DOCTOR OF PHILOSOPHY

Tissue Transglutaminase (TG2) is a potential therapeutic target in the treatment of chemoresistant breast cancer

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Thesis Summary

Tissue transglutaminase (TG2) has been suggested to be a key player in the progression and metastasis of chemoresistant breast cancer. One of the foremost survival signalling pathways implicated in causing drug resistance in breast cancer is the constitutive activation of NFκB (Nuclear Factor -kappa B) induced by TG2. This study provides a mechanism by which TG2 constitutively activates NFκB which in turn confers chemoresistance to breast cancer cells against doxorubicin. Breast cancer cell lines with varying expression levels of TG2 as well as TG2 null breast cancer cells transfected with TG2 were used as the major cell models for this study. This study made use of cell permeable and impermeable TG2 inhibitors, specific TG2 and Rel A/ p65 targeting siRNA, TG2 functional blocking antibodies, IKK inhibitors and a specific targeting peptide against Rel A/p65 to investigate the pathway of activation involved in the constitutive activation of NFκB by TG2 leading to drug resistance. Crucial to the activation of Rel A/p65 and drug resistance in the breast cancer cells is the interaction between the complex of IκBα and Rel A/p65 with TG2 which results in the dimerization of Rel A/p65 and polymerization of IκBα. The association of TG2 with the IκBα-NFκB complex was determined to be independent of IKKα/β function. The polymerized IκBα is degraded in the cytoplasm by the μ-calpain pathway which allows the cross linked Rel A/ p65 dimers to translocate into the nucleus. Using R283 and ZDON (cell permeable TG2 activity inhibitors) and specific TG2 targeting siRNA, the Rel A/ p65 dimer formation could be inhibited. Co-immunoprecipitation studies confirmed that the phosphorylation of the Rel A/p65 dimers at the Ser536 residue by IKKε took place in the cell nucleus. Importantly, this study also investigated the transcriptional regulation of the *TGM2* gene by the pSer536 Rel A/ p65 dimer and the importance of this TG2-NFκB feedback loop in conferring drug resistance to breast cancer cells. This data provides evidence that TG2 could be a key therapeutic target in the treatment of chemoresistant breast cancer.

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Chapter 1: Introduction

1. Introduction

Breast cancer still remains as one of the most commonly diagnosed diseases among women and the social as well as economic impact of this malignancy is still enormous (Youlden, *et al.* 2012). Preventing breast cancer progression is an idea that dates back through history and there has been a lot of proof to show that the interaction between the independent and the environment they live in, can impact on the risk of breast cancer development (Cazzaniga & Bonanni, 2012). In spite of the development of numerous anticancer drugs, chemoresistance remains a major obstacle that results in the failure of treatment regimens. Some breast cancer cells maybe inherently resistant to drugs while others develop the resistance post chemotherapy. Chemoresistance can be caused by multiple factors: inactivation of the drug by detoxifying enzymes, changes in tumour suppressor genes, drug efflux by transport mechanisms, altered expression of anti and pro apoptotic proteins as well as protection from DNA damage mechanisms (LaPensee & Jonathan, 2010). Overcoming this chemoresistance has become a major focus of many studies.

Previously it has been shown that breast cancer cells selected out for drug resistance have high levels of the multifunctional protein cross linking enzyme tissue transglutaminase (TG2), however, no direct link has been established between TG2 and drug resistance. Studies undertaken in numerous breast cancer cell lines have shown that higher expression of TG2 makes the cancer cells more invasive and metastatic as well as conferring resistance to various drugs (Chen, *et al.* 2002). Many hypotheses have been put forward indicating that this could be due to the interaction between TG2 and the transcription factor Nuclear Factor-kappa B (NFκB). Literature has shown that TG2 expression leads to constitutive activation of NFκB via a pathway independent of IKK (Inhibitor-kappa Kinase). In numerous cancers such as breast, ovarian, cervical etc., the expression of TG2 has been correlated with constitutive activation of NFκB (Mann, *et al.* 2006)

1.1 Transglutaminases: A family portrait

Transglutaminases or TGs are a family of enzymes predominantly found in plants, micro-organisms, invertebrates and mammals. Multiple distinct forms of TGs have been identified in mammals. The term Transglutaminases was first coined by Clarke and colleagues in 1957 to illustrate the transamidating activity observed in guinea-pig liver (Clarke, *et al.* 1957). Studies done later showed that TGs cross linked proteins through an acyl-transfer reaction involving the γ-carboxamide group of a peptide-bound glutamine and the ε-amino group of a peptide bound lysine, which results in the formation of ε-(γ-glutamyl) lysine isopeptide bond (Griffin, *et al.* 2002) (Figure 1.1).

(Chandrashekar & Mehta, 2000)

Figure 1.1 Cross **linking reaction catalysed by Transglutaminases**

TGs are known to accelerate the post-translation modification of proteins by transamidation of the free glutamine residues. The resulting covalent stable isopeptide bond is resistant to proteolysis, chemical, mechanical and enzymatic disruption (Greenberg, *et al.* 1991). So far nine members of the family have been identified all of which have now been characterized at the protein level. In mammals other than erythrocyte band 4.2 which is inactive, the other TG family members require Ca^{2+} for the transamidating activity. The family members are Factor XIII A, keratinocyte transglutaminase (TG1), tissue transglutaminase (TG2), epidermal transglutaminase (TG3), prostate transglutaminase (TG4), Transglutaminases X, Y and Z (TG5,

TG6 and TG7) and the non-catalytically active erythrocyte band 4.2 (Iismaa, *et al.* 2009) (Table 1.1).

(Iismaa, *et al.* 2009)

Table 1.1 Functions of members of Transglutaminase (TG) family

Some of the common features seen in each of the members include the lack of glycosylation sites and cysteine bonds in spite of the presence of N-linked glycosylation sites and large numbers of cysteine residues in such proteins. Also, of the TGs, that are secreted, TG2 and Factor XIIIA that are associated with the plasma membrane lack the presence of the N-terminal hydrophobic sequence and are not secreted via the Golgi /ER pathway. The overall primary structure of the TG enzymes varies among the members; however, they all share the same amino acid sequence in the active site (Griffin, *et al.* 2002).

1.1.1 Factor XIII

Factor XIII (FXIII) is a major blood coagulation factor which is a pro-transglutaminase that circulates in the plasma in a tetrameric form (FXIII-A2-B2) (Muszbek, *et al.* 2008). FXIII comprises of two catalytically active A subunits, FXIIIA (~83kDa) and two inhibitor or carrier B subunits, FXIIIB (~80kDa). In the plasma, FXIIIB is found in excess and almost half of this exists in a non-complex free form. Dimers of FXIIIA denoted as FXIIIA2 are present within the cytoplasm of numerous cells such as macrophages and platelets, which is not characteristic of FXIIIB. FXIII is a well-known zymogen, which in its active form (FXIIIA) can function as a transglutaminase that catalyses the formation of ε(γ-glutamyl) lysyl bonds between polypeptide chains. FXIIIA plays a very important role in the regulation of fibrinolysis and homeostasis as well as in a wide range of physiological and pathological processes (Muszbek, *et al.* 2011).

Factor XIII A forms a dimeric structure (unlike the other transglutaminase members) and comprises of five major structural domains: an activation peptide at the NH_2- terminal, β-sandwich, catalytic core and β-barrel 1 and 2. The monomers of FXIIIA subunit are arranged as a dimeric structure wherein the central core domains are surrounded by β-sheet domains (Facchiano & Facchiano, 2009). On the other hand FXIIIB is a glycoprotein that consists of ten short tandem repeats known as GP1 structures, each containing ~60 amino acids joined together by two internal disulphide bonds (Ichinose, *et al.* 1990).

Pro-FXIII is activated during the late stages of the coagulation process by either Ca^{2+} or thrombin. Thrombin has been shown to cleave the activated peptide present on the NH2-terminus of FXIIIA, which results in the formation of the active truncated form of FXIIIA, FXIIIa (Muszbek, *et al.* 2011).

Contractile and adhesive proteins as well as components of the fibrinolytic clotting system form protein substrates of FXIIIa. One of the main physiological functions of FXIII in the plasma is the cross linking and incorporation of plasminogen in activator into fibrin, so as to protect it from plasmin. Plasma FXIII is also involved in tissue repair and wound healing. Cellular FXIII, when present on the surface of the cells, can support and maintain homeostatic functions (Torocsik, *et al.* 2005).

FXIIIa has also been demonstrated to limit bacterial infections in the wound and incorporates macromolecules to support cell survival and migration. The complex formed between α5β3 integrin and VEGF receptor 2 is crucial for formation of granulation tissue and angiogenesis is mediated by FXIIIa. Reduced FXIII levels have been observed in plasma during chronic inflammatory conditions that involve blood loss and subsequent activation of the coagulation cascade (Soendergaard, *et al.* 2013).

1.1.2 Keratinocyte transglutaminase

Keratinocyte Transglutaminase (TG1) is also known as transglutaminase epidermal type 1 and expressed mainly in the stratified squamous epithelia of coverings, the lower female genital tract and the upper digestive tract. Three response elements similar to AP-2, approximately 0.5 kb from the transcription initiation site are promoters of TG1. Proteolytic cleavage, TIG-3 protein and Ca^{2+}, activate the catalytic functions of TG1. Phorbol esters are known to induce the expression of TG1 while RA (Retinoic acid) down regulates the expression of TG1 (Mehta, 2005).

Studies done on the time course of TG1 expression after incision on the dorsal skin of neonatal mice has provided great insights into the role of TG1 in cutaneous healing (Inada, *et al.* 2000). Using *in situ* hybridization, TG1 was found to be expressed in the suprabasal keratinocytes next to the wound edge, two hours post the wound and its expression increased in the subsequent hour near the edge of the injury. TG1 is also known to be expressed in the wound site much before the infiltration of leucocytes, which implied that TG1 is essential during very early stages of wound repair and also in preparation for the remodelling of stratum corneum. TG1 is found to be highly expressed in the leading edges of migrating keratinocytes, when re-epithelialisation was completed. Within these migrating keratinocytes, the expression of TG1 was seen to be concurrent to its cross linking substrate, involucrin on the plasma membrane (Inada, *et al.* 2000).

The gene encoding TG1 is localized to chromosome 14 q11.2-13, and is reported to comprise of 15 exons and 14 introns. At the tissue level, the localization of TG1 has been identified to the granular layer at the later stages of differentiation using immunochemical and immunohistological methods, while specific antibodies against the N-terminal fragments of TG1 showed that TG1 was also present in the spinous and suprabasal layers (Kim, *et al.* 1992; Steinert, *et al.* 1996). Mutations that include a C-to-T change in the binding site of the transcription factor Sp1 within the promoter region and the Gly143-to-Glu mutation in exon 3, Val382-to-Met mutation in exon 7 of the *TGM1* gene result in autosomal recessive lamellar ichthyosis which is a rare keratinisation disorder of the skin. Patients of this disorder exhibit extremely decreased TG1 activity with an absence of any detectable TG1 polypeptide (Jessen, *et al.* 2000)

1.1.3 Epidermal transglutaminase

Epidermal transglutaminase or TG3, was first characterised by Buxman and Wuepper in 1975 and purified in 1976, and still is one of the least understood members of the family of TGs (Buxman & Wuepper, 1976). The human TG3 gene is localized into chromosome 20q11-12

(Wang, *et al.* 1994). The sequences that are between the 128 and 91 base pairs upstream of the transcription initiation site represent the proximal promoter region of the *TGM3* gene. The binding of the Ets and Sp1 motifs to their respective cognate binding factors is essential for the transcription of *TGM3* (Mehta, 2005).

The TG3 protein is an intact, latent pro-enzyme with low specific activity and of size, 77kDa. After the cleavage of TG3 into two 30kDa and 47kDa fragments, the enzyme becomes activated by the non-covalent interaction between the two fragments. A recent study done by Cheng *et al.* in 2006, cathepsin L was shown to cleave TG3 *in vitro*. The binding of Ca^{2+} to TG3 can increase the specific activity of the enzyme, similar to the other TGs (Cheng, *et al.* 2006)

The use of polyclonal antibodies indicated that during the later stages of differentiation, TG3 could be detected in the epidermis (Lee, *et al.* 1996) as well as in the small intestine, testis, brain and fore stomach (Hitomi, *et al.* 1999). Using a monoclonal antibody, the cytoplasmic distribution of TG3 in the cornified and granular layer was enumerated which implied that TG3 played a role in the early phase of cornified cell envelope formation. Activated TG3 can cross link CE (cornified envelope) proteins such as trichohyalin, SPRs 1, 2 and 3 and loricrin which occurs during epidermal terminal differentiation (Hitomi, *et al.* 2003)

TG3 cross links the keratin and trichohyalin intermediate filaments to harden the inner root sheath, which is very crucial for hair fibre morphogenesis. TG3 is also responsible for cell envelope formation during the latter stages of differentiation in the hair follicle and epidermis. The $TG3^{-/-}$ mice embryos fail to get implanted which suggested that this gene plays a very pivotal role in the early developmental stages (John, *et al.* 2012). Other studies have also indicated that $TG3^{-/-}$ mice developed curled whiskers and fur as well as high protein extractability showing irregular structures in the cross linking of structural hair proteins (Thiebach, *et al.* 2007). TG3 along with TG1 is known to participate in the maturation of the epithelium by catalysing the specific cross linking of numerous structural proteins present in the

cell envelope (SRPs, involucrin, filaggrin, desmoplakin, keratins, loricrin and envoplakin) (Ahvazi, *et al.* 2002).

1.1.4 Prostate transglutaminase (TG4)

Prostate transglutaminase or TG4 was previously known as dorsal prostate protein 1 (DP1) or even androgen regulated major secretary protein because the main site of expression of this protein was in prostatic fluids, seminal plasma and the prostate. The regions between the positions of -113 and -61 base pairs upstream of the *TGM4* gene promoter are very crucial for the core promoter activity. The transcriptional regulation of *TGM4* promoter is the Sp1 binding motif at positions -96 to -87 base pairs upstream. The positions from -1276 to -563 harbour the promoter for a cyclophilin pseudo gene (Mehta, 2005).

The rat homologue of TG4 has been shown to be responsible for the cross linking during the formation of the copulatory plug and also may be involved in sperm cell motility as well as immunogenicity to a certain degree (Davies, *et al.* 2007). Initial studies done showed that TG4 expression was only confined to luminal epithelial cells. The function of this enzyme in prostate cancer cells has been linked to invasiveness and the regulation of the interactions between the endothelial cells and prostate cancer cells (Ablin, *et al.* 2011). Recently, variants of TG4 have also been reported in malignant and benign human prostate tissues (Cho, *et al.* 2010). This molecule has a diverse impact on prostate cancer cell growth, invasion, and migration and also involved in epithelial to mesenchymal transition and tumour-endothelial interaction. TG4 also interacts with other molecular complexes, thus implicating it as a biomarker of aggressive cancer as well as a therapeutic target (Jiang & Ablin, 2011).

1.1.5 Transglutaminases 5, 6 and 7:

Transglutaminase 5 (TGX), Transglutaminase 6 (TGY) and Transglutaminase 7 (TGZ) are the three recently discovered transglutaminases.

Transglutaminase 5 is encoded on human chromosome 15q15.1 (molecular weight 81kDa) and requires proteolytic processing for the activation of TG5 cross linking activity (Pietroni, *et al.* 2008). In cultured keratinocytes, TG5 has been found to be co localized with vimentin during the formation of filament network, when undergoing EMT (Epithelial to Mesenchymal transition) (Candi, *et al.* 2001). Even though TG2 was found to be expressed in numerous adult and foetal tissues, the exact cells that express the proteins in tissues are unknown (Candi, *et al.* 2004). However, the loss of function or mutated forms of TG5 has been reported to be the cause of developing acral peeling of skin syndrome, which implements its role in the formation of cornified cell envelops of keratinocytes (Cassidy, *et al.* 2005).

Similar to TG5, TG6 is also activated by proteolytic processing and comprises of two polypeptide chains which are synthesized as a precursor form consisting of a single polypeptide. TG6 is mainly responsible for the late phases of formation of cell envelop in the hair follicle and epidermis. TG7 is expressed ubiquitously in testis and lung. The transcription level of TG7 has been shown to be increased in breast cancer patients with a poor prognosis (Mehta, 2005).

1.1.6 Erythrocyte band 4.2

The human erythrocyte band 4.2 is a crucial membrane-associated protein present at approximately 200,000 copies per cell of 72 kDa in size. Band 4.2 is particularly important in normal erythrocyte function as patients who lack band 4.2, are anaemic due to the increased erythrocyte destruction with abnormally shaped and fragile erythrocytes. However, the exact function of band 4.2 in erythrocytes remains unclear. Even though the amino acid sequence homology between tissue transglutaminase and Factor XIIIA spans the regions containing the active sites, band 4.2 has no detectable cross linking activity when tested *in vitro* (Korsgren & Cohen, 1991).

Erythrocyte band 4.2 is known to be one of the major components of the red blood cell member and is located on the interior surface of the cells, where, the protein binds to the cytoplasmic domain of the anion exchange protein, band 3, with high affinity. The gene of human

erythrocyte protein 4.2 maps to chromosome 15 q15-q21 and contains 13 exons (Najfeld, *et al.* 1992).

The interaction of band 4.2 with ankyrin and spectrin seems to suggest that this protein plays a functional role in stabilizing the links between the cytoskeleton and the over laying membrane. Studies (Mouro-Chanteloup, *et al.* 2003) have also shown that band 4.2 interacts with CD47 which further contributes to the anchoring of the Rh molecule with the RBC skeleton (Dahl, *et al.* 2004). The interaction of band 4.2 with the remaining RBC proteins indicates its importance in maintaining the stability, integrity and flexibility of RBCs. Two isoforms of band 4.2 have been described, a protein fragment of 691 amino acids with a molecular weight of 72kDa encoded for by the short isoform cDNA and a protein of 721 amino acids with a molecular weight of 74kDa encoded for by the long isoform cDNA (Sung, *et al.* 1992 ; Zhu, *et al.* 1998)

1.2 Tissue Transglutaminase

Tissue transglutaminase or TG2 is the most ubiquitous member of the TG family. TG2 is a multifunctional protein that is involved in various cellular functions at different cellular locations. In contrast to the other transglutaminase family members, TG2 is involved in diverse roles in living and dying cells because of its different functions (Fesus & Piacentini, 2002).

TG2 is a multifunctional protein and bi-functional enzyme that acts as a transglutaminase in the presence of Ca^{2+}, but also as a GTPase/ ATPase, protein kinase and protein disulphide isomerase. It is involved in a vast array of cellular processes such as cell migration, survival, growth, adhesion, differentiation, ECM (Extracellular matrix) organization and apoptosis. Such an impact of TG2 in these functions suggests that this protein plays a crucial role in pathological states and physiological responses which contributes greatly toward inflammation, wound healing, tumour growth, metastasis, neurodegeneration, vascular remodelling and fibrosis (Nurminskaya & Belkin, 2012; Wang & Griffin, 2013; Wang & Griffin, 2012)

1.2.1 Structure of TG2

The nature of the enzymatic activity of TG2 is reliant on the type and amount of binding ligands as well as structural status of the protein (Bergamini, 2007). TG2 has been shown to exist in both open and closed conformation and comprises four sequential domains: β-sandwich at the N-terminal, catalytic core domain and two β-barrels at the C-terminal. The active site is within the catalytic core, formed of Cys277, Asp358 and His 335, present at the base of a cavity and buried under two loops TG2 is a monomeric protein of 685-691 amino acids and has a molecular mass of 76-85kDa. TG2 also entails the same four structural domains as the other TG members. Domain 1 covers amino acids 1-139 and consists of a flexible loop, a short helix, an isolated β-strand, five tightly packed anti-parallel strands in β-sandwich conformation and a another short strand that covers the lower end of the β-sandwich. In the second domain, the peptide chain folds into two more β-strands that are directed downwards and upwards along the core domain. This domain spans amino acids 140-454 and contains Ser 171 and Lys 173 that are required for GTP binding. Four additional β-strands and α-helixes also span the domain. The first three α-helixes are arranged in a triangular fashion which contains the active site triad (Cis, His and Asp) whereas the last helix is close to the core domain and is involved in Ca^{2+} binding activity. Amino acids 454-478 is the site that codes for the isoenzymes specialized function. Domains 3 and 4 are C-terminal domains and are crucial in regulating GTPase and transamidating activity (Griffin, *et al.* 2002; Nurminskaya & Belkin, 2012; Wang & Griffin, 2013) (Figures 1.2 A & B)

The four distinct domains of TG2 have unique properties. The N-terminal β-sandwich domain contains the binding site for FN and promotes the attachment of cells to the ECM. When induced by $Ca^{2+,}$ the catalytic core domain extends and is responsible for cross-linking cellular proteins. The barrel 1 domain contains the GTP/ATP binding site that allows TG2-mediated signalling pathways to proceed. The C-terminal Barrel 2 domain in its GTP bound form can recruit and activate phospholipase C (PLC) and contributes to the pro-inflammatory properties of TG2 (Mehta, *et al.* 2010). More recently, novel TG2 HS binding sites have been identified

which confirmed the interaction between syndecan 4 and matrix bound TG2. This binding site has also been demonstrated to be crucial for regulating the externalization of TG2 into the ECM via a method that involves the shedding of cell surface HS (Wang, *et al.* 2012) (Figure 1.2 C)

(A)

(B)

(C)

Figure 1.2: Structure of TG2: (A) TG2 structure in the absence of ligands bound to it. (B) Structure of TG2 (produced by using Rasmol) showing domains 1-4 coloured in Magenta, orange, blue and green respectively. Regulatory loop is coloured red (between Domain 2 and 3),

Active site amino acids forming the triad is in yellow, amino acids involved in Ca^{2+} binding in black and those interacting with GTP are in light grey (Chen & Mehta, 1999) (C) Structural and functional domains of TG2 (Mehta, *et al.* 2010)

1.2.2 Cellular distribution of TG2:

The possible reason that TG2 is engaged in various biological processes is due to its tissue wide distribution. Different cells and tissues constitutively express TG2, while in others, TG2 can be induced as a part of the maturation phase or induced by numerous external stimuli which induce cell stress or wound healing. TG2 is widely distributed in mammalian tissues with high levels found in smooth and vascular endothelial muscle cells (Thomazy & Fesus, 1989).

At the cellular level, majority of the enzyme is present in cellular cytosolic compartments. However, detectable amounts of TG2 are also found associated with subcellular fractions such as the plasma membrane, nuclei, mitochondria and ECM. The mitochondrial location of TG2 is thought not to be related to TG2 activity but to its PDI (protein disulphide isomerase) activity (Krasnikov, *et al.* 2005)

1.2.2.1 Cytoplasm

In theory, GTPase activity of TG2 should represent the main function of the enzyme as intracellular Ca^{2+} levels are low, but TG2 has also been reported to act as a transamidating enzyme by catalysing the cross linking of intracellular proteins when Ca^{2+} rises (Nicholas, *et al.* 2003). Furthermore, most of the potential substrates of TG2 are all primarily cytoplasmic proteins. TG2 transamidating function can be induced by numerous factors such as ROS, chemokines and growth factors which can increase intracellular Ca^{2+} by driving the release of Ca^{2+} from reserve sources (Facchiano & Facchiano, 2009).

Using a FRET based technique; the conformational changes of TG2 in living cells have also been studied. Significant amounts of TG2 that was shown to be present in the perinuclear recycling compartment (PNRC) had a "closed" conformation. On the other hand, the TG2

located on the cell membrane had an "open" conformation, which is potentially active, catalytically. Moreover, results have also illustrated that when cell membrane TG2 gets internalized, the enzyme accumulates in the PNRC, rather than undergoing degradation. Once apoptosis is induced, the conformation of cytoplasmic TG2 changes and becomes activated, even so, the TG2 present within the endosomes was maintained in the inactive conformation even in the late stages of cell death (Pavlyukov, *et al.* 2012).

Other small molecules can even interact with TG2 and alter the conformation of the protein (Nurminskaya & Belkin, 2012). Initial studies done by Singh & Cerione (1996) in HeLa cells revealed that TG2 was present latent in the cytoplasm as a part of a cytoplasmic complex multi-protein that induced the GTPase function of TG2 (Singh & Cerione, 1996). In the case of EGF induction, a similar result of TG2 shift from the cytoplasm to the cell membrane was observed (Antonyak, *et al.* 2009).Other than the GTPase and transamidating functions of TG2, the scaffolding functions of the protein have also been documented (Park, *et al.* 2010). TG2 modulated Rho family of GTPases via a variety of unrelated but distinct mechanisms such as the activation of RhoA in a non-enzymatic manner by integrin cluster mediated by TG2 and the serotonylation of cytosolic Rac1 and RhoA induced by TG2 (Walther, *et al.* 2011). In addition to this, other cytosolic signalling proteins such as 14-3-3 and PKA/AKAP13 (Lewis, *et al.* 2005), have been shown to interact with TG2 in the cytoplasm by binding to the PKA produced Ser 216 and pSer 212 residues on TG2 (Mishra & Murphy, 2004). Contradictory findings have also been reported as cytoplasmic TG2 was observed to decrease the activity of adenylyl cyclase activity in fibroblasts, but on the other hand was found to activate the adenylyl cyclase activity in neuroblastoma cells (Tucholski & Johnson, 2003)

1.2.2.2 Nuclear

TG2 can also be translocated into the nucleus with the help of importin-α-3 and studies have even shown that TG2 was localized to the mitochondrial membrane, which has been suggested to be vital in maintaining mitochondria function by regulating the activity of ANT1 activity.

Nuclear TG2 only represents about 5-7% of the total TG2 within a cell. Numerous stimuli such as increase in intracellular Ca^{2+} levels or cell stress can augment the TG2 concentration in the nucleus (Mehta, *et al.* 2006a/2006b). Increasing evidence is being provided to support the importance of nuclear TG2 in the function of regulating gene expression by either interacting with transcription factors or post translational modification of nuclear proteins such as histones (Mishra, *et al.* 2006), hypoxia-inducible factor 1 (Filiano, *et al.* 2008), Sp1 (Han & Park, 1999), *Rb* and E2F1 (Mishra, *et al.* 2007). Using these mechanisms, TG2 can control the survival, growth, apoptosis and differentiation that are involved in various physiological as well as pathological processes (Kuo, *et al.* 2011).

1.2.2.3 Extracellular matrix (ECM)

Ample evidence is available for the association of TG2 with the plasma membrane. The translocation of TG2 to the plasma membrane takes place by the binding of TG2 to the internal membrane surface, where it is not only adsorbed, but some of it is translocated to the external surface, by a process which is not well understood (Balklava, *et al.* 2002). TG2 lacks the classic hydrophobic leader sequence and so it cannot be secreted by the endoplasmic- Golgi dependent mechanism, even though it has been detected abundantly in the ECM and cell membrane fractions (Gaudry, *et al.* 1999; Stephens, *et al.* 2004). Extracellular TG2 is either retained on the cell surface or deposited into the ECM, which includes interaction with its high affinity binding partners such as FN (Fibronectin) and HS (Heparan sulphate) (Janiak, *et al.* 2006; Collighan & Griffin, 2009).

TG2 has also been identified as an important enzyme involved in matrix turnover. At the cell surface as well as in the matrix, TG2 has been thought to be involved in cell adhesion on FN by acting as an integrin co-receptor (Akimov, *et al.* 2000). FN bound to TG2 via cell surface HS in a manner independent of transamidase activity promotes cell adhesion as well as survival mechanisms. The cell surface heparan sulphate proteoglycan syndecan-4 interacts with TG2 which in turn activates its co-receptors, such as syndecan-2 and α5β1 integrins. The activation

of α5β1 involves the activation and binding of PKCα and phosphorylation of FAK and activation of ERK1/2 (Verderio *et al.* 1998) (Wang *et al.* 2010).

1.2.3 Regulation of TG2 expression and activity

Expression of TG2 is controlled by the TG2 promoter and encoded by *TGM2* gene which comprises of 13 exons (Gentile, 1994). Classic CAAT and TATAA regions are present upstream of the open reading frame (ORF) where sequences are present to which Sp-1 and NFκB transcription factors bind (Ai, *et al.* 2012). Also, it has been reported that the TG2 promoter may be highly methylated at the CpG islands, and this specific hyper methylation can silence the *TGM2* gene with respect to tissue differentiation (Ai, *et al.* 2008). Elevated expression of TG2 protein has been demonstrated in smooth muscle, and endothelial cells which are widely distributed throughout all organs (Thomazy & Fesus, 1989). Induction of increased TG2 expression in parenchymal and stromal cells in organs may be due to inflammatory cytokines such as IL-1, IL-6, NFκB, TNF-α and TGF-β as well as retinoids and Vitamin D which induce expression due to a response element on the *TGM2* gene promoter (Iismaa, *et al.* 2009).

Numerous heterogeneous factors regulate the activity of TG2, once the protein is expressed. Acting as a G-coupled protein, TG2 binds to GTP and activates phospholipase C which allows transmission of signals from trans-membrane helix receptors (Iismaa, *et al.* 2000). In the presence of high levels of Ca^{2+}, the GTP/ GDP molecules are released from the TG2 molecule, which inhibits signalling and promotes transamidating activity (Lorand & Graham, 2003). Interaction of TG2 with certain molecules like sphingosylphosphocholine (lyso-SM) can greatly reduce the calcium requirement. In the absence of molecules such as lyso-SM, almost 160μM of Ca^{2+} was required for TG2 activity which could be achieved at 10μM of Ca^{2+} in the presence of lyso-SM (Lesort, *et al.* 2000). In the extracellular environment, Ca^{2+} is present abundantly (approximately 1.2mM), whilst intracellularly, the levels of Ca^{2+} are very low (approximately 100nM). Generally it was considered that under normal physiological conditions, TG2 is not

active intracellularly due to the low Ca^{2+} concentrations and high GTP. However, recent evidence has come to light which suggests that living cells can induce a decrease in their intracellular GTP levels by almost 20% and up regulate Ca^{2+} levels by almost 10 fold. Moreover, molecular interactions that take place between TG2 and other factors may render the enzyme sensitive to low concentrations of Ca^{2+} (Kiraly, *et al.* 2011)

Another molecule that has a strong influence on the activity of TG2 is NO as almost 15 cysteine residues can be denitrosylated or nitrosylated in a manner dependent on Ca^{2+}, which would activate and inhibit the enzyme respectively (Lai, *et al.* 2001; Telci, *et al.* 2009). Induction by RA can modify RhoA, which is a member of Rho GTPases family involved with rearrangements of the cytoskeleton (Singh, *et al.* 2003). TG2 also possesses nuclear localization sequence (NLS) and with the importin α3 can translocate into the nucleus (Peng, *et al.* 1999). Even in the nucleus, TG2 can act as a G-protein and transamidating enzyme. In the presence of nuclear Ca^{2+}, TG2 can cross link transcriptional factors and histones (Ballestar, *et al.* 2001; Oliverio, *et al.* 1997).

1.2.4 Role of TG2 in cell death:

The multifunctional characteristic of TG2 complicates the role it plays in the complex circuitry of cell survival and death. TG2 can be activated by various apoptotic stimuli and has been reported to play numerous roles in cell death. Early studies reported that TG2 can regulate PCD (programmed cell death) in a caspase-dependent manner (Milakovic, *et al.* 2004; Tucholski & Johnson, 2002). Also, there is evidence that points to the role of TG2 in modulating other modes of controlled cell death. The transamidating activity of TG2 can induce the release of AIF (apoptosis inducing factor) from the mitochondrial compartments of pancreatic ductal adenocarcinoma cells and activate apoptosis independent of caspase action (Fok & Mehta, 2007). Similarly, in the same cell lines, TG2 was found to block autophagy (Akar, *et al.* 2007). Another report suggested that in the absence of TG2, autophagosomes could not mature and subsequent acidification does not happen (D'Eletto, *et al.* 2009). More recently, studies have

demonstrated that the transamidating activity of TG2 might be responsible for the final stages of maturation of the autophagosome and so is involved in autophagy (Rossin, *et al.* 2011).

To support the pro apoptotic functions, inhibiting the expression of TG2 in U937 cells in response to apoptotic stimuli prevented death (Oliverio, *et al.* 1999). It has also been illustrated that in NIH 3T3 cells that have been treated with Calphostin C, TG2 induced apoptosis was induced by TG2 by catalysing the cross linking of DLK (dual leucine zipper bearing kinase), which is a pro apoptosis kinase (Robitaille, *et al.* 2008). In the pancreatic cancer cell line, Panc-28, Fok & Mehta reported that Ca^{2+} induced apoptosis was facilitated by TG2 (Fok & Mehta, 2007). Once a cell begins to undergo PCD, TG2 is activated within the cell to cross link intracellular proteins in order to prevent the leakage of the harmful contents of the cell (Nicholas, *et al.* 2003). This insoluble protein scaffold stabilizes the integrity of the apoptotic cells prior to being cleared by phagocytosis, thus preventing the non-specific release of any harmful intracellular components as well as subsequent inflammatory responses and scar formation in the nearby tissue (De Laurenzi & Melino, 2001). In the later phases of apoptosis, TG2 can be cleaved by caspase 3, which disable the cross linking function of TG2. This implies that there exists a functional relationship between the members of the apoptotic machinery and TG2 (Nanda, *et al.* 2001). $TG2^{-/-}$ knockout mice also appear to have defective clearance of apoptotic cells (De Laurenzi & Melino, 2001). Peptides on the cell membrane derived from the TG2 sequence can cause conformational changes to Bax and result in its translocation into the mitochondria and subsequently release cytochrome c. The mutant of the active site of TG2 (C277S) promoted cell death in U937 and HL-60 cancer cell lines, which suggests that the transamidating function of TG2 is not essentially responsible for the initiation of apoptosis. Instead, the BH3 domain of TG2 has been concerned in inducing apoptosis (Nardacci, *et al.* 2003) TG2 and Bax have been shown to interact through their BH3 domains (Szondy, *et al.* 2003). Due to this interaction, at the mitochondrial level, Bax serves as a substrate for TG2. During apoptosis, mitochondria with Bax polymerized by TG2, is clustered in specific cytoplasmic regions, with dense matrix and few cristae. A TG2- dependent shift of

mitochondria occurs to a high polarized state prior to the loss of mitochondrial transmembrane potential. Also, the induction of TG2 by RA in neuroblastoma and adenocarcinoma cell lines sensitized these cancer cell lines to apoptosis due to the hyper polarization of the mitochondria (Szondy, *et al.* 2003). Recently, it has been identified that a pro-apoptotic TG2 isoform, known as TGase-S, that lacks the 3'C-terminal end has been drawn into the cell death process. This isoform is inducible by TNF-α and can be detected in brain tissue derived from patients with Alzheimer disease, where in it promoted apoptosis via the formation of large size oligomers that are toxic to the cell (Antonyak, *et al.* 2006).

In contradiction, TG2 expression has also been shown to protect cells from apoptosis. Rapidly dividing cancer cells are known to over express TG2. Cancer cells isolated from metastatic sites or that exhibit resistance to chemotherapeutic drugs have increased levels of TG2 expression (Chhabra, *et al.* 2009) TG2 is known to post-translationally modify the Rb protein, which is a vital suppressor of apoptosis (Oliverio, *et al.* 1997). The TG2/Rb interaction increases tremendously, parallel to a decrease in apoptosis, which is an important aspect of the anti-apoptotic effects of TG2. Experiments in which $Rb^{-/-}$ fibroblasts were used, demonstrated that Rb is essential for TG2 to reveal anti apoptotic activity in response to RA treatment (Milakovic, *et al.* 2004). It was also hypothesized that TG2 may protect the tumour cells from apoptosis by nuclear translocation. A controversy still prevails to whether TG2 activity in the nucleus is essential for binding and protecting Rb from degradation during apoptosis. Exposure of TG2 expressing cells to LY29402, the PI3K inhibitor, decreases the ability of TG2 to bind to GTP. This suggests that PI3K regulates the switch between the GTP binding activity and transamidating activity of TG2. The Ras-ERK pathway greatly influences the switch between the pro-apoptotic transamidating activity and pro-survival TG2/GTPase activity (Antonyak, *et al.* 2003). The binding of GTP is known to convert the enzyme from an open conformation to a closed protein conformation which is able to provide protection against any apoptotic stimuli. The other hypothesis put forward is that the expression of the full length form of TG2 in tumour cells confers protection against apoptosis whereas the expression of a shorter version of

TG2 which is truncated at the 3'end (TGase-S) and does not bind to GTP, is cytotoxic. The TGase-S shows no GTP binding capability, implying that the ability of TG2 to induce cell death is due to the inability of TG2 to bind to GTP (Antonyak, *et al.* 2006).

Thus, the exact mechanisms by which TG2 modulates cell apoptosis or facilitates protection from cell death is specific to cell type and two widely described mechanisms exploited by TG2 are (a) controlling transcription factors either directly or indirectly promote apoptosis or survival and (b) mediating ECM contacts with the cell consequently promoting cell survival cues (Gundemir, *et al.* 2012).

1.2.5 Pathological roles of TG2

1.2.5.1 Coeliac disease:

The post translational modification of proteins catalysed by TG2 can generate auto-antibodies, just as in auto immune disorders such as coeliac disease (CD) (Molberg, *et al.* 2000). Gluten sensitive enteropathy or coeliac disease is a multifactorial chronic disease which is caused by a permanent intolerance to ingested wheat gluten or similar proteins from barley and rye (Kagnoff, 2007). About 1% of the total population of children and adults are affected and the most conventional treatment is a gluten-free diet (GFD) (Kagnoff, 2007)

One of the primary initiators of the inflammatory response to gluten in CD patients is a 33-amino acid peptide which is resistant to digestion by enzymes of the small intestinal mucosa of humans, both *in vitro* and *in vivo* (Shan, *et al.* 2002). As a result of abnormal $CD4^+$ T-cell-initiated immune response to gluten chronic inflammation develops in the small intestine. This results in villous atrophy as well as flattening of the mucosa (Sollid, 2002). Genes encoding for HLA-DQ2 and HLA-DQ8 are strongly associated with the pathology of CD (Sollid, 2000). Intestinal T cells recognize gluten peptides when glutamines are converted into glutamic acid. The deamination of gliadins by TG2, results in an epitope that binds very efficiently to the HLA receptors DQ2. This is recognized by T cells derived from the gut, thus starting the cascade of

inflammation that would consequently lead to mucosal damage (Molberg, 1998). TG2, itself, is an auto-antigen that is characteristic of CD. Patients of CD have high levels of serum antibodies to both gluten and TG2 (Dieterich, 1997). It has been speculated that TG2 can cross link itself to gliadin, resulting in the production of auto antibodies, apart from its deamidation of the gliadin peptide. The generation of anti-TG2 IgA antibodies could also depend on the gliadin specific T cells that are specific to TG2. IgG and IgA anti TG2 antibodies are found in the majority of patients with CD, which makes them a very powerful diagnostic tool (Tommasini, *et al.* 2004). Also, studies done by Myrsky *et al.* (2008) have demonstrated that the coeliac disease specific antibodies that target TG2 interrupted several stages in the angiogenesis cascade. Moreover, these auto antibodies can disorganize the actin cytoskeleton in capillary cell types. Such a disruption in angiogenesis could lead to disturbance of the mucosal vasculature which is observed in coeliac disease patients on a diet that contains gluten (Myrsky, *et al.* 2008)

1.2.5.2 Neurodegeneration:

Four transglutaminases have been shown to be expressed in brain: TG1, TG2, TG3 (Kim, 1999) and TG6 (Hadjivassiliou, 2008) and TG2 has been implicated in various processes in the peripheral and central nervous systems.

The involvement of transglutaminase in the pathophysiology of Alzheimer's disease (AD), was initially suggested by Selkoe *et al.* in 1982 (Selkoe, *et al.* 1982). It was also hypothesized that TG2 facilitates the formation of insoluble aggregated amyloid beta (Ab) peptide, which are neurotoxic in an aggregated state (Selkoe, 2000). Studies have shown that TG2 can cross link Ab1-28 (Ikura, *et al.* 1993), Ab1-42 (Dudek & Johnson, 1994) and APP (Ho, *et al.* 1994). Tau protein has also been shown to be an excellent substrate for TG2 both *in vitro* and *in vivo*. Research conducted on human specimens indicates that TG2 could be involved in the cross linking of tau, as seen in AD pathology. TG2 activity was almost three fold greater in the prefrontal cortex of AD samples and recent findings seem to indicate that almost a fivefold increase is seen in TG2 in the brains of AD patients that is co-localized with neurofibrillary

tangles (Johnson, *et al.* 1997). These neurofibrillary tangles serve as the sites of γ-glutamyl-ε-lysine cross links in AD patients (Citron, *et al.* 2001). It has also been reported that TG2 undergoes alternative splicing in AD brains due to the intron- exon swapping of TG2 mRNA which results in a short and long isoforms (Citron, *et al.* 2002).

Howard Green was the first to suggest that an increase in the number of glutamines beyond a threshold would result in a protein becoming a substrate for TG2 and hence contributed to aggregate formation in Huntington's disease (HD). Studies conducted *in vitro* confirmed that polyQ peptides were good substrates of TG2, as in the presence of TG2, it forms insoluble aggregates along with the proteins of brain extracts (Kahlem, *et al.* 1996). It was also demonstrated that Htt proteins with long polyamine expansions, formed cross linked polymers, when incubated in the presence of TG2 (Kahlem, *et al.* 1998). Even though, TG2 expression and activity levels are increased in HD brains, recent studies have shown that TG2 may not be essential for the formation of Huntington aggregates in the brain. Studies carried out on human neuroblastoma cell line, SH-SY5Y demonstrated that TG2 does not modify Htt, and that even in the absence of TG2, Htt aggregates are formed (Chun, *et al.* 2001). Experiments done on $TG2^{-/-}$ crossed with HD mice, suggests that the formation of the aggregates may not depend on TG2 activity although cell survival may since $TG2^{-/-}$ /HD mice showed a considerable delay in the initiation of death and motor dysfunction. It is therefore, currently unclear, if the enzymatic activity of TG2 plays a role in the formation of Htt aggregate formation, and if so, what role it plays in the pathogenesis of HD (Bailey & Johnson, 2005)

1.2.5.3 Fibrosis

The pathological process of fibrosis is usually characterized by the accumulation of ECM proteins and fibroblasts that destroy normal tissue function and structure. Numerous organs can be affected by this disease such as kidney, liver and lungs (Wynn, 2007) and TG2 has been implicated to play a regulatory role in most of the above. TG2 can promote fibrosis is several ways due to its multifunctional roles. TG2 has been reported to augment the production as well

as maturation of fibrillar FN in a transamidating-independent manner (Akimov & Belkin, 2001) and increase deposition of collagen both directly via cross linking and by activation of matrix bound TGF β. Moreover, studies done by Chau, *et al.* in 2005, demonstrated that TG2 cross linked collagen had greater resistance to proteases secreted by the cell which results in protection from cell mediated degradation (Chau, et al, 2005)

In the rat and mouse models of liver and kidney fibrosis, TG2 enhances the process. Even in human kidney fibrosis, expression in TG2 is enhanced (Grenard, *et al.* 2001 ; Shweke, *et al.* 2008). Initial studies done on mouse models of renal fibrosis demonstrated that the progress of the disease leads to profound changes in the cellular location, synthesis and subsequent activity of TG2. This causes both intracellular and extracellular changes wherein the enzyme can play a role in cell death as well as in matrix stabilization and deposition that can lead to the activation of TGF-β, thus effecting the progression of renal fibrosis. Due to this, TG2 has been implicated as a therapeutic target to control renal scarring and subsequent failure (Johnson, *et al.* 1999). Also, in patients that experienced nephropathies in the tubulointerstitium and glomeruli, an up regulation of insoluble and soluble TG2 was observed. The insoluble TG2 forms were detected extracellularly, while the soluble counterparts were mainly found inside the cell (Johnson, *et al.* 2003). With the use of site directed and irreversible TG2 activity inhibitors, kidney function has been shown to improve and also prevent renal remodelling in rat models of kidney fibrosis and scarring. This further confirmed the role of TG2 as a potential target to prevent scarring of tissue (Johnson, *et al.* 2007).

Using human lung biopsy sections of pulmonary fibrosis patients, cultured primary human lung fibroblasts and mouse models of fibrosis, TG2 has also been reported to play a major role in pulmonary fibroblast characteristics as well as pulmonary fibrosis regulated by TGF-β (Olsen, *et al.* 2011). Also, $TG2^{-/-}$ mice were protected from the progress of fibrotic lesions in models of obstructive nephropathy. This defence may result in decreased myofibroblast and macrophage infiltration. Reduce rates of collagen I synthesis were also observed due to low levels of TGF-β activation (Shweke, *et al.* 2008).

In the case of liver fibrosis, TG2 activity was found to be up regulated and the TG2 mRNA levels were in correlation to the expression of genes related to fibrosis. In knock out TG2 mouse models, hepatic TG2 activity was observed to be significantly low, but on administering TAA or CCl_4, a pattern of liver fibrosis was revealed. This seems to imply that even though TG2 was increased during progression of hepatic fibrosis, it plays no role in stabilization of the collagen matrix or fibrogenesis (Popov, *et al.* 2011). Literature seems to clearly indicate that evidence provided for the role of TG2 in fibrosis is strong for kidney and lung models of fibrosis while on the other hand; involvement of TG2 in liver fibrosis remains under researched.

1.2.5.4 Cancer progression:

The progression of cancer is very similar to inflammatory responses (Mantovani, *et al.* 2008) and conflicting reports have indicated that TG2 may be down regulated or up regulated in the process (Mehta, 2008). In primary tumours, TG2 expression is usually reduced in the phases of tumour progression, while on the other hand, increased TG2 expression is observed in chemoresistant and secondary metastatic tumours (Kotsakis & Griffin, 2007). A recent study demonstrated that hyper methylation of CpG islands occurred. The CpG islands have been shown to overlap the transcriptional and translation promoter sites of the *TGM2* gene (Ai, *et al.* 2008). In a similar manner, in neuroblastoma cells, over expression of N-myc was illustrated to reduce TG2 expression by the recruitment of HDAC and Sp1 transcription factor to the respective binding sites of the *TGM2* gene promoter (Liu, *et al.* 2007). Also, injecting TG2 into tumours of mice inhibited the growth of CT26 colon carcinoma (Jones, *et al.* 2006).

Conversely, TG2 has been reported to be up regulated in numerous cancers such as ovarian, breast, colon and pancreatic cancers, increasing the invasive potential of tumours and promoting resistance against chemo therapeutic drugs (Wang, *et al.* 2012; Khanna, *et al.* 2011). Coherently, inhibiting TG2 with siRNA or specific TG2 activity inhibitors, reduced tumour cell migration as well as increased sensitivity to cell death induced by chemotherapy (Yuan, *et al.* 2007). At a more molecular level, TG2 is known to be constitutively activated by several pro

survival factors such as FAK/Akt and NFκB (Mehta, 2005). In drug resistant cancers, TG2 is up regulated in mRNA, protein as well as enzyme activity levels. In PC-14 lung cancer cells, Han and Park observed that TG2 expression increased after selection for doxorubicin resistance. Furthermore, inhibiting TG2 confers drug sensitivity to the PC-14 lung cancer cells, which clearly suggested that TG2 played a crucial role in cancer cells acquiring drug resistance (Han & Park, 1999).

1.2.5.4.1 Regulation of cell survival in cancer cells by TG2

Evidence has also been collected to show that intracellular TG2 can act as an anti- as well as pro-apoptotic factor in which the anti-apoptotic activity of TG2 can be attributed to NFκB activation (Mann, *et al.* 2006; Condello, *et al.* 2008). On the other hand, extracellular TG2 has an important role in cell survival because of its interaction with integrins (Kotsakis & Griffin, 2007) or syndecans to enhance cell adhesion, thereby rescuing cells from anoikis (Verderio, *et al.* 1998). The role of TG2 has been known to vary depending on the stages of tumour progression as well as its metastatic ability. With regard to proliferation and apoptosis, intracellular TG2 has been related to transmitting anti-growth signals during the growth of the tumour (Birckbichler & Patterson, 1978)

The link between TG2 and apoptosis has been well documented. Augmented expression of TG2 results in rapid cell death or leaves the cell susceptible to agents that induce cell death (Mehta, *et al.* 2004). In contradiction, high TG2 expression can also lengthen cell survival by protecting it from apoptosis. To support this, various experiments have been conducted with drug resistant and metastatic cancer cell lines which have showed high expression of TG2. In fibroblasts, increased TG2 expression is known to protect the cells against apoptosis by retinoic acid. Additionally, TG2 links with the integrin family of proteins which prevents apoptosis (Antonyak, *et al.* 2001; Boehm, *et al.* 2002)

In carcinomas such as breast, pancreatic and non-small cell lung cancer, TG2 is over expressed which in turn are thought to cause activation of NFκB by cross-linking its inhibitor IκBα. This

is however dependent on the intracellular enzymatic activity of TG2 through its activation by Ca^{2+} which then proceeds to constitutively activate NFκB. In HL60 leukaemia cells, TG2 promotes cell survival by protecting the *Rb* gene product from caspase induced degradation by its transamidating activity. This results in cell survival. Also, in colon cancer cells, TG2 suppresses apoptosis by cross-linking of caspase-3 which prevents its cleavage and activation. Moreover, this enzyme is involved in attaching epithelial cells to the ECM. This activates 'outside-in' signalling which promotes cell survival (Cao, *et al.* 2008).

1.2.5.4.2 Role of TG2 in invasion and adhesion of cancer cells:

A huge amount of evidence has been reported to prove that increased TG2 expression causes enhanced cell attachment and spreading and under expression of TG2 can result in the disruption of these functions. Cell surface TG2 can act to either help or resist cell migration but this difference lies mainly on the composition of the extracellular matrix (Kotsakis & Griffin, 2007). When cell surface TG2 undergoes proteolytic degradation by membrane type matrix metalloproteinases (MT-MMPs) in gliomas and fibrosarcomas, it causes decreased cell adhesion and movement of tumour cells on FN. However, it increases tumour cell motility on collagen matrices because cross-linking of collagen results in increased cell adhesion (Akimov, *et al.* 2000). Studies on NIH 3T3 cell lines have showed that when oncogenes (such as H-Ras and Raf-1) are activated, there is decreased TG2-mediated cell adhesion as TG2 biosynthesis and surface expression decreases (Akimov, *et al.* 2000). TG2 can act as a substrate for c-CAM which is a ubiquitously expressed cell adhesion molecule that slows down tumour growth (Hunter, *et al.* 1998)

1.2.5.4.3 Role of TG2 in angiogenesis during cancer progression:

Under normal physiological conditions, the TG2 cross linking activity within the cell is suppressed due to the low concentrations of Ca^{2+} and lack of nucleotides required for activation. Once TG2 is brought onto the surface it is activated by Ca^{2+} and via protein cross linking brings about a change in the ECM (Belkin, 2012). The ECM plays an important role in tumour

angiogenesis and tumour cell migration as its main role is to provide mechanical support to tumour cells (Kotsakis & Griffin, 2007). A stable ECM displays anti-angiogenic properties and can inhibit proliferation and spread of malignant cells mainly due to the collagen cross-linking caused by TG2 (Jones, *et al.* 2006). Experiments have showed that over production of collagen due to TG2 stabilizes the ECM preventing tumour progression. It has also been reported that as primary tumours increase in size, TG2 activity and expression is down regulated. This is known to favour progression of the tumour as the decreased TG2 activity helps disrupt matrix stability aiding angiogenesis and increased spread of tumour cells (Kotsakis & Griffin, 2007). Increased expression of TG2 in the tissue surrounding tumours can therefore stabilize the ECM and cut off the tumour cells to the rest of the body by inducing fibrosis (Haroon, *et al.* 1999).

Angiogenesis is a very important stage in tumour development as tumour cells require their own blood supply to grow and metastasize (Folkman, 1990). Inhibiting angiogenesis will result in lack of growth and endothelial cell migration. Most of the functions of TG2 seem to be similar to that of angiogenesis involving cell migration, adhesion and ECM stabilization. Even so, the exact role of TG2 in angiogenesis has still not been determined (Kotsakis & Griffin, 2007). In epithelial cells, the ECM proteins are cross linked by TG2 which is thought to play an important role in stabilization of the basement membrane. However, no vascular abnormalities have been observed in TG2 knockout mice. TG2 was shown to be down regulated in a gene array analysis study to check for gene expression during capillary morphogenesis (Bell, *et al.* 2001).This could mean that TG2 is not required for the initial stages of angiogenesis such as capillary formation. Since the migration of epithelial cells and formation of blood vessels is dependent on the composition of the endothelial basement membrane, TG2 activity might inhibit the endothelial mobilization process causing increased cell adhesion. TG2 is known to cause a change in the ECM which regulates capillary tube development. With the help of angiogenesis assays, it has been shown that administration of TG2 can stop angiogenesis without causing cell death. This is done by increasing the protein build up in the ECM (Jones, *et al.* 2006).With all

this evidence, it can be concluded that large amounts of TG2 activity is involved in suppressing angiogenesis in tumour cells.

1.2.5.4.4 Role of TG2 in metastasis of cancer cells

In pathologies such as cancer which is matrix associated, the transamidating and GTP-binding properties of TG2 aid in the proliferation and invasion of malignant cells into the surrounding tissue (Collighan & Griffin, 2009). Experiments undertaken on mutant malignant hamster fibrosarcoma cells have shown that reduced TG2 activity can be correlated to metastasis and growth of tumours (Knight, *et al.* 1991). The primary tumour undergoes a very rigid selection procedure due to which there is a higher expression in secondary tumours as compared to primary tumours (Kotsakis & Griffin, 2007). This correlates with the metastasis studies done in human breast cancer, hemangioblastomas and in melanomas (Fok, *et al.* 2006 ; Mehta, *et al.* 2012 ; Mizoguchi, *et al.* 1998). TG2 is known to be up regulated in cell lines with high metastatic ability as compared to those with low metastasizing ability (Jiang, *et al.* 2003).This may in part be attributed to the role TG2 plays in strengthening points of contact for the tumour cells on the sub-endothelial matrix (Kotsakis & Griffin, 2007). Integrin β1 and Syndecan-4 present on the cell surface is known to form complexes with TG2 present on the cell surface. This interaction is known to facilitate adhesion, spreading and movement of cells (Verderio, *et al.* 1998). Also, TG2 when deposited in the matrix associates with FN through an RGD independent pathway and this interaction between TG2, integrins, syndecans and FN can cause a higher expression of TG2 in cancer cells resistant to drugs (Herman, *et al.* 2006).

1.2.5.4.5 Role of TG2 in chemoresistance:

A major obstacle in the successful treatment of cancer is the development of resistance to chemotherapeutic drugs and increased metastatic ability. High levels of TG2 are expressed in cancer cells selected for resistance or isolated from metastatic tumour sites. In breast cancer, TG2 promotes cell survival by associating with members of the integrin family (Herman, *et al.*

2006). All this gives a clear indication that TG2 is involved in promoting cancer progression and hence makes it of interest as a target for anti-cancer therapy.

A number of studies have demonstrated that there was a key relationship between drug resistance against doxorubicin and expression of TG2. Epigenetic analysis and proteomic profiling studies conducted on breast cancer cells resistant to doxorubicin implicated TG2 as an important target protein that increases doxorubicin resistance (Chekhun, *et al.* 2007; Park, *et al.* 2007). Activation of EGF signalling in breast cancer cells is another mechanism of TG2 induction which contributes towards the oncogenic potential of these cells (Antonyak, *et al.* 2004). Furthermore, in conditions of stress, several pathways and transcription factors can regulate the expression of TG2 (Jang, *et al.* 2010; Mehta, *et al.* 2010).

1.2.5.4.6 Role of TG2 in EMT

Recent studies show that Epithelial to Mesenchymal Transition (EMT) is important during progression of diseases such as inflammation, cancer and fibrosis (Sarkar, *et al.* 2009). EMT has been shown to be vital in making cancer cells resistant to drugs and in helping early stage tumours to become more invasive (Thiery, *et al.* 2009). TG2 is also known to be up regulated in tumour cells and to play a crucial role in promoting inflammation and fibrosis. Links have been made between EMT and TG2 to suggest that TG2 participates in tumour progression by regulating EMT. Studies performed on ovarian cancer cells show that high TG2 expression regulates EMT and this increases invasiveness and metastasis of the cancer cells (Shao, *et al.* 2009). TG2 is also responsible for promoting the autonomy of cancer cells from growth regulatory mechanisms and supporting anchorage-independent growth (Mehta, *et al.* 2010).

1.3 Nuclear Factor- kappa B

NFκB is a cytoplasmic transcription factor that regulates over 200 genes. It is involved in many cellular and organismal processes including immune and inflammatory responses, developmental processes, cellular growth and apoptosis. It has also been implicated in a large

number of disease states such as cancer, arthritis, chronic inflammation, asthma, neurodegeneration and heart diseases (Beyaert, 2004).

NFκB as a transcription factor has many diverse functions. At the cellular level, the activation of NFκB inhibits programmed and elicited cell death. This protects the cell and allows it to survive. It also induces expression of crucial genes that encode for anti-apoptotic proteins such as Bcl-XL, c-FLIP, A1/Bfl etc. This upholds cell survival and also controls the expression of anti-oxidant genes such as ferritin heavy chain (Lin & Karin, 2003; Pham, *et al.* 2004) and superoxide dismutase 2 that add to its pro-survival functions (Kamata, *et al.* 2005).

1.3.1 NFκB family members

The NFκB family is composed of five members: p65 or RelA, RelB, cRel, *nfκb1* (p50/p105) which is a combination of p50 and its precursor p105, *nfκb*2 (p52/p100) which is a complex between p52 and its precursor 100. The inactive precursor, p100 and p105 are inhibitory proteins that maintain the NFκB dimers in the cytosol of a resting cell (Pereira & Oakley, 2008). They have been characterized as such due to the presence of an evolutionarily conserved 300-amino acid Rel Homology Domain (RHD) positioned at their N-terminal ends (Hayden & Ghosh, 2004). Rel-A, Rel-B and c-Rel have transcriptional activation domains (TADs) in their C-terminal ends that allow them to activate target gene expression. On the other hand, *nfκb1* and *nfκb*2 do not contain TADs and so they repress transcription unless they are bound to a protein that has a TAD such as the other three NFκB family members (Hoffmann, *et al.* 2006). The target genes coded for by NFκB can be classified into four groups: immunoregulatory and inflammatory genes, anti-apoptotic genes, genes that positively regulate cell proliferation and genes that encode for negative regulators of NFκB. All of these groups contribute to tumourigenesis (Karin, *et al.* 2002). The NFκB family members exist as either homodimers or heterodimers bound to the inhibitory family of proteins in an unstimulated cell. The IκB family restricts the translocation of NFκB to the nucleus thereby maintaining it in its inactive state. Members of the IκB family have many copies of a sequence called the ankyrin repeat domains

that cover the nuclear localization sequence of the NFκB members and prevent them from entering into the nucleus (Hayden & Ghosh, 2004)

1.3.2 Activation of NFκB

NFκB is usually present in its latent, inactive form unless stimulated by an environmental response. Through the binding of NFκB to T-cell receptors, B-cell receptors, TNFR, CD40, BAFFR, LTβR and Toll/IL-1R family, the function of various cytokines, growth factors and effector enzymes get regulated. It can also control the expression of genes and factors outside the immune system (Hayden & Ghosh, 2004). NFκB activation is initiated by the signal induced degradation of IκB proteins by the activation of a kinase called the IκB kinase or IKK. The IKK proteins phosphorylated the IκB followed by quick ubiquitin-dependent degradation by the 26S proteosome (Karin & Ben-Neriah, 2001). The IKK is made up of IKKα and IKKβ which are two catalytic subunits as well as IKKγ or NEMO (NFκB essential modulator) which is a vital non-enzymatic regulatory subunit (Lin & Karin, 2003)

When the cell receives any one of a multitude of external stimuli or certain receptors are recognized, NFκB signalling can occur either by the classical pathway (canonical) or the alternate pathway (non-canonical) (Hayden & Ghosh, 2004; Karin, 1999).

(A) **(B)**

(Hayden & Ghosh, 2004)

Figure 1.3 (A) Canonical & (B) Non-canonical pathway of NFκB activation

The classical pathway gets activated by cytokines such as IL-1 and TNF-α or by bacterial products such as LPS or when a ligand binds to one of the TLR super family receptors. Activation of the receptor causes proteins like TRAF to get recruited to the cytoplasmic domain of the receptor. These adaptor proteins cause the activation of the IKK complex which in the canonical pathway consists of one molecule of IKKα, one molecule IKKβ and two molecules of NEMO. IKKβ phosphorylates IκBα at serine residues 32 and 36 resulting in poly-ubiquitination followed by degradation by the 26S proteosome. This releases NFκB which is predominantly p50:p65 from its inhibitor IκBα which then translocates into the nucleus and activates the target genes regulated by the κB sites (Hayden & Ghosh, 2004; Perkins, 2006). Dimers made up of RelA/p65, RelB, cRel and p50 are usually activated by the classical pathway.

The non-canonical pathway activates NFκB complexes of p100/RelB and mainly occurs during the development of lymphoid organs responsible for the production of T and B lymphocytes.

Very few stimuli can activate NFκB through this pathway such as B cell activating factor, Lymphotoxin B and CD40. The IKK complex in this pathway is only made up of two subunits of IKKα. Activation by the ligand stimulates NFκB inducing kinase or NIK which in turn phosphorylates the IκB site of p100 which results in the processing and release of the p52/Rel-B active heterodimer. This active heterodimer translocates into the nucleus and causes target gene expression (Moynagh, 2005).

1.3.3 Significance of NFκB in breast cancer progression

Numerous studies have demonstrated that in both primary human breast cancer cells and mammary carcinoma cell lines, activation of NFκB is prominent (Cogswell, *et al.* 2000; Sovak, *et al.* 2000). Even so, the exact mechanism that underlies the activation of NFκB has yet to be understood. Almost a decade ago, NFκB was implicated to have an important role to play in driving the progression of breast cancer. The primary subunits of NFκB that are activated in breast cancer cells appears to be p50 and p65 homodimers and heterodimers (Karin, 2006). Studies also reported that breast cancer progression from a hormone-dependent non- metastatic grade to a higher hormone-independent metastatic form was reliant on the elevation of NFκB activity. Moreover, the constitutive activation of NFκB was demonstrated to occur frequently in ER (oestrogen receptor)- negative basal like breast cancer cells (Biswas, *et al.* 2000; Biswas, *et al.* 2004)

In relation with these findings that indicated that NFκB was constitutively activated in metastatic breast cancer, several target genes of the oncogenic transcription factor were activated which would enhance the metastatic and invasive potential of breast cancer cells. For example, IL-8 was illustrated to increase in breast cancer cells *in vivo* and *in vitro* (Wu, *et al.* 2008) and is also increased in the serum of patients who were experiencing breast cancer cell dissemination (Benoy, *et al.* 2004). Another well-known target of NFκB, TNF-α was exhibited to increase the invasiveness when used to treat breast cancer cell lines (Cho, *et al.* 2009; Kim, *et al.* 2008). Finally, IL-6 has also demonstrated to induce motility in breast cancer cells and

proposed to be a negative prognostic marker of metastasis (Knupfer & Preiss, 2007). The involvement of Rel A/ p65 in human carcinomas is still a major subject of debate. Previous studies conducted with antisense oligonucleotides that inhibited Rel A/p65 expression reported that Rel A/ p65 played a role in the neoplastic alteration of transformed cancer cell lines. These studies demonstrated decreased cancer cell adhesion, growth and progression (Rayet & Gelinas, 1999). The over expression of Rel A/ p65 was noted in adenocarcinomas of stomach and breast as well as squamous carcinomas of the neck and head (Mantovani, 2010)

1 .4 Relationship between tissue transglutaminase (TG2) and NFκB

The over expression of TG2 in several cancer cells is often related to the constitutive activation of NFκB (Verma & Mehta, 2007a/2007b). Also, inhibiting the activity of TG2 using specific inhibitors resulted in the down regulation of NFκB activity (Cao, *et al.* 2008; Kim, *et al.* 2006). The ability of TG2 to promote activity of NFκB to induce chemoresistance against doxorubicin in breast cancer cells was observed both in EGFR negative and positive cell lines (Kim, *et al.* 2006). In epithelial ovarian cancer cells, combination treatment of TG2 inhibitor, KCC009 and cisplatin, illustrated that TG2 enzymatic activity was responsible in modulating the activity of NFκB and promoting cell survival (Cao, *et al.* 2008).

TG2 has been shown to form a direct complex with NFκB in the cytoplasmic fractions and subsequently modify the affinity of NFκB for IκBα (Verma & Mehta, 2007a/2007b). IκBα has also been described as a substrate for TG2 activity and this association could lead to the formation of an insoluble polymer in the cytosol that is unable to bind to NFκB (Lee, *et al.* 2004). In the nucleus, TG2 can directly form a complex with Rel A/ p65 which is directed to target genes, including *TGM2*, which leads to a positive regulatory feedback loop in doxorubicin resistant breast cancer cell lines (Verma & Mehta, 2007a/2007b). Many hypotheses have been established with regard to interaction between the two proteins and efforts are being made to clearly define the mechanistic detail behind it. Cancer cells selected for being resistant to chemotherapy as well as cancer cells with high metastatic ability show high levels of TG2

expression (Herman, *et al.* 2006). This shows a direct link between TG2 activity, metastasis and drug resistance. This could be suggesting that the presence of TG2 constitutively activates NFκB to confer drug resistance, metastatic and apoptosis evading properties to the cancer cells. Active NFκB in cancer cells prevents the apoptotic response of cells to anti-cancer drugs and radiation. Anti-apoptotic genes are also regulated by NFκB. The process of metastasis involves the movement of cancer cells into, through and out of the blood vessels. Adhesion molecules required for this process are directly linked to NFκB (Karin & Lin, 2002).

There is a high expression of TG2 in many drug-resistant and metastatic forms of cancer. NFκB is also constitutive in numerous hostile and metastatic tumours. With this theory, it could be implied that TG2 plays a role in constitutive activation of NFκB (Mann, *et al.* 2006). Experiments have been done where a calcium ionophore was used to increase Ca^{2+} levels in cancer cell lines with low and high levels of TG2 and where TG2 was absent. The increase in Ca^{2+} brought about increased activity of TG2 in the cell lines which had low and high basal levels of TG2 while the TG2 negative cell lines remained unaffected. When the *in situ* levels of TG2 were measured, there seemed to be a strong correlation with NFκB levels. Increasing the concentration of calcium ionophore seemed to increase NFκB activation. The TG2 negative cell lines had no noticeable NFκB activation. This goes to show that the transamidation activity and high expression of TG2 were crucial for activating NFκB (Mann, *et al.* 2006).

