Transglutaminase in the Life and Death of the Pancreatic β-cell

Shakthi Dookie

Thesis submitted in partial fulfillment with the requirements of Nottingham Trent University for the degree of Doctor of Philosophy

June 2010

ABSTRACT

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Tissue transglutaminase (TG2) is a ubiquitous enzyme that catalyses both the Ca^{2+}-dependent formation of protein cross-links via intermolecular isopeptide bonds, and the Ca^{2+}-independent hydrolysis of GTP. The multifunctional nature of the TG2 protein has been reported in numerous intracellular mechanisms, cell-surface associations, and extracellular matrix (ECM) interactions. In the pancreas, the expression of TG may be fundamental to the insulin-secretion function of β-cells, and associated diabetic disorders. The functional roles of TG2 in the pancreas were investigated in the present study using *in vitro* models of rat pancreatic insulinoma β-cells (BRIN-BD11), and *ex vivo* islet of Langerhans models from human, rat, $TG2^{(-/-)}$ knockout mice and their wild-type counterparts. The importance of ECM-associated TG2 in the maintenance of β-cell survival and function was also investigated using an *in vitro* human urinary bladder carcinoma support matrix (5637 cells). Biochemical analysis of both clonal BRIN-BD11 and islet β-cells showed a thiol-dependent TG2 activity mechanism that was reciprocally regulated by Ca^{2+} and GTP. Intracellular TG2 cross-linking activity was up-regulated in the presence of glucose and retinoic acid, while cell-surface associated TG2 activity was reduced in the presence of membrane proteases. The *in vitro* application of irreversible active-site directed TG inhibitors (R281, R283 and BOC-peptide) strongly supported a role for TG2 in the glucose-stimulated insulin secretion function of BRIN-BD11 β-cells and rat islets, with a possible survival role for the enzyme under conditions of β-cell oxidative stress. Further characterisation of the pancreatic β-cell TG led to the discovery of a novel short-form (~60-kDa) TG2 protein expressed in BRIN-BD11, human and mouse islets that was immunoreactive to CUB7402 antibody, and showed GTP-binding potential. Analysis of the BRIN-BD11 proteome using 2-D SDS-PAGE and western blotting exhibited possible phosphorylation of the TG2 protein at ~60-kDa (pI 4-6), with an additional 120-kDa and ~35-kDa protein. Molecular screening using northern blot analysis of BRIN-BD11 mRNA confirmed the presence of two short-form TG2 transcripts at ~2.5-kb and ~1.0-kb in addition to the full length 3.5-kb TG2 transcript, while RT-PCR analysis using C-terminal directed primers revealed a short-form BRIN-BD11 product similar to an alternatively spliced shorter TG2 isoform found in rat brain. Modifications to ECM-associated TG2 in an *in vitro* 3-day 5637 support matrix demonstrated the importance of this protein in cell adhesion, cell spreading and aggregation in the maintenance of BRIN-BD11 survival and function. Preliminary studies produced an up-regulation of β-cell focal adhesion kinase, and actin-stained focal adhesion points, in response to the TG2-rich support matrices. The 5637 pre-conditioned matrix also supported greater β-cell viability in response to diabetic conditions such as hyperglycaemia, oxidative stress, and hyperlipidaemia. These findings demonstrate that the primary role for TG2 in pancreatic β-cells may be attributed to insulin secretion and related diabetic stress survival mechanisms. This study also reports the discovery of a shortened TG2 isoform unique to pancreatic β-cells that could lend further insight into TG alternative splicing mechanisms as it relates to structure-function complexity and transcriptional regulation.

DECLARATION

This work has not been accepted in substance for any other degree, and is not being concurrently submitted in candidature for any other degree.

This is to certify that the work presented here was carried out by the candidate herself. Due acknowledgement is made of all the assistance received.

Signed …….

(Candidate)

Signed …….

(Director of Studies)

DEDICATION

In memory of

Dr. Richard A. Jones
(1968 – 2006)

Thank you for being the light and love that guides me through this journey.

From the unreal lead me to the real;
From darkness lead me to light;
From death lead me to deathlessness.
-The Upanishads

ACKNOWLEDGEMENTS

I wish to express immense gratitude toward my supervisors:
Dr. Elisabetta Verderio-Edwards who accepted me as her first PhD student. Thank you for your patience, understanding and time.
Prof. Martin Griffin for his vision, guidance and inspiring discussions which have lit an eternal flame of enthusiasm for science within me. It has been a privilege being in your company both at Nottingham Trent and now Aston University (Birmingham). Thank you also for the access to human islets from the UK Tissue Bank, and the supply of TG inhibitors: R281 and R283.
Prof. Noel Morgan for his encouragement and kindness during my time spent at the Peninsula Medical School (Plymouth). Thank you for teaching me how to isolate Islets of Langerhans, providing the pancreatic models used in this study, and for the β-cell and insulin advice through the years.
Dr Alan Hargreaves and **Prof. Ellen Billet** for all their support during the final and most challenging stretch of my PhD. I am most grateful for your time and patience.

