NOTTINGHAM TRENT UNIVERSITY

# Modulation of transglutaminase 2 activity in H9c2 cells by protein kinase A and protein kinase C signalling

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**By**

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*First and foremost, praises and thanks to Allah almighty, for His showers of blessings and lightning of my way through my research work to complete this project successfully*

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**Declaration**

This submission is the result of my work. All help and advice, other than that received from tutors, has been acknowledged and primary and secondary sources of information have been properly attributed. Should this statement prove to be untrue, I recognise the right and duty of the Board of Examiners to recommend what action should be taken in line with the University's regulations on assessment contained in the Handbook.

Signed ..... Date 26/ 06/ 2014

**Dedication**

*To my beloved mother and the soul of beloved father*
*To my husband and children*
*To all my brothers, sisters and dearest friends for their love, support and encouragement, without whom after Allah this thesis could never been completed*

## Abstract

Transglutaminase 2 (TG2; EC 2.3.2.13) has been shown to protect cardiomyocytes against ischaemia and reperfusion-induced cell death and to mediate cell survival in many cell types. Given the prominent role of PKA and PKC in cardioprotection, this study investigated whether TG2 was involved in the cytoprotection induced by activation of these two kinases in cardiomyocyte-like H9c2 cells.

Cultured H9c2 cells were extracted following stimulation with activators of PKC (phorbol-12-myristate-13-acetate; PMA) and PKA (forskolin; FK). Transglutaminase 2 activity was determined using an amine incorporating (*in vitro* and *in situ*) and a protein crosslinking assays. Different protein kinase inhibitors were used to determine the involvement of PKC and PKA in the activation of TG2 in H9c2 cells. To confirm the involvement of TG2 activity via PKC and PKA, TG2 specific (Z-DON and R283) inhibitors were used. Western blot analysis revealed the presence of TG2 and TG1 (TG2 >> TG1) protein, but not TG3. Since the $H_2O_2$, a major contributor to reactive oxygen species following damage was used to induce oxidative stress. The role of TG2 in PMA- and forskolin-induced cytoprotection was investigated by monitoring $H_2O_2$-induced oxidative stress in H9c2 cells. The identification of TG2 substrates in H9c2 cells was investigated using pull down assay coupled with proteomic analysis techniques.

The PMA and FK-induced time and concentration-dependent increases in TG2 catalysed biotin cadaverine incorporation in H9c2 cells. Forskolin but not PMA also increased TG2 catalysed protein crosslinking. The PKC (Ro-31 8220) and PKA (KT 5720 and Rp-8-Cl-cAMP$_S$) inhibitors, blocked PMA and FK-induced TG2 activity. Immunocytochemistry using ExtrAvidin®-FITC revealed *in situ* TG2-mediated biotin cadaverine incorporation into protein substrates following stimulation of PMA, FK and their receptor agonists. The TG2 inhibitors Z-DON and R283 attenuated the PMA- and FK-induced increases in TG2 activity. Pre-treatment with PMA and FK reversed $H_2O_2$-induced cell death as judged by a MTT reduction assay and the release of cellular LDH. The TG2 inhibitors R283 and Z-DON blocked PMA and FK-induced cytoprotection. Proteomic analysis identified more than 25 proteins that serve as intracellular substrates for TG2 following PMA and FK stimulation. Some of these identified proteins have already been reported as TG2 substrates, but not in H9c2 cells e.g. tubulin while others e.g. α-actinin have not been identified before.

In summary, these data have shown TG2 activity to be stimulated via PKA and PKC-dependent signalling pathways in H9c2 cells and suggest a role for TG2 in cytoprotection-induced via these two protein kinases.

## Publication

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## Abstract

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Modification of transglutaminase 2 activity in H9c2 cardiomyocytes after treatment with activators of protein kinase A and C. Proceedings of the 5$^{th}$ Saudi International Conference, the University of Warwick, Coventry, the United Kingdom, June 2011, Poster presentation, abstract, P73, Volume: ISBN :978-0-9569045-0-8.

Modulation of transglutaminase 2 activity in H9c2 cardiomyocytes by activators of protein kinase A and protein kinase C. Proceedings of the 6$^{th}$ Saudi Scientific International Conference, Brunel University, London, the United Kingdom, October 2012, Poster presentation, abstract no:340, P230, Volume: ISBN-10: 0956904505.

Proteomic analysis of transglutaminase 2 substrates modulated by PKC and PKA activation in H9c2 cardiomyocytes, Proceedings of the 7$^{th}$ Saudi Scientific International Conference, Edinburgh University, the United Kingdom, January 2014, Poster presentation, abstract no:333, P123, Volume: ISBN-14: 9780956904522.

Cytoprotective role of transglutaminase 2 activity mediated by protein kinase C and A in H9c2 cardiomyocytes against oxidative stress. Proceedings of the GRC on Transglutaminases in Human Disease Processes, Tuscany Il Ciocco Resort in Lucca (Barga), Italy, June 2014, Poster presentation.

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**Abbreviations**

AC, adenylate cyclase
Akt, serine/threonine-specific protein kinase
APS, ammonium persulphate
BAX, bcl-2-associted x protein
Bcl-2, B-cell lymphoma 2
BTC, biotin cadaverine
BSA, bovine serum albumin
cAMP, cyclic adenosine monophosphate
DMSO, dimetilsulfoxide
dNTPs, deoxynucleotides
DTT, dithiothreitol
EDTA, ethylenediaminetetraacetic acid
ECM, extracellular matrix
ER, endoplasmic reticulum
ERK, extracellular-signal-regulated kinase
FK, forskolin
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GPCRs, G protein-coupled receptors
HRP, horseradish peroxidase
HSP, heat shock protein
JAKs, janus kinases
JNK, c-Jun amino-terminal kinase
LDH, lactate dehydrogenase
mAb, monoclonal antibody
MTT, tetrazolium salt, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltertra-zolium bromide
MW, molecular weights
MAPKs, mitogen-activated protein kinases
NBT, nitro-blue tetrazolium
O/N, over night
PBS, phosphate buffered saline
PCR, polymerase chine reaction
PI3K, phosphatidylinositol-3-kinase
PKA, protein kinase A
PKC, protein kinase C
PLC, phospholipase C
PMA, phorbol-12-myristate-13-acetate
R283, 1,3-dimethyl-2[(oxopropyl)thio]imidazolium
ROS, reactive oxygen species
RT, room temperature
SDS-PAGE, sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SR, sarcoplasmic reticulum
STS, staurosporine
TMB, tetrametilbenzidine
TG2, Transglutaminase 2
Z-DON, Benzyloxycarbonyl-(6-Diazo-5-oxonorleucinyl)-L-Valinyl-L-Prolinyl-L-Leucinmethylester

# CHAPTER I:

# GENERAL INTRODUCTION

# 1. Introduction

## *1.1. History of transglutaminase (Transglutaminases in cell and organisms)*

The transglutaminase (TG) enzymes were first discovered 55 years ago in mammalian guinea pig liver homogenates. They were shown to have the ability to catalyse the calcium-dependent formation of covalent bonds between small molecule amines and definite proteins, along with the release of free ammonia (Clarke et al., 1959; Mycek et al., 1959). However, when the activity of transglutaminases was explored in blood plasma, it was found that factor XIIIa had the ability to cross link and stabilise fibrin monomers as part of the blood coagulation mechanism (Pisano et al., 1969), establishing the idea that these enzymes are able to modify proteins and act as biological glues (Griffin et al., 2002). Further studies revealed different TG isoenzymes and nine different genes encoding for TGs were identified in mammalian cells, some of which have been studied at the protein level (Grenard et al., 2001). Different transglutaminases can be categorised by their characteristic tissue distribution and they can also be found together in a number of diverse tissue types (Grenard et al., 2001). Since the complete sequence of TG2 was characterised in guinea pig liver (Ikura et al., 1988), and from TG2 cDNA clone of human endothelial cells and mouse macrophages (Gentile et al., 1991), transglutaminases have been shown to be highly conserved among different species (Makarova et al., 1999) and widely distributed in various cell types. Examples include, endothelial and smooth muscle cells in arteries, veins, and mesangial cells (like smooth muscle cells that are usually found around the kidney-blood vessels), renomedullary interstitial tumour cells or differentiation of cells to enterocytes in small intestine (Thomázy & Fésüs, 1989). In addition, catalytic activities of transglutaminase have been detected in a wide range of organisms, including invertebrates (Zanetti et al., 2004), unicellular primitive eukaryotes (Wada et al., 2002), plants (Serafini-Fracassini & Del Duca, 2008), amphibians (Zhang & Masui, 1997), birds (Puszkin & Raghuraman, 1985) and fish (Lin et al., 2008). The enzymatic functions of TGs generally involve either tissue assembly or repair (Kim et al., 2002), making them of particular interest for researchers.

### *1.2. Reactions catalysed by transglutaminases*

Transglutaminases (TG; Protein glutamine γ-glutamyl-transferases, EC 2.3.2.13) are a family of $Ca^{2+}$ dependent enzymes that catalyse the posttranslational modification of proteins. Once $Ca^{2+}$ ions bind to TG catalytic core domain, a cysteine residue is exposed at the active site of the enzyme leading to the formation of a bond between ε-amide (as an isodipeptide or polyamine bond) and the γ-carboxamide of protein bound glutamine residues (Lorand & Conrad, 1984). There are at least five different catalytic reactions known for these enzymes. These can be classified into three types; transamidation, deamidation (or hydrolysis) and esterification (Lorand & Graham, 2003). In the TG transamidation reaction, acyl transfer between the γ-carboxamide group of a protein bound glutamine residue and the ε-amino group of a protein containing lysine residue results in the crosslinking of proteins (Fig. 1.1/1a). Moreover, incorporation of monoamines or polyamines by attachment to the γ-carboxamide of a glutaminyl residue and acylation of lysyl residue are two more transamidation reactions (Fig. 1.1/1b,c). However, in esterification reactions, an alcohol substrate binds to protein glutamine resulting in an esterified product (Fig. 1.1/2), while hydrolysis occurs in the presence of $H_2O$ and results either in deamidation through the replacement of the $NH_2$ group with an -OH group or cleavage of an isopeptide bond (Fig. 1.1/3a,b; Iismaa et al., 2009).

Additional activities have been revealed for TG2, which will be discussed later in section (1.3.7), including its ability to act as a G protein (Nakaoka et al., 1994; Prasanna Murthy et al., 1999), protein disulphide isomerase (Ferrari & Söling, 1999) and protein kinase (Mishra & Murphy, 2004).

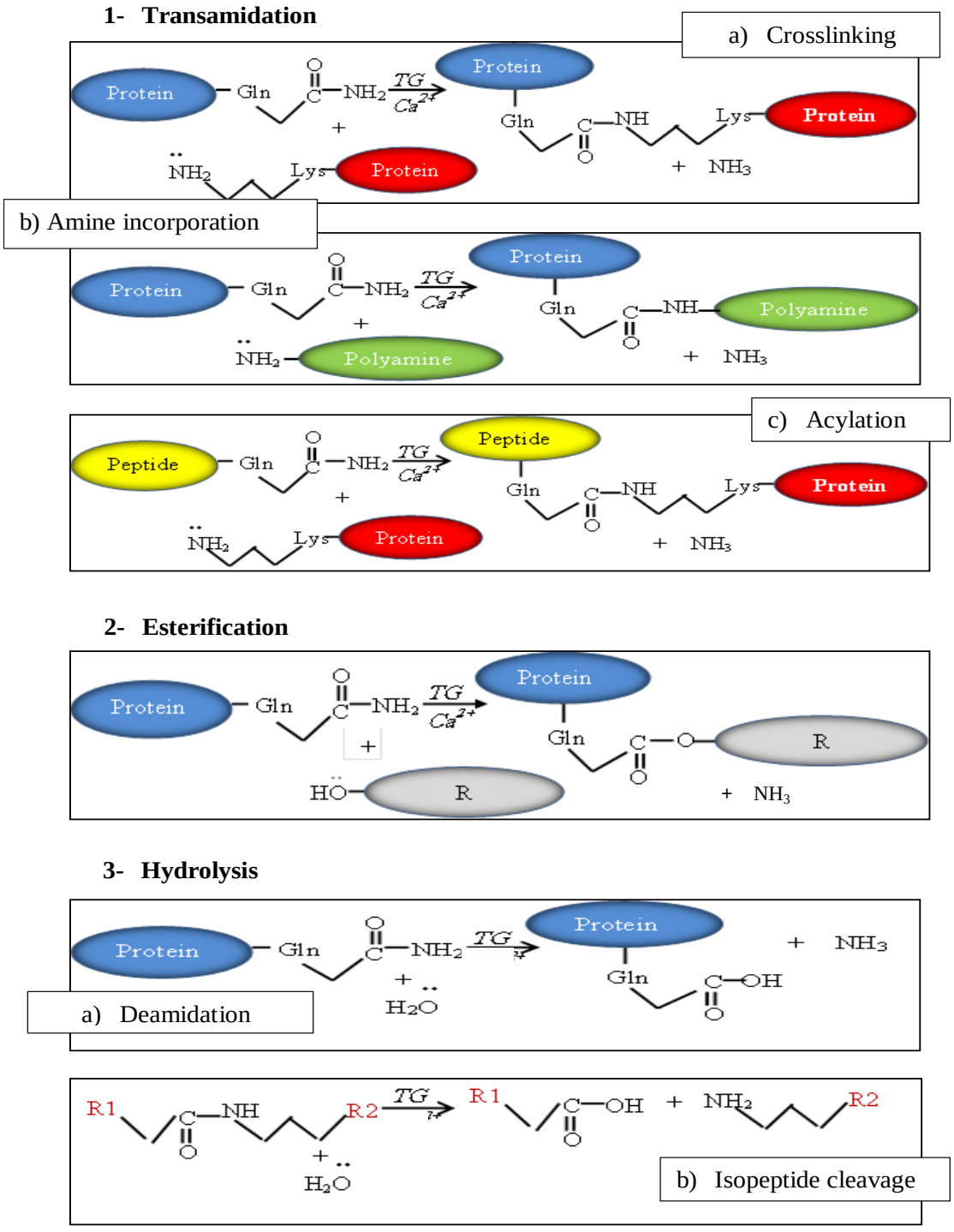



**Figure 1.1 Posttranslational reactions catalysed by transglutaminases**

The different TG-catalytic reactions. Shown are acceptor glutamine (Gln) residue of one protein (blue oval) and the lysine (Lys) donor residue another (red oval), a Gln-containing peptide (green oval) and an alcohol substrate (grey oval). R1 and R2, represents the side chains in branched isopeptides. Figure modified from Lorand & Graham (2003).

### *1.3. Transglutaminase family members*

At least nine different genes encoding for TG isoenzymes have been identified in mammalian cells (Grenard et al., 2001), though only seven have been studied at the protein level (Table 1.1). Each TG can be characterised via its own distinctive tissue distribution (Grenard et al., 2001). However, they also appear in a number of diverse tissue types frequently in combination with other TG family members. Transglutaminases have particular functions in the cross linking of specific proteins or tissue structures. For example, blood plasma transglutaminase (Factor XIIIa) is essential for the formation and stabilisation of fibrin clots during haemostasis, and TGs 1, 3, and 5, are mostly expressed in the skin epidermis, contributing towards the correct formation of the cornified cell envelope (Eckert et al., 2005). Band 4.2 protein, which has no catalytic activity due to the absence of a catalytic core domain (Iismaa et al., 2009), is a component of the cytoskeleton (Aeschlimann et al., 1998). Transglutaminase 2 (TG2) is expressed ubiquitously and is implicated in a wide range of cellular processes, such as programmed cell death (Akar et al., 2007b), cell differentiation (Singh et al., 2003) and tumour growth (Verma et al., 2008). It has been suggested that TG2 may act as an apoptotic inhibitor of retinoblastoma protein regulation during the cell cycle (Antonyak et al., 2001; Boehm et al., 2002; Tucholski, 2010).

**Table 1.1 Transglutaminase enzymes family**

| TGs | Molecular mass in kDa | Biological function and/or location | Gene location | Reference |
|---|---|---|---|---|
| **Factor XIIIa Plasma Platelet** | 360<br>166 | Blood clotting, angiogenesis and wound healing | 6p24-25<br>1q28 | ( Olaisen et al., 1985; Dardik et al., 2006; Dardik et al., 2007) |
| **TG 1 (Keratinocyte TG, kTG)** | 106 | Keratinocyte differentiation and correct formation of the cornified cell envelope | 14q11.2 | (Yamanishi et al., 1992; Jans et al., 2007) |
| **TG 2 (Tissue TG, tTG, cTG)** | 78 | Apoptosis and cell differentiation, matrix stabilization, adhesion protein and signal transduction | 20q11-12 | (Gentile, et al., 1994; Siegel & Khosla, 2007; Chhabra et al., 2009) |
| **TG 3 (Epidermal TG, eTG)** | 77 | Epidermal, nail and hair follicle differentiation | 20q11-12 | (Wang et al., 1994; Zhang, et al., 2005; Cheng et al., 2008) |
| **TG 4 (Prostate TG, pTG)** | 80 | Suppression of sperm immunogenicity & rodent fertility | 3q21-22 | (Dubbink et al., 1998; Ablin et al., 2011) |
| **TG 5 (TG X)** | 81 | Expressed in epithelial tissues and involved in differentiation. | 15q15.2 | (Aeschlimann et al., 1998; Thibaut et al., 2005) |
| **TG 6 (TG Y)** | 80 | Expressed in cortical and cerebellar neurons | 20q11 15 | (Grenard et al., 2001; Thomas et al., 2013) |
| **TG 7 (TG Z)** | 80 | Not characterized | 15q15.2 | (Grenard et al., 2001; Thomas et al., 2013) |
| **Protein 4.2** | 74 | Involved in formation of membrane & cytoskeleton components of red cells and blood vessels. | 15q15.2 | (Sung et al., 1992; Mouro-Chanteloup et al., 2003) |

Table 1.1 Classification of TGs isoenzymes is summarised, according to their molecular mass, known gene location, cell or tissue localisation and biological functions (Lorand & Graham, 2003).

Apart from Protein 4.2, gene and protein structure of all TG family members consists four conserved domains; a) an amino-terminal β-sandwich, b) a core domain which contains a catalytic triad of cysteine (Cys), histidine, (His) and aspartate (Asp) residues and a transition stabilising site tryptophan (Trp), and c) two COOH-terminal β-barrel domains (barrel 1 and barrel 2). However, TG1 and Factor XIIIa family members have an additional N-terminal pro-peptide sequence that can be cleaved to activate the enzyme (Fig. 1.2; Lorand & Graham, 2003).

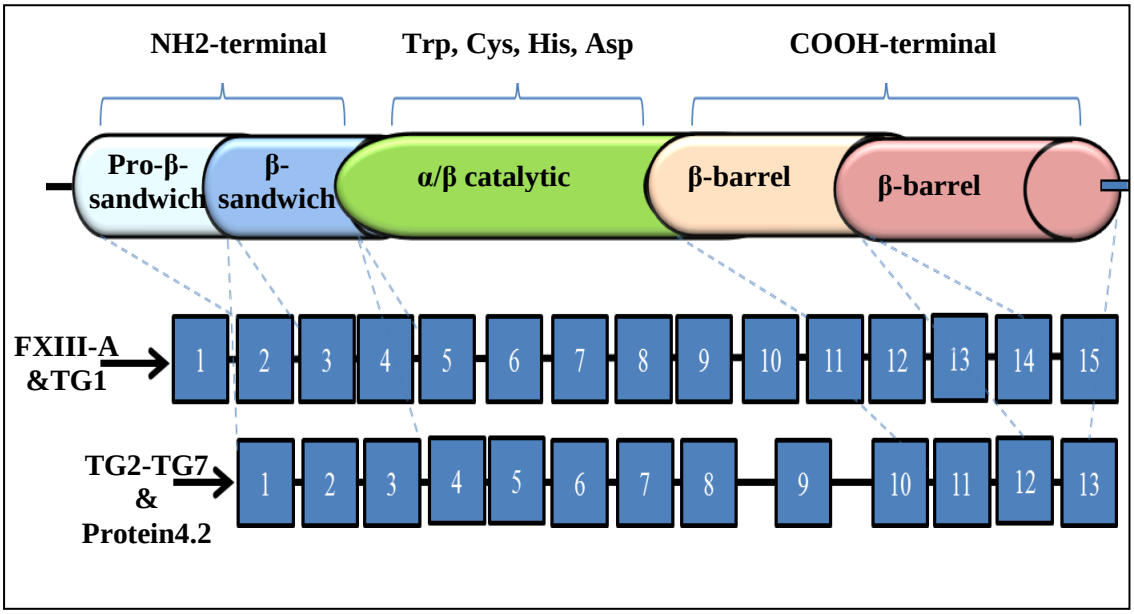



**Figure 1.2 Transglutaminase protein domains and genomic organisation**

Diagram shows the four structural domains of the protein, namely the $NH_2$-terminal β-sandwich, two COOH terminal and α/β catalytic core domain that contain essential cysteine (Cys), histidine (His), aspartate (Asp) and tryptophan (Trp) residues. Additional pro-peptide sequences ($NH_2$-terminal) for TG1 and Factor XIIIa are also indicated. Dotted lines represent the relative location of exons encoding structural domains. Fifteen exons (numeral) and fourteen introns of genes encoding FXIII-a and TG1 (starting from exon 2). Genes with 13 exons and 12 introns encode isoforms TG2 to TG7 and protein 4.2 (Lorand & Graham, 2003).

### 1.3.1. Keratinocyte Transglutaminase (TGK)

Keratinocyte transglutaminase (TGK), also known as transglutaminase epidermal type I, is encoded in the human gene TGM1 (Phillips et al., 1992). This is a membrane-

bound transglutaminase that is able to link to the membrane by an esterified fatty acid (Chakravarty & Rice, 1989). This membrane anchorage region is hypersensitive to proteolysis by trypsin, plasmin or $Ca^{2+}$ and temperature dependent proteolysis, resulting in release of the enzyme in a soluble form (Rice et al., 1990). Moreover, these features are important in the formation of cross-linked envelopes at the cell periphery upon calcium activation during terminal differentiation of human epidermal cells (Thacher & Rice, 1985), and in stabilising internal structures (Rice et al., 1990). In addition to activation by calcium, 12-O-tetradecanoylphorbol-13-acetate (TPA) can activate TG1 and elevate its mRNA expression while retinoic acid down-regulates its expression in cultured human keratinocytes (Liew & Yamanishi, 1992). Interaction between TG1 and tazarotene-induced gene 3 protein leads to its activation to regulate keratinocyte terminal differentiation of human foreskin keratinocytes (Jans et al., 2007). Tazarotene-induced gene 3 acts in epithelial cancer cells as a class II tumour suppressor to impede cell proliferation (Deucher et al., 2000) and it is play an important role in survival of human keratinocytes via controlling TG1 activity (Sturniolo et al., 2003; Sturniolo et al., 2004). The epidermis is the first physical barrier for the protection of organisms from pathogen invasion and dehydration (Candi et al., 2005). In order to exert its protective barrier function, a complex balance between the proliferation and differentiation components is required during the formation of the cornified envelope process (Terrinoni et al., 2012). Thus, any such mutation or abnormality in these compartments can cause skin pathogenesis. It has been reported that specific deletions or mutations in the TG1 gene can result in a rare keratinisation disorder called Lamellar ichthyosis, which is characterised by abnormal cornified envelope formation (Terrinoni et al., 2012).

### 1.3.2. Epidermal Transglutaminase (TG3)

Transglutaminase type 3 (TG3) is another member of the transglutaminase TG family, that is commonly expressed during the late stage of terminal differentiation and more most likely to be found in epidermis (Martinet et al., 1988; Zhang et al., 2005), hair follicles and nails (Martinet et al., 1988; Cheng et al., 2008), keratinocytes, and brains (Hitomi et al., 2001). However, it also has been suggested that TG3 has an essential role during early embryogenesis at the developmental stage of mouse limb bud skin formation (Zhang et al., 2005).

Similar to TG1, TG3 is involved in regulation of the cornified cell envelope through interacting with and mediating the crosslinking of various protein structures (small proline-rich proteins, involucrin and loricrin) that are important in assembly of cornified cell envelope during the terminal differentiation stage of skin epidermal cells (Kalinin, et al., 2001; Kalinin et al., 2002). In addition, TG3 enzyme is thought to participate in shape determination or hardening of the inner root sheath through crosslinking of intermediate filaments and trichohyalin protein to the inner root sheath cell of hair follicles and the granular layers of the epidermis that is essential for hair cortical cells morphogenesis (Lee et al., 1993). The molecular mass of TG3 has been reported to be 77 kDa in human as well as in mouse tissues (Kim et al., 1993). The protein can be cleaved *in vitro* by cathepsin L (Cheng et al., 2006) and by proteinase K, trypsin, and thrombin (Kim et al., 1990), into a 50 kDa N-terminal fragment, which is the catalytically active form, and a 27 kDa C-terminal fragment, which is the non-catalytic form (Hitomi et al., 2003).

Unlike TG1, no mutation in TG3 has been linked to any human disease, although the failure in implantation of the TG3 knockout mouse blastocyst shows that it is essential in the earliest stages of embryo development (Ahvazi et al., 2004). Transglutaminase 3 has been suggested to be involved in aggregation and crosslinking of mutant huntingtin protein into intranuclear inclusions in patients with Huntingdon's disease (Zainelli et al., 2005). It is believed that TG3 is an auto-antigenic target in coeliac patients with dermatitis herpetiformis, a blistering skin disease (Sárdy et al., 2002). It is worth to note that both these transglutaminase isoenzymes have never been investigated in cardiomyocytes, however, TG1 but not TG3 has been shown to be expressed in the vena cava and aortic smooth muscle cells (Johnson et al., 2012).

### 1.3.3. Transglutaminase 4 (TG4)

An alternative name for TG4 is prostate-specific transglutaminase, since it is predominantly secreted in the prostate gland. However, it is also found at low levels in other tissue types (Gentile et al., 1995; Dubbink et al., 1998; An et al., 1999). Transglutaminase 4 has been shown to display GTPase and protein crosslinking activities in rat coagulatory gland secretions (Spina et al., 1999). These activities have been linked to its N-terminal end domain, which was demonstrated by the analysis of

different TG4 mutants (Mariniello et al., 2003). The rat dorsal prostate TG or dorsal protein 1 is a homologue of TG4 that has molecular mass of 62 kDa (Wilson & French, 1980).

Despite the lack of studies at molecular level, TG4 has been suggested to be up regulated by androgens in both the rat dorsal prostate and coagulating gland (Steinhoff et al., 1994; Dubbink et al., 1999). The transamidation activity of TG4 has been shown to be important in copulatory plug formation in human and rat sperm cells (Williams-Ashman, 1984) and in the immunogenicity and motility of tumour cells (Ablin & Jiang, 2011). In addition, its expression level was reported to be strongly associated with the invasiveness of human prostate cancer cells (Davies et al., 2007) in which TG4 transfected prostate cancer cells shed increased invasiveness. In prostate cancer, overexpression of TG4 has a potential role in activation and adherence of endothelial cells and these effects were reduced when TG4 expression was knocked down (Jiang et al., 2009).

### 1.3.4. Transglutaminases 5-7 (TGs5-7)

Transglutaminase 5 (TGX) plays a role in cornified cell envelope (CE) formation in human epidermis and keratinocyte differentiation through *in vitro* crosslinking of the specific epidermal substrates loricrin, involucrin and small proline-rich proteins (Candi et al., 2001). During hair follicle homeostasis, TG5 was significantly expressed as well as TG3, which suggests that they possibly play a balancing function in hair follicle homeostasis, hair shaft differentiation and construction, and could also participate in the crosslinking of these structures (Thibaut et al., 2005). In normal human skin tissue, TG5 was detected in the upper layers by immunofluorescence, being concentrated in the spinous and granular layers, while low levels of TG5 were detected in the basal layer (Candi et al., 2002). Both haematoxylin-eosin and immunofluorescence staining techniques have revealed that over expression of TG5 was indirectly implicated with many incidences of pathologic human epidermis including, psoriasis, ichthyosis vulgaris and Darier's disease (Candi et al., 2002). The secretion of TG5 is not restricted to keratinocytes and epidermis, it has also been detected in other types of human cells e.g. in erythroleukemia (a pre-leukemic state),

osteosarcoma (a type of bone tumour) and dermal fibroblasts (Aeschlimann et al., 1998).

Transglutaminase 5 as well as TG6 and TG7 have molecular weights of approximately 80-81 kDa (Grenard et al., 2001). Recently, physiological functions of TG6 have been identified; Thomas and colleagues revealed that TG6 was extensively expressed in neuronal cells of mouse brain and in a human carcinoma cell line (Thomas et al., 2013). In addition, the biochemical analysis in the same study indicated the possible presence of $Ca^{2+}$ and GDP biding sites similar to those present in TG2 and TG3 (Thomas et al., 2013). The involvement of TG6 in coeliac disease autoantibody mediated gluten ataxia has been demonstrated (Stamnaes et al., 2010). By exome sequencing, Wang and his colleagues identified the presence of a mutation in the TG6 gene in patients with familial ataxia (a genetic neurodegenerative disorder characterised by incoordination of gait, hands, speech and limb that affects diverse regions within the brain cerebellum; Matilla-Dueñas et al., 2010; Wang et al., 2010). In mouse cerebral cortex cells, NGF (nerve growth factor) and dibutyryl cAMP are both able to up-regulate TG6 expression, suggesting involvement in neural cell differentiation (Thomas et al., 2013). Transglutaminase 7 is mainly expressed in lungs and testis (Grenard et al., 2001). However, this TG isoenzyme is still not fully investigated.

### 1.3.5. Blood plasma transglutaminase (Factor XIII)

Factor XIII is a combination of two dimers, one contains two catalytic subunits (FXIIIa) and two non-catalytic subunits (FXIIIb), playing an essential role in blood circulation and coagulation (Schwartz et al., 1973; Lorand, 2001). The enzyme is a well characterised member among the TG family. In addition, genomic sequences have revealed the localisation of factor XIIIa in human chromosome 6 p24-25, and the protein encoded by this gene has a molecular weight of 83 kDa (Ichinose, et al., 1986; Ichinose et al., 1990). Factor XIIIb subunit has a molecular weight of 80 kDa and the gene encoded to this protein is located in human chromosome 1q31-32.1 (Bottenus et al., 1990). Factor XIII has been shown to be synthesised in the liver and placenta (Iismaa et al., 2009). At the cellular level, factor XIIIa is mainly expressed in

monocytes, hepatocytes, macrophages, platelets, and endothelial cells (Iismaa et al., 2009).

The activation of factor XIII by calcium ionophore in human monocytes led to the production of covalently cross-linked angiotensin II type 1 ($AT_1$) receptor (which binds a vasopressor hormone controlling blood pressure of the cardiovascular system) creating homodimer in patients with atherosclerosis (thickening in arterial wall due to fatty material accumulation) (AbdAlla et al., 2004). Apolipoprotein E is a glycoprotein that is believed to play a role in cholesterol homeostasis and inflammatory responses associated with atherosclerotic vessels (Curtiss & Boisvert, 2000). In Apolipoprotein E-deficient mice, the inhibition of factor XIII activity or release of angiotensin II prevent this crosslinking formation and thus adhesion of monocyte to endothelial cells, and symptoms of atherosclerosis suggested the involvement at the onset of atherosclerosis (AbdAlla et al., 2004). Factor XIII plays a role in angiogenesis (regeneration of blood vessel) of endothelial cells through its pro-angiogenic activity (Dardik et al., 2006) and tissue repair through triggering cell migration and proliferation, and repressing apoptosis of monocytes and fibroblasts (Dardik et al., 2007). The blood plasma TG (factor XIII A) knockout animal model exhibited significantly reduced reproduction, and uterine bleeding was observed in female mouse models (Koseki-Kuno et al., 2003). *In situ* hybridisation for factor XIII revealed restricted expression in skeletal elements of zebrafish and the inhibition of factor XIII activity by KCC-009 (TG2 irreversible acivicin derived inhibitor) reduces average vertebrae mineralisation, suggesting a vital role of factor XIII in bone mineralisation (Deasey et al., 2012).

### 1.3.6. Protein 4.2

Also known as human erythrocyte band 4.2, the gene encoding this protein is located on human chromosome 15 and the protein has a molecular weight of 72-74 kDa (Sung et al., 1992; Zhu et al., 1998). Protein 4.2 has been shown to be subject to fatty acid modification (myristylation) at an N-terminal glycine and exists at several cytoskeletal locations within red blood cells associated with cell membranes (Risinger et al., 1992). This suggests that it might play a role in growth monitoring and signal transduction. Protein 4.2 is one of the red cell skeleton proteins and contributes to

stabilising the linkages between the cytoskeleton and the erythrocyte membrane (Golan et al., 1996). Thus it has been implicated in haemolytic anaemia through its interaction with CD47 as one of Rhesus complex proteins in red blood cells membrane (Mouro-Chanteloup et al., 2003). Unlike the other TG family members, band 4.2 protein preferentially binds ATP instead of GTP (Azim et al., 1996).

### 1.3.7. Transglutaminase 2 EC 2.3.2.13

Transglutaminase 2 is probably the most interesting member of the TG for many reasons (Facchiano et al., 2006). It displays a number of enzymatic functions and many different molecules (proteins, amines, nucleotides, drugs) can acts as substrates for TG2. Transglutaminase 2 may act at different cellular sites for recognising these substrates, further extending its range of actions. It is not only present in the intracellular environment, as it was initially defined as "cytosolic" TG, it is also found in the nuclear and extracellular environments. This member of the TG family has been shown to catalyse some of the chemical reactions that are associated with human diseases, opening new pathogenic perceptions and probably new therapeutic approaches. Transglutaminase 2 has also been shown to be of use in biotechnological applications such as in pharmaceutical and food industry (Facchiano et al., 2006). The ability of the enzyme to crosslink a verity of keratinocyte proteins makes them one of the major components present in cosmetic and pharmaceutical products e.g. sunscreens, hair condition agents, perfume and anti-inflammatory and anti-oxidant drugs. In the food industry, many meat proteins are TG substrates including collagen, fibrin fibronectin, and the ability of the enzyme to crosslink these substrates enhances sausage binding and texturing (Mariniello & Porta, 2005).

Transglutaminase 2 or tissue transglutaminase (tTG) (also known as transglutaminase C) is a protein-glutamine gamma-glutamyltransferase and is universally expressed in almost all tissues and organ-specific cell types (Iismaa et al., 2009; see section 1.3). It is found in endothelial cells, fibroblasts cells and smooth muscles (Thomazy & Fesus, 1989) either outside or inside the cells. In the human genome, TG2 is encoded by the TGM2 gene and is located in chromosome 20q12 (Gentile et al., 1994) with a size of 32.5kb consisting 13 exons and 12 introns (Fraij & Gonzales, 1997; Gentile et al.,

1991) as shown in figure 1.2. The full length of the TG2 protein is 687 amino-acids with a molecular weight of approximately 78 kDa.

The x-ray α-crystal structure of TG2 has been reported previously and it has a complex and unique structure that makes it a multifunctional enzyme (Liu et al., 2002). Transglutaminase 2 has two forms; inside the cell it is known as an intracellular TG2 and outside the cell it is known as extracellular TG2 (Fig. 1.3). Inside the cell, TG2 adopt a compact conformation, in which it binds to guanosine-5´-triphosphate (GTP) and crystallises in a condition where the active site is covered (Liu et al., 2002). Thus, inside the cell, the normally high concentrations of GTP inhibit TG2´s catalytic activity, allowing the enzyme to function as a G-protein in membrane signal-transduction pathways by the activation of phospholipase C-δ (Nakaoka et al., 1994; Prasanna Murthy et al., 1999). Outside the cell, TG2 adopts an extended conformation in the presence of $Ca^{2+}$ and the active site cysteine thiol group is displayed and interacts with the glutamine protein bond carboxamide, resulting in thioester intermediate formation and thus it becomes active (Griffin et al., 2002; Pinkas et al., 2007).

In its active form, TG2 performs extracellular functions by binding to integrins on the cell surface and fibronectin in the extracellular matrix. Thus, it regulates cell adhesion, movement, signalling, proliferation, and differentiation (Siegel & Khosla, 2007). However, there is evidence showing that the extracellular TG2 remains in an inactive form even in the presence of $Ca^{2+}$ and that this is due to the redox environment that enhances the formation of disulphide bonds between cysteine residues (Pinkas et al., 2007; Jin et al., 2011; DiRaimondo et al., 2012).

Transglutaminase 2 plays an important role in stabilisation of the extracellular matrix via its transamidation activity, which is involved in bone remodelling, wound healing and angiogenesis (Griffin et al., 2002). By contrast, its intracellular role is thought to be mainly crosslinking activity, regulating apoptosis under harsh conditions (Iismaa et al., 2009). However, the intracellular roles of TG2-mediated polyamine incorporation activity have not been fully investigated. Therefore, the aim of this study was to focus on the intracellular role of TG2-mediated polyamine incorporation in cardiomyocytes in response to protein kinase activation.

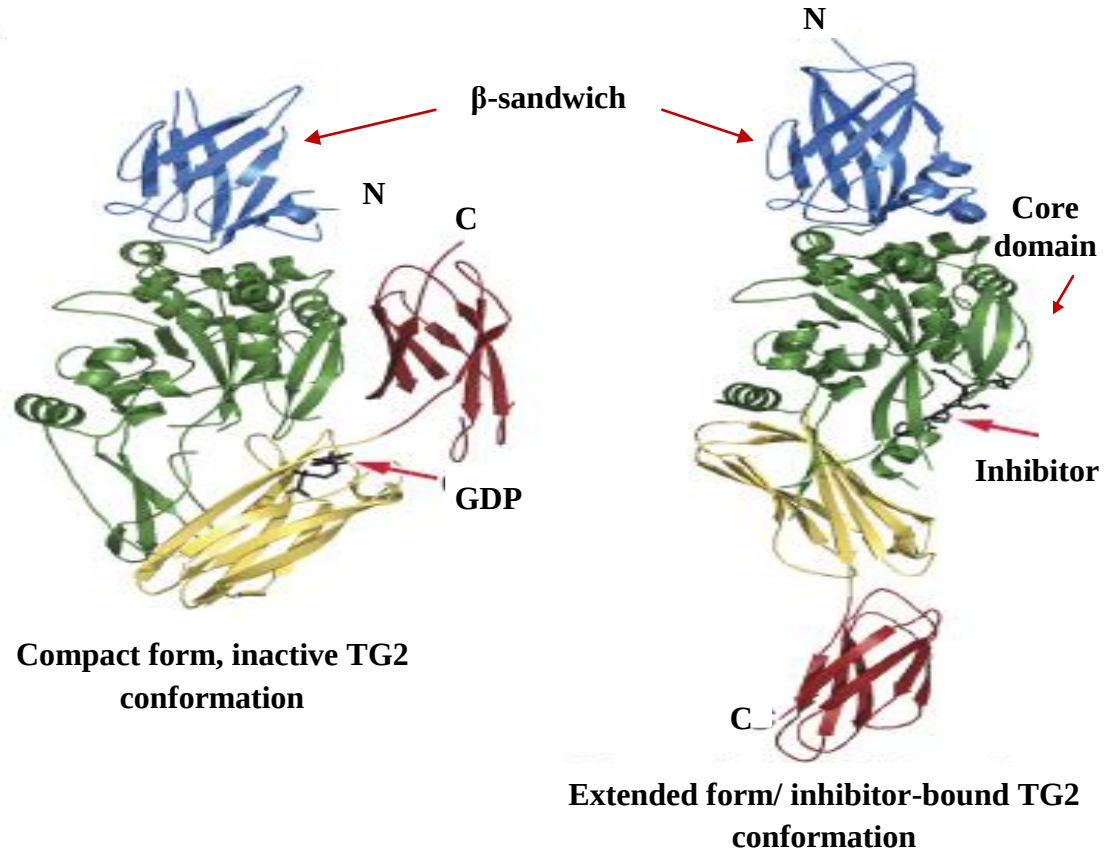



**Figure 1.3 The crystal structures of TG2**

The N-terminal β-sandwich is shown in blue (N), and the C-terminal β-barrels in red (C). (A) Compact form, GDP-bound TG2. (B) Extended form, TG2 inhibited with the active-site inhibitor; modified from Pinkas et al., (2007).

Transglutaminase 2 has been shown to have a number of additional activities, one of which is that of a G protein (Gh). TG2 can hydrolyse GTP to GDP and act as G protein in association with the plasma membrane associated $\alpha_1$ adrenergic receptor with transfer of signalling to the activation of phospholipase C (Nakaoka et al., 1994; Vezza et al., 1999). However, the exact biochemical mechanism is not clear but is thought to involve PLC-δ (Nanda et al., 2001). One more activity identified for TG2 is protein kinase activity, through which TG2 can phosphorylate proteins such as retinoblastoma protein in fibroblasts (Mishra et al., 2007), histone proteins in breast cancer cells (Mishra et al., 2006) and p53 (Mishra & Murphy, 2006). The first paper published on TG2 kinase activity was reported by Mishra et al., (2004) in breast cancer cells, resulting in phosphorylation of insulin-like growth factor-binding

protein-3 (IGFBP-3) enhancing the affinity of insulin-like growth factors (IGF)-I protein to bind to IGFBP-3, thus attenuating its pro-apoptotic effects (Mishra & Murphy, 2004). Kinase activity has also been implicated in mouse embryonic fibroblasts, in which protein kinase A and cAMP enhance TG2 kinase activity and phosphorylation, while increasing $Ca^{2+}$ level is able to inhibit this activity (Mishra et al., 2007). This kinase activity has also been reported for factor XIII (Mishra & Murphy, 2006).

Protein disulphide isomerase (PDI) is a member of the thioredoxin super-family present in the lumen of the endoplasmic reticulum (ER). It is involved in the correct introduction of disulphide bridges within polypeptides and correct construction and conformation for diverse proteins (Ferrari & Söling, 1999). Protein disulphide isomerase has been identified as another activity for TG2 (Hasegawa et al., 2003). This activity is $Ca^{2+}$ and nucleotide independent, being modulated by oxidant and antioxidant concentrations (Ferrari & Söling, 1999). The discovery of this activity-resulted in the generation of the hypotheses that TG2 may be able to act as PDI in cytosol, where almost all TG2 is present, $Ca^{2+}$ concentration is low and nucleotide concentration is high (Hasegawa et al., 2003). It has been reported that TG2 regulates the ADP/ATP transporter role in mitochondria through its PDI activity (Malorni et al., 2009).

#### 1.3.7.1. Calcium-dependent activity of TG2

In eukaryotes, $Ca^{2+}$ is required for transglutaminase to crosslink proteins through a catalytically active conformation. Specific glutamic and aspartic acid residues are essential for $Ca^{2+}$ binding (Josse et al., 2001). Based on TG2 sequence homology to blood factor XIIIa transglutaminase (see figure 1.2), $Ca^{2+}$ binding sites on TG2 are primarily suggested to be between amino acids 427 and 455 (Islamovic et al., 2007). The majority of studies agree that TG2 has six $Ca^{2+}$ binding sites, which are all located in the catalytic domain and five of them are able to influence its transamidation activity (Király et al., 2009).

Crystallisation studies of factor XIIIa showed that glutamic acid (E) E490, E485, aspartic acid (D) D438, and alanine (A) A457 are $Ca^{2+}$-binding sites. They are involved in the formation of this negatively charged site and are conserved in other

TG structures (Fox et al., 1999). However, in TG2, site directed mutagenesis of these $Ca^{2+}$ binding sites resulted in merely decreased sensitivity to $Ca^{2+}$ activation when compared to wild type TG2 (Ikura et al., 1995). Similar residues revealed in TG3 include E448, E443, and A393 and the resolution of its crystal structure has shown that, when $Ca^{2+}$ binds, a channel opens and exposes to tryptophan residues to manage access of the substrate to the active site (Ahvazi et al., 2002).

The concentration of $Ca^{2+}$ required for TG2 activation varies *in vitro* and *in vivo*. For example, 3-4 μM $Ca^{2+}$ is required for activation of purified TG2, while more than 100 μM is required for activation of recombinant TG2 (Lai et al., 1997). A huge $Ca^{2+}$ concentration gradient is maintained in cells across the plasma membrane, approximately 100 nM inside and 2 mM outside the cell (O'Malley et al., 1999). In normal cellular conditions, where the $Ca^{2+}$ concentration is low, TG2 is maintained in its folded form, while under stress or signalling conditions that elevate intracellular free $Ca^{2+}$ concentrations results in the opened conformation of TG2, revealing its active site. These facts lead to the suggestion that TG2 is catalytically inactive in a normal cellular environment and it is activated under extreme or fatal conditions, such as in necrosis and apoptosis (Nicholas et al., 2003; Pinkas et al., 2007).

#### 1.3.7.2. Transglutaminase 2 substrate properties

The recognition of proteins that act as TG2 substrates and the target amino acids on such proteins are of critical importance for studying TG2's biological roles in different cell types and tissues. A significant number of proteins have been identified as TG2 substrates, which are listed in Table (1.2); these include extracellular and intracellular structural proteins, hormones, enzymes and small heat shock proteins.

Approximately, 46 TG2 interacting proteins have been identified according to the Human Protein Reference online database http://genomics.dote.hu/wiki/. The TRANSDAB database reports 155 TG2 substrates, which are mostly located in the cytoplasm, in addition to five TG2 substrates involved in its kinase activity and two in its deamidase activity. Transglutaminases modify both lysine and glutamine residues. However, they are much more selective toward glutamine residues than toward amine donor lysine residues (Esposito & Caputo, 2005; Lorand & Graham, 2003). It has

been proposed that glutamine residues should be exposed at the target surface of protein's surface to act as a TG2 substrate.

**Table 1.2 Transglutaminase 2 substrates**

| Protein substrates | Reactive site | Associated disease |
|---|---|---|
| ***Cytoskeleton proteins*** | | |
| Actin<br>Tau<br>β-tubulin<br>Vimentin | Glutamine and lysine<br>Glutamine and lysine<br>Glutamine and lysine<br>Glutamine and lysine | Alzheimer's disease |
| ***Heat shock proteins*** | | |
| alpha B-crystallin<br>Hsp60<br>Hsp70<br>Hsp90 | Lysine | Neurological diseases |
| ***Enzyme*** | | |
| Aldolase<br>GAPDH<br>Small GTPase RohA | <br><br>Lysine | Metabolic and endocrinology diseases, genetic disease<br>Huntington's disease |
| ***Crystallins*** | | |
| βB3-crystallin<br>βBp (βB2)-crystalline | Glutamine<br>Glutamine | Chronic liver disease. |
| ***Others*** | | |
| Amines (monoamines, diamines, polyamines): cadaverine, histamine, putrescine, serotonin, spermidine, spermine | Act as amine donor | |
| Cytochrome C<br>Collagen alpha 1(III) | Glutamine<br>Glutamine | Chronic liver disease. |
| Gluten proteins | Glutamine | Coeliac disease |
| Fibrinogen A-α | Glutamine and lysine | Other autoimmune, inflammatory and related diseases |

The table 1.2 showing the different protein substrates, their reactive sites for mammalian TG2 catalysed crosslinking, and related diseases were identified in cells via different proteomic analysis methods. Modified from Esposito & Caputo, (2005) and Boros (2008).

The effectiveness of glutamine as a substrate is favoured when glycine or asparagine precedes or follows the glutamine residue (Pastor et al., 1999). Therefore, if it is

located at the N- or C-terminal, or between two proline residues and it will not be identified as a substrate (Pastor et al., 1999).

The sequences of different amino acids around glutamine residues were extensively studied to identify their effect on substrate efficiency. For example, Gorman and Folk (1984) studied the effect of the deletion or replacement of amino acids around glutamine residues from synthetic peptides derived from β-casein (Fig. 1.4; predicted amino acid sequences). They suggested that the positions of valine at -5, leucine at -2, lysine at +2, valine at +3, leucine at +4 and proline at +5 are important in determining the ability of a specific glutamine (Fig. 1.4a) to be a TG2 substrate (Gorman & Folk, 1984).

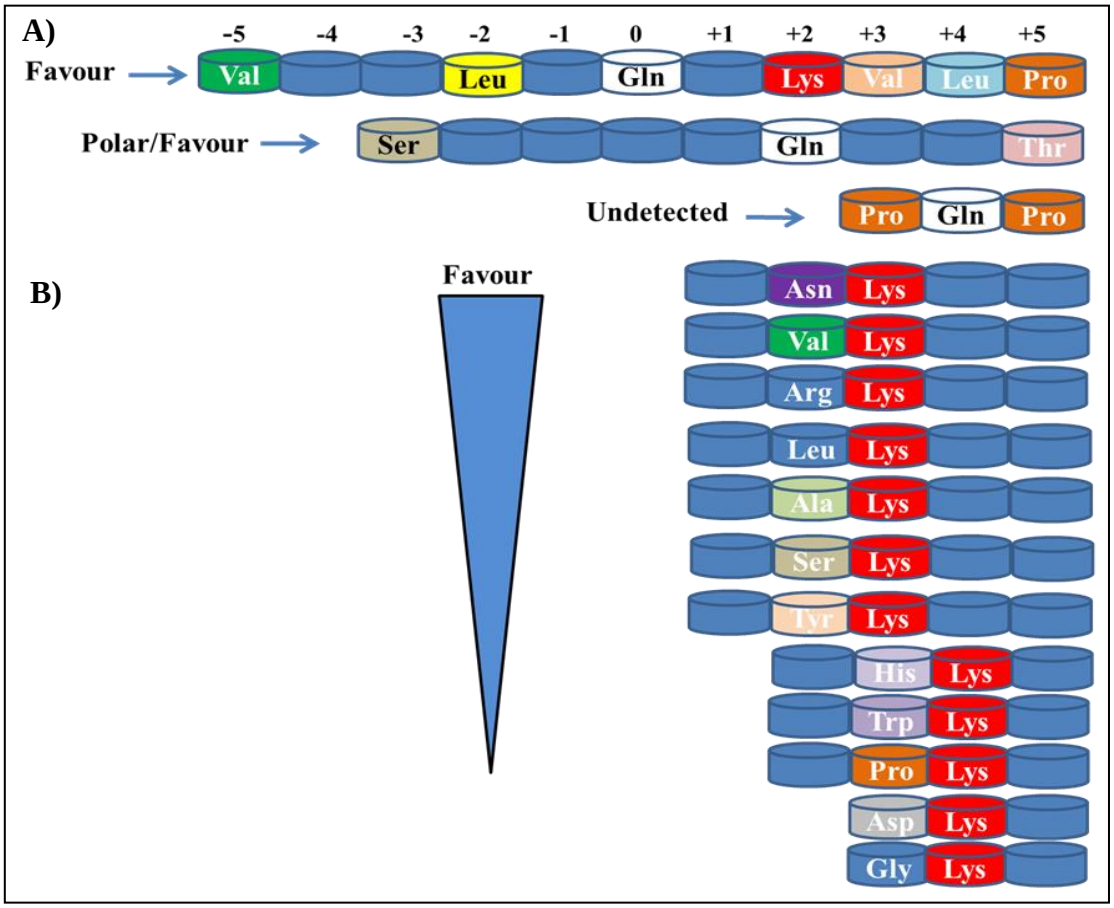



**Figure 1.4 The amino acid sequences of peptides which TG2 favours for a substrate (predicted sequence)**

A) Preferred amino acid sequences surrounding a glutamine residue. B) Preferred amino acid sequences surrounding a lysine residue.

In addition, these amino acid sequences act as acceptor sites for TG2 in cross linking reactions and the adjacent glutamine residues are the most polar and highly charged amino acids e.g. charged residue (lysine at +2) and polar (serine at +5, glutamine at +2 and threonine at −3) (Aeschlimann et al., 1992). The influence of amino acids around lysine (Fig. 1.4b) has also been established. The modification of lysine-surrounded sequences in α-crystallin revealed that the presence of aspartate or glycine amino acids before lysine had unfavourable properties on substrate reactivity, whereas tryptophan, proline and histidine were less unfavourable. In contrast, asparagine, valine, arginine, alanine, leucine, tyrosine and serine have been found to have a positive effect in enhancing the substrate reactivity (Grootjans et al., 1995).

#### 1.3.7.3. TG2 and its substrates in cellular biological functions

Both experimental studies and mass spectrometry coupled with bioinformatics analysis allow better insight and understanding into potential TG2 substrates. Many of these substrates have been shown to be involved in controlling cell function and to be involved in human diseases (Facchiano et al., 2006). Some of the cellular biological functions controlled by TG2 through its substrates are cell survival and death, signal transduction, cytoskeleton regulation, membrane trafficking and function and ECM-cell interaction and stabilisation (see the next sections).

##### 1.3.7.3.1. Cell death and cell survival

Cell death and cell survival are major biological phenomena that can occur either voluntarily or sometimes accidentally. The molecular pathways that regulate both of these phenomena are evolutionarily conserved and their components can exist in single-cell organisms. In addition, their molecular mechanisms are complicated and often entangled with other cellular mechanisms, such as cell proliferation and cell differentiation, thus moulding a broadly related signalling network (Johnson, 2013). Transglutaminase has been shown to regulate the balance between cell survival and cell death through binding to cathepsin D (CTSD; an aspartyl protease) and crosslinking pro-survival proteins results in attenuated CTSD levels as well as apoptosis in mouse embryonic fibroblasts (Kim et al., 2013). Breakpoint cluster region protein (Bcr), a GTPase-activating protein for Rac which that negatively regulates acute inflammatory responses (Cunnick et al., 2009), was identified as a

TG2 substrate. Transglutaminase 2-induced aggregation of Bcr in primary human pulmonary artery endothelial (HPAECs) cells occurs under extreme stress conditions induced by a hypoxia-mimetic agent such as $CoCl_2$ (Yi et al., 2011).

*In vivo* TG2 has been shown to be able to modify retinoblastoma protein (RB: a key modulator of cell growth and death) in promonocytic cells undergoing apoptosis through polymerisation of RB protein (Oliverio et al., 1997). Furthermore, TG2 may crosslink the pro-apoptotic enzyme dual leucine zipper-bearing kinase modulating its function and enhancing its kinase activity resulting in the activation of c-Jun amino-terminal kinase (JNK) (Robitaille et al., 2004). These data suggest that TG2 is involved indirectly in regulating signal-transduction cascade by modifying the activity of enzyme substrate itself.

#### 1.3.7.3.2. Signalling transduction

Transglutaminase 2 is a GTP-binding and hydrolysing protein (G alpha h) (Nakaoka et al., 1994). The activation of TG2 in HeLa cells has been shown to increase transamidation of RhoA (a member of the low molecular weight Ras superfamily of G-proteins) (Bishop & Hall, 2000) and stimulate its binding to RhoA-associated kinase-2 (ROCK-2), thus promoting cell adhesion and the forming of stress fibres (Singh et al., 2001). Transglutaminase 2 can also alter the signalling function of some of its substrates proteins that act as a growth and differentiation factors such as epidermal growth factor (EGF; Antonyak et al., 2009), vascular endothelial growth factor receptor 2 (VEGFR-2; Dardik & Inbal, 2006), the bifunctional ectoenzyme human cyclic ADP ribose hydrolase (CD38; Umar et al., 1996) that is essential for intracellular $Ca^{2+}$ regulation (Malavasi et al., 2008). Similarly, insulin-like growth factor-binding protein-3 (IGFBP-3; Mishra & Murphy, 2004), (IGFBP-1; Sakai et al., 2001) and neurite growth-promoting factor 2 (NEGF2) that act as heparin-binding polypeptide (Iwasaki et al., 1997; Kojima et al., 1997) are all growth factor proteins whose signalling functions can be altered by TG2.

#### 1.3.7.3.3. Cytoskeleton and membrane trafficking regulation

Many TG2 substrates are involved in cytoskeletal regulation. For example, the activation of TG2 by $Ca^{2+}$ allows it to crosslink and rearrange intracellular and

extracellular cytoskeleton adapter proteins such as radixin, ezrin and moesin (Orrù et al., 2003). Other examples include, the crosslinking of microtubule-associated protein tau and neurofilament proteins (heavy, middle and light chain) that act as pathological markers for Alzheimer's disease (Prasanna Murthy et al., 1999; Grierson et al., 2001) and cytoplasmic actin in human leukemia cells (Nemes et al., 1997). However, β-tubulin is the best acyl donor of TG2 substrates in early embryogenesis (Maccioni & Arechaga, 1986). Recently, spectrin and myosin were identified as TG2 substrates in human intestinal epithelial cells (Orrù et al., 2003).

Membrane trafficking is a crucial process for all aspects of cell physiology including cell function, growth, death, signalling and development (Di Paolo & De Camilli, 2006). Valosin, clathrin and importin are proteins that are involved in the membrane trafficking processes and have been identified as TG2 substrates (Orrù et al., 2003). Indeed, the involvement of TG2 in cell trafficking regulation has also been described in Huntington's disease, since the overexpression of TG2 blocks the secretion of brain-derived neurotrophic factor (BDNF) from the Golgi region through formation of clathrin-coated vesicles containing-BDNF (Borrell-Pagès et al., 2006).

#### 1.3.7.3.4. ECM-cell interaction and stabilisation

The exact mechanism by which TG2 is secreted out of the cell to the extracellular environment remains unknown. The normal trafficking pathway for any proteins requires them to have a leader sequence and usually go through the endoplasmic reticulum, Golgi apparatus, and plasma membrane to the extracellular space (Chou et al., 2011). The fact that TG2 has no leader sequence suggests that it must have a non-classical releasing pathway (Akimov & Belkin, 2001a; Collighan & Griffin, 2009). Recent studies proposed that the membrane trafficking of TG2 and its biological activity were linked to its binding to cell-surface heparan sulphate proteoglycans such as syndecan-4 (Scarpellini et al., 2009). However, other studies have suggested the involvement of TG2 crosslinking activity in its secretion outside smooth muscle cells through the microparticles (van den Akker et al., 2012). Another study strongly believes that this trafficking is heavily linked to a specific sequence within the β-sandwich domain of TG2 structure (Chou et al., 2011).

The ability of TG2 to be secreted out of the cell, where high $Ca^{2+}$ concentration can activate it and allow it to stabilise and crosslink ECM proteins, makes this a fertile area for researchers to investigate. Some of the critical TG2 substrates identified within ECM proteins are fibronectin, laminin and collagen (Mosher, 1984). Several laboratories have shown that the majority of TG2 protein is localised in ECM and on the plasma membrane in different cell types and tissues (Zemskov, et al., 2006). Depending on cell types, TG2 can enzymatically (posttranslational modification of ECM) (Akimov & Belkin, 2001b), or non-enzymatically (cell adhesion, migration and growth) (Akimov & Belkin, 2001a) function at these locations. This can be either by crosslinking various ECM proteins or by non-covalent modulation of cell-ECM interactions with growth factors, consequently, regulating β1 and β3 subfamilies of integrins (Akimov et al., 2000), cell adhesion molecules of the immunoglobulin superfamily (Hunter et al., 1998), heparan sulphate proteoglycan e.g. syndecan-4 (Zemskov et al., 2006; Telci et al., 2008), platelet derived growth factor receptor (Zemskov et al., 2009) and VEGFR-2 (Dardik & Inbal, 2006).

#### 1.3.7.4. Transglutaminase 2 in disease states

Transglutaminase 2 has been implicated in numerous pathological conditions, such as; inflammatory diseases, neurodegenerative disorders (Hoffner & Djian, 2005), diabetes (Bernassola et al., 2002) some cancers (Mangala & Mehta, 2005) and autoimmune disorders (Molberg et al., 2000). The involvement of TG2 and its substrates in these diseases will be discussed below.

##### 1.3.7.4.1. Gluten sensitivity diseases

The catalytic function of TG2 results in posttranslational modifications of proteins that are thought to contribute to the generation of autoantibodies such as those in gluten sensitivity disease or coeliac disease (CD). Coeliac disease is an autoimmune disorder with a genetic element caused by dietary exposure to gluten from barley, wheat, and rye flour (Di Sabatino & Corazza, 2009). Some gluten proteins are resistant to gastrointestinal proteases, which result in the accumulation of immuno-toxic peptides in the lower intestine and thus the triggering of inflammatory responses (Matysiak-Budnik et al., 2008). Thereby, the immune system attacks villous atrophy (of the small intestinal mucosa) which results in villous flattening in chronic diarrhoea

and malabsorption of food (Matysiak-Budnik et al., 2008). A covalently cross-linked bond between the gliadin peptide and TG2 itself has been reported in this disease (Sollid et al., 1997). In this process, TG2 acts as a hapten-carrying gliadin in a manner that an elicits immune response and generates autoantibodies. Transglutaminase 2 modifies specific gluten peptides-sequences through its deamidation activity; the modified epitope generated binds efficiently to DQ2 of a HLA receptor of intestinal cell lines (Fleckenstein et al., 2002). This immune complex is recognised by circulating T cells, generating autoantibodies (IgA) against TG2, which is commonly used as a diagnostic marker for coeliac disease (Dieterich et al., 1997; Koning et al., 2005). The role of TG2 in coeliac disease is not only in triggering auto-antigen and in modifying gliadin pathogenic epitope generation, but it also has a further role in regulating lymphocyte migration and controlling the early immune response stages of coeliac disease (Maiuri et al., 2005).

#### 1.3.7.4.2. Neurodegenerative diseases

Transglutaminase 2 activity has been implicated in the pathogenesis of a number of neurodegenerative diseases. Protein aggregation in damaged neural tissue is the major characteristic linked with these diseases. For example, Alzheimer's disease (AD) is associated with the destruction of nerve tissues in the cortex and hippocampus of brain (responsible for memory) (Graeber et al., 1998). Abnormal clusters of senile plaques and tangles are the main cause of cell death and tissue loss in AD patients (McKhann et al., 1984). The aggregation of β-amyloid has been shown to be associated with extracellular senile plaques that block signals between neural cells (Glenner & Wong, 1984; Masters & Beyreuther, 2006), while aggregation of hyper-phosphorylated tau proteins was linked with the formation of neurofibrillary tangles that disrupt cell nutrients (Buée et al., 2000; Lee et al., 2001). It has been demonstrated that almost all neurofilament polypeptides act as substrates for TG2 promotes its cross linking function (Miller & Anderton, 1986; Grierson et al., 2001). Many different studies have confirmed that TG2 is involved in the aggregation of tau protein and tangles through its crosslinking activity (Appelt & Balin, 1997; Halverson, et al., 2005; Wilhelmus et al., 2009).

Huntington's disease (HD) is another neurodegenerative disorder characterised by choreia (abnormal and writhing movements of limbs and facial muscles) and decline in mental processes and functions. Huntington's disease is caused by a dominant mutation, which occurs in an autosomal gene huntingtin (htt). This mutation results in over repeating of CAG trinucleotide in the first exon of the htt gene, which results in a long polyglutamine (polyQ) expansion at the N-terminus of huntingtin (Andrew et al., 1993). The role of TG in this disease is not clear, some studies hypothesised that TG2 protein cross linking-activity may contribute to the formation of the aggregated proteins (Gentile et al., 1998; Cooper et al., 1999). Moreover, polyglutamine has been found to be an excellent substrate for TG2 (Kahlem et al., 1996) and, based on this finding, further studies revealed significant increases in TG2 expression and activity in HD brain (Cooper et al., 1999; Cooper et al., 2002). Another study by Karpuj et al. (2002) confirmed these findings using a non-specific TG2 inhibitor (cystamine) in animal models of HD. It was found that the formation of the aggregated proteins was inhibited and the survival rates were improved (Karpuj et al., 2002). However, a study in human neuroblastoma SH-SY5Y cells revealed that TG2 was not involved in HD protein aggregation as mutant truncated huntingtin aggregation protein was present in the absence of TG2 (Chun, et al., 2001). It is clear that more work is needed to reveal the exact role of TG2 in this disease.

#### 1.3.7.4.3. Inflammation and tumour progression

Transglutaminase 2 has been implicated in the main phases of inflammation and tumour progression processes, including ECM homeostasis, cell adhesion, cell migration, apoptosis and angiogenesis (Kotsakis & Griffin, 2007). Inflammation is a response of the immune system to cell or tissue-infection, injury or any other stress (Medzhitov, 2010). This can result in severe pathological conditions, such as those, which appear with cancer and degenerative fibrotic diseases. The inflammatory process is usually involved in tissue repair and wound healing (Kiritsy & Lynch, 1993). Transglutaminase 2 has been shown to be involved in the initial phases of inflammation and the wound healing process. For example, during the initial phase of cell damage, the release of cytokines and growth factors has been shown to regulate TG2 synthesis (Verderio et al., 2004). An increase in TG2 synthesis was observed in response to the cytokines interleukin IL-1β and tumour necrosis factor TNF-α in rat

brain astrocytes (Monsonego et al., 1997). Transglutaminase 2 has been shown to promote inflammation by activating the nuclear factor kappa B (NF-κB) cascade in tissue macrophages in the brain (Lee et al., 2004).

The involvement of TG2 in the wound healing processes, its expression and crosslinking activity were also shown to be associated with tissue fibrosis and scarring. A lot of research has focused on the relevance of TG2 in fibrosis in different organs, including liver (Mirza et al., 1997; Grenard et al., 2001), renal tissue (Skill et al., 2001; Johnson et al., 2003), lung (Griffin, et al., 1978), and heart (Small et al., 1999). Transglutaminase 2 can directly bind to cell-surface heparin sulphate chains which promote Arg-Gly-Asp (RGD)-independent cell adhesion mechanism, with the subsequent activation of protein kinase C, focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) (Telci et al., 2008).

Inflammatory responses and TG2 expression play an essential role during different cancer phases (initiation, progression, invasion and spreading). Chronic expression of TG2 may induce cell growth, survival and allow accumulation of oncogenic mutations, and consequently tumour progression (Mehta & Han, 2011). Several studies have implicated up-regulation of TG2 expression in carcinomas including pancreatic (Akar et al., 2007; Cheung et al., 2008), breast (Mehta et al., 2004), glioblastoma (Yuan et al., 2005; Yuan et al., 2006) and skin tumours (Fok et al., 2006). The over expression of TG2 in carcinomas was linked with its anti-apoptotic role (Boehm et al., 2002) and chemotherapeutic drug resistance (Kim et al., 2006). The expression of TG2 in pancreatic cancer cells has been connected with activation of FAK, Akt, and NF-kB signalling pathways and inhibition of the ability of the phosphatase and tensin homologue located on chromosome 10 (PTEN) gene to act as a tumour suppressor gene (Verma et al., 2008). The mediation of these oncogenic signalling pathways by TG2 possibly causes resistance to chemotherapy and amplifies invasiveness of cancer cells. A study in mouse pancreatic cancer has suggested that inhibition of TG2 by small-interfering RNA (siRNA) can be used as a therapeutic approach to improve pancreatic cancer treatment (Verma et al., 2008).

#### 1.3.7.4.4. Heart diseases

Various heart cells abundantly express TG2 including endothelial smooth muscle cells, cardiomyocytes and vascular cells (Thomazy & Fesus, 1989). A large number of research studies have pointed to the critical roles of TG in cardiac biology and pathophysiology. Transglutaminase has been cited to be involved in regulation of myocardium growth, fibrosis and wound healing (Sane et al., 2007). Away from its enzymatic activity, TG2 likely influences hypertension (high blood pressure) of vascular smooth muscle cells by enhancing vasoconstriction (Janiak et al., 2006). In this case, TG2 stimulates the binding of RhoA/ROCK-2 kinase and auto-phosphorylation of ROCK-2 (Rho-associated coiled-coil containing protein kinase) through direct binding between cell surface TG2 and fibronectin (Janiak et al., 2006).

The crosslinking activity of TG2 has been shown to contribute to inward remodelling (a reduction in lumen diameter of vessels) of arteries. Rat blood flow inward remodelling was lowered when TG2 was inhibited via exposure to nitric oxide, whereas it was increased by retinoic acid treatment to enhance TG2 expression (Bakker et al., 2005). This could be a novel therapeutic target for chronic vasoconstriction or any inward remodelling pathogenesis. Another study by Engholm et al. (2011) in spontaneously hypertensive rats (SHR) has shown that inhibition of TG2 with cystamine results in reduction of inward remodelling (Engholm et al., 2011).

Transglutaminase 2 has been shown to be linked to cardiac hypertrophy. Cardiac hypertrophy (heart enlargement) is one potential risk factor associated with heart failure and ischaemic heart disease (Shiojima et al., 2005). Up-regulation of the TG2 gene has been reported in cardiac hypertrophy and cardiac failure in rat models (Iwai et al., 1995). In failing human heart tissue of both ischaemic and dilated cardiomyopathy, GTP-binding and TG2 activities were decreased while TG2 protein levels were increased in dilated heart (Hwang et al., 1996). In transgenic mouse models, GTP-binding and TG2 over-expression regulate activation of cyclooxygenase (COX-2) (Zhang et al., 2003). The up-regulation of the COX-2 gene was linked to cardiac failure (Abassi et al., 2001) and cell survival (Adderley & Fitzgerald, 1999). Therefore, TG2-mediated COX-2 activation may differentially modulate

cardiomyocyte death or survival. Recently, Li et al. (2009) demonstrated that TG2 over-expression, but not its activity, was implicated in hypertrophic agonist-induced (endothelin (ET) 1) cardiac hypertrophy, which suggests the involvement of TG2 in signalling activity (Li et al., 2009).

Atherosclerosis is a condition in which plaques (accumulation of fatty substances) build up on and thicken arterial walls (Ismail & Peden, 2011). Since inflammation has been shown to play a significant role in this disease (Libby, 2006), the involvement of TG2 in its inflammation process may be an important factor. The presence of TG2 in human coronary artery was reported (Sumi et al., 2002) and isodipeptide epsilon (gamma-glutamyl) lysine crosslinking generated by TG2 was isolated from rabbit atherosclerotic aorta (Bowness et al., 1994). In aorta of cholesterol-fed rabbits, TG2 protein levels and activity were increased (Wiebe et al., 1991). Leukocytes or white blood cells have been the main source of TG2 in atherosclerotic injuries. *In vitro*, macrophage TG2 expression induces apoptotic cell clearance and decreases atherosclerotic lesion size *in vivo* (Abedin et al., 2004).

In the case of ischaemic /reperfusion injury (where the return of blood to ischaemic damaged tissue results in more damage), the infarct size was shown to be increased in TG2 knockout mouse heart, combined with a serious failure in ATP level (Szondy et al., 2006). This suggests the action of TG2 at a mitochondrial level under physiological conditions. It has been previously shown that TG2 over-expression hyperpolarises mitochondria and drives cells to apoptosis (Grazia Farrace et al., 2002).

#### 1.3.7.5. Apoptotic and anti-apoptotic role of TG2

Various experimental systems have established the involvement of TG2 in apoptosis (Piacentini et al., 1991). In the majority of cells, TG2 protein is barely detectable and its messenger RNA is transcribed as a signal of apoptosis (Nagy et al., 1997; Verderio et al., 1998). Over-expression of TG2 has been largely used as a specific marker of cells undergoing the apoptosis process. This could be because its over expression drives cells to suicide, as clones resistant to TG2 transfection show greatly reduced cell growth *in vitro* (Piredda et al., 1997) and *in vivo* (Piacentini et al., 1996). Since TG2 crosslinking activity is inhibited by GTP, the aggregation of its protein substrates

in cells should not necessarily correlate with its crosslink activity (Fesus et al., 1991; Melino & Piacentini, 1998). A study by Fabbi et al., (1999) established that the loss of TG2 crosslink activity during apoptosis is due to cleavage by caspase-3 (Fabbi et al., 1999), which is a member of the cytosolic aspartate–specific cysteine protease family (Alnemri et al., 1996). However, the activation of TG2 via $Ca^{2+}$ in dying cells results in the irreversible accumulation of an insoluble cross-linked protein scaffold that stops the leakage of intracellular macromolecules (Piredda et al., 1997; Melino & Piacentini, 1998). This insoluble protein scaffold could stabilise the integrity of cells undergoing apoptosis before phagocytosis clearance, consequently preventing the release of harmful components such as nucleic acids, lysosomal enzymes etc., that elicit inflammatory responses (Knight et al., 1993).

Transglutaminase 2 has been shown to have both pro- and anti-apoptotic roles that depend on cell stimulation and the cell type (Antonyak et al., 2001; Antonyak et al., 2002; Gundemir & Johnson, 2009). One possible anti-apoptotic role is the ability of TG2 to prevent the degradation of retinoblastoma protein (a tumour suppressor that acts to prevent cell growth) and thus sustain its anti-apoptotic role during cell death (Boehm et al., 2002; Milakovic et al., 2004). In contrast, TG2 has been reported to promote the release of cytochrome *C* and Bax (an apoptosis regulator) through conformational changes using its BH3-like (Bcl-2 homology) domain (Rodolfo et al., 2004). In addition, the induction of TG2 has been shown to be paralleled to down-regulation of Bcl-2, but is not affected by its inhibition (Melino et al., 1994). The overexpression of TG2 inhibited apoptotic cell death induced by $Ca^{2+}$ overload through Bax suppression, which in turn decreased activation of caspase-3 and -9, secretion of cytochrome *C*, and mitochondrial permeability transition (Cho et al., 2010). This revealed the protective and anti-apoptotic role of TG2 in diseases involving $Ca^{2+}$ overload.

As TG2 transamidation activity is strongly dependent on the concentration of intracellular $Ca^{2+}$, its activity is also linked to the apoptotic process by inhibiting the release of DNA and intracellular proteins from dying cells (Fesus et al., 1991). Moreover, $Ca^{2+}$ overload induced cell death and apoptosis triggering was detected in cells under hypoxic or oxidative stress conditions (Orrenius et al., 2003). However, the disruption of $Ca^{2+}$ homeostasis in cells can affect other enzymes that are $Ca^{2+}$-

dependent, such as endonuclease, phospholipases and calpain which are all involved in cell injury and death (Dong et al., 2005). Therefore, the exact apoptotic and anti-apoptotic role of TG2 are still unclear and need further investigation. In addition, because of the multiple functions of TG2 and its ability to take part in signal transduction, different roles in apoptosis have been suggested that depend on the type of cell and stress involved (Fesus & Szondy, 2005).

In human neuroblastoma SH-SY5Y cells, different types of stress stimuli have displayed a variable effect on apoptotic processes, in which staurosporine and osmotic stress treatments caused a significant increase in caspase-3 activity and apoptotic nuclear changes, in combination with induction of TG2 transamidating activity, whereas heat shock stress did not (Tucholski & Johnson, 2002). This suggested that apoptotic and anti-apoptotic effects of TG2 are not constant in identical cell types.

#### 1.3.7.6. Protective role of TG2

Despite the fact that TG2 is involved in diseases processes, it also plays a protective role in some diseases. Transglutaminase 2 elicited liver protection against hepatitis C virus in which its expression and localisation at in the ECM were decreased in the advanced stages of fibrosis in hepatitis C virus-infected patients (Nardacci et al., 2003). In addition, carbon tetrachloride induced liver injury in TG2 knockout mice failed to clear necrotic tissue in comparison with wild-type mice, suggesting its role in protection was through tissue stabilisation and repair. In neuroblastoma cells, TG2 overexpression induced by retinoic acid or staurosporine elevated apoptosis via crosslinking of glutathione S-transferase P1-1, histone H2B and β-tubulin (Piredda et al., 1999). Conversely, TG2 activation by retinoic acid-induced differentiation inhibited apoptosis in human promyelocytic leukemia (HL60) cells, while monodansylcadaverine (MDC; an inhibitor of TG2) eliminated this protective effect (Antonyak et al., 2001). This suggested an important role of TG2 enzymatic function in cells under differentiation and stress condition. In the same context, retinoic acid treatment inhibited cell death caused by TNF-α and this effect was also abolished by MDC in SH-SY5Y human neuroblastoma cells (Kweon et al., 2004). It is already known that treatment of cells with retinoic acid results in the activation of the pro-

survival protein phosphatidylinositol 3-kinase (PI3K)–Akt and that this requires TG2 induction and activation (Antonyak et al., 2002).

The involvement of TG2 and its substrates in membrane-mediated glucose-stimulated insulin secretion in rat pancreatic islets has been reported (Gomis et al., 1989) to be induced by retinoic acid or vitamin A (Driscoll et al., 1997). The aggregation and internalisation of insulin receptors have been linked to cells or tissues that display high levels of TG2-crosslinking activity, e.g. human fibroblasts (Baldwin, et al., 1980). The TG2 knockout mice show a high level of glucose concentrations correlated with decreases in TG2 activity compared to wild-type animals. The knockout mice show a decrease in glucose-stimulated insulin release, suggesting a role for TG2 in glucose regulation and metabolism processes (Bernassola et al., 2002).

There is a strong relationship between deficits in glucose-induced insulin secretion resulting in mitochondrial dysfunction through the loss of ATP production (Maechler & Wollheim, 2001), and involvement of TG2 in mitochondrial hyperpolarisation (change in mitochondrial membrane voltage) (Grazia Farrace et al., 2002). Szondy and colleagues linked this relationship to a cardioprotective role of TG2 against ischaemia and reperfusion-induced cell death. They found that the deletion of TG2 lead to a major drop in ATP production along with significant increase in the infarct size (Fig. 1.5), suggesting the involvement of TG2 catalytic activity in the posttranslational modification of some essential mitochondrial regulatory proteins (Szondy et al., 2006). In the SH-SY5Y neuroblastoma cell line, up-regulation of TG2 selectively decreases the oxygen and glucose deprivation (OGD) -induced hypoxia inducible factor 1 (HIF1) and thus protected cells from OGD induced cell death. The protective role of TG2 here is due to interaction with HIF1β and prevention of the formation of the heterodimeric form of (HIF1) which consists from HIF1α and HIF1β (Filiano et al., 2008) and is thought to be responsible for activation of pro-apoptosis genes (Filiano et al., 2010). Transglutaminase 2 protected NIH3T3 fibroblasts from glucose deprivation (GD)-induced apoptosis in WT TG2 mice and it was more effective in the presence of 5 μM retinoic acid that results in up-regulation of TG2 expression and GTP binding activity (Antonyak et al., 2003).

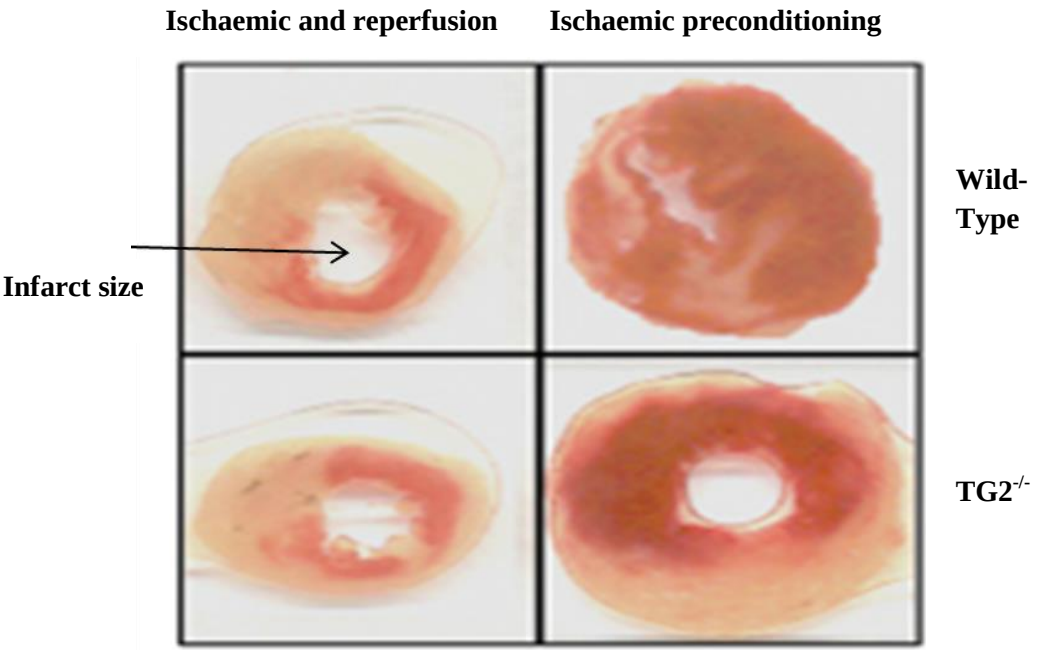



**Figure 1.5 Protective role of TG2 in heart tissue**

The figure shows the limitation in infarct size in heart of WT mice in comparison to TG2-/-after ischaemia/reperfusion. Adapted from Szondy et al., (2006).

In human embryonic kidney 293 cells, the overexpression of TG2 inhibited apoptotic cell death induced by $Ca^{2+}$ overload through Bax suppression, which in turn decreased activation of caspase-3 and -9, secretion of cytochrome *C*, and mitochondrial permeability transition (Cho et al., 2010). This revealed the protective and anti-apoptotic roles of TG2 in diseases involving $Ca^{2+}$ overload. TG2 can protect cells from apoptosis by modification of the tumour suppressor protein p110 Rb (Boehm et al., 2002). Neuroblastoma cells transfected with an active form of TG2 were protected against DNA-damage-induced stress by inhibition of p53 (tumour suppressor protein) activation (Tucholski, 2010). Interestingly, p53 is a target for TG2 kinase activity. Transglutaminase 2 contributes to the protection of aortic walls during remodelling of the abdominal aortic aneurysms (AAAs) (Munezane et al., 2010) in which the proteins expression of potential biomarkers for AAAs (TNF-α; matrix metalloproteinases 2 and 9 (MMP-2 and -9)) (Longo et al., 2002) were attenuated by exogenous TG2 in isolated tissue culture (Munezane et al., 2010) .

### *1.4. Myocardial cell injury and cell death*

Many different types of myocardial cell injury and cell death are triggered in response to pathological processes associated with oxidative stress, ischaemic reperfusion or other cardiac diseases. Such injuries can induce necrosis, apoptosis and more recently autophagy. Necrosis is evident when cells or subcellular organelles are swelling via cell membrane disruption (Trump et al., 1997). Since ischaemic reperfusion injury alters the cells from reversible to irreversible mode, a severe defect in membrane permeability develops that permits the uncontrolled flow of $Ca^{2+}$, alterations in electrolyte channels and loss of $Mg^{2+}/K^{+}$ ions, resulting in the swelling of cytoplasm, mitochondria and other organelles (Buja, et al., 1993; McCully et al., 2004). This results in physical defects, such as holes in the cell membrane and fractures due to the cells swelling. Moreover, the release of cellular components in and around tissues activates the inflammatory response and neutrophil influx that could lead to damage of neighbouring cells.

Apoptosis is another type of cell death (see section 1.6) that is dependent on energy to eliminate the damaged cells without triggering an inflammatory responses (Elmore, 2007). Apoptosis has been known as a process that can be provoked by external stimuli that are either physiological or pathological (Kettleworth, 2007). The importance of the pathogenic process of apoptosis is well established in the development of myocardial disease therapies. This involves caspase pathway activation by Fas (apo1)/TNFR-1 signalling (Kaufmann & Hengartner, 2001) such pathway is blocked by the ubiquitin-like protein (sentrin) (Okura et al., 1996). During apoptosis, many different gene products are regulated, including an inner mitochondrial protein (bcl-2), p53 and c-myc a member of an oncogene family (Hoffman & Liebermann, 2008).

In addition, mitochondria contribute to the apoptosis process (Fig. 1.6), being altered via free radicals or other pathological processes resulting in release of cytochrome *C* that binds to the apoptotic protease (Apaf-1). This leads to cell death through generation of apoptosomes that activate both caspase and apoptotic pathways (Iliodromitis et al., 2007). Over expression of bcl-2 (anti-apoptotic protein), which is

located in mitochondrial membranes is responsible in the blockage of such a pathway (Hetts, 1998; Marani et al., 2002). Apoptosis results in shrinking of cells, which is due

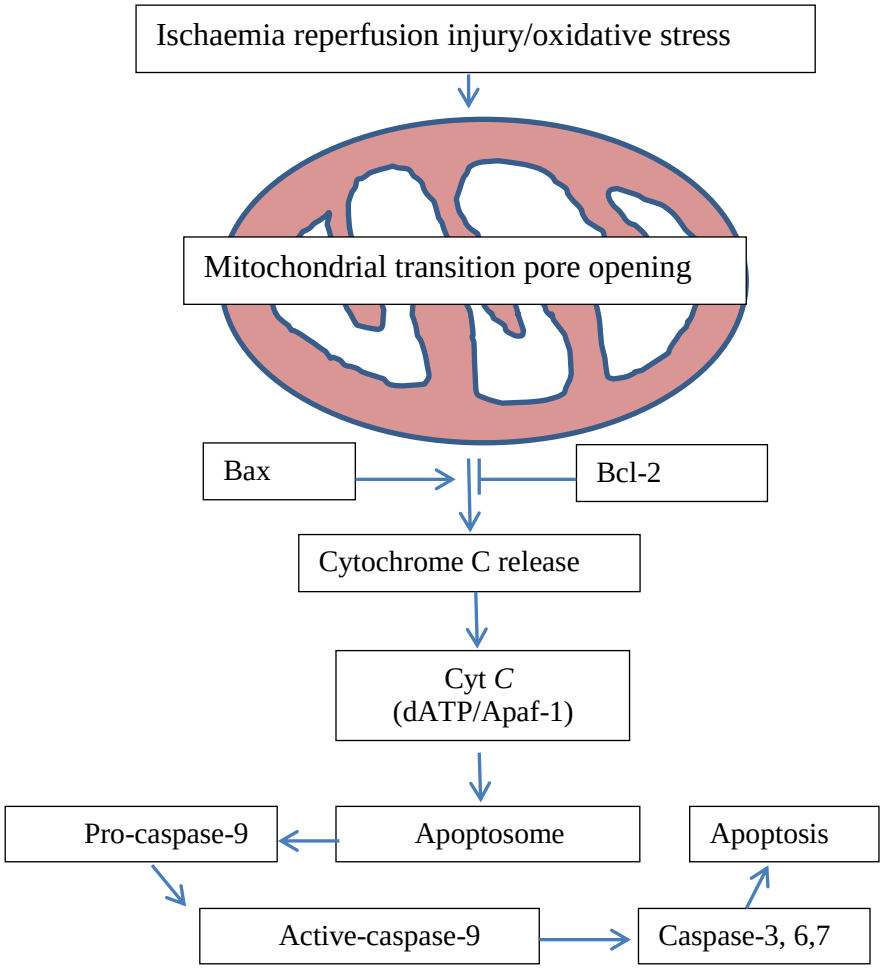



**Figure 1.6 The mitochondrial death pathway**

The schematic diagram outlining the mitochondrial death pathway in response to pathological processes associated with ischaemia reperfusion injury and oxidative stress. The translocation of pro-apoptotic Bax to the mitochondrial membrane promotes mitochondrial transition pore opening and thus cytochrome *c* release. Cyt C binds to apoptotic protease (Apaf-1/dATP) in turn this leads to cell death through generation of apoptosomes that activate caspases and trigger apoptosis. Anti-apoptotic Bcl-2 can prevent Bax and mitochondrial membrane association. Scheme modified from Iliodromitis et al., (2007).

to activation of TG2 (see section 1.3.7.5) and protease, producing cytoplasmic protein crosslinking (Nemes et al., 1996; Szondy et al., 1997; Grabarek et al., 2002). The activation of TG2 in cells undergoing apoptotic death results in the irreversible accumulation of a cross-linked protein scaffold that stops the leakage of intracellular macromolecules (Piredda et al., 1997; Melino & Piacentini, 1998). Consequently, rapid phagocytoses are triggered without the elicitation of inflammatory responses (Falasca et al., 2005).

The autophagy induction has been shown to be associated with up-regulation of the mitochondrial pro-apoptotic protein BNIP3 in glioma cells (Daido et al., 2004). Autophagy has been shown to have a key role in cellular homeostasis or clearance of damaged organelles, cellular response to stress conditions (Levine, 2005), cancer (Liang et al., 1999), neurodegenerative disorders (Yuan et al., 2003), and cardiomyopathy disorders (Shimomura et al., 2001). Moreover, BNIP3 has been shown to be involved in ischaemic reperfusion injury, mediating the up-regulation of autophagy as a protective response in HL-1 cardiac cells thus, inducing mitochondrial dysfunction (Hamacher-Brady et al., 2006).

### *1.5. Protein kinases in ischaemic/ pharmacological preconditioning*

Over the past 20 years, a significant body of research has focused on ways to prevent or block irreversible ischaemic injury associated with heart disease or cardiac surgery. It is known that brief periods of ischaemia before reperfusion (ischaemic preconditioning; IPC) is an effective mechanism that is capable of protecting the heart from myocardial ischaemic injury (Murry et al., 1986). Pharmacological preconditioning (PPC) acts as an alternative approach for IPC, in which cardioprotective effects are triggered pharmacologically (Kloner & Jennings, 2001). A number of pharmacological preconditioning agents have been identified, including agonists of G protein-coupled receptors (GPCRs) such as the adenosine $A_1$ receptor (Yellon & Downey, 2003).

Reversible protein phosphorylation plays a key role in signal transduction pathways and a growing number of protein kinases have been shown to be involved in IPC and PPC including protein kinase C (PKC), (Yoshida et al., 1997; Hassouna et al., 2004), mitogen-activated protein kinases (MAPKs) and protein kinase B (PKB; or Akt)

(Armstrong, 2004; Hausenloy & Yellon, 2004). The three major MAPK families (p38 MAPK, extracellular signal-regulated kinases (ERK1 and ERK2) and c-Jun N-terminal kinases (JNK1 and JNK2)) and PKB are triggered in response to both ischaemia and reperfusion (Armstrong, 2004; Hausenloy & Yellon, 2004). It is commonly acknowledged that PKB and ERK1/2 are cardioprotective and trigger anti-apoptotic survival pathways, whereas p38 MAPKs and JNK are thought to stimulate cell death (Abe et al., 2000; Sugden & Clerk, 2001). Nevertheless, there is also evidence that p38 MAPK and JNK are implicated in cardioprotection (Dougherty et al., 2002; Steenbergen, 2002). Protein kinase B, ERK1/2 and p38 MAPK have been reportedly involved in PPC and IPC (Dana et al., 2000; Punn et al., 2000; Brar et al., 2002; Chanalaris et al., 2003). In addition to serine/threonine kinases (PKC, PKB, and MAPKs), non-receptor tyrosine kinases including Src, Bmx and Janus kinases (JAKs) are implicated in cardioprotection (Vondriska et al., 2001; Bolli et al., 2003; Zhang et al., 2004).

### *1.6. Protein kinase A and protein kinase C*

In a variety of eukaryotic genomes, protein kinases account for ~2 % of genes and are found to be one of the major families of proteins (Manning et al., 2002). Up to 30 % of human cellular proteins are under the control of protein kinases that are considered to act as major regulatory mechanisms directing the basic cellular processes and signal transduction of complex pathways (Ficarro et al., 2002; Manning et al., 2002). By mass spectrometric analysis of protein phosphorylation, it has been revealed that the majority of cellular mechanisms are regulated by the reversible phosphorylation of proteins on more often with serine, threonine, and less on tyrosine residues (Mann et al., 2002).

Protein kinase A and protein kinase C both belong to the AGC family of protein kinases (Pearce et al., 2010). These kinases are a group of enzymes that chemically modify a specific protein at its serine and threonine residues transferring the terminal phosphate group of ATP to protein bound serine, threonine or tyrosine residues in an action called phosphorylation. Activation of these kinases results in phosphorylation of target proteins (substrates) and regulates their enzymatic activity, localisation and

function, thereby, orchestrating almost all the essential aspects of cellular function and processes in living organism (Cohen, 2000).

### 1.6.1. Protein kinase A: Structure, function and regulation

The heterotetrameric form of protein kinase A is an inactive state of the enzyme composed of two subunits, a catalytic subunit which contains the ATP binding domain and a regulatory binding domain. The regulatory subunit also contains two domains, one to bind cyclic AMP and the other to inhibit the active subunit of PKA. Following G-protein-coupled GPCR activation, adenylyl cyclase is activated in downstream signalling. This results in the production of cAMP, which in turn binds to PKA regulator subunits and releases the catalytic subunits to phosphorylate target proteins (Taylor et al., 1990). PKA-kinase anchor proteins control the subcellular localisation of the enzyme and up to 50 different types of these multidomain scaffolding proteins have been reported and all can localise PKA to particular subcellular organelles (cytoskeleton, plasma membrane, Golgi apparatus, mitochondria, ion channels, and centrosomes) within different cell types (Wong et al., 2002).

Protein kinase A modulates various biological functions in cells and this depends on the type of stimulator (hormones and GPCRs) and cell type. For example, the activation of PKA through the stimulation of β- adrenergic receptor by the hormone adrenaline in cardiomyocytes results in glucose production and phosphorylation of glycogen phosphorylase and acetyl- CoA carboxylase (Rang, et al., 2003). In contrast, the activation of PKA with catecholamine also stimulates β- adrenergic receptor in the same cells resulting in $Ca^{2+}$ repositioning at the sarcoplasmic reticulum and phosphorylation of phospholamban (Rang et al., 2003). The activation of PKA can enhance cardiac myocytes by phosphorylation of troponin I/C, L-type $Ca^{2+}$ channel and phospholamban which serve to control $Ca^{2+}$ concentration and myofilament sensitivity to $Ca^{2+}$ (Xiang & Kobilka, 2003; De Arcangelis et al., 2008). Protein kinase A is also known to be involved in gene regulation. For example, phosphorylation of cAMP response-element binding protein (CREB) by PKA allows it to initiate the transcription of Pax3 (paired box gene) and Myf5 (myogenic

regulatory factor 5) associated genetic factors involved in muscular tissue formation (Chen et al., 2004).

Immunocytochemistry analysis of rat brain and cultures of brain cells has revealed the accumulation and localisation of protein kinase A in the Golgi-centrosomal area and microtubule-organising centres thus suggesting the involvement of PKA in metabolism regulation, cellular mobility/trafficking and microtubule stability (De Camilli et al., 1986). Protein kinase A has also been shown to mediate pancreatic β-cell proliferation stimulated by exendin-4 making it a potential therapeutic target for treating inadequate β-cell mass in both diabetes types (Song et al., 2008).

### 1.6.2. Protein kinase C: Structure, function and regulation

Protein kinase C is a crucial enzyme in the biochemical mechanism of signal transduction and is implicated in a variety of cellular functions (Nishizuka, 1986). The activation of phospholipase C (PLC) through $Ca^{2+}$ ionophore exposure can directly cleave inositol phospholipids to produce inositol phosphates and diacylglycerol (DAG) necessary for PKC activation (Huang & Cabot, 1990; Peterson & Walter, 1992). Over 10 protein kinase C isozymes have been identified (conventional PKCα, PKCβI/II and PKCγ; novel PKCδ, PKCε, PKCη and PKCθ and a typical PKCζ and PKCλ/ι) based on differences in their crystal structure and cofactor regulation (Nishizuka, 1995; Parker & Murray-Rust, 2004). These PKC isoforms require $Ca^{2+}$ alone or $Ca^{2+}$ and diacylglycerol (DAG) for activation. This serine/threonine kinase is activated by production of DAG in response to Gq protein–coupled receptor activation by α-adrenergic agonists, autocrine factor endothelin and angiotensin II (Dorn II & Brown, 1999; Robu et al., 2003). Immunofluorescence and Western blotting analyses of NIH 3T3 fibroblasts revealed that after activation of PKC by phorbol ester 12- O-tetradecanoylphorbol-13-acetate (PMA), PKCδ, PKCγ and PKCη were localised at the Golgi apparatus while PKCα PKC-βII, PKCε accumulated in the endoplasmic reticulum, the cytoskeleton and nuclear membranes, respectively (Goodnight et al., 1995). These different locations usually occur due to binding of isoenzymes to specific scaffold proteins. For example, RACK1 can bind to active PKC isoform, modifying its signalling activity and trans-locating it to a particular subcellular region (Schechtman & Mochly-Rosen, 2001).

Some of these scaffold proteins (plakophilins) are specific to one PKC isoenzyme (PKCα) that regulates cell junction assembly (Bass-Zubek et al., 2008). While others can bind to and localise many kinase isoenzymes such as AKAP12, which localises both PKCα and PKC βII (Piontek & Brandt, 2003). In addition, AKAP79 can localise and maintain the function of protein phosphatase-2B, PKA and PKC in neuromuscular junctions (Perkins et al., 2001). All PKC isoforms have a critical role in a multitude of cell biological functions. For example, PKCα has been shown to be involved in cell growth, division, differentiation, adhesion and apoptosis (Dempsey et al., 2000; Parker & Murray-Rust, 2004). Furthermore, PKCα can act as immuno-regulator through T cell-interferon production (Pfeifhofer et al., 2006). Protein kinase Cγ has been linked to brain functions associated with learning and memory, while PKCε has been shown to play a role in GABA receptor function in brain (Brose & Rosenmund, 2002).

### 1.6.3. Cardioprotection mediated by PKA and PKC

The protective role of PKC has been well studied and characterised in models of ischaemic preconditioning against ischaemic injury (see section 1.5). The protein kinase C isozyme PKCε has been shown to be critical in prompting of ischaemic and anaesthetic cardioprotective effects (Liu et al., 1999; Toma et al., 2004). The desflurane-induced preconditioning in ischaemia and reperfusion heart tissue by activation of PKCε was shown to be in correlation with ERK activation and infarct size limitation (Toma et al., 2004). Other studies reported an association with the activation of PKCδ, PKCε, as well as Src family protein tyrosine kinase with ischaemic preconditioning in response to isoflurane treatment of intact rat heart. This was in correlation with PKC activation of upstream transduction events including mitochondrial ATP–sensitive potassium channel-opening and reactive oxygen species production (Ludwig et al., 2004). A study by Zatta et al., (2006) proposed the involvement of PKC in post-conditioning (brief repetition of the early moments of reperfusion induce cardioprotection) that results in reduction of cardiomyocyte damage (Zatta et al., 2006). It is also believed that the protective effect of PKC could be due to permeability transition pore (mPTP) blocking in cardiac mitochondrial function (Baines et al., 2003). Conversely, PKCδ activation has been linked with ischaemic reperfusion injury, since the inhibition of this PKC isozyme by selective

PKCδ inhibitor (δV1-1) enhanced cardioprotection effects and reduced reperfusion injury in isolated perfused rat heart (Inagaki et al., 2003). In addition, rapid translocation and accumulation of PKCδ has been observed in the mitochondria of reperfusion heart in association with superoxide anion generation and apoptosis induction (Churchill & Szweda, 2005). Activation of PKA and PKC both are part of the cardioprotective properties of exendin-4 (peptide drug acting as glucagon-like peptide-1 (GLP-1) important in controlling levels of glucose in blood), which also protects myocardium from acute ischaemic–reperfusion injury (Hausenloy & Yellon, 2008; Hausenloy & Yellon, 2012).

Protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) are two major mediators of signal transduction pathways associated with ischaemic preconditioning and pharmacological preconditioning induced cardioprotection. Figure 1.7 represents the protective mechanisms mediated by activation of PKA and PKC induced by the pharmacological preconditioning agent (exenatide) against ischaemia–reperfusion injury. Many potential cardioprotective signalling pathways elicit in responses to exenatide as pro-survival signalling cascades PI3K–Akt and adenylyl cyclase-cAMP–PKA), ROS generation, endothelial nitric oxide synthase, and PKC translocation (Hausenloy & Yellon, 2008; Hausenloy & Yellon, 2012). These signalling cascades can exert cardioprotective effects through a number of vital cellular mechanisms such as glucose up-take, mPTP inhibition, apoptosis reduction and production of cardioprotective gene factors (Hausenloy & Yellon, 2012). Volatile anaesthetic agents may also induce cardioprotection mediated by PKA and PKC signal transduction activation (Frässdorf et al., 2009).

Similarly, chronic morphine treatment exerts cardioprotective phenotype mediated by a PKC-independent pathway involving Gs coupled (stimulated adenylyl cyclase) protein, PKA and β-adrenergic receptors, whereas acute morphine induced preconditioning is mediated by Gi coupled (inhibited adenylyl cyclase) G-protein and PKC (Peart & Gross, 2006). In the case of ischaemic preconditioning, PKA activation in combination with p38 MAPK can provide a dual role in cardioprotection (Makaula et al., 2005). Interestingly, in the same study the activation of PKA by forskolin (FK; preconditioning-mimetic agent) prior to ischaemia reduces the infarct size whereas the non-selective PKA inhibitor H-89 when given to perfused

rat heart half an hour before global ischaemia-reperfusion, also decreases the infarct size and improves post-ischaemic function.

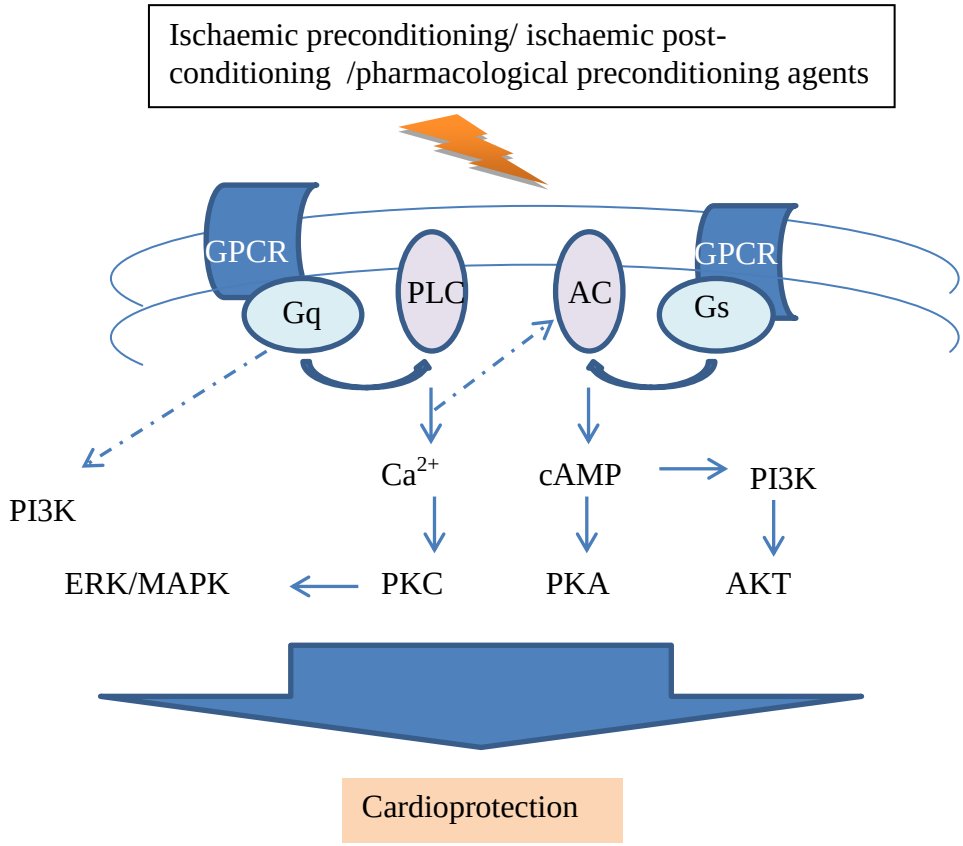



**Figure 1.7 The cardioprotective mechanisms mediated by PKA and PKC**

Shown is a schematic diagram representing the protective mechanisms mediated by activation of PKA and PKC induced by ischaemic preconditioning/ischaemic post-conditioning or pharmacological preconditioning agents against ischaemia–reperfusion injury.

Although downstream signalling of PKA activation remains unclear, a study in intact dog heart suggested that the protective effect of PKA could be through attenuation of calpain-dependent degradation of sarcolemmal proteins (Inserte et al., 2004). Another study showed that protective effects of PKA appeared to be correlated with inhibition of small GTPase Rho and its kinases (Sanada et al., 2001).

### *1.7. Protein kinases and their cellular link with TG2?*

Protein kinases are one of the most important protein families (Huston & Krebs, 1968; Manning et al., 2002), since the majority of cellular proteins undergo phosphorylation by protein kinases, which can directly control many basic cellular processes and signal transduction pathways (Ficarro et al., 2002; Manning et al., 2002). Protein kinase A and PKC are both involved in posttranslational modification of proteins mainly through phosphorylation that can result in regulation of specific protein enzymatic activity, localisation and function (Cohen, 2000).

As posttranslational modification of proteins by phosphorylation plays a critical role in the regulation of their cellular functions (Walsh, 2006; Pearce et al., 2010). Similarly, TG2 is a ubiquitous enzyme that mediates posttranslational modifications of protein, and protein-protein interactions (Fesus & Piacentini, 2002; Lorand & Graham, 2003). Transglutaminase 2 is able to crosslink or activate a variety of signalling molecules involved in cell death, cell survival and cell proliferation. For instance, the activation of TG2 and its transamination activity was shown to modulate regulation of RhoA, ERK1/2, JNK1, and p38 MAP kinases in neuronal differentiation (Singh et al., 2003). Transglutaminase 2 catalysed reactions have been shown to modulate posttranslational modification of retinoblastoma gene product (pRB) playing a role in programing cell death (Oliverio et al., 1997). At the molecular level, TG2 overexpression modulates the phosphorylation and activation of the cyclic AMP-response element (CRE)-binding protein, an event that contributes to neuronal differentiation (Tucholski & Johnson, 2003). Both over-expression and activation of TG2 enhances cAMP levels and thus, PKA activation, consequently exerting its pro-apoptotic role in tumour development (Caraglia et al., 2002). Alpha-(1B)-adrenoreceptor facilitated intracellular $Ca^{2+}$ signalling was reported to be mediated by the interaction of TG2 with phospholipase C (PLC)-$_{\delta 1}$ (Kang et al., 2002) . Although, TG2 can modulate signal transduction of different proteins either through posttranslational modification or through protein-protein interaction, the exact mechanisms by involved remains unresolved. This could be due to the protein phosphorylation events, which are responsible for the activation of different signalling pathways simultaneously.

Transglutaminase 2 possesses multiple enzymatic activities (including a kinase function; see section 1.2), and has been shown to be activated by PKA (Mishra et al., 2007). It has been reported that PKA phosphorylate TG2 at serine-216 ($Ser^{216}$) residue facilitating protein-protein interaction, increasing its kinase activity and inhibiting its transamidation activities (Mishra & Murphy, 2006; Mishra et al., 2007). A more recent study suggests that this phosphorylation is also mediated by the activation of the NF-$_k$B and its inhibition of PTEN protein has a critical role in tumour invasiveness (Wang et al., 2012). Transglutaminase 2 expression has been shown to be regulated by PKCδ activation in pancreatic cancer cells, which in turn inhibit a type II, programmed cell death (autophagy), reflecting the important role of PKC and TG2 expression in mediating autophagy (Akar et al., 2007).

Although activation of TG2 has been linked to PKC and PKA in some cell lines (Akar et al., 2007), it has not yet been investigated in the rat H9c2 cardiomyocyte derived cell lines. In addition, TG2 has many enzymatic activities, not all of which have been investigated. For example, its crosslinking activity was extensively studied in many cells and tissues with respect to its involvement in disease processes (Ruan & Johnson, 2007; Sane et al., 2007) or in protection from diseases (Datta et al., 2006; Munezane et al., 2010). However, the polyamine incorporation activity of TG2 and its role in modulating biological cellular functions has had less consideration from scientific researchers. The intracellular role of TG2 was restricted to its crosslinking activity and apoptosis regulation in response to different stressors (Iismaa et al., 2009). As the role of TG2-mediated polyamination of intracellular proteins had not been fully investigated, the focus of this current study was to determine the intracellular role of TG2-mediated polyamine incorporation in H9c2 cells in order to address this gap in TG research.

### *1.8. Cardiomyocytes function and properties*

Cardiac diseases remain the major causes of death word wide, more than three million of them occur after age sixty (Mendis et al., 2011). However, atherosclerosis and diabetes are precursors that begin earlier in life making an advanced prevention of cardiac diseases essential from childhood (McGill et al., 2008). The heart is an important organ responsible in the supply of different organs with oxygen and nutrition needed through pumping blood in blood vessels by repeated contacting and

relaxing of its cardiac muscles and associated connective tissues (Ganong & Barrett, 2005; John, 2011). In mammalia, the heart composes of four chambers derived from the mesoderm layer that differentiated later to form mesothelial pericardium. This forms the layer that coats the outer area of the heart, while the endothelium layer coats the inner area of the heart and also forms lymphatic and blood vessels (van Wijk et al., 2007). A network of cardiac neurons is also present in the heart to generate and conduct electrical action potential (Ganong & Barrett, 2005; John, 2011).

Two different types of cells exist in the heart including cardiomyocytes and cardiac pacemaker cells that are responsible for generating and transferring of the electric impulses from cell to cell (Klabunde, 2011). Cardiomyocytes are cardiac muscle cells that compose of a long chains of cardiac-contractile units (sarcomeres) forming myofibrils (Bird et al., 2003). Two types of myofibril filaments are found in sarcomeres; thick filaments composed of myosin proteins and thin filaments composed of actin proteins (Severs, 2000). Actin and alpha-actinin proteins are bound to Z-discs that form borders of sarcomeres. Myosin proteins have a long fibrous tail with a spherical head that has actin and ATP binding sites and is usually hidden by tropomyosin proteins. In the presence of $Ca^{+2}$, the tropomyosin conformation is altered and myosin head exposed and bound to actin results in the sliding of thin and thick filaments over each other and causes cardiac myocytes contraction (Alberts et al., 2002). In contrast, binding of myosin to ATP results in dissociation of actin and myosin causes cardiomyocyte relaxation (Solaro & Rarick, 1998; Alberts et al., 2002).

Cardiomyocytes are similar to skeletal muscles cells with one nucleus and contain a large number of mitochondria allowing high and rapid production of ATP (Olivetti et al., 1996). Both atria and ventricles are made up from cardiomyocytes that enable them to flexibly stretch by shortening and lengthening their fibres, a function that is critical for proper heart beating (Severs, 2000).Vimentin and desmin are two main components present in cytosol of cardiomyocytes that are responsible for holding cellular organelles and giving more flexibility to the cells (Sampayo-Reyes et al., 2006). Adult cardiomyocytes have a cylindrical shape and it has been shown that the shape of cardiomyocytes affects their electrical properties as arterial myocytes (Munk et al., 1996). Different cardiac cells are connected to each other with gap conjunctions

that permit different ions ($Na^+$, $K^+$ and $Ca^{2+}$) to diffuse into the cells which facilitates cellular depolarisation and polarisation (Martini, 2007).

The mechanism of excitation-contraction coupling is tightly controlled by calcium concentration and handling, however, mishandling and uncontrolled calcium in myocytes has been shown to be the major causes of contractile dysfunction and cardiac dysrhythmia in pathophysiological disorders (Pogwizd et al., 2001). Calcium concentration and handling are regulated by sarcoplasmic reticulum (SR), upon member depolarisation, the $Ca^{2+}$ enters the cells through voltage-gated L-type calcium channels, activated ryanodine receptors on the sarcoplasmic reticulum results in release of $Ca^{2+}$ in a process called calcium-induced calcium release (CICR) (Bers, 2002). The increase of intracellular calcium concentration allows cardiomyocyte filaments to contract, where decline of it results in relaxing (Bers, 2002). This later can be achieve by transport $Ca^{2+}$ out of cells through four different pathways including mitochondrial $Ca^{2+}$ uniporter, sarcolemmal $Na^+/Ca^{2+}$ exchange, sarcolemmal $Ca^{2+}$ -ATPase and SR-$Ca^{2+}$ -ATPase (Bers, 2000). It has been shown that the major removal of calcium (~70 %) from cytosol is through SR $Ca^{2+}$ -ATPase pump, in rabbit ventricular myocytes (Bassani et al., 1994).

The amount of $Ca^{2+}$ entry through voltage-gated L-type calcium channels is limited by calmodulin binding to C-terminal of $Ca^{2+}$ channel itself (Peterson et al., 1999). In addition, SR $Ca^{2+}$ release has a negative feedback on voltage-gated L-type calcium channels influx (Sipido et al., 1995). The $Na^+$/ $Ca^{2+}$ exchange also contributes in $Ca^{2+}$ influx mode either by elevating $Na^+$ e.g. blockage of $Na^+/K^+$-ATPase via digitalis glycosides drug or by inhibition of voltage-gated L-type calcium channels and activation of SR $Ca^{2+}$ release (Dipla et al., 1999). The sensitivity of cardiomyocytes toward $Ca^{2+}$ is decreased by high phosphate and $Mg^{2+}$ concentrations or by acidity environment of cells, as in ischaemic condition. Moreover, the activation of β-adrenergic receptors can reduced cardiomyocytes $Ca^{2+}$ sensitivity, where caffeine and certain inotropic drugs can reversed this effect (Santana et al., 1998).

B-adrenergic receptors also contribute in cardiac contraction-relaxation in which they stimulate activation of GTP-binding protein (Gs) that turns to activate adenylyl

cyclase to produce cAMP-induced PKA activation (Greenstein et al., 2004). The later kinase results in phosphorylation of many proteins that are essential for excitation–contraction coupling mechanism including, myosin binding protein C, phospholamban, L-type $Ca^{2+}$ channels, ryanodine receptor, and troponin I (Bers, 2002). For example, the phosphorylation of troponin I and phospholamban by PKA accelerates the reuptake of $Ca^{2+}$ by SR and thus dissociation of it from myofilaments results in myocardial relaxing. While phosphorylation of RyR2 by PKA at ser2809 results in dissociation of peptidyl-prolyl cis-trans isomerase (FKBP12.6) from the receptor and elevating open probability of RyR2 $Ca^{2+}$ channel (Marx et al., 2000; Wehrens et al., 2003). The activation of B-adrenergic receptors have shown to be associated with heart failure where phosphorylation level of RyR2 by PKA is high (Marx et al., 2000). Hyperphosphorylation of RyR2 by PKA induced disassociation of RyR2/FKBP12.6 complex (Marx et al., 2000) and leakage of $Ca^{2+}$ in canine heart failure model (Ono et al., 2000).

In cardiomyocytes, the phosphorylation of RyR2 by calcium/calmodulin-dependent protein kinase II (CaMKII) activates RyR2 $Ca^{2+}$ channel in manner independent of FKBP12.6. This effect showed in parallel with elevation of heart rate and $Ca^{2+}$ release (Wehrens et al., 2004). Inositol (1,4,5)-trisphosphate (InsP3) are able to induce $Ca^{2+}$ release from SR in cardiomyocytes (arterial cells), however the level of $Ca^{2+}$ release is lower and has less potential action than those release by (CICR) (Lipp et al., 2000). Although, α1- adrenergic and muscarinic agonists can trigger InsP3 production and contractile force, this effect is mediated mainly by PKC activation rather than InsP3 (Wu et al., 2006).

### *1.9. Cardiomyocytes cell culture*

Many potential strategies have been developed to address the threat of heart diseases. The use of animal models by scientific researchers has helped in understanding the underlying causes of cardiac diseases (Dhein et al., 2005; Gross, 2009). This method is used to to overcome several ethical concerns associated to the usage of human tissue. In addition, cultures of cardiomyocytes have been used as simple model for studying the alterations that occur at the cellular level under different conditions. Cardiomyocytes were isolated from both murine and rodent animals. Culturing

cardiomyocytes have been increased in the last decade due to the convenience, flexibility and economic advantages that they have over whole-animal heart experiments (Mitcheson et al., 1998). These also help in elimination of the contamination of other cell types such as, endothelium and fibroblast cells that can reduce the effects of experiments on a specific target cells types (Diaz & Wilson, 2006). Many different studies prefer pure cardiomyocytes including, cell signalling mediated ischaemia and preconditioning, subcellular components and toxicity-based studies (Dhein et al., 2005; Gross, 2009). The culture of primary cardiomyocytes has been applied for different cardiac conditions include myocardial ischemic, hypertrophy, heart failure and arrhythymias (Dhein et al., 2005).

Although primary cardiomyocyte cultures have many advantages, some drawbacks have also reported for them includes heterogeneous properties, inconsistent results and low reproducibility are often low in toxicity tests (Astashkina et al., 2012). In addition, primary cardiomyocytes are limited by shorter culture duration and suitable control is need for experiments (Louch et al., 2011). Therefore, alternative strategies have been developed to minimise these limitations. These alternative strategies are directed to develop immortalised cardiomyocyte cell lines that are able to proliferate and maintain differentiated phenotype in culture (White et al., 2004). AT-1 is the first cardiomyocyte cell line which was derived from atrial tumour of transgenic mouse. These cells have some difficulty to deal with, they must use as a primary cells because they need serial propagation, they are not suitable for passaging or recovering from cryopreserved stocks (Lanson et al., 1992). Therefore, HL-1 cell lines have been generated from AT-1and many aspects have improved. These cell lines can be serially passaged and can be also be recovered from frozen stocks. The HL-1 cardiomyocyte lines that can contract and display an adult cardiomyocyte-like pattern and similar expression of cardiac-specific receptors and proteins (Claycomb et al., 1998). Moreover, they have been employed for studying cardiac pathology, cellular signalling, electrophysiology, toxicology, cell cycle, apoptosis and calcium regulation (Strigun et al., 2012; White et al., 2004).

The cardio-myoblast H9c2 cell line, is embryonic progenitor cells derived from neonatal rat heart, has been widely used as an in vitro model as it displays a biochemical and electrophysiological properties similar to those that appear in both

cardiac and skeletal tissues (Sardão et al., 2007). Although, the H9c2 cells are non-beating cells but they have many aspects identical to primary cardiomyocytes making them a useful model for cardiovascular researchers to investigate the cellular and molecular processes involved in hypertrophy, toxicology, differentiation, and apoptosis (Pereira et al., 2011; Watkins et al., 2011). The H9c2 cells have preserved many components of hormonal and electrical signalling pathways that exist in adult cardiomyocytes (Hescheler et al., 1991). The ability of H9c2 cells to divide in comparison to terminal-differentiated cardiomyocyte makes them as an animal-free alternative (Koekemoer et al., 2009).

The role of TG2 in cardioprotection is limited to molecular regulation where its biological activities still need further research. The TG2-catalyzed posttranslational modification of the substrate proteins, through incorporation of monoamines or polyamines, can modify the physical-chemical features of the substrates (Fesus & Piacentini, 2002; Park et al., 2010; Gundemir et al., 2012), hence playing a crucial role in controlling their biological activity. Some of these targeted TG2 substrates mentioned above (section 1.3.7.3) are cytoskeleton proteins, heat shock proteins, transport proteins etc. that may also play an essential role in cardiomyocytes function. These TG2 substrates have never been investigated in cardiomyocytes and none has been linked to its cellular function. Therefore, this study investigated for the first time the modulation of TG2 in cardiomyocytes in response to phorbol-12-myristate-13-acetate (PMA) and forskolin (FK) treatments and the affected target TG2 substrate proteins.

Since it is clear that TG2, PKC, and PKA play distinct role in mediating cardioprotection during ischaemia-reperfusion injury, it was important to assess the possible relation between these protein kinases and TG2 in cardiomyocytes. The present study investigated whether TG2 activity was modulated by PKC and PKA activation in H9c2 cardiomyocyte-like cells. This would helps to determine whether the PKC and PKA signalling cascades were involved in modulating TG2-mediated cytoprotection and facilitate identification of targeted proteins. Thus, this study hypothesis that TG2 can modulate its protective effect in the heart via activation of PKA and PKC.

**Main aim**

The overall aims of this study were to investigate the modulation of TG2 activity in response to protein kinase A and protein kinase C activation in cardiomyocytes, and its role in cytoprotection.

**The specific aims were:**

- To investigate the effect of PMA and FK as PKC and PKA activators respectively on TG2 cellular protein level and activities in the H9c2 cardiomyocyte cell line.
- To study the effect of protein kinase inhibitors on TG2 activity and cellular protein level in H9c2 cardiomyocyte cell line.
- To investigate the ability of H9c2 cellular proteins to act as substrates for endogenous TG2-catalyzed polyamine incorporation reactions before and after treatment with PMA and FK.
- To study the effect of protein kinase activators and inhibitors on purified guinea pig liver TG2 activities.
- To characterise the effect of oxidative stress on TG2 activities and protein level before and after PMA/FK induced cytoprotection in H9c2 cells.
- To detect and identify the targeted TG2 substrates in the H9c2 cardiomyocyte cell line in response to PMA and FK and their protective effect against $H_2O_2$.
- To analyse the data and write up the PhD thesis.

# CHAPTER II:

# MATERIAL AND METHODS

## 2. Materials and Methods

### *2.1. Material*

#### 2.1.1. Cell culture reagents

Dulbecco's modified eagle's medium (DMEM), foetal calf serum, trypsin (10X), L-glutamine (200 mM), penicillin (10,000 $Uml^{-1}$)/streptomycin (10,000 $\mu gml^{-1}$) were purchased from BioWhittaker Ltd., Lonza, UK, Phosphate buffered saline (PBS) and serum-deficient DMEM (Gibco) were obtained from Life Technologies (Invitrogen, UK).

#### 2.1.2. Plastic ware

All sterile plastic ware was tissue culture treated and supplied by Sarstedt, UK. Cryotube vials (Nunc brand products), supplied by Merck Ltd., UK. Nunc Lab-Tek II CC2 Chamber was supplied by Thermo Fisher Scientific Inc, UK. T75 flask, 6 well plates, 24 well plates, 96 well plates, cells scraper supplied by Sarstedt, UK.

#### 2.1.3. Inhibitors

##### 2.1.3.1. Protein kinase inhibitors

Chelerythrine, Gö 6983 ({2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide}), H-89, and Ro-31-8220 ({3-[1-[3-(amidinothio) propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide bisindolylmaleimide IX, methanesulfonate}), KT 5720 and Rp-8-Cl-cAMPS were purchased from Calbiochem, UK.

##### 2.1.3.2. Transglutaminase inhibitors

The irreversible TG2 inhibitor Z-DON (Benzyloxycarbonyl-(6-Diazo-5-oxonorleucinyl)-L-Valinyl-L-Prolinyl-L-Leucinmethylester) Z-DON-Val-Pro-Leu-OMe) was obtained from Zedira (GmbH, Germany). R283 (1,3-dimethyl-2[(oxopropyl)thio]imidazolium) was synthesized by Dr I Coutts at Nottingham Trent University, UK.

#### 2.1.3.3. Protease and phosphatase inhibitors

Protease inhibitor cocktail, phosphatase inhibitor 2 and 3 cocktails were obtained from Sigma-Aldrich Company Ltd., UK.

### 2.1.4. Transglutaminase substrates

Casein and N′,N′-dimethylcasein were obtained from Sigma-Aldrich Company Ltd., UK. Biotin-TVQQEL was purchased from Pepceuticals, UK. Trifluoroacetic acid salt (biotin-X-cadaverine) (5-(((N-(biotinoyl)amino)hexanoyl)amino) pentylamine, trifluoroacetic acid salt) and biotin-cadaverine (N-(5-aminopentyl)biotinamide, trifluoroacetic acid salt) were ordered from Invitrogen, UK.

### 2.1.5. Agonist and antagonist

N6-cyclopentyadenosine (CPA) or isoproterenol (ISO) 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) adenosine A1 receptor antagonist were obtained from Sigma-Aldrich Company Ltd. UK. Phorbol-12-myristate-13-acetate (PMA) was purchased from Tocris Bioscience, UK and Forskolin (FK) was ordered from Sigma-Aldrich, UK.

### 2.1.6. Antibodies

#### 2.1.6.1. Primary antibodies

Primary antibodies that have been used in this study are listed in table (2.1) including working dilutions for both Western blotting and immunocytochemistry techniques.

**Table 2.1 Primary antibodies and working dilutions required for Western blotting and immunocytochemistry techniques**

| Antibody | Working dilution (Western blotting) | Working dilution (immuno-cytochemistry) | Company/Catalogue number |
|---|---|---|---|
| Mouse GAPDH **(monoclonal)** | 1:200 | N/A | Santa Cruz Biotechnology, UK. (sc-32233) |
| Mouse CUB-7402 **(monoclonal)** | 1:1000 | N/A | Abcam, UK (ab2386) |
| Mouse anti-α-tubulin (B512) **(monoclonal)** | 1:2000 | 1:20 | Sigma-Aldrich, UK (T9026) |
| Mouse anti-TGase 1 **(monoclonal)** | 1:1000 | N/A | Abcam, UK (ab167657) |
| Rabbit anti-TGase 3 **(polyclonal)** | 1:1000 | N/A | Abcam, UK (ab27001) |
| Mouse anti-phosphotyrosine **(monoclonal)** | 1:1000 | N/A | Cell Signalling Technology Inc, UK (9411) |
| Mouse anti-phosphothreonine **(monoclonal)** | 1:1000 | N/A | Cell Signalling Technology Inc, UK (9391) |
| Mouse Anti-phosphoserine **(monoclonal)** | 1:1000 | N/A | Cell Signalling Technology Inc, UK (9606) |
| Mouse anti-phospho-specific ERK1/2 ($Thr^{202}/Tyr^{204}$) **(monoclonal)** | 1:2000 | N/A | Sigma-Aldrich, UK (E7028) |
| Mouse anti-pAKT **(monoclonal)** | 1:2000 | N/A | Cell Signalling Technology Inc, UK (4051) |
| Rabbit anti (MAO-B) **(monoclonal)** | 1:1000 | 1:20 | Abcam, UK (ab125010) |
| Mouse anti-Calnexin (AF18) **(monoclonal)** | 1:1000 | 1:20 | Santa Cruz Biotechnology, UK. (sc-23954) |
| Mouse anti-α-actinin **(monoclonal)** | 1:1000 | 1:20 | Sigma-Aldrich, UK (A5044) |
| Rabbit anti-active caspase3 **(monoclonal)** | 1:5000 | 1:10 | Cell Signalling Technology Inc, UK (9664) |
| Mouse anti-lamin **(polyclonal)** | 1:2000 | N/A | Sigma-Aldrich, UK (L1293) |
| Mouse anti-TG2 (ID10) **monoclonal** | 1:200 | N/A | antibodies (Griffin, School of Biomedical and Natural Sciences, NTU), |

### 2.1.6.2. Secondary antibodies

Secondary antibodies that have been used in this study are listed in table (2.2) including working dilutions for both Western blotting and immunocytochemistry techniques.

**Table 2.2 Secondary antibodies and working dilutions required for Western blotting and immunocytochemistry techniques**

| Antibody | Working dilution (Western blotting) | Working dilution (immuno-cytochemistry) | Company/ Catalogue number |
|---|---|---|---|
| peroxidase-conjugated®ExtrAvidin | 1:5000 | N/A | Sigma-Aldrich, UK (E2886) |
| anti-mouse IgG-HRP | 1:5000 | N/A | Sigma-Aldrich, UK (A4416) |
| anti-rabbit IgG-HRP | 1:5000 | N/A | Sigma-Aldrich, UK (A0545) |
| ExtrAvidin®-FITC | N/A | 1:200 | Sigma-Aldrich, UK (E2761) |
| anti-mouse-Alexa 568 | N/A | 1:200 | Molecular Probes (Invitrogen, UK) (A-11031) |
| anti-rabbit-Alexa 568 | N/A | 1:200 | Molecular Probes (Invitrogen, UK) (A10042) |

### 2.1.7. Chemical reagents

Ammonium persulphate (APS), bromophenol blue, bicinchoninic acid (BCA), 3-((3-cholamidopropyl) dimethylammonium)-1-propanesulfonate (CHAPS), copper (II) phthalocyanine, β-mercaptoethanol, iodoacetamide, mineral oil, sodium hydroxide (NaOH), sodium chloride, tris (hydroxymethyl) aminomethane, triton X-100, TWEEN®80, urea are from Sigma-Aldrich, UK. AccuGel™ 29:1 acrylamide, electrophoresis running buffer (10x), electrophoresis transfer buffer (10x), protogel® resolving buffer (4x), protogel®stacking buffer are from Geneflow, UK. Acetone, dimethyl sulfoxide (DMSO), ethanol, glacial acetic acid, glycerol, methanol, sodium azide, sodium phosphate dibasic, sodium phosphate monobasic are all from Fisher Scientific, UK. Dithiothreitol (DTT), bovine serum albumin (BSA), sodium dodecyl sulphate (SDS) are from Melford Laboratories Ltd., UK. Carrier ampholytes (Bio-Rad Laboratories Ltd., UK), enhanced chemiluminescence reagent (ECL) and Brillant

Blue R-250 Protein Gel Stain are from Uptima, Interchim, France, N,N,N',N'-tetramethylethylenediamine (TEMED) (National Diagnostics, USA) and blueye prestained protein ladder (Geneflow Limited., UK).

### *2.2. Methods*

#### 2.2.1. Cell Culture

All cell culture was carried out using aseptic techniques and all cells were grown in T75 culture flasks. H9c2 cell line (rat neonatal ventricular myocytes) was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). They were cultured in DMEM, supplemented with 2 mM of L-glutamine, 10 % (v/v) (FCS) foetal calf serum (Biosera, UK), 100 $\mu gml^{-1}$ of streptomycin and 100 $Uml^{-1}$ of penicillin. Cells were grown in a humidified atmosphere of 5 % (v/v) $CO_2$ at 37°C until 80 % confluence was reached. The medium was removed and the cells were rinsed by adding 10 ml of sterile phosphate buffered saline (PBS) and then they were incubated with 2 ml of (1x) trypsin (Lonza, UK). The cell flask was then incubated for 2-3 min at 37°C and monitored under the light microscope at (100x) magnification (Olympus CK40-SLP, Japan). A ten ml of medium was added to the flask, the cells were removed and then centrifuged at 5000 xg for 5 min using Sanyo Harrier 18/80 refrigerated centrifuge (Sanyo Gallenkamp, UK) at 25°C. The pellets were re-suspended in 1 ml of medium. The cell suspensions were split and divided into four fresh flasks. The rate of H9c2 cell growth was also measured by counting cells (see section 2.2.2) every day for 7 days.

#### 2.2.2. Cells count

The amount of viable cells was determined prior to any treatment. Trypan blue exclusion assay a method described previously by Wang (2006) was used to estimate viable cell count. The H9c2 cell pellets were re-suspended in 1.0 ml of medium that was prepared during sub-culturing (section 2.2.1); 10 µl of cell re-suspension was mixed with 10 µl trypan blue dye. This mixture was pipette onto an Improved Neubauer haemocytometer (Camlab, UK) (0.1 mm depth, 400 $mm^{-2}$) and the cells were counted in the four (0.1 $mm^3$) corner squares, including those touching the left and bottom wells using a light microscope at (100x) magnification (Olympus CK40-

SLP, Japan). The cell density per ml was then calculated according to the following formula;

Cell density = cell number (mean from four fields) x $10^4$ x dilution factor

### 2.2.3. Cell treatments

The steps for each treatment incubation are shown in figure 2.1

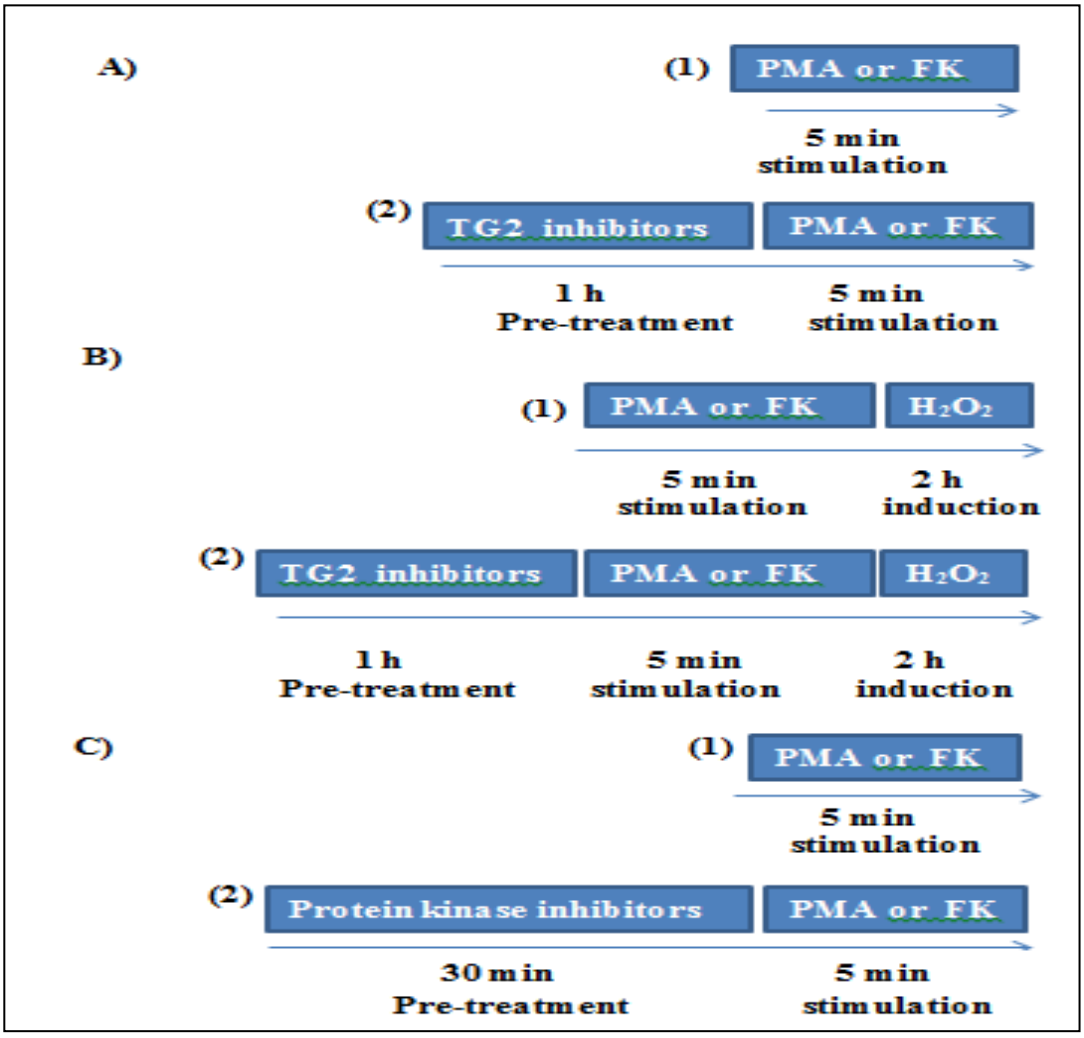



**Figure 2.1 The flow diagram represents experimental incubation steps for different treatments**

- **A)** Protein kinase activators treatment in presence (2) or absence (1) of TG2 inhibitors.
- **B)** Oxidative stress-induced cell death: PMA and FK-induced cytoprotection in presence (2) or absence (1) of TG2 inhibitors.
- **C)** Protein kinase activators treatment in presence (2) or absence (1) of protein kinase inhibitors.

#### 2.2.3.1. Protein kinase activators treatment

In various experiments, H9c2 cells were treated at 80 % confluence with or without 1 μM phorbol-12-myristate-13-acetate (PMA; Tocris Bioscience, UK) or 10 μM Forskolin (FK; Sigma, UK) (from a 10 mM stock solution that was dissolved in dimethyl sulfoxide (DMSO) and further diluted with DMEM. For time courses experiments, cells were treated with either PMA or FK for 1, 5, 10, 20 & 40 min. In other experiments, cells were also incubated for 5 min with different concentrations of PMA (0.005, 0.010, 0.032, 0.05, 0.100, 0.3161 and 1.000 μM) or B) FK (0.100, 0.30, 0.50, 1.00, 3.02, 5.01 and 10 μM). Control cells were also treated with the appropriate volume of DMSO equal to PMA and FK volumes that was also compared to cells untreated (with DMEM). Changes in H9c2 cell morphology were monitored and visualised using an inverted light microscope (Olympus CKX31SF, Philippine).

#### 2.2.3.2. Protein kinase inhibitors treatment

All protein kinase inhibitors were obtain from Calbiochem, UK and prepared as a 10 mM stock solutions dissolved in DMSO and further diluted with DMEM to obtain various final concentrations.

Cultures of H9c2 cells (80 % confluent) were pre-incubated in DMEM medium with or without the kinase inhibitors (5 μM Gö 6983 {2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide} a protein kinase C inhibitor, 10 μM RO-31-8220 {3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide bisindolylmaleimide IX, methanesulfonate} a protein kinase C inhibitor, 1 μM chelerythrine chloride a protein kinase C inhibitor, 1 μM H-89 dihydrochloride 5 μM KT 5720 and 50 μM Rp-8-Cl-cAMPS a protein kinase A inhibitor for 30 min. Medium containing different inhibitors was removed and replaced with medium containing 1 μM PMA or 10 μM FK and incubated for 5 min. Control cells were treated also with the appropriate volume of DMSO equal to each drugs volumes (the vehicle-treated control). Cells untreated (with DMEM) served as control.

#### 2.2.3.3. Oxidative stress-induced cell death: PMA and FK-induced cytoprotection

H9c2 cells in fully supplemented DMEM were pre-treated for 5 min with 1 μM PMA or 10 μM FK prior to 2h exposure to 600 μM $H_2O_2$. Where appropriate, cells were also treated for 1h with the TG2 inhibitors Z-DON (Z-DON-Val-Pro-Leu-OMe) (150

μM) or R283 1,3-dimethyl-2[(oxopropyl)thio]imidazolium (200 μM) before the addition of PMA or FK. Medium containing PMA/FK and TG2 inhibitors was removed and replaced with fresh fully supplemented DMEM prior to $H_2O_2$ treatment.

### 2.2.4. Determination of H9c2 morphological change

Coomassie blue staining for morphological change detection is a method described by Mochizuki & Furukawa (1987). To observe the cell morphological change, H9c2 cells were plated in 24-well flat bottomed plates (25,000 cells per well) and allowed to recover for 24h in fully supplemented DMEM. They were then exposed to different treatments as described previously (section 2.2.3.3). Growth medium was aspirated from the H9c2 cells using vacuum. Cells were rinsed twice with 500 μl of chilled PBS flowed by fixation for 10 min with 500 μl per well of ice-cold 90 % (v/v) methanol/PBS. Fixing solution was removed by aspiration and cells were stained for ~10 min at room temperature (25°C) in 0.25 % (w/v) Coomassie blue stain in 25 % (v/v) ethanol, 10 % (v/v) glacial acetic acid aqueous solution. Cells were rinsed twice in distilled water to remove excess stain and left to air-dry. In order to determined morphological change, cells were observed using an inverted light microscope at (100x) magnification and digital images were captured on a Canon PC 1200 camera.

### 2.2.5. Cell extraction

Cultures of H9c2 cells were rinsed twice with 2 ml of chilled PBS, lysed with 500 μl of ice-cold lysis buffer containing; 50 mM Tris-HCl pH 8.0, 0.5 % (w/v) sodium deoxycholate, protease inhibitors 5 mg $ml^{-1}$ with or without 10 mg $ml^{-1}$ phosphatase inhibitors cocktail 2 and 3 (Sigma, UK). Cell lysates were scraped and clarified by centrifugation at 4°C for 20 min at 14000 xg (Scientific Laboratory, UK). Supernatants were collected in new sterile 1.5 ml Eppendorf tubes (Fisher Scientific, UK) and stored in at -20°C.

### 2.2.6. Acetone precipitation

The proteins solubility could be dropped and precipitated and this could be induced by reducing the effective dielectric constant of the media. This was usually achieved by adding a water-soluble solvent with a small relative permittivity (dielectric constant), such as acetone, to an aqueous solution of protein (Jiang et al., 2004). This method was used when sample concentration was required. In brief, nine volumes of

ice cold (-20°C) acetone were added to the sample, vortex mixed for 15 seconds and left overnight to precipitate at -20°C. The samples were centrifuged at 300 xg for 20 min at 4°C, the supernatant was removed and the pellet was air dried for 20 min at 20°C. The pellet was subsequently suspended in an appropriate volume of buffer.

### 2.2.7. Protein estimation

Bicinchoninic acid (BCA) protein assay was based on the method of Stoscheck (1990) using a kit from Sigma (Poole, UK) was used to estimate protein concentration in H9c2 extracts. Kits contain; 1 % (w/v) bicinchoninic acid solution (reagent A), 4% (w/v) copper (II) sulphate pentahydrate (reagent B) and protein standard solution (bovine serum albumin). Reagent C was prepared by mixing reagent A with reagent B at 50:1 ratio. Bovine serum albumin (BSA) protein standards were prepared in a range of 0–1 mg $ml^{-1}$ in an appropriate volume of buffer. In a 96-well plate (Nunc), a volume of 25 µl of either BSA protein or unknown sample were incubated with 200 µl of reagent C at 37°C for 30 min. The absorbance of the samples was measured at 570 nm using a plate reader (Expert 96, Scientific laboratory, UK) and a calibration graph was plotted for protein range 0-1 m $gml^{-1}$.

### 2.2.8. Subcellular fractionation

To determine subcellular distribution of TG2 in H9c2 cells, H9c2 cells were sub-fractionated. H9c2 cells were grown T125 culture flasks and then treated with 1 µM PMA or 10 µM FK (section 2.2.3.1). After which they were lysed with 500 µl of subcellular fractionation buffer of 20 mM HEPES (pH 7.4) containing; 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA and freshly 1 mM DTT and (10 µl) 10 mg $ml^{-1}$ phosphatase inhibitors cocktail 2 and 3 were added. The plates were scraped immediately and cells lysate were transferred to 1.5 ml eppendorf tubes and placed in ice. Cells lysate was homogenised and passed through a 25 Ga needle (10x) using a 1 ml syringe, and incubated in ice for 20 min. The nuclear pellet was centrifuged out at 720 xg for 5 min and supernatant was transferred to a new 1.5 ml microfuge tube. The pellet was then washed (1x) by subcellular fractionation buffer and centrifuged again at 720 xg for 10 min. The washing buffer was removed and pellet was re-suspended in lysate buffer (section 2.2.5). The supernatant was centrifuged at 10,000 xg for 20 min to separate the crude

mitochondria. The pellet was then washed as was with nuclear pellet and re-suspended in lysate buffer. The supernatant was centrifuged for a further 1h at 80,000 xg to isolate the mixed microsomal fraction. The pellet was washed as before and re-centrifuged for further 45 min. The ER pellet was re-suspended in the same buffer as used for the above fractions. The supernatant (containing cell cytosol) was subjected to acetone precipitation (section 2.2.6) and the precipitated pellet was re-suspended in lysate buffer. Protein concentration was then determined for subcellular fractions (section 2.2.7). The subcellular fractions were analysed by Western blotting (section, 2.2.12) using monoclonal antibodies to TG2 (Cub7402), calnexin (Sigma, UK), lamin and α-tubulin (B512) (Sigma, UK).

### 2.2.9. Transglutaminase activity

#### 2.2.9.1. *In vitro* TG2 activity

Transglutaminase activity was monitored by two different assays;

##### 2.2.9.1.1. Biotin cadaverine-incorporation assay

The assay was performed as the method described by Slaughter et al., (1992) with modifications of Lilley et al., (1998). Briefly, 96 well microtitre plates (Maxisorp Nunc, UK) were coated overnight at 4°C with 250 µl of N′,N′-dimethylcasein (5 ml of 10 mg $ml^{-1}$ in 50 ml of 100 mM Tris-HCl, pH 8.0). After discarding the unbound protein the plate was washed with pure water and blocked with 250 µl of 3 % (w/v) BSA in 0.1 M Tris-HCl, pH 8.5 and incubated for half an hour at 37°C. The plate was washed and 20 µl of biotin-cadaverine (25 mg $ml^{-1}$ stock in 50 mM Tris-HCl pH 8.0) and 5 µl of β-mercaptoethanol (Sigma, UK) were freshly added to both 10 ml of 6.67 mM calcium chloride or 13.3 mM EDTA dissolved in 100 mM Tris-HCl pH 8.0. For each well 150 µl of these buffers were added separately and 50 µl of samples or diluted standard TG2 (Guinea pig liver TGase; Sigma, UK) (1 mg $ml^{-1}$; standard TG2 that was prepared in 50 mM Tris–HCl, pH 8.0, and stored in aliquots at -80°C) along with negative control (100 mM Tris buffer). The plate was then incubated for an hour at 37°C and washed as before. Then, a volume of 200 µl of 100 mM Tris-HCl pH 8.0 containing 1:5 dilution of ExtrAvidin® peroxidase was added to each well and the plate was incubated at 37°C for 45 min then washed as before. The plate was developed with 200 µl of developing buffer (75 µl of 10 mg $ml^{-1}$ TMB and 1.5 µl of 3

% (v/v) hydrogen peroxide were added to 10 ml of 100 mM sodium acetate, pH 6.0) that was freshly made and incubated at room temperature for 5-15 min. The reaction was terminated by adding 50 µl of 5 M sulphuric acid. The absorbance was read at 450 nm using a plate reader (Expert 96, Scientific laboratory, UK). One unit of transglutaminase activity was defined as a change in absorbance of 0.01 at 450 nm per hour. The specific activity of TG in different samples was calculated as follow;

Average signal of calcium in three wells – Average signal of EDTA in three wells X 100 / 2.216 (equation of purified TG activity standard curve of biotin cadaverine-incorporation) / protein concentration of sample.

2.2.9.1.2. Biotin-TVQQEL crosslinking assay

The assay was performed according to the method of Trigwell et al., (2004) with minor modifications. Briefly, casein (sodium salt) was dissolved in 10 mM Tris-HCl pH 8.0, at 5 mg $ml^{-1}$ and stored in aliquots at -20°C until required. Microtitre plate 96 wells (Maxisorp Nunc, UK) were coated and incubation overnight at 4°C with casein at 1.0 mg $ml^{-1}$ in 100 mM Tris-HCl, pH 8.0 (250 µl per well). Wells were washed two times with distilled water, before the addition of 250 µl of blocking solution (100 mM Tris-HCl, pH 8.0 containing 0.1 % (w/v) BSA. The plate was incubated at 37°C for 1h. The wells were washed as before; then 150 µl of reaction buffer (100 mM Tris–HCl (pH 8.5) containing; 5 µl of β-mercaptoethanol (Sigma, UK), 5 µl biotin-TVQQEL (Pepceuticals, UK) (25 mg $ml^{-1}$ stock in 50 mM Tris-HCl pH 8.0) and either 6.7 mM $CaCl_2$ or 13.3 mM EDTA were added to each well. The reaction was started by the addition of 50 µl of extract samples or diluted standard TG2 to each well and allowed to proceed for 1h at 37°C. The reaction development and termination were performed as described in (section 2.2.8.1.1). The specific activity of TG in different samples was calculated as follow; Average signal of calcium in three wells – Average signal of EDTA in three wells X 100 / 2.269 (equation of purified TG activity standard curve of Biotin-TVQQEL crosslinking) / protein concentration of sample.

2.2.9.2. *In situ* TG2 activity

H9c2 cells were seeded on 8-well chamber slides (Thermo Fisher Scientific, UK) at density of ($1.5 \times 10^4$ cells/well) and cultured for 24h in fully supplemented DMEM.

The medium was then removed and adherent cells gently washed with PBS and slides incubated for 4h with 1 mM biotin-X-cadaverine (5-(((N-(biotinoyl)amino)hexanoyl)amino) pentylamine, trifluoroacetic acid salt) (1:100 (v/v) in DMEM). Cells were then treated for 1h with the TG2 inhibitors Z-DON (Z-DON-Val-Pro-Leu-OMe) (150 μM) before the addition of either 1 μM PMA or 10 μM FK for 5, 10, or 20 min. Following stimulation, cells were fixed with 3.7 % (w/v) paraformaldehyde in PBS for 15 min at room temperature and permeabilised with 0.1 % (v/v) Triton-X100 in PBS for 15 min at room temperature. Each step was followed by (3x) 5 min washes with PBS. Finally, cells were blocked with 3 % (w/v) BSA for 1h at room temperature and transamidated and cross-linked cadaverine substrates detected by 1:200 (v/v) ExtrAvidin®-FITC (green fluorescence; Sigma-Aldrich, UK). Nuclei were stained with blue fluorescence (DAPI; Invitrogen, UK) and viewed at (400x) magnification using fluorescence microscope (Olympus BX51, Japan).

### 2.2.10. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecylsulphate-polyacrylamide gel electrophoresis was performed by the method described by Laemmli (1970) with modifications. For making 10.0 % (SDS-PAGE) in 10 ml, the resolving gel was prepared by combining 3.38 ml acrylamide (AccuGel 29:1 30 %; National Diagnostic, USA), 4 ml distilled water, 2.5 ml of (4x) resolving buffer (National Diagnostic, USA), 100 μl of freshly made 10 % (w/v) ammonium persulphate (APS) and 15 μl N,N,N',N'-tetramethylethylenediamine (TEMED) were mixed in a beaker and poured quickly into the gel casting mould and 2 cm below the bottom of the comb was left for the stacking gel. The bubbles that were generated were removed by overlaying with water. This was left for 30 min until the gel was polymerized completely. For making 10 ml stacking gel, 1.3 ml acrylamide, 6.1 ml distilled water, 2.5 ml of (4x) stacking buffer (National Diagnostic, USA), 100 μl of 10 % (w/v) APS and 40 μl TEMED were mixed in beaker and poured quickly into the gel casting assembly upon the resolving gel and left for 30 min to completely polymerize. For 50 μg protein sample, 20 μl of (4x) sample buffer were added to 60 μl of extracted protein and boiled for 5 min; after cooling the denatured samples were loaded in gel wells along with 3 μl of molecular weight standards Precision Plus Protein standards (Bio-Rad, UK). Using the Bio-Rad

(Bio-Rad Laboratories, USA) apparatus, the gel was subjected to electrophoresis at 175 v for one hour.

### 2.2.11. Agarose gel electrophoresis

#### 2.2.11.1. Preparation and casting

The mini gel-casting tray (Bio-Rad Laboratories, Hercules, USA) was rinsed with 95 % (v/v) ethanol and dried. The gel-casting tray was then assembled and the comb level was adjusted so that it resets with a few mm of space between the comb teeth and the casting tray. The 2.5 % (w/v) agarose gel was prepared by mixing 2.5 g of agarose powder (Bioline Reagents Ltd., UK) in 100 ml of (1x) TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH of 8.3) that was made freshly from (10x) TBE buffer. The mixture was melted in microwave for 2-3 min with frequently gentle mix until no particles appear. When the melted agarose gel was cold down to ~40°C, a 5 µl of SYBR Safe DNA gel stain (Life Technologies, Invitrogen, UK) was added. A 50 ml of melted agarose gel was poured into the casting tray and was left for 10-15 min until the gel was solidifying completely.

#### 2.2.11.2. Loading and running the agarose gel

A 5 µl of loading dye (Bioline Reagents Ltd., UK) was added to each sample. Samples were mix and briefly centrifuged. A casting tray containing the agarose gel into the electrophoresis chamber and the (1x) TBE (Tris-Borate-EDTA; electrophoresis buffer) was gradually added to the chamber until the buffer just covered the gel. Samples along with 5 µl DNA ladder day (Bioline Reagents Ltd., UK) were loaded and the gel was run at 100 v for 1h or till the dye has migrated to within ¾ of the length of the gel.

### 2.2.12. Western blot analysis

To investigate the presence of TGs and phosphoproteins, the protein extracts (50 µg per lane) and 100 nmol standard TG2 were separated by 10 % (w/v) SDS-PAGE (section 2.2.9) and transferred onto a nitrocellulose membrane by using the wet-transferring system (Bio-Rad Laboratories, Hercules, USA) as described by Towbin et al. (1979). The membrane was blocked with 3 % (w/v) skimmed milk powder in TBS containing 0.1 % (v/v) Tween-20 and probed with (1:1000, v/v) anti-phosphotyrosine,

anti-phosphothreonine, anti-phosphoserine, (1:2000, v/v) phospho-specific ERK1/2, (ID10) monoclonal antibodies, anti-TG2 (TG 100), anti-TG2 (CUB 7402) anti-TG1 or anti-TG3. Horseradish peroxidase-conjugated anti-mouse IgG, Horseradish peroxidase-conjugated anti- rabbit IgG, Horseradish peroxidase-conjugated anti- goat IgG and goat anti-mouse IgG Horseradish peroxidase-conjugated were used as appropriate secondary antibodies. Immunoreactive proteins were detected by enhanced chemiluminescence (ECL). The ECL detection was performed using the Fujifilm Intelligent dark box system (Fujifilm, UK) according to the manufacturer's instructions one volume of ECL reagent A and two volumes of ECL reagent B were mixed to a final volume of 1 ml. Mixed ECL substrate was spread well and incubated with blotted nitrocellulose membrane for one minute. Excess ECL substrate was then drained off using filter paper, the nitrocellulose was placed directly into the dark box and chemiluminescence was detected following the manufacturer's instructions. Band intensities were quantified by densitometry (Adobe Photoshop CS4). The histogram within the software reported the intensity for each band. This was divided by GAPDH band intensity (normalisation) and then relative intensity to control was calculated (normalised intensity / control intensity X 100).

### 2.2.13. Stripping and reprobing of Western blots

Stripping and re-probing of western blots was carried out as described by Kaufmann et al., (1987) with modifications. Membranes were submerged in stripping buffer (100 mM β-mercaptoethanol, 2 % (w/v) SDS, 62.5 mM Tris-HCl at pH 6.7) and incubated for 30 min at 50°C. The membranes were then washed twice for ten min in TBS-Tween. The membranes were then blocked and probed as described in (section 2.2.11).

### 2.2.14. Two-dimensional gel electrophoresis

Two-dimensional electrophoresis was carried out according to Nirmalan et al., (2004). The proteins that were extracted from H9c2 treated cells were acetone precipitated, and the proteins pellet was dissolved in rehydration buffer containing; 8 M urea, 4% (w/v) CHAPS, 50 mM DTT, 0.2 % (v/v) carrier ampholytes, 0.0002 % (w/v) Bromophenol Blue. In the first-dimension isoelectric focusing, an equal amount of protein was loaded on immobilized pH gradient (IPG) strips with a pH range of 3 to

10 and focused with a Protean isoelectric focusing cell (Bio-Rad Laboratories, Hercules, USA). For second-dimension, the IPG strips were equilibrated for 15 min with 10 % (w/v) DTT (as a reducing agent to break disulfide bonds) in 10 ml of an equilibration buffer (50 mM Tris base, pH 8.8, 6 M urea, 30 % (v/v) glycerol and 2 % (w/v) SDS) and for further 15 min with 25 % (w/v) iodoacetamide (to prevent any reformed disulfide bonds) in the equilibration buffer. Each IPG strip was loaded onto a gel of the appropriate percentage of acrylamide, sealed with 1 % (w/v) agarose, and polyacrylamide gel electrophoresis was performed as describe in section (2.2.10). After electrophoresis, the proteins were fixed and visualized using PlusOne silver staining kit (GE Healthcare life science, UK) according to the manufacturer's suggested protocols (section 2.2.16). Gels were imaged in Biomolecular Imagers (FLA 7000, FUJIFILM, life sciences, UK).

### 2.2.15. Phosphorylated protein and total protein stains

In order to evaluate phosphorylation events in H9c2 after different treatments, cell lysates of treated cells were assayed for protein concentration using the BCA assay (section 2.2.7), denatured with (4x) SDS sample buffer at 95°C for 5 min and separated by 10 % SDS-PAGE (section 2.2.10). To visualise proteins, electrophoresed gels were stained with 0.2 % (w/v) Coomassie blue (Uptima, Interchim, France) and then destained with $ddH_2O$. However, to visualise phosphoproteins, the gel was stained with Pro-Q®Diamond phosphoprotein gel stain (Invitrogen, UK) and subsequently with SYPRO®Ruby Protein Gel Stain (Invitrogen, UK) to quantify the total proteins and to determine the ratio of phospho protein to total protein ratio. The method was performed according to the supplemented protocol of Invitrogen Detection Technologies. Briefly, the separated proteins were fixed with ~100 ml of fix solution (freshly prepared from 50 % (v/v) methanol, 10 % (v/v) acetic acid and made up to 100 ml $ddH_2O$) for 1 hour at R/T. The gel was washed with ~100 ml of ultrapure water for (3x) 10 min and was stained with 60 ml of Pro-Q®Diamond phosphoprotein gel stain with gentle agitation in the dark for ~90 min. To visualize the gel, it was destained with 80–100 ml of destain solution (50 ml of 1 M sodium acetate, pH 4.0, 750 ml of ultrapure water, and 200 ml of acetonitrile) for (3x) 30 min at R/T, with protection from light. Each gel was washed twice with ultrapure water at R/T for 5 min per wash. Then the gels were imaged in a Biomolecular Imagers (FLA

7000, FUJIFILM, life sciences, UK). For total-protein stain, the gel was rinsed with ultrapure water twice for 5 min and then incubated directly in 60 ml of SYPRO®Ruby-gel stain solution for 24 h with protection from light. To visualize the gel, it was destained with 80 ml of wash solution (freshly prepared from 10 % (v/v) methanol, 7 % (v/v) acetic acid and made up to 100 ml dd$H_2O$) for (3x) 30 min at R/T. The gels were then scanned in a Biomolecular Imagers (FLA 7000, FUJIFILM, life sciences, UK).

### 2.2.16. Silver stain

After resolving protein in 1D or 2D gel, the proteins were visualised by silver stain (PlusOne™Silver Staining Kit, Protein, GE Healthcare, UK). The method was performed according to the GE Healthcare protocol, briefly; the polyacrylamide gels were fixed with ~250 ml of fix solution (freshly prepared from 40 % (v/v) ethanol, 10 % (v/v) acetic acid and made up to 250 ml dd$H_2O$) for 2 hours R/T. These after which the fixation solution was removed and replaced by 250 ml of sensitizing solution containing; 35 % (v/v) ethanol, 5 % (w/v) sodium thiosulphate, 0.8497 M sodium acetate, 25 % (w/v) glutaraldehyde and up to 250 ml dd$H_2O$ and left in shaking for at least 30 min. The gel was washed with ~100 ml of ultrapure water for (3x) 10 min. The gel was stained with 250 ml 2.5 % (w/v) silver nitrate solution with gentle agitation in the dark for ~90 min. The silver solution was removed and gel rinsed twice in distilled water for one minute each time. To visualise the gel, a 250 ml of developing solution containing; 0.239 M sodium carbonate, 37 % (w/v) formaldehyde and made up to 250 ml dd$H_2O$ were added and left shaking for 2 to 5 min. When the bands or spots reach a desired intensity, gel was then transferred to stopping solution containing; 39.22 mM EDTA-$Na_2$•$2H_2O$ in 250 ml dd$H_2O$. Each gel was washed twice with ultrapure water at R/T for 5 min per wash. Then gels were imaged in Biomolecular Imagers (FLA 7000, FUJIFILM, life sciences, UK). Subsequently, the gels were analysed by Progenesis SameSpots software, UK.

### 2.2.17. Biotinylation and fractionation of TG2 substrates

Biotin-X-cadaverine (5-(((N-(biotinoyl)amino)hexanoyl)amino) pentylamine, trifluoroacetic acid salt) represents the acyl-acceptor probe for TG2 (Ruoppolo et al., 2003), was used for labelling TG2 substrate proteins in PMA treated H9c2 cells. 180

mM stock of Biotin-X-cadaverine was prepared at 0.1 mg $ml^{-1}$ in DMSO, 10 µl of which were further diluted in 2 ml of complete DMEM medium to reach final concentration of 1 mM and pre-incubated with H9c2 monolayers in T75 flasks for 4h. After discarding the labelling medium and washing with PBS, H9c2 cells were then treated with 1 µM of PMA in fresh medium for 5 min and proteins were extracted as described earlier (section 2.2.5). CaptAvidin®agarose sedimented bead suspension (Invitrogen, UK) was then used to isolate the bound biotinylated ligands (Fig. 2.2) as follows; 200 µl CaptAvidin beads were suspended in 800 µl biotin-binding buffer (50 mM citrate phosphate buffer, pH 6.0) and mixed well. Cell lysate proteins (500 µg) were re-suspended in 500 µl of biotin-binding buffer and incubated with 100 µl CaptAvidin beads overnight at 4°C with gentle agitation. Samples were centrifuged at 3000 xg for 15 min and unbound material (supernatant) was collected. CaptAvidin beads were washed with 10 mM Tris-HCl, pH 8.0 and centrifuged at 3000 xg for 3 min and the supernatant was collected (this step was repeated twice). The biotinylated polypeptides were released from the beads using 50 mM sodium bicarbonate pH 10 buffer and boiling in 30 µl of SDS sample buffer for 5 min at 95°C (section 2.2.10). The bound and unbound material were resolved in 4-15 % gradient gel (Bio-Rad, UK) followed by Western blotting (section 2.2.12) and probing with HRP-conjugated®ExtrAvidin-peroxidase to detect TG2 substrate proteins.

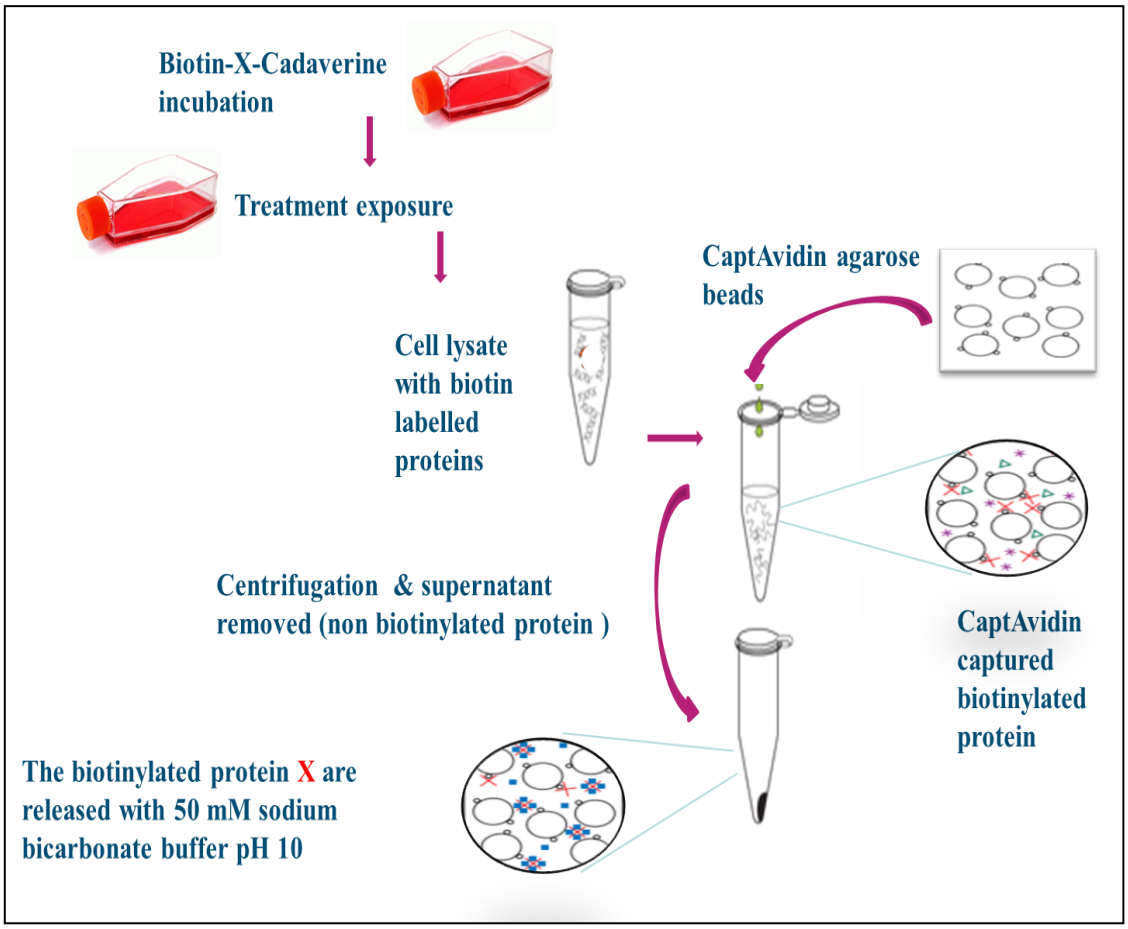



**Figure 2.2 The flow diagram represents fractionation steps of acyl-acceptor binding TG2 substrates**

As scheme in figure 2.1, H9c2 cells were pre-incubated with 1 mM biotin-X-cadaverine for 4h in culture hood. After different treatment biotin labelled proteins in cells lysate was subjected to pull down assay in which CaptAvidin®agarose sedimented beads suspension was used to isolate the bound biotinylated (biotin-cadaverine labelled) proteins. Biotinylated proteins were then recovered by centrifugation and released from the agarose beads with 50 mM sodium bicarbonate buffer pH 10.

2.2.18. Dot blot

To detect biotinylated (biotin-cadaverine labelled) proteins in culture medium after different treatments, the culture medium and proteins from biotinylated experiment (section 2.2.17) was subjected to dot blot. The nitrocellulose membrane was placed inside the manifold of a 96 well dot–blot system (Bio-Rad Laboratories, Hercules, USA). An equal amount (10 µg) of proteins of each sample was spotted onto the nitrocellulose membrane and then was allowed to dry for 2 hours at room

temperature. The nitrocellulose membrane was removed from the dot–blot apparatus and treated as previously described in (section 2.2.12). The nitrocellulose membrane was probed with HRP- ExtrAvidin®peroxidase to detect TG2 substrate proteins. The biotinylated proteins were visualised by ECL as described in (section 2.2.12).

### 2.2.19. Measurement of proteins serving as substrates for TG2 in the presence of calcium and EDTA

The ability of cellular proteins to act as substrates for endogenous TG2-catalysed polyamine incorporation reactions was investigated as described previously by Singh et al. (1995). Briefly, cell extracts containing equal amounts (200 μg) of proteins were incubated in 100 μl of 100 mM Tris-HCl (pH 8.5) buffer containing; 1 mM biotin cadaverine and 5 mM of either $CaCl_2$ or EDTA (background control). The reaction mixture (50 μl) was removed at different time points and directly mixed with (4x) sample buffer to stop the reaction. The reaction mixtures were separated by SDS-PAGE (section 2.2.10) and transferred on to nitrocellulose membrane filters. The membrane was probed with HRP- ExtrAvidin®peroxidase and biotinylated amine incorporation detected by enhanced by ECL as described in (section 2.2.12).

### 2.2.20. Immunoprecipitation

Protein G-Sepharose beads 4 fast flow (Amersham Biosciences AB, Uppsala Sweden) were washed twice and re-suspended in lysis buffer. Total cell lysate protein (500 μg) of Biotin-X-cadaverine labelled sample was pre-clarified by incubation with 50 μl protein G-Sepharose beads for 1 hour at 4°C with gentle rotation using rotating wheel mixer (Stuart Scientific Blood Tube Rotator SB1, Jencons- PLS, UK) and beads were spun down by centrifugation at 3000 x g for 2 min at 4°C. The supernatants were transferred to a fresh tube and beads were discarded. The pre-clarified lysates were incubated overnight with 1μg of anti-α-actinin mAb (Sigma, UK) antibody at 4°C with gently rotation. After incubation overnight, the lysates were mixed with 90-100 μl of protein G-Sepharose beads and incubated one again overnight, at 4°C with gently rotation. The beads were spun down by centrifugation at 3000 xg for 2 min at 4°C and the supernatant was removed. The beads were washed 3x with lysis buffer and centrifuged for 2 minutes at 3000 xg. Bound proteins were eluted from beads with 4x sample buffer and analysed by

Western blotting (section 2.2.11). To confirm α-actinin as TG2 substrate proteins, the immunocomplex proteins were resolved in 10 % SDS followed by Western blotting (section 2.2.12) and probing with HRP-conjugated®ExtrAvidin-peroxidase.

### 2.2.21. Cell viability measurement

#### 2.2.21.1. MTT assay

In order to identify the viability of cells in response to different treatments, the tetrazolium salt, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltertra-zolium bromide MTT assay was performed. This assay measured the reduction of MTT compound by mitochondrial succinate dehydrogenase (Denizot & Lang, 1986). This reaction only occured in metabolically active cells. Briefly, the 80 % confluent H9c2 cells were cultured in 24 wells plates overnight, at a density of (25,000 cells per well). At the end of each treatment period, a volume of 50 µl (1:10 in DMEM) of 5 mg $ml^{-1}$ MTT (Sigma, UK), was added to each well and the plate was incubated at 37°C for 2h. After the medium was removed, 500 µl of DMSO was added to each well to dissolve the formazan. The magnitude of the reduction reaction was determined by monitoring the absorbance of the solubilised formazan product at 570 nm using a plate reader (Expert 96, Scientific laboratory, UK). The MTT data was expressed as percentage of control (untreated cells). Data calculated as follow; average of the four wells absorbance of each sample / average of the four wells absorbance of control sample X100.

#### 2.2.21.2. Lactate dehydrogenase (LDH) assay

The death of H9c2 cells in response to $H_2O_2$ and different treatments was evaluated by a lactate dehydrogenase assay (LDH), a method as described previously (Decker & Lohmann-Matthes, 1988). Cardiomyocytes H9c2 cells were grown in 96-well plates at a density of $1\times10^5$ cells/ml (5,000 cells per well) overnight. Following $H_2O_2$ exposure the activity of lactate dehydrogenase (LDH) released into the culture medium was detected colourimetrically using the CytoTox 96®Non-Radioactive a cytotoxicity assay kit (Promega, UK). The LDH assay was performed according to the manufacturer's instructions. Briefly, after each treatments exposure the cell culture plate was centrifuged for 5 min at 1000 xg using the plate centrifuge 5430 (Eppendorf AG, Germany) and 50 µl of medium was transferred to a 96 wells flat bottomed micro

plates and 50 µl of assay mixture of LDH was added to each well. The cell culture plate was protected from light and incubated at 25°C for 30 min. The reaction mixtures were stopped by 50 µl of the provided kit stop solution and the absorbance recorded at 490 nm. The amount of LDH released into the medium was expressed as percentage of control (untreated cells). Data calculated as follow; average of the four wells absorbance of each sample / average of the four wells absorbance of control sample X100.

### 2.2.22. Caspase-3 activity

The induction of apoptosis in H9c2 cells in response to $H_2O_2$ and different treatments was evaluated by a colorimetric caspase-3 assay as described by Sordet et al. (2002) with minor modifications. After each treatment exposure, cells were rinsed twice with 2 ml of chilled PBS, lysed with 500 µl of ice-cold caspase lysis buffer of 50 mM HEPES (pH 7.4) containing; 5 mM CHAPS and 5 mM DTT (Sigma, UK). Cell lysates were scraped and clarified by centrifugation at 4°C for 20 min at 14000 xg (Sigma, UK). The supernatant (75 µl) were loaded into 96-well plates. The reaction started by the addition of 20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1 % (w/v) CHAPS, 5 mM DTT and 100 µM caspase-3 peptide substrate Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA; Sigma, UK) to each wells. After one hour incubation at 37°C, the absorbance of the p–nitroanilide (pNA) release correlating to caspase-3 activity was read at 405 nm using a plate reader (Expert 96, Scientific Laboratory, UK). The enzyme activity is expressed as a percentage increase over control. Cells treated with 1 µM staurosporine (STS) for 2 hours, were used as a positive control.

### 2.2.23. DNA fragmentation assay

Genomic DNA fragmentation is a consequence of apoptosis cell death, a method described previously and modified by Zhou et al., (1998) and Lee (2001) determined fragmented DNA after treatment cells with harmful agents. Briefly, H9c2 cells were plated out at density of 50,000 cells/ml in sterile 6 well culture dishes and incubated for a further 24h to allow recovery. Cells were subjected to oxidative stress-induced cell death/ PMA and FK-induced cytoprotection as described previously (section 2.2.3.3). The monolayer was rinsed twice with 2 ml of chilled PBS, lysed with 200 µl of ice-cold lysis buffer of 0.2 % (w/v) Tris-HCl (pH 8.5) containing; 10 mM NaCl, 1

mM EDTA, and 1 % (w/v) SDS. After adding 0.1 mg $ml^{-1}$ of proteinase K (Sigma, UK), the cell lysates were then incubated at 60°C for 2h. The reaction was stopped by adding 0.3 M sodium acetate into the cell lysates and incubated on ice for 30 min. The cell lysates supernatants were clarified by centrifugation at 4°C for 15 min at 20,000 xg (Scientific laboratory, UK). The resultant supernatants were transferred to a new sterile tubes and the DNA was precipitated by the addition of five volumes of ice cold (-20°C) 100 % (v/v) ethanol and incubated overnight at -20°C. After centrifugation for 30 min at 20,000 xg, DNAs pellet was washed again with 70 % (v/v) ethanol, re-suspended in 100 µl of distilled water supplemented with 0.2 mg $ml^{-1}$ of RNase A (Sigma, UK) and incubated at 55°C for 1h. The DNA fragmentation was then detected by loading 20 µg of DNA into 1.5 % (w/v) agarose gel (section 2.2.11). The DNA bands were visualised under UV light.

### 2.2.24. Immunocytochemistry analysis

H9c2 cells were seeded on 8-well chamber slides (Thermo Fisher Scientific, UK) at density of ($1.5 \times 10^4$ cells/well) and cultured for 24h in fully supplemented DMEM. The medium was then removed and adherent cells were gently washed with PBS and slides incubated (or not) for 4h with 1 mM biotin-X-cadaverine (1/100 in DMEM). Cells were then treated with 1 µM PMA or 10 µM FK for 5 min. The stimulation cells were fixed with 3.7 % (w/v) paraformaldehyde in PBS for 15 min at room temperature and permeabilised with 0.1 % (v/v) Triton-X100 in PBS for 15 min at room temperature. Each step was followed by (3x) 5 min washes with PBS. Finally, cells were blocked with 3 % (w/v) BSA for 1h at room temperature. For mitochondrial detection, cells were incubated with rabbit anti-monoamine oxidases B (MAO-B) mAb, and anti-rabbit-Alexa568 secondary antibody (red). Endoplasmic reticulum (ER) was detected by mouse anti-calnexin (AF18) antibody mAb and the cytoskeleton was detected by mouse anti-α-actinin antibody mAb and both were visualised by anti-mouse-Alexa568 secondary antibody (red) (Molecular probes, Invitrogen, Carlsbad, CA). Active caspase-3 was detected by rabbit anti-active caspase-3 antibody mAb (Cell Signalling, UK) and visualised by anti-rabbit-Alexa568 secondary antibody (red). Nuclei were stained with DAPI (blue) and the slide viewed at (400x) magnification using fluorescence microscope (Olympus BX51, Japan).

### 2.2.25. Messenger RNA detection and quantification

#### 2.2.25.1. Reverse transcription polymerase chain reaction

To detect and semi-quantify protein expression of TG2 after PMA and FK exposure, reverse transcription polymerase chain reaction (RT-PCR) was conducted. Total RNA was extracted from control and treated cells using a RNeasy Mini isolation Kit (Qiagen, UK) according to the manufacturer's protocol. The concentration and purity of RNA in each samples was determined by NanoDrop ND-2000c spectrophotometer (Thermo Scientific, Labtech, UK). For RT-PCR, specific primers for TG2 and GAPDH were designed using Primer3 software that yielded products spanning exon-intron boundaries to ensure that products were derived from mRNA only. MyTaq One-Step RT-PCR Kit (Bioline Reagents Ltd., UK) was used to reverse transcribe RNA to cDNA ready for PCR amplification. Briefly, a mixture of 12.5 µl 2xMyTaq One-Step Mix, 1 µl of 10 µM of both forward and reverse primers (Table 2.3), 0.5 µl RiboSafe RNase Inhibitor, 0.25 µl reverse transcriptase, 2.5 µl RNA and $ddH_2O$ in a total volume of 25 µl. Amplification was performed using the Platinum PCR SuperMix (Invitrogen, UK). RT-PCR condition was carried out as follow; 1 cycle for reverse transcription at 45°C for 20 min, 1 cycle for polymerase activation at 95°C for 1 min followed by 40 cycles at 95°C for 10s (denaturation), 60°C for 10s (annealing), 72°C for 30s (extension) and a final extension at 72°C for 10 min. The PCR products along with the DNA ladder (Bioline Reagents Ltd., UK) were analysed by 2.5 % (w/v) agarose gel (Bioline Reagents Ltd., UK) electrophoresis and visualized with SYBR Safe DNA gel stain (Life Technologies, Invitrogen, UK) under UV light. The densitometry values of the bands were quantified and analysed using (Adobe Photoshop CS4). The expression of GAPDH was used to normalize variable template loading.

**Table 2.3 Table shown forward and reverse primers for TG2 and GAPDH used in this study**

| mRNA | Oligo name | Sequence | Amplicon size (bp) | Company |
|---|---|---|---|---|
| TG2 | NM_019386.2for<br><br>NM_019386.2rev | AGCCAACCACCT GAACAAAC<br>CAGGGTCAGGTT GATGTCCT | 226 | Eurofins MWG operon, USA |
| GAPDH | NM_017008.4for<br><br>NM_017008.4rev | GAGAAGGCTGGG GCTCAC<br>GTTGTCATGGAT GACCTTGGC | 186 | Sigma, UK |

The table 2.3 shows the primers' size, sequences and companies.

2.2.25.2. Quantitative polymerase chain reaction

2.2.25.2.1. Reverse transcription

After total the RNA was extracted from control and treated cells as described in (section 2.2.25.1) 2 µg total RNA was reverse transcribed into DNA using 0.5 µg of the random hexamer primers (Oligo (dT)), $ddH_2O$ was added to give a final volume of 15 µl. The samples were then heated using UNO-Thermoblock to 70°C for 5 min to melt secondary structures. Samples were then immediately put on ice and a mixture of 5 µl of moloney murine leukemia virus (M-MLV, 5x) reaction buffer (Promega, UK), 1 µl of 40 mM deoxynucleotide triphosphates (dNTP's), 0.7 µl RNasin ribonuclease inhibitor, 1 µl M-MLV reverse transcriptase and 2.3 µl $ddH_2O$ in a total volume of 25 µl. Samples were mixed gently and incubated for 80 min at 40°C in a water bath. After incubation, samples were finally heated using an UNO-Thermoblock to 95°C for 5 min and immediately stored at -20°C ready for qRT PCR.

2.2.25.2.2. Quantitative RT-PCR

QRT-PCR was set up into PCR tubes using Sybr Green SuperMix (Applied Biosystems, UK) following manufacture's protocol described. An equivalent volume (0.5 µL) of cDNA was used as the template for PCR using (0.5 µL) gene-specific primer sets for both TG2 and GAPDH and added to final volume of 12.5 µL. In cases of non-template controls (NTC), cDNA template was substituted with $ddH_2O$. Thermocycling conditions used for qPCR were one cycle for polymerase activation at

95°C for 10 min, followed by 40 cycles at 95°C for 15s (denaturation), 60°C for 30s (annealing), 72°C for 30s (extension) and a final extension at 72°C for 10 min. Thermocycling was applied using a Bio-Rad MiniOpticon System (Bio-Rad Laboratories, UK). Samples were run on a qRT-PCR Thermal Cycler. Samples were run in duplicate and repeated at least 3 times to ensure validity of results, which were then analysed using as delta Ct (DCt) equation. Cycle threshold (Ct) was calculated using supplied software and the transcript abundance of TG2 relative to transcript abundance of GAPDH were calculated and used to determine changes in TG2 mRNA expression.

### 2.2.26. Sample preparation for MALDI-TOF Mass spectrophotometry analysis

The protein bands were excised from 4–15% precast polyacrylamide gel (Mini-PROTEAN® TGX™ Precast Gel, Bio-Rad, UK; Fig. 2.3).

#### 2.2.26.1. In gel digestion with trypsin

##### 2.2.26.1.1. Gel fragment preparation

Protein bands from 1D gel or protein spots from 2D gel were excised using a sterile scalpel and placed into a low protein-binding microfuge tube (LoBind, Eppendorf) and were cut it into 1 mm pieces. Gel pieces were washed with >10 volumes of Millipore water (~200 µl) for 30 seconds, to wash out any acetic acid.

##### 2.2.26.1.2. Destaining and dehydrating

For Coomassie blue staining, gel pieces were destained two times for 5 min until colourless with 200 µl of 100 mM ammonium bicarbonate ($(NH_4)HCO_3$) and 50 % (v/v) methanol. For silver staining gel pieces were destained by a freshly prepared 1:1 solution of 100 mM sodium thiosulphate ($Na_2S_2O_3$) and 30 mM potassium ferricyanide ($K_3Fe(CN)_6$). The reaction was stopped and silver ions were removed by washing twice for 2 min with 500 µl of ultra-pure water. Gel pieces were dehydrated for 15 min at 37°C with 200 µl of 50 mM $(NH_4)HCO_3$ in 50 % (v/v) acetonitrile. The dehydration solution was removed and 100 % (v/v) acetonitrile was added for 30s or until the gel pieces shrunk and became white.

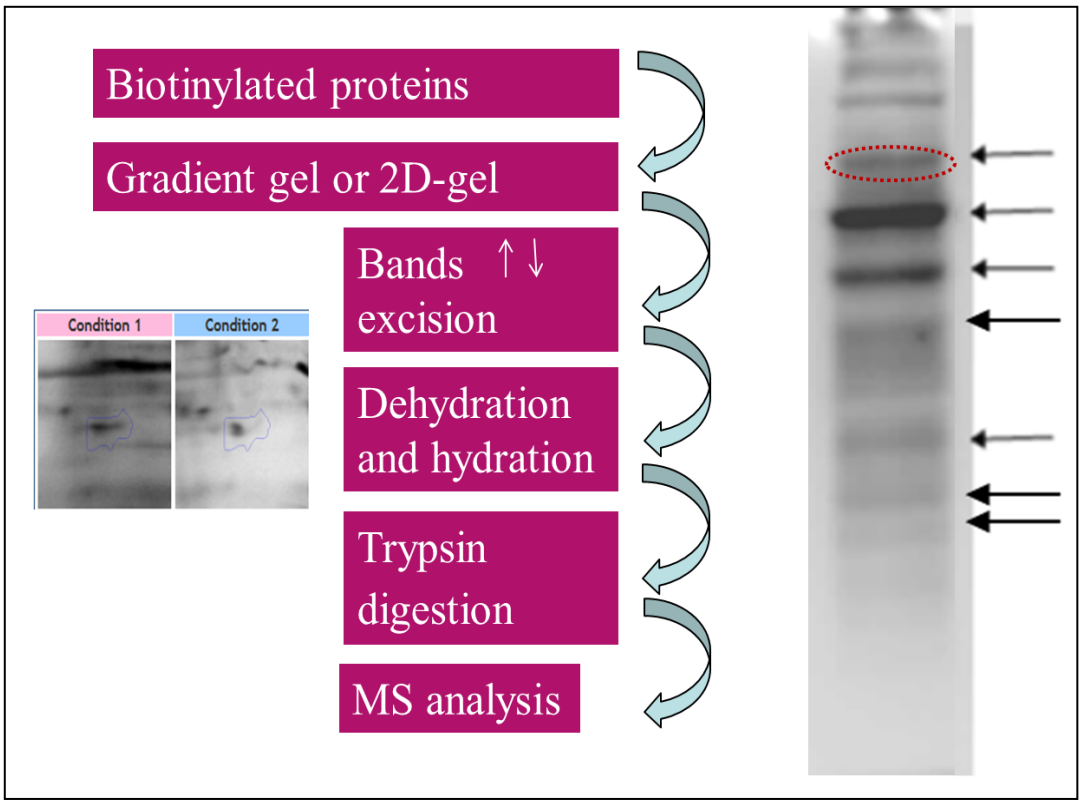



**Figure 2.3 The flow diagram represents the identification of TG2 substrate proteins' protocol used for mass spectrophotometry**

The proteins were extracted from the gel by dehydration and hydration methods (section 2.2.26), following with trypsin digestion. Samples were directly analyzed by LC-MSMS.

2.2.26.1.3. Rehydrating

Dehydrated gel pieces were rehydrated in 200 µl of 5 mM $(NH_4)HCO_3$ for 5 min and then an equal amount of 100 % (v/v) acetonitrile was added and incubated with gentle shaking for further 15 min. This solution was removed and the gel pieces were once more covered by 100 % (v/v) acetonitrile and mixed until gel pieces shrank. Acetonitrile was removed and gel particles were dried by pipetting excess liquid. Gel particles rehydrated by adding 200 µl ultra-pure water.

2.2.26.1.4. Trypsin digestion

After the water was removed from the gel particles, proteins in gel were digested with trypsin over night at 37°C in 20 µl digestion solution containing; 200 ng trypsin gold (Promega, UK) in 100 mM $(NH_4)HCO_3$. The reaction was terminated by adding 1 %

(v/v) trifluroacetic acid (TFA) and mixed for 5 min. Digested proteins were recovered by centrifuging for 10 min at max speed ~8000 xg (Scientific Laboratory, UK). Supernatant containing digestion solution transfer to a new tube, and sample was ready for LC-based mass analysis. This was carried out in the John van Geest Cancer Research Centre at Nottingham Trent University, Bruker Daltonics were analysed using LC-MALDI-TOFTOF (UltrafleXtreme, Bruker Daltonics, Germany). LC-fractions were controlled using WARP-LC software (version 3.2, Bruker Daltonics) and FlexControl software (version 3.3, Bruker Daltonics). Data acquired were searched against rat (Rattus norvegicus) in SWISSPROT using MASCOT (version 2.3 server, Matrix Science).

#### 2.2.26.2. Peptide purification

The digested peptides were purified by ZipTip-$C_{18}$ column (Millipore, UK), Zip-Tips are pipette tips that contain 0.5 µl volume immobilized chromatography media (C18, resin) attached at their distal end that used for clean-up before spotting onto MALDI plate. A P20 pipetter was set to 10 µl for the Zip-Tips and the Zip-Tip was washed 5 times with 0.1 % (v/v) trifluroacetic acid (TFA) in acetonitrile, followed with 5 times wash with 0.1 % (v/v) TFA in 1:1 acetonitrile: water. The Zip-Tip was equilibrated twice with 0.1 % (v/v) TFA in water and digested peptides were passed through the Zip-Tips repeatedly by pipetting in and out to bind the sample to the resin. This was followed by wash three times with 0.1 % TFA and 5 % (v/v) methanol in water to remove unbound material. The sample was eluted directly from the Zip-Tip in 3 µl in 80 % (v/v) acetonitrile through 15 aspirating and dispensing cycles. A 1.0 µl of eluted sample was mixed with 1.1 µl of matrix, typically alpha-cyano-4-hydroxycinnamic acid in 0.1 % (v/v) TFA 80 % (v/v) acetonitrile and spotted in triplicate on the MALDI-TOF sample plate.

### Statistical analysis

All graphs were prepared using Graph Pad Prism software, while statistical analysis of data was performed by both One way ANOVA following by "Tukey's Multiple Comparison Test“, Dunnett comparison test" , and Two way ANOVA for group comparison. Results represent mean ± SEM and p-value less than 0.05 were considered statistically significant.

# CHAPTER III:

# *IN VITRO* MODULATION OF TG2 ACTIVITY BY PKC AND PKA

## 3. Introduction

Transglutaminase 2 has been known as a molecular "Swiss army knife" that have multiple enzymatic functions which include transamidation, protein kinase and protein disulphide isomerase activity (Gundemir et al., 2012). It also acts as a G-protein, which is independent of its transamidation activity, mediating signal transduction pathways prompted by the G-protein coupled receptor family such as the $\alpha_{1B}$-adrenergic receptor (Nakaoka et al., 1994). Interestingly, the activity of TG family members can be regulated by protein kinases. For example, TG2 phosphorylation by PKA increases its kinase activity but inhibits in transamidating activity (Mishra et al., 2007). The protein crosslinking activity of TG1 is activated by phorbolester that induced PKC and ERK1/2 activation (Bollag et al., 2005). In addition, TG2 expression in pancreatic cancer cells has been shown to be regulated by PKCδ, which in turn inhibit a type II programmed cell death (autophagy) (Akar et al., 2007). This revealed the important role of PKC in mediating autophagy and TG2 expression. Phosphorylation of TG1 ($Ser^{-82}$ and $Ser^{-85}$) by PKC has already been demonstrated in keratinocytes (Chakravarty, et al., 1990; Rice et al., 1996). Overall, these observations suggest that both PKA and PKC-dependent signalling pathways can regulate the activity and expression of specific TG isoenzymes.

The activation of both PKC and PKA is involved in cardioprotection mechanisms stimulated by different agents. The translocation of PKCε from cytosol to membrane can trigger cardioprotection effects stimulated by ischaemic preconditioning (see section 1.5) or anaesthetic, such as activation of pro-survival proteins ERK1/2 and reduction in ischaemic cell injury (Liu et al., 1999; Toma et al., 2004). Other protein kinase C isoenzymes (PKCδ, PKCε) are involved in ischaemic heart preconditioning in response to isoflurane treatment in which their activation is associated with upstream transduction events including; mitochondrial $K_{ATP}$ channel-opening and reactive oxygen species production (Ludwig et al., 2004; Frässdorf et al., 2009). Volatile anaesthetic agents and chronic morphine application may also induce cardioprotection mediated by PKA and PKC signalling transduction activation (Peart & Gross, 2006). The activation of PKA in combination with p38 MAPK can provide a dual role in cardioprotection (Makaula et al., 2005). Although the mechanism of downstream signalling of PKA activation remains unclear, it is believed that the

protective effect of PKA appears to be correlated with inhibition of the small GTPase Rho and its kinases (Sanada et al., 2001).

Phorbol ester is a natural extracted from croton oil *californicus* and it is frequently used in cell culture as a PKC activator (Saitoh & Dobkins, 1986). In addition, it has been reported to stimulate PKC dependent signalling pathways involved in ischaemic preconditioning, which facilitates activation of cardioprotective ATP-sensitive potassium channels, facilitating the induction of larger sarcolemma $K_{ATP}$ channel currents (Nishizuka, 1995). Forskolin is a natural product extracted from the *Coleus forskohlii* herb and it is widely used in medical research (Pradeep et al., 2006). Forskolin acts as an adenylyl cyclase activator, which increases the turnover of cyclic adenosine monophosphate (cAMP; Morimoto et al., 2001). Forskolin has been shown to inhibit colon cancer cell growth (McEwan et al., 2007) and improve heart function (Roth et al., 2002). It has also been used to simulate PKA to mimic ischaemic preconditioning (Makaula et al., 2005).

PKC and PKA are two major mediators of signal transduction pathways associated with ischaemic preconditioning and pharmacological preconditioning induced cardioprotection (Yellon & Downey, 2003; Sanada et al., 2011). Interestingly, TG2 has been shown to mediate cardioprotection against ischaemia and reperfusion-induced cell death by regulating ATP synthesis in cardiomyocytes (Szondy et al., 2006). Similarly, increased TG2 expression protects neuronal cells from oxygen and glucose deprivation-induced cell death (Filiano et al., 2008). Given the emerging role of TG2 in cardioprotection coupled with is regulation by protein kinases associated with cardioprotection, one of the aims of this study was to investigate the regulation of TG2 by PKA and PKC in the H9c2 rat embryonic cardiomyoblast-derived cell line (Kimes & Brandt, 1976).

The H9c2 cells are derived from embryonic rat heart tissue (Kimes & Brandt, 1976) and are extensively used as an *in vitro* model for investigating and studying cardioprotection events since they display similar morphological, biochemical and electrophysiological properties to primary cardiac myocytes (Hescheler et al., 1991). Although the regulation of TG isoenzymes by PKA and PKC has been studied in other cell lines (Bollag et al., 2005; Mishra et al., 2007), the regulation of TG2 has not

been investigated in this cardiomyocytes cell lines. Therefore, the primary aims of this study were to investigate; the activation of TG2 in response to phorbol-12-myristate-13-acetate (PMA; a PKC activator) and forskolin (FK; a PKA activator).

### *3.1. Aim*

The main aim of the work presented in this chapter was to determine the effects of a phorbol ester (PMA; a protein kinase C activator) and forskolin (FK; protein kinase A activator) of on TG2 activity and its protein level in rat embryonic cardiomyoblast-derived cell line (H9c2).

### *3.2. Methods*

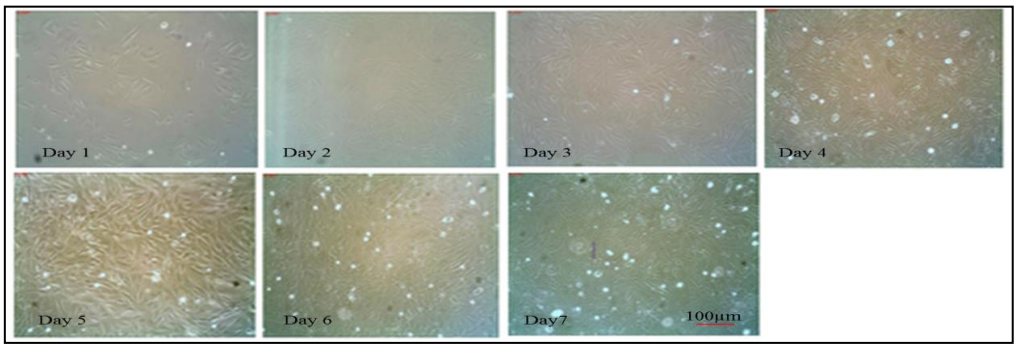
As described in chapter 2 of this study (section 2.2).

### *3.3. Results*

#### 3.3.1. H9c2 cell in culture

To determine the cell growth characteristics of H9c2 rat embryonic cardiomyoblast-derived cell line, a standard growth curve was generated (section 2.2.1).

**A)**




**B)**

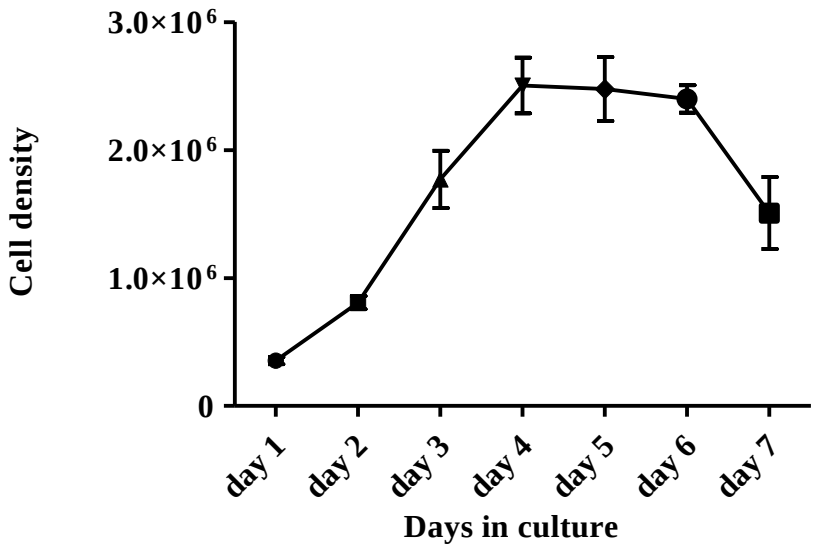



**Figure 3.3.1 The photograph and growth curve of H9c2 in culture.**

H9c2 cells were plated in equal density of 5 x $10^5$ cells per 75 flask cultured in DMEM media of complete condition supplemented with 10 % FBS. A) Photograph of H9c2 cells during seven days culturing were monitored under the inverted light microscope at (100x) magnification. Scale bar 100 µm. B) H9c2 growth curve of seven days and cells density (cell number) was determined using haemocytometer. Data points represent the mean ± SEM of 4 determinations from 3 independent experiments of three different passage numbers.

Figure 3.3.1 shows a photograph and growth curve of H9c2 in culture of seven days. As shown in Figure 3.3.1A, the H9c2 cells exhibited spindle fibroblast-like shape that

rapidly attached to culture flask walls in the first day after of culturing. Many gaps were observed at this day with fewer connected cells. Cells showed rapid division and proliferation during the following two days and they form a monolayer in which cells become connected to each other with fewer gaps. In days 4-6, cells reached 100 % confluence and the flask becomes crowded. In days 6-7, the colour of culture media changed (yellowish) due to pH drop and some dead cells start floating.

As shown figure 3.3.1B, the H9c2 cells density after 24 hours (day one) culturing was similar or less to the starting density ~ 4 x $10^5$ cells. However, in the two following days of culturing, the cells density was shown to gradually increase (doubling time). At day four, significant increase approximately 4 fold (~ 3 x$10^6$) in the cells density was observed and this shown to be steady for 2 more days followed with decline.