There are various pathways by which NFκB can get activated without IKK activation. NFκB can get activated via the MKK3/6-p38 mitogen activated protein kinase. UV can bring about rapid degradation of IκBα and mutant IκBα. Reactive Oxygen intermediates can also activate the NFκB pathway. However, the major IKKα/β-independent pathway is the signal dependent lysosomal degradation of IκBα (Lee, *et al.* 2004). In human breast cancer cell lines, it has been shown that the presence of TG2 will increase their resistance to chemotherapeutic drugs such as doxorubicin. Also, a shift in the accumulation of NFκB from the cytosol to the nucleus was observed. Increasing TG2 in these cells also showed an increase in NFκB activation that co-related with low levels of IκBα (Kim, *et al.* 2009). Analysis of IκBα and TG2 levels in breast

cancer cell lines showed that in the presence of TG2, IκBα expression was low and vice-versa. Such a low level of IκBα predominantly indicates the activation and release of NFκB. Western blot analysis conducted for pIκBα, illustrated no expression of this protein, which indicates that IκBα does not deplete because of phosphorylation (Kim, *et al.* 2006)

Mobility shift assays done by Mann, *et al.* in 2006, illustrate that halting TG2 expression would stop the constitutive activation of NFκB. Immunoprecipitation studies done with anti-IκBα antibody on TG2 cells showed that IκBα formed dimeric forms. Further treating the cells with Ca^{2+} increased the formation of dimeric and polymeric IκBα. Similar results were obtained with TG2- specific inhibitors. This concludes that TG2 is closely associated with IκBα and that IκBα acts as a substrate for TG2 reactions (Mann, *et al.* 2006).

Even nuclear localization of TG2 with Rel A/p65 has been reported with confocal microscopy. TG2 has properties to serve as a serine-threonine kinase and on the other hand Rel A/p65 undergoes phosphorylation at its serine residues. These results could imply that Rel A/ p65 acts as a possible substrate for TG2 kinase activity (Mann, *et al.* 2006). TG2 transfection studies done by Lee *et al.* (2004) on microglial cells portray increase in NFκB activation. TG2 also seems to activate the NFκB pathway which in turn exacerbates inflammation. Finally, it can be hypothesized that TG2 polymerizes IκBα and reduces its binding capacity to NFκB. Numerous stimuli that prompt NFκB activation have common characteristics with stimuli driven by TG2. This goes to show that NFκB and TG2 induce each other (Lee, *et al.* 2004).

In liver cells, TNF-α induces the synthesis of TG2 via the activation of IκBα phosphorylation. This results in NFκB dissociating and becoming active to translocate into the nucleus. Following translocation, it binds to the TG2 promoter and induces its expression. In the similar way, TG2 can also cross link IκBα and cause NFκB activation (Kim, *et al.* 2010). Multiple cancer cell lines acquire resistance to drugs and metastatic ability due to the increased expression of TG2 and NFκB. The signalling of both these molecules is associated with

increased survival of cancer cells and invasiveness. TG2 expression activates FAK and Akt and NFκB down regulates tumour suppressor PTEN (Wang, *et al.* 2012).

Due to the extensive role of TG2 in cancer progression, this protein has been hypothesized to also play a role in EMT. The NFκB, FAK and Akt pathways are known to be closely associated with the modulation of EMT, conferring drug resistance and promoting metastasis (Kalluri & Weinberg, 2009). NFκB has been shown to induce EMT through the expression of *Zeb1* and *Zeb2* (Chua, *et al.* 2007) as well as by stabilizing Snail and preventing its ubiquitination (Wu, *et al.* 2009). Based on the above observations, it has been reasoned that TG2 induced EMT is due to the constitutive activation of NFκB which has been supported along with increased in *Snail, Zeb1* and *Zeb2* expression (Shao, *et al.* 2009).

All of the above studies seem to suggest that there is a deep molecular as well as mechanistic connection between NFκB, TG2 and the cancer progression cascade. Further research on this topic can help reveal valuable information on how to reverse the chemoresistance in breast cancer cells.

1.5 Aims of the project

Previous work suggests that TG2 causes the constitutive activation of NFκB which allows breast cancer cells to exhibit a chemoresistant phenotype against drugs such as doxorubicin. However, the actual mechanism behind the interaction between TG2 and NFκB subunits to elicit a cell survival response and chemoresistance is still not fully understood. The aims of the project are as follows:

(a) To establish a relationship between TG2 and chemoresistance in breast cancer cells and to verify whether this phenotype can be reversed in the presence of TG2 activity inhibitors and specific siRNA.

(b) To study the involvement of TG2 activity on the constitutive activation of NFκB and investigate whether this is an extracellular or intracellular phenomenon.

(c) To characterize the pathway involved in the constitutive activation of NFκB induced by TG2

(d) To determine the presence of NFκB subunits on the *TGM2* gene promoter, which may contribute to a TG2-NFκB feedback loop in breast cancer cells

Chapter 2: Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Cell lines

The cell lines used in this study were breast cancer cell lines. The MDA MB 231 WT cells were a kind gift from Dr. Stephane Gross (Aston University, UK). The MDA MB 231 Clone 9 and MDA MB 231 Clone 16 cells were a kind gift from Dr. Kapil Mehta (Texas University, USA). The MCF7 cell lines was purchased from ATCC (American Type Culture condition). The SKBR3 and T47D cell lines were obtained from Birmingham University, Birmingham, UK. The doxorubicin resistant breast cancer cell lines, MCF7/RT, SKBR3/RT and T47D/RT used in this study were developed by selecting out surviving cells in the presence of increasing concentrations of doxorubicin, until the final cell lines were resistant and were able to survive in 1µg/ml of doxorubicin. The complete growth medium that the resistant cell lines were cultured in was always supplemented with doxorubicin, so that the cell lines can maintain their chemoresistant phenotypes.

2.1.2 List of antibodies

Neomarkers, Pierce, UK

- Mouse monoclonal TG2 Antibody (Cub 7402) (1:1000)
- Mouse monoclonal TG2 Antibody (TG100) (1:1000)

Cell signalling, UK

- Rabbit polyclonal Rel A/p65 antibody (1:1000)
- Rabbit polyclonal Rel B antibody (1:1000)
- Rabbit polyclonal c-Rel antibody (1:1000)
- Rabbit polyclonal p100/p52 antibody (1:1000)

- Rabbit polyclonal E-Cadherin antibody (1:1000)
- Mouse monoclonal N-Cadherin antibody (1:1000)
- Rabbit polyclonal phospho-Serine 536-Rel A/p65 antibody (1:1000)

Santa Cruz, UK

- Rabbit polyclonal p105/p50 antibody (1:500)
- Rabbit polyclonal IκBα antibody (1:1000)
- Mouse α-Tubulin antibody (1:1000)

Sigma Aldrich, UK

- Rabbit polyclonal Lamin A antibody (1:1000)
- Rabbit polyclonal Fibronectin antibody (1:2000)
- Goat anti-mouse HRP conjugated secondary antibody (1:1000)

Dako, Denmark

- Swine anti- rabbit HRP conjugated secondary antibody (1:1000)
- Rabbit anti-mouse FITC conjugated secondary antibody (1: 1000)

2.1.3 Chemicals

The cell culture media, RPMI-1640 and DMEM was purchased from PAA cell Culture Company. The distilled water (dH_2O) used in the experiments was obtained from Milli Q water purifier (Millipore/Water, Watford, UK). The chemicals and reagents used in the experiments were obtained from the following companies:

Zedira, Darmstadt, Germany

- Purified guinea pig liver transglutaminase (gplTG)
- Biotin-X-Cadaverine
- Z-DON-Val-Pro-Leu-OMe (Z-DON)

Cleaver Scientific Ltd, Rugby, UK

- BLUE Wide Range Pre-stained Protein Ladder

Bio-Rad, Hemel Hempstead, UK

- Bio-Rad protein assay kit

Calbiochem (EMB Merck Millipore) , Nottingham, UK

- Genecticin (G418 Sulphate)
- Calpeptin (cell permeable Calpain inhibitor)
- NFκB SN50 : {H-(AAVALLPAVLLALLAPVQRKRQKLMP)-OH} (cell permeable inhibitor peptide)

Sigma-Aldrich, Poole, Dorset, UK

- Tumour Necrosis Factor a (TNF-α)
- Geneticin (G418 Sulphate)
- PS-1145 dihydrochloride *(N-(6-Chloro-9H-pyrido[3,4-b]indol-8-yl)-3-pyridinecarboxamide dihydrochloride);* IKK inhibitor

Imgenex, San Diego, USA

- NFκB/SEAPorter Assay kit
- NFκB p65 (Ser 529/536) Inhibitory peptide set : (1mg of lyophilized peptide)

❖ *Inhibitor peptide (DRQIKIWFQNRRMKWKKNGLLSGDEDFSS); Rel A/p65 sequence is underlined*

❖ *Control peptide (DRQIKIWFQNRRMKWKK)*

R&D Systems Europe Ltd., Abingdon, UK

- Human RelA/NFκB p65 ExactaChIP Chromatin IP kit

Axon Medchem BV, Groningen, Netherlands

- BX795 (Pyrrolidine-1-carboxylic acid [3-(5-iodo-4-{3-[(thiophene-2-carbonyl)-amino]-propylamino}-pyrimidin-2-ylamino)-phenyl]-amide); Potent and very specific inhibitor of IKKε

Vector Laboratories, UK

- Vectashield mounting medium

Roche Applied Science, Burgess Hill, UK

- Cell Proliferation Kit II (XTT) : Sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium-bis (4-methoxy-6-nitor) benzene sulfonic acid hydrate reagents

Life Technologies Ltd/ Invitrogen, Paisley, UK

- Lipofectamine 2000 Transfection Reagent

Qiagen

- Human TG2 targeting siRNA
- Human Rel A/p65 targeting siRNA.

GE Healthcare

- A and G-Sepharose bead slurry
- ECL Chemiluminiscence development kit

Aston university, Birmingham, UK

- R283 : 1,3-dimethyl-2[(oxopropyl)thio]imidazolium derivative site directed TG2 irreversible inhibitor which is cell permeable (Balklava, *et al.* 2002)
- R294: non-permeable TG2 inhibitor which is known to block the cross linking activity of cell surface TG2 (Griffin, *et al.* 2008)

2.1.4 PCR primers

Gene	Primer sequences	Annealing Temperature
TGM2	Forward 5'-ATGAGAAATACCGTGACTGCCTTAC-3'	**63°C**
	Reverse 5'- CAGCTTGCGTTTCTGCTTGG-3'	
Rel A/p65	Forward 5'- ACGATCTGTTTCCCCTCATC-3'	**55°C**
	Reverse 5'-TGCTTCTCTCCCCAGGAATA-3'	
-κB site on *TGM2* gene promoter	Forward 5'-TACTTAGGTGGCTCCCTGTCTTC-3'	**52°C**
	Reverse 5'-CCAGATGAAAGGATGGGTTTGG-3'	
p21	Forward 5'-CCAGCCCTTGGATGGTTT-3'	**60°C**
	Reverse 5'-GCCTCCTTTCTGTGCCTGA-3'	
GAPDH	Forward 5'-TGCACCACCAACTTGCTTAGC-3'	**55-60°C**
	Reverse 5'- GGCATGGACTGTGGTCATGAG-3'	

Table 2.1: List of primers

2.1.5 Equipment

Beckman Coulter Instruments Ltd, High Wycombe, UK

- Spectrophotometer Model DU-7
- Beckman Allegra X-15R centrifuge

Bio-Rad, Hemel Hempstead, UK

- Glass plates (1.5mm)
- Protein III Minigel Vertical electrophoresis apparatus
- Western blot wet transfer system

Corning, Staffs, UK

- pH meter

Sanyo, UK

- CO_2 incubator Model IG150

Nikon Inc, Badhoevedorp, Netherlands

- Inverted Phase Microscope model TM-100

Leica Microsystems Ltd, Milton Keynes, UK

- SP5 TCS II MP Leica Laser Confocal Microscope
- DMI4000 B Automated Inverted Leica Fluorescent Microscope

IMPLEN GmbH, Munchen, Germany

- NanoPhotometer Pearl

2.2 METHODS

2.2.1 Cell culture techniques

2.2.1.1 Culture conditions of cells

The cells used in this project included MDA MB 231 Wild Type, MDA MB 231 Clone 9, MDA MB 231 Clone 16, MCF7/Wild type, MCF7/ Doxorubicin Resistant (MCF7/RT), SKBR3/Wild Type, SKBR3/ Doxorubicin Resistant (SKBR3/RT), T47D/Wild Type , T47D/Doxorubicin Resistant (T47D/RT) breast cancer cell lines. All of the above mentioned breast cancer cell lines were cultured in a humidified atmosphere at 37^{o}C and 5% (v/v) CO2. The MDA MB 231 Wild Type, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were cultured in complete RPMI-1640 containing 10% FBS, 25mM L-Glutamine and penicillin/streptomycin (100U/ml and 100µg/ml respectively) and used for experiments, unless otherwise indicated. The SKBR3/WT, SKBR3/RT, T47D/WT and T47D/RT cells were cultures in completed Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1% (v/v) nonessential amino acids and penicillin/streptomycin (100U/ml and 100µg/ml respectively) and used for experiments.

2.2.1. 2 Passaging of cell lines

The complete cell culture medium (DMEM or RPMI-1640) and 0.25% (w/v) trypsin were pre warmed prior to use. The adherent confluent (at least 90% confluency) cells were washed once with serum free DMEM or RPMI-1640 to remove dead cells and debris. The cells were then treated with Trypsin for 5 min at 37^{o}C for the cells to detach. The cells were then collected in complete medium (RPMI-1640 or DMEM) to inactivate the trypsin and centrifuged at 300xg for 5 min. The supernatant was then discarded and the cell pellet was resuspended in complete growth medium. Subsequently the cells were seeded into tissue culture flasks to obtain the desired confluency.

2.2.1.3 Cell counting using haemocytometer

The cell lines were collected as described in detail in **Section 2.2.1.2**. To count the cells, 10µl of each cell line suspension was added into each side of the haemocytometer. Using a phase contrast microscopy on an inverted microscope, the cells were counted in four separate chambers, neglecting the cells present on the top and right corner lines. The cell numbers obtained after counting was multiplied with 10^4 to determine the cell number present per ml of the cell suspension.

2.2.1.4 Freezing and defrosting of cell lines

The cell lines were trypsinized and counted as described above in **Sections 2.2.1.2 and 2.2.1.3** and subsequently re-suspended in freezing mixture which is composed of 10% (v/v) cell culture grade DMSO in heat inactivated FBS. The cells were then distributed as 1ml aliquots into cryogenic vials and kept at -20^{o}C for 1 h. After this, the cryogenic vials were kept in -80^{o}C for a few days, following which, the vials were transferred into a liquid nitrogen storage container for long term storage.

To defrost the frozen cells, the cryogenic vials of the cell lines in liquid nitrogen were removed and placed in the cell culture incubator at 37^{o}C until the freezing mixture and cells are completely thawed. The cell suspension was then carefully transferred into sterile Falcon tubes and 10 mls of complete growth medium (RPMI-1640 or DMEM) was added drop wise, with gentle mixing after every drop to make sure that the cells were diluted slowly. The cell and medium suspension was then transferred into a tissue culture flask. The medium was changed in the next 12- 24 h and the cell lines were passaged (**Section 2.2.1.2**) at least once prior to using them for an experiment.

2.2.1.5 SiRNA transfections

In order to silence the expression of TG2 and Rel A/p65 in the breast cancer cell lines, different small interfering RNA (siRNA) sequences that target human TG2 and Rel A/p65 were obtained from Qiagen. The sequences for the siRNA are listed below:

TG2 siRNA:

TGM2-1 CCGCGTCGTGACCAACTACAA

TGM2-6 CACAAGGGCGAACCACCTGAA

Rel A/p65 siRNA:

Hs_RELA_5 AAGATCAATGGCTACACAG

Hs_RELA_7 CCGGATTGAGGAGAAACGT

Hs_RELA_8 ATGGAGTACCCTGAGGCTA

The universal negative control siRNA was purchased from Qiagen and used as control treatments. 1nmol of the lyophilized siRNA were dissolved in 100µl of sterile, RNase free water to obtain a 10µM solution which was stored at -20^{o}C. The transfections were performed using Lipofectamine 2000 in accordance to the manufacturer's protocol. Prior to transfection, breast cancer cell lines were seeded (5x10^{5} cells into each well of a 6-well plate and 3000 cells into each well of a 96 well plate) for 24 h to reach 60-70% confluency. Before transfection, the cells were washed with serum free medium and replaced with pre-warmed fresh complete medium. Each siRNA was diluted with serum free medium to reach a final concentration of 100nM. The siRNA and Lipofectamine 2000 transfection reagent was prepared as directed by the manufacturer by incubating the mixture at room temperature for 15-20 min to form the transfection complex. The complex was then added in a drop wise manner on to the seeded cells with gentle swirling of the plate to make sure that the transfection complex is uniformly distributed (in accordance to manufacturers' instructions). After 48 h of incubating the cells

with the siRNA at 37^{o}C, the cells were used in western blotting, activity and cell viability assays.

2.2.1.6 Cell transfection of TG2 and vector control plasmid

2.2.1.6.1 Kill curves

3000 cells of SKBR3 and MCF7 cell lines were seeded into per well of a 96-well plate and treated with increasing concentrations of G418 (250µg/ml, 500µg/ml, 750µg/ml, 1mg/ml and 1.5 mg/ml). After 24, 48 and 72 h of incubation with the antibiotic, 30µl of XTT reagents (**Section 2.2.13**) mixture was added into each well and incubated with the cells for 4 h at 37^{o}C. The absorbance was read at 490nm and 750 nm with a help of a Spectrafluor plate reader. The absorbances were expressed as absorbance of 490nm-750nm. The percentage of cell viability in the presence of increasing concentrations of the antibiotic was calculated.

2.2.1.6.2 Transfection of the breast cancer cell lines with plasmids using Lipofectamine 2000 and selection using G418

The cells were seeded into appropriate tissue culture flasks depending on the experiment to be conducted (5x10^{5} cells per well of a 6 well plate and 3000 cells per well of a 96 well plate) and allowed to adhere overnight and reach 70-80% confluency. The next day, the plasmids and Lipofectamine 2000, depending on the tissue culture flasks, were diluted into OptiMEM medium according to the manufacturer's instructions. The diluted Lipofectamine 2000 and diluted plasmid DNA were combined and allowed to incubate at room temperature to for 15-20 min to allow the formation of the transfection complex. The existing growth medium from the wells was aspirated and replaced with fresh pre-warmed complete medium. After the incubation time, the transfection complex was added drop wise on to the cells with gentle swirling. The cells were incubated along with the transfection complex for 48 h after which the clones were selected for further experiments using 500µg/ml of G418 for 48 h. Once transfected with the

plasmids, the cells were maintained in cell culture medium containing 250 µg/ml of G418 for further experiments. The sub cellular fractions of the transfected breast cancer cell lines were collected as detailed in **Section 2.2.2** and the fractions were *analysed* for TG2 and NFκB expression as well as activity.

2.2.2 Preparation of whole cell lysate, cytoplasmic and nuclear fractions of cells

The cell lines were fractionated into the sub cellular fractions (whole cell lysate, cytoplasmic and nuclear fractions) as established previously (Suzuki, *et al.* 1999). The breast cancer cell lines were seeded and the cell monolayer was washed once with ice cold PBS after which the cells were collected using a cell scraper into 1 ml of PBS. The cells were then centrifuged at 1500 x g 10 min and washed twice with ice cold PBS. The cells were again centrifuged at 2000 x g for 5min at 4^{o}C and the supernatant was discarded. The pellet was re-suspended in 200µl of ice cold Buffer A (1M Hepes/KOH, pH 7.9, 1M KCl, 2mM EDTA, 50mM EGTA, 0.1mM PMSF and 1% (v/v) Protease Inhibitor cocktail) and mixed well by pipetting. The cells in Buffer A were kept on ice for 15 min and then 100 µl of 10% NP-40 was added and vortexed vigorously until the cellular membrane is destroyed. A small amount of the cells and lysis buffer mixture was taken to be used as the whole cell lysate. The samples were then centrifuged at 2500 x g for 5mins at 4^{o}C and the supernatant was collected and used as the cytosolic fraction. The pellet was then re-suspended in 150µl of BL buffer (10mM potassium phosphate, 10mM Tris-HCl, pH 8.0, 0.4M LiCl, 0.1% NP-40, 0.1mM PMSF and 1% (v/v) protease cocktail inhibitor) by pipetting and then vortexed for proper mixing after which the samples were left on ice for 15 min. The nuclear fraction is the supernatant collected by centrifugation of the samples at high speed (≥15,000x g) for 1 h at 4^{o}C. The cytosolic and nuclear fractions were *analysed* for the expression of TG2 and Rel A/p65 by western blot.

2.2.3 Protein Concentration

The protein concentration of the sub cellular extracts were determined using the commercial kit available from Bio-Rad based on the Lowry protein concentration method (Lowry, *et al.* 1951).

Using BSA as a standard, 5µl of BSA at different concentrations (ranging from 0.1-1.5mg/ml) was added to a 96-well plate. 25µl of Reagent A was added to the wells followed by 200µl of Reagent B and incubated for 15min at room temperature. The absorbance was read at 750nm using a plate reader and the values were plotted as a standard graph to aid in determining the protein concentration of the cell samples.

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

After the protein concentration assay (**Section 2.2.3**), the protein extracts that contain the desired amount of protein was dissolved in a 1:1 ratio with 2x strength reducing Laemmli buffer (125mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (v/v) SDS, 10% (v/v) 2-β-mercaptoethanol and 0.004% bromophenol blue) (Laemmli, *et al.* 1970) and boiled to denature for 5 min and then stored at -80^{o}C until needed to be used or else resolved by the SDS-PAGE method. The gels consisted of a 10% (w/v) polyacrylamide separating gel which contained 0.75M Tris 0.2% (w/v) SDS stock solution, pH 8.8 and 3% (w/v) polyacrylamide stacking gel which consisted of 0.2M Tris 0.2% (w/v) SDS stock solution, pH 6.8. The polymerization of the gels were initiated by the addition of freshly prepared 10% ammonium persulphate and N, N, N', N' - Tetramethylethylenediamine (TEMED).

The separating and stacking gels were prepared as indicated in the table below. The separating gels (80 x 60 x 1.5mm) were cast using the Atto-mini gel system and isopropanol was poured on top of the gel to fill the gel system and provide the gel with a flattened upper surface. The gels were then allowed to polymerise for 45 min to 1 h at room temperature.

After polymerization of the separating gel, the upper surface was washed thoroughly using distilled water and the edge of the gels were blotted dry. The stacking gels were prepared and poured between the glass plates and either a 10-well comb or 15-well comb was used to form the sample wells. Following polymerization of the gel, the sample well comb was carefully removed and the wells were washed as well as filled with (25mM) Tris- (192mM) Glycine- (0.1% (w/v)) SDS electrode running buffer, pH 8.5. The desired protein concentration was

loaded into each well. Electrophoresis was conducted at 90V through the stacking gel and then 120V through the separating gel until the bromophenol blue tracking dye is seen to reach the bottom of the separating gel.

Running buffer (pH 8.5)

- 25mM Tris
- 192mM Glycine
- 0.1% (w/v) SDS

Separating Gel

	8% gel	**10% gel**
30% Acrylamide	4.00 ml	5.00 ml
4x Tris HCl/SDS, pH 8.8	3.75 ml	3.75 ml
dH20	7.25 ml	6.25 ml
10% Ammonium Persulphate	0.05 ml	0.05 ml
TEMED	0.01 ml	0.01 ml

Stacking Gel

30% Acrylamide	0.65 ml
Tris HCl/SDS, pH6.8	1.25 ml
dH2O	3.05 ml
10% Ammonium Persulphate	0.025 ml
TEMED	0.005 ml

Table 2.2 Composition of polyacrylamide gels

2.2.5 Western Blotting of Proteins

The proteins previously resolved by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes using a Bio-Rad wet blot system as per previously established protocols (Towbin, *et al.* 1979). The fibre pads, nitrocellulose sheets and electroblotting papers were soaked in ice cold transfer buffer. The gel holder cassette was placed open and the filter pads, pre-soaked in transfer buffer were placed on the black coloured surface of the cassette. The wet electrode paper was then placed on the filter pad, on top of which the gel was placed. The soaked nitrocellulose paper was then laid on top of the gel followed by wet electrode paper and finally the second pre-soaked filter pad. A glass roller was used to remove any trapped air bubbles in the set up. The cassettes were then assembled and inserted into the apparatus used for blotting in such a way that the membrane side of the set up faces the anode electrode. The apparatus was then filled with ice cold transfer buffer. The electro-transfer of the proteins in the gel was performed for 2 h at 200mA. The success of the transfer of proteins on to the nitrocellulose membrane was confirmed by the presence of high molecular weight markers on the membrane.

Transfer Buffer (pH 9.7)

- 48.8mM Tris
- 39mM glycine
- 20% (v/v) methanol

2.2.6 Immunodevelopment of western blots

After transferring the membranes were stained with Ponceau S stain to make sure that the proteins had been transferred correctly onto the membrane. The membrane is then destained using 1x TBS-Tween. After destaining, the membrane was blocked with 5% blocking buffer (5% Marvel in 1xTBS-Tween) probed with different primary antibodies overnight with shaking at 4^{o}C. The next day, the membrane was washed 4 times for 15 min each and then probed with the suitable secondary antibody for 2 h with shaking at room temperature. The membrane is then washed again with 1x TBS-Tween, 4 times for 15 min each. Immuno-detection of the blots was completed using the Amersham enhanced Chemiluminescence (ECL) system kit in accordance to the manufacturers' protocol. The two reagents, A and B were mixed in an equal ratio and 1ml of the mixture was added onto each blot. After 1 min of incubation the membranes were wrapped in cling film and placed in an autoradiography cassette. The exposure of the blot was performed in a dark room using either Amersham Hyperfilm ECL (GE Healthcare, UK) or Kodak BioMax XAR film (Sigma-Aldrich, UK) exposed to the nitrocellulose membranes for varying time lengths depending on the intensity of the emitted signal The films were then developed using GBX developer and fixer (Sigma-Aldrich, UK), after which the films were extensively washed with water before air-drying.

2.2.7 Stripping and re-probing of nitrocellulose membrane

To make sure that the proteins loaded on the SDS-PAGE gel were equal, the primary and secondary antibodies were taken off from the blot using stripping buffer. The nitrocellulose membranes were immersed in stripping buffer and incubated between 50-60^{o}C for 30min in an

oven, with occasional shaking. The stripping buffer was then discarded and the blots were washed four times (15 min each) with 1x TBS-Tween, pH 7.4 at room temperature with gentle agitation. The membrane was then blocked as described above and primary antibodies that will allow determination is equal protein loading (Anti-α-Tubulin and anti-Lamin-A) as well as the appropriate secondary antibodies were applied onto the membrane. The immunodetection of the blots was carried out as described in **Section 2.2.6** using the ECL system.

Stripping buffer

- 65 mM Tris HCl, pH 6.7
- 2% (w/v) SDS
- 100mM 2-mercaptoethanol

2.2.8 Co-Immunoprecipitation

The breast cancer cells were seeded into 60mm petridishes (1x 10^6 cells/ dish), and left to become 60-70% confluent. The next day, the breast cancer cell lines were treated with the various inhibitors as per required. The subcellular fractions of the cell lines were collected as described in **Section 2.2.2**. 250µg of subcellular protein was then pre-cleared with 50µl of Protein A- or G- Sepharose bead slurries on a rocking platform by incubation for 2 h at 4^{o}C. The precleared nuclear were then incubated with 0.5µg of the appropriate antibody for 90 min, with gentle shaking on a rocking platform at 4^{o}C. The immune complexes were then precipitated with 50µl of Protein A or G-Sepharose bead slurries overnight at 4^{o}C on a shaker. The next day, the beads were washed with the nuclear lysis buffer and extracted into 30µl of 2x Laemmli sample buffer by boiling for 5 min. Samples were resolved by SDS gel electrophoresis, transferred onto nitrocellulose membrane and then immunoprobed with the target antibodies (**Sections 2.2.4-2.2.6**).

2.2.9 Cellular localization of TG2 transamidating activity via incorporation of FITC-Cadaverine

The *in situ* TG2 mediated cross links in the breast cancer cell lines were measured by incorporation of the fluorescent primary amine substrate, FITC-Cadaverine into cells and detected using fluorescent microscopy. The breast cancer cell lines (80,000 cells) were seeded into an 8-well chamber slide and incubated overnight. The next day, the cells were exposed to fresh complete medium that contains 0.5mM fluorescein Cadaverine and incubated for 16 h (Nicholas, *et al.* 2003). Following the incubation, the medium was removed and the cells were washed gently three times with PBS, pH 7.4 and then fixed with pre-chilled methanol (-20^{o}C) and then incubated for 15 min at -20^{o}C. The cells were then washed three times with PBS, pH 7.4 and air dried. Drops of Vectashield mounting medium containing DAPI was added onto the cells and subsequently covered with a cover slip and sealed using nail varnish. The slides were then viewed by automated inverted microscope Leica DM1400B fluorescent microscopy and the resulting images were *analysed* using Leica LAS AF Image browser software.

2.2.10 Detection of TG2 using confocal microscopy after immunocytochemistry

The breast cancer cells were seeded into 8-well glass chamber slides (1x10^{5} cells per well) and allowed to attach and become confluent overnight. The next day, the cells were washed thrice (three min per wash) with PBS, pH 7.4. 100µl of 3.7% paraformaldehyde in PBS was added into each well and incubated at 37^{o}C for 20 min to fix the cells. The breast cancer cell lines were then washed with PBS, pH 7.4 three times (three min per wash). 100µl of 0.5% Triton-X-100 in PBS was added into each well and incubated for 15 min to permeablize the cells, after which the cells were washed with PBS, pH 7.4 as describe above. The wells were then blocked with 100µl of 10% horse serum (heat inactivated) in PBS for 2 h at 37^{o}C after which the wells were washed again with PBS, pH 7.4. The mouse anti-human TG2 antibody Cub7402 (1:100 dilution) in 1% horse serum in PBS, at a final volume of 100µl was added into each well and incubated for 45 min at 37^{o}C. The wells were washed thrice with PBS, pH 7.4 (five min per

wash) and then incubated with the secondary anti-mouse FITC (1:200 dilution) in 1% goat serum in PBS for 45 min at 37°C. The cells were washed thrice with PBS. pH 7.4 (five min per wash) and then rinsed briefly in water. The samples were then air dried after which a drop of DAPI in Vectashield mounting medium was added onto the cells and sealed with a cover slip with the help of nail varnish. The slide was stored in the fridge (4°C) until different fields were captures using SP5 TCS II MP Laser confocal microscope made by Leica Microsystems and the resulting images were analysed using Leica LAS AF Image Browser software.

2.2.11 Detection of the cell surface and whole cell lysate TG2 activity via Biotin Cadaverine incorporation into Fibronectin

The transglutaminase activity of TG2 was measured via biotin-cadaverine incorporation into FN as described previously by Jones, *et al.* in 2006 (Jones, *et al.* 2006). 50µl of 5 µg/ml of FN in 50mM Tris-HCl, pH 7.4 was used to pre-coat the 96 well plates and left over night at 4°C. After washing the wells three times with 50mM Tris-HCl, pH 7.4, the wells were blocked with 3% BSA in PBS, blocking buffer, pH 7.4 for 1 h at 37°C. To determine the cell surface TG2 activity of the breast cancer cells, $2x10^4$ cells in 100µl of serum free medium was seeded into each well and incubated at 37°C with 5% (v/v) CO_2 for 2 h in the presence of 0.1 mM biotin-X-cadaverine, 1 mM DTT and 10mM $CaCl_2$. To measure the whole cell lysate activity of TG2 within the breast cancer cell lines, 50µg of whole cell lysate protein (obtained as described in **Section 2.2.2**) was diluted in serum free medium to a final volume of 100µl along with 0.1 mM biotin-X-cadaverine, 1mM DTT and 10mM $CaCl_2$. 50 ng/well of guinea pig liver TG2 in serum free medium containing 1mM DTT, 0.1 mM biotin-X-cadaverine, 10mM EDTA (negative control) or 10mM $CaCl_2$ (positive control) was used as the control samples. After incubation, for 2 h, the reaction was stopped by adding 100µl of 2 mM EDTA in PBS, pH 7.4 into each well and the cells were detached using 100µl of 0.1 % (w/v) deoxycholate in 2mM EDTA in PBS, pH 7.4 for 10 min at room temperature, with gentle shaking. the supernatant was discarded and the remaining fibronectin layer was washed with 50mM Tris-HCl pH 7.4, three

times and then blocked with blocking buffer for 30 min at 37°C. The biotin-X- cadaverine incorporated into FN was incubated with Extravidin-peroxidase conjugate (Sigma-Aldrich, UK) diluted in blocking buffer (1:1000) for 1 h at 37°C. The wells were again washed with 50mM Tris-HCl, pH 7.4, three times and the reaction was developed by the addition of 100µl of developing buffer containing SIGMAFAST OPD (o-Phenylenediamine dihydrochloride) tablets in 20mls of distilled water to yield a ready to use buffered solution that contains OPD and urea hydrogen peroxide. The development of colour was terminated using 50µl 3N HCl and the absorbance was read at 490nm using a Spectrafluor plate reader. The results are expressed as percentage of TG2 activity with respect to the positive control samples.

2.2.12 NFκB SEAPorter Assay Kit

The NFκB SEAPorter assay kit has been designed to measure NFκB activation using the SEAP protein secreted into the culture medium as a read out. The concentration of SEAP found in the medium is directly proportional to the NFκB activity levels within the cell lines. The assay kit contains a pNFκB/SEAP plasmid which is known to express the SEAP protein, under the regulation of the NFκB promoter. The NFκB SEAPorter assay was performed as per the manufacturers' protocol (Cullen & Malim, 1992)

2.2.12.1 Cell culture and transfection using the NFκB SEAPorter Assay Kit

Approximately $5x10^5$ cells were seeded per well of a 6-well tissue culture plate containing 2ml DMEM supplemented with 10% FBS, non-essential amino acids. The cells were then incubated at 37°C in a CO_2 cell culture incubator, overnight, so that the cells become 70-80% confluent. The next day, 4µg of plasmid DNA (pNFκB/SEAP plasmid) was diluted into 250µl of serum free medium that contained non-essential amino acids and for each transfection, 10µl of Lipofectamine 2000 was diluted into serum free medium. The two above solutions were mixed and then incubated at room temperature for 15-20 min. The existing medium in the plate was removed and replaced with 1.5ml DMEM with 10% FBS. 500µl of the plasmid-lipid mixture was added onto the plate drop by drop with gentle mixing. The cells were then incubated at

37°C in CO_2 incubator for 48 h. After this incubation, the cells were treated with various inhibitors for the desired time course. Subsequent to the treatments, the cell supernatant was collected for the SEAP assay.

2.2.12.2 Measurement of SEAP

The standards of SEAP that were provided with the assay kit were used as the controls and allowed the quantitative measurement of SEAP protein in the 96-well micro titre plate. The supernatant of the transfected samples were diluted (1:10) using the dilution buffer provided along with the kit. 10µl of both the SEAP standards and the samples were added into each well in triplicates. Each well containing either a sample or a standard was supplemented with 10µl of water. The micro titre plate was then sealed and incubated at 65°C for 30 min to activate any endogenous alkaline phosphatase present in the supernatant. This allowed precise and accurate quantification of SEAP. The plate was then spun briefly to ensure that all of the liquid returned to the bottom of the well. 100µl of 1mg/ml PNPP substrate solution was added into each well and incubated at room temperature for 30 min, after which the absorbance reading was taken at 405nm using the Spectafluor plate reader. The results have been represented with respect to the concentration of SEAP (standard) in ng/ml present in the media after accounting for the dilution factor.

2.2.13 Cell viability analysis using XTT Assay

The XTT assay was used to measure the drug sensitivity of the breast cancer cell lines. The XTT assay quantifies the viable cell numbers in the presence of chemotherapeutic drugs such as doxorubicin. The XTT assay is based on the principle that the cleavage of the yellow tetrazolium salt, XTT to form an orange formazen dye only transpires in metabolically viable cells and was performed in accordance to the manufacturers' instructions (Roche Applied Sciences, UK). The formazen dye formed can be directly quantified using a Spectrafluor plate reader. The breast cancer cell lines were seeded into 96- well plates (3000 cells per well) at a volume of 100µl with complete medium and left over night in a humidified atmosphere at 37°C

and 5% CO_2. The cells were incubated with the relevant treatments for a time course depending on the treatments and cell lines used. To perform the cell proliferation assay, 5 mls of XTT labelling agent was mixed with 0.1ml of electron coupling reagent. After the treatments were performed on the breast cancer cell lines, 30µl of the mixed XTT reagents, at a final concentration of 0.3mg/ml, were added into each well and incubated for 4 h in a humidified atmosphere at 37^{o}C and 5% CO_2. Following the incubation period, the formation of orange formazen dye was formed. The absorbance of the samples was measured using a Spectrafluor plate reader. The wavelength used to measure the formazen product is 450m, and the reference wavelength used was 750nm. The background wavelength of 750nm was subtracted from the absorbance values obtained from 450nm. The untreated cell lines were assumed to be 100% viable and the subsequent percentage of viable cells was determined.

2.2.14 TGF-β1 Activity Assay

The relative TGF-β1 activity of the breast cancer cell lines was detected using the Human/ Mouse TGF beta1 ELISA Ready-SET-Go (2nd generation) from eBiosciences. The assay was used to detect the endogenous human TGF beta 1 using the medium supernatant collected (Fantini, *et al.* 2006). A micro-titre 96 well plate was coated with 100µl/well of capture antibody diluted in coating buffer according to the instructions provided with the kit. The plate was then sealed and incubated overnight at 4^{o}C. The next day, the wells were aspirated and washed five times with 1x wash buffer (approximately 250µl of wash buffer per well) and allowed to soak for at least 1 min during each wash and blotted to remove any residual buffer. The wells were then blocked with 200µl/well of 1x assay diluent and incubated at room temperature for 1 h. The wells were then aspirated and washed as per described above. Since animal serum that is used in the culture medium may contain very high levels of latent TGF beta 1, the latent TGF beta 1 was activated to the immunoreactive form by acidification and then neutralized subsequently. For every 100µl of the sample (medium supernatant), 20µl of HCl was added and incubated at room temperature for 10 min and then subsequently

neutralized with 20µl of 1N NaOH, which would dilute the sample to a factor of 1;1:4. 100µl of diluted acid activated sampled was added into wells in triplicates. The standards provided with the assay kit were also diluted using 1x assay diluent and 100µl of the standard was added into the wells. Following this, the plate was sealed and incubated overnight at 4^{o}C to ensure maximum sensitivity of the assay. The next day, the wells were washed five times with 1x wash buffer and 100µl of detection antibody diluted in 1x assay diluent was added into each well and then incubated for 1 h at room temperature. The wells were washed as described previously after which 100µl of Avidin-HRP diluted in 1x Assay diluent was added to each well of the micro titre plate and incubated at room temperature for 30 min The wells were washed 7 times, with a soaking period of 2 min between washed. 100µl of substrate solution was added into each well and incubated at room temperature for 15 min and finally the reaction was stopped using 50µl of stop solution. The micro titre plate was then read at 450nm.

2.2.15 Polymerase chain reaction

2.2.15.1 Preparation of RNA

TRIZOL Reagent was purchased from Invitrogen (Life Technologies) and used to rapidly isolate the total RNA from adherent cell monolayer as instructed in the manufacturers' protocol (Chomczynski & Mackey, 1995). Approximately, 5x10^{6} breast cancer cell lines were seeded per well into 6-well plates and allowed to become 80-90% confluent. Once the required confluency was attained, the cell monolayer was washed once with ice cold PBS. The cells were lysed directly in the tissue culture dish by adding 1 ml of TRIZOL reagent and scraped with the cell scraper. The cell solution in TRIZOL was passed several times through a pipette and vortexed thoroughly. To the cell lysate, 0.2 mls of chloroform was added and vortexed vigorously for 15 seconds after which the samples were incubated for 2 to 3 min at room temperature. The samples were then centrifuged at 12,000x g for 15 min using a refrigerated centrifuge (4^{o}C). Subsequent to centrifugation, the lysate separates into a colourless upper aqueous phase, an interphase and the red phenol-chloroform phase which is the lower phase.

The RNA that needs to be extracted is present in the aqueous phase, exclusively. The upper aqueous phase was carefully separated and collected in a fresh tube. The volume of the aqueous phase was measured and mixed with 0.5 mls of isopropyl alcohol and used to precipitate the RNA. The samples were then incubated between 20-30^{o}C for 10 min and then centrifuged at 12,000x g for 10 min in a refrigerated centrifuge (4^{o}C). The RNA precipitated is seen as a gel like pellet on the side of the tube. Following centrifugation, the supernatant was completely discarded and the RNA pellet was washed once with 1ml of 75% ethanol. The samples were vortexed and again centrifuged at 7,500x g for 5 min at 4^{o}C. The supernatant was discarded and the ethanol wash step was repeated once more. The RNA pellet was then air dried for 15 min and then subsequently dissolved in DEPC-treated RNase free water by gentle pipetting. The dissolved RNA samples were analysed spectrophotometrically using a Nanophotometer (IMPLEN, Munchen, Germany) to determine the purity of the RNA (A260/A280) obtained as well as the concentration of RNA in the sample.

2.2.15.2 Setting up of PCR

After the extraction of RNA from the breast cancer cell lines, the PCR reaction was performed using Taq PCR Core kit from Qiagen according to the manufacturer's instructions. The solutions were initially kept on ice after being thawed completely. The reaction mix for the PCR was made up as:

- PCR buffer - 5µl (1x)
- Forward Primer - 0.5µM
- Reverse Primer - 0.5µM
- dNTP mix - 1µl (200µM)
- Taq DNA polymerase - 0.25µl (2.5 units/ reaction)
- Template DNA - 100ng
- Distilled water to make the final reaction volume 50µl

All of the solutions, with exception of the template DNA was mixed depending on the number of PCR reactions that need to be conducted. The master mix was mixed thoroughly by pipetting and then distributed among pre-chilled PCR tubes. The template DNA was then added into the individual PCR tubes that contained the master mix. A typical PCR thermal cycler program was set up as described in the table below:

Denaturation	15 min	95°C
Annealing	1 min	Depending on the primers used (Table 2.2)
Extension	1 min	72°C
Number of cycles	40 cycles	
Final extension	10 min	72°C
Hold	Infinite time	4°C

Table 2.3 Typical PCR program set up

The samples were always analysed in triplicate. After the PCR reaction was performed, the PCR samples were mixed with the loading dye and run on a 1.5% agarose gel in TAE buffer and analysed by gel electrophoresis.

2.2.16 Human Rel A Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP assay) is a very useful technique to identify target genes of specific transcription factors, study mechanisms of gene expression and categorize proteins that regulate transcription as well as genetic networks. This assay will allow determination if a particular DNA-protein interaction is present at a certain location, time point and treatment conditions. According to the manufacturers' instructions the DNA-protein complexes were fixed using formaldehyde cross linking, the chromatin was sheared by sonication and the protein-DNA complex of interest was immunoprecipitated using specific antibodies. The DNA bound to protein fragments were then identified by PCR (Solomon & Varshavsky, 1985)

2.2.16.1 Assay procedure

The ChIP assay was performed to determine the interaction between the dimeric Rel A/p65 forms and DNA. The breast cancer cells were seeded into 60mm petridishes and left to become 80-90% confluent. Once the cells reached the desired confluency, the cells were fixed by adding 37% (w/v) formaldehyde to a 1% (w/v) final concentration (40µl of formaldehyde for 1ml of complete media) and the samples were kept on the shaker for 15 min at room temperature. The formaldehyde was quenched by adding 1 M glycine to a final concentration of 125 mM (141µl of glycine for 1 ml of complete media). The samples were kept on the shaker for 5 min at room temperature, pelleted and the media was removed. The cell pellet was resuspended in 500 µl of Lysis Buffer, resuspended by pipetting and then incubated on ice for 10 min. The samples were then sonicated to shear chromatin (Output 3, 10 cycles, and 30 seconds "ON", samples on ice for 2 min before next cycle). The lysates were then centrifuged for 10 min at 12,000 x g at 4^{o}C. The supernatant was collected and the pellet was discarded. The supernatant by diluted adding 1 mL of Dilution Buffer and 5 µl of the Anti-RelA Antibody or Normal Sheep IgG (control) to the samples and incubated overnight at 4^{o}C on the shaker. The next day 50µl Protein A-Sepharose bead slurry was added to the samples and incubated for 30 min at 4^{o}C with gentle shaking. The beads were then collected by centrifugation at 12,000 x g for 1 min. A series of washes were then performed with the four wash buffers provided with the assay kit by adding 1ml of the wash buffer into the beads and pipetting up and down between each wash. The beads were then collected by centrifugation at 12,000xg for 1 min. After the last wash, 100µl of Chelating Resin Solution was added directly to the beads and boiled for 10 min in a water bath. The samples were then centrifuged at 12,000xg for 1 min at room temperature and the supernatant was transferred into a clean tube. The final volume of the samples collected was approximately 60-80µl. To clean up and concentrate the obtained DNA, 0.1 volumes of 3M sodium acetate buffer solution, pH5.2 and 0.7 volumes of isopropanol was added to collected samples. The samples were mixed well and centrifuged at 15,000xg for 30 min after which the supernatant was discarded. The DNA pellet was then rinsed in 1.5ml of 70% ethanol and

centrifuged as previously for 10 min. The supernatant was discarded and the pellet was air dried until it was free of ethanol. The DNA pellet was then resuspended in 100µl of endotoxin free water and the concentration and purity of the ChIP samples was determined using a Nano Photometer.

2.2.16.2 PCR analysis of ChIP samples

The protein-DNA complexes obtained from chromatin immunoprecipitation were used in subsequent PCR reactions with appropriately designed primers. The *p21* promoter primers provided along with the kit was used as the positive control. The PCR was carried out for the -κB site on the *TGM2* gene promoter and *GAPDH* as specified in **Section 2.2.15.2**.

2.2.17 Statistical analysis

Any statistically significant differences between data sets in experiments were calculated using ANOVA and *post hoc* tests were performed on the significant data

Chapter 3: Relationship between TG2 and chemoresistance in breast cancer cells

3. Relationship between TG2 and chemoresistance in breast cancer cells

3.1 INTRODUCTION

Metastasis and chemoresistance are known to be the major hurdles in the successful treatment of breast cancer. A very common feature in metastatic and drug resistant tumour cells is that they display amplified resistance to apoptosis. Chemotherapy is a crucial component in the treatment regime for breast cancers. Despite a rapid reduction in tumour mass subsequent to chemotherapeutic cycles, cancers may persist and develop distal metastasis afterwards (Gong, *et al.* 2010). Data exists supporting the hypothesis that the expression of particular oncogenes or aberrant expression of tumour suppressor genes encourages aggressiveness, tumour cell growth and also relative expression of drug resistance. In addition, metastatic cancers demonstrate elevated resistance to radiation and drug therapies than their primary tumour counterparts (Fesik, 2005). Understanding the molecular factors responsible for chemoresistance and metastasis of breast cancer cells is therefore essential for the development of successful and novel therapeutic approaches.

It is very reasonable to contemplate that the over expression of TG2 signifies a link between metastatic and chemoresistant tumour cells. Many cancer cell lines, such as malignant melanoma, glioma, lung adenocarcinoma, breast carcinoma, pancreatic ductal adenocarcinoma, prostate carcinoma, ovarian carcinoma and prostate carcinoma, chosen for drug resistance, express high levels of TG2 (Verma & Mehta, 2007a). In the same way, metastatic cancer cells derived from patients with malignant melanoma (Fok, *et al.* 2006), breast cancer (Mehta, *et al.* 2004) and ovarian cancer (Hwang, *et al.* 2008); demonstrate a considerable increase in TG2 levels over their primary tumour cells. Reduction of or inhibiting TG2 in cancer cells has been demonstrated to be concurrent with susceptibility to cell death induced by chemotherapeutic drugs and also inhibit invasion (Yuan, *et al.* 2007). In parallel with these results, increased expression of TG2 in ovarian carcinoma enhanced cell adhesion to FN and promoted cell

migration (Satpathy, *et al.* 2007). In ovarian cancer patients, increased TG2 expression in tumour cell samples was associated with considerably worse prognosis in both multivariate and univariate analysis (Hwang, *et al.* 2008). Increased expression of TG2 in pancreatic tumour cells has been related to lymphovascular invasion and nodal metastasis (Verma, *et al.* 2006). All of the above findings indicated that presence of TG2 in cancer cells was abnormal and concerned with promoting a metastatic and a chemoresistant phenotype.

Previous studies undertaken have shown that drug resistance and metastatic breast cancer cells, expressed very high levels of TG2 which may imply that TG2 contributes to the development of a chemoresistant phenotype. A further understanding of TG2 mediated cellular interactions with the ECM and the signalling pathways induced in response to any such interaction may provide new targets for the treatment and intervention of metastatic and chemoresistant breast cancers (Mangala, *et al.* 2007)

Ai *et al.* in 2008 hypothesized that the epigenetic silencing of the *TGM2* gene in breast cancer cells could serve as a marker for chemotherapeutic drug sensitivity (Ai, *et al.* 2008). The down regulation of TG2 using siRNA or antisense resulted in the reversal of chemoresistance, while the ectopic expression conferred resistance to drugs in melanoma, glioblastoma, ovarian and breast cancer cells (Verma & Mehta, 2007a). TG2 expression can protect cancer cells from both apoptosis and autophagy. Inhibiting TG2 expression using siRNA could also result in the induction of autophagy in cancer cell lines and eventually cell death. Inhibiting the PKCδ activity by siRNA or rottlerin has been associated with a parallel decrease in the expression of TG2 and induction of autophagy in pancreatic cancer cells (Akar, *et al.* 2007)

The aim of the work contained in this chapter was to identify the potential involvement of TG2 expression and activity in chemoresistance against doxorubicin in breast cancer cells. Further studies were conducted to find out whether supplementing chemotherapeutic agents with TG2 cross linking inhibitors or silencing TG2 expression could decrease the chemoresistance phenotype.

3.2 METHODS:

3.2.1 TG2 whole cell lysate and cell surface activity assay

Cells ($2x10^4$ cells/ml and add 100µl/well) or 50µg of whole cell lysate protein, along with 0.1mM Biotin-X- Cadaverine, 10mM $CaCl_2$ or 10mM EDTA and 1mM DTT was seeded into 96-well plates pre-coated with 5µg/ml of Fibronectin and allowed to incubate for 2 h at 37^oC. The TG2 whole cell lysate and cell surface activity of the breast cancer cells in the presence of inhibitors was measured as detailed above (**Section 2.2.11**).

3.2.2 *In situ* TG2 activity using FITC-Cadaverine incorporation:

To investigate the *in situ* TG2 activity within the breast cancer cells with varying expressions of TG2, FITC Cadaverine was incorporated into the breast cancer cell lines and then subsequently analysed using Fluorescence microscopy (**Section 2.2.9**)

3.2.3 Immunostaining of TG2:

The breast cancer cells were stained using anti-TG2 Cub 7402 antibody (1:100) and anti-mouse FITC (1:200) secondary antibody was used. The cells were then subsequently analysed using confocal microscope (**Section 2.2.10**)

3.2.4 Cell viability analysis:

Breast cancer cell lines treated with TG2 inhibitors (R283, R294 and Z-DON) or TG2 siRNA and doxorubicin for time points of 24, 48 and 72 h culture periods were incubated with XTT reagents (Roche, UK) and the signals were detected using plate reader at 490 and 750 nm (background reading, which was erased from the absorbance 490nm) as described in detail in **Section 2.2.13**.

3.2.5 SiRNA transfection:

TG2 specific targeting siRNA was used to inhibit the expression of TG2 in breast cancer cells using Lipofectamine 2000 (Invitrogen) as the transfection reagent. Two different siRNA sequences that targeted human TG2 was obtained from Qiagen. The sequences are listed below:

TGM2-1 CCGCGTCGTGACCAACTACAA (denoted as (i))

TGM2-6 CACAAGGGCGAACCACCTGAA (denoted as (ii))

A stock of lyophilized siRNA was dissolved in 100µl of sterile RNase-free water to obtain a 10µM solution and then stored at -20^{o}C. The transfections were performed according to the manufacturer's protocol. Prior to transfection, breast cancer cells, were seeded into plates overnight to reach a confluency of 70-80%. The next day, the cells were washed with serum free medium and replaced with pre- warmed fresh complete growth medium. The siRNA were diluted in serum free medium to reach a final concentration of 100nM. After pre-incubation with Lipofectamine 2000 (Invitrogen) for 10-15 min, the transfection complex containing Lipofectamine 2000 and TG2 siRNA was added drop wise onto the cells seeded in the plates, along with gentle swirling to make sure that the transfection complex is uniformly distributed. 48 h after transfection, further experiments were performed on the cell lines.

3.2. 6 Detection of siRNA transfection efficiency:

After 48 h of incubation with TG2 targeting siRNA or negative control siRNA with breast cancer cells, the whole cell lysate, cytoplasmic and nuclear fractions were collected and the protein concentration was detected as described in **Sections 2.2.2-2.2.3**. Western blotting was conducted to detect the presence of TG2 using mouse anti-TG2 monoclonal antibody (Cub 7402) (1:1000) and anti-mouse secondary antibody (1:1000) dilution (**Sections 2.2.4-2.2.6**). Signals were detected using ECL system kit.

3.3 RESULTS:

3.3.1 Development of doxorubicin resistant cell lines

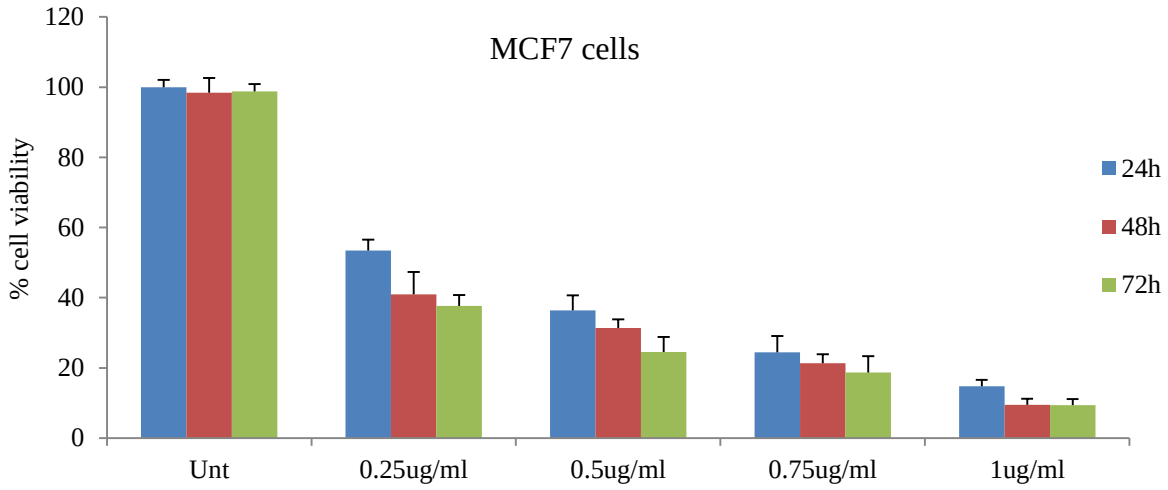
3.3.1.1 Cell viability analysis of TG2 null breast cancer cell lines in the presence of doxorubicin

The TG2-null MCF7/WT, SKBR3/WT and T47D/WT breast cancer cell lines were treated with increasing concentrations of doxorubicin (0.25, 0.5, 0.75 and 1μg/ml) in 96-well plates for time points of 24, 48 and 72 h. The XTT assay which is designed to spectrophotometrically quantify viable cell numbers on treating the cells with cytotoxic agents was applied as described previously in **Section 2.2.13**.

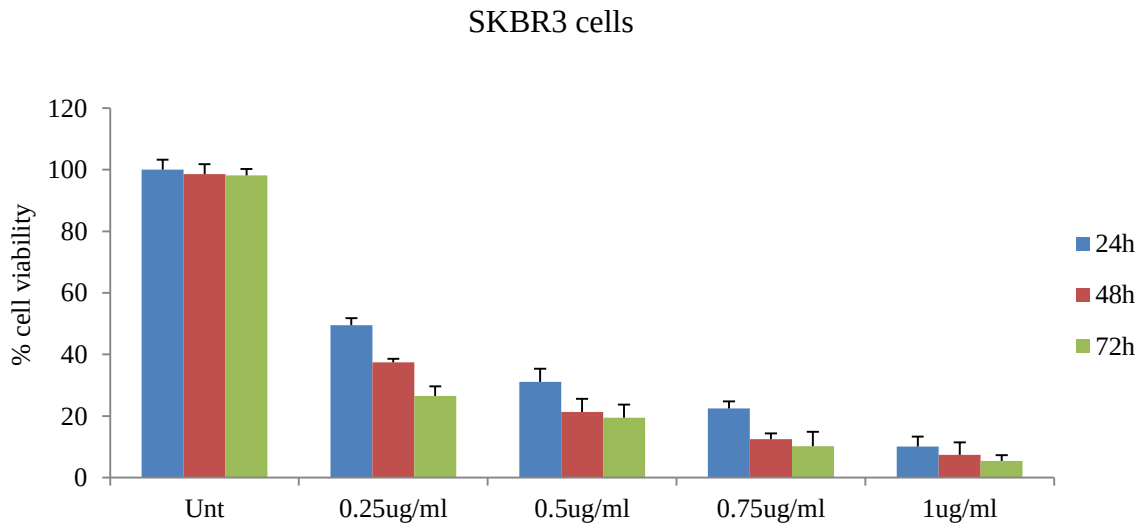
Earlier studies have shown that low or null- TG2 breast cancer cell lines were more chemo susceptible to even very low concentrations of doxorubicin (Verma & Mehta, 2007a/2007b).In agreement, the MCF7/WT, SKBR3/WT and T47D/WT breast cancer cell lines were demonstrated to be chemosusceptible to doxorubicin which might be attributed to the absence of endogenous TG2 in these breast cancer cell lines.

Treating the MCF7 cells with increasing concentrations of doxorubicin, demonstrated an initial decrease in the viability of the cells over the treatment time period (Figure 3.1 A). Initially, the susceptibility of the T47D to 0.25μg/ml of doxorubicin was less compared to the SKBR3 cells; however successive increases in the concentration of doxorubicin to 0.5μg/ml of doxorubicin rendered both the T47D and SKBR3 breast cancer cell lines equally susceptible to the chemotherapeutic agent (Figure 3.1 B & C).

(A)


MCF7 cells
% cell viability
120
100
80
60
40
20
0
Unt
0.25ug/ml
0.5ug/ml
0.75ug/ml
1ug/ml
24h
48h
72h

(B)


SKBR3 cells
% cell viability
120
100
80
60
40
20
0
Unt
0.25ug/ml
0.5ug/ml
0.75ug/ml
1ug/ml
24h
48h
72h

(C)

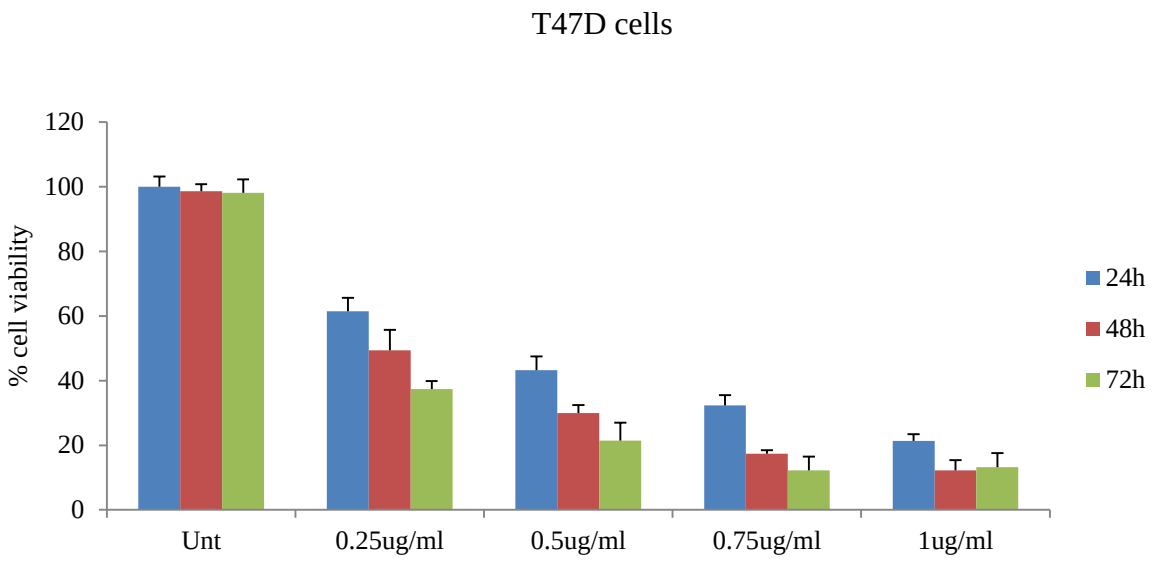


Figure 3.1 Cell viability analysis of MCF7/WT (A), SKBR3/WT (B) and T47D/WT (C) cells in the presence of increasing concentrations of doxorubicin.

The MCF7/WT, SKBR3/WT and T47D/WT cells (3000 cells/ well of a 96 well plate) were treated with increasing concentrations of doxorubicin (0.25, 0.5, 0.75 and 1µg/ml). After 4, 48 and 272 h of incubation with doxorubicin, the XTT assay was performed and the absorbance was read at 490nm as well as 750nm using a Spectrafluor plate reader (as introduced in Section 2.2.13). The data shown above is mean percentage of viable cells + SEM with respect to the untreated cells (taken as 100% viability) of independent experiments (n=3).

3.3.1.2 Western Blot analysis of TG2 in the presence of increasing concentration of doxorubicin

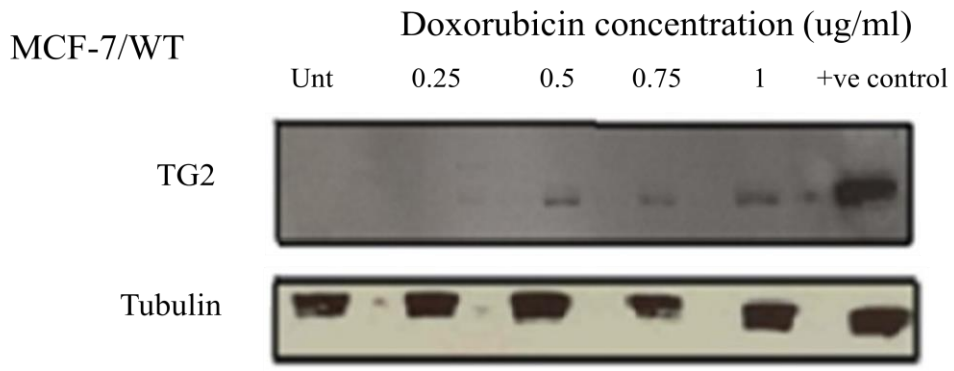
The MCF7/WT, SKBR3/WT and T47D/WT were cultured initially in the lowest concentration (0.25µg/ml) of doxorubicin and half of the surviving cells were lysed and separated into their cytoplasmic and nuclear fractions and the rest of the cells were grown in the next increasing concentration of doxorubicin (0.5µg/ml). This was repeated until the final cells obtained were resistant and able to survive in 1µg/ml of doxorubicin. The surviving cells in 1µg/ml of doxorubicin were termed MCF7/RT, SKBR3/RT and T47D/RT. The cellular fractions collected were *analysed* by Western Blotting using anti-TG2 antibody (Cub 7402; TG100) (1:1000) as described in **Section 2.2.4-2.2.6**. The MDA MB 231 Clone 16 cells that are known to express high levels of TG2 were used as the positive control for the western blot experiments.

The western blot data indicated a clear gradual increase in the TG2 expression observed in the MCF- cells (Figure 3.2 A). The cytoplasmic fractions of the MCF7 cells treated with the initial concentrations of (0.25µg/ml) doxorubicin demonstrated no detectable TG2 expression, which was similar to the parental MCF7 cell line. From concentrations of 0.5µg/ml onwards, the expression of TG2 was observed. The MCF7 cells resistant to 1µg/ml of doxorubicin (MCF7/RT), showed significant TG2 expression. This seems to suggest that in the presence of escalating concentrations of doxorubicin, chemoresistance of the breast cancer cell lines increased in the MCF7 breast cancer cell line concurrent with increase in TG2 levels. These results suggest that TG2 may be responsible for rendering MCF7 cells resistant to chemotherapeutic agents such as doxorubicin.

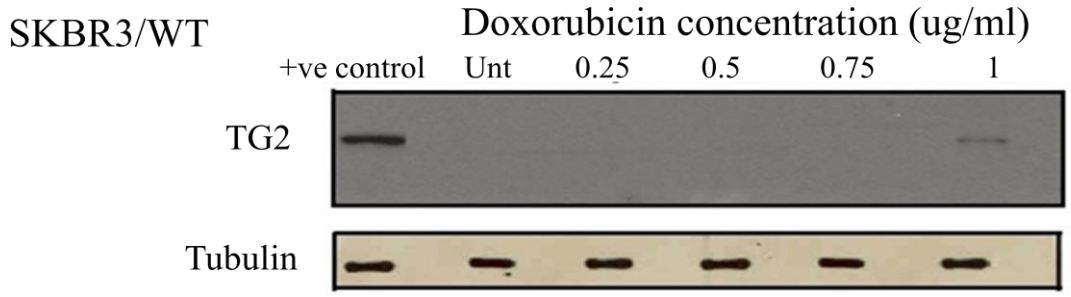
With the SKBR3 cells, no detectable TG2 expression was observed in the cell lines obtained when resistant up to 0.75µg/ml of doxorubicin. The SKBR3/RT cells that are resistant to 1µg/ml of doxorubicin (Figure 3.2 B), demonstrated a slight expression of TG2 in their cytoplasmic fractions. On the other hand, the T47D cells that are resistant to 1µg/ml of doxorubicin showed no increase in their TG2 expression (Figure 3.2 C). This could suggest that the mechanism of chemoresistance within the T47D/RT cell line functions via a pathway that is

independent of TG2 activity and expression. The above findings seem to indicate the mechanism of chemoresistance inculcated by TG2 in breast cancer cell lines may be dependent on numerous other cell characteristics and could be specific to cell type. Also, since TG2 expression in the SKBR3 cells was only observed at 1μg/ml but the MCF7 cells increased their TG2 expression at lower concentrations (0.5μg/ml) of doxorubicin, other survival pathways might also be at play in these SKBR3 cells cultured in low concentrations of doxorubicin.