Special thanks to Prof. Glen McHale, Reverend Dr. Richard Davey, Prof. Bob Rees, Prof. Colin Creaser, and Imelda Borland for their compassion during those unexpected PhD intermissions.

In the numerous labs I've worked in, I would like to thank the many people who so generously gave of their time to share their insights with me. At Nottingham Trent, my deepest gratitude goes to my partner in crime, Dr. Richard Jones (who sadly died of cancer during my PhD) for my early TG experimental training, and for all I know about the Confocal Microscope! Thanks to Dr. Panos Kotsakis and Wayne Harris, for all the laughs during the 2D-gel electrophoresis experiments. Cheers to Iain Walker in Area 2 for assistance with the animal experiments, and to Sara Croft for lovely chats during our RT-PCR experimental time. Outside Nottingham, thanks go to Dr. Tim Johnson and Dr. Linghong Huang for their great help during my time spent at the Hallamshire and Northern General Hospitals (Sheffield) while learning the Northern Blotting technique. Thank you to Dr. Russell Collighan for the 6:30am drives in to Aston University (Birmingham) from Nottingham in the middle of those winter months, and for teaching me the qPCR technique (special thanks also to Lin, Dilek and Vivienne for their kindness during this time). Thank you to Dr. Hannah Welters for teaching me the Insulin Secretion Assay and ^{125}[I]-Insulin Radioimmunoassay at Peninsula Medical School (Plymouth). An acknowledgement goes to Prof. Gerry Melino (University Tor Vergata, Italy) for the TG2$^{-/-}$ mice.

I have been in Biochemistry Research for so many years now, that it would be impossible to mention everyone. I will attempt to thank some very special people who have encouraged me along the way, and made me smile even when it seemed like the end of the world:
- For those crazy indoor coffee-break discussions: David Chau, Richard Parsons, Sohail Mushtaq, Danielle Barry (1981–2006, may you rest in peace), Alessandra Scarpellini and Emma Storey.
- For the liberating outdoor coffee break discussions: Joao Cortez, Maria Kontovraki, Amarnath Talasila, Michael Brice, Julia Fitzgerald, Anne Darlison, Lesley Foulds, Begona Caneda-Ferron, Renee Germack, Laurice Fretwell and James Hind.
- To my lovely friends in the postgraduate room: Shiva, Tina, Susan, Indy, Michelle, Steph, Flo, David, Heidi, Ash, Amy, and my MSc students: Javeiria, Shamas, Tayyba, Mrunalini and Anoop.

Outside work, a heartfelt thank you to Richard Jones' parents: Margaret and Terry Jones for taking me under their protective wings, and making Sheffield my second home. Thank you also to Richard's friends: Gary, Jade, Ian, Kirsty and David Oldfield for all the lovely memories. Special mention goes to the kind and compassionate people at the Buddhist Centre (Nottingham) who brought me deeper understanding.

Thank you to Krishan Kapoor for being a pillar of strength through all the PhD-related stress.

A special thank you goes to Spud.

As always, thank you to my 'larger than life' family in South Africa: most fondly my mum, dad, 3 brothers and grandparents for their love, support and constant faith in my potential. Though we are far apart, I remain with you in spirit everyday. Mum, this one's for you: Happy Birthday!

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ADP	adenosine-5'-diphosphate
ATCC	American tissue culture collection
ATP	adenosine-5'-triphosphate
B4.2	band 4.2 protein
BCA	bicinchoninic acid
BSA	bovine serum albumin
Biotin-X-cadaverine	5((N-biotinoyl)amino)hexanoyl)amino)pentylamine tifluoroacetate salt
bp	base pairs
BM	basement membrane
cAMP	adenosine 3',5'-cyclic monophosphate
CD	coeliac disease
cDNA	complementary deoxyribonucleic acid
DMEM	Dulbeco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
DTT	dithiothreitol
CE	cornified envelope
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbant assay
FAK	focal adhesion kinase
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FN	fibronectin
FXIII	factor XIII
Gαh	TG2 α-subunit
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine-5'-diphosphate
gplTG2	guinea pig liver tissue transglutaminase
GTP	guanosine-5'-triphosphate
GTPase	guanosine-5'-triphosphatase
HBSS	Hank's balanced salt solution
HD	Huntington's disease
HRP	horseradish peroxidise
HSPG	heparin sulphate proteoglycan
IL	interleukin
IgG	immunoglobulin
IP_3	inositol triphosphate
kDa	kilodaltons
LTBP-1	Latent transforming TGF-1 binding protein1
M	molar