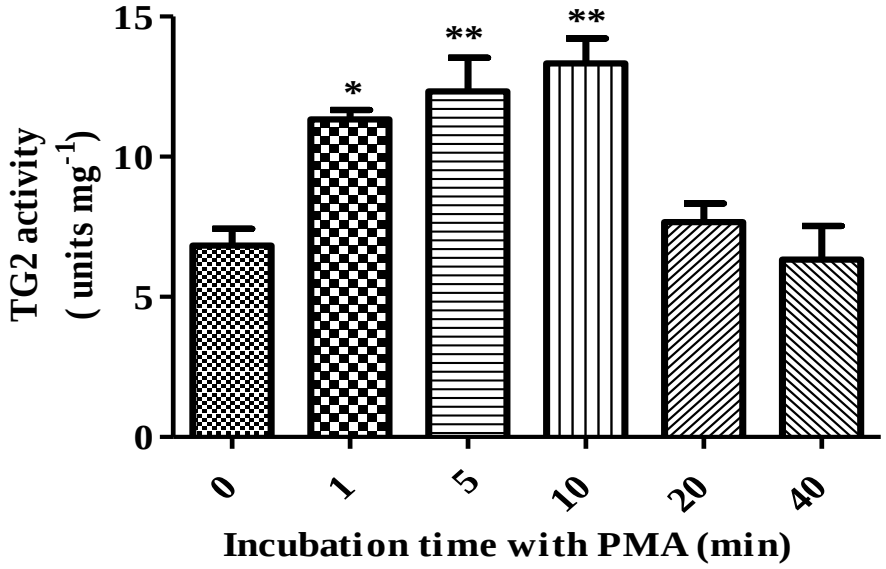
### 3.3.2. The effect of protein kinase activators on biotin cadaverine incorporation TG2 activity

To study transglutaminase activity in H9c2 cells in response to PMA and FK treatments, H9c2 cells were treated with 1 µM PMA (Ertracht et al., 2011; Aggeli et al., 2008; Reilly et al., 1998) or 10 µM FK (Leung et al., 2007) for different incubation times. The cells were then subjected to TG2 biotin cadaverine incorporation assay as described in methods (section 2.2.9.1.1).

#### 3.3.2.1. Time dependent effects of PMA and FK on biotin cadaverine incorporation TG2 activity

A time course exposure of H9c2 cells to PMA or FK showed a statistically significant transient increase ($n = 3$, $^{**}p < 0.01$) in TG2 catalysed biotin cadaverine incorporation peaking at 5 and 10 min exposure to PMA and 1 and 5 min exposure to FK (Fig. 3.3.1). However, significant decreases ($n = 3$, $^{**}p < 0.01$) were observed after 10 min incubation in cell treated with PMA. In contrast, cells that were treated with FK showed a gradual decrease after this time point returning to basal levels.

A)




B)

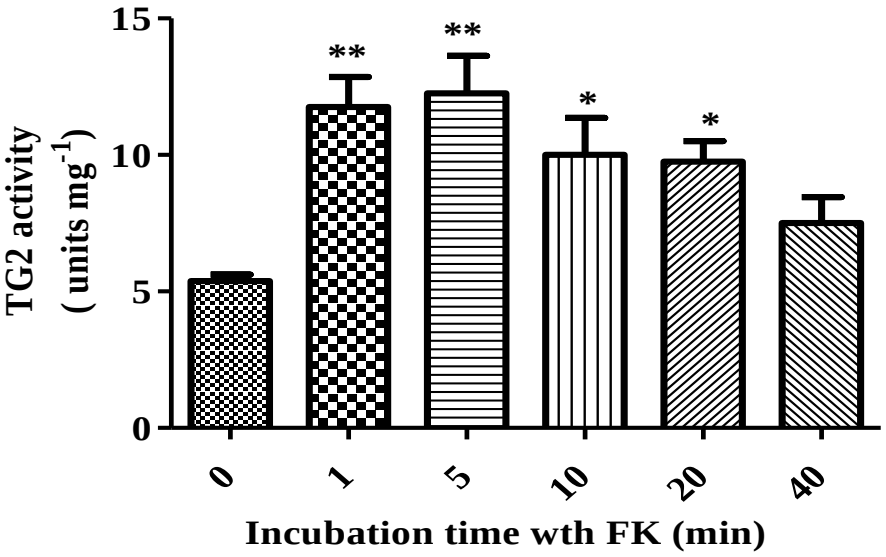



**Figure 3.3.2 Time dependent effects of PMA and FK on cadaverine incorporation TG2 activity**

H9c2 cells were incubated with A) 1 μM PMA or B) 10 μM FK for the indicated time periods, harvested and lysed with 0.1 M Tris buffer containing protease and phosphatase inhibitors. The cell lysates were clarified by centrifugation and subjected to biotin cadaverine incorporation assay. Data points represent the mean ± SEM TG2 specific activity from 3 (A) or 4 (B) independent experiments. Data analysis of 0 min (control) vs. PMA or FK incubation time was performed using "Dunnett comparison test" where statistical significance was accepted at $^{**}p < 0.01$ or $^{*}p < 0.05$. Purified guinea pig liver was used as positive control and the mean ± SEM of TG2 activity was 19.72±2.37 units $mg^{-1}$.

### 3.3.2.2. Concentration dependent effects of PMA and FK on biotin cadaverine incorporation TG2 activity

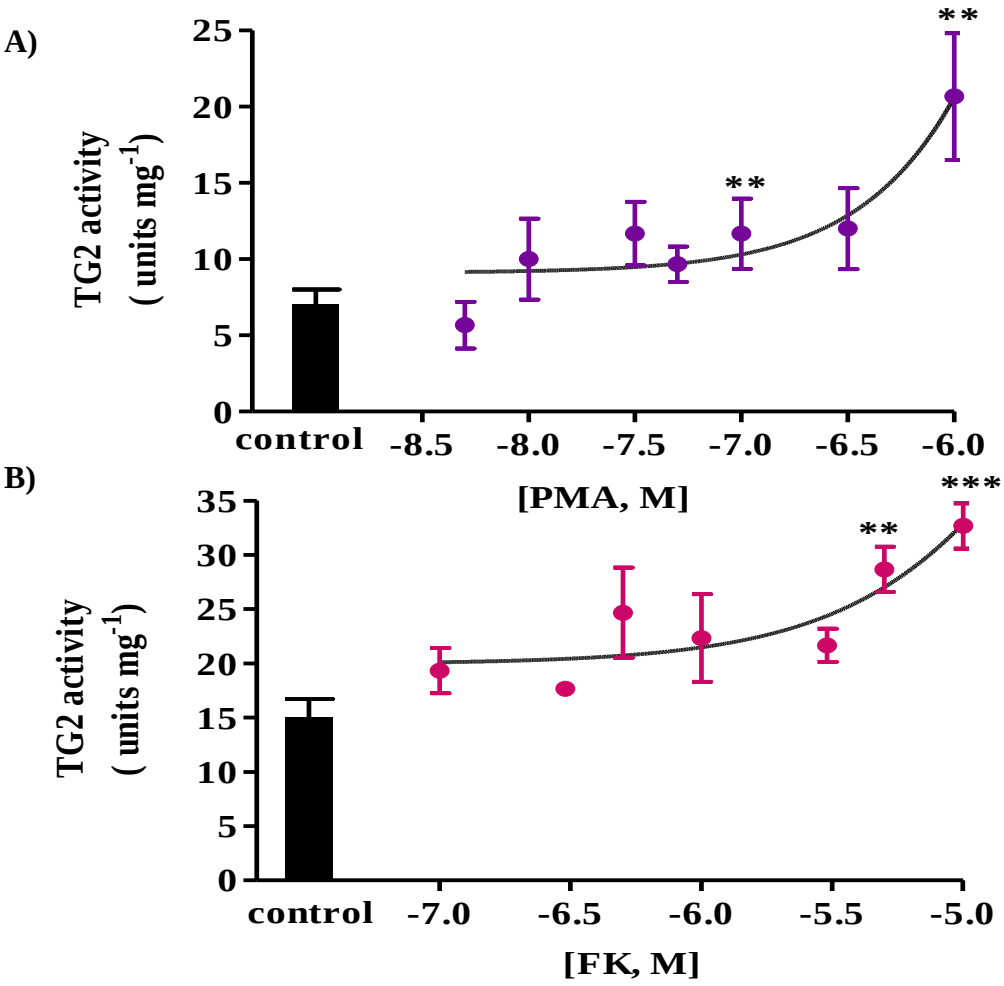



**Figure 3.3.3 Concentration dependent effects of PMA and FK on biotin cadaverine incorporation TG2 activity**

H9c2 cells were treated for 5 min with various concentrations of A) PMA (0.005-1 µM) or B) FK (0.05-10 µM) were harvested and lysed with 0.1 M Tris buffer containing protease and phosphatase inhibitors. The cell lysates were subjected to biotin cadaverine incorporation assay. Control cells were treated with the appropriate volume of DMSO equal to PMA and FK volumes for 5 min and no significant differences compared to control-unstimulated. Graph plotted using Nonliner regression curve fit, "log(agonist) vs. response". Data points represent the mean ± SEM TG2 specific activity from 3 independent experiments. Data analysis of control vs. PMA or FK different concentration was performed using "Dunnett comparison test" to compare control vs. FK. Statistical significance was accepted at ***$p < 0.001$, **$p < 0.01$ or *$p < 0.05$. Purified guinea pig liver was used as positive control and the mean ± SEM of TG2 activity was 24.73 ± 3.20 units $mg^{-1}$.

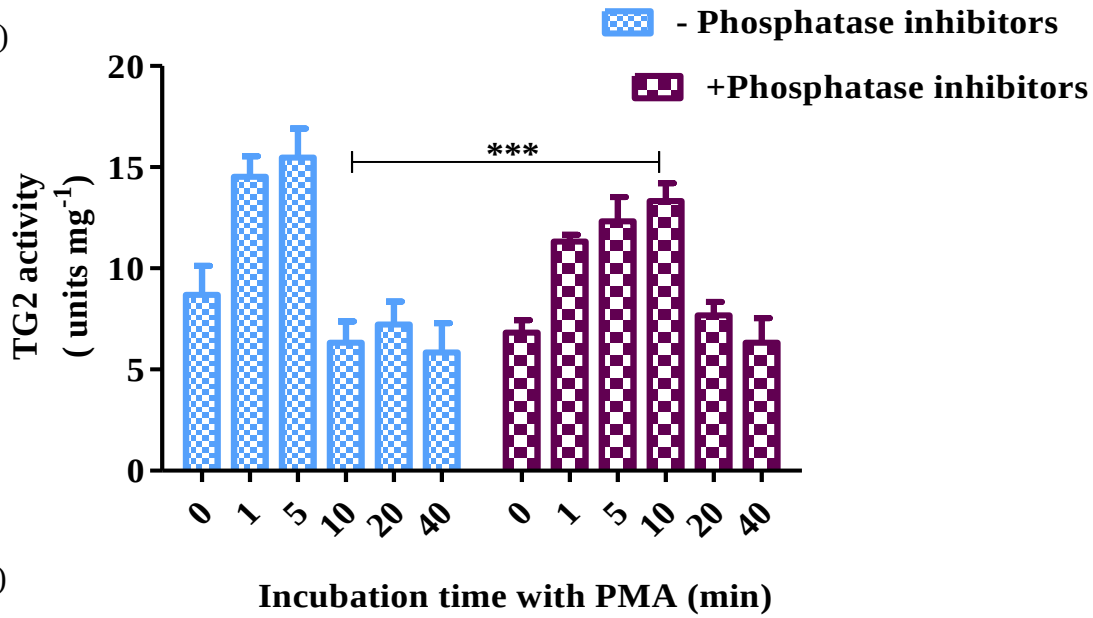
Since an increase in TG2 activity was observed at an early incubation time, cells were treated with different concentration of PMA and FK for 5 min and subjected to biotin cadaverine incorporation assay. A gradual increase in TG2 activity was observed with increasing PMA (Fig. 3.3.3A) or FK concentrations (Fig. 3.3.3B).

### 3.3.2.3. Effect of phosphatase inhibitors on biotin cadaverine incorporation TG2 activity

In order to determine if phosphatase inhibitors have an effect on transglutaminase activity, two different extractions were made for cells treated with PMA, one in the presence and one in absence of phosphatase inhibitors. These different samples were subjected to enzyme assay.

In H9c2 cells with or without phosphatase inhibitors, the specific activity of TG2 initially increased. Cells that were extracted without adding phosphatase inhibitors showed a significant increase ($n = 3$, $^{**}p < 0.01$) after (5 min) over untreated cells (0 min) (Fig. 3.3.4). This transient increase decreased over the 40 min incubation period. In contrast, cells that were extracted with buffer containing (50 μM) phosphatase inhibitors showed maximum increase ($n = 3$, $^{**}p < 0.01$) in 10 min followed by a decrease. However, two-way ANOVA analysis (Table 3.3.1) indicated phosphatase inhibitors had no effect on the ability of PMA to induce TG2 activity (P-value = 0.8957). Moreover, P-values for the interaction and PMA incubation time is less than 0.0001, which shows that interaction between phosphatase inhibitors and PMA incubation time has a statistically significant impact on the TG2 catalysed biotin cadaverine incorporation reaction. The presence of phosphatase inhibitors results in prolonging of TG2 activity until 10 min incubation and shown significant increase (10 min; $n = 3$, $^{***}p < 0.001$) in this time point compared to phosphatase inhibitors untreated sample at the same time point.

A)




B)

**Incubation time with PMA (min)**

**Table 3.3.1 Two-way ANOVA analysis of the effect of phosphatase inhibitors**

| Two-way ANOVA | | |
|---|---|---|
| Source of Variation | % of total variation | P |
| Interaction | 25.07 | P <0.001 |
| phosphatase inhibitors | 0.00 | 0.8957 |
| PMA incubation time | 68.50 | P <0.001 |

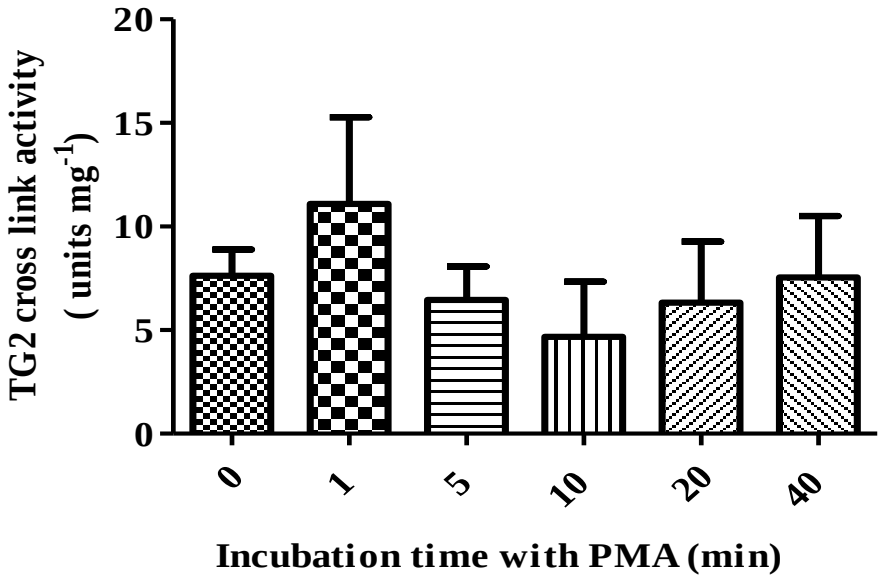
**Figure 3.3.4 Effect of phosphatase inhibitors on the activation of TG2 by PMA**

H9c2 cells were incubated with 1 µM PMA for the time indicated, then harvested and lysed with 0.1 M Tris buffer containing proteases and with (Brown bars; + phosphatase inhibitors) or without (Blue bars; - phosphatase inhibitors) phosphatase inhibitors. The cell lysates were subjected to biotin cadaverine incorporation assay. Data points represent the mean ± SEM TG2 specific activity from 3 independent experiments. ). ***P < 0.001, "- Phosphatase inhibitors vs. + Phosphatase inhibitors " using Two-way ANOVA following by "Bonferroni post-tests". (B) **Table 3.3.1** shown the Two-way ANOVA output indicating the use of phosphatase inhibitors has no significant effect on the results (P-value = 0.8957). However, interaction between phosphatase inhibitors and PMA incubation time has statistically significant impact (***p <0.001) on the TG2 catalysed biotin cadaverine incorporation reaction. Two-way ANOVA showed significant effect of PMA incubation ($F$= 51.11, $dF$=5, 24, ***p < 0.001), no statistical significant of the phosphatase inhibitors ($F$= 0.02, $dF$ =1, 24, p<0.89) and statistical significant of the interaction of PMA incubation/ phosphatase inhibitors ($F$ = 18.70, $dF$ =5, 24, ***p < 0.001).

### 3.3.3. The effect of protein kinase activators on TG2 protein crosslinking activity

Transglutaminase 2 activity in H9c2 cells was assayed in the presence of PMA or FK using protein crosslinking activity assay (Trigwell et al., 2004) via the acyl-donor probe biotin-TVQQEL (Ruoppolo et al., 2003) as describe in methods (section 2.2.9.1.2) . Data analysis indicated a significant increase ($n = 4$, $*p < 0.05$) in TG2 mediated protein crosslinking activity in H9c2 cells that were treated with FK (Fig. 3.3.5B). This increase was observed at 20 min incubation time. In contrast, cells that were treated with PMA did not show significant changes in TG2 protein crosslinking activity (Fig. 3.3.5A).

**A)**




**B)**

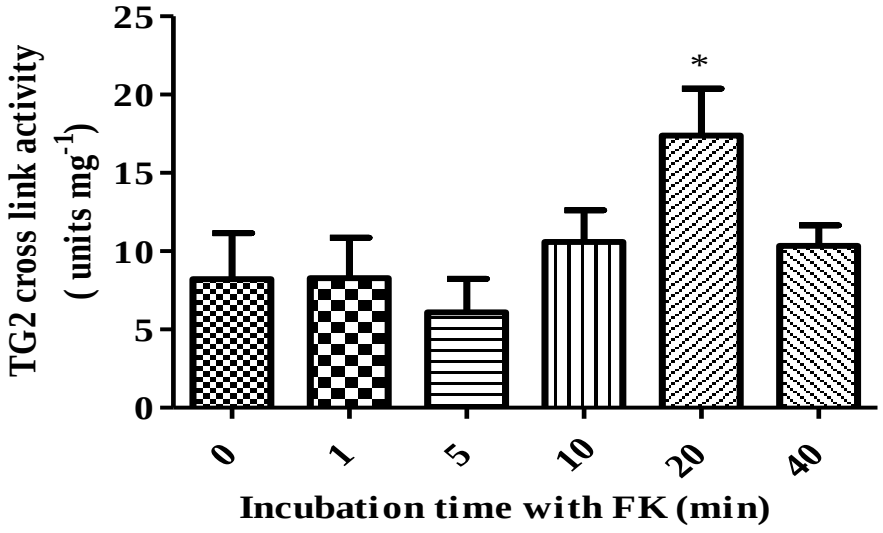



**Figure 3.3.5 Time dependent effects of PMA and FK on TG2 protein crosslinking activity**

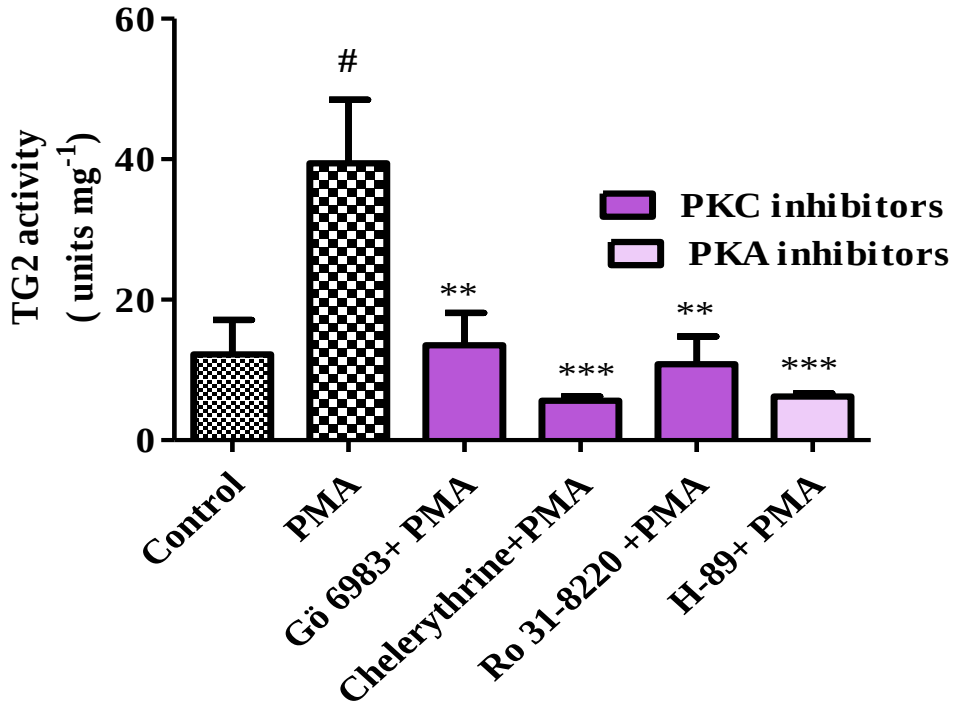
H9c2 cells were incubated with A) 1 μM PMA or B) 10 μM FK at the times indicated, then harvested and lysed with 0.1 M Tris buffer containing protease and phosphatase inhibitors. The cell lysates were subjected to TVQQEL-crosslinking assay. Data points represent the mean ± SEM TG2 specific activity from 4 independent experiments. Data analysis of 0 min (control) vs. PMA or FK incubation time was performed using "Dunnett comparison test" where statistical significance was accepted at $*p < 0.05$.

### 3.3.4. The effect of PKA and PKC inhibitors on TG2 activity stimulated with PMA and FK in H9c2 cells

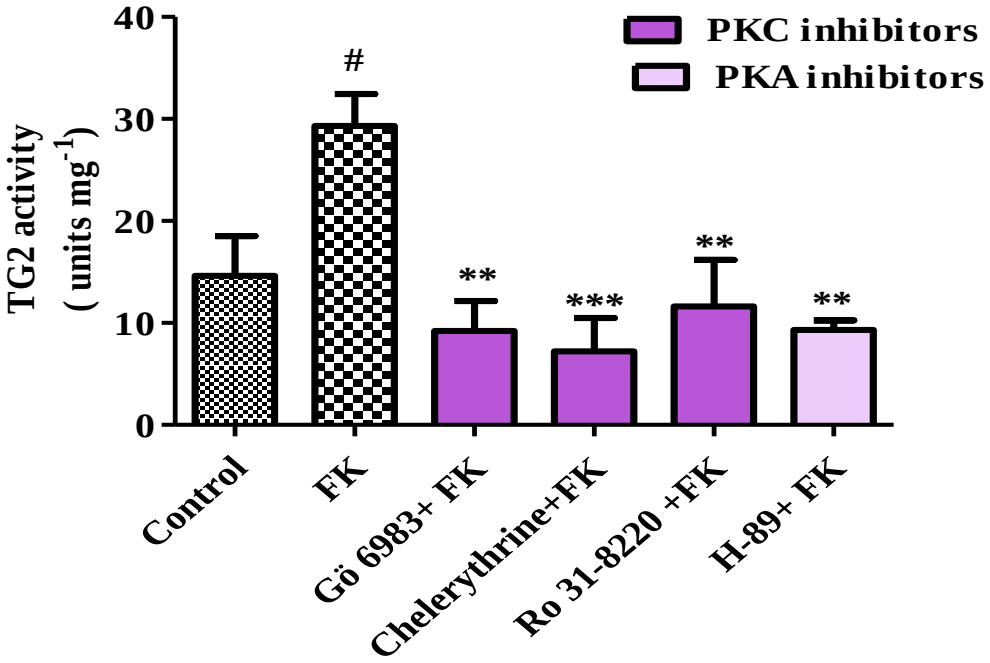
In order to confirm whether activation of PKC or PKA were associated with PMA or FK-stimulated TG activity in cardiomyocytes, inhibitors for these kinases were tested. H9c2 cells were pre-incubated with different protein kinase inhibitors before treatment with PMA or FK (section 2.2.3.2 and fig. 2.1C) and then cells lysed subjected to biotin cadaverine incorporation assay (section 2.2.9.1.1).

Data of this study suggest that both treatments showed a significant decrease in TG2 catalysed biotin cadaverine incorporation in the presence of different protein kinase inhibitors (Fig. 3.3.6). A 50 % decrease relative to protein kinase activator treated cells, returned the activity to a value not significantly ($p > 0.05$ different from the control (untreated cells) level. However, apart from chelerythrine chloride, cells that were treated with protein kinase inhibitors alone showed no change in TG2 activity (Fig. 3.3.6D).

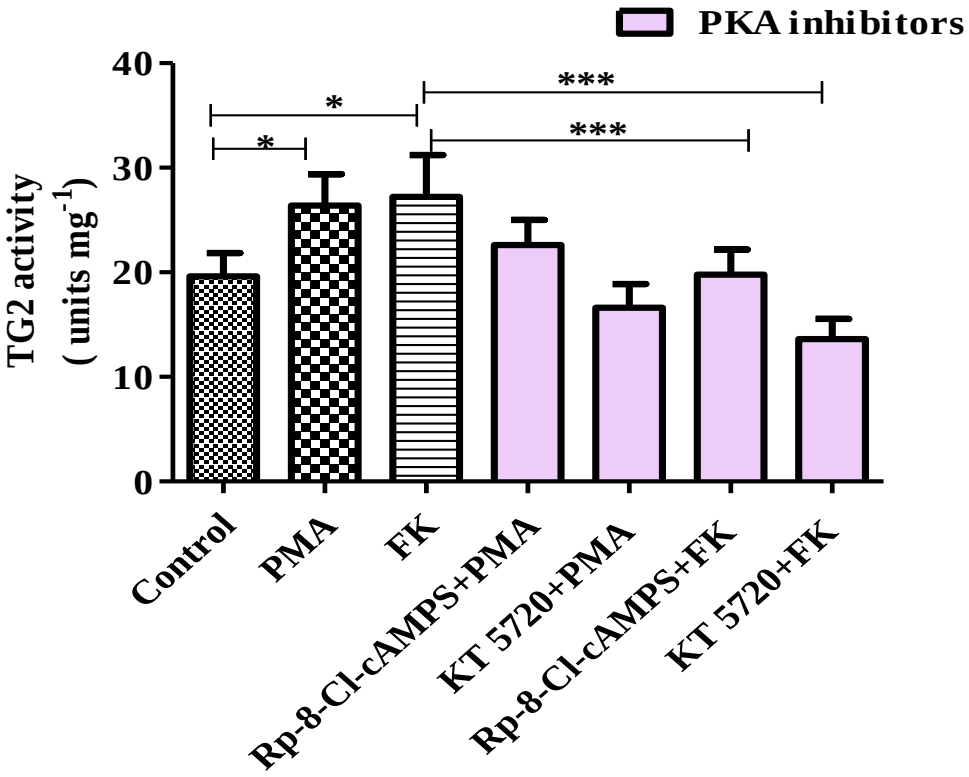
**A)**



B)


PKC inhibitors
PKA inhibitors
TG2 activity
( units mg-1)
40
30
20
10
0
#
**
***
**
**
Control
FK
Gö 6983+ FK
Chelerythrine+FK
Ro 31-8220 +FK
H-89+ FK

C)


PKA inhibitors
TG2 activity
( units mg-1)
40
30
20
10
0
*
*
***
***
Control
PMA
FK
Rp-8-Cl-cAMPS+PMA
KT 5720+PMA
Rp-8-Cl-cAMPS+FK
KT 5720+FK

**D)**

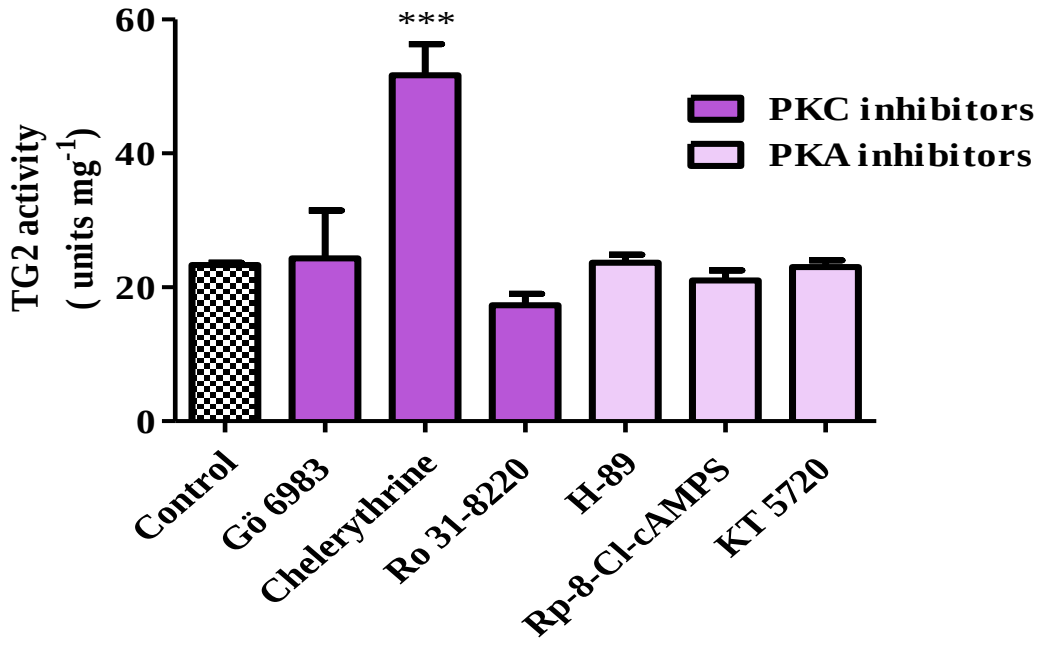



**Figure 3.3.6 The effect of PKA and PKC inhibitors on TG2 activity stimulated with PMA and FK in H9c2 cells**

H9c2 cells were pre-treated for 30 min with the PKC inhibitors Go 6983 (5 µM), Ro 31-8220 (10 µM), chelerythrine (1 µM) and the PKA inhibitor H-89 (1 µM) prior to stimulation for 5 min with A) 1 µM PMA, B) 10 µM FK for 5 min or D) without. C) The effect of PKA inhibitors KT 5720 (5 µM) and Rp-8-Cl-cAMPS (50 µM) on TG2 activity stimulated with PMA and FK. Cell lysates were subjected to biotin cadaverine incorporation assay. Data points represent the mean ± SEM TG2 specific activity from 5 independent experiments. Data analysis of (A, B & C) was performed using "Bonferroni's multiple comparison test" to compare control vs. either PMA or FK and later to protein kinase inhibitors. Data analysis of (D) was performed using "Dunnett comparison test" to compare control vs. protein kinase inhibitors. Statistical significance was accepted at ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Significant activity induced by PMA and FK alone in compared to control were shown (#$p < 0.05$).

On the other hand, chelerythrine chloride treated cells showed a significant increase ($n = 5$, ***$p < 0.001$) in TG2 catalysed biotin cadaverine incorporation activity (Fig. 3.3.6D). H9c2 cells were pre-treated for 30 min with the PKA inhibitors KT 5720 (5 µM) and Rp-8-Cl-cAMPs (50 µM; Dodge-Kafka et al., 2005; Kwak et al., 2008; Galliher-Beckley et al., 2011) prior to 5 min exposure to PMA or FK. PMA-and FK-induced TG2 catalysed biotin-cadaverine incorporation (Fig. 3.3.6C). KT 5720 and Rp-8-Cl-cAMPs significantly ($n = 5$, ***$p < 0.001$) reduced and blocked FK-induced

TG2 activity, but had no effect on PMA responses, confirming the involvement of PKA in TG2 activation.

### 3.3.5. The effects of protein kinase activators and inhibitors on purified guinea pig liver transglutaminase activity

To study possible direct effect of protein kinase activators and inhibitors on TG2 activity, transglutaminase activity purified guinea pig liver TG2 was assayed in the presence of both groups of protein kinase inhibitors using the biotin cadaverine incorporation assay and biotin-peptide crosslinking assay as described in materials and methods (section 2.2.9.1).

#### 3.3.5.1. The effects of protein kinase activators and inhibitors on purified guinea pig liver transglutaminase activity determined by cadaverine-incorporation assay

Initially the direct effect of PMA and FK on purified guinea pig liver transglutaminase activity was determined using the biotin cadaverine incorporation assay. As shown in figure 3.3.7A, PMA (1 μM) and FK (10 μM) had no significant effect on guinea pig liver transglutaminase activity. In marked contrast, purified transglutaminase activity was significantly inhibited by the protein kinase inhibitors Gö 6983 (5 μM; Gschwendt et al., 1996), chelerythrine (1 μM; Herbert et al., 1990; Chijiwa et al., 1990), and H-89 (1 μM; Chijiwa et al., 1990) but not by PKC inhibitor; Ro 31-8220 (10 μM; Davis et al., 1989) or by PKA inhibitors; KT 5720 (5 μM) and Rp-8-Cl-cAMPs (50 μM) (Dodge-Kafka et al., 2005; Kwak et al., 2008; Galliher-Beckley et al., 2011) (see Fig. 3.3.7B).

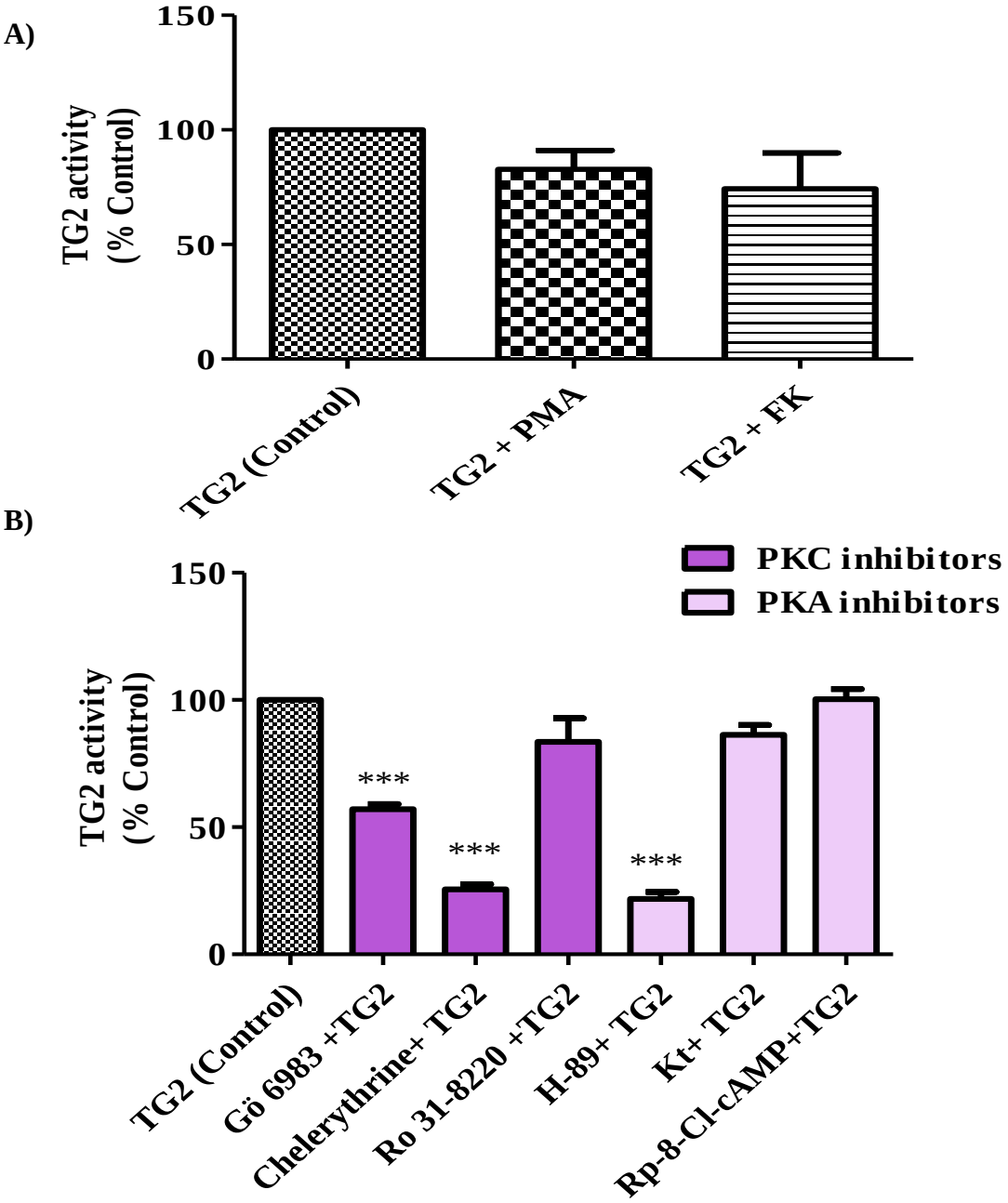



**Figure 3.3.7 The effects of protein kinase activators and inhibitors on purified guinea pig liver transglutaminase activity determined by cadaverine-incorporation assay**

A) Effects of 1 µM PMA and 10 µM FK on guinea pig liver transglutaminase activity. B) Effects of protein kinase inhibitors on guinea pig liver transglutaminase activity. Data points represent the mean ± SEM TG2 activity from 4 independent experiments at basal level of purified guinea pig liver transglutaminase (TG2 Control = 100). Data analysis was performed using "Dunnett comparison test" to compare TG2 control vs. either TG2 + protein kinase activators or TG2 + protein kinase inhibitors. Statistical significance was accepted at ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

### 3.3.5.2. The effects of protein kinase activators and inhibitors on purified guinea pig liver transglutaminase activity determined by TG2 protein crosslinking activity assay

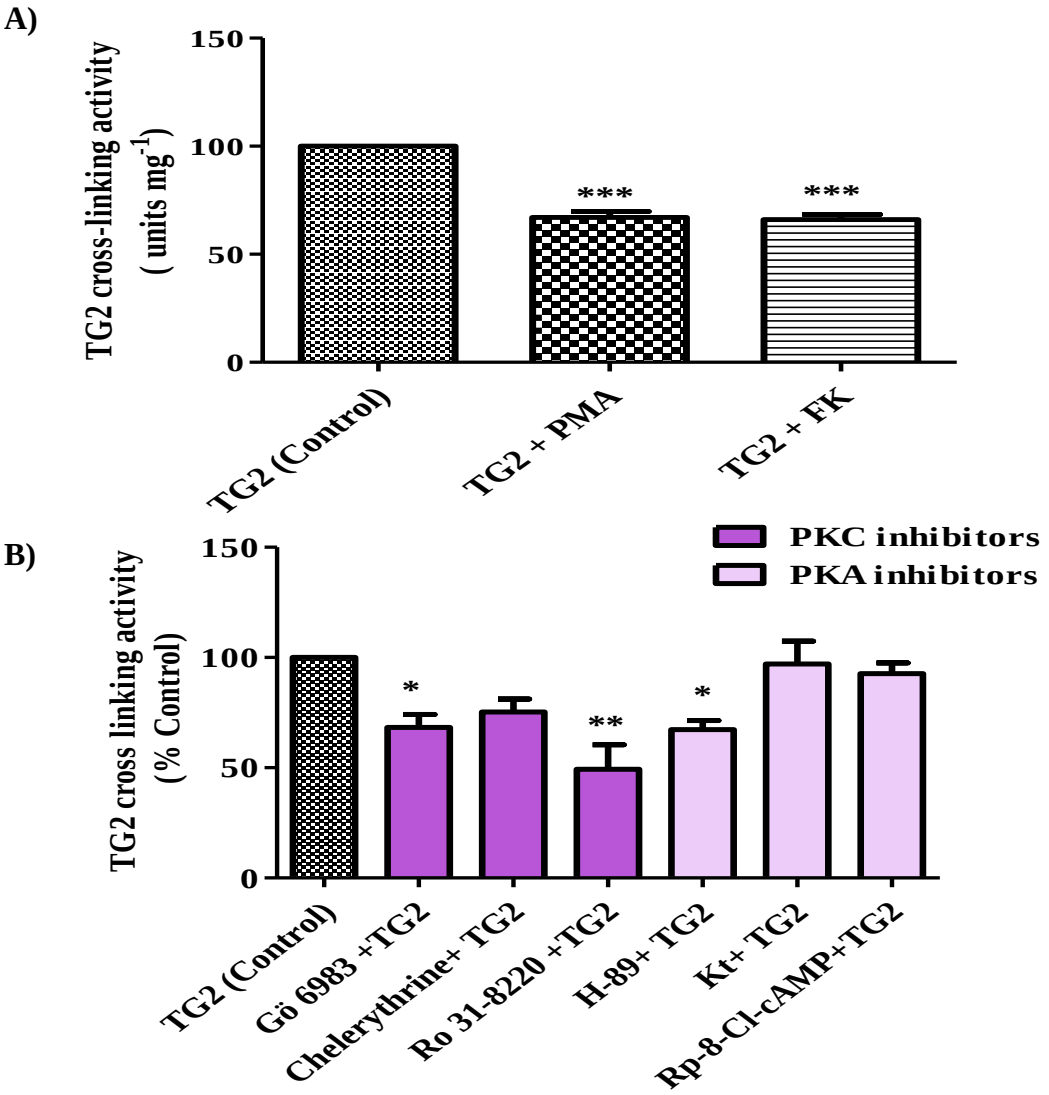



**Figure 3.3.8 The effects of protein kinase activators and inhibitors on purified guinea pig liver transglutaminase activity determined by TG2 protein crosslinking activity**

A) Effects of 1 µM PMA and 10 µM FK on guinea pig liver transglutaminase activity. B) Effects of protein kinase inhibitors on guinea pig liver transglutaminase activity. Data points represent the mean ± SEM TG2 specific activity from 4 independent experiments at basal level of purified guinea pig liver TG2 (control = 100). Data analysis was performed using "Dunnett comparison test" to compare TG2 control vs. TG2 + either protein kinase activators or protein kinase inhibitors. Statistical significance was accepted at ***p <0.001, ** p < 0.01, *p < 0.05.

The data suggest that there is a significant decrease (n = 4, ***p < 0.001) on purified guinea pig liver transglutaminase activity in presence of 1 µM PMA and 10 µM FK using the peptide crosslinking assay compared to untreated purified guinea pig liver transglutaminase (Fig. 3.3.8). Apart from chelerythrine, KT 5720 and Rp-8-Cl-cAMPs, all other protein kinase inhibitors significantly attenuated guinea pig liver transglutaminase activity. As RO-31-8220 induces a significant decrease (n = 3, **p < 0.001) in TG2 protein crosslinking activity assay (Fig. 3.3.8B).

### 3.3.6. Effect of protein kinase activators on protein level of TG2

#### 3.3.6.1. Screening cells for presence of transglutaminase family

Cardiomyocyte H9c2 cells were probed for the presence of different members of the transglutaminase family (TG1, TG2 and TG3) using SDS page (section 2.2.10) and Western blot (section 2.2.12) techniques as described in material and methods. The following figure of Western blot reveals that H9c2 cells can express TG1 (Fig. 3.3.9A) and TG2 (Fig. 3.3.9A and B). By contrast, TG3 was not detected in H9c2 cells as shown in (Fig. 3.3.9A).

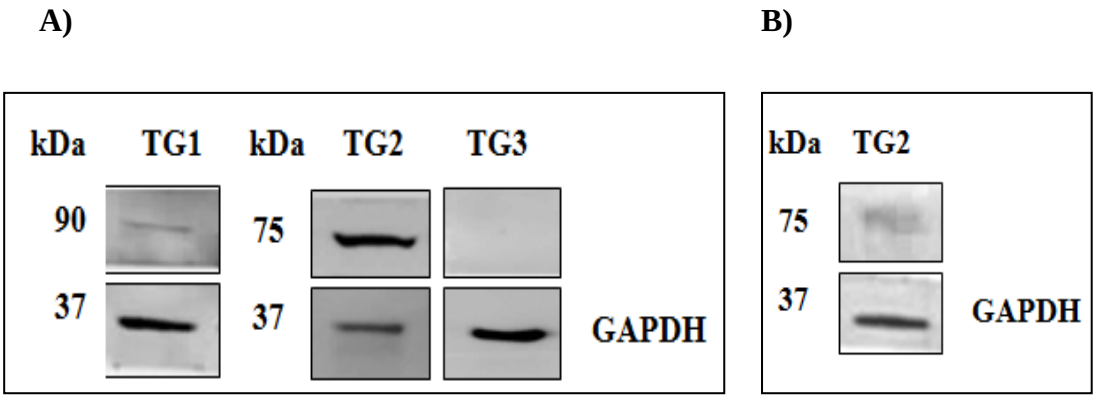


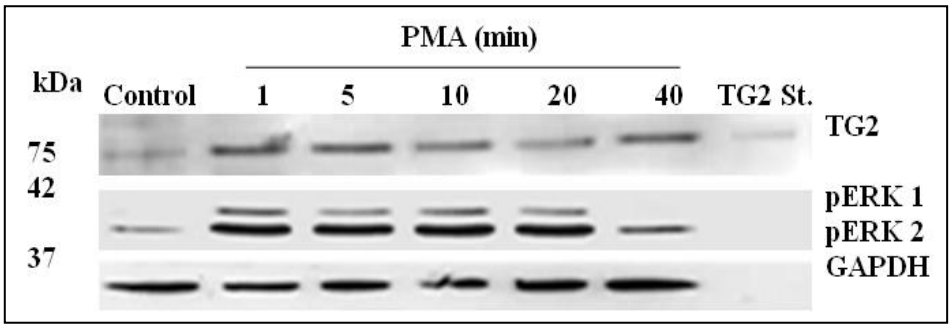

**Figure 3.3.9 Detection of transglutaminase family following in H9c2 cells**

The total protein extract (50 µg) from H9c2 cells was analysed by Western blotting for (A) TG1 by Anti-TG1 mAb (A), TG2 by Anti-TG2 (CUB 7402) mAb and TG3 by Anti-TG3 mAb (B) Anti-TG2 (ID10) mAb. Anti-GAPDH mAb was used as a loading control for the total amount of cellular protein. The results are typical of 4 independent experiments

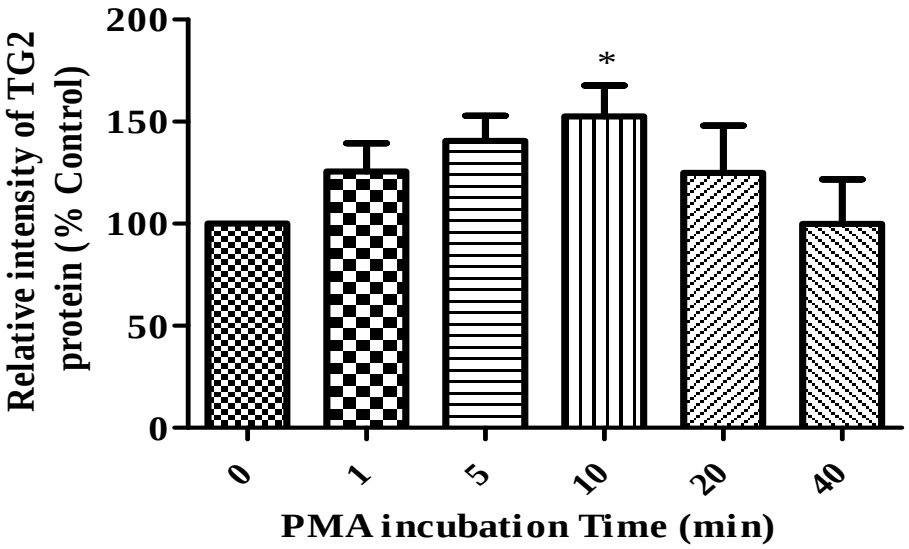
### 3.3.6.2. Levels of TG2 protein following PMA and FK exposure

Western blotting analysis of H9c2 cell extracts indicated that the levels of TG2 might alter following exposure to PMA (Fig. 3.3.10A) or FK (Fig. 3.3.10C) using anti-TG2 mAb (CUB 7402). Densitometry results for protein quantification of PMA treated H9c2 cells revealed strong significant increase (n=6, *p < 0.05) in TG2 protein level at 10 min incubation (Fig. 3.3.10B). However, cells that were treated with FK showed significant increases (n = 5, **p < 0.01) in TG2 protein level at both 5 and 10 min exposure time (Fig. 3.3.10D).

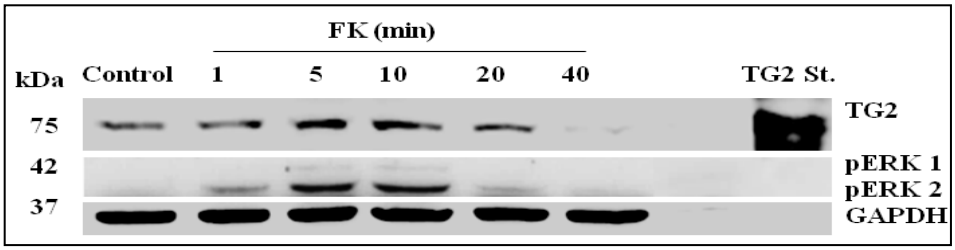
**A)**




**B)**



**C)**




**D)**

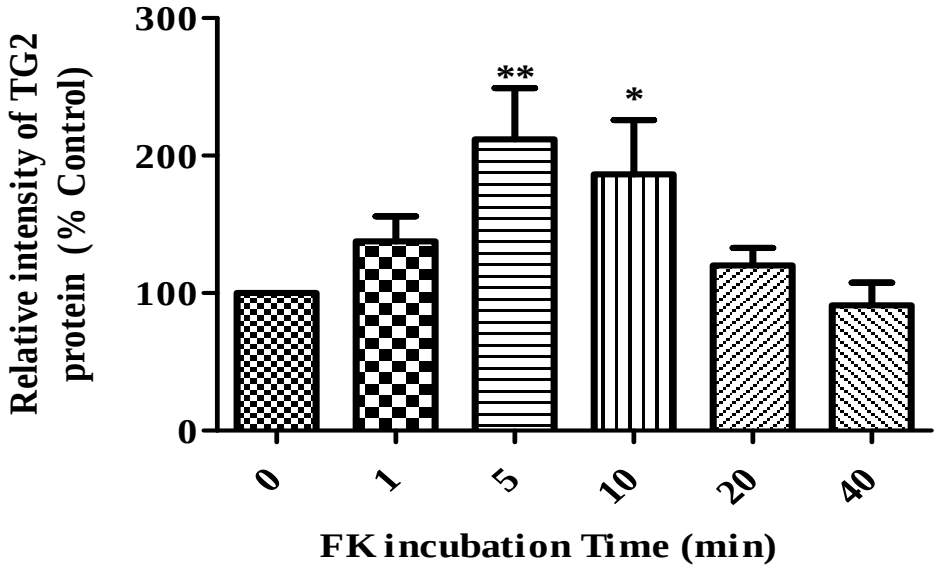
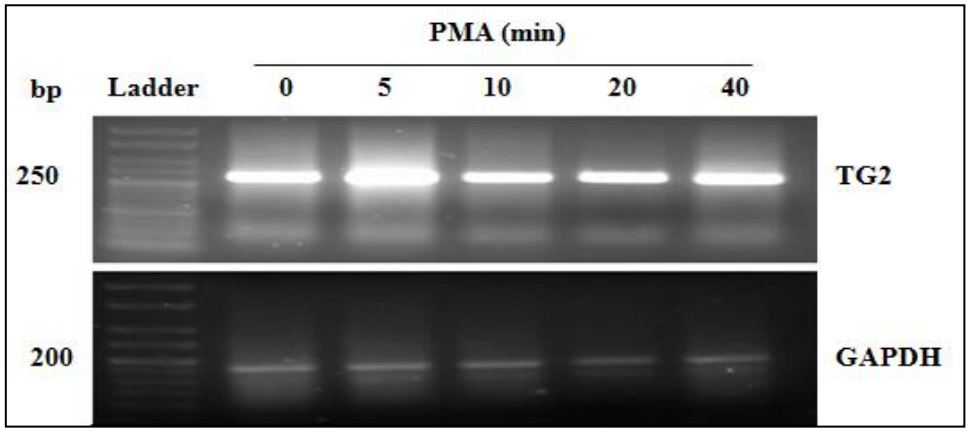



**Figure 3.3.10 Levels of TG2 protein following PMA and FK exposure**

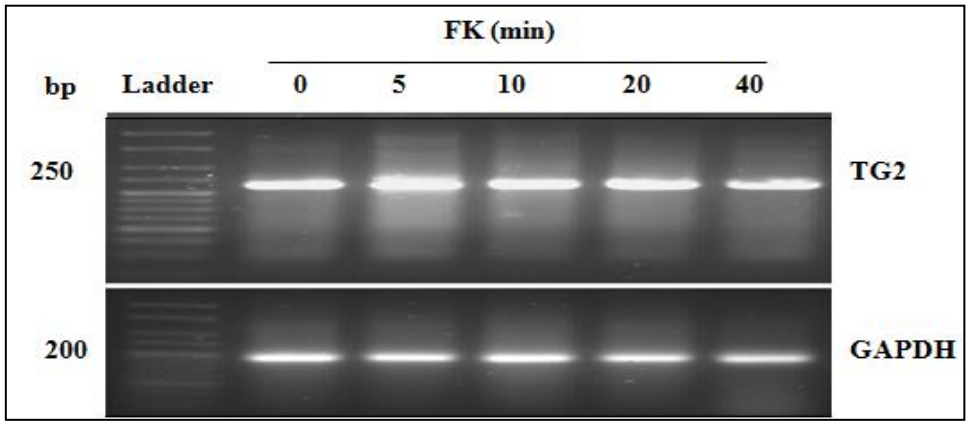
H9c2 cells were treated with A) 1 µM PMA or C) 10 µM FK for the times indicated. The total protein extract (50 µg) was analysed by Western blotting for TG2 by probing with primary antibody CUB 7402 and anti-GAPDH mAb was used as a control of the total amount of cellular protein and anti-pERK 1/2 as a control for protein kinase activators. (B-D) Densitometry was carried out in Adobe Photoshop CS4 and values plotted as relative intensity versus the treatment incubation time. Results represent mean ± SEM of the optical density ratio from 6 (B) or 5 (D) independent experiments. Data are expressed as the percentage of TG2 protein at basal level in the untreated cells (0 min) after GAPDH normalisation. Data analysis was performed using "Dunnett comparison test" to compare 0 min (control) vs. either PMA or FK incubation time. Statistical significance was accepted at *$p < 0.05$, **$p < 0.01$.

Transglutaminase 2 mRNA expression after PMA and FK treatment was also detected using reverse transcription polymerase chain reaction (RT-PCR). The PCR products along with the DNA ladder were resolved in 2.5 % (w/v) agarose gel electrophoresis (w/v) and visualized under UV light (see section 2.2.11). Transglutaminase 2 mRNA expression was also quantified by quantitative polymerase chain reaction (qRT-PCR) using Sybr Green (see section 2.2.25). The transcript abundance of TG2 relative to GAPDH transcript abundance was calculated and used to calculate changes in TG2 mRNA expression. Both results are shown in figure 3.3.11. In gel, the expression of TG2 mRNA was shown to increase after 5 min exposure to PMA or FK followed by a decrease (Fig. 3.3.11A and B).

**A)**




**B)**



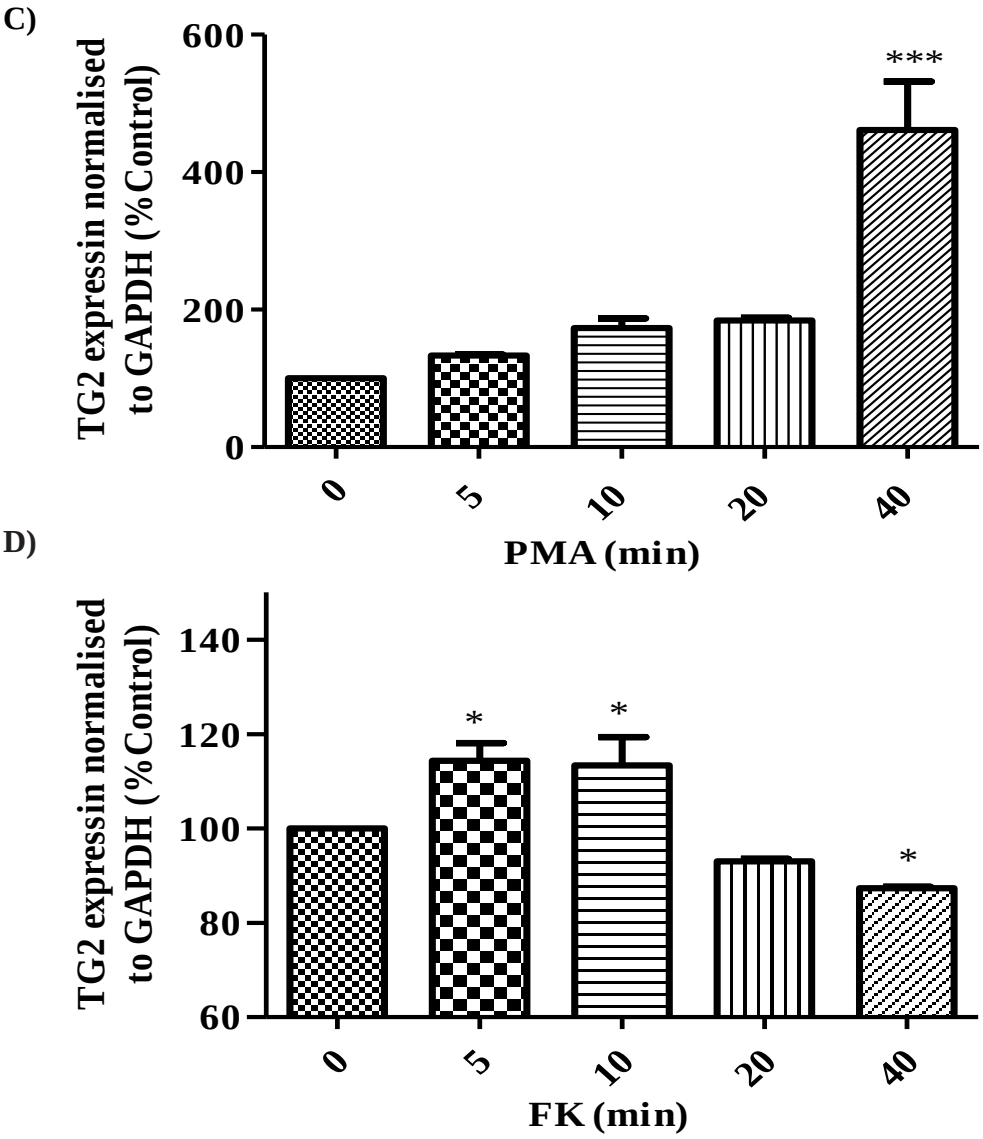



**Figure 3.3.11 Expression of TG2 mRNA after PMA and FK exposure using RT-PCR and qPCR**

A) H9c2 cells were treated with A) 1 µM PMA or B) 10 µM FK over time. Total RNA was extracted and the expression of TG2 mRNA was evaluated by RT-PCR. An equal load of PCR products were resolving in 2.5 % (w/v) agarose gel and visualised under UV light. The expression of GAPDH was used to normalize variable template loading. C-D) Quantification of TG2 mRNA expression by qPCR. Results represent mean ± SEM of TG2 mRNA expression from 3 independent experiment performed in triplicate. Data are expressed as the percentage of TG2 mRNA expression at basal level in the untreated cells (0 min) after GAPDH normalization. Data analysis was performed using "Dunnett comparison test" to compare 0 min (control) vs. either PMA (C) or FK (D) incubation time. Statistical significance was accepted at *$p < 0.05$, ***$p < 0.001$.

The TG2 mRNA expression results from qRT-PCR revealed increases in TG2 mRNA expression after 5 and 10 min exposure to FK, which was similar to the trends observed on probed western blots. However, the expression level of TG2 mRNA in samples treated with PMA showed a gradual, but not significant increase in expression. There was a significant increase ($n = 3$, $^{***}p < 0.001$) in TG2 mRNA expression detected after prolonged incubation with PMA at 40 min (Fig. 3.3.11C and D).

### 3.3.7. Levels of TG2 protein following PMA and FK exposure in the absence and presence of protein kinase inhibitors

Western blot analysis following PMA and FK exposure with pre-incubation of different protein kinase inhibitors showed a decrease in TG2 protein level compared to cells activated with either PMA (Fig. 3.3.12) or FK (Fig. 3.3.13) alone, GAPDH was used as intracellular control for cytoplasm protein, while pERK 1/2 was used as a control for protein kinase activators. A significant decrease ($n = 5$, $^{**}p < 0.01$) in TG2 expression was shown with FK /chelerythrine (CC) but not PMA and FK /RO-31-8220 compare to FK treated cells (Fig. 3.3.13A and B). In PMA treated cells, pERK 1/2 showed a significant increase compared to both control ($n = 4$, $^{***}P < 0.001$) and inhibitor treatments ($n = 4$, $^{*}p < 0.05$) (Fig. 3.3.12A and B). In FK treated cells, pERK 1/2 also showed a significant increase compared to both control ($n = 4$, $^{*}p < 0.05$) and inhibitors ($n = 4$, $^{**}p < 0.01$) (Fig. 3.3.13A and C).

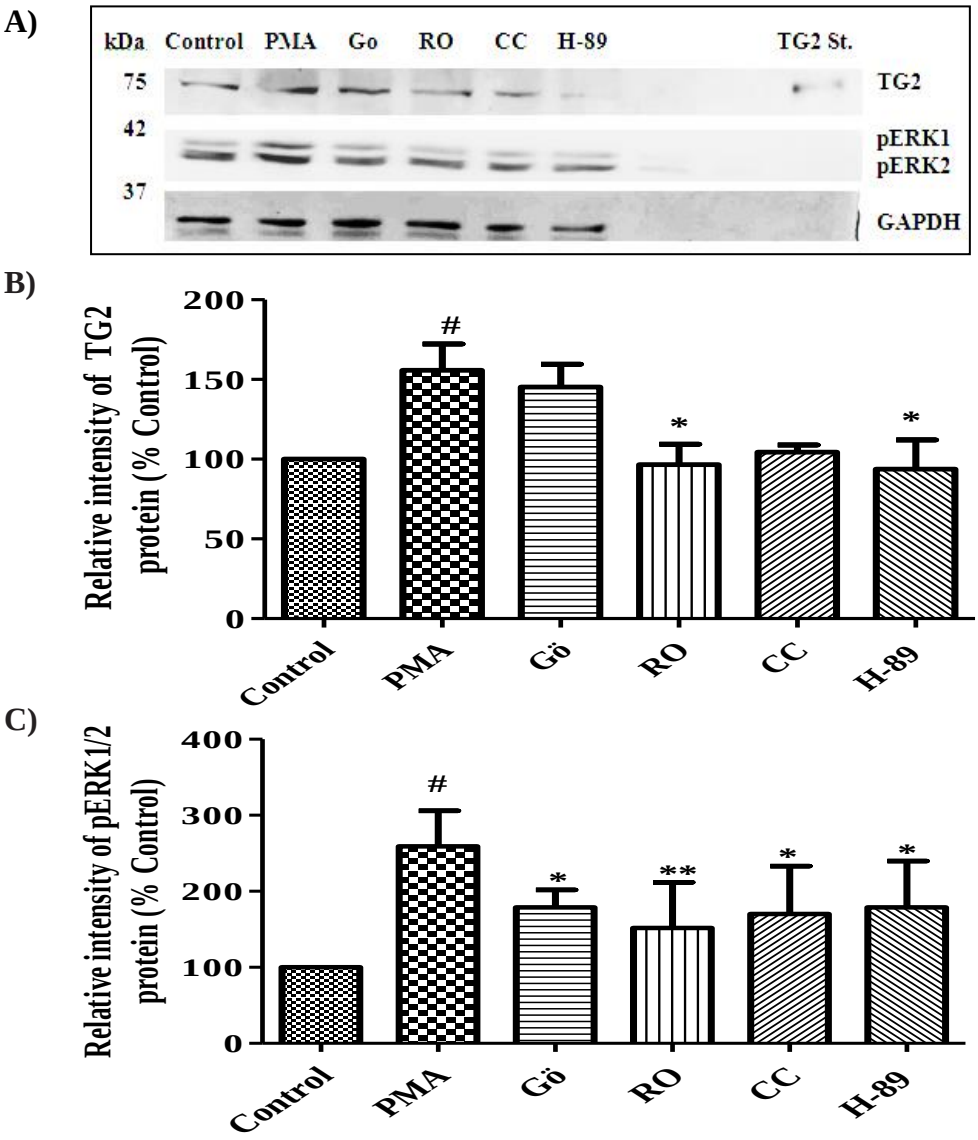



**Figure 3.3.12 Levels of TG2 protein following PMA exposure in the absence and presence of protein kinase inhibitors**

H9c2 cells were pre-incubated with different protein kinase inhibitors for 30 min followed by exposure for 5 min with 1 µM PMA. (A) The total protein extract (30 µg) was analysed by Western blotting for TG2 by anti-TG2 mAb (CUB 7402), anti-pERK 1/2 as a control for protein kinase activators and anti-GAPDH mAb was used as a control of the total amount of cellular protein. (B & C) densitometry was carried out in Adobe Photoshop CS4 and values plotted as relative intensity versus the treatment incubation time. Results represent mean ± SEM of the optical density ratio from 5 independent experiments. Data expressed as the percentage of TG2 protein at basal level of control after GAPDH normalisation. Data analysis was performed using "Bonferroni's multiple comparison test" to compare control vs. PMA or PMA vs. protein kinase inhibitors. Statistical significance was accepted at *$p < 0.05$, **$p < 0.01$. Significant protein level induced by PMA alone in compared to control were shown (#$p < 0.05$ (B) and (#$p < 0.001$ (C)).Chelerythrine (CC).

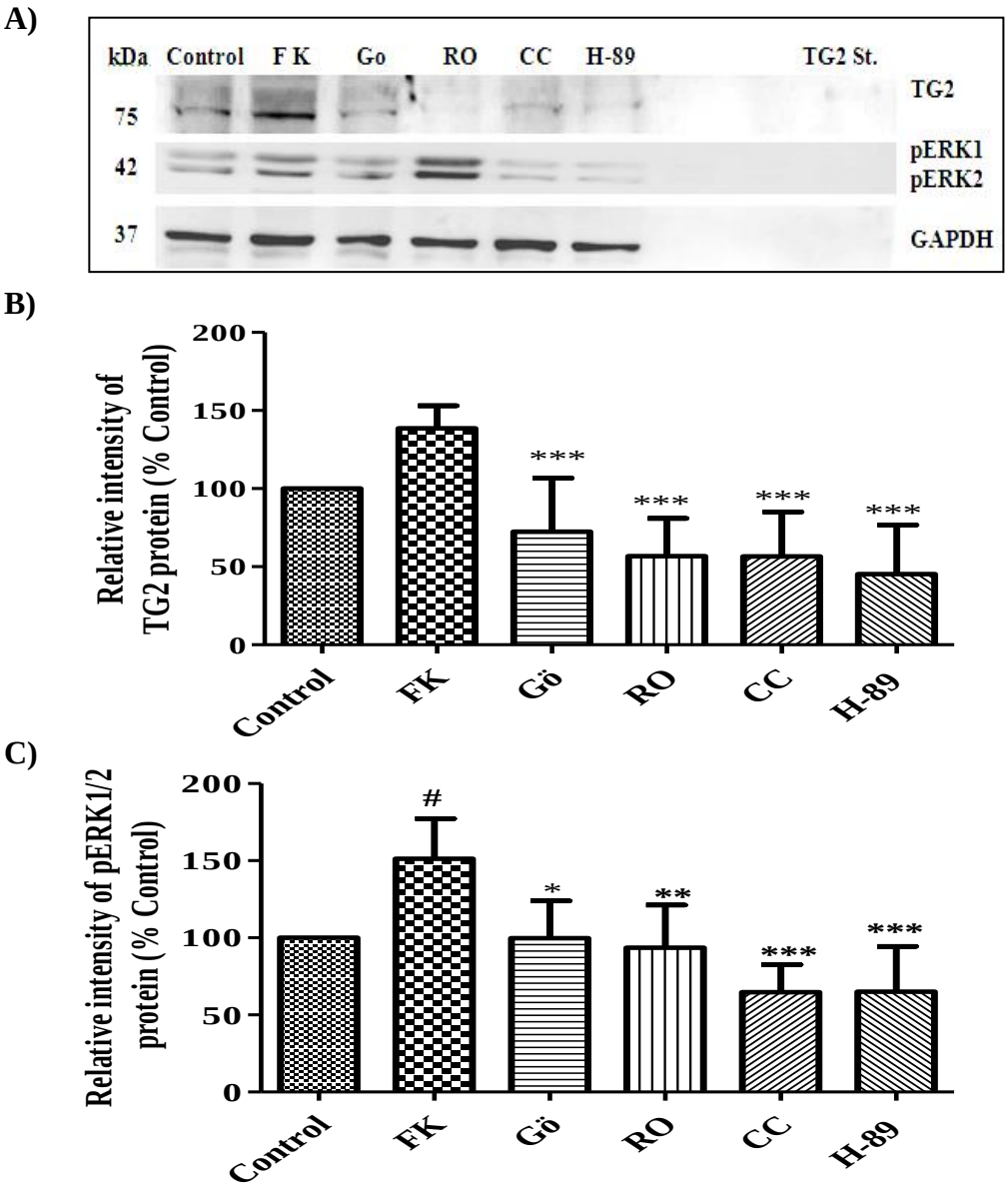



**Figure 3.3.13 Levels of TG2 protein following FK exposure in the absence and presence of protein kinase inhibitors**

H9c2 cells were pre-incubated with different protein kinase inhibitors for 30 min followed by exposure for 5 min with 10 µM FK. (A) The total protein extract (30 µg) was analysed by Western blotting for TG2 by anti-TG2 mAb (CUB 7402), anti-pERK 1/2 as a control for protein kinase activators and anti-GAPDH mAb was used as a control of the total amount of cellular protein. (B & C) densitometry was carried out in Adobe Photoshop CS4 and values plotted as relative intensity versus the treatment incubation time. Results represent mean ± SEM of the optical density ratio from 6 independent experiments. Data expressed as the percentage of TG2 protein at basal level of control after GAPDH normalisation. Data analysis was performed using "Bonferroni's multiple comparison test" to compare control vs. FK or FK vs. protein kinase inhibitors. Statistical significance was accepted at* $p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Significant protein level induced by FK alone in compared to control were shown (#$p < 0.05$). Chelerythrine (CC).

### *3.4. Discussion*

Transglutaminases are regulated by PKA and PKC in non-cardiomyocyte cell types (Bollag et al., 2005; Mishra et al., 2007). However, the regulation of TG2 by PKA- and PKC-dependent signalling in cardiomyocytes had not been reported prior to the current study. Therefore, the modulation of TG2 activity by these protein kinase activators was measured using two different TG2 transamination assays.

It is of essential to maintain and record the cell line growth and characteristics before setting any of experiment that require the use of cell line either *in vitro* or *in situ* investigation. Any abnormal alteration of cell growth can have significant effect on the experimental results (Wang, 2006). Therefore, initially, the growth of H92 cells was monitored and appropriate density of cells was defined. In this study, according to cell growth standard curve (Fig. 3.3.1B) the optimal day for sub-culturing and further experimental treatments of H9c2 cells was shown to be the third day of cell growth. A typical standard growth curve for different cultured cell lines has four different phases include, lag phase, log or growth phase, stationary phase and decline phase (Freshney, 2006; Wang, 2006). The third day was shown to be at the growth phase of H9c2 cardiomyocyte cells in which cells displayed rapid division and proliferation and form a monolayer of approximately 80-90 % confluent (Fig. 3.3.1A). This stage has shown to be the recommended phase for cell lines to be maintained in and to be assessed for different cellular functions before they enter the stationary phase and monolayer becomes 100 % confluent (Freshney, 2006) as in days 4-6 (Fig. 3.3.1). The sub-culturing and further experimental treatments of cells need to be setup in this phase to ensure cellular viability and enzymatic function, phenotypic and genetic stability (Budde et al., 1998). Accordingly, the third day of cells growth was adopted for subsequent experiments measuring the TG2 activity in H9c2 cells.

Initially, the direct effect of PMA and FK on purified guinea pig liver transglutaminase activity was investigated for comparison. In the biotin cadaverine incorporation activity assay (Slaughter et al., 1992), no significant effect was observed on purified guinea pig liver transglutaminase (GPL) activity in the presence of protein kinase activators (PMA and FK) (Fig. 3.3.7A). However, a significant decrease ($n = 4$, $^{***}p < 0.001$) was shown using the peptide crosslinking activity

assay (Trigwell et al., 2004) in comparison to purified guinea pig liver transglutaminase activity (Fig. 3.3.8). Conversely, the TG2 amine biotin cadaverine incorporation activity of H9c2 cells was shown to increase ($n = 3$, $^{**}p < 0.01$) in the presence of protein kinase activators (PMA and FK) at the early treatment time points (Fig. 3.3.2). This response was subsequently shown to be concentration dependent (Fig. 3.3.3). This suggests a relatively wide range of PMA or FK concentrations is able to induce an increase in TG2 incorporation activity within H9c2 cells.

Conversely, a significant reduction in TG2 protein crosslinking activity was noticed in early time point exposure of H9c2 cells to FK but not with PMA (Fig. 3.3.5A). It known that protein crosslinking requires high levels of $Ca^{2+}$ (Trigwell et al., 2004); therefore, the decrease could be due to either the concentration of calcium ions in cells not being at a high enough level to enhance TG2 crosslinking activity. Otherwise, the enzyme may have switched away from protein crosslinking to amine incorporation as a result of modification (phosphorylation). Moreover, since the substrate probe that was used in measurement of TG2 crosslinking activity is biotin-TVQQEL, which can incorporate into lysine residue substrates (Ruoppolo et al., 2003). It could be that H9c2 cells contain more many Glu-residue continuing substrates than Lys-residue continuing substrates.

The fact that the protein kinase activators used in this study interacted differently with transglutaminase, could be due to involvement of these protein kinase activators in cell signalling alteration (Chemin et al., 2007) in a manner that changes the action of TG2 inside the cells. Furthermore, it could be possible that the protein kinase activators alter the conformation of potential TG2 substrates in cells, resulting in an increase in available substrate contributing to the higher TG2 activity observed. Indeed, it is already known that activation of protein kinases results in protein phosphorylation which in turn can directly affect the activity of target proteins by causing a conformational change in these target proteins (Cohen, 2000). Alternatively, the phosphorylated amino can bind to specific protein or other substrate and alter its activity and function (Pearce et al., 2010). Therefore, this study investigated the phosphorylation events induced by these two protein kinase activators in H9c2 cells. The activation of protein phosphorylation resulting from PMA induced-PKC

activation agrees with other studies that have reported phosphorylation effects of phorbol esters (Jacquier-Sarlin et al., 1995; Teixeira et al., 2003; Bollag et al., 2005).

The present results have demonstrated that both PMA and FK induce protein phosphorylation in H9c2 cells. Following electrophoresis or polyacrylamide gels that were stained with Pro-Q Diamond fluorescent stain to evaluate protein phosphorylation and subsequently stained with SYPRO Ruby stain for total protein (see section 2.2.15). The ratio of total protein-to-protein phosphorylation was found to change significantly within 5 min after treatment with PMA and FK compared to the control cells (see appendix Fig. 8.1 and Fig. 8.2). The increase was most prominent after 5 and 10 min in cells that were pre-incubated with MA or FK. It had already been demonstrated that the exposure of cardiomyocytes to PMA for 5 min leads to increased protein phosphorylation mainly through activation of MAPK and mitogen-activated extracellular signal-regulated kinase (MEK) 10 fold (Lazou et al., 1994; Lazou et al., 1998). The increased phosphorylation of proteins relative to the control in samples treated with PMA at the early time points was also confirmed in the current work. This may indicate a rapid increase ($n = 4$ ***$P < 0.001$) of phosphorylation events in cardiomyocyte-like H9c2 cells in response to PMA treatment (Fig. 8.1). This was also true when cells were treated with FK (Fig. 8.2). Increased protein phosphorylation has been reported in reperfused heart tissue in response to FK (England & Shahid, 1987). Additional reports have detailed the phosphorylation of the cAMP response element binding protein (CREB) in cardiac myocytes (Goldspink & Russell, 1994) and $ser^{16}$ phosphorylation in swine artery HSP20 (Meeks et al., 2008).

The level of protein phosphorylation in PMA or FK treated H9c2 cells at different time points was also monitored by western blot analysis using different anti-phospho-amino acid antibodies (see appendix Fig. 8.3). The current study found increased phosphorylation of target proteins in serine, threonine and tyrosine residues. Western blots of H9c2 cell extracts treated with either PMA or FK at different time points were probed with anti-phosphoserine (Fig. 8.3A) and anti-phosphothreonine antibodies (Fig. 8.2B). In general, the results demonstrate an increase in band intensity of proteins containing either phosphoserine or phosphothreonine in treated samples over time compared to the control cells (0 min). Interestingly, on the

nitrocellulose membrane filters that were probed with anti-phosphoserine (Fig. 8.3A). There was a slight increase in band intensity ~74 kDa, which corresponded to standard TG2, in comparison to control and one more band at 50 kDa. However, when membrane was stripped and re-probed with anti-phosphothreonine antibodies, the TG2 standard no longer appeared but a new band corresponding to ~70 kDa was detected. Another western blot of the sample was probed with anti-phosphotyrosine antibodies (Fig. 8.3C). This showed an increase in band intensity of proteins that corresponded to size of~100 kDa and ~77 kDa over the same incubation times.

These observations demonstrated that a wide range of proteins can be phosphorylated in H9c2 cells following exposure to these kinase activators. Thus, it could be possible that this phosphorylation event results in conformational changes of these target proteins or in the affinity of specific proteins to recognise and bind to other proteins and thus affect the regulation of TG2 activity. Indeed, it has been suggested that PMA can alter polyamine levels in human promyelocytic leukemia cells, affecting transglutaminase activity (Huberman et al., 1981).

Activity in both protein crosslinking and biotin cadaverine incorporation assay of purified guinea pig liver TG2 was shown to decrease in the presence of certain protein kinase inhibitors. The biotin cadaverine incorporation assay (Fig. 3.3.7B) shows a significant ($n = 4$, $^{***}P < 0.001$) decrease in the presence of the protein kinase C inhibitors Gö 6983 (Peterman et al., 2004) and chelerythrine (Herbert et al., 1990), and the protein kinase A inhibitor H-89 (Makaula et al., 2005; Asai et al., 2009). By contrast, the protein crosslinking activity assay (Fig. 3.3.8B) shows a significant ($n = 3$, $^{***}p < 0.001$) decrease in activity with RO-31-8220, a PKC or PKA inhibitor (Davies et al., 2000), whereas no significant decrease was observed with chelerythrine. The purified transglutaminase was shown to react to protein kinase inhibitors in the same manner to that of the TG2 assayed H9c2 cells before and after exposure to PMA or FK (Fig. 3.3.6). This indicates that protein kinase inhibitors may be able to interact directly with TG2, resulting in its inhibition. However, not all the protein kinase inhibitors induced changes in TG2 activity except chelerythrine, which showed a significant increase in TG2 catalysed biotin cadaverine incorporation activity of untreated cells with protein kinase activators (Fig. 3.3.6C). This suggests that chelerythrine reacts differently with TG2 following protein kinase activators pre-

treatment in H9c2 cells, possibly interacting with TG2 kinase site. Indeed, the chelerythrine chloride is a selective-reversible inhibitor for protein kinase C that is able to inhibit the enzyme activity by two different ways, either competitively with phosphate acceptor or uncompetitively with ATP binding site of the enzyme (Herbert et al., 1990). These properties of the inhibitor can reduce its specificity toward PKC as it is appeared from this study that the inhibitor can block PKA activity as well. The inhibitor is also able to independently activate MAPK of PKC inhibition. This can explain the activation of TG2 incorporation activity of cells stimulated with chelerythrine chloride (CC) alone (Fig. 3.3.6C). However, Kinetic analysis of the effects of the inhibitors may help to elucidate their mode of action.

PKA inhibitors H-89 (1 μM), KT5720 (5 μM) and Rp-cAMPs (50 μM) have been already used in H9c2 and in HeLa cells to block cAMP and forskolin activated PKA (Dodge-Kafka et al., 2005; Kwak et al., 2008; Galliher-Beckley et al., 2011). In addition, both these protein kinase inhibitors H-89 and KT5720 have reversed the protective effects induced by either DBcAMP (a cAMP analogue) or forskolin in H9c2 cells (Chae et al., 2004). Due to PKA heterotetramirc form, the inhibition of the enzyme can be achieved by two distinct ways either via inhibitors that have structure analogue to cAMP such as, Rp-8-Cl-cAMPS or via inhibitors structure analogues to ATP such as, H-89 and KT 5720 (Christensen et al., 2003; Christensen et al., 2003; Lochner & Moolman, 2006). Since Rp-8-Cl-cAMPS are able to block the regulatory subunit of PKA preventing holoenzyme dissociation, thus it is work in very earlier step to block the enzyme (Davies et al., 2000). This unique difference in acting site by Rp-8-Cl-cAMPS to inhibit PKA makes it more specific than the other PKA inhibitors (Daugirdas et al., 1991; Hughes et al., 1997). Interestingly, PKA inhibitors KT 5720 and Rp-8-Cl-cAMPS blocked FK induced TG2 activity (Fig. 3.3.6D), confirming the involvement of PKA in FK-mediated responses.

These results are of particular interest, as the increase in TG2 activity at early time points occurs at a critical time in ischaemic preconditioning of heart tissue (Murry et al., 1986). A previous study by Tucholski & Johnson (2003) provided evidence that TG2 mediates adenylyl cyclase activity in human SH-SY5Y neuroblastoma cells. Another study in mouse embryonic fibroblasts showed that activation of PKA with dibutyryl-cAMP triggered phosphorylation of both TG2 at serine residue $Ser^{216}$

(Mishra & Murphy, 2006) and retinoblastoma protein (Rb) at $Ser^{780}$, but PKA kinase activity was inhibited by H-89 a PKA inhibitor (Mishra et al., 2007). In addition, phorbol esters have been implicated in the stimulation of transglutaminase activity in keratinocytes through activation of PKC (Chakravarty et al., 1990; Rice et al., 1996). In this study, PMA and FK modulated the levels of TG2 protein as verified by Western blotting (Fig. 3.3.10) and activity alterations that were impeded using different PKC and PKA inhibitors (Fig. 3.3.6). The altered expression of TG2 mRNA was also confirmed using semi- and qRT-PCR (Fig. 3.3.11). The discrepancy between PCR image results and quantification results is due to the limitation of traditional PCR that depends on size discrimination of agarose gel results, which is obtained from reaction end point (Heid et al., 1996; Schmittgen & Livak, 2008). The qPCR is unlike traditional PCR, its results are obtained from exponential phase, which is the optimal point for data analysis that representing the actual relation between the mount of starting sample and amplification of PCR product at different cycle number (Heid et al., 1996; Schmittgen & Livak, 2008). Although, the TG2 expression varies from sample to sample, detection of reaction end point in agarose gel is not able to resolve this variation. However, qPCR results confirmed these differences (Fig. 3.3.11B).

Previous studies in a human endometrial adenocarcinoma cell line indicated that induction of TG2 expression was mediated by activation with calcitonin. This polypeptide hormone has been implicated in regulation of $Ca^{2+}$ homeostasis through its cell surface receptor utilizing both cAMP and $Ca^{2+}$ signalling pathways (Li et al., 2002; Li et al., 2006). This expression was inhibited upon treatment with H-89, a PKA inhibitor but not with calphostin C a PKC inhibitor (Li et al., 2006).

A phorbol ester (PMA) has also been used to investigate protein kinase C activation and ERK1/2 phosphorylation (Nanzer et al., 2004). Protein kinase C inhibitors impede PMA-induced phospholipase D (PLD) activation and ERK-1/2 phosphorylation in keratinocytes with a profile that associates with their ability to inhibit PMA-stimulated TG1 activity (Bollag et al., 2005). In addition, FK-stimulated cAMP significantly increased ERK1/2 phosphorylation in ventricular myocytes (Wang et al., 2009).

Extracellular signal-regulated kinases (ERK 1 and 2) are one of the major MAPK families that have already been shown to have a protective role in cardiomyocytes via activation of anti-apoptotic pro-survival pathways (Abe et al., 2000; Sugden & Clerk, 2001). These kinases also reported to be involved in ischaemic and pharmacological preconditioning (Chesley et al., 2000; Punn et al., 2000). It has been reported that over-expression and activation of the G protein Gαh (i.e. TG2) stimulates norepinephrine-induced ERK activation and is inhibited by an alpha-adreno-receptor blocker (prazosin) in neonatal rat cardiomyocytes (Lee et al., 2003). One study has shown that in H9c2 cells over expression of TG2 induced by retinoic acid (RA) induces phosphorylation of the MAPKs ERK1/2, and JNK; inhibition of TG2 by anti-tTGase antibody resulted in an inverse effects on those kinases (Kim, 2009).

Our study has shown that activation of TG2 via PKC and PKA activators leads to phosphorylation of ERK 1 and 2 in the response at PMA and FK (Fig. 3.3.10). Analysis of PKC and PKA downstream signalling pathways showed that constitutive activation or transient stimulation of TG2 activity were associated with increases in ERK1/2 activation in cardiomyocytes (Fig. 3.3.12 and Fig. 3.3.13).

Modulation of TG2 by ERK signalling has been demonstrated in fibroblasts (Wang, 2010). It has been reported that increased activation of the ERK pathway stimulates TG2 mRNA expression and biosynthesis, whereas inhibition of ERK results in the opposite effect (Akimov & Belkin, 2003). The effects of pharmacological inhibitors on different protein kinases in H9c2 cells resulted in inhibition of both ERK phosphorylation (Fig. 3.3.12 and Fig. 3.3.13) and transglutaminase activity. This suggests that the increase in TG2 activity following PMA or FK exposure could be a protective cellular response in H9c2 cardiomyocyte.

In conclusion, these results suggest that a time course exposure of H9c2 cells to PMA or FK showed a significant increase in TG2 catalysed biotin cadaverine incorporation after 5 min exposure. This response was subsequently shown to be concentration dependent. Conversely, using different PKC and PKA inhibitors, this activation could be moderated. These data suggested modulation of TG2 *in vitro* by these protein kinase signalling pathways.

# CHAPTER IV:

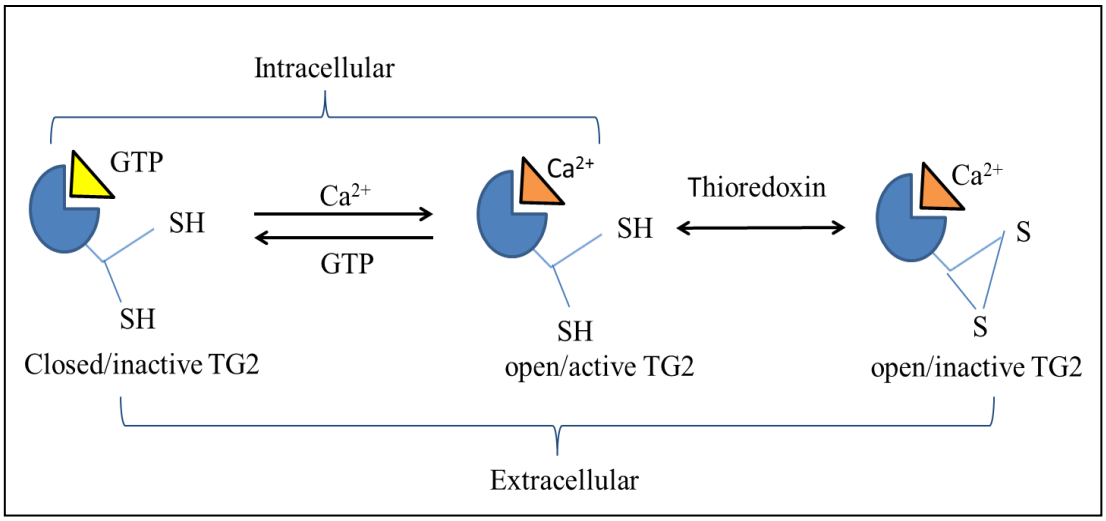
# *IN SITU* MODULATION OF TG2 ACTIVITY BY PKC / PKA AND THEIR RECEPTORS

## 4. Introduction

Transglutaminase 2 activity is tightly regulated and controlled by posttranslational modification mechanisms. The extensively studied mechanism involves $Ca^{2+}$ ions, guanine nucleotides and the redox state of the enzyme's regulator environment. The activation of TG2 requires the binding of $Ca^{2+}$ ions at multiple calcium binding sites (Bergamini, 1988; Király et al., 2009). The presence of the guanine nucleotide molecules GTP or GDP can inhibit $Ca^{2+}$ ion binding to TG2 and thus inactivate it. However, high concentration of calcium and low GTP/GDP is needed for activation. The binding and release of these molecules with TG2 enzyme are associated with a large conformational changes in its structure (Fig. 4.1.1a). The active and inactive forms of TG2 have helped in understanding the reason why the enzyme remains inactive inside the cell under normal conditions (low free $Ca^{2+}$ and high GTP/GDP; Siegel et al., 2008). The redox state of the enzyme environment has also helped in clarifying inactivation of TG2 in the extracellular environment (high calcium and low GTP/GDP; Siegel et al., 2008). This is due to the higher oxidative environment of the extracellular matrix, which maintains TG2 in its inactivate state by the formation of a disulphide bond between vicinal residues maintaining $Ca^{2+}$ bound TG2 in an inactive state (Stamnaes et al., 2010; Jin et al., 2011). However, the protein cofactor thioredoxin-1 can transiently activate TG2 in a biological mechanism that could involve interferon-γ, a cytokine that helps immunity against infections and for tumour it is also key activator of macrophages (Abassi et al., 2001; Schroder et al., 2004; Jin et al., 2011).

This mechanism was also shown to be responsible in activation of extracellular TG2, mediating the deamidation of gliadin in coeliac disease patients (Nadalutti et al., 2013). Once activation of transglutaminase has occurred, the active site cysteine thiol residue is displayed and interacts with the glutamine protein bond carboxamide, resulting in ammonia release (Fig. 4.1.1b). Subsequently, the generated thioester intermediate can then be attacked by an ε-amino group from a lysine residue, converting glutamine to glutamate. This results in the formation of a stable ε(γ-glutamyl)-lysine isopeptide bond that is resistant to proteolysis (Griffin et al., 2002).

**A)**




**B)**

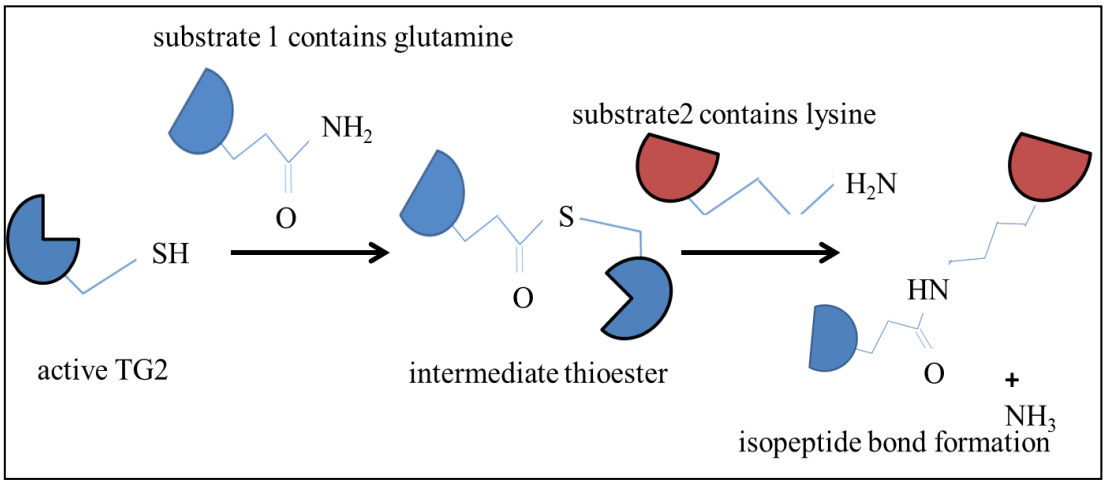



**Figure 4.1.1 Conformation state of transglutaminase and its activity**

A) Transglutaminase 2 states under different physiological conditions and their activity. B) the catalytic mechanism of TG2, the active site cysteine attacks the glutamine residue of acyl donor substrate 1, generating an intermediate thioester that can be attacked by the amine contains a lysine of an acyl acceptor substrate 2, forming an isopeptide bond and ammonia released.

The transamidation activity of TG2 is of pharmacological importance, as it is believed that this activity contributes to a wide range of essential physiological processes and the pathogenesis of diseases. The enzymatic activity allows TG2 to crosslink proteins at diverse subcellular location and thus regulates cell adhesion, cell signalling,

apoptosis and differentiation (Spom & Roberts, 1983; Melino & Piacentini, 1998; Lorand & Graham, 2003; Zemskov et al., 2006).

Many different methods have been developed to measure the enzymatic activity of TG2 ether *in vitro* or *in situ*. One of these is the using of a microplate *in vitro* TG assay which, usually utilizes N,N′-dimethylcasein as acyl donor and 5-(biotinamido)pentylamine as the substrate (Slaughter et al., 1992) (section 2.2.3). The assay used to measure the incorporation of polyamines into proteins as signal absorbance (Piacentini et al., 1988; Fesus & Tarcsa, 1989; Esposito et al., 2003). The other approach is detection of *in situ* activity, which is unlike the *in vitro* assay as it depends on unknown substrates. The benefit of *in situ* experimentation is that it measures TG2 activity in its natural environments. In addition, this method is more visual than the *in vitro* assay and using it can help in the detection and localisation of TG substrates in different cell compartments. The *in situ* detection of transamidation activity of TG2 can be carried out by the incorporation of artificial substrates, e.g. monodansyl-cadaverine or 5-biotinamidopentylamine into protein in either permeabilised or intact cells. The incorporated substrates is then detected in SDS-PAGE or *in situ* using antibodies (Jeon et al., 1989; Slaughter et al., 1992). Moreover, for the rapid and sensitive measurement of *in situ* activity, biotinylated or fluorescent amine incorporation into tissue (Lesort et al., 2000) or cells (Yamane et al., 2010; Itoh et al., 2011) has been used.

The inhibition of enzymatic activity of TG2 is of therapeutic interest in the treatment of certain diseases (Yuan et al., 2005). Therefore many classes of TG2 irreversible inhibitors have been synthesised (Wodzinska, 2005; Siegel & Khosla, 2007). Some of these inhibitors are dihydroisoxazole small-molecule inhibitors, such as KCA075 and KCC009 that can highly specifically and irreversibly inhibit human TG2 (Choi et al., 2005; Yuan et al., 2006). In addition, the membrane permeable irreversible inhibitors of transglutaminase R283 and ZDON (Fig. 4.1.2) were also used to confirm a reduction in *in vitro* and *in situ* TG2 activity in cells or tissue sections (Maiuri et al., 2008; McConoughey et al., 2010).

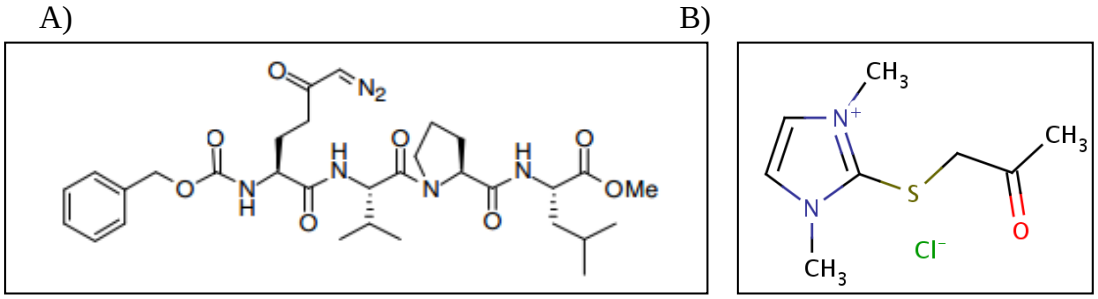



**Figure 4.1.2 The chemical structure of membrane permeable irreversible transglutaminase inhibitors**

A) Z-DON; Benzyloxycarbonyl-(6-diazo-5-oxonorleucinyl)-L-Valinyl-L-Prolinyl-L-Leucinmethylester a peptide-based irreversible, active site directed inhibitor. It contains a 6-diazo-5-oxonorleucinyl (DON) core mimics the gluten peptide and mediates the alkylation of active-site cysteine in TG2 that attack the carbonyl group, resulting in nitrogen release. B) R283; 1,3-dimethyl-2[(oxopropyl)thio]imidazolium) a potent irreversible, active site directed inhibitor. The reaction mediates the acetonylation of active-site cysteine in TG2 that attack the carbonyl group (functional group of the enzyme) mimic glutamine residue. Both inhibitors reaction resulting in a stable thioether adduct. Adapted from McConoughey et al., (2010).

Beta-adrenergic receptors (β-AR) belong to the G-protein coupled receptor (GPCR) superfamily. There are three subtypes of β-AR: $\beta_1$, $\beta_2$ and $\beta_3$ all of which couple to $G_s$-proteins and the $\beta_1$-AR is the main subtype expressed in the mammalian heart. Accumulating evidence has shown that activation of β-AR is associated with the increasing of intracellular levels of cAMP in animal heart models (Xiang & Kobilka, 2003; Dorn et al., 2008). Signalling by cardiac β-receptors has been extensively studied. The common pathway is initiated from stimulation of β-ARs coupled to Gs (an alpha subunit of the stimulatory G protein) which activate adenylyl cyclase (AC) and thus increases cAMP-production to activate PKA (Lefkowitz, 2007). The main target for cAMP is PKA, which represents a key signalling mechanism for neuro-hormonal stimulation regulating diverse cardio-physiological processes from contraction and energy metabolism to heart performance. Upon activation of PKA in cardiac myocytes, two major changes are observed; a) improvement of cardiac contraction; and this involves phosphorylation of two essential ion channels on the plasma membrane and sarcoplasmic reticulum in addition to phosphorylation of myofibril contractile proteins (Xiang & Kobilka, 2003). b) The negative stimulation of ligand-bound β-ARs occurs by phosphorylation of activated receptors (Lefkowitz,

2007). Some of the protein targets phosphorylated following PKA activation are L-type calcium channels (Zhao et al., 1994), troponin I, phospholamban (Sulakhe & Vo, 1995) and MyBP-C a myosin binding protein-C (Kunst et al., 2000). It is of interest to notice that some of these essential proteins are also TG2 substrates, such as myosin (Orrù et al., 2003) and troponin I (McDonough et al., 1999).