(A)



(B)



(C)

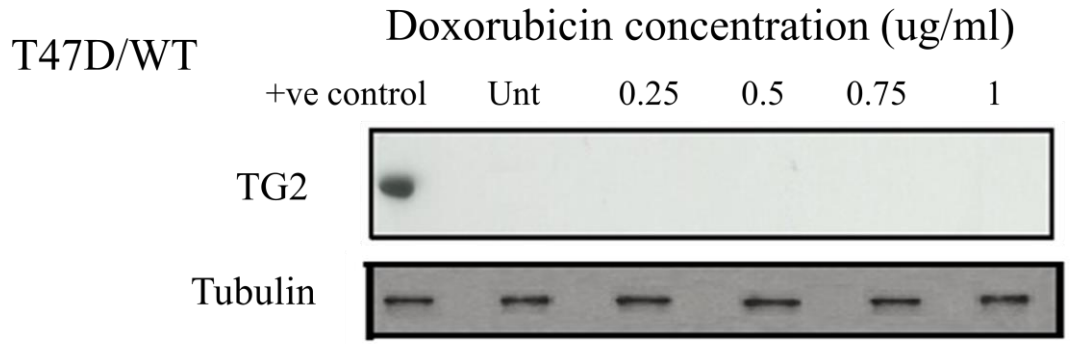


Figure 3.2 Detection of TG2 expression in MCF7, SKBR3 and T47D cells cultured in the presence of increasing concentrations of doxorubicin using western blot

The MCF7 (A), SKBR3 (B) and T47D (C) (2x10^6 cells/ 100mm petridish) were cultured from the lowest to the highest concentration of doxorubicin, where the surviving cells from the lower doxorubicin concentrations were subsequently cultured in higher concentrations, until the final

surviving wild type breast cancer cells were resistant to 1μg/ml of doxorubicin (Section 3.3.1). The cells were then fractionated into the whole cell lysate fractions (Section 2.2.2) and analysed by western blotting and detected using anti-TG2 antibody (Cub 7402; TG100) (1:1000) and anti-α-Tubulin for equal loading. The positive control used in the experiments was MDA MB 231 Clone16 cells, which are known to express very high levels of TG2 protein (Mehta, *et al.* 2004).

3.3.2 Chemoresistant breast cancer cell lines have high TG2 expression and activity

3.3.2.1 Detection of cell surface and whole cell lysate TG2 activity in the presence of TG2 activity inhibitors

Cells surface and whole cell lysate TG2 activity was measured using the Biotin-X- Cadaverine incorporation assay as described in **Section 2.2.11**. The MDA MB 231 WT, MCF7/WT, SKBR3/WT, T47D/WT and T47D/RT lack TG2 activity in their whole cell lysates. The MDA MB 231 Clone 9 (Mehta, *et al.* 2004) and SKBR3/RT cells illustrated very low whole cell lysate TG2 activity, while the MDA MB 231 Clone 16 (Mehta *et al.* 2004) and MCF7/RT cells demonstrated fairly high WCL TG2 activity. Treating the TG2-null breast cancer cell lines with TG2 activity cell permeable inhibitors, R283 and Z-DON as well as cell impermeable inhibitor, R294, did not show any change in the TG2 activity status in these cells. The SKBR3/RT, MDA MB 231 Clone 9, MDA MB 231 Clone 16 and MCF7/RT cells, on being treated with the above TG2 inhibitors, displayed a decrease in TG2 whole cell lysate activity (Figure 3.3).

With respect to the cell surface TG2 activity of these breast cancer cell lines, the MDA MB 231 WT, MDA MB 231 Clone 9, MCF7/WT, SKBR3/WT, SKBR3/RT, T47D/WT and T47D/RT illustrate no detectable TG2 activity on their cell surface, which is concurrent with the TG2 whole cell lysate activity data obtained (Figure 3.4)

The MDA MB 231 Clone 16 cells that demonstrated high TG2 whole cell lysate activity also had cell surface TG2 activity. As depicted in Figure 3.4, treating the MDA MB 231 Clone 16 cells with R283, R294 and Z-DON, led to a significant decrease, in the cell surface TG2 activity of the Clone 16 cells. On the other hand, no detectable cell surface TG2 activity was observed in the MCF7/RT cells (Figure 3.4)

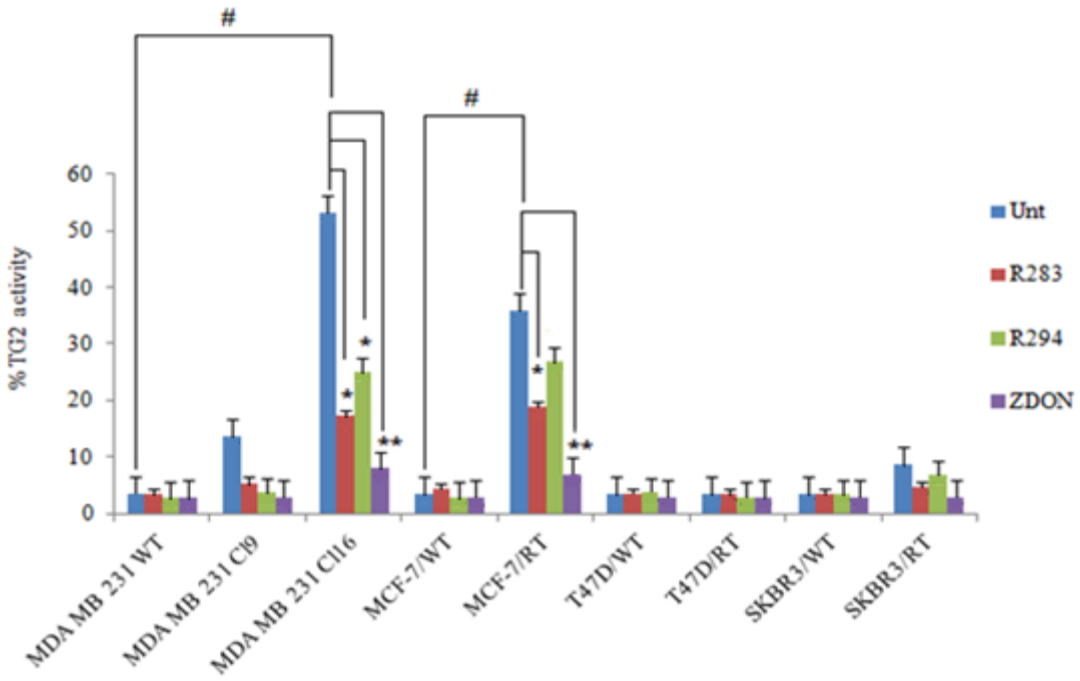


Figure 3.3 Detection of TG2 activity in whole cell lysates of breast cancer cell lines in the presence of TG2 activity inhibitors.

*The levels of TG2 activity was analysed by Biotin-X- Cadaverine incorporation into fibronectin. The breast cancer cell lines were treated with the TG2 inhibitors, R283 (500µM), R294 (500µM) and Z-DON (50µM) for 72h and analysed as described in Section 2.2.11. The positive control (50 ng/ml gplTG + 10mM $CaCl_2$) was taken as 100% TG2 activity (1.1893 ± 0.12739). Data shown is the mean percentage of TG2 activity + SEM from 3 independent experiments, with respect to the positive control (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * p<0.05; ** p<0.01 between untreated and inhibitor treated cells. # p<0.01 between cell groups.*

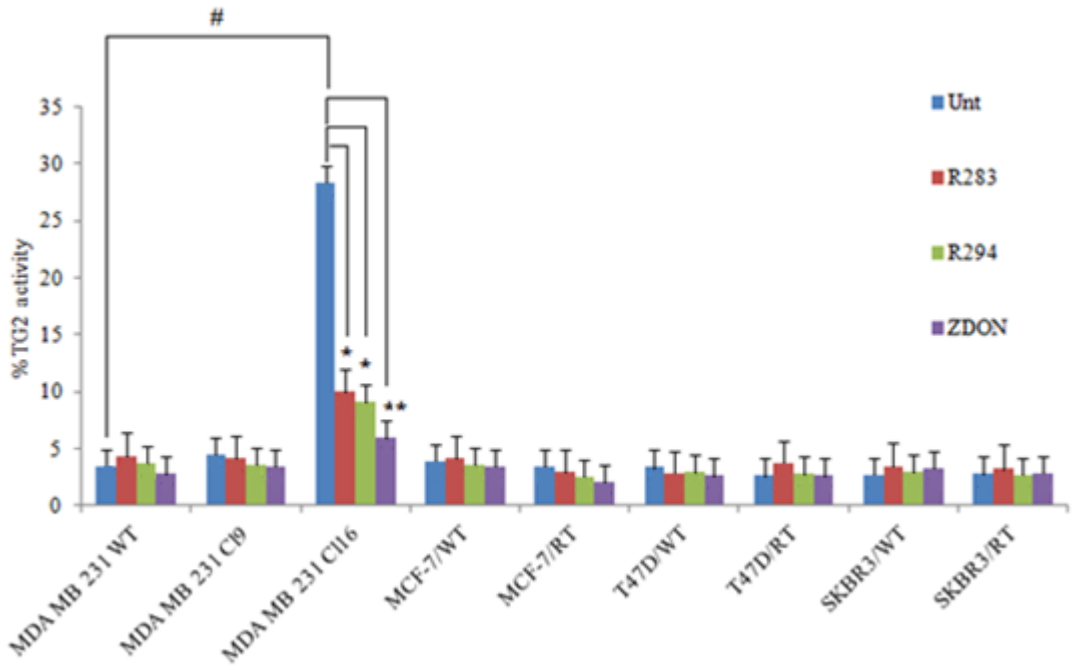


Figure 3.4 Detection of cell surface TG2 activity in breast cancer cell lines.

*The breast cancer cell lines were analysed for the cell surface TG2 activity in the presence of TG2 activity inhibitors as described in Section 2.2.11. Positive control (50 ng/ml gplTG + 10mM $CaCl_2$) was taken to be 100% TG2 activity (1.21874±0.19475). Data shown is the mean percentage of TG2 activity + SEM from 3 independent experiments, with respect to the positive control (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * $p<0.05$; ** $p<0.01$ between untreated and inhibitor treated cells.*

3.3.2.2 Detection of TG2 expression in breast cancer cells after exposure toTG2 activity inhibitors

The breast cancer cell lines were treated with TG2 inhibitors, R283, R294 and Z-DON, after which the whole cell lysate, cytoplasmic and nuclear fractions of the cells were collected as described in **Section 2.2.2**. 50µg of protein of each fraction was collected using Lowry protein concentration (**Section 2.2.3**) and analysed by Western Blot using anti-TG2 antibody (Cub 7402; TG100) to determine TG2 expression levels with the breast cancer cells. The MDA MB 231 WT, MCF7/WT, SKBR3/WT and T47D/WT cells did not exhibit any TG2 protein expression in their whole cell lysate fractions (Figures 3.5). The MDA MB 231 Clone 9 and MDA MB 231 Clone 16 illustrate low and high expression of TG2 respectively in their whole cell lysate fractions (Figure 3.5). On treating the TG2 expressing cells with the cell permeable activity inhibitors, R283 and Z-DON, a significant decrease was observed in the cytoplasmic TG2 expression of these breast cancer cells (Figure 3.6). The cell impermeable inhibitor, R294, failed to change the expression of TG2 in the TG2 expressing breast cancer cells lines. The MCF7/RT cell lines (that were derived as explained in **Section 3.3.1**), also illustrated TG2 expression, which on being treated with cell permeable TG2 activity inhibitors (R283 and Z-DON) decreased significantly, while the cell impermeable inhibitor, R294, did not affect the expression of TG2. The SKBR3/RT and MDA MB 231 Clone 9 cells that demonstrated low TG2 expression, also showed a decrease in TG2 expression levels, on being treated with R283 and Z-DON (Figure 3.6). A time course dependent treatment of the MDA MB 231 Clone 16 cells with R283 and Z-DON exhibited a decrease in TG2 expression, following 48 h of treatment. This pattern continues onto the 72 h treated samples (Figure 3.7). These results may suggest that the decrease in TG2 expression in the high TG2 breast cancer cells, on being treated with TG2 cell permeable inhibitors, could be as a result of inhibiting TG2 activity within the cells.

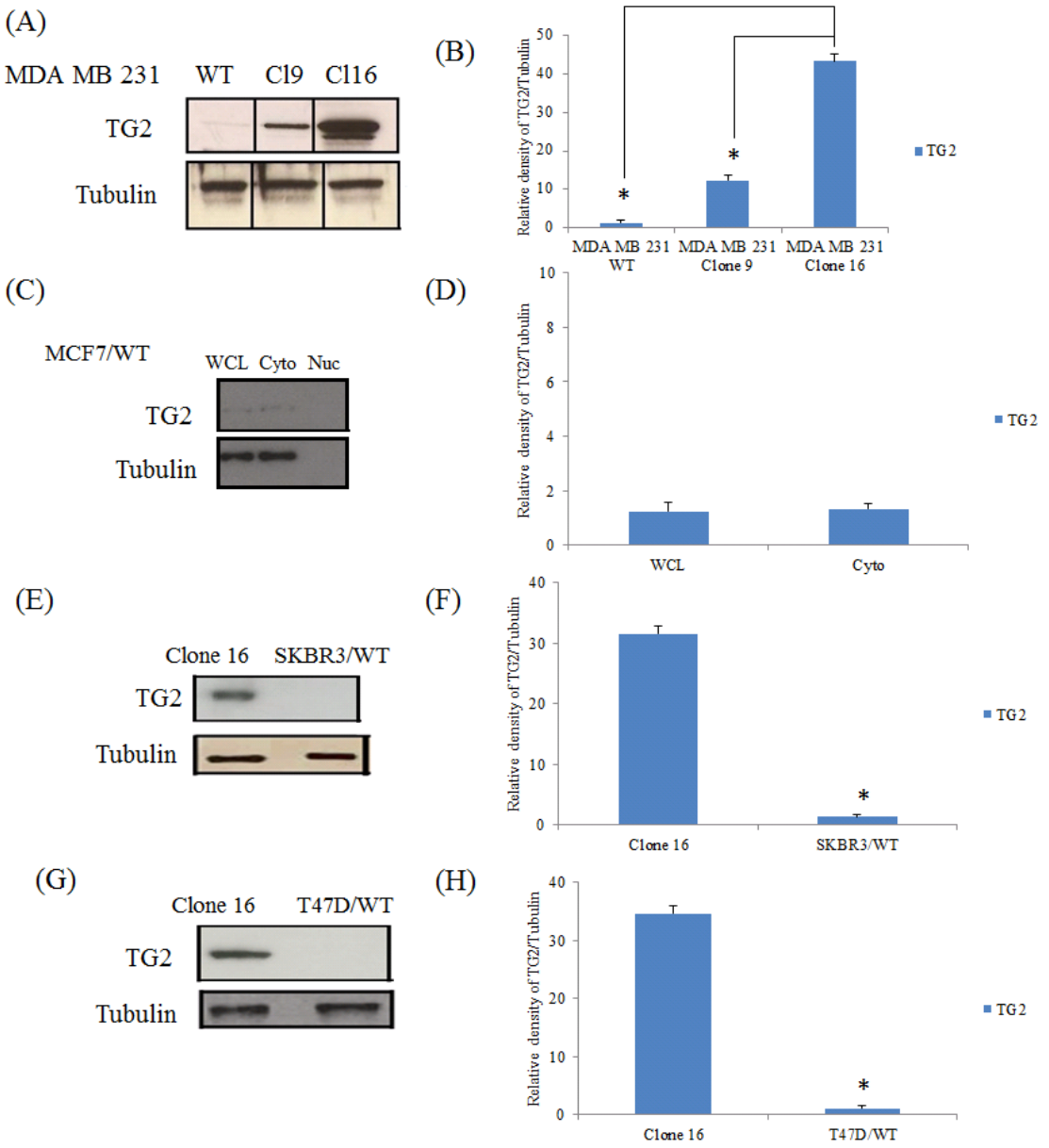


Figure 3.5 Western blot detection of TG2 expression in breast cancer cell lines.

The MDA MB 231 WT, Clone 9 and Clone 16 (A), MCF7/WT (C), SKBR3/WT (E) and T47D/WT (G) cells were seeded into 60mm petridishes (1x10^6 cells/ plate) and allowed to become confluent after which the whole cell lysate, cytoplasmic and nuclear fractions were collected (Section 2.2.2) and analysed for TG2 expression using Cub 7402 (1:1000) and TG100 (1:1000) ***(A, C, E & G)*** *The western blot data shown is a representation of three independent experiments (n=3).* ***(B, D, F & H)*** *Densitometry analysis was performed using image J and is represented as the mean densitometry of three blots + SEM. * $p<0.05$.*

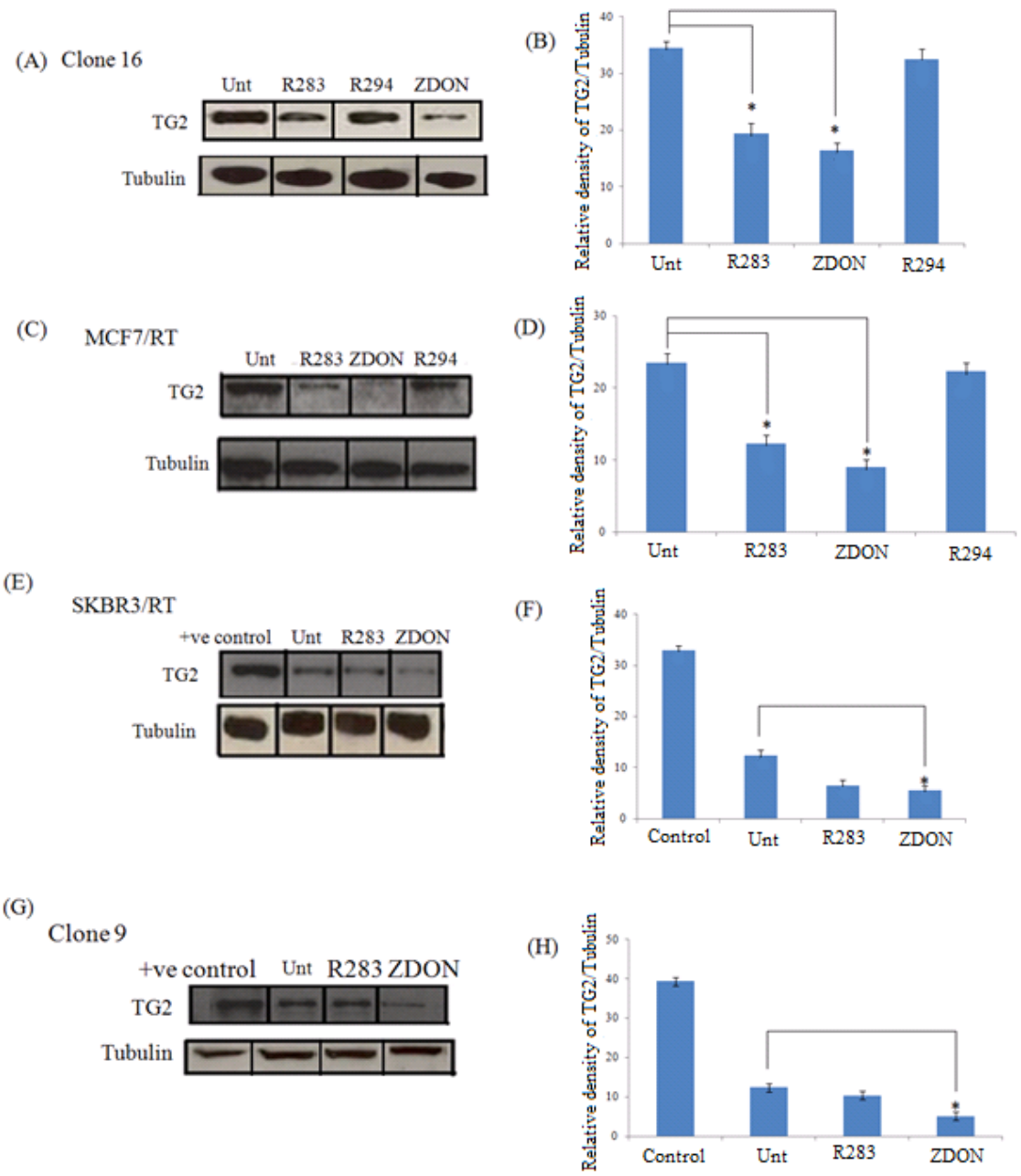


Figure 3.6 Treatment of TG2 expressing breast cancer cells with TG2 activity inhibitors

The MDA MB 231 Clone 16 (A), MCF7/RT (C), SKBR3/RT (E) and MDA MB 231 Clone 9 (G) cells were treated with R283, Z-DON and R294 for a time course of 72 h after which the whole cell lysate, cytoplasmic and nuclear fractions of the breast cancer cells were collected (Section 2.2.2) and analysed for the TG2 expression levels by western blotting using anti-TG2 antibody (Cub 7402; TG100) (1:1000). Anti-α-Tubulin was used as the equal loading marker. ***(A, C, E & G)*** *The western blot data shown is a representation of three independent experiments (n=3).* ***(B,***

***D, F & H**) Densitometry analysis was performed using image J and is represented as the mean densitometry of three blots + SEM, * $p<0.05$*

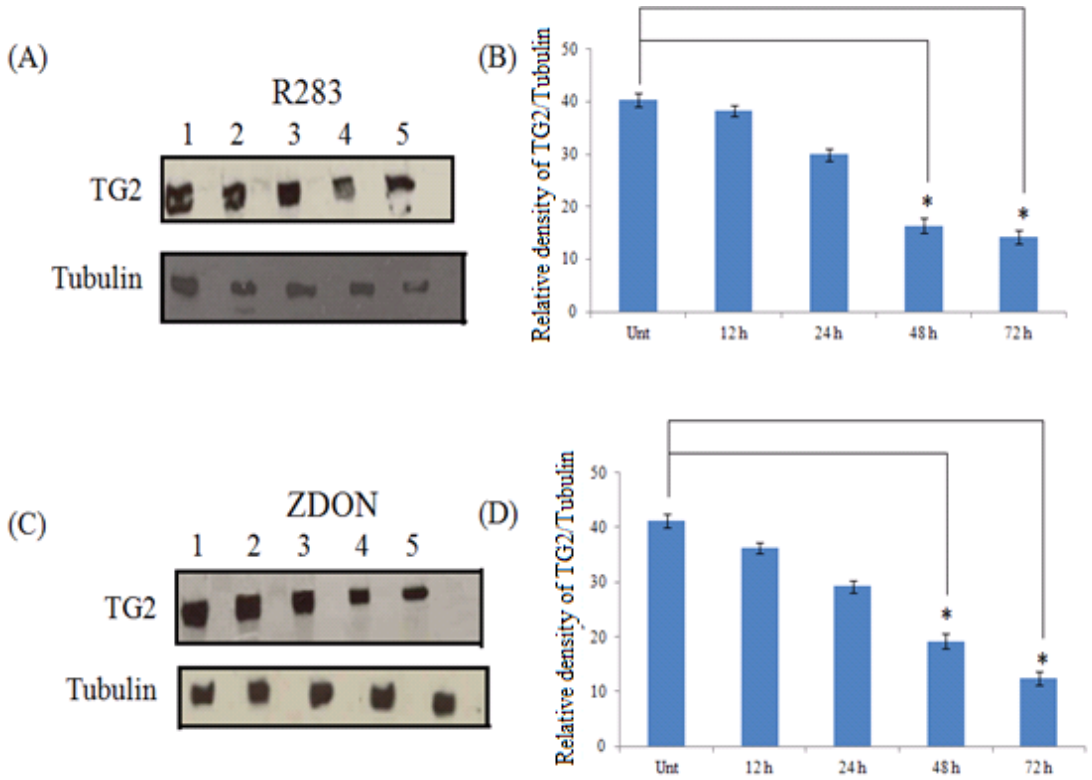


Figure 3.7 Detection of TG2 expressions in MDA MB 231 Clone 16 cells on treatment with TG2 inhibitors over a time course

*The MDA MB 231 Clone 16 cells (1x10^6 wells/ dish of 60mm petridish) were treated with R283 (500µM) and Z-DON (50µM) and after time points of 12, 24, 48 and 72 h, the whole cell lysate fractions of the Clone 16 cells were collected as introduced in Section 2.2.2 for the protein expression of TG2 using Cub 7402 (1:1000). Lane 1 represents Untreated cells, Lane 2 represents cells treated with TG2 inhibitor for 12 h, Lane 3 represents treatment time course of 24 h, Lane 4 represents treatment time course of 48 h and Lane 5 represents treatment time course of 72 h. The membranes were subsequently analysed using anti-α-Tubulin to ensure equal loading of protein. (**A&C**) The western blot data shown is a representation of three individual experiments (n=3). (**B&D**) Densitometry analysis was performed using image J and is represented as the mean densitometry of three blots + SEM. * p<0.05.*

3.3.2.3 Detection of TG2 *in situ* activity by FITC-Cadaverine Incorporation and Fluorescent microscopy.

In order to investigate the location of the TG2 activity within these breast cancer cell lines, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were incubated with 0.5mM FITC-Cadaverine for 16 h in 8-well chamber slides. The fluorescent signals were detected using the fluorescence microscope as described in **Section 2.2.9**. As shown in Figure 3.8, the MDA MB 231 Clone 16 and MCF7/RT cells showed high TG2 activity. The MDA MB 231 WT and MCF7/WT illustrated no measurable fluorescent signal.

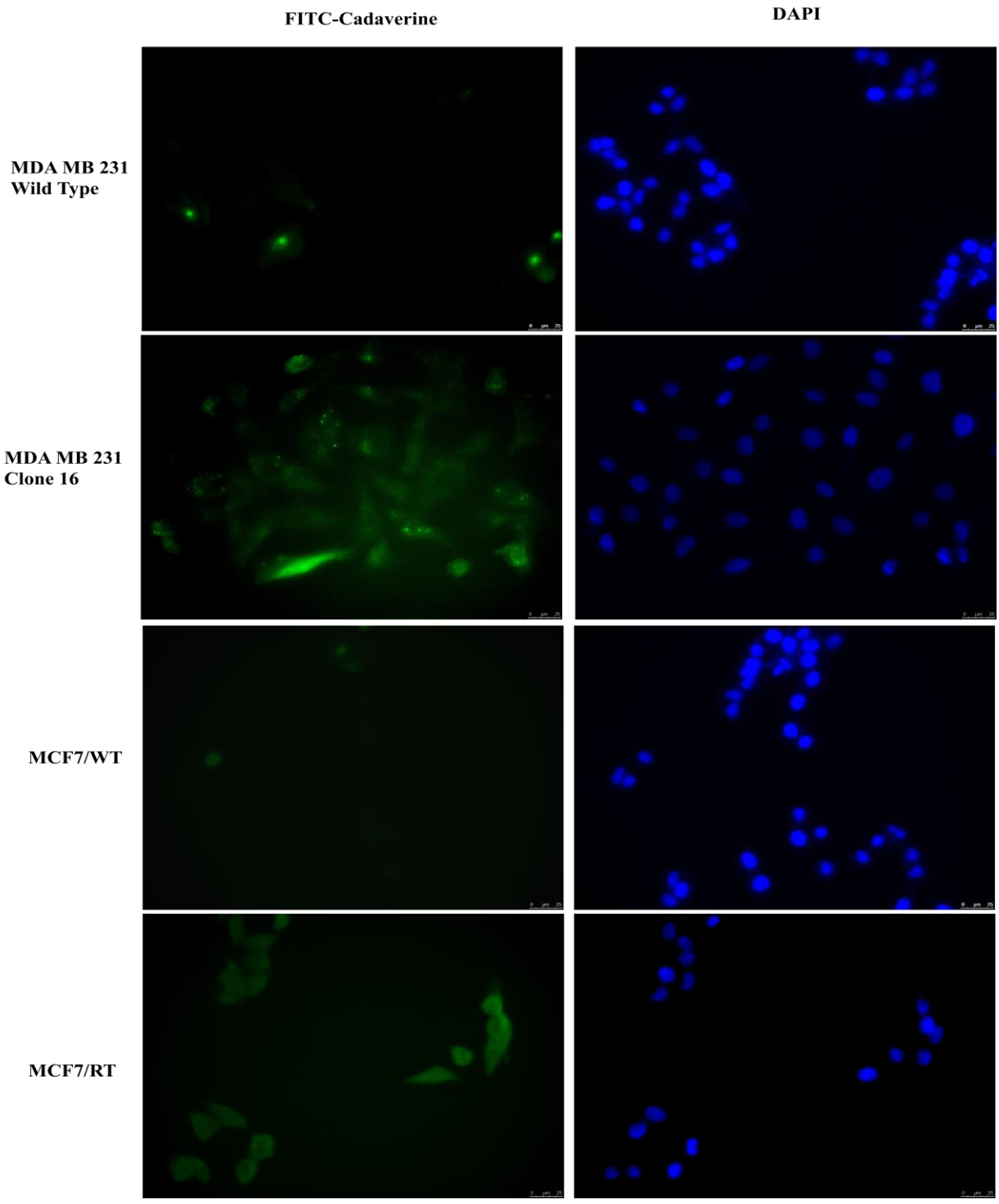


Figure 3.8 Cellular localization of TG2 activity in breast cancer cell lines using fluorescent microscopy

The cellular localization of TG2 in MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells was analysed by FITC- Cadaverine incorporation into cells. The live cells were

incubated with FITC- Cadaverine for 16 h and subsequently analysed by fluorescence microscopy (Section 2.2.9) (n=3)

3.3.2.4 Detection of TG2 using Immunocytochemistry and confocal microscopy

The cellular localization of TG2 within the breast cancer cell lines were further investigated using anti-TG2 (Cub7402) and secondary anti-mouse FITC conjugated antibody as described in detail previously in **Section 2.2.10**. The distribution of TG2 protein with the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF/RT was detected using confocal microscopy. No fluorescence signal was detected in the MDA MB 231 WT and MCF7/WT cells and an equal distribution of TG2 antigen was illustrated in the cytoplasmic fraction of the MDA MB 231 Clone 16 and MCF7/RT cell lines with no detectable TG2 seen in the nucleus (Figure 3.9)

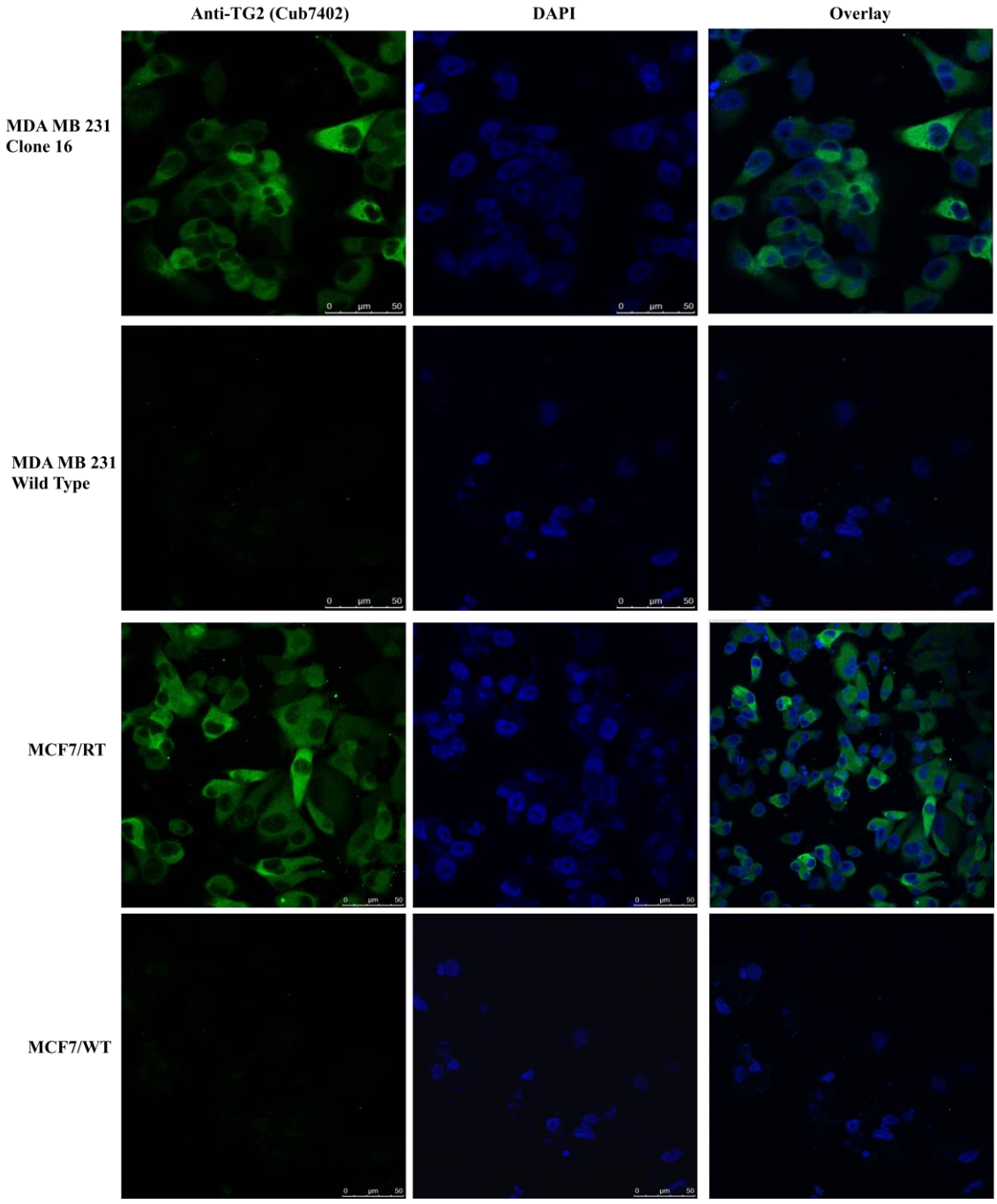


Figure 3.9 Cellular localization of TG2 protein expression in breast cancer cells using confocal microscopy

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were stained as described in Section 2.2.10. 1x10^5 cells were seeded into 8-well chamber slides overnight. The next day the cells were fixed and blocked, subsequently after which the cells were incubated with primary anti-TG2 (Cub7402) (1:100) for 45 min and secondary mouse anti-FITC (1:200)

for 45 min at 37^{o}C. The slides were then mounted with Vectashield mounting medium containing DAPI after which the fluorescent signals were detected using laser confocal microscopy (n= 3).

3.3.2.5 Cell viability analysis of breast cancer cell lines in the presence of doxorubicin

To decipher the relationship between TG2 activity and chemoresistance in breast cancer cell lines, the MDA MB 231 WT, MDA MB 231 Clone 9, MDA MB 231 Clone 16, MCF7/WT, SKBR3/WT, T47D/WT and their respective resistant cell lines, MCF7/RT, SKBR3/RT, and T47D/RT that were derived as described in detail in **Section 3.3.1** were cultured in the presence of increasing concentrations of doxorubicin (0.25- 1µg/ml) and then with 1µg/ml of doxorubicin along with the TG2 cell permeable inhibitors, R283 and Z-DON as well as the cell impermeable, R294 for a time course of 72 h. A dose-dependent decrease was observed in the cell viability of all the breast cancer cells, however, the TG2 expressing breast cancer cell lines clearly seemed to be more chemoresistant to doxorubicin than the low TG2 expressing and TG2 null breast cancer cell lines (Figure 3.10).

The susceptibility of these breast cancer cell lines varied according to their relative expressions of TG2. The MDA MB 231 WT, MCF7/WT, SKBR3/WT, T47D/WT and MDA MB 231 Clone 9 cells demonstrated low cell viability in the presence of doxorubicin due to the lack of or low TG2 expression and activity (Figure 3.10). On the other hand, the MDA MB 231 Clone 16, MCF7/RT cells that expressed high TG2 activity, showed higher cell viability as compared to the MDA MB 231 WT and MCF7/RT cells, on being treated with 1µg/ml of doxorubicin. Treatment of the MDA MB 231 Clone 16 and MCF7/RT cells with R283 and Z-DON caused a significant decrease in the cell viabilities of the high TG2 expressing cell lines. R294, the cell impermeable inhibitor did not show any considerable decrease in the cell viability of the Clone 16 and MCF7/RT cells as compared to the cell permeable inhibitors (Figure 3.11).

The SKBR3/RT cells showed very low TG2 expression in the presence of 1µg/ml of doxorubicin and subsequent treatment of the cells surviving in 1µg/ml doxorubicin in combination with cell permeable TG2 inhibitors did however show a significant decrease in cell viability. The T47D/RT cells did not show any TG2 expression even in the presence of

doxorubicin and, combination treatments of the cells with TG2 inhibitors and doxorubicin, did not show any decrease in the cell viability of T47D/RT cells (Figure 3.11)

This could imply that TG2 activity caused the MDA MB 231 Clone 16, MCF7/RT and SKBR3/RT cells to become more resistant to doxorubicin. Using cell permeable TG2 inhibitors that targeted the activity of TG2, the chemoresistance of these breast cancer cell lines against doxorubicin could be reduced. High TG2 activity causes these breast cancer cells to become more resistance to chemotherapeutic drugs such as doxorubicin. This also shows a positive correlation between chemoresistance and TG2 activity/ expression levels.

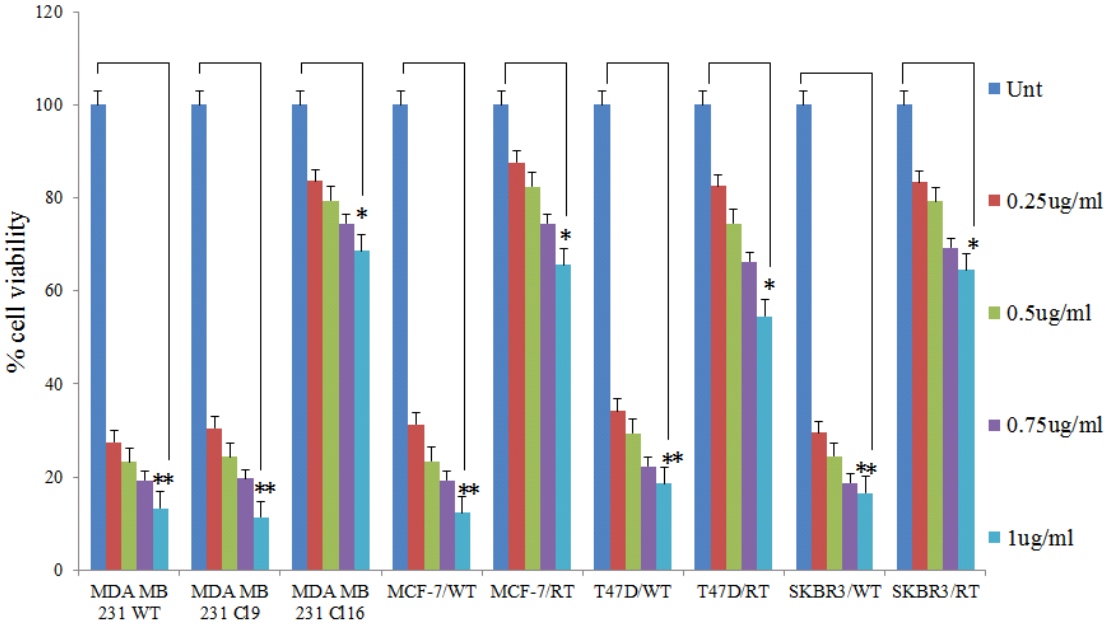
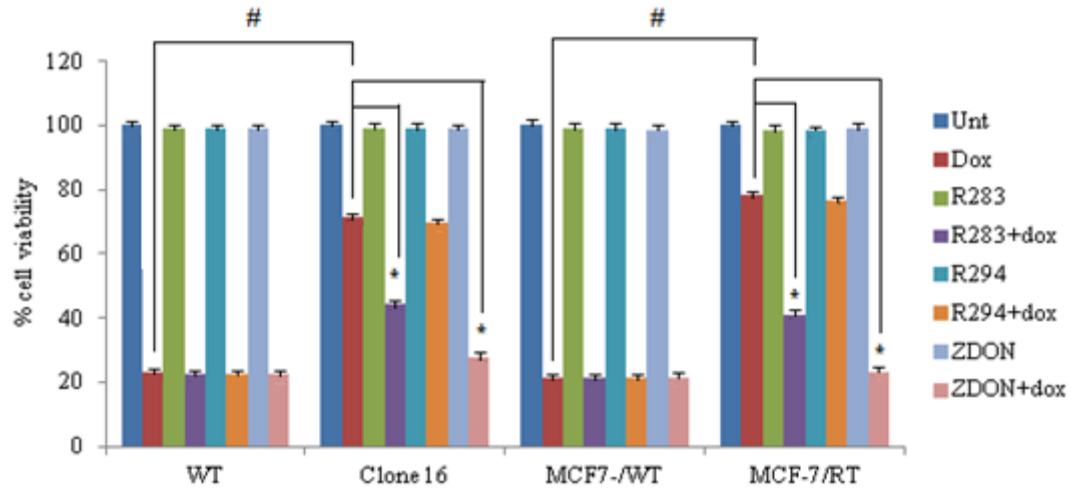


Figure 3.10 Cell viability analyses of breast cancer cell lines in the presence of increasing concentrations of doxorubicin

*The breast cancer cells were seeded (3000 cells per well) into 96 well plates and treated over a time course of 72 h and treated with increasing concentrations of doxorubicin (0.25, 0.5, 0.75 and 1µg/ml). After the treatment time course, the cells were analysed by using XTT reagents and the relative absorbance was detected as 490nm and 750nm. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Bonferroni post-hoc test. * p<0.05; **p<0.01.*

(A)



(B)

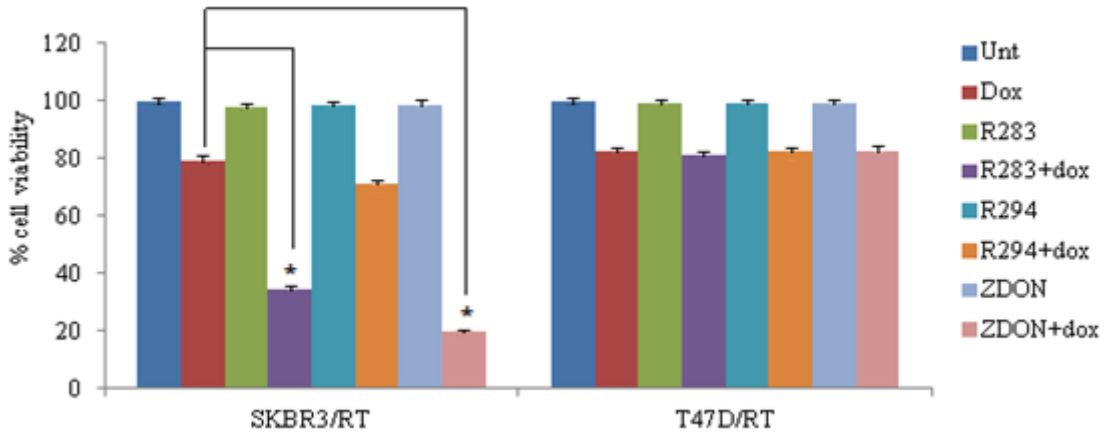


Figure 3.11 (A & B) Detection of cell viability in breast cancer cell lines in the presence of doxo☆ubicin and TG2 inhibitors

*The breast cancer cells (3000 cells per well of 96 well plate) were treated with doxorubicin (1µg/ml) as well as TG2 inhibitors (R283 {500µM}, R294 {500µM} and Z-DON {50µM}). After 72 h of treatment, the cell viability of the breast cancer cells were detected by XTT analysis and the absorbance was measured at 490 nm and 750 nm. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * p<0.01 between untreated and treated cells; # p<0.01 between cell groups.*

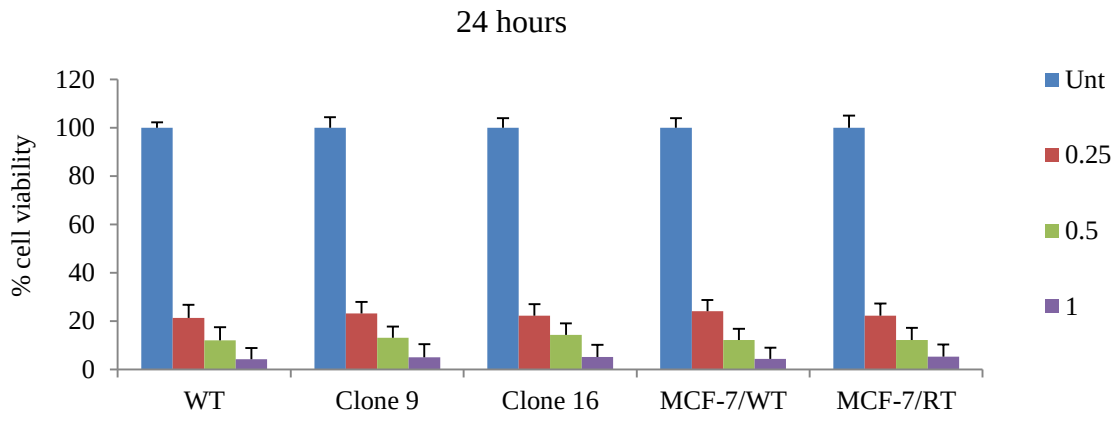
3.3.2.6 Cell viability analysis of breast cancer cell lines in the presence of cisplatin

The MDA MB 231 Clone 16 and MCF7/RT cells have been shown to be resistant to doxorubicin, as depicted in Figure 3.10. To verify whether the increased TG2 activity and expression within these cells would confer chemoresistance to other chemotherapeutic agents, the cell viability of these breast cancer cell lines in the presence of cisplatin was tested .

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT were treated with increasing concentrations (0.25µg/ml, 0.5µg/ml and 1µg/ml) of cisplatin for time courses of 24 and 48 h. The cell viability of the breast cancer cells was detected as described previously in **Section 2.2.13**.

Even though the MDA MB 231 Clone 16 and MCF7/RT cells were shown to have resistance against doxorubicin, treatment with only 0.25µg/ml of cisplatin for a time period of 24 h showed extremely low cell survival rates. The TG2-null MCF7/WT and MDA MB 231 WT cells were also very susceptible to treatment with low concentrations of cisplatin. As the results indicated, the high TG2 and TG2-null cells illustrated similar susceptibility to cisplatin, suggesting that chemoresistance against cisplatin in these breast cancer cell lines may be independent of TG2 expression and activity (Figure 3.12).

(A)



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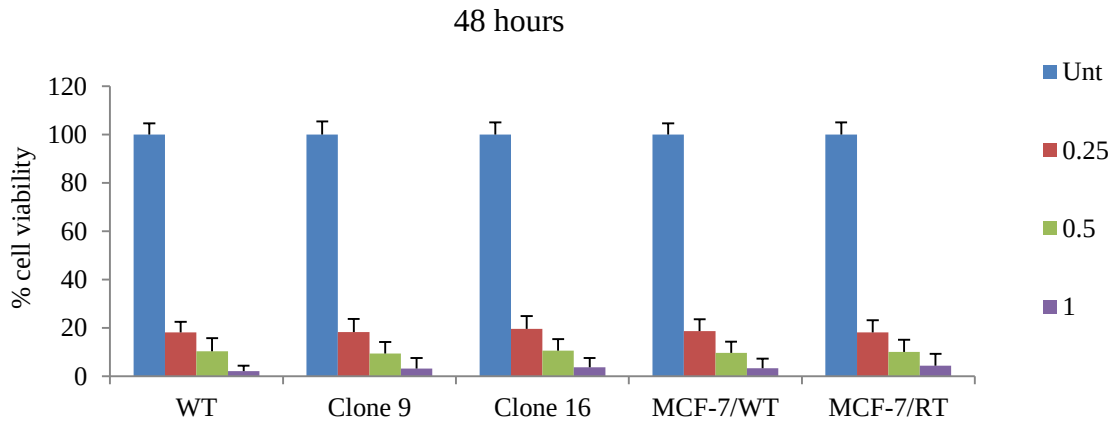


Figure 3.12 Cell viability analysis of breast cancer cell lines in the presence of increasing concentrations of cisplatin over a time course

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells (3000 cells / well in a 96 well plate) were treated with increasing concentrations of cisplatin (0.25, 0.5, 1µg/ml) over a time course. After 24 h and 48 h, the cell viability of the breast cancer cells were analysed by XTT analysis as introduced in Section 2.2.13. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3).

3.3.3 Inhibition of TG2 using TG2 specific siRNA

3.3.3.1 Susceptibility of breast cancer cell lines treated with TG2 specific siRNA in the presence of doxorubicin

To verify whether decreasing the expression of TG2 in breast cancer cell lines conferred them more susceptible to doxorubicin, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were treated with doxorubicin (1µg/ml) after transfection with TG2 specific siRNA. The viable cells were determined using the XTT assay as detailed above in **Section 2.2.13**. As illustrated in Figure 3.13, the MDA MB 231 Clone 16 and MCF7/RT cells demonstrated a significant decrease in their cell viable numbers, on being treated with a combination of doxorubicin and TG2 specific siRNA, TGM2-6 (ii), as compared to the cells treated only with doxorubicin. Decreasing the expression of TG2 using TG2 specific siRNA, resulted in the decrease of the chemoresistance against doxorubicin in the high TG2 expressing cell lines.

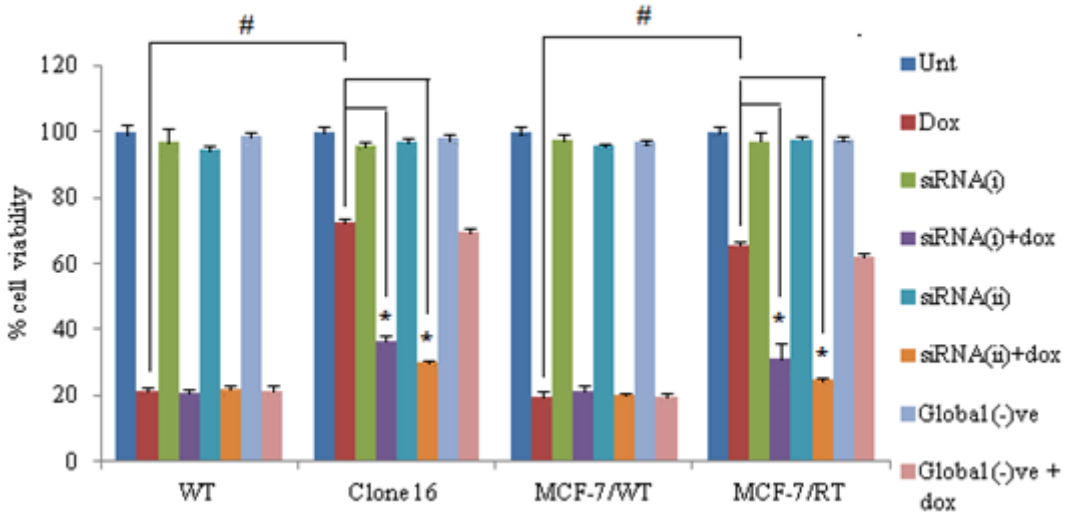


Figure 3.13 Cell viability of breast cancer cells after treatment with doxorubicin and TG2-specific siRNA

*The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT (3000 cells per well of 96 well plate) cells were treated with TG2 specific siRNA for 48 h (final concentration of 100nm) and doxorubicin (1µg/ml) for a time course of 72 h after which the viable cells were detected using XTT analysis. Global negative siRNA control was used as the negative control. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * $p<0.01$ between cells transfected and non-transfected with siRNA in the presence of doxorubicin. # $p<0.05$ between cell groups.*

3.3.3.2 TG2 expression and activity decreases on treating the breast cancer cell lines with TG2 siRNA2

3.3.3.2.1 The inhibitory effect of TG2 targeting siRNA on TG2 expression

TG2 specific targeting siRNA were used to inhibit the expression of TG2 in the MDA MB 231 WT, MDA MB 231 Clone 16, and MCF7/WT and MCF7/RT cell lines as introduced in **Section 3.2.5**. After 48 h of incubation with the siRNA (final concentration of 100nM), the cytoplasmic and nuclear fractions were collected and Western blotting was performed to detect the presence of TG2 antigen in the cellular fractions (**Sections 2.2.2-2.2.6**). As shown, no effect of the control global negative siRNA was found on the expression of TG2. Treating the TG2 expressing MDA MB 231 Clone 16 and MCF7/RT cell lines with TGM2-6 (ii) and TGM2-1 (i) exhibited significant reduction in the endogenous TG2 expression of these breast cancer cell lines (Figure 3.14).

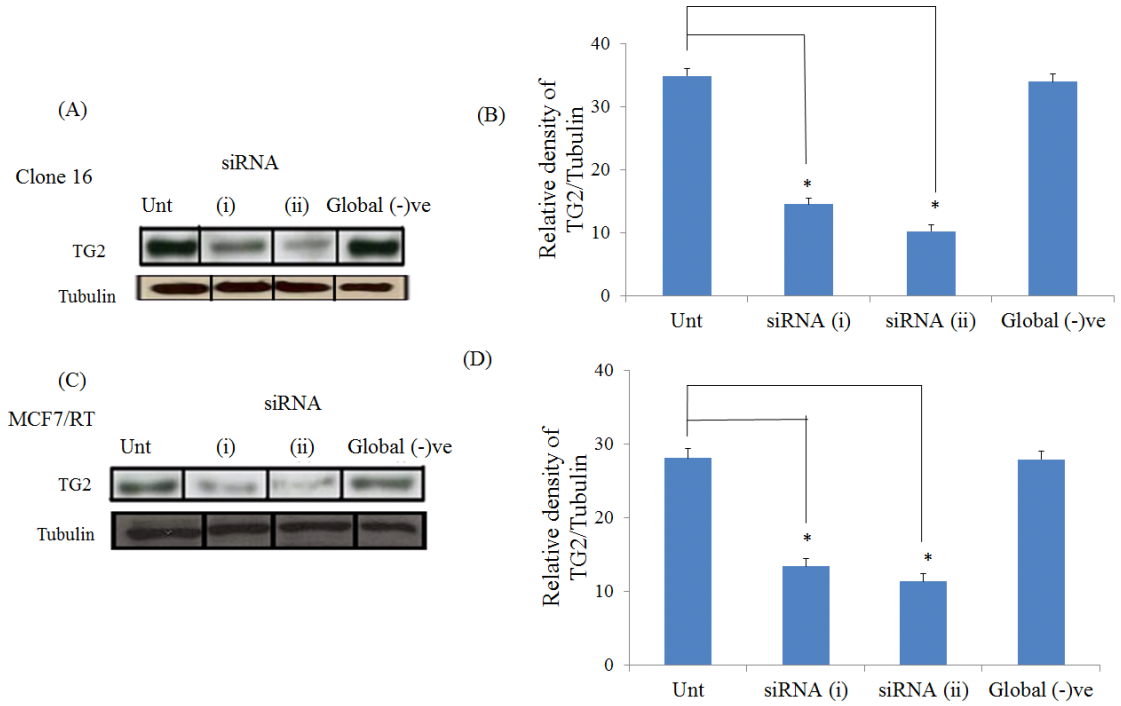


Figure 3.14 Analysis of the expression of TG2 in MDA MB 231 Clone 16 and MCF7/RT cells in the presence of different TG2 specific siRNA.

*The MDA MB 231 Clone 16 and MCF7/RT cells were ($5x10^5$ cells/well) seeded into 6- well plates and treated with TG2 specific siRNA (final concentration of 100nM) for 48 h, after which the cytoplasmic fractions of the MDA MB 231 Clone 16 and MCF7/RT cells were analysed for the relative TG2 expression. Global negative siRNA was used as the negative control. Anti-α-Tubulin was used as the marker for equal loading of protein. (**A&C**) The western blot data shown is representative of three independent experiments (n=3). (**B&D**) Densitometry analysis was performed using image J and is the mean densitometry of three blots + SEM. * $p<0.05$.*

3.3.3.2.2 The inhibitory effect of TG2 targeting siRNA on TG2 whole cell lysate activity.

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were *analysed* for differences in the TG2 activity in their whole cell lysate on being treated for 48 h with various TG2 specific siRNA as described previously in **Section 3.2.5**. The TGM2-6 (iii) demonstrated the most significant decrease in the whole cell lysate TG2 activity of the MDA MB 231 Clone 16 and MCF7/RT cells, followed by TGM2-1 (i), which seems to be consistent with the decrease in the TG2 expression within these cells as showed in the western blot (Figure 3.15). These results suggest that inhibiting the expression of TG2 using TG2 specific siRNA consequently reduces the whole cell lysate TG2 activity of the MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines.

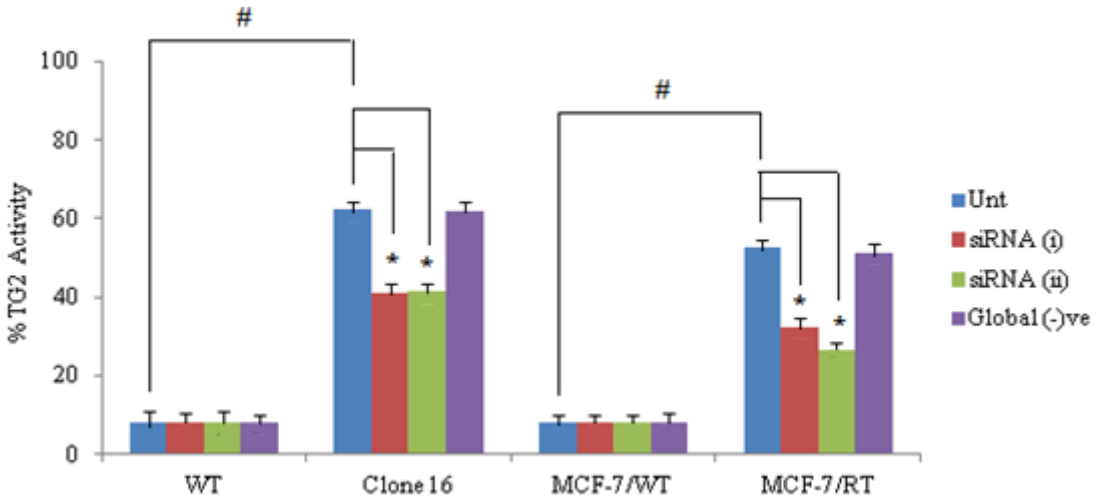


Figure 3.15 Investigation of TG2 whole cell lysate activity in the presence of TG2-specific siRNA

*The whole cell lysates of MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT after treatment with 100nM of TG2 specific siRNA for 48 h were collected and analysed for the TG2 activity as described in Section 2.2.11 by Biotin-X- Cadaverine incorporation into fibronectin. Positive control (50 ng/ml gplTG + 10mM $CaCl_2$) was taken to be 100% TG2 activity (1.29574±0.199473). Data shown above is mean percentage TG2 activity + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * $p<0.05$ between cells transfected and non-transfected with siRNA. # $p<0.05$ between cell groups.*

3.3.4 Transfection of TG2 plasmid into TG2-null breast cancer cell lines

3.3.4.1 Susceptibility of MCF7/WT and SKBR3/WT cells to G418

Previous studies have shown that when TG2-null cell lines were transfected with TG2, their chemoresistance against chemotherapeutic drugs increased significantly (Verma A, 2006) To verify this, the TG2 null MCF7/WT and SKBR3/WT breast cancer cell lines were transiently transfected with TG2 plasmid and a Vector control plasmid. For the establishment of the transiently transfected cell lines, MCF7/WT and SKBR3/WT cell lines were cultured in a range of G418 concentrations (250µg/ml, 500µg/ml,750µg/ml, 1mg/ml and1.5mg/ml) and used to identify the lowest possible concentration that would induce at least about 80% of cell death within 48-72 h as introduced in Section 2.2.1.6. The resulting killing curve suggested that the concentration of 500µg/ml of G418 was the optimal concentration for the selection of MCF7 and SKBR3 TG2 containing clones (Figure 3.16)

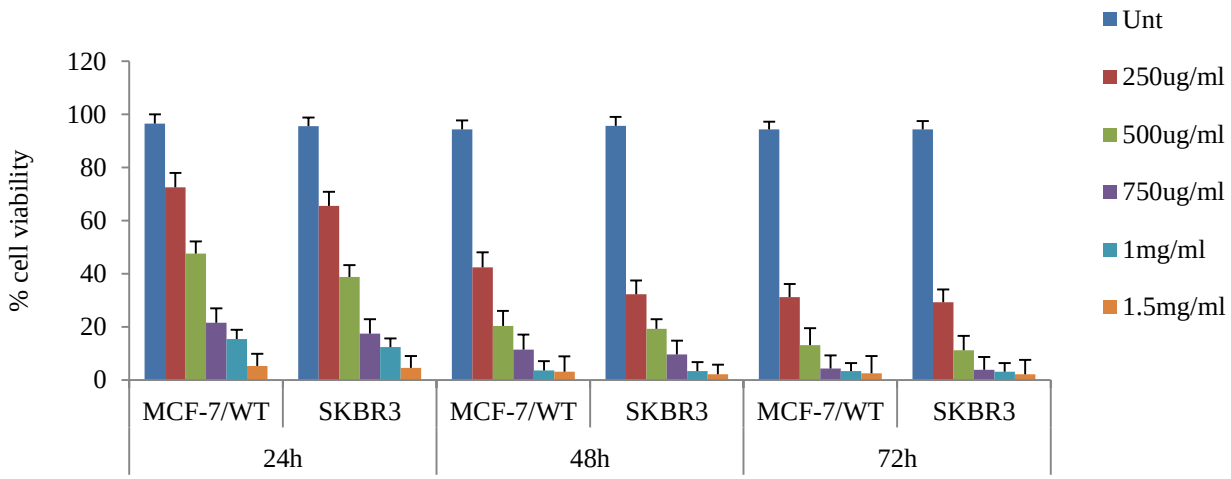


Figure 3.16 The effect of G418 on TG2 null MCF7 and SKBR3 cell proliferation illustrated via XTT assay.

The TG2-null MCF7/WT and SKBR3/WT cells (3000 cells per well of 96 well plate) were cultured with increasing concentrations of G418 (250µg/ml, 500µg/ml, 750µg/ml, 1mg/ml and 1.5 mg/ml). After 24 h, 48 h and 72 h of incubation with G418, XTT assay was conducted and absorbance was measured at 490nm and 750nm using a plate reader. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3).

3.3.4.2 Transfecting breast cancer cell lines with TG2 plasmid increased TG2 activity and expression.

The MCF7 and SKBR3 cells were transiently transfected with TG2 and empty vector control as introduced in **Section 2.2.1.6** and grown in cell medium supplemented with 500µg/ml of G418 to select the transfected cells. For all subsequent experiments performed, the transfected cells were maintained with 250µg/ml of G418 in the cell culture medium. The effect of the transfection was studied by detecting the levels of TG2 expression via Western Blotting. In the MCF7/WT, SKBR3/WT, MCF7 and SKBR3 cells transfected with empty vector plasmid, TG2 antigen was not detected by Western Blot in the cytoplasmic fractions as demonstrated in Figure 3.17. After transfecting the cells with TG2 carrying plasmid, high levels of TG2 antigen was detected using a TG2 specific (TG100) monoclonal antibody against TG2. These results prove the successful establishment of the TG2 transfected MCF7 and SKBR3 cell lines, which provide an ideal model to investigate the role of endogenous TG2 in the chemoresistant process against doxorubicin, since the potential influence of exogenous TG2 was ruled out in the previous Section. The SKBR3 cells transfected with the TG2 plasmid were also treated with cell permeable inhibitors, R283 and Z-DON. Both the cell permeable inhibitors knocked down TG2 expression significantly in these cell lines (Figure 3.18)

In order to confirm the results from the Western blotting of the presence of the TG2 antigen within the TG2 transfected MCF7 and SKBR3, whole cell lysate TG2 activity was measured via the biotinylated cadaverine incorporation assay as described in **Section 2.2.11**. As illustrated in Figure 3.19, the whole cell lysate TG2 activity was only detected in the TG2 transfected MCF7 and SKBR3 cells but not in the MCF7/WT and SKBR3/WT cells, which further confirmed the presence of TG2 activity due to the expression of TG2 plasmid in the MCF7 and SKBR3 breast cancer cell lines. Moreover, treating the TG2 transfected MCF7 and SKBR3 cells with TG2 inhibitors, R283 and Z-DON, showed a significant decrease in the TG2 expression as well as TG2 activity within these breast cancer cell lines. These results clearly suggest that the transfection of TG2 plasmid into TG2-null MCF7 and SKBR3 cells resulted in

the endogenous expression and activity of TG2 in the cytoplasmic fractions of the breast cancer cell lines.