MAb	monoclonal antibody
MDC	monodansylcadaverine
ml	milliliters
mM	millimolar
MMP	matrix metalloproteinase
µl	microliter
µM	micromolar
MOPS	3-(4-morphonyl) 1-propanesulphonic acid
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
nM	nanomolar
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFM	parafolmaldehyde
pH	negative log of hydrogen ion concentration
PKC	protein kinase C
PLC	phospholipase C
PMSF	Phenyl methyl sulfonyl fluoride
pTG	prostate transglutaminase
RA	retinoic acid
RGD	arginine-glycine-aspartic acid
SD	standard deviation
SDS	sodium dodecyl sulphate
TAE	Tris-Acetate-EDTA
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	N,N,N,N'-tetramethylene diamine
TG	transglutaminase
TGFβ1	transforming growth factor β1
TMB	3,3',5,5'- tetramethylbenzidine
TNF	tumour necrosis factor
TRITC	tetramethyl rhodamine B isothiocyanate
Triton X-100	t-ocylphenoxypolyethoxyethanol
UV	ultra violet
VEGF	vascular endothelial growth factor

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COMMUNICATIONS AND ABSTRACTS

Dookie, S.; Griffin, M.; Ali, T.; Chokhandre, M.; Singh, A.; Billet, E.E.; Morgan, N.G.; Verderio, E.A.M.; Matrix-associated Transglutaminase in Pancreatic β-cell Function and Survival; *9th International Conference for Protein Crosslinking and Transglutaminases*, Marrakech (Morocco), Sep 1-5th, 2007. (Poster Presentation)

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Chapter One:

Introduction

Matt Ridley from *Genome*

"In the beginning was the word. The word proselytized the sea with its message, copying itself unceasingly and forever. The word discovered how to rearrange chemicals so as to capture little eddies in the stream of entropy and make them live. The word transformed the land surface of the planet from a dusty hell to a verdant paradise. The word eventually blossomed and became sufficiently ingenious to build a porridgy contraption called a human brain that could discover and be aware of the word itself. My porridgy contraption boggles every time I think this thought…

'…As the earth and ocean were probably peopled with vegetable productions long before the existence of animals; and many families of these animals long before other families of them, shall we conjecture that one and the same kind of living filament is and has been the cause of all organic life?' asked the polymathic poet and physician Erasmus Darwin in 1794. Yet how can a filament make something live?

Life is a slippery thing to define, but it consists of two very different skills: the ability to replicate and the ability to create order… The key to both of these features of life is information. The ability to replicate is made possible by the existence of a recipe, the information that is needed to create a new body…The ability to create order through metabolism also depends on information – the instructions for building and maintaining the equipment that creates the order.

The filament of DNA is information, a message written in a code of chemicals, one chemical for each letter…Heredity is a modifiable stored program; metabolism is a universal machine. In the beginning was the word. The word was not DNA. That came afterwards, when life was already established, and when it had divided the labour between two separate activities: chemical work and information storage, metabolism and replication. But DNA contains a record of the word, faithfully transmitted through all subsequent aeons to the astonishing present."

Ridley, Matt (1958-), Zoologist and Science Writer. *Genome*, Fourth Estate, 1999. Quoted from *The Oxford Book of Modern Science Writing* by Richard Dawkins, Oxford University Press, 2008.

Chapter One:

Introduction

1.1. Introduction to the Transglutaminase

It has been just over half a century since the first published discovery of the protein, transglutaminase (TG). Since then, this enigmatic TG enzyme has been studied extensively within an accumulating and complex knowledge base, growing in conjunction with the latest biochemical and molecular techniques over time (for relevant reviews see: Zemskov *et al*., 2006; Esposito and Caputo, 2005; Verderio *et al*., 2004; Lorand and Graham, 2003; Fesus and Piacentini, 2002; Griffin *et al*., 2002; Aeschlimann and Thomazy, 2000; Chen and Mehta, 1999; Folk, 1980). Insight into TG's role and activity has become essential for a fuller understanding of numerous hereditary diseases and various autoimmune, inflammatory and degenerative conditions. Between 1957 to 1960 Waelsch and co-workers, while studying this enzyme's activity in the liver of the guinea pig, coined the term 'trans-glutamin-ase' for the first time (Sarkar *et al*., 1957) to describe it's main function; i.e. the Ca^{2+}-dependent transamidation of glutamine side chains, which results in the irreversible cross-linking of proteins into stabilised matrices (Neidle *et al*., 1958; Clarke *et al*., 1959; Mycek *et al*., 1959; Mycek and Waelsch, 1960).

1.1.1. The Transglutaminase Family

In man, the TG family of intracellular and extracellular enzymes consist of nine TG genes, eight of which encode active enzymes (Grenard *et al*., 2001), but of which only six have been isolated and characterised to date. The uncovered TG family in humans (Table 1.1) features: i) factor XIII (fXIII) found in blood; ii) band 4.2, which maintains erythrocyte membrane integrity and has lost its enzymatic activity; iii) TG1, TG3 and TG5 isoforms found intracellularly in epithelial tissue; iv) TG2 which is

Table 1.1

expressed ubiquitously in tissues and takes on both intracellular and extracellular forms; and (v) TG6 and TG7, the tissue distribution of which remains unknown. The TG enzyme activity has also been identified in other mammals (Chung, 1972