion.

Next, we measured the effects of TG2 expression and enzymatic function on NF-κB activity. Using a reporter assay, we found that the activity of the NF-κB complex was decreased 2-fold in cells stably transfected with AS-TG2 compared with controls (Figure 51C). Likewise, inhibition of TG2 enzymatic function by KCC009 led to decreased NF-κB activity in SKOV3 cells (Figure 51F). This

inhibition of the NF-κB promoter activity was paralleled by a corresponding increase in expression levels of inhibitory IκBα level in cells transfected with AS-TG2 versus controls (Figure 51D). The expression level of IκBα was also increased by treatment with the TG2 enzyme inhibitor KCC009 (Figure 51E). This suggested that the expression and the enzymatic function of TG2 are essential in regulating the expression level of the inhibitory unit IκBα and subsequently the activity of the NF-κB complex.

To investigate whether these observations have therapeutic relevance, we measured the effects of TG2 inhibitor KCC009 on OC cell proliferation. The TG2 inhibitor KCC009 had anti-proliferative effects in SKOV3 cells, with an IC50 of <250 μM (Figure 51G). This effect may be partly explained by the inhibition of the NF-κB survival pathway, but other contributory mechanisms cannot be excluded. These results suggested that inhibition of TG2 may serve as a novel modality to enhance sensitivity of ovarian cancer cells to cisplatin. We therefore tested whether the combination of KCC009 and cisplatin exerts synergistic effects in SKOV3 cell proliferation. Measurement of growth inhibition indices after treatment with each agent and with the two-drug combination led to a calculated combination index (CI) of 0.89 (Figure 51H), using the median-effect principle of Chou et al (Chou and Talalay 1984). This CI value indicates a modest synergistic effect between cisplatin and the TG2 inhibitor.