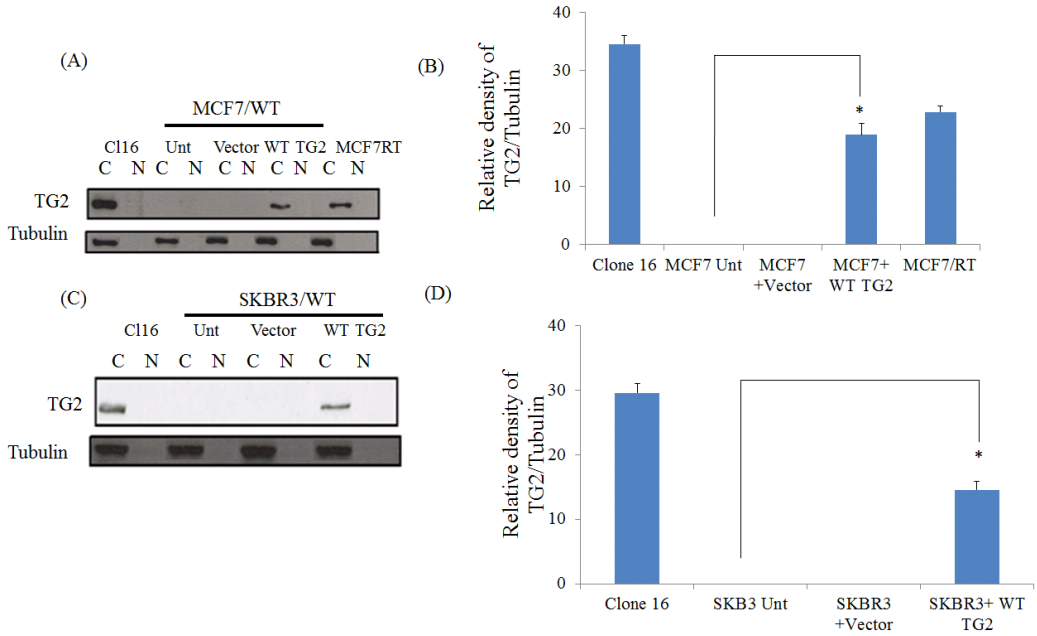


Figure 3.17 Detection of TG2 in TG2 transfected MCF7 (A) and SKBR3 (C) cells by western blot

*Western blotting was conducted with the cytoplasmic (C) and nuclear (N) fractions to confirm the presence of TG2 in the TG2 and empty vector transfected MCF7 and SKBR3 cells using Lipofectamine as described in Section 2.2.1.6. The membranes were reprobed with anti-α-Tubulin antibody to check for equal loading of protein. (**A&C**) The western blot data shown is a representation of three independent experiments (n=3). (**B&D**) Densitometry analysis was performed using Image J and is represented as the mean densitometry of three blots + SEM. * p< 0.05.*

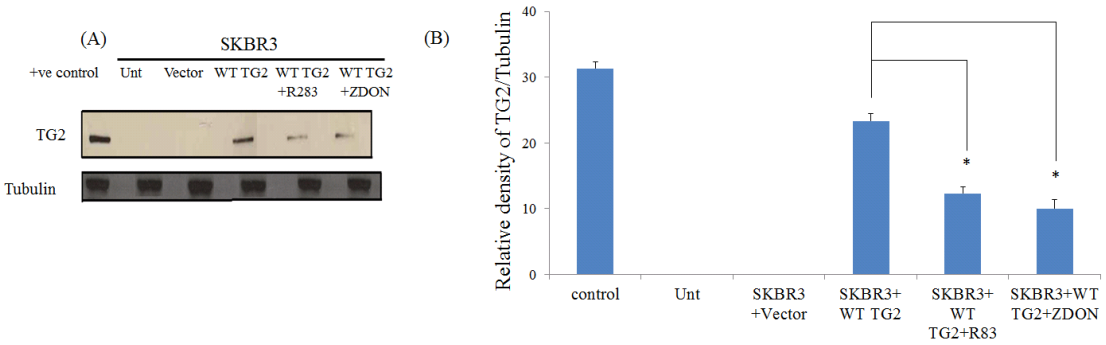


Figure 3.18 Detection of TG2 in the TG2 transfected SKBR3 cells on being treated with TG2 inhibitors by Western blot

*Western blotting was conducted on the cytoplasmic and nuclear fractions of the TG2 and Vector plasmid transfected cells, treated with TG2 inhibitors, R283 (500μM) and Z-DON (50μM) for 72 h. The membranes were reprobed with anti-α-Tubulin antibody to check for equal loading of protein. (**A**) The western blot data shown is a representation of three independent experiments (n=3). (**B**) Densitometry analysis was performed using image J and is represented as the mean densitometry of three blots ± SE. * $p<0.05$.*

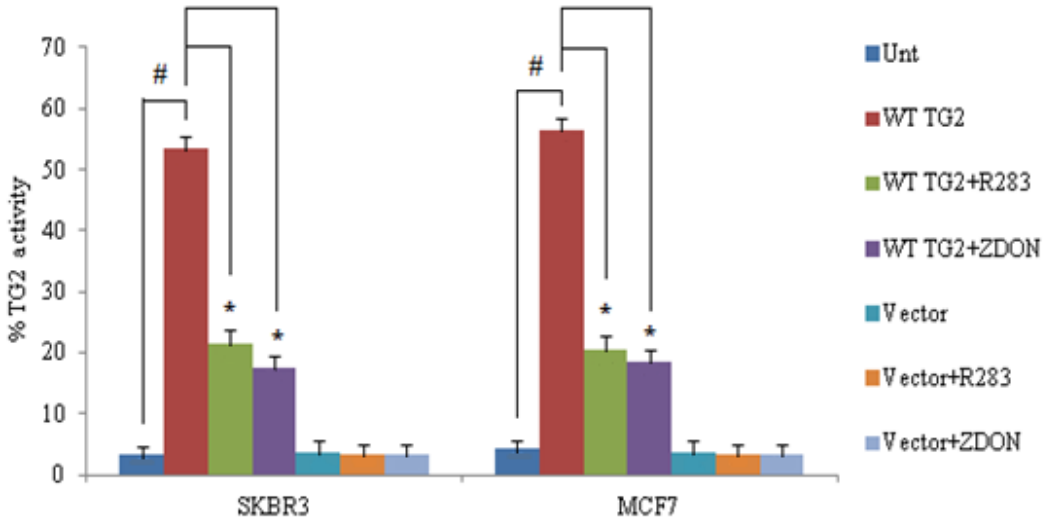


Figure 3.19 Detection of whole cell lysate TG2 activity in MCF7 and SKBR3 transfected breast cancer cell lines.

*The establishment of the TG2 transfected MCF7 and SKBR3 cells was accomplished as mentioned in Section 2.2.1.6. The whole cell lysate TG2 activity of these breast cancer cells was measured via Biotin-X-Cadaverine incorporation into fibronectin. Positive control (50 ng/ml gplTG + 10mM $CaCl_2$) was taken to be 100% TG2 activity (1.38136±0.23753). Data shown above is mean percentage TG2 activity + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * $p<0.01$ between TG2 plasmid transfected cells untreated and treated with TG2 inhibitors. # $p<0.01$ between non transfected and TG2 plasmid transfected cells.*

3.3.4.3 TG2 transfected breast cancer cell lines are more resistant to doxorubicin

The effect of TG2 expression and activity on the chemoresistant nature of breast cancer cell lines was investigated using TG2 transfected SKBR3 cells lines, while the empty vector transfected cells were used as the control group. As shown in Figure 3.20, transfecting TG2 into SKBR3 cell lines seems to increase the chemoresistance against increasing concentrations of doxorubicin. Treating the TG2 transfected SKBR3 with the TG2 activity inhibitors, R283 and Z-DON, demonstrated significant decrease in the viable cells in the presence of 1μg/ml of doxorubicin (Figure 3.20). These results indicate that the presence of endogenous TG2 expression is sufficient to confer chemoresistance to breast cancer cell lines and that treating these transfected cell lines with TG2 cell permeable inhibitors, facilitates the cell lines susceptible to chemotherapeutic drugs such as doxorubicin. This further suggests that the presence of TG2 confers chemoresistance to these breast cancer cell lines against doxorubicin.

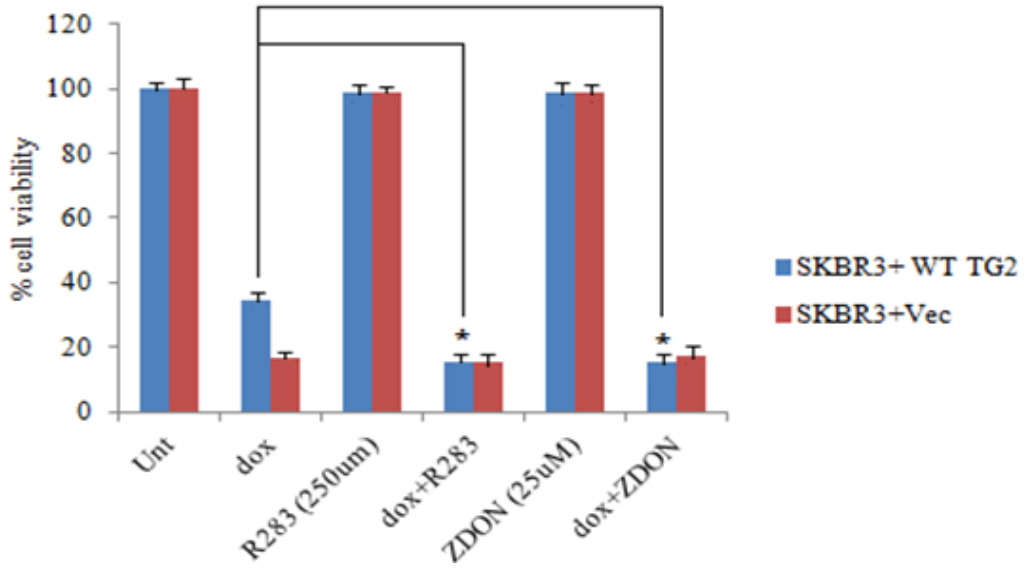


Figure 3.20 Detection of cell viability of TG2-null SKBR3 and MCF7 cells after transfection with TG2

*The SKBR3 cells (3000 cells / well in a 96 well plate) that were transfected as previously described (Section 2.2.1.6) were analysed by XTT in the presence of doxorubicin (1μg/ml) along with the cell permeable TG2 inhibitors, R283 (250μM) and Z-DON (25μM) to check for chemoresistant nature of the TG2 transfected cells. These transfected cells were then subsequently analysed by the XTT assay. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Bonferroni post-hoc test. * $p<0.01$ between TG2 plasmid transfected cells treated with and without inhibitors in the presence of doxorubicin.*

3.4 DISCUSSION

Tumour progression and drug resistance creates a key clinical obstacle in the treatment of breast cancer. Understanding the molecular mechanisms that underlie the development of the metastatic phenotype and drug resistance is crucial for the development of effective therapies against breast cancer treatment (Mehta, *et al.* 2004). The above findings provide evidence that TG2 expression and activity is up-regulated in drug resistant breast cancer cells. Therefore, increased expression and activity of TG2 may serve as a prognostic marker for drug resistant tumours and play an important role in the establishment of these phenotypes.

The MDA MB 231 Clone 9 and MDA MB 231 Clone 16 cells were derived from the parental MDA MB 231 as described previously by Mehta *et al.* (2004). The MDA MB 231 WT cells did not express any TG2 while the clones, MDA MB 231 Clone 9 and MDA MB 231 Clone 16, isolated from the metastatic parent cell line MDA MB 231, display low and high TG2 expression respectively . It should be noted that the MDA MB 231 WT cells obtained from Aston University did not express any endogenous TG2 which was contradictory to the results published by Mehta *et al.* in 2004 (Mehta, *et al.* 2004). However, the chemosensitive nature of these MDA MB 231 WT cells in the presence of doxorubicin was similar to what was observed by Mehta *et al.* (Mehta, *et al.* 2004). Following treatment with the TG2 cell permeable inhibitors (R283 and Z-DON), a clear and significant decrease was observed in the endogenous TG2 expression, of the high TG2 and chemoresistant breast cancer cell line. This is agreement with the data obtained from the Biotin -X- Cadaverine Incorporation assay, where the inhibitors, R283 and Z-DON, illustrated significant reduction in the TG2 whole cell lysate and cell surface activity of MDA MB 231 Clone 16.

The MCF7/RT cell line was derived from the parental MCF7/WT cells by culturing the TG2-null cell line in the presence of increasing concentrations of doxorubicin (0.25, 0.5, 0.75 and 1μg/ml). The MCF7 cells illustrate an increase in TG2 expression, as the cells begin to gain resistance to 0.75 μg/ml of doxorubicin. The TG2 expression further increases when the doxorubicin concentration was increased to 1μg/ml, thus ensuring that the MCF7/RT cell lines

derived did express significant levels of TG2 protein expression as well as TG2 whole cell lysate activity. Similar to the MDA MB 231 Clone 16 cells, the MCF7/RT cells also, became more susceptible to doxorubicin treatment, on being treated with the cell permeable TG2 activity inhibitors. This chemosusceptible nature of the MCF7/RT cells could be attributed to the decrease observed in the endogenous TG2 expression and activity levels.

MDA MB231 and MCF7 breast cancer cell lines are adenocarcinomas that are cancers of the epithelial tissue that originated from the mammary gland. The MDA MB 231 breast cancer cell line was derived from a metastatic carcinoma, while the MCF7 cell line was derived from an *in situ* carcinoma, which has not yet invaded surrounding tissue (Alkhalaf & El-Mowafy, 2003). Clinically, studies have shown that cancers of the MDA MB 231 cell line is much harder to treat as compared to the MCF7 cell line as the MDA MB 231 cells are more resistant to chemotherapy and even grow exponentially faster. Also, the MCF7 cell line expresses both oestrogen as well as progesterone receptors, which is absent in the MDA MB 231 breast cancer cells (Sorlie, *et al.* 2009). Similar to the MDA MB 231 and MCF7 breast cancer cell lines, the SKBR3 breast cancer cell line is also an adenocarcinoma derived from metastatic sites. Contrary to the above three breast cancer cells, the T47D cell line is of ductal carcinoma origin (Trempe, 1976). This could account for the difference in chemoresistance patterns as well as TG2 expression between the T47D and the other three breast cancer cell lines.

It should be further noted that the expression of endogenous TG2 protein and activity in the chemoresistant breast cancer cells, was confined to the cytoplasmic fraction of these cells. This was verified using *in situ* FITC-cadaverine incorporation and immunocytochemistry for TG2 antigen respectively. The majority of the TG2 activity and expression observed in the MDA MB 231 Clone 16 and MCF7/RT cells, was localized to the cytoplasmic fractions with no TG2 activity or expression detected in the nuclear fractions

The SKBR3/RT and T47D/RT breast cancer cell lines were derived from the parent SKBR3/WT and T47D/WT using the same method as the MCF7/RT cell lines were obtained.

The SKBR3 cell line does not express any TG2, till the cell lines show resistance to1µg/ml of doxorubicin. The SKBR3 cells resistant to doxorubicin concentrations lower than 1µg/ml do not demonstrate any TG2 expression. Conversely, the T47D cells derived that are known to be resistant to 1µg/ml of doxorubicin, do not express any TG2 protein. This could suggest that the mechanism of chemoresistance in the T47D breast cancer cell lines could be independent of TG2 function. These results were supported by previous studies that have demonstrated that, independent of their type and source, cancer cells selected out for resistance against chemo therapeutic drugs display only a moderate increase in TG2 levels (Budillon, *et al.* 2013)

Based on previous observations and current data that breast cancer cells, resistant to chemotherapeutic drugs, express high levels of TG2, it can be speculated that TG2 may be protecting cancer cells from chemically or drug induced apoptosis. A reduction in the levels of TG2, by transfection with TG2-specific siRNA was shown to reduce chemoresistance in MDA MB 231 Clone 16 and MCF7/RT cell lines. Conversely, transfecting TG2 plasmid into TG2-null MCF7 and SKBR3 breast cancer cell lines, increased chemoresistance against doxorubicin, which can be reversed by treatment with TG2 cell permeable inhibitors (R283 and Z-DON), which clearly suggests that TG2 plays a major role in the acquisition of chemoresistance. This has been shown in PC-14/ADR lung cancer cells as well, where the reduction in TG2 levels due to transfection with TG2 specific antisense can reverse the drug resistance (Han & Park, 1999)

In a study conducted by Jiang *et al.* in 2003, to identify the metastasis-associated proteins using proteomic analysis, TG2 was observed to be one of the eleven proteins that were amplified selectively in metastatic human lung cancer (Jiang, *et al.* 2003a). Comparable, to these findings, comprehensive research of more than 30,000 genes using three different methods suggested that TG2 was one of the most prominently expressed genes in pancreatic tumours (Iacobuzio-Donahue, *et al.* 2003).Treating cancer cells with EGF induced the expression of TG2 and subsequently subverted apoptosis induced by doxorubicin (Antonyak, *et al.* 2004). These above studies clearly implicate TG2 to be playing a major role in the acquisition of chemoresistance by cancer cells.

Founded on the information and results attained this chapter, it can be speculated that TG2 is a very promising therapeutic target to reverse chemoresistance in breast cancer cells. With respect to this, the use of TG2 specific siRNA and TG2 cell permeable activity inhibitors, to silence the endogenous TG2 expression and activity holds great promise in developing new therapeutics required to overcome drug resistant in metastatic cancer cells.

These findings of increased TG2 levels in drug resistant breast cancer cells have amplified interest towards understanding the contributions of TG2 in the development of chemoresistance. TG2 is reputed to cause the constitutive activation of the NFκB and FAK/PI3K/AKT cell survival signalling pathways (Mehta, *et al.* 2004) the next chapter will concentrate on elucidating the mechanisms behind the conferment of drug resistant phenotype by TG2 in breast cancer cell lines. Individually or collectively, these pathways have been known to confer resistance to chemotherapy by avoiding autophagic or apoptotic death (Mehta, *et al.* 2010). This increased threshold of cancer cells to undergo apoptosis, allows them to display resistance against numerous anticancer drugs as well as to survive and proliferate successfully in the stressful microenvironment following metastasis to foreign tissues. TG2 thus represents a crucial target for further investigation in order overcome chemoresistance in breast cancer cells.

Chapter 4: TG2 activity results in aberrant NFκB activation through an intracellular mechanism

4. TG2 activity results in aberrant NFκB activation through an intracellular mechanism

4. 1 INTRODUCTION:

The previous chapter provides evidence that the increased expression and activity of TG2 can promote survival, growth and chemoresistance of breast tumour cells and as such TG2 could represent a potential therapeutic target to provide improved results for patient outcomes. Deciphering the molecular mechanisms that supports the development of metastasis and drug resistance in cancer cells can offer new targets for the treatment of disease.

The transcription factor, nuclear factor-kappaB (NFκB) is known to play a crucial role in the regulation of apoptosis, cell growth and metastatic functions. Even though the constitutive activation of NFκB has been observed in various cancers, the molecular mechanisms that result in such an activation remain unknown (Nakanish & Toi, 2005). On the basis of previous studies done, metastatic and drug resistant cancers express very high levels of TG2 which can confer chemoresistance to cancer cells (Antonyak, *et al.* 2004; Mangala & Mehta, 2005). It could therefore be hypothesized that TG2 may contribute to the constitutive activation of NFκB. Many reports suggest that the over expression of TG2 is linked to the constitutive activation of NFκB (Mann, *et al.* 2006). Over expression of TG2 is concurrent with constitutive NFκB activity in tumour cell lines. Activation of TG2 results in the activation of NFκB and conversely, the down regulation of TG2 using small interfering RNA decreases the activation of NFκB. This may point to TG2 inhibition as an alternate therapy to prevent the constitutive activation of NFκB and leave cancer cells sensitized to effective anti-cancer therapy (Mehta, *et al.* 2010).

In pancreatic and breast cancer cells, TG2 is known to activate NFκB by the cross linking of the IκBα subunit, which leads to the polymerization of IκBα and the subsequent translocation of the

NFκB subunit to the nucleus. This polymerization of IκBα is dependent on the enzymatic activity of TG2 which leads to the activation of NFκB that is sustained and constitutive (Kim, *et al.* 2006 ; Mann, *et al.* 2006). EMSA and Western blot studies conducted on cancer cell lines with varying TG2 expressions demonstrated that in the presence of increased levels of TG2, there was a parallel increase in the activation as well as enzymatic activity of NFκB. With these results, it was hypothesized that there could be a direct correlation between the expression levels of TG2 and the activation of NFκB in numerous cancer cells (Mann, *et al.* 2006).

In spite of the noteworthy advances in primary treatment of breast cancer, prevention and prediction of metastasis and drug resistance remains a discouraging clinical problem. To improve progress in the field of chemotherapy, it is necessary to understand the basic molecular mechanism that is known to regulate the progression of a primary tumour to a metastatic disease. Several reports as well as results from Chapter III have clearly suggested that tissue transglutaminase (TG2) plays an important role in the development of metastatic and drug resistant cancer phenotype (Cao, *et al.* 2008 ; Grigoriev & al., 2001 ; Kim & al., 2006)

Mehta *et al.* (2004) isolated two clones, MDA MB 231 Clone 9 and Clone 16 from a metastatic parental breast cancer cell line, MDA MB 231 Wild Type based on their different TG2 expression levels and sensitivity to doxorubicin (Mehta, *et al.* 2004). The highly drug resistant clone, MDA MB 231 Clone 16 demonstrated almost a 12-15 fold increased expression of TG2 as compared to the drug sensitive MDA MB 231 Clone 9. Results obtained in the previous chapter (Chapter III), have illustrated that the parental MDA MB 231 WT obtained from Aston University did not show any detectable endogenous TG2 expression and activity. The MDA MB 231 Clone 9 demonstrated very low TG2 expression and activity while the MDA MB 231 Clone 16 cells illustrated the highest TG2 expression and activity of all the breast cancer cell lines. Prior evidence has suggested that without any relation to the type and source, the cancer cells that have been selected out for the chemoresistance exhibited an immoderate increase in the levels of TG2 (Verma, *et al.* 2006 ; Chhabra, *et al.* 2009). On the basis of the current observations made and the previous published results, chemoresistant breast cancer cells

express high levels of TG2 protein, which may imply that TG2 can protect breast cancer cell lines from apoptosis induced by stress as well as chemotherapy (Mehta, *et al.* 2004).

The molecular details behind the regulation of TG2 by NFκB (Rel A/p65) has been initially elucidated in Raw cells (with and without LPS treatment) using a ChIP based promoter. The ChIP promoter using Rel A/p65 specific antibody showed that Rel A/p65 was recruited to the -630 and -839 region of the *TGM2* gene promoter which is enhanced further in the presence of LPS. Parthenolide effectively blocked the binding of the Rel A/p65 to this region of the *TGM2* promoter (Ghanta, *et al.* 2011).

Increased TG2 activity was also known to trigger NFκB activation by causing the polymerization of the NFκB inhibitor, IκBα, as opposed to the stimulation of IKK (Kim, 2006). This polymerization of the IκBα subunit causes the consecutive activation of NFκB, which would result in the activation of target genes which are involved in inflammatory process (Falasca, *et al.* 2008 ; Kim, 2006). The lack of TG2 is an actual advantage, during the endotoxic shock as this deficiency seems to be related with the activation of NFκB which will allow the restoration of the immunological equilibrium (Ghanta, *et al.* 2011)

In view of the above observations, studies in this chapter will attempt to determine how the cross linking activity of TG2 may be responsible to promote oncogenic and drug resistant mechanisms in breast cancer cell lines. Evidence will be provided to understand the mechanistic detail behind the constitutive activation of NFκB by TG2 in breast cancer cells and how this aberrant activity of NFκB will lead to a chemoresistant phenotype. Studies will also be done to comprehend whether inhibiting TG2 cross linking activity using cell permeable and cell impermeable inhibitors and inhibition of mRNA TG2 expression will down regulate the constitutively activated NFκB and consequently decrease chemoresistance in breast cancer cell lines.

4.2 METHODS

4.2.1 Sub-cellular fractionation of breast cancer cell lines:

The whole cell lysate, cytoplasmic and nuclear fractions of the breast cancer cell lines were collected as described in detail in **Section 2.2.2.**

4.2.2 Western Blot analysis:

The subcellular components of the breast cancer cells were *analysed* for their protein concentration and 50µg of protein from each fraction was loaded onto an SDS-Page gel and then transferred onto a nitrocellulose membrane. The various primary antibodies and the complementary secondary antibodies were used to immuno probe the nitrocellulose membrane after appropriately blocking the membrane. The membranes were then developed using ECL from GE Healthcare and then subsequently exposing them to film (**Sections 2.2.3-2.2.6**). To ensure that the changes observed were significant, the results described in this study have been obtained from at least three independent experiments. Using anti-α-Tubulin as a loading marker for the cytoplasmic fractions and anti-Lamin-A as a loading marker for the nuclear fractions, equal loading of proteins was ensured. With the help of Image J software, the reported increase/ decrease in protein band densities were statistically assessed after densitometry analysis.

4.2.3 NFκB Activity Assay:

The NFκB activity assay was performed using the NFκB SEAPorter assay kit from IMGENEX. The assay kit includes pNFκB/SEAP plasmid that expresses the SEAP protein under the control of the NFκB promoter.

The protocol used was in accordance with the manufacturer's protocol (**Section 2.2.12)** .

4.2.4 Detection of the interaction between Rel A/ p65 and TG2 in the nucleus via co-immunoprecipitation

Co-immunoprecipitation assays were performed to detect the interaction between Rel A/ p65 and TG2 in the nucleus of high and no TG2 expressing breast cancer cell lines as introduced in **Section 2.2.8.** The rabbit anti-Rel A/ p65 polyclonal antibody and protein A beads were used to pull down the Rel A/ p65 immuno-complex in the pre-cleared (with Protein A beads) nuclear fraction samples. Western blotting (**Section 2.2.3-2.2.6)** was used to detect the presence of Rel A/ p65 dimers and monomers in the nucleus as well as the presence of TG2 in the immune complex using anti-TG2 monoclonal antibody (Cub 7402) (1:1000).

4.2.5 SiRNA transfection:

Rel A/p65 specific targeting siRNA was used to inhibit the expression of Rel A/p65 in the breast cancer cell lines using Lipofectamine 2000 transfection method, according to the manufacturers' instructions (Invitrogen). Three different siRNA sequences that target human Rel A/p65 were obtained from Qiagen. The sequences are listed as below:

Hs_RELA_5 AAGATCAATGGCTACACAG (denoted as (i))

Hs_RELA_7 CCGGATTGAGGAGAAACGT (denoted as (ii))

Hs_RELA_8 ATGGAGTACCCTGAGGCTA (denoted as (iii))

Universal negative control siRNA was also used to treat the cells as a control, to ensure that any changes observed were due to the action of the siRNA and no other factors relating to transfection procedure. The lyophilized siRNA was dissolved in 100 µl of sterile DNase / RNase free water to obtain a 10µM stock solution which was then stored at -20^{o}C. Prior to transfection, the breast cancer cell lines were seeded in plates and allowed to become 60-70% confluent, overnight. The cells were then washed with PBS, pH 7.4 and the medium was replaced with pre-warmed fresh complete growth medium. The siRNA were diluted in serum free growth medium for the siRNA to reach a final concentration of 100nM. The siRNA were

then pre-incubated with Lipofectamine 2000 in serum-free medium at room temperature for 15-20 min for the transfection complex to form. The complex was then added drop wise onto the cells while gently swirling the plate to make sure that the transfection complex was uniformly distributed. 48 h after transfection, the breast cancer cells were *analysed* for the Rel A/p65 expression in the subcellular fractions, relative NFκB activity levels and TG2 activity and expression levels in the cells.

4.3 RESULTS:

4.3.1 Aberrant Rel A/p65 expression and activity in the nuclear fractions of breast cancer cell lines that exhibit high levels of TG2.

4.3.1.1 Decreased NFκB activity in breast cancer cell lines in the presence of TG2 inhibitors

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT, MCF7/RT, SKBR3 and MCF7 cells transfected with TG2 plasmid and empty vector control plasmid were *analysed* for the relative NFκB activity levels in the presence of various TG2 inhibitors.

The NFκB SEAPorter Assay is designed to measure the level of NFκB activation using secreted alkaline phosphatase in the medium as a read out. The NFκB/SEAP plasmid expresses SEAP protein under the control of the NFκB promoter. Ligands and inhibitors added to the cells that alter NFκB activity can be screened for by measuring the SEAP present in the cell culture medium (**Section 2.2.12).**

The TG2 expressing MDA MB 231 Clone 16, MCF7/RT and TG2 transfected SKBR3 and MCF7 illustrated a higher activation of NFκB as compared to the MDA MB 231 WT, MCF7/WT and empty vector transfected SKBR3 and MCF7 cells. On treating the MDA MB 231 Clone 16, MCF7/RT and TG2 transfected SKBR3 and MCF7 cells with the TG2 cell permeable inhibitor, Z-DON, maximum inhibition of NFκB activity was observed, followed by R283. The cell impermeable TG2 specific inhibitor, R294 did not show any significant decrease in NFκB activity compared to the cell permeable inhibitors (Figures 4.1). This could imply that the activation of NFκB in these MDA MB 231 Clone 16, MCF7/RT and TG2 transfected SKBR3 and MCF7 cells could be due to the role of intracellular activity of TG2. These cell permeable TG2 inhibitors illustrated a significant difference in both the TG2 activity, TG2

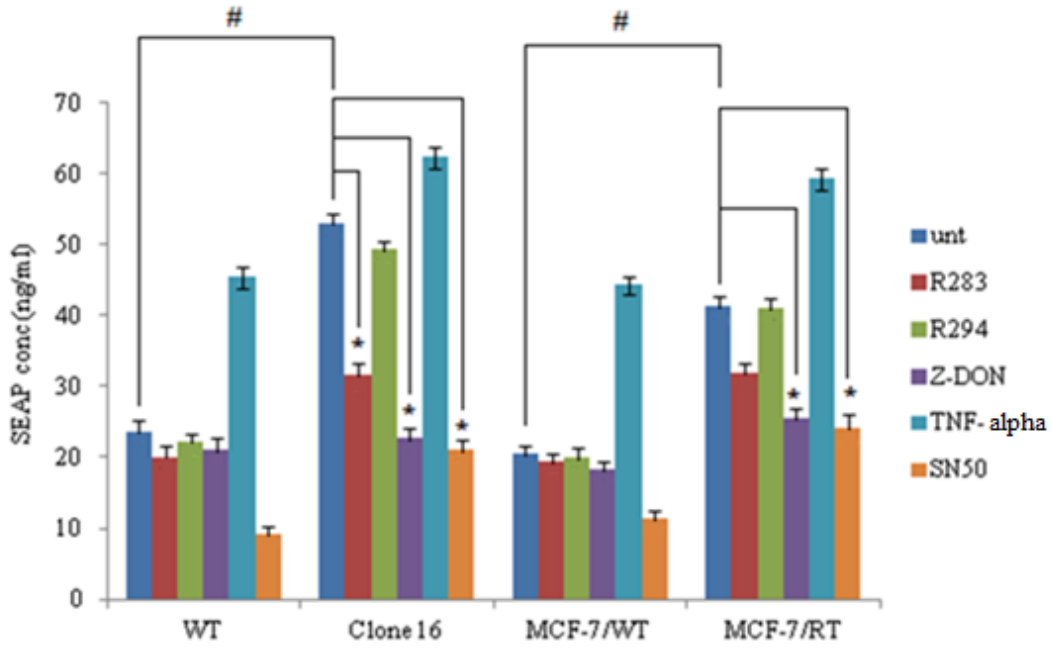
expression and subsequently the basal NFκB activity levels measured in these breast cancer cell lines.

The low and no TG2 expressing breast cancer cell lines (MDA MB 231 WT, MCF7/WT, SKBR3/WT, SKBR3/RT, T47D/WT, T47D/RT and empty vector transfected SKBR3 and MCF7 cells) that have been treated with the TG2 inhibitors, did not show any significant change in the NFκB activity (Figures 4.1). This could be attributed to the lack of TG2 expression as well as TG2 activity within these breast cancer cell lines.

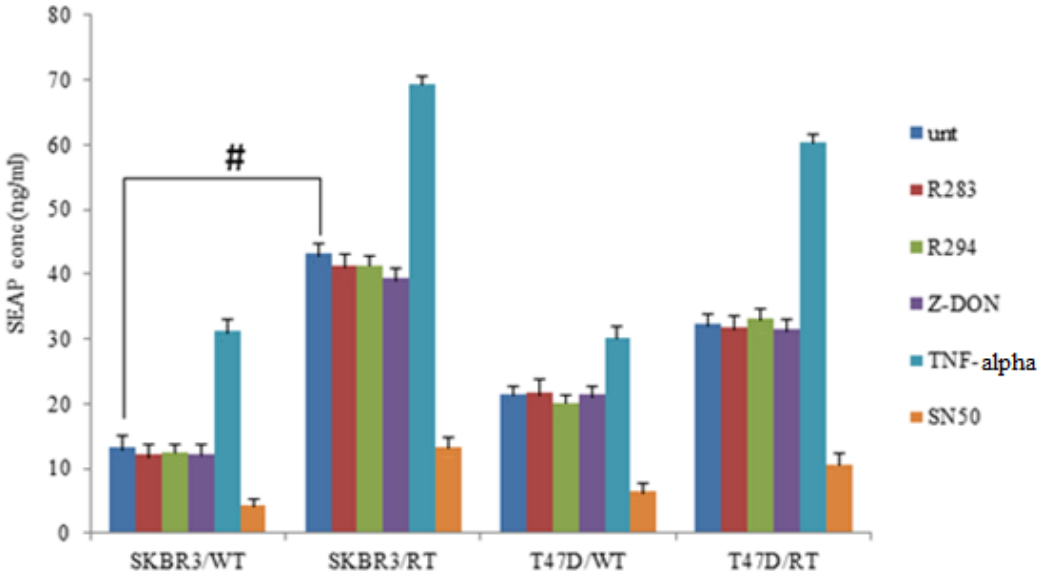
Also, on treating the high TG2 and TG2-null breast cancer cell lines with TNF-α, the NFκB activity increased significantly. Furthermore, a considerable decrease was seen in the NFκB activity of these breast cancer cells on being treated with SN50 which is an NFκB inhibitor that inhibits the translocation of NFκB into the nucleus. TNF-α and SN50 were used as the positive and negative controls respectively to determine the efficiency of the assay.

These results strongly indicate that the constitutive activation of NFκB in breast cancer cell lines can be attributed to the transamidating activity of TG2. In the absence of TG2, the SKBR3 and MCF7 cells illustrate low levels of NFκB activity while transfecting these cell lines with the TG2 plasmid demonstrated an increase in NFκB activity levels, which could be constitutively induced due to the presence of TG2. This increased NFκB activity in the high TG2 breast cancer cell lines could be reduced significantly in the presence of cell permeable TG2 activity inhibitors.

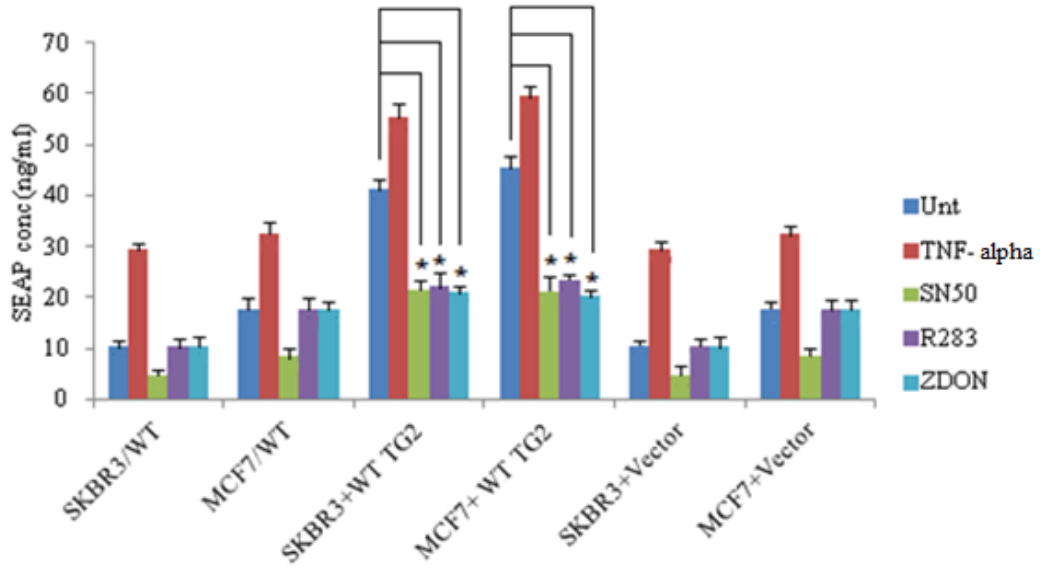
(A)


#
#
70
60
50
40
30
20
10
0
SEAP conc (ng/ml)
*
*
*
*
unt
R283
R294
Z-DON
TNF- alpha
SN50
WT
Clone 16
MCF-7/WT
MCF-7/RT

(B)


80
70
60
50
40
30
20
10
0
SEAP conc (ng/ml)
#
unt
R283
R294
Z-DON
TNF-alpha
SN50
SKBR3/WT
SKBR3/RT
T47D/WT
T47D/RT

(C)



Figures 4.1 (A, B and C) Analysis of NFκB activity levels in breast cancer cell lines in the presence of TG2 activity inhibitors

*The NFκB activity of the breast cancer cell lines were analysed using the NFκB/SEAP Reporter Assay. 5x10^5 cells of each breast cancer cell line was seeded into 6-well plates and left overnight. The next day the cells were transfected with NFκB/SEAP plasmid. 48 h after transfection, the breast cancer cell lines were treated with TG2 inhibitors, R283 (500μM), R294 (500μM) and Z-DON (50μM). The supernatant was then collected for the SEAP assay as described in detail in Section 2.2.12. Data shown is mean concentration of SEAP + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * p< 0.05 between untreated and TG2 inhibitor treated cells. # p<0.05 between cell groups.*

4.3.1.2 Detection of Rel A/p65 expression in the cytoplasmic and nuclear fractions of breast cancer cell lines in the presence of TG2 inhibitors.

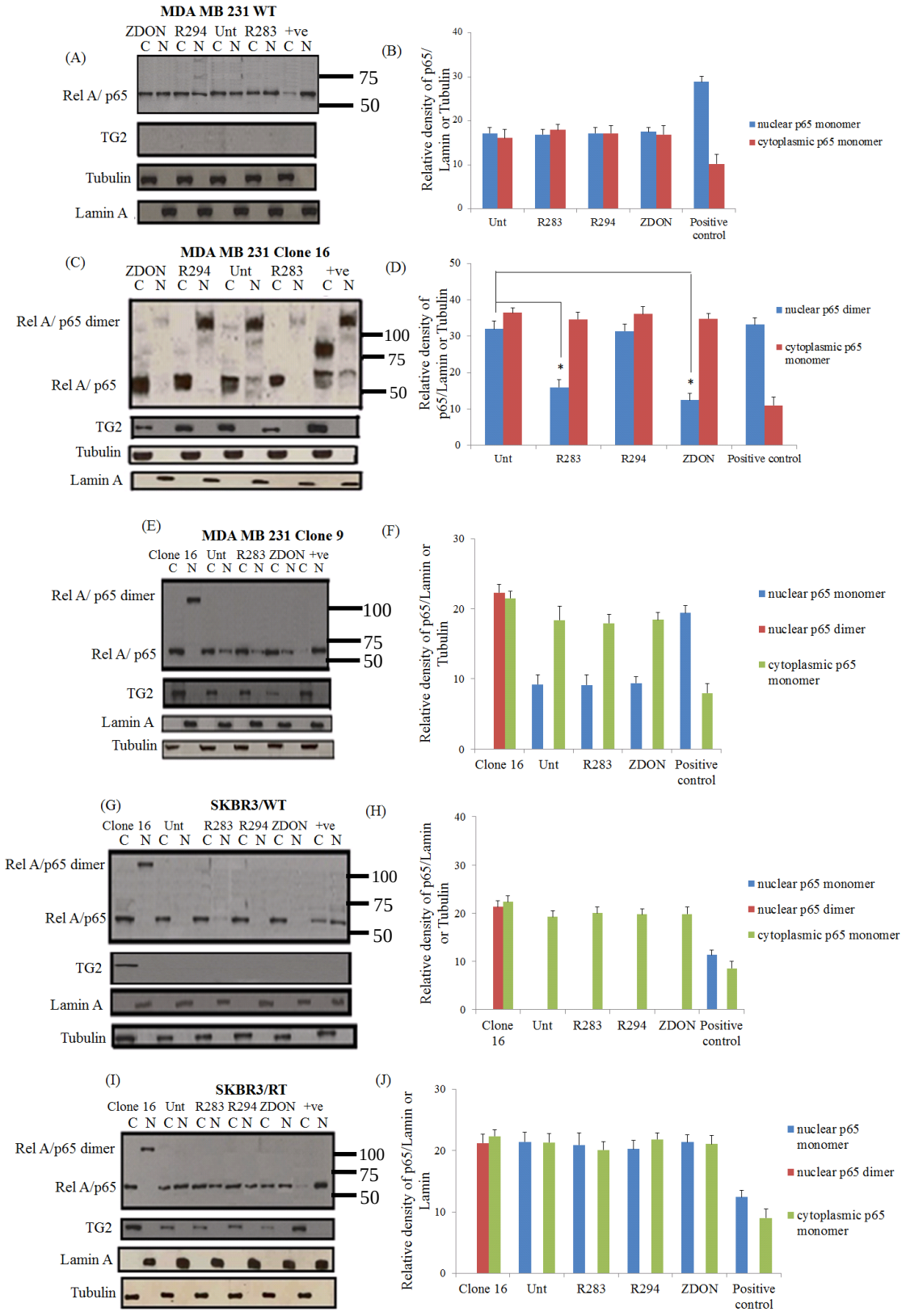
The decrease observed in the expression of TG2 within the breast cancer cells on treating them with TG2 activity inhibitors indicated that another factor may be involved in regulating TG2 expression within these cells. Also, previous data has shown that TG2 is responsible for the constitutive activation of NFκB within these cells (Mann, *et al.* 2006). The breast cancer cell lines were seeded and allowed to become confluent (70-80%) overnight. After treating the cells for 72 h with TG2 inhibitors, the samples were collected and fractionated into their cytoplasmic and nuclear fractions as described in **Section 2.2.2** and analysed by Western blotting.

In the western blot data obtained, monomeric Rel A/p65 in the cytoplasm of the breast cancer cells at 65kDa was observed. However, in the nucleus of TG2 expressing MDA MB 231 Clone 16 and MCF7/RT cell lines as well as TG2 transfected MCF7 and SKBR3 cells, the Rel A/p65 band was observed at a higher molecular higher weight. The Rel A/p65 in the nucleus appears to be approximately 130kDa in size which may indicate that this could be a high molecular weight form of Rel A/p65. Also, this high molecular weight Rel A/p65 observed in the nuclear fractions could be representative of a cross linked form of RelA/p65 (Figure 4.2) as a result of TG2 activity within these high TG2 breast cancer cell lines. Treating the MDA MB 231 Clone 16, MCF7/RT and TG2 transfected SKBR3 and MCF7 breast cancer cell lines with cell permeable TG2 activity inhibitors (R283 and Z-DON) demonstrated a considerable decrease in the high molecular weight Rel A/p65, which was not observed on treatment with R294 (cell impermeable TG2 activity inhibitor). The decrease observed in the high molecular weight Rel A/p65 form in the nucleus is concurrent with the decrease in the TG2 expression in the cytoplasmic fraction of MDA MB 231 Clone 16, MCF7/RT and TG2 transfected SKBR3 and MCF7 cell lines. Also, as demonstrated in Chapter 3, the TG2 activity of the MDA MB 231 Clone 16, MCF7/RT and TG2 transfected SKBR3 and MCF7 cells also decreased on treatment with cell permeable TG2 activity inhibitors, R283 and Z-DON. This could imply that intracellular TG2 activity is responsible for the constitutive activation of NFκB in these breast

cancer cell lines as both the TG2 cell permeable activity inhibitors seem to reduce the constitutive activation of NFκB more significantly than the cell impermeable TG2 activity inhibitor, R294.

The TG2 null MDA MB 231 WT, MCF7/WT and SKBR3/WT cells did not illustrate any high molecular weight Rel A/p65 in the nucleus and only a monomeric Rel A/p65 was observed in the cytoplasmic and nuclear fractions. Treating these no or low expressing TG2 breast cancer cell lines with TG2 inhibitors, does not cause any change in the Rel A/p65 expression within these cells. On transfecting the MCF7 and SKBR3 cells with TG2 as introduced in **Section 2.2.1.6**, the Rel A/p65 high molecular weight form was observed in the nuclear fractions which can be attributed to the expression and activity of TG2 observed within these breast cancer cells. The empty vector control plasmid does not induce the expression and activity of TG2 and in these cells no high molecular weight Rel A/p65 form was observed (Figure 4.2).

Even though the MDA MB 231 Clone 9 and SKBR3/RT cells do illustrate low TG2 expression and activity, no high molecular weight Rel A/p65 forms were observed in the nuclear fraction. Also, treatment with the TG2 inhibitors (R283 and Z-DON) did not demonstrate any difference in the cytoplasmic and nuclear expression of Rel A/p65, even though there was a clear decrease observed in the TG2 expression in the MDA MB 231 Clone 9 and SKBR3/RT cells. This may imply that the amount of TG2 expression levels and activity within breast cancer cell lines was crucial to determine if the Rel A/p5 high molecular weight form will be observed in the nuclear fraction. Also, these results indicate that high expression and activity of TG2 was essential for the formation of the Rel A/p65 high molecular weights in the nucleus. The breast cancer cell lines that have previously been shown to be resistant to doxorubicin, demonstrate high expression and activity of TG2 as well as a high molecular weight Rel A/p65 form in the nuclear fractions. This aberrant form of Rel A/p65 could therefore be responsible for rendering these breast cancer cell lines resistant to doxorubicin as Rel A/p65 has been shown previously to confer drug resistance (Kim, *et al.* 2006)


MDA MB 231 WT
ZDON R294 Unt R283 +ve
C N C N C N C N C N
(A)
Rel A/ p65
TG2
Tubulin
Lamin A
75
50
(B)
Relative density of p65/ Lamin or Tubulin
nuclear p65 monomer
cytoplasmic p65 monomer
Unt
R283
R294
ZDON
Positive control
MDA MB 231 Clone 16
(C)
Rel A/ p65 dimer
Rel A/ p65
100
(D)
Relative density of p65/Lamin or Tubulin
nuclear p65 dimer
cytoplasmic p65 monomer
MDA MB 231 Clone 9
(E)
Clone 16 Unt R283 ZDON +ve
(F)
Relative density of p65/Lamin or Tubulin
SKBR3/WT
(G)
Clone 16 Unt R283 R294 ZDON +ve
Rel A/p65 dimer
Rel A/p65
(H)
SKBR3/RT
(I)
(J)

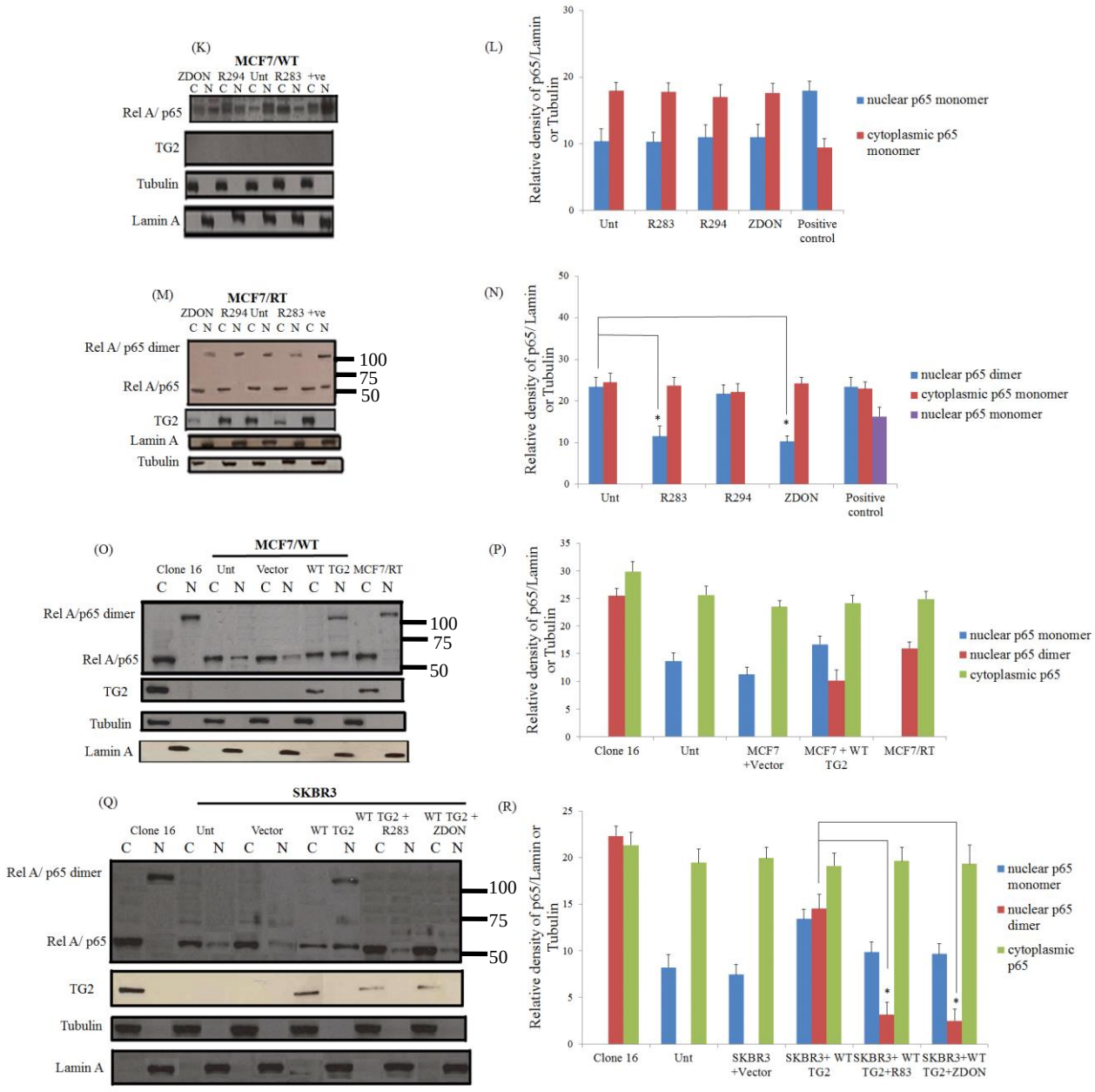


Figure 4.2 (A-R) Analysis of the expression of Rel A/p65 in the cytoplasmic and nuclear fractions of breast cancer after treatment with TG2 activity inhibitors using western blot.

The MDA MB 231 WT (A), MDA MB 231 Clone 16 (C),MDA MB 231 Clone 9 (E), SKBR3/WT (G), SKBR3/RT (I), MCF7/WT (K), MCF7/RT (M) and TG2 and vector plasmid transfected MCF7 (O) and SKBR3 (Q) cancer cell lines (1x10^6 cells per well) were seeded into 60mm petridishes and left overnight to become confluent. The next day, the cells were treated with R283 (500μM), R294 (500μM) and Z-DON (50μM) for a time course of 72 h. After treatment,

*the cells were fractionated into the cytoplasmic and nuclear fractions as described in detail in Section 2.2.2. The sub cellular fractions were then analysed by western blotting for the expression of Rel A/p65 using rabbit polyclonal anti-Rel A/p65 antibody (1:1000) and anti-TG2 antibody (Cub 7402; TG100) (1:1000). The membranes were reprobed with anti- Lamin A and anti-α-Tubulin antibody to check for equal loading of protein. The breast cancer cells were treated with 10ng/ml of TNFα for 24 h and the sub cellular fractions were collected and used as the positive control. (**A, C, E, G, I, K, M, O & Q**) The data shown above is taken from three independent experiments (n=3). Differences in expression (**B, D, F, H, J, L, N & P**) as calculated by Image J analysis is representative of the mean densitometry reading obtained from three independent experiments + SEM. * p<0.05*

4.3.1.3 Co-immunoprecipitation of Rel A/p65 and TG2 in the nuclear fraction of breast cancer cell lines after treatment with TG2 inhibitors.

To determine the association between Rel A/ p65 high molecular weights and TG2 in the nucleus, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were treated with TG2 activity inhibitors R283, R294 and Z-DON. 72 h after treatment, the nuclear fractions of these cells were collected as described in **Section 2.2.2**. Using anti- Rel A/p65 antibody and Protein A beads, the Rel A/p65 complex was immunoprecipitated out as described in **Section 2.2.8**.

In the nuclear fractions of the MDA MB 231 WT and MCF7/WT cells, only a monomeric form of Rel A/p65 (~ 65kDa) was observed with no significant change in the nuclear Rel A/ p65 in the presence of TG2 inhibitors. This can clearly be attributed to the absence of TG2 expression and activity within these breast cancer cell lines (Figures 4.3).

On the other hand, in the nuclear fractions of the MDA MB 231 Clone 16 and MCF7/RT cells, it was observed that two Rel A/ p65 bands were pulled down using the rabbit polyclonal anti-Rel A/p65 antibody, a monomeric Rel A/p65 (~65kDa) and a high molecular weight Rel A/p65 (~130kDa). On treating these breast cancer cells with cell permeable, TG2 inhibitors, the high molecular weight Rel A/p65 form decreased significantly, with no change observed in the monomeric Rel A/p65 (Figures 4.3). Also, on reprobing the membrane with anti-TG2 antibody, no nuclear TG2 expression was observed. This was in correlation to the localization studies done in Chapter III, which demonstrated that the expression and activity of TG2 within the TG2 expressing breast cancer cell lines was primarily concentrated in the cytosol. The cell impermeable TG2 inhibitor, R294, failed to show any significant decrease in both the monomeric and high molecular weight form of Rel A/p65. These results suggest that the high molecular weight form of Rel A/p65 observed in the nucleus fractions was as a result of cytosolic TG2 cross linking activity, as TG2 expression is completely absent in the nucleus of these breast cancer cell lines.

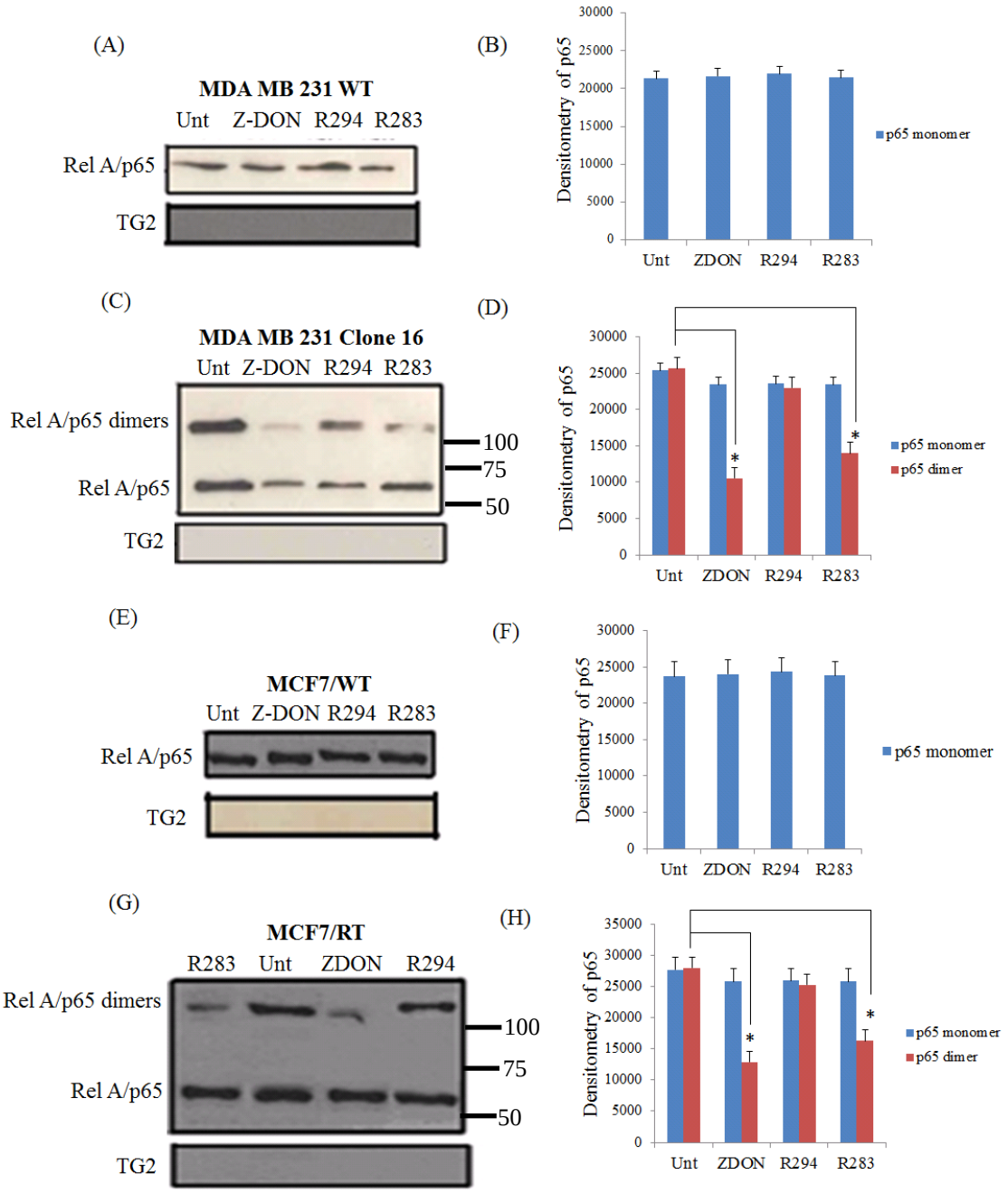


Figure 4.3 (A-H) Detection of the interaction between TG2 and Rel A/p65 in the nuclear fractions of breast cancer cell lines

The MDA MB 231 WT (A), MDA MB 231 Clone 16 (C), MCF7/WT (E) and MCF7/RT (G) cells (1x10^6 cells/ well) were seeded into 60 mm petridishes and left overnight. The next day these breast cancer cell lines were treated with R283 (500μM), R294 (500μM) and Z-DON (50μM) over a time course of 72 h. After treatment, the breast cancer cells were fractionated into the cytoplasmic and nuclear fractions as described in Section 2.2.2. The nuclear fractions of the

*breast cancer cell lines were used in co-immunoprecipitation experiments as introduced in Section 2.2.8. After collection of the nuclear fraction and pre-clearing using protein A beads, rabbit anti-Rel A/p65 polyclonal antibody (0.5µg/sample) and protein A beads were used to pull down the Rel A/ p65 immunocomplex and (**A, C, E, &G**) western blotting was then used to detect the presence of Rel A/p65 (1:1000) and TG2 (1:1000) within the immunocomplex collected from the various cell samples (n=3). (**B, D, F &H**) Differences in expression as calculated by Image J analysis is representative of the mean densitometry reading obtained from three independent experiments + SEM. * $p<0.05$*

4.3.2 Expression of NFκB family members in breast cancer cell lines

4.3.2.1 Western blot detection of p105/p50 in breast cancer cell lines in the presence of TG2 inhibitors.

In order to determine if the expression and activity of TG2 in breast cancer cell lines affected the status of the other NFκB family members in these breast cancer cell lines, the MDA MB 231 Wild Type and MDA MB 231 Clone 16 were analysed by western blot (introduced in **Sections 2.2.4-2.2.6**) to examine the relative expression of p105/p50 in the cytoplasmic and nuclear fractions of the breast cancer cell lines. After 72 h of treating these breast cancer cell lines with TG2 activity inhibitors, R283, R294 and Z-DON, the cells were fractionated into the cytoplasmic and nuclear fractions (**Section 2.2.2**). On analysis of the sub cellular fractions by western blotting using anti- p105/p50 antibody (1:500), no difference was observed between the TG2 null MDA MB 231 WT and high TG2 expressing MDA MB 231 Clone 16 cells. Subsequent treatment of the breast cancer cell lines with the various TG2 inhibitors (R283, R294 and Z-DON), failed to demonstrate any significant change in the expression levels of p105/p50, both in the cytoplasmic as well as nuclear fraction of the cell lines (Figures 4.4). These results clearly indicate that the activity and expression of TG2 within the breast cancer cell lines did not have any effect on the expression of p105/p50. Also, this could be indicative that the constitutive activation of NFκB members by TG2 did not involve p105/ p50.

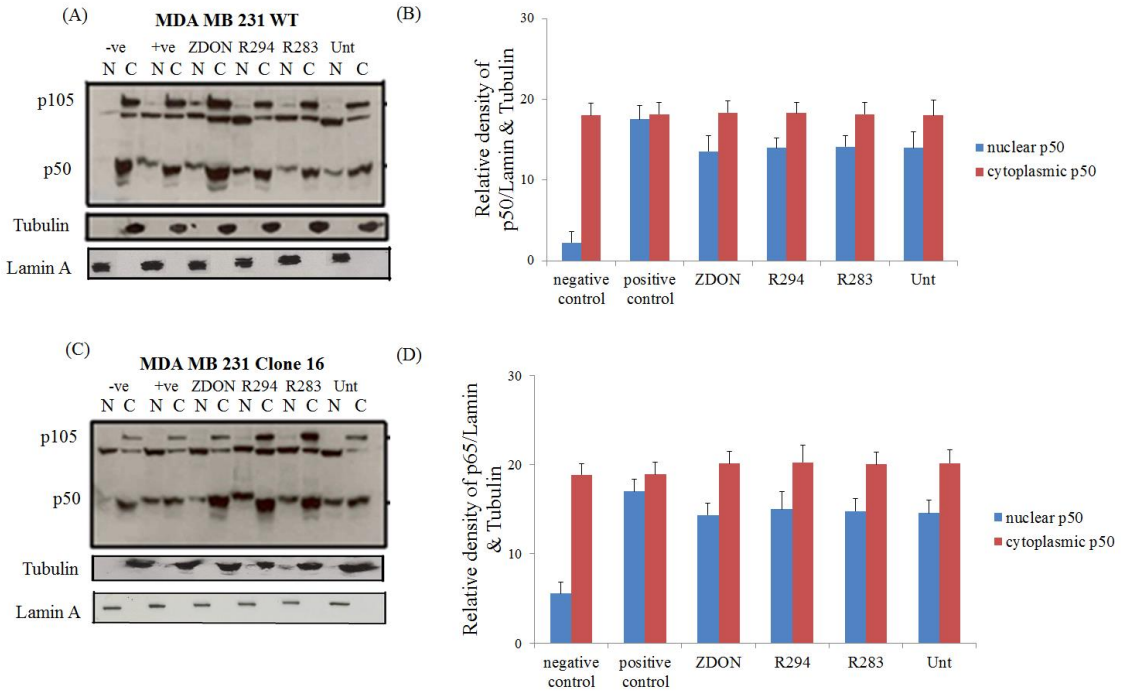


Figure 4.4 Analysis of p105/p50 in the cytoplasmic and nuclear fractions of breast cancer cell lines in the presence of TG2 activity inhibitors by western blot

*The MDA MB 231 WT (A) and MDA MB 231 Clone 16 (C) (1x10^6 cells/ well) were seeded into 60 mm petridishes and left overnight. The next day, the cells were treated with R283 (500μM), R294 (500μM) and Z-DON (50μM) for a time course of 72 h. After treatment, the cells were fractionated into the cytoplasmic and nuclear fractions as described in detail in Section 2.2.2. TNF-α and SN50 treated cells were taken as the positive and negative controls respectively. **(A&C)** The sub cellular fractions were then analysed by western blot (Section 2.2.4- 2.2.6) for the expression of p105/ p50 using rabbit polyclonal anti-p105/p50 antibody (1:500). The membranes were reprobed with anti-α-Tubulin and Lamin A antibody to check for equal loading of protein. The data shown is a representation of three independent experiments (n=3). **(B&D)** Differences in expression as calculated by Image J analysis is representative of the mean densitometry reading obtained from three independent experiments + SEM.*

4.3.2.2 Expression of Rel B and c-Rel in the presence of TG2 inhibitors.

To effectively determine whether TG2 expression and activity had any significant effect on the expression of the other NFκB family members, the relative levels of Rel B, c-Rel and p52/p100 were determined in the whole cell lysate fractions of the MDA MB 231 WT and MDA MB 231 Clone 16 cells. The WCL fractions of the breast cancer cell lines were collected as described in **Section 2.2.2** and 50µg of whole cell lysate protein was *analysed* by western blot using anti-Rel B, anti-c-Rel and anti-p52/p100 antibodies (Figure 4.5). The MDA MB 231 WT and MDA MB 231 Clone 16 cells did not demonstrate any difference in the expression levels of Rel B and c-Rel. Also, treating these breast cancer cell lines with R283, R294 and Z-DON, did not alter the protein levels of Rel B or c-Rel. The MDA MB 231 WT and MDA MB 231 Clone 16 cells did not express any p100/p52, which remained unchanged, in the presence of TG2 inhibitors. This could signify that the expression and activity of TG2 within breast cancer cell lines only caused the constitutive activation of the Rel A/p65 member of the NFκB family and had no effect at all on the other family members.

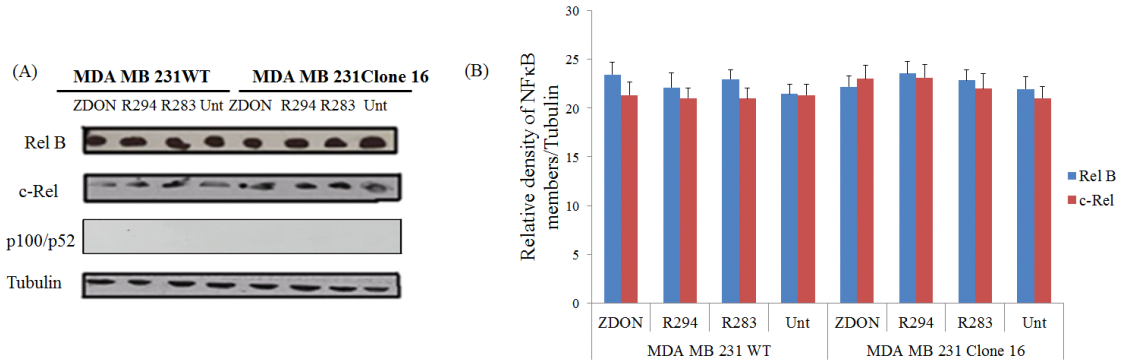


Figure 4.5 Analysis of Rel B, c-Rel and p100/p52 in the whole cell lysate fractions of breast cancer cell lines in the presence of TG2 activity inhibitors by western blot

The MDA MB 231 WT and MDA MB 231 Clone 16 (1x10^6 cells/ well) were seeded into 60 mm petridishes and left overnight. The next day, the cells were treated with R283 (500µM), R294 (500µM) and Z-DON (50µM) for a time course of 72 h. After treatment, the whole cell lysate fractions of the breast cancer cells were collected as described in detail in Section 2.2.2. The whole cell lysate fractions(50µg protein) were then analysed by ***(A)*** *western blot (Section 2.2.4-2.2.6) for the expression of Rel B, c-Rel and p100/p52 using rabbit polyclonal anti-Rel B antibody (1:1000), rabbit polyclonal anti-c-Rel antibody (1:1000) and rabbit polyclonal anti-p100/p52 antibody (1:1000). The membranes were reprobed with anti-α-Tubulin antibody to check for equal loading of protein (n=3).* ***(B)*** *The data shown is a representation of three independent experiments. Differences in expression as calculated by Image J analysis is representative of the mean densitometry reading obtained from three independent experiments + SEM.*

4.3.3 Constitutive activation of Rel A/p65 decreased in the presence of TG2 specific siRNA

4.3.3.1 Decreased Rel A/p65 high molecular weight in the presence of TG2 siRNA

To determine if inhibiting the mRNA expression of TG2 would cause an effect on the formation of Rel A/ p65 high molecular weights, the MDA MB 231 Clone 16 and MCF7/RT cells were transfected with 100nm of TG2 specific siRNA as well as global negative control siRNA for a time course of 48 h as described previously using Lipofectamine (**Section 2.2.1.5**) After the treatment, these cells were collected and fractionated into the cytoplasmic and nuclear fractions as explained in **Section 2.2.2.** Subsequently, western blot was used to detect the presence of Rel A/p65 high molecular weights in the nucleus as well as TG2 expression in the cytosolic fraction

The MDA MB 231 Clone 16 and MCF7/RT cells that have been known previously to express high levels of TG2, illustrated a significant decrease in the TG2 expression levels on being treated with the two TG2 specific siRNA. Consequently, the Rel A/p65 high molecular weight observed in the nuclei of untreated and global negative siRNA treated samples, decreased and a monomeric form of Rel A/p65 in the nuclei was seen, on treating the breast cancer cell lines with TG2 specific siRNA (Figures 4.6).

With these results, it may be concluded, that the Rel A/ p65 high molecular weights seen in the MDA MB 231 Clone 16 and MCF7/RT nuclei is an effect of TG2 transamidating activity within these cells. Treating these cells with TG2 siRNA shows a decrease in the high molecular weight Rel A/ p65 form seen in the nuclei of these cells. There is also a subsequent increase in the expression of Rel A/p65 monomers in the nucleus which was previously not observed in the MDA MB 231 Clone 16 and MCF7/RT cells on being treated with cell permeable TG2 activity inhibitors.