The $A_1$-adenosine receptor is one of the G-protein coupled receptor superfamily and it is a member of four-adenosine receptor subtypes ($A_1$, $A_{2A}$, $A_{2B}$ and $A_3$) that have been cloned and designated (Mubagwa & Flameng, 2001). Adenosine receptors have been shown to be expressed in ventricular cardiomyocytes and regulate different cellular functions (Auchampach & Bolli, 1999). Activation of the adenosine A1 receptor using different pharmacological agents on myocytes through binding to the (α) subunit of the heterotrimeric G protein (Gi) results in adenylyl cyclase inhibition and thus decreases concentration of cAMP, while the binding to (β or δ) subunits leads to PLC-β activation (Dickenson & Hill, 1997; Dickenson et al., 2012). This results in an increase of inositol triphosphate (IP3) and diacylglycerol (DAG) concentration (Wu et al., 1992; Terzic et al., 1993). Furthermore, the role of different adenosine subtype receptors in cardioprotection has been demonstrated in different species (Fredholm, et al., 2001). The adenosine receptor has been considered as a potential trigger for cardioprotection mechanism against ischaemic damage. However, the underlying mechanisms of adenosine-mediated cardioprotection remain unclear, but appear to involve PKC, since different protein kinase inhibitors e.g. (chelerythrine, polymyxin B and staurosporine) attenuate cardioprotection effects induced by adenosine receptor activation (De Jong et al., 2000).

Agonists such as diazoxide will pharmacologically precondition the heart against the effects of ischaemia and thus reduce infarct size (area of ischaemic necrosis in issue), that is similar to the one induced by ischaemic preconditioning (Forbes et al., 2001). This diazoxide-induced activation of mitochondrial $K_{ATP}$ channel is triggered by adenosine and nitric oxide (NO) (Lochner, et al., 2002). The adenosine allows mitochondrial $K_{ATP}$ channel opening via diazoxide, while nitric oxide improves mitochondrial $K_{ATP}$ channel activation by diazoxide (Sato et al., 2000). The opening of mitochondrial $K_{ATP}$ results in elevated levels of ROS and reactive nitrogen species (RNS) that are required for the signalling cascade. This in turn activates a wide range

of kinases, such as PKC, JAK/STAT, and p38 MAPK that are also involved in cardiac protection (Nakano et al., 2000). Moreover, the translocation of heat shock proteins was also shown to be a downstream target for adenosine receptor or PKC activation in the cardioprotection process (Sakamoto et al., 2000). However, receptor-mediated cardiomyocyte signalling pathways involved in TG2 activation have not been investigated before.

Taken together, the above considerations coupled with the importance of both of these G protein-coupled receptors in cardioprotection mechanism and the fact that they are both downstream targets for PKA (β-adrenergic receptor) and PKC ($A_1$-adenosine receptor) activation suggest that further investigation would be worthwhile. Therefore, the possible link between the stimulation of these receptors to induce PKC or PKA and TG2 activation mediated by this pathway was investigated.

### *4.1. Aims*

The main aims of the work presented in this chapter were to determine the effects of receptors activation and PMA/FK on TG2 activity *in situ in present and absence of TG2 inhibitors*.

### *4.2. Methods*

As described in chapter 2 of this study (section 2.2).

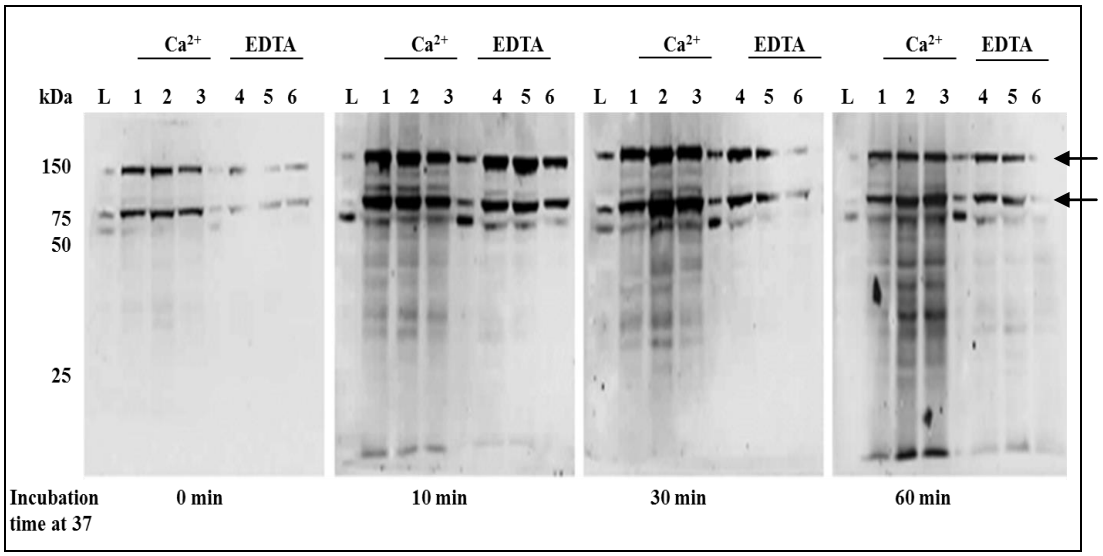
### *4.3. Results*

In the previous chapter, the data presented showed that time dependent exposure of H9c2 cells to PMA or FK showed a significant transient increase in TG2 catalysed biotin cadaverine incorporation after 5 min. This response was subsequently shown to be concentration-dependent. Conversely, using different PKC and PKA inhibitors, this activation could be moderated. However, western blotting analyses of H9c2 cell extracts and qRT-PCR indicated that the protein and mRNA levels of TG2 might alter following exposure to PMA or FK and protein kinase inhibitors. In this chapter, TG2 activity was investigated in intact cells.

#### 4.3.1. Activation of endogenous TG2 in response to PMA and FK in a calcium-dependent manner

To determine whether PMA or FK-induced TG2 activation in H9c2 cells was related to activation of endogenous TG2 in a calcium-dependent manner, equal amounts of cell proteins were incubated in the presence of 1 mM biotin cadaverine (BTC) which represents the acyl-acceptor probe in the presence of either 5 mM $Ca^{2+}$ or EDTA (background control). Reaction mixtures were subjected to immuno-blotting and the membranes were probed with ExtrAvidin®peroxidase as described in materials and methods (section 2.2.19). The acyl-acceptor probe biotin cadaverine was incorporated into endogenous protein substrates of TG2 in H9c2 cells. The results showed calcium dependent incorporation of biotin cadaverine into numerous proteins in H9c2 cells (Fig. 4.3.1). More importantly, treatment of H9c2 cells with PMA and FK in the presence of 5 mM $CaCl_2$ resulted in significant labelling of cellular proteins while control cells that were not treated with PMA or FK were shown to have less biotin cadaverine labelling in the presence or absence of calcium (Fig. 4.3.1).

A)


Ca2+
EDTA
kDa
L 1 2 3 4 5 6
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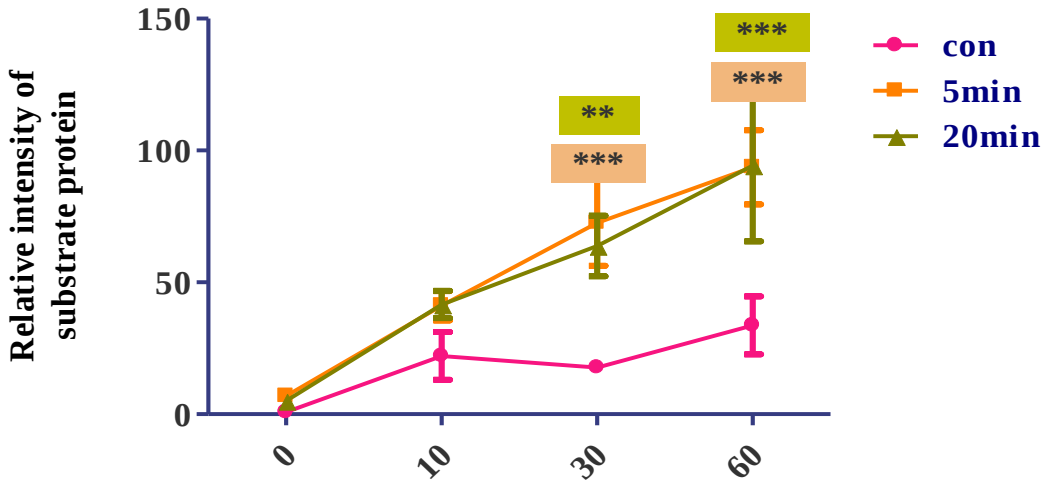
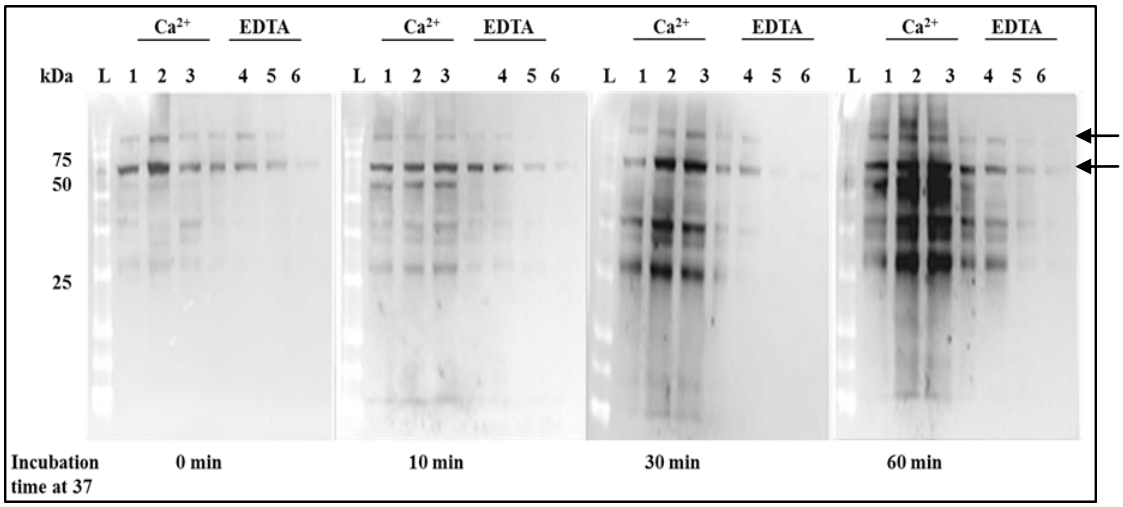
75
50
25
Incubation time at 37
0 min
10 min
30 min
60 min

PMA

B)


Relative intensity of substrate protein
150
100
50
0
**
***
***
***
con
5min
20min
0
10
30
60
Incubation time at 37 °C (min)

C)


Ca2+
EDTA
kDa
L 1 2 3 4 5 6
75
50
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Incubation time at 37
0 min
10 min
30 min
60 min

D)

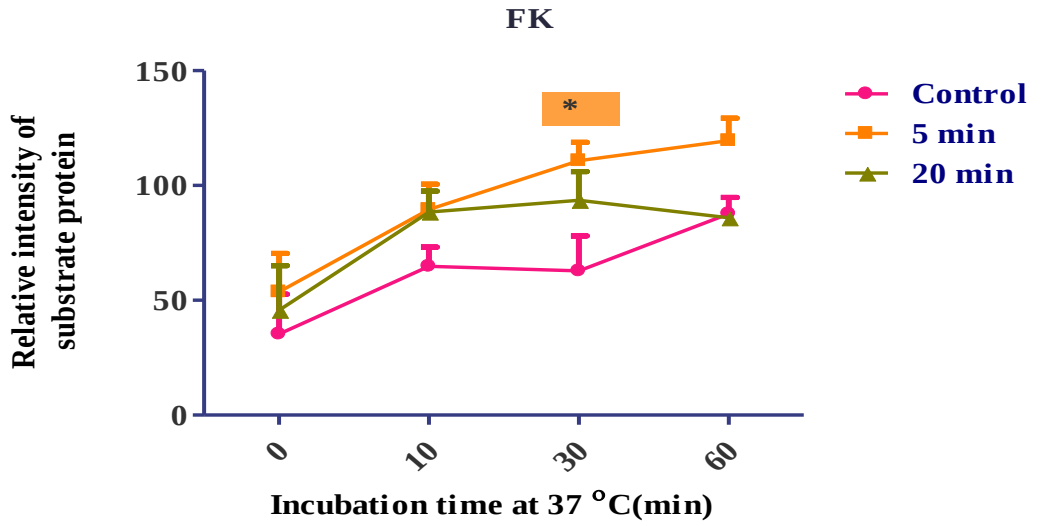



**Figure 4.3.1 Activation of endogenous TG2 in response to PMA and FK in a calcium-dependent manner**

Labbling of endogenous protein substrates in (A) PMA-treated H9c2 and (C) FK-treated H9c2, control (lanes 1 and 4), 5 min treated cells (lanes 2 and 5), and 20 min treated cell (lanes 3 and 6) and ladder (L). Activity was analysed by incubating total cell lysate proteins in the presence of 1 mM BTC (biotin cadaverine) and 5 mM $CaCl_2$ (lanes 1–3) or EDTA (lanes 4–6). At the indicated times, 4x sample buffer was added to stop the reaction, and the reaction mixtures (50 µg per lane) were subjected to SDS-PAGE followed by Western blotting and then analysed for TG2-catalyzed conjugation of BTC into proteins using ExtrAvidin®-peroxidase as a probe. (B &D) Densitometry results represent mean ± SEM of the relative intensity versus the incubation time at 37°C from 3 independent experiments. (B) Two-way ANOVA showed significant effect of $Ca^{+2}$ incubation ($F$= 8.92, $dF$ =3, 16, **p<0.01) and PMA treatment ($F$= 22.59, $dF$ =2, 16, ***p < 0.001), and statistical significant of the interaction of PMA/ $Ca^{+2}$ ($F$ = 3.38, $dF$ =6, 16,*p < 0.05). ***P < 0.001, control vs 5 min PMA, ***P < 0.001 and **P < 0.01 control vs 20 min PMA as determined by "Bonferroni post-tests". (D) Two-way ANOVA showed significant effect of $Ca^{+2}$ incubation ($F$ = 10.60, $dF$ =3, 24, ***p < 0.001) and FK treatment ($F$=6.21, $dF$ =2, 24, **p < 0.01), and no statistical significant of the interaction of FK/ $Ca^{+2}$. *P < 0.05 compered of FK at 5 min with control as determined by "Bonferroni post-tests". Arrow indicated to biotin conjugated proteins (non-specific bands).

### 4.3.2. Visualisation of endogenous *in situ* TG2 activity following PMA and FK exposure

An assay was developed using a biotinylated probe to assess *in situ* TG2 activity in H9c2 cells in response to PMA and FK. A biotinylated primary amine such as a biotin-X-cadaverine (BTC) acts as the acyl-acceptor in the transamidating reaction catalysed by TG2 and becomes incorporated into the endogenous intracellular protein substrates of TG2 (Lee et al., 1993). H9c2 cells were pre-incubated with 1 mM biotin-X-cadaverine for 4h at 37°C prior to treatment with 1 μM PMA or 10 μM FK for 5, 10, or 20 min. After fixation and permeabilisation, intracellular proteins with covalently attached biotin-X-cadaverine, as a result of PMA/FK-induced TG2 activity, were visualized using ExtrAvidin®-FITC (section 2.2.9.2). As shown in Figure 4.3.2, PMA and FK-induced time dependent increases were observed in biotin-X-cadaverine incorporation into endogenous protein substrates of TG2 in H9c2 cells. The incorporation was most evident after 5 and 10 min incubation. This biotinylation in living cells were reduced after 20 min incubation with PMA or FK (Fig. 4.3.2). These data are comparable to TG2 transamidation activity observed *in vitro* (see Fig. 3.3.1). Surprisingly, given the covalent nature of biotin-X-cadaverine incorporation, fluorescence staining returned to control levels after 20 min incubation with PMA and FK.

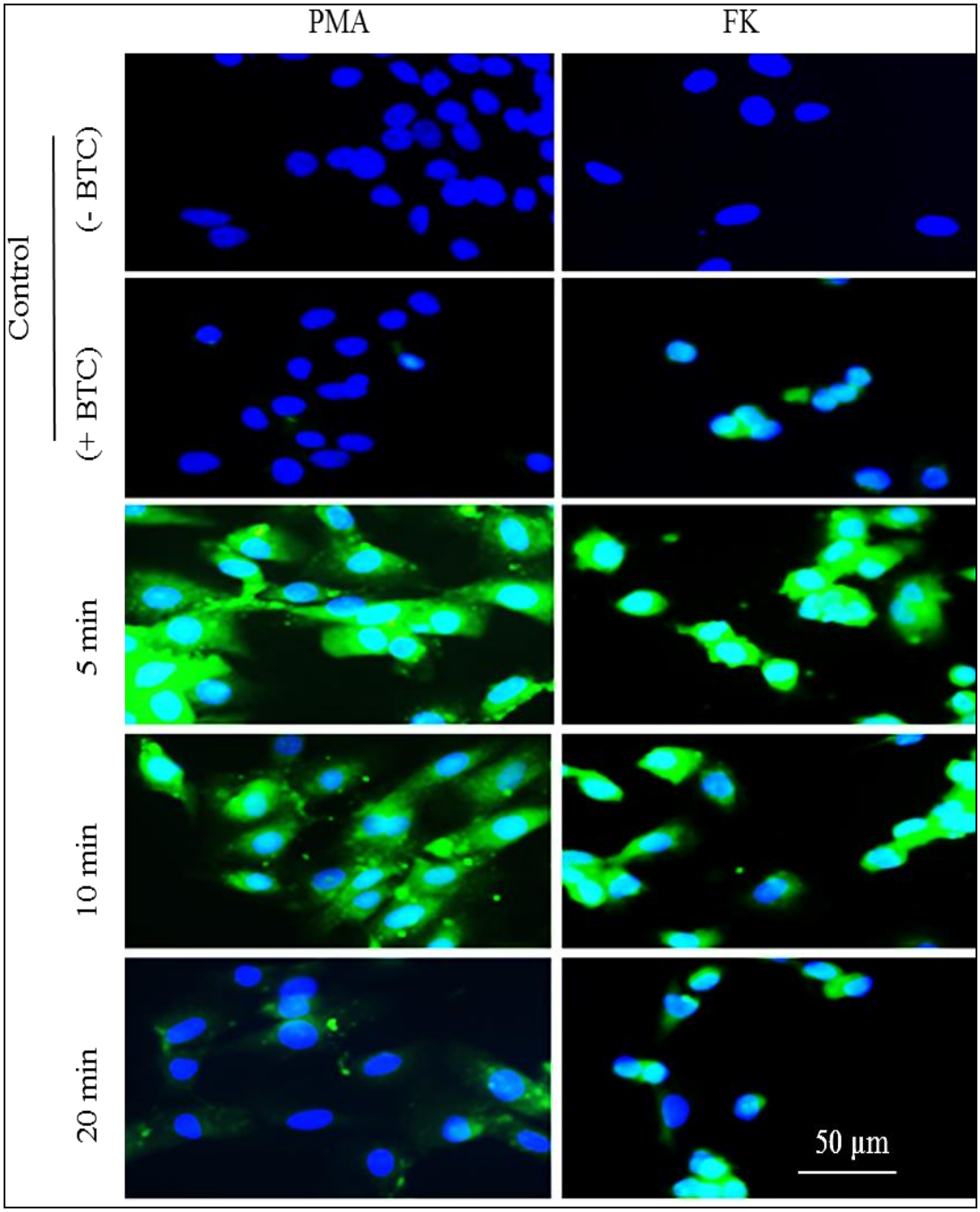


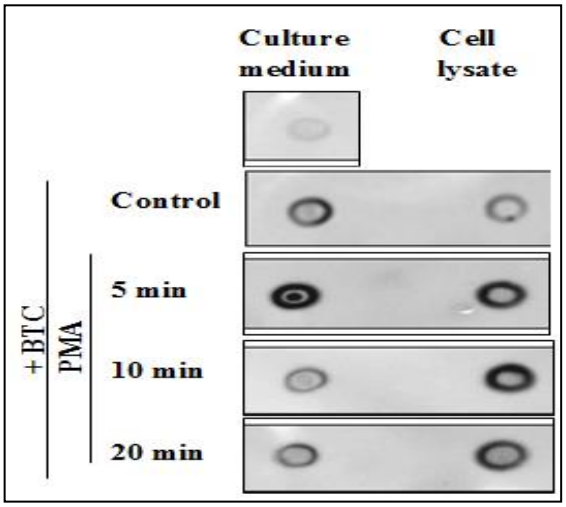

**Figure 4.3.2 Visualisation of endogenous *in situ* TG2 activity in H9c2 cells following PMA and FK exposure**

Cells cultured in chamber slides were incubated with 1 mM biotin-X-cadaverine (BTC) for 4 hours. Cells were then treated with 1 µM PMA or 10 µM FK for 5, 10, 20 min. Untreated cells used as control either in presence of BTC (+) or in absence of BTC (-), The TG2 mediated biotin-X-cadaverine incorporation into intracellular proteins was visualised with ExtrAvidin®-FITC (green). Nuclei were stained with DAPI (blue). The original magnification of the images was 400x. The results are typical of 4 independent experiments.

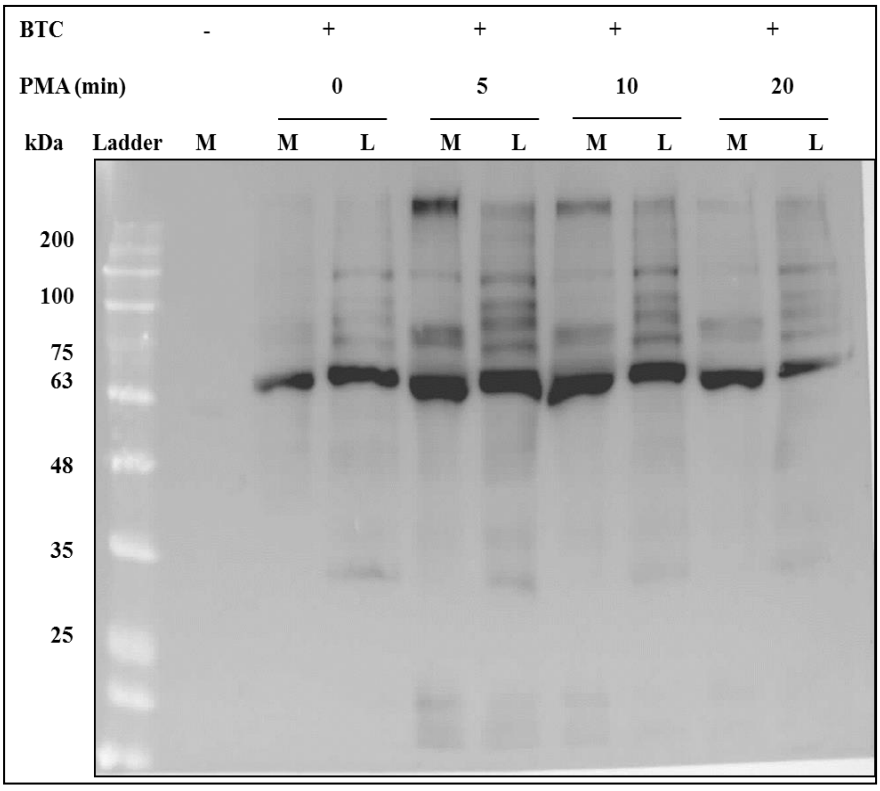
### 4.3.3. Identification and fractionation of acyl-donor TG2 proteins in extra- and intracellular proteins

In order to investigate the reduction in TG2 incorporation activity (biotinylated products) in living cells after 20 min incubation with PMA or FK, the presence of biotinylated (biotin-cadaverine labelled) proteins in the culture medium (M) and lysates (L) of treatment sample at different time point was investigated by immuno-dotblotting.

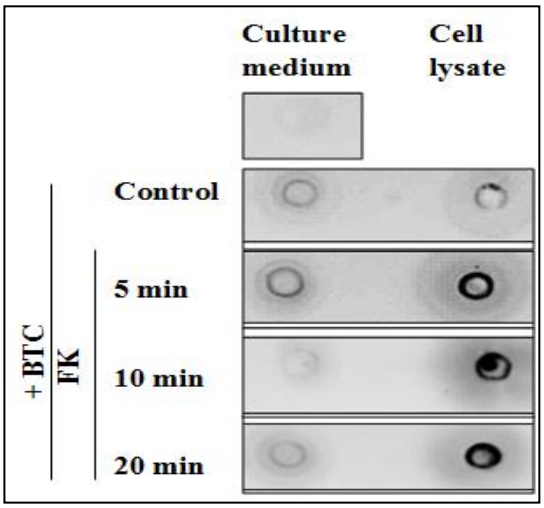
**A)**




**B)**



**C)**




**D)**

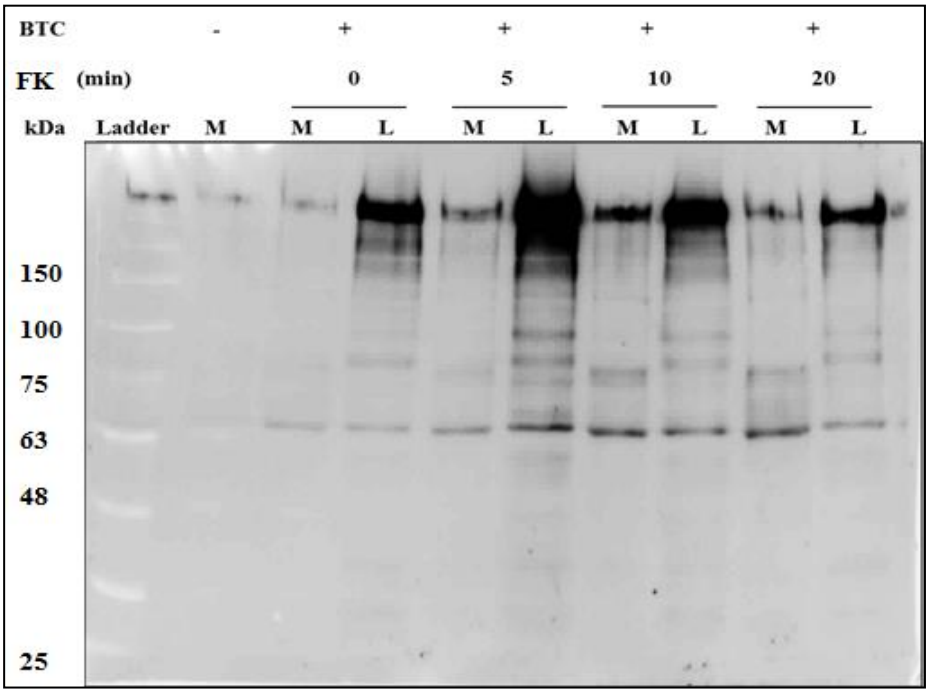



**Figure 4.3.3 Identification and fractionation of acyl-donor TG2 proteins in extra- and intra-cellular proteins**

H9c2 cells were pre-incubated with 1 mM biotin-X-cadaverine (BTC) for 4 h in culture hood. Cells were then treated (A & B) with 1 µM PMA or (C & D) with 10 µM FK at the indicated time points, while untreated cells (0 min) used as control. The culture medium (M) and extracted protein lysate (L) from H9c2 cells following a biotinylating experiment were then collected at different time points. An equal amount (500 µg) of biotinylated (biotin-cadaverine labelled) proteins were extracted using Captavidin® agarose beads. The captured biotinylated proteins were either (15 µl) dot-blotted onto nitrocellulose filters (A & C) or (30 µl) resolved by SDS-PAGE followed by Western blotting (B & D). TG2 transamidating activity and protein substrates were detected using ExtrAvidin®-peroxidase. The results are typical of 3 independent experiments. (-) without BTC while, (+) with BTC.

The cell culture medium from the incubated cells at different time's incubation with PMA or FK was collected and passed through Captavidin®agarose beads (section 2.2.17). The eluted biotin-cadaverine labelled proteins were subjected to dot blotting assay (section 2.2.18) or SDS-PAGE followed by Western blotting and probing with ExtrAvidin®-peroxidase. Biotin-cadaverine labelled proteins were visualised in the culture medium after 5 min incubation, suggesting that they were rapidly externalised by the H9c2 cells (Fig. 4.3.3).

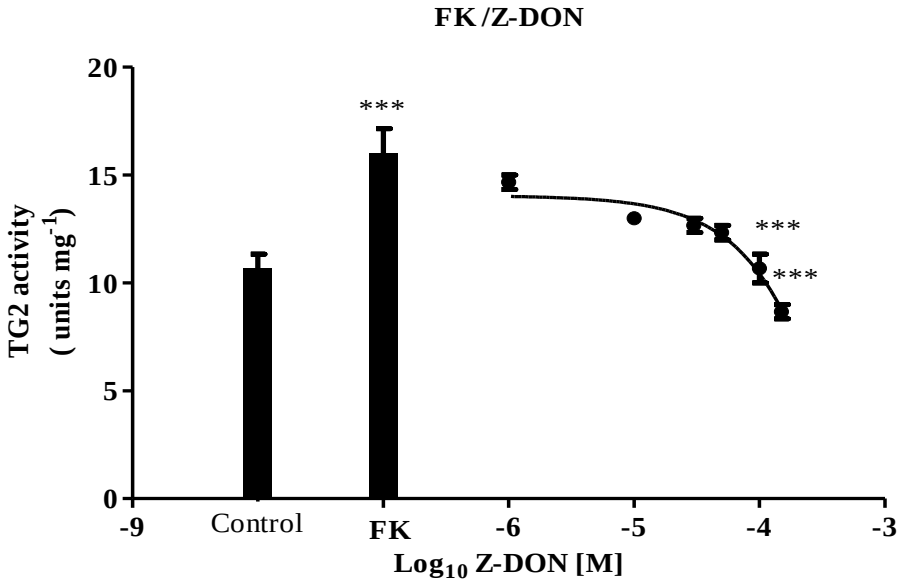
### 4.3.4. The effect of TG2 inhibitors on PMA and FK-induced TG2 activity

To confirm that TG2 is responsible for PMA and FK-stimulated TG2 activity in H9c2 cardiomyocytes, two structurally different specific TG2 inhibitors were tested. R283 (1,3-dimethyl-2 [(oxopropyl)thio]imidazolium) derivative is a cell permeable and irreversible TG2 inhibitor (Freund et al., 1994; Balklava et al., 2002), whereas Z-DON (Z-DON-Val-Pro-Leu-OMe) is a peptide-based cell permeable inhibitor, which irreversibly alkylates the active site of TG2 (Schaertl et al., 2010).

#### 4.3.4.1. The effect of different concentrations of TG2 inhibitors on TG2 biotin cadaverine incorporation activity stimulated with FK in H9c2 cells

To identify the effective TG2 inhibitor concentrations that were able to block TG2 activity induced by protein kinase activators, H9c2 cardiomyocyte cells were pre-treated for 1h with a wide concentration range (1-150 μM) of the TG2 inhibitors Z-DON or R283 (1-200 μM) prior to 5 min exposure to 10 μM FK. The cells were then lysed and subjected to the biotin cadaverine incorporation assay.

**A)**




**B)**

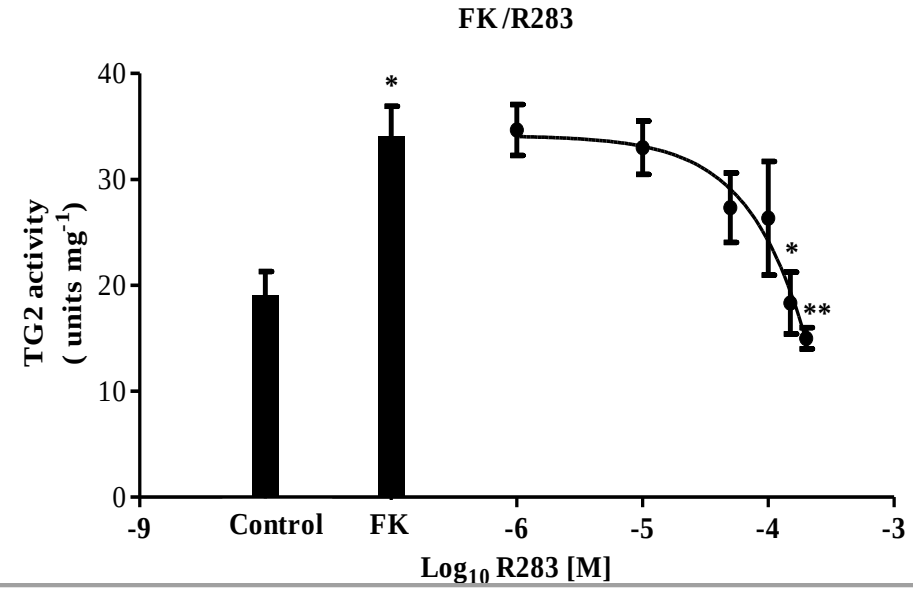



**Figure 4.3.4 The effect of TG2 inhibitors on TG2 biotin cadaverine incorporation activity stimulated with FK in H9c2 cells**

H9c2 cells were pre-treated with A) 1-150 μM (Z-DON) or B) 1-200 μM (R283) TG2 inhibitors for 1h and then stimulated with 10 μM FK for 5 min. Cell lysates were subjected to biotin cadaverine incorporation assay. Graph plotted using Nonliner regression curve fit, "log(inhibitor) vs. response". Data points represent the mean ± SEM TG2 specific activity from 3 independent experiments. Data analysis was performed using "Bonferroni's multiple comparison test" to compare control (untreated cells) vs. FK and FK vs. Z-DON or R283. Statistical significance was accepted at ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

As shown in Figure 4.3.4, Z-DON completely blocks FK-induced TG2 biotin cadaverine incorporation activity, which returned to basal level at concentration of 100-150 μM and 150-200 μM with R283. The data show that both treatments induced a significant decrease (n = 3, ***p < 0.001 *versus* FK+ 150 μM Z-DON (Fig. 4.3.4A) and n = 3, **p < 0.01 *versus* FK+ 200 μM R283 (Fig. 4.3.4B) in TG2 catalysed biotin cadaverine incorporation activity. These data further suggest that Z-DON at a concentration less than 100 μM and R283 at 150 μM have no significant effect on the FK-induced transglutaminase amine incorporation activity of H9c2 cells, compared to untreated cells (control), suggesting that 150 μM/Z-DON and 200 μM /R283 were appropriate concentrations to use to block TG2 activity in this cell line. Accordingly, these two concentrations (150 μM/Z-DON and 200 μM /R283) were adopted for subsequent experiments measuring the TG2 activity in H9c2 cells.

#### 4.3.4.2. The effect of TG2 inhibitors on TG2 biotin cadaverine incorporation activity stimulated with PMA and FK in H9c2 cells

To confirm that TG2 was responsible for PMA and FK-stimulated TG2 activity in H9c2 cardiomyocytes, two structurally different specific, cell permeable and irreversible TG2 inhibitors R283 and Z-DON were tested. Cardiomyocyte H9c2 cells were pre-treated for 1h with the TG2 inhibitors Z-DON (150 μM) or R283 (200 μM) prior to 5 min exposure to 1 μM PMA or 10 μM FK as described in methods section (Fig. 2.1A). The cells were then lysed and subjected to the biotin cadaverine incorporation assay. As shown in Figure 4.3.5, Z-DON and R283 completely block PMA and FK-induced TG2 incorporation activity confirming the involvement of TG2. The data suggest that both treatments showed a significant decrease (n = 5, **p < 0.01 *versus* PMA+ Z-DON *p < 0.05 *versus* FK+ Z-DON (Fig. 4.3.5A) and n = 3, ***p < 0.001 *versus* PMA+ R283, and **p < 0.01 *versus* FK+ R283 (Fig. 4.3.5B) in TG2 catalysed biotin cadaverine incorporation in the presence of TG2 inhibitors.

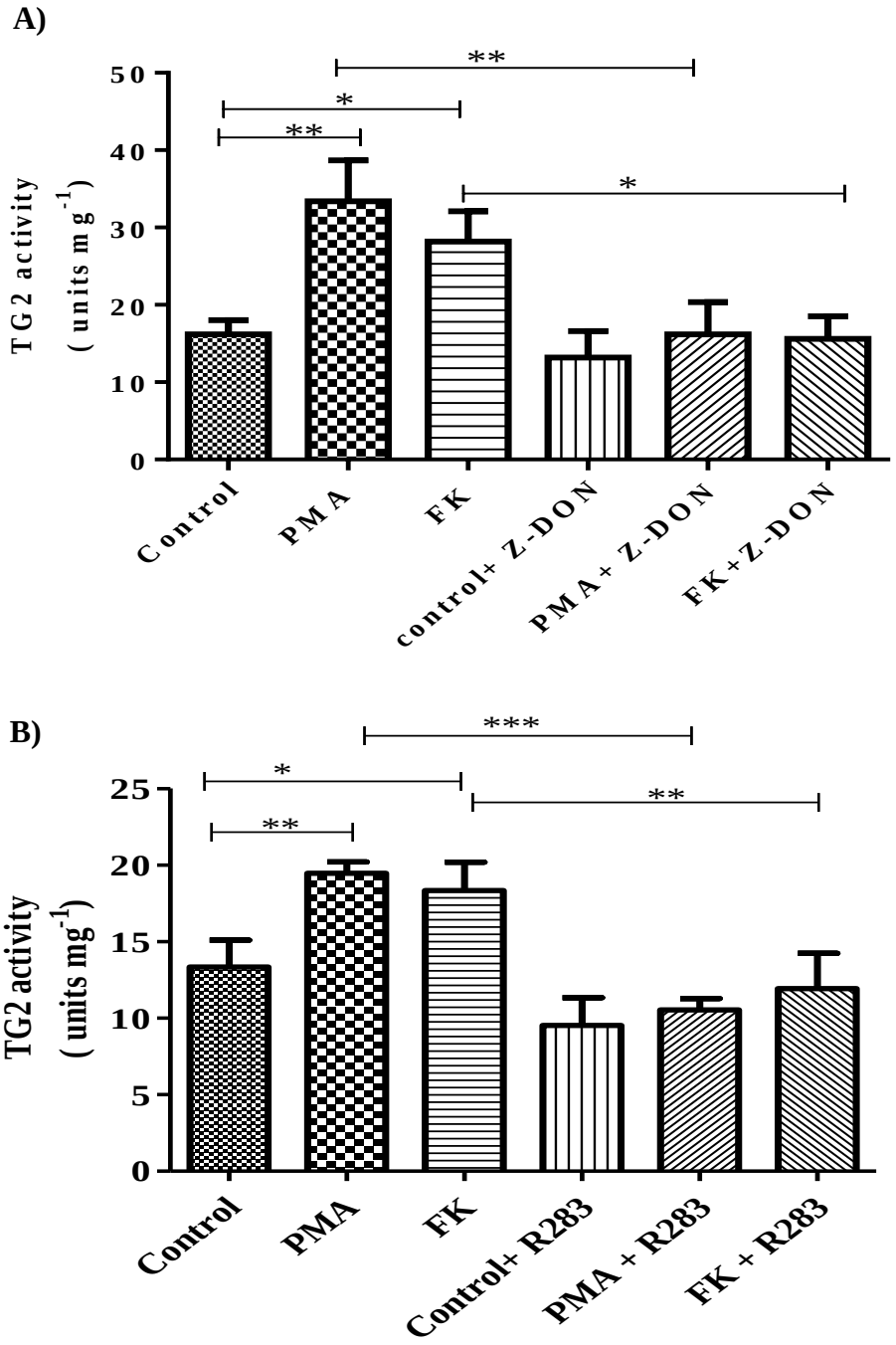



**Figure 4.3.5 The effect of TG2 inhibitors on TG2 biotin cadaverine incorporation activity stimulated with PMA and FK in H9c2 cells**

H9c2 cells were pre-treated with A) 150 μM (Z-DON) or B) 200 μM (R283) TG2 inhibitors for 1h and then stimulated with 1 μM PMA or 10 μM FK for 5 min. Cell lysates were subjected to biotin cadaverine incorporation assay. Data points represent the mean ± SEM TG2 specific activity from 5 (a) or 3 (b) independent experiments. Data analysis was performed using "Bonferroni's multiple comparison test" to compare control (untreated cells) vs. either PMA or FK and these later vs. Z-DON or R283. Statistical significance was accepted at ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

### 4.3.4.3. The effect of TG2 inhibitor on *in situ* TG2 activity stimulated with PMA and FK in H9c2 cells

To confirm the involvement of TG2 activation, cells were also treated with 150 μM site specific inhibitor of TG2 (Z-DON) prior to incubation with either 1 μM PMA or 10 μM FK for 5 min. The presence of this inhibitor in treated cells resulted in complete inhibition of TG2 activity and prevented BTC being incorporated into endogenous protein substrates (green) of cytoplasmic compartment and cytoskeletal elements (Fig. 4.3.6).

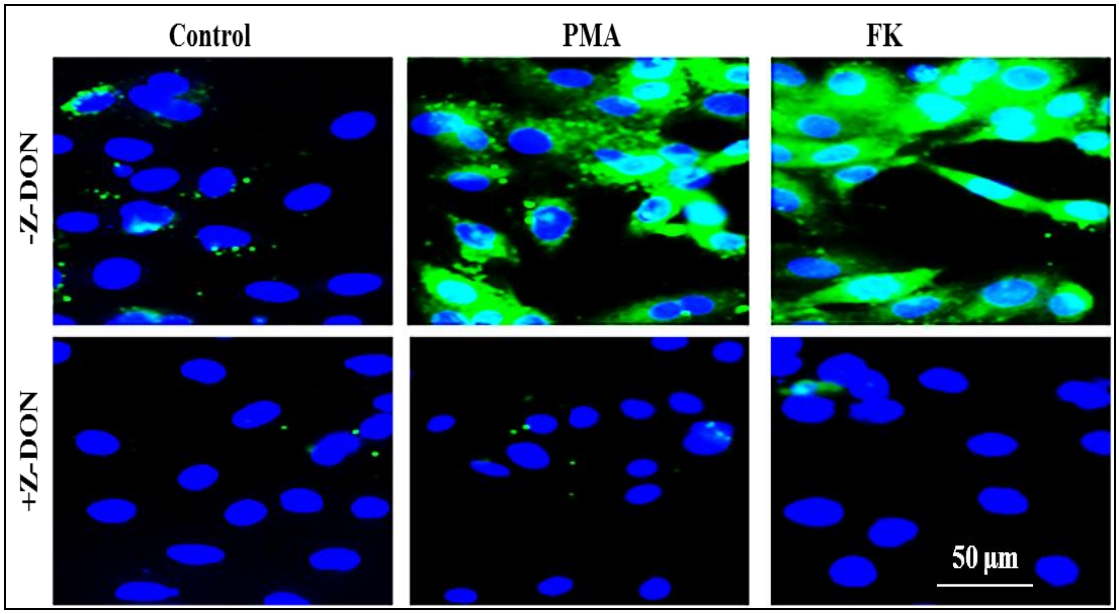



**Figure 4.3.6 The effect of TG2 inhibitor on *in situ* TG2 activity stimulated with PMA and FK in H9c2 cells**

Cells cultured in chamber slides were incubated with 1 mM biotin-X-cadaverine (BTC) for 4 hours. Cells were then incubated either with (+) or without (-) 150 μM (Z-DON) TG2 inhibitor for 1h prior to PMA/FK treatments for 5 min, while untreated cells used as control. The TG2 mediated biotin-X-cadaverine incorporation into intracellular proteins was visualised with ExtrAvidin®-FITC (green). Nuclei were stained with DAPI (blue). The original magnification of the images was 400x. The results are typical of 4 independent experiments.

### 4.3.5. The effect of the selective adenosine $A_1$ receptor agonist $N^6$-cyclopentyadenosine and the non-selective β-adrenergic receptor agonist isoprenaline on *in situ* TG2 activity

Since TG2 activity was shown to be elevated in the presence of activators of protein kinase A and C, it could be activated in presence of their receptor activators. Receptors mediated by these protein kinases were also investigated by *in situ* TG2 amine incorporation activity. A selective adenosine $A_1$ receptor agonist $N^6$-cyclopentyadenosine (CPA) and the non-selective β-adrenergic receptor agonist isoprenaline (ISO) were used. Transglutaminase 2 transamidation activity was found to be elevated in the presence of 1 μM CPA or ISO for 5 min and BTC was incorporated into endogenous protein substrates of TG2 in response to both treatments (Fig. 4.3.7). Expectedly, the presence of TG2 inhibitor (Z-DON) blocked this activation and prevented BTC being incorporated into TG2 substrate proteins in H9c2 cells.

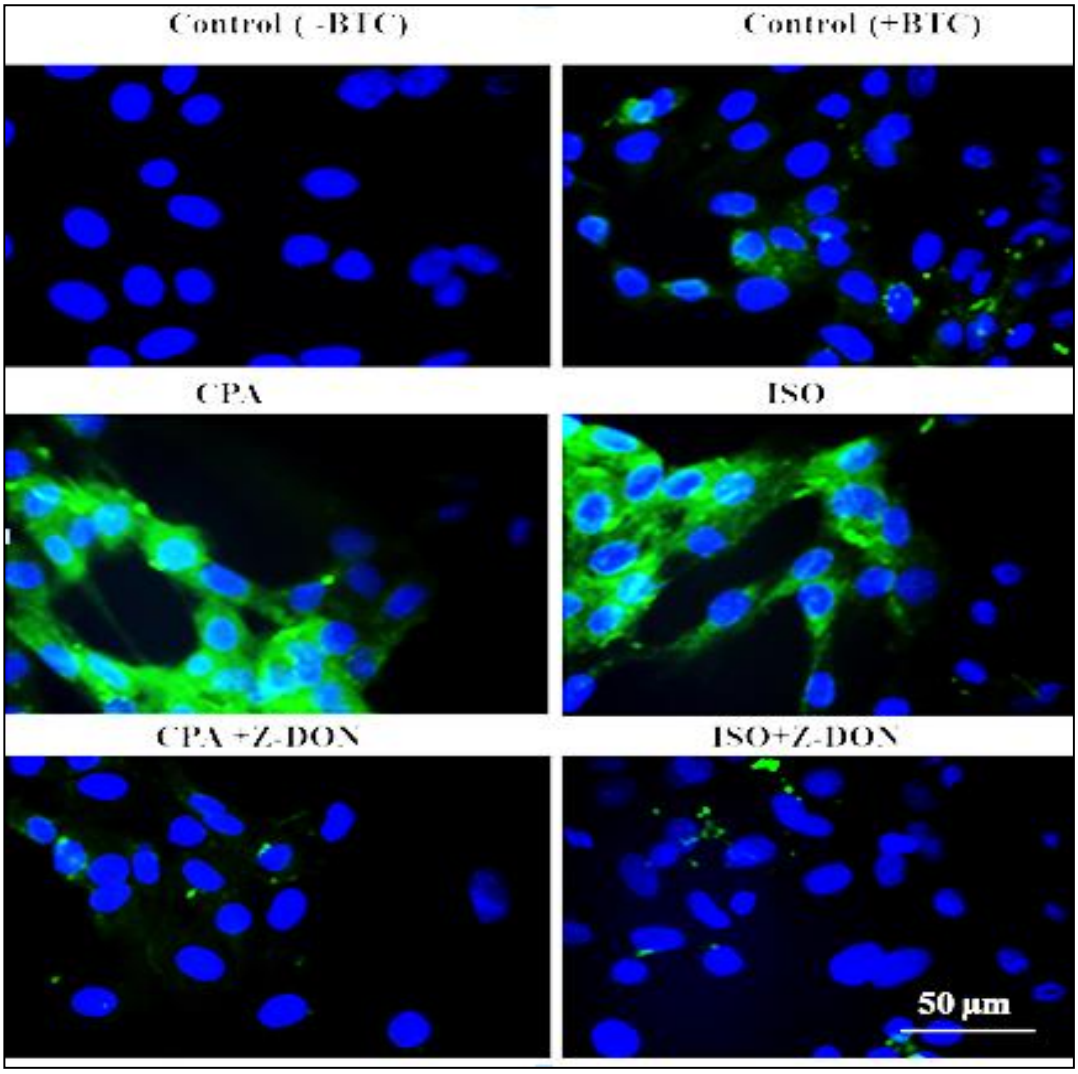



**Figure 4.3.7 Endogenous *in situ* TG2 activity following CPA and ISO exposure visualised by biotin cadaverine incorporation activity**

Cells cultured in chamber slides were incubated with 1 mM biotin-X-cadaverine (BTC) for 4h. Cells were then incubated either with (+) or without (-) 150 µM (Z-DON) TG2 inhibitor for 1h prior to 1 µM of either $N^6$-cyclopentyadenosine (CPA) or isoproterenol (ISO) for 5 min. Untreated cells (control) in absence of BTC (-) or in presence of BTC (+). The TG2 mediated biotin-X-cadaverine incorporation into intracellular proteins was visualised with ExtrAvidin®-FITC (green). Nuclei were stained with DAPI (blue). The original magnification of the images was 400x. Untreated cells (control) in absence of BTC (-) or in presence of BTC (+).The results are typical of 3 independent experiments.

### 4.3.6. The effect of adenosine $A_1$ receptor antagonist *in situ* TG2 activity following CPA exposure

In order to confirm whether a selective adenosine $A_1$ receptor agonist $N^6$-cyclopentyadenosine (CPA) modulated TG activity in H9c2 cardiomyocytes, antagonists for this receptor was also tested.

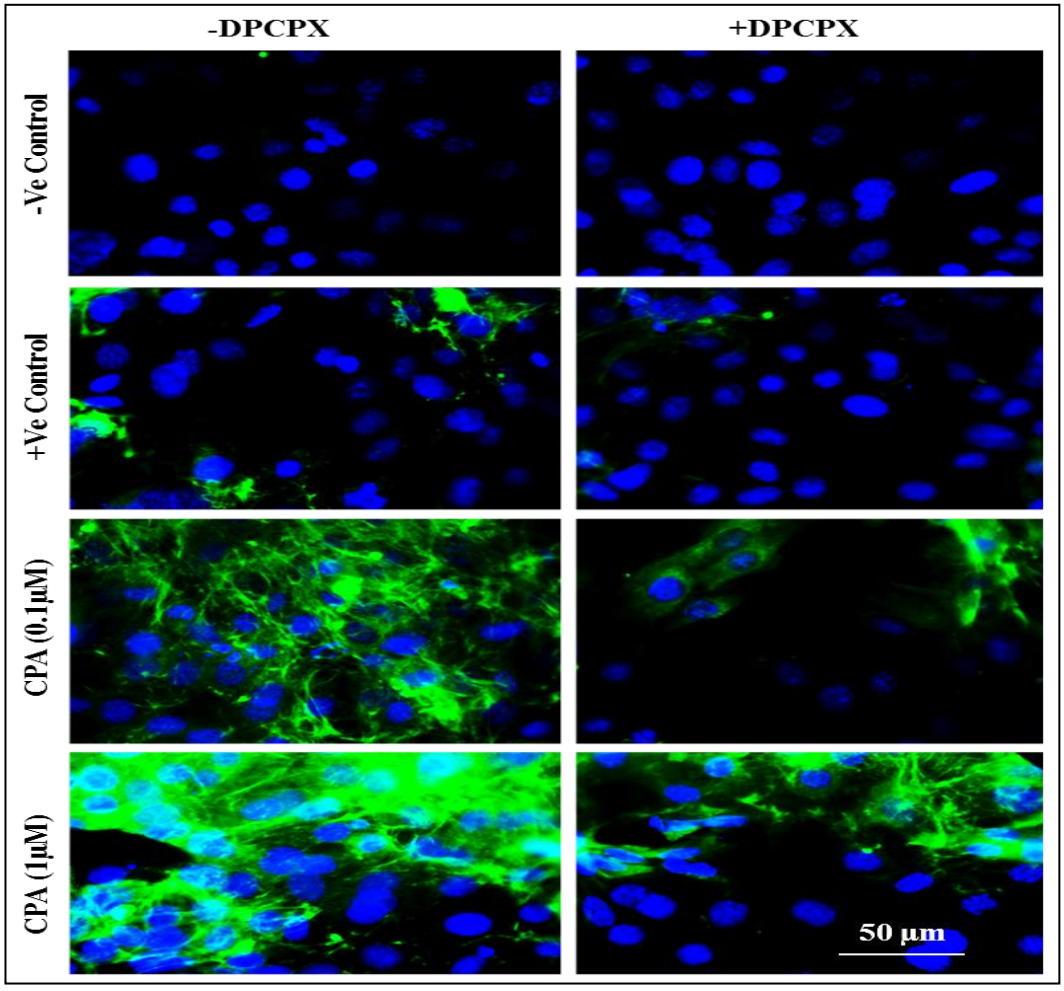



**Figure 4.3.8 The effect of adenosine $A_1$ receptor antagonist *in situ* TG2 activity following CPA exposure visualised by biotin cadaverine incorporation activity**

Cells cultured in chamber slides were incubated with 1 mM biotin-X-cadaverine (BTC) for 4h. Cells were then incubated either with (+) or without (-) 10 µM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) adenosine $A_1$ receptor antagonist prior of 1 µM & 0.1 µM $N^6$-cyclopentyadenosine (CPA) for 5 min. Untreated cells used as a control in absence of BTC (–ve) or in presence of BTC (+ve). The TG2 mediated biotin-X-cadaverine incorporation into intracellular proteins was visualised with Extravidin®-FITC (green). Nuclei were stained with DAPI (blue). The original magnification of the images was 400x.The results are typical of 3 independent experiments.

Cells cultured in chamber slides were incubated with 1 mM biotin-X-cadaverine (BTC) for 4h in cell culture incubator. H9c2 cells were then treated with 10 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) adenosine $A_1$ receptor antagonist prior to the addition of 1 μM and 0.1 μM CPA for 5 min. The TG2 mediated biotin-X-cadaverine incorporation into intracellular proteins was visualised with Extravidin®-FITC (green). As shown in Figure 4.3.8, the adenosine $A_1$ receptor antagonist DPCPX was able to partially block *in situ* TG2 transamidation activity that was elevated in the presence of both concentrations of CPA and prevent BTC incorporated into endogenous protein substrates of TG2.

### 4.3.7. The detection of TG2 activity in mitochondria and endoplasmic reticulum

To detect some of cell compartments that could be co-localised with TG2 incorporation activity, anti-monoamine oxidase B (MAO-B) mAb was used as a mitochondrial marker, while calnexin (AF18) was used as an endoplasmic reticulum (ER) marker.

The monoamine oxidases are considered to be enzyme located on the outer membrane of mitochondria (Berry et al., 1994; Abell & Kwan, 2000). Merging of the images demonstrates the co-localization of amine incorporation activity in the mitochondria of PMA/FK-treated cells (red + green = yellow/orange; Fig. 4.3.9). Transglutaminase 2 activity co-localised in ER (Fig. 4.3.10) was also shown to be enhanced by PMA and FK treatment.

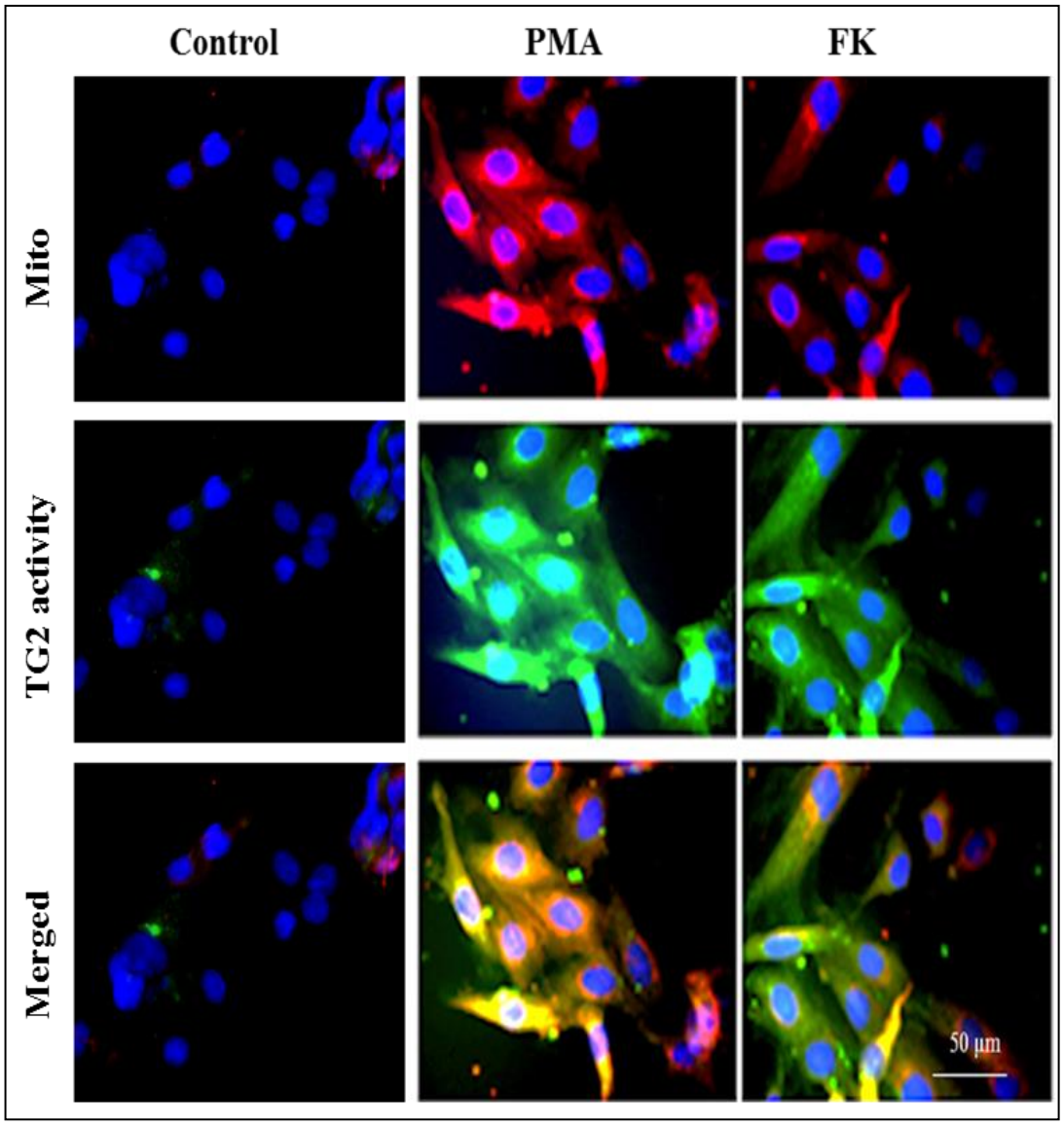



**Figure 4.3.9 Assessment of TG2 activity in mitochondria**

Cells cultured in chamber slides were incubated with 1 mM biotin-X-cadaverine for 4h. Cells were then treated with either 1 µM PMA or 10 µM FK, while untreated cells used as a control. The TG2-mediated biotin-X-cadaverine incorporation into intracellular proteins was visualised with Extravidin®-FITC (green). Nuclei were stained with DAPI (blue). Mitochondria (Mito) were detected by rabbit anti-monoamine oxidases B (MAO-B) mAb and visualised by red (anti-rabbit-Alexa 568 secondary antibody). The original magnification of the images was 400x. The results are typical of 3 independent experiments.

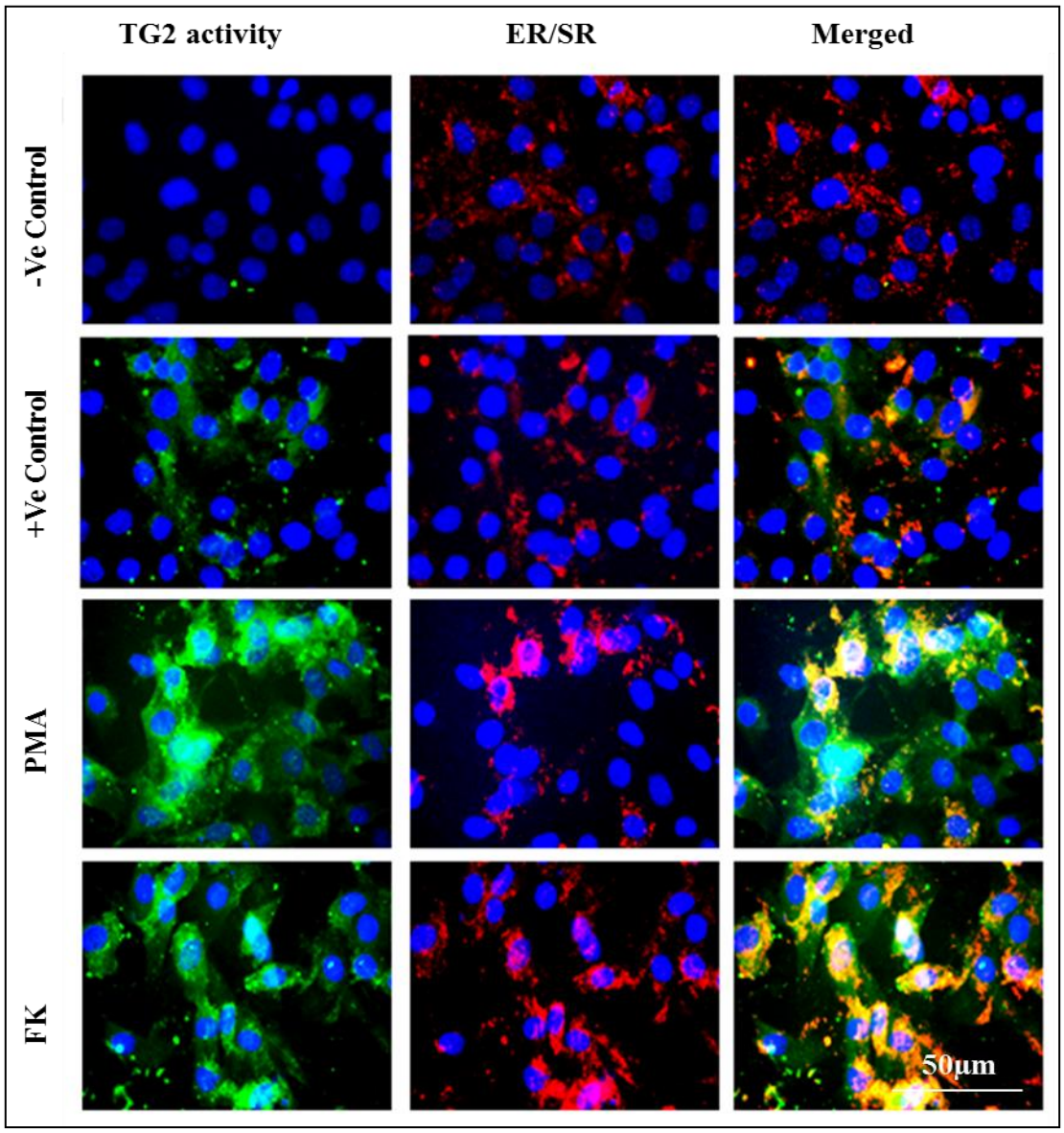



**Figure 4.3.10 The co-localisation of TG2 activity in endoplasmic/sarcoplasmic reticulum**

Cells cultured in chamber slides were incubated with 1 mM biotin-X-cadaverine for 4h. Cells were then treated with either 1 µM PMA or 10 µM FK while, untreated cells used as a control in absence of BTC (–ve) or in presence of BTC (+ve). The TG2 mediated biotin-X-cadaverine incorporation into intracellular proteins was visualised with Extravidin®-FITC (green). Endoplasmic/sarcoplasmic reticulum (ER/SR) was detected by mouse anti-Calnexin (AF18) antibody mAb and visualised by red (anti-mouse-Alexa568 secondary antibody). Nuclei were stained with DAPI (blue). The original magnification of the images was 400x. Co-localisation of endoplasmic reticulum (red) and TG2 activity (green) stained yellow shown in the merged photograph. The results are typical of 3 independent experiments.

### 4.3.8. The detection of TG2 in mitochondria and sarcoplasmic/endoplasmic reticulum fraction

Since TG2 activity was also shown to target mitochondria and sarcoplasmic/endoplasmic reticulum proteins, the presence of TG2 in these two cellular organelles was also investigated for comparison. For this, extracts from H9c2 cells before and after PMA/FK treatments were sub-fractionated by differential centrifugation as described in material and methods (section 2.2.8). The results show the presence of TG2 in endoplasmic reticulum and mitochondria as well as cytosol (Fig. 4.3.11). Anti-tubulin antibodies were used as a marker for cytosol, anti-lamin antibodies were used as marker for nucleus while calnexin antibodies were used as a marker for ER/SR.

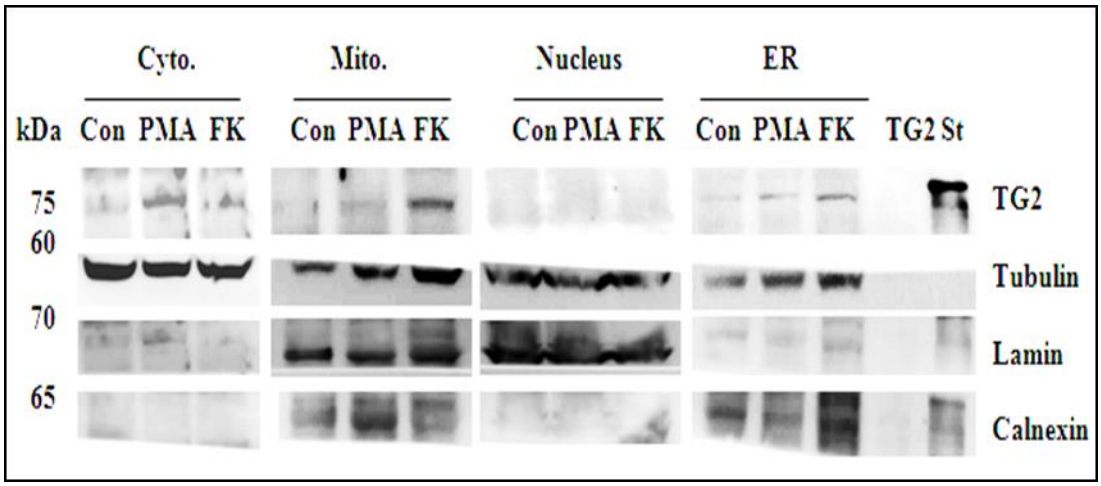



**Figure 4.3.11 Detection of TG2 in subcellular fractions of H9c2 cells after PMA /FK treatment**

H9c2 cells were treated with either 1 µM PMA or 10 µM FK, after which they were lysed with 500 µl of subcellular fractionation buffer, homogenized and subjected to subcellular fractionation by centrifugation. Untreated cells used as a control (Con). An equal amount of subcellular fraction protein (30 µg) were analysed by Western blotting using monoclonal antibodies to TG2 (CUB 7402) calnexin, lamin, and α-tubulin (B512). The results are typical of 3 independent experiments. Cytosol (Cyto.), mitochondria (Mito.) sarcoplasmic/endoplasmic reticulum (ER/SR) and purified guinea pig liver transglutaminase used as stander (TG2 St).

### *4.4. Discussion*

The data presented in this and other studies clearly indicate that TG2 activity can be regulated by PKA and PKC-dependent signalling pathways (Mishra & Murphy, 2006; Mishra et al., 2007). However, since TG2 is a calcium-activated enzyme (Griffin et al., 2002), it is not clear if modulation of TG2 activity by protein kinases occurs independently of its regulation by calcium. To address this, this study monitored TG2 activity, following PMA and FK treatment, in the presence and absence of calcium. From the data presented, it appears that PMA and FK-induced TG2 activity depends on the continued presence of calcium (Fig. 4.3.1).

In this study, the acyl-acceptor probe biotin cadaverine (Smethurst et al., 1993) was incorporated into endogenous protein substrates of TG2 in H9c2 cell lysates in the presence of calcium and EDTA. Since TG2 is a calcium-activated enzyme (Hand et al., 1985), TG2 biotin cadaverine incorporation activity was elevated in the presence of calcium after 30 min incubation at 37C° of both PMA and FK treated and untreated cells (Fig. 4.3.1). Importantly, this increase was statistically significant for both PMA and FK treatments. The results showed calcium dependent incorporation of biotin cadaverine into numerous proteins in H9c2 cells because none or less proteins were labelled in the presence of EDTA. Control cells that were not treated with either PMA or FK showed less biotin cadaverine labelling in the presence or absence of calcium. In addition, it was shown that there was significant (***$p < 0.001$) labelling of cellular proteins after an hour incubation at 37C°. This confirms the previous *in vitro* results of TG2 biotin cadaverine incorporation assay (see Fig. 3.3.1) indicating that PMA or FK-induced TG2 activation in H9c2 cells was related to activation of endogenous TG2 in a calcium dependent manner.

In chapter three, the study showed that a time-dependent exposure of H9c2 cells to PMA or FK caused a significant increase in TG2 catalysed biotin cadaverine incorporation after 5 min exposure *in vitro* (see Fig. 3.3.1). Conversely, using different PKC and PKA inhibitors, this activation was moderated (see Fig. 3.3.6). In this chapter, the study confirmed this activation in intact cells in the presence of TG2 inhibitor. An immunocytochemistry-based assay was developed that enabled the visualisation of *in situ* TG2 activity.

Initially, the effective TG2 inhibitor concentrations that were able to block TG2 activity induced by protein kinase activators was determined. The data show that Z-DON at a concentration lower than 100 μM and R283 at 150 μM had no significant effect on the FK-induced transglutaminase amine incorporation activity of H9c2 cells, compared to untreated cells (control) (Fig. 4.3.4). This suggests that a concentration of 150 μM/Z-DON and 200 μM/R283 were appropriate concentrations to be used to block TG2 activity in these cells. A higher concentration of R283 (250 μM) has been used to block TG2 activity in other cell lines such as in human SH-SY5Y neuroblastoma cells with no apparent toxicity (Beck et al., 2006). Thus, this suggests that 200 μM R283 is also able to inhibit TG2 activity of H9c2 cells and may not affect cell viability either. On the other hand, a lower concentration for Z-DON (50 μM) has been used to attenuate TG2 activity in some cells, including YAC128 primary neurons (McConoughey et al., 2010) and rat vena cava smooth muscle cells (Johnson et al., 2012). However, it is possible that this concentration of Z-DON could block TG2 activity if used for long period of incubation as shown in YAC128 primary neurons after 12h and for 24h in wild type (Q7) and mutant HD (Q111) striatal cells (McConoughey et al., 2010). Furthermore, this concentration can partially attenuate TG2 activity as shown in rat vena cava smooth muscle cells (Johnson et al., 2012). The results from these previous studies and this study suggest that the concentration of Z-DON needed can vary between different cell types and could be affected by the incubation period.

The TG2 biotin cadaverine incorporation activity stimulated by protein kinase A and C activators showed a significant decrease ($n = 5$, ** $P < 0.01$ *versus* PMA+ Z-DON, *$P < 0.05$ *versus* FK+ Z-DON) in the presence of TG2 inhibitor (Z-DON; Fig. 4.3.5A). There was a 50 % decrease relative to the protein kinase activator treated cells, but no significant difference from the control (untreated cells) level. The inhibitor Z-DON is an irreversible TG2 inhibitor that attaches covalently to the TG2 active site cysteine (Choi et al., 2005; Schaertl et al., 2010; Verhaar et al., 2011). Similarly, R283, a cell permeable and irreversible TG2 inhibitor (Freund et al., 1994; Balklava et al., 2002), completely blocks PMA and FK-induced transglutaminase amine incorporation activity confirming the involvement of TG2 (Fig. 4.3.5B).

Most cells take up polyamines (Seiler et al., 1996) and biotin-X-cadaverine has been used to label cells *in situ* and to visualise proteins that are targeted by transglutaminase (Perry et al., 1995). *In situ* TG2′s transamidation activity (Fig. 4.3.2) appeared variable between different treatments. However, biotin-X-cadaverine was found to be predominantly incorporated into endogenous protein substrates of TG2 in PMA or FK treated H9c2 cells. It was seen prominently as a punctate pattern in the cytoplasm and cytoskeletal elements with a bright nucleus after 5 and 10 min incubation. This biotinylation in living cells showed a reduction after 20 min incubation with PMA or FK (Fig. 4.3.2), in agreement with measurements of TG2 transamidating activity in the *in vitro* assay (see chapter 3) and TG2 substrates detection (Fig. 4.3.1).

However, given the covalent nature of biotin-X-cadaverine incorporation into protein substrates, it was surprising to observe *in situ* TG2 activity returning to basal levels after 20 min (Fig. 4.3.2). The question where now raised here is; How do the biotinylated proteins at 5 and 10 min disappear within 20 min showing a reduction in labelling? This suggested that some proteins could be expelled from the cell (e.g. via exosomes) or that they could be rapidly degraded by the proteasome or another proteolytic network. Therefore, the substrate proteins (biotin-cadaverine labelled proteins) in the cell culture medium and cell lysate from the treated cells were collected and biotin-cadaverine labelled proteins captured with Captavidin®agarose beads. Biotin cadaverine labelled proteins were visualised in the culture medium after 5 min incubation suggesting that the biotinylated proteins were rapidly externalized by the H9c2 cells. The presence of biotinylated proteins in the culture medium decreased after 10 and 20 min incubation (Fig. 4.3.3) suggesting that it may be they are degraded outside the cells.

The presence of TG2 inhibitor in intact cells resulted in complete inhibition of TG2 activity and prevented BTC incorporation into protein substrates (Fig. 4.3.6). This suggests a relationship between protein kinase activation and TG2 which may be mediated by their G protein coupled receptors (GPCRs). The activation of PKA has shown to be mediated by a non-selective β-adrenergic receptor (β-AR) agonist (isoproterenol) that elevates cAMP (Lohse et al., 2003). It has been suggested that adenosine $A_1$ receptor-mediated activation of PKC induces cardioprotection (Dana et

al., 2000; Kudo et al., 2002). Although these receptors have important roles in cardiac physiological function and protection, as has TG2, the link between them and TG2 has not been investigated to this date. This study has shown that the selective adenosine $A_1$ agonist $N^6$-cyclopentyladenosine (CPA; Elzein & Zablocki, 2008), and β-adrenergic receptor (β-AR) agonist (isoproterenol) can both stimulate TG2 incorporation activity *in situ* (Fig. 4.3.7) and these were reversed by the TG2 inhibitor (Z-DON). However, an antagonist for selective adenosine $A_1$ (8-cyclopentyl-1,3-dipropylxanthine (DPCPX)) was tested and showed its ability to block this activation, suggesting modulation of TG2 activity by this receptor (Fig. 4.3.8). These results presented suggest a novel role for TG2 in mediating adenosine and β-adrenergic receptor. However, a further study for *in vitro* TG2 activity and target substrates via these receptors agonists is still needed.

Transglutaminase 2 incorporation activity as induced by either PMA or FK was shown to be co-localised in mitochondria of H9c2, confirmed by co-localisation with anti-monoamine oxidases B (MAO-B; Fig. 4.3.9). This enzyme has been detected in cardiomyocytes of spontaneously hypertensive rat (Pino et al., 1997) and in different human tissues (Rodríguez et al., 2001). Interestingly, the present results have shown that MAO-B was elevated in the presence of protein kinase activators compared to untreated H9c2 cells. This agrees with other studies that have reported that PMA treatment can elevate both MAO-B gene and protein levels in human hepatocytoma cells (Wong et al., 2002; Shih & Chen, 2004). In addition, MAO-B's activity has been shown to be increased in aging tissues (Diez & Maderdrut, 1977; Shih et al., 1999). This suggests that the role of TG2 is mediated by protein kinase activators in cell proliferation and differentiation. It is possible that MAO-B could be one of TG2 substrate proteins. Thus, further work is needed to detect this protein among the biotinylated target proteins in PMA/FK treated H9c2 cells identified by western blotting.

It is already known that the endoplasmic/sarcoplasmic reticulum (ER/SR) acts as an intracellular calcium store that helps to maintain a steady state low concentration of intracellular free $Ca^{2+}$ and this in turn participates in the rapid of release of $Ca^{2+}$ in response to signalling (Brown & Loew, 2012; Lukyanets & Lukyanetz, 2013). Transglutaminase 2 has not been detected either in the ER or Golgi compartment in

previous studies (Lorand & Graham, 2003; Iismaa et al., 2009). However, the ER may possibly contribute in the activation of TG2 through release of calcium in the cytoplasm (Jeitner et al., 2009). In this study, calnexin was used as an endoplasmic reticulum marker (Volpe et al., 1992; Kleizen & Braakman, 2004) and TG2 activity was co-localised to the endoplasmic reticulum (Fig. 4.3.10).Moreover, calnexin detection was also shown to be enhanced in the presence of PMA and FK. Calnexin is also often found on the mitochondria-associated ER membrane, suggesting its role in regulation of ER $Ca^{2+}$ signalling (Myhill et al., 2008). The presence of calnexin in the mitochondrial fraction could be due to palmitoylation, which is covalent attachment of fatty acids to cysteine or serine and threonine residues of membrane proteins (Lynes et al., 2011). Although there was a lamine-contamination in mitochondria fractions, TG2 was shown in the enrichment compared to the nucleus fractions. Subcellular fractionation also confirmed the presence of TG2 in ER/SR of H9c2 cells, which was more abundant in PMA and FK treated cells (Fig. 4.3.11). This is in agreement with more recent observation showing that the activation of TG2 was associated with the ER in differentiated SH-SY5Y cells. In addition, the accumulation of TG2 on the surface of ER membranes following exposure to MPP+ (1-methyl-4-phenylpyridinium a toxic molecule can cause ATP depletion and cell death), suggesting its possible role in Parkinson's disease (Verhaar et al., 2012). Since mitochondria and sarcoplasmic reticulum have shown to have an important role in cardiomyocytes signaling (Maechler & Wollheim, 2001; Lukyanets & Lukyanetz, 2013), it would be worth in future work to investigate TG2 activity in each fractions. The identification of the TG2 substrates in different organelles would help to establish the role of TG2 in cardiomyocyte.

In conclusion, the results presented in this chapter of study have shown that TG2 activity is also mediated by PKC/PKA and their G-protein coupled receptors in intact cells. In addition, they provided a strong evidence for a so far undetected, localisation of TG2 in the ER/SR, at least in cardiomyocyte cells. This suggests that activation of TG2 may have a direct influence on posttranslational modification of ER/SR proteins. However, the ER/SR and mitochondrial membranes have not been extensively characterised for cross contamination by other cellular structures and remains a limitation of this study.

# CHAPTER V:

# PROTECTIVE ROLE OF TG2 IN THE CARDIOMYOCYTE RESPONSE TO OXIDATIVE STRESS

## 5. Introduction

Oxidative stress is a term that can be used to describe an imbalance between the systematic production of reactive oxygen species (ROS) and the capability of a biological system to repair the resultant damage (Maritim et al., 2003). In cells or tissues, oxidative stress can either increase oxidizing species generation or significantly decrease antioxidant defences (Schafer & Buettner, 2001). Generally, the disruption of cellular homeostasis of redox state by pathogens or stress stimuli can cause toxic effects through the triggering of elevated ROS production (Giordano, 2005). These are short-lived oxygen derived species and include peroxides and free radicals. These latter reactive species are responsible for damage to all of the cells components including DNA, proteins and lipids (Evans & Cooke, 2004).

Superoxide is one of the less reactive species that can be also converted to more violent reactive oxidants by redox (reduction-oxidation) cycling compounds results in massive cellular damage (Valko et al., 2005). However, low concentrations of ROS can act as cell signalling molecules involved in protein synthesis. In contrast, at high levels, they can cause cell injury via inducing oxidation and lipid peroxidation of cardiac proteins, stimulating apoptosis (Kwon et al., 2003). Therefore, oxidative stress is able to interact with cellular signalling as well (Giordano, 2005). In addition, its effects on cells are dependent upon the level of toxic stress generated and the ability of the cells to overcome this perturbation. Under normal conditions, cells produce a low level of oxygen-derived species through normal aerobic metabolism and these are usually destroyed by normal cellular defence mechanisms. This may involve regeneration of antioxidant molecules either enzymatically by thioredoxin and thioredoxin reductase, or non-enzymatically by intracellular antioxidants such as the vitamins E, C, and β-carotene (Conrad et al., 2004). However, more severe oxidative stress can drive cells to death. For example, a modest oxidative stress can induce apoptosis, while strong stresses can result in necrosis by causing a significant reduction in ATP production that prevents the normal control of apoptotic cell death (Lennon et al., 1991; Lelli et al., 1998).

Oxidative stress is suspected to contribute to the pathogenesis of numerous diseases. For example, production of ROS and reactive nitrogen in association with a reduction

of antioxidant activity and energy metabolism have been detected in neurodegeneration (Guidi et al., 2006). The accumulation of oxidative stress and mitochondrial dysfunction were reported as biomarkers for Alzheimer's disease and Parkinson's disease (Ramalingam & Kim, 2012). Oxidative stress is believed to be linked to certain cancers, in which it acts as a mutagen resulting in DNA damage and it can also suppress apoptosis, thus enhancing tumour cell proliferation and invasiveness (Halliwell, 2007).

It is widely accepted that oxidative stress is linked to cardiovascular diseases through oxidation of small particles of lipoproteins, which are also used as a marker for coronary artery disease (Holvoet et al., 2001). These small particles of lipoproteins can be transported to the artery wall resulting in plaque formation, stopping blood flow and thus increasing the risk for atherosclerosis, heart attack , stroke, myocardial infarction and subsequently cardiac death (Carmena et al., 2004). Oxidative stress is a major component of ischaemia/reperfusion injury, as cells under the ischaemia-reperfusion state prefer to convert the less reactive oxidants, such as hydrogen peroxide or nitric oxide, to more reactive species such as hydroxyl radicals or peroxynitrite (Wang & Zweier, 1996). This is due to the acidic and reducing environment associated with the ischaemia-reperfusion state that can result in the release of ferric and ferrous ions from metallo-proteins, which in turn can catalyse the less reactive oxidants to from more reactive species (Goswami et al., 2007). For the period of ischaemia-reperfusion, the increase in ROS level and extended intracellular free-radical system can lead to cellular damage. It has also been reported that ROSs are involved in alteration of cation homeostasis via membrane proteins that regulate lipid peroxidation and cation transport resulting in membrane permeability changes (Buja, 2005). Moreover, oxidative stress has been shown to be involved in eliciting the inflammatory response against ischaemia reperfusion through leukocyte activation (Granger et al., 1989; Kurose et al., 1999). An accumulation of evidence has implicated oxidative stress in many other cardiac diseases, including myocardial infarction (heart attack) (Jones et al., 2001), myocardial stunning (contractile dysfunction) (Lefer & Granger, 2000), and heart failure (Byrne et al., 2003).

There is a strong relationship between TG2 overexpression and oxidative stress, which can result in either cell death or cell survival. This depends upon the cell type,

stress levels, the length of stress, as well as the TG2 location and its state of activation (Ientile et al., 2007; Iismaa et al., 2009). Both activation and up-regulation of TG2 has been detected in almost all of the neurodegenerative disorders in which oxidative stress is considered as a key factor. These include ischaemia, Huntington's, Alzheimer's and Parkinson's diseases (Caccamo et al., 2004; Ruan & Johnson, 2007; Caccamo et al., 2010). *In situ* increased TG2 activity has been reported in response to $H_2O_2$ induced ROS activation in Swiss 3T3 fibroblasts (Lee et al., 2003). This activation was blocked in the presence of ROS scavengers such as *N*-acetyl-L-cysteine (NAC) and cystamine (also a TG2 inhibitor). Since oxidative stress-induced ROS accumulation can cause either direct or indirect cell signalling cascades involving programmed cell death, researchers have focused on oxidative stress-induced TG2 activation resulting in cell injury (Fesus & Szondy, 2005). It has been reported that during under stress stimuli, TG2 can differentially affect the cell's response, driving it to either apoptosis or survival (Fesus & Szondy, 2005; Song et al., 2011). The $H_2O_2$-induced oxidative stress in cardiomyocytes was shown to result in TG2 up-regulation, in correlation with an increase in the expression of apoptotic markers such as caspase-3, Bax and cytochrome *C* (Song et al., 2011). This suggested a role for TG2 in cardiomyocyte apoptosis in response to oxidative stress induced by ischaemic injury. However, the protective role of TG2 in cardiomyocyte apoptosis remains unclear.

The protective role of PKA activation induced by FK against oxidative stress has been reported in neurones and the rat PC12 adrenal pheochromocytoma cell line (Kamata et al., 1996; Jin et al., 2010; Park et al., 2012). Similarly, the activation of PKC using PMA has a neuroprotective effect against $H_2O_2$-induced toxicity on rat hippocampal and cortical neuronal cells (Doré et al., 1999). Moreover, the activation of both PKC and the MAPK pathway with PMA seem to inhibit cell death induced by $H_2O_2$ in mutant (ST111/111Q) huntingtin striatal cells (Ginés et al., 2010). Thus, it is possible that both these protein kinase activators can induce cardioprotective effects against oxidative stress. Previous studies have shown that TG2 protects cardiomyocytes from ischaemia/reperfusion-induced injury (Szondy et al., 2006). Since PKC and PKA are two key mediators of ischaemic preconditioning and pharmacological preconditioning in cardiomyocytes (Yellon & Downey, 2003; Sanada et al., 2011). This study

investigated the role of TG2 in PMA and FK-induced cytoprotection against oxidative stress in H9c2 cells.

### *5.1. Aims*

The aim of the work in this chapter of the study was to determine whether TG2 played a role in PKC/PKA-mediated cardioprotection in H9c2 cells. The work focused on the effects of PMA/ FK induced cytoprotection against oxidative stress induced by $H_2O_2$ and the activity of TG2. Furthermore, for comparison the effects of its inhibitors in these protective effects was were assessed.

### *5.2. Methods*

As described in chapter 2 of this study (section 2.2).

## *5.3. Results*

### The role of TG2 in PMA and FK-induced cytoprotection

In order to study the involvement of TG2 in cardioprotection of H9c2 cardiomyocytes, $H_2O_2$ was used to mimic schaemia-like stress, after pre-treatment with PMA and FK.

### 5.3.1. The effect of the TG2 inhibitors on oxidative stress-induced cell death: PMA and FK-induced cytoprotection

The H9c2 cells were pre-treated with or without PMA or FK for 5 min alone or in the presence or absence of 150 μM TG2 site specific inhibitor (Z-DON-Val-Pro-Leu-OMe) for 1h followed by incubation with 600 μM $H_2O_2$ (Chanoit et al., 2011; Daubney et al., 2014; Mao et al., 2014) for 2h as described in methods (section 2.2.3.3 and fig. 2.1B). The lysed cells were subjected to biotin cadaverine incorporation assay as method described by Slaughter et al. (1992). The exposure of H9c2 cells to $H_2O_2$ caused an increase in TG2 catalysed biotin cadaverine incorporation activity but this was not a statistically significant increase (Fig. 5.3.1A). However, a significant increase was shown in samples pretreated with PMA but not with FK ($n = 5$, $^*P < 0.05$). In contrast, the use of TG2 inhibitor (Z-DON) resulted in a significant reduction ($n = 5$, $^*P < 0.05$ v*ersus* $H_2O_2$ and $^{**} P < 0.01$ v*ersus* PMA+$H_2O_2$) of the activation. However, using the protein crosslinking TG2 assay (Trigwell et al., 2004), $H_2O_2$ treatment in the presence of TG2 inhibitor caused an increase in TG2 cross linking activity compared to $H_2O_2$ treated cells (Fig. 5.3.1B).

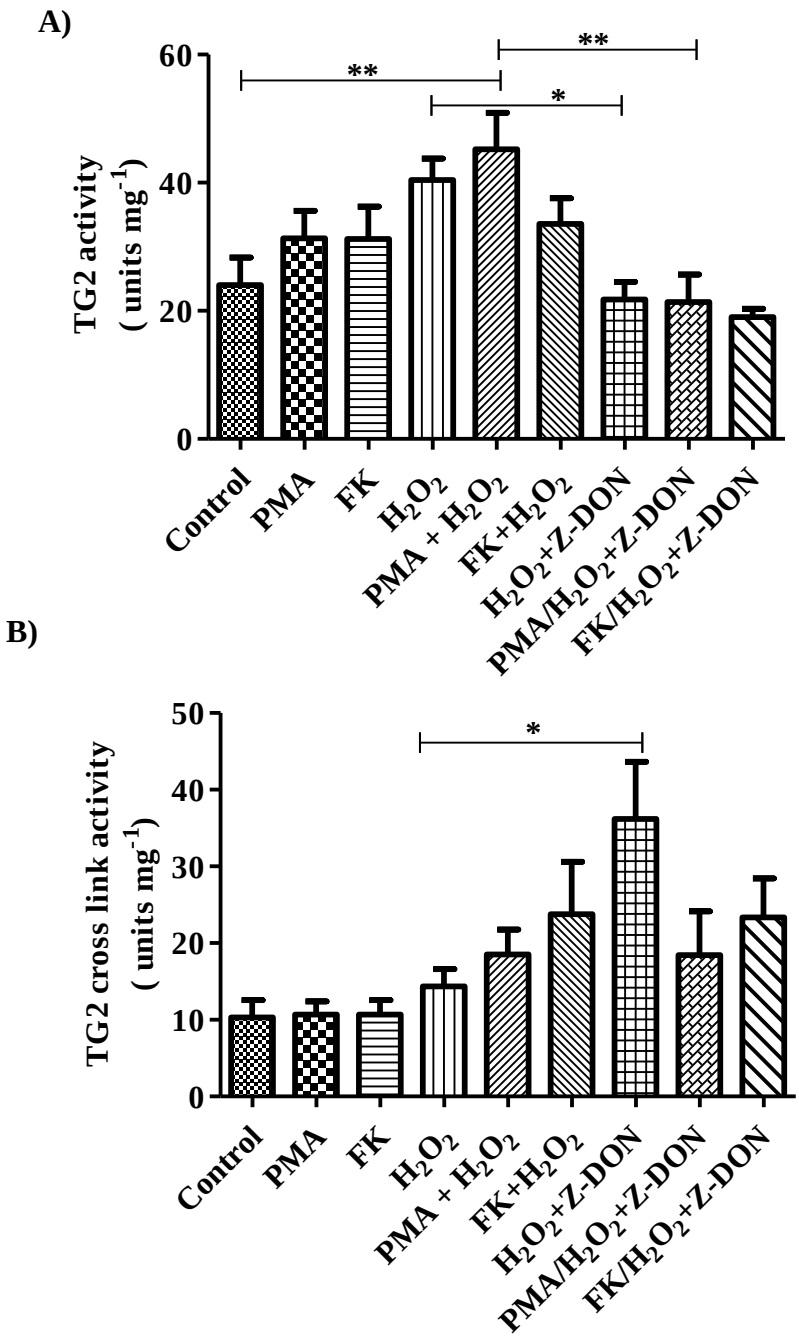



**Figure 5.3.1 The effect of the TG2 inhibitor on oxidative stress-induced cell death and PMA and FK-induced cytoprotection**

H9c2 cells were pre-treated with or without 150 μM TG2 inhibitor (Z-DON) for 1h and stimulated with either 1 μM PMA or 10 μM FK for 5 min alone or followed by induction of 600 μM $H_2O_2$ for 2h while, unstimulated cells was used as control. Cell lysates were subjected to A) biotin cadaverine incorporation assay and B) protein crosslinking assay. Data points represent the mean ± SEM TG2 specific activity from 5 (a) or 6 (b) independent experiments. Data analysis was performed using "Bonferroni's multiple comparison test". Statistical significance was accepted at ** $P < 0.01$, *$P < 0.05$.

### 5.3.2. Endogenous *in situ* amine incorporation into intracellular H9c2 cell proteins following PMA/FK treatment and $H_2O_2$ exposure

This activity was also measured *in situ and* visualised by TG2-meditated biotin-X-cadaverine incorporation into protein in the presence of the TG2 inhibitor Z-DON. The results are shown below in Fig. 5.3.2.

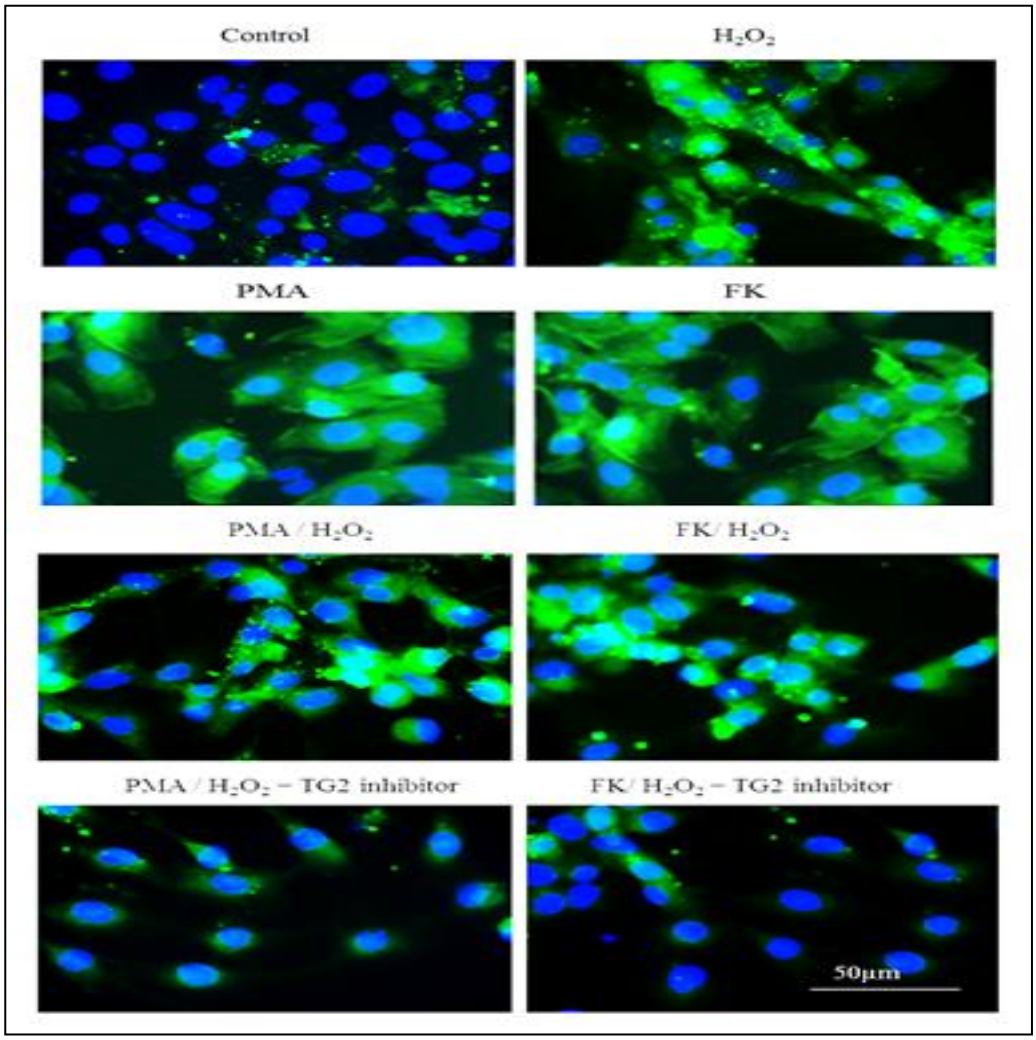



**Figure 5.3.2 Endogenous *in situ* labelling of intracellular H9c2 cell proteins by TG2 following PMA/FK treatment and $H_2O_2$ exposure**

Cells cultured in chamber slides were incubated with 1 mM biotin-X-cadaverine for 4h. Cells were then treated either with 1 µM PMA or 10 µM FK alone or followed by exposure to 600 µM $H_2O_2$ for 2h in the presence or absence of 150 µM Z-DON while, unstimulated cells was used as control. The TG2 mediated biotin-X-cadaverine incorporation into intracellular proteins was visualised with Extravidin®-FITC (green). Nuclei were stained with DAPI (blue). The original magnification of the images was 400x. The results are typical of 3 independent experiments.

Cells in the chamber were incubated with 1 mM biotin-X-cadaverine for 4h. They were then treated with either 1 μM PMA or 10 μM FK alone or followed by the addition of $H_2O_2$ for 2h in presence or absence of 150 μM TG2 inhibitor. After fixation and permeabilisation, intracellular H9c2 proteins with covalently attached biotin-X-cadaverine as a result of PMA/FK-induced TG2 activity were visualised following incubation with ExtrAvidin–FITC using a florescence microscope (section 2.2.9.2). TG2-mediated biotin-X-cadaverine incorporation was found to be predominantly associated with endogenous protein substrates in response to oxidative stress and PMA or FK/ pretreatment against this stress (Fig. 5.3.2). Pre-treatment with the TG2 inhibitor Z-DON prevented biotin-X-cadaverine incorporation (green) into the endogenous protein substrates of the cytoplasmic compartment and cytoskeletal elements. Thus, the presence of the TG2 inhibitor Z-DON in treated cells resulted in reduction of TG2 incorporation activity. As a result of this series of experiments, it was important to consider whether the TG2 inhibitors blocked PMA/FK induced cardioprotection.

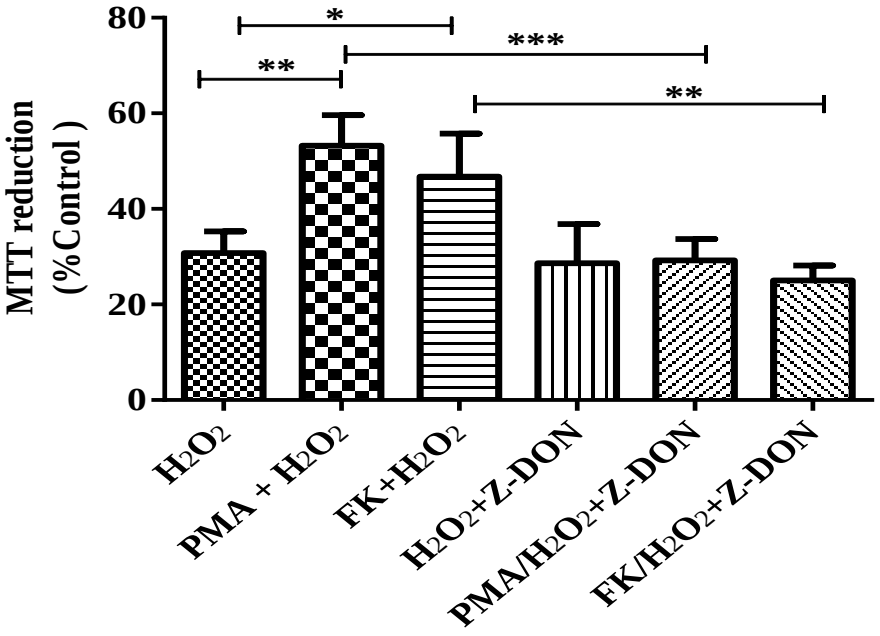
### 5.3.3. Effect of the TG2 inhibitor Z-DON on PMA and FK-induced cytoprotection of H9c2 against $H_2O_2$ determined by MTT and LDH assay

To investigate the possible role of TG2 in cardioprotection of H9c2 cardiomyocytes, the effect of TG2 inhibitors in H9c2 cardiomyocyte cell death were tested. Cellular viability was determined by MTT reduction assay (section 2.2.21.1) and cytotoxicity was measured with LDH activity assay (section 2.2.21.2). H9c2 were pre-incubated either with or without 150 μM TG2 inhibitor (Z-DON) for 1h prior to 5 min treatment either with 1 μM PMA or 10 μM FK followed by 600 μM $H_2O_2$ for 2h.