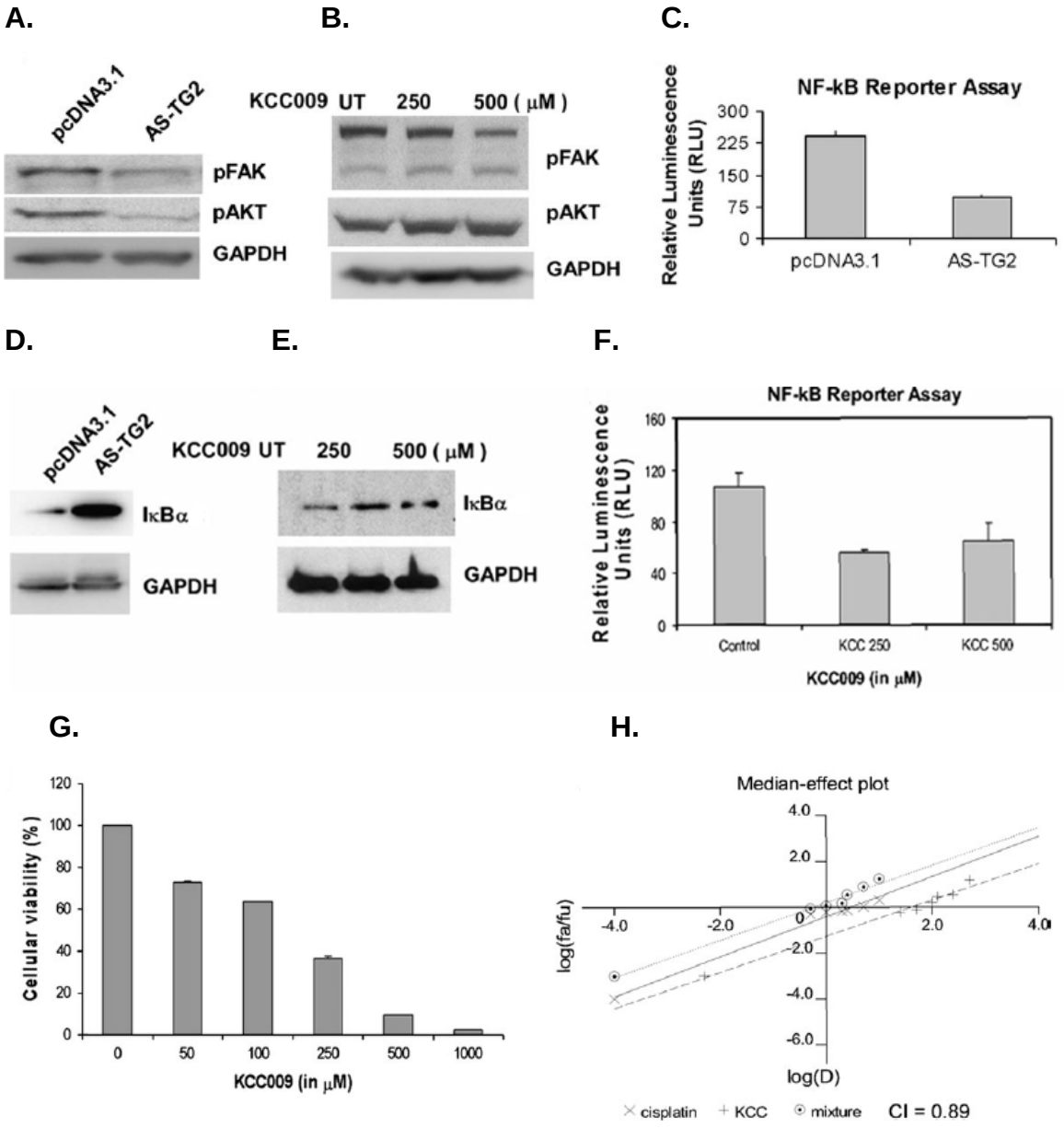

Figure 51: TG2 modulates Akt and NF-κB activity in OC cells and TG2 inhibitors sensitize OC cells to cisplatin.

Figure 51: TG2 modulates Akt and NF-κB activity in OC cells and TG2 inhibitors sensitize OC cells to cisplatin. A. Western blot for Ser473Akt, phosphorylated FAK and GAPDH in SKOV3 cells stably transfected with AS-TG2 and vector. B. Western blot for Ser473Akt, phosphorylated FAK and GAPDH in SKOV3 cells treated with KCC009 for 24 h at a concentration of 250 and 500 μM. C. NF-κB promoter activity measured by a reporter assay in SKOV3 cells stably transfected with AS-TG2 and vector. D. Western blot for IκBα in SKOV3 cells stably transfected with AS-TG2 and vector. E. Western blot for IκBα in SKOV3 cells treated with KCC009 for 24 h at a concentration of 250 and 500 μM. F. NF-κB promoter activity measured by a reporter assay in SKOV3 cells treated with the TG2 inhibitor KCC009 at a concentration of 250 and 500 μM. G. Effects of KCC009 on ovarian cancer cell proliferation were measured by MTT assay. Data represent average of triplicate experiments ± SEM. H. KCC009 has synergistic effects when combined with cisplatin (CI=0.89) in SKOV3 cells. Cell proliferation was measured by MTT assay after 48 h treatment with cisplatin, KCC009 and the combination of two drugs and the CI was measured based on the median-effect principle of Chou et al. The median-effect plot was analyzed using Calcusyn.

CHAPTER 4: DISCUSSION

4.1 Summary of results

In this study we showed that TGF-β1 secreted in the OC milieu regulates the expression and function of TG2 in OC cells but not in normal ovarian surface epithelial cells. This regulation proceeds primarily through the canonical Smad signaling pathway, which can be blocked by a TGF-β neutralizing antibody, a TGF-β receptor kinase inhibitor, or siRNA targeting the Smad family of transcription factors. Additionally, non-Smad signaling (TAK1 and NF-κB) is also involved in TG2 upregulation by TGF-β1. TGF-β induced TG2 promotes EMT by negatively regulating E-cadherin expression. More specifically, TG2 induces E-cadherin transcription suppressor Zeb1 by activating NF-κB signaling pathway. This leads to increased cell invasiveness *in vitro* and tumor metastasis *in vivo*. The N-terminal FN-binding domain of TG2 (tTG 1-140), lacking both enzymatic and GTPase function, induced EMT in OC cells. Increased TG2 expression also correlates with increased spheroid formation, enriched OCICs population, and enhanced expression of the stem cell-specific transcription factors Nanog, Oct3/4, Sox-2, and STAT3. A TGF-β receptor kinase inhibitor SD-208 blocked OC peritoneal metastasis in a manner consistent with suppression of TG2 expression levels. Further, we showed that TG2 protects ovarian cancer cells from cisplatin-induced apoptosis by regulating NF-κB activity. Therefore, we propose a model whereby TGF-β-induced TG2 modulates EMT, metastasis, OCIC homeostasis and chemoresistance (Figure 52). These findings contribute to a better understanding of the mechanisms of OC metastasis modulated by TG2 and point to

potential therapeutic interventions targeting TG2 or TGF-β signaling to interrupt OC tumor progression.