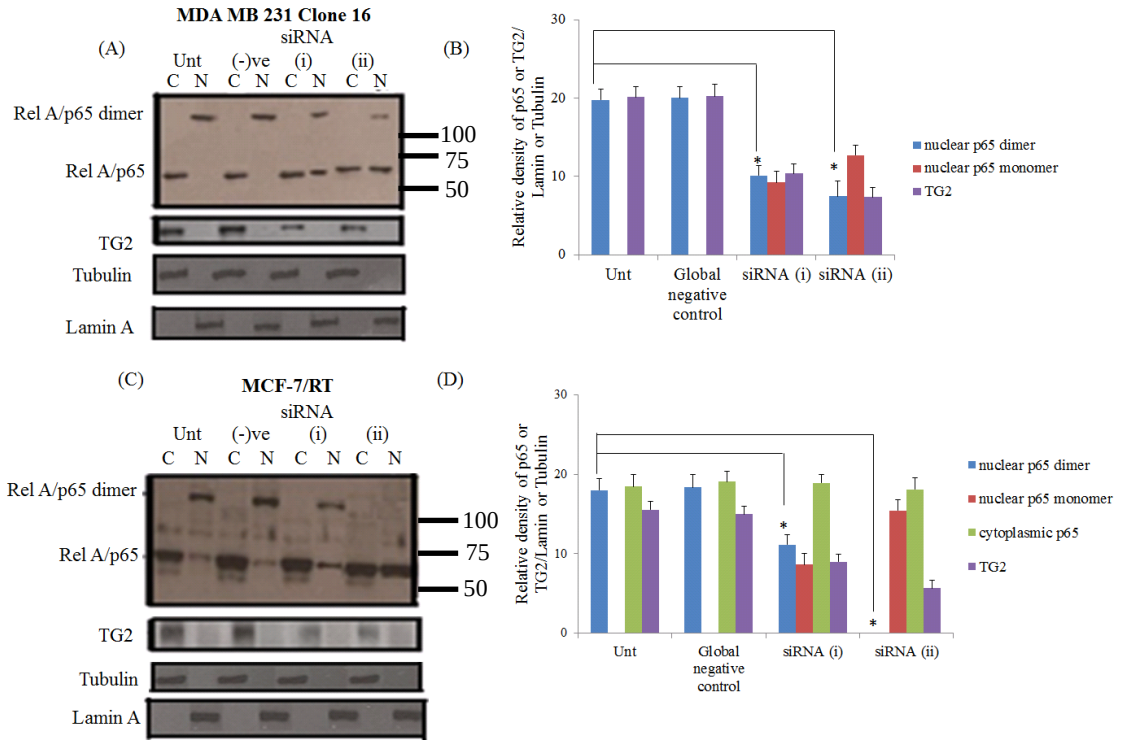


Figure 4.6 Analysis of the expression of Rel A/p65 in the cytoplasmic and nuclear fractions of breast cancer after transfection with TG2 specific siRNA using western blot.

*TG2 siRNA targeting human TG2 were used to inhibit the mRNA expression of TG2 in the MDA MB 231 Clone 16 (A) and MCF7/RT(C) cell lines. 5x10^{5}cells (per well) were seeded into 6 well plates and left overnight. The next day, the cells were transfected with two TG2 specific siRNA (final concentration of 100nm) for a time course of 48 h (Section 2.2.1.5). Global negative control siRNA was used as the negative control. After treatment, the cells were fractionated into the cytoplasmic and nuclear fractions as described in detail in Section 2.2.2. (**A &C**) The sub cellular fractions were then analysed by western blot for the expression of Rel A/p65 using rabbit polyclonal anti-Rel A/p65 antibody (1:1000). The membranes were reprobed with anti-Lamin A and anti-α-Tubulin antibody to check for equal loading of protein. The data shown are representative westerns from three independent experiments (n=3). (**B&D**) Differences in expression as calculated by Image J analysis is taken from the mean densitometry reading obtained from three independent experiments + SEM. * p<0.05*

4.3.3.2 NFκB activity decreases in breast cancer cell lines in the presence of TG2 siRNA

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were assayed for the respective NFκB activity levels as described previously in **Section 2.2.12**. The NFκB reporter assay showed that on treating the high TG2 breast cancer cell lines with TG2 siRNA, the NFκB activity within these cells decreased considerably. This correlated with the cell viability assay in the presence of doxorubicin, wherein the breast cancer cells lost chemoresistance to doxorubicin on being treated with TG2 siRNA. These results could indicate that the presence of TG2 in these breast cancer cell lines constitutively activates the Rel A/ p65 subunit of the NFκB family members in an aberrant manner, which decreases on being treated with the TG2 specific siRNA. The MDA MB 231 WT and MCF7/WT cells demonstrated very low levels of NFκB activity, which remained unchanged on being treated with TG2 specific siRNA. This could imply that the aberrant and constitutive activation of Rel A/ p65 observed in the MDA MB 231 Clone 16 and MCF7/RT cells was as a result of TG2 expression and activity within these breast cancer cell lines. Treatment with TG2 specific siRNA clearly shows a significant decrease in the NFκB activity levels in these breast cancer cells, which is similar to the basal levels of NFκB activity observed in the chemosusceptible parent cell lines (Figure 4.7).

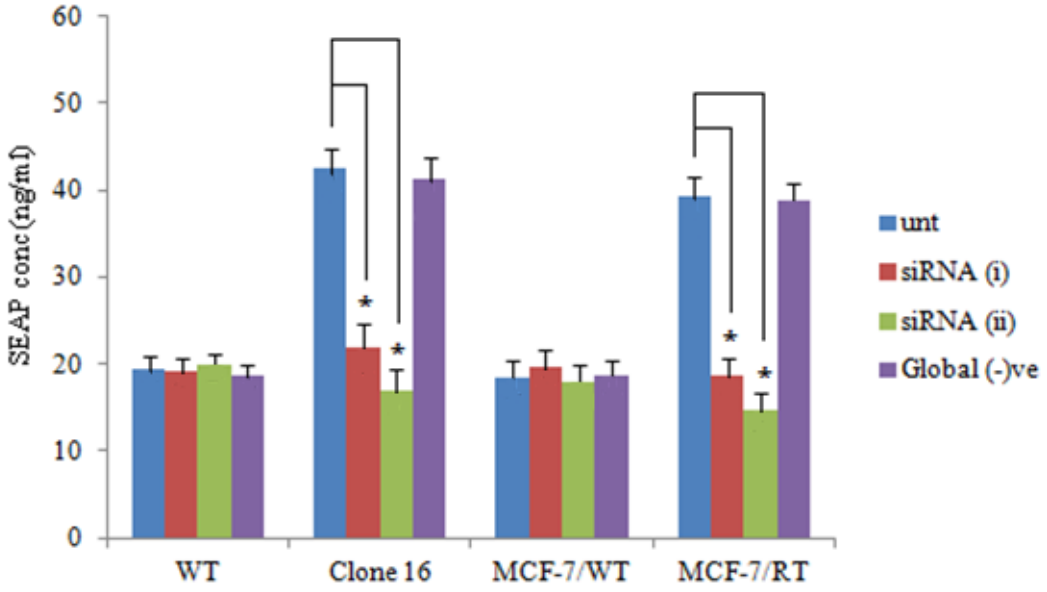


Figure 4.7 Analysis of NFκB activity levels in breast cancer cell lines in the presence of TG2 specific siRNA.

*The NFκB activity of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines were analysed using the NFκB/SEAP Reporter Assay. $5x10^5$ cells of each breast cancer cell line were seeded into 6-well plates. The next day, the cells were transfected with NFκB/SEAP plasmid. 48 h after transfection, the breast cancer cell lines were treated with TG2specific siRNA (at a final concentration of 100nM). The supernatant was then collected for the SEAP assay as described in detail in Section 2.2.12. Data shown is mean concentration of SEAP + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * $p < 0.05$ between cells transfected and non-transfected with siRNA.*

4.3.4 Treatment of breast cancer cells with Rel A/p65 specific siRNA

4.3.4.1 Decreased expression of Rel A/p65 and TG2 in the cytoplasmic and nuclear fractions of breast cancer cell lines on being treated with Rel A/p65 specific siRNA

The MDA MB 231 WT and MDA MB 231 Clone 16 cells were transfected with Rel A/p65 specific siRNA as described in **Section 2.2.1.5** and subsequently fractionated into the cytoplasmic and nuclear fractions (**Section 2.2.2**). The fractions were then *analysed* by western blotting to detect the presence of Rel A/p65 and TG2. The MDA MB 231 WT cells demonstrated a clear decrease in the Rel A/p65 expression in both the cytoplasmic and nuclear fractions on being treated with Rel A/p65 specific siRNA for 48 h. The MDA MB 231 WT cells did not express any TG2, which does not change on to transfection with Rel A/p65 specific siRNA (Figure 4.8). The MDA MB 231 Clone 16 cells demonstrated a significant decrease in the monomeric and high molecular weight Rel A/p65 in the cytoplasm and nucleus respectively on being transfected with Rel A/p65 specific siRNA (Figure 4.8). The TG2 levels in the cytoplasm of the MDA MB 231 Clone 16 cells also decreases, subsequent to Rel A/p65 siRNA transfection, with the most significant decrease observed with Hs_RELA_5 (i), followed by Hs_RELA_7 (ii) and the least decrease was demonstrated by Hs_RELA_8 (iii) (Figure 4.8). The global negative control siRNA does not show any difference in the Rel A/p65 expression levels in the TG2 expressing and TG2 null breast cancer cell lines. These results seem to imply that the expression of TG2 in the MDA MB 231 Clone 16 was dependent on transcriptional activity of Rel A/p65. Inhibiting the mRNA expression of Rel A/p65 in the MDA MB 231 Clone 16 cells resulted in the decrease of TG2 expression within these high TG2 expressing breast cancer cells. The membranes were then reprobed with anti-α-Tubulin and anti-Lamin A to determine equal loading of protein.

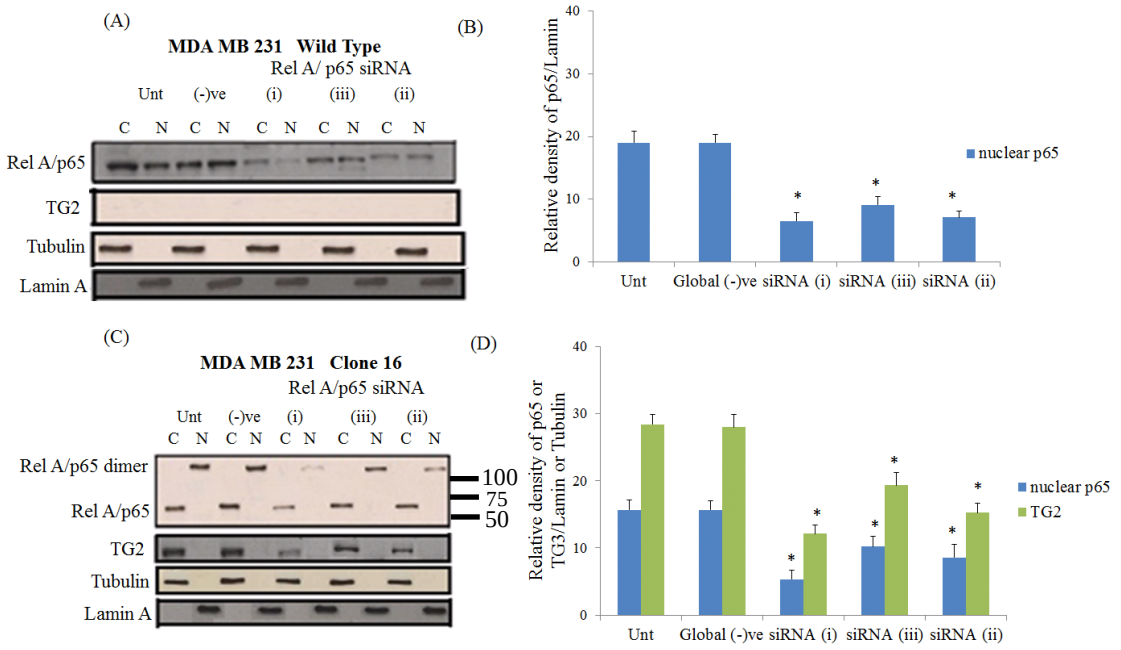


Figure 4.8 Analysis of the expression of Rel A/p65 and TG2 in the cytoplasmic and nuclear fractions of breast cancer after transfection with Rel A/ p65 specific siRNA using western blot.

*Rel A/p65 siRNA targeting human p65 were used to inhibit the expression of Rel A/p65 in the MDA MB 231 WT (A) and MDA MB 231 Clone 16 (C) cell lines. 5x10^{5}cells (per well) were seeded into 6 well plates and left overnight. The next day, the cells were transfected with three different Rel A/p65 specific siRNA (final concentration of 100nM) for a time course of 48 h (Section 2.2.1.5). Global negative control siRNA was used as the negative control. After treatment, the cells were fractionated into the cytoplasmic and nuclear fractions as described in detail in Section 2.2.2. The sub cellular fractions were then analysed by western blot for the expression of Rel A/p65 using rabbit polyclonal anti-Rel A/p65 antibody (1:1000) as well as mouse monoclonal anti-TG2 antibody (1:1000). The membranes were reprobed with anti-Lamin A and anti-α-Tubulin antibody to check for equal loading of protein. (**A &C**)The western blot data shown is a representative of three independent experiments (n=3). (**B &D**) Differences in expression as calculated by Image J analysis is representative of the average densitometry readings obtained from three independent experiments + SEM. * p<0.05*

4.3.4.2 Decreased NFκB activity of breast cancer cell lines in the presence of Rel A/p65 specific siRNA

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were transfected with Rel A/p65 specific siRNA and then *analysed* for the NFκB activity levels using the NFκB/SEAP reporter assay as described previously in **Section 2.2.12**. The SEAP reporter assay demonstrated a considerable decrease in the NFκB activity levels of the breast cancer cell lines on being transfected with the Rel A/p65 specific siRNA (Figure 4.9), which is in comparison to the data obtained by western blot analysis (Figure 4.8). The Hs_RELA_5 (i) demonstrated the most significant decrease in NFκB activity, followed by Hs_RELA_7 (ii) and finally Hs_RELA_8 (iii) in all of the breast cancer cell lines. The remaining NFκB activity observed in these breast cancer cell lines could be representative of the activity of the other NFκB family members in the breast cancer cell lines. These results seem to imply that the majority of the NFκB activity observed in the MDA MB 231 Clone 16 and MCF7/RT cells is as a result of constitutive activation of the Rel A/p65 member of the NFκB family due to the cross linking activity of TG2. Also, treating the MDA MB 231 Clone 16 and MCF7/RT cells with Rel A/p65 specific siRNA reduces the NFκB activity levels within these breast cancer cells to similar levels observed in the untreated MDA MB 231 WT and MCF7/WT cells. This clearly suggests that the aberrant activation of NFκB within the MDA MB 231 Clone 16 and MCF7/RT from the basal levels could be attributed to the endogenous transamidating activity of TG2 within these breast cancer cell lines.

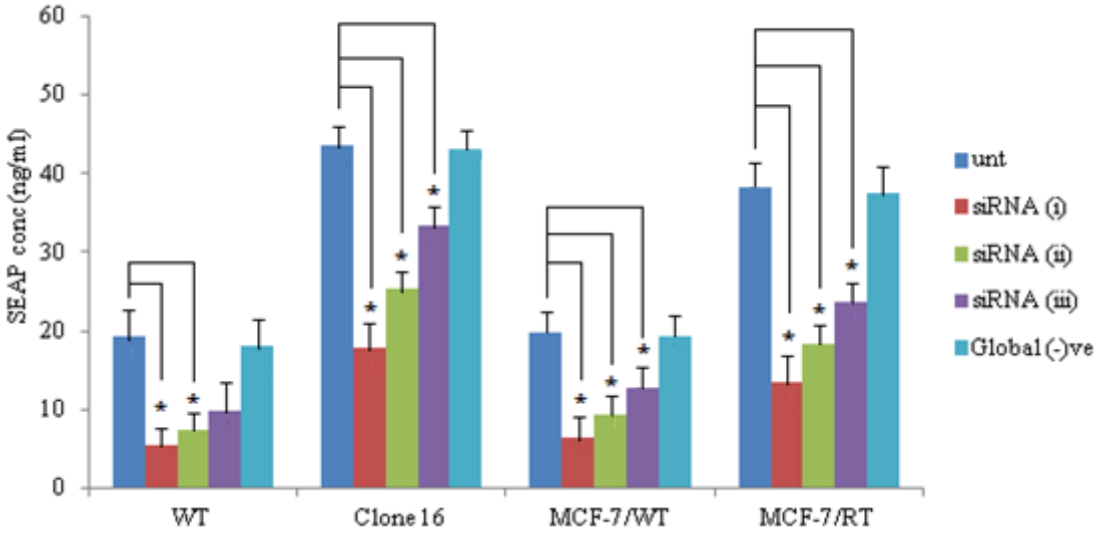


Figure 4.9 Analysis of NFκB activity levels in breast cancer cell lines in the presence of Rel A/p65 specific siRNA.

*The NFκB activity of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines were analysed using the NFκB/SEAP Reporter Assay. $5x10^5$ cells of each breast cancer cell line was seeded into 6-well plates and left overnight. The next day, the cells were transfected with NFκB/SEAP plasmid. 48 h after transfection, the breast cancer cell lines were treated with three different Rel A/p65 specific siRNA as well as global negative control siRNA (at a final concentration of 100nM). The supernatant was then collected for the SEAP assay as described in detail in Section 2.2.12. Data shown is mean concentration of SEAP ± SE from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * p< 0.05 between cells transfected and non-transfected with siRNA.*

4.3.4.3 Detection of TG2 activity of breast cancer cell lines on treatment with Rel A/ p65 targeting siRNA

As described in **Section 2.2.1.5**, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were treated with Rel A/p65 specific siRNA and the whole cell lysates of these breast cancer cell lines were collected (**Section 2.2.2).** The whole cell lysate fractions of the breast cancer cell lines treated with Rel A/p65 specific siRNA and global negative control siRNA were *analysed* for TG2 activity (**Section 2.2.11**). The MDA MB 231 WT and MCF7/WT cells do not express any TG2 activity in the whole cell lysate fractions, which remains unchanged on transfection with Rel A/p65 specific siRNA. On the other hand, the MDA MB 231 Clone 16 and MCF7/RT cells that are known to exhibit TG2 activity show a significant decrease in the TG2 activity levels on being transfected with Rel A/p65 specific siRNA. The Hs_RELA_5 (i) shows the most significant decrease, followed by Hs_RELA_7 (ii) and finally Hs_RELA_8 (iii) (Figure 4.10). These results are in correlation with the decrease observed in the NFκB activity (Figure 4.9) and expression levels as well as TG2 expression within the MDA MB 231 Clone 16 and MCF7/RT cell lines (Figure 4.8) . The global negative control siRNA does not demonstrate any change in the TG2 activity levels within the above mentioned breast cancer cell lines. These results seem to indicate that inhibiting the mRNA expression of Rel A/p65 using Rel A/p65 targeting siRNA in the high TG2 expressing breast cancer cell lines could lead to a significant decrease in both the TG2 activity (Figure 4.10) as well as TG2 expression of the MDA MB 231 Clone 16 and MCF7/RT cell lines (Figure 4.8).With this, it is possible to speculate that Rel A/p65 has a response element on the *TGM2* gene promoter which would allow control of the expression and subsequent activity of TG2 within these cells.

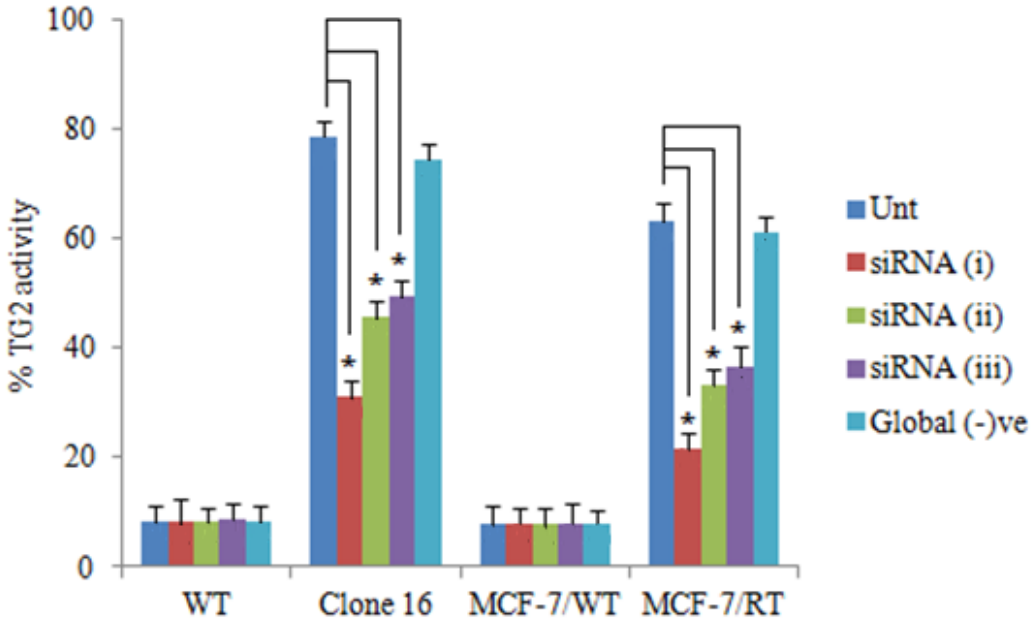


Figure 4.10 Investigation of TG2 whole cell lysate activity in the presence of Rel A/p65 -specific siRNA

*The whole cell lysate fractions of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT after treatment with three different Rel A/p65 specific siRNA over a time course of 48 h were collected and analysed for the TG2 activity as described in Section 2.2.11 by biotinylated cadaverine incorporation into fibronectin. Positive control (50 ng/ml gplTG + 10mM $CaCl_2$) was taken to be 100% TG2 activity (1.13258±0.20936). Data shown above is mean percentage TG2 activity + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * $p<0.05$ between cells transfected and non-transfected with siRNA.*

4.3.4.4 Increased susceptibility of chemoresistant breast cancer cell lines to doxorubicin on treatment with Rel A/p65 specific siRNA

To determine the effect of inhibiting mRNA expression of Rel A/p65 using Rel A/p65 specific siRNA on the chemoresistance of the breast cancer cell lines, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines were transfected with Rel A/p65 specific siRNA in the presence of doxorubicin. The viability of the breast cancer cell lines was then determined using the XTT assay as described in detail in **Section 2.2.13**. The MDA MB 231 WT and MCF7/WT cells were chemosusceptible to doxorubicin and subsequent transfection with Rel A/p65 siRNA and global negative control siRNA maintains the chemosensitive phenotype of the TG2 null breast cancer cell lines. On the other hand, the untreated and global negative control siRNA transfected MDA MB 231 Clone 16 and MCF7/RT cell lines have been shown to be chemoresistant against doxorubicin. Transfecting these breast cancer cell lines with Rel A/p65 siRNA demonstrated a significant decrease in the cell viability of the high TG2 breast cancer cell lines, in the presence of doxorubicin. It was also noted that transfecting the high TG2 breast cancer cells with Rel A/ p65 siRNA induced toxicity in the cells (Figure 4.11).

Previous studies have shown that the activation of NFκB can attenuate the apoptotic response of breast cancer cells against chemotherapeutic drugs and subsequently inhibiting the constitutive activation of NFκB can greatly sensitize tumour cells to chemotherapy (Karin, *et al.* 2002). Also, one of the major drivers of constitutive NFκB activity in cancer cell lines has been identified as TG2 (Mangala & Mehta, 2005). Correlating these findings with the results obtained in the current study, it can be hypothesized that in the high TG2 breast cancer cell lines, MDA MB 231 Clone 16 and MCF7/RT, the chemoresistant phenotype against doxorubicin can be a feature of constitutively activated Rel A/p65 induced by TG2 transamidating activity.

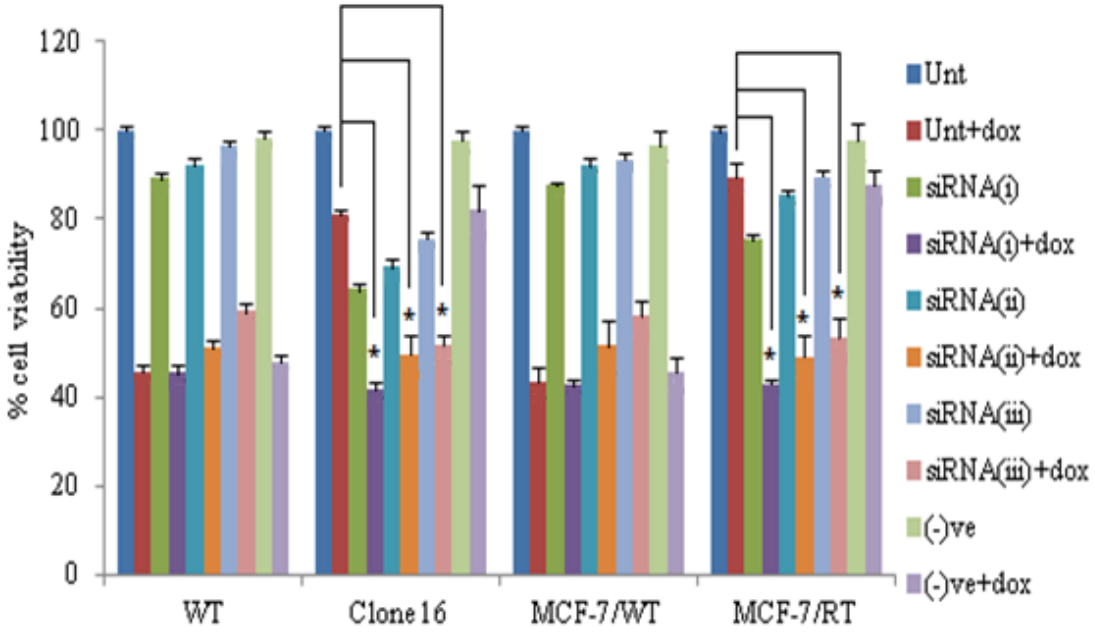


Figure 4.11 The effect of transfecting breast cancer cell lines with Rel A/p65 specific siRNAs in the presence of doxorubicin as illustrated via XTT assay.

*The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells (3000 cells per well) were seeded into 96-well plates and left overnight. The next day the cells were treated with three different Rel A/p65 specific siRNA for 48 h (final concentration of 100nM) and doxorubicin (1μg/ml) for a time course of 72 h after which the viable cells were detected using XTT analysis. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * $p<0.05$ between cells transfected and non-transfected with siRNA in the presence of doxorubicin.*

4.3.5 Aberrant Rel A/p65 is not altered on treatment with various TG2 inactivating antibodies.

4.3.5.1 Detection of TG2 cell surface and whole cell lysate activity on treatment with different TG2 antibodies.

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were *analysed* for the whole cell lysate and cell surface TG2 activity assay as described in **Section 2.2.11**, subsequent to treatment with TG2 antibodies. The three TG2 antibodies used include mouse monoclonal ID10, mouse monoclonal, transglutaminase-inactivating antibody (TG2 In Ac Ab) and mouse monoclonal Cub 7402. Mouse monoclonal IgG isotype was used as the negative control for the experiment.

With respect to the cell surface TG2 activity in the breast cancer cell lines, the MDA MB 231 Clone 16 cell line are the only breast cancer cells that express cell surface TG2 activity. On being treated with the three different TG2 antibodies and the mouse IgG isotype, the MDA MB 231 Clone 16 cells demonstrated a significant decrease in the cell surface TG2 activity. The most significant decrease was observed when the MDA MB 231 Clone 16 cells were treated with mouse monoclonal transglutaminase-inactivating antibody (TG2 In Ac Ab), followed by mouse monoclonal ID10 antibody and finally mouse monoclonal Cub 7402 antibody. The mouse IgG isotype did not cause any change in the cell surface TG2 activity of the MDA MB 231 Clone 16 cells. The MDA MB 231 WT, MCF7/WT and MCF7/RT cells did not express any cell surface TG2 activity and so, there was no change on being treated with the TG2 antibodies and the mouse IgG isotype (Figure 4.12).

To determine the whole cell lysate activity of TG2 in the breast cancer cell lines on being treated with the various TG2 antibodies as well as mouse IgG isotype, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were seeded into 6-well plates ($5x10^5$ cells/ well), and subsequently treated with 25ng/ml of the TG2 antibodies as well as Mouse IgG isotype for a time course of 24 h. After treatment, the whole cell lysate fractions of the breast

cancer cell lines were obtained as described in **Section 2.2.2**. 50μg of whole cell lysate protein of each treated cell line was used to determine the whole cell lysate TG2 activity as described in detail in **Section 2.2.11**.

The MDA MB 231 WT and MCF7/WT cell lines did not demonstrate any TG2 whole cell lysate activity and so remain unaffected by the treatment with TG2 antibodies as well as mouse IgG isotype (Figure 4.13). The TG2 expressing MDA MB 231 Clone 16 and MCF7/RT cells illustrated no significant change in the whole cell lysate TG2 activity on being treated with the various TG2 antibodies. The mouse IgG was used as the negative control for the TG2 antibodies.

These results signified that the TG2 antibodies used were cell impermeable and so only affected the cell surface TG2 activity of the MDA MB 231 Clone 16 cells. Also, the whole cell lysate activity of the high TG2 expressing MDA MB 231 Clone 16 and MCF7/RT cells remain unaltered on treatment with the TG2 antibodies.

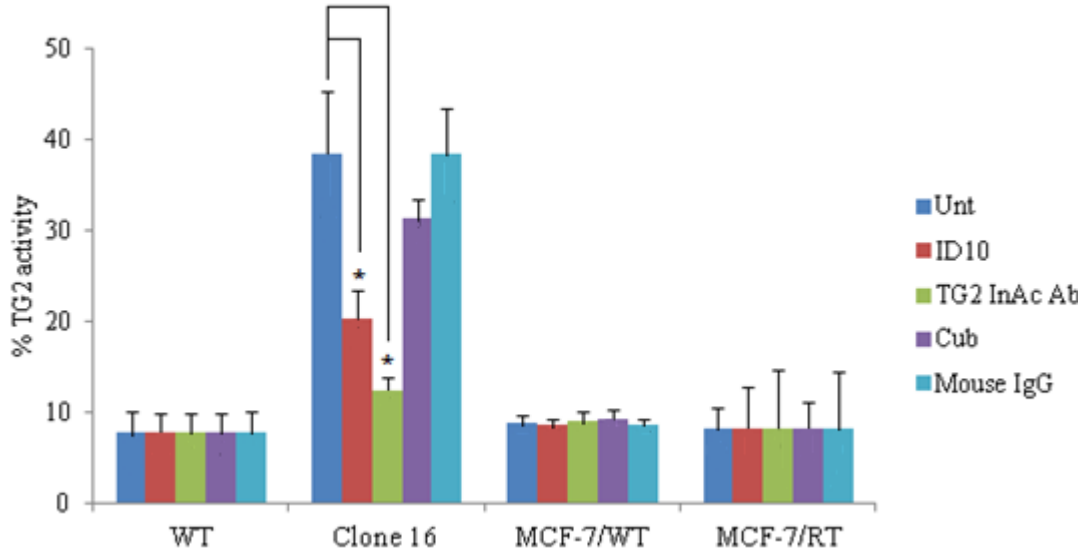


Figure 4.12 Investigation of TG2 cell surface activity in the presence of TG2 antibodies

*The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT (2X 10^4 per 100µl of medium) were seeded into 96-well plates and analysed for the cell surface TG2 activity by biotinylated cadaverine incorporation into fibronectin as introduced in Section 2.2.11. Positive control (50 ng/ml gplTG + 10mM $CaCl_2$) was taken to be 100% TG2 activity (1.2947 ± 0.199364). Data shown above is mean percentage TG2 activity + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * $p<0.05$ between untreated and TG2 antibody treated cells.*

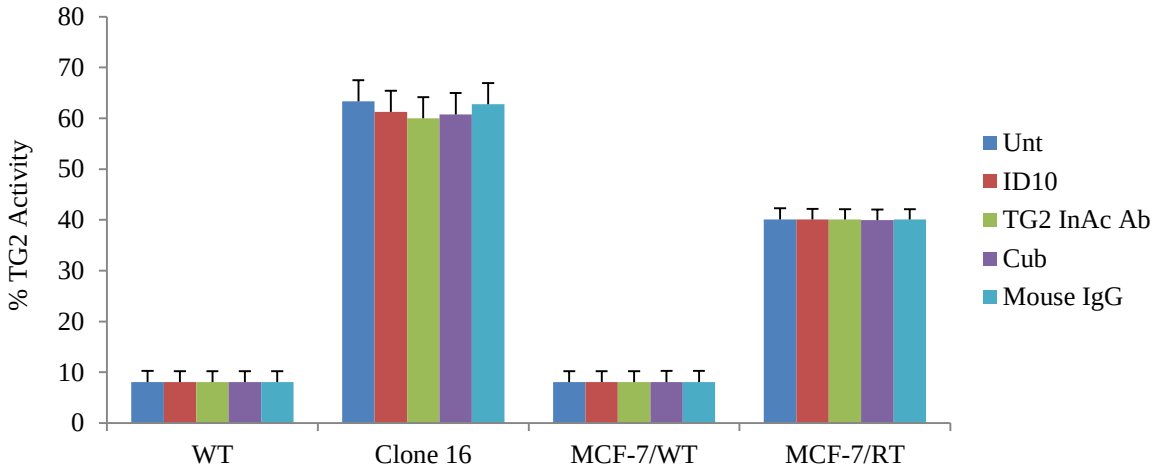


Figure 4.13 Investigation of TG2 whole cell lysate activity in the presence of TG2 antibodies

The whole cell lysates of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT after treatment of cells with TG2 antibodies, ID10 (25ng/ml), transglutaminase-inactivating antibody (TG2 In Ac Ab) (25ng/ml), Cub 7402(25ng/ml) and mouse IgG (25ng/ml) for a time course of 24 h were collected and 50µg of whole cell lysate protein was analysed for the TG2 whole cell lysate activity as described in Section 2.2.11 by biotinylated cadaverine incorporation into fibronectin. Positive control (50 ng/ml gplTG + 10mM $CaCl_2$) was taken to be 100% TG2 activity (1.24345 ±0.254846). Data shown above is mean percentage TG2 activity + SEM from 3 independent experiments (n=3).

4.3.5.2 NFκB activity and expression on treatment with TG2 antibodies

To confirm that extracellular TG2 activity observed in the MDA MB 231 Clone 16 cells was not responsible for the formation of the Rel A/p65 high molecular weights as well as the aberrant activation of Rel A/p65 in these breast cancer cell lines, TG2 targeting antibodies were used. The mouse monoclonal antibody ID10, mouse monoclonal transglutaminase-inactivating (TG2 In Ac Ab) antibody as well as mouse monoclonal Cub 7402 were applied to the breast cancer cell lines. Mouse IgG was used as the negative control antibody. The MDA MB 231 WT and MDA MB 231 Clone 16 cells were treated with the various TG2 antibodies (final concentration of 25ng/ml) and negative control mouse IgG (25ng/ml) for a time course of 24 h. After treatment, the expression of Rel A/p65 in the cytoplasmic and nuclear fractions was determined by western blot (**Section 2.2.2**) using polyclonal rabbit anti-Rel A/p65 antibody.

The untreated MDA MB 231 WT cells only express monomeric Rel A/p65 in the cytoplasmic and nuclear fractions. No change was observed in the Rel A/p65 monomer in the cytoplasmic and nuclear fractions, on treatment with the various TG2 antibodies. This could be attributed to the lack of TG2 expression and activity within these cells (Figure 4.14). The negative control mouse IgG, also did not demonstrate any change in the Rel A/p65 expression within these breast cancer cell lines.

In the untreated MDA MB 231 Clone 16 cells, Rel A/p65 high molecular weights were observed in the nuclear fractions and the monomeric Rel A/p65 form was observed in the cytoplasmic fractions (Figure 4.14) . As described previously, this high molecular weight form of p65 could be representative of the high TG2 cross linking activity within these cells and this high molecular weight Rel A/p65 has also been shown to decrease in the presence of TG2 specific cell permeable inhibitors. On treating the MDA MB 231 clone 16 cells with the various TG2 antibodies for a time course of 24 h, no significant change was observed in the Rel A/p65 high molecular weight in the nucleus or even the monomeric Rel A/p65 in the cytoplasm.

These results seem to imply that the high molecular weight Rel A/ p65 band observed was specifically due to intracellular TG2 activity. Interfering with extracellular TG2 either through the binding of various antibodies (transglutaminase-inactivating antibody [(TG2 In Ac Ab)], ID10 and Cub 7402) did not illustrate any significant difference in the RelA/ p65 high molecular weight band. The negative control mouse IgG isotype treated MDA MB 231 Clone 16 cells demonstrated no change as compared to the untreated Clone 16 cells. The increased constitutive activation of Rel A/p65 in the MDA MB 231 Clone 16 cells can be thus clearly attributed to the increased expression and intracellular activity of TG2 within these breast cancer cell lines.

To further substantiate the results obtained by western blot, the NFκB/SEAP reporter assay (described in **Section 2.2.12**) was performed on the MDA MB 231 WT, MDA MB 231 Clone 16, MCF/WT and MCF7/RT cells, on treatment with the various TG2 antibodies. Both the TG2 expressing MDA MB 231 Clone 16 and MCF7/RT as well as TG2 null MDA MB 231 WT and MCF7/WT cells did not illustrate any significant change in the NFκB activity levels. The breast cancer cell lines treated with the various TG2 antibodies, on comparison with the untreated cell lines, display almost comparable levels of NFκB activity (Figure 4.15)

With the above findings, it can be clearly established that the high and constitutive activation of NFκB by TG2 is an intracellular phenomenon in the MDA MB 231 Clone 16 and MCF7/RT cell lines. The TG2 antibodies target and inactivate the activity of TG2 on the cell surface does not demonstrate any significant change in the NFκB activity levels in the high TG2 breast cancer cell lines. This completely rules out the possibility that inhibiting extracellular TG2 would decrease the aberrant and constitutive activation of NFκB.

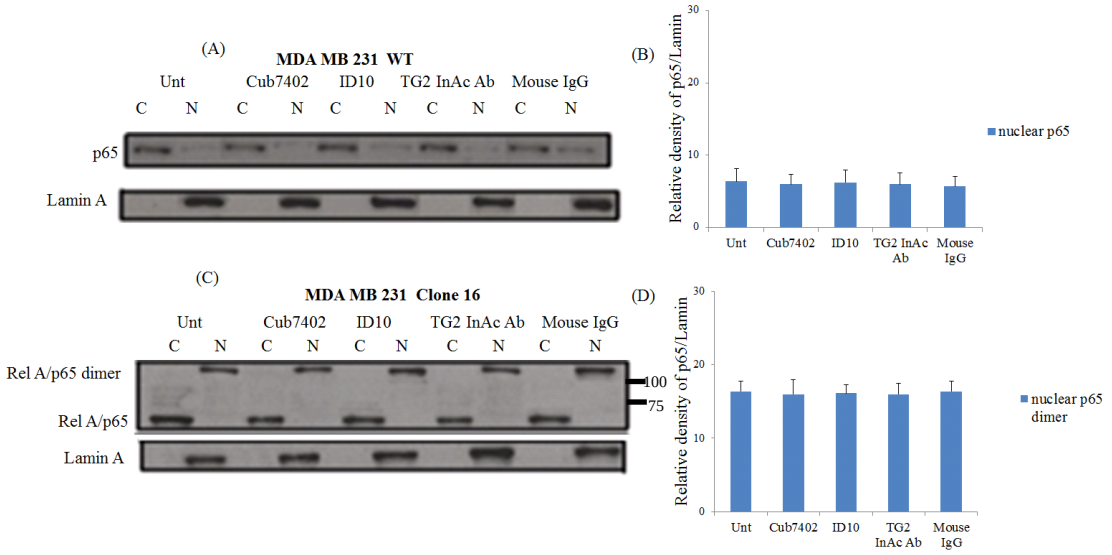


Figure 4.14 Analysis of the expression of Rel A/p65 in the cytoplasmic and nuclear fractions of breast cancer after treatment with various TG2 antibodies using western blot.

*The MDA MB 231 WT (A) and MDA MB 231 clone 16 (C) cells (1x10^6 cells) were seeded into 60 mm petridishes and left overnight. The next day, the cells were treated with ID10, transglutaminase-inactivating antibody (TG2 In Ac Ab), Cub 7402 and mouse IgG for a time course of 24 h. After treatment, the cells were fractionated into the cytoplasmic and nuclear fractions as described in detail in Section 2.2.2. The sub cellular fractions were then analysed by western blot ting for the expression of Rel A/p65 using rabbit polyclonal anti-Rel A/p65 antibody (1:1000). The membranes were reprobed with anti- Lamin A antibody to standardise for equal loading of nuclear protein. (**A & C**) The western blot data shown is a representation of three independent experiments (n=3). (**B &D**) Differences in expression as analysed by Image J analysis is representative of the mean densitometry reading obtained from three independent experiments + SEM.*

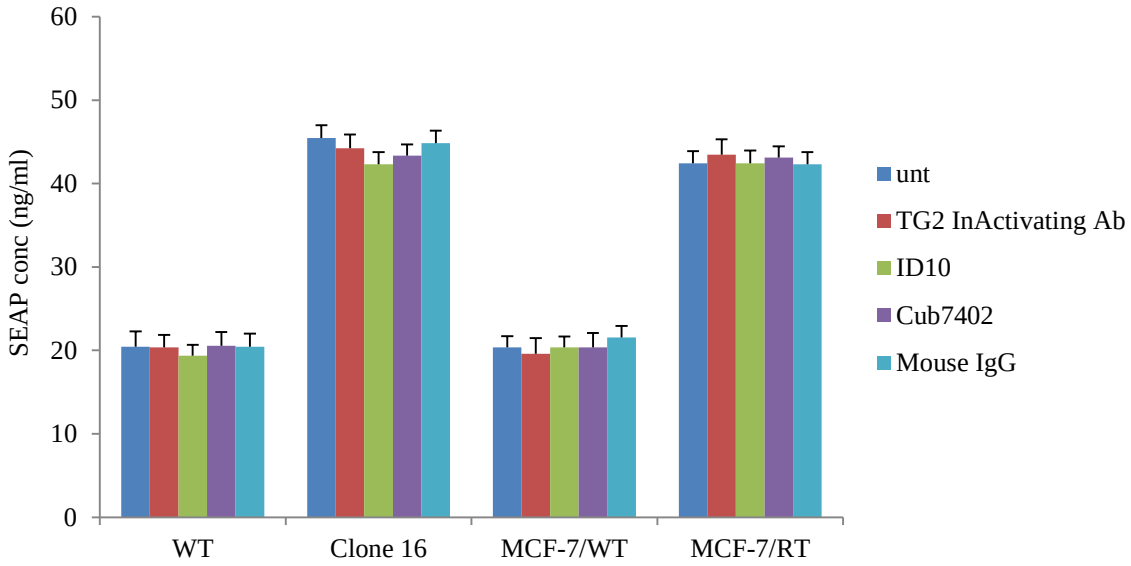


Figure 4.15 Analysis of NFκB activity levels in breast cancer cell lines on treatment with various TG2 antibodies.

The NFκB activity of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines were analysed using the NFκB/SEAP Reporter Assay (Section 2.2.12). 5x10^5 cells of each breast cancer cell line were seeded into 6-well plates and transfected with NFκB/SEAP plasmid. 48 h after transfection, the breast cancer cell lines were treated with three TG2 antibodies, ID10 (25ng/ml), transglutaminase-inactivating antibody (TG2 In Ac Ab) (25ng/ml) and Cub 7402 (25ng/ml) as well as mouse IgG (25ng/ml) for 24 h. The supernatant was then collected for the SEAP assay. Data shown is mean concentration of SEAP + SEM from 3 independent experiments (n=3).

4.3.5.3 Cell viability analysis of breast cancer cell lines in the presence of doxorubicin and TG2 antibodies

From the results observed in **Section 4.3.4**, it can be hypothesized that inhibiting cell surface TG2 activity of the breast cancer cell lines, did not decrease the aberrant and constitutive activation of the Rel A/p65 subunit of the NFκB family. To determine whether any changes may be observed in the chemoresistant nature of these breast cancer cell lines, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were treated with the different TG2 antibodies (ID10, transglutaminase-inactivating antibody (TG2 In Ac Ab), Cub 7402 and Mouse IgG isotype (25ng/ml), for a time course of 24 h in the presence of doxorubicin. The high TG2 expressing MDA MB 231 Clone 16 and MCF7/RT cells have previously been shown to be highly chemoresistant to doxorubicin and this chemoresistance was reduced significantly on treating these breast cancer cell lines with cell permeable TG2 activity inhibitors (R283 and Z-DON). On treating the high TG2 expressing MDA MB 231 Clone 16 and MCF7/RT cells with the various TG2 antibodies in the presence of doxorubicin, no change was observed in the viability of the cell lines as compared to the untreated cell lines. The chemoresistant nature of the MDA MB 231 Clone 16 and MCF7/RT cells was still maintained even on treatment with the TG2 antibodies, in the presence of doxorubicin. The mouse IgG isotype also does not affect the chemoresistant nature of the MDA MB 231 Clone 16 and MCF7/RT against the chemotherapeutic drug, doxorubicin (Figure 4.16). The cell viability of the high TG2 expressing breast cancer cell lines also did not decrease in the presence of doxorubicin and TG2 antibodies. This was in positive correlation with the unchanged Rel A/p65 high molecular weights observed in the nucleus on being treated with the TG2 antibodies.

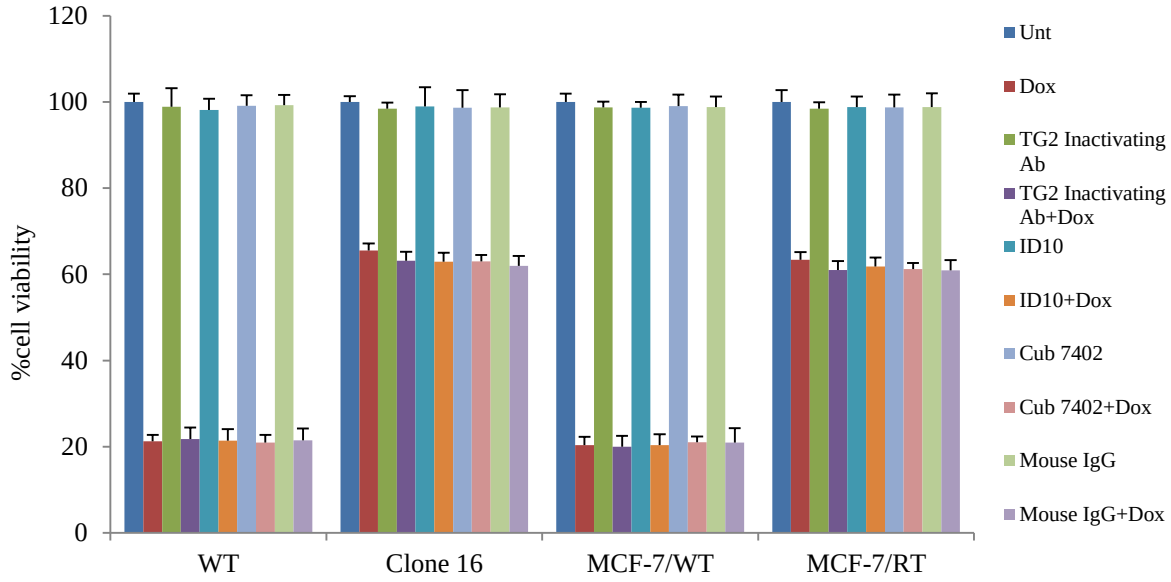


Figure 4.16 The effect of treating breast cancer cell lines with TG2 antibodies in the presence of doxorubicin as illustrated via XTT assay.

The MDA MB 231WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells (3000 cells per well) were seeded into 96-well plates and left overnight. The next day the cells were treated with three different TG2 antibodies and mouse IgG (25ng/ml) for a time course of 24 h and doxorubicin (1µg/ml) for a time course of 72 h after which the viable cells were detected using XTT analysis. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3).

4.3.6 In-vitro cross linking of recombinant Rel A/p65 using guinea pig liver tissue transglutaminase (TG2)

To determine if TG2 can cross link Rel A/ p65 protein *in vitro*, recombinant human Rel A/p65 (100ng/ml) was treated with increasing concentrations of guinea pig liver TG2 (0.01ng/ml-10ng/ml). The reaction was conducted in PBS (final volume of 20µl) and supplemented with 10mM $CaCl_2$ and 1mM DTT. To check the effect of inhibiting TG2 activity, R294 and Z-DON were used in the reaction, as the negative control, the reaction was supplemented with 10mM EDTA. Western Blotting analysis of the recombinant Rel A/p65 illustrated a monomeric band at 65kDa corresponding to Rel A/ p65. On being treated with a low concentration of TG2 (0.01ng/ml), the high molecular weight Rel A/ p65 band starts appearing. Increasing the concentrations of TG2, showed an increase in the Rel A/p65 high molecular weight band observed at approximately 130kDa. The monomeric Rel A/p65 band started to disappear, showing high molecular weight polymers as the TG2 concentration increases from 0.01ng/ml to 10ng/ml. When 10mM EDTA was added to the reaction tube, no high molecular weight p65 band was observed. Also, treatment with TG2 activity inhibitors, Z-DON and R294 showed a decrease in the Rel A/p65 high molecular weight band as compared to the samples that had not been treated with inhibitors. The blot was then reprobed for TG2 to make sure that the concentrations of TG2 that were added corresponded to the increasing high molecular weight band and the decreasing monomeric band (Figure 4.17).

These results illustrated that the presence of these Rel A/p65 high molecular weights could be due to TG2 activity *in vitro*. The Rel A/p65 high molecular weight bands seen in the TG2 high Clone 16 and MCF7/RT cells also could be due to the TG2 activity within these cells. This *in vitro* experiment showed that the high molecular weight bands observed are due to TG2 activity which can be inhibited using inhibitors of TG2 activity such as EDTA, Z-DON and R294.

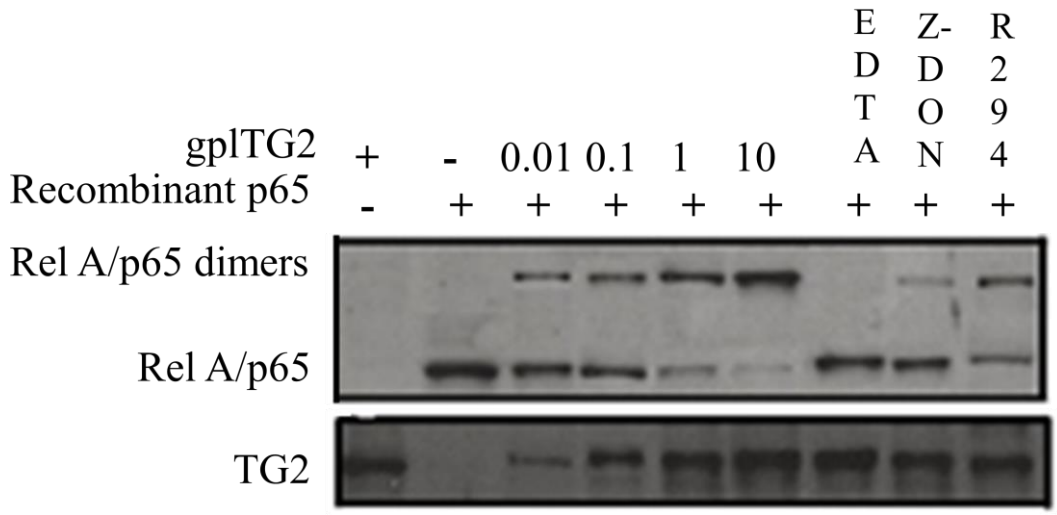


Figure 4.17 Western blot analysis of *in vitro* cross linking of recombinant Rel A/p65 protein using guinea pig liver TG2.

100ng/ml of recombinant human Rel A/ p65 protein was taken in 1.5ml tubes for the various treatments. Different concentrations of guinea pig liver TG2 ranging from 0.01ng/ml to 10ng/ml were used along with 1mM DTT and 10mM $CaCl_2$. The negative control used was the recombinant human Rel A/p65 protein, 10ng/ml gplTG, 1mM DTT and 10mM EDTA. Two different TG2 inhibitors (R294 and Z-DON) were used as well to see if they would inhibit the cross linking. The reaction tubes were set up to a final volume of 20µl with PBS. The reaction was allowed to take place at room temperature for 1 h. After the time period, 500µM of TG2 inhibitor R283 was added into all of the reaction tubes to stop the reaction. 20µl of Laemmli buffer was added into the samples and boiled for 5 min. The samples were then loaded onto an 8% SDS PAGE gel and analyzed by western blot using rabbit polyclonal anti-Rel A/ p65 as well as mouse monoclonal anti-TG2 antibodies. The data presented is a representation of three independent experiments (n=3).

4.4 DISCUSSION:

The key obstacles to successful cancer therapy include the development of metastasis and drug resistance. The most prevalent feature of metastatic and drug resistant tumour cells is that the cells become increasingly resistant to induction of apoptosis (Kerbel, *et al.* 1994). It has become very evident that certain oncogenic mutations interrupt programmed cell death, which subsequently initiates tumour development, further progression, drug resistance as well as metastasis. Numerous epigenetic and genetic disruptions have been identified in advanced stages of cancer and thereby confer resistance against apoptotic mechanisms. One of the most commonly observed features of drug resistant cancer cells is the activation of NFκB (Fesik, 2005). The capability of cancer cells to evade apoptosis allows them to demonstrate resistance to chemo therapeutic drugs as well as allow tumour cells to survive and grow in unfavourable micro environments and in distant tissue sites (Chhabra, *et al.* 2009).

Many recent studies have clearly implicated that TG2 plays a major role, in the progression and growth of cancer cells. Cancer cells that have been selected out for the chemoresistant nature displayed elevated levels of TG2 (Verma & Mehta, 2007a/2007b ; Fok, *et al.* 2006 ; Mehta, *et al.* 2004 ; Verma, *et al.* 2006 ; Satpathy, *et al.* 2007). Results obtained in Chapter III were in agreement with the previous published studies. The MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines exhibited high levels of TG2 expression and activity. This elevated expression and activity of TG2 caused these breast cancer cell lines to become chemoresistant to doxorubicin, and this chemoresistance was reduced on treating the MDA MB 231 Clone 16 and MCF7/RT cells with cell permeable TG2 activity inhibitors (R283 and Z-DON). Also, decreasing endogenous TG2 activity and expression using TG2 specific small interfering RNA (siRNA) reversed this chemoresistance against doxorubicin in the MDA MB 231 Clone 16 and MCF7/RT cells. As shown in Chapter III, the expression of TG2 was also down regulated, when the high TG2 expressing breast cancer cell lines were treated in the presence of cell permeable TG2 inhibitors. This decrease in TG2 expression, on inhibiting TG2 activity within

the breast cancer cell lines, signified that another factor with a response element on the *TGM2* gene promoter was causing this change in TG2 expression.

The oncogenic transcription factor, NFκB, has been known to play a crucial role in the regulation of genes that are concerned with apoptosis, drug resistance, metastasis and cell growth (Karin, *et al.* 2002). Also, NFκB has been shown to be constitutively activated in numerous cancers, and the role of NFκB in metastasis and chemoresistance has been well documented (Naugler & Karin., 2008). In the context of these breast cancer cell lines, it is very appealing to conclude that the expression and activity of TG2 resulted in the constitutive activation of NFκB. Therefore, it can be hypothesized that tumour cell types with elevated expression and activity of TG2, display increased NFκB activity (Mann, *et al.* 2006)

Essentially, the down regulation of TG2 using cell permeable inhibitors (R283 and Z-DON) as well as TG2 specific siRNA resulted in a significant decrease in the activity of NFκB. Furthermore, aberrant high molecular weight forms of Rel A/p65 were observed in the nuclear fractions of the TG2 expressing breast cancer cells, which have been observed to decrease significantly in the presence of cell permeable TG2 inhibitors, R283 and Z-DON. Treating the TG2 breast cancer cell lines with TG2 specific siRNA also resulted in the decrease of the high molecular weight Rel A/p65, and a subsequent increase in the monomeric form of Rel A/p65 in the nucleus, which was not observed in the untreated or global negative siRNA treated breast cancer cells. The other NFκB family members such as p105/p50, Rel B and c-Rel were observed to be very similar in both high and no TG2 breast cancer cell lines. Moreover, treating the breast cancer cell lines with the TG2 activity inhibitors did not illustrate any changes in the levels of the other NFκB family members. The transamidating activity of TG2 was proven to be specific for the constitutive activation of the Rel A/p65 subunit of NFκB

A study, conducted by Kim *et al.* (Kim, *et al.* 2006) suggested that inhibiting the cross linking activity of TG2, reversed the chemoresistance of breast cancer cells lines against doxorubicin, mainly due to the subsequent attenuation of NFκB activation. Also, a similar conclusion was

suggested by Cao *et al.* (Cao, *et al.* 2008), stating that in ovarian cancer cell lines, cisplatin induced apoptosis was circumvented due to the constitutive activation of the NFκB survival pathway.

Under resting conditions, NFκB can induce inflammation by its activation which is transient and known to be crucial for the survival and growth of normal cells. The constitutive activation of NFκB by TG2 due to the cross linking activity of TG2 may be one of the key pathways responsible for conferring metastatic and chemoresistant phenotypes (Verma, *et al.* 2008). In the data represented in this chapter, this hypothesis is supported and also suggests that reducing the endogenous activity of TG2, can result in the down regulation of NFκB constitutive activity and thus increases the chemosensitivity of drug-resistant breast cancer cells.

The hypothesis that intracellular TG2 activity is responsible for the constitutive activation of Rel A/p65 as well as the high molecular weight form of Rel A/p65 observed in the nucleus was further confirmed on treating the breast cancer cell lines with various TG2 antibodies such as ID10, transglutaminase-inactivating antibody (TG2 In Ac Ab) as well as Cub 7402. Even though these TG2 antibodies caused a significant decrease in the cell surface TG2 activity of the MDA MB 231 Clone 16 cells, the NFκB activity levels, Rel A/p65 high molecular weight in the nucleus as well as the chemoresistant phenotype of the high TG2 breast cancer cell lines, remains unaltered.

These results clearly suggest that endogenous TG2 activity was responsible for the constitutive activation of NFκB in breast cancer cell lines, which confers chemoresistance to breast cancer cell lines against doxorubicin. The next chapter will focus on elucidating the mechanistic detail behind the constitutive activation of NFκB by TG2 and to decipher whether the activation of NFκB within chemoresistant breast cancer cell lines take place through the canonical or non-canonical pathway of NFκB activation.

To sum up the above work, it was demonstrated that in high TG2 expressing breast cancer cell lines, the cross linking activity of TG2 caused the constitutive activation of the Rel A/p65

subunit of the NFκB family and does not affect the other NFκB family members such as p105/p50, Rel B, c-Rel and p100/p52. This process of constitutive activation by TG2 was predominantly due to the cross linking of Rel A/p65 to allow the formation of Rel A/p65 high molecular weight forms in the nucleus of the high TG2 expressing breast cancer cell lines. Treating the high TG2 breast cancer cell lines with TG2 cell permeable inhibitors as well as TG2 specific siRNA resulted in the down regulation of the activity of NFκB within these cell lines and also resulted in a significant decrease in the high molecular weight form of Rel A/p65 observed in the nucleus. The NFκB activity and Rel A/p65 high molecular weights remain unaltered on being treated with cell impermeable inhibitors as well as TG2 targeting antibodies, which are known to target and inhibit the cell surface activity of TG2 and do not alter endogenous TG2 activity. Treating the breast cancer cell lines with Rel A/p65 specific siRNAs demonstrated that inhibiting the mRNA expression of Rel A/p65 reduced the NFκB activity levels in the TG2 expressing breast cancer cell lines to similar levels as the parental wild type cells. Moreover, a significant decrease was observed in the nuclear Rel A/p65 high molecular weights indicating that the high molecular weights were only made up by Rel A/p65 subunit of NFκB. The Rel A/p65 siRNAs also reduced the expression of TG2 within the high TG2 breast cancer cell lines. This could indicate that a self-regulating molecular response loop where TG2 constitutively activates NFκB (Rel A/p65) which in turn, directly increases the transcription of the *TGM2* gene. Also, cell viability studies showed that inhibiting the endogenous activity of TG2, caused the breast cancer cell lines to become chemo sensitive to chemotherapeutic drugs such as doxorubicin, which could be implicated as one of the potential phenotypic consequences of inhibiting the TG2-NFκB molecular loop.

There are still remaining questions to be addressed such as: (1) What is the pathway of NFκB activation that TG2 exercises? The classical or canonical pathway of NFκB activation that involves IKK or a Non-canonical pathway of NFκB activation?; (2) Does the Rel A/p65 high molecular weight bind to DNA and if this Rel A/p65 high molecular weight undergoes any post translational modifications in the cytoplasm or nucleus prior to binding to DNA?; (3) What is

the nature of the signal transduction that activates the TG2-NFκB loop and key events stemming from this loop such as triggering of epithelial-mesenchymal transition (EMT)? The next chapters will explore the signalling pathway, DNA binding and post translational process mediated by constitutive activation of Rel A/p65 by TG2.

Chapter 5: Constitutive activation of NFκB by TG2 is independent of IKKα/β function

5. Constitutive activation of NFκB by TG2 is independent of IKKα/β function

5.1 INTRODUCTION

The family members of the NFκB proteins are known to share approximately 300 amino acids in their N-terminal domain known as the Rel homology domain (RHD) which contains crucial sequences for DNA binding, IκB binding and also sites for dimerization (Baeuerle & Henkel, 1994). The different members vary in their C-terminal domain. Rel A/p65, Rel B as well as c-Rel demonstrate trans-activating functions, while on the other hand the p105/p50 and p100/p52 members include inhibitory domains. p100 and p105 are the inactive precursors of the p52 and p50 proteins, respectively (Baldwin, 1996).

In a resting un-stimulated cell, the NFκB family members are localized in the cytoplasm. Proteolytic processing will allow the removal of the C-terminal inhibitory domains, which consequently allows the proteins to translocate and enter the nucleus. p52 and p50 are known to form heterodimers or homodimers with Rel A/p65, Rel B and c-Rel which have a transactivation domain (Hayden & Ghosh, 2008). The NFκB family members are sequestered in the cytoplasm, by their association with IκBα, IκBβ and IκBγ, which are the members of the inhibitory κB family, IκB (Oeckinghaus & Ghosh, 2009). The activation of NFκB can result in numerous stimuli, which includes the activation of membrane receptors such as tumour necrosis factor receptors (TNFR) and B cell receptors (BCR) as well as a variety of extracellular stimuli such as osmotic shock and inflammatory cytokines (Ghosh & Karin, 2002)

The activation of these NFκB signalling cascades is due to the phosphorylation of two vital serine residues present on the IκB proteins. This phosphorylation modification of IκB can trigger the ubiquitination and degradation of IκB proteins through proteosome destruction machinery (Ghosh & Hayden, 2008). As an outcome, the free NFκB protein enters into the nucleus which subsequently activates the transcription of a variety of genes which is involved in

pathways that control cellular growth, apoptosis, inflammatory and immune responses as well as developmental processes (Karin & Ben-Neriah, 2000). The above events particularly depend on the activation of upstream IKK complex, which is multimeric. The IKK complex is composed of two highly similar kinases known as IKKα and IKKβ, one non-enzymatic protein called NFκB essential modulator (NEMO) or IKKγ as well as a recently identified protein known as ELKS (Madonna, *et al.* 2012). The regulatory component, NEMO is considered absolutely essential for the formation of the IKK complex by binding to specific conserved residues present on the carboxyl terminal of both the IKKα and IKKβ (Zandi, *et al.* 1997).

There are two major activation pathways for NFκB that have been characterized, which have been regarded as the canonical or classical pathway and the non-canonical or alternative pathway. Both of the above pathways have been shown to rely on phosphorylation, induced by external signals and the degradation of the inhibitory molecule, which releases the NFκB protein which shuttles into the nucleus (Pahl, 1999). The signals that prompt activation, identity of the kinases that are activated, the inhibitory molecules and NFκB proteins involved in both the pathways are very different. The classical pathway is usually triggered by pro inflammatory cytokines (IL-1β or TNF-α), which finally leads to the degradation of IκBα, which is the inhibitory NFκB molecule by IKKγ/NEMO complex via a TAK1-dependent pathway (Shostak & Chariot, 2011). The non-canonical pathway involves the degradation partially of p100 precursor to form the active p52 through a NIK-dependent pathway. This pathway depends on IKKα homo-dimer and leads ultimately to the nuclear migration of Rel B/p52 hetero-dimers. This pathway plays a vital role in adaptive immunity (Naumann, *et al.* 1993). In the canonical pathway of NFκB activation, the IKK complex phosphorylates IκB which then induces the proteasomal degradation via ubiquitination. The heterodimers and homodimers of the NFκB family are then capable to migrate into the nucleus and cause the activation of target genes. The classical or canonical pathway involves Rel A: p50 and c-Rel: p50 hetero dimers and strongly depends on the activity of IKKβ activity (Zandi, *et al.* 1998; Karin & Delhase, 2000; Senftleben, *et al.* 2001)

Chapter 5: Constitutive activation of NFκB by TG2 is independent of IKKα/β function

A very common feature found in breast cancer tumour types is the constitutive activation of NFκB, which is known to play a crucial role in cell survival, inflammation, immunity as well as proliferation (Hayden & Ghosh, 2008). Aberrant activation of NFκB results in the continuous and persistent localization of the NFκB proteins to the nucleus. This translocation has been shown to disrupt the balance between cell death and cell proliferation, mainly via the up regulation of anti-apoptotic pathways and proteins (Karin & Lin, 2002). In some breast cancer cells, other mechanisms and pathways are in play that can activate the NFκB pathway. EGF in breast cancer cells has been shown to set off IκBα phosphorylation specifically on tyrosine 42 via a pathway that is independent of IKKα/β. Previous studies have shown that EGF-induced activation of NFκB occurs without the phosphorylation of serine residues and the subsequent ubiquitination of IκBα, which is independent of IKK (Sethi, *et al.* 2007)

Literature has shown that another prominent feature of TG2 cross-linking activity is the constitutive activation of NFκB in a mechanism by which cytoplasmic IκBα is polymerized by TG2 and then subsequently degraded. The TG2 catalyzed cross-linking of IκBα causes the depletion of the inhibitory -κB protein, without affecting its phosphorylation status. This leads to an aberrant activation of the NFκB pathway which is thought to contribute to inflammation as well as cancer progression (Lee, *et al.* 2004).

The aim of this chapter is to further study the pathway that mediates the constitutive activation of Rel A/ p65 by TG2 and the involvement of IKKα/β and IκBα in this mechanism. Using μ-calpain inhibitor, calpeptin and IKKα/β phosphorylation inhibitor, PS1145, western blot, TG2 whole cell lysate and cell surface activity and NFκB reporter assay were performed to detect the relative levels of activation and expression of TG2 and NFκB. The impact of these inhibitors on the chemoresistant phenotype of the high TG2 breast cancer cell lines against doxorubicin was also studied by XTT analysis.