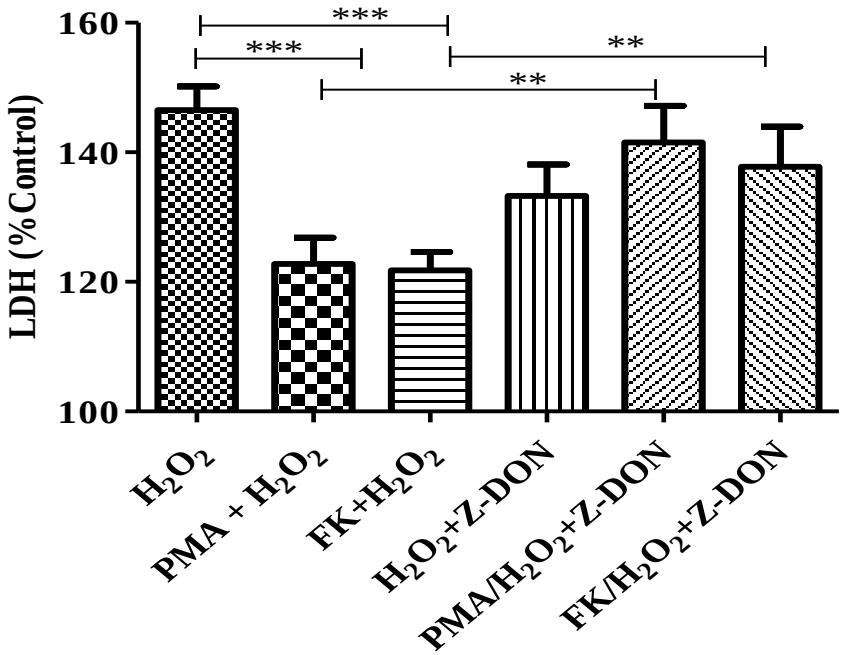
The results show that $H_2O_2$ can induce a significant reduction in cell viability (***$P < 0.001$ *versus* untreated cells; Fig. 5.3.3A). Pretreatment of cells with either PMA or FK significantly reversed the $H_2O_2$-induced cell death (** $P < 0.01$ and *$P < 0.05$ v*ersus* $H_2O_2$, respectively), TG2 inhibitor blocks this protection (*** $P < 0.001$ v*ersus* PMA+ $H_2O_2$, **$P < 0.01$ v*ersus* FK+ $H_2O_2$; Fig. 5.3.3A). Cytotoxicity was measured using the release of lactate dehydrogenase. $H_2O_2$ induced a significant release of LDH in the H9c2 medium (*** $P < 0.0001$ v*ersus* untreated cells). Pretreatment of cells with PMA and FK significantly reversed the $H_2O_2$-induced cell death (**$P < 0.01$

v*ersus* $H_2O_2$). TG2 inhibitor (Z-DON) blocked this protection (*P < 0.05 v*ersus* PMA+$H_2O_2$; Fig. 5.3.3B). However, Z-DON alone had no significant effect on H9c2 cell viability either in the presence or absence of PMA and FK (Fig. 5.3.3C and D).

**A)**




**B)**



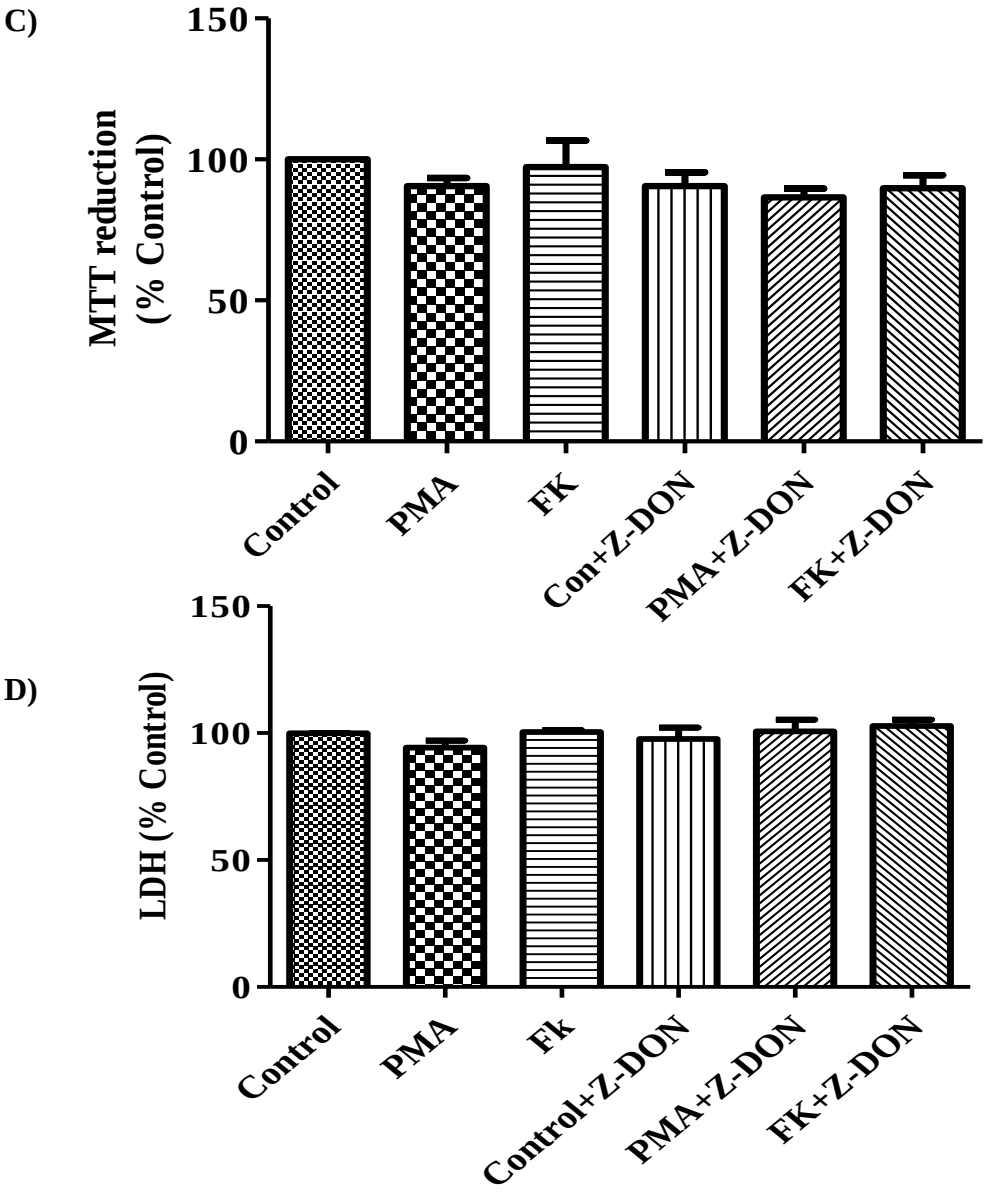


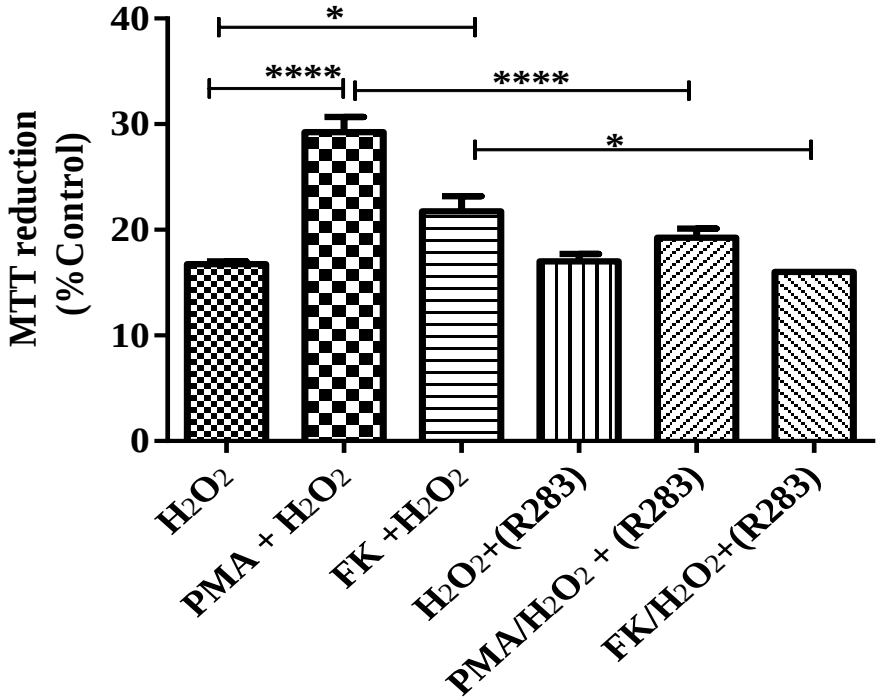

**Figure 5.3.3 Effect of the TG2 inhibitor Z-DON on PMA and FK-induced cytoprotection of H9c2 against $H_2O_2$ determined by MTT and LDH assay**

H9c2 cells were pre-incubated with or without 150 μM TG2 inhibitor (Z-DON) for 1h prior to 5 min with either 1 μM PMA or 10 μM FK followed by 600 μM $H_2O_2$ for 2h while, unstimulated cells was used as control. A) Cell viability was determined by MTT assay. B) The release of lactate dehydrogenase was determined by LDH assay. H9c2 were pre-incubated with or without 150 μM TG2 inhibitor (Z-DON) for 1h prior of 5 min 1 μM PMA or 10 μM FK and cell viability was determined by either MTT assay (C) or LDH (D). Data points represent the mean ± SEM from 5 (A & B), 4 (C) or 3 (D) independent experiments. Data analysis was performed using "Bonferroni's multiple comparison test" where the statistical significance was accepted at ***P < 0.001, ** P < 0.01 and *P < 0.05.

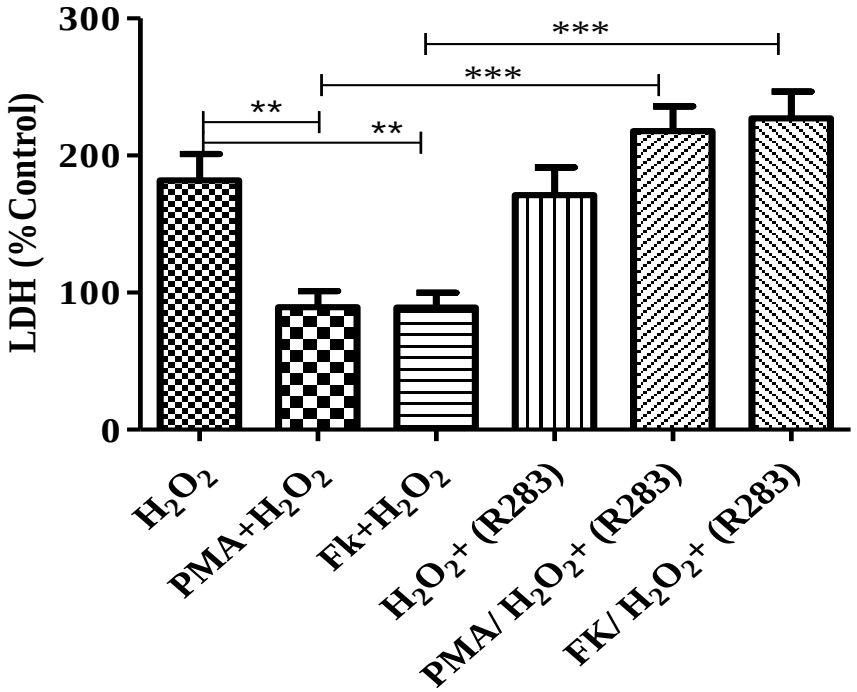
### 5.3.4. Effect of the TG2 inhibitor R283 on PMA and FK-induced cytoprotection of H9c2 cells against $H_2O_2$ determined by MTT assay

For confirmation of the results in Figure 5.3.3, an irreversible, site specific TG2 inhibitor 1,3,dimethyl-2-[(2-oxopropyl) thio] imidazolium chloride (R283; Freund et al., 1994) was used and cell viability was measured by MTT reduction assay and cytotoxicity was measured with LDH activity assay. The results are shown in Figure 5.3.4 and they show that $H_2O_2$ induced a significant reduction in the cell viability (***P < 0.001 versus untreated control).

**A)**




**B)**



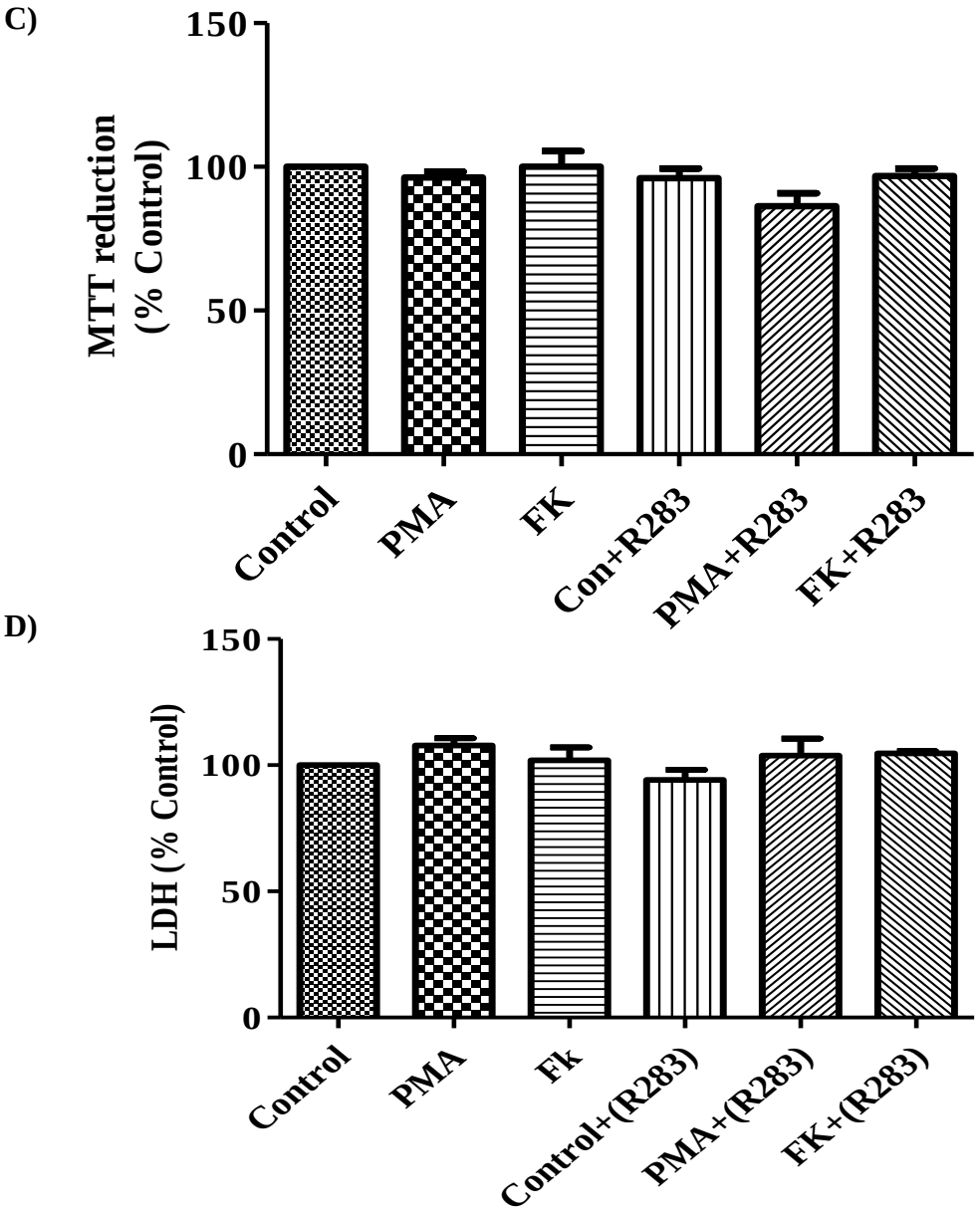



**Figure 5.3.4 Effect of the TG2 inhibitor R283 on PMA and FK-induced cytoprotection of H9c2 against $H_2O_2$ determined by MTT assay and LDH assay**

H9c2 cells were pre-incubated with or without 200 μM R283 for 1h prior to 5 min with either 1 μM PMA or 10 μM FK followed by 600 μM $H_2O_2$ for 2 h while, unstimulated cells was used as control. A) Cell viability was determined by MTT reduction assay. B) The release of lactate dehydrogenase was determined by LDH assay. H9c2 cells were pre-incubated with or without 200 μM R283 for 1h prior to 5 min of either 1 μM PMA or 10 μM FK and cell viability was measured by either MTT assay (C) or LDH (D). Data points represent the mean ± SEM from 4 (A & C), or 3 (B & D) independent experiments. Data analysis was performed using "Bonferroni's multiple comparison test" where the statistical significance was accepted at ***P < 0.001, ** P < 0.01 and *P < 0.05.

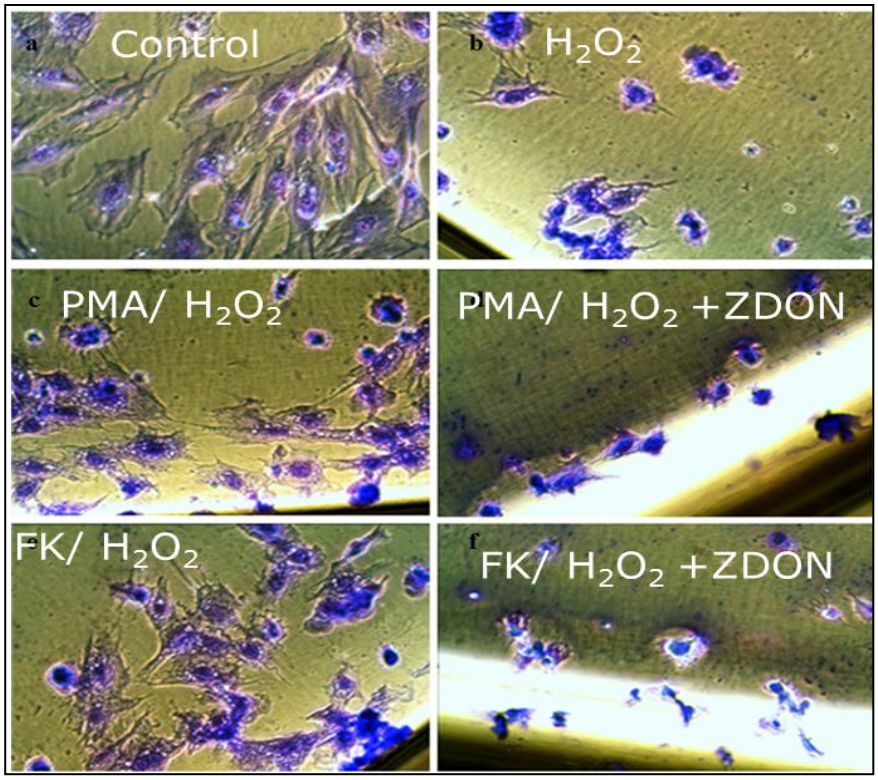
Pre-treatment of cells with PMA and FK significantly reversed the $H_2O_2$ can induce cell death (***P < 0.001,*P < 0.05 versus $H_2O_2$). The cell-permeable TG2 inhibitor (R283; Griffin et al., 2008) blocks this protection (***P < 0.001 versus PMA+$H_2O_2$, *P < 0.05 versus FK+ $H_2O_2$). $H_2O_2$ induced a significant release of LDH in the H9c2 medium (***P < 0.001 versus untreated (control) =100 %). Pre-treatment of cells with PMA and FK significantly reversed the $H_2O_2$-induced cell death (***P < 0.001 versus $H_2O_2$). TG2 inhibitor (R283) blocks this protection (**P < 0.01 versus PMA+ $H_2O_2$ and FK+ $H_2O_2$; Fig. 5.3.4B). However, R283 alone had no significant effect on H9c2 cell viability ether in presence or absence of PMA and FK (Fig. 5.3.4C and D).

### 5.3.5. Effect of the TG2 inhibitor Z-DON on PMA and FK-induced cytoprotection against $H_2O_2$ determined by cell morphological change

To observe any morphological change in the cells after oxidative stress induced by $H_2O_2$ and the effect of TG2 inhibitors (Z-DON) on cytoprotection by PMA and FK, Coomassie blue staining of living cell (Mochizuki & Furukawa, 1987) was performed. The H9c2 cells were exposed to different treatments stained Coomassie brilliant blue and morphological change were observed using an inverted light microscope at x 100 magnification as described previously (section 2.2.4). The morphological change was also monitored in cell culture as well (Fig.5.3.5B).

As shown in Figure 5.3.5, typical cardiomyocytes presented stretched pseudopodia, connected to each other with cell junction and a confluent monolayer. When the cells were treated with 600 μM $H_2O_2$, the cardiomyocytes exhibited retracted pseudopodia and some vacuoles. Many of the cells could not attach to the surface, with granular material in the remaining cardiomyocytes. The extent of these morphological change was more evident in the cells treated with TG2 inhibitors. Cardiomyocytes pretreated with either PMA or FK showed slightly retracted pseudopodia and had less vacuoles compared with cells treated with 600 μM $H_2O_2$, which indicated that the oxidative damage of cardiomyocytes was reduced by PMA and FK, confirming a protective effect of this reagents (Fig. 5.3.5).

A)




B)

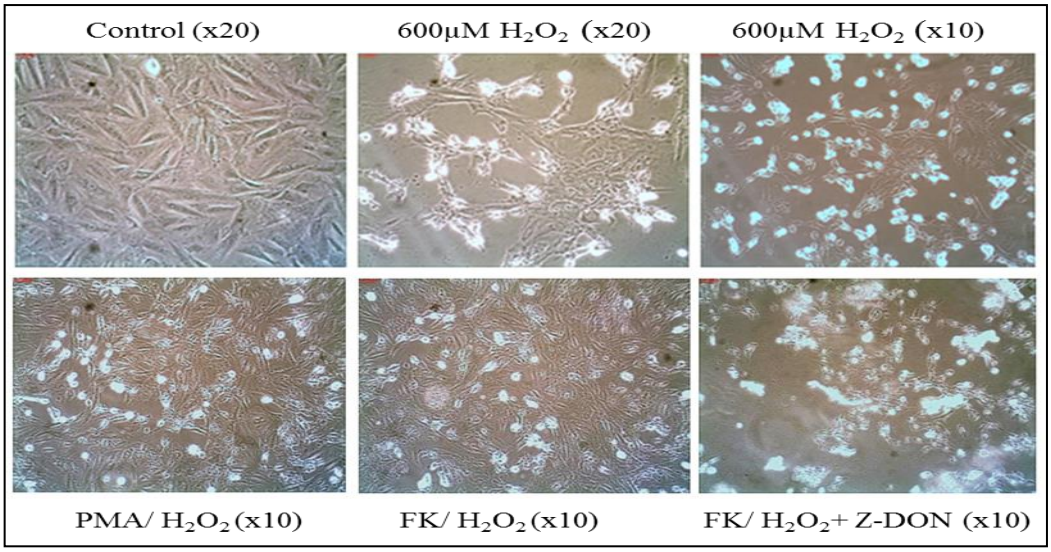


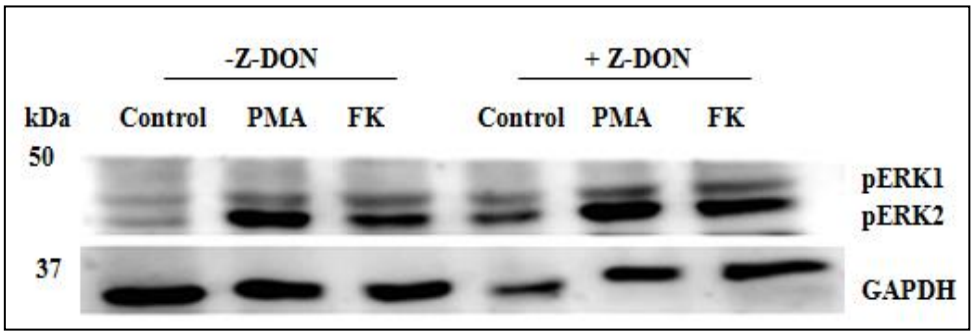

**Figure 5.3.5 Morphological change of H9c2 cardiomyocytes**

A) H9c2 cells were pre-incubated with or without 150 μM Z-DON for 1h prior to 5 min stimulation with either 1 μM PMA or 10 μM FK alone or followed by 600 μM $H_2O_2$ for 2h while, unstimulated cells was used as control. Cells were visualised either B) in culture or A) after staining with Coomassie blue. Morphological changes of cells were observed using an inverted light microscope at x 100 magnification and digital images were captured on a Canon PC 1200 camera. The results are typical of 3 independent experiments.

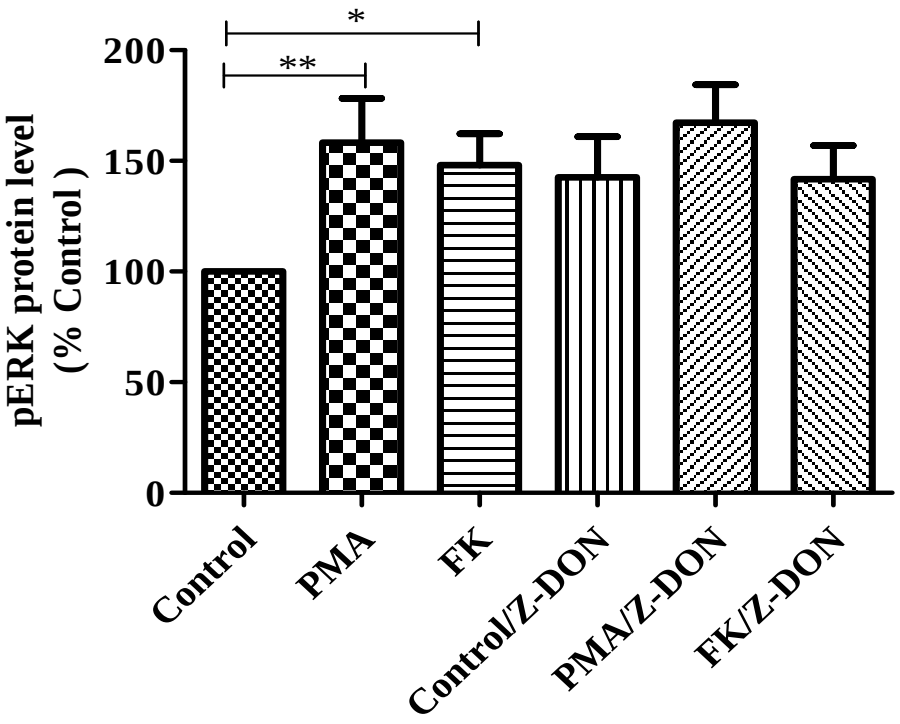
### 5.3.6. The effects of Z-DON and R283 on PMA and FK-induced ERK1/2 activation

The reversal of PMA and FK-induced cytoprotection by R283 and Z-DON may be a consequence of these TG2 inhibitors possessing PKC/PKA inhibitor activity. To address this important consideration, this study determined the effect of R283 and Z-DON on PMA and FK-induced ERK1/2 activation. As shown in Figure 5.3.6, pre-treatment of H9c2 cells with R283 (200 μM; 1h) had no significant effect on PMA or forskolin-induced ERK1/2 activation (Fig. 5.3.6C & D). Similarly, Z-DON (150 μM; 1h) did not reverse PMA or FK-induced ERK1/2 activation. These data suggest that R283 and Z-DON do not function as inhibitors of PKC or PKA (Fig. 5.3.6A & B).

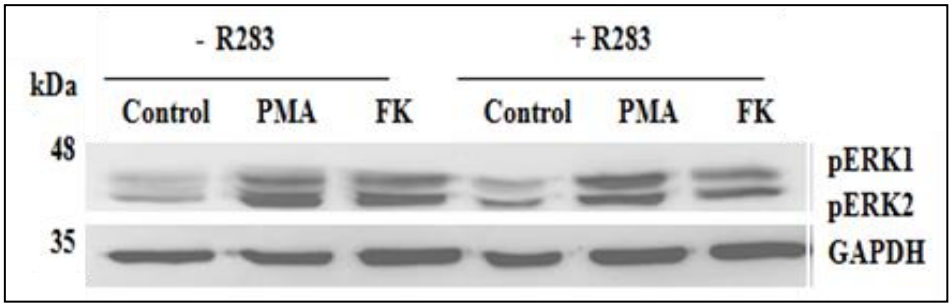
**A)**




**B)**



**C)**




**D)**

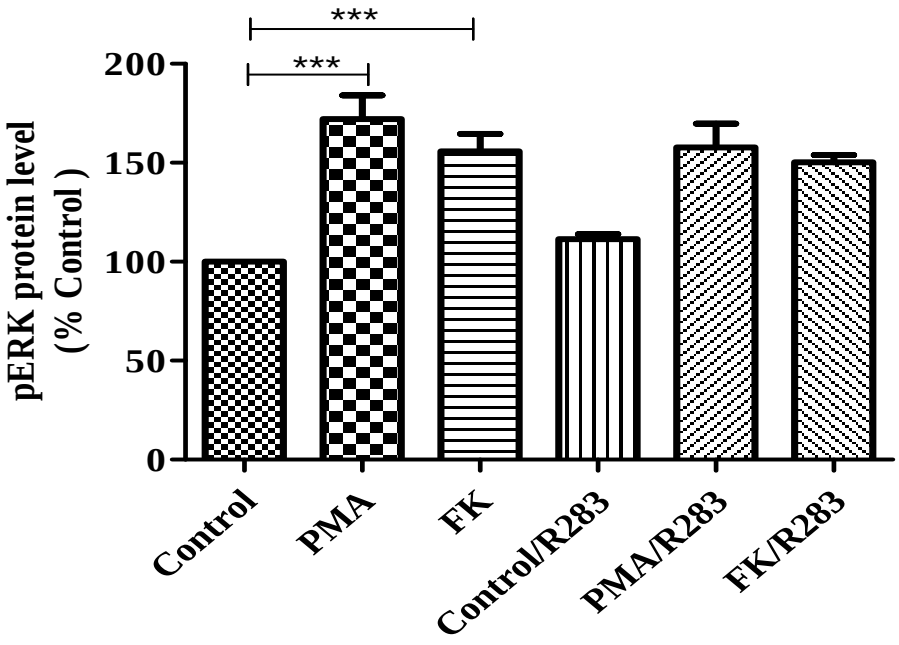



**Figure 5.3.6 Effect of the TG2 inhibitors on PMA and FK-induced ERK1/2 activation**

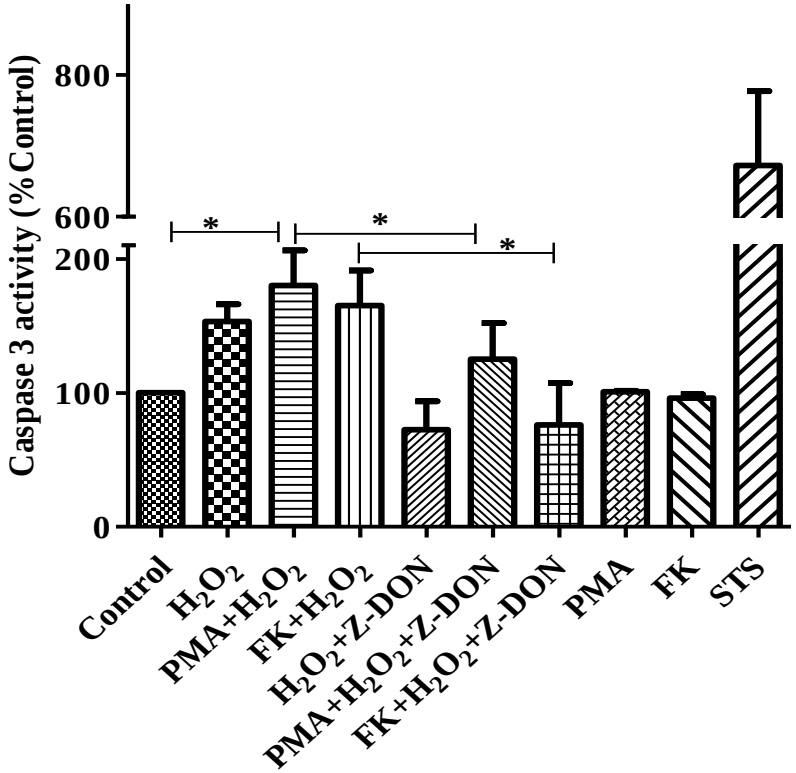
H9c2 cells were pretreated for 1h with (+) or without (-)TG2 inhibitors A) R283 (200 μM) or C) Z-DON (150 μM) prior to 5 min stimulation with either PMA (1 μM) or FK (10 μM). Following PMA and FK exposure, cell lysates (50 μg per lane) were analysed by Western blotting for activation of ERK1/2 using a phospho-specific antibody. Samples were also analysed on separate blots using antibodies that recognise TG2 and GAPDH (to confirm equal protein loading). B and D) Quantified data are expressed as the percentage of control cell values and represent the mean ± SEM of 3 independent experiments. Data analysis was performed using "Bonferroni's multiple comparison test" where the statistical significance was accepted at $***P < 0.001$, $**P < 0.01$ and,$*P < 0.05$.

### 5.3.7. Effect of the TG2 inhibitor Z-DON on PMA and FK-induced cytoprotection against $H_2O_2$ determined by caspase-3 activity

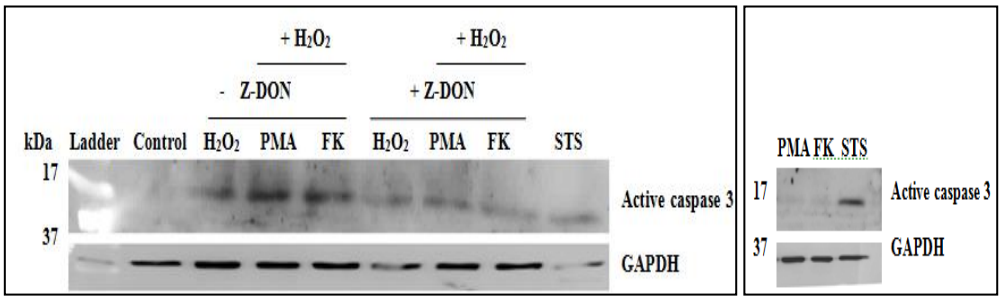
Since both TG2 inhibitors were shown to block the protective role of PMA and FK against $H_2O_2$-treated cells resulting in increased cell death, the activity of caspase-3 which is an enzyme responsible for the induction of programmed cell death (Cohen, 1997; Nicholson, 1999) was measured.

The H9c2 cells were pre-treated either with or without PMA or FK for 5 min in either the presence or absence of 150 μM TG2 site-specific inhibitor Z-DON for 1h, followed by incubation with 600 μM $H_2O_2$ for 2h. The lysed cells were subjected to colorimetric caspase-3 assay (section 2.2.22) as described by Sordet et al. (2002). The exposure of H9c2 cells to $H_2O_2$ resulted in an increase in caspase-3 activity that was not statistically significant. However, Z-DON significantly decreased this activation ($n = 5$, $^*P < 0.05$). Interestingly, a significant increase was shown in samples pretreated via PMA, but not FK ($n = 5$, $^*P < 0.05$). In contrast, the use of Z-DON resulted in a reduction that was significant with FK+$H_2O_2$ ($n = 5$, $^*P < 0.05$ v*ersus* $H_2O_2$), but not with PMA+$H_2O_2$ (Fig. 5.3.7A). Western blotting analysis of H9c2 cell extracts indicated that the levels of active caspase-3 were significantly increased in cells treated with $H_2O_2$ and pretreated with PMA but not FK ($n = 3$ $^*P < 0.05$ versus untreated control); Fig. 5.3.7B & C).

**A)**



**B)**




**C)**

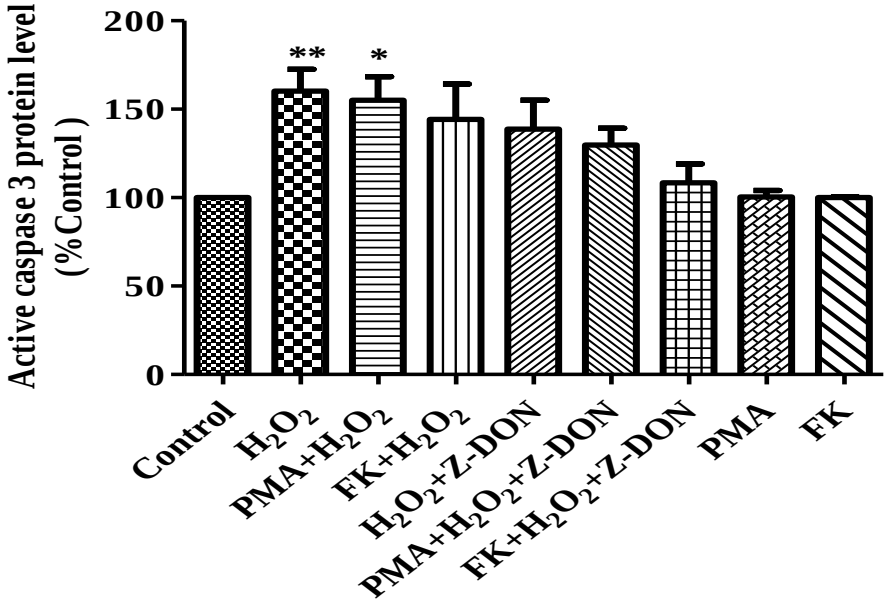



**Figure 5.3.7 Effect of the TG2 inhibitor Z-DON on PMA and FK-induced cytoprotection against $H_2O_2$ determined by caspase-3 activity**

H9c2 cells were treated with 1 µM PMA or 10 µM FK for 5 min followed by 600 µM $H_2O_2$ for 2h in presence or absence of 150 µM Z-DON. A 1 µM staurosporine-treated cells was used as positive control. A) pNA release correlated to caspase-3 activity was determined by colorimetric assay. B) The total protein extract (50 µg per lane) was resolved by SDS-PAGE and transferred on to nitrocellulose filters. Western blotting for caspase-3 was detected by rabbit anti-caspase-3 mAb and anti-GAPDH mAb was used as a control of the total amount of the collected protein. Data are expressed as the percentage of caspase-3 at basal level in the untreated cells (control). Values are means ± SEM of 5 (A) or 3 (C) independent experiments. Data analysis was performed using "Bonferroni's multiple comparison test" where the statistical significance was accepted at *P < 0.05, **P < 0.01.

### 5.3.8. *In situ* analysis for caspase-3 activation in response to the TG2 inhibitor Z-DON on PMA and FK-induced cytoprotection against $H_2O_2$

This activity was also measured *in situ* and visualised by active caspase-3 antibody probing of western blots from cells treated in the presence of the TG2 inhibitor Z-DON.

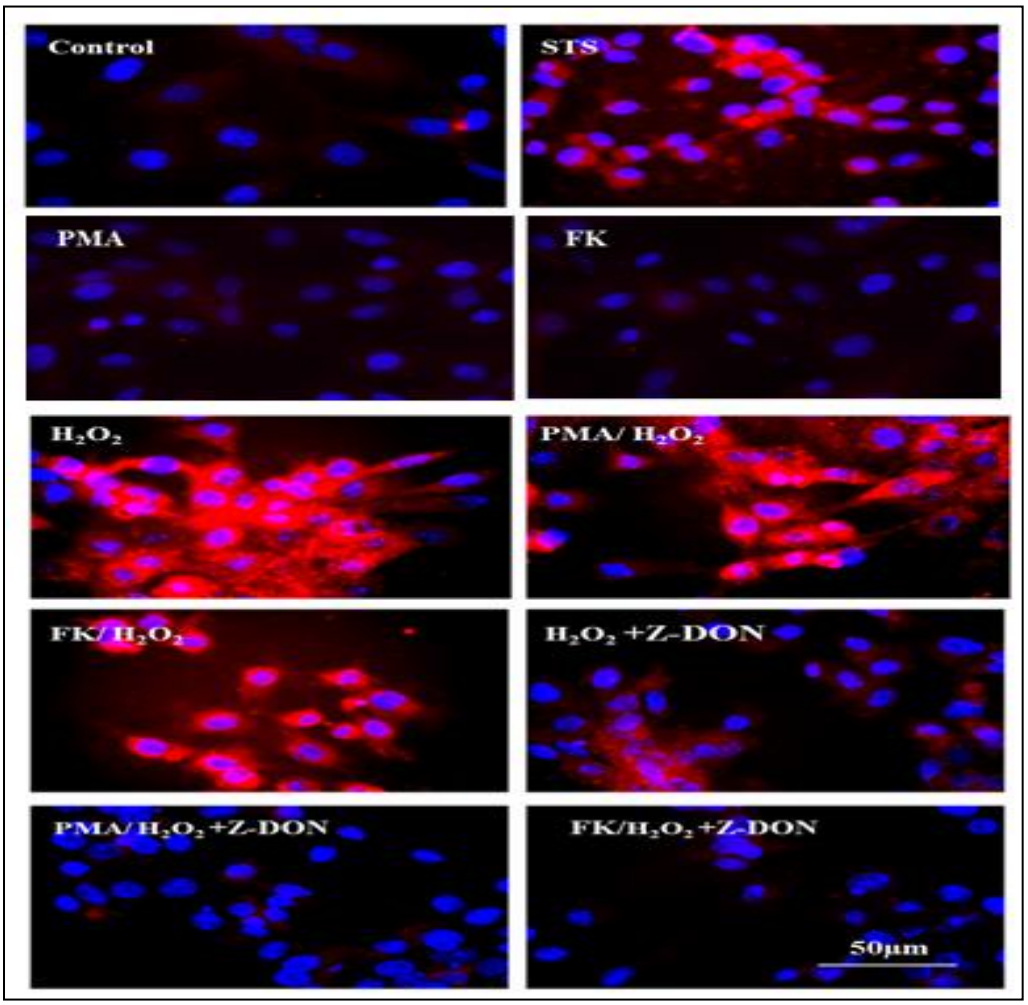



**Figure 5.3.8 The detection of active caspase-3 in H9c2 treated cells**

Cells in chamber slides were treated either with 1 µM PMA or 10 µM FK alone or followed by addition of 600 µM $H_2O_2$ for 2h in the presence or absence of 150 µM Z-DON for 1h. Cells treated with 1 µM staurosporine (STS) for 2h, were used as a positive control, while, unstimulated cells was used as control. Caspase-3 activity was detected by rabbit anti-active caspase-3 antibody mAb and visualised by red (anti-rabbit-Alexa 568 secondary antibody). Nuclei were stained with DAPI (blue). The original magnification of the images was 400x. The results are typical of 3 independent experiments.

The results are shown in Fig. 5.3.8. Cells incubated in chamber slides were treated with either 1 μM PMA or 10 μM FK followed by induction of 600 μM $H_2O_2$ for 2h in presence or absence of 150 μM TG2 inhibitor. Cells treated with 1 μM staurosporine for 2 h were used as positive control. After fixation, permeabilisation and blocking, cell slides were incubated overnight at 4 °C with anti-active caspase-3 antibodies as shown in Fig. 5.3.8. The active caspase-3 was shown to be dominant in response to oxidative stress and less so in PMA or FK-pretreated cells (Fig. 5.3.8). The presence of TG2 inhibitor in treated cells resulted in a reduction of active caspase-3 staining.

### 5.3.9. Effect of the TG2 inhibitor Z-DON on PMA and FK-induced cytoprotection against $H_2O_2$ determined by DNA fragmentation

In order to confirm the possible role of TG2 in H9c2 cardiomyocytes protection, DNA fragmentation (section 2.2.23) was also investigated. DNA fragmentation (smeared bands) was observed in extracts from cells treated with $H_2O_2$ alone or in cells pre-incubated with Z-DON (Fig. 5.3.9).

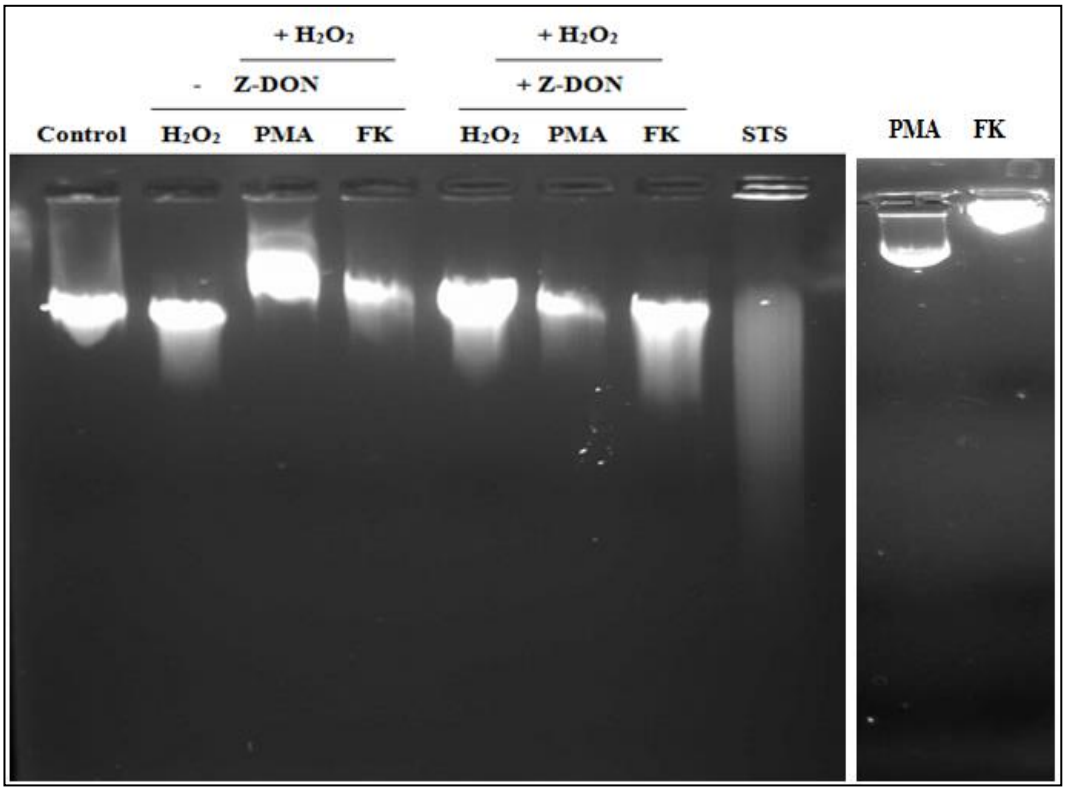



**Figure 5.3.9 Effect of the TG2 inhibitor Z-DON on PMA and FK-induced cytoprotection against $H_2O_2$ determined by DNA fragmentation assay**

H9c2 cells were treated with 1 μM PMA or 10 μM FK for 5 min either alone or followed by 600 μM $H_2O_2$ for 2h in the presence or absence of 150 μM Z-DON for 1h. A 1 μM staurosporine (STS)-treated culture was used as positive control while, unstimulated cells was used as control. DNA was then extracted and a mass of 30 μg extracted DNA was separated by electrophoresis in a 1.5 % (w/v) agarose gel and then visualized under UV light. The results are typical of 3 independent experiments.

### 5.3.10. The detection of TG2 protein level in H9c2 cells pre-treated with PMA and FK following $H_2O_2$ exposure

In order to find out if there was a correlation between TG2 activity and TG2 protein level in H9c2 cells pre-treated with either PMA or FK following $H_2O_2$ exposure, TG2 protein was detected after Western blotting. Western blotting analysis of H9c2 cell extracts indicated that the level of TG2 protein increased in the presence of PMA or FK, but this level did not show significant in change response to $H_2O_2$ alone or in cells pretreated with PMA but not FK (Fig. 5.3.10A & B) using the anti-TG2 mAb (CUB 7402). Densitometry results for protein quantification of FK treated H9c2 cells

following $H_2O_2$ exposure revealed strong significant increase (n = 3, P < 0.01) in TG2 protein level that showed a significant decrease in presence of Z-DON.

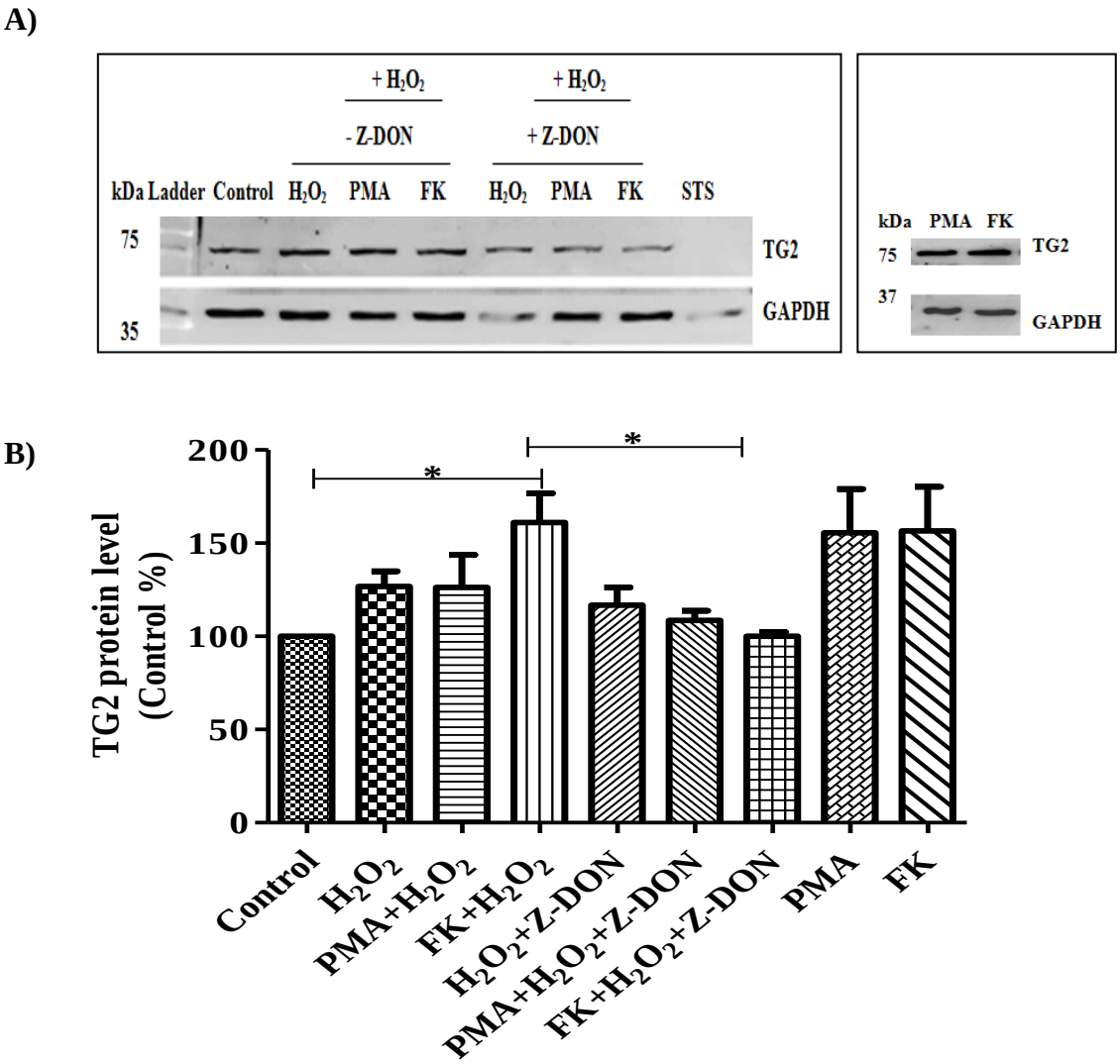


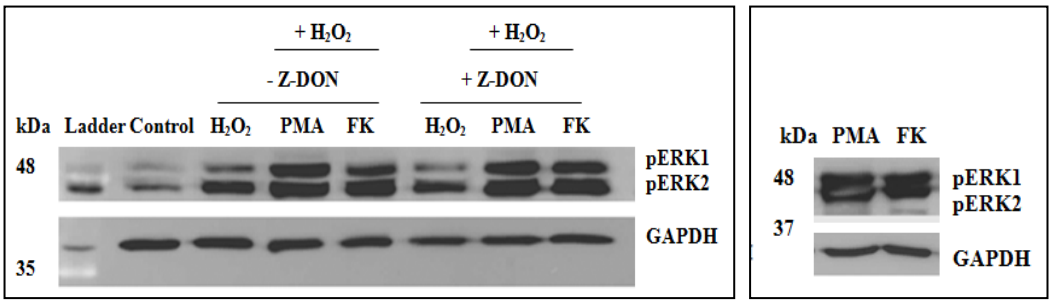

**Figure 5.3.10 The detection of the TG2 protein level in H9c2 cells pretreated with PMA and FK following $H_2O_2$ exposure**

H9c2 cells were treated with or without 150 µM Z-DON for 1h. Cells were then given 5 min with 1 µM PMA or 10 µM FK treatment either alone or prior to the addition of 600 µM $H_2O_2$ for 2h. The total protein extract (50 µg per lane) was resolved by SDS-PAGE and transferred onto nitrocellulose filters. A) Western blotting for TG2 by anti-TG2 mAb (CUB 7402); anti-GAPDH mAb was used as a control of the total amount of the collected protein. B) Densitometry was carried using Adobe Photoshop CS4 and the values were plotted as relative intensity versus different treatments. Data are expressed as the percentage of TG2 proteins at basal level in the untreated cells (control). Values are means ± SEM of 3 independent experiments. Data analysis was performed using "Bonferroni's multiple comparison test" where the statistical significance was accepted at *P < 0.05.

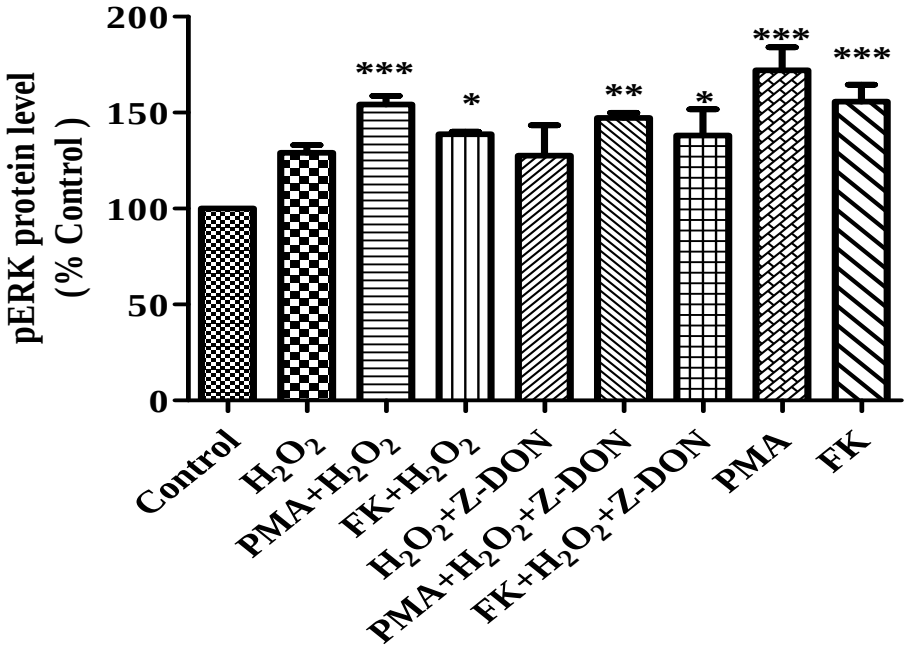
### 5.3.11. The effect of Z-DON on survival proteins (pERK1/2 and pAKT) in H9c2 cells pre-treated with PMA and FK before $H_2O_2$ exposure

The effects of $H_2O_2$ and Z-DON on the activity of cell survival proteins such as pERK1/2 and pAKT were also investigated. Data presented in Figure 5.3.11 indicated that cells pre-treated with PMA and FK followed by $H_2O_2$ showed a significant increase in pERK (n = 3, P < 0.0001for PMA and P < 0.001 for FK) compared to control and $H_2O_2$ treated cells. In the presence of Z-DON these activation did not show any significant attenuation (Fig. 5.3.11A & B). However, pAKT showed a significant increase in cell pre-treated with PMA and FK prior to $H_2O_2$, which was also significantly decreased in presence of Z-DON (Fig. 5.3.11C & D).

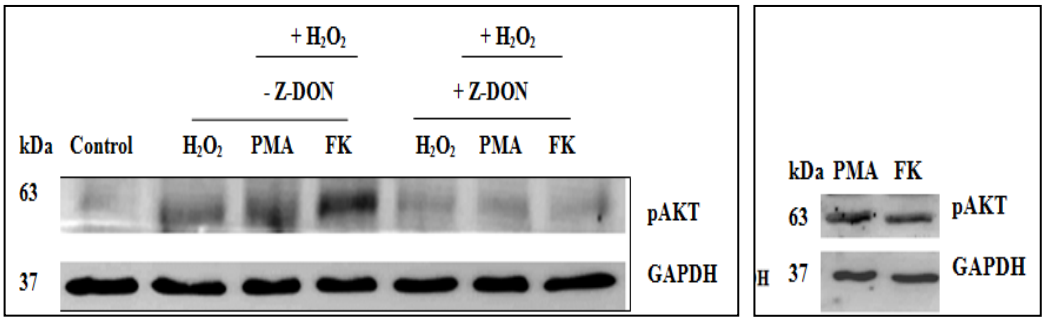
**A)**




**B)**



C)




D)

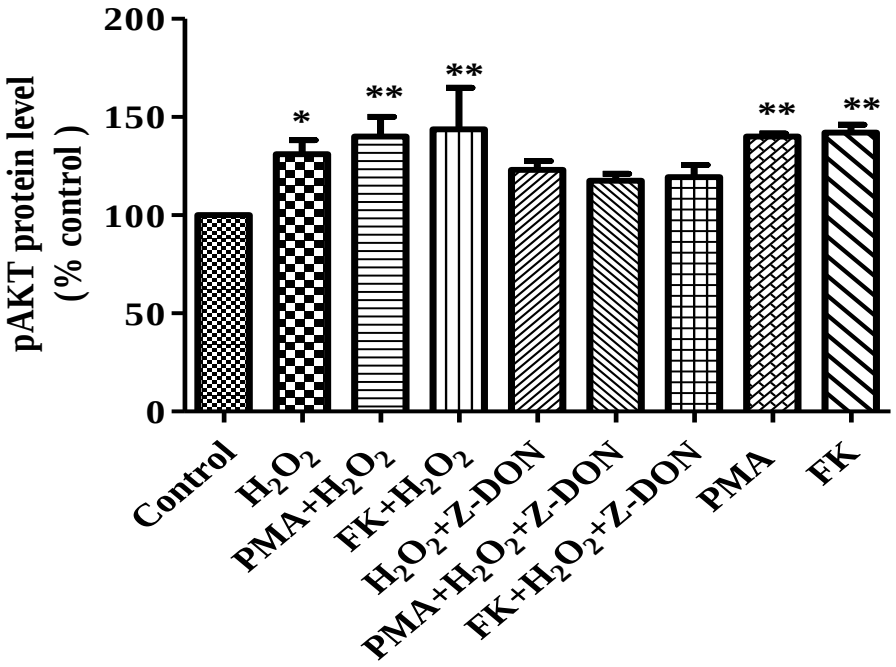



**Figure 5.3.11 The effect of Z-DON on survival proteins (pERK1/2 and pAKT) in H9c2 cells pre-treated with PMA and FK followed by $H_2O_2$ exposure**

H9c2 cells were treated either with or without 150 µM Z-DON for 1h. They were then incubated for 5 min with either 1 µM PMA or 10 µM FK alone or prior to the addition of 600 µM $H_2O_2$ for 2h. The total protein extract was resolved by SDS-PAGE and transferred onto nitrocellulose filters. A) Western blotting for anti-phospho-specific ERK1/2 mAb, C) anti-phospho-specific Akt where, anti-GAPDH mAb was used as a control of the total amount of the collected protein. B) and D) Densitometry was carried using Adobe Photoshop CS4 and values plotted as relative intensity versus the different treatments. Data are expressed as the percentage of proteins at basal level in the untreated cells (control). Values are means ± SEM of 3 independent experiments, Data analysis was performed using "Bonferroni's multiple comparison test" where the statistical significance was accepted at *P < 0.05, **P < 0.01, ***P < 0.001.

### *5.4. Discussion*

The data presented in this study provides provide evidence that the activity of TG2 is modulated in H9c2 cells by PMA and FK and in turn, this seems to have an involvement in the protection of H9c2 cells against oxidative stress.

Transglutaminase 2 (TG2) has been suggested to be involved in many patholoGlogical condition including neurodegenerative disorders, cardiac-vesicular diseases some cancers and coeliac disease (Verma et al., 2008). It has also been shown to protect mouse cardiomyocytes against ischaemia and reperfusion-induced cell death by regulating ATP synthesis (Szondy et al., 2006). Protein kinase C (PKC) and PKA have been shown to be modulators of ischaemic preconditioning (IPC) and pharmacological preconditioning (PPC) in cardiomyocytes of different animals (Yellon & Downey, 2003). In the previous chapters, the results of this study have shown that the activity of TG2 is modulated in H9c2 cells treated with either PMA or FK. Moreover, this was confirmed by an *in situ* assay in the presence of TG2 inhibitors. In this chapter, the possible role of TG2 in cytoprotection against oxidative stress was also investigated for comparison.

Hydrogen peroxide ($H_2O_2$) has been widely used to induce oxidative stress in order to mimic the condition of ischaemia-reperfusion injury in cardiac cells (Kurose et al., 1999). Oxidative stress has been shown to contribute to the pathogenesis of numerous cardiovascular diseases, including ischaemic heart disease (Wang & Zweier, 1996), atherosclerosis heart attack, stroke (Carmena et al., 2004), heart failure and sudden cardiac death (Byrne et al., 2003). In this study, a concentration of $H_2O_2$ has been optimised by our lab group and significant death has been observed in H9c2 cells at this concentration (600 µM) (Daubney et al., 2014). Similarly, another study by Mao et al. (2014) has shown that the ranges of $H_2O_2$ concentration 200-1000 µM are able to induce-oxidative stress in H9c2 cells result in mitochondrial dysfunction and apoptosis (Mao et al., 2014). In addition, the pre-treatment of cells by Levocarnitine has shown to reverse this effect and protect H9c2 cells (Mao et al., 2014). In addition, 600 µM of $H_2O_2$ has shown to induce mitochondrial permeability transition pore (mPTP) opening in H9c2 cells (Chanoit et al., 2011). Therefore, this concentration has

been adapted to induce oxidative stress in H9c2 to study effect of the TG2 inhibitor on oxidative stress-induced cell death and PMA and FK-induced cytoprotection.

The activation of *in situ* TG2 activity following oxidative stress induced either by $H_2O_2$ or UV irradiation has been reported in human lens epithelial cells (HLE-B3; Shin et al., 2004). The present data show that oxidative stress is induced in H9c2 cells by 600 µM $H_2O_2$. In turn, this results in stimulation of TG2 biotin cadaverine incorporation activity in the *in situ* (Fig. 5.3.2) and *in vitro* assays but not significantly as in the later (Fig. 5.3.1A). However, it did not stimulate protein crosslinking activity (Fig. 5.3.1B). Indeed, it has been reported that oxidative stress via $H_2O_2$ could not elevate *in vitro* TG activity as this depends on the cell type and the existence of specific cellular factors (Shin et al., 2004; Park et al., 2010). However, when the oxidative stress was induced followed by pre-treatment with PMA, but not FK, there was a statistically significant increase in TG2 catalysed biotin cadaverine incorporation both *in vitro* (Fig. 5.3.1A) and *in situ* with both treatments (Fig. 5.3.2). In contrast, treatment with the TG2 inhibitor (Z-DON) resulted in a reduction of this activation *in vitro* (Fig. 5.3.1) and in intact H9c2 cells (Fig. 5.3.2). This suggested that this the activity was due to activation of endogenous TG2 in H9c2 cells.

The level of TG2 protein increased in the presence of with PMA or FK, but this level did not show a significant increase in response to $H_2O_2$ alone or in pretreatment by PMA, but not FK (Fig. 5.3.10A & B). This confirms the previous suggestion by Shin et al., (2004) that there is no correlation between TG2 protein level and its activity (Jeon et al., 2004). Although in this study, no correlation has been observed between TG2 protein level and its *in vitro* activity in the presence of $H_2O_2$, there was a correlation between TG2 protein level and its *in situ* activity (Fig. 5.3.2). This is in disagreement with a previous observation in SH-SY5Y neuroblastoma cells treated with retinoic acid that showing correlation between TG2 protein level and *in vitro* but not *in situ* activity (Zhang et al., 1998). However, these opposing views could be due to the fact that the level of TG2 activation depends upon cell and stress types (Shin et al., 2004). Until now, the molecular mechanism (s) by which intracellular TG2 is regulated is not clear. While some TG family members have been reported to have a proteolysis site that activates the intracellular enzymes (Lorand & Graham, 2003), no proteolytic activation has been reported in TG2 regulation (Jeon & Kim, 2006).

However, phosphorylation of TG2 by PKA has been reported (Mishra et al., 2007) TG2-SUMOylation (SUMO is small ubiquitin-like modifier that covalently attaches to and detaches from cellular proteins altering their functions) in response to oxidative stress increases its proteins level and enhances its activity in CF airway epithelial cells (Luciani et al., 2009). In this study, therefore, it is possible that TG2 is regulated by posttranslational modification by these kinase activators. This may explained the reduction of TG2 protein level by Z-DON, when the oxidative stress was induced in cells pre-treatment with FK (Fig. 5.3.10A & B). This reduction could be either due to reduction in its protein levels itself or due to phosphorylation by PKA that alter the binding of anti-TG2 mAb to its target results in reduction in band intensity. Further study is needed it to investigate if the anti-TG2 mAb showed differential binding in the presence or absence of a posttranslational modification of TG2.

To investigate the possible role of TG2 in the cytoprotection of H9c2 cardiomyocytes to oxidative stress, the effect of TG2 inhibitors in H9c2 cardiomyocyte cell viability were tested. Lactate dehydrogenase is an enzyme widely expressed in mammalian cells and commonly used as marker for cell damage (Cho et al., 2008). This enzyme is not released from the cytoplasm under normal physiological conditions. Thus, it is an ideal enzyme for measurement of cellular cytotoxicity as a consequence of membrane insult (Cho et al., 2008; Kim et al., 2009). Thus, the release of LDH into H9c2 cell culture medium can reflect the amount of damage occurring in the presence of $H_2O_2$. The present results here indicated that $H_2O_2$ can induce a significant decrease on in H9c2 cell viability as shown by inhibition of MTT reduction (Fig. 5.3.3A) and a large release of LDH into the culture medium (***$P < 0.001$ v*ersus* untreated control) (Fig. 5.3.3B). This in agreement with many studies that used a similar concentrate of $H_2O_2$ in this cell lines (Chanoit et al., 2011; Daubney et al., 2014; Mao et al., 2014), Pretreatment of cells with PMA and FK reversed the $H_2O_2$-induced cell death. This may be due to the ability of PMA to directly activate PKC and mimic ischaemic preconditioning in reperfusion heart (Kuno et al., 2007; Liu et al., 2008). Thus, it may be generating a signalling cascade that reverses the cell injury induced by oxidative stress. Treatment with the TG2 inhibitors Z-DON or R283 (Griffin et al., 2008), significantly blocked this protection against LDH release (Fig. 5.3.4). These results

indicate a significant role for TG2 in modulating both PKC and PKA signalling and its protective effect through this signalling pathway.

The recommended $IC_{50}$ for Z-DON to be cell permeable is 50 μM. However, in the current study used a higher concentration (150 μM) of Z-DON because lower concentrations were not able to block TG2 activity (Fig. 4.3.4A). Therefore, this study considered that this dose might result in cell death, as had been reported in a Huntington's disease (HD) transgenic model where high concentrations of Z-DON mainly above 80 μM were shown to be associated with toxicity in some cells (Schaertl et al., 2010). Another study, on the other hand, has used 125 μM Z-DON in food given to fruit flies (Drosophila model of HD) which, showed a ~80 % decrease in TG activity with no obvious toxic effects (McConoughey et al., 2010). Cell viability was also measured with Z-DON and R283 alone or in presence of PMA and FK. The result indicated that 150 μM of Z-DON had no significant effect on H9c2 cell viability either in presence or absence of PMA and FK (Fig. 5.3.3C). This observation could be due to the cell type and the cytotoxicity assay that has been used. Similar observations were made with 200 μM R283 (Fig. 5.3.4C) and this is consistent with evidence that R283 ranging from 50-250 μM has no effect on viability of human SH-SY5Y neuroblastoma cells (Beck et al., 2006).

The cell morphology changes after oxidative stress induced by $H_2O_2$ and the effect of TG2 inhibitors (Z-DON) on cytoprotection by PMA and FK was observed. The stretched pseudopodia and connected cells that are usually observed in normal H9c2 cells were retracted, disconnected and many granular materials and vacuoles were formed when the cells were exposed to 600 μM $H_2O_2$ (Fig. 5.3.5). However, these effects were attenuated by PMA and FK and were more pronounced in the presence of the TG2 inhibitor Z-DON. This observation has indicated an important role of TG2 in modulating PMA and FK inducing cytoprotection against oxidative stress.

DNA fragmentation is a characteristic phenomenon of cell undergoing apoptosis. Moreover, it can be generated by activation of caspase activated DNase (CAD) by caspase-3 (Enari et al., 1998). DNA fragmentation and caspase activation induced by oxidative stress have been already detected in H9c2 cells under oxidative stress (Turner et al., 1998). The using of agarose gel electrophoresis for DNA fragmentation

detection is a common method in apoptotic cells due to simplicity and feasibility (August & Kaufmann, 1997). However, the method is limited by DNA recovering and thus it requires a high amount of cells to allow DNA to be detectible, these makes the method less sensitive and only qualitative (Collins et al., 1992; Gavrieli et al., 1992). In the current study, the DNA fragmentation was shown as a smear band but not as a laddering in cells when exposed with $H_2O_2$ alone and in cells pre-incubated with Z-DON (Fig. 5.3.9). Indeed, this could be either due to unsufficient DNA recovery or due to DNA fragmentation at an earlier time point and appeared as DNA smearing associated with necrosis (Portera-Cailliau et al., 1995).

Since caspases are enzymes responsible for the induction of cell death by apoptosis (Cohen, 1997; Nicholson, 1999), caspase-3 activity and its proteins level were investigated. The exposure of H9c2 cells to $H_2O_2$ resulted in an increase in caspase-3 activity, although this was not statistically significant. However, the TG2 inhibitor Z-DON significantly decreased this activation. Interestingly, a significant increase was shown in pretreatment via PMA, but not FK. In contrast, the use of Z-DON resulted in a reduction in caspase activation that was significant compared with FK+$H_2O_2$, but not PMA+$H_2O_2$ (Fig. 5.3.7A). Both immunoblotting and immunohistochemistry analyses of H9c2 cells indicated the presence of active caspase-3 when treated with $H_2O_2$ and pretreated with either PMA or FK. Active caspase-3 was shown to be predominant in response to oxidative stress and less in PMA or FK/ pretreated cells (Fig. 5.3.8). The presence of TG2 inhibitor in treated cells resulted in reduction of caspase-3 activation. Indeed, a recent study in rat neural cells has revealed that caspase-3 activation acts as downstream event of ischaemic preconditioning in parallel with CAD, in which preconditioning intervenes to prevent apoptotic cell death (Tanaka et al., 2004). Another study referred that activation of caspase-3 and some elements that are normally associated with cell death, such as ROS, are essential in neuroprotection for up-regulation of the protective protein HSC70 (McLaughlin et al., 2003). Furthermore, they suggested that the activation of caspase-3 in preconditioning was prevented from eliciting cell death. Therefore, the blocking of this activation might prevent protective proteins from being synthesised. Moreover, it could be possible that the complete inhibition of caspase-3 in cells treated with Z-DON (TG2 inhibitor) results in a shift of cells death from apoptosis to necrosis, as

previously reported in ATP depletion stimulated B lymphocyte apoptotic cell death (Lemaire et al., 1998).

The effect of $H_2O_2$ and Z-DON on survival proteins such as pERK1/2 and pAKT were also investigated. The activation and phosphorylation of ERK1/2 and AKT are crucial for survival signalling associated with cardioprotective mechanism (Kilter et al., 2009; Kim et al., 2012). The present results indicated that cells pretreated with either PMA or FK alone or in the presence of $H_2O_2$ showed significant increase in pERK compare to control and $H_2O_2$ treated cells (Fig. 5.3.11A & B). In the presence of Z-DON this activation did not show significant attenuation. However, pAKT showed a significant increase in cells pretreated with PMA and FK in presence of $H_2O_2$ that was also significantly decreased in the presence of Z-DON (Fig. 5.3.11C & D). These results suggest that PMA and FK can activate AKT signalling under $H_2O_2$ induced oxidative stress and that TG2 plays a role in this protective effect, as confirmed by the reversal of this effect in the presence of Z-DON.

Although the data failed to shows apoptosis and conventional DNA fragmentation ladder, the cell death was evident by MTT, LDH and by morphological change data. Thus, the results of this chapter clearly suggest that TG2 activity can be regulated via PKC and PKA-dependent signaling. Nonetheless, TG2 activity modulates PMA /FK- has cytoprotection effect against oxidative stress induced by $H_2O_2$ suggesting a cell survival (protective) role for TG2 in H9c2 cells through these signalling.

# CHAPTER VI:

# IDENTIFICATION OF TG2 SUBSTRATES IN H9c2 CELLS

## 6. Introduction

Transglutaminase 2 (TG2) is widely expressed in certain mammalian tissues and has been shown to possess many enzymatic function. For example, it is able to catalyse a transamidating, a deamidating, GTP-binding/hydrolyzing activity, an intrinsic kinase and isopeptidase activities (Griffin et al., 2002; Mishra & Murphy, 2004). Therefore, it has been assigned in many fundamental biological processes including proliferation, differentiation and apoptosis (Aeschlimann & Paulsson, 1994; Griffin et al., 2002). The first suggestion of the involvement of TG2 activity in cellular apoptosis was observed in rat liver with hyperplasia inducing cell death (Fesus et al., 1987). The crosslinking activity of TG2 is thought to be responsible in stabilising dying cells through the formation of intracellular crosslinked protein structures, which prevent leakage of apoptotic cell components (Fesus & Szondy, 2005), thus inhibiting the inflammatory response. In addition, the presence of TG2 outside the cell has implicated it in the formation and repair of extracellular matrix (Aeschlimann & Thomazy, 2000). Transglutaminase 2 has also been reported to be involved in cell adhesion and the migration of monocytic cells through fibronectin matrices during inflammation (Akimov & Belkin, 2001a). The involvement of TG2 in signal transduction through α1-adrenergic receptors has also been reported (Nakaoka et al., 1994) where it has been implicated in the protection of mouse hepatocytes against Fas-mediated cell death (Sarang et al., 2005). In addition, TG2 can also modify specific proteins through its catalytic action that incorporates polyamines into acyl-donor substrates. This catalytic action can alter the structure and function of specific proteins or kinases thus, triggering cascades. An example includes, TG2 catalysed polyamine incorporation of spermine and spermidine into the neurotransmitter peptide substance P, providing enriched resistance to proteases action *in vitro* (Esposito et al., 1999). A similar polyamination reaction has also shown to be responsible for inducing cell death in human vascular and melanoma cells (Facchiano et al., 2001). Moreover, TG2 has been reported to mediate the polyamination of human and rat vasoactive intestinal peptide (VIP), enhancing its ability to bind to and activate pituitary adenylyl cyclase activating peptide receptor (VPAC1), which is an important hypophysiotropic hormone acting as a neuromodulator (De Maria et al., 2002).

Recently, TG2 was found to be implicated in a wide variety of pathological states such as coeliac disease, inflammation, cancer, fibrosis, neurodegenerative disorders e.g. Alzheimer's and Huntington's diseases (Cooper et al., 2002; Kim et al., 2002). In human cardiovascular pathology, the induction of TG2 activity enhanced cell matrix crosslinking causing vascular stiffening in aging (Santhanam et al., 2010). In cardiomyocytes, TG2 knockout mice display sensitivity to ischaemia/reperfusion injury compare to wild type mice in correlation with a significant decrease in ATP (Mastroberardino et al., 2006; Szondy et al., 2006). Furthermore, this research group has found that adenine nucleotide translocator 1 (ANT1) can act as a TG2 substrate for its protein crosslinking activity, thus modulating mitochondrial ADP/ATP exchange in apoptosis (Malorni et al., 2009). Elafin also known as trappin is a TG2 extracellular substrate (Schalkwijk et al., 1999) and the TG2 crosslinking activity results in aggregation of this substrate and the formation of plaques in atherosclerotic human coronary artery (Sumi et al., 2002).

Another TG2 substrate and possibly related to cell fate is RhoA, which is a member of the Ras superfamily of G-proteins, plays a significant role in cell growth and actin cytoskeleton regulation. *In vivo* TG2 can modulate the transamidation of RhoA induced by retinoic acid, stimulating stress fibre and focal adhesion complex formation in HeLa cells (Singh et al., 2001). RhoA has also been shown to promote cytoskeleton rearrangement and activation of the MAP kinase pathway in SH-SY5Y neuroblastoma cells induced to differentiate by retinoic acid (Singh et al., 2003). Recently, TG2 activity was shown to be associated with the inward remodelling of smooth muscle cells therefore the extracellular protein that may act as TG2 substrates were investigated (van den Akker et al., 2011). Transglutaminase 2 activity was stimulated by DTT in smooth muscle cells, while TG2 substrates were labelled with the amine donor biotin cadaverine. This was analysed by mass spectrometry for identification of labelled proteins. Collagen, fibulin-2 and nidogen-1 were identified as glutamine donors for transamidation activity of TG2 in these smooth muscle cells (van den Akker et al., 2011).

TG2 is capable of catalysing the incorporation of polyamines into a wide range of substrates including proteins, peptides, mono- and polyamines and nucleotides. Thus, identification of these substrates is of critical importance in understanding the role of

TG2 in cell physiological function and in disease states (Esposito & Caputo, 2005). In addition, the identification of theses substrates may have a significant impact on resolving the complexity of this multifunctional enzyme in cardiomyocytes and provide a guide for new diagnostic markers and drug targets in heart disease.

Many approaches have been developed for the detection and identification of TG substrates. An indirect method for recognizing substrates is the detection of protein crosslinking using SDS-PAGE and Western blotting, and inhibition of protein crosslinking with amines or incorporation of glutamine-rich peptides (Butler & Landon, 1981; Groenen et al., 1992; Lajemi et al., 1997). The labelling of TG substrates with radioactive amines, FITC-cadaverine and biotinylated glutamine-containing peptides have been used to identify TG substrates both *in vitro* and *in vivo* (Nemes et al., 1997; Csosz et al., 2002). These detection methods can be also validated by utilising the *in situ* assay in which biotinylated amines or glutamine-containing peptides are used to localise different substrates within the organelles of cells. This strategy can be used in conjunction with affinity chromatography for the isolation of substrates followed by identification of the labelled TG-reactive protein using mass spectrophotometry. This approach has been applied in placenta and has yielded information on TG2-catalyzed posttranslational modification of specific proteins or peptides e.g. actin, annexin, integrins alpha V/alpha IIb and monoamine oxidase type A (Robinson et al., 2007).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is one of the most commonly used proteomics techniques to separate proteins according to their variation in isoelectric point (pI) and their molecular weights. This separation method is carried out in two dimensions, the first of which separates proteins by isoelectric-focussing, whereas the second dimension uses SDS-PAGE to separate polypeptides according to their relative molecular mass. Using these methods, a thousand different proteins in a lysate sample can be characterized, separated, resolved, and detected (Issaq & Veenstra, 2008). Various staining method can then be applied to visualise the separated proteins by probing the protein itself using different dyes such as, Coomasse blue and silver stain. This approach has also been applied to verify TG substrates in bone (Kaartinen et al., 2002).

Together, with developments in mass spectrometry techniques and software tools, the identification of proteins from complex mixtures of biological origins becomes more stringent and accurate. There are two approaches for characterizing the proteins either by ionization of whole proteins via matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) and then analysed by mass spectrometry (Preisler et al., 2000). This approach is known as a "top-down" protein analysis strategy. The other approach is a "bottom-up" proteomic strategy, in which proteins are digested by protease enzyme such as, trypsin to generate smaller peptides then they are also introduced to a mass spectrometry and thus identification can be done using peptide mass fingerprinting or tandem mass spectrometer through database comparison (Henzel et al., 1993). The MALDI time-of-flight (MALDI-TOF) instrument is commonly used in peptide mass analysis.

Since TG has been shown to be having pathological and protective roles in different diseases, it is essential to have a better insight in TG2 substrates, target sites and interacting proteins, which may act as novel drug targets or new diagnostic markers. Thus, this chapter is focused on detection of TG2 substrates in response to PMA and FK and their protective effect against $H_2O_2$-induced stress. The fractionation of acyl-donor TG2 substrate proteins was analysed in H9c2 cells, using a pull down assay followed by protein separation in 1D/2D PAGE, and subsequent analysis by Western blotting and immunoprobing techniques. Subsequently, identification of target substrates by MS-MALDI was performed. In some cases, TG2 activity was also confirmed by immunofluorescence staining techniques.

### *6.1. Aims*

The main aim of the work in this chapter was to identify and fractionate acyl-donor TG2 substrates in H9c2 cardiomyocytes. The work focused on detection of TG2 substrates in response to PMA and FK and their protective effect against $H_2O_2$-induced cytotoxicity.

### *6.2. Methods*

As described in chapter 2 of this study (section 2.2).

## *6.3. Results*

### 6.3.1. Identification of proteins that serve as substrates for TG2

#### 6.3.1.1. Detection of TG2 activity and protein substrates following PMA and FK exposure in the presence and absence of TG2 inhibitor

To detect TG2 protein substrates and activity in PMA and FK treated cells, equal amounts of whole cell extract proteins from control and stimulated cells were resolved by SDS-PAGE and transferred onto nitrocellulose membrane filters. Proteins conjugated with biotin-X-cadaverine by *in situ* TG2 activity were visualized by probing with ExtrAvidin®-peroxidase. The acyl-acceptor probe biotin cadaverine was incorporated into endogenous protein substrates of TG2 in H9c2 cells as described in Material and Methods (section 2.2.17).

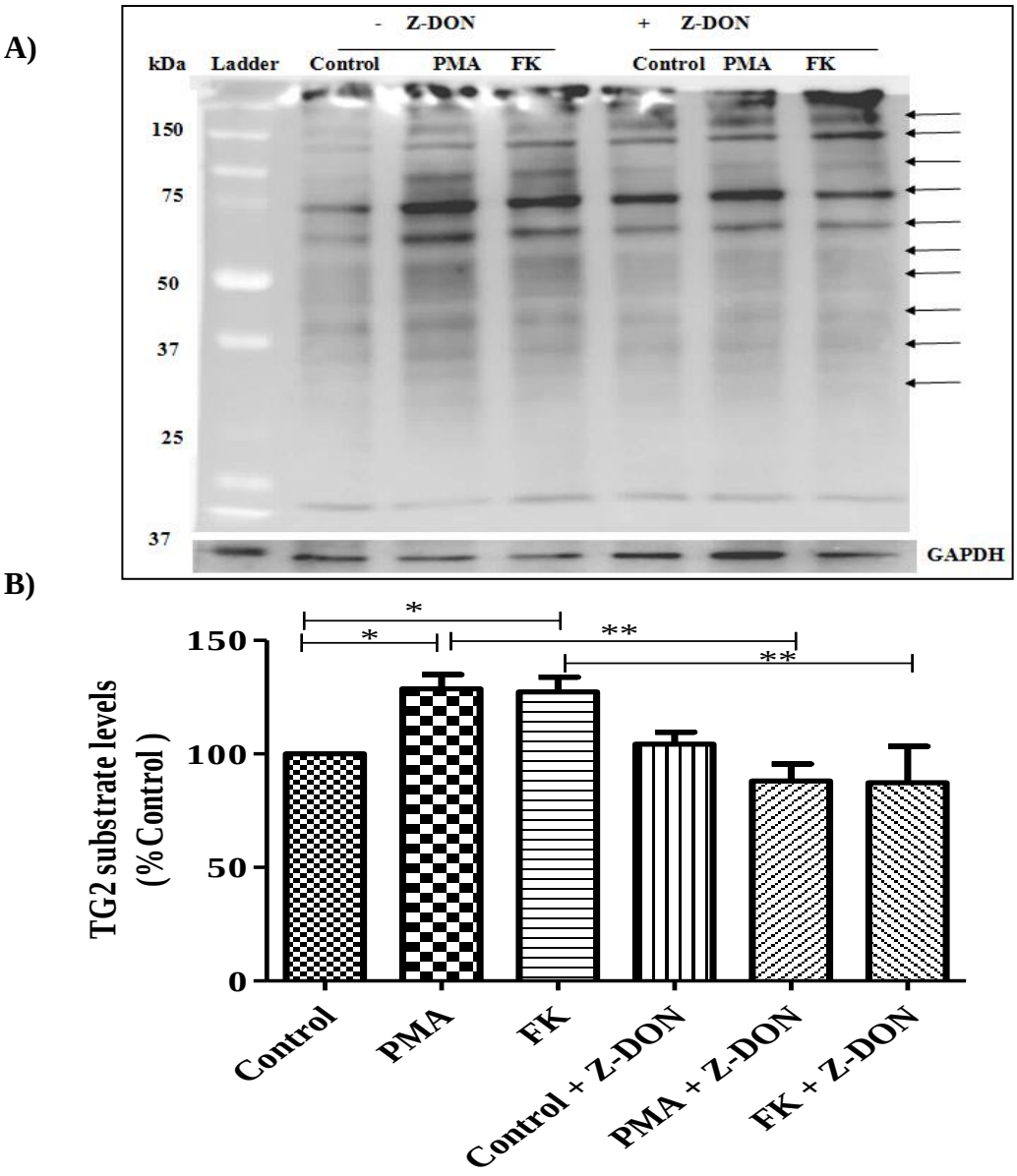



**Figure 6.3.1 Detection of TG2 activity and protein substrates following PMA and FK exposure in the presence and absence of Z-DON**

H9c2 cells were pre-incubated with 1 mM biotin-X-cadaverine for 4h. They were then treated with 150 µM Z-DON for 1h prior to either 1 µM PMA or 10 µM FK treatments for 5 min. The total protein extract (50 µg) was resolved by SDS-PAGE and transferred on to nitrocellulose membrane filters. A) TG2 transamidating activity and protein substrates were detected with ExtrAvidin®-peroxidase. Anti-GAPDH mAb was used as a control of the total amount of cellular protein. The arrows point to the changed proteins. B) Densitometry was carried out in Adobe Photoshop CS4 and the values were plotted as relative intensity versus the treatments. Data are expressed as a percentage of TG2 substrate proteins at basal level in the untreated cells (control) after GAPDH normalisation. Values are means ± SEM of 3 independent experiments. Data analysis was performed using "Bonferroni's multiple comparison test" where the statistical significance was accepted at * $p<0.05$ and ** $p < 0.01$.

The results in fig. 6.3.1 show elevated biotin-X-cadaverine incorporation into numerous proteins in PMA and FK-stimulated compared to untreated control H9c2 cells, with molecular weights ranging from ~25 to 200 kDa and higher molecular-masses sometimes observed. (n = 3, *p < 0.05; Fig. 6.3.1B & A, lane 3 & 4). The biotin cadaverine labelled proteins also showed reduced biotinylation in the presence of the TG2 inhibitor Z-DON (n = 3, **p < 0.01; Fig. 6.3.1B & A, lane 6 and 7).

### 6.3.1.2. Detection of TG2 protein substrates following PMA/FK treatment and $H_2O_2$ exposure

To detect biotinylated TG2 substrate proteins following PMA/FK treatment and $H_2O_2$ exposure, an equal amounts of protein extracted from whole cells were resolved by SDS-PAGE and transferred onto nitrocellulose membrane filters. Proteins conjugated with biotin-X-cadaverine by *in situ* TG2 activity were visualized by probing with ExtrAvidin®-peroxidase. The acyl-acceptor probe biotin cadaverine was incorporated into endogenous protein substrates of TG2 in H9c2 cells.

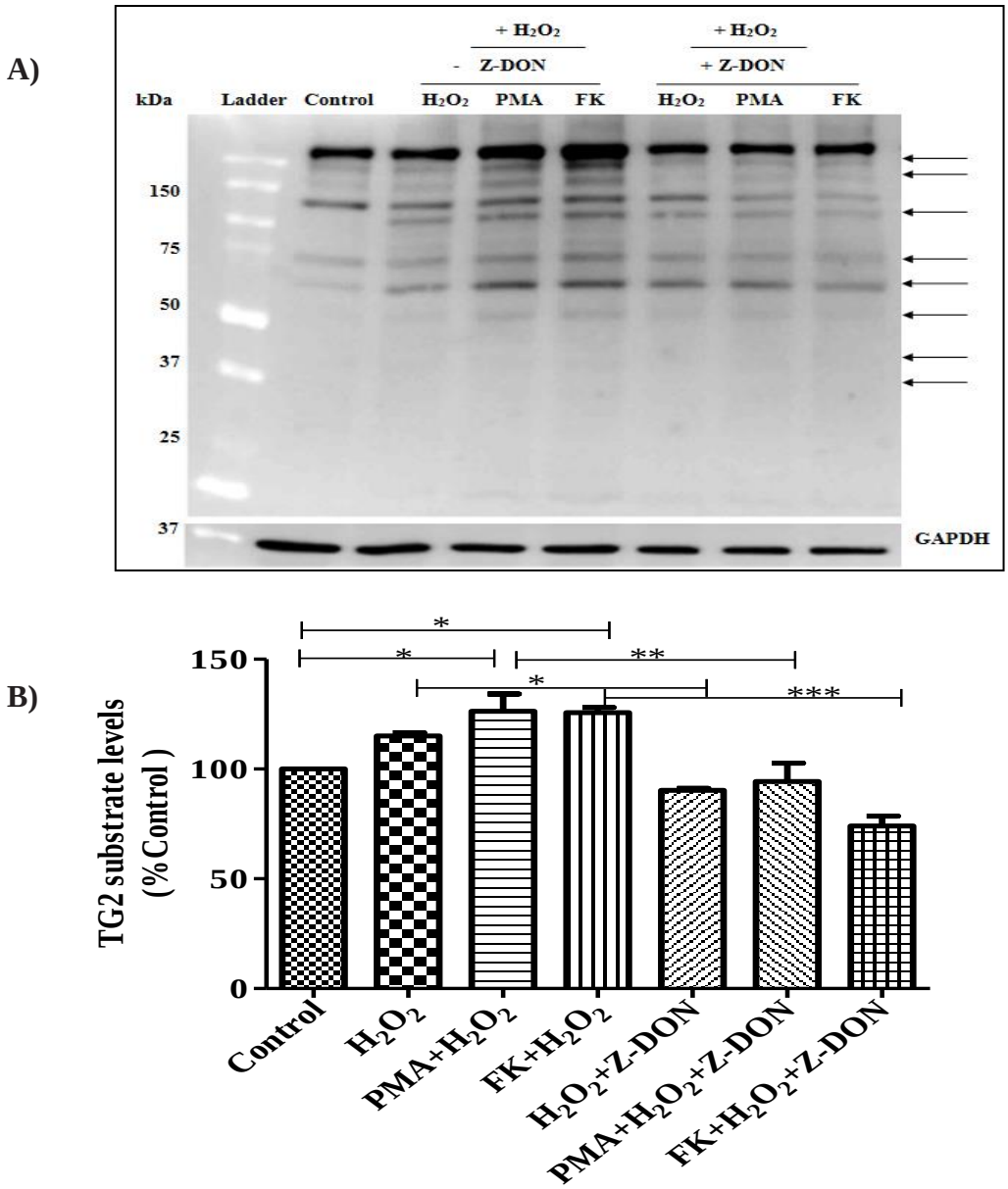



**Figure 6.3.2 Detection of TG2 protein substrates following PMA/FK treatment and $H_2O_2$ exposure**

H9c2 cells were pre-incubated with 1 mM biotin-X-cadaverine for 4h then treated with or without 150 µM Z-DON for 1h. They were then treated for 5 min either with 1 µM PMA or 10 µM FK prior to the addition of 600 µM $H_2O_2$ for 2h. The total protein extract (50 µg) was resolved by SDS-PAGE and transferred onto nitrocellulose filters. A) TG2 transamidating activity and protein substrates were detected with ExtrAvidin®-peroxidase. Anti-GAPDH mAb was used as a control of the total amount of cellular protein. The arrows point to the changed proteins. B) Densitometry was carried out in Adobe Photoshop CS4 and values were plotted as relative intensity versus the treatments. Data are expressed as a percentage of TG2 substrate proteins at basal level in the untreated cells (control). Values are means ± SEM of 3 independent experiments. Data analysis was performed using "Bonferroni's multiple comparison test" where the statistical significance was accepted at *p < 0.05, **p <0.01 and ***P < 0.001.

The results in fig. 6.3.2 show biotin cadaverine incorporation into numerous proteins in H9c2 cells, which was elevated in response to either PMA or FK-induced cytoprotection against $H_2O_2$ stress compared to untreated cells (control) (n = 3, *P < 0.05; Fig. 6.3.2B & A, lanes 4 & 5). The labelling of proteins was reduced in the presence of the TG2 inhibitor Z-DON (n = 3, *P < 0.05 *versus* $H_2O_2$, **P < 0.01 versus PMA+ $H_2O_2$, ***P < 0.001 versus FK+ $H_2O_2$; Fig. 6.3.2B & A, lane 6-8). A similar banding pattern of biotin-cadaverine labelled proteins to that observed in response to PMA or FK alone (Fig. 6.3.1) was found in cells treated with either PMA and FK followed by $H_2O_2$ insult.

### 6.3.2. Fractionation and identification of acyl-donor (Gln-donor) TG2 substrate proteins

To isolate TG2 substrate proteins after treatment by either PMA or FK, the biotin-cadaverine labelled target proteins in treated H9c2 cells were captured with CaptAvidin beads as described in Materials and Methods (section 3.9 and figure 2.2.2) and subjected to SDS-PAGE on a 4-15 % polyacrylamide gradient gel and visualised by Coomassie Blue-stain.

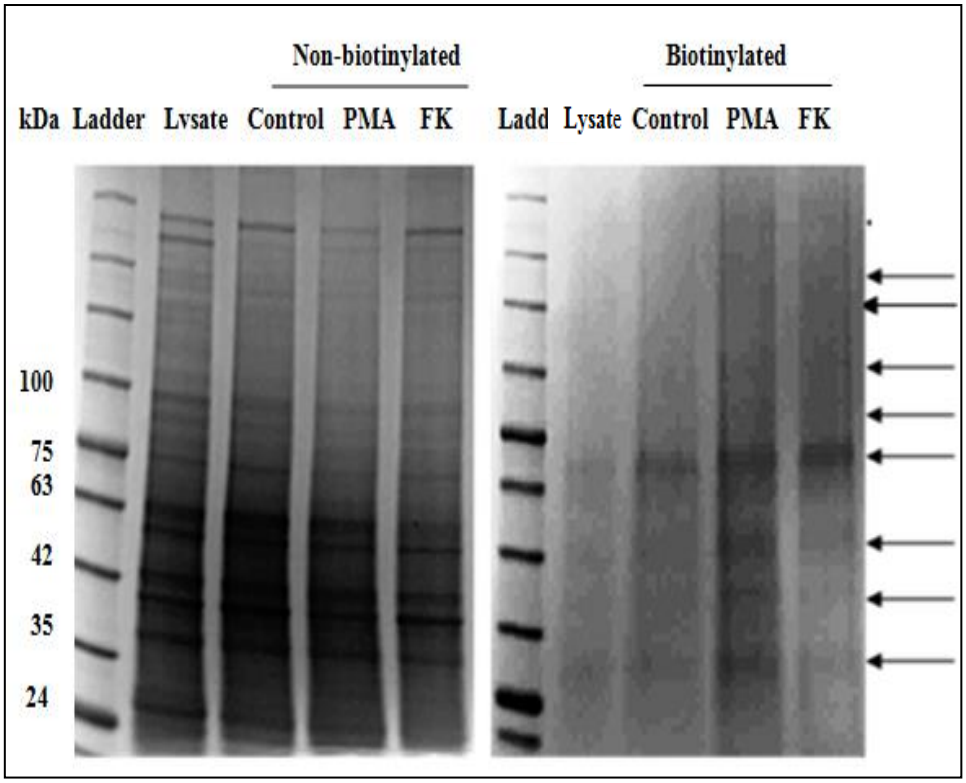



**Figure 6.3.3 TG2-mediated labelling after PMA and FK treatments in H9c2 cells with the acyl-acceptor probe (biotin-X-cadaverine)**

The treated H9c2 proteins were fractionated on CaptAvidin beads, equal amounts of bound (right panel) and unbound materials (left panel) were loaded onto 4-15 % gradient gels stained with Coomassie Blue-stain. The arrows indicate excised bands that were selected for protein identification. The lanes in right panel represented, ladder (lane 1), total cell lysate (lane 2), control untreated cells (lane 3), PMA treated cells (lane 4) and FK treated cells (lane5). The lanes in left panel represented, ladder (lane 1), CaptAvidin captured cell lysate (lane 2), control untreated cells (lane 3), PMA treated cells (lane 4) and FK treated cells (lane5). The results are typical of 4 independent experiments.

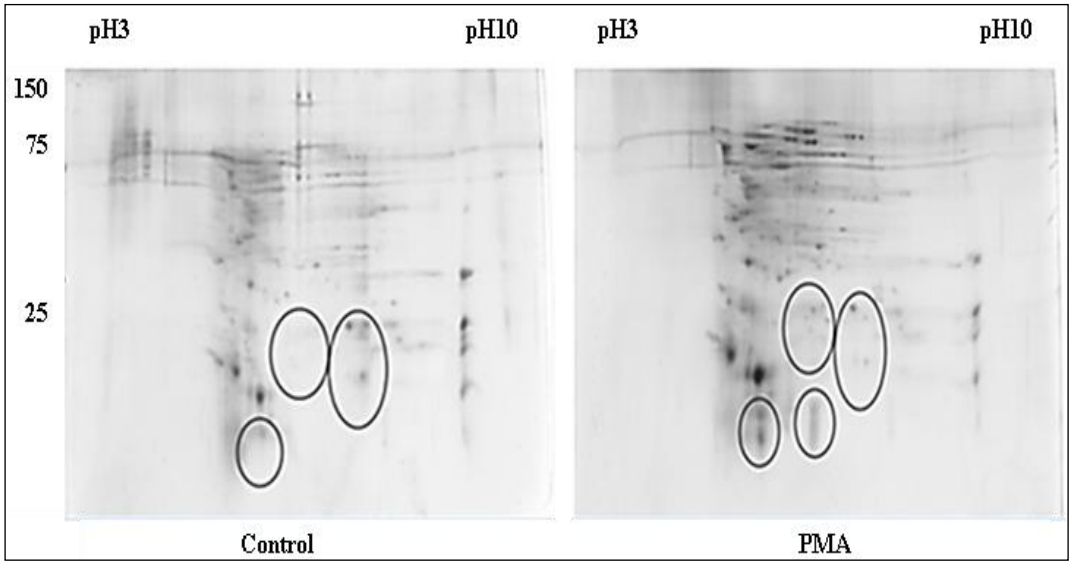
Figure 6.3.3 shows a biotinylated and non-biotinylated fraction of TG substrates detected in PMA/ FK H9c2 treated cells. Many proteins were detected and differences were shown compared to control. It was clear that CaptAvidin beads selectively isolated and captured biotin-X-cadaverine labelled proteins since the large number of bands that appeared in whole cell lysate seemed to disappear from the non-biotinylated (supernatant) fractions (Fig. 6.3.3). These missing bands were recovered in the eluted fraction and represent biotin cadaverine labelled substrates (Fig. 6.3.3).

There were less biotin-cadaverine labelled products remaining in the supernatant and wash fractions when they were analysed by Western blotting and probed with ExtrAvidin®-peroxidase (see appendix Fig. 8.4).

### 6.3.3. Detection of TG2 substrate protein in PMA treated H9c2 cells using 2D-PAGE

In order to gain a better resolution of TG2 substrate proteins in response to PMA treatments, biotin-X-cadaverine labelled proteins in PMA treated cells were fractionated on CaptAvidin beads, then subjected to 2D-PAGE (section 2.2.14) and visualised by either silver stain (section 2.2.16) or by Western blotting.

**A)**




**B)**

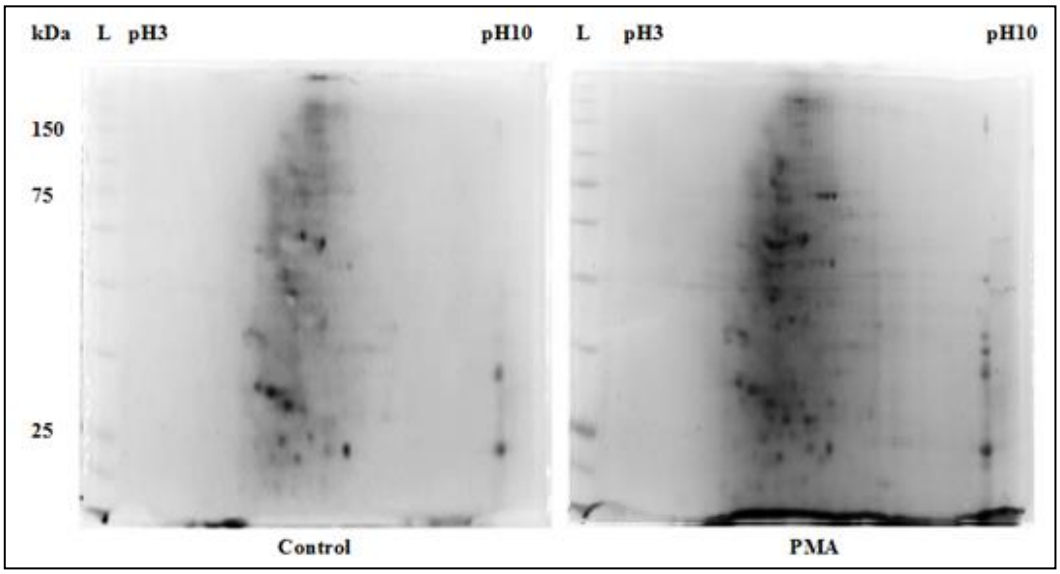


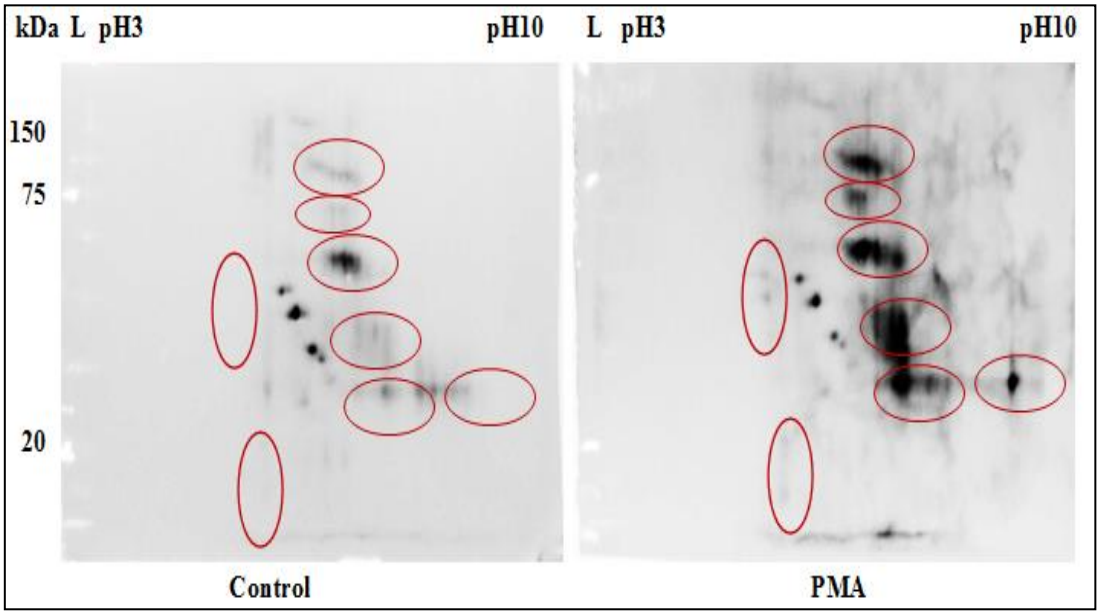

**Figure 6.3.4 Detection of TG2 substrate proteins in PMA treated H9c2 cells by 2D-PAGE**

For the first-dimension of isoelectric focusing protein was loaded onto immobilized pH gradient (IPG) strips with a pH range of 3 to 10. After subsequent SDS-PAGE in the second dimension, the proteins were fixed and visualized using silver staining kit. A) 2D-PAGE of total proteins (50 µg) after biotin-X-cadaverine labelling in untreated H9c2 cells (left panel) and PMA-treated H9c2 cells (right panel). B) Biotinylated control and PMA treated H9c2 proteins isolated with CaptAvidin beads. The results are typical of 5 independent experiments.

In the current study, the 2D-PAGE technique was used to investigate the activity of TG2 in response to PMA in H9c2. On silver stained gels, many protein spots were detected and differences were shown compared to control (Fig. 6.3.4). However, it was clear that CaptAvidin beads were selectively isolating and capturing biotin-X-cadaverine labelled protein since the amount and profile of protein spots that were present before CaptAvidin fractionation (Fig. 6.3.4A) changed dramatically after CaptAvidin fractionation (Fig. 6.3.4B).

These captured proteins that resolved by 2D-PAGE were also analysed by Western blotting and detected with ExtrAvidin®-peroxidase (Fig. 6.3.5). The results of Western blotting revealed that many protein spots showed increased intensity in response to PMA (circled in Fig. 6.3.5A) which reflected increasing TG2 incorporation activity. Changes in TG2 incorporation activity were quantified by Progenesis same spots software.

**A)**




**B)**

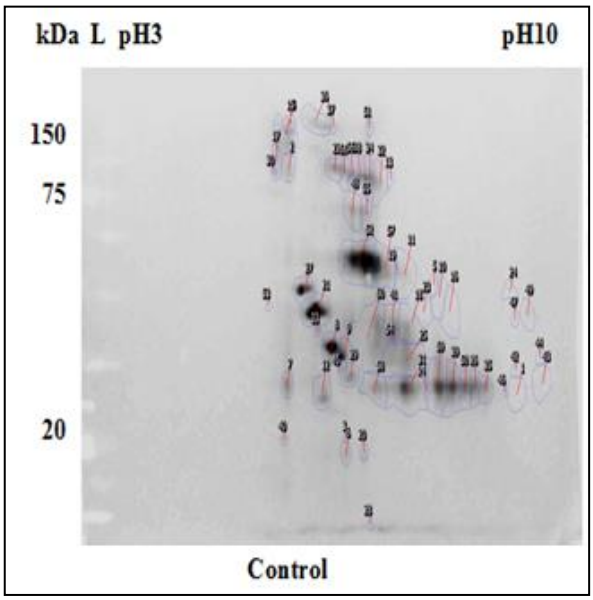



**Figure 6.3.5 Biotin cadaverine labelled PMA treated H9c2 proteins detected with HRP-ExtrAvidin-peroxidase**

H9c2 cells were pre-incubated with 1 mM biotin-X-cadaverine for 4h. They were then treated A) without (left panel) or with 1 µM PMA (right panel) for 5 min. The biotinylated (biotin-cadaverine labelled) proteins (500 µg) were isolated with CaptAvidin beads and 50 µg subjected to PAGE, transferred onto nitrocellulose membrane filters and the captured TG2 substrates were detected with ExtrAvidin®-peroxidase. Red circle represent the changed TG2 substrates. B) Western blot analysis image of the biotin cadaverine labelled proteins. Control (untreated sample) was used as reference for 2D-PAGE analysis image in Progenesis same spots software. The results are typical of 5 independent experiments.

**Table 6.3.1 The 2D-PAGE analysis data of TG2 substrate proteins in PMA treated H9c2 cells**

| TG2 activity | Protein spots | Anova (P) | Fold change |
|---|---|---|---|
| **Increased Proteins** | 1 | 0.016 | 9.1 |
| | 5 | 0.009 | 5.9 |
| | 10 | 0.001 | 4.3 |
| | 11 | 9.457e-004 | 4.2 |
| | 13 | 8.850e-004 | 3.4 |
| | 14 | 2.410e-004 | 3.2 |
| | 16 | 0.009 | 3.0 |
| | 18 | 0.002 | 2.9 |
| | 19 | 0.003 | 2.8 |
| | 23 | 2.828e-004 | 2.7 |
| | 25 | 0.001 | 2.6 |
| | 31 | 0.005 | 2.1 |
| | 32 | 0.008 | 2.0 |
| | 34 | 0.003 | 1.9 |
| | 38 | 0.002 | 1.6 |
| | 41 | 0.021 | 1.4 |
| | 48 | 0.054 | 1.8 |
| **Decreased Proteins** | 4 | 0.001 | 6.4 |
| | 6 | 1.657e-004 | 5.4 |
| | 7 | 0.004 | 4.8 |
| | 8 | 3.306e-004 | 4.6 |
| | 9 | 3.600e-005 | 4.6 |
| | 12 | 2.605e-005 | 3.6 |
| | 15 | 0.002 | 3.1 |
| | 17 | 2.552e-004 | 3.0 |
| | 20 | 2.830e-004 | 2.8 |
| | 21 | 0.003 | 2.7 |
| | 27 | 0.002 | 2.6 |
| | 29 | 1.981e-004 | 2.2 |

Data table represent activated and decreased protein spots reflecting altered TG2 activity. Data values of 3 accumulated 2D-PAGE, *P < 0.05 was viewed as significant. Fold change is shown.

Approximately 60 protein spots were detected from the isolated biotinylated TG2 substrates in PMA treated H9c2 cells when sufficient protein was applied on 2D-PAGE. Fourteen protein spots represented in Figure 6.3.5 and listed in Table 6.3.1 were shown to exhibit significant increases in compared to control sample by ≥ 2.00 fold (Fig. 6.3.5B) and eight more protein spots showed increases that were not significantly different from the control. However, twelve protein spots showed a significant decrease by ≥ 2.00 fold in compered to control (see fig. 6.3.5B and Table 6.3.1).

### 6.3.4. Identification of TG2 substrates

The protein bands that showed activation and inhibition were excised from gels according to their molecular weight, judging from molecular weight ladder (Fig. 6.3.1). The proteins were then extracted from gel by dehydration and hydration, followed with trypsin digestion as described in Material and Methods (section 2.2.26 and figure.2.2.2). Samples were then directly analyzed by LC-MS/MS in which multiple proteins were identified within a single protein band. Proteins with a high score and sequence coverage (see appendices section fig. 8.6) following peptide mapping are listed in Table 6.3.2. More than 25 proteins that can serve as acyl-donor for transglutaminase were identified, which ranged in molecular weight from 25 to 100 kDa. These targets include cytoskeletal organizing proteins, chaperone proteins, $Ca^{2+}$ and phospholipid binding proteins and proteins involved in vesicle transport processes. Some of these proteins were also identified from silver stained 2D-PAGE (see appendix Fig. 8.5).

**Table 6.3.2 Functional classification of identified acyl-donor TG2 protein substrates in H9c2 treated cells**

| Functional group | Proteins | MW [kDa] | SC [%] | Known substrate* |
|---|---|---|---|---|
| **Cytoskeleton network** | α-actinin-1 | 102 | 26 | No |
| | Actin; cytoplasmic 1, | 41 | 31 | Yes |
| | Alpha cardiac muscle 1& | 41 | 29 | No |
| | Aortic smooth muscle. | 41 | 29 | No |
| | Tubulin | 50 | 31 | Yes |
| | Myosin-9 | 226 | 31 | Yes |
| | Elongation factor 1-alpha 1 | 50 | 23 | Yes |
| | Tropomyosin | 32 | 29 | Yes |
| | Vimentin, | 54 | 21 | Yes |
| | Prelamin | 74 | 22 | Yes |
| **$Ca^{2+}$and phospholipid binding protein** | Annexin; A2 | 38 | 31 | No |
| | & A3 | 36 | 33 | No |
| **Protein folding** | Heat shock protein HSP 90-alpha, beta | 84<br>83 | 26<br>22 | Yes<br>Yes |
| | Heat shock cognate 71 kDa | 70 | 25 | Yes |
| | protein, 78 kDa glucose-regulated protein | 72 | 24 | No |
| | Malectin | 32 | 28 | No |
| | Serpin H1 | 46 | 31 | No |
| **Energy metabolism** | Prohibitin-2 | 33 | 39 | No |
| | Voltage-dependent anion-selective channel protein 1 | 31 | 44 | No |
| **Miscellaneous** | Guanine nucleotide-binding protein subunit beta-2-like 1 | 35 | 18 | No |
| **Endomembrane vesicle trafficking** | Arf-GAP -containing protein 1 (ASAP1) | 127 | 23 | No |
| | Ras-related protein Rab-35 | 25 | 30 | No |
| **mRNA metabolism and transport** | Heterogeneous nuclear ribonucleoprotein A1 | 34 | 39 | Yes |
| | 60S ribosomal protein L5 | 34 | 35 | No |

The biotin cadaverine labelled proteins were analysed by mass spectrometry. Data of identified proteins are reported according to high score and sequence coverage percentage (SC%). Molecular weight (MW) is also indicated in kDa. (*) According to the TRANSDAB database (http://genomics.dote.hu/wiki/index.php/).

### 6.3.5. Co-localisation of α-actinin and tubulin with TG2 activity

Work to this point has established the modulation of TG2 activity following PKA and PKC activation in H9c2 and their protective role against oxidative stress mediated by TG2 and identified target substrate proteins. However, it was important to confirm whether selected target proteins could be localised in intact cells. One of the main functional classifications of TG2 substrates in the H9c2 PMA/FK treated cells were cytoskeleton organising proteins, suggesting a role for TG2 in the organization and turnover of cardiomyocytes plasma membranes and its associated cytoskeleton.

Alpha actinin and tubulin are among the most abundant TG2 target protein substrates observed in PMA/FK treated H9c2 cells (Table 6.3.1). Evidence of direct α-actinin biotin cadaverine labelling in stimulated cells was obtained by immunological techniques using anti-α-actinin 1 antibody.

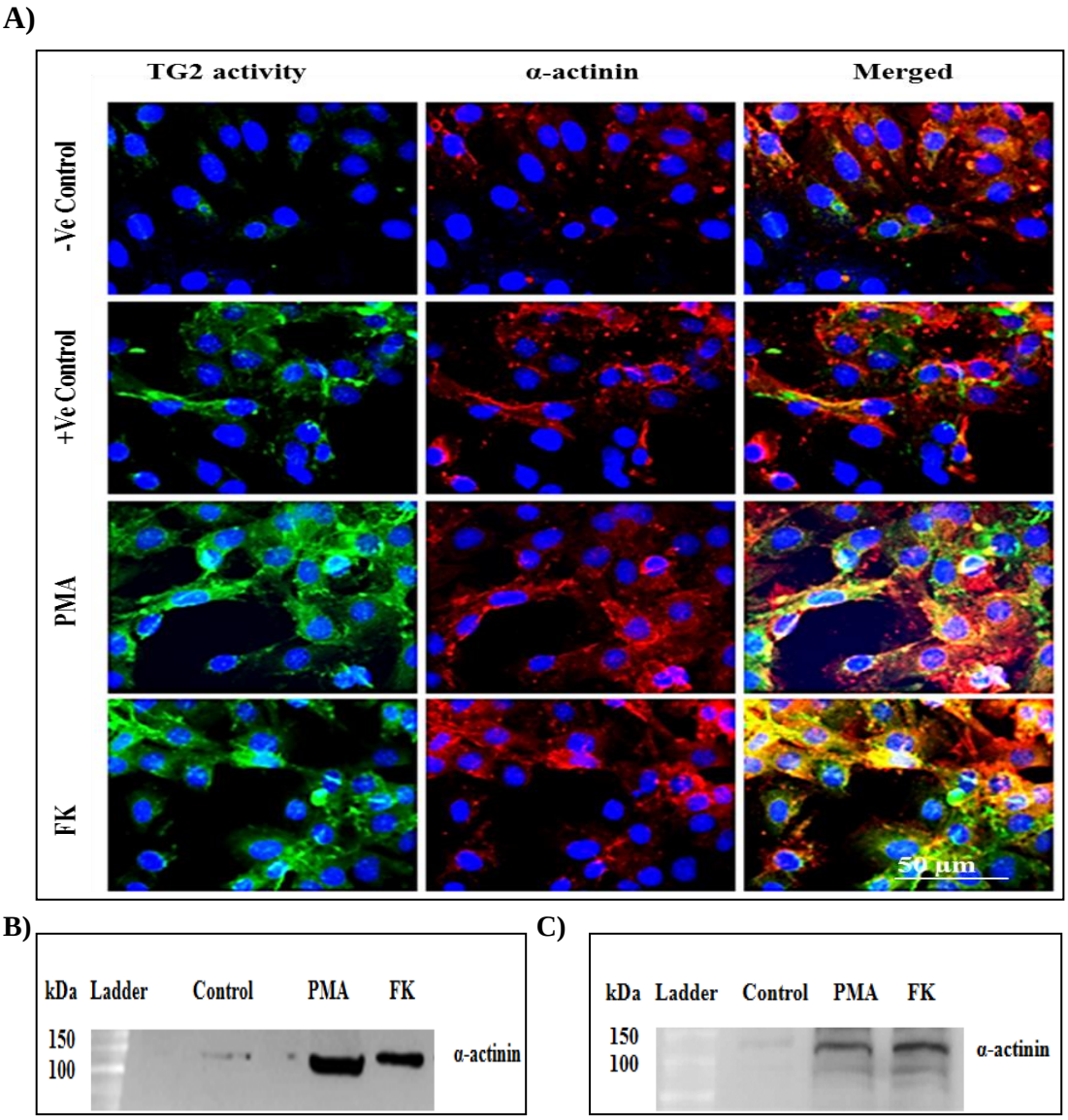
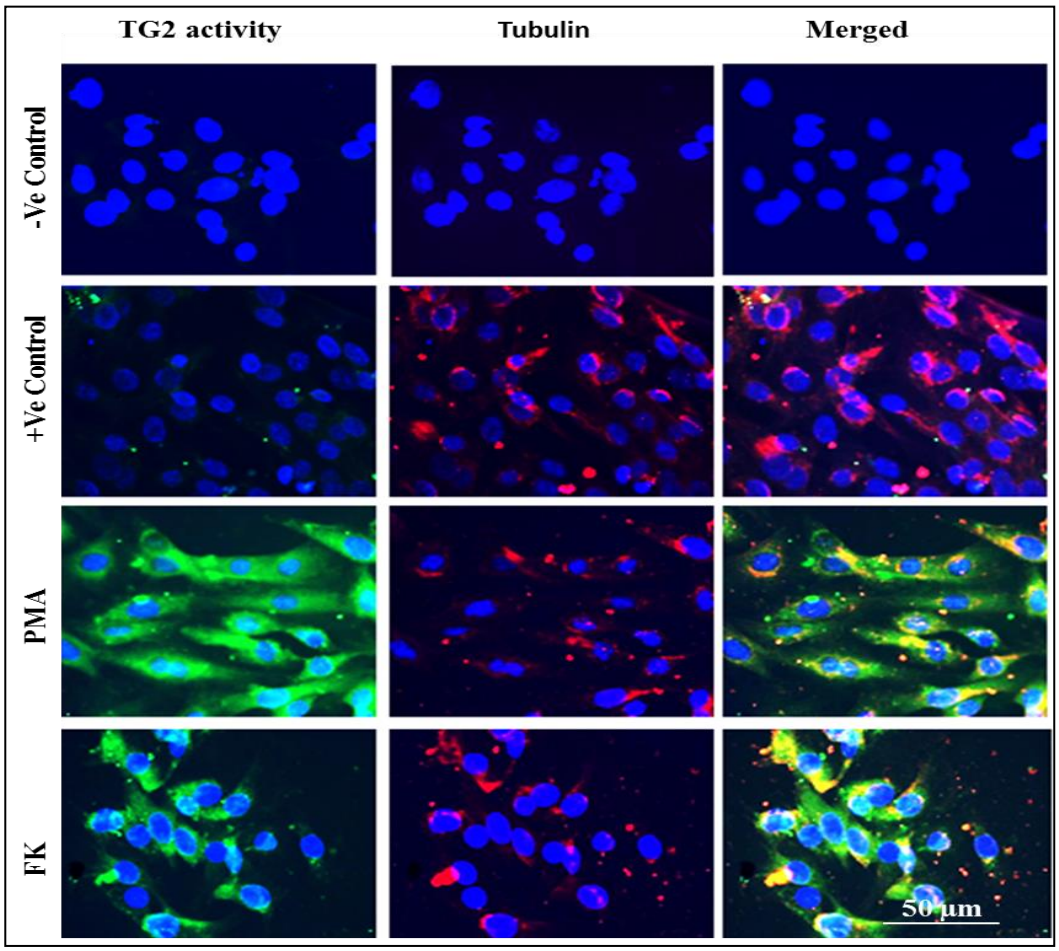



**Figure 6.3.6 The co-localisation of α-actinin with TG2 activity as TG2 cytoskeleton substrate**

A) Cells in chamber slides were incubated with 1 mM biotin-X-cadaverine for 4h and then treated with either 1 µM PMA or 10 µM FK while, unstimulated cells used as control in absence of BTC (–ve) or in presence of BTC (+ve). The TG2 mediated biotin-X-cadaverine incorporation into intracellular proteins was visulaised with Extravidin®-FITC (green). Actinin was detected by mouse anti-α-actinin mAb and visualised by anti-mouse-Alexa 568 secondary antibody (red). Nuclei were stained with DAPI (blue). The original magnification of the images was 400x. Co-localisation of α-actinin (red) and TG2 activity (green) stained yellow is shown in the merged photograph. B) The biotin-cadaverine labelled proteins were isolated with CaptAvidin beads subjected to SDS-PAGE and transferred onto nitrocellulose membrane filters the captured α-actinin substrates were detected with anti-α-actinin mAb. C) immunoprecipitation of biotin-cadaverine labelled proteins using α-actinin mAb and detected with HRP-conjugated®ExtrAvidin-peroxidase. The results are typical of 3 independent experiments.

A)




B)

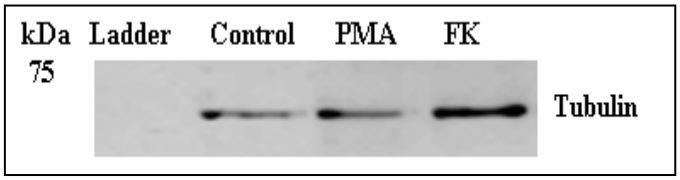



**Figure 6.3.7 The co-localisation of α-tubulin with TG2 activity as TG2 cytoskeleton substrate**

Cells in chamber slides were incubated with 1 mM biotin-X-cadaverine for 4h. They were then treated with either 1 µM PMA or 10 µM FK, while unstimulated cells used as control in absence of BTC/ α-tubulin (–ve) or in presence of BTC/α-tubulin (+ve). The TG2 mediated biotin-X-cadaverine incorporation into intracellular proteins was visualised with Extravidin®-FITC (green). The Microtubules were detected by mouse anti-tubulin mAb and visualised by anti-mouse-Alexa 568 secondary antibody (red). Nuclei were stained with DAPI (blue). The original magnification of the images was 400x. Co-localisation of tubulin (red) and TG2 activity (green) stained yellow is shown in the merged image. B) an equal amount (500 µg) of the biotin-cadaverine labelled proteins were isolated with CaptAvidin beads subjected to SDS-PAGE and transferred onto nitrocellulose membrane filters, and the captured α-tubulin substrates were detected with anti-α-tubulin mAb. The results are typical of 3 independent experiments.

Immunoblotting detection of α-actinin 1 was carried out from PMA/FK treated H9c2 cells, and the presence of elevated levels of a biotin cadaverine labelled immunoreactive band was verified in stimulated cell extracts by probing blots with anti-α-actinin antibody (Fig. 6.3.6B). No other bands appeared on the probed nitrocellulose membrane, suggesting that the CaptAvidin beads were selective and the antibodies were specific. The immunoprecipitate of α-actinin from biotin cadaverine labelled samples (section 2.2.20) was subjected to SDS-PAGE, transferred to nitrocellulose membrane and detected using HRP-conjugated®ExtrAvidin-peroxidase (Fig. 6.3.6C). The result showed a similar band and enrichment in PMA and FK stimulated cell extracts that was detected with anti α-actinin mAb, further confirming the previous data.

To confirm that α-actinin serves as TG a substrates, immunolocalisation of selected α-actinin to TG2 activity in the presence or absence of PMA and FK was carried out using a double staining immunohistochemistry technique. Merging of the images demonstrates the co-localisation of alpha actinin to TG2 incorporation activity in PMA/FK-treated cells (red + green = yellow/orange; Fig. 6.3.6A).

Tubulin was also confirmed as a TG2 substrate by *in vitro* and *in situ* activity (Fig. 6.3.7A). Immunoblotting of α-tubulin was carried out from H9c2 PMA/FK treated cells, and a biotin cadaverine labelled immunoreactive band was verified by the binding to the anti-α-tubulin antibody (Fig. 6.3.7B). Again, the elevated levels of antibody reactivity were suggestive of increased amine incorporation into tubulin in PMA and FK-stimulated cells.

### 6.3.6. The effect of TG2 inhibitor on α-actinin distribution in response to PMA/FK and $H_2O_2$ exposure

To investigate the effects of TG2 inhibitors on α-actinin expression following PKA and PKC activation in H9c2 and their protection against oxidative stress, an equal amount of protein extracted from whole cells was resolved by SDS-PAGE and transferred onto nitrocellulose membrane filters. The level of α-actinin protein was detected by anti-α-actinin antibody. As shown in Figure 6.3.8 both PMA and FK treatments of H9c2 cells resulted in increased band intensity of α-actinin protein while TG2 inhibitors either Z-DON (Fig. 6.3.8B) or R283 (Fig. 6.3.8C) treatments result in

reduced levels of α-actinin. However, the increase of α-actinin protein was more evident in cells treated with PMA than in FK treated cells. Similar observations were recorded in PMA/FK treated samples followed by $H_2O_2$ insult (Fig. 6.3.8A).

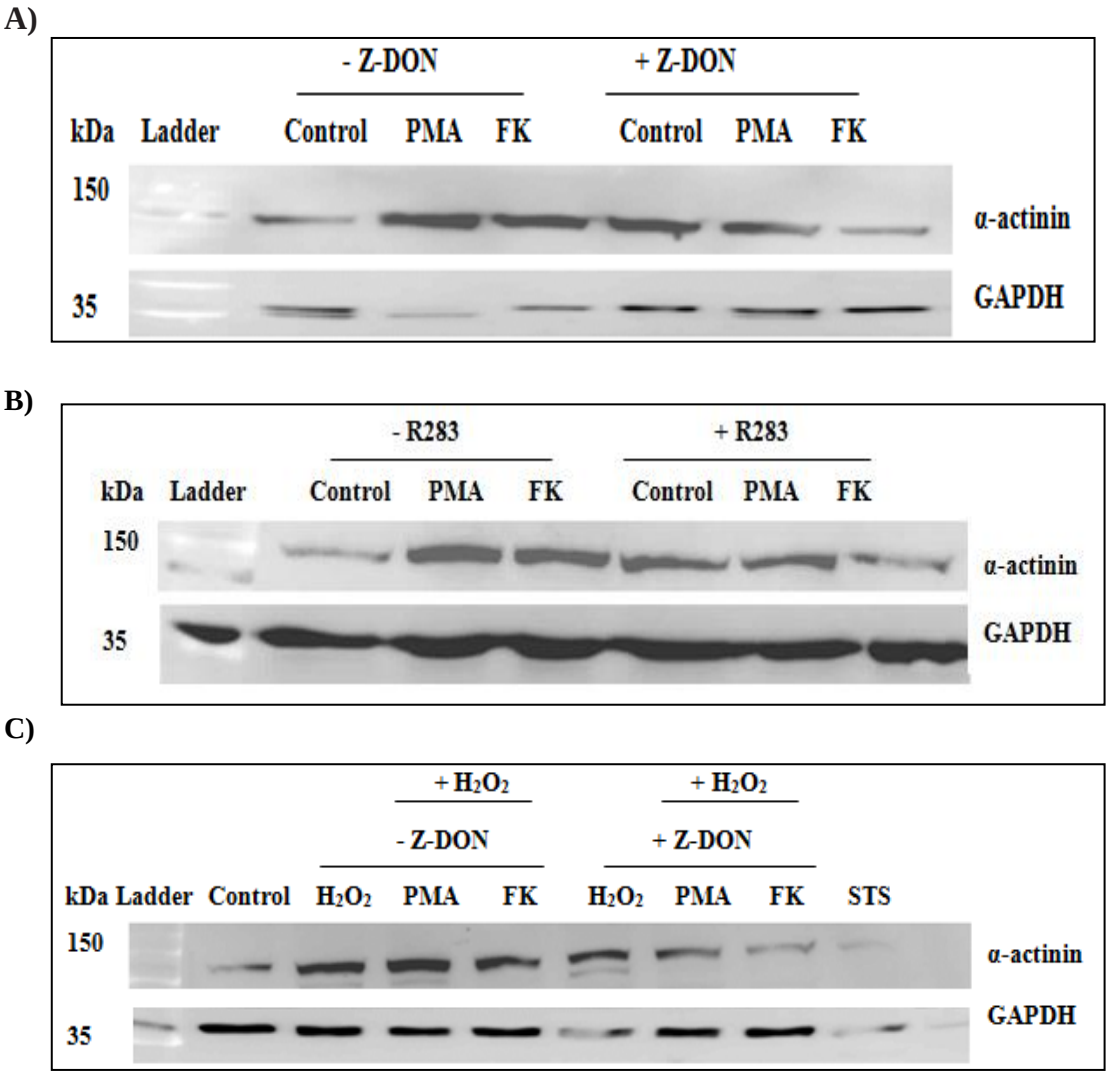



**Figure 6.3.8 Detection of α-actinin in H9c2 cells in response to PMA, FK and $H_2O_2$ stress in the presence and absence of TG2 inhibitors**

H9c2 cells were treated with or without A) 150 µM Z-DON or B) 200 µM R 283 for 1h. Cell were stimulated for 5 min with either 1 µM PMA or 10 µM FK treatment alone or C) prior to 600 µM $H_2O_2$ exposure for 2h while, unstimulated cells used as control. The total protein extract (50 µg) was resolved by SDS-PAGE and transferred onto nitrocellulose filters. Western blots were probed with anti-α-actinin and anti-GAPDH mAb was used as a loading control. The results are typical of 3 independent experiments.

### *6.4. Discussion*

The results in the previous chapters show the modulating of TG2 in both PKC and PKA signalling and its cytoprotective role through these signalling molecules. Transglutaminase 2 protein substrates and activity were detected in H9c2 treated cells. Biotin cadaverine was incorporated into endogenous protein substrates of TG2 in H9c2 cells. The results show biotin cadaverine incorporated into numerous proteins in H9c2 cells which exhibit increased amine incorporation following PMA or FK treatments (Fig. 6.3.1) or during PMA or FK mediated cytoprotection against $H_2O_2$ stress in comparison to untreated cells (Fig. 6.3.1). Biotin cadaverine labelling of proteins decreased in the presence of the TG2 inhibitor Z-DON. Comparing the labelled proteins detected in the *in situ* assay (Fig. 6.3.1) to the labelled proteins found *in vitro* in the presence of $Ca^{2+}$ (see chapter 4, Figure 4.3.1), a similarity in distribution and the number of bands was observed. This observation suggests that the substrates that were labelled *in vitro* assay might be obtainable also by *in situ* assay.

In the current study for first time this activity was also detected by 2D-PAGE and Western blotting analysis. This 2D-PAGE technique was used to investigate the activity of TG2 in response to PMA in H9c2 cells using a pull-down assay. The quantification of proteins spots from 2D-PAGE results revealed that up to 30 protein spots were shown to increase or decrease in response to PMA treatment (Fig. 6.3.5B and Table 6.3.1).

Many intra-and extra cellular proteins can interact with TG2 acting as substrates that are modulated by amine incorporation (Esposito & Caputo, 2005). This interaction may be essential for those cellular proteins to perform their biological functions. The TG2-catalyzed posttranslational modification of the substrates through incorporation of polyamines can modify the physical-chemical properties of proteins. For example, this modification would alter their isoelectric point by changing the surface charge through the addition of extra positively by charged amine groups, which may control their biological activity (Aeschlimann & Thomazy, 2000; Fesus & Piacentini, 2002; Griffin et al., 2002; Lorand & Graham, 2003). The recognition of proteins that act as TG2 substrates is important in the study of biological roles of TG2. For example, fibronectin, fibulin-2, nidogen-1 and alpha-1 chain of collagen type I were identified

as extracellular TG2 substrates that may play a role in remodeling of smooth muscle cells (van den Akker et al., 2011).

Since TG2 has been shown to have pathological and a protective role against different diseases, it is therefore essential to have a better knowledge and understanding of its substrates, target sites and interacting proteins, which may act as novel drug targets or new diagnostic markers. Although protein kinases have been shown to provide cardioprotection signalling in response to ischaemic preconditioning (Helen et al., 2012; Kloner & Jennings, 2001) and TG2 has also shown to protect heart from ischaemic reperfusion injured (Szondy et al., 2006), no one has investigated the TG2 substrates that may involved in this protective mechanism. In addition, the substrates of TG2 in H9c2 cardiomyocytes have not been previously studied.

Therefore, the detection some of TG2 substrate proteins which were exhibited increased amine incorporation in response to PMA and FK treatment in either the presence or absence of $H_2O_2$ and those which were inhibited via a TG2 inhibitor blocked protective action is of interest. The biotin-X-cadaverine labelled target proteins in treated H9c2 cells were captured with CaptAvidin beads and analyzed by LC-MS/MS. This enabled multiple proteins to be identified within a single protein band. Proteins with a high score and high sequence coverage of peptide mapping report are listed in Table 6.3.2. More than 25 proteins were identified which serve as acyl-donor substrates for transglutaminase.

Using SDS-PAGE various targets were identified, this approach was used in the past to determine TG2 protein substrates (Orrù et al., 2003; Robinson et al., 2007). However, the advantage of the current approach was to use the cell penetrating synthetic TG substrate biotin-X-cadaverine as a probe to identify TG2 protein substrates. This amine substrate does not interfere with normal cell processes and does not require the addition of calcium activation buffer (Esposito & Caputo, 2005). In addition, using this approach for labelling of TG2 substrates does not require extensive proteolysis and HPLC analysis that is needed with [$^{14}$C]putrescine labelling (Folk et al., 1980).

According to the TRANSDAB database (http://genomics.dote.hu/wiki/index.php/), some of the identified proteins were already reported as TG2 substrates, but not in

H9c2 cells; these included heat shock proteins 70 and 90, actin, tubulin, myosin-9, elongation factor 1-alpha 1, tropomyosin, vimentin, heterogeneous nuclear ribonucleoprotein A1. However, some of the proteins have not been previously identified as TG substrates either in H9c2 or in other cell lines.

Relative to their biological functions, the identified target proteins were classified into seven groups. These include cytoskeletal organizing proteins, chaperone proteins, $Ca^{2+}$ and phospholipid binding proteins, proteins involved in energy metabolism, miscellaneous proteins, and proteins involved in vesicle transport processes.

The first classification group of identified TG2 substrates is cytoskeleton organising proteins (Table 6.3.2). The cardiomyocyte cytoskeleton is an important biological structure because of its complexity and the numerous roles it plays (Sarantitis et al., 2012). The cytoskeleton contributes to the maintenance of myocardial cell structural and functional integrity. It can also participate in cell division, cell migration, vesicle transport, receptors localisation, the cell function and communication, and signalling transduction (Schweitzer et al., 2001; Pyle & Solaro, 2004).

The thin filaments in sarcomeric cytoskeleton of cardiomyocyte consist of actin and tropomyosin proteins while myosin protein is present in the thick filaments (Modica-Napolitano & Singh, 2002; Camelliti et al., 2006). In this study, the actin (cytoplasmic 1, alpha cardiac muscle 1 and aortic smooth muscle), tropomyosin and myosin 9 all were shown to act as TG2 substrates in H9c2 cells (Table 6.3.2). These proteins have been shown to play an important role in cardiac contraction performance (Noguchi et al., 2004). Moreover, the connection of tropomyosin with cardiac tropomodulin complex has been shown to thin filament and thus stopping actin depolymerisation of cardiomyocyte (Goodwin & Muntoni, 2005; Camelliti et al., 2006). Interestingly, proteomic analyses of H9c2 cells has shown that the level of cytoplasmic actin decreased in response to oxidative stress induced by $H_2O_2$ treatment (Chan, 2013).

This study identified α-actinin as a glutamine donor TG2 substrate. Alpha actinin is an actin microfilament binding protein, which regulates microfilament function and organisation (Gregorio & Antin, 2000; Calaghan et al., 2004). Immunofluorescence images showed co-localisation of α-actinin with TG2 activity induced by PMA or FK, confirming it as TG2 substrate (Fig. 6.3.6A). The α-actinin was also confirmed as

TG2 substrate on a Western blot following a pull down assay (Fig. 6.3.6B) and immunoprecipitation of α-actinin (Fig. 6.3.6C). It has been reported that the activation of PKC by phorbol esters results in enrichment of the actin-associated protein α-actinin and PKC isoform translocation in the human neutrophil cytoskeleton (Niggli et al., 1999). Alpha actinin has been shown to play an essential role in the contractile function of smooth muscle cells, modulating cytoskeleton restructuring (Fultz et al., 2000). However, the accumulation of α-actinin protein was observed in the sarcoplasmic reticulum of myocytes from patients with failing hearts, but not as a toxic effect (Fultz et al., 2000, Hein et al., 2009). Alpha actinin is located at the Z-line of the sarcomere, where PKCε can be translocated and exert its cardioprotection effects through maintenance of the contractile apparatus (Robia et al., 2001). Therefore, the depletion of α-actinin by TG2 inhibitors (Fig. 6.3.8) could possibly affect PKCε translocation and activation, thus removing its protective effects.

The interaction between α-actinin and annexin A6 has been detected in cardiomyocytes suggesting an important role for annexin A6 in excitation and contraction process (Mishra et al., 2011). Therefore, altered levels of α-actinin might disrupt the cardiac excitation and contraction cycle.

Tubulin was also among the identified TG2 substrates and was confirmed as a TG2 substrate for *in vitro* and *in situ* TG2 activity (Fig. 6.3.7). Tubulins are the main structural protein of cardiac-microtubules in which α- and β-tubulin heterodimers are polymerized (Gregorio & Antin, 2000). Because of their dynamic features of rapid de-polymerisation and re-polymerisation, they have the ability to alter the cytoskeleton's flexibility, thus contributing to myocardial cell contractile activity (Severs et al., 2006). Together with cardiac-microtubule associated proteins, the α- and β-tubulin can stabilise cardiomyocytes by connecting the subcellular structure and thus play an important role in the transmission of chemical and mechanical signalling within- and between cells (Gregorio & Antin, 2000; Schweitzer et al., 2001; Severs et al., 2006). Furthermore, this study was identified the protein vimentin as a TG2 substrate (Table 6.3.2); this is known to form intermediate filaments that contribute to structural organisation in the cytoplasm (Schaper et al., 1991). An increase in tubulin and vimentin protein levels has been observed in patients with dilated cardiomyopathy (a condition in which the heart becomes enlarged; Schaper et al., 1991; Di Somma et al.,

2000). However, it was suggested that the increase in these cytoskeleton elements could be an action taken by myocardium cells when contractile material is impaired to compensate for the lack of cellular stability (Heling et al., 2000). Interestingly, PKCε activation has been shown to mediate the phosphorylation of vimentin and regulation of β1-integrin (a cell surface receptor) compartment, recycling and motility in fibroblast cell lines (Ivaska et al., 2005) or human kidney (HEK-293) cells (Kim et al., 2010). This observation is of interest, as it supports the present data showing that TG2 mediated the incorporation of biotin into vimentin in response to PMA activated PKC. In addition, it could be possible that phosphorylation of TG2 substrates by PKC facilitates TG2 biotin cadaverine incorporation activity.

In the current study, prelamin A was also detected and identified as a TG2 substrate. Prelamin A is an immature form of lamin A that requires serial posttranslational modifications in its carboxyl-terminal to become a mature lamin (Davies et al., 2009). Type A lamin is a nuclear cytoskeleton proteins belonging to the intermediate filament family and is commonly observed in differentiated cardiomyocytes (Mudry et al., 2003; Kong & Kedes, 2004). Nuclear lamin has been shown to have multiple functions; it is involved in DNA replication and repair, transcription, chromatin organisation, apoptosis, nuclear growth and cell differentiation (Dechat et al., 2010).

Elongation factor 1α, a multifunctional protein that acts as actin-binding protein, peptide synthesis promoter and substrate for Rho-associated kinase (Izawa et al., 2000) was identified as a TG2 substrate in H9c2 cells. Moreover, a significant reduction in elongation factor 1α expression has been shown to be associated with cell death that can eliminate abnormal tetraploid cells (which have mis-segregated chromosomes due to a fault in the compact of chromatids during mitosis) and inhibit tumorigenesis (Kobayashi & Yonehara, 2008). Oxidative stress can result in down regulation of elongation factor 1α while pre-treatment with quercetin (a natural polyphenolic compound that has anti-inflammatory and anti-oxidant effects; Boots et al., 2008) can reverse this effect (Chan, 2013).

The second classification of TG2 substrates included $Ca^{2+}$ and phospholipid binding proteins. Annexin is a calcium-dependent cytoskeleton and phospholipid-binding protein involved in several biological events (Gerke et al., 2005). It can interact with

various cell-membrane components regulate cellular structural organisation, control cell growth mediate intracellular signalling and it also can act as a typical calcium channels (Moss & Morgan, 2004). Different types of annexin have been identified in myocardium these include annexin A1, A2, A4, A5, A6 and A7 but not annexin A3 (Camors et al., 2005). In this study, two annexins type A2 and a novel annexin type A3 were identified as TG2 substrates in H9c2 cells.

Interestingly, the actin, elongation factor 1-alpha 1, annexin, lamin-A and myosin-9 have all been shown to be a target for quercetin's protective effect against oxidative stress in H9c2 cells (Chan, 2013).

The third group of TG2 substrates (Table 6.3.2) represents proteins involved in protein folding machinery (chaperones) including heat shock protein HSP 90-α and β, heat shock cognate 71 kDa protein, 78 kDa glucose-regulated protein, malectin, and serpin H1. The heat shock protein HSP 90-α and β, both belong to the heat shock family of stress proteins and are mainly found in the cytoplasmic (Benjamin & McMillan, 1998). One more function known for chaperones *in vivo* is to prevent aggregation of proteins under stress conditions and to stimulate reparation of the enzymatic activity of the denatured substrates such as citrate synthase, β-galactosidase, or luciferase on removal of stress (Benjamin & McMillan, 1998). Heat shock protein 90 is one of the chaperone proteins that facilitate the folding, gathering, and segregating of other proteins (Benjamin & McMillan, 1998). The HSP 90 chaperone protein is considered to be a key regulator in cell physiology and can mediate various processes including signal transduction and differentiation beside its role in protein folding. This could be due to the fact that the majority of cellular proteins require HSP 90s to reach their final conformation such as growth factor receptors (Sawai et al., 2008), kinases (Yun & Matts, 2005), and many carcinogenic proteins (da Silva & Ramos, 2012). Recently, HSP 90 has been reported to mediate the cardioprotection effects stimulated by hydrogen sulphide ($H_2S$) against chemical hypoxia-induced injury in H9c2 cardiomyocytes through the attenuation of oxidative stress and enhancement of mitochondrial function (Yang et al., 2011). This chaperone has been shown to be involved in regulation of oestrogen receptor function by modulating its binding to its cognate DNA (Sabbah et al., 1996).

Heat shock protein 71 is a constitutive chaperone, which shares ~90 % identical sequence homology with rat HSP70 (Morshauser et al., 1999). This heat shock protein also functions inside the cell as a chaperone involved in protein folding and transport, beside its function in tissue protection against stress and injury (Srivastava, 2002). Moreover, it has an extracellular function in cell signalling mediated stress (Calderwood et al., 2007). The HSC 70 released in response to ischaemia-reperfusion suggested that it might have a crucial role in the myocardial inflammatory response and cardiac dysfunction (Zou et al., 2008).

The 78 kDa glucose-regulated proteins are sarcoplasmic/endoplasmic reticulum chaperones and they are of particular clinical interest, because of the important functions of sarcoplasmic/endoplasmic reticulum in repair or degradation of misfolded proteins as a result of cell injury after myocardial ischaemia or mutations (Glembotski, 2008). The induction of 78 kDa glucose-regulated proteins has been reported in response to ischaemic pre-conditioning in brain tissue to protect from further ischaemic damage by reducing sarcoplasmic/endoplasmic reticulum stress and preventing delayed neuronal cell death (Hayashi et al., 2003).

Proteomic analysis of H9c2 cells revealed that the heat shock protein 71 kDa was up-regulated while 78 kDa glucose-regulated protein was down regulated in response to doxorubicin-induced damage in cardiomyocytes (Bao et al., 2012). Both HSPs and PKC have been well documented to be cardioprotective against ischaemia-reperfusion injury (Dorn et al., 1999; Fryer et al., 2001; Coaxum et al., 2007). The PMA induced activation of PKC results in increased HSP 70 and HSP 90 expression either due to mRNA stabilisation in human blood monocytes (Jacquier-Sarlin et al., 1995) or to transcriptional activation in H9c2 cardiomyocytes cells (Coaxum et al., 2007). Since all of these HSPs have been identified as TG2 substrates in the current study, this may suggest that the increase in TG2 activity following PMA and FK exposure is in fact, a cardioprotective cellular response. However, this cannot rule out the possibility that the presence of HSPs may be due to the biotin-X-cadaverine itself.

The fourth group of identified TG2 substrates (Table 6.3.2) contains proteins involved with metabolism and energy involved production proteins such as prohibitin and voltage-dependent anion-selective channel protein 1. The latter is of particular interest

as it acts as $Ca^{2+}$ regulator for transportation in-and out of the mitochondrial membrane and extensively communicates with metabolic enzymes (e.g. pyruvate dehydrogenase) that use $Ca^{2+}$ as a cofactor in metabolic processes (Zaid et al., 2005). However, it also plays a role in apoptosis by facilitating the release of cytochrome *C* (Lemasters & Holmuhamedov, 2006). Proteomic analysis of H9c2 cells has revealed that down regulation of prohibitin and voltage-dependent anion-selective channel protein 1 was associated with doxorubicin-induced damage in cardiomyocytes (Bao et al., 2012). The role of mitochondria in triggering necrosis and apoptosis pathway has been extensively studied in cardiomyocytes under oxidative stress (Borutaite & Brown, 2003). Prohibitin has been known as a mitochondrial chaperone protein involved in its structure and function (Nijtmans et al., 2002; Kasashima et al., 2008) and the Ras-raf signal transduction pathway (Rajalingam & Rudel, 2005). The protective role of this protein has been reported in H9c2 cardiomyocytes in response to oxidative stress-induced cell injury (Liu et al., 2009). Prohibitin transfected H9c2 cells showing overexpression of this protein suppressed the mitochondria-mediated apoptosis pathway through inhibition of cytochrome *C* release from mitochondria to cytoplasm and by alteration of the mitochondrial membrane permeability transition pore (Liu et al., 2009). Similarly, it has also been reported to protect cardiomyocytes against hypoxia induced cell death (Muraguchi et al., 2010).

The fifth group of identified TG2 substrates (Table 6.3.2) contains endomembrane vesicle trafficking proteins including the Arf-GAP-containing protein 1 and Ras-related protein Rab-35. The Arf-GAP-containing protein 1 is a members of the ADP-ribosylation factor (ARF) family of G proteins that regulates membrane traffic and organelle structure by engaging vesicle coat proteins, mediating membrane lipid conformation and interacting with G proteins regulators (Donaldson & Jackson, 2011). The Arf-GAP-containing protein 1 is a prototype of the peripheral ARF GAPs, serving to organise the cell signalling effects of platelet-derived growth factor, the actin cytoskeleton and membrane trafficking during cell movement (Randazzo et al., 2000).

Ras-related protein Rab-35 is one of Rab proteins from the branch of the small G protein superfamily proteins that are known to regulate intracellular vesicle trafficking (Takai et al., 2001). The functional role of Rab35 has been reported to be the

regulation of actin filament assembly during bristle progression in Drosophila by recruiting the actin-bundling protein fascin as an effector protein (Zhang et al., 2009). The proteomic analysis of purified exosomes from oligodendroglial cells has revealed that the majority of identified proteins are Rab GTPases and Rab-35 is the most abundant protein suggesting, a role in exosome secretion (Hsu et al., 2010).

The final group (Table 6.3.2) represents the proteins involve in mRNA metabolism and transport, which consist of heterogeneous nuclear ribonucleoprotein A1 and 60S ribosomal protein L5. The heterogeneous nuclear ribonucleoprotein A1 belongs to the RNA-binding protein family and has key roles in gene expression regulation at the transcriptional level (He & Smith, 2009). The role of heterogeneous nuclear ribonucleoprotein A1 in differentiation of smooth muscle cell from the stem cells and in cardiovascular regenerative medicine has been identified (Wang et al., 2012; Huang et al., 2013). The 60S ribosomal protein L5 is one of the ribosomal proteins important in coordinating cell growth and cell division (Donati et al., 2013). It plays an important role in activation of tumor suppressor protein (p53) under stress conditions through binding to and inhibition of the ubiquitin E3 ligase to induce cell cycle arrest and apoptosis (Sun et al., 2010). Interestingly, some of these identified proteins were also identified in 2D-PAGE (see appendix, Fig.8.5), including (1)78 kDa glucose-regulated protein, (2) tubulin, (3) annexin; A2, (4) actin, (5) voltage-dependent anion-selective channel protein 1, (6) α-actinin, (7) vimentin and (8-9) 60S ribosomal protein L5.

The main functional classifications of TG2 substrates in the H9c2 PMA/FK treated cells were cytoskeletal organising proteins (Table 6.3.2), suggesting a role for TG2 in the organization and turnover of the cardiomyocyte cytoskeleton. Alpha actinin and tubulin are among the most abundant target protein families observed in the H9c2 cells. In conclusion, the present results have shown that TG2-catalyses posttranslational modification of the target substrates that are predominately involved in cytoskeletal organisation, protein folding or endomembrane vesicle trafficking, through polyamine incorporation activity.

# CHAPTER VII:

# GENERAL DISCUSSION AND FUTURE WORKS

## 7. General discussion and future work

The history of transglutaminase can be traced back to the mid-1950s when a new enzyme (called transglutaminase) extracted from liver was discovered with the establishment of its reaction catalysis (Sarkar et al., 1957). The transamidation activity remained the only function known for transglutaminase until the discovery of the ability of TG2 to bind and hydrolyse GTP, which then defined its GTPase function (Achyuthan & Greenberg, 1987). Since that time, several more enzymatic functions for TG2 have been reported and different isoenzymes have been discovered. However, the impact of TG2 and its activities in cellular responses until now are still under investigation.

Transglutaminases (TGs) are a family of $Ca^{2+}$ dependent enzymes that catalyse the posttranslational modification of proteins. Several classes of this enzyme have been identified (TGs 1-7) (Lorand & Graham, 2003). Transglutaminase 2, which is ubiquitously expressed, possesses multiple enzymatic activities, including transamidation, deamidation, protein disulphide isomerase, esterase, nucleosidase and protein kinase, acting as a G-protein in trans-membrane signalling (Gundemir et al., 2012). The cytoplasmic form of TG2 has been ascribed a role in apoptosis and cell signalling whereas the extracellular TG2 has roles in ECM stabilisation, cell adhesion and migration (Iismaa et al., 2009; Nadalutti, et al., 2011). TG2 has been shown to mediate cardioprotection against ischaemia and reperfusion-induced cell death (Szondy et al., 2006). Similarly, increased TG2 expression protects neuronal cells from oxygen and glucose deprivation-induced cell death (Filiano et al., 2008) and protects cells from DNA degradation (Tucholski, 2010).

Protein kinase C and PKA are two major mediators of signal transduction pathways associated with ischaemic preconditioning and pharmacological preconditioning induced cardioprotection (Yellon & Downey, 2003; Hassouna et al., 2004; Sanada et al., 2011). Transglutaminase 2 and TG1 can be regulated by PKA and PKC in some cell lines (Bollag et al., 2005; Akar et al., 2007b; Mishra et al., 2007). However, the modulation of by TG2 activity in cardiomyocytes by these protein kinases has been never investigated. The impact of this modulation on cell survival as key role in cardioprotection has never been reported. Thus, the purpose of this study in H9c2 cells was to investigate the activation of TG2 in response to phorbol-12-myristate-13-acetate (PMA) acts as a PKC activator and forskolin (FK) acts a PKA activator and to

determine whether TG2 played a role in PKC/PKA-mediated cytoprotection. The H9c2 cells are derived from embryonic rat heart tissue (Kimes & Brandt, 1976) and are extensively used as an *in vitro* model for investigating and studying cardioprotection events since they display similar morphological, biochemical and electrophysiological properties to primary cardiac myocytes (Hescheler et al., 1991). The data presented here provide evidence that TG2 activity is modulated in H9c2 cells by signalling pathways induced by PMA and FK. Moreover, the inhibition of TG2 activity decreased PMA and FK-mediated cytoprotection against $H_2O_2$-induced oxidative stress suggesting a cardioprotection role for TG2.

***Modulation of TG2 by PKC and PKA-dependent signalling in H9c2 cells in vitro and in situ activity***

As detailed in the introduction, the activity of TG2 and other TG family members can be regulated by PKA and PKC (Bollag et al., 2005; Mishra et al., 2007). However, at present the regulation of TG2 by both PKA and PKC-dependent signalling in cardiomyocytes has not been reported. Hence, in this study, the potential regulation of TG2 by PKC and PKA-dependent signalling was investigated in H9c2 cells treated with PMA and FK. PMA and FK treatments triggered significant time and concentration-dependent increases in TG2-mediated biotin cadaverine incorporation activity in H9c2 cells. Forskolin but not PMA also induced a time-dependent increase in TG2-mediated protein crosslinking activity. Transglutaminase crosslinking activity involves the formation of a covalent bond between glutamine and lysine residues in adjacent proteins, whereas TG amine activity refers to incorporation of primary amines into protein substrates. The regulation of TG2 activity in H9c2 cells by FK is in agreement with previous studies in mouse embryonic fibroblasts (MEF), which have shown TG2 becomes phosphorylated at $Ser^{216}$ in response to PKA activation (Mishra & Murphy, 2006; Mishra & Murphy, 2006). The phosphorylation of TG2 by PKA appears to have several consequences, including the enhancement of protein-protein interactions, promotion of its kinase activity and inhibition of protein crosslinking activity. However, it is notable that in this study FK-treatment enhanced TG2-mediated crosslinking activity in mouse embryonic fibroblasts (Mishra & Murphy, 2006; Mishra et al., 2007). It should be noted that the inhibition of TG2

protein crosslinking activity reported by Mishra et al. (2007) was detected with histidine-tagged TG2 immobilised on nickel-agarose and incubated with the PKA catalytic subunit, whereas in the current H9c2 cells were treated with FK prior to measurement of TG2 protein crosslinking activity. Hence, the effects of FK on TG2 observed in the current may by PKA-independent (e.g., FK triggers a robust increase in ERK1/2 phosphorylation).

Importantly, PMA and FK-stimulated transglutaminase activity in H9c2 cardiomyocytes was inhibited by Z-DON and R283, two structurally different (see section 4) TG2 inhibitors (Freund et al., 1994; Schaertl et al., 2010) confirming the modulation of TG2. Although the expression TG2 is regulated by PKCδ in pancreatic cancer cells (Akar et al., 2007) there does not appear to be any published data regarding the regulation of TG2 activity by PKC-dependent pathways. Hence, the data presented in the current study have shown for the first time that TG2 activity (amine incorporation but not crosslinking) can be stimulated following treatment of H9c2 cells with the PKC activator PMA. However, previous studies have shown that the crosslinking activity of TG1 is enhanced by PMA-induced PKC activation (Bollag et al., 2005). It is interesting to note that TG1 activation by PMA is sensitive to the MEK1 inhibitor PD98059, suggesting the involvement of ERK1/2 in PMA-induced TG1 activation (Bollag et al., 2005). Interestingly, TG1 but not TG3 for the first time was also detected in H9c2 cells; however, its level of protein expression was not altered by these kinase activators. This observation could eliminate the possibility that TG1 has a role in contributing this activity. Future experiments could explore the potential involvement of ERK1/2 (and possibly other potential downstream protein kinases) in PMA-induced TG2 activation in H9c2 cells.

To confirm the involvement of PKA in FK and PKC in PMA-mediated TG2 activation, inhibitors for these kinases were tested for their ability to inhibit PMA or FK-induced TG2 activity. The PKC inhibitors Gö 6983 (5 μM) (Gschwendt et al., 1996), Ro 31-8220 (10 μM) (Davis et al., 1989), chelerythrine (1 μM) (Herbert et al., 1990; Chijiwa et al., 1990) and the PKA inhibitor H-89 (1 μM) (Chijiwa et al., 1990) significantly blocked both PMA and FK-induced TG2 catalysed biotin cadaverine incorporation. These observations suggest either a lack of protein kinase inhibitor selectivity or a possibly a direct effect of the inhibitors on TG2 activity. Interestingly, Gö 6983, H-89 and chelerythrine significantly inhibited the activity of purified

guinea-pig liver TG2, revealing of a direct interaction of these inhibitors with TG2. Although Ro-318220 did not attenuate purified TG2 activity, it is still unclear as to why this PKC inhibitor blocked FK-induced TG2 activity since it does not significantly attenuate PKA activity (Davies et al., 2000). However, PKA inhibitors KT 5720 and Rp-8-Cl-cAMPS blocked FK induced TG2 activity, confirming the involvement of PKA in FK-mediated responses. Further studies will investigate a wider range of PKA and PKC inhibitors not only on the activity of purified guinea-pig liver TG2 and also on their ability to inhibit PMA and FK-induced TG2 activity in H9c2 cells. The possibility that PMA or FK might directly influence purified TG2 activity was also studied. PMA and FK did not influence the activity of purified TG2, suggesting that these protein kinase activators do not directly interact with TG2.

Extracellular signal-regulated kinases (ERK 1 and 2) are one of the major mitogen-activated protein kinase (MAPK) families that have a protective role in cardiomyocytes (Abe et al., 2000; Sugden & Clerk, 2001). The phosphorylation of ERK1/2 by PKA and PKC activation via FK and PMA, respectively, has been reported (Nanzer et al., 2004; Wang et al., 2009). In the current work, a study of the effect of different protein kinase inhibitors in pERK1/2 activation has revealed that TG2 polyamine incorporation activity modulated by PMA and FK is a downstream target for PKA and PKC activation. Different protein kinase inhibitors induced significant decreases in TG2 activity and protein levels in conjunction with pERK 1/2 reduction which was verified by Western blotting. Conversely, both TG2 specific inhibitors failed to block ERK1/2 activation.

The results presented in this thesis demonstrate that PMA and FK treatments were able to increase the detectable TG2 protein level. These increases were prevented by protein kinase inhibitors. Although PMA/FK mediated TG2 incorporation activity was in correlation with increase of TG2 protein and mRNA levels, it is also likely that the activation of TG2 is not entirely due to induction of its gene. However, it is might be due to either TG2 protein being covalently modified (e.g. phosphorylation) or interaction with a regulatory protein induced by these kinase activators that increases its activity. The presented results have demonstrated that both PMA and FK induce a wide range of protein phosphorylation in H9c2 cells and this agrees with other studies (Jacquier-Sarlin et al., 1995; Teixeira et al., 2003; Bollag et al., 2005). Thus, it could be possible that these phosphorylation events result in conformational changes of

these target proteins or in altered affinity of specific proteins to recognise and bind to other proteins and thus affect regulation of TG2 activity. Phosphorylation of TG2 itself may also alter anti-TG2 mAb binding to give a perceived increase in protein as a result of increased antibody binding.

Polyamines are required for almost all cellular processes including cell signalling, phosphorylation, protein synthesis and gene expression (Igarashi & Kashiwagi, 2000). In cells, polyamine levels could be altered due to transportation, biosynthesis and degradation (Pegg, 1988; Giordano et al., 2010). When the concentration of polyamines in the cells or tissues is in the millimolar range, TG2 can catalyse the incorporation of these polyamines into intracellular proteins that act as specific acyl-donor substrates (Jeon et al., 2003). This polyamination reaction can result in covalent modification of many proteins in intact cells and modulate the function and metabolism of such proteins. Although intracellular polyamine levels are < 1.0 mM in cardiac tissue (Wang et al., 2010), different stimuli may disturb polyamine homeostasis and thus crosslinking of intracellular proteins is unlikely to occur (Song et al., 2013). This can be verified by the present observations in which TG2 amine incorporation was more dominant than TG2 crosslinking activity.

Interestingly, using a cell permeable biotinylated substrate of TG2 (biotin-X-cadaverine) it is possible to visualise the protein targets of TG2 activity after 5 min following activation of PKA and PKC with pharmacological activators. The results were comparable to PMA- and FK-induced amine incorporation activity observed *in vitro*. Increases in transglutaminase activity were seen as early as 5 min following stimulation with PMA or FK, and the effect was maximal by 10 min in both *in vitro* and *in situ* activity. This suggests that no intermediate steps may be beyond these early responses for the activation of the transglutaminase. However, as TG2 is a calcium-activated enzyme (Griffin et al., 2002), it is possible that direct release of intracellular calcium following treatment with protein kinase activators is responsible for the activation of transglutaminase. It may be that there are areas of the cytoplasm where $Ca^{2+}$ ions are released that may have elevated levels of $Ca^{2+}$ sufficient to drive protein crosslinking as well. Indeed, PMA has been reported to stimulate, ATP-dependent calcium transport within 2 min in neutrophil plasma membranes (Lagast et al., 1984) and cause a dose-dependent influx of calcium in human red blood cells (Andrews et al., 2002). Moreover, both PKA and PKC activation are responsible for

increases of cytosolic calcium ion levels from extra- (via voltage-gated channels) and intracellular (mobilisation in ER) sources in rat PC12 pheochromocytoma cells (Dermitzaki et al., 2004). From the data presented, it appears that PMA and FK-induced TG2 activity depends on the continued presence of calcium. Therefore, it would be interesting to confirm these observations *in situ* by pre-treating H9c2 cells with the intracellular $Ca^{2+}$ chelator (BAPTA) prior to PMA and FK stimulation (Robinson & Dickenson, 2001) or to follow the release of $Ca^{2+}$ using $Ca^{2+}$ fluo-dyes. The use of different and differentiating cardiomyocytes cell lines would determine whether the effects of PMA and FK on *in situ* TG activity are a common observation in heart tissue.

However, given the covalent nature of biotin-X-cadaverine incorporation into protein substrates, it was unexpected to observe that *in situ* TG2 activity reversed to basal level after 20 min. Explanations of this result could be that the biotin-cadaverine labelled proteins were either targeted for degradation or that they were rapidly expelled from the cell. Further results implicated the latter, since labelled proteins were detected in the culture medium. However, it cannot be ruled out the possibility that these two processes occur simultaneously. Another explanation could be that the concentration of endogenous polyamines in the H9c2 cells were increased and accumulated after 10 min treatments with PMA and FK; thus, during the incubation time some of these free endogenous polyamines exported outside the cells resulting in an apparent reduction of TG2 activity over time and depletion of biotin-cadaverine labelled proteins. The prolonged treatment of HL-60 cells with PMA resulted in an increased level of the polyamines spermidine and putrescine but decreased levels of spermine (Gavin et al., 2004). This also suggests that PMA treatment may result in the degradation of endogenous polyamine (spermine) in H9c2 cells. Therefore, it would be of value to determine the concentration of different polyamines in H9c2 cells before and after PMA and FK treatments at different time point using high-pressure liquid chromatography (Wang et al., 2010).

This is of interest, as TG2 activity has not been detected in cardiomyocyte culture medium before, yet this finding has been supported by another study that also detected TG2 activity in normal human and glaucomatous cultured trabecular meshwork cells and tissues (Tovar-Vidales et al., 2008).

***Role of TG2 in PMA and FK-induced cytoprotection***

Recent evidence suggests that TG2 has a cardioprotective role against ischaemia and reperfusion-induced cell death by regulating ATP synthesis in cardiomyocytes (Szondy et al., 2006). Since TG2 activity appears to be regulated by protein kinases associated with cardioprotection (PKA and possibly PKC) this study investigated whether it plays a role in PMA and FK-induced cytoprotection. The data presented have shown for the first time that inhibition of TG2 activity decreased PMA and FK-mediated cytoprotection against $H_2O_2$-induced oxidative stress.

The role of TG2 in modulating protection induced by PMA and FK against oxidative stress was addressed using different cytotoxic measurement approaches. Firstly, the morphological changes in H9c2 cells were monitored before and after PMA and FK treatments in the presence and absence of TG2 inhibitors. The pseudopodia of the cells retracted and both granules and were formed in the presence of $H_2O_2$. These effects were attenuated by PMA and FK and but more pronounced in the presence of Z-DON TG2 inhibitor. Secondly, cell viability following $H_2O_2$ exposure was assessed by monitoring MTT reduction and lactate dehydrogenase (LDH) release. The fact that $H_2O_2$ exposure resulted in morphological alteration, cell survival reduction and LDH release from H9c2 cardiomyocytes had already been reported (Liang et al., 2010). As expected, pre-treatment with PMA and FK reversed $H_2O_2$-induced inhibition of MTT reduction and release of LDH. Finally, the DNA fragmentation in cells was evident when exposed with $H_2O_2$ alone and in cells pre-incubated with Z-DON. The TG2 inhibitors R283 and Z-DON blocked PMA and FK-induced cytoprotection suggesting a protective role for TG2 in mediated PKC and PKA-induced cell survival. Although, Z-DON treatment decreased caspase-3 activation, it did not secure cells from death. This suggests that the complete inhibition of caspase-3 by TG2 inhibitor results in a shift of cell death from apoptosis to necrosis, similar to previous reports on ATP depletion stimulated B lymphocyte apoptotic cell death (Lemaire et al., 1998). It would also be of interest to confirm this protection by measuring another endpoint e.g. ROS production.

Although, the protective effect of PMA and FK against oxidative stress induced by $H_2O_2$ has not previously reported in H9c2 cardiomyocytes, the results of this study are in agreement with other studies that document the protective effects of these agents

against cell injury induced by $H_2O_2$ in neural cells (Kamata et al., 1996; Ginés et al., 2010; Jin et al., 2010; Park et al., 2012). Thus, it is possible that both of these protein kinase activators can induce cardioprotection against oxidative stress. However, it is important to note that both PMA and FK induce a robust increase in ERK1/2 activation in H9c2 cells. The ERK1/2 pathway is also a prominent protein kinase associated with cardioprotection (Hausenloy & Yellon, 2004) and hence the cardioprotective effects of PMA and FK observed in H9c2 cells may involve ERK1/2. The results in the current study indicated that cells pre-treated with PMA and FK in the presence of $H_2O_2$ showed a significant increase in ERK activation compared to control, and $H_2O_2$ treated cells. In the presence of Z-DON this activation did not show significant attenuation. This suggests that the ERK pathway might not be involved in cardioprotection modulated by TG2 activation. On the other hand, up-regulation of pAKT has also been shown to be associated with cardioprotection mechanism against ischaemia-reperfusion injury in animals (Hausenloy & Yellon, 2006). However, pAKT (PKB) activation showed a significant increase in cells pre-treated with PMA and FK in the presence of $H_2O_2$, which was also significantly decreased in the presence of Z-DON. These results suggest that PMA and FK activate pAKT signalling under $H_2O_2$ induced oxidative stress and the possibility that TG2 could modulate this protective effect through pAKT signalling was supported by the reversal of this effect in the presence of Z-DON.

Figure 7.1 shows the proposed cascade of signalling events in H9c2 cardiomyocytes involved in cardioprotection modulated by TG2 activation using data presented in this thesis and other literature. This proposed model (Fig. 7.1) indicates that the beneficial effects of PMA and FK in protecting cardiac cells from $H_2O_2$-induced cell injury require AKT activation mediated by TG2 activity as demonstrated by using theTG2 inhibitor Z-DON.

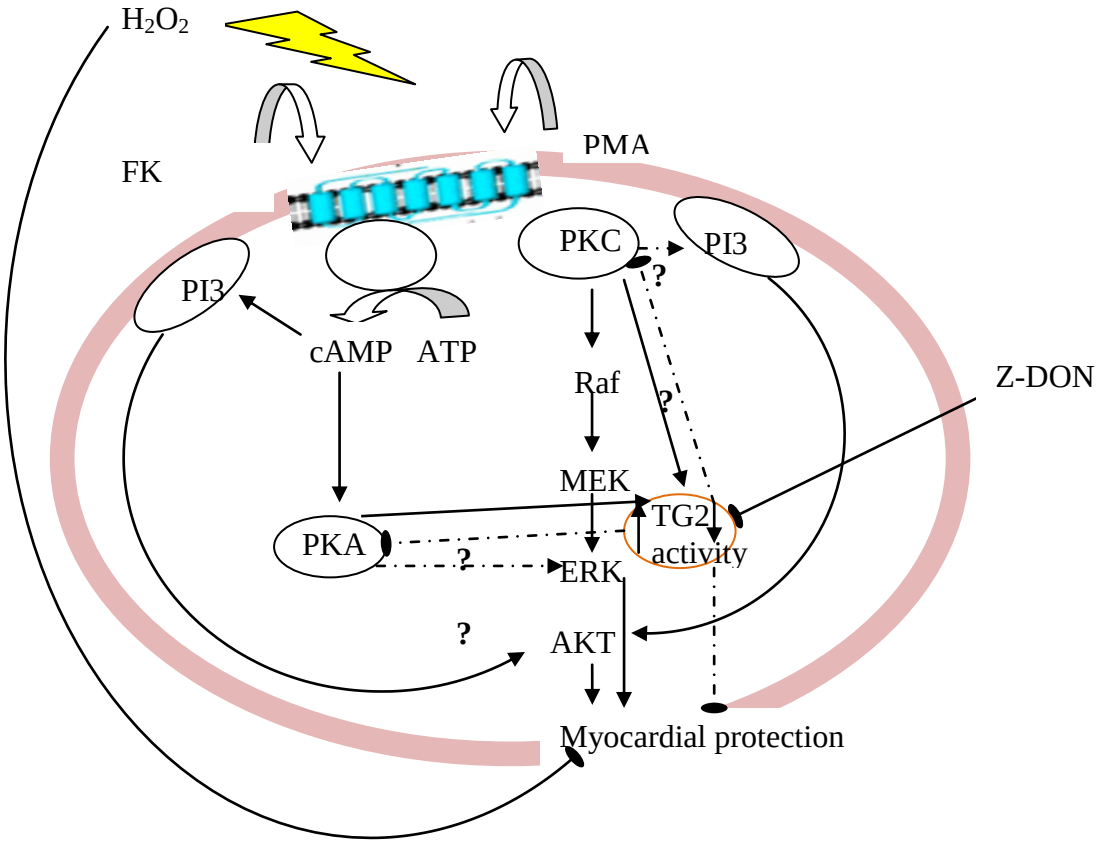



**Figure 7.1 Proposed cascade of signalling events in H9c2 cardiomyocytes involved in cardioprotection modulated by TG2 activation**

Forskolin (FK) activates adenylate cyclise (AC) to generate cAMP, stimulating both PKA and PI3/Akt activities (Wang & Yang, 2009). Upon activation of PKC by PMA, both the MAPK/ERK and PI3/Akt pathways are induced (Lallemend et al., 2005) and possibly mediated by TG2 activation resulting in protection of myocardial cells from $H_2O_2$ induced cell death and DNA degradation. ? Sign for an unknown pathway.

Although the PI3/Akt signalling mediated-PKC activation is not a classical pathway, it could be linked by a non-receptor tyrosine kinase PYK2 that can be phosphorylated and activate the PI3/Akt pathway (Sayed et al., 2000; Shi & Kehrl, 2001; Sarkar et al., 2002). Alternatively, the activation of this pathway could be due to oxidative stress (e.g. induced by $H_2O_2$) in H9c2 cells (Hong et al., 2001; Singla et al., 2008). Obviously, further studies exploring the effect of inhibitors for PKA, PKC, ERK1/2 and pAKT would either confirm or eliminate the involvement of these exact kinases in PMA and FK-mediated cytoprotection in H9c2 cells. These pathways may also

contribute to TG2-mediated protection against $H_2O_2$ in response to PMA and FK treatments. In order to identify the mechanism by which TG2 protects cells against $H_2O_2$ induced stress, an ideal approach is to inhibit each of the pathways, separately and see whether TG2 is still protective. The diminishing of the pathways could be achieved by several means dependent on the pathway such as using specific inhibitors for these affected kinases or using small interfering RNA (Milhavet et al., 2003) to knock down important proteins involved in specific pathways.

In spite of indecision surrounding the precise identity of the protein kinases involved in PMA and FK-induced cytoprotection, it is apparent that TG2 activation is involved in their cardioprotective actions. The phosphorylation of TG2 by PKA has several consequences, including the promotion of protein-protein interactions and enhancement of TG2 kinase activity, both of which might underlie its protective role (Mishra & Murphy, 2006; Mishra et al., 2007). For example, the phosphorylation of TG2 at $Ser^{216}$ by PKA forms a binding site for the adaptor protein 14-3-3 (Mishra & Murphy, 2006). Interestingly, 14-3-3 proteins regulate various cellular functions including signalling pathways that are associated with cell survival (Mackintosh, 2004). Although not associated with PKA phosphorylation and independent of its transamidating activity, TG2-mediated protection of neuronal cells against hypoxia and glucose deprivation-induced cell death through its interaction with hypoxia inducible factor 1β (HIF1β) which in turn reduces HIF1 signalling (Filiano et al., 2008). It would be interesting in future work to identify TG2 interacting proteins in cardiomyocytes following PMA and FK stimulation.

TG2 also has serine/threonine kinase activity and it has been involved in phosphorylation of insulin-like growth factor-binding protein 3, p53 and retinoblastoma protein *in vitro* (Gundemir et al., 2012). Both retinoblastoma and p53 proteins are important regulators of apoptosis and thus it is believable that they are associated with TG2- modulated cardioprotection in H9c2 cells. Furthermore, the anti-apoptotic effects of TG2 implicate the crosslinking of retinoblastoma protein (Boehm et al., 2002) and hence it would be interesting to determine if retinoblastoma protein is a TG2 substrate in H9c2 cells. Moreover, the phosphorylation of TG2 by PKA at $Ser^{216}$ appears to play an important role in TG2 mediated activation of NF-κB and PKB in fibroblast cells (Wang et al., 2012). Again, both of these signalling

pathways are associated with cardioprotection (Mishra et al., 2003; Hausenloy & Yellon, 2007) and it is possible they are regulated by TG2 in H9c2 cardiomyocytes following FK stimulation. This would need to be confirmed by an *in vivo* assay. In summary, TG2 is able of eliciting several pro-cell survival pathways related to cardioprotection that are either independent of its transamidating activity that require the prior phosphorylation by PKA or depend on its protein kinase function.

The activity of TGs are inhibited by GTP/GDP and evidence suggests that TG2 when bound to GTP/GDP functions as a G-protein (known as $G\alpha_h$; (Mhaouty-Kodja, 2004)). Indeed, several members of the G-protein coupled receptor (GPCRs) family including the α1B-adrenergic receptor, thromboxane A2 receptor and oxytocin receptor couple to $G\alpha_h$ when activated, promoting exchange of GDP for GTP (Gundemir et al., 2012). Activated $G\alpha_h$-GTP stimulates phospholipase Cδ1 promoting phosphoinositide hydrolysis and stimulating increases in intracellular $Ca^{2+}$ (Gundemir et al., 2012). An increasing number of membrane-bound receptors belonging to the GPCR superfamily have been implicated in cardioprotection including the adenosine $A_1$ receptor and members of the adrenergic receptor family (Sanada et al., 2011). These receptors trigger cardioprotection via the activation of signalling pathways involving PKC and PKA (Sanada et al., 2011). Indeed the data presented in this study have shown that the selective adenosine $A_1$ receptor agonist $N^6$-cyclopentyadenosine and the non-selective β-adrenergic receptor agonist isoprenaline trigger *in situ* increases in TG2 activity in H9c2 cells and is reversed by the inhibitor Z-DON. On-going work is currently exploring the role of TG2 in cardioprotection triggered by the adenosine $A_1$ receptor.

In the current study, it has shown that TG2 activation prior to $H_2O_2$-induced oxidative stress is cytoprotective. There is growing evidence in the literature that oxidative stress promotes the up-regulation of TG2, which may promote cell survival or apoptosis depending on cell type (Caccamo et al., 2012). Interestingly, oxidative stress up-regulates TG2 expression in rat neonatal cardiomyocytes, contributing to $H_2O_2$-induced apoptosis (Song et al., 2011). It is worth noting that in this study it was observed an increase in TG2 activity in H9c2 cells following $H_2O_2$ stimulation. The present results have shown that oxidative stress induced by $H_2O_2$ results in stimulation of the biotin amine incorporation but not the protein crosslinking TG2 activity in

H9c2 cells, as shown by *in situ* and *in vitro* assays. This is in agreement with previous studies, which have reported that oxidative stress via $H_2O_2$ could not elevate *in vitro* TG2 activity, which depends on the cell type and the existence of specific cellular factors (Shin et al., 2004; Park et al., 2010). However, when the oxidative stress followed pre-treatment with PMA but not FK, there was a statistically significant increase in TG2 catalysed biotin cadaverine incorporation detected *in vitro* and *in situ* with both treatments. Both *in vitro* and in *in situ*, different TG2 inhibitors also reversed this activation. This suggests that this activity was due activation of endogenous TG2 in H9c2 cells and that this may contribute to cytoprotection.

There were no correlations between the *in vitro* activation but not *in situ* and TG2 protein level in response to $H_2O_2$ alone or in pre-treatment cells by PMA but not FK. This confirms the previous suggestion by Shin et al. (2004) in which there is not always any correlation between TG2 protein level and its activity (Jeon et al., 2004). This suggests that the level of TG2 activation is dependent on cell and stress types (Shin et al., 2004) possibly due to $Ca^{2+}$ level. Until now, the molecular mechanisms by which intracellular TG2 is regulated are not clear. Some TG family members have been reported to undergo proteolytic cleavage to activate the intracellular enzymes (Lorand & Graham, 2003); no proteolytic activation has been reported in the case of TG2 regulation (Jeon & Kim, 2006). However, phosphorylation of TG2 by PKA and TG2 SUMOylation in response to oxidative stress has been reported (Mishra et al., 2007; Luciani et al., 2009). In this study, therefore, it is possible that TG2 is posttranslationally modified by these kinases. Further work will be undertaken to establish whether TG2 is modified by a posttranslational modification such as phosphorylation mediated by protein kinases A and C.

Despite the fact that TG2 is unlikely to be localised in the endoplasmic reticulum (Lorand & Graham, 2003; Iismaa et al., 2009), in this study, TG2 activity was shown to target mitochondria and endoplasmic reticulum proteins. Significantly, the likelihood, that TG2 functions to facilitate survival or death could be highly dependent on its cellular localisation and substrate accessibility (Esposito & Caputo, 2005; Park et al., 2010). It is worth mentioning that a recent study by Szondy and his group suggests that the abolition of TG2 in knockout mice resulted in a serious failure of ATP production and a significant increase in heart infarct size (Szondy et al., 2006).

This suggests the involvement of TG2 catalytic activity in the posttranslational modification of some essential mitochondrial regulatory proteins (Szondy et al., 2006). Although there is no report of TG2 translocation onto different organelle membranes, the interaction of isoforms of PKC with different organelles depends on the signal transduction and cell types (Schechtman & Mochly-Rosen, 2001). This may explain the co-localisation of TG2 onto the endoplasmic reticulum and mitochondria (Fig. 4.3.11). Therefore, it is possible that TG2 is transiently localised to the mitochondria or endoplasmic reticulum, where it can interact with and modify substrates, which may potentially play a role in cardioprotection. It will be of value in future studies to determine TG2 activity in each cellular organelle (mitochondria and endoplasmic reticulum) of heart tissue/cells to further explore its function modulated by PKA and PKC activation. Hence, it is interesting to speculate that TG2, when activated promotes cell survival if activated prior to an oxidative insult, whereas it may participate in cell death when activated following exposure to the stress stimulus. Future experiments will seek to address this potential dual role of TG2 in cardiomyocytes.

**TG2 substrates induced by PKA and PKC activation in H9c2 cells**

Most cells take up polyamines (Seiler et al., 1996) and biotin-X-cadaverine has been used to label cells and visualise proteins that are targeted by transglutaminase (Perry et al., 1995). Using this cell permeable biotinylated substrate of TG2, it is possible to visualise the protein targets of TG2 activity after 5 min treatment following oxidative stress or activation of PKA and PKC with pharmacological activators. Many intra and extra-cellular proteins can interact with TG2 acting as substrates that are modulated by its activities (Esposito & Caputo, 2005). This interaction may be important for those cellular proteins to perform their biological functions. The recognition of proteins that act as TG2 substrates is of critical importance for studying TG2's biological role in different cell types and tissues (Facchiano et al., 2006).

Posttranslational modifications of proteins through incorporation of polyamines or crosslinking mediated by TG2 have been implicated in a wide range of physiological functions, including ECM stabilisation and formation (Grenard et al., 2001), angiogenesis progression (Wang et al., 2013), cell adhesion and survival regulation

(Wang et al., 2011) and apoptosis (Rossin et al., 2012). Therefore, the biotin-X-cadaverine labelled proteins in treated H9c2 cells were captured with CaptAvidin beads and analysed by LC-MSMS. Twenty-five proteins were identified as TG2 substrates and some of these protein targets were confirmed by immunofluorescence staining and Western blotting techniques. These targets include cytoskeletal organising proteins, chaperone proteins, $Ca^{2+}$ and phospholipid binding proteins and proteins involved in membrane transport processes.

These data suggest the association of TG2 with the ER, since ER chaperone proteins HSP 90 and a novel TG2 substrate 78 kDa glucose-regulated protein were also among identified TG2 substrates targeted by this activity. Proteomic analysis of human CaCo-2 intestinal epithelial cells also revealed that HSP 90 was a TG2 substrate located on the ER (Orrù et al., 2003). The 78 kDa glucose-regulated protein is one of many ER chaperones that play a significant role in cardioprotection (Glembotski, 2008; Yang et al., 2011). Several mitochondrial matrix proteins have been shown to be TG2 substrates and were identified in the current and other studies, including HSP 70, HSP 90 organising protein, prohibitin (Orrù et al., 2003; Park et al., 2010) and two more novel mitochondrial proteins malectin, and serpin H1. Indeed, the involvement of these essential proteins in the modulation of TG2 activity induced by PKC and PKA signalling pathway suggests a role for this activity in cardioprotective cellular responses.

Several cytoskeleton organising proteins were identified as TG2 substrates in the current study; among them α-actinin and tubulin. Both of these proteins have an essential role in the contractile function of smooth muscle cells modulating cytoskeleton restructuring (Fultz et al., 2000). Alpha actinin is also known as a marker for the Z-line of the sarcomere and the translocation of PKCε to this region is of importance to cardioprotection promotion (Robia et al., 2001). This suggests that the depletion of amine incorporation into α-actinin by TG2 inhibitors may affect the translocation and activation of PKCε, eliminating its protective effects. The polyamination of tubulin by TG2 has been reported in neuronal cells both *in vitro* and *in vivo*, in which the inhibition of transglutaminase activity or polyamine synthesis result in a significant decrease in neuronal microtubule stability (Song et al., 2013). Cardiac-microtubules support intracellular transport, facilitate cell growth, and form a basis for cardio morphology (Gregorio & Antin, 2000; Schweitzer et al., 2001; Severs

et al., 2006). Thus, it may be that polyamination of these cytoskeleton proteins is essential for cardiomyocyte microtubule stabilisation and thus necessary for unique cardiomyocyte structures and functions. Further confirmation will be necessary to verify these candidates as genuine substrates of TG2.

Endomembrane vesicle trafficking proteins including the Arf-GAP -containing protein 1 and Ras-related protein Rab-35 were also shown to be TG2 substrates in H9c2 cells. This finding suggests a possible role of TG2 mediated by PMA and FK in organising the actin cytoskeleton (Randazzo et al., 2000) and regulating intracellular vesicle trafficking (Takai et al., 2001) as well as exosome secretion (Hsu et al., 2010).

The present data support the hypothesis that TG2 catalyses the posttranslational polyamination of target substrates involved in cytoskeletal organisation, protein folding machinery or endomembrane vesicle trafficking, and that this is likely to modify the physical-chemical properties of these target proteins. In turn, this is likely to influence their interactions with other proteins and control their biological activity with respect to cardioprotection.

In the current study, TG2-mediated changes to a specific substrate protein mediated induced by protein kinase activators have not always been linked to its cellular function. It would be of interest to make this correlation via identification of these TG2 substrates *in vivo* in combination with a structural and functional proteomic approach. This could help in identification of these TG2 substrate proteins in relation to physiology and cardiac disease, allowing one to explore the crosslinking or interaction in such conditions as normal verses ischaemic or preconditioned cells or tissues, and normal verses differentiated cells or even cells undergoing necrotic or apoptotic processes. In addition, would also be of interest to screen for differences in TG substrates between various stimuli.

In conclusion, the results presented in this thesis demonstrate that TG2 activity is modulated in H9c2 cells by PMA- and FK-mediated signalling pathways. Importantly, the study has shown for the first time that inhibition of TG2 attenuates PMA and FK-mediated cytoprotection against $H_2O_2$-induced oxidative stress suggesting a cardioprotective role for this multi-functional enzyme. This TG2 transamidation activity is mainly a polyamine incorporation activity that results in posttranslational modification of intercellular proteins. The main target proteins labelled following activation by these protein kinase activators are involved in cytoskeletal organising,

protein folding machinery or endomembrane vesicle trafficking. Overall, this study has made a significant contribution to the understanding of the intracellular roles of TG2 polyamine incorporation activity, which remains largely an unploughed field. Moreover, it has revealed the relationship between PKC, PKA, their receptors and TG2 transamidation activity in cardiomyocytes. Figure 7.2 summarizes proposed mechanisms of TG2 activation modulated by PKA and PKC, protecting cardiac cells from $H_2O_2$-induced cell injury based on the data presented in this and other published work.

Future work aims to identify TG2 substrates in H9c2 cells following PMA and FK stimulation by 2D-PAGE and to compare the effect of each treatment on these substrates by linking them to their cellular function, the potential regulation of TG2 activity by GPCRs associated with cardioprotection and to explore further the potential mechanisms of TG2-mediated cardioprotection. In addition, it is of importance to measure calcium release before and after each treatment and determination of TG2 activity in subcellular fractions. In the future, it may be possible to exploit the new set of information for the treatment of ischaemic heart disease using heart tissue.

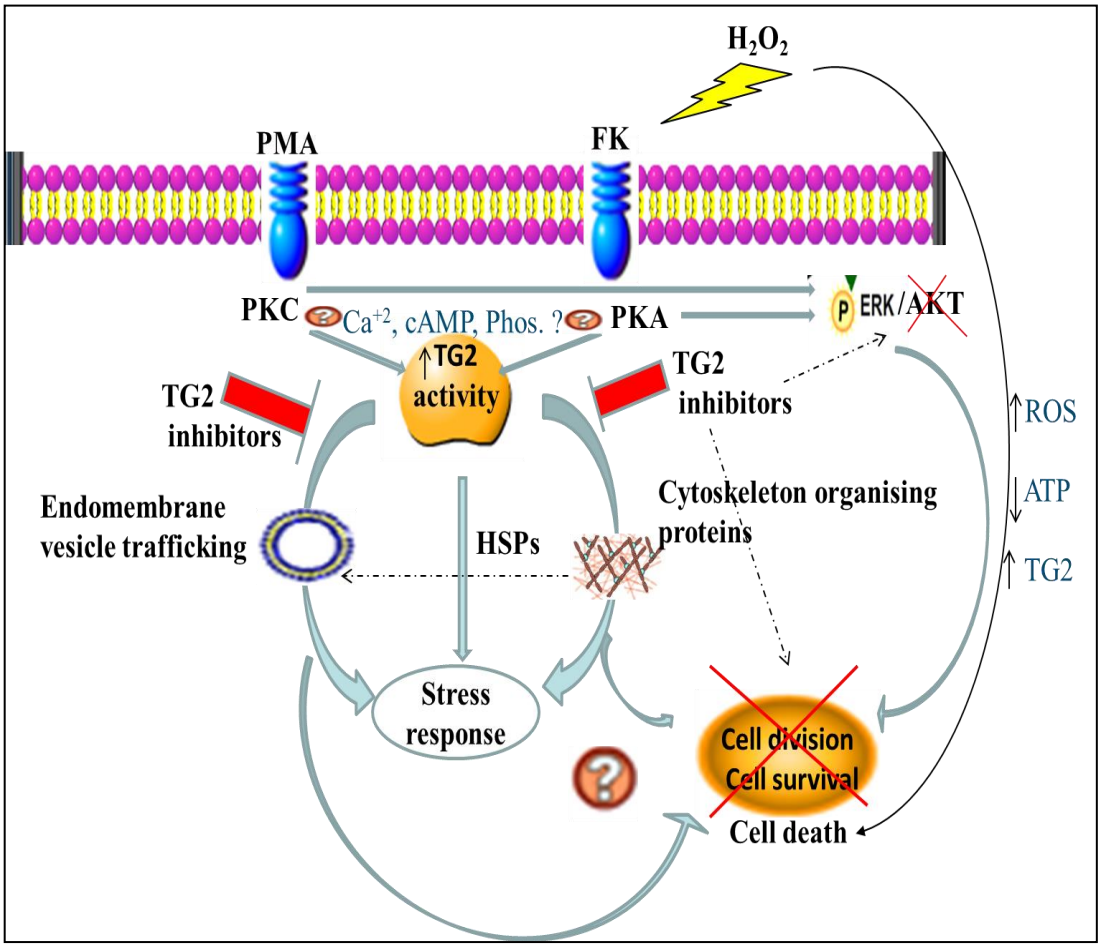



**Figure 7.2 Hypothetical model of proposed mechanisms of TG2 activation modulated PKA and PKC protecting cardiac cells from $H_2O_2$-induced cell injury based on the data presented in the current study and other published data**

Forskolin (FK) and PMA increase TG2 polyamine incorporation activity through an unknown mechanism possibly involving elevated $Ca^{2+}$ (Lagast et al., 1984; Andrews et al., 2002; Griffin et al., 2002; Dermitzaki et al., 2004), cAMP elevation (Szondy et al., 2006; Obara et al., 2012) or phosphorylation of TG2 or its substrates proteins (Mishra & Murphy, 2006; Mishra & Murphy, 2006; Mishra et al., 2007). The increased TG2 polyamine incorporation activity results in posttranslational modification of intracellular proteins (Fesus & Piacentini, 2002; Park et al., 2010; Gundemir et al., 2012). The main target proteins by of TG2 activation are cytoskeletal organising, protein folding (HSPs) or endomembrane vesicle trafficking proteins. Oxidative stress induced by $H_2O_2$ promotes cell death and apoptosis increased levels of ROS (Lee et al., 2003), decreased mitochondrial ATP production (Lennon et al., 1991; Lelli et al., 1998) or TG2 overexpression (Song et al., 2011). The pre-exposure of cells to PMA and FK induces cell survival pathways resulting in cytoprotection against $H_2O_2$ through ERK/PI3 phosphorylation (Lallemend et al., 2005; Wang et al., 2009) and TG2 polyamine incorporation activity. The TG2 inhibitors block these protective effects (TG2 activity and pAKT but not pERK) resulting in cell death.

# CHAPTER VIII:

# REFERENCES

## 8. References


Abassi, Z., Brodsky, S., Gealekman, O., Rubinstein, I., Hoffman, A. & Winaver, J. (2001). Intrarenal expression and distribution of cyclooxygenase isoforms in rats with experimental heart failure. *American Journal of Physiology-Renal Physiology, 280*(1), F43-F53.

AbdAlla, S., Lother, H., Langer, A., Faramawy, Y. & Quitterer, U. (2004). Factor XIIIA transglutaminase crosslinks AT1 receptor dimers of monocytes at the onset of atherosclerosis. *Cell, 119*(3), 343-354.

Abe, J., Baines, C. P. & Berk, B. C. (2000). Role of mitogen-activated protein kinases in ischemia and reperfusion injury: The good and the bad. *Circulation Research, 86*(6), 607-609.

Abedin, M., Tintut, Y. & Demer, L. L. (2004). Vascular calcification. *Arteriosclerosis, Thrombosis, and Vascular Biology, 24*(7), 1161-1170.

Abell, C. W. & Kwan, S. W. (2000). Molecular characterization of monoamine oxidases A and B. *Progress in Nucleic Acid Research and Molecular Biology, 65*, 129-132.

Ablin, R. J., Kynaston, H. G., Mason, M. D. & Jiang, W. G. (2011). Prostate transglutaminase (TGase-4) antagonizes the anti-tumour action of MDA-7/IL-24 in prostate cancer. *Journal of Translational Medicine, 9*(1), 49-75.

Ablin, R. & Jiang, W. (2011). The role of transglutaminases in the pathophysiology of prostate cancer. *Current Oncology, 18*(5), 241-242.

Achyuthan, K. & Greenberg, C. (1987). Identification of a guanosine triphosphate-binding site on guinea pig liver transglutaminase. role of GTP and calcium ions in modulating activity. *Journal of Biological Chemistry, 262*(4), 1901-1906.

Adderley, S. R. & Fitzgerald, D. J. (1999). Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. *Journal of Biological Chemistry, 274*(8), 5038-5046.

Aeschlimann, D., Koeller, M. K., Allen-Hoffmann, B. L. & Mosher, D. F. (1998). Isolation of a cDNA encoding a novel member of the transglutaminase gene family from human keratinocytes. *Journal of Biological Chemistry, 273*(6), 3452-3460.

Aeschlimann, D., Paulsson, M. & Mann, K. (1992). Identification of Gln726 in nidogen as the amine acceptor in transglutaminase-catalyzed cross-linking of laminin-nidogen complexes. *Journal of Biological Chemistry, 267*(16), 11316-11321.

Aeschlimann, D. & Paulsson, M. (1994). Transglutaminases: Protein cross-linking enzymes in tissues and body fluids. *Thrombosis and Haemostasis, 71*(4), 402-415.

Aeschlimann, D. & Thomazy, V. (2000). Protein crosslinking in assembly and remodelling of extracellular matrices: The role of transglutaminases. *Connective Tissue Research, 41*(1), 1-27.

Aggeli, I. S., Beis, I. & Gaitanaki, C. (2008). Oxidative stress and calpain inhibition induce alpha B-crystallin phosphorylation via p38-MAPK and calcium signalling pathways in H9c2 cells. *Cellular Signalling, 20*(7), 1292-1302.