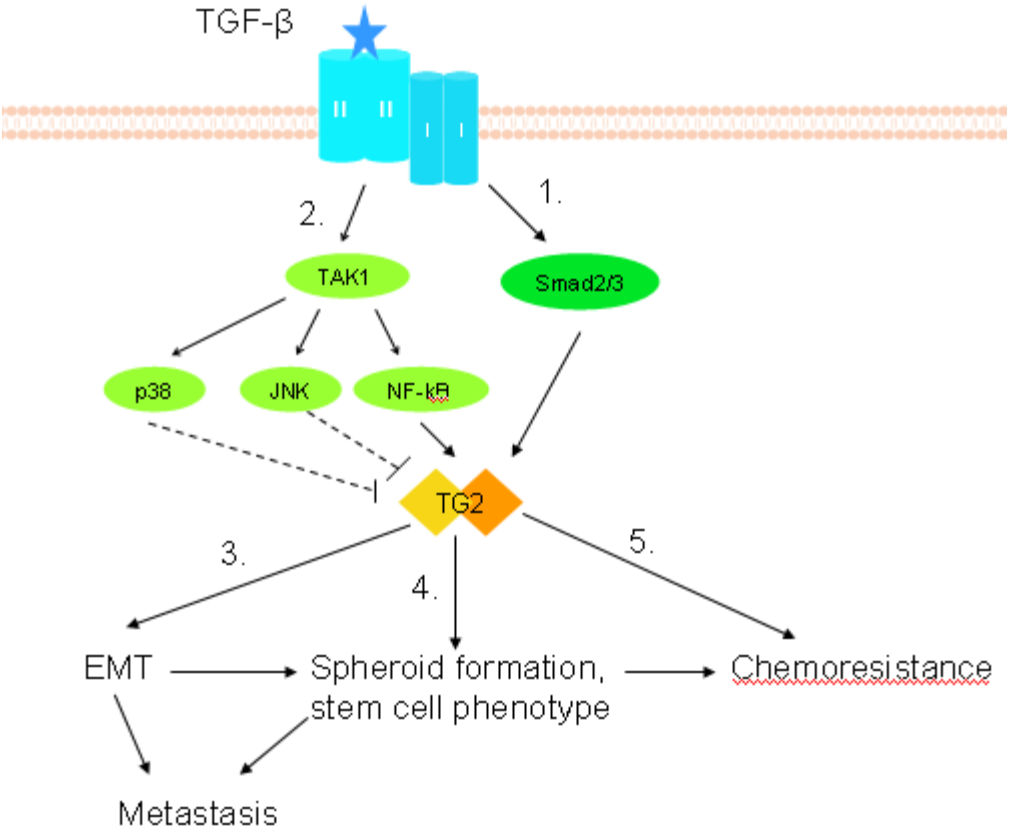

Figure 52: Proposed mechanisms by which TGF-β-induced TG2 regulates EMT, spheroid formation, metastasis and chemoresistance. 1. TGF-β1 activates Smad-dependent signaling pathway to upregulate TG2 expression in OC cells. 2. TGF-β1 activates TGF-β-activated kinase 1 (TAK1), which activates downstream NF-κB to upregulate TG2 expression in OC cells. The other two TAK1 downstream signaling, p38 and JNK, might be involved in inhibition of TG2 upregulation by TGF-β1 in OC cells. 3. By modulating E-cadherin transcription repressor Zeb1, overexpressed TG2 in OC cells induces EMT to promote tumor metastasis. 4. Overexpressed TG2 in OC cells induces spheroid formation and a stem cell phenotype. 5. Overexpressed TG2 protects OC cells from cisplatin-induced apoptosis by regulating NF-κB activity.

4.2 TGF-β1 induces TG2 overexpression in OC cells

First, we demonstrated that TGF-β1 is secreted in the OC milieu in an autocrine manner. TGF-β1 is secreted at higher concentrations by OC cells compared to non-transformed NOSE cells (Figure 8A), and similarly, higher TGF-β1 levels were detected in OC associated ascites compared to non-malignant effusions (Figure 8B). These data are in agreement with other studies showing that TGF-β1 is secreted in malignant OC ascites (Abendstein et al 2000) (Santin et al 2001). While we found that TGF-β1 secretion in OC associated ascites at an average level around 1719 pg/mL, Abenstein et al reported an average TGF-β1 secretion level around 5443 pg/mL in 10 OC ascites samples (Abendstein et al 2000), and Santin et al reported an average secreted TGF-β1 level around 407 pg/mL in 28 OC asitic fluid samples (Santin et al 2001). These differences may be due to different techniques of measurement and different methods of collection and storage for the specimens analyzed. Further, by stimulating OC cells with conditioned media, we found that TG2 expression level was increased, and that this increase could be blocked by a TGF-β receptor kinase inhibitor (Figure 13). These data suggested that TGF-β1 is secreted by OC cells in an autocrine manner.

Second, activation of TGF-β signaling in OC cells induced the expression and activity of TG2, an enzyme overexpressed in OC and linked to OC metastasis (Satpathy et al 2007). The specificity of TGF-β's effect was demonstrated as TG2 induction was blocked by a neutralizing antibody or a TGF-β receptor kinase inhibitor. Interestingly, we observed that TGF-β1 did not induce

TG2 expression or enzymatic activity in non-transformed NOSE and LP9 cells. Like OC cells, these cells express type I and II TGF-β1 receptors and the other elements of the signaling cascade, as previously reported (Yamada et al 1999) (Dunfield et al 2002). Our results showed that TG2 is not induced by TGF-β1 in NOSE cells, which coincided with attenuated Smad and TAK1 activation. We speculate that the lesser extent of activation of Smad2/3 in NOSE cells may be due to increased expression levels of the inhibitory Smad7 in these cells, as we show in Figure 11.