5.2 METHODS

5.2.1 Treatment of breast cancer cell lines with Calpeptin

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT ($1x10^6$ cells/ dish) were seeded into 60-mm petridishes and grown overnight in 60mm petridishes to become 70-80% confluent. The next day, the cells were treated with 50μM of calpeptin for 6 h as described previously by Kim *et al.* (Kim, *et al.* 2010). After treatment, the breast cancer cell lines were collected and *analysed* by western blot for IκBα and α-Tubulin.

5.2.2 Treatment of breast cancer cell lines with PS1145

3000 cells each of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT were seeded into each well of a 96- well plate and grown to 70-80% confluency overnight. The next day the breast cancer cells were treated with increasing concentrations of PS1145 (10μM, 15μM and 20μM) for a time course of 24, 48 and 72 h. After the treatment, the breast cancer cell lines were *analysed* using XTT to determine the lowest possible concentration of PS1145 that would maintain the cell viability of the breast cancer cell lines. The optimum concentration of PS1145 to treat the breast cancer cells, without any cytotoxicity to the cells was determined. The results were expressed as percentage of viable cells.

Subsequent to determining the optimum concentration and time course for PS1145 treatment, the breast cancer cell lines ($1x10^6$ cells/ dish) were seeded into 60-mm petridishes and treated with PS1145. After the treatment time course, the cells were *analysed* using western blot for IκBα, Rel A/p65, TG2, α-Tubulin and Lamin A, TG2 whole cell lysate and cell surface activity and NFκB activity.

5.3 RESULTS

5.3.1 Detection of IκB in the cytoplasmic fractions of the breast cancer cell lines in the presence of TG2 inhibitors

To determine the status of IκBα in these breast cancer cell lines, the cytoplasmic fractions of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT, MCF7/RT and the TG2 plasmid and vector plasmid transfected SKBR3 and MCF7 cell lines were *analysed* by Western blot using anti-IκBα antibody (described previously in **Sections 2.2.3-2.2.6**). The TG2 null MDA MB 231 WT, MCF7/WT, SKBR3/WT and empty vector transfected SKBR3 and MCF7 cell lines illustrated the presence of monomeric IκBα forms in their cytoplasmic fractions. The TG2 expressing MDA MB 231 Clone 16, MCF7/RT and TG2 transfected SKBR3 and MCF7 cell lines also demonstrated monomeric IκBα. However, upon extended exposure time, high molecular weight forms of IκBα (approximately 66kDa) were also observed in the cytoplasmic fractions of the high TG2 expressing MDA MB 231 Clone 16, MCF7/RT and TG2 plasmid transfected SKBR3 and MCF7 breast cancer cell lines. Treating the MDA MB 231 Clone 16 and MCF7/RT cell lines with the cell permeable TG2 activity inhibitor, Z-DON, these dimeric forms of IκBα decreased significantly. The cell permeable inhibitor, Z-DON, does not alter the monomeric IκBα forms observed. The cell impermeable TG2 activity inhibitor, R294, did not cause any change in the IκBα dimers observed the MDA MB 231 Clone 16 and MCF7/RT cell lines (Figure 5.1). These results seem to imply that there is a correlation between the levels of TG2 and the presence of IκBα dimers in the cytoplasm of breast cancer cell lines. Also as shown previously, the high TG2 breast cancer cell lines that express the IκBα dimers are chemoresistant to doxorubicin this chemoresistance exhibited could be as a result of the formation of IκBα dimeric forms. Inhibiting the activity of TG2 in the MDA MB 231 Clone 16 and MCF7/RT cell lines using Z-DON significantly decreased the activity of TG2 (Chapter III) as well as the presence of the IκBα dimers, consequently reducing the chemoresistant phenotype of these breast cancer cell lines. Studies done previously by Kim, *et al.*(2010), has

demonstrated that treating TG2 transfected MCF7 cell lines with cystamine (a TG inhibitor), significantly inhibited the formation of these IκBα dimers (Kim, *et al.* 2010).

TG2 in these breast cancer cells seems to mediate the activation of Rel A/ p65 by catalysing the cross linking of the key inhibitor of NFκB, IκBα. Even in literature, Rel A/p65 cross linking has been shown to be mediated by TG2 along with the degradation of IκBα, which is a known TG2 substrate (Kim, 2006).Therefore, it may be hypothesized that TG2 binds to the Rel A/p65: IκBα complex, cross linking the inhibitor protein, IκBα as well as the Rel A/p65 to form dimers.

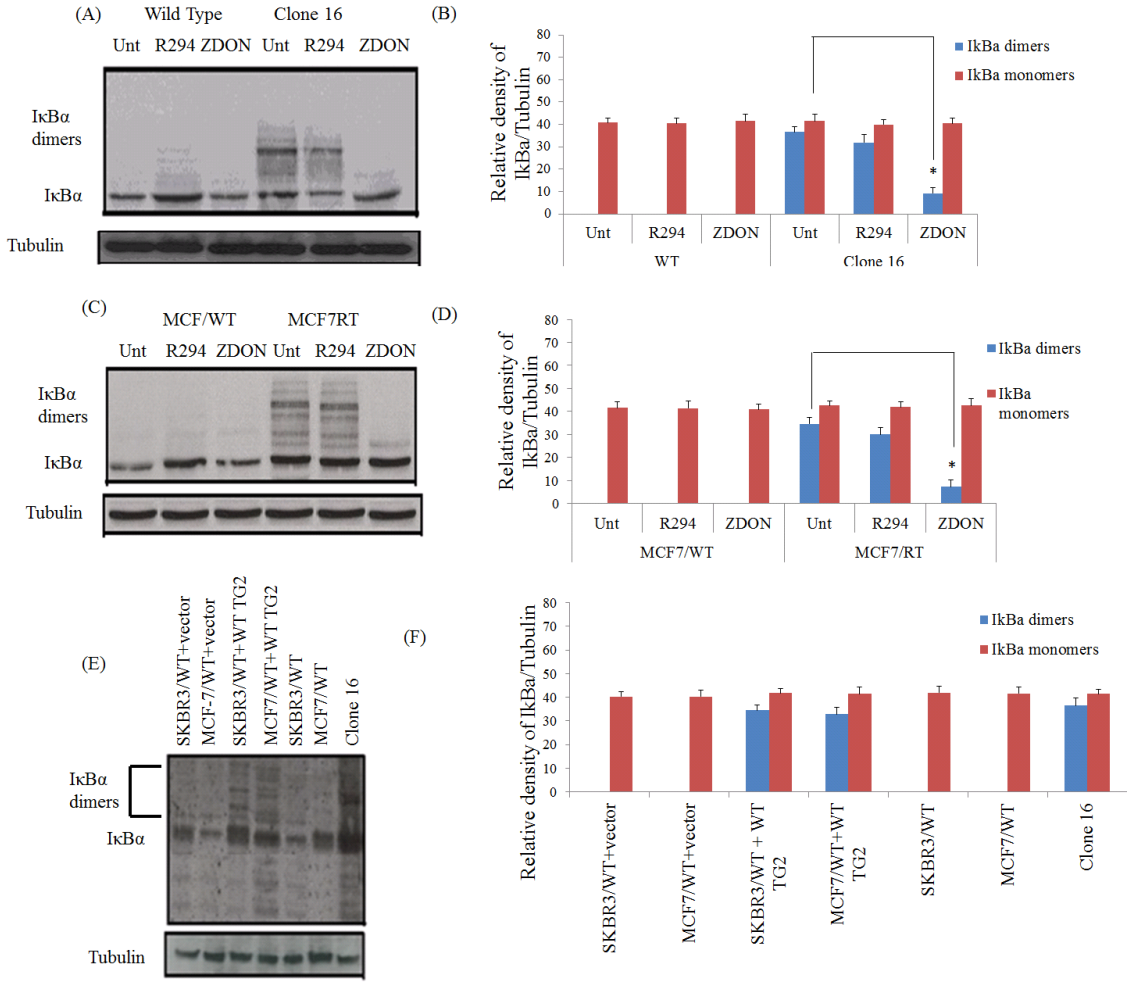


Figure 5.1 Analysis of IκBα in the cytoplasmic fractions of breast cancer cell lines in the presence of TG2 activity inhibitors

The presence of free IκBα was detected in the cytoplasmic fractions of the breast cancer cell lines using western blot (anti-IκBα antibody). 1x 10^6 cells of each breast cancer cell line was seeded into 60-mm petridishes and grown overnight to reach 70-80% confluency. The next day the breast cancer cell lines were treated with 500μM of R294 and 50μM of Z-DON. 72 h after treatment, the cytoplasmic fractions of the breast cancer cell lines were collected as described in Section 2.2.2. The sub-cellular fractions were analysed using anti-IκBα antibody (1:1000). The membranes were reprobed with anti-α-Tubulin to ensure that the proteins had been equally

*loaded. (**A, C, E**) The western blot data shown above is representative of two independent experiments (n=2). (**B, D, F**) Differences in expression of the IκBα dimers and monomers as calculated by Image J analysis is represented as mean densitometry readings + SEM. *p <0.05*

5.3.2 Western blot analysis of free IκBα in the presence of Calpeptin.

Previous studies have suggested that TG2 mediated activation of NF-κB was accompanied by the formation of the IκBα polymers. These polymers have also been shown to be degraded through the μ-calpain system. These IκBα polymers induced by TG2 have been shown to not accumulate in the breast cancer cells. Inhibiting the degradation of these IκBα polymers with Calpeptin caused the accumulation of these IκBα polymers in the cytoplasmic fraction of cells (Kim *et al.* 2010). The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines were treated with 50μM of calpeptin for a time course of 6 h and the whole cell lysate and cytoplasmic fractions of the breast cancer cell lines were *analysed* by western blot using anti- IκBα antibody. The high TG2 breast cancer cell lines, MDA MB 231 Clone 16 and MCF7/RT, illustrated the presence of high molecular weight protein bands which were seemingly characteristic of IκBα polymerized forms (Figure 5.2). These results seem to indicate that calpain is very important for the degradation of the polymerized IκBα form and inhibiting calpain using calpeptin increased the levels of IκBα polymers present in the MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines. Activity of TG2 in breast cancer cell lines might result in the polymerization of IκBα which does not accumulate within the cells and undergoes rapid degradation. This could explain why only dimeric forms of IκBα were initially detected within the cytoplasmic fractions of the high TG2 breast cancer cell lines (Figure 5.1). There seems to be a strong relationship between the formation of IκBα polymers and expression of TG2. This link can be further confirmed on treating the Clone 16 cells with various TG2 inhibitors and seeing if inhibiting TG2 plays a role in the amount of free IκBα present in the cell.

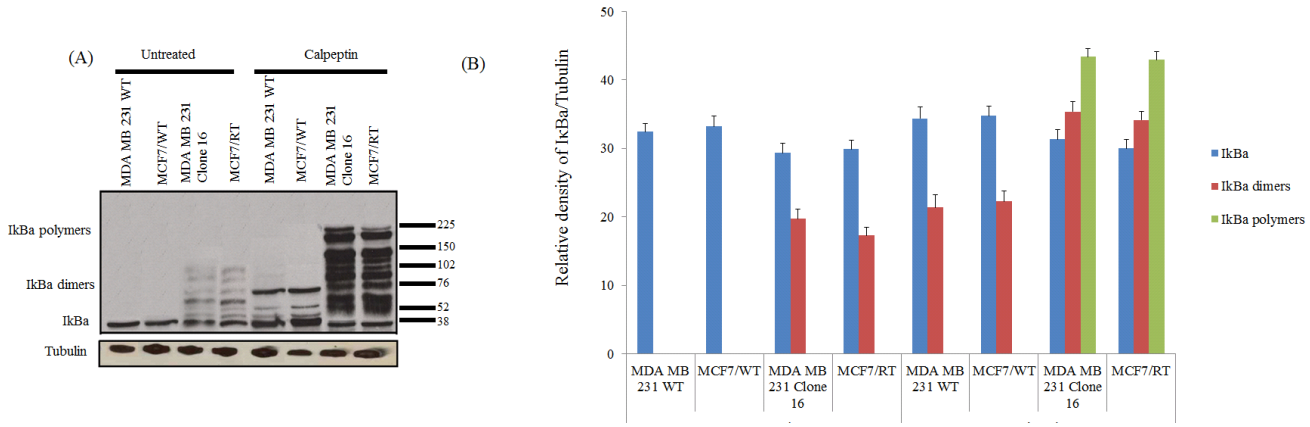


Figure 5.2: Evidence for calpain mediated degradation of IκBα polymers induced by TG2 activity in breast cancer cell lines

*The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were seeded in 60mm petridishes (1x10^6 cells/plate) and allowed to become confluent overnight. The breast cancer cell lines were then treated with 50μM of Calpeptin for 6 h. The cytoplasmic fractions of the breast cancer cell lines were collected and subsequently analysed for IκBα expression by western blot using anti-IκBα (1:1000). The membranes were reprobed with anti-α-Tubulin to check the equal loading of proteins. (**A**) The western blot data shown above is a representation of two independent experiments (n=2). (**B**) Densitometry analysis was performed and is represented as the mean densitometry + SEM. * p<0.05*

5.3.3 IKK inhibitor, PS1145 does not block the constitutive activation of NFκB by TG2

5.3.3.1 Killing curve to determine optimum concentration of PS1145

To establish the optimum concentration of PS1145 to treat the breast cancer cells without the inhibitor being cytotoxic, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines were treated with increasing concentrations of PS1145 (10μM, 15μM and 20μM) as described in **Section 5.2.2**. Using this experiment, 10μM of PS1145 for a time course of 24 h was selected to be the optimum concentration and longest possible time to treat the breast cancer cell lines, without being cytotoxic to the cells (Figure 5.3)

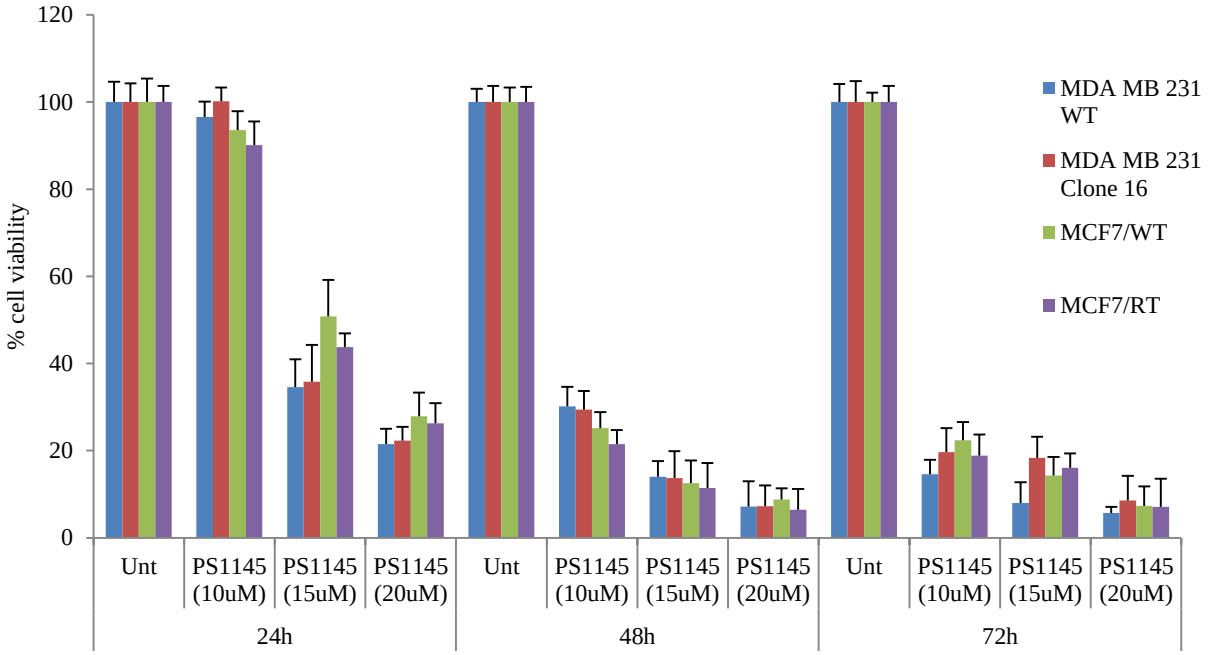


Figure 5.3 Cell viability analyses of breast cancer cell lines in the presence of increasing concentrations of PS1145 over a time course

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines (3000 cells/well) were seeded into 96 well plates and allowed to become 70-80% confluent overnight. The next day the breast cancer cell lines were treated with increasing concentrations, 10μM, 15μM and 20μM of IKK inhibitor, PS1145. After 24, 48 and 72 h of treatment, the cell viability of the breast cancer cell lines was analysed using XTT (Section 2.2.13). Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3).

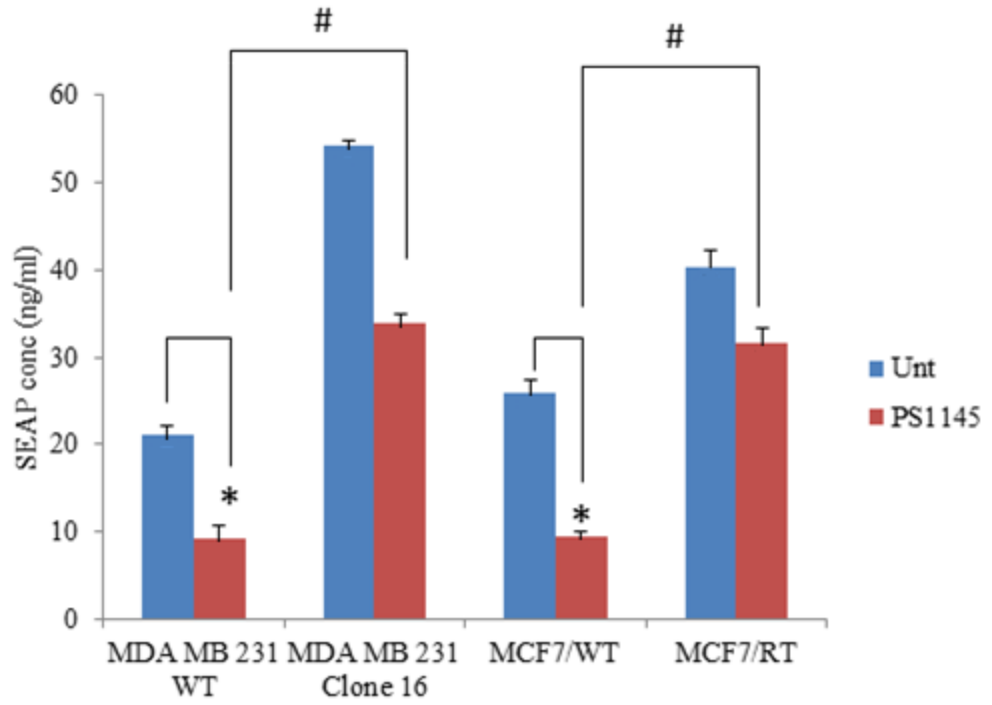
5.3.3.2 Activity and expression of NFκB in the presence of PS1145

The breast cancer cell lines were treated with the IKKα/β inhibitor, PS1145 to determine the effect of inhibiting the canonical pathway of NFκB activation on the levels of NFκB activity within the cell lines. In order to test the NFκB activity levels within the breast cancer cell lines, NFκB/SEAP reporter assay was carried out as described in detail previously **(Section 2.2.12)**. After transfecting the breast cancer cells with the NFκB/SEAP reporter plasmid, the cells were subsequently treated with 10μM of PS1145 for a time course of 24 h. The medium that the breast cancer cells were grown in was collected and assayed for the relative amount of SEAP. The MDA MB 231 WT, MCF7/WT, SKBR3/WT, SKBR3/RT, T47D/WT, T47D/RT and empty vector transfected SKBR3 cells demonstrated a considerable decrease in the NFκB activity levels on being treated with PS1145. This observation was comparable to the initial finding that these breast cancer cell lines did not express any TG2. On the other hand, the TG2 expressing MDA MB 231 Clone 16, MCF7/RT and TG2 transfected SKBR3 breast cancer cell lines did not illustrate a significant decrease in the NFκB activity levels on being treated with PS1145 (Figures 5.4 A, B & C). The slight decrease demonstrated in the NFκB activity could be attributed to the minimal effect of IKKα/β. These results could imply that, other than the canonical pathway of NFκB activation, another unconventional pathway of NFκB activation could be active in the chemoresistant cell lines. Blocking the conventional pathway of NFκB activation using a IKKα/β inhibitor, PS1145, does not seem to change the NFκB activity levels within the high TG2 breast cancer cell lines, which may indicate that the high levels of NFκB activity within these cell lines is due to the constitutive activation of Rel A/p65 induced by TG2.

After determining the NFκB activity levels within these breast cancer cell lines, the relative expression of Rel A/p65 in the cytoplasmic and nuclear fractions of these breast cancer cell lines was detected as well. The MDA MB 231 WT and MDA MB 231 Clone 16 breast cancer cells were treated with 10μM of PS1145 for 24 h, after which the cytoplasmic and nuclear fractions of the cells were collected as described in **Section 2.2.2**. The sub cellular fractions

were then analysed using anti-Rel A/p65 antibody by western blot. The MDA MB 231 WT cells demonstrated a clear shift in the levels of protein expression from the nucleus to the cytoplasm of Rel A/p65 on being treated with PS1145. This implied that PS1145 was blocking the phosphorylation of IκBα by IKKα/β and the subsequent release of Rel A/p65 into the nucleus, which accounted for the decrease in Rel A/p65 nuclear expression. With respect to the MDA MB 231 Clone 16, the aberrant Rel A/p65 dimer seen in the nucleus remains significantly unchanged on PS1145 treatment (Figure 5.5). These results could indicate that in the high TG2 expressing breast cancer cells lines, the canonical pathway of NFκB activation using IKKα/β is not involved in the activation of the aberrant Rel A/p65 dimer. The constitutive activation of Rel A/p65 in the TG2 expressing breast cancer cell lines can thus be suggested to be completely independent of IKKα/β and dependent on the intracellular transamidating activity of TG2.

(A)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SEAP conc (ng/ml)
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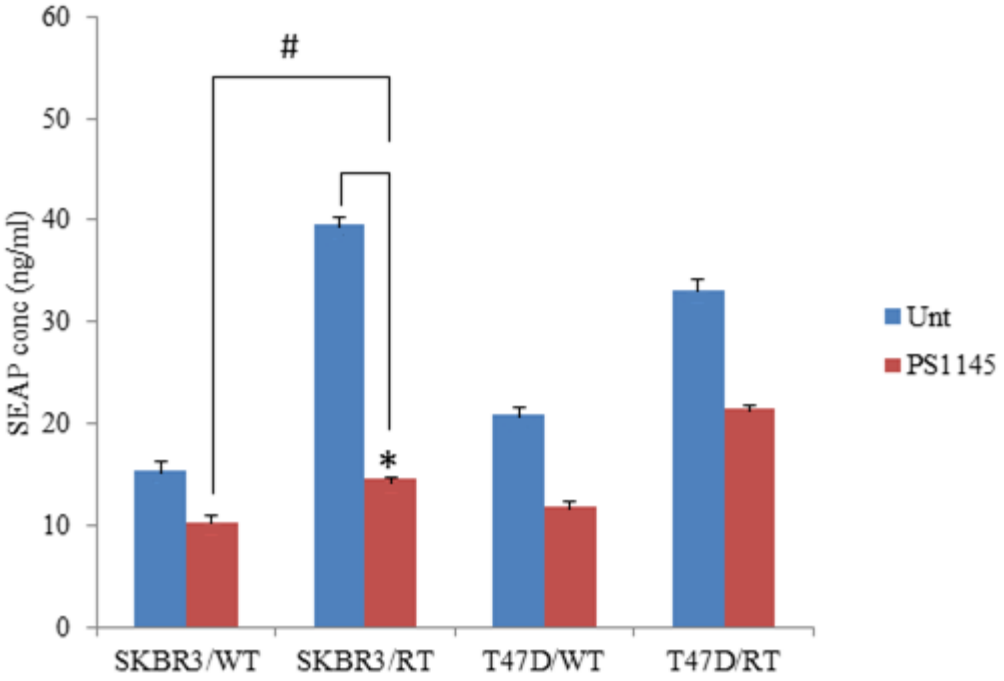
(B)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T47D/RT

(C)

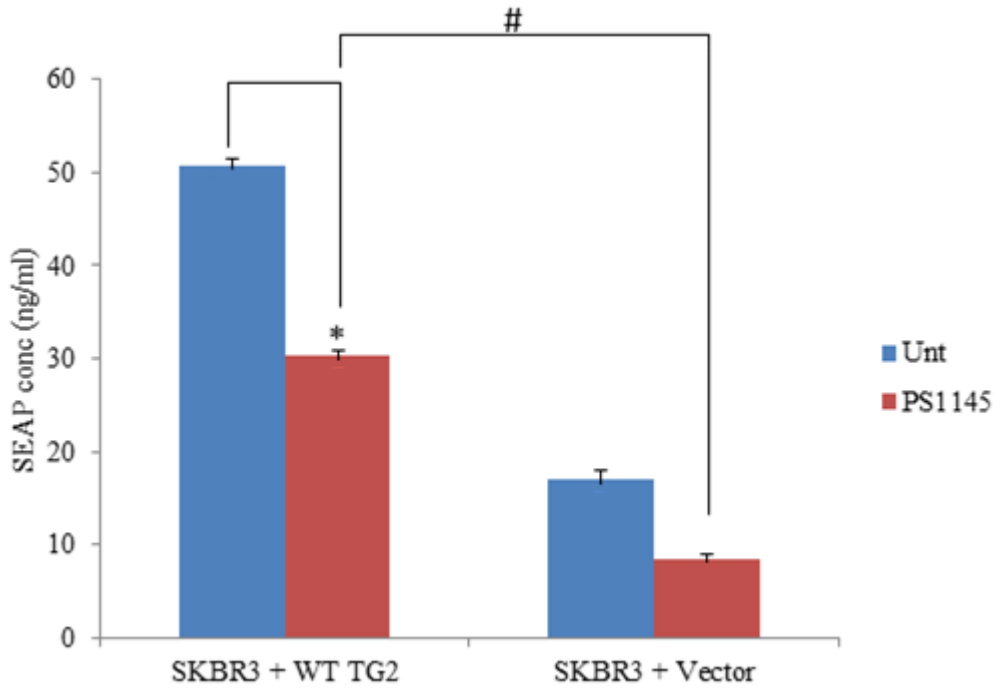


Figure 5.4 (A, B & C) Analysis of NFκB activity levels in breast cancer cell lines on treatment with IKK inhibitor, PS1145

*The NFκB activity levels of the breast cancer cell lines were analysed using the NFκB/SEAP Reporter Assay. As described previously in Section 2.2.12, $5x10^5$ cells of each breast cancer cell line was seeded into each well of a 6-well plate along and left overnight to attain confluency. The next day, the breast cancer cell lines were transfected with NFκB/SEAP plasmid. 48 h after transfection, the breast cancer cell lines were treated with 10μM of PS1145 over a time course of 24 h. The culture medium was then collected and used for subsequent analysis. Data shown is mean concentration of SEAP ± SE from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * p< 0.05 between untreated and PS1145 treated cells. # p<0.05 between cell groups.*

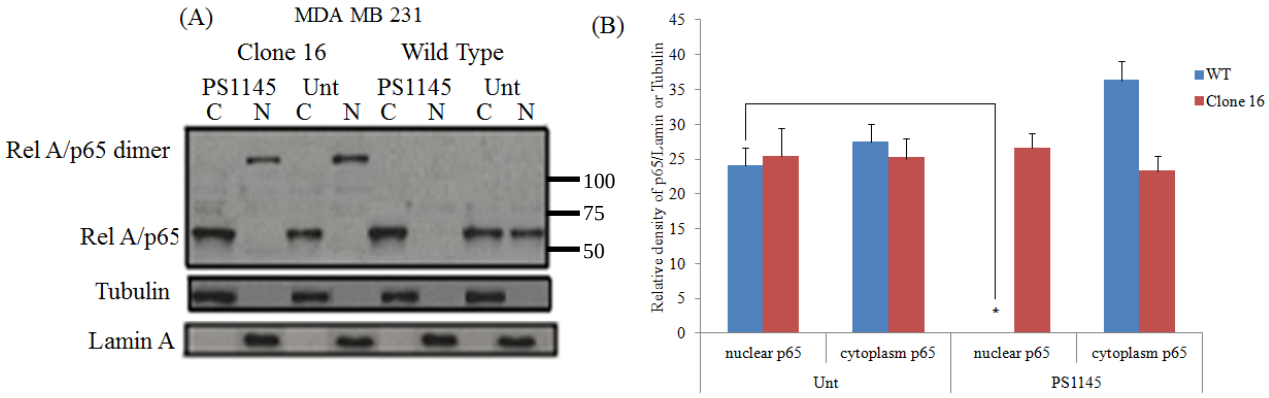


Figure 5.5 Western blot analysis of Rel A/p65 in the sub cellular fractions of breast cancer cell lines on treatment with PS1145

*The IKKα/β inhibitor, PS1145 was used to inhibit the phosphorylation of IκBα by IKKα/β and subsequent activation of NFκB (Rel A/p65) via a canonical or classical pathway in the breast cancer cell lines. 1x10^6 cells of each breast cancer cell line was seeded into 60-mm petridishes and left to become 70-80% confluent overnight. The next day the sub cellular fractions of the cell lines were obtained as described in Section 2.2.2. The sub cellular fractions were then analysed for the expression of Rel A/p65 using western blotting and anti-Rel A/p65 antibody. Anti-α-Tubulin and Lamin A were used as the markers of equal protein loading for the cytoplasmic and nuclear fractions and also to ensure that the fractions had not been cross contaminated. (**A**) The western blot data shown above is a representation of three independent experiments (n=3). (**B**) Densitometry scans were done of the western blots and are represented as the mean of the densitometry readings + SEM.* $p<0.05$*

5.3.3.3 NFκB activity and expression in the presence of PS1145 and TG2 inhibitors

To confirm if the constitutively activated NFκB in the high TG2 expressing breast cancer cell lines could be decreased using a combinatorial treatment of both TG2 inhibitors as well as PS1145, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT breast cancer cell lines were treated with 10μM PS1145 for 24 h as well as R283 (500μM) and Z-DON (50μM) for a time course of 72 h. The breast cancer cell lines were then *analysed* for the NFκB activity levels by using the NFκB/SEAP reporter assay (described in **Section 2.2.12**). The MDA MB 231 WT and MCF7/WT cell lines have been previously shown to express no TG2 as well as decreased NFκB activity levels as compared to the respective high TG2 clones. On treating the MDA MB 231 WT and MCF7/WT breast cancer cell lines with 10μM of PS1145 for 24 h resulted in a significant decrease in the NFκB activity within these cells. The cell permeable TG2 inhibitors, R283 and Z-DON, do not show any change in the NFκB activity levels in the TG2 null breast cancer cell lines. Combination treatment, including PS1145 and cell permeable TG2 inhibitors, R283 and Z-DON, resulted in the NFκB activity remaining comparable to the decrease observed on treatment with only PS1145, without any effect of the TG2 activity inhibitors. Conversely, the high TG2 expressing MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines, did not demonstrate a noteworthy decrease in the NFκB activity levels. Treating the breast cancer cell lines with TG2 cell permeable inhibitors, R283 and Z-DON, drastically decreased the NFκB activity levels in the MDA MB 231 Clone 16 and MCF7/RT cell lines (Figure 5.6). A combination treatment of PS1145 and TG2 inhibitors, R283 and Z-DON, demonstrated a similar decrease in the NFκB activity which was comparable to the NFκB activity of the MDA MB 231 Clone 16 and MCF7/RT cells that were treated with the TG2 cell permeable inhibitors only. These results seem to indicate clearly that the major amount of NFκB activity observed in the MDA MB 231 Clone 16 and MCF/RT breast cancer cell lines can be attributed to the high TG2 expression and activity levels within these breast cancer cell lines.

The sub cellular fractions of the MDA MB 231 WT and MDA MB 231 Clone 16 breast cancer cell lines were also collected in order to determine the expression of Rel A/p65 in the nuclear fractions, in the presence of TG2 inhibitors as well as PS1145, by western blotting using anti-Rel A/ p65 antibody. The TG2 null MDA MB 231 WT illustrated a clear increase in the Rel A/ p65 expression in the cytoplasmic fraction and a subsequent decrease of Rel A/p65 expression in the nuclear fractions, on being treated with the IKKα/β inhibitor, PS1145. On treating the TG2- null breast cancer cell line with TG2 inhibitors, no significant change was observed in the Rel A/p65 expression in the cytoplasmic or nuclear fractions. A combined treatment of PS1145 and TG2 inhibitors on the MDA MB 231 WT cell line, did not show any additional increase in the cytoplasmic expression of Rel A or decrease in the nuclear expression of Rel A/p65, to the change observed on treating these breast cancer cell lines with only PS1145. The high TG2 expressing MDA MB 231 Clone 16 cells do not show any significant change in the aberrant Rel A/p65 dimer observed in the nucleus or the monomeric Rel A/p65 observed in the cytoplasm, on being treated only with the IKKα/β inhibitor, PS1145. On treating these high TG2 breast cancer cell lines with PS1145 as well as TG2 cell permeable inhibitors, R283 and Z-DON, there was a significant decrease observed in the dimeric Rel A/p65 band in the nucleus of the MDA MB 231 Clone 16 cells (Figure 5.7). Also, treating the high TG2 breast cancer cell lines with cell permeable TG2 inhibitors, R283 and Z-DON, illustrated a decrease in the aberrant nuclear Rel A/p65 dimer concurrent with the results of Chapter IV. The decrease observed in the nuclear Rel A/p65 dimer of the MDA MB 231 Clone 16 breast cancer cell line, on being treated with only the cell permeable TG2 inhibitors as well as in combination of cell permeable TG2 inhibitors and PS1145 is very comparable. This could imply that the decrease observed is only as an effect of the TG2 inhibitors and the IKK inhibitor, PS1145 does not cause any change in the dimeric Rel A/p65 form in the nucleus. These results seem to clearly indicate that the presence of the dimeric Rel A/p65 forms in the nucleus of the high TG2 breast cancer cell lines is independent of the conventional NFκB activation pathway wherein IKKα/β is known to be a key player. IKKα/β is responsible for phosphorylation of inhibitor of NFκB, IκBα, which

allows the free NFκB complex to translocate from the cytoplasm to the nucleus of the breast cancer cell lines. Since PS1145 only seems to inhibit the activity of NFκB in the TG2- null breast cancer cells lines, it can be inferred that the high TG2 expressing MDA MB 231 Clone 16 and MCF7/RT display constitutively activated NFκB in the form of Rel A/ p65 dimers, which is independent of IKKα/β phosphorylation activity. Also, the unconventional pathway observed in the MDA MB 231 Clone 16 and MCF7/RT cell lines has been demonstrated to be completely dependent on TG2 as decreasing the intracellular activity of TG2 using R283 and Z-DON, significantly decreased the NFκB activity levels as well as the aberrant Rel A/p65 dimer observed in the nucleus.

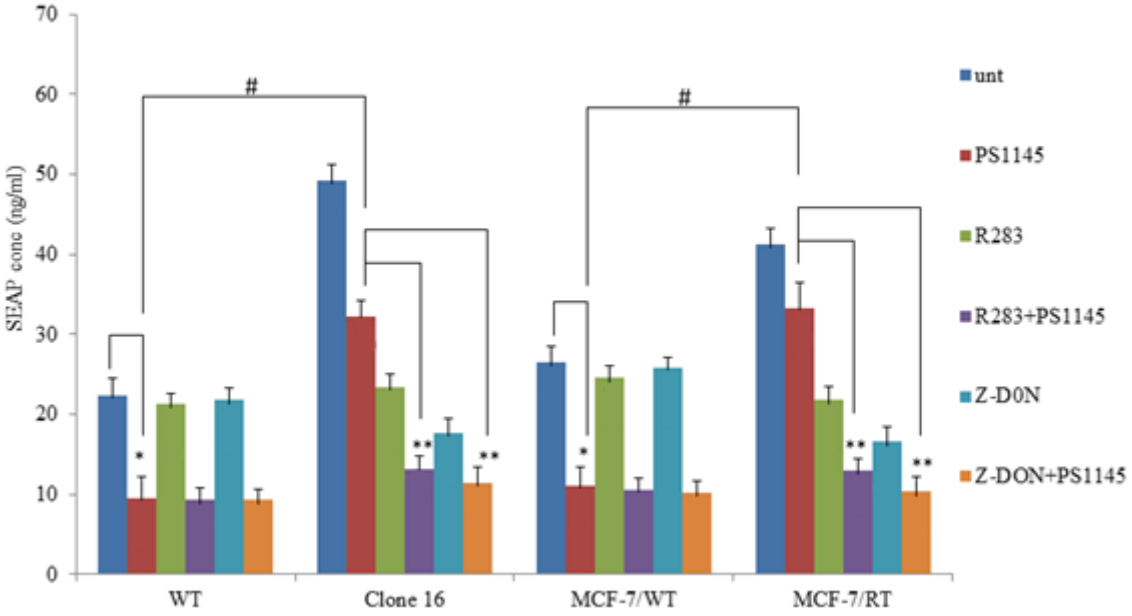


Figure 5.6 Analysis of NFκB activity levels in breast cancer cell lines on being treated with PS1145 and TG2 inhibitors

*The breast cancer cell lines were analysed for the NFκB activity levels on treatment with PS1145 using the NFκB/SEAP Reporter assay. $5x10^5$ cells of each breast cancer cell line was seeded into 6-well plates and transfected with NFκB/SEAP plasmid. 48 h after transfection, the breast cancer cell lines were treated with 10μM of PS1145 for 24 h as well as TG2 inhibitors, R283 (500μM) and Z-DON (50μM) for 72 h and the supernatant was collected for SEAP analysis as described in detail in Section 2.2.12. Data shown is mean concentration of SEAP ± SE from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * p< 0.05 between untreated and PS1145 treated cells; **p<0.05 between untreated and TG2 inhibitor treated cells in the presence of PS1145. # p<0.05 between cell groups treated with PS1145.*

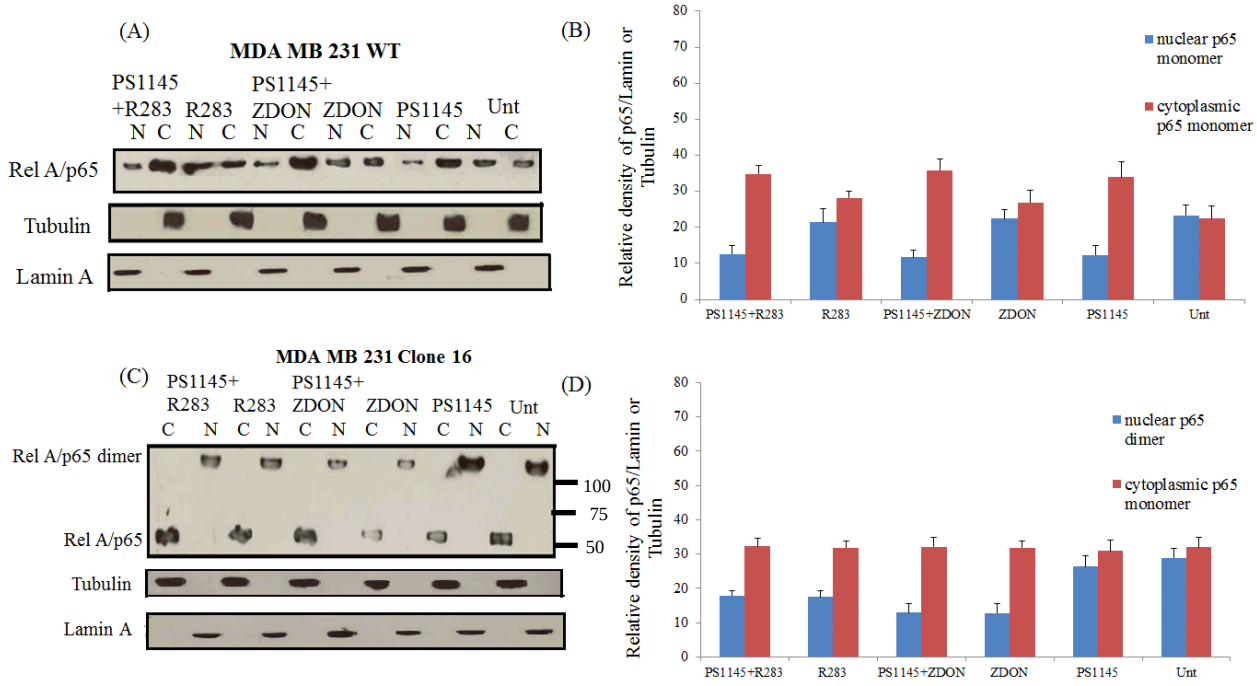


Figure 5.7 Western blot analysis of Rel A/p65 in the cytoplasmic and nuclear fractions of breast cancer cell line on being treated with PS1145 and TG2 inhibitors

*The MDA MB 231 WT (A) and MDA MB 231 Clone 16 (C) cells (1x10^6 cells per dish) were seeded into 60-mm petridishes and left overnight to become confluent. The following day, the cells were treated with 10μM of PS1145 for 24 h and TG2 inhibitors, R283 (500μM) and Z-DON (50μM) for 72 h. After the treatment, the cells were fractionated into the cytoplasmic and nuclear fractions as previously described in Section 2.2.2. The sub cellular fractions were then analysed by western blot for the expression of Rel A/p65 using rabbit polyclonal anti- Rel A/p65 antibody (1:1000). The membranes were then reprobed for anti-Lamin A antibody to check for equal protein loading. (**A & C**) The western blot data is a representation of three independent experiments (n=3). (**B & D**) Differences in expression of Rel A/p65 as calculated by Image J analysis is representative of the mean densitometry readings + SEM.*

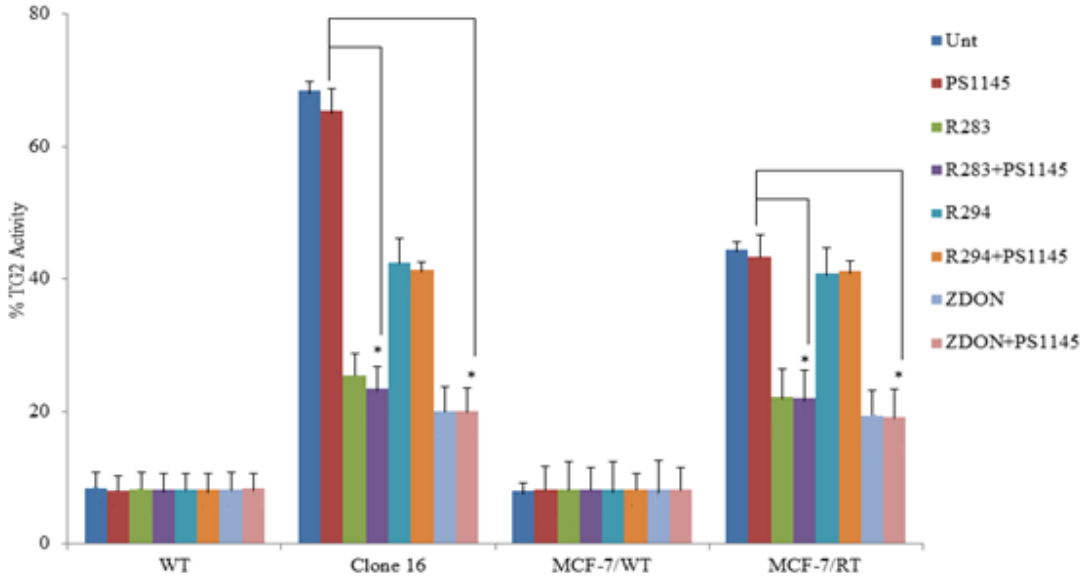
5.3.3.4 Activity and expression of TG2 in breast cancer cell lines on treatment with IKK inhibitor, PS1145 and TG2 inhibitors

The breast cancer cell lines were *analysed* for the expression of TG2 in their cytoplasmic fractions as well as the TG2 activity at the cell surface and in their whole cell lysates, on being treated with TG2 activity inhibitors and PS1145. The cell surface and whole cell lysate activity of TG2 in the breast cancer cell lines on being treated with TG2 inhibitors and PS1145 was performed as previously described in **Section 2.2.11**. The TG2 null MDA MB 231 WT, MCF7/WT did not display any cell surface or whole cell lysate TG2 activity and on treating these breast cancer cell lines with PS1145 and TG2 activity inhibitors no change in the TG2 activity of these cell lines was observed . The MDA MB 231 Clone 16 cells demonstrated high cell surface TG2 activity as well as whole cell lysate TG2 activity. Treating the MDA MB 231 Clone 16 cells with PS1145 did not alter the TG2 activity levels within this cell line. A combination treatment of TG2 activity inhibitors and PS1145 decreased the TG2 cell surface and whole cell lysate activity of the MDA MB 231 Clone 16 cells to a similar level as only treatment with TG2 activity inhibitors. The MCF7/RT cells only demonstrated whole cell lysate TG2 activity, which remains unaltered on treatment with PS1145. Treating the MCF7/RT cell lines with both PS1145 and TG2 activity inhibitors illustrated a decrease in the TG2 whole cell activity of these cells, however, only to the same extent as that of the TG2 inhibitors (Figure 5.8). These results seem to imply that the activity of TG2 seen within these breast cancer cell lines is independent of IKKα/β function and so treating these cell lines with PS1145 does not affect the TG2 activity levels.

To verify the results obtained by the TG2 cell surface and whole cell lysate activity assay, western blot analysis was conducted on the breast cancer cell lines to determine the level of TG2 expression in the cytoplasmic fractions on being treated with PS1145. The MDA MB 231 WT and MDA MB 231 Clone 16 breast cancer cell lines were treated with 10μM of PS1145 for 24 h after which the cytoplasmic fractions of the cell lines were obtained as described in S**ection 2.2.2**. Analysis of the sub cellular fraction using anti-TG2 antibody by western blot

revealed no change in the expression of TG2 in the cytoplasmic fractions of the PS1145 treated and untreated MDA MB 231 Clone 16 (Figure 5.9). With the above results, the suggestion that phosphorylation of IκBα by IKKα/β was not essential for the constitutive activation of NFκB as well as expression and activity of TG2 within these breast cancer cell lines can be put forward.

(A)



(B)

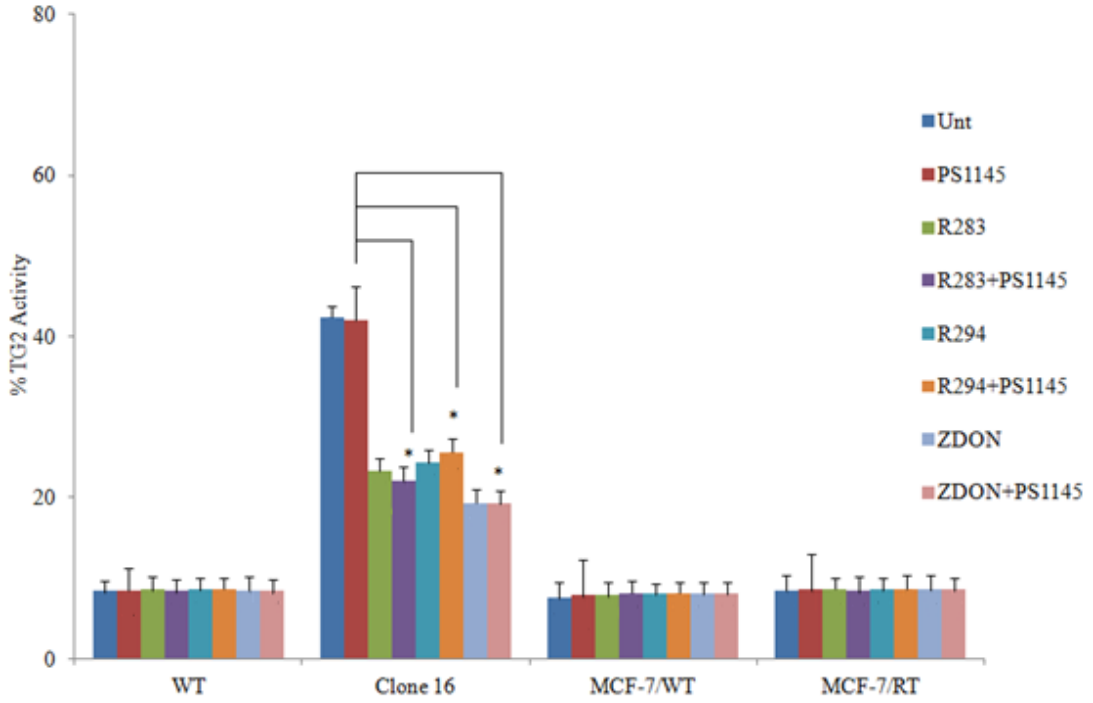


Figure 5.8 Analysis of the whole cell lysate (A) and cell surface (B) TG2 activity in breast cancer cell lines on being treated with PS1145 and TG2 activity inhibitors

The cell surface and whole cell lysate TG2 activity assay of the breast cancer cell lines were analysed by the incorporation of biotinylated cadaverine into fibronectin. Positive control (50

*ng/ml gplTG + 10mM $CaCl_2$) was taken to be 100% TG2 activity (1.28576 ± 0.29475(A); 1.214834 ± 0.218435 (B) . Data shown above is mean percentage TG2 activity + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * p<0.05 between untreated and TG2 inhibitor treated cells in the presence on PS1145.*

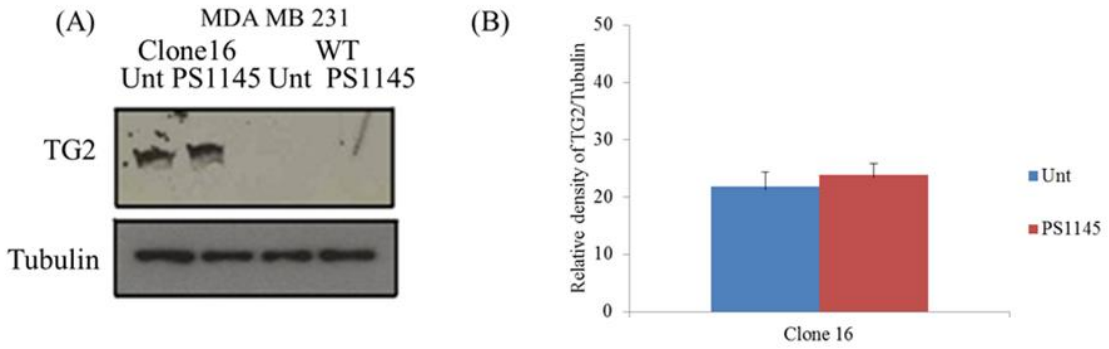


Figure 5.9 Analysis of the expression of TG2 in the cytoplasmic fractions of breast cancer cell lines on treatment with PS1145

*The MDA MB 231 WT and MDA MB 231 Clone 16 (1x10^6 cells per dish) were seeded into 60-mm petridishes and left overnight to become 70-80% confluent. The next day the breast cancer cell lines were treated with 10μM of PS1145 for 24 h after which the cytoplasmic fractions of the cells were obtained as described in Section 2.2.2. The sub cellular fraction was then analysed for the expression of TG2 by western blot using mouse monoclonal anti-TG2antibody (Cub 74202)(1:1000). The membranes were reprobed with anti-α-Tubulin antibody to ensure equal loading of protein. (**A**) The western blot data shown is a representation of three independent experiments (n=3). (**B**) Densitometry analysis was performed using image J and is represented as the mean densitometry + SEM.*

5.3.3.5 Chemo sensitivity of breast cancer cell lines in the presence of doxorubicin on being treated with PS1145

As an important intracellular molecule in the NFκB signalling pathway, the role of IKKα/β was investigated using a IKKα/β specific inhibitor, PS1145 to treat the breast cancer cell lines. XTT analysis of the breast cancer cell lines in the presence of PS1145 and doxorubicin was done to investigate whether the presence of PS1145 would decrease chemoresistance of the high TG2 breast cancer cell lines to doxorubicin treatment.

The TG2 null MDA MB 231 WT and MCF7/WT cell lines showed a considerable loss in cell viability, in the presence of doxorubicin (1µg/ml). Treating the MDA MB 231 WT and MCF7/WT cell lines with PS1145 in the presence of doxorubicin, further decreased the cell viability. On the other hand the TG2 high MDA MB 231 Clone 16 and MCF7/RT cell lines demonstrated reduced loss in cell viability in the presence of doxorubicin (1µg/ml). Even on treating these high TG2 breast cancer cell lines with PS1145 in the presence of doxorubicin, no significant decrease was demonstrated in cell viability (Figure 5.10)

These results could imply that blocking the basal activation of NFκB by the canonical pathway in the TG2 null MDA MB 231 WT and MCF7/WT cells rendered these cells more susceptible to doxorubicin. Also since the MDA MB 231 Clone 16 and MCF7/RT cells demonstrated no significant decrease in cell viability when treated with PS1145 in the presence doxorubicin, the expression and activity of TG2 in the cells could be contributing to constitutively activating the NFκB survival pathway in a manner independent to IKKα/β.

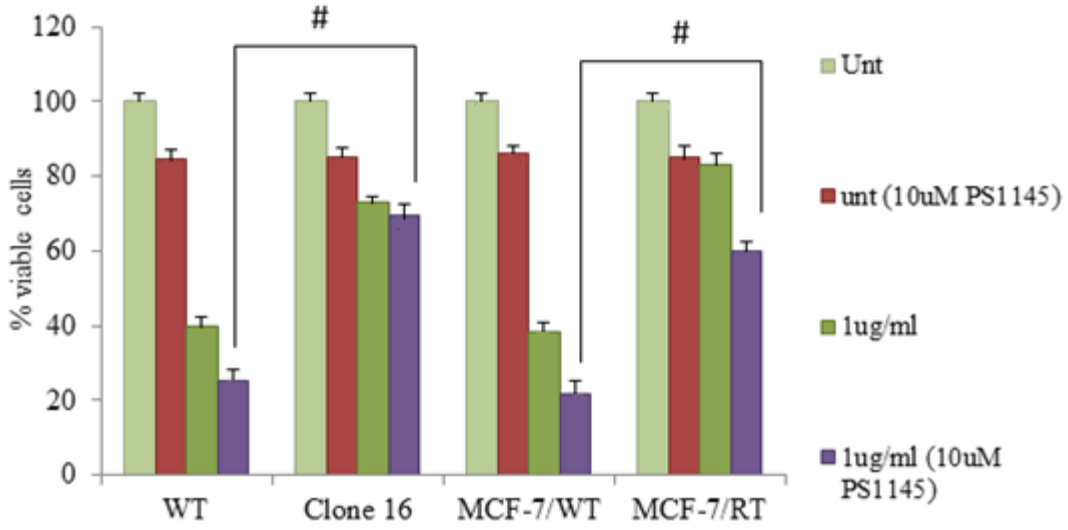


Figure 5.10 The effect of PS1145 treatment on the cell viability of breast cancer cell lines in the presence of doxorubicin

3000 cells each of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were seeded per well into a 96-well plate and left overnight. The next day the cells were treated with doxorubicin (1μg/ml) for 72 h and 10μM of PS1145 for 24 h. After treatment, the cell viability of the breast cancer cell lines was analysed using XTT assay. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability +SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA. # $p < 0.05$ between cell groups treated with PS1145 in the presence of doxorubicin.

5.4 DISCUSSION

Calpeptin is a cell-permeable inhibitor of μ-calpain, which is a calcium-dependent neutral non-lysosomal thiol protease that has been found in numerous eukaryotic cells (Suzuki, *et al.* 2004). Previous studies have shown that μ-calpain is responsible for the regulated signalling of NFκB in skeletal muscles. Also, reports have also shown that μ-calpain can direct free IκBα into the PEST domain for subsequent degradation, thus implicating calpain to be an important player in the activation of NFκB. In human HepG2 cells, Han *et al.* in 1999 demonstrated that calpain provided a proteolytic degradation pathway which was parallel to the ubiquitin pathway followed by TNFα mediated IκBα degradation (Han, *et al.* 1999). In WEH1231 B cells, the rapid degradation of IκBα was also shown not dependent on the ubiquitin proteosome pathway, but regulated by calpain inhibitors or even calcium chelating agents (Miyamoto, *et al.* 1998).

In the high TG2 expressing breast cancer cell lines, constitutive activation of NFκB was observed as previously demonstrated in chapter 4. In the cytoplasmic fractions of the MDA MB 231 Clone 16 and MCF7/RT cells, IκBα dimers were observed as opposed to the monomeric forms of free IκBα in the TG2 null breast cancer cell lines. Inhibiting TG2 in the MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines has previously been shown to decrease NFκB activity within these cells. On treatment with the cell-permeable TG2 inhibitor, Z-DON, the dimeric IκBα complex has also been demonstrated to decrease. The TG2 null breast cancer cell lines did not demonstrate any IκBα dimers which may imply that the IκBα dimers were characteristic to transamidating activity of TG2.

In the high TG2 expressing MDA MB 231 Clone 16 and MCF7/RT cell lines, there is evidence to suggest that a novel system for degradation of IκBα was in function. Previous studies done by Kim, *et al.*(2010) also reported that a TG2-calpain system was active in high TG2 expressing breast cancer cell lines which may be a possible mechanism by which NFκB could be constitutively activated in these cells. In this study, treating the high TG2 breast cancer cell lines with the μ-calpain inhibitor calpeptin increased the presence of IκBα polymers in the MDA MB 231 Clone 16 and MCF7/RT cells. Also, TG2 has been shown to catalyze the cross

linking of IκBα protein molecules at lysine 33, 36 and 177 as well as glutamines 313, 267 and 266 (Folk & Chung, 1985). This cross-linking of IκBα mediated by TG2 would result in the release of the NFκB (Rel A/p65) molecule and subsequent degradation by the μ-calpain proteosomal degradation pathway, which would result in accumulation of the IκBα dimers within the cytoplasmic fractions of the high TG2 breast cancer cell lines. In this way, TG2 could regulate the activation of NFκB in breast cancer cell lines independent of ubiquitination.

To determine whether the constitutive activation of NFκB (Rel A/p65) as well as the polymerization and subsequent degradation of IκBα was dependent on the phosphorylation of IκBα by IKKα/β or independent of IKKα/β and dependent on TG2, the breast cancer cell lines were treated with the IKK phosphorylation inhibitor, PS1145. Studies determined that in the high TG2 breast cancer cell lines, treatment with PS1145 did not significantly decrease the NFκB activity or the formation of the Rel A/ p65 dimers in the nuclear fractions. On the other hand, in the TG2 null breast cancer cell lines, PS1145 significantly reduced the NFκB activity of the cells and also prevented the translocation of monomeric Rel A/p65 from the cytoplasmic and nuclear fraction. This could imply that the activity of NFκB in the TG2 breast cancer cell lines was reliant on TG2 activity and expression, as opposed to the classical pathway present in the TG2 null breast cancer cell lines, wherein the NFκB activity is dependent on phosphorylation of IκBα by IKK α/β. These results suggest that in breast cancer cell lines that display high levels of TG2, a calpain and TG2-based pathway might provide an alternative degradation pathway to the classical ubiquitin - proteosome pathway of IκBα degradation (Han, *et al.* 1999). Since the NFκB family member activated in the high TG2 breast cancer cell lines has been determined to be Rel A/p65 dimeric forms, which is independent of IκBα phosphorylation by IKK, the constitutive activation of Rel A/ p65 dimers can be attributed to a canonical pathway which is non-conventional. The effect of PS1145 treatment on the expression and activity levels of TG2 was further validated by western blot analysis, whole cell lysate and cell surface TG2 activity assays. No significant change was observed in the expression or activity levels of TG2 in the breast cancer cell lines on treatment with PS1145.

Chapter 5: Constitutive activation of NFκB by TG2 is independent of IKKα/β function

The NFκB family member Rel A/p65 has been previously shown to cause TG2 mRNA expression in cultured breast cancer cell lines as the *TGM2* gene has been revealed to contain two NFκB consensus binding sites (Ai, *et al.* 2012 ; Caccamo, *et al.* 2005). Moreover, the chemoresistant properties of the high TG2 breast cancer cell lines could not be reversed with PS1145 treatment. This provides evidence to support that the constitutive activation of Rel A/ p65 dimers that confer chemoresistant phenotype to TG2 expressing breast cancer cell lines is independent of IKK function, enumerating a deviation from the classical NFκB activation pathway.

To summarize the above observations, this chapter's work demonstrates that the constitutive activation of Rel A/ p65 dimers mediated by TG2 is via the cross linking of IκBα to form dimers and polymers which are degraded spontaneously through the μ-calpain proteosomal degradation pathway and is also independent of IKKα/β phosphorylation function. Importantly, inhibiting IKKα/β function using PS1145 did not reduce the chemoresistant phenotype of the high TG2 breast cancer cell lines or decrease the activity and expression of TG2.

Chapter 6: Phosphorylated status of the cross linked Rel A/ p65 dimer is necessary for *TGM2* gene expression and chemoresistance

6. Phosphorylated status of the cross linked Rel A/p65 dimer is necessary for *TGM2* gene expression and chemoresistance

6.1 INTRODUCTION

The activation of the NFκB signalling pathway is dependent on various external stimuli (Hayden & Ghosh, 2004). The activation of this pathway will allow the translocation of the NFκB transcription factors from the cytoplasm to the nucleus of cells. The NFκB subunits, either homo-dimers or hetero-dimers, are held in the cytoplasm by a family of proteins known as the IκBα or inhibitors of NFκB. On receiving external stimuli, the IκBα proteins are phosphorylated by IKKα, IKKβ or IKKγ, ubiquitinylated and subsequently degraded proteolytically. This allows the NFκB subunits to move into the nucleus (Karin & Ben-Neriah, 2000).

Several studies have shown that the NFκB family of proteins can be modified post-translationally and these alterations can greatly influence the transcriptional activity of the NFκB subunits (Chen, *et al.* 2001) For example, the S- nitrosylation of cysteine 65 of the p50 subunit has been shown to greatly affect the binding of the p50 member to DNA (Marshall & Stamler, 1999) . Similarly, reports have demonstrated that Rel A/p65 can undergo phosphorylation as a response to some external stimulating factors (Vermeulen, *et al.* 2002). Phosphorylation of Serine 276 in Rel A/p65 by protein kinase A has been shown to be essential during IκBα degradation as well as for the recruitment of p300/ CREB binding protein to Rel A/p65 in order to activate transcription (Zhong, *et al.* 2002) The phosphorylation of serine 536 by IκB kinases and serine 529 by casein kinase II (Wang, *et al.* 2000) after either LPS or TNF-α stimulation has also been demonstrated to increase transcriptional activity (Okazaki, *et al.* 2003). Studies done by Sasaki *et al.* in 2005 clearly demonstrated that the phosphorylation of

serine residues of Rel A/p65 were not associated with either p50 or IκBα and MG-132 which is a known proteosome inhibitor, reduced the translocation of Rel A/p65 to the nucleus but the phospho-Rel A/p65 remained unaffected (Sasaki, *et al.* 2005). Also, dominant-negative mutants of IκBa were shown to decrease the transcriptional activity of Rel A/p65 but the phospho-dimeric form of serine 536 p65 remained unaltered. This seems to suggest that phospho-Ser 536-p65 translocates into the nucleus immediately after activation and was not regulated by IκBα function. The implications of serine phosphorylation at residue 536 in the regulation of the transactivating potential of TG2 has not yet been clearly defined (Sasaki, *et al.* 2005). The direct post translational modification of Rel A/ p65 subunits of the NFκB family involves the phosphorylation of the C- terminal domain (Harris, *et al.* 2006).

Numerous serine 536 kinases have been described such as IKKα, IKKβ, IKKε as well as NFκB activating kinase (Vermeulen, *et al.* 2003; Viatour, *et al.* 2005; Sakurai, *et al.* 1999; Sizemore, *et al.* 2002; Jiang, *et al.* 2003; Buss, *et al.* 2004; Lawrence, *et al.* 2005). Even though IKKε is structurally very similar to IKKα and IKKβ with a C-terminal leucine domain, N-terminal kinase domains as well as helix-loop-helix domains, IKKε only shares about 27% of primary sequence identity with IKKα (Kishore, *et al.* 2002). Studies done previously have determined that IKKε can directly phosphorylate Rel A/ p65, even though the exact physiological relevance of these events remains to be established. Mutation of the Ser 536 site to Alanine has also been shown to decrease the phosphorylation induced by IKKε, which may implicate that the phosphorylation site of IKKε on Rel A/ p65 was specific to Serine 536 (Buss, *et al.* 2004). Following genotoxic stress in cells, IKKε can enter into the nucleus of the cell in a kinase dependent manner, wherein IKKε can phosphorylate PML nuclear bodies allowing the retention of the IKKε complex in the nucleus. Within these sub nuclear bodies, IKKε can undergo SUMO modification which will subsequently allow the phosphorylation of the nuclear substrate including Rel A/ p65, that will therefore contribute to the anti-apoptotic and chemoresistant properties in response to damaged DNA (Renner, *et al.* 2010)

Chapter 6: Phosphorylated status of the cross linked Rel A/p65 dimer is necessary for TGM2 gene expression and chemoresistance

Various stimuli can induce the phosphorylation of Rel A/ p65 at serine 536 and the pathways that are responsible for this post-translational modification have to yet be deciphered. Previous results have reported that using RNA interference to block IKKα/β; dominant-negative mutants of IKK as well as SC-514 (a specific IKKβ inhibitor) did not block the Ser 536 phosphorylation of Rel A/ p65. On the other hand, over expression of IKKε has been shown to phosphorylate Ser 536 both *in vivo* and *in vitro* (Buss, *et al.* 2004). Studies conducted by Adli and Baldwin in 2006, demonstrated that IKKε was expressed highly in breast cancer cell lines and also involved in the regulation of NFκB activity via the ability to control the constitutive as well as basal phosphorylation of Rel A/ p65 at Serine 536 (Adli & Baldwin, 2006). Decreasing the expression of IKKε or expressing a S536A mutant form of Rel A/ p65 suppressed the proliferation of cancer cells which clearly indicated that IKKε played a crucial role in the regulation of the constitutive activation of NFκB activity (Adli & Baldwin, 2006).

Studies conducted on breast cancer cell lines illustrated that Rel A/ p65 interacted with two separate NFκB binding sites within the *TGM2* gene promoter. In conditions of genotoxic stress, breast cancer cell lines demonstrated a high association of Rel A/ p65 with the *TGM2* promoter. Also, mutating the -κB binding sites or use of NFκB pharmacological inhibitors decreased the *TGM2* promoter activity significantly (Ai, *et al.* 2012). With this, a novel loop wherein Rel A/p65 and TG2 activated each other resulted in a sustained activation of Rel A/ p65 as well as a chemoresistant phenotype could be hypothesized. Numerous literature reports have also suggested that NFκB has transcriptional control over the baseline expression of the *TGM2* gene in numerous cancer cells (Chen, *et al.* 2008 ; Kim, 2011).

In this chapter, IKKε was identified as the Rel A/ p65 kinase that induces the phosphorylation of Ser 536 in response to constitutive activation by TG2. Using BX795, which is a specific IKKε inhibitor, the phosphorylated status of Serine 536 in Rel A/p65 was determined using western blot. In Chapter IV, inhibiting Rel A/p65 using siRNA demonstrated a decrease in the activity and expression of TG2 within breast cancer cell lines. In order to further confirm these findings and study the post-translational modifications of the Rel A/ p65 dimer, a NFκB p65

(Ser529/536) inhibitory peptide was used to detect the binding of the dimers to -κB binding sites on DNA.

6.2 METHODS

6.2.1 Treatment of breast cancer cell lines with BX795

BX795 was developed as a small molecule inhibitor which could significantly inhibit IKKε levels at low concentrations *in vitro* (Bain, *et al.* 2007) and was specific to IKKε as BX795 did not inhibit other protein kinases such as IKKα /IKKβ (Clark, *et al.* 2009). Using BX795 the role and regulation of IKKε in breast cancer cell lines can be determined. 3000 cells each of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines were seeded into each well of a 96- well plate and grown to 70-80% confluency overnight. The next day the breast cancer cells were treated with increasing concentrations of BX795 (10μM, 15μM and 20μM) for time courses of 24, 48 and 72 h. After the treatment, the breast cancer cell lines were *analysed* using XTT to determine the appropriate concentration of BX795 that would not be cytotoxic to the cells. These results were expressed as the percentage of viable cells.

After determining the optimum concentration and time course of BX795 treatment, the breast cancer cell lines ($1x10^6$ cells/dish) were seeded into 60 mm petridishes and treated with BX795. After the time course of treatment, the breast cancer cell lines were subsequently used for further experiments.

6.2.2 Treatment of breast cancer cell lines with NFκB p65 (Ser529/536) inhibitory peptide

The NFκB p65 (Ser529/536) inhibitory peptide set that contains Rel A/p65 inhibitor peptide as well as a control peptide was purchased from IMGENEX (Catalogue Number: IMG-2003). The NFκB p65 (Ser529/536) inhibitory peptide blocks Rel A/ p65 Serine 529/536 phosphorylation during NFκB activation which inhibits the binding of Rel A/ p65 to DNA. The main function of the NFκB p65 (Ser529/536) inhibitory peptide is to inhibit the activation of NFκB. The sequences of the inhibitory peptides are as follows:

NFκB p65 (Ser529/536) inhibitory peptide: DRQIKIWFQNRRMKWKKNGLLSGDEDFSS

(p65 sequence is underlined)

Control Peptide: DRQIKIWFQNRRMKWKK

According to the manufacturer's instructions, 100μM of both the NFκB p65 (Ser529/536) inhibitory peptide and the control peptide was used to treat the breast cancer cell lines for a time course of 1 h. The NFκB p65 (Ser529/536) inhibitory peptide contains the PTD (protein transduction sequence) - DRQIKIWFQNRRMKWKK, renders the peptide cell permeable. The Control peptide in this set only consists of the PTD sequence.

Subsequently, the cytoplasmic and nuclear fractions of the breast cancer cell lines were collected as described in **Section 2.2.2**. Western blot analysis was performed on the sub cellular fractions to analyse the expression of TG2, α-Tubulin as well as TG2 whole cell lysate activity assay.

6.2.3 Detection of the phosphorylated status of Rel A/ p65 dimers via co-immunoprecipitation

Co-immunoprecipitation assays were performed to detect the phosphorylated status of Rel A/ p65 dimers in the breast cancer cell lines that were treated with BX795 for a time course of 48 h and subsequently fractionated into the cytoplasmic and nuclear fractions (**Section 2.2.2**). Briefly, rabbit anti- Rel A/ p65 polyclonal antibody and protein A beads were used to pull down the Rel A/ p65 immunocomplex in the pre-cleared cytoplasmic and nuclear samples. Western blotting was used to detect the presence of serine phosphorylation in the immunoprecipitated complex using mouse anti-phosphoserine monoclonal antibody (**Section 2.2.8**).

6.2.4 Chromatin Immunoprecipitation (ChIP) and PCR

The translocation of the NFκB subunit Rel A/p65 to the -κB sites of particular NFκB gene promoter targets in the breast cancer cell lines was assessed via ChIP assay using the

ExactaChIP ™ Human Rel A Chromatin Immunoprecipitation Kit (Catalogue Number EC5078). The ChIP assay was performed as described in detail in **Section 2.2.16.1**. The immunoprecipitated DNA was subjected to PCR as introduced in **Section 2.2.16.2**. The primer pairs used for the PCR analysis used for the *TGM2, p21 and GAPDH* gene promoter is as summarized in **Section 2.1.3**. The *p21* gene promoter was used as a positive control and *GAPDH* was used as the house keeping gene. The PCR samples were then further *analysed* by 1.5% Agarose gel electrophoresis (in 1x TAE).

6.3 RESULTS

6.3.1 Phosphorylated status of the Rel A/p65 dimer to DNA in breast cancer cell lines

NFκB activity is controlled in several stages by post-translational modifications including the phosphorylation of the transactivating Rel A/p65 subunit at various serine sites. To determine if the Rel A/ p65 dimers underwent phosphorylation at serine sites, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT breast cancer cell lines were immunoprecipitated with anti- Rel A/ p65 antibody and the resulting immunoprecipitates were subjected to immunoblotting using anti-phosphoserine antibody.

Results shown in Figure 6.1 clearly indicated that Rel A/ p65 was phosphorylated at a serine residue in the nucleus of high TG2 breast cancer cell lines as revealed by the presence of a distinct phosphoserine pull down band in the Rel A/ p65 immunoprecipitates. The TG2-null MDA MB 231 WT and MCF7/WT cell lines did not display any pull down phosphoserine band. Furthermore, in the MDA MB 231 Clone 16 and MCF7/RT cell lines, the dimeric Rel A/ p65 form observed in the nucleus was found to be phosphorylated at a serine residue, while the monomeric Rel A/p65 in the cytoplasmic fraction as well as the nuclear fraction did not undergo the post translational modification. These results clearly indicate that in the high TG2 breast cancer cell lines, the Rel A/ p65 dimers present in the nuclear fractions underwent post translational modification on a serine site.

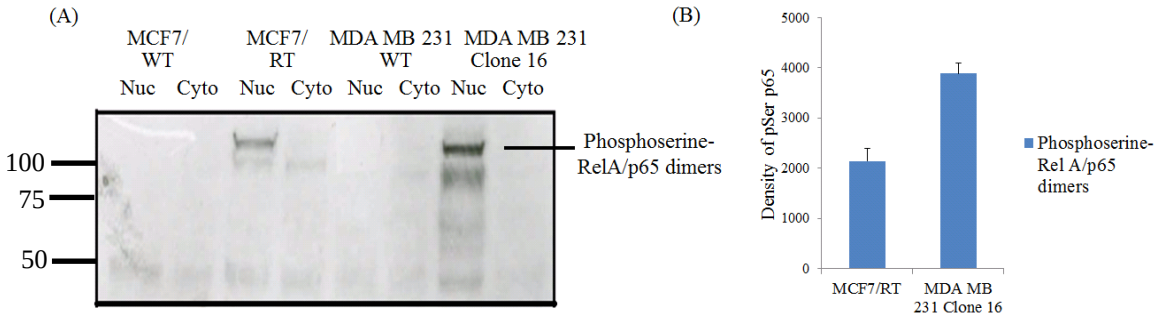


Figure 6.1 Analysis of the phosphorylated serine status of Rel A/ p65 dimers in the cytoplasmic and nuclear fractions of breast cancer cell lines using co-immunoprecipitation.

*The phosphorylated status of the Rel A/ p65 dimers was detected in the cytoplasmic and nuclear fractions of the breast cancer cell lines using western blotting (anti-phosphoserine antibody). $1x10^6$ cells of each breast cancer cell line was seeded into 60-mm petridishes and grown overnight to reach 70-80% confluency. The next day, the breast cancer cell lines were fractionated into the cytoplasmic and nuclear fractions as described in Section 2.2.2. The sub cellular fractions were then immunoprecipitated using anti-Rel A/p65 antibody and the immune complex then analysed by western blotting using anti-phosphoserine antibody (1:1000). (**A**) The western blot data shown above is a representation of three independent experiments (n=3). (**B**) Densitometry analysis was performed using Image J and the data shown is represented as the mean densitometry ± SE.*

6.3.2 Treatment of breast cancer cells with IKKε phosphorylation specific inhibitor, BX795

6.3.2.1 Killing curve to determine optimum concentration of BX795

To establish the optimum concentration of BX795 to treat the breast cancer cell lines without the inhibitor being cytotoxic, the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines were treated with increasing concentrations of BX795 (10µM, 15µM and 20µM) over a time course as described in **Section 6.2.1**. 10µM of BX795 over a time course of 48 h was selected to be the optimum concentration and longest possible time course to treat the breast cancer cell lines, without causing cytotoxicity to the cells (Figure 6.2)

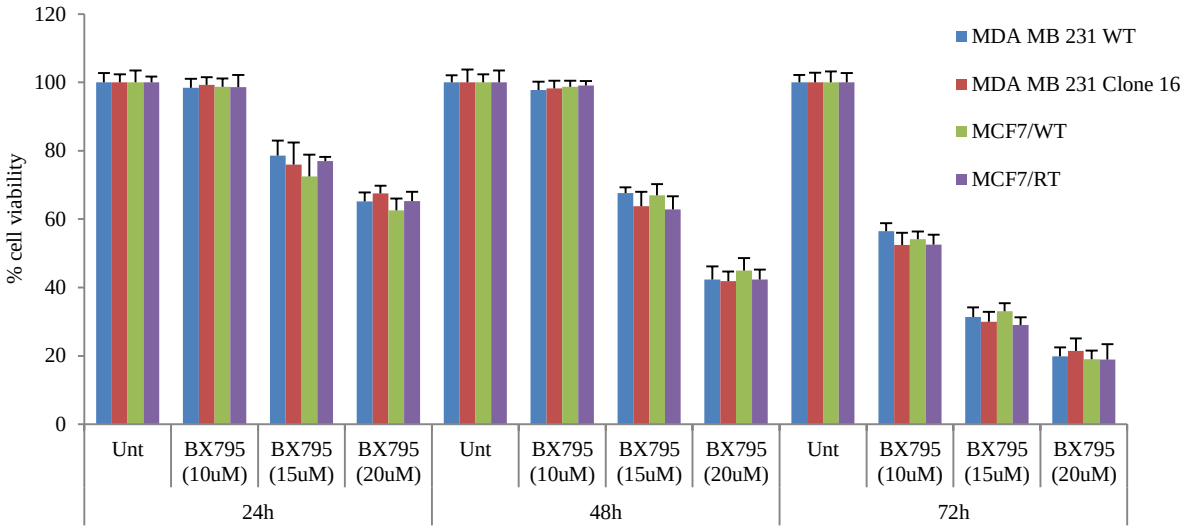


Figure 6.2 Cell viability analyses of breast cancer cell lines in the presence of increasing concentrations of BX795 over a time course

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines (3000 cells/well) were seeded into 96 well plates and allowed to become 70-80% confluent overnight. The next day the breast cancer cell lines were treated with increasing concentrations, 10μM, 15μM and 20μM of IKKε inhibitor, BX795. After 24, 48 and 72 h of treatment, the cell viability of the breast cancer cell lines was analysed using XTT (Section 2.2.13). The data shown is the percentage of viable cells measured in triplicate + SEM. The untreated cells (Unt) have been taken as 100% cell viability.

6.3.2.2 Detection of the phosphorylated serine status of Rel A/ p65 dimers by co-immunoprecipitation in the presence of BX795

Results from the previous chapters indicated that IKKα/β was not involved in the constitutive activation of Rel A/p65 dimers by TG2 as PS1145 failed to demonstrate any significant decrease in the Rel A/p65 dimers, NFκB activity, TG2 protein expression and activity as well as chemoresistance. To determine whether the dimeric Rel A/ p65 form was phosphorylated on serine residues by IKKε present in the nucleus, the effect of the IKKε phosphorylation inhibitor, BX795 was studied. The high TG2 expressing MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines were treated with 10μM of BX795 for 48 h and immunoprecipitated using anti-Rel A/ p65 antibody (0.5μg/sample). Protein A beads were then used to pull down the Rel A/ p65 immune complex which was subsequently *analysed* by western blotting using anti-phosphoserine antibody to detect the presence of the phosphorylated serine antigen. The results obtained clearly indicated that treating the MDA MB 231 Clone 16 and MCF7/RT with BX795 decreased the phosphorylated serine status of the Rel A/p65 dimers in the nucleus of these breast cancer cell lines (Figure 6.3). This also implies that the phosphorylation of a serine residue of Rel A/p65 dimers was as a result of IKKε activity as BX795 inhibited the serine phosphorylation.

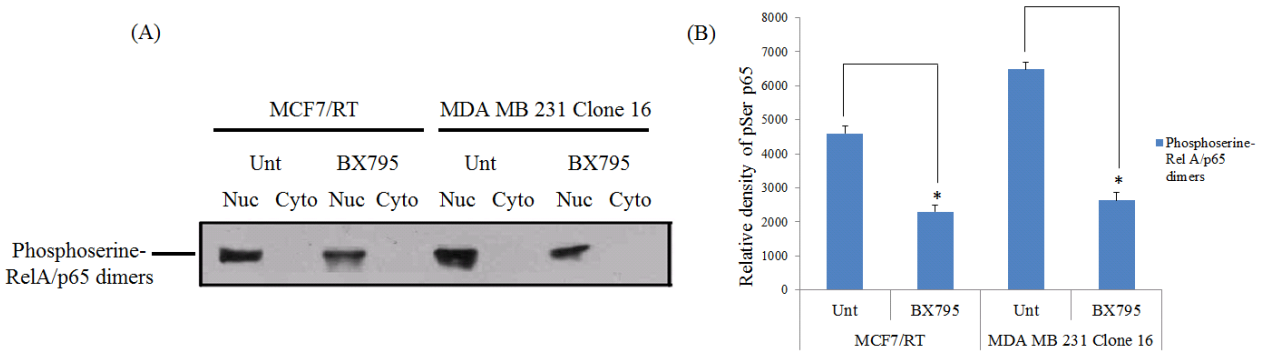


Figure 6.3 Analysis of phosphorylated serine status of Rel A/ p65 dimers in the cytoplasmic and nuclear fractions of breast cancer cell lines using co-mmunoprecipitation, in the presence of BX795

*The phosphorylated status of the Rel A/ p65 dimers was detected in the cytoplasmic and nuclear fractions of the breast cancer cell lines using western blot (anti-phosphoserine antibody {1:1000}). 1x10^6 cells each of MDA MB 231 Clone 16 and MCF7/RT cell line was seeded into 60-mm petridishes and grown overnight to reach 70-80% confluency. The next day, the breast cancer cell lines were treated with 10µM of BX795 for a time course of 48 h. After the treatment, the breast cancer cell lines were fractionated into the cytoplasmic and nuclear fractions as described in Section 2.2.2. The sub cellular fractions were then immunoprecipitated using anti-Rel A/p65 antibody and the immune complex was then analysed by western blot using anti-phosphoserine antibody (1:1000) (**A**). The western blot data shown above is representative of three independent experiments (n=3). Densitometry scans were quantified (**B**) by Image J analysis and are represented as mean of densitometry + SEM. * p< 0.05.*

6.3.2.3 Western blot analysis of pSer536-Rel A/p65 in breast cancer cell lines

Previously published literature has established that IKKε phosphorylates the serine 536 residue on Rel A/ p65, on being constitutively activated as a response to various cellular stimuli. To identify this, the cytoplasmic and nuclear fractions of the MDA MB 231 WT, Clone 16, MCF7/WT and MCF7/RT cells were treated with the IKKε inhibitor, BX795 as well as the cell permeable TG2 activity inhibitor, Z-DON and then *analysed* by western blotting using anti-pSer536- Rel A/p65 antibody. The TG2 low MDA MB 231 WT and MCF7/WT cells did not express any pSer536- Rel A/p65 in the cytoplasmic and nuclear fractions as described in previous sections. On the other hand, the high TG2 expressing MDA MB 231 Clone 16 and MCF7/RT cells demonstrated phosphorylation at the Serine 536 residue of the Rel A/ p65 dimer in the nuclear fraction. The IKKε phosphorylation inhibitor, BX795 decreased the serine 536 phosphorylation of the Rel A/p65 dimers. Also, as previous results indicated, the cell permeable TG2 activity inhibitor, Z-DON, decreased the Rel A/ p65 dimers which subsequently decreased the pSer 536- Rel A/p65 dimers (Figure 6.4) .These results clearly imply that the serine 536 residue of the Rel A/ p65 dimer of the high TG2 breast cancer cell lines was phosphorylated as a post translational modification. Also, this phosphorylated serine 536 residues seemed to be a characteristic of the nuclear Rel A/ p65 dimers. This phosphorylation of the Rel A/ p65 dimer at the serine 536 residue can be attributed to the kinase activity of the IKKε member of the IKK family of proteins as BX795, which is a specific IKKε phosphorylation inhibitor, decreases the phosphorylated status of the Rel A/ p65 dimer.

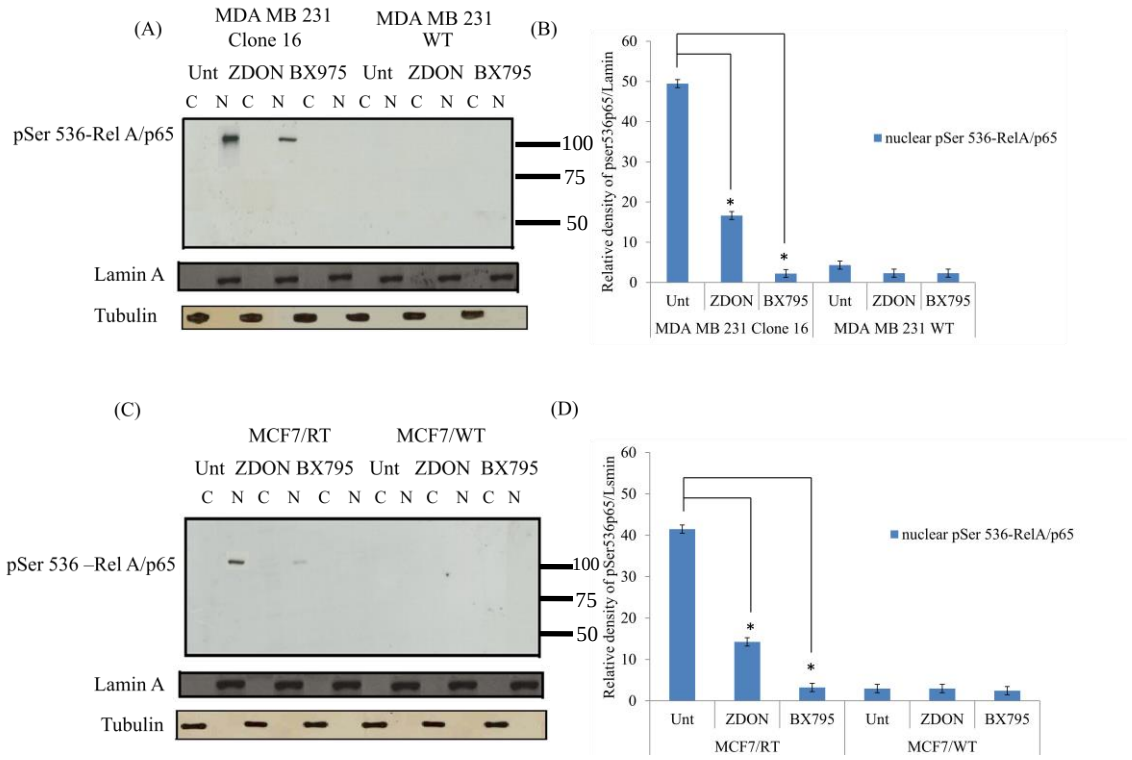


Figure 6.4 Analysis of pSer 536 - Rel A/ p65 dimers in breast cancer cell lines in the presence of BX795

*The MDA MB 231 WT, MDA MB 231 Clone 16 (A), MCF7/WT and MCF7/RT (C) breast cancer cell lines (5x10^5 cells/well) were seeded into 6-well plates and left overnight to become 70-80% confluent. The next day, the breast cancer cell lines were treated with 10µM of BX795 for 48 h and 50µM of Z-DON for 72 h. After the treatment, the breast cancer cell lines were fractionated into the cytoplasmic and nuclear fractions as described in Section 2.2.2. The sub cellular fractions were then analysed by western blotting (**A & C**) using anti-pSer 536- RelA/ p65 antibody (1:1000). The membranes were then reprobed using anti-Lamin A and anti-α-Tubulin antibody to ensure that the protein is equally loaded. The western blot data shown above is a representation of three independent experiments (n=3).(**B &D**) Differences in the expression of pSer 536- RelA/ p65 dimers in the treated and untreated samples was calculated using Densitometry was carried out and data is shown as the mean densitometry + SEM. * p< 0.05*

6.3.2.4 Decreased TG2 expression and activity in breast cancer cell lines treated with BX795

To determine if the phosphorylated serine status of the Rel A/p65 dimers was crucial for TG2 expression and activity, the breast cancer cell lines were *analysed* for the expression of TG2 by western blotting in the cytoplasmic fractions as well as the whole cell lysate.TG2 activity was also measured on being treated with IKKε inhibitor, BX795. The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were treated with 10μM of BX795 for a time course of 48 h and *analysed* by western blot for TG2 expression and TG2 whole cell lysate activity as described in **Sections 2.2.3 - 2.2.6** and **Section 2.2.11** respectively. On analysing the TG2 expression within the MDA MB 231 Clone 16 and MCF7/RT cell lines, there was a clear indication that BX795 treatment decreased the TG2 expression within these high TG2 breast cancer cell lines. Inhibiting the phosphorylation of the Rel A/p65 dimer at the serine 536 site seems to reduce the expression of TG2. This could be because the post translational modification of the Rel A/ p65 dimer is essential for the expression of TG2 (Figure 6.5). To further verify these results, the TG2 whole cell lysate activity of the breast cancer cell lines were *analysed*. The TG2 null MDA MB 231 WT and MCF7/WT did not demonstrate any TG2 whole cell lysate activity and on treating these breast cancer cell lines with BX795, no change in the TG2 levels within the cells was found The MDA MB 231 Clone 16 and MCF7/RT cell lines demonstrated a high level of whole cell lysate TG2 activity and treatment with BX795 illustrated a considerable decrease in the TG2 activity within these cells (Figure 6.6). These results clearly indicated that the serine 536 phosphorylation of the Rel A/ p65 dimer by IKKε in the nucleus was essential for the expression and subsequent activity of TG2 within the MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines.

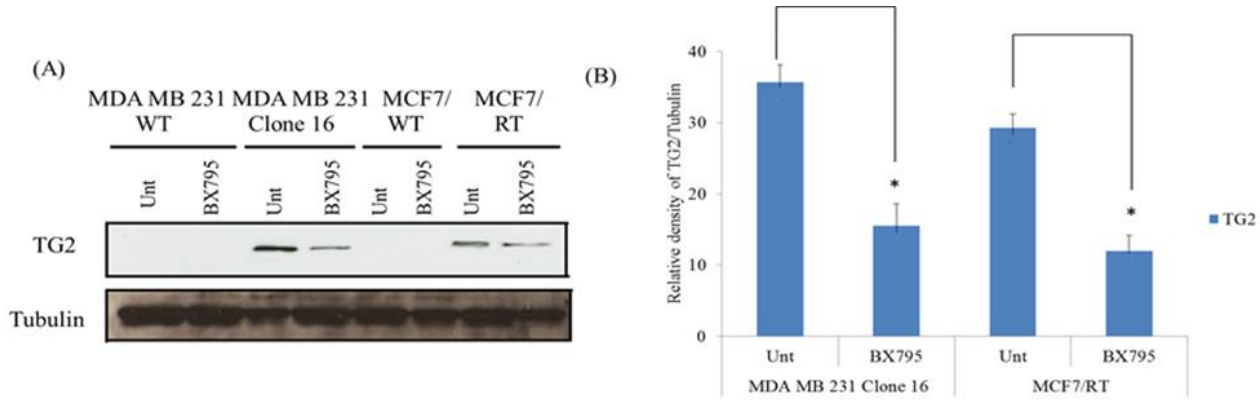


Figure 6.5 Analysis of the expression of TG2 in the cytoplasmic fractions of breast cancer cell lines on treatment with BX795

*The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines (5x10^5 cells per well) were seeded into a 6 well plate and left overnight to become confluent. The next day the breast cancer cell lines were treated with10μM of BX795 for 48 h after which the cytoplasmic fractions of the breast cancer cell lines were obtained as described in Section 2.2.2. (**A**) The cytoplasmic fractions were then analysed for the expression of TG2 by western blot using monoclonal anti-TG2 antibody (TG100) (1:1000). The membranes were reprobed with anti-α-Tubulin antibody to correct for protein loading. The western blot data shown is representative of three independent experiments (n=3). (**B**) Densitometry analysis was performed using image J and is represented as mean densitometry + SEM. * p< 0.05*

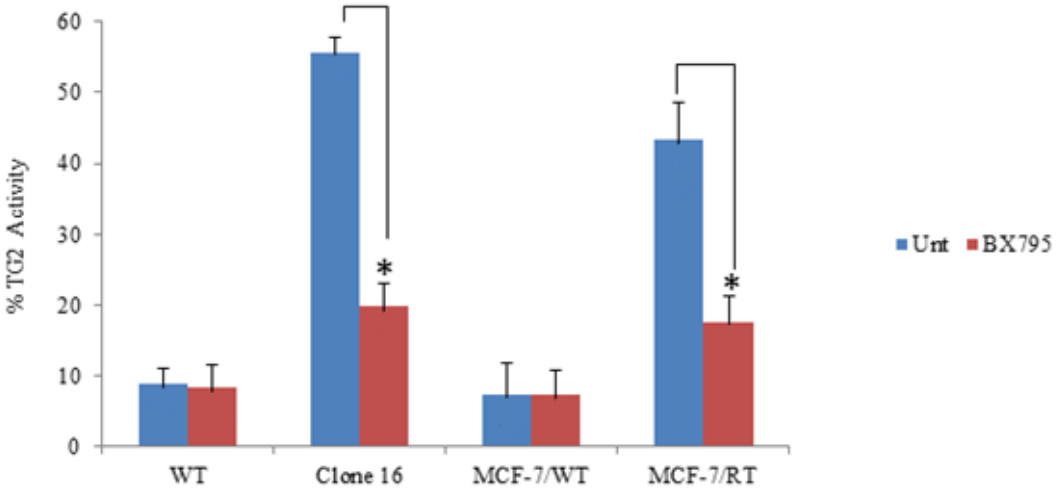


Figure 6.6 Analysis of whole cell lysate TG2 activity in breast cancer cell lines on being treated with BX795

*The whole cell lysate TG2 activity of the breast cancer cell lines were analysed using the incorporation of biotinylated cadaverine into fibronectin as described in Section 2.2.11. Positive control (50 ng/ml gplTG + 10mM $CaCl_2$) was taken to be 100% TG2 activity (1.31038 ± 0.401384). Data shown above is mean percentage TG2 activity + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA. * $p<0.05$ between untreated and BX795 treated cells.*

6.3.2.5 Analysis of IκBα dimers and p50/p105 NFκB subunits in breast cancer cell lines on being treated with BX795

To determine if treatment with IKKε phosphorylation inhibitor, BX795 had any effect on the IκBα dimer and p50/p105 expression, western blot analysis was performed on the cytoplasmic and nuclear fractions of the breast cancer cell lines. The MDA MB 231 WT and MDA MB 231 Clone 16 breast cancer cell lines were treated with 10μM of BX795 for a time course of 48 h and then *analysed* by western blotting using anti-IκBα antibody and anti-p50/p105 antibody. On analysis of the cytoplasmic fractions of the breast cancer cell lines using anti-IκBα antibody, the TG2 null MDA MB 231 WT cells illustrated the presence of monomeric IκBα, which does not change on being treated with BX795. As previously demonstrated, the high TG2 MDA MB 231 Clone 16 showed the presence of IκBα dimers in the cytoplasmic fractions. Treating the MDA MB 231 Clone 16 cells with BX795 did not change the status of the IκBα dimers within these cells (Figure 6.7 A). This could imply that inhibiting the phosphorylation of IKKε did not cause any change in the IκBα dimers in the high TG2 breast cancer cell lines. Furthermore, the p50/p105 subunit also remains unchanged in the high and low TG2 expressing breast cancer cell lines. These results indicate that inhibiting the kinase function of IKKε does not have any effect on the p50/p105 subunit of NFκB (Figure 6.7 C)

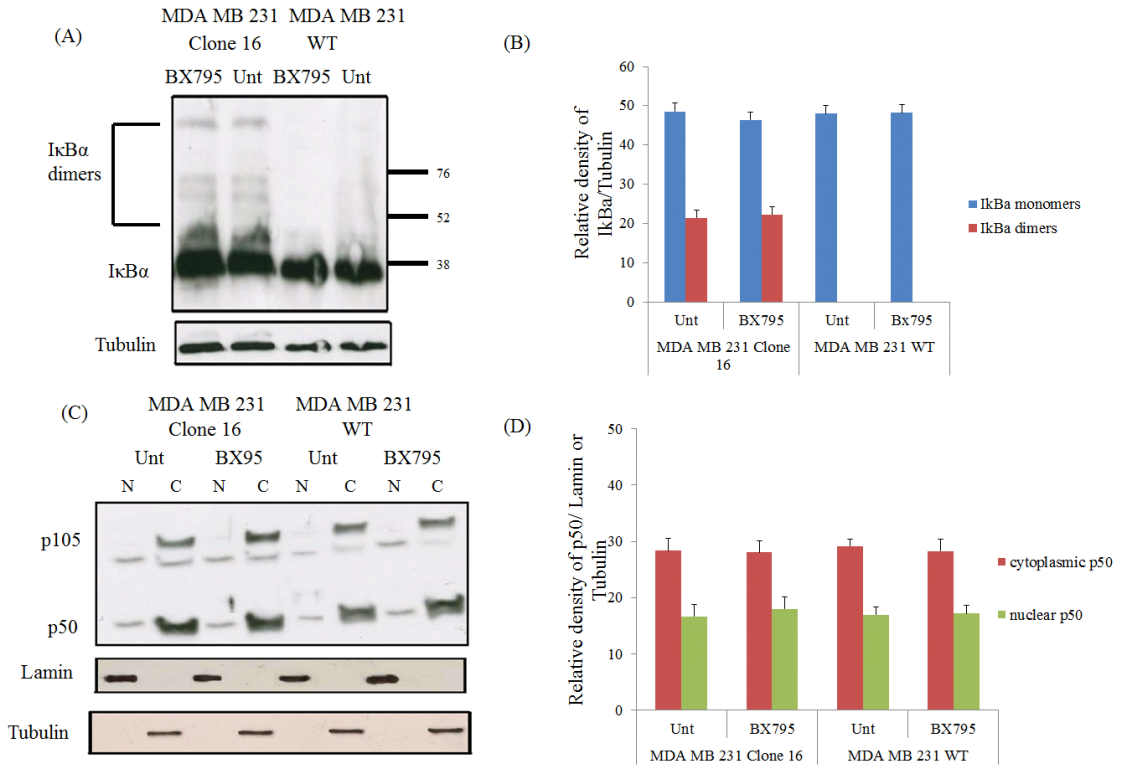


Figure 6.7 Western blot analysis of IκBa and p50/p105 NFκB subunit of NFκB in breast cancer cells on being treated with BX795

The MDA MB 231 WT and MDA MB 231 Clone 16 cells were seeded into 6-well plates ($5x10^5$ cells/well) and left overnight to become 70-80% confluent. The next day the breast cancer cell lines were treated with 10μM of BX795 for 48 h after which the cytoplasmic and nuclear fractions were derived as described in section 2.2.2 and analysed by western blotting ***(A & C)*** *using anti-IκBa antibody (1:1000) and anti- p50/p105 antibody (1:500). The membranes were then reprobed with anti-α- Tubulin and anti- Lamin A to correct for protein loading. The western blot data shown above is a representation of two independent experiments (n=2).* ***(B& D)*** *Densitometry analysis was performed using image J and represented as mean densitometry + SEM.*

6.3.2.6 Chemosensitivity of breast cancer cell lines on treatment with BX795 and TG2 inhibitors in the presence of doxorubicin.

XTT analysis of the breast cancer cell lines in the presence of doxorubicin and BX795 and TG2 cell permeable inhibitors (R283 and Z-DON) was performed to investigate whether treatment with BX795 would increase the chemosensitivity of high TG2 breast cancer cell lines in response to doxorubicin treatment in a comparable manner to the TG2 cell permeable inhibitors as described previously. The TG2 low MDA MB 231 WT and MCF7/WT cell lines demonstrated a considerable decrease in the loss of cell viability, in the presence of 1µg/ml of doxorubicin and further treatment with 10µM of BX795 or TG2 inhibitors, R283 (500µM) and Z-DON (50µM) did not change the chemosensitive phenotype of these breast cancer cell lines. On the other hand, the high TG2 expressing MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines that were inherently resistant to 1µg/ml of doxorubicin illustrated a considerable loss in the cell viability on being treated with 10µM of BX795 (Figure 6.8). This decrease in cell viability was consistent with the decrease observed on decreasing the TG2 activity levels within these high TG2 breast cancer cell lines. These results are clearly indicative that the phosphorylation of serine 536 of the Rel A/ p65 dimer is essential for the chemoresistance against doxorubicin in the MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines.

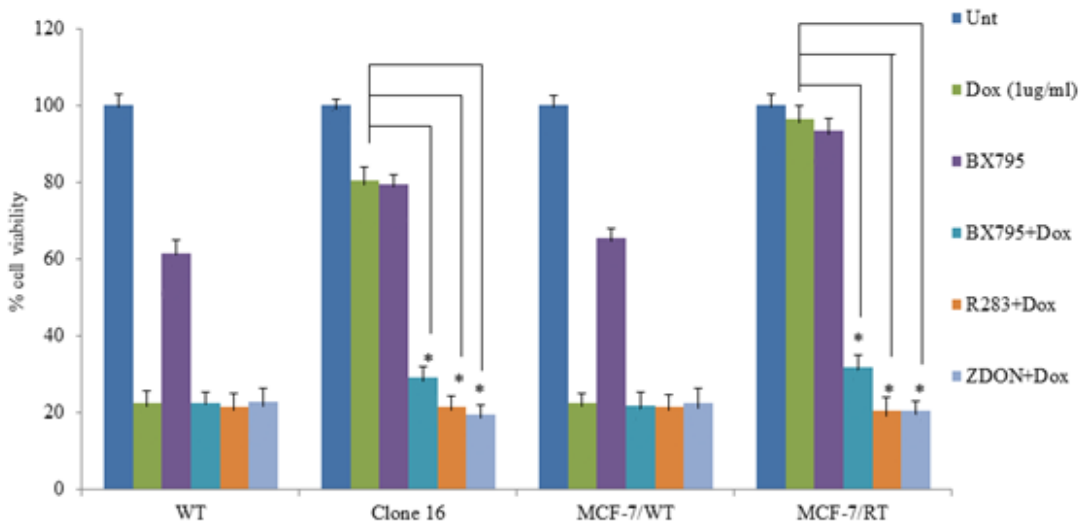


Figure 6.8 The effect of BX795 and TG2 cell permeable inhibitor treatment on the cell viability of breast cancer cell lines in the presence of doxorubicin

*3000 cells each of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were seeded per well into a 96-well plate and left overnight. The next day the cells were treated with 10μM of BX795 for 48 h, 500μM of R283 and 50μM of Z-DON for 72 h in the presence of 1μg/ml of doxorubicin for 72 h. After treatment, the cell viability of the breast cancer cell lines was analysed using XTT assay. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA with Dunnett's post-hoc test. * $p<0.01$ between cells treated with either BX795 or TG2 inhibitors in the presence of doxorubicin.*

6.3.3 Treatment of breast cancer cell lines with NFκB p65 (Ser529/536) inhibitory peptide

6.3.3.1 Decreased TG2 expression and activity in the presence NFκB p65 (Ser529/536) inhibitory peptide

Previous published data (Kim, *et al.* 2006), has clearly shown that during a DNA damage response, expression of TG2 is significantly up regulated and the use of pharmacological inhibitors of NFκB can inhibit this event. This could imply that high levels of Rel A/p65 sub unit could bind to the *TGM2* gene promoter (Ai, *et al.* 2012) The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were transfected with NFκB p65 (Ser529/536) inhibitory peptide as described in **Section 6.2.2** and the whole cell lysates of these breast cancer cell lines were collected (**Section 2.2.2**). The whole cell lysate fractions of these breast cancer cell lines treated with NFκB p65 (Ser529/536) inhibitory peptide and negative control peptide were *analysed* for TG2 activity (**Section 2.2.11**) and the cytoplasmic and nuclear fractions were *analysed* for the TG2 expression using western blot analysis (**Sections 2.2.3-2.2.6**). The MDA MB 231 WT and MCF7/WT cells do not express any TG2 activity in the whole cell lysate fractions, which remains unchanged on being treated with NFκB p65 (Ser529/536) inhibitory peptide. On the contrary, the MDA MB 231 Clone 16 and MCF7/RT cells that are known to exhibit TG2 activity show a significant decrease in the TG2 activity levels on being treated with the NFκB p65 (Ser529/536) inhibitory peptide (Figure 6.9). The negative control inhibitory peptide does not cause any change in the TG2 activity levels of the breast cancer cell lines. Similarly, the MDA MB 231 Clone 16 and MCF7/RT cells also illustrated a significant decrease in the expression of TG2 on being treated with the NFκB p65 (Ser529/536) inhibitory peptide (Figure 6.10). These results seem to clearly indicate that inhibiting the binding of Rel A/p65 dimers to DNA causes a significant decrease in the TG2 activity and expression in the high TG2 breast cancer cell lines. This further confirms that Rel A/ p65 has a -κB binding site

on the *TGM2* gene promoter which allows the control of TG2 expression and activity within the high TG2 breast cancer cell lines.

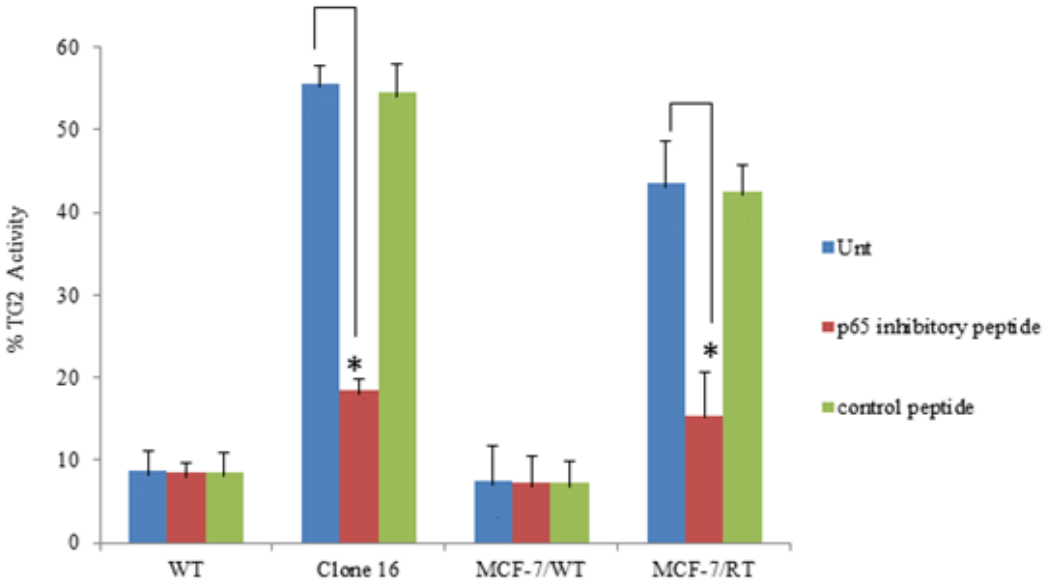


Figure 6.9 Analysis of TG2 whole cell lysate activity in the presence of NFκB p65 (Ser529/536) inhibitory peptide

*The whole cell lysate fractions of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT after treatment with 100μM of NFκB p65 (Ser529/536) inhibitory peptide and negative control inhibitory peptide over a time course of 1 h were collected and analysed for the TG2 activity as described in Section 2.2.11 by biotinylated cadaverine incorporation into fibronectin. Positive control (50 ng/ml gplTG + 10mM $CaCl_2$) was taken to be 100% TG2 activity (1.29836 ± 0.31932). Data shown above is mean percentage TG2 activity ± SE from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA. * $p<0.05$ between untreated and p65 inhibitory peptide treated cells.*

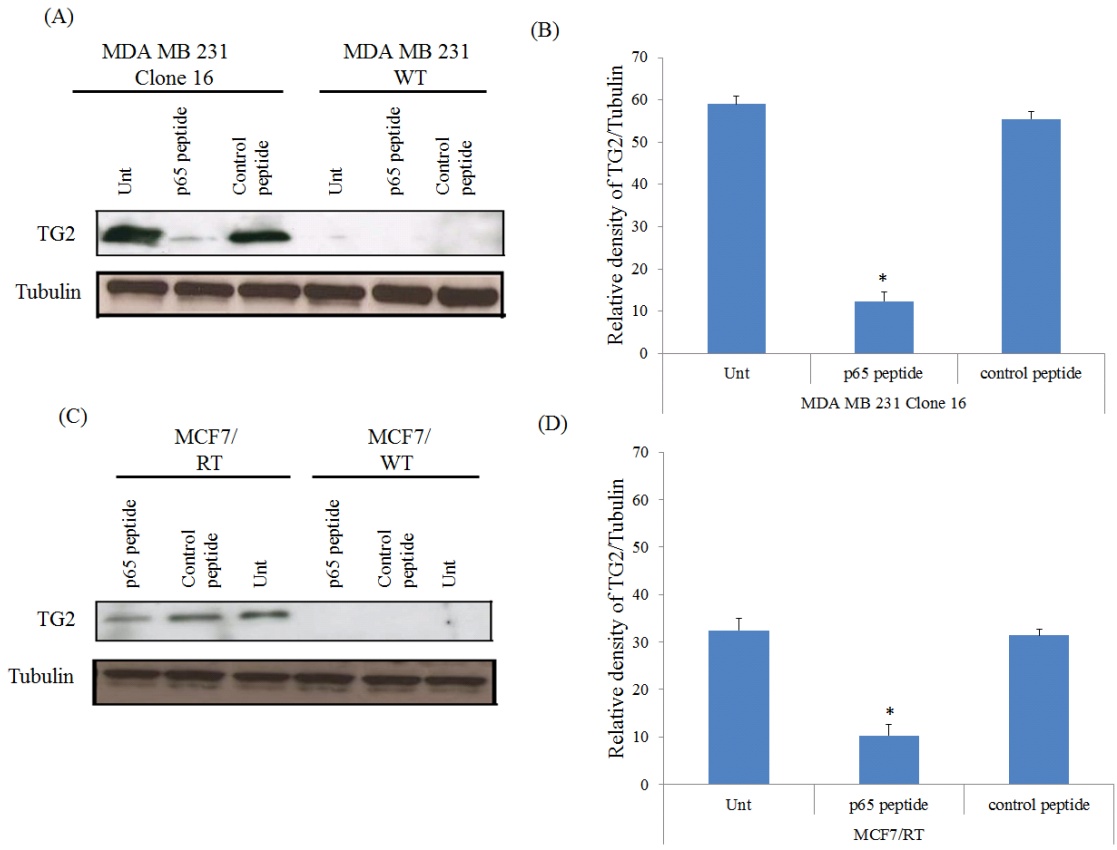


Figure 6.10 Western blot analysis of TG2 expression in breast cancer cell lines in the presence of NFκB p65 (Ser529/536) inhibitory peptide

*NFκB p65 (Ser529/536) inhibitory peptide was used to inhibit the binding of Rel A/p65 dimers to the -κB site on the TGM2 gene promoter. 5x10^5 cells (per well) each of MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT were seeded into 6 well plates and left overnight. The next day, the breast cancer cells were treated with 100μM of NFκB p65 (Ser529/536) inhibitory peptide and negative control inhibitory peptide over a time course of 1 h. After the treatment, the cytoplasmic fractions of the breast cancer cell lines were collected as described in detail in Section 2.2.2. The sub cellular fraction was then analysed for TG2 expression by western blot as described in sections 2.2.3-2.2.6 using mouse monoclonal anti-TG2antibody (1:1000). The membranes were reprobed with anti-α-Tubulin antibody to correct for equal protein loading. (**A & C**) The western blots shown here is a representation of three independent experiments (n=3). (**B & D**) Densitometry analysis was performed using Image J and is represented as the mean densitometry + SEM. * p< 0.05.*

6.3.3.2 Reduced chemoresistance of breast cancer cell lines on being treated with NFκB p65 (Ser529/536) inhibitory peptide in the presence of doxorubicin

In order to analyse if the binding of the pSer 536 Rel A/ p65 dimers was crucial for the chemoresistant phenotype of the high TG2 breast cancer cell lines, XTT analysis was performed after treating the cell lines with NFκB p65 (Ser529/536) inhibitory peptide in the presence of doxorubicin (1µg/ml).The TG2 null MDA MB 231 WT and MCF7/WT cells illustrated a considerable loss in the cell viability in the presence of 1µg/ml of doxorubicin, which also decreases similarly on being treated with the NFκB p65 (Ser529/536) inhibitory peptide. Previous results have already established that the MDA MB 231 Clone 16 and MCF7/RT breast cancer cells were chemoresistant to 1µg/ml of doxorubicin. On being treated with NFκB p65 (Ser529/536) inhibitory peptide, the TG2 high MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines demonstrated a decrease in the cell viability in the presence of doxorubicin (Figure 6.11). These results clearly imply that blocking the binding of Rel A/p65 dimer to DNA using the NFκB p65 (Ser529/536) inhibitory peptide increased the chemosensitivity of the high TG2 breast cancer cell lines. Inhibiting the binding of the Rel A/p65 dimer to the -κB binding site of the *TGM2* gene promoter decreased the TG2 expression and activity within the MDA MB 231 Clone 16 and MCF7/RT cell line which subsequently reduced the chemoresistance of these breast cancer cell lines. The TG2 expression and activity within the MDA MB 231 Clone 16 and MCF7/RT cell lines seems to be key in conferring drug resistance against doxorubicin in these cell lines. This also suggests that there is a regulatory loop between TG2 and Rel A/ p65 dimers and sustained activation of this TG2/NFκB loop has effects on breast cancer behaviour such as chemoresistance against doxorubicin.

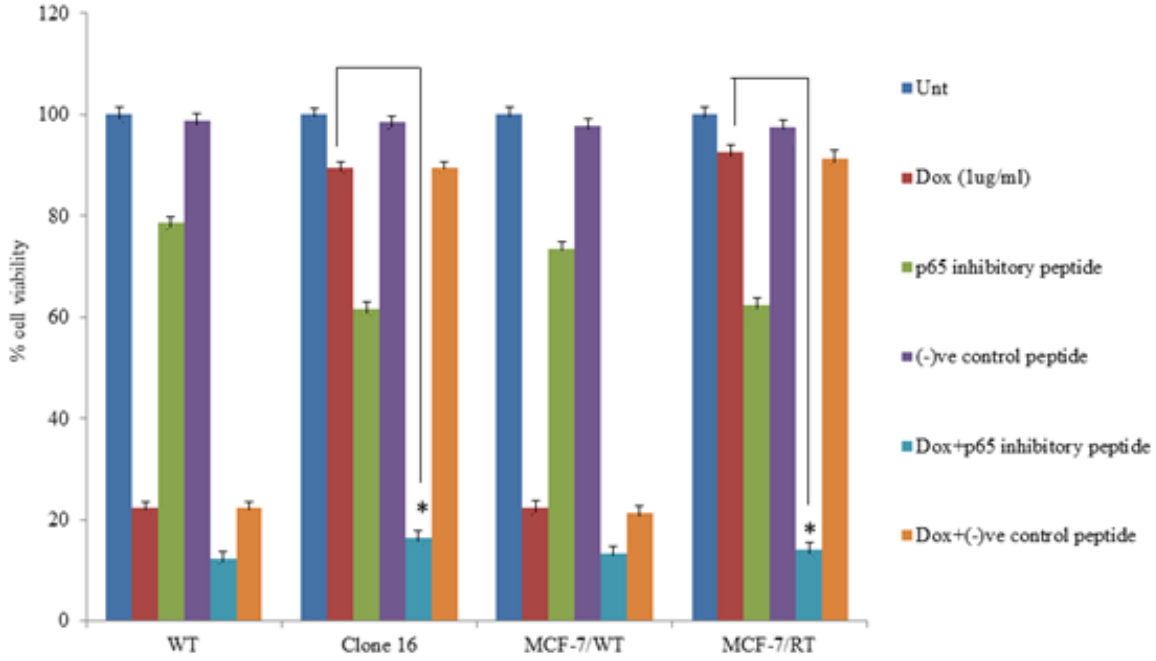


Figure 6.11 The effect of NFκB p65 (Ser529/536) inhibitory peptide on the cell viability of breast cancer cell lines in the increasing concentrations of doxorubicin

*3000 cells each of the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were seeded per well into a 96- well plate and left overnight. The next day the cells were treated with 100μM of NFκB p65 (Ser529/536) inhibitory peptide for 1 h and 1μg/ml of doxorubicin for a time course of 72 h. After treatment, the cell viability of the breast cancer cell lines were analysed using XTT. Untreated cells were taken as 100% viable. Data shown above is mean percentage of cell viability + SEM from 3 independent experiments (n=3). Statistical analysis was performed using ANOVA. * $p<0.01$ between cells untreated and treated with p65 inhibitory peptide in the presence of doxorubicin.*

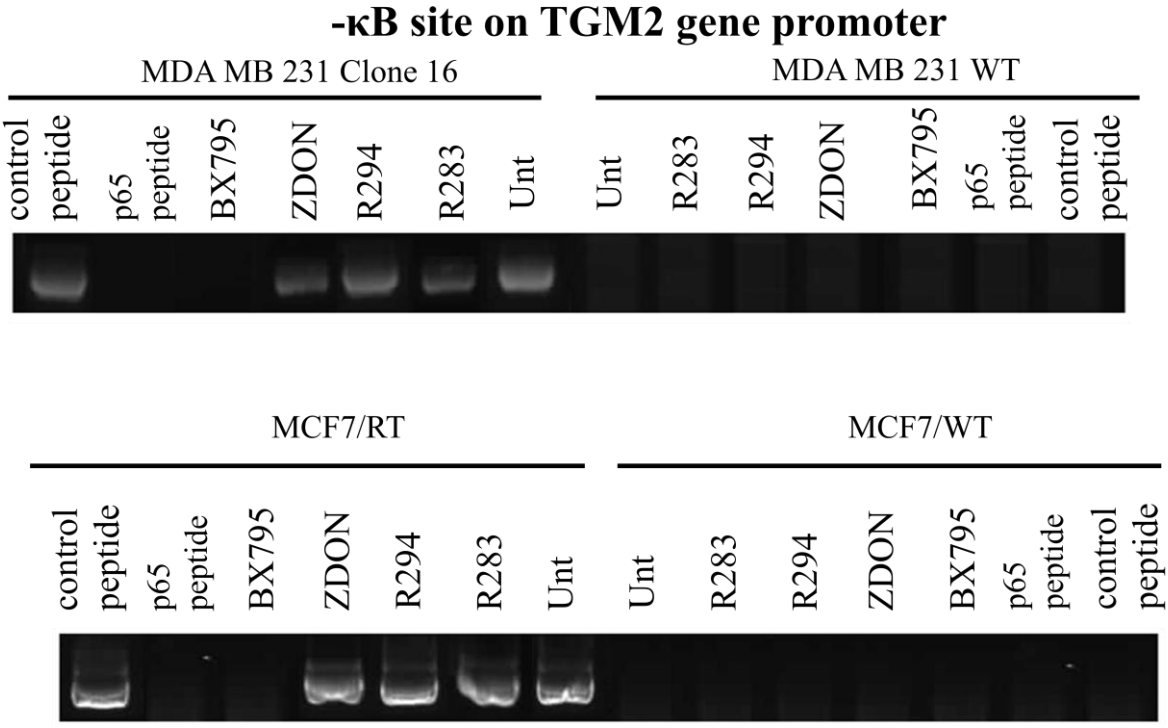
6.3.4 Binding of p-Ser536-RelA/ p65 dimer to the -κB site on the *TGM2* gene promoter.

6.3.4.1 Chromatin Immunoprecipitation of Rel A/p65 bound to the -κB site on the *TGM2* promoter

To determine whether binding of pSer536 Rel A/p65 dimers was required to drive the expression of TG2 in the breast cancer cell lines, the Rel A/ NFκB p65 ExactaChIP Chromatin IP kit (Catalogue #: ECP5078) was utilized to study the binding of the p-Ser536 Rel A/p65 dimer to the -κB site on the *TGM2* promoter in the breast cancer cell lines. The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT breast cancer cell lines were treated with cell permeable and cell impermeable inhibitors TG2 activity inhibitors, IKKε phosphorylation inhibitor, NFκB p65 (Ser529/536) inhibitory peptide and control peptide over specific time courses. Subsequently, chromatin immunoprecipitation was performed on the breast cancer cell lines as described in **Section 6.2.4**. The isolated ChIP-DNA was used to perform the PCR as described above (**Section 6.2.4**) with the primers that are specific for the -κB site on the *TGM2* promoter. The -κB binding site present on the *p21* promoter was used as the positive control for the ChIP-DNA. The breast cancer cells that lacked expression and activity of TG2 (MDA MB 231 WT and MCF7/WT) failed to show any binding activity of Rel A/p65 to the *TGM2* gene promoter. Results shown in Figure 6.12 clearly revealed that in the high TG2 breast cancer cell lines, anti- Rel A/p65 antibody could pull down the -κB binding site on the *TGM2* promoter. Treating the MDA MB 231 Clone 16 and MCF7/RT cells with NFκB p65 (Ser529/536) inhibitory peptide and BX795 inhibited this binding completely. This further validated that the *TGM2* gene promoter had an NFκB binding site which would allow the continuous regulation of a TG2-NFκB cycle. Also, this seems to suggest that the phosphorylated serine 536 status of the Rel A/ p65 dimers was crucial to allow binding of the NFκB subunit to the *TGM2* gene promoter site.

However, the MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines did not show any decrease or loss in DNA binding, on being treated with the cell permeable inhibitors R283 and Z-DON. As per the results obtained in the previous Chapters, treating the high TG2 breast cancer cell lines with cell permeable TG2 activity inhibitors (R283 and Z-DON) demonstrated a decrease in the TG2 activity and protein expression as well as a decline in the formation of the Rel A/ p65 dimers within these cell lines. This could imply that even though the formation of Rel A/ p65 dimers was inhibited using R283 and Z-DON, it can be hypothesized that the non-cross linked Rel A/p65 could still bind to the -κB site on the *TGM2* promoter. Nevertheless, in spite of the binding of Rel A/p65 subunits to the -κB site on the *TGM2* gene promoter, the breast cancer cell lines treated with R283 and Z-DON illustrated a decrease in the TG2 protein expression within the cells.

(A)



(B)

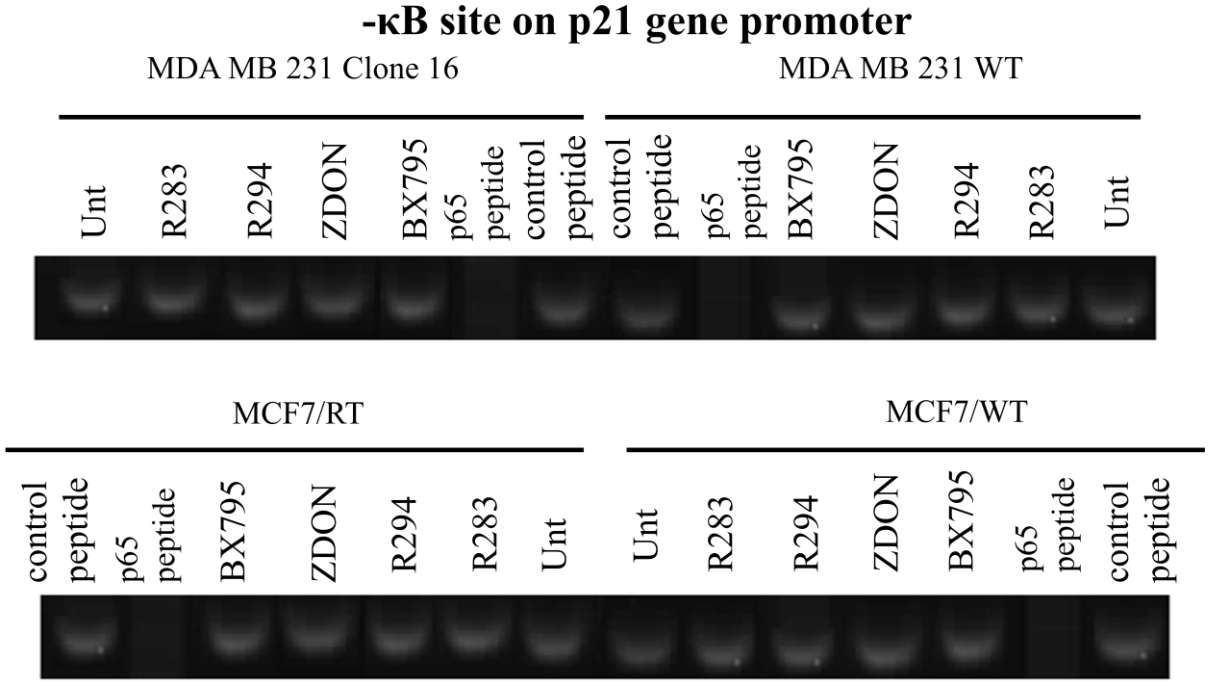


Figure 6.12 (A & B) Analysis of Rel A/p65 dimers binding to -κB site on *TGM2* (A) and *p21* gene (B) promoter using Chromatin Immunoprecipitation (ChIP)

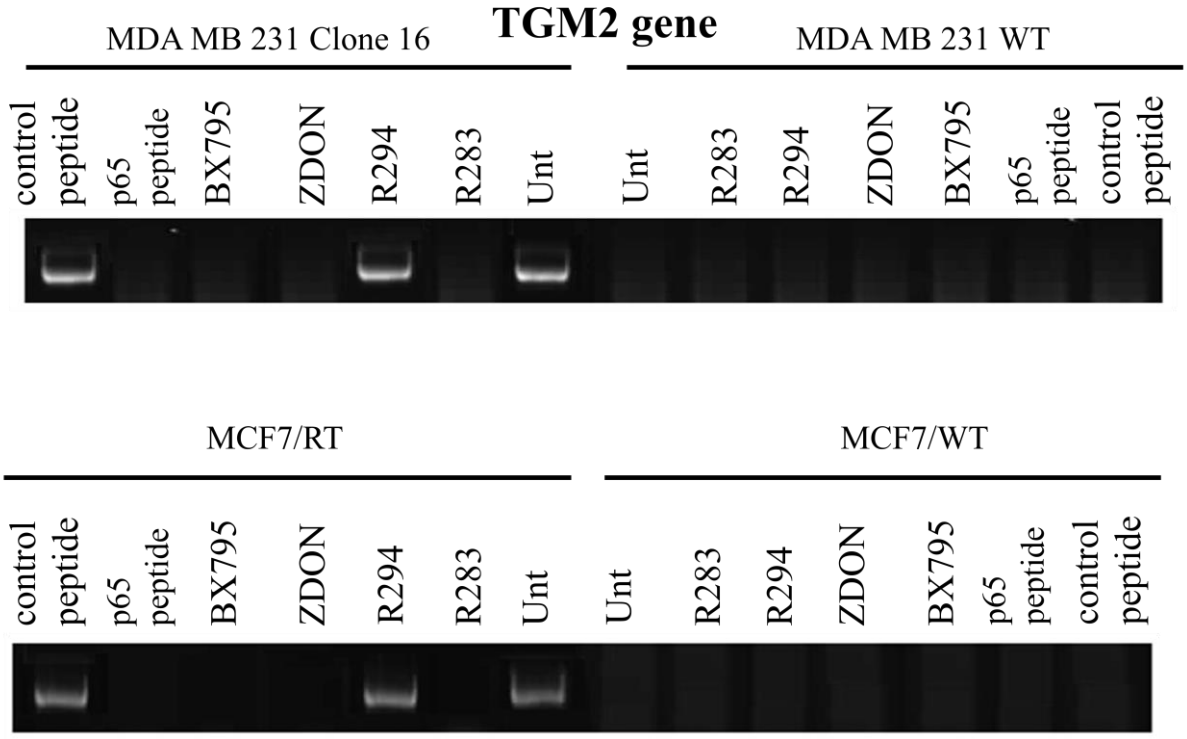
Chromatin Immunoprecipitation was performed on formaldehyde fixed MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT breast cancer cell extracts using Rel A/p65 specific antibody 2.2.15.1. PCR was carried out on the immunoprecipitates, using specific sets of primers (Shown in Section 2.2.15.2), to amplify the TGM2 promoter sequence containing the -κB binding sites as well as the p21 promoter sequence containing the -κB binding sites. The data presented is a representation of three independent experiments (n=3).

6.3.4.2 PCR for TG2 mRNA expression

Results from the previous section demonstrated that the pSer536 Rel A/p65 dimers were able to bind to the -κB site on the *TGM2* gene promoter, even on being treated with TG2 cell permeable inhibitors, R283 and Z-DON. However, this did not agree with the results obtained from previous chapters as treating the breast cancer cell lines with R283 and Z-DON clearly decreased the TG2 protein expression within the high TG2 and chemoresistant breast cancer cell lines. To verify the previous obtained results the mRNA expression of TG2 protein was determined by PCR. Total RNA was extracted from the breast cancer cell lines using TRIZOL reagent (Invitrogen) according to the manufacturers' instructions and PCR was subsequently performed. The PCR was conducted using *TGM2* gene and *GAPDH* gene primers as detailed in **Section 2.2.14**. The PCR product was then subsequently *analysed* by agarose gel electrophoresis using 1.5% agarose gel in 1x TAE.

The results contained from the PCR illustrated that treating the high TG2 MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines with cell-permeable inhibitors R283 and Z-DON completely inhibited the mRNA expression of *TGM2*. The NFκB p65 (Ser529/536) inhibitory peptide as well as IKKε kinase inhibitor BX795 had a similar effect on the *TGM2* gene expression. The cell impermeable inhibitor, R294 did not inhibit the mRNA expression of TG2, which was similar to results obtained from the western blot analysis of TG2 protein levels in the MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines (Figure 6. 13). This may imply that the Rel A/p65 observed bound to the -κB site on the *TGM2* gene promoter was a monomer and since the *TGM2* gene expression and subsequent protein expression was inhibited on treatment with R283 and Z-DON cell permeable TG2 activity inhibitors the cross linked dimeric form of Rel A/p65 could thereby be considered crucial for the gene expression of *TGM2*.

(A)



(B)

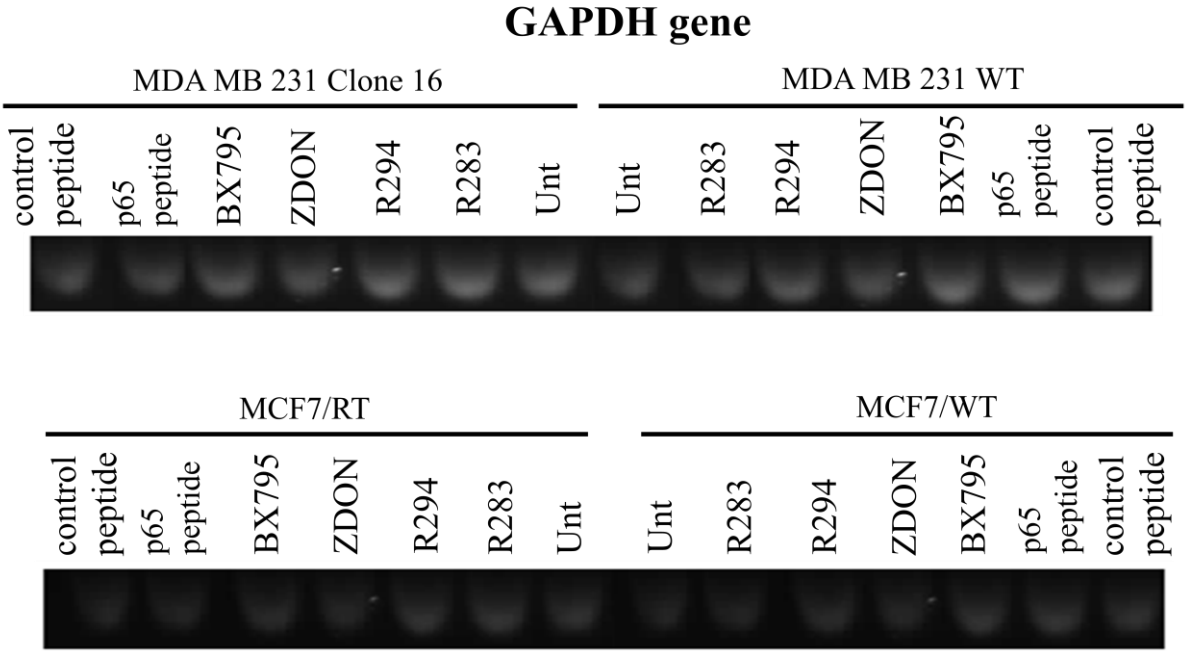


Figure 6.13 PCR analysis of the *TGM2* and *GAPDH* gene expression in breast cancer cell lines in various inhibitor treatments

The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT breast cancer cell lines ($5x10^5$ cells/well) were seeded into 6-well plates and left overnight to become confluent. The next day the breast cancer cell lines were treated with 500μM of R283, 500μM of R294 and 50μM Z-DON for 72 h, 10μM of BX795 for 48 h and NFκB p65 (Ser529/536) inhibitory peptide as well as control peptide for 1 h. After the treatments, RNA was prepared (Section 2.2.14.1) and then subsequently analysed by PCR using TGM2 and GAPDH gene primers. The results shown above are a representation of two independent experiments conducted (n=2).

6.4 DISCUSSION

Numerous nuclear modifications of the NFκB subunits can greatly affect DNA binding as well as interactions with repressors and activators of gene expression (Chen, *et al.* 2005). The protein modifications of the NFκB subunits may vary between cell lines as well as variation in the NFκB subunit inducing stimuli. Indeed, the results obtained in this Chapter demonstrate that the Rel A/p65 dimer phosphorylation at the Ser 536 residue is essential for the expression of the *TGM2* gene as well as inducing chemoresistance in the high TG2 breast cancer cell lines. Co-immunoprecipitation experiments have shown that the Rel A/ p65 dimers were phosphorylated at a serine residue. Since the previous chapters indicated that PS1145 which was an IKKα/β specific inhibitor did not alter the status of the Rel A/p65 dimers or even the TG2 activity and expression within the high TG2 breast cancer cell lines, an IKKε kinase inhibitor, BX795 was applied. Blocking the phosphorylation of the Rel A/ p65 dimer using BX795 resulted in decreased TG2 expression and activity within the high TG2 MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines. Concurrent with this decrease in TG2 expression and activity, the chemosensitivity of the high TG2 breast cancer cell lines against doxorubicin was also reduced. These results clearly indicated that the serine phosphorylation of the nuclear Rel A/ p65 dimer by IKKε is essential for the expression and activity of TG2 as well as chemoresistance in these breast cancer cell lines.

Literature seems to suggest that IKKε kinase function is most characteristic of a Ser 536 residue phosphorylation (Perkins, 2006). To verify if the case was similar in the breast cancer cell lines, NFκB p65 (Ser529/536) inhibitory peptide which would specifically inhibit the phosphorylation of Serine 536, which would subsequently impede the binding of Rel A/p65 to DNA was utilized. The NFκB p65 (Ser529/536) inhibitory peptide significantly decreased the TG2 activity as well as expression within the breast cancer cell lines. Furthermore, the chemoresistant phenotype of the MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines was also altered considerably. These results seem to clearly indicate that the phosphorylation of Rel A/p65 dimers at the Ser 536 residue was crucial for the chemoresistance against

doxorubicin as well as TG2 expression within the breast cancer cell lines. Further studies were conducted with ChIP assay to identify gene networks as well as expression mechanisms by determining whether Rel A/p65 dimers would interact with the -κB binding site on the *TGM2* gene promoter. The NFκB p65 (Ser529/536) inhibitory peptide as well as IKKε kinase inhibitor, BX795 clearly inhibited the binding of the Rel A/p65 dimers to the -κB site on the *TGM2* gene promoter. However, the cell permeable TG2 inhibitors, R283 and Z-DON which previously demonstrated reduced the TG2 protein expression levels, did not inhibit the binding of Rel A/p65 dimers to the -κB site on the *TGM2* gene promoter.

To verify this, PCR analysis was conducted on RNA samples collected from the breast cancer cell lines using *TGM2* gene primers. The NFκB p65 (Ser529/536) inhibitory peptide and BX795 inhibited the expression of *TGM2* gene in MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines. Also, the cell permeable TG2 inhibitors, R283 and Z-DON inhibited the expression of the *TGM2* gene. This clearly indicated that there was a self-regulating molecular feedback loop wherein TG2 constitutively activates NFκB and in turn, NFκB directly up regulates the gene transcription of *TGM2*. A clear relationship between the expression and activity of TG2 in cultured breast cancer cell lines and the increased binding of Rel A/p65 binding to DNA has been characterised using EMSA (Mann, *et al.* 2006). Other groups have also documented that there is an intricate relationship between the protein abundance and transcript levels of TG2 as well as NFκB activity (Ai, *et al.* 2012; Kim, *et al.* 2006). Furthermore, the pharmacological inhibition and RNAi mediated knockdown of TG2 results in decreased DNA interaction with NFκB (Mann, *et al.* 2006). With these results, it clearly seems that TG2 is essential to maintain the constitutive activation of NFκB.

To summarize the above observations, the work in this chapter demonstrated that the serine phosphorylation of Rel A/p65 dimers by IKKε was vital for the TG2 expression and activity levels within the breast cancer cell lines. Also, blocking the serine phosphorylation reduced the chemoresistance of the high TG2 breast cancer cell lines against doxorubicin. Furthermore, NFκB p65 (Ser529/536) inhibitory peptide was used to detect if Ser 536 was specifically the

residue phosphorylated on the Rel A/p65 dimers. This inhibitory peptide blocks the binding of the Rel A/p65 dimers to the -κB binding site on the *TGM2* gene promoter as well as reduces chemoresistance to doxorubicin. Analysis of the *TGM2* gene expression demonstrated that inhibiting TG2 activity using R283 and Z-DON, BX795 and NFκB p65 (Ser529/536) inhibitory peptide decreased the *TGM2* gene expression within the breast cancer cell lines. Importantly, a novel TG2-NFκB regulatory loop seems to be playing a vital role maintaining the continuous expression and activity of TG2 which constitutively activates NFκB in chemoresistant breast cancer cells.

Chapter 7: Epithelial to Mesenchymal Transition in breast cancer cells

7. Epithelial to Mesenchymal Transition in Breast Cancer cell lines

7.1 INTRODUCTION

Epithelial to Mesenchymal Transition can be defined as the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype. During this process, cancer cells attain molecular changes that allow aberrant cell to cell adhesive interactions and dysfunctional cell junctions. These characteristics tend to promote cancer cell invasion and subsequent progression into the surrounding microenvironment. Such a transformation has profound implications in the process of breast cancer metastasis and there is evidence to support that numerous tumours contain a unique subpopulation of stem - like cells with mesenchymal attributes that are chemoresistant (Foroni, *et al.* 2012). EMT is known to play a very important role in the process of tumour metastasis and invasion. Also, it has been hypothesized that EMT is likely to occur in particular stages of tumour progression such as intravasation and invasion wherein tumour cells dissociate and move to organ/tissue sites distant from the primary site (Gupta & Massague, 2006 ; Thompson, *et al.* 2005). EMT is a crucial developmental process in which epithelial cells lose their polarity and epithelial nature and acquire a mesenchymal phenotype which possesses increase motility behaviour (Thiery & Sleeman, 2006).

EMT is characterized by the loss of adhesion markers such as occludins and E-cadherin, decrease of epithelial markers such as cytokeratins, increase of mesenchymal markers such as vimentin, up regulated invasiveness, motility and metastasis. Furthermore, a process called "cadherin-switching" which involves the up-regulation of mesenchymal cadherins such as N-cadherin and down regulation of E-cadherin has also been associated with EMT (Thiery, 2002 ; Maeda, *et al.* 2005). As demonstrated in previous Chapters, there is significant evidence to support that increased levels of TG2 activity and expression confers drug resistance as well as metastatic properties to breast cancer cells (Mehta, *et al.* 2004; Mangala, *et al.* 2007). Stable

expression of TG2 in mammary epithelial cells is known to be associated with EMT and also concurrent with the constitutive activation of NFκB (Kumar, *et al.* 2010; Verma, *et al.* 2008; Mann, *et al.* 2006; Kim, *et al.* 2006).

TGF-β1 has been implicated in inducing TG2 expression (Quan, *et al.* 2005) and TG2 can activate TGF-β1 (Nunes, *et al.* 1997). In TG2 transfected MCF10A epithelial cells, no activation of TGF-β signalling was observed and also in the absence of TG2, TGF-β1 failed to induce EMT, implying that TGF-β1 has no role to play in inducing EMT in the absence of TG2 (Kumar, *et al.* 2010). This suggested that TG2 is a crucial downstream effector of TGF-β induced EMT. Supplementary studies are required to further validate tumour potential of MCF10A transfected with TG2 cells, even though *in vitro* data is clearly available to support the hypothesis that stable transfection of TG2 is enough to induce a drug resistant and metastatic phenotype (Kumar, *et al.* 2010). EMT induced by TG2 has been largely associated with TGF- β1 signalling with the increase of $CD44^{high}/CD24^{-/low}$ cell population, the ability to self-renew and form mammospheres (Kumar, *et al.* 2010).

Previous studies have demonstrated that the dysfunctional expression of TG2 in epithelial cells causes the constitutive activation of Akt, NFκB and FAK (Mann, *et al.* 2006 ; Kim, *et al.* 2006 ; Verma, *et al.* 2008). All of these above pathways are known to be closely related to promoting metastasis, conferring drug resistance and regulation of EMT (Wang, *et al.* 2009 ; Kalluri & Weinberg, 2009). Active NFκB is thought to be the hallmark of advanced stage tumours (Karin, *et al.* 2002 ; Parsad, *et al.* 2010). Constitutive activation of NFκB is known to function in the chemoresistance and death inducing stimuli and also promotes EMT and induces metastasis (Huber, *et al.* 2004; Orlowski, *et al.* 2002). The induction of EMT by NFκB has been accredited to the increased stability of Snail along with the increased production of ICOP2 signalosome 2 that is known to inhibit the ubiquitination and degradation of *Snail*. The constitutive activation of NFκB in MCF10A mammary epithelial cells, induces EMT due to an increase in the expression in *Zeb1* and *Zeb2* expression (Kumar, *et al.* 2010). With regard to these observations,, it can be hypothesized that EMT induced by TG2 can result in the

constitutive activation of NF-κB and subsequently the increased expression of *Snail, Zeb 1* and *Zeb 2* (Chua, *et al.* 2007). Also, in recent studies, TG2 has been shown to associate with NF-κB and the recruitment of TG2 to the promoter region of Snail which leads to the regulation of transcription (Kim, *et al.* 2010).

The aim of this chapter is to further investigate the influence of increased TG2 expression and activity on the EMT like phenotype of the breast cancer cell lines. Using breast cancer cell lines that express varying levels of TG2, western blot analysis of various epithelial and mesenchymal markers such as E- cadherin, N- cadherin and fibronectin was performed to detect the effect of high TG2 levels in inducing EMT in breast cancer cells. TGF-β1 activity assay following the use of TG2 inhibitors (R283, R294 and Z-DON) will also be used to investigate whether TG2 induced EMT in these breast cancer cell lines was due to activation of the TGF-β1 signalling pathway.

7.2 METHODS

7.2.1 Detection of Epithelial and Mesenchymal markers in breast cancer cell lines

After 72 h of incubation with TG2 cell permeable (R283 and Z-DON) and cell impermeable (R294) activity inhibitors, whole cell lysates were collected and the protein concentration was detected as introduced in **Section 2.2.3**. Western blot was performed to detect the presence of E- cadherin, N- cadherin, fibronectin and TGF-β receptor I and II using appropriate primary and secondary antibodies (**Section 2.2.4- 2.2.6**). Signals were further detected using the Chemiluminescence (ECL) system kit.

7.2.2 TGF-β1 Activity Assay

Using the Human/Mouse TGF β 1 ELISA kit from e-Bioscience (88-8350), the breast cancer cell lines were assayed for TGF-β1 in the medium. The MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were seeded into 24-well plates and left overnight as per the instructions of the kit. The breast cancer cells were initially grown in serum replacement medium and treated with cell permeable (R283 and Z-DON) and cell impermeable (R294) TG2 activity inhibitors over a time course of 72 h. After the treatment, the cell medium was collected from each cell line and assayed for TGF-β1. The Corning Costar 9018 plate provided along with the kit was coated with 100µl/ well of capture antibody diluted in coating buffer, sealed and incubated overnight at 4^{o}C. The wells were washed 5 times with wash buffer (250µl/well). The wells were then blocked with 1x assay diluent (200µl/well) and incubated at room temperature for 1 h. The wells were washed 5 times again with wash buffer. The samples (culture medium) were then acidified to be activated and subsequently neutralized. To 100µl of sample, 20µl of 1N HCl was added and incubated at room temperature for 10 min and then neutralized with 20µl of 1N NaOH. Standards were also diluted using the assay diluent and 100µl of standard was added per well. 100µl of sample was added into each well and incubated at room temperature for 2 h. The wells were then washed five times with Wash Buffer. 100µl of

Avidin-HRP was added per well and incubated at room temperature for 30 min. The wells were then washed seven times with wash buffer. 100µl of substrate solution was added into each well and incubated at room temperature for 15 min after which 50µl of stop solution was added to each well. The plate was then read using a plate reader at 450nm.

7.3 RESULTS

7.3.1 TGF-β induced Epithelial to Mesenchymal Transition (EMT) in breast cancer cell lines

Recent studies have indicated that the EMT gene signature of TG2 transfected MCF10A mammary epithelial cells closely resembled TGF-β induced EMT (Xu, *et al.* 2009). To verify this, the expression of TGF-β receptor I and II was determined in the MDA MB 231 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cell lines (Figure 7.1)

Furthermore, the MDA MB 21 WT, MDA MB 231 Clone 16, MCF7/WT and MCF7/RT cells were assayed for the relative TGF-β1 presence in the medium in the presence of TG2 activity inhibitors as described in **Section 7.2.2**. Both the high and low TG2 breast cancer cell lines did not express any TGF-β1 activity. The cell permeable and cell impermeable TG2 activity inhibitors subsequently had no effect on the TGF-β1 activity levels of the breast cancer cell lines (Figure 7.2). These results clearly indicate that the chemoresistant phenotype, that could be as a result of EMT in these breast cancer cell lines, is completely independent of TGF-β1 signalling and activity.

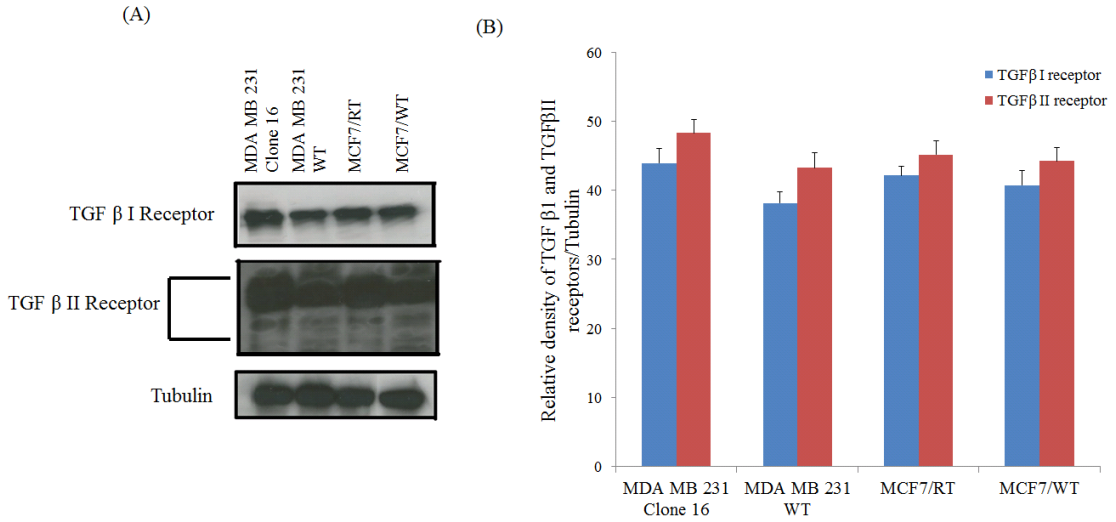


Figure 7.1 Analysis of expression of TGF-β Type I and II receptors of breast cancer cell lines using Western blot

*The breast cancer cell lines ($1x10^6$ cells per well) were seeded into 60mm petridishes and left to become confluent. The whole cell lysate fractions of the breast cancer cell lines were collected as described in detail in Section 2.2.2. 50µg of whole cell lysate proteins were then analysed by western blot for the expression of TGF-β Type I and II receptors using rabbit polyclonal anti-TGF-β Type I and II receptor antibody (1:1000). The membranes were then reprobed with anti-α-Tubulin antibody to correct for equal loading of protein. (**A**)The western blot data shown is a representation of three independent experiments (n=3). (**B**) Densitometry of the westerns was quantified by Image J analysis is represented as mean densitometry + SEM.*

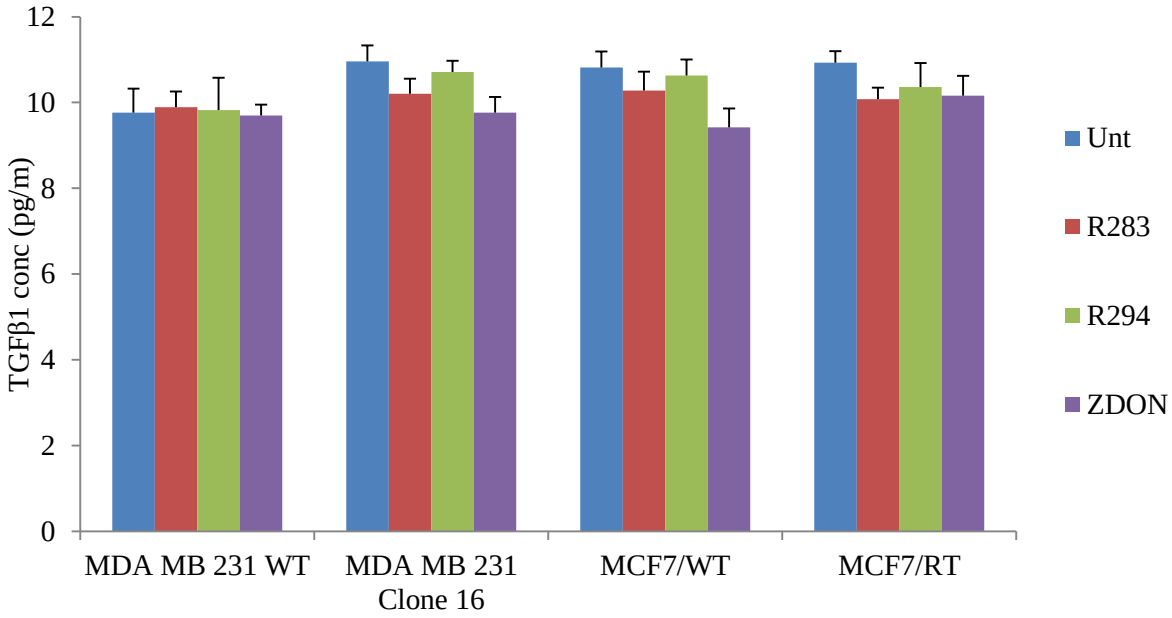


Figure 7.2 Analysis of TGF-β1 levels in breast cancer cell lines in the presence of TG2 activity inhibitors

The TGF-β1 levels in the cell culture medium of the breast cancer cell lines were analysed using the Human/ Mouse TGF β 1 ELISA (2nd Generation) kit from eBioscience. 0.5 x 10^5 cells of each breast cancer cell line was seeded into 24- well plates with serum-replacement medium and treated for 72 h with R283 (500µM), R294 (500µM) and Z-DON (50µM). After the time course of treatment, the supernatant was collected and analysed as described in Section 7.2.1. The concentration of TGF-β1 (pg/ml) calculated in the culture supernatant is represented as the mean + SEM from three independent experiments (n=3).

7.3.2 Analysis of various Epithelial to Mesenchymal Transition (EMT) markers in breast cancer cell lines

Previous studies have reported that chemoresistant and metastatic breast cancer cell lines express high levels of TG2 and this high expression and activity of TG2 contributed to increased invasion, survival and drug resistance (Mehta, *et al.* 2004; Mangala, *et al.* 2007). In order to understand the role of TG2 in metastatic and mesenchymal transformation, breast cancer cell lines with varying expression levels of TG2 were *analysed* using western blotting. The high TG2 expressing MDA MB 231 Clone 16 cells did not express any E-cadherin or N-cadherin which was similar to the TG2 null MDA MB 231 WT breast cancer cells. On the other hand, the TG2 null MCF7/WT breast cancer cells expressed more E- cadherin as compared to their high TG2 drug resistant counterpart MCF7/RT cells. Moreover, the drug resistant MCF7/RT cells also expressed N- cadherin. The fibronectin expression in the TG2 null MDA MB 231 WT and MCF7/WT cells was less than the TG2 expressing MDA MB 231 Clone 16 and MCF7/RT cells (Figure 7.3).

These results seem to imply that TG2 expression does not correlate with the expression of EMT markers in the MDA MB 231 WT and MDA MB 231 Clone 16 breast cancer cell lines. The decrease or loss of epithelial cell markers like E- cadherin and the *de novo* increase in expression of mesenchymal markers such as N- cadherin and fibronectin are the molecular changes that drive the morphological changes during EMT (Thiery, *et al.* 2009).The expression of E- cadherin and N- cadherin seemed to correlate with the TG2 expression only in the MCF7/WT and MCF7/RT breast cancer cell lines. Even though the high TG2 breast cancer cell lines were resistant to doxorubicin, TG2- induced expression of EMT markers did not promote this phenotype. Also, TG2 induced expression of EMT markers seems to be unique to the MCF7 cell line as the MDA MB 231 WT and MDA MB 231 Clone 16 do not show any difference in the expressions of these markers.

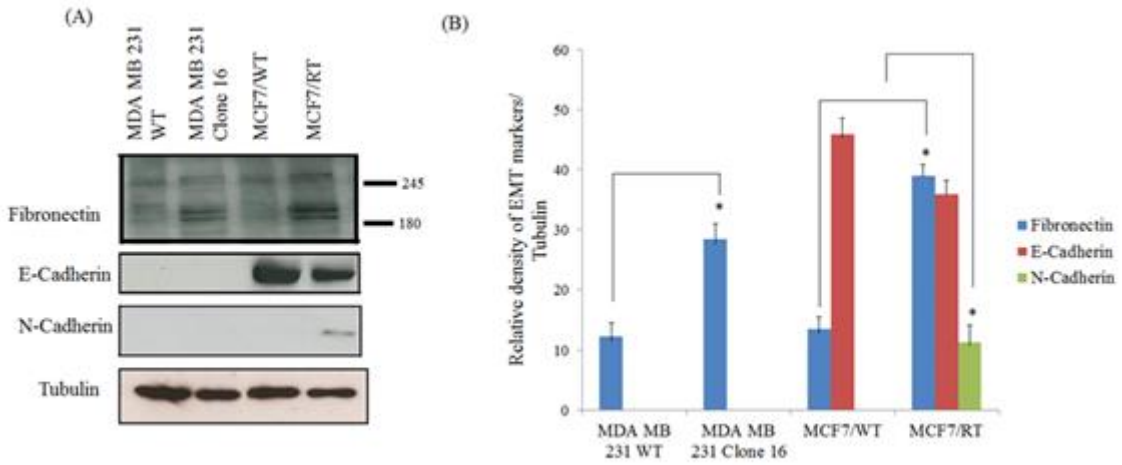


Figure 7.3 Analysis of the expression of epithelial and mesenchymal markers in the whole cell lysate fractions of breast cancer cell lines using western blot

*The breast cancer cells (5x10^5 cells per well) were seeded into 6-well petridishes and left to become confluent. The whole cell lysate fractions of the breast cancer cell lines were analysed by western blot for the expression of E- cadherin, N- cadherin, and fibronectin using rabbit polyclonal anti-E-cadherin (1:1000), mouse monoclonal anti-N-cadherin (1:1000) and rabbit polyclonal anti- fibronectin (1:2000) antibodies respectively. The membranes were then reprobed with anti-α-Tubulin antibody to correct for equal loading of protein. (**A**) The western blot data shown is a representation of three independent experiments (n=3). (**B**) Differences in expression are calculated by Image J analysis and is represented as mean densitometry + SEM. *p <0.05.*

7.4 DISCUSSION

Recent studies of the underlying molecular pathways that regulate the progression of tumours to a drug resistant and metastatic phenotype points to EMT as a key event (Thiery, *et al.* 2009). Accumulating evidence points to prolonged EMT as being important in cancer progression by altering the relatively immobile epithelial cells into fibroblast like cells with decreased intracellular adhesion, increased invasiveness and motility of cancer cells (Micalizzi & Ford, 2009 ; Wang , 2009 ; Kalluri, 2009 ; Kalluri & Weinberg, 2009). Experiments conducted by Kumar *et al.* in 2010 (Kumar, *et al.* 2010) illustrated that expression of TG2 confers drug resistance and promotes invasiveness in transformed as well as normal epithelial cells by the induction of EMT. This could suggest that TG2 serves as the main driver of the metastatic and drug resistant phenotype in cancer cells. A similar observation was also noted between metastasis and EMT induced by TG2 in ovarian cancer cells (Shao, *et al.* 2009). Numerous cancer cells relies deeply on the EMT programme for successful invasion, intravasation, extravasation as well as subsequent colonization by cancer cells (Thiery, *et al.* 2009). EMT causes the loss of epithelial markers such as desmocollin, occludin, desmoplakin and E-cadherin which decreases the cell to cell adhesion and allows the motility of these cancer cells (Thiery, 2002). In order to investigate whether TG2 was responsible for driving any of the EMT induced molecular alterations in the chemoresistant and metastatic breast cancer cell lines, the protein expression of various EMT markers such as FN, E- cadherin and N- cadherin was *analysed* using western blotting. Importantly, the high TG2 MDA MB 231 Clone 16 cell failed to display any difference in the expression of the mesenchymal markers that were a signatory of EMT in spite of the high chemoresistance against doxorubicin as compared to the TG2 null MDA MB 231 WT breast cancer cells. The MCF7/ RT cell line illustrated a loss in expression of E-Cadherin and an increase in N-Cadherin expression as compared to its drug sensitive low TG2 counterpart, MCF7/WT. The other EMT marker, FN was expressed less in both the TG2 null MDA MB 231 WT and MCF7/WT as compared to the high TG2 expressing MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines.

In the same manner, TGF-β1 has been considered to be a strong inducer of EMT in both normal as well as pathological conditions (Xu, *et al.* 2009; Thiery, *et al.* 2009). Addition of TGF-β1 to epithelial cells induced EMT in culture. Classical TGF-β signalling requires the binding of TGF-β to Type II receptors, phosphorylation of Type I receptors and consequently the phosphorylation of Smad 3 and Smad 2. This phosphorylated Smad 2/3 forms a trimeric form with Smad 4 which then translocates into the nucleus wherein interaction between co-activators, co-repressors and transcription factors occurs to promote expression of mesenchymal markers and suppression of epithelial markers. Furthermore, activation of Rho GTPases, ERL MAP kinases and PI3 Kinase/ AKT has been hypothesized to be implicated in EMT induced by TGF-β (Deng, *et al.* 2010; Santibáñez, *et al.* 2010). It has also been demonstrated by previous studies that TGF-β1 induces the expression of TG2 (Quan, *et al.* 2005) and TG2 can in turn activate matrix bound TGF-β1 (Nunes, *et al.* 1997), implicating that there is cross talk between TG2 and TGF-β1. The high TG2 breast cancer cell lines did not show any difference in the TGF β Type I and II receptors as compared to the TG2 null breast cancer cell lines. No increase of TGF-β1 activity was observed in the high TG2 MDA MB 231 Clone 16 and MCF7/RT breast cancer cell line and inhibiting TG2 activity with cell permeable inhibitors did not alter the TGF-β1 activity levels within these cells. This seems to suggest that TG2 was not a downstream mediator of TGF-β1 induced EMT in these breast cancer cell lines. In contrast, studies conducted by Kumar *et al.* in 2010 reported that in the absence of TG2 expression, TGF-β1 failed to induce EMT in breast cancer cell lines (Kumar, *et al.* 2010) (Mann, *et al.* 2012).

Chapter 8: Discussion

8. Discussion

One of the major obstacles that limit the success of current chemotherapy in the treatment of breast cancer is the acquisition of cancer drug resistance. Mechanisms that may contribute to the chemoresistant phenotype could be inherent biological features or can be induced as a response to anti-tumour drugs (Sui, *et al.* 2011). Developing methods to inhibit and overcome such a chemoresistance phenotype in breast cancer cells have become the topic of immense research.

Tissue transglutaminase (TG2) is the most ubiquitous and diverse member of the TG family that can catalyze the post-translational modification of proteins through the incorporation of a primary amine at glutamine residues or cross link proteins by the formation of ε-(γ-glutamyl) lysine isopeptide bonds in the presence of Ca^{2+} (Mehta, *et al.* 2010). Moreover, TG2 can bind as well as hydrolyze ATP and GTP which allows it to function as a G- protein in numerous cell signalling processes (Iismaa, *et al.* 1997 ; Baek, *et al.* 2001). Within the intracellular environment of a cell, the transamidating activity of TG2 is rigidly modulated by the binding of GTP or GDP to the protein, while in the extracellular environment the transamidating activity of TG2 is detected since activating levels of Ca^{2+} are present (Collighan & Griffin, 2009). TG2 can also function as a kinase, protein disulphide isomerase as well as a scaffolding protein (Wang, *et al.* 2012 ; Agnihotri, *et al.* 2013). TG2 has been considered to be a multi functional protein as it can localize into the nucleus, cytoplasm and also be exported outside from the cell (Chen & Mehta, 1999 ; Griffin, *et al.* 2002)

Key roles of TG2 in regulating physiological processes includes protection of cells from external and endogenous stress, tissue repair to sustain homeostasis in response to cell stress and damage as well as forms a component of the cell defence mechanism (Agnihotri, *et al.* 2013). One of the most interesting features of TG2 is that it can modulate opposing functions depending on the predominant activity and cellular localization. GTP/ GDP, Ca^{2+} and redox potential are the three main regulators of TG2 activity. When Ca^{2+} ions bind to TG2, the

enzyme becomes catalytically active and is found in the 'open' confirmation. Since intracellular Ca^{2+} levels are usually low, TG2 is found in the closed conformation and functions as a scaffolding protein where it may be involved in regulating intracellular signalling pathways. Alterations in the intracellular calcium levels as a response to cell damage would induce catalytic activity of TG2 that results in the cross linking of intracellular proteins and subsequently cell death (Kiraly, *et al.* 2011; Nicholas, *et al.* 2003).

In many inflammatory diseases, increased expression and transamidating activity of TG2 was observed to be a common feature (Kim, 2006). It is increasingly (Kiraly, *et al.* 2011) evident that inflammatory responses play a crucial role during the process of tumour progression, invasion and metastasis. Modulation of the survival, growth and progression patterns of the cancer cells is achieved by cross talk with the immune and stromal cells that infiltrate around the tumour cells (Grivennikov, *et al.* 2010). In this manner, numerous findings have demonstrated that the presence of increased levels of TG2 in cancer cells could have a link with increased metastasis, chemoresistance as well as a poor prognosis for the patient. (Verma & Mehta, 2007a ; Mangala, *et al.* 2007 ; Satpathy, *et al.* 2007 ; Park, *et al.* 2010). These reports suggest that a link exists between inflammation, TG2 and the progress of chemoresistant and metastatic cancer.

Published literature has shown that TG2 is over expressed in various cancer cell types such as malignant melanoma (Fok, *et al.* 2006), lung carcinoma (Park, *et al.* 2010), breast carcinoma (Mehta, *et al.* 2004), glioblastoma (Yuan, *et al.* 2007), ovarian carcinoma (Hwang, *et al.* 2008) and pancreatic carcinoma (Verma, *et al.* 2006). Experimental studies carried out on metastatic human lung carcinoma clearly implicated TG2 as one of the 11 selectively amplified genes (Jiang, *et al.* 2003) and also *TGM2* gene was demonstrated to be expressed differentially in pancreatic cancers (Iacobuzio-Donahue, *et al.* 2003).

Current data clearly supports that TG2 plays a very crucial, but under recognized role in shaping of the breast cancer phenotype. Many groups have illustrated that TG2 expression

widely varies within panels of mammary epithelial cell lines as cultured breast cancer cells (Ai, *et al.* 2008; Mehta, *et al.* 2004). Previous studies have reported that breast cancer cells that had been selected out for chemoresistance displayed high levels of the Ca^{2+} -dependent protein cross linking enzyme, TG2, however the mechanistic detail behind the link between TG2 and resistance is still to be established (Devarajan, *et al.* 2002 ; Herman, *et al.* 2006). Ectopically expressing recombinant TG2 in MCF10A mammary epithelial cell line resulted in gene expression alterations, which was concurrent with the cells acquiring an invasive phenotype (Kumar, *et al.* 2010). Further studies also indicated that the expression of TG2 in the MCF10A cells lines also increased the stem cell like properties($CD44^{high}$/ $CD24^{low/-}$ such as augmented self-renewal and growth in the form on mammospheres (Kumar, *et al.* 2010). This observation was in line with the studies done using MDA MB 231 cells which established that high expression of TG2 was associated with increased cell motility *in vitro* (Mehta, *et al.* 2004). All of the above findings clearly paved the way to a possibility that the expression of TG2 could be used as a biomarker for determining prognostic significance of breast cancer and also associated with increased malignancy.

Another very significant finding that revolved around the acquisition of the tumorigenic phenotype was observed in the doxorubicin resistant MCF7 breast cancer cell lines that expressed heightened levels of TG2 in comparison to the doxorubicin sensitive MCF7 parental line (Mehta, 1999). Consistent with this finding, other labs have also demonstrated the effect of TG2 in doxorubicin resistance using diverse cell model systems. The chemoresistant phenotype was clearly due to high levels of TG2 as the knock down of TG2 mediated by siRNA expression, reduced the doxorubicin resistance (Yuan, *et al.* 2005 ; Han & Park, 1999; Herman, *et al.* 2006 ; Yuan, *et al.* 2007 ; Cao, *et al.* 2008).

The exact mechanism that regulates induction of TG2 expression and chemoresistance has yet to be understood. A straightforward relationship between chemoresistance against doxorubicin and expression levels of TG2 has been reported by several groups. Epigenetic profiling and proteomic analysis of breast cancer cells resistant to doxorubicin validated the function of TG2

to increase doxorubicin resistance (Park, *et al.* 2009 ; Chekhun, *et al.* 2007). Activation of EGF signalling is another mechanism of TG2 induction that could contribute to the tumorigenic potential of breast cancer by increasing doxorubicin chemoresistance (Antonyak, *et al.* 2004). Furthermore, under conditions of stress, TG2 expression can also modulate activation of different epigenetic mechanism, survival pathways and transcription factors like NFκB and HIF-1α (Dyer, *et al.* 2011 ; Jang, *et al.* 2010 ; Mehta, *et al.* 2010)

The over expression of TG2 in many cancer cells has often been correlated with the constitutive activation of NFκB (Verma & Mehta, 2007a). In addition, specific inhibitors of TG2 activity can decrease the activation of NFκB. The ability of TG2 to constitutively activate NFκB has been illustrated in doxorubicin resistant breast cancer cells. Fascinatingly, the NFκB activity mediated by TG2 induction was seen in both EGFR negative and positive breast cancer cells (Kim, *et al.* 2006). NFκB is an oncogenic transcription factor that activates numerous genes responsible for cancer cell survival, proliferation, angiogenesis, and metastasis and drug resistance. The main modulation of NFκB occurs via the association with IκB proteins. As a response to external stimuli, the IκBα bound to NFκB is phosphorylated and then degraded by the proteasomal degradation pathway. This results in the release of free NFκB dimers that translocate into the nucleus and transcribe genes that contain the -κB response element (Karin, 2006).

p53 is a prominent tumour suppressor that regulates cell cycle and controls the process of aging in mammals. Several cell lines have wild type p53 which is required to initiate DNA damage induced apoptosis. p53 has been shown to be crucial in regulating steps that are crucial for apoptosis, such as the transcriptional activation of Bax (Yin, *et al.* 1997), generation of ROS (Polyak *et al.* 1997), mitochondrial cytochrome c release as well as caspase activation (Henkels and Turchi, 1999). Even though much is known about p53 dependent apoptotic pathways, p53 independent pathways of DNA damage induced apoptosis in cells that lack expression of p53, or even have mutated or non-functional p53has yet to be deciphered (Kita and Nakashima, 2002; Matsui *et al.* 2001). Studies have shown that in cell lines that lack p53, doxorubicin can

induce apoptosis by increasing peroxide levels, mitochondrial depolarization, activate cytoplasmic caspase 3 as well as elicit cytochrome c release from mitochondria (Tsang, *et al.* 2003).

Studies conducted by Mishra and Murphy in 2006 (Mishra & Murphy, 2006), demonstrated that TG2 can phosphorylated p53 at serine residues 20 and 15. By phosphorylation of p53 induced by TG2, the interaction between Mdm2 and p53 significantly reduces, which subsequently disrupts apoptosis (Mishra & Murphy, 2006).

In agreement to this, the MDA MB 231 and MCF7 cell lines differ greatly in the type of p53 protein expressed. The MDA MB 231 cells have been shown to have a mutant p53 protein as a result of an arginine to lysine mutation at residue 280, whereas the MCF7 cells express wild type p53 (Gurtner, *et al.* 2010). In cancers where mutant p53 is expressed, the aberrant form of p53 is commonly known to be more stable that the wild type p53 and so is present at higher levels (Hupp, *et al.* 2000). Such a mutant form of p53 does not only not retain its tumour suppressive nature but can also sometimes illustrate increased oncogenic characteristics (Hui, *et al.* 2006).

TG2 has been demonstrated to regulate NFκB activity via different mechanisms. TG2 can directly form a complex with p50/p65 dimers in the cytosol, which modifies the affinity of the NFκB molecule to its inhibitory substrate, IκBα (Verma & Mehta, 2007a). IκBα has also been shown to be a good substrate for TG2 transamidating activity and the cross linking of IκBα protein creates an insoluble polymer in the cytoplasm that can no longer bind to the NFκB subunits (Lee, *et al.* 2004). However activation of TG2 in an intracellular environment where TG2 is tightly controlled needs to take place for this mechanism. In the nucleus, TG2 has been shown to associate with Rel A/ p65 in the nucleus and can target non- canonical genes, including TG2 that results in a positive regulatory loop in chemoresistant cells (Verma & Mehta, 2007a).

In spite of these developments, the exact mechanistic detail as to how TG2 induces the constitutive activation of NFκB which leads to the acquisition of chemoresistance against doxorubicin in breast cancer cells is not known. The aim of this project was to establish the mechanistic detail behind the drug resistance against doxorubicin and TG2 levels in breast cancer cells.

In order to establish the role of TG2 in conferring chemoresistance to doxorubicin, breast cancer cell lines that expressed varying levels of TG2 were used. No detectable TG2 antigen was observed in the MDA MB 231 WT, MCF7/WT, SKBR3/WT and T47D/WT cells, which provide an ideal model to study the role of TG2 in chemoresistance against doxorubicin. The MDA MB 231 Clone 16 and MDA MB 231 Clone 9 were selected out from their parental wild type and expressed high and low TG2 respectively (Mehta, *et al.* 2004). On culturing the MCF7/WT, SKBR3/WT and T47D/WT in the presence of increasing concentrations of doxorubicin, the TG2 expression in the MCF7/WT and SKBR3/WT cell lines gradually increased. This was not the case with the T47D cell lines, which seems to suggest that another mechanism of chemoresistance against doxorubicin in these breast cancer cell lines. The TG2 expressing MCF7 cells and low TG2 SKBR3 were established as MCF7/RT and SKBR3/RT (doxorubicin resistant) cells. Furthermore, in the TG2 plasmid transfected MCF7 and SKBR3 cells, TG2 expression and activity could be detected in the cell lysates. Detection of the cellular localization of TG2 activity and expression in the breast cancer cells suggested that TG2 expression and transamidating activity was predominant in the cytoplasm. The cell viability analysis conducted on the breast cancer cell lines in the presence of increasing concentrations of doxorubicin suggested that the high TG2 expressing breast cancer cells were more chemoresistant to doxorubicin as compared to the TG2 null breast cancer cell lines. In the presence of cell permeable TG2 activity inhibitors (R283 and Z-DON), the chemoresistance of the high TG2 breast cancer cells decreased significantly. However no significant decrease was shown with the cell impermeable inhibitor, R294 .With these results, a key relationship between the expression and intracellular activity levels of TG2 and chemoresistance against doxorubicin

in breast cancer could be established. Concurrent with this decrease in TG2 activity and chemoresistance against doxorubicin of the breast cancer cell lines, treatment with the cell permeable R283 and Z-DON also causes a down regulation in the expression levels of TG2. This seems to suggest that the regulation of TG2 expression in the breast cancer cells is under the control of a "factor" whose activation was dependent on the intracellular activity of TG2. Similar results were observed in the TG2 transfected MCF7 and SKBR3, where the presence of TG2 activity and expression conferred these cells resistant to doxorubicin, which could be reversed in the presence of cell permeable TG2 inhibitors (R283 and Z-DON). Down regulating the mRNA TG2 levels in the high TG2 breast cancer cells using TG2 specific siRNA illustrated the same effect on the chemoresistant phenotype of the cells. An intracellular pathway that is dependent on the activity and expression of TG2 in breast cancer cells seems to be a crucial event in mediating chemoresistance against doxorubicin. However, it should be noted that the high and low TG2 expressing breast cancer cells were susceptible to cisplatin, suggesting that TG2 expression and activity may be specific to conferring chemoresistance against doxorubicin in breast cancer.

Among the well-known regulators of TG2, Rel A/ p65 has been well document as studied (Brown, 2013). Even though previous work has documented that TG2 constitutively activates Rel A/ p65 in breast cancer cells, the exact mechanism of activation remains unknown. The high TG2 breast cancer cell lines as well as TG2 transfected breast cancer cell lines that demonstrated to be chemoresistant to doxorubicin, proved to have higher levels of NFκB activity as compared to their TG2 null parental cell lines. In the presence of cell permeable TG2 inhibitors, R283 and Z-DON, the NFκB activity of the high TG2 breast cancer cells decreases significantly. Further investigation of Rel A/ p65 protein expression in the cytoplasmic and nuclear fractions of the breast cancer cell lines revealed that in the nucleus of the high TG2 breast cancer cell lines, Rel A/ p65 was present at a molecular weight of approximately 130kDa that represented the dimeric form of Rel A/ p65. This Rel A/ p65 dimer was also found to be absent in the cytoplasmic fractions of the high TG2 breast cancer cells as well as the TG2 null

breast cancer cell lines. In addition to this, treating the high TG2 breast cancer cells with cell permeable TG2 activity inhibitors, R283 and Z-DON, resulted in a significant decrease of the Rel A/ p65 dimers. This decrease of Rel A/ p65 dimers was concurrent with the decrease in the TG2 expression and activity levels within the cells and also loss of chemoresistance against doxorubicin. These results seem to implicate the transamidating activity of TG2 in the constitutive activation of Rel A/ p65 which in turn may be related to the expression of TG2. Cell impermeable TG2 activity inhibitor, R294, did not demonstrate such a decrease, suggesting that the constitutive activation of Rel A/ p65 and formation of Rel A/p65 dimers was dependent on intracellular TG2 activity as opposed to extracellular activity. This clearly implies that intracellular TG2 activity was responsible for the aberrant Rel A/ p65 dimer formation and also for conferring chemoresistance against doxorubicin in breast cancer.

Co-immunoprecipitation assays were used to pull down the TG2- Rel A/ p65 complex in the nuclear fractions of the high TG2 MDA MB 231 Clone 16 and MCF7/RT. However, no interaction was observed in the nuclear fractions between TG2 and Rel A/ p65. Hence the possibility of any direct interaction between Rel A/ p65 dimers and TG2 in the nucleus of the breast cancer cells was ruled out completely. Since TG2 expression and activity was observed only in the cytoplasm, as determined by microscopy techniques as well as western blot, the formation of the Rel A/ p65 dimer was thought to occur in the cytoplasm and then subsequently translocated into the nucleus, where it could be detected. The presence of these aberrant Rel A/ p65 dimers in the nucleus of the high TG2 breast cancer cells could account for the increased constitutive activation of NFκB.

To further investigate these Rel A/ p65 dimers were in fact only of Rel A/p65 the mRNA expression of Rel A/ p65 was blocked using specific targeting siRNAs. Inhibiting Rel A/ p65 clearly reduced the expression of both the Rel A/ p65 monomers and polymers in the high and low/no TG2 breast cancer cells, indicating that the protein band observed in the nuclear fractions of the high TG2 breast cancer cell lines was likely to be composed of only Rel A/ p65 and no other NFκB family members. The remaining NFκB activity observed in the high TG2

breast cancer cells could be accounted for by the other NFκB family members. To further rule out the involvement of other NFκB family members in the high TG2 breast cancer cell lines, the levels of p50/p105, Rel B, c-Rel and p52/p100 was determined after treatment with TG2 activity inhibitors. No change was observed in any other NFκB family member other than Rel A/ p65, implying that the constitutive activation of NFκB induced by TG2 is primarily the Rel A/ p65 subunit. Knocking down the TG2 mRNA expression in the high TG2 MDA MB 231 Clone 16 and MCF7/RT cells using TG2 specific siRNA resulted in the decrease of the Rel A/ p65 dimers and a concurrent increase in the monomeric Rel A/ p65 of the nuclear fractions. Also, inhibiting TG2 mRNA expression down regulated the NFκB activity levels as well as chemoresistance against doxorubicin. Investigation of the regulation of sustained TG2 expression in chemoresistant breast cancer cells revealed that inhibiting the intracellular transamidating activity of TG2 with the cell permeable inhibitors R283 and Z-DON, decreased the protein expression of TG2.Furthermore, treating the high TG2 MDA MB 231 Clone 16 and MCF7/RT breast cancer cells with Rel A/ p65 siRNAs considerably decreased the activity and expression of TG2, consequently reversing the chemoresistant phenotype. Along with the findings about Rel A/ p65 dimers, it is therefore possible that the Rel A/ p65 dimers may be responsible in regulating transcription of TG2 within these breast cancer cells.

IκBα is the main inhibitory protein responsible for sequestering NFκB subunits in the cytoplasm in a resting cell. In response to external stimuli, IκBα proteins are phosphorylated and subsequently degraded, releasing the free and active NFκB subunits to translocate into the nucleus and bind to the -κB sites on the promoters of target genes (Bergqvist, *et al.* 2008). Numerous reports have suggested that cytoplasmic IκBα can be polymerized by TG2 and subsequently degraded. The cross linking of IκBα induced by TG2 causes the depletion of IκBα without the affecting the phosphorylation status and leads to the activation of NFκB subunits, Rel A/ p65. This can contribute to the increase in inflammation and progression of cancer (Kim, 2006; Mehta & Han., 2011). To determine the interaction between IκBα and TG2 in the cytoplasmic fraction, the breast cancer cell lines were treated with TG2 activity inhibitors and

TG2 specific siRNA. The high TG2 breast cancer cell lines showed the presence of polymeric forms of IκBα in the cytoplasm which was inhibited on treating the cells with only the cell permeable TG2 inhibitors, R283 and Z-DON. On the other hand, in the low/null TG2 breast cancer cells only IκBα was observed in the cytoplasm. These results seem to suggest that the transamidating activity of cytoplasmic TG2 was responsible for the polymerization of IκBα, which resulted in the constitutive activation of NFκB and hence continued chemoresistance in the breast cancer cells. Inhibiting the μ-calpain proteasomal degradation pathway with Calpeptin illustrated the increasing presence of IκBα polymers in the cytosolic fractions of the high TG2 breast cancer cell lines which was absent in the TG2 null cells. Previous studies have shown that TG2 can catalyse the formation of cross links between free IκBα molecules at lysines 33, 36 and 177 and glutamines 266, 267, 313 (Park, *et al.* 2006).

To confirm that the constitutive activation of Rel A/p65 induced by TG2 was via a unconventional pathway that does not involve IKKα/β, the breast cancer cells were treated with the kinase inhibitor PS1145. The NFκB activity of the high TG2 breast cancer cell lines were not significantly reduced, but the low/null TG2 cell lines showed greatly reduced NFκB activity. Furthermore, treating the high TG2 breast cancer cells with PS1145 did not alter the status of the Rel A/p65 dimers in the nucleus, TG2 expression and activity levels also remained unchanged. Inhibiting IKKα/β with PS1145 also did not significantly reduce chemoresistance against doxorubicin in high TG2 breast cancer cells, indicating that the constitutive activation of NFκB mediated by TG2 was not dependent on IKKα/β function. Similar results were also reported in studies conducted by Kumar & Mehta in 2012 where mammary epithelial breast cells, MCF10A cells that were transfected with TG2 and treated with parthenolide (IκB kinase inhibitor), showed no difference in the target EMT genes that are regulated by NFκB (Kumar & Mehta, 2012). These observations clearly indicated that the TG2 induced constitutive activation of NFκB is independent on the conventional IKK pathway.

Studies have demonstrated that the phosphorylation of the NFκB protein was essential for regulating NFκB activation. This phosphorylation can occur in the nucleus or in the cytoplasm

and has also been shown to be cell type specific as well as stimuli specific. Also, the specific site of phosphorylation differs and also leads to a specific effect of NFκB on target gene activation (Ryu, *et al.* 2011 ; Viatour, *et al.* 2005). To determine whether, the Rel A/ p65 dimers undergo any post translational modification, the high TG2 MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines were *analysed* by co-immunoprecipitation assays. With this, the possibility that the Rel A/ p65 dimers were phosphorylated at a serine residue was confirmed. Also, the phosphoserine Rel A/ p65 was only seen in the nuclear fractions of the high TG2 breast cancer cell lines. This clearly suggested that the Rel A/ p65 dimers underwent phosphorylation at a serine residue in order to increase transcriptional activation of the NFκB subunits. The phosphorylation /dephosphorylation of Rel A/ p65 at serine residues can be carried out by a variety of different kinases and phosphatases respectively (Perkins, 2012), which raises the possibility that specific kinases have functions of special significance in activating defined signalling pathways (Buss, *et al.* 2012). Since, the co-immunoprecipitations clearly revealed that the phosphorylation of the Rel A/ p65 dimers occurs only in the nucleus, kinases that are found in the nuclear compartment were further *analysed*. Such a phosphorylation of Rel A/ p65 in the nucleus has been demonstrated previously by several groups (Madrid, *et al.* 2001 ; Jiang, *et al.* 2003 ; Yang, *et al.* 2003 ; Sasaki, *et al.* 2005) . Co-immonoprecipitation studies have also shown that phospho-Rel A/ p65 does not associate with IκBα or p50, both in the nucleus and cytoplasm. Additionally, phospho- Rel A/ p65 has been implicated to exist independent of the canonical NFκB complex of p65 and p50 (Sasaki, *et al.* 2005).

Among the well characterized phosphoserine sites on nuclear Rel A/ p65 is serine 536 which is mainly mediated by IKKε which is a well known non canonical IKK (Adli & Baldwin, 2006 ; Mattioli, *et al.* 2004). Experiments using IKKε specific inhibitor BX795 showed that inhibiting IKKε activity decreased the phosphoserine status of the nuclear Rel A/ p65 dimers. To further confirm if the nuclear Rel A/ p65 dimer was phosphorylated at the Serine 536 site, a specific pSer 536 p65 antibody was used. As expected the nuclear Rel A/ p65 dimers were specifically

phosphorylated at the serine 536 site, which could be inhibited by the IKKε inhibitor, BX795. In addition to the reduction on the phosphoserine status of the Rel A/ p65 dimers, the TG2 expression and activity in the high TG2 breast cancer cell lines also decreased significantly. Moreover, the chemoresistant nature of the high TG2 breast cancer cell lines in the presence of doxorubicin was also reversed. The phosphorylation of Rel A/ p65 in the nucleus by IKKε and its relevance in the progression of cancer has been reported previously. Overexpression of IKKε and Rel A/ p65 in cancer cell lines has been accounted for leading to the phosphorylation of Rel A/ p65 specifically at Serine 536 (Buss, *et al.* 2004 ; Adli & Baldwin, 2006). The regulation of the phosphorylation of Ser 536 in Rel A/ p65 subunits occurs on the C- terminal of the TAD and originally demonstrated by Sakurai *et al.* in 1999 (Sakurai, *et al.* 1999). Ever since, numerous other groups have confirmed that IKKs are directly responsible for phosphorylating p65 at Ser 536 (Sizemore, *et al.* 2002 ; Sakurai, *et al.* 2003 ; Kishore, *et al.* 2003). Also, even in cell lines thay were deficient for IKKα or IKKβ or transfected with dominant negative forns of IKKα and IKKβ, pSer 536 p65 was still observed (Yang, *et al.* 2003; Sizemore, *et al.* 2002). Phosphorylation of serine 536 on Rel A/ p65 by IKKε accounts for the post translational modification specifically in cases where Rel A/ p65 was not bound to IκB or dependent on IKK for activation (Buss, *et al.* 2004). As a response to DNA damaging agents that can activate NFκB in the absence of IKKα/β function, IKKε plays a crucial role in the phosphorylation Rel A/ p65 (Tergaonkar, *et al.* 2003). Although, it had become apparent that IKKε was responsible for phosphorylation of Ser 536 in Rel A/ p65, the contribution of such a post translational modification on Rel A/ p65 transactivation through this site was yet to be stabished by analyzing NFκB target genes.

Initial results had illustrated that inhibiting the transamindating activity of intracellular TG2 using R283 and Z-DON as well as Rel A/ p65 specific siRNA to inhibit Rel A/ p65 mRNA expression, greatly reduced the TG2 protein levels in the high TG2 breast cancer cell lines. This clearly suggested that Rel A/ p65 was essential to regulate the expression of TG2 in chemoresistant breast cancer cell lines. The Rel A/ p65 subunit has also been shown previously

to be sufficient as well as necessary to drive the expression of TG2 within cultured breast cancer cell lines (Ai, *et al.* 2012). The effects of the transactivation of Rel A/ p65 on TG2 expression was found to be quite direct as the *TGM2* gene contains a -κB binding site consensus (Ai, *et al.* 2008) and Rel A/ p65 was shown to associate with these binding sites as evaluated by ChIP assay. Using the NF-κB p65 (Ser529/536) inhibitory peptide, the serine 536 phosphorylation of the Rel A/ p65 was inhibited which consequently inhibited the binding of the Rel A/ p65 dimers to the -κB binding sites on their target genes. The expression and activity along with the chemoresistance of the high TG2 breast cancer cell lines was inhibited and this clearly suggested that the phosphorylation of serine 536 residues on Rel A/ p65 dimers was essential for these NFκB subunits to bind to the -κB site on the *TGM2* gene promoter. Furthermore, ChIP analysis confirmed that both the IKKε inhibitor, BX795 and NF-κB p65 (Ser529/536) inhibitory peptide inhibited the binding of Rel A/ p65 dimers to the -κB site on the *TGM2* gene promoter. However, the cell permeable TG2 activity inhibitors, R283 and Z-DON, that down regulated the formation of the Rel A/ p65 dimers and TG2 expression, failed to block the binding of the Rel A p65 to the -κB site on the *TGM2* gene promoter. The *TGM2* gene expression was determined subsequently in the presence of the cell permeable TG2 activity inhibitors, R283 and Z-DON, and a clear significant decrease was observed in the *TGM2* gene expression in the high TG2 breast cancer cell lines, This could account for the consequent decrease in the TG2 protein and activity levels as well as chemoresistance against doxorubicin observed in the high TG2 breast cancer cells. The results of these experiments were further confirmed by reference to previous work conducted that reported the presence of the single -κB binding site within the *TGM2* gene promoter as deciphered by EMSA (Electrophoretic mobility shift assay) (Caccamo, *et al.* 2005 ; Mirza, *et al.* 1997).

Taken together, all this current evidence clearly supports the existence of a novel regulatory feedback loop wherein TG2 transamidating activity was responsible for constitutively activating Rel A/ p65 by cross linking and these Rel A/ p65 dimers were phosphorylated at the serine 536 residue which in turn drives the expression of TG2. Additionally, high expression and activity

of TG2 and constitutive activation of NFκB has been demonstrated to take place both in primary cultured breast cancer cells as well as breast tumours (Ai, *et al.* 2012). This regulatory loop seems to be self-sustaining because the expression of TG2 and activity of NFκB is substantially lower in normal epithelial cell lines as compared to cultured cancer cell lines. Such a self-amplifying regulatory-loop results in an obvious chemoresistant phenotype in high TG2 expressing breast cancer cells (Brown, 2013). NFκB directs the expression of various pro survival and anti-apoptotic genes as a response to cytotoxic damage induced by chemotherapeutic agents (Karin, 2006).

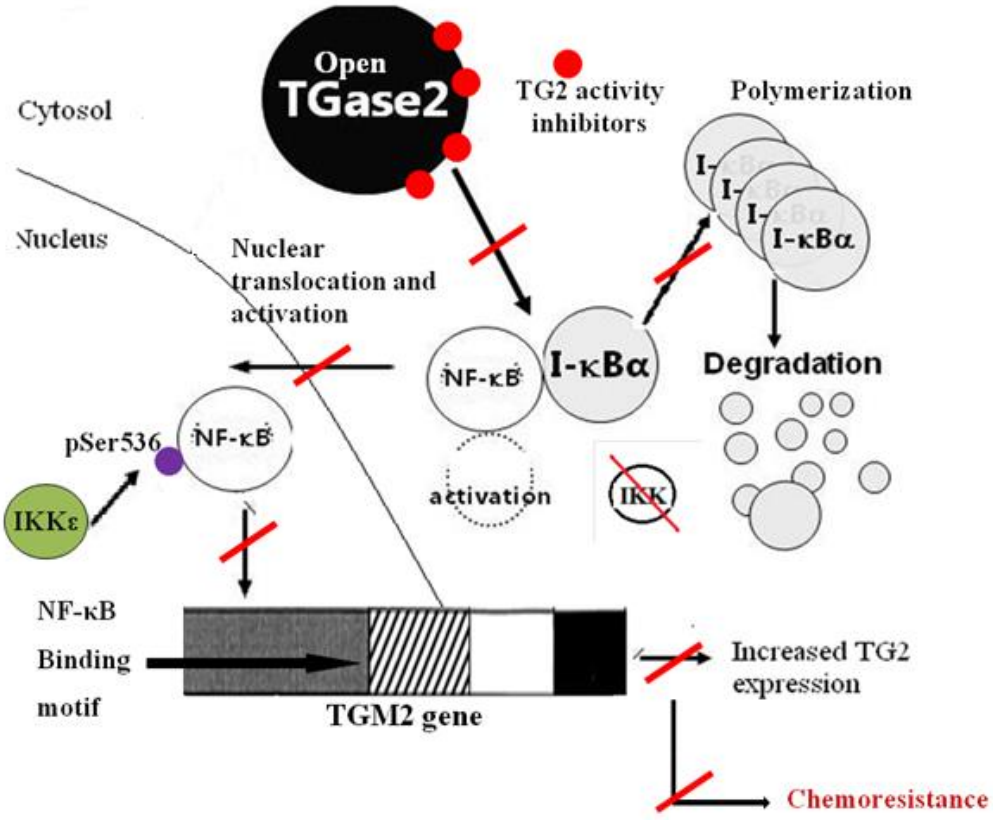


Figure 8.1 Schematic representation of TG2-NFκB regulatory loop

Another key event reported to be regulated by this TG2/ NFκB feedback loop is the activation of EMT (Epithelial to Mesenchymal transition), by down regulation of genes responsible for maintaining the epithelial integrity, E- Cadherin and increase in the molecules required for mesenchymal and invasive phenotypes such as TGF-β1, N- Cadherin, Vimentin and FN (Kumar, *et al.* 2010). However, in spite of the presence of the self-sustained TG2/ NFκB regulatory loop in the chemoresistant breast cancer cell lines, there was no significant difference observed in the EMT markers (E-cadherin, N-cadherin and TGFβ1 activity) between the high and null TG2 MDA MB 231 breast cancer cell lines (Clone 16 and WT respectively. The MCF7/WT cells did express high levels of E-cadherin which seems to have decreased in the MCF7/RT breast cancer cell lines. Similarly, in the MCF/RT cell lines, expression of N-cadherin was observed. Such a "cadherin-switch" could account for the EMT characteristics in the MCF7 cell lines. Also, studies have demonstrated that expression of TG2 in MCF10A cells

resulted in the reduction of E- cadherin expression and up regulation of various EMT markers (Kumar, *et al.* 2010).

The continuous activation of the TG2/ NFκB loop might even have other effects on the behaviour of tumour cells other than the ones detailed above. Via this loop TG2 expression may affect the structure of the ECM, and the increased expression of extracellular TG2 may change the motility and invasiveness of the cancer cells. Furthermore, the activity of extracellular TG2 has been shown to cross link integrins which results in the continuous activation of FAK at the membrane of the cell which supports the activation of AKT kinase (Verma, *et al.* 2006 ; Kang, *et al.* 2004). Recently, a study conducted by Wang and Griffin demonstrated that inhibiting the cell surface TG2 activity of MDA MB 231 Clone 16 breast cancer cells resulted in a decrease in S100A4 polymer formation as well as subsequent cell migration. Furthermore, this study also revealed that activation of PKCα can link the α5β1 integrin and syndecan 4 co signalling pathways in facilitating the cell migration mediated by S100A4 and TG2 (Wang & Griffin, 2013).

From the viewpoint of therapeutics, switching off the TG2/ NFκB regulatory loop has an apparent benefit for treating chemoresistant and invasive breast cancer. A lot of effort has been put into developing IKK kinase activity inhibitors that would block the constitutive activation of NFκB subunits (Gilmore & Herscovitch, 2006). However, since current research has indicated that the transamidating activity of TG2 is responsible for constitutively activating Rel A/ p65 via a mechanism that is independent of IKK function, such inhibitory molecules would not have any effect on the TG2/ NFκB regulatory loop. The development and use of novel cell permeable TG2 activity inhibitors in combination with chemotherapeutic drugs holds considerable promise in disrupting this signalling loop and improving the therapeutic measures for treatment of chemoresistant breast cancer.

Chapter 9: Future Direction

9. Future Direction

Following the investigations that have been described in this thesis, a number of future projects could be taken up, which involve using breast cancer tissue samples as well as knock out mouse models:

- Other breast cancer cell lines which are from both of glandular as well as ductal origin should be tested for TG2 activity, Rel A/p65 high molecular weight forms as well as chemoresistance against doxorubicin to investigate if the chemoresistant phenotype was specific to certain breast cancer cell line origins.
- The MDA MB 231 Clone 16 and MCF7/RT breast cancer cells that have been determined to be chemoresistant to doxorubicin, but chemosusceptible to cisplatin. These high TG2 expressing breast cancer cells should be repeatedly cultured in increasing concentrations of doxorubicin so as to develop cisplatin resistant breast cancer cell lines. Subsequent protein analysis of TG2 levels and Rel A/p65 high molecular weight forms could help determine if the chemoresistant phenotype of breast cancer cells induced by TG2 was specific to doxorubicin or similar anthracylin drugs.
- Transient transfection of Wild Type TG2 plasmid into TG2 null breast cancer cell lines to establish stable breast cancer cell lines that express high amount of TG2. Protein expression and activity assays and well as cell viability tests could then be used to determine whether similar result are observed as with the high TG2 MDA MB 231 Clone 16 and MCF7/RT breast cancer cell lines.
- It would of further interest to transfect the TG2 null breast cancer cell lines with mutant forms of TG2 pertaining to the active catalytic site as well as the GTP binding site to confirm that TG2 activity was chiefly responsible to confer chemoresistant phenotype against doxorubicin as well as the formation of high molecular weight Rel A/p65 as well as IκBα forms.

- Further upstream inhibitors of the NFκB activation pathways could be used to further confirm the exact activation pathway, either canonical or non-canonical, of Rel A/p65 high molecular weight forms.
- Mass Spectrometry analysis of the high molecular weight Rel A/p65 form could help establish the exact nature of these supposedly dimeric forms.
- Obtaining metastatic breast cancer tissue samples and immunohistochemistry staining with different antibodies specific for TG2 and Rel A/p65.
- Using these metastatic breast cancer tissues, staining of inflammatory cells such as macrophages and neutrophils will allow determining infiltration of cells into these tumour sites.
- DNA micro array studies would further help determine gene expression patterns of chemoresistant genes or drug efflux genes that may be responsible in conferring a chemoresistant phenotype against doxorubicin in highTG2 breast cancer cells

In summary, such future studies would further help understand the exact mechanistic phenomenon behind chemoresistant breast cancer cell lines and allow development of therapeutics which would help increase prognosis.

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Appendix 1: List of Abbreviations

AD	Alzheimer's disease
ATP	Adenosine 5'- triphosphate
Aβ	Amyloid beta- protein
BAFF	B cell activating factor
BCR	B cell receptor
BSA	Bovine Serum Albumin
Ca^{2+}	Free Calcium ions
$CaCl_2$	Calcium Chloride
CCl_4	Carbon tetrachloride
CD	Celiac disease
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin Immunoprecipitation
CO_2	Carbon dioxide
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DEPC	Diethylpyrocarbonate
DEPC	Diethyl pyrocarbonate
DLK	Dual leucine zipper bearing kinase
DMEM	Dulbecco's modified Eagles' medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
Dox	Doxorubicin
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EGF	Epithelial growth factor
EGTA	Ethylene glycol tetra acetic acid
EMT	Epithelial to mesenchymal transition
ER	Oestrogen receptor
ERK	Extracellular signal regulated kinase
FAK	Focal adhesion kinase
FBS	Foetal Bovine serum
FITC	Fluorescein isothiocyanate
FN	Fibronectin
FXIII	Factor XIII
FXIII A	Factor XIII subunit A
FXIII B	Factor XIII subunit B
GDP	Guanosine 5'diphosphate
GFD	Gluten free diet
gplTG	Guinea pig liver transglutaminase
GTP	Guanosine 5'- triphosphate
GTPase	Guanosine 5'- triphosphatase
h	hour/ hours
HD	Huntington's disease
HDAC	Histone deacetlyase
HRP	Horseradish peroxidase
HS	Heparan sulphate
IgG	Immunoglobulin
IKK	Inhibitor of -κB kinase
IL-1	Interleukin -1
IL-1β	Interleukin -1 beta

IL-6	Interleukin - 6
IκB	Inhibitor of -κB
KCl	Potassium chloride
kDa	Kilodalton
KOH	Potassium hydroxide
LiCl	Lithium Chloride
LPS	Lipopolysaccharides
M	Molar
MAPK	Mitogen activated protein kinase
MEK	MAPK kinase 1
min	minute/ minutes
ml	Milliliters
mM	Millimolar
mRNA	Messenger ribonucleic acid
MT-MMPs	Memebrane type- metalloproteinase
NEMO	NFκB essential modulator
NFκB	Nuclear factor kappa - light chain enhancer of activated B cells
NIK	NFκB inducing kinase
nM	Nanomolar
nm	Nanometer
NO	Nitric oxide
^{o}C	Degree Celcius
OPD	*o*-phenylenediamine
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
pH	Negative log of hydrogen ion concentration
PI3K	Phosphoinositide 3- kinases
PKCα	Protein kinase C alpha
PKCδ	Protein kinase C delta
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PNRC	Perinuclear recycling compartment
PNPP	para-Nitrophenylphosphate
PS1145	n-(6-Chloro-9H-pyrido[3,4-b]indol-8-yl)-3-pyridinecarboxamide dihydrochloride
pSer536	Phosphorylates serine 536 residue
RA	Retinoic acid
Rb	Retinoblastoma gene
RBC	Red blood cells
RHD	Rel homology domain
RNase	Ribonuclease
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
SDS- PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SEAP	Secreted alkaline phosphatase
siRNA	Small interfering ribonucleic acid
TAA	Triazole acetic acid
TAD	Transactivation domain

TEMED	N,N,N',N'-tetramethylene diamine
TG	Transglutaminase
TG2	Tissue transglutaminase
TGFβ1	Transforming growth factor beta 1
TLR	Toll like receptor
TNFR	Tumour necrosis factor receptor
TNFα	Tumour necrosis factor - alpha
Tris	Tris (Hydroxymethyl)-amino ethane
Triton X -100	t-Ocylphenoxypolyethoxyethanol
Unt	Untreated
v/v	volume/volume
w/v	weight/ volume
WCL	Whole cell lysate
x g	G- force
XTT	Sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate
Z-DON	Z-DON-Val-Pro-Leu-OMe
µ	Micro
µl	Microlitre
µM	Micromolar