Ahvazi, B., Kim, H. C., Kee, S. H., Nemes, Z. & Steinert, P. M. (2002). Three-dimensional structure of the human transglutaminase 3 enzyme: Binding of calcium ions changes structure for activation. *The EMBO Journal, 21*(9), 2055-2067.

Ahvazi, B., Boeshans, K. M. & Rastinejad, F. (2004). The emerging structural understanding of transglutaminase 3. *Journal of Structural Biology, 147*(2), 200-207.

Akar, U., Ozpolat, B., Mehta, K., Fok, J., Kondo, Y. & Lopez-Berestein, G. (2007). Tissue transglutaminase inhibits autophagy in pancreatic cancer cells. *Molecular Cancer Research, 5*(3), 241-249.

Akimov, S. S. & Belkin, A. M. (2003). Opposing roles of Ras/Raf oncogenes and the MEK1/ERK signaling module in regulation of expression and adhesive function of surface transglutaminase. *Journal of Biological Chemistry, 278*(37), 35609-35619.

Akimov, S. S., Krylov, D., Fleischman, L. F. & Belkin, A. M. (2000). Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. *The Journal of Cell Biology, 148*(4), 825-838.

Akimov, S. S. & Belkin, A. M. (2001a). Cell surface tissue transglutaminase is involved in adhesion and migration of monocytic cells on fibronectin. *Blood, 98*(5), 1567-1576.

Akimov, S. S. & Belkin, A. M. (2001b). Cell-surface transglutaminase promotes fibronectin assembly via interaction with the gelatin-binding domain of fibronectin a role in TGFβ-dependent matrix deposition. *Journal of Cell Science, 114*(16), 2989-3000.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P. (2002). *Molecular Biology of the Cell*. 4th Ed. New York: Garland Science; 2002. The Cytoskeleton and Cell Behavior. Available from: http://www.ncbi.nlm.nih.gov/books/NBK26930/

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W. & Yuan J. (1996). Human ICE/CED-3 protease nomenclature. *Cell, 87*(2), 171-171.

An, G., Meka, C., Bright, S. P. & Veltri, R. W. (1999). Human prostate-specific transglutaminase gene: Promoter cloning, tissue-specific expression, and down-regulation in metastatic prostate cancer. *Urology, 54*(6), 1105-1111.

Andrew, S. E., Goldberg, Y. P., Kremer, B., Telenius, H., Theilmann, J., Adam, S., Starr, E., Squitieri, F., Lin, B., Kalchman, M. A., Graham, R. K. & Hayden, M. R. (1993). The relationship between trinucleotide (CAG) repeat length and clinical features of huntington's disease. *Nature Genetics, 4*(4), 398-403.

Andrews, D. A., Yang, L. & Low, P. S. (2002). Phorbol ester stimulates a protein kinase C–mediated agatoxin-TK–sensitive calcium permeability pathway in human red blood cells. *Blood, 100*(9), 3392-3399.

Antonyak, M. A., Boehm, J. E. & Cerione, R. A. (2002). Phosphoinositide 3-kinase activity is required for retinoic acid-induced expression and activation of the tissue transglutaminase. *Journal of Biological Chemistry, 277*(17), 14712-14716.

Antonyak, M. A., Singh, U. S., Lee, D. A., Boehm, J. E., Combs, C., Zgola, M. M., Page, R. L. & Cerione, R. A. (2001). Effects of tissue transglutaminase on retinoic acid-induced cellular differentiation and protection against apoptosis. *Journal of Biological Chemistry, 276*(36), 33582-33587.

Antonyak, M. A., Li, B., Regan, A. D., Feng, Q., Dusaban, S. S. & Cerione, R. A. (2009). Tissue transglutaminase is an essential participant in the epidermal growth factor-stimulated signaling pathway leading to cancer cell migration and invasion. *Journal of Biological Chemistry, 284*(27), 17914-17925.

Antonyak, M. A., McNeill, C. J., Wakshlag, J. J., Boehm, J. E. & Cerione, R. A. (2003). Activation of the ras-ERK pathway inhibits retinoic acid-induced stimulation of tissue transglutaminase expression in NIH3T3 cells. *Journal of Biological Chemistry, 278*(18), 15859-15866.

Appelt, D. M. & Balin, B. J. (1997). The association of tissue transglutaminase with human recombinant tau results in the formation of insoluble filamentous structures. *Brain Research, 745*(1-2), 21-31.

Armstrong, S. C. (2004). Protein kinase activation and myocardial ischemia/reperfusion injury. *Cardiovascular Research, 61*(3), 427-436.

Asai, M., Tsukamoto, O., Minamino, T., Asanuma, H., Fujita, M., Asano, Y., Takahama, H., Sasaki, H., Higo, S., Asakura, M., Takashima, S., Hori M. & Kitakaze, M. (2009). PKA rapidly enhances proteasome assembly and activity in in vivo canine hearts. *Journal of Molecular and Cellular Cardiology, 46*(4), 452-462.

Astashkina, A., Mann, B. & Grainger, D. W. (2012). A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity. *Pharmacology & Therapeutics, 134*(1), 82-106.

Auchampach, J. A. & Bolli, R. (1999). Adenosine receptor subtypes in the heart: Therapeutic opportunities and challenges. *American Journal of Physiology-Heart and Circulatory Physiology, 276*(3), H1113-H1116.

August, J. T. & Kaufmann, S. H. (1997). *Apoptotis: Pharmacological implications and therapeutic opportunities: Apoptosis: Pharmacological implications and therapeutic opportunities*. San Diego, Academic press.

Azim, A. C., Marfatia, S. M., Korsgren, C., Dotimas, E., Cohen, C. M. & Chishti, A. H. (1996). Human erythrocyte dematin and protein 4.2 (pallidin) are ATP binding proteins. *Biochemistry, 35*(9), 3001-3006.

Baines, C. P., Song, C., Zheng, Y., Wang, G., Zhang, J., Wang, O., Guo, Y., Bolli, R., Cardwell, E.M. & Ping, P. (2003). Protein kinase Cε interacts with and inhibits the permeability transition pore in cardiac mitochondria. *Circulation Research, 92*(8), 873-880.

Bakker, E. N. T. P., Buus, C. L., Spaan, J. A. E., Perree, J., Ganga, A., Rolf, T. M., Sorop, O., Bramsen, L. H., Mulvany, M., J. & VanBavel, E. (2005). Small artery remodeling depends on tissue-type transglutaminase. *Circulation Research, 96*(1), 119-126.

Baldwin, D., Prince, M., Marshall, S., Davies, P. & Olefsky, J. M. (1980). Regulation of insulin receptors: Evidence for involvement of an endocytotic internalization pathway. *Proceedings of the National Academy of Sciences, USA, 77*(10), 5975-5978.

Balklava, Z., Verderio, E., Collighan, R., Gross, S., Adams, J. & Griffin, M. (2002). Analysis of tissue transglutaminase function in the migration of swiss 3T3 fibroblasts the active-state conformation of the enzyme does not affect cell motility but is important for its secretion. *Journal of Biological Chemistry, 277*(19), 16567-16575.

Bao, G., Wang, H., Shang, Y., Fan, H., Gu, M., Xia, R., Qin, Q. & Dang, A. M. (2012). Quantitative proteomic study identified cathepsin B associated with doxorubicin-induced damage in H9c2 cardiomyocytes. *Bioscience Trends, 6*(6), 283-287.

Bassani, J. W., Bassani, R. A. & Bers, D. M. (1994). Relaxation in rabbit and rat cardiac cells: Species-dependent differences in cellular mechanisms. *The Journal of Physiology, 476*(2), 279-293.

Bass-Zubek, A. E., Hobbs, R. P., Amargo, E. V., Garcia, N. J., Hsieh, S. N., Chen, X., Whal lll, J. K., Denning, M. F. & Green, K. J. (2008). Plakophilin 2: A critical scaffold for PKCα that regulates intercellular junction assembly. *The Journal of Cell Biology, 181*(4), 605-613.

Beck, K. E., De Girolamo, L. A., Griffin, M. & Billett, E. E. (2006). The role of tissue transglutaminase in 1-methyl-4-phenylpyridinium (MPP )-induced toxicity in

differentiated human SH-SY5Y neuroblastoma cells. *Neuroscience Letters, 405*(1-2), 46-51.

Benjamin, I. J. & McMillan, D. R. (1998). Stress (heat shock) proteins molecular chaperones in cardiovascular biology and disease. *Circulation Research, 83*(2), 117-132.

Bergamini, C. M. (1988). GTP modulates calcium binding and cation-induced conformational changes in erythrocyte transglutaminase. *FEBS Letters, 239*(2), 255-258.

Bernassola, F., Federici, M., Corazzari, M., Terrinoni, A., Hribal, M. L., De Laurenzi, V., Ranalli, M., Massa, Q., Sesti, G., McLean, W., H., Citro, G., Barbetti, F. & Melino, G. (2002). Role of transglutaminase 2 in glucose tolerance: Knockout mice studies and a putative mutation in a MODY patient. *The FASEB Journal, 16*(11), 1371-1378.

Berry, M., Juorio, A. & Paterson, I. (1994). The functional role of monoamine oxidases A and B in the mammalian central nervous system. *Progress in Neurobiology, 42*(3), 375-391.

Bers, D. M. (2000). Calcium fluxes involved in control of cardiac myocyte contraction. *Circulation Research, 87*(4), 275-281.

Bers, D. M. (2002). Cardiac excitation–contraction coupling. *Nature, 415*(6868), 198-205.

Bird, S. D., Doevendans, P. A., van Rooijen, M. A., Brutel de la Riviere, A., Hassink, R. J., Passier, R. & Mummery, C. L. (2003). The human adult cardiomyocyte phenotype. *Cardiovascular Research, 58*(2), 423-434.

Bishop, A. L. & Hall, A. (2000). Rho GTPases and their effector proteins. *Biochemical Journal, 348*(2), 241-255.

Boehm, J. E., Singh, U., Combs, C., Antonyak, M. A. & Cerione, R. A. (2002). Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 rb. *Journal of Biological Chemistry, 277*(23), 20127-20130.

Bollag, W. B., Zhong, X., Dodd, M. E., Hardy, D. M., Zheng, X. & Allred, W. T. (2005). Phospholipase d signaling and extracellular signal-regulated kinase-1 and-2 phosphorylation (activation) are required for maximal phorbol ester-induced transglutaminase activity, a marker of keratinocyte differentiation. *Journal of Pharmacology and Experimental Therapeutics, 312*(3), 1223-1231.

Bolli, R., Dawn, B. & Xuan, Y. T. (2003). Role of the JAK–STAT pathway in protection against myocardial ischemia/reperfusion injury. *Trends in Cardiovascular Medicine, 13*(2), 72-79.

Boots, A. W., Haenen, G. R. & Bast, A. (2008). Health effects of quercetin: From antioxidant to nutraceutical. *European Journal of Pharmacology, 585*(2), 325-337.

Boros, S. (2008). *The Gordian Knot of Small Heat Shock Proteins and Tissue Transglutaminase* (Doctoral dissertation, Radboud University Nijmegen, Netherlands). Retrieved from http://hdl.handle.net/2066/32056.

Borrell-Pagès, M., Canals, J. M., Cordelières, F. P., Parker, J. A., Pineda, J. R., Grange, G., Bryson, E. A., Guillermier, M., Hirsch, E., Hantraye, P., Cheetham, M.E., Néri, C., Alberch, J., Brouillet, E., Saudou, F. & Humbert, S. (2006). Cystamine and cysteamine increase brain levels of BDNF in huntington disease via HSJ1b and transglutaminase. *Journal of Clinical Investigation, 116*(5), 1410-1424.

Borutaite, V. & Brown, G. C. (2003). Mitochondria in apoptosis of ischemic heart. *FEBS Letters, 541*(1), 1-5.

Bottenus, R. E., Ichinose, A. & Davie, E. W. (1990). Nucleotide sequence of the gene for the b subunit of human factor XIII. *Biochemistry, 29*(51), 11195-11209.

Bowness, J. M., Venditti, M., Tarr, A. H. & Taylor, J. R. (1994). Increase in [epsilon]([gamma]-glutamyl) lysine crosslinks in atherosclerotic aortas* 1. *Atherosclerosis, 111*(2), 247-253.

Brar, B. K., Stephanou, A., Knight, R. & Latchman, D. S. (2002). Activation of protein kinase B/Akt by urocortin is essential for its ability to protect cardiac cells against hypoxia/reoxygenation-induced cell death. *Journal of Molecular and Cellular Cardiology, 34*(4), 483-492.

Brose, N. & Rosenmund, C. (2002). Move over protein kinase C, you've got company: Alternative cellular effectors of diacylglycerol and phorbol esters. *Journal of Cell Science, 115*(23), 4399-4411.

Brown, S. & Loew, L. M. (2012). Computational analysis of calcium signaling and membrane electrophysiology in cerebellar purkinje neurons associated with ataxia. *BMC Systems Biology, 6*(1), 70.

Budde, I. P., Rohde, B. H., Bender, C. L. & Ullrich, M. S. (1998). Growth phase and temperature influence promoter activity, transcript abundance, and protein stability during biosynthesis of the pseudomonas syringae phytotoxin coronatine. *Journal of Bacteriology, 180*(6), 1360-1367.

Buée, L., Bussiere, T., Buée-Scherrer, V., Delacourte, A. & Hof, P. R. (2000). Tau protein isoforms, phosphorylation and role in neurodegenerative disorders1. *Brain Research Reviews, 33*(1), 95-130.

Buja, L. M. (2005). Myocardial ischemia and reperfusion injury. *Cardiovascular Pathology, 14*(4), 170-175.

Buja, L. M., Eigenbrodt, M. L. & Eigenbrodt, E. H. (1993). Apoptosis and necrosis. basic types and mechanisms of cell death. *Archives of Pathology & Laboratory Medicine, 117*(12), 1208-1214.

Butler, S. J. & Landon, M. (1981). Transglutaminase-catalysed incorporation of putrescine into denatured cytochrome c: Preparation of a mono-substituted derivative reactive with cytochrome c oxidase. *Biochimica et Biophysica Acta (BBA)-Protein Structure, 670*(2), 214-221.

Byrne, J., Grieve, D., Cave, A. & Shah, A. (2003). Oxidative stress and heart failure. *Archives Des Maladies Du Coeur Et Des Vaisseaux, 96*(3), 214-221.

Caccamo, D., Campisi, A., Curro, M., Volti, G. L., Vanella, A. & Ientile, R. (2004). Excitotoxic and post-ischemic neurodegeneration: Involvement of transglutaminases. *Amino Acids, 27*(3-4), 373-379.

Caccamo, D., Currò, M., Condello, S., Ferlazzo, N. & Ientile, R. (2010). Critical role of transglutaminase and other stress proteins during neurodegenerative processes. *Amino Acids, 38*(2), 653-658.

Caccamo, D., Currò, M., Ferlazzo, N., Condello, S. & Ientile, R. (2012). Monitoring of transglutaminase2 under different oxidative stress conditions. *Amino Acids, 42*(2-3), 1037-1043.

Caccamo, D., Curro, M. & Ientile, R. (2010). Potential of transglutaminase 2 as a therapeutic target. *Expert Opinion on Therapeutic Targets, 14*(9), 989-1003.

Calaghan, S., Le Guennec, J. & White, E. (2004). Cytoskeletal modulation of electrical and mechanical activity in cardiac myocytes. *Progress in Biophysics and Molecular Biology, 84*(1), 29-59.

Calderwood, S. K., Mambula, S. S., Gray Jr, P. J. & Theriault, J. R. (2007). Extracellular heat shock proteins in cell signaling. *FEBS Letters, 581*(19), 3689-3694.

Camelliti, P., Green, C. R. & Kohl, P. (2006). Structural and functional coupling of cardiac myocytes and fibroblasts. *Advances in Cardiology, 42*, 132-149.

Camors, E., Monceau, V. & Charlemagne, D. (2005). Annexins and $Ca^{2+}$ handling in the heart. *Cardiovascular Research, 65*(4), 793-802.

Candi, E., Schmidt, R. & Melino, G. (2005). The cornified envelope: A model of cell death in the skin. *Nature Reviews Molecular Cell Biology, 6*(4), 328-340.

Candi, E., Oddi, S., Paradisi, A., Terrinoni, A., Ranalli, M., Teofoli, P., Citro, G., Scarpato, S., Puddu, P. & Melino, G. (2002). Expression of transglutaminase 5 in normal and pathologic human epidermis. *Journal of Investigative Dermatology, 119*(3), 670-677.

Candi, E., Oddi, S., Terrinoni, A., Paradisi, A., Ranalli, M., Finazzi-Agró, A. & Melino, G. (2001). Transglutaminase 5 cross-links loricrin, involucrin, and small proline-rich proteins in vitro. *Journal of Biological Chemistry, 276*(37), 35014-35023.

Caraglia, M., Marra, M., Giuberti, G., D'Alessandro, A. M., Beninati, S., Lentini, A., Pepe, S., Boccellino, M. & Abbruzzese, A. (2002). Theophylline-induced apoptosis is paralleled by protein kinase A-dependent tissue transglutaminase activation in cancer cells. *Journal of Biochemistry, 132*(1), 45-52.

Carmena, R., Duriez, P. & Fruchart, J. (2004). Atherogenic lipoprotein particles in atherosclerosis. *Circulation, 109*(23), III-2-III-7.

Chae, H., Chae, S. & Kim, H. (2004). Cyclic adenosine monophosphate inhibits nitric oxide-induced apoptosis of cardiac muscle cells in a c-jun N-terminal kinase-dependent manner. *Immunopharmacology and Immunotoxicology, 26*(2), 249-263.

Chakravarty, R., Rong, X. & Rice, R. (1990). Phorbol ester-stimulated phosphorylation of keratinocyte transglutaminase in the membrane anchorage region. *Biochemical Journal, 271*(1), 25-30.

Chakravarty, R. & Rice, R. H. (1989). Acylation of keratinocyte transglutaminase by palmitic and myristic acids in the membrane anchorage region. *Journal of Biological Chemistry, 264*(1), 625-629.

Chanalaris, A., Lawrence, K., Stephanou, A., Knight, R., Hsu, S., Hsueh, A. & Latchman, D. S. (2003). Protective effects of the urocortin homologues stresscopin (SCP) and stresscopin-related peptide (SRP) against hypoxia/reoxygenation injury in rat neonatal cardiomyocytes. *Journal of Molecular and Cellular Cardiology, 35*(10), 1295-1305.

Chanoit, G., Zhou, J., Lee, S., McIntosh, R., Shen, X., Zvara, D. A. & Xu, Z. (2011). Inhibition of phosphodiesterases leads to prevention of the mitochondrial permeability transition pore opening and reperfusion injury in cardiac H9c2 cells. *Cardiovascular Drugs and Therapy, 25*(4), 299-306.

Chemin, J., Mezghrani, A., Bidaud, I., Dupasquier, S., Marger, F., Barrère, C., Nargeot, J. & Lory, P. (2007). Temperature-dependent modulation of CaV3 T-type calcium channels by protein kinases C and A in mammalian cells. *Journal of Biological Chemistry, 282*(45), 32710-32718.

Chen, A. E., Ginty, D. D. & Fan, C. (2004). Protein kinase A signaling via CREB controls myogenesis induced by wnt proteins. *Nature, 433*(7023), 317-322.

Chen, Y., Chou, H., Lin, S., Chen, Y., Chang, Y., Chen, L. & Chan, H. (2012). Cardioprotective effects of quercetin in cardiomyocyte under Ischemia/Reperfusion injury. *Evidence-Based Complementary and Alternative Medicine: ECAM, 2013*, 364519-364519.

Cheng, T., Hitomi, K., van Vlijmen-Willems, I. M., de Jongh, G. J., Yamamoto, K., Nishi, K., Watts, C., Reinheckel, T., Schalkwijc, J. & Zeeuwen, P. L. (2006). Cystatin M/E is a high affinity inhibitor of cathepsin V and cathepsin L by a reactive site that is distinct from the legumain-binding site. A novel clue for the role of cystatin M/E in epidermal cornification. *Journal of Biological Chemistry, 281*(23), 15893-15899.

Cheng, T., van Vlijmen-Willems, I. M., Hitomi, K., Pasch, M. C., van Erp, P. E., Schalkwijk, J. & Zeeuwen, P. L. (2008). Colocalization of cystatin M/E and its target proteases suggests a role in terminal differentiation of human hair follicle and nail. *Journal of Investigative Dermatology, 129*(5), 1232-1242.

Chesley, A., Lundberg, M. S., Asai, T., Xiao, R. P., Ohtani, S., Lakatta, E. G. & Crow, M. T. (2000). The {beta} 2-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through Gi-dependent coupling to phosphatidylinositol 3'-kinase. *Circulation Research, 87*(12), 1172-1179.

Cheung, W., Darfler, M. M., Alvarez, H., Hood, B. L., Conrads, T. P., Habbe, N., Krizman, D. B., Mollenhauer, J., Feldmann, G. & Maitra, A. (2008). Application of a global proteomic approach to archival precursor lesions: Deleted in malignant brain tumors 1 and tissue transglutaminase 2 are upregulated in pancreatic cancer precursors. *Pancreatology, 8*(6), 608-616.

Chhabra, A., Verma, A. & Mehta, K. (2009). Tissue transglutaminase promotes or suppresses tumors depending on cell context. *Anticancer Research, 29*(6), 1909-1919.

Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. & Hidaka, H. (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H-89), in PC12D pheochromocytoma cells. *Journal of Biological Chemistry, 265*(9), 5267-5272.

Cho, M., Niles, A., Huang, R., Inglese, J., Austin, C. P., Riss, T. & Menghang, X. (2008). A bioluminescent cytotoxicity assay for assessment of membrane integrity using a proteolytic biomarker. *Toxicology in Vitro, 22*(4), 1099-1106.

Cho, S. Y., Lee, J. H., Bae, H. D., Jeong, E. M., Jang, G. Y., Kim, C. W., Shin, D. M., Jeon, J. H. & Kim, I. G. (2010). Transglutaminase 2 inhibits apoptosis induced by calciumoverload through down-regulation of bax. *Experimental & Molecular Medicine, 42*(9), 639-650.

Choi, K., Siegel, M., Piper, J. L., Yuan, L., Cho, E., Strnad, P., Rich, K. M. & Khosla, C. (2005). Chemistry and biology of dihydroisoxazole derivatives: Selective inhibitors of human transglutaminase 2. *Chemistry & Biology, 12*(4), 469-475.

Chou, C., Streets, A. J., Watson, P. F., Huang, L., Verderio, E. A. & Johnson, T. S. (2011). A crucial sequence for transglutaminase type 2 extracellular trafficking in

renal tubular epithelial cells lies in its N-terminal β-sandwich domain. *Journal of Biological Chemistry, 286*(31), 27825-27835.

Christensen, A. E., Selheim, F., de Rooij, J., Dremier, S., Schwede, F., Dao, K. K., Martinez, A., Maenhaut, C., Bos, J. L., Genieser, H. G & Doskeland, S. O. (2003). cAMP analog mapping of Epac1 and cAMP kinase. discriminating analogs demonstrate that epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *The Journal of Biological Chemistry, 278*(37), 35394-35402.

Chun, W., Lesort, M., Tucholski, J., Ross, C. A. & Johnson, G. (2001). Tissue transglutaminase does not contribute to the formation of mutant huntingtin aggregates. *The Journal of Cell Biology, 153*(1), 25-34.

Churchill, E. N. & Szweda, L. I. (2005). Translocation of δPKC to mitochondria during cardiac reperfusion enhances superoxide anion production and induces loss in mitochondrial function. *Archives of Biochemistry and Biophysics, 439*(2), 194-199.

Clarke, D., Mycek, M., Neidle, A. & Waelsch, H. (1959). The incorporation of amines into protein. *Archives of Biochemistry and Biophysics, 79*, 338-354.

Claycomb, W. C., Lanson, N. A., Stallworth, B. S., Egeland, D. B., Delcarpio, J. B., Bahinski, A. & Izzo, N. J. (1998). HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proceedings of the National Academy of Sciences of the United States of America, 95*(6), 2979-2984.

Coaxum, S. D., Griffin, T. M., Martin, J. L. & Mestril, R. (2007). Influence of PKC-α overexpression on HSP 70 and cardioprotection. *American Journal of Physiology-Heart and Circulatory Physiology, 292*(5), H2220-H2226.

Cohen, G. (1997). Caspases: The executioners of apoptosis. *Biochem Journal, 326*(1), 1-16.

Cohen, P. (2000). The regulation of protein function by multisite phosphorylation–a 25 year update. *Trends in Biochemical Sciences, 25*(12), 596-601.

Colligan, R. & Griffin, M. (2009). Transglutaminase 2 cross-linking of matrix proteins: Biological significance and medical applications. *Amino Acids, 36*(4), 659-670.

Collins, R. J., Harmon, B. V., Gobé, G. C. & Kerr, J. F. (1992). Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *International Journal of Radiation Biology, 61*(4), 451-453.

Conrad, M., Jakupoglu, C., Moreno, S. G., Lippl, S., Banjac, A., Schneider, M., Beck, H., Hatzopoulos, A. K., Just, U. & Sinowatz, F. (2004). Essential role for mitochondrial thioredoxin reductase in hematopoiesis, heart development, and heart function. *Molecular and Cellular Biology, 24*(21), 9414-9423.

Cooper, A. J. L., Jeitner, T. M., Gentile, V. & Blass, J. P. (2002). Cross linking of polyglutamine domains catalyzed by tissue transglutaminase is greatly favored with pathological-length repeats: Does transglutaminase activity play a role in (CAG) n/Qn-expansion diseases? *Neurochemistry International, 40*(1), 53-67.

Cooper, A. J. L., Sheu, K. F. R., Burke, J. R., Strittmatter, W., Gentile, V., Peluso, G., & Blass, J. P. (1999). Pathogenesis of inclusion bodies in (CAG) n/Qn-Expansion diseases with special reference to the role of tissue transglutaminase and to selective vulnerability. *Journal of Neurochemistry, 72*(3), 889-899.

Csosz, E., Keresztessy, Z. & Fesus, L. (2002). Transglutaminase substrates: From test tube experiments to living cells and tissues. *Minerva Biotecnologica, 14*(2), 149-154.

Cunnick, J. M., Schmidhuber, S., Chen, G., Yu, M., Yi, S., Cho, Y. J., Kaartinen, V., Minoo, P., Warburton, D. & Groffen, J. (2009). Bcr and abr cooperate in negatively regulating acute inflammatory responses. *Molecular and Cellular Biology, 29*(21), 5742-5750.

Curtiss, L. & Boisvert, W. (2000). Apolipoprotein E and atherosclerosis. *Current Opinion in Lipidology, 11*(3), 243-251.

da Silva, V. C. & Ramos, C. H. (2012). The network interaction of the human cytosolic 90 kDa heat shock protein Hsp90: A target for cancer therapeutics. *Journal of Proteomics, 75*(10), 2790-2802.

Daido, S., Kanzawa, T., Yamamoto, A., Takeuchi, H., Kondo, Y. & Kondo, S. (2004). Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. *Cancer Research, 64*(12), 4286-4293.

Dana, A., Skarli, M., Papakrivopoulou, J. & Yellon, D. M. (2000). Adenosine A1 receptor induced delayed preconditioning in rabbits: Induction of p38 mitogen-activated protein kinase activation and Hsp27 phosphorylation via a tyrosine kinase–and protein kinase C–dependent mechanism. *Circulation Research, 86*(9), 989-997.

Dardik, R. & Inbal, A. (2006). Complex formation between tissue transglutaminase II (tTG) and vascular endothelial growth factor receptor 2 (VEGFR-2): Proposed mechanism for modulation of endothelial cell response to VEGF. *Experimental Cell Research, 312*(16), 2973-2982.

Dardik, R., Krapp, T., Rosenthal, E., Loscalzo, J. & Inbal, A. (2007). Effect of FXIII on monocyte and fibroblast function. *Cellular Physiology and Biochemistry, 19*(1-4), 113-120.

Dardik, R., Leor, J., Skutelsky, E., Castel, D., Holbova, R., Schiby, G., Shaish, A., Dickneite, G., Loscalzo, J. & Inbal, A. (2006). Evaluation of the pro-angiogenic effect of factor XIII in heterotopic mouse heart allografts and FXIII-deficient mice. *Thrombosis and Haemostasis, 95*(3), 546-550.

Datta, S., Antonyak, M. A. & Cerione, R. A. (2006). Importance of $Ca^{2+}$-dependent transamidation activity in the protection afforded by tissue transglutaminase against doxorubicin-induced apoptosis. *Biochemistry, 45*(44), 13163-13174.

Daubney, J., Bonner, P. L., Hargreaves, A. J. & Dickenson, J. M. (2014). Cardioprotective and cardiotoxic effects of quercetin and two of its in vivo metabolites on differentiated H9c2 cardiomyocytes. *Basic & Clinical Pharmacology & Toxicology,* 13 OCT 2014 DOI: 10.1111/bcpt.12319.

Daugirdas, J., Zhou, H., Tamulaitis, V., Nutting, C. & Fiscus, R. (1991). Effect of H-8, an isoquinolinesulfonamide inhibitor of cyclic nucleotide-dependent protein kinase, on cAMP-and cGMP-mediated vasorelaxation. *Journal of Vascular Research, 28*(5), 366-371.

Davies, B. S., Fong, L. G., Yang, S. H., Coffinier, C. & Young, S. G. (2009). The posttranslational processing of prelamin A and disease. *Annual Review of Genomics and Human Genetics, 10*, 153-174.

Davies, S. P., Reddy, H., Caivano, M. & Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal, 351*(1), 95-105.

Davies, G., Ablin, R. J., Mason, M. D. & Jiang, W. G. (2007). Expression of the prostate transglutaminase (TGase-4) in prostate cancer cells and its impact on the invasiveness of prostate cancer. *Journal of Experimental Therapeutics & Oncology, 6*(3), 257-264.

Davis, P. D., Hill, C. H., Keech, E., Lawton, G., Nixon, J. S., Sedgwick, A. D., Wadsworth, J., Westmacott, D. & Wilkinson, S. E. (1989). Potent selective inhibitors of protein kinase C. *FEBS Letters, 259*(1), 61-63.

De Arcangelis, V., Soto, D. & Xiang, Y. (2008). Phosphodiesterase 4 and phosphatase 2A differentially regulate cAMP/protein kinase a signaling for cardiac myocyte contraction under stimulation of β1 adrenergic receptor. *Molecular Pharmacology, 74*(5), 1453-1462.

De Camilli, P., Moretti, M., Donini, S. D., Walter, U. & Lohmann, S. (1986). Heterogeneous distribution of the cAMP receptor protein RII in the nervous system: Evidence for its intracellular accumulation on microtubules, microtubule-organizing centers, and in the area of the golgi complex. *The Journal of Cell Biology, 103*(1), 189-203.

De Jong, J., De Jonge, R., Keijzer, E. & Bradamante, S. (2000). The role of adenosine in preconditioning. *Pharmacology & Therapeutics, 87*(2), 141-149.

De Maria, S., Metafora, S., Metafora, V., Morelli, F., Robberecht, P., Waelbroeck, M., Stiuso, P., De Rosa, A., Cozzolino, A. & Esposito, C. (2002). Transglutaminase-mediated polyamination of vasoactive intestinal peptide (VIP) Gln16 residue modulates VIP/PACAP receptor activity. *European Journal of Biochemistry, 269*(13), 3211-3219.

Deasey, S., Grichenko, O., Du, S. & Nurminskaya, M. (2012). Characterization of the transglutaminase gene family in zebrafish and in vivo analysis of transglutaminase-dependent bone mineralization. *Amino Acids, 42*(2-3), 1065-1075.

Dechat, T., Adam, S., Taimen, P., Shimi, T. & Goldman, R. (2010a). Nuclear lamins. *Cold Spring Harbor Perspectives in Biology, 2*(11), a000547-a000547.

Decker, T. & Lohmann-Matthes, M. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *Journal of Immunological Methods, 115*(1), 61-69.

Dempsey, E. C., Newton, A. C., Mochly-Rosen, D., Fields, A. P., Reyland, M. E., Insel, P. A. & Messing, R. O. (2000). Protein kinase C isozymes and the regulation of diverse cell responses. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 279*(3), L429-L438.

Denizot, F. & Lang, R. (1986). Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods, 89*(2), 271-277.

Dermitzaki, I., Tsatsanis, C., Alexaki, V. I., Castanas, E. & Margioris, A. N. (2004). Roles of protein kinase A (PKA) and PKC on corticotropin-releasing hormone (CRH)-induced elevation of cytosolic calcium from extra-and intra-cellular sources. *Hormones (Athens, Greece), 3*(4), 252-258.

Deucher, A., Nagpal, S., Chandraratna, R., Di Sepio, D., Robinson, N. A., Dashti, S. R. & Eckert, R. L. (2000). The carboxy-terminal hydrophobic domain of TIG3, a class II tumor suppressor protein, is required for appropriate cellular localization and optimal biological activity. *International Journal of Oncology, 17*(6), 1195-

Dhein, S., Mohr, F. W. & Delmar, M. (2005). *Practical methods in cardiovascular research*. Heidelberg. Springer, pp. 215 -453.

Diaz, R. J. & Wilson, G. J. (2006). Studying ischemic preconditioning in isolated cardiomyocyte models. *Cardiovascular Research, 70*(2), 286-296.

Dickenson, J. M. & Hill, S. J. (1997). Transfected adenosine A1 receptor-mediated modulation of thrombin-stimulated phospholipase C and phospholipase A2 activity in CHO cells. *European Journal of Pharmacology*, 321(1), 77-86.

Dickenson, J., Freeman, F., Mills, C., L., Sivasubramaniam, S. & Thode, C. (2013). *Molecular pharmacology: From DNA to drug discovery*. Hoboken, NJ: Wiley. 1203.

Di Paolo, G. & De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature, 443*(7112), 651-657.

Dipla, K., Mattiello, J. A., Margulies, K. B., Jeevanandam, V. & Houser, S. R. (1999). The sarcoplasmic reticulum and the Na+/Ca2+ exchanger both contribute to the Ca2+ transient of failing human ventricular myocytes. *Circulation Research, 84*(4), 435-444.

Di Sabatino, A. & Corazza, G. R. (2009). Coeliac disease. *The Lancet, 373*(9673), 1480-1493.

Di Somma, S., Marotta, M., Salvatore, G., Cudemo, G., Cuda, G., De Vivo, F., Di Benedetto, M., Ciaramella, F., Caputo, G. & de Divitiis, O. (2000). Changes in myocardial cytoskeletal intermediate filaments and myocyte contractile dysfunction in dilated cardiomyopathy: An in vivo study in humans. *Heart, 84*(6), 659-667.

Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E. O. & Schuppan, D. (1997). Identification of tissue transglutaminase as the autoantigen of coeliac disease. *Nature Medicine, 3*(7), 797-801.

Diez, J. A. & Maderdrut, J. L. (1977). Development of multiple forms of mouse brain monoamine oxidase in vivo and in vitro. *Brain Research, 128*(1), 187-192.

DiRaimondo, T. R., Klöck, C. & Khosla, C. (2012). Interferon-γ activates transglutaminase 2 via a phosphatidylinositol-3-kinase-dependent pathway: Implications for coeliac sprue therapy. *Journal of Pharmacology and Experimental Therapeutics, 341*(1), 104-114.

Donaldson, J. G. & Jackson, C. L. (2011). ARF family G proteins and their regulators: Roles in membrane transport, development and disease. *Nature Reviews Molecular Cell Biology, 12*(6), 362-375.

Donati, G., Peddigari, S., Mercer, C. A. & Thomas, G. (2013). 5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2-p53 checkpoint. *Cell Reports, 4*(1), 87-98.

Dong, Z., Saikumar, P., Weinberg, J. & Venkatachalam, M. (2005). Calcium in cell injury and death. *Annual Review of Pathology, 1*, 405-434.

Doré, S., Takahashi, M., Ferris, C. D., Hester, L. D., Guastella, D. & Snyder, S. H. (1999). Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. *Proceedings of the National Academy of Sciences USA, 96*(5), 2445-2450.

Dorn II, G. W. & Brown, J. H. (1999). Gq signaling in cardiac adaptation and maladaptation. *Trends in Cardiovascular Medicine, 9*(1), 26-34.

Dorn, G. W., Souroujon, M. C., Liron, T., Chen, C., Gray, M. O., Zhou, H. Z., Csukai, M., Wu, G., Lorenz, J. N. & Mochly-Rosen, D. (1999). Sustained in vivo cardiac protection by a rationally designed peptide that causes ε protein kinase C translocation. *Proceedings of the National Academy of Sciences USA, 96*(22), 12798-12803.

Dorn, I., Gerald, W. & Liggett, S. B. (2008). Pharmacogenomics of β-Adrenergic receptors and their accessory signaling proteins in heart failure. *Clinical and Translational Science, 1*(3), 255-262.

Dougherty, C. J., Kubasiak, L. A., Prentice, H., Andreka, P., Bishopric, N. H. & Webster, K. A. (2002). Activation of c-jun N-terminal kinase promotes survival of cardiac myocytes after oxidative stress. *Biochemical Journal, 362*(3), 561-571.

Dodge-Kafka, K. L., Soughayer, J., Pare, G. C., Michel, J. J. C., Langeberg, L. K., Kapiloff, M. S. & Scott, J. D. (2005). The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. *Nature, 437*(7058), 574-578.

Driscoll, H. K., Adkins, C. D., Chertow, T. E., Cordle, M. B., Matthews, K. A. & Chertow, B. S. (1997). Vitamin A stimulation of insulin secretion: Effects on transglutaminase mRNA and activity using rat islets and insulin-secreting cells. *Pancreas, 15*(1), 69-77.

Dubbink, H. J., de Waal, L., van Haperen, R., Verkaik, N. S., Trapman, J. & Romijn, J. C. (1998). The human prostate-specific transglutaminase gene (TGM4): Genomic organization, tissue-specific expression, and promoter characterization. *Genomics, 51*(3), 434-444.

Dubbink, H. J., Hoedemaeker, R. F., Van der Kwast, T., Schröder, F. & Romijn, J. C. (1999). Human prostate-specific transglutaminase: A new prostatic marker with a unique distribution pattern. *Laboratory Investigation, 79*(2), 141-150.

Eckert, R. L., Sturniolo, M. T., Broome, A. M., Ruse, M. & Rorke, E. A. (2005). Transglutaminase function in epidermis. *Journal of Investigative Dermatology, 124*(3), 481-492.

Elmore, S. (2007). Apoptosis: A review of programmed cell death. *Toxicologic Pathology, 35*(4), 495-516.

Elzein, E. & Zablocki, J. (2008). A1 adenosine receptor agonists and their potential therapeutic applications. *Expert Opinion on Investigational Drugs, 17*(12), 1901-1910.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. & Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature, 391*(6662), 43-50.

Engholm, M., Eftekhari, A., Chwatko, G., Bald, E. & Mulvany, M. (2011). Effect of cystamine on blood pressure and vascular characteristics in spontaneously hypertensive rats. *Journal of Vascular Research, 48*(6), 476-484.

England, P. & Shahid, M. (1987). Effects of forskolin on contractile responses and protein phosphorylation in the isolated perfused rat heart. *Biochemical Journal, 246*(3), 687-965.

Esposito, C. & Caputo, I. (2005). Mammalian transglutaminases. *FEBS Journal, 272*(3), 615-631.

Esposito, C., Costa, C., Amoresano, A., Mariniello, L., Sommella, M., Caputo, I. & Porta, R. (1999). Transglutaminase-mediated amine incorporation into substance P protects the peptide against proteolysis in vitro. *Regulatory Peptides, 84*(1), 75-80.

Esposito, C., Paparo, F., Caputo, I., Porta, R., Salvati, V. M., Mazzarella, G., Auricchio, S. & Troncone, R. (2003). Expression and enzymatic activity of small intestinal tissue transglutaminase in coeliac disease. *The American Journal of Gastroenterology, 98*(8), 1813-1820.

Ertracht, O., Liani, E., Bachner-Hinenzon, N., Bar-Am, O., Frolov, L., Ovcharenko, E. & Amit, T. (2011). The cardioprotective efficacy of TVP1022 in a rat model of ischaemia/reperfusion. *British Journal of Pharmacology, 163*(4), 755-769.

Evans, M. D. & Cooke, M. S. (2004). Factors contributing to the outcome of oxidative damage to nucleic acids. *BioEssays, 26*(5), 533-542.

Fabbi, M., Marimpietri, D., Martini, S., Brancolini, C., Amoresano, A., Scaloni, A., Bargellesi, A. & Cosulich, E. (1999). Tissue transglutaminase is a caspase substrate during apoptosis. Cleavage causes loss of transamidating function and is a biochemical marker of caspase 3 activation. *Cell Death and Differentiation, 6*(10), 992-1001.

Facchiano, F., Facchiano, A. & Facchiano, A. M. (2006). The role of transglutaminase-2 and its substrates in human diseases. *Front Biosci, 11*, 1758-1773.

Facchiano, F., D'Arcangelo, D., Riccomi, A., Lentini, A., Beninati, S. & Capogrossi, M. C. (2001). Transglutaminase activity is involved in polyamine-induced programmed cell death. *Experimental Cell Research, 271*(1), 118-129.

Falasca, L., Iadevaia, V., Ciccosanti, F., Melino, G., Serafino, A. & Piacentini, M. (2005). Transglutaminase type II is a key element in the regulation of the anti-inflammatory response elicited by apoptotic cell engulfment. *The Journal of Immunology, 174*(11), 7330-7340.

Ferrari, D. M. & Söling, H. (1999). The protein disulphide-isomerase family: Unravelling a string of folds. *Biochemical Journal, 339*(1), 1-10.

Fesus, L., Davies, P. & Piacentini, M. (1991). Apoptosis: Molecular mechanisms in programmed cell death. *European Journal of Cell Biology, 56*(2), 170-177.

Fesus, L., Thomazy, V. & Falus, A. (1987). Induction and activation of tissue transglutaminase during programmed cell death. *FEBS Letters, 224*(1), 104-108.

Fesus, L. & Tarcsa, E. (1989). Formation of N epsilon-(gamma-glutamyl)-lysine isodipeptide in chinese-hamster ovary cells. *Biochem Journal, 263*, 843-848.

Fesus, L. & Piacentini, M. (2002). Transglutaminase 2: An enigmatic enzyme with diverse functions. *Trends Biochem Sci, 27*(10), 534-539.

Fesus, L. & Szondy, Z. (2005). Transglutaminase 2 in the balance of cell death and survival. *FEBS Lett, 579*(15), 3297-3302.

Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F. & White, F. M. (2002). Phosphoproteome analysis by mass spectrometry and its application to saccharomyces cerevisiae. *Nature Biotechnology, 20*(3), 301-305.

Filiano, A., Tucholski, J., Dolan, P., Colak, G. & Johnson, G. (2010). Transglutaminase 2 protects against ischemic stroke. *Neurobiology of Disease, 39*(3), 334-343.

Filiano, A. J., Bailey, C. D., Tucholski, J., Gundemir, S. & Johnson, G. V. (2008). Transglutaminase 2 protects against ischemic insult, interacts with HIF1β, and attenuates HIF1 signaling. *The FASEB Journal, 22*(8), 2662-2675.

Fleckenstein, B., Molberg, Ø., Qiao, S., Schmid, D. G., von der Mülbe, F., Elgstøen, K., Jung, G. & Sollid, L. M. (2002). Gliadin T cell epitope selection by tissue transglutaminase in coeliac disease. *Journal of Biological Chemistry, 277*(37), 34109-34116.

Fok, J. Y., Ekmekcioglu, S. & Mehta, K. (2006). Implications of tissue transglutaminase expression in malignant melanoma. *Molecular Cancer Therapeutics, 5*(6), 1493-1503.

Folk, J., Park, M., Chung, S., Schrode, J., Lester, E. & Cooper, H. (1980). Polyamines as physiological substrates for transglutaminases. *Journal of Biological Chemistry, 255*(8), 3695-3700.

Forbes, R. A., Steenbergen, C. & Murphy, E. (2001). Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. *Circulation Research, 88*(8), 802-809.

Fox, B. A., Yee, V. C., Pedersen, L. C., Le Trong, I., Bishop, P. D., Stenkamp, R. E., & Teller, D. C. (1999). Identification of the calcium binding site and a novel ytterbium site in blood coagulation factor XIII by x-ray crystallography. *Journal of Biological Chemistry, 274*(8), 4917-4923.

Fraij, B. M. & Gonzales, R. A. (1997). Organization and structure of the human tissue transglutaminase gene. *Biochimica Et Biophysica Acta (BBA)-Gene Structure and Expression, 1354*(1), 65-71.

Frässdorf, J., De Hert, S. & Schlack, W. (2009). Anaesthesia and myocardial ischaemia/reperfusion injury. *British Journal of Anaesthesia, 103*(1), 89-98.

Fredholm, B. B., IJzerman, A. P., Jacobson, K. A., Klotz, K. & Linden, J. (2001). International union of pharmacology. XXV. nomenclature and classification of adenosine receptors. *Pharmacological Reviews, 53*(4), 527-552.

Freshney, R. I. (2006). Basic principles of cell culture. Culture of Cells for Tissue Engineering.Ed.John Wiley & Sons, New Jersey.Inc.Hobokan.

Freund, K. F., Doshi, K. P., Gaul, S. L., Claremon, D. A., Remy, D. C., Baldwin, J. J., Pitzenberger, S. M. & Stern, A. M. (1994). Transglutaminase inhibition by 2-[(2-oxopropyl) thio] imidazolium derivatives: Mechanism of factor XIIIa inactivation. *Biochemistry, 33*(33), 10109-10119.

Fryer, R. M., Wang, Y., Hsu, A. K. & Gross, G. J. (2001). Essential activation of PKC-δ in opioid-initiated cardioprotection. *American Journal of Physiology-Heart and Circulatory Physiology, 280*(3), H1346-H1353.

Fultz, M., Li, C., Geng, W. & Wright, G. (2000). Remodeling of the actin cytoskeleton in the contracting A7r5 smooth muscle cell. *Journal of Muscle Research & Cell Motility, 21*(8), 775-787.

Galliher-Beckley, A. J., Williams, J. G. & Cidlowski, J. A. (2011). Ligand-independent phosphorylation of the glucocorticoid receptor integrates cellular stress pathways with nuclear receptor signaling. *Molecular and Cellular Biology, 31*(23), 4663-4675.

Ganong, W. F. & Barrett, K. E. (2005). *Review of medical physiology* (Vol 22). New York: McGraw-Hill Medical.

Gavin, I. M., Glesne, D., Zhao, Y., Kubera, C. & Huberman, E. (2004). Spermine acts as a negative regulator of macrophage differentiation in human myeloid leukemia cells. *Cancer Research, 64*(20), 7432-7438.

Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *The Journal of Cell Biology, 119*(3), 493-501.

Gentile, V., Davies, P. & Baldini, A. (1994). The human tissue transglutaminase gene maps on chromosome 20q12 by in situ fluorescence hybridization. *Genomics, 20*(2), 295-297.

Gentile, V., Grant, F. J., Porta, R. & Baldini, A. (1995). Localization of the human prostate transglutaminase (type IV) gene (TGM4) to chromosome 3p21.33-p22 by fluorescence in situ hybridization. *Genomics, 27*(1), 219-220.

Gentile, V., Saydak, M., Chiocca, E., Akande, O., Birckbichler, P., Lee, K., et al. (1991). Isolation and characterization of cDNA clones to mouse macrophage and human endothelial cell tissue transglutaminases. *Journal of Biological Chemistry, 266*(1), 478-483.

Gentile, V., Sepe, C., Calvani, M., Melone, M. A. B., Cotrufo, R., Cooper, A. J. L., et al. (1998). Tissue transglutaminase-catalyzed formation of high-molecular-weight aggregatesin VitroIs favored with long polyglutamine domains: A possible mechanism contributing to CAG-triplet diseases. *Archives of Biochemistry and Biophysics, 352*(2), 314-321.

Gerke, V., Creutz, C. E. & Moss, S. E. (2005). Annexins: Linking $Ca^{2+}$ signaling to membrane dynamics. *Nature Reviews Molecular Cell Biology, 6*(6), 449-461.

Ginés, S., Paoletti, P. & Alberch, J. (2010). Impaired TrkB-mediated ERK1/2 activation in huntington disease knock-in striatal cells involves reduced p52/p46 shc expression. *Journal of Biological Chemistry, 285*(28), 21537-21548.

Giordano, E., Flamigni, F., Guarnieri, C., Muscari, C., Pignatti, C., Stefanelli, C. & Caldarera, C. M. (2010). Polyamines in cardiac physiology and disease. *Open Heart Fail J, 3*, 25-30.

Giordano, F. J. (2005). Oxygen, oxidative stress, hypoxia, and heart failure. *Journal of Clinical Investigation, 115*(3), 500-508.

Glembotski, C. C. (2008). The role of the unfolded protein response in the heart. *Journal of Molecular and Cellular Cardiology, 44*(3), 453-459.

Glenner, G. G. & Wong, C. W. (1984). Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochemical and Biophysical Research Communications, 120*(3), 885-890.

Golan, D. E., Corbett, J. D., Korsgren, C., Thatte, H. S., Hayette, S., Yawata, Y. & Cohen, C. M. (1996). Control of band 3 lateral and rotational mobility by band 4.2 in intact erythrocytes: Release of band 3 oligomers from low-affinity binding sites. *Biophysical Journal, 70*(3), 1534-1542.

Goldspink, P. H. & Russell, B. (1994). The cAMP response element binding protein is expressed and phosphorylated in cardiac myocytes. *Circulation Research, 74*(6), 1042-1049.

Gomis, R., Casanovas, A. & Malaisse, W. (1989). Transglutaminase-catalyzed incorporation of [2, 5-3H] histamine into a mr 84000 particulate protein in pancreatic islets. *Bioscience Reports, 9*(1), 55-61.

Goodnight, J. A., Mischak, H., Kolch, W. & Mushinski, J. F. (1995). Immunocytochemical localization of eight protein kinase C isozymes overexpressed in NIH 3T3 fibroblasts. isoform-specific association with microfilaments, golgi, endoplasmic reticulum, and nuclear and cell membranes. *The Journal of Biological Chemistry, 270*(17), 9991-10001.

Goodwin, F. C. & Muntoni, F. (2005). Cardiac involvement in muscular dystrophies: Molecular mechanisms. *Muscle & Nerve, 32*(5), 577-588.

Gorman, J. & Folk, J. (1984). Structural features of glutamine substrates for transglutaminases. role of extended interactions in the specificity of human plasma factor XIIIa and of the guinea pig liver enzyme. *Journal of Biological Chemistry, 259*(14), 9007-9010.

Goswami, S. K., Maulik, N. & Das, D. K. (2007). Ischemia-reperfusion and cardioprotection: A delicate balance between reactive oxygen species generation and redox homeostasis. *Annals of Medicine, 39*(4), 275-289.

Grabarek, J., Ardelt, B., Kunicki, J. & Darzynkiewicz, Z. (2002). Detection of in situ activation of transglutaminase during apoptosis: Correlation with the cell cycle phase by multiparameter flow and laser scanning cytometry. *Cytometry, 49*(2), 83-89.

Graeber, M., Kösel, S., Grasbon-Frodl, E., Möller, H. & Mehraein, P. (1998). Histopathology and APOE genotype of the first Alzheimer disease patient, Auguste D. *Neurogenetics, 1*(3), 223-228.

Granger, D. N., Benoit, J. N., Suzuki, M. & Grisham, M. B. (1989). Leukocyte adherence to venular endothelium during ischemia-reperfusion. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 257*(5), G683-G688.

Grazia Farrace, M., Piredda, L., Matarrese, P., Ciccosanti, F., Falasca, L., Rodolfo, C., Giammarioli, A. M., Verderio, E., Griffin, M. & Malorni, W. (2002). Transglutaminase overexpression sensitizes neuronal cell lines to apoptosis by increasing mitochondrial membrane potential and cellular oxidative stress. *Journal of Neurochemistry, 81*(5), 1061-1072.

Greenstein, J., Tanskanen, A. & Winslow, R. (2004). Modeling the actions of beta-adrenergic signaling on excitation--contraction coupling processes. *Ann NY Acad Sci, 1015*, 16-27.

Gregorio, C. C. & Antin, P. B. (2000). To the heart of myofibril assembly. *Trends in Cell Biology, 10*(9), 355-362.

Grenard, P., Bates, M. K. & Aeschlimann, D. (2001). Evolution of transglutaminase genes: Identification of a transglutaminase gene cluster on human chromosome 15q15. *Journal of Biological Chemistry, 276*(35), 33066-33078.

Grenard, P., Bresson-Hadni, S., El Alaoui, S. I., Chevallier, M., Vuitton, D. A. & Ricard-Blum, S. (2001). Transglutaminase-mediated cross-linking is involved in the stabilization of extracellular matrix in human liver fibrosis. *Journal of Hepatology, 35*(3), 367-375.

Grierson, A. J., Johnson, G. V. W. & Miller, C. C. J. (2001). Three different human tau isoforms and rat neurofilament light, middle and heavy chain proteins are cellular substrates for transglutaminase. *Neuroscience Letters, 298*(1), 9-12.

Griffin, M., Barnes, R. N., Wynne, J. & Williams, C. (1978). The effects of bleomycin and copper bleomycin upon transglutaminase enzymes. *Biochemical Pharmacology, 27*(8), 1211-1219.

Griffin, M., Casadio, R. & Bergamini, C. M. (2002). Transglutaminases: Nature's biological glues. *Biochemical Journal, 368*(2), 377-396.

Griffin, M., Mongeot, A., Collighan, R., Saint, R. E., Jones, R. A., Coutts, I. G. C. & Rathbone, D. L. (2008). Synthesis of potent water-soluble tissue

transglutaminase inhibitors. *Bioorganic & Medicinal Chemistry Letters, 18*(20), 5559-5562.

Groenen, P. J., Bloemendal, H. & de Jong, W. W. (1992). The carboxy-terminal lysine of alpha B-crystallin is an amine-donor substrate for tissue transglutaminase. *European Journal of Biochemistry, 205*(2), 671-674.

Grootjans, J. J., Groenen, P. J. T. A. & de Jong, W. W. (1995). Substrate requirements for transglutaminases. *Journal of Biological Chemistry, 270*(39), 22855-22858.

Gross, D. R. (2009). *Animal models in cardiovascular research*., New York, NY. Springer Science and Business Media LLC.

Gschwendt, M., Dieterich, S., Rennecke, J., Kittstein, W., Mueller, H. & Johannes, F. (1996). Inhibition of protein kinase C μ by various inhibitors. differentiation from protein kinase c isoenzymes. *FEBS Letters, 392*(2), 77-80.

Guidi, I., Galimberti, D., Lonati, S., Novembrino, C., Bamonti, F., Tiriticco, M., Fenoglio, C., Venturelli, E., Baron, P. & Bresolin, N. (2006). Oxidative imbalance in patients with mild cognitive impairment and alzheimer's disease. *Neurobiology of Aging, 27*(2), 262-269.

Gundemir, S., Colak, G., Tucholski, J. & Johnson, G. V. (2012). Transglutaminase 2: A molecular Swiss army knife. *Biochimica Et Biophysica Acta (BBA)-Molecular Cell Research, 1823*(2), 406-419.

Gundemir, S. & Johnson, G. (2009). Intracellular localization and conformational state of transglutaminase 2: Implications for cell death. *PLoS One, 4*(7), e6123-e6129.

Halliwell, B. (2007). Oxidative stress and cancer: Have we moved forward? *Biochemical. Journal 401*(1), 1-11.

Halverson, R. A., Lewis, J., Frausto, S., Hutton, M. & Muma, N. A. (2005). Tau protein is cross-linked by transglutaminase in P301L tau transgenic mice. *The Journal of Neuroscience, 25*(5), 1226-1233.

Hamacher-Brady, A., Brady, N., Logue, S., Sayen, M., Jinno, M., Kirshenbaum, L., Gottlieb, R. & Gustafsson, Å. B. (2006). Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. *Cell Death & Differentiation, 14*(1), 146-157.

Hand, D., Bungay, P., Elliott, B. & Griffin, M. (1985). Activation of transglutaminase at calcium levels consistent with a role for this enzyme as a calcium receptor protein. *Bioscience Reports, 5*(12), 1079-1086.

Hasegawa, G., Suwa, M., Ichikawa, Y., Ohtsuka, T., Kumagai, S., Kikuchi, M., Sato, Y. & Saito, Y. (2003). A novel function of tissue-type transglutaminase: Protein disulphide isomerase. *Biochemical Journal, 373*(3), 793-803.

Hassouna, A., Matata, B. M. & Galiñanes, M. (2004). PKC-ε is upstream and PKC-α is downstream of mitoKATP channels in the signal transduction pathway of ischemic preconditioning of human myocardium. *American Journal of Physiology-Cell Physiology, 287*(5), C1418-C1425.

Hausenloy, D. J. & Yellon, D. M. (2004). New directions for protecting the heart against ischaemia–reperfusion injury: Targeting the reperfusion injury salvage kinase (RISK)-pathway. *Cardiovascular Research, 61*(3), 448-460.

Hausenloy, D. J. & Yellon, D. M. (2006). Survival kinases in ischemic preconditioning and postconditioning. *Cardiovascular Research, 70*(2), 240-253.

Hausenloy, D. J. & Yellon, D. M. (2007). Preconditioning and postconditioning: United at reperfusion. *Pharmacology & Therapeutics, 116*(2), 173-191.

Hausenloy, D. J. & Yellon, D. M. (2008). GLP-1 therapy beyond glucose control. *Circulation: Heart Failure, 1*(3), 147-149.

Hausenloy, D. J. & Yellon, D. M. (2012). Taking lizard saliva to heart. *European Heart Journal, 33*(12), 1426-1430.

Hayashi, T., Saito, A., Okuno, S., Ferrand-Drake, M. & Chan, P. H. (2003). Induction of GRP78 by ischemic preconditioning reduces endoplasmic reticulum stress and prevents delayed neuronal cell death. *Journal of Cerebral Blood Flow & Metabolism, 23*(8), 949-961.

He, Y. & Smith, R. (2009). Nuclear functions of heterogeneous nuclear ribonucleoproteins A/B. *Cellular and Molecular Life Sciences, 66*(7), 1239-1256.

Heid, C. A., Stevens, J., Livak, K. J. & Williams, P. M. (1996). Real time quantitative PCR. *Genome Research, 6*(10), 986-994.

Hein, S., Block, T., Zimmermann, R., Kostin, S., Scheffold, T., Kubin, T., Klövekorn, W. & Schaper, J. (2009). Deposition of nonsarcomeric alpha-actinin in cardiomyocytes from patients with dilated cardiomyopathy or chronic pressure overload. *Experimental & Clinical Cardiology, 14*(3), e68-75.

Helen, V. E., John, D. S. & George, S. B. (2012). The A-kinase-anchoring protein AKAP-lbc facilitates cardioprotective PKA phosphorylation of Hsp20 on Ser16. *Biochemical Journal, 446*(3), 437-443.

Heling, A., Zimmermann, R., Kostin, S., Maeno, Y., Hein, S., Devaux, B., Bauer, E., Klövekorn, W., Schlepper, M. & Schaper, W. (2000). Increased expression of cytoskeletal, linkage, and extracellular proteins in failing human myocardium. *Circulation Research, 86*(8), 846-853.

Henzel, W. J., Billeci, T. M., Stults, J. T., Wong, S. C., Grimley, C. & Watanabe, C. (1993). Identifying proteins from two-dimensional gels by molecular mass

searching of peptide fragments in protein sequence databases. *Proceedings of the National Academy of Sciences USA, 90*(11), 5011-5015.

Herbert, J., Augereau, J., Gleye, J. & Maffrand, J. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochemical and Biophysical Research Communications, 172*(3), 993-999.

Hescheler, J., Meyer, R., Plant, S., Krautwurst, D., Rosenthal, W. & Schultz, G. (1991). Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. *Circulation Research, 69*(6), 1476-1486.

Hetts, S. W. (1998). To die or not to die. *JAMA: The Journal of the American Medical Association, 279*(4), 300-307.

Hitomi, K., Ikeda, N. & Maki, M. (2003). Immunological detection of proteolytically activated epidermal-type transglutaminase (TGase 3) using cleavage-site-specific antibody. *Bioscience, Biotechnology, and Biochemistry, 67*(11), 2492-2494.

Hitomi, K., Horio, Y., Ikura, K., Yamanishi, K. & Maki, M. (2001). Analysis of epidermal-type transglutaminase (TGase 3) expression in mouse tissues and cell lines. *The International Journal of Biochemistry & Cell Biology, 33*(5), 491-498.

Hoffman, B. & Liebermann, D. (2008). Apoptotic signaling by c-MYC. *Oncogene, 27*(50), 6462-6472.

Hoffner, G. & Djian, P. (2005). Transglutaminase and diseases of the central nervous system. *Front Biosci, 10*, 3078-3092.

Holvoet, P., Mertens, A., Verhamme, P., Bogaerts, K., Beyens, G., Verhaeghe, R., Collen, D., Muls, E. & Van de Werf, F. (2001). Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. *Arteriosclerosis, Thrombosis, and Vascular Biology, 21*(5), 844-848.

Hong, F., Kwon, S. J., Jhun, B. S., Kim, S. S., Ha, J., Kim, S., Sohn, N.W., Kang, C. & Kang, I. (2001). Insulin-like growth factor-1 protects H9c2 cardiac myoblasts from oxidative stress-induced apoptosis via phosphatidylinositol 3-kinase and extracellular signal-regulated kinase pathways. *Life Sciences, 68*(10), 1095-1105.

Hsu, C., Morohashi, Y., Yoshimura, S., Manrique-Hoyos, N., Jung, S., Lauterbach, M. A., Bakhti, M., Grønborg, M., Möbius, W. & Rhee, J. (2010). Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A–C. *The Journal of Cell Biology, 189*(2), 223-232.

Hughes, S. J., Hollingsworth, M. & Elliott, K. R. (1997). The role of a cAMP-dependent pathway in the uterine relaxant action of relaxin in rats. *Journal of Reproduction and Fertility, 109*(2), 289-296.

Huang, C. & Cabot, M. (1990). Phorbol diesters stimulate the accumulation of phosphatidate, phosphatidylethanol, and diacylglycerol in three cell types. evidence for the indirect formation of phosphatidylcholine-derived diacylglycerol

by a phospholipase D pathway and direct formation of diacylglycerol by a phospholipase C pathway. *Journal of Biological Chemistry, 265*(25), 14858-14863.

Huang, Y., Lin, L., Yu, X., Wen, G., Pu, X., Zhao, H., Fang, C., Zhu, J., Ye, S. & Zhang, L. (2013). Functional involvements of heterogeneous nuclear ribonucleoprotein A1 in smooth muscle differentiation from stem cells in vitro and in vivo. *Stem Cells, 31*(5), 906-917.

Huberman, E., Weeks, C., Herrmann, A., Callaham, M. & Slaga, T. (1981). Alterations in polyamine levels induced by phorbol diesters and other agents that promote differentiation in human promyelocytic leukemia cells. *Proceedings of the National Academy of Sciences USA, 78*(2), 1062-1066.

Hughes, S. J., Hollingsworth, M. & Elliott, K. R. (1997). The role of a cAMP-dependent pathway in the uterine relaxant action of relaxin in rats. *Journal of Reproduction and Fertility, 109*(2), 289-296.

Hunter, I., Sigmundsson, K., Beauchemin, N. & Öbrink, B. (1998). The cell adhesion molecule C-CAM is a substrate for tissue transglutaminase. *FEBS Letters, 425*(1), 141-144.

Huston, R. & Krebs, E. (1968). Activation of skeletal muscle phosphorylase kinase by calcium ions. II. identification of the kinase activating factor as a proteolytic enzyme. *Biochemistry, 7*(6), 2116-2122.

Hwang, K. C., Gray, C. D., Sweet, W. E., Moravec, C. S. & Im, M. J. (1996). {Alpha} 1-adrenergic receptor coupling with gh in the failing human heart. *Circulation, 94*(4), 718-726.

Ichinose, A., Bottenus, R. E. & Davie, E. W. (1990). Structure of transglutaminases. *The Journal of Biological Chemistry, 265*(23), 13411-13414.

Ichinose, A., Hendrickson, L. E., Fujikawa, K. & Davie, E. W. (1986). Amino acid sequence of the a subunit of human factor XIII. *Biochemistry, 25*(22), 6900-6906.

Ientile, R., Caccamo, D. & Griffin, M. (2007). Tissue transglutaminase and the stress response. *Amino Acids, 33*(2), 385-394.

Igarashi, K. & Kashiwagi, K. (2000). Polyamines: Mysterious modulators of cellular functions. *Biochemical and Biophysical Research Communications, 271*(3), 559-564.

Iismaa, S. E., Mearns, B. M., Lorand, L. & Graham, R. M. (2009). Transglutaminases and disease: Lessons from genetically engineered mouse models and inherited disorders. *Physiological Reviews, 89*(3), 991-1023.

Ikura, K., Nasu, T., Yokota, H., Tsuchiya, Y., Sasaki, R. & Chiba, H. (1988). Amino acid sequence of guinea pig liver transglutaminase from its cDNA sequence. *Biochemistry, 27*(8), 2898-2905.

Ikura, K., Yu, C., Nagao, M., Sasaki, R., Furuyoshi, S. & Kawabata, N. (1995). Site-directed mutation in conserved anionic regions of guinea pig liver transglutaminase. *Archives of Biochemistry and Biophysics, 318*(2), 307-313.

Iliodromitis, E. K., Lazou, A. & Kremastinos, D. T. (2007). Ischemic preconditioning: Protection against myocardial necrosis and apoptosis. *Vascular Health and Risk Management, 3*(5), 629-637.

Inagaki, K., Hahn, H. S., Dorn, G. W. & Mochly-Rosen, D. (2003). Additive protection of the ischemic heart ex vivo by combined treatment with δ-protein kinase C inhibitor and ε-protein kinase C activator. *Circulation, 108*(7), 869-875.

Inserte, J., Garcia-Dorado, D., Ruiz-Meana, M., Agulló, L., Pina, P. & Soler-Soler, J. (2004). Ischemic preconditioning attenuates calpain-mediated degradation of structural proteins through a protein kinase A-dependent mechanism. *Cardiovascular Research, 64*(1), 105-114.

Islamovic, E., Duncan, A., Bers, D. M., Gerthoffer, W. T. & Mestril, R. (2007). Importance of small heat shock protein 20 (Hsp20) C-terminal extension in cardioprotection. *Journal of Molecular and Cellular Cardiology, 42*(4), 862-869.

Ismail, N., & Peden, E. K. (2011). *Atherectomy and plaque analysis. In: Mark, G. D., Alan, B. L., William, E. K. &, Ioannis, K. (Eds.), Pumps and Pipes: Proceedings of the Annual Conference*, 99-104, New York, NY: Springer.

Issaq, H. J. & Veenstra, T. D. (2008). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): Advances and perspectives. *BioTechniques, 44*(5), 697-698.

Itoh, M., Kawamoto, T., Tatsukawa, H., Kojima, S., Yamanishi, K. & Hitomi, K. (2011). In situ detection of active transglutaminases for keratinocyte type (TGase 1) and tissue type (TGase 2) using fluorescence-labelled highly reactive substrate peptides. *Journal of Histochemistry & Cytochemistry, 59*(2), 180-187.

Ivaska, J., Vuoriluoto, K., Huovinen, T., Izawa, I., Inagaki, M. & Parker, P. J. (2005). PKCϵ-mediated phosphorylation of vimentin controls integrin recycling and motility. *The EMBO Journal, 24*(22), 3834-3845.

Iwai, N., Shimoike, H. & Kinoshita, M. (1995). Genes up-regulated in hypertrophied ventricle. *Biochemical and Biophysical Research Communications, 209*(2), 527-534.

Iwasaki, W., Nagata, K., Hatanaka, H., Inui, T., Kimura, T., Muramatsu, T., Yoshida, K., Tasumi, M. & Inagaki, F. (1997). Solution structure of midkine, a new heparin-binding growth factor. *The EMBO Journal, 16*(23), 6936-6946.

Izawa, T., Fukata, Y., Kimura, T., Iwamatsu, A., Dohi, K. & Kaibuchi, K. (2000). Elongation factor-1α is a novel substrate of rho-associated kinase. *Biochemical and Biophysical Research Communications, 278*(1), 72-78.

Jacquier-Sarlin, M. R., Jornot, L. & Polla, B. S. (1995). Differential expression and regulation of Hsp 70 and Hsp90 by phorbol esters and heat shock. *Journal of Biological Chemistry, 270*(23), 14094-14099.

Janiak, A., Zemskov, E. A. & Belkin, A. M. (2006). Cell surface transglutaminase promotes RhoA activation via integrin clustering and suppression of the src-p190RhoGAP signaling pathway. *Molecular Biology of the Cell, 17*(4), 1606-1619.

Jans, R., Sturniolo, M. T. & Eckert, R. L. (2007). Localization of the TIG3 transglutaminase interaction domain and demonstration that the amino-terminal region is required for TIG3 function as a keratinocyte differentiation regulator. *Journal of Investigative Dermatology, 128*(3), 517-529.

Jeitner, T. M., Muma, N. A., Battaile, K. P. & Cooper, A. J. (2009). Transglutaminase activation in neurodegenerative diseases. *Future Neurology, 4*(4), 449-467.

Jeon, J. H., Lee, H. J., Jang, G. Y., Kim, C. W., Shin, D. M., Cho, S. Y., Yeo, E. J., Park, S. C. & Kim, I. G. (2004). Different inhibition characteristics of intracellular transglutaminase activity by cystamine and cysteamine. *Experimental & Molecular Medicine, 36*(6), 576-581.

Jeon, J., Kim, C., Shin, D., Kim, K., Cho, S., Kwon, J., Choi, K., Kang, H. & Kim, I. (2003). Differential incorporation of biotinylated polyamines by transglutaminase 2. *FEBS Letters, 534*(1), 180-184.

Jeon, W. M., Lee, K. N., Birckbichler, P. J., Conway, E. & Patterson Jr, M. K. (1989). Colorimetric assay for cellular transglutaminase. *Analytical Biochemistry, 182*(1), 170-175.

Jeon, J. H. & Kim, I. G. (2006). Role of protein modifications mediated by transglutaminase 2 in human viral diseases. *Frontiers in Bioscience : A Journal and Virtual Library, 11*, 221-231.

Jiang, L., He, L. & Fountoulakis, M. (2004). Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *Journal of Chromatography A, 1023*(2), 317-320.

Jiang, W. G., Ablin, R. J., Kynaston, H. G. & Mason, M. D. (2009). The prostate transglutaminase (TGase-4, TGaseP) regulates the interaction of prostate cancer and vascular endothelial cells, a potential role for the ROCK pathway. *Microvascular Research, 77*(2), 150-157.

Jin, C., Yang, Y., Huang, H., Kai, M. & Lee, M. (2010). Mechanisms of L-DOPA-induced cytotoxicity in rat adrenal pheochromocytoma cells: Implication of oxidative stress-related kinases and cyclic AMP. *Neuroscience, 170*(2), 390-398.

Jin, X., Stamnaes, J., Klöck, C., DiRaimondo, T. R., Sollid, L. M. & Khosla, C. (2011). Activation of extracellular transglutaminase 2 by thioredoxin. *Journal of Biological Chemistry, 286*(43), 37866-37873.

John, E. (2011). Guyton and hall textbook of medical physiology. Jackson, Mississippi. Elsevier Health Sciences.

Johnson, D. E. (2013). *Cell death signaling in cancer biology and treatment*. doi: 10.1007/978-1-4614-5847-0.

Johnson, K. B., Petersen-Jones, H., Thompson, J. M., Hitomi, K., Itoh, M., Bakker, E. N., Johnson, G. V., Colak, G. & Watts, S. W. (2012). Vena cava and aortic smooth muscle cells express transglutaminases 1 and 4 in addition to transglutaminase 2. *American Journal of Physiology-Heart and Circulatory Physiology, 302*(7), H1355-H1366.

Johnson, T. S., El-Koraie, A. F., Skill, N. J., Baddour, N. M., El Nahas, A. M., Njloma, M., Adam, A. G. & Griffin, M. (2003). Tissue transglutaminase and the progression of human renal scarring. *Journal of the American Society of Nephrology, 14*(8), 2052-2062.

Jones, S. P., Girod, W. G., Marotti, K. R., Aw, T. Y. & Lefer, D. J. (2001). Acute exposure to a high cholesterol diet attenuates myocardial ischemia-reperfusion injury in cholesteryl ester transfer protein mice. *Coronary Artery Disease, 12*(1), 37-44.

Josse, D., Lockridge, O., Xie, W., Bartels, C. F., Schopfer, L. M. & Masson, P. (2001). The active site of human paraoxonase (PON1). *Journal of Applied Toxicology, 21*(1), S7-S11.

Kaartinen, M. T., El-Maadawy, S., Räsänen, N. H. & McKee, M. D. (2002). Tissue transglutaminase and its substrates in bone. *Journal of Bone and Mineral Research, 17*(12), 2161-2173.

Kahlem, P., Terre, C., Green, H. & Djian, P. (1996). Peptides containing glutamine repeats as substrates for transglutaminase-catalyzed cross-linking: Relevance to diseases of the nervous system. *Proceedings of the National Academy of Sciences USA, 93*(25), 14580-14585.

Kalinin, A. E., Kajava, A. V. & Steinert, P. M. (2002). Epithelial barrier function: Assembly and structural features of the cornified cell envelope. *BioEssays, 24*(9), 789-800.

Kalinin, A., Marekov, L. N. & Steinert, P. M. (2001). Assembly of the epidermal cornified cell envelope. *Journal of Cell Science, 114*(17), 3069-3070.

Kamata, H., Tanaka, C., Yagisawa, H. & Hirata, H. (1996). Nerve growth factor and forskolin prevent H2O2-induced apoptosis in PC12 cells by glutathione independent mechanism. *Neuroscience Letters, 212*(3), 179-182.

Kang, S. K., Kim, D. K., Damron, D. S., Baek, K. J. & Im, M. J. (2002). Modulation of intracellular $Ca^{2+}$ via [alpha] 1B-adrenoreceptor signaling molecules, G [alpha] h (transglutaminase II) and phospholipase C-[delta] 1. *Biochemical and Biophysical Research Communications, 293*(1), 383-390.

Karpuj, M. V., Becher, M. W. & Steinman, L. (2002). Evidence for a role for transglutaminase in Huntington's disease and the potential therapeutic implications. *Neurochemistry International, 40*(1), 31-36.

Kasashima, K., Sumitani, M., Satoh, M. & Endo, H. (2008). Human prohibitin 1 maintains the organization and stability of the mitochondrial nucleoids. *Experimental Cell Research, 314*(5), 988-996.

Kaufmann, S. H. & Hengartner, M. O. (2001). Programmed cell death: Alive and well in the new millennium. *Trends in Cell Biology, 11*(12), 526-534.

Kaufmann, S. H., Ewing, C. M. & Shaper, J. H. (1987). The erasable western blot. *Analytical Biochemistry, 161*(1), 89-95.

Kettleworth, C. R. (2007). *Cell apoptosis research advances.* New York. Nova Biomedical Book.

Kilter, H., Werner, M., Roggia, C., Reil, J., Schäfers, H., Kintscher, U. & Böhm, M. (2009). The PPAR-γ agonist rosiglitazone facilitates akt rephosphorylation and inhibits apoptosis in cardiomyocytes during hypoxia/reoxygenation. *Diabetes, Obesity and Metabolism, 11*(11), 1060-1067.

Kim, D. S., Park, S. S., Nam, B. H., Kim, I. H. & Kim, S. Y. (2006). Reversal of drug resistance in breast cancer cells by transglutaminase 2 inhibition and nuclear factor-κB inactivation. *Cancer Research, 66*(22), 10936-10943.

Kim, H., Lewis, M., Gorman, J., Park, S. C., Girard, J., Folk, J. & Chung, S. (1990). Protransglutaminase E from guinea pig skin. isolation and partial characterization. *Journal of Biological Chemistry, 265*(35), 21971-21978.

Kim, H., Nakamura, F., Lee, W., Hong, C., Pérez-Sala, D. & McCulloch, C. A. (2010). Regulation of cell adhesion to collagen via β1 integrins is dependent on interactions of filamin A with vimentin and protein kinase C epsilon. *Experimental Cell Research, 316*(11), 1829-1844.

Kim, H., Yoon, S. C., Lee, T. Y. & Jeong, D. (2009). Discriminative cytotoxicity assessment based on various cellular damages. *Toxicology Letters, 184*(1), 13-17.

Kim, I., Gorman, J., Park, S., Chung, S. & Steinert, P. (1993). The deduced sequence of the novel protransglutaminase E (TGase3) of human and mouse. *Journal of Biological Chemistry, 268*(17), 12682-12690.

Kim, S. O. (2009). Cell surface tissue transglutaminase-induced activation of phosphoinositol 3-Kinase/Akt pathway. *Korean Journal of Physical Anthropology, 22*(3), 245-254.

Kim, S. Y., Jeitner, T. M. & Steinert, P. M. (2002). Transglutaminases in disease. *Neurochemistry International, 40*(1), 85-103.

Kim, S., Kim, K., Ahn, E., Yoo, B. C. & Kim, S. (2013). Depletion of cathepsin D by transglutaminase 2 through protein cross-linking promotes cell survival. *Amino Acids, 44*(1), 73-80.

Kim, Y., Park, K., Song, J., Shim, T., Islam, K. N., Bae, J., Kim, S., Lee, S., Hwang, K. & Kim, D. (2012). The PPARγ agonist protects cardiomyocytes from oxidative stress and apoptosis via thioredoxin overexpression. *Bioscience, Biotechnology, and Biochemistry, 76*(12), 2181-2187.

Kimes, B. & Brandt, B. (1976). Properties of a clonal muscle cell line from rat heart. *Experimental Cell Research, 98*(2), 367-381.

Király, R., Csősz, É., Kurtán, T., Antus, S., Szigeti, K., Simon-Vecsei, Z., Korponay-Szabó, I. R., Keresztessy, Z. & Fésüs, L. (2009). Functional significance of five noncanonical $Ca^{2+}$-binding sites of human transglutaminase 2 characterized by site-directed mutagenesis. *FEBS Journal, 276*(23), 7083-7096.

Kiritsy, C. P. & Lynch, S. E. (1993). Role of growth factors in cutaneous wound healing: A review. *Critical Reviews in Oral Biology & Medicine, 4*(5), 729-760.

Klabunde, R. (2011). *Cardiovascular physiology concepts (2nd Ed)*. Phlidilphia PA. Lippincott Williams & Wilkins.

Kleizen, B. & Braakman, I. (2004). Protein folding and quality control in the endoplasmic reticulum. *Current Opinion in Cell Biology, 16*(4), 343-349.

Kloner, R. A. & Jennings, R. B. (2001). Consequences of brief ischemia: Stunning, preconditioning, and their clinical implications. *Circulation, 104*(25), 3158-3167.

Knight, R. L., Hand, D., Piacentini, M. & Griffin, M. (1993). Characterization of the transglutaminase-mediated large molecular weight polymer from rat liver; its relationship to apoptosis. *European Journal of Cell Biology, 60*(1), 210-216.

Kobayashi, Y. & Yonehara, S. (2008). Novel cell death by downregulation of eEF1A1 expression in tetraploids. *Cell Death & Differentiation, 16*(1), 139-150.

Koekemoer, A. L., Chong, N. W., Goodall, A. H. & Samani, N. J. (2009). Myocyte stress 1 plays an important role in cellular hypertrophy and protection against apoptosis. *FEBS Letters, 583*(17), 2964-2967.

Kojima, S., Inui, T., Muramatsu, H., Suzuki, Y., Kadomatsu, K., Yoshizawa, M., Hirose, S., Kimura, T., Sakakibara, S. & Muramatsu, T. (1997). Dimerization of midkine by tissue transglutaminase and its functional implication. *Journal of Biological Chemistry, 272*(14), 9410-9416.

Kong, K. Y. & Kedes, L. (2004). Cytoplasmic nuclear transfer of the actin-capping protein tropomodulin. *Journal of Biological Chemistry, 279*(29), 30856-30864.

Koning, F., Schuppan, D., Cerf-Bensussan, N. & Sollid, L. M. (2005). Pathomechanisms in coeliac disease. *Best Practice & Research Clinical Gastroenterology, 19*(3), 373-387.

Koseki-Kuno, S., Yamakawa, M., Dickneite, G. & Ichinose, A. (2003). Factor XIII A subunit-deficient mice developed severe uterine bleeding events and subsequent spontaneous miscarriages. *Blood, 102*(13), 4410-4412.

Kotsakis, P. & Griffin, M. (2007). Tissue transglutaminase in tumour progression: Friend or foe? *Amino Acids, 33*(2), 373-384.

Kudo, M., Wang, Y., Xu, M., Ayub, A. & Ashraf, M. (2002). Adenosine A1 receptor mediates late preconditioning via activation of PKC-δ signaling pathway. *American Journal of Physiology-Heart and Circulatory Physiology, 283*(1), H296-H301.

Kuno, A., Critz, S. D., Cui, L., Solodushko, V., Yang, X., Krahn, T., Albrecht, B., Philipp, S., Cohen, M. V. & Downey, J. M. (2007). Protein kinase C protects preconditioned rabbit hearts by increasing sensitivity of adenosine A2b-dependent signaling during early reperfusion. *Journal of Molecular and Cellular Cardiology, 43*(3), 262-271.

Kunst, G., Kress, K. R., Gruen, M., Uttenweiler, D., Gautel, M. & Fink, R. H. (2000). Myosin binding protein C, a phosphorylation-dependent force regulator in muscle that controls the attachment of myosin heads by its interaction with myosin S2. *Circulation Research, 86*(1), 51-58.

Kurose, I., Wolf, R., Cerwinka, W. & Granger, D. N. (1999). Microvascular responses to ischemia/reperfusion in normotensive and hypertensive rats. *Hypertension, 34*(2), 212-216.

Kurose, H., Nishida, M. & Nagao, T. (1999). Beta gamma subunit of heterotrimeric G protein as a new target molecule for drug development. *Nihon Yakurigaku Zasshi.Folia Pharmacologica Japonica, 114*(1), 75-80.

Kwak, H., Park, K., Choi, H., Chung, K., Lim, H. & Park, H. (2008). PDE4 inhibitor, roflumilast protects cardiomyocytes against NO-induced apoptosis via activation of PKA and epac dual pathways. *Cellular Signalling, 20*(5), 803-814.

Kweon, S., Lee, Z., Yi, S., Kim, Y., Han, J., Paik, S. & Ha, K. (2004). Protective role of tissue transglutaminase in the cell death induced by TNF-alpha in SH-SY5Y neuroblastoma cells. *Journal of Biochemistry and Molecular Biology, 37*(2), 185-191.

Kwon, S. H., Pimentel, D. R., Remondino, A., Sawyer, D. B. & Colucci, W. S. (2003). $H_2O_2$ regulates cardiac myocyte phenotype via concentration-dependent activation of distinct kinase pathways. *Journal of Molecular and Cellular Cardiology, 35*(6), 615-621.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680-685.

Lagast, H., Pozzan, T., Waldvogel, F. & Lew, P. (1984). Phorbol myristate acetate stimulates ATP-dependent calcium transport by the plasma membrane of neutrophils. *Journal of Clinical Investigation, 73*(3), 878-883.

Lai, T. S., Bielawska, A., Peoples, K. A., Hannun, Y. A. & Greenberg, C. S. (1997). Sphingosylphosphocholine reduces the calcium ion requirement for activating tissue transglutaminase. *Journal of Biological Chemistry, 272*(26), 16295-16300.

Lajemi, M., Demignot, S., Borge, L., Thenet-Gauci, S. & Adolphe, M. (1997). The use of fluoresceincadaverine for detecting amine acceptor protein substrates accessible to active transglutaminase in living cells. *The Histochemical Journal, 29*(8), 593-606.

Lallemend, F., Hadjab, S., Hans, G., Moonen, G., Lefebvre, P. P. & Malgrange, B. (2005). Activation of protein kinase CβI constitutes a new neurotrophic pathway for deafferented spiral ganglion neurons. *Journal of Cell Science, 118*(19), 4511-4525.

Lanson, N. A.,Jr, Glembotski, C. C., Steinhelper, M. E., Field, L. J. & Claycomb, W. C. (1992). Gene expression and atrial natriuretic factor processing and secretion in cultured AT-1 cardiac myocytes. *Circulation, 85*(5), 1835-1841.

Lazou, A., Fuller, S. J., Bogoyevitch, M. A., Orfali, K. A. & Sugden, P. H. (1994). Characterization of stimulation of phosphoinositide hydrolysis by alpha 1-adrenergic agonists in adult rat hearts. *American Journal of Physiology-Heart and Circulatory Physiology, 267*(3), H970-H978.

Lazou, A., Sugden, P. H. & Clerk, A. (1998). Activation of mitogen-activated protein kinases (p38-MAPKs, SAPKs/JNKs and ERKs) by the G-protein-coupled receptor agonist phenylephrine in the perfused rat heart. *Biochemical Journal, 332*(2), 459-465.

Lee, J., Kim, Y. S., Choi, D. H., Bang, M. S., Han, T. R., Joh, T. H. & Kim, S. Y. (2004). Transglutaminase 2 induces nuclear factor-κB activation via a novel pathway in BV-2 microglia. *Journal of Biological Chemistry, 279*(51), 53725-53735.

Lee, J. C. (2001). Inhibitory effects of the ethanol extract of ulmus davidiana on apoptosis induced by glucose-glucose oxidase and cytokine production in cultured mouse primary immune cells. *Biochemistry and Molecular Biology Reports, 34*(5), 463-471.

Lee, K. H., Lee, N., Lim, S., Jung, H., Ko, Y. G., Park, H. Y., Jang, Y., Lee, H. & Hwang, K. C. (2003). Calreticulin inhibits the MEK1, 2-ERK1, 2 pathway in [alpha] 1-adrenergic receptor/Gh-stimulated hypertrophy of neonatal rat cardiomyocytes. *The Journal of Steroid Biochemistry and Molecular Biology, 84*(1), 101-107.

Lee, K. N., Arnold, S. A., Birkbichler, P. J., Patterson Jr, M. K., Fraij, B. M., Takeuchi, Y. & Carter, H. A. (1993). Site-directed mutagenesis of human tissue transglutaminase: Cys-277 is essential for transglutaminase activity but not for GTPase activity. *Biochimica Et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 1202*(1), 1-6.

Lee, S., Kim, I., Marekov, L., O'keefe, E., Parry, D. & Steinert, P. (1993). The structure of human trichohyalin. potential multiple roles as a functional EF-hand-like calcium-binding protein, a cornified cell envelope precursor, and an intermediate filament-associated (cross-linking) protein. *Journal of Biological Chemistry, 268*(16), 12164-12176.

Lee, V. M. Y., Goedert, M. & Trojanowski, J. Q. (2001). Neurodegenerative tauopathies. *Annual Review of Neuroscience, 24*(1), 1121-1159.

Lee, Z., Kwon, S., Kim, S., Yi, S., Kim, Y. & Ha, K. (2003). Activation of in situ tissue transglutaminase by intracellular reactive oxygen species. *Biochemical and Biophysical Research Communications, 305*(3), 633-640.

Lefer, D. J. & Granger, D. N. (2000). Oxidative stress and cardiac disease. *The American Journal of Medicine, 109*(4), 315-323.

Lefkowitz, R. (2007). Seven transmembrane receptors: Something old, something new. *Acta Physiologica, 190*(1), 9-19.

Lelli, J. L., Becks, L. L., Dabrowska, M. I. & Hinshaw, D. B. (1998). ATP converts necrosis to apoptosis in oxidant-injured endothelial cells. *Free Radical Biology and Medicine, 25*(6), 694-702.

Lemaire, C., Andréau, K., Souvannavong, V. & Adam, A. (1998). Inhibition of caspase activity induces a switch from apoptosis to necrosis. *FEBS Letters, 425*(2), 266-270.

Lemasters, J. J., & Holmuhamedov, E. (2006). Voltage-dependent anion channel (VDAC) as mitochondrial governator--thinking outside the box. *Biochimica Et Biophysica Acta, 1762*(2), 181-190.

Lennon, S., Martin, S. & Cotter, T. (1991). Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Proliferation, 24*(2), 203-214.

Lesort, M., Tucholski, J., Zhang, J. & Johnson, G. V. (2000). Impaired mitochondrial function results in increased tissue transglutaminase activity in situ. *Journal of Neurochemistry, 75*(5), 1951-1961.

Leung, G. P., Tse, C. & Man, R. Y. (2007). Characterization of adenosine transport in H9c2 cardiomyoblasts. *International Journal of Cardiology, 116*(2), 186-193.

Levine, B. (2005). Eating oneself and uninvited guests: Autophagy-related pathways in cellular defense. *Cell, 120*(2), 159-162.

Li, Q., Bagchi, M. K. & Bagchi, I. C. (2006). Identification of a signaling pathway involving progesterone receptor, calcitonin, and tissue transglutaminase in ishikawa endometrial cells. *Endocrinology, 147*(5), 2147-2154.

Li, Q., Wang, J., Armant, D. R., Bagchi, M. K. & Bagchi, I. C. (2002). Calcitonin down-regulates E-cadherin expression in rodent uterine epithelium during implantation. *Journal of Biological Chemistry, 277*(48), 46447-46455.

Li, X., Wei, X. L., Meng, L. L., Chi, M. G., Yan, J. Q., Ma, X. Y., Jia, Y. S., Liang, L., Yan, H. T. & Zheng, J. Q. (2009). Involvement of tissue transglutaminase in endothelin 1-induced hypertrophy in cultured neonatal rat cardiomyocytes. *Hypertension, 54*(4), 839-844.

Liang, Q., Yu, X., Qu, S., Xu, H. & Sui, D. (2010). Acanthopanax senticosides B ameliorates oxidative damage induced by hydrogen peroxide in cultured neonatal rat cardiomyocytes. *European Journal of Pharmacology, 627*(1), 209-215.

Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H. & Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature, 402*(6762), 672-675.

Libby, P. (2006). Inflammation and cardiovascular disease mechanisms. *The American Journal of Clinical Nutrition, 83*(2), 456S-460S.

Liew, F. M. & Yamanishi, K. (1992). Regulation of transglutaminase 1 gene expression by 12-O-tetradecanoylphorbol-13-acetate, dexamethasone, and retinoic acid in cultured human keratinocytes. *Experimental Cell Research, 202*(2), 310-315.

Lilley, G. R., Skill, J., Griffin, M. & Bonner, P. L. R. (1998). Detection of Ca2 - dependent transglutaminase activity in root and leaf tissue of monocotyledonous and dicotyledonous plants. *Plant Physiology, 117*(3), 1115-1123.

Lin, X., Söderhäll, K. & Söderhäll, I. (2008). Transglutaminase activity in the hematopoietic tissue of a crustacean, pacifastacus leniusculus, importance in hemocyte homeostasis. *BMC Immunology, 9*(1), 58.

Lipp, P., Tovey, S., Burrell, K., Berridge, M., Li, W., & Bootman, M. (2000). Functional InsP3 receptors that may modulate excitation-contraction coupling in the heart. *Current Biology, 10*(15), 939-942.

Liu, G. S., Cohen, M. V., Mochly-Rosen, D. & Downey, J. M. (1999). Protein kinase C-ξ is responsible for the protection of preconditioning in rabbit cardiomyocytes. *Journal of Molecular and Cellular Cardiology, 31*(10), 1937-1948.

Liu, X., Ren, Z., Zhan, R., Wang, X., Wang, X., Zhang, Z., Leng, X., Yang, Z. & Qian, L. (2009). Prohibitin protects against oxidative stress-induced cell injury in cultured neonatal cardiomyocyte. *Cell Stress and Chaperones, 14*(3), 311-319.

Liu, Y., Yang, X. M., Iliodromitis, E. K., Kremastinos, D. T., Dost, T., Cohen, M. V., & Downey, J. M. (2008). Redox signaling at reperfusion is required for protection from ischemic preconditioning but not from a direct PKC activator. *Basic Research in Cardiology, 103*(1), 54-59.

Liu, S., Cerione, R. A. & Clardy, J. (2002). Structural basis for the guanine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity. *Proceedings of the National Academy of Sciences USA, 99*(5), 2743-2747.

Lochner, A., Marais, E., Toit, E. D. & Moolman, J. (2002). Nitric oxide triggers classic ischemic preconditioning. *Annals of the New York Academy of Sciences, 962*(1), 402-414.

Lochner, A. & Moolman, J. (2006). The many faces of H89: A review. *Cardiovascular Drug Reviews, 24*(3-4), 261-274.

Lohse, M. J., Engelhardt, S. & Eschenhagen, T. (2003). What is the role of β-adrenergic signaling in heart failure? *Circulation Research, 93*(10), 896-906.

Longo, G. M., Xiong, W., Greiner, T. C., Zhao, Y., Fiotti, N. & Baxter, B. T. (2002). Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *Journal of Clinical Investigation, 110*(5), 625-632.

Lorand, L. & Conrad, S. M. (1984). Transglutaminases. *Molecular and Cellular Biochemistry, 58*(1), 9-35.

Lorand, L. & Graham, R. M. (2003). Transglutaminases: Crosslinking enzymes with pleiotropic functions. *Nature Reviews Molecular Cell Biology, 4*(2), 140-156.

Lorand, L. (2001). Factor XIII: Structure, activation, and interactions with fibrinogen and fibrin. *Annals of the New York Academy of Sciences, 936*(1), 291-311.

Luciani, A., Villella, V. R., Vasaturo, A., Giardino, I., Raia, V., Pettoello-Mantovani, M., D'Apolito, M., Guido, S., Leal, T. & Quaratino, S. (2009). SUMOylation of tissue transglutaminase as link between oxidative stress and inflammation. *The Journal of Immunology, 183*(4), 2775-2784.

Ludwig, L. M., Weihrauch, D., Kersten, J. R., Pagel, P. S. & Warltier, D. C. (2004). Protein kinase C translocation and src protein tyrosine kinase activation mediate isoflurane-induced preconditioning in vivo: Potential downstream targets of mitochondrial adenosine triphosphate–sensitive potassium channels and reactive oxygen species. *Anesthesiology, 100*(3), 532-539.

Lukyanets, I. A. & Lukyanetz, E. A. (2013). Modulation of calcium signaling by the endoplasmic reticulum in carassius neurons. *Biochemical and Biophysical Research Communications, 433*(4), 591-594.

Lynes, E. M., Bui, M., Yap, M. C., Benson, M. D., Schneider, B., Ellgaard, L., Berthiaume, L. G. & Simmen, T. (2011). Palmitoylated TMX and calnexin target to the mitochondria-associated membrane. *The EMBO Journal, 31*(2), 457-470.

Maccioni, R. B. & Arechaga, J. (1986). Transglutaminase (TG) involvement in early embryogenesis. *Experimental Cell Research, 167*(1), 266-270.

Mackintosh, C. (2004). Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. *Biochemical Journal, 381*(2), 329-342.

Maechler, P. & Wollheim, C. B. (2001). Mitochondrial function in normal and diabetic β-cells. *Nature, 414*(6865), 807-812.

Maiuri, L., Luciani, A., Giardino, I., Raia, V., Villella, V. R., D'Apolito, M., Pettoello-Mantovani, M., Guido, S., Ciacci, C. & Cimmino, M. (2008). Tissue transglutaminase activation modulates inflammation in cystic fibrosis via PPARγ down-regulation. *The Journal of Immunology, 180*(11), 7697-7705.

Maiuri, L., Ciacci, C., Ricciardelli, I., Vacca, L., Raia, V., Rispo, A., Griffin, M., Issekutz, T., Quaratino, S. & Londei, M. (2005). Unexpected role of surface transglutaminase type II in coeliac disease. *Gastroenterology, 129*(5), 1400-1413.

Makaula, S., Lochner, A., Genade, S., Sack, M. N., Awan, M. M. & Opie, L. H. (2005). H-89, a non-specific inhibitor of protein kinase A, promotes post-ischemic cardiac contractile recovery and reduces infarct size. *Journal of Cardiovascular Pharmacology, 45*(4), 341-347.

Malavasi, F., Deaglio, S., Funaro, A., Ferrero, E., Horenstein, A. L., Ortolan, E., Vaisitti, T. & Aydin, S. (2008). Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiological Reviews, 88*(3), 841-886.

Malorni, W., Farrace, M., Matarrese, P., Tinari, A., Ciarlo, L., Mousavi-Shafaei, P., D'Eletto, M., Di Giacomo, G., Melino, G. & Palmieri, L. (2009). The adenine nucleotide translocator 1 acts as a type 2 transglutaminase substrate: Implications for mitochondrial-dependent apoptosis. *Cell Death & Differentiation, 16*(11), 1480-1492.

Mangala, L. S. & Mehta, K. (2005). Tissue transglutaminase (TG2) in cancer biology. *Progress in Experimental Tumor Research, 38*, 125-138.

Mann, M., Ong, S., Grønborg, M., Steen, H., Jensen, O. N. & Pandey, A. (2002). Analysis of protein phosphorylation using mass spectrometry: Deciphering the phosphoproteome. *Trends in Biotechnology, 20*(6), 261-268.

Manning, G., Plowman, G. D., Hunter, T. & Sudarsanam, S. (2002). Evolution of protein kinase signaling from yeast to man. *Trends in Biochemical Sciences, 27*(10), 514-520.

Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. (2002). The protein kinase complement of the human genome. *Science, 298*(5600), 1912-1934.

Mao, C., Lu, H., Kong, N., Li, J., Liu, M., Yang, C. & Yang, P. (2014). Levocarnitine protects H9c2 rat cardiomyocytes from H2O2-induced mitochondrial dysfunction and apoptosis. I*nternational Journal of Medical Sciences, 11*(11), 1107-1115.

Marani, M., Tenev, T., Hancock, D., Downward, J. & Lemoine, N. R. (2002). Identification of novel isoforms of the BH3 domain protein bim which directly activate bax to trigger apoptosis. *Molecular and Cellular Biology, 22*(11), 3577-3589.

Mariniello, L., Esposito, C., Caputo, I., Sorrentino, A. & Porta, R. (2003). N-terminus end of rat prostate transglutaminase is responsible for its catalytic activity and GTP binding. *The International Journal of Biochemistry & Cell Biology, 35*(7), 1098-1108.

Mariniello, L. & Porta, R. (2005). Transglutaminases as biotechnological tools. P*rogress in Experimental Tumor Research, 38*, 174-191.

Maritim, A., Sanders, R. & Watkins, 3. J. (2003). Diabetes, oxidative stress, and antioxidants: A review. *Journal of Biochemical and Molecular Toxicology, 17*(1), 24-38.

Martinet, N., Kim, H. C., Girard, J., Nigra, T., Strong, D. H., Chung, S. & Folk, J. (1988). Epidermal and hair follicle transglutaminases. partial characterization of soluble enzymes in newborn mouse skin. *Journal of Biological Chemistry, 263*(9), 4236-4241.

Masters, C. L. & Beyreuther, K. (2006). Pathways to the discovery of the Aβ amyloid of alzheimer's disease. *Journal of Alzheimer's Disease, 9*(3), 155-161.

Mastroberardino, P. G., Farrace, M. G., Viti, I., Pavone, F., Fimia, G. M., Melino, G., Rodolfo, C. & Piacentini, M. (2006). "Tissue" transglutaminase contributes to the formation of disulphide bridges in proteins of mitochondrial respiratory complexes. *Biochimica Et Biophysica Acta (BBA)-Bioenergetics, 1757*(9), 1357-1365.

Matilla-Dueñas, A., Sánchez, I., Corral-Juan, M., Dávalos, A., Alvarez, R. & Latorre, P. (2010). Cellular and molecular pathways triggering neurodegeneration in the spinocerebellar ataxias. *The Cerebellum, 9*(2), 148-166.

Matysiak-Budnik, T., Moura, I. C., Arcos-Fajardo, M., Lebreton, C., Ménard, S., Candalh, C., Ben-Khalifa, K., Dugave, C., Tamouza, H. & Van Niel, G. (2008). Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in coeliac disease. *The Journal of Experimental Medicine, 205*(1), 143-154.

McConoughey, S. J., Basso, M., Niatsetskaya, Z. V., Sleiman, S. F., Smirnova, N. A., Langley, B. C., Mahishi, L., Cooper, A. J. L., Antonyak, M. A. & Cerione, R. A. (2010). Inhibition of transglutaminase 2 mitigates transcriptional dysregulation in models of Huntington's disease. *EMBO Molecular Medicine, 2*(9), 349-370.

McCully, J. D., Wakiyama, H., Hsieh, Y. J., Jones, M. & Levitsky, S. (2004). Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury. *American Journal of Physiology-Heart and Circulatory Physiology, 286*(5), H1923-H1935.

McDonough, J. L., Arrell, D. K. & Van Eyk, J. E. (1999). Troponin I degradation and covalent complex formation accompanies myocardial ischemia/reperfusion injury. *Circulation Research, 84*(1), 9-20.

McEwan, D. G., Brunton, V. G., Baillie, G. S., Leslie, N. R., Houslay, M. D. & Frame, M. C. (2007). Chemoresistant KM12C colon cancer cells are addicted to low cyclic AMP levels in a phosphodiesterase 4–Regulated compartment via effects on phosphoinositide 3-kinase. *Cancer Research, 67*(11), 5248-5257.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D. & Stadlan, E. M. (1984). Clinical diagnosis of Alzheimer's disease. *Neurology, 34*(7), 939-944.

McLaughlin, B., Hartnett, K. A., Erhardt, J. A., Legos, J. J., White, R. F., Barone, F. C., & Aizenman, E. (2003). Caspase 3 activation is essential for neuroprotection in preconditioning. *Proceedings of the National Academy of Sciences USA, 100*(2), 715-720.

Medzhitov, R. (2010). Inflammation 2010: New adventures of an old flame. *Cell, 140*(6), 771-776.

Meeks, M. K., Han, S., Tucker, A. L. & Rembold, C. M. (2008). Phospholemman does not participate in forskolin-induced swine carotid artery relaxation. *Physiological research/Academia Scientiarum Bohemoslovaca, 57*(5), 669-675.

Mehta, K., Fok, J., Miller, F. R., Koul, D. & Sahin, A. A. (2004). Prognostic significance of tissue transglutaminase in drug resistant and metastatic breast cancer. *Clinical Cancer Research, 10*(23), 8068-8076.

Mehta, K. & Han, A. (2011). Tissue transglutaminase (TG2)-induced inflammation in initiation, progression, and pathogenesis of pancreatic cancer. *Cancers, 3*(1), 897-912.

Melino, G., Annicchiarico-Petruzzelli, M., Piredda, L., Candi, E., Gentile, V., Davies, P. & Piacentini, M. (1994). Tissue transglutaminase and apoptosis: Sense and antisense transfection studies with human neuroblastoma cells. *Molecular and Cellular Biology, 14*(10), 6584-6596.

Melino, G. & Piacentini, M. (1998). Tissue'transglutaminase in cell death: A downstream or a multifunctional upstream effector? *FEBS Letters, 430*(1), 59-63.

Mhaouty-Kodja, S. (2004). Ghα/tissue transglutaminase 2: An emerging G protein in signal transduction. *Biology of the Cell, 96*(5), 363-367.

Milakovic, T., Tucholski, J., McCoy, E. & Johnson, G. V. W. (2004). Intracellular localization and activity state of tissue transglutaminase differentially impacts cell death. *Journal of Biological Chemistry, 279*(10), 8715-8722.

Milhavet, O., Gary, D. S. & Mattson, M. P. (2003). RNA interference in biology and medicine. *Pharmacological Reviews, 55*(4), 629-648.

Miller, C. C. J. & Anderton, B. H. (1986). Transglutaminase and the neuronal cytoskeleton in Alzheimer's disease. *Journal of Neurochemistry, 46*(6), 1912-1922.

Mirza, A., Liu, S., Frizell, E., Zhu, J., Maddukuri, S., Martinez, J., Davies, P., Schwarting, R., Norton, P. & Zern, M. (1997). A role for tissue transglutaminase in hepatic injury and fibrogenesis, and its regulation by NF-kappaB. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 272*(2), G281-G288.

Mishra, S., Melino, G. & Murphy, L. J. (2007). Transglutaminase 2 kinase activity facilitates protein kinase A-induced phosphorylation of retinoblastoma protein. *Journal of Biological Chemistry, 282*(25), 18108-18115.

Mishra, S. & Murphy, L. J. (2004). Tissue transglutaminase has intrinsic kinase activity. *Journal of Biological Chemistry, 279*(23), 23863- 23868.

Mishra, S. & Murphy, L. J. (2006). The p53 oncoprotein is a substrate for tissue transglutaminase kinase activity. *Biochemical and Biophysical Research Communications, 339*(2), 726-730.

Mishra, S., Chander, V., Banerjee, P., Oh, J. G., Lifirsu, E., Park, W. J. Kim, D. H. & Bandyopadhyay, A. (2011). Interaction of annexin A6 with alpha actinin in cardiomyocytes. *BMC Cell Biology, 12*(1), 7-8.

Mishra, S. & Murphy, L. J. (2006). Phosphorylation of transglutaminase 2 by PKA at Ser216 creates 14-3-3 binding sites. *Biochemical and Biophysical Research Communications, 347*(4), 1166-1170.

Mishra, S. & Murphy, L. J. (2004). Tissue transglutaminase has intrinsic kinase activity: Identification of transglutaminase 2 as an insulin-like growth factor-binding protein-3 kinase. *Journal of Biological Chemistry, 279*(23), 23863-23868.

Mishra, S., Melino, G. & Murphy, L. J. (2007). Transglutaminase 2 kinase activity facilitates protein kinase A-induced phosphorylation of retinoblastoma protein. *Journal of Biological Chemistry, 282*(25), 18108-18115.

Mishra, S., Saleh, A., Espino, P. S., Davie, J. R. & Murphy, L. J. (2006). Phosphorylation of histones by tissue transglutaminase. *Journal of Biological Chemistry, 281*(9), 5532-5538.

Misra, A., Haudek, S. B., Knuefermann, P., Vallejo, J. G., Chen, Z. J., Michael, L. H., Sivasubramanian, N., Olson, E. N., Entman, M. L. & Mann, D. L. (2003). Nuclear factor-κB protects the adult cardiac myocyte against ischemia-induced apoptosis in a murine model of acute myocardial infarction. *Circulation, 108*(25), 3075-3078.

Mochizuki, Y. & Furukawa, K. (1987). Application of Coomassie brilliant blue staining to cultured hepatocytes. *Cell Biology International Reports, 11*(5), 367-371.

Modica-Napolitano, J. S. & Singh, K. (2002). Mitochondria as targets for detection and treatment of cancer. *Expert Reviews in Molecular Medicine, 4*(09), 1-19.

Molberg, Ø., McAdam, S. N. & Sollid, L. M. (2000). Role of tissue transglutaminase in coeliac disease. *Journal of Pediatric Gastroenterology and Nutrition, 30*(3), 232-240.

Monsonego, A., Shani, Y., Friedmann, I., Paas, Y., Eizenberg, O. & Schwartz, M. (1997). Expression of GTP-dependent and GTP-independent tissue-type transglutaminase in cytokine-treated rat brain astrocytes. *Journal of Biological Chemistry, 272*(6), 3724-3732.

Morimoto, C., Kameda, K., Tsujita, T. & Okuda, H. (2001). Relationships between lipolysis induced by various lipolytic agents and hormone-sensitive lipase in rat fat cells. *Journal of Lipid Research, 42*(1), 120-127.

Morshauser, R. C., Hu, W., Wang, H., Pang, Y., Flynn, G. C. & Zuiderweg, E. R. (1999). High-resolution solution structure of the 18 kDa substrate-binding domain of the mammalian chaperone protein Hsc70. *Journal of Molecular Biology, 289*(5), 1387-1403.

Mosher, D. F. (1984). Cross-linking of fibronectin to collagenous proteins. In *Transglutaminase* (pp. 63-68). Springer US.

Moss, S. E. & Morgan, R. O. (2004). The annexins. *Genome Biology, 5*(4), 219.

Mouro-Chanteloup, I., Delaunay, J., Gane, P., Nicolas, V., Johansen, M., Brown, E. J., Peters, L. L., Le Van Kim, C., Cartron, J. P. & Colin, Y. (2003). Evidence that the red cell skeleton protein 4.2 interacts with the rh membrane complex member CD47. *Blood, 101*(1), 338-344.

Mubagwa, K. & Flameng, W. (2001). Adenosine, adenosine receptors and myocardial protection an updated overview. *Cardiovascular Research, 52*(1), 25-39.

Mudry, R. E., Perry, C. N., Richards, M., Fowler, V. M. & Gregorio, C. C. (2003). The interaction of tropomodulin with tropomyosin stabilizes thin filaments in cardiac myocytes. *The Journal of Cell Biology, 162*(6), 1057-1068.

Munezane, T., Hasegawa, T., Tanaka, A., Okada, K. & Okita, Y. (2010). Activation of transglutaminase type 2 for aortic wall protection in a rat abdominal aortic aneurysm formation. *Journal of Vascular Surgery, 52*(4), 967-974.

Muraguchi, T., Kawawa, A. & Kubota, S. (2010). Prohibitin protects against hypoxia-induced H9c2 cardiomyocyte cell death. *Biomedical Research, 31*(2), 113-122.

Murry, C. E., Jennings, R. B. & Reimer, K. A. (1986). Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation, 74*(5), 1124-1136.

Mycek, M., Clarke, D., Neidle, A. & Waelsch, H. (1959). Amine incorporation into insulin as catalyzed by transglutaminase. *Archives of Biochemistry and Biophysics, 84*(2), 528-540.

Myhill, N., Lynes, E. M., Nanji, J. A., Blagoveshchenskaya, A. D., Fei, H., Simmen, K. C., Cooper, T. J., Thomas, G. & Simmen, T. (2008). The subcellular distribution of calnexin is mediated by PACS-2. *Molecular Biology of the Cell, 19*(7), 2777-2788.

Nadalutti, C. A., Korponay-Szabo, I. R., Kaukinen, K., Wang, Z., Griffin, M., Mäki, M., & Lindfors, K. (2013). Thioredoxin is involved in endothelial cell extracellular transglutaminase 2 activation mediated by coeliac disease patient IgA. *PloS One, 8*(10), e77277.

Nadalutti, C., Viiri, K. M., Kaukinen, K., Maki, M. & Lindfors, K. (2011). Extracellular transglutaminase 2 has a role in cell adhesion, whereas intracellular transglutaminase 2 is involved in regulation of endothelial cell proliferation and apoptosis. *Cell Prolif, 44*(1), 49-58.

Nagy, L., Thomazy, V. A., Saydak, M. M., Stein, J. P. & Davies, P. J. (1997). The promoter of the mouse tissue transglutaminase gene directs tissue-specific, retinoid-regulated and apoptosis-linked expression. *Cell Death and Differentiation, 4*(7), 534-547.

Nakano, A., Baines, C. P., Kim, S. O., Pelech, S. L., Downey, J. M., Cohen, M. V., & Critz, S. D. (2000). Ischemic preconditioning activates MAPKAPK2 in the isolated rabbit heart evidence for involvement of p38 MAPK. *Circulation Research, 86*(2), 144-151.

Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M. J. & Graham, R. M. (1994). Gh: A GTP-binding protein with transglutaminase activity and receptor signaling function. *Science, 264*(5165), 1593-1596.

Nanda, N., Iismaa, S. E., Owens, W. A., Husain, A., Mackay, F. & Graham, R. M. (2001). Targeted inactivation of Gh/tissue transglutaminase II. *Journal of Biological Chemistry, 276*(23), 20673-20678.

Nanzer, A. M., Khalaf, S., Mozid, A. M., Fowkes, R. C., Patel, M. V., Burrin, J. M., Grossman, A. B. & Korbonits, M. (2004). Ghrelin exerts a proliferative effect on a rat pituitary somatotroph cell line via the mitogen-activated protein kinase pathway. *European Journal of Endocrinology, 151*(2), 233-240.

Nardacci, R., Lo Iacono, O., Ciccosanti, F., Falasca, L., Addesso, M., Amendola, A., Antonucci, G., Craxì, A., Fimia, G. M. & Iadevaia, V. (2003). Transglutaminase type II plays a protective role in hepatic injury. *The American Journal of Pathology, 162*(4), 1293-1303.

Nemes Jr, Z., Friis, R., Aeschlimann, D., Saurer, S., Paulsson, M. & Fésüs, L. (1996). Expression and activation of tissue transglutaminase in apoptotic cells of involuting rodent mammary tissue. *European Journal of Cell Biology, 70*(2), 125-133.

Nemes, Z., Adány, R., Balázs, M., Boross, P. & Fésüs, L. (1997). Identification of cytoplasmic actin as an abundant glutaminyl substrate for tissue transglutaminase in HL-60 and U937 cells undergoing apoptosis. *Journal of Biological Chemistry, 272*(33), 20577-20583.

Nicholas, B., Smethurst, P., Verderio, E., Jones, R. & Griffin, M. (2003). Cross-linking of cellular proteins by tissue transglutaminase during necrotic cell death: A mechanism for maintaining tissue integrity. *Biochemical Journal, 371*(2), 413-422.

Nicholson, D. (1999). Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death and Differentiation, 6*(11), 1028-1042.

Niggli, V., Djafarzadeh, S. & Keller, H. (1999). Stimulus-induced selective association of actin-associated proteins (α-actinin) and protein kinase C isoforms with the cytoskeleton of human neutrophils. *Experimental Cell Research, 250*(2), 558-568.

Nijtmans, L., Sanz, M. A., Grivell, L. & Coates, P. (2002). The mitochondrial PHB complex: Roles in mitochondrial respiratory complex assembly, ageing and degenerative disease. *Cellular and Molecular Life Sciences CMLS, 59*(1), 143-155.

Nirmalan, N., Sims, P. F. & Hyde, J. E. (2004). Quantitative proteomics of the human malaria parasite plasmodium falciparum and its application to studies of development and inhibition. *Molecular Microbiology, 52*(4), 1187-1199.

Nishizuka, Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. *The FASEB Journal, 9*(7), 484-496.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. *Science, 233*(4761), 305-312.

Noguchi, T., Hünlich, M., Camp, P. C., Begin, K. J., El-Zaru, M., Patten, R., Leavitt, B. J., Ittleman, F. P., Alpert, N. R. & LeWinter, M. M (2004). Thin filament-based modulation of contractile performance in human heart failure. *Circulation, 110*(8), 982-987.

Nurminskaya, M. V. & Belkin, A. M. (2012). Cellular functions of tissue transglutaminase. *International Review of Cell and Molecular Biology, 294*, 1-97.

Okura, T., Gong, L., Kamitani, T., Wada, T., Okura, I., Wei, C. F., Chang, H. M. & Yeh, E. (1996). Protection against Fas/APO-1-and tumor necrosis factor-mediated cell death by a novel protein, sentrin. *The Journal of Immunology, 157*(10), 4277-4281.

Olaisen, B., Gedde-Dahl Jr, T., Teisberg, P., Thorsby, E., Siverts, A., Jonassen, R., & Wilhelmy, M. (1985). A structural locus for coagulation factor XIIIA (F13A) is located distal to the HLA region on chromosome 6p in man. *American Journal of Human Genetics, 37*(1), 215-220.

Oliverio, S., Amendola, A., Di Sano, F., Farrace, M. G., Fesus, L., Nemes, Z., Piredda, L., Spinedi, A. & Piacentini, M. (1997). Tissue transglutaminase-dependent posttranslational modification of the retinoblastoma gene product in promonocytic cells undergoing apoptosis. *Molecular and Cellular Biology, 17*(10), 6040-6048.

O'Malley, D. M., Burbach, B. J. & Adams, P. R. (1999). Fluorescent calcium indicators: Subcellular behavior and use in confocal imaging. *Methods in Molecular Biology (Cl*ifton, N.J.), 122, 261-303.

Orrenius, S., Zhivotovsky, B. & Nicotera, P. (2003). Regulation of cell death: The calcium–apoptosis link. *Nature Reviews Molecular Cell Biology, 4*(7), 552-565.

Orrù, S., Caputo, I., D'Amato, A., Ruoppolo, M. & Esposito, C. (2003). Proteomics identification of acyl-acceptor and acyl-donor substrates for transglutaminase in a human intestinal epithelial cell line. *Journal of Biological Chemistry, 278*(34), 31766-31773.

Park, D., Choi, S. S. & Ha, K. S. (2010). Transglutaminase 2: A multi-functional protein in multiple subcellular compartments. *Amino Acids, 39*(3), 619-631.

Park, K. H., Park, H. J., Shin, K. S., Choi, H. S., Kai, M. & Lee, M. K. (2012). Modulation of PC12 cell viability by forskolin-induced cyclic AMP levels through ERK and JNK pathways: An implication for L-DOPA-induced cytotoxicity in nigrostriatal dopamine neurons. *Toxicological Sciences, 128*(1), 247-257.

Parker, P. J. & Murray-Rust, J. (2004). PKC at a glance. *Journal of Cell Science, 117*(2), 131-132.

Pastor, M. T., Diez, A., Pérez-Payá, E. & Abad, C. (1999). Addressing substrate glutamine requirements for tissue transglutaminase using substance P analogues. *FEBS Letters, 451*(3), 231-234.

Pearce, L. R., Komander, D. & Alessi, D. R. (2010). The nuts and bolts of AGC protein kinases. *Nature Reviews Molecular Cell Biology, 11*(1), 9-22.

Peart, J. N. & Gross, G. J. (2006). Cardioprotective effects of acute and chronic opioid treatment are mediated via different signaling pathways. *American Journal of Physiology-Heart and Circulatory Physiology*, *291*(4), H1746-H1753.

Pegg, A. E. (1988). Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Research*, *48*(4), 759-774.

Perkins, G. A., Wang, L., Huang, L. J., Humphries, K., Yao, V. J., Martone, M., Deerinck, T. J., Barraclough, D. M., Violin, J. D. & Smith, D. (2001). PKA, PKC, and AKAP localization in and around the neuromuscular junction. *BMC Neuroscience*, *2*(1), 17.

Perry, M., Mahoney, S. A. & Haynes, L. (1995). Transglutaminase C in cerebellar granule neurons: Regulation and localization of substrate cross-linking. *Neuroscience*, *65*(4), 1063-1076.

Peterman, E. E., Taormina, P. I. I., Harvey, M. & Young, L. H. (2004). Go 6983 exerts cardioprotective effects in myocardial ischemia/reperfusion. *Journal of Cardiovascular Pharmacology*, *43*(5), 645-656.

Peterson, M. W. & Walter, M. E. (1992). Calcium-activated phosphatidylcholine-specific phospholipase C and D in MDCK epithelial cells. *American Journal of Physiology-Cell Physiology*, *263*(6), C1216-C1224.

Pfeifhofer, C., Gruber, T., Letschka, T., Thuille, N., Lutz-Nicoladoni, C., Hermann-Kleiter, N., N., Braun, U., Leitges, M. & Baier, G. (2006). Defective IgG2a/2b class switching in PKCα−/− mice. *The Journal of Immunology*, *176*(10), 6004-6011.

Phillips, M., Stewart, B. & Rice, R. (1992). Genomic structure of keratinocyte transglutaminase. recruitment of new exon for modified function. *Journal of Biological Chemistry*, *267*(4), 2282-2286.

Piacentini, M., Fesus, L., Farrace, M., Ghibelli, L., Piredda, L. & Melino, G. (1991). The expression of" tissue" transglutaminase in two human cancer cell lines is related with the programmed cell death (apoptosis). *European Journal of Cell Biology*, *54*(2), 246-254.

Piacentini, M., Piredda, L., Starace, D., Annicchiarico-Petruzzelli, M., Mattei, M., Oliverio, S., Farrace, M. & Melino, G. (1996). Differential growth of N-and S-type human neuroblastoma cells xenografted into scid mice. correlation with apoptosis. *The Journal of Pathology*, *180*(4), 415-422.

Piacentini, M., Martinet, N., Beninati, S. & Folk, J. (1988). Free and protein-conjugated polyamines in mouse epidermal cells. effect of high calcium and retinoic acid. *Journal of Biological Chemistry*, *263*(8), 3790-3794.

Piacentini, M., D'Eletto, M., Falasca, L., Grazia Farrace, M. & Rodolfo, C. (2011). Transglutaminase 2 at the crossroads between cell death and survival. *Advances in Enzymology and Related Areas of Molecular Biology*, *78*, 197-246.

Pinkas, D. M., Strop, P., Brunger, A. T. & Khosla, C. (2007). Transglutaminase 2 undergoes a large conformational change upon activation. *PLoS Biol, 5*(12), 2788-2796.

Pino, R., Failli, P., Mazzetti, L. & Buffoni, F. (1997). Monoamine oxidase and semicarbazide-sensitive amine oxidase activities in isolated cardiomyocytes of spontaneously hypertensive rats. *Biochemical and Molecular Medicine, 62*(2), 188-196.

Piontek, J. & Brandt, R. (2003). Differential and regulated binding of cAMP-dependent protein kinase and protein kinase C isoenzymes to gravin in human model neurons: Evidence that gravin provides a dynamic platform for the localization for kinases during neuronal development. *The Journal of Biological Chemistry, 278*(40), 38970-38979.

Piredda, L., Farrace, M. G., Lo Bello, M., Malorni, W., Melino, G., Petruzzelli, R., & Piacentini, M. (1999). Identification of 'tissue' transglutaminase binding proteins in neural cells committed to apoptosis. *FASEB Journal, 13*(2), 355-364.

Piredda, L., Amendola, A., Colizzi, V., Davies, P. J., Farrace, M. G., Fraziano, M., Gentile, V., Uray, I., Piacentini, M. & Fesus, L. (1997). Lack of 'tissue' transglutaminase protein cross-linking leads to leakage of macromolecules from dying cells: Relationship to development of autoimmunity in MRLIpr/Ipr mice. *Cell Death and Differentiation, 4*(6), 463-472.

Pisano, J. J., Finlayson, J. S. & Peyton, M. P. (1969). Chemical and enzymic detection of protein cross-links. measurement of. epsilon.-(gamma-glutamyl) lysine in fibrin polymerized by factor XIII. *Biochemistry, 8*(3), 871-876.

Portera-Cailliau, C., Hedreen, J. C., Price, D. L. & Koliatsos, V. E. (1995). Evidence for apoptotic cell death in huntington disease and excitotoxic animal models. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 15*(5 Pt 2), 3775-3787.

Pradeep, N., Murali, B., Rajasekharan, P., Prakashkumar, R., Revadigar, V. & Shashidhara, S. (2006). Variability in the chemical constituents in the roots of coleus forskohlii from different geographical regions of india. *The Utilization, 765*(1), 245-254.

Prasanna Murthy, S., Lomasney, J. W., Mak, E. C. & Lorand, L. (1999). Interactions of G(h)/transglutaminase with phospholipase Cdelta1 and with GTP. *Proceedings of the National Academy of Sciences USA, 96*(21), 11815-11819.

Preisler, J., Hu, P., Rejtar, T. & Karger, B. L. (2000). Capillary electrophoresis-matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a vacuum deposition interface. *Analytical Chemistry, 72*(20), 4785-4795.

Punn, A., Mockridge, J. W., Farooqui, S., Marber, M. S. & Heads, R. J. (2000). Sustained activation of p42/p44 mitogen-activated protein kinase during recovery

from simulated ischaemia mediates adaptive cytoprotection in cardiomyocytes. *Biochemical Journal, 350*(3), 891-899.

Puszkin, E. G. & Raghuraman, V. (1985). Catalytic properties of a calmodulin-regulated transglutaminase from human platelet and chicken gizzard. *Journal of Biological Chemistry, 260*(29), 16012-16020.

Pyle, W. G. & Solaro, R. J. (2004). At the crossroads of myocardial signaling the role of Z-discs in intracellular signaling and cardiac function. *Circulation Research, 94*(3), 296-305.

Rajalingam, K. & Rudel, T. (2005). Ras-raf signaling needs prohibitin. *Cell Cycle, 4*(11), 1503-1505.

Ramalingam, M. & Kim, S. (2012). Reactive oxygen/nitrogen species and their functional correlations in neurodegenerative diseases. *Journal of Neural Transmission, 119*(8), 891-910.

Randazzo, P. A., Andrade, J., Miura, K., Brown, M. T., Long, Y., Stauffer, S., Roller, P. & Cooper, J. A. (2000). The arf GTPase-activating protein ASAP1 regulates the actin cytoskeleton. *Proceedings of the National Academy of Sciences USA, 97*(8), 4011-4016.

Rang, H., Dale, M., Ritter, J. & Moore, P. (2003). Pharmacology. 5th Edition. Edinburgh: Churchill Livingstone, *Elsevier Science Ltd., 37*(6) 378-392.

Reilly, B. A., Brostrom, M. A. & Brostrom, C. O. (1998). Regulation of protein synthesis in ventricular myocytes by vasopressin. the role of sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ stores. *The Journal of Biological Chemistry, 273*(6), 3747-3755.

Rice, R., Mehrpouyan, M., Quin, Q., Phillips, M. A. & Lee, Y. (1996). Identification of phosphorylation sites in keratinocyte transglutaminase. *Biochemical Journal, 320*(2), 547-550.

Rice, R., Rong, X. & Chakravarty, R. (1990). Proteolytic release of keratinocyte transglutaminase. *Biochemical Journal, 265*(2), 351-357.

Risinger, M., Dotimas, E. & Cohen, C. (1992). Human erythrocyte protein 4.2, a high copy number membrane protein, is N-myristylated. *Journal of Biological Chemistry, 267*(8), 5680-5685.

Robia, S. L., Ghanta, J., Robu, V. G. & Walker, J. W. (2001). Localization and kinetics of protein kinase C-epsilon anchoring in cardiac myocytes. *Biophysical Journal, 80*(5), 2140-2151.

Robinson, A. J. & Dickenson, J. M. (2001). Activation of the p38 and p42/p44 mitogen-activated protein kinase families by the histamine H1 receptor in DDT1 mF-2 cells. *British Journal of Pharmacology, 133*(8), 1378-1386.

Robinson, N. J., Baker, P. N., Jones, C. J. & Aplin, J. D. (2007). A role for tissue transglutaminase in stabilization of membrane-cytoskeletal particles shed from the human placenta. *Biology of Reproduction, 77*(4), 648-657.

Robitaille, K., Daviau, A., Tucholski, J., Johnson, G., Rancourt, C. & Blouin, R. (2004). Tissue transglutaminase triggers oligomerization and activation of dual leucine zipper-bearing kinase in calphostin C-treated cells to facilitate apoptosis. *Cell Death & Differentiation, 11*(5), 542-549.

Robu, V. G., Pfeiffer, E. S., Robia, S. L., Balijepalli, R. C., Pi, Y., Kamp, T. J., & Walker, J. W. (2003). Localization of functional endothelin receptor signaling complexes in cardiac transverse tubules. *Journal of Biological Chemistry, 278*(48), 48154-48161.

Rodolfo, C., Mormone, E., Matarrese, P., Ciccosanti, F., Farrace, M. G., Garofano, E., Piredda, L., Fimia, G.M., Malorni, W. & Piacentini, M. (2004). Tissue transglutaminase is a multifunctional BH3-only protein. *Journal of Biological Chemistry, 279*(52), 54783-54792.

Rodríguez, M. J., Saura, J., Billett, E. E., Finch, C. C. & Mahy, N. (2001). Cellular localization of monoamine oxidase A and B in human tissues outside of the central nervous system. *Cell and Tissue Research, 304*(2), 215-220.

Rossin, F., D'Eletto, M., Macdonald, D., Farrace, M. G. & Piacentini, M. (2012). TG2 transamidating activity acts as a rheostat controlling the interplay between apoptosis and autophagy. *Amino Acids, 42*(5), 1793-1802.

Roth, D. M., Bayat, H., Drumm, J. D., Gao, M. H., Swaney, J. S., Ander, A., & Hammond, H.K. (2002). Adenylyl cyclase increases survival in cardiomyopathy. *Circulation, 105*(16), 1989-1994.

Ruan, Q. & Johnson, G. V. (2007). Transglutaminase 2 in neurodegenerative disorders. *Frontiers in Bioscience, 12*, 891-904.

Ruoppolo, M., Orrù, S., D'Amato, A., Francese, S., Rovero, P., Marino, G. & Esposito, C. (2003). Analysis of transglutaminase protein substrates by functional proteomics. *Protein Science, 12*(6), 1290-1297.

Sabbah, M., Radanyi, C., Redeuilh, G. & Baulieu, E. (1996). The 90 kDa heat-shock protein (Hsp90) modulates the binding of the oestrogen receptor to its cognate DNA. *Biochemical Journal, 314*(1), 205-213.

Saitoh, T. & Dobkins, K. R. (1986). Protein kinase C in human brain and its inhibition by calmodulin. *Brain Research, 379*(1), 196-199.

Sakai, K., Busby, W. H., Clarke, J. B. & Clemmons, D. R. (2001). Tissue transglutaminase facilitates the polymerization of insulin-like growth factor-binding protein-1 (IGFBP-1) and leads to loss of IGFBP-1's ability to inhibit insulin-like growth factor-I-stimulated protein synthesis. *Journal of Biological Chemistry, 276*(12), 8740-8745.

Sakamoto, K., Urushidani, T. & Nagao, T. (2000). Translocation of HSP27 to sarcomere induced by ischemic preconditioning in isolated rat hearts. *Biochemical and Biophysical Research Communications, 269*(1), 137-142.

Sanada, S., Kitakaze, M., Papst, P. J., Asanuma, H., Node, K., Takashima, S., Asakura, M., Ogita, H., Liao, Y. & Sakata, Y. (2001). Cardioprotective effect afforded by transient exposure to phosphodiesterase III inhibitors the role of protein kinase A and p38 mitogen-activated protein kinase. *Circulation, 104*(6), 705-710.

Sanada, S., Komuro, I. & Kitakaze, M. (2011). Pathophysiology of myocardial reperfusion injury: Preconditioning, postconditioning, and translational aspects of protective measures. *American Journal of Physiology-Heart and Circulatory Physiology, 301*(5), H1723-H1741.

Sane, D. C., Kontos, J. L. & Greenberg, C. S. (2007). Roles of transglutaminases in cardiac and vascular diseases. *Frontiers in Bioscience: A Journal and Virtual Library, 12*, 2530-2545.

Santhanam, L., Tuday, E. C., Webb, A. K., Dowzicky, P., Kim, J. H., Oh, Y. J., Sikka, G., Kuo, M., Halushka, M. K. & Macgregor, A. M. (2010). Decreased S-nitrosylation of tissue transglutaminase contributes to age-related increases in vascular stiffness. *Circulation Research, 107*(1), 117-125.

Sarang, Z., Molnár, P., Németh, T., Gomba, S., Kardon, T., Melino, G., Cotecchia, S., Fésüs, L. & Szondy, Z. (2005). Tissue transglutaminase (TG2) acting as G protein protects hepatocytes against Fas-mediated cell death in mice. *Hepatology, 42*(3), 578-587.

Sarantitis, I., Papanastasopoulos, P., Manousi, M., Baikoussis, N. G. & Apostolakis, E. (2012). The cytoskeleton of the cardiac muscle cell. *Hellenic J Cardiol, 53*(5), 367-379.

Sárdy, M., Kárpáti, S., Merkl, B., Paulsson, M. & Smyth, N. (2002). Epidermal transglutaminase (TGase 3) is the autoantigen of dermatitis herpetiformis. *The Journal of Experimental Medicine, 195*(6), 747-757.

Sarkar, N. K., Clarke, D. D. & Waelsch, H. (1957). An enzymically catalyzed incorporation of amines into proteins. *Biochimica Et Biophysica Acta, 25*(2), 451-452.

Sarkar, S., Svoboda, M., de Beaumont, R. & Freedman, A. S. (2002). The role of AKT and RAFTK in β1 integrin mediated survival of precursor B-acute lymphoblastic leukemia cells. *Leukemia & Lymphoma, 43*(8), 1663-1671.

Sato, T., Sasaki, N., O'Rourke, B. & Marbán, E. (2000). Adenosine primes the opening of mitochondrial ATP-sensitive potassium channels A key step in ischemic preconditioning? *Circulation, 102*(7), 800-805.

Sawai, A., Chandarlapaty, S., Greulich, H., Gonen, M., Ye, Q., Arteaga, C. L., Sellers, W., Rosen, N. & Solit, D. B. (2008). Inhibition of Hsp90 down-regulates mutant epidermal growth factor receptor (EGFR) expression and sensitizes EGFR mutant tumors to paclitaxel. *Cancer Research, 68*(2), 589-596.

Sayed, M. R., Sheid, M. P., Stevens, C. M. & Duronio, V. (2000). Thrombin-stimulated phosphatidylinositol 3-kinase activity in platelets is associated with activation of PYK2 tyrosine kinase: Activation of both enzymes is aggregation independent. *Journal of Cellular Physiology, 183*(3), 314-320.

Scarpellini, A., Germack, R., Lortat-Jacob, H., Muramatsu, T., Billett, E., Johnson, T., & Verderio, E. A. (2009). Heparan sulfate proteoglycans are receptors for the cell-surface trafficking and biological activity of transglutaminase-2. *Journal of Biological Chemistry, 284*(27), 18411-18423.

Schaertl, S., Prime, M., Wityak, J., Dominguez, C., Munoz-Sanjuan, I., Pacifici, R. E., Courtney, S., Scheel, A. & Macdonald, D. (2010). A profiling platform for the characterization of transglutaminase 2 (TG2) inhibitors. *Journal of Biomolecular Screening, 15*(5), 478-487.

Schafer, F. Q. & Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biology and Medicine, 30*(11), 1191-1212.

Schalkwijk, J., Wiedow, O. & Hirose, S. (1999). The trappin gene family: Proteins defined by an N-terminal transglutaminase substrate domain and a C-terminal four-disulphide core. *Biochemical Journal, 340*(3), 569-577.

Schaper, J., Froede, R., Hein, S., Buck, A., Hashizume, H., Speiser, B., Friedl, A. & Bleese, N. (1991). Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy. *Circulation, 83*(2), 504-514.

Schechtman, D. & Mochly-Rosen, D. (2001). Adaptor proteins in protein kinase C-mediated signal transduction. *Oncogene, 20*(44), 6339-6347.

Schmittgen, T. D. & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols, 3*(6), 1101-1108.

Schroder, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. (2004). Interferon-γ: An overview of signals, mechanisms and functions. *Journal of Leukocyte Biology, 75*(2), 163-189.

Schwartz, M. L., Pizzo, S. V., Hill, R. L. & McKee, P. A. (1973). Human factor XIII from plasma and platelets. molecular weights, subunit structures, proteolytic activation, and cross-linking of fibrinogen and fibrin. *The Journal of Biological Chemistry, 248*(4), 1395-1407.

Schweitzer, S. C., Klymkowsky, M. W., Bellin, R. M., Robson, R. M., Capetanaki, Y. & Evans, R. M. (2001). Paranemin and the organization of desmin filament networks. *Journal of Cell Science, 114*(6), 1079-1089.

Seiler, N., Delcros, J. & Moulinoux, J. P. (1996). Polyamine transport in mammalian cells. an update. *The International Journal of Biochemistry & Cell Biology, 28*(8), 843-861.

Serafini-Fracassini, D. & Del Duca, S. (2008). Transglutaminases: Widespread cross-linking enzymes in plants. *Annals of Botany, 102*(2), 145-152.

Severs, N. J., Dupont, E., Thomas, N., Kaba, R., Rothery, S., Jain, R., Sharpey, K. & Fry, C. H. (2006). Alterations in cardiac connexin expression in cardiomyopathies. *Advances in Cardiology, 42*, 228-242.

Shi, C. & Kehrl, J. H. (2001). PYK2 links gqα and G13α signaling to NF-κB activation. *Journal of Biological Chemistry, 276*(34), 31845-31850.

Shih, J. & Chen, K. (2004). Regulation of MAO-A and MAO-B gene expression. *Current Medicinal Chemistry, 11*(15), 1995-2005.

Shih, J., Chen, K. & Ridd, M. (1999). Monoamine oxidase: From genes to behavior. *Annual Review of Neuroscience, 22*, 197-217.

Shimomura, H., Terasaki, F., Hayashi, T., Kitaura, Y., Isomura, T. & Suma, H. (2001). Autophagic degeneration as a possible mechanism of myocardial cell death in dilated cardiomyopathy. *Japanese Circulation Journal, 65*(11), 965-968.

Shin, D. M., Jeon, J. H., Kim, C. W., Cho, S. Y., Kwon, J. C., Lee, H. J., Choi, K. H., Park, S. C. & Kim, I. G. (2004). Cell type-specific activation of intracellular transglutaminase 2 by oxidative stress or ultraviolet irradiation. *Journal of Biological Chemistry, 279*(15), 15032-15039.

Shiojima, I., Sato, K., Izumiya, Y., Schiekofer, S., Ito, M., Liao, R., Colucci, W. S. & Walsh, K. (2005). Disruption of coordinated cardiac hypertrophy and anglogenesis contributes to the transition to heart failure. *Journal of Clinical Investigation, 115*(8), 2108-2118.

Siegel, M. & Khosla, C. (2007). Transglutaminase 2 inhibitors and their therapeutic role in disease states. *Pharmacology & Therapeutics, 115*(2), 232-245.

Siegel, M., Strnad, P., Watts, R. E., Choi, K., Jabri, B., Omary, M. B. & Khosla, C. (2008). Extracellular transglutaminase 2 is catalytically inactive, but is transiently activated upon tissue injury. *PLoS One, 3*(3), e1861-e1861.

Siegel, M. & Khosla, C. (2007). Transglutaminase 2 inhibitors and their therapeutic role in disease states. *Pharmacol Ther, 115*(2), 232-245.

Singh, R. N., McQueen, T. & Mehta, K. (1995). Detection of the amine acceptor protein substrates of transglutaminase with 5-(biotinamido) pentylamine. *Analytical Biochemistry, 231*(1), 261-263.

Singh, U. S., Kunar, M. T., Kao, Y. & Baker, K. M. (2001). Role of transglutaminase II in retinoic acid-induced activation of RhoA-associated kinase-2. *The EMBO Journal, 20*(10), 2413-2423.

Singh, U. S., Pan, J., Kao, Y., Joshi, S., Young, K. L. & Baker, K. M. (2003). Tissue transglutaminase mediates activation of RhoA and MAP kinase pathways during retinoic acid-induced neuronal differentiation of SH-SY5Y cells. *Journal of Biological Chemistry, 278*(1), 391-399.

Singla, D. K., Singla, R. D. & McDonald, D. E. (2008). Factors released from embryonic stem cells inhibit apoptosis in H9c2 cells through PI3K/Akt but not ERK pathway. *American Journal of Physiology-Heart and Circulatory Physiology, 295*(2), H907-H913.

Skill, N. J., Griffin, M., El Nahas, A. M., Sanai, T., Haylor, J. L., Fisher, M., Jamie, M. F., Mould, N. N. & Johnson, T. S. (2001). Increases in renal epsilon-(gamma-glutamyl)-lysine crosslinks result from compartment-specific changes in tissue transglutaminase in early experimental diabetic nephropathy: Pathologic implications. *Laboratory Investigation, 81*(5), 705-716.

Slaughter, T. F., Achyuthan, K. E., Lai, T. S. & Greenberg, C. S. (1992). A microtiter plate transglutaminase assay utilizing 5-(biotinamido) pentylamine as substrate. *Analytical Biochemistry, 205*(1), 166-171.

Small, K., Feng, J. F., Lorenz, J., Donnelly, E. T., Yu, A., Im, M. J., Dorn, G. W. & Liggett, S. B. (1999). Cardiac specific overexpression of transglutaminase II (gh) results in a unique hypertrophy phenotype independent of phospholipase C activation. *Journal of Biological Chemistry, 274*(30), 21291-21296.

Smethurst, P. A., Bungay, P. J. & Griffin, M. (1993). The use of a biotin-labelled amine to identify endogenous substrates of transglutaminase in pancreatic islets. *Biochemical Society Transactions, 21*(4), 424S-424S.

Sollid, L., Molberg, Ø., McAdam, S. & Lundin, K. (1997). Autoantibodies in coeliac disease: Tissue transglutaminase—guilt by association? *Gut, 41*(6), 851-852.

Song, H., Kim, B., Chang, W., Lim, S., Song, B., Cha, M., Jang, Y. & Hwang, K. (2011). Tissue transglutaminase 2 promotes apoptosis of rat neonatal cardiomyocytes under oxidative stress. *Journal of Receptors and Signal Transduction, 31*(1), 66-74.

Song, W., Schreiber, W. E., Zhong, E., Liu, F., Kornfeld, B. D., Wondisford, F. E. & Hussain, M. A. (2008). Exendin-4 stimulation of cyclin A2 in β-cell proliferation. *Diabetes, 57*(9), 2371-2381.

Song, Y., Kirkpatrick, L. L., Schilling, A. B., Helseth, D. L., Chabot, N., Keillor, J. W., Johnson, G. V. & Brady, S. T. (2013). Transglutaminase and polyamination of tubulin: Posttranslational modification for stabilizing axonal microtubules. *Neuron, 78*(1), 109-123.

Sordet, O., Rébé, C., Plenchette, S., Zermati, Y., Hermine, O., Vainchenker, W., Garrido, C., Solary, E. & Dubrez-Daloz, L. (2002). Specific involvement of caspases in the differentiation of monocytes into macrophages. *Blood, 100*(13), 4446-4453.

Spina, A., Esposito, C., Pagano, M., Chiosi, E., Mariniello, L., Cozzolino, A., Porta, R. & Illiano, G. (1999). GTPase and transglutaminase are associated in the secretion of the rat anterior prostate. *Biochemical and Biophysical Research Communications, 260*(2), 351-356.

Spom, M. B. & Roberts, A. B. (1983). Role of retinoids in differentiation and carcinogenesis. *Cancer Research, 43*(7), 3034-3040.

Srivastava, P. (2002). Roles of heat-shock proteins in innate and adaptive immunity. *Nature Reviews Immunology, 2*(3), 185-194.

Stamnaes, J., Pinkas, D. M., Fleckenstein, B., Khosla, C. & Sollid, L. M. (2010). Redox regulation of transglutaminase 2 activity. *Journal of Biological Chemistry, 285*(33), 25402-25409.

Steenbergen, C. (2002). The role of p38 mitogen-activated protein kinase in myocardial ischemia/reperfusion injury; relationship to ischemic preconditioning. *Basic Research in Cardiology, 97*(4), 276-285.

Steinhoff, M., Eicheler, W., Holterhus, P., Rausch, U., Seitz, J. & Aumüller, G. (1994). Hormonally induced changes in apocrine secretion of transglutaminase in the rat dorsal prostate and coagulating gland. *European Journal of Cell Biology, 65*(1), 49-59.

Stoscheck, C. M. (1990).Quantitation of protein. *Methods in Enzymology, 182*, 50-68.

Sturniolo, M. T., Chandraratna, R. A. & Eckert, R. L. (2004). A novel transglutaminase activator forms a complex with type 1 transglutaminase. *Oncogene, 24*(18), 2963-2972.

Sturniolo, M. T., Dashti, S. R., Deucher, A., Rorke, E. A., Broome, A., Chandraratna, R. A., Keepers, T. & Eckert, R. L. (2003). A novel tumor suppressor protein promotes keratinocyte terminal differentiation via activation of type I transglutaminase. *Journal of Biological Chemistry, 278*(48), 48066-48073.

Sugden, P. H. & Clerk, A. (2001). Akt like a woman: Gender differences in susceptibility to cardiovascular disease. *Circulation Research, 88*(10), 975-977.

Sulakhe, P. V. & Vo, X. T. (1995). Regulation of phospholamban and troponin-I phosphorylation in the intact rat cardiomyocytes by adrenergic and cholinergic stimuli: Roles of cyclic nucleotides, calcium, protein kinases and phosphatases and depolarization. *Molecular and Cellular Biochemistry, 149*(1), 103-126.

Sumi, Y., Inoue, N., Azumi, H., Seno, T., Okuda, M., Hirata, K., Kawashima, S., Hayashi, Y., Itoh, H. & Yokoyama, M. (2002). Expression of tissue

transglutaminase and elafin in human coronary artery:: Implication for plaque instability. *Atherosclerosis, 160*(1), 31-39.

Sun, X., Wang, Y., Xirodimas, D. P. & Dai, M. (2010). Perturbation of 60 S ribosomal biogenesis results in ribosomal protein L5-and L11-dependent p53 activation. *Journal of Biological Chemistry, 285*(33), 25812-25821.

Sung, L., Chien, S., Fan, Y., Lin, C., Lambert, K., Zhu, L., Lam, J. & Chang, L. S. (1992). Human erythrocyte protein 4.2: Isoform expression, differential splicing, and chromosomal assignment. *Blood, 79*(10), 2763-2770.

Szondy, Z., Mastroberardino, P., Varadi, J., Farrace, M., Nagy, N., Bak, I., Viti, I., Wieckowski, M., Melino, G. & Rizzuto, R. (2006). Tissue transglutaminase (TG2) protects cardiomyocytes against ischemia/reperfusion injury by regulating ATP synthesis. *Cell Death & Differentiation, 13*(10), 1827-1829.

Szondy, Z., Molnar, P., Nemes, Z., Boyiadzis, M., Kedei, N., Tóth, R. & Fésüs, L. (1997). Differential expression of tissue transglutaminase during in vivo apoptosis of thymocytes induced via distinct signaling pathways. *FEBS Letters, 404*(2-3), 307-313.

Takai, Y., Sasaki, T. & Matozaki, T. (2001). Small GTP-binding proteins. *Physiological Reviews, 81*(1), 153-208.

Tanaka, H., Yokota, H., Jover, T., Cappuccio, I., Calderone, A., Simionescu, M., Bennett, M. V. & Zukin, R. S. (2004). Ischemic preconditioning: Neuronal survival in the face of caspase-3 activation. *The Journal of Neuroscience, 24*(11), 2750-2759.

Taylor, S. S., Buechler, J. A. & Yonemoto, W. (1990). cAMP-dependent protein kinase: Framework for a diverse family of regulatory enzymes. *Annual Review of Biochemistry, 59*(1), 971-1005.

Teixeira, C., Stang, S. L., Zheng, Y., Beswick, N. S. & Stone, J. C. (2003). Integration of DAG signaling systems mediated by PKC-dependent phosphorylation of RasGRP3. *Blood, 102*(4), 1414-1420.

Telci, D., Wang, Z., Li, X., Verderio, E. A. M., Humphries, M. J., Baccarini, M., Basaga, H. & Griffin, M. (2008). Fibronectin-tissue transglutaminase matrix rescues RGD-impaired cell adhesion through syndecan-4 and β1 integrin co-signaling. *Journal of Biological Chemistry, 283*(30), 20937-20947.

Terrinoni, A., Serra, V., Codispoti, A., Talamonti, E., Bui, L., Palombo, R., Sette, M., Campione, E., Didona, B. & Annicchiarico-Petruzzelli, M. (2012). Novel transglutaminase 1 mutations in patients affected by lamellar ichthyosis. *Cell Death & Disease, 3*(10), e416-e416.

Terzic, A., Puceat, M., Vassort, G. & Vogel, S. (1993). Cardiac alpha 1-adrenoceptors: An overview. *Pharmacological Reviews, 45*(2), 147-175.

Thacher, S. M. & Rice, R. H. (1985). Keratinocyte-specific transglutaminase of cultured human epidermal cells: Relation to cross-linked envelope formation and terminal differentiation. *Cell, 40*(3), 685-695.

Thibaut, S., Candi, E., Pietroni, V., Melino, G., Schmidt, R. & Bernard, B. A. (2005). Transglutaminase 5 expression in human hair follicle. *Journal of Investigative Dermatology, 125*(3), 581-585.

Thomas, H., Beck, K., Adamczyk, M., Aeschlimann, P., Langley, M., Oita, R. C., Thiebach, L., Hils, M. & Aeschlimann, D. (2013). Transglutaminase 6: A protein associated with central nervous system development and motor function. *Amino Acids, 44*(1), 161-177.

Thomázy, V. & Fésüs, L. (1989). Differential expression of tissue transglutaminase in human cells. *Cell and Tissue Research, 255*(1), 215-224.

Toma, O., Weber, N. C., Wolter, J. I., Obal, D., Preckel, B. & Schlack, W. (2004). Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and extracellular signal-regulated kinase 1 and 2 in the rat heart in vivo. *Anesthesiology, 101*(6), 1372-1380.

Tovar-Vidales, T., Roque, R., Clark, A. F. & Wordinger, R. J. (2008). Tissue transglutaminase expression and activity in normal and glaucomatous human trabecular meshwork cells and tissues. *Investigative Ophthalmology & Visual Science, 49*(2), 622-628.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences USA, 76*(9), 4350-4354.

Trigwell, S., Lynch, P., Griffin, M., Hargreaves, A. & Bonner, P. (2004). An improved colorimetric assay for the measurement of transglutaminase (type II)-(γ-glutamyl) lysine cross-linking activity. *Anal.Biochem, 330*(1), 164-166.

Trump, B. E., Berezesky, I. K., Chang, S. H. & Phelps, P. C. (1997). The pathways of cell death: Oncosis, apoptosis, and necrosis. *Toxicologic Pathology, 25*(1), 82-88.

Tucholski, J. (2010). TG2 protects neuroblastoma cells against DNA-damage-induced stress, suppresses p53 activation. *Amino Acids, 39*(2), 523-532.

Tucholski, J. & Johnson, G. V. W. (2002). Tissue transglutaminase differentially modulates apoptosis in a stimuli-dependent manner. *Journal of Neurochemistry, 81*(4), 780-791.

Tucholski, J. & Johnson, G. V. W. (2003). Tissue transglutaminase directly regulates adenylyl cyclase resulting in enhanced cAMP-response element-binding protein (CREB) activation. *Journal of Biological Chemistry, 278*(29), 26838-26843.

Turner, N. A., Xia, F., Azhar, G., Zhang, X., Liu, L. & Wei, J. Y. (1998). Oxidative stress induces DNA fragmentation and caspase activation via the c-jun NH2-terminal kinase pathway in H9c2 cardiac muscle cells. *Journal of Molecular and Cellular Cardiology, 30*(9), 1789-1801.

Umar, S., Malavasi, F. & Mehta, K. (1996). Post-translational modification of CD38 protein into a high molecular weight form alters its catalytic properties. *Journal of Biological Chemistry, 271*(27), 15922-15927.

Valko, M., Morris, H. & Cronin, M. (2005). Metals, toxicity and oxidative stress. *Current Medicinal Chemistry, 12*(10), 1161-1208.

van den Akker, J., van Weert, A., Afink, G., Bakker, E. N., van der Pol, E., Böing, A. N., Nieuwland, R. & VanBavel, E. (2012). Transglutaminase 2 is secreted from smooth muscle cells by transamidation-dependent microparticle formation. *Amino Acids, 42*(2-3), 961-973.

van den Akker, J., VanBavel, E., van Geel, R., Matlung, H. L., Tuna, B. G., Janssen, G. M., van Veelen, P. A., Boelens, W. C., De Mey, J. G. and Bakker, E. N. (2011). The redox state of transglutaminase 2 controls arterial remodeling. *PloS One, 6*(8), e23067-e23067.

Verderio, E., Nicholas, B., Gross, S. & Griffin, M. (1998). Regulated expression of tissue transglutaminase in swiss 3T3 fibroblasts: Effects on the processing of fibronectin, cell attachment, and cell death. *Experimental Cell Research, 239*(1), 119-138.

Verderio, E., Johnson, T. & Griffin, M. (2004). Tissue transglutaminase in normal and abnormal wound healing: Review article. *Amino Acids, 26*(4), 387-404.

Verhaar, R., Jongenelen, C. A., Gerard, M., Baekelandt, V., Van Dam, A. M., Wilhelmus, M. M., & Drukarch, B. (2011). Blockade of enzyme activity inhibits tissue transglutaminase-mediated transamidation of alpha-synuclein in a cellular model of Parkinson's disease. *Neurochemistry International, 58*(7), 785-793.

Verhaar, R., Drukarch, B., Bol, J. G., Jongenelen, C. A., Musters, R. J. & Wilhelmus, M. M. (2012). Increase in endoplasmic reticulum-associated tissue transglutaminase and enzymatic activation in a cellular model of Parkinson's disease. *Neurobiology of Disease, 45*(3), 839-850.

Verma, A., Guha, S., Diagaradjane, P., Kunnumakkara, A. B., Sanguino, A. M., Lopez-Berestein, G., Sood, A. K., Aggarwal, B. B., Krishnan, S. & Gelovani, J. G. (2008). Therapeutic significance of elevated tissue transglutaminase expression in pancreatic cancer. *Clinical Cancer Research, 14*(8), 2476-2483.

Verma, A., Guha, S., Wang, H., Fok, J. Y., Koul, D., Abbruzzese, J. & Mehta, K. (2008). Tissue transglutaminase regulates focal adhesion kinase/AKT activation by modulating PTEN expression in pancreatic cancer cells. *Clinical Cancer Research, 14*(7), 1997-2005.

Vezza, R., Habib, A. & FitzGerald, G. A. (1999). Differential signaling by the thromboxane receptor isoforms via the novel GTP-binding protein, gh. *Journal of Biological Chemistry, 274*(18), 12774-12779.

Volpe, P., Villa, A., Podini, P., Martini, A., Nori, A., Panzeri, M. C. & Meldolesi, J. (1992). The endoplasmic reticulum-sarcoplasmic reticulum connection: Distribution of endoplasmic reticulum markers in the sarcoplasmic reticulum of skeletal muscle fibers. *Proceedings of the National Academy of Sciences USA, 89*(13), 6142-6146.

Vondriska, T. M., Zhang, J., Song, C., Tang, X. L., Cao, X., Baines, C. P., Pass, J. M., Wang, S., Bolli, R. and Ping, P. (2001). Protein kinase C ε–Src modules direct signal transduction in nitric Oxide–Induced cardioprotection: Complex formation as a means for cardioprotective signaling. *Circulation Research, 88*(12), 1306-1313.

Wada, F., Nakamura, A., Masutani, T., Ikura, K., Maki, M. & Hitomi, K. (2002). Identification of mammalian-type transglutaminase in physarum polycephalum. *European Journal of Biochemistry, 269*(14), 3451-3460.

Walsh, C. (2006). *Posttranslational modifications of proteins: Expanding nature's inventory*. Roberts and Company Publishers.

Wang, F. (2006). Culture of animal cells: A manual of basic technique. *In Vitro Cellular & Developmental Biology-Animal, 42*(5), 169-169.

Wang, G., Xiao, Q., Luo, Z., Ye, S. & Xu, Q. (2012). Functional impact of heterogeneous nuclear ribonucleoprotein A2/B1 in smooth muscle differentiation from stem cells and embryonic arteriogenesis. *Journal of Biological Chemistry, 287*(4), 2896-2906.

Wang, J. L., Yang, X., Xia, K., Hu, Z. M., Weng, L., Jin, X., Jiang, H., Zhang, P., Shen, L. & Guo, J. F. (2010). TGM6 identified as a novel causative gene of spinocerebellar ataxias using exome sequencing. *Brain, 133*(12), 3510-3518.

Wang, M., McBride, O., Kim, I. & Steinert, P. (1994). Assignment of the human transglutaminase 2 (TGM2) and transglutaminase (TGM3) genes to chromosome 20q11. 2. *Genomics, 23*(3), 721-722.

Wang, P. & Zweier, J. L. (1996). Measurement of nitric oxide and peroxynitrite generation in the postischemic heart evidence for peroxynitrite-mediated reperfusion injury. *Journal of Biological Chemistry, 271*(46), 29223-29230.

Wang, S. M. & Yang, W. (2009). Circulating hormone adrenomedullin and its binding protein protect neural cells from hypoxia-induced apoptosis. *Biochimica Et Biophysica Acta (BBA)-General Subjects, 1790*(5), 361-367.

Wang, Y., Klein, J. D., Blount, M. A., Martin, C. F., Kent, K. J., Pech, V., Wall, S. M. & Sands, J. M. (2009). Epac regulates UT-A1 to increase urea transport in inner

medullary collecting ducts. *Journal of the American Society of Nephrology, 20*(9), 2018-2024.

Wang, Y. X., Li, H., Zhao, Y. & Xu, C. (2010). Downregulation of the ornithine Decarboxylase/polyamine system inhibits angiotensin-induced hypertrophy of cardiomyocytes through the NO/cGMP-dependent protein kinase type-I pathway. *Cell Physiol Biochem, 25*(4-5), 443-450.

Wang, Y., Ande, S. & Mishra, S. (2012). Phosphorylation of transglutaminase 2 (TG2) at serine-216 has a role in TG2 mediated activation of nuclear factor-kappa B and in the downregulation of PTEN. *BMC Cancer, 12*(1), 277.

Wang, Z., Collighan, R. J., Gross, S. R., Danen, E. H., Orend, G., Telci, D. & Griffin, M. (2010). RGD-independent cell adhesion via a tissue transglutaminase-fibronectin matrix promotes fibronectin fibril deposition and requires syndecan-4/2 alpha5beta1 integrin co-signaling. *The Journal of Biological Chemistry, 285*(51), 40212-40229.

Wang, Z., Perez, M., Caja, S., Melino, G., Johnson, T., Lindfors, K. & Griffin, M. (2013). A novel extracellular role for tissue transglutaminase in matrix-bound VEGF-mediated angiogenesis. *Cell Death & Disease, 4*(9), e808.

Wang, Z., Telci, D. & Griffin, M. (2011). Importance of syndecan-4 and syndecan-2 in osteoblast cell adhesion and survival mediated by a tissue transglutaminase−fibronectin complex. *Experimental Cell Research, 317*(3), 367-381.

Wiebe, R. I., Tarr, A. H. & Bowness, J. M. (1991). Increased transglutaminase in the aortas of cholesterol-fed rabbits: Occurrence of buffer soluble and insoluble forms and an inhibitor. *Biochemistry and Cell Biology, 69*(12), 821-827.

Wilhelmus, M. M. M., Grunberg, S., Bol, J. G. J. M., Van Dam, A. M., Hoozemans, J. J. M., Rozemuller, A. J. M. & Drukarch, B. (2009). Transglutaminases and Transglutaminase-Catalyzed Cross-Links colocalize with the pathological lesions in alzheimer's disease brain. *Brain Pathology, 19*(4), 612-622.

Williams-Ashman, H. G. (1984). Transglutaminases and the clotting of mammalian seminal fluids. *Transglutaminase* (pp. 51-61) Springer US.

Wilson, E. M. & French, F. (1980). Biochemical homology between rat dorsal prostate and coagulating gland. purification of a major androgen-induced protein. *Journal of Biological Chemistry, 255*(22), 10946-10953.

Wodzinska, J. M. (2005). Transglutaminases as targets for pharmacological inhibition. *Mini Reviews in Medicinal Chemistry, 5*(3), 279-292.

Wong, W. K., Ou, X. M., Chen, K. & Shih, J. C. (2002). Activation of human monoamine oxidase B gene expression by a protein kinase C MAPK signal transduction pathway involves c-jun and egr-1. *Journal of Biological Chemistry, 277*(25), 22222-22230.

Wu, D., Katz, A., Lee, C. H. & Simon, M. I. (1992). Activation of phospholipase C by alpha 1-adrenergic receptors is mediated by the alpha subunits of gq family. *Journal of Biological Chemistry, 267*(36), 25798-25802.

Xiang, Y. & Kobilka, B. K. (2003). Myocyte adrenoceptor signaling pathways. *Science, 300*(5625), 1530-1532.

Yamane, A., Fukui, M., Sugimura, Y., Itoh, M., Alea, M. P., Thomas, V., El Alaoui, S., Akiyama, M. & Hitomi, K. (2010). Identification of a preferred substrate peptide for transglutaminase 3 and detection of in situ activity in skin and hair follicles. *FEBS Journal, 277*(17), 3564-3574.

Yamanishi, K., Inazawa, J., Liew, F., Nonomura, K., Ariyama, T., Yasuno, H., Abe, T., Doi, H., Hirano, J. & Fukushima, S. (1992). Structure of the gene for human transglutaminase 1. *Journal of Biological Chemistry, 267*(25), 17858-17863.

Yang, Z., Yang, C., Xiao, L., Liao, X., Lan, A., Wang, X., Guo, R., Chen, P., Hu, C. & Feng, J. (2011). Novel insights into the role of HSP90 in cytoprotection of H2S against chemical hypoxia-induced injury in H9c2 cardiac myocytes. *International Journal of Molecular Medicine, 28*(3), 397-403.

Yellon, D. M. & Downey, J. M. (2003). Preconditioning the myocardium: From cellular physiology to clinical cardiology. *Physiological Reviews, 83*(4), 1113-1151.

Yi, S., Groffen, J. & Heisterkamp, N. (2011). Bcr is a substrate for transglutaminase 2 cross-linking activity. *BMC Biochemistry, 12*(1), 8-17.

Yoshida, K., Kawamura, S., Mizukami, Y. & Kitakaze, M. (1997). Implication of protein kinase C-α, δ, and ε isoforms in ischemic preconditioning in perfused rat hearts. *Journal of Biochemistry, 122*(3), 506-511.

Yuan, J., Lipinski, M. & Degterev, A. (2003). Diversity in the mechanisms of neuronal cell death. *Neuron, 40*(2), 401-413.

Yuan, L., Siegel, M., Choi, K., Khosla, C., Miller, C., Jackson, E., Piwnica-Worms, D. & Rich, K. (2006). Transglutaminase 2 inhibitor, KCC009, disrupts fibronectin assembly in the extracellular matrix and sensitizes orthotopic glioblastomas to chemotherapy. *Oncogene, 26*(18), 2563-2573.

Yuan, L., Choi, K., Khosla, C., Zheng, X., Higashikubo, R., Chicoine, M. R. & Rich, K. M. (2005). Tissue transglutaminase 2 inhibition promotes cell death and chemosensitivity in glioblastomas. *Molecular Cancer Therapeutics, 4*(9), 1293-1302.

Yun, B. & Matts, R. L. (2005). Differential effects of Hsp90 inhibition on protein kinases regulating signal transduction pathways required for myoblast differentiation. *Experimental Cell Research, 307*(1), 212-223.

Zaid, H., Abu-Hamad, S., Israelson, A., Nathan, I. & Shoshan-Barmatz, V. (2005). The voltage-dependent anion channel-1 modulates apoptotic cell death. *Cell Death & Differentiation, 12*(7), 751-760.

Zainelli, G. M., Dudek, N. L., Ross, C. A., Kim, S. & Muma, N. A. (2005). Mutant huntingtin protein: A substrate for transglutaminase 1, 2, and 3. *Journal of Neuropathology & Experimental Neurology, 64*(1), 58-65.

Zanetti, L., Ristoratore, F., Bertoni, A. & Cariello, L. (2004). Characterization of sea urchin transglutaminase, a protein regulated by guanine/adenine nucleotides. *Journal of Biological Chemistry, 279*(47), 49289-49297.

Zatta, A. J., Kin, H., Lee, G., Wang, N., Jiang, R., Lust, R., Reeves, J. G., Mykytenko, J., Guyton, R. A. & Zhao, Z. (2006). Infarct-sparing effect of myocardial postconditioning is dependent on protein kinase C signaling. *Cardiovascular Research, 70*(2), 315-324.

Zemskov, E. A., Janiak, A., Hang, J., Waghray, A. & Belkin, A. M. (2006). The role of tissue transglutaminase in cell-matrix interactions. *Front Biosci, 11*, 1057-1076.

Zemskov, E. A., Loukinova, E., Mikhailenko, I., Coleman, R. A., Strickland, D. K. & Belkin, A. M. (2009). Regulation of platelet-derived growth factor receptor function by integrin-associated cell surface transglutaminase. *Journal of Biological Chemistry, 284*(24), 16693-16703.

Zhang, J., Lesort, M., Guttmann, R. P. & Johnson, G. V. W. (1998). Modulation of the in situ activity of tissue transglutaminase by calcium and GTP. *Journal of Biological Chemistry, 273*(4), 2288-2295.

Zhang, J., Hui Ying, Z., Fang, D., Ai Ping, L. & LIU, Z. H. (2005). Transglutaminase 3 expression in C57BL/6J mouse embryo epidermis and the correlation with its differentiation. *Cell Research, 15*(2), 105-110.

Zhang, J. & Masui, Y. (1997). Role of amphibian egg transglutaminase in the development of secondary cytostatic factor in vitro. *Molecular Reproduction and Development, 47*(3), 302-311.

Zhang, J., Fonovic, M., Suyama, K., Bogyo, M. & Scott, M. P. (2009). Rab35 controls actin bundling by recruiting fascin as an effector protein. *Science, 325*(5945), 1250-1254.

Zhang, Y., Blattman, J. N., Kennedy, N. J., Duong, J., Nguyen, T., Wang, Y., Davis, R. J., Greenberg, P. D., Flavell, R. A. & Dong, C. (2004). Regulation of innate and adaptive immune responses by MAP kinase phosphatase 5. *Nature, 430*(7001), 793-797.

Zhang, Z., Vezza, R., Plappert, T., McNamara, P., Lawson, J. A., Austin, S., Pratico, D., Sutton, M. S. J. & FitzGerald, G. A. (2003). COX-2-dependent cardiac failure in Gh/tTG transgenic mice. *Circulation Research, 92*(10), 1153-1161.

Zhao, X. L., Gutierrez, L. M., Chang, C. F. & Hosey, M. M. (1994). The alpha 1-subunit of skeletal muscle L-type ca channels is the key target for regulation by A-kinase and protein phosphatase-1C. *Biochemical and Biophysical Research Communications, 198*(1), 166-173.

Zhou, H., Summers, S. A., Birnbaum, M. J. & Pittman, R. N. (1998). Inhibition of akt kinase by cell-permeable ceramide and its implications for ceramide-induced apoptosis. *Journal of Biological Chemistry, 273*(26), 16568-16575.

Zhu, L., Kahwash, S. B. & Chang, L. (1998). Developmental expression of mouse erythrocyte protein 4.2 mRNA: Evidence for specific expression in erythroid cells. *Blood, 91*(2), 695-705.

Zou, N., Ao, L., Cleveland, J. C., Yang, X., Su, X., Cai, G., Banerjee, A., Fullerton, D. A. and Meng, X. (2008). Critical role of extracellular heat shock cognate protein 70 in the myocardial inflammatory response and cardiac dysfunction after global ischemia-reperfusion. *American Journal of Physiology-Heart and Circulatory Physiology*, *294*(6), H2805-H2813.

# CHAPTER IX:

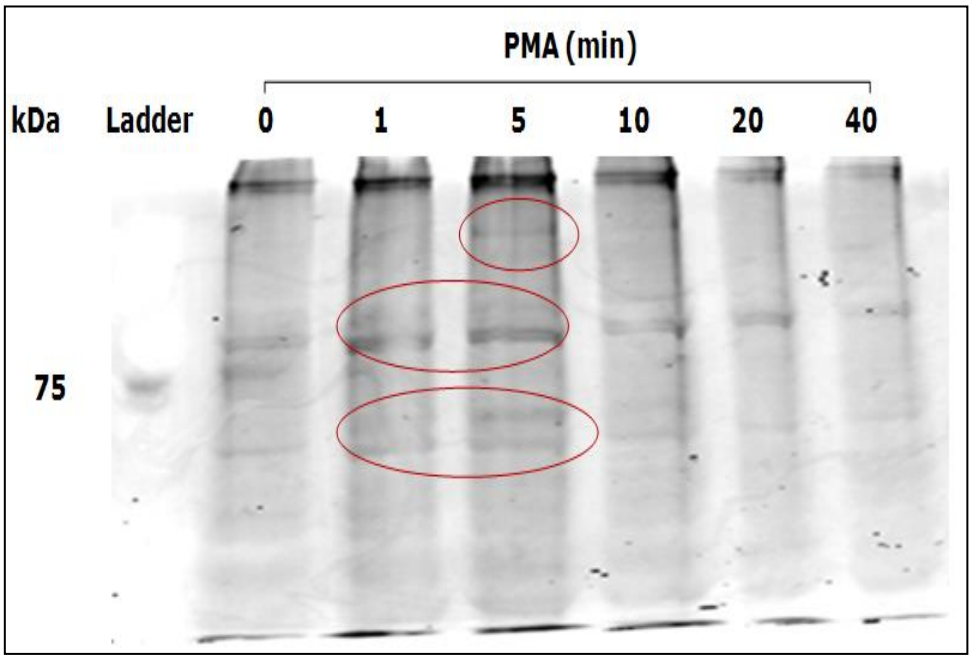
# APPENDICES

## 9. Appendices

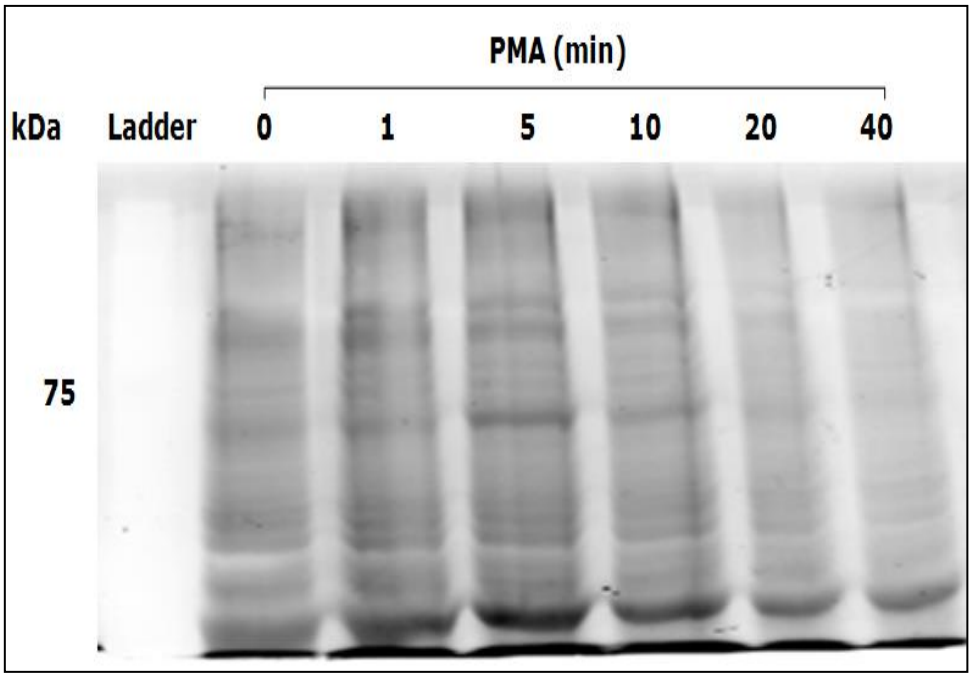
***Protein kinase activator stimulated protein phosphorylation in H9c2 cells***

Initial experiments concentrated on evaluation of phosphorylation events in H9c2 cells via different protein stains after treatment with protein kinase activators.

**A)**




**B)**



**C)**

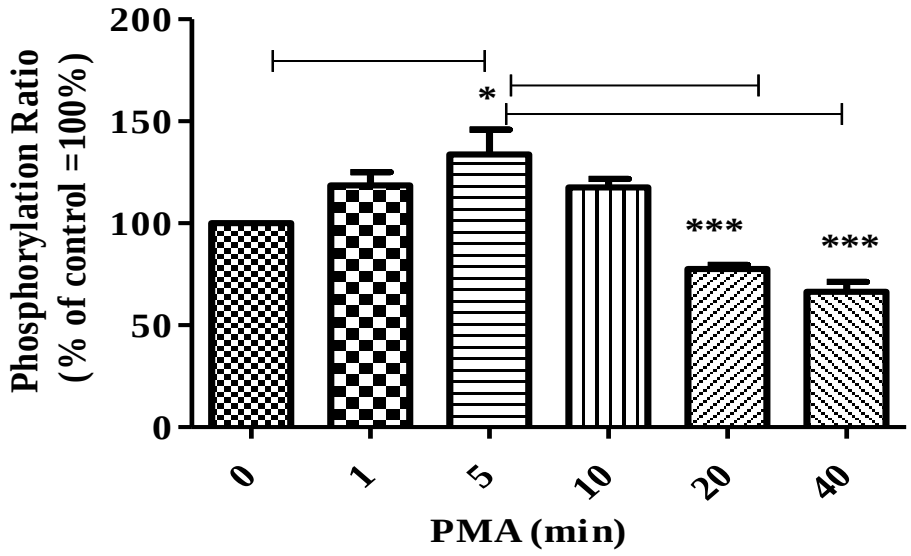
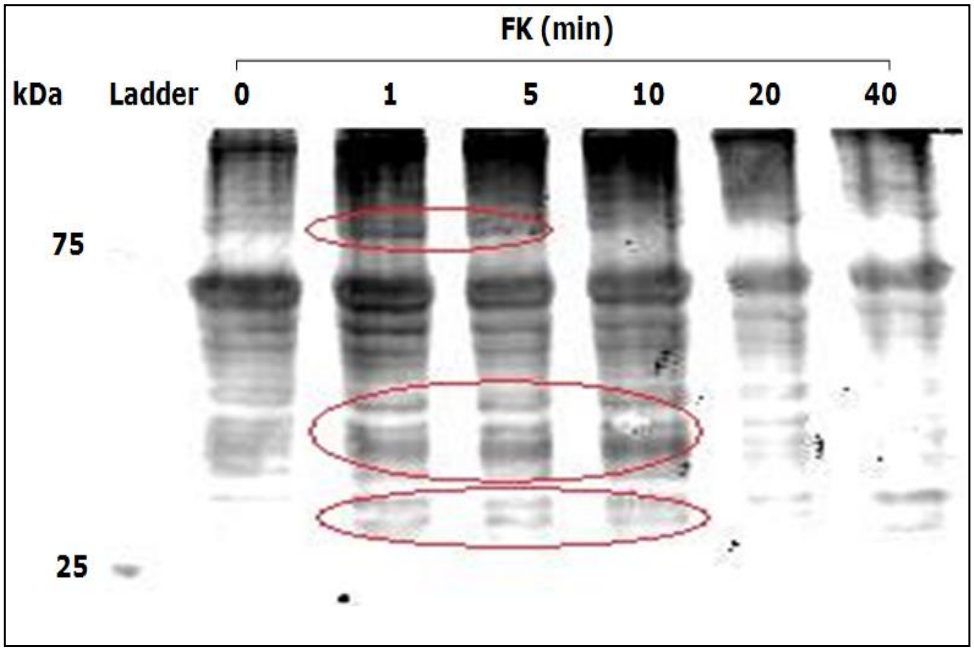


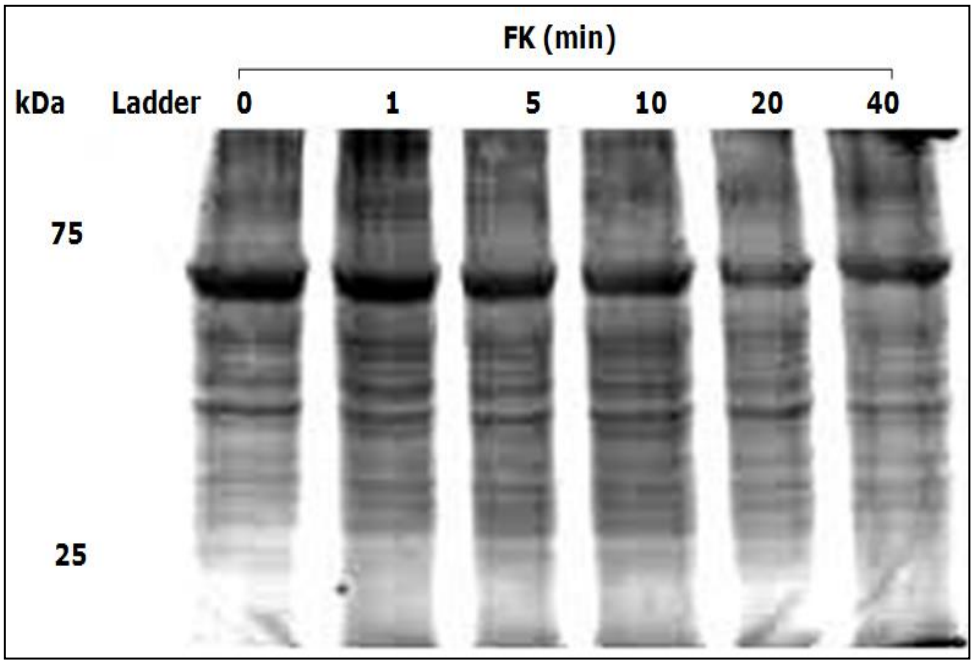

**Figure 8.1 Quantification of protein phosphorylation in response to PMA in H9c2 cells by Pro.Q diamond phospho-stain and SYPRO Ruby protein stain**

H9c2 cell lines were incubated with 1 µM PMA for the times indicated and were then harvested, lysed and denatured at 95°C in hot Laemmli buffer. Lysates were resolved by SDS-PAGE and proteins were revealed with Pro-Q Diamond phosphoprotein stain (A) and subsequently with SYPRO Ruby total protein gel stain (B). Protein marker lane 1, Control (0 min) lane 2, 1 µM PMA treated H9c2 cells in time course; lanes 3-7, respectively. (C) Densitometry was carried out in Adobe Photoshop CS4 and values plotted as relative intensity versus the treatment incubation time. Results represent mean ± SEM of the optical density ratio from three independent experiments. Data are expressed as the percentage of phosphorylation of proteins at basal level in the untreated cells (0 min). *P < 0.05,***P <0.001. Proteins showing increased phosphorylation are highlighted by red circles.

**A)**


FK (min)
kDa
Ladder
0
1
5
10
20
40
75
25

**B)**


FK (min)
kDa
Ladder
0
1
5
10
20
40
75
25

**C)**

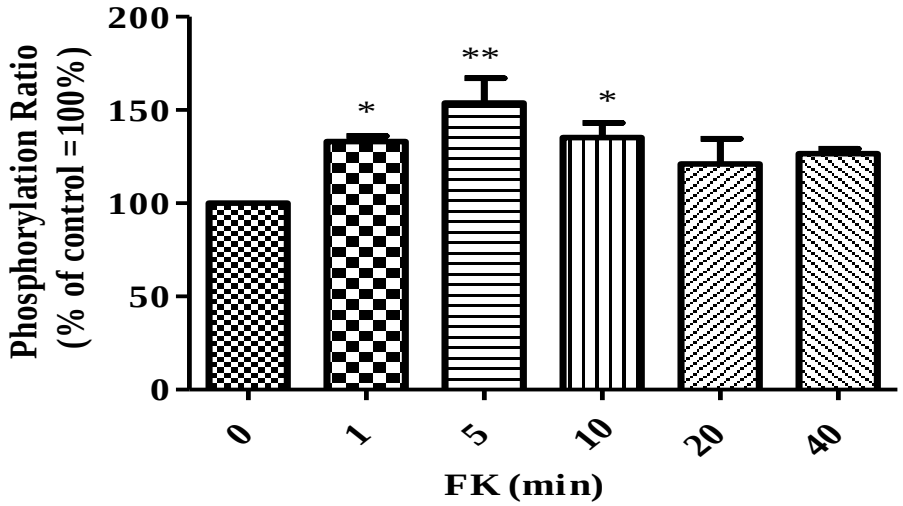



**Figure 8.2 Quantification of protein phosphorylation in response to FK in H9c2 cells by Pro.Q diamond phospho-stain and SYPRO® Ruby protein stain**

H9c2 cell lines were incubated with 10 µM FK for the times indicated and were then harvested, lysed and denatured at 95°C in hot Laemmli buffer. Lysates were resolved by SDS-PAGE and proteins were revealed with Pro-Q Diamond phosphoprotein stain (A) and subsequently with SYPRO Ruby total protein gel stain (B). Protein marker lane 1, Control (0 min) lane 2, 10 µM FK treated H9c2 cells in time course; lanes 3-7, respectively. (C) Densitometry was carried out in Adobe Photoshop CS4 and values plotted as relative intensity versus the treatment incubation time. Results represent mean ± SEM of the optical density ratio from three independent experiments. Data are expressed as the percentage of phosphorylation of proteins at basal level in the untreated cells (0 min). *P < 0.05,**P <0.01. Proteins showing increased phosphorylation are highlighted by red circles.

The results demonstrate (Fig. 8.1) an increase in band intensity of phpophoproteins in PMA treated samples over time compared to the control cells (0 min). The increase was most prominent after 5 & 10 min (Fig. 8.1a). In addition, when samples were subsequently stained for protein with SYPRO Ruby stain (Fig. 8.1b), the ratio of protein phosphorylation was determined from both stains and calculated relative to control. This shows an increase in protein phosphorylation of the samples that were treated with PMA at the early time points (Fig. 8.1c).

This was also true when H9c2 cells were treated with FK as a protein kinase A activator (Fig. 8.2).

***Western blot analysis of phosphorylated protein***

Western blots of H9c2 cell extracts treated with PMA or FK in different time points were probed with anti-phosphoserine (Fig. 8.3a) and anti-phosphothreonine antibodies (Fig. 8.3b). In general, the results demonstrate an increase in band intensity of proteins containing either phosphoserine or phosphothreonine in treated samples over time compared to the control cells (0 min). Interestingly, on the filter paper that was probed with anti-phosphoserine (Fig. 8.3a) there was a slight increase in band intensity ~74 kDa that corresponded to standard TG2 in comparison to control and one more band in 50 kDa. However, when membrane was stripped and re-probed with phosphothreonine antibodies, TG2 the standard no longer appeared but a new band corresponding to ~70 kDa was detected. Another western blot of the sample probed with anti-phosphotyrosine antibodies (Fig. 8.3c). This showed an increase in band intensity of proteins that corresponded to size of~100 kDa and ~77 kDa over the incubation time.

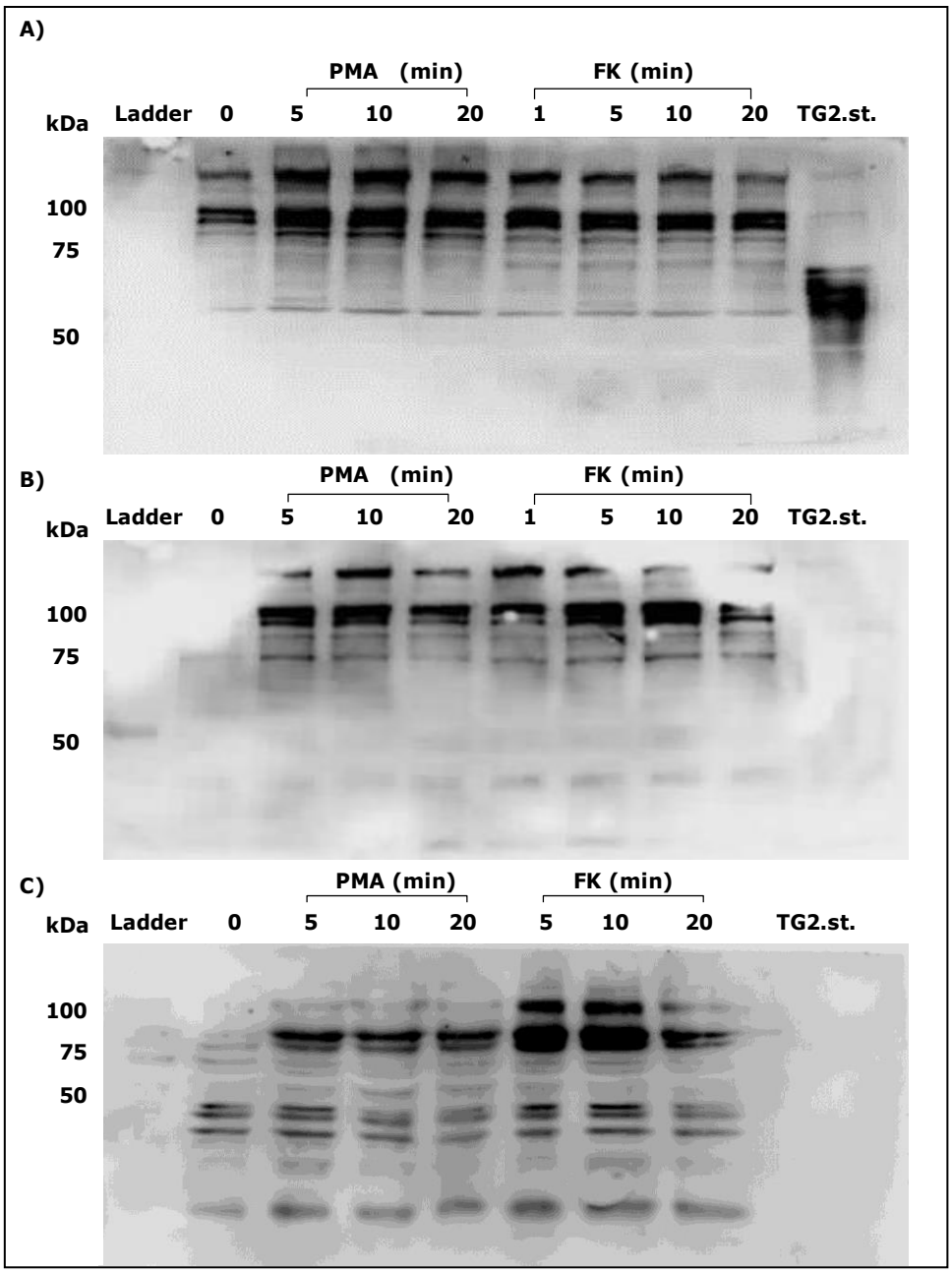



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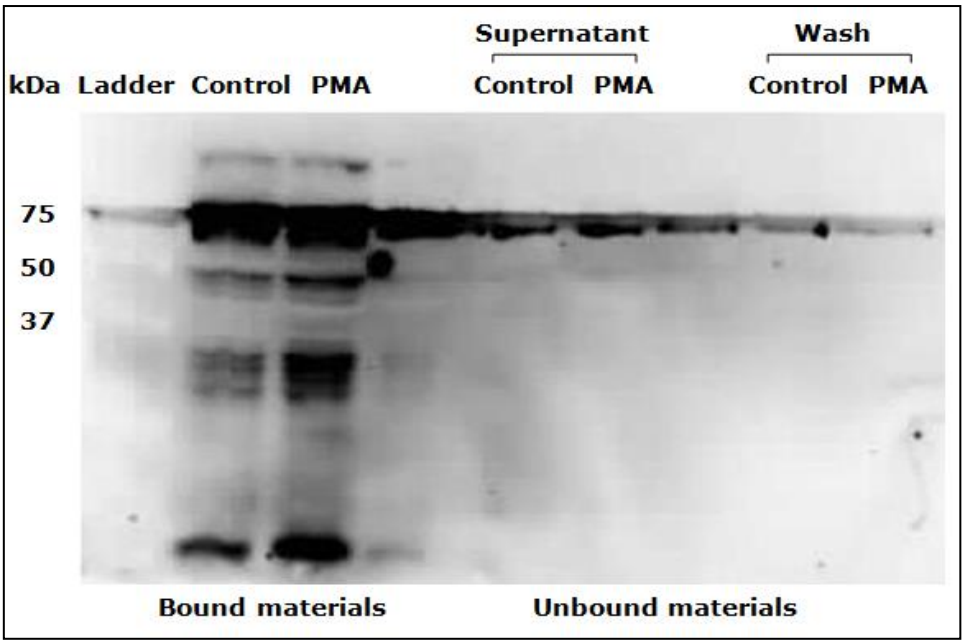
**Figure 8.3 Detection of protein bound phospho-tyrosine, phospho-serine and phospho-threonine in PMA and FK treated H9c2 cells**

H9c2 cell lines were incubated with 1 μM PMA or 10 μM FK for the times indicated and were then harvested, lysed and denatured at 95°C in hot Laemmli buffer. Lysates were loaded and resolved by SDS-PAGE and transferred onto nitrocellulose membrane filters. Filters were then blocked and probed with (A) anti-phosphoserine, (B) anti-phosphothreonine (C) anti-phosphotyrosine. Protein marker lane 1, control (untreated cells = 0 min) lane 2, PMA treated H9c2 cells in time course lanes 3-5 respectively and FK treated H9c2 cells in time course lanes 6-9 respectively and TG2 lane 10.

**A)**




**B)**

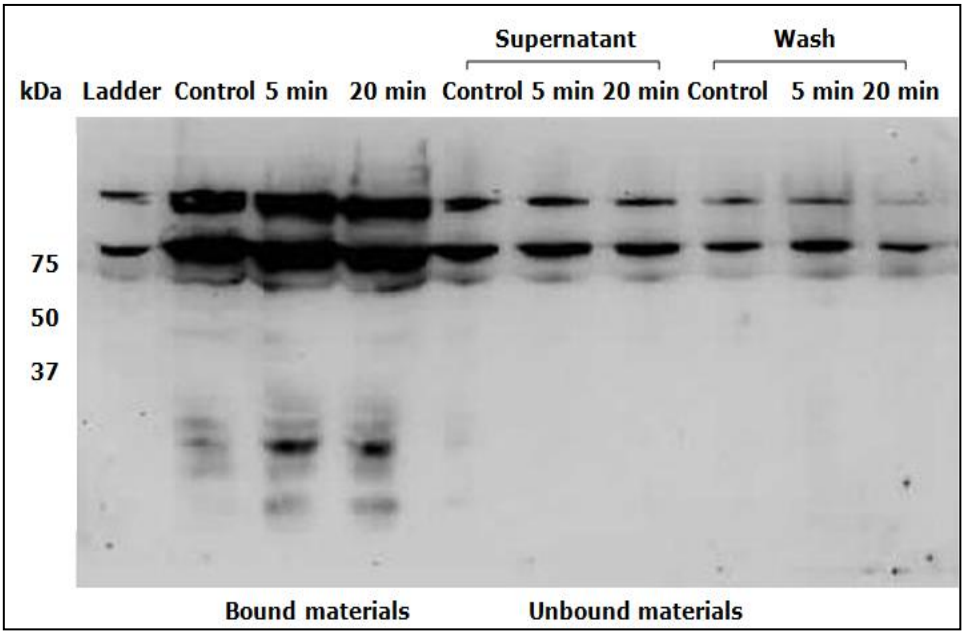



**Figure 8.4 TG2-mediated labelling of PMA/FK treated H9c2 cells with the acyl-acceptor probe biotin-X-cadaverine**

PMA/FK treated biotin-X-cadaverine labelled H9c2 proteins were fractionated on CaptAvidin beads; equal amounts of bound and unbound materials were resolved by SDS-PAGE and analysed by Western blotting. The resultant blots of H9c2 proteins were probed with ExtraAvidin peroxidase. A) biotin-X-cadaverine proteins isolated with CaptAvidin beads from PMA treated cells. B) biotin-X-cadaverine proteins isolated with CaptAvidin beads from FK treated cells.

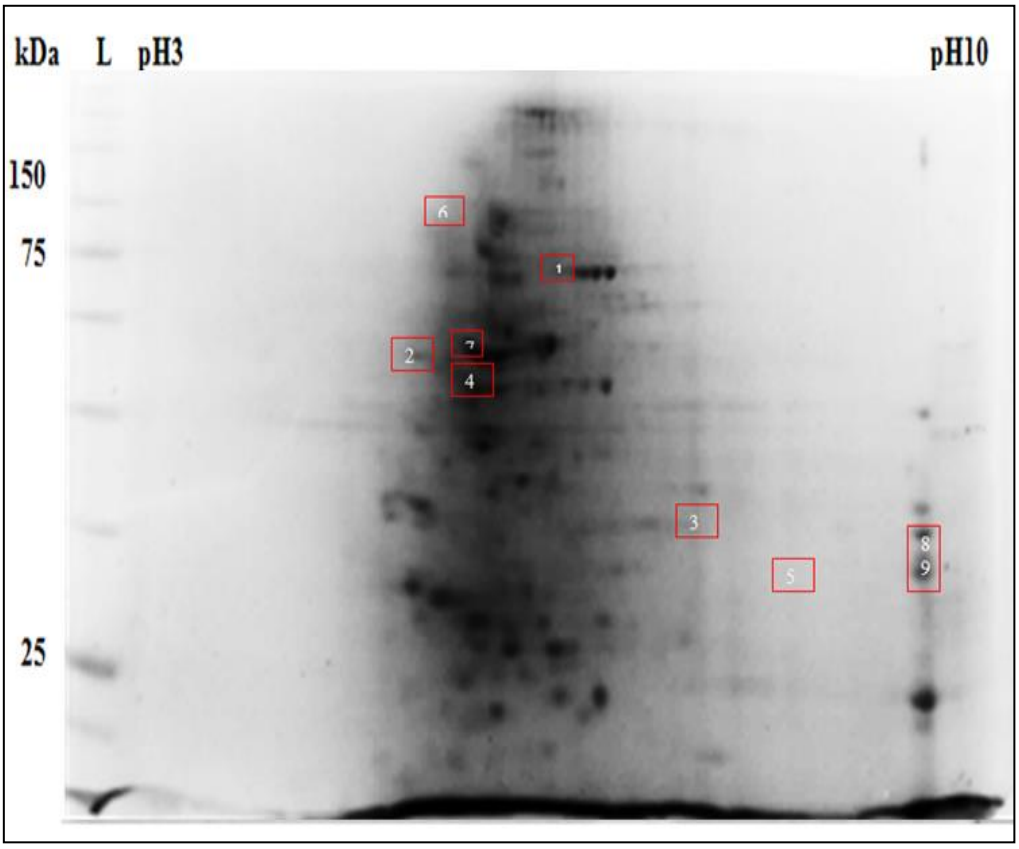



**Figure 8.5 Identification of TG2 substrate proteins in PMA treated H9c2 cells from 2D-PAGE**

H9c2 cells were pre-incubated with 1 mM biotin-X-cadaverine for 4h. They were then treated with 1 µM PMA for 5 min. The biotin-cadaverine labelled proteins were isolated with CaptAvidin beads, subjected to 2D-PAGE and the spots aligned to those on western blots (see Fig 6.2.5). Spots of interest were extracted, digested with trypsin and subjected to mass spectrophotometry. The numbered spots were identified as (1) 78 kDa glucose-regulated protein, (2) tubulin, (3) annexin; A2, (4) actin, (5) voltage-dependent anion-selective channel protein 1, (6) α-actinin, (7) vimentin and (8-9) 60S ribosomal protein L5.

MATRIX SCIENCE *Mascot Search Results*

### 1) Protein View

Match to: **VDAC1_RAT** Score: **194**
**Voltage-dependent anion-selective channel protein 1 OS=Rattus norvegicus GN=Vdac1 PE=1 SV=4**

Nominal mass ($M_r$): **30737**; Calculated pI value: **8.62**
NCBI BLAST search of VDAC1_RAT against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Rattus norvegicus
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **44%**

Matched peptides shown in **Bold Red**

**1** MAVPPTYADL GKSARDVFTK GYGFGLIKLD LKTKSENGLE FTSSGSANTE
**51** TTKVNGSLET KYR**WTEYGLT FTEKWNTDNT LGTEITVEDQ LAR**GLK**LTFD**
**101** **SSFSPNTGK**K NAKIKTGYKR EHINLGCDVD FDIAGPSIRG ALVLGYEGWL
**151** AGYQMNFETS KSR**VTQSNFA VGYKTDEFQL HTNVNDGTEF GGSIYQK**VNK
**201** **KLETAVNLAW TAGNSNTR**FG IAAKYQVDPD ACFSAK**VNNS SLIGLGYTQT**
**251** **LKPGIKLTLS ALLDGK**NVNA GGHKLGLGLE FQA

### 2) Protein View

Match to: **ACTB_RAT** Score: **185**
**Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1**

Nominal mass ($M_r$): **41710**; Calculated pI value: **5.29**
NCBI BLAST search of ACTB_RAT against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Rattus norvegicus
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **31%**

Matched peptides shown in **Bold Red**

**1** MDDDIAALVV DNGSGMCK**AG FAGDDAPRAV FPSIVGRPR**H QGVMVGMGQK
**51** DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEK**IWHHTF YNELRVAPEE**
**101** **HPVLLTEAPL NPK**ANREKMT QIMFETFNTP AMYVAIQAVL SLYASGRTTG
**151** IVMDSGDGVT HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTER**GYSF**
**201** **TTTAER**EIVR DIKEKLCYVA LDFEQEMATA ASSSSLEK**SY ELPDGQVITI**
**251** **GNER**FRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR K**DLYANTVLS**
**301** **GGTTMYPGIA DR**MQKEITAL APSTMKIK**II APPER**KYSVW IGGSILASLS
**351** TFQQMWISK**Q EYDESGPSIV HR**KCF

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## Figure 8.6 An example of Mascot fingerprinting reports for some of identified TG2 substrates

Report shows fingerprinting of 1) voltage-dependent anion-selective channel protein 1, and 2) actin, cytoplasmic 1, the score and % sequence coverage are highlighted in yellow.