Third, we point to the canonical Smad signaling pathway as the principal mechanism of TG2 expression regulation by TGF-β1 in OC cells. By using siRNA targeting Smad2/3, we found that knock down of Smad2/3 not only blocked TG2 upregulation by TGF-β1, but it also decreased the basal expression level of TG2. The ChIP assay further confirmed that TG2 expression is transcriptionally regulated through Smad signaling pathway by demonstrating direct binding of pSmad3 to the TG2 promoter. This binding was enhanced in response to TGF-β stimulation. Previously a TGF-β1/BMP4 response element had been identified in the mouse TG2 gene promoter. This element was considered responsible for the stimulatory effect of TGF-β1 and the inhibitory effect of BMP4 on transglutaminase in a mink lung cell line Mv1Lu cells (Ritter and Davies 1998). However, others have shown that the effect of TGF-β1 on TG2 expression is cell-specific. For example, TGF-β1 stimulates TG2 expression in Mv1Lu cells, but inhibits TG2 expression in the mouse preosteoblastic cell line MC3T3 E1 (Ritter and Davies 1998). It has been reported that TGF-β1 can

induce TG2 enzymatic activity in normal and transformed human epidermal keratinocytes, and that this enhanced activity is related to increased TG2 expression levels, which is consistent with our findings. However, the authors claimed that TGF-β1 regulates TG2 expression through a mechanism dependent on protein synthesis (George et al 1990). In human subconjunctival fibroblasts, it was reported that TGF-β2, but not TGF-β1, induces TG2 expression and activity through PI3K-Akt signaling pathway (Jung et al 2007). Likewise, it was TGF-β2, but not TGF-β1, that has been shown to induce TG2 activity in human lens epithelial cell line HLE-B3 (Shin et al 2008). Therefore, our data are the first to show that TGF-β1 transcriptionally regulates TG2 in human ovarian cancer cells via Smads binding elements within the promoter of the gene. Moreover, the positive correlation we found between TG2 expression and Smad activation in human ovarian tumors suggested that the mechanism is operative *in vivo* (Figure 18).

On the other hand, the non-canonical TAK1 pathway was also engaged in the regulation of TG2 in OC cells. By using specific silencing or overexpression of TAK1 targets, we demonstrated that the main TG2 transcriptional regulator in the non-canonical pathway is the NF-κB complex. This is consistent with previous report that the tumor necrosis factor (TNF)-α transcriptionally modulates TG2 expression via a NF-κB motif in the promoter of the gene in liver cells (Yee et al 1997). TGF-β can activate NF-κB through Smad-dependent signaling pathway in keratinocytes (Lopez-Rovira et al 2000). It also can activate NF-κB through a Smad-independent TAK1 signaling pathway in osteoclasts (Gingery et

al 2008). In our study, we demonstrated cooperation between Smads and the NF-κB complex in the process of TG2 transcription regulation by TGF-β1 in ovarian cancer cells. By crosslinking NF-κB inhibitory protein IκB and promoting its degradation, TG2 is known to induce NF-κB activity (Mann et al 2006). Thus, a feed forward loop may exist between TG2 and NF-κB.

TG2 is required for activation of the large latent complex (LLC) in bovine endothelial cells (Kojima et al 1993). LTBP, the latent TGF-β binding protein, which is essential for correct folding and efficient secretion of TGF-β1, is cross-linked to the ECM by TG2 and thus targets the LLC to the matrix, where TGF-β1 can be activated (Nunes et al 1997). In addition, NF-κB is an important mediator that induces TGF-β expression and activity in monocytes (Rameshwar et al 2000) and fibrosarcoma cells (Perez et al 1994). Therefore, the feed forward loop between TG2 and NF-κB may be expanded to a large network between TGF-β1, NF-κB and TG2. Indeed, the relationship between TG2, NF-κB and TGF-β1 was further confirmed in Swiss 3T3 cells, in which TG2 induced the expression and bioactivity of TGF-β1, a process mediated by NF-κB (Telci et al 2009).

4.3 TG2 induces EMT in OC cells by modulating E-cadherin transcriptional repressor Zeb1

First, by using knockdown and overexpression of TG2 in OC cells, we demonstrated that TG2 expression level in OC cells is correlated with an elongated mesenchymal morphology accompanied by the loss of epithelial marker E-cadherin and gain of the mesenchymal markers N-cadherin and vimentin. This phenotypical change led to increased migration and invasion of

OC cells in matrigel. Our study showed for the first time that TG2 promotes EMT in cancer cells (Shao et al 2009). Our findings were later confirmed in a breast cancer model (Kumar et al 2010) and in an epidermoid carcinoma model (Lin et al 2011), suggesting that the phenomenon observed is more general, and not OC restricted. As EMT is required for initiation of tumor metastasis, this novel function of TG2 sheds new light on the mechanism by which overexpressed TG2 enhances metastasis of ovarian cancer (Satpathy et al 2007), breast cancer (Mehta et al 2004) (Mangala et al 2007), pancreatic ductal adenocarcinoma (Verma et al 2006), and melanoma (Fok et al 2006).

Second, by exploring the transcriptional suppressors of E-cadherin, we found that TG2 expression is correlated with the expression of zinc finger transcription factor Zeb1. By knockdown and overexpression of Zeb1 in OC cells with and without TG2 expression, we identified Zeb1 as an important mediator of TG2-induced EMT. Further, we demonstrated that TG2 activates NF-κB to induce Zeb1 upregulation. Thus, for the first time, we reported that TG2 induced EMT by modulating E-cadherin transcriptional suppressor Zeb1, a process which is mediated by NF-κB. This mechanism was later reproduced in breast cancer (Kumar et al 2010) and epidermoid carcinoma (Lin et al 2011).

Zeb1 represses E-cadherin transcriptional expression by binding to the E-box element on E-cadherin promoter (Eger et al 2005). It can also repress expression of genes that regulate cell polarity, such as Crumbs3, HUGL2 and Pals1-associated tight junction protein (Aigner et al 2007). Therefore, Zeb1 is an important player in regulating epithelial cell polarity and tumor progression.

Several signaling pathways have been reported to regulate Zeb1 expression to induce EMT in various cancer cells, such as TGF-β1 in breast cancer (Shirakihara et al 2007), NF-κB in breast cancer (Chua et al 2007), IGF-1 in prostate cancer (Graham et al 2008), and estrogen in ovarian cancer (Hurt et al 2008). Members of microRNA (miR)-200 family have been reported to be suppressors of Zeb1 and Zeb2 to inhibit EMT (Korpal et al 2008) (Park et al 2008). In our study, we demonstrated that NF-κB regulates Zeb1 expression to promote EMT in OC cells. Given the network between TG2, NF-κB and TGF-β1, and the fact that Zeb1 is a downstream target of TGF-β1, we can not exclude a direct effect of TGF-β1 on Zeb1 in OC cells. In addition to Zeb1, we also observed increased Zeb2 and Slug expression in OC cells overexpressing TG2. This suggested that Zeb2 and Slug may also be involves in TG2 induced EMT, but the exact mechanism needs to be further studied.

Third, by introducing wild-type TG2, N-terminal FN-binding domain (tTG1-140), TG2 enzymatic mutant (C277S) and TG2 GTPase mutant (R580A) into the TG2-null OC cell line OV90, we found that tTG1-140 is sufficient to induce EMT. This was demonstrated by decreased E-cadherin, increased Zeb1, and increased migration and invasion in matrigel compared with control cells. As this fragment is important in cell adhesion to FN, these data suggest that the TG2-mediated interaction between the tumor cells and the ECM may be involved in the process of EMT induction.

Cell surface TG2 acts as a co-receptor for β1 and β3 integrin to bind FN, and thus TG2, FN, and β1 or β3 integrin form a tertiary adhesion complex to

promote cell adhesion and migration (Akimov et al 2000). Except for being a co-receptor, TG2 also modulates β1 integrin expression level by inhibiting β1 integrin degradation through calpain-induced proteolysis (Satpathy et al 2007). Integrins are a superfamily of transmembrane glycoproteins that mediate cell adhesion to the ECM. About 18 α and 8 β subunits have been described and these subunits can form up to 24 heterodimers, each with a distinct function. β1 integrin has been reported to be an important mediator for TGF-β-induced EMT (Bhowmick et al 2001). TGF-β directly upregulates β1 integrin expression and evidence for binding of Smad3/4 to the promoter of β_1 integrin has been provided (Yeh et al 2010). In addition, αV integrins, such as αV β_1 and αV β_3 can interact with the arginine-glycine-aspartic acid (RGD) sequence of LAP. This protein associates with TGF-β, induce its conformational change, leading to its activation (Ludbrook et al 2003). My work and these previous studies suggest that TGF-β signaling, TG2, and integrins are interconnected at multiple levels.

As transmembrane receptors on the cell surface, integrins connect the ECM with the intracellular cytoskeleton. Upon binding to the ECM, integrins cluster to form focal adhesion complexes. This process initiates outside-in signaling. On the other hand, intracellular signaling can change the conformation of integrins and induce inside-out signaling. These signaling pathways include focal adhesion kinase (FAK), integrin-linked kinase (ILK), Src, Rac, Rho, and cdc42 small GTPases. Engagement of integrins can also activate cell surface receptor tyrosine kinases such as PDGFR, EGFR, VEGFR, and FGFR even in the absence of ligand binding, and this integrin-receptor interaction further

activates downstream PI3K/Akt and Ras/Raf/MAPK signaling pathways (Yamada and Even-Ram 2002).

Integrin signaling is important for OC progression. For instance, ILK has been reported to be overexpressed in OC (Lossner et al 2009) and to promote OC metastasis (Ahmed et al 2003). Moreover, ILK is involved in TGF-β induced migration and invasion of OC cells (Lin et al 2007). Recently, Zeb1 was reported to be a downstream mediator of ILK to promote EMT in bladder cancer (Matsui et al 2012). Taken together, we speculate that via engagement of integrins and perhaps by activating downstream ILK signaling, tTG1-140 upregulates Zeb1 expression and induces EMT. Future work investigating how tTG1-140 activates ILK needs to be performed to confirm this proposed hypothesis.

However, both the TG2 enzymatic mutant (C277S) and the GTPase mutant (R580A) contain a functional FN-binding domain couldn't induce EMT in OV90 cells. As TG2 has no secretory signals, it is delivered to cell surface by interacting with β1 integrin in perinuclear recycling endosomes (Zemskov et al 2011). We also know that the tTG1-140 fragment is secreted, as it is detectable in the conditioned media of stably transduced OV90 cells. We speculate that the two other mutants may not be transferred to cell surface successfully after protein synthesis, and perhaps are not able to engage the integrins on the cell surface and fail to initiate integrin downstream signaling. Interestingly, mutation of Arg-579 in rat TG2 (Arg-580 in human TG2) to Ala induces a conformational change of TG2 (Begg et al 2006a). This GTP-independent mutant adopts a semi-compact conformation, which is different from the compact form of GTP-bound

wild-typeTG2. Likewise, Cys-277 also involves in allosteric regulation of TG2 by forming a stabilizing H-bond between Cys-277 and Tyr-516. Substitution of Cys-277 with Ala or Ser disrupts the stability of compact TG2 (Begg et al 2006a). The conformational change of C277S and R580A may block the transfer of TG2 to the plasma membrane. This proposed explanation remains to be proved by using IF tracking to follow cellular localization of the mutant proteins.

4.4. TG2 induces an ovarian cancer stem cell phenotype

The concept of cancer stem cells was first defined in leukemia (Bonnet and Dick 1997) and later on was applied to solid tumors. Ovarian cancer stem cells were first identified using the cell surface markers CD44 and CD117 (Zhang et al 2008). One important property of stem cells is self-renewal, as evidenced by the ability to form spheroids *in vitro* (Dontu et al 2003). We demonstrated here that TG2 expression level was increased in ovarian cancer stem cells, both in OC cells grown as spheroids and in the CD44+/CD117+ population isolated from human ovarian tumors. Knock-down of TG2 decreased the percentage of CD44+/CD117+ cells in cancer cell lines and spheroid formation by OC cells. These data suggested that TG2 is required for the maintenance of the OC stem cell phenotype. As we showed previously that TG2 induces EMT in OC cells, our findings are in agreement with the previous report that EMT generates the breast cancer stem cell phenotype (Mani et al 2008). Indeed, we also observed increased vimentin and decreased E-cadherin expression in cells grown as spheroids. In ovarian cancer, it is possible that only cells undergoing EMT and

acquiring migratory abilities can be shed from the primary tumor site, can self-renew, survive in the peritoneal cavity (via spheroid formation) and can form metastatic tumors in distant organs.

The process of self-renewal is important for stem cells to maintain pluripotency and represents one of the key characteristics of “stemness”. A set of transcription factors including Nanog, Oct3/4, Sox-2, and STAT3 play critical roles in the maintenance of stem cells’ self-renewal (Niwa 2007). Oct3/4 and Sox-2, together with c-Myc and klf4, known as Yamanaka factors, can induce pluripotency in both mouse and human somatic cells (Takahashi and Yamanaka 2006) (Takahashi et al 2007). Oct3/4 and Sox-2, together with Nanog and Lin28, were also reported to induce pluripotent stem cells from human somatic cells (Yu et al 2007). These studies indicate that Oct3/4 and Sox-2 are essential to maintain stem cell pluripotency and “stemness”. We showed here that the expression level of these stem cell specific transcription factors Nanog, Oct3/4, Sox-2, and STAT3 are upregulated in TG2 expressing ovarian cancer cells. These data suggest that TG2 might induce a stem cell phenotype in ovarian cancer cells by modulating stem cell specific transcriptional factors.

Except for these transcriptional factors, several signaling pathways, such as TGF-β, Wnt, Notch, and hedgehog (Hh), are also critical regulators of stem cell self-renewal, pluripotency, and differentiation (Liu et al 2007) (Takebe et al 2011). We showed that β-catenin, the downstream effector of canonical Wnt signaling pathway, is upregulated in TG2 expressing ovarian cancer cells. We also demonstrated that TGF-β induces spheroid formation and increases the

CD44+/CD117+ population in OC cells, and these effects are blocked in cells where TG2 is knocked down. These data further support that TG2 plays an important role in the maintenance of ovarian cancer stem cell homeostasis.

After ovarian cancer stem cells were first isolated using the cell surface markers CD44 and CD117, other markers have been described to identify ovarian cancer cells with stem cell features, e.g. CD133, ALDH1, and others (see Introduction: section 1.5). Ovarian cancer cells bearing those markers possess some of the same stem cell characteristics: self-renewal, ability to initiate tumors, and chemoresistance. At present, there is no accepted consensus on a defined marker or a combination of several markers as the common standard marker for ovarian cancer stem cells, as the field is evolving. All these markers need to be tested by further studies. One limitation of our study is that we limited the CSC markers studied to CD44/CD117 based on our best interpretation of the available literature. In future work, to better understand the mechanism by which TG2 modulates ovarian cancer stem cell homeostasis, some of other marker, such as CD133 or ALDH1 might be used alone or in combination with CD4/CD117 to isolate ovarian cancer stem cells

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4.5. TG2 induces chemoresistance in OC cells

By knocking down and overexpressing TG2 in OC cells, we demonstrated that TG2 expression levels in OC cells correlates with OC cells' survival after cisplatin treatment, suggesting that TG2 mediates resistance of these cells to cisplatin. This finding is consistent with TG2's role in maintenance of ovarian

cancer stem cells described above, as cancer stem cells are known for chemoresistance. There are several mechanisms that might mediate chemoresistance of cancer stem cells. First, CSCs can exist in a long-term quiescent or dormant state (Li and Bhatia 2011). The resting state renders CSCs resistant to agents that block DNA synthesis in highly proliferating cells, such as cancer cells. Second, CSCs express high levels of adenosine triphosphate (ATP)-binding cassette (ABC) transporters that regulate the efflux of chemotherapy agents from cells (Moitra et al 2011). This ability has been exploited to isolate ovarian CSCs as they can effectively exclude the Hoechst 33342 dye, and these CSCs have been named the “side population” (Szotek et al 2006). There are 8 subfamilies of ABC transporters: ABCA to ABCG, arsenite and antimonite transporter (ANSA) (Mahadevan and List 2004). It has been demonstrated that CD44+/CD117+ ovarian CSCs exhibit upregulated ABCG2 (Zhang et al 2008). Third, CSCs express regulators of drug metabolism such as aldehyde dehydrogenase isoform 1 (ALDH1), which induces chemoresistance in hematopoietic cells (Magni et al 1996). Increased expression of ALDH1 has been detected in several types of CSCs such as breast (Ginestier et al 2007) and ovary stem cells (Silva et al 2011). Fourth, CSCs demonstrate enhanced expression/activation of growth factors and anti-apoptotic signaling pathways (Gangemi et al 2009). In ovarian cancer, both protein kinase B (Akt)- and nuclear factor-kappa B (NF-κB)-regulated survival pathways are reported to be implicated in acquired cisplatin resistance (Yang et al 2006) (Mabuchi et al 2004). Here we

showed that TG2 induces chemoresistance by regulating NF-κB activity in OC cells.

TG2 has been implicated in doxorubicin resistance in breast cancer cells (Kim et al 2006) and its role in this process was linked to adhesion-mediated activation of intracellular signaling (Herman et al 2006). We also found that TG2 knockdown led to decreased levels of Akt phosphorylation in SKOV3 cells, in parallel with decreased levels of FAK activation. However, reconstitution of Akt activity through overexpression of CA-Akt in AS-TG2 cells altered only partially cisplatin-induced activation of caspases-9 and -3, suggesting that Akt was not the primary pathway through which TG2 protects cancer cells from chemotherapy-induced apoptosis. While activation of Akt by TG2 in SKOV3 cells may be related to facilitation of integrin engagement with subsequent FAK phosphorylation, our findings do not exclude other non-adhesion-mediated mechanisms leading to Akt activation. It is interesting that TG2 inhibition by KCC009 had discrepant effects on the levels of phosphorylated FAK compared with the levels of phosphorylated Akt in SKOV3 cells, suggesting that perhaps Akt activation in these cells may be modulated through other pathways. Indeed, a recent report suggests that the phosphatase and tensin homolog (PTEN) is a direct TG2 substrate in pancreatic cancer cells (Verma et al 2008). It is possible that such a mechanism is operative in ovarian cancer cells as well.

In contrast to the inability of CA-Akt to fully reduce cisplatin-mediated apoptosis in the absence of TG2, overexpression of constitutively active p65 subunit of NF-κB alone significantly reduced apoptosis induced by platinum in

AS-TG2 cells. This finding implicates activation of the NF-κB survival pathway by TG2 as the main escape mechanism from cisplatin-induced apoptosis. Given that in certain cellular contexts Akt plays a role in activating NF-κB (Gustin et al 2006), the discrete roles played by the two pathways may not be fully separated. We further examined the activity of the NF-κB complex in ovarian cancer cells and found that NF-κB activity is modulated by the level and function of TG2. This is consistent with prior studies in pancreatic cancer cells, where TG2 has been implicated in constitutive activation of the NF-κB complex, presumably through destabilization of IκBα. The inhibitory IκBα subunit appears to be a direct substrate for cross-linking mediated by TG2 in malignant pancreatic cells (Mann et al 2006). Abnormally cross-linked IκBα would then be targeted for proteosomal degradation and displaced from the NF-κB complex, leading to its activation. We found increased levels of IκBα levels in SKOV3 cells where TG2 has been knocked down, suggesting that a similar mechanism of TG2-mediated NF-κB activation is functional in EOC cells.

Furthermore, we demonstrate for the first time that KCC009, a dihydroisoxazole inhibitor of TG2 (Choi et al 2005), inhibits directly NF-κB and increases levels of IκBα in SKOV3 cells. KCC009 was investigated as a potential anticancer agent in glioblastoma, its effects being attributed to disruption of the extracellular matrix (Yuan et al 2005) and to inhibition of the Akt survival pathway and its targets (Yuan et al 2007). Interestingly, KCC009 did not significantly affect activation of Akt in ovarian cancer cells, contrasting the findings in glioblastoma. This may be due to the fact that Akt is constitutively active in SKOV3 cells

(Altomare et al 2004) (Arboleda et al 2003) and in ovarian tumors (Cheng et al 1992) and thus may be less dependent on outside-in signaling compared with glioblastoma cells. Constitutive activation of Akt in ovarian cancer cells has been attributed to low levels of expression of the phosphatase PTEN (Arboleda et al 2003) or to Akt gene amplification. Our data suggest that the cytotoxic effects of KCC009 in SK0V3 ovarian cancer cells and its modest synergistic activity in combination with cisplatin may be due to its inhibitory effect on the NF-κB survival pathway.

4.6. Concluding remarks and future studies

In summary, in these studies we investigated the mechanisms of TG2 upregulation in OC and the pathways by which TG2 induces OC metastasis and chemoresistance. We demonstrated that TGF-β1, a cytokine involved in tumor progression, is secreted in the OC milieu in an autocrine manner, and that TGF-β1 induces TG2 expression and enzymatic function in OC cells. Aside from TGF-β1, there are many other pathways that regulate EMT, tumor metastasis, cell survival, and chemoresistance. These pathways include, but are not limited to Wnt, Notch, Hedgehog, PDGF, EGF, and VEGF. Some of these growth factors or ligands are expressed or secreted in the OC milieu through autocrine or paracrine mechanisms. Future work will explore whether TG2 may be inducible by other pathways in the OC tumor milieu and dissect the mechanisms involved.

We also showed that TGF-β1-induced TG2 promotes EMT in OC cells and plays an important role in the homeostasis of OCICs, which are critical for OC

peritoneal metastasis and chemoresistance. These findings indicate that TG2 is a critical player implicated in ovarian cancer progression and suggest that the protein may be a target for future drug development, particularly focusing on agents that block OC progression. To proceed to this next step, it will be important to understand which part and function of the molecule is critical to the induction of EMT. My work suggests that the FN-binding domain of TG2 is implicated in EMT in ovarian cancer. If these findings are confirmed, then the focus can be placed on discovering small molecules that inhibit the interaction between TG2 and FN. As this site of protein-protein interaction has been biochemically mapped to a well-defined β5-β6 domain that inserts tightly into a pocket of FN, it may be possible to design inhibitors blocking this site of interaction. This could be performed by designing an assay that quantitatively measures the binding between TG2 and FN that can be adapted to high throughput screening of large libraries of compounds. It would be anticipated that by blocking TG2-FN binding, these small molecules will inhibit cell binding to the matrix. By inhibiting cellular adhesion, such agents could have a direct effect on peritoneal metastasis, as a critical step in this process is represented by cell adhesion to the ECM. In addition, my data suggest that inhibitors of TG2-FN interaction could also block EMT, and therefore they would have effects on OCICs and metastasis.

To determine the role of TG2 in OC progression, different OC cell lines were engineered to either overexpress or to express low levels of TG2. This approach changed the expression level of TG2 expression intracellularly and

made possible the study of cellular TG2's effects on metastasis and cancer cell properties. But TG2 can also be secreted into the extracellular microenviroment, and our group has previously shown that TG2 is detectable in malignant OC ascites. The role of TG2 in the extracellular space has not been elucidated, particularly as it pertains to cancer progression. Importantly, TG2 functions as an enzyme in the extracellular millieu, where Ca^{2+} concentrations are high and GTP concentrations are low. It is therefore possible that the phenotype induced by soluble TG2 is dependent on the enzymatioc function of this protein. Examples of experiments that will be carried out are *in vitro* and *in vivo* assays utilizing ovarian cancer cells which do not express TG2 and recombinant purified TG2. Effects of soluble TG2 on tumor metastasis in vivo, cell adhesion, cell migration and invasion *in vitro* will be measured. Additionally it will be important to understand whether TG2 affects the extracellular matrix through crosslinking and transamidation. Effects of soluble TG2 on proliferation and survival of normal cells in the matrix (fibroblasts, endothelial cells) will be evaluated. A possibility is that TG2 may facilitate the transition of endothelial cells to mesenchymal cells. This can be investigated by using similar procedures we used to measure EMT, such as morphology of the cells, measurement of markers of differentiation, and other functional assays (endothelial tubules formation).

Lastly, my work has begun dissecting the mechanisms of EMT in ovarian cancer by focusing on the effects of TG2 in this process. The observations made here can be further extended to better understand E-cadherin expression regulation in OC and how this impacts cellular plasticity, as the cancer cells

transition between the primary tumor, spheroids in the peritoneal fluid, and metastatic sites. This dynamic process is likely to involve context-dependent transcriptional regulation for the critical elements that determine cell polarity and interaction. By understanding this multi-step process, we can uncover how metastasis proceeds and how its critical steps may be intercepted to block cancer progression.

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PUBLICATIONS

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CONFERENCE PRESENTATIONS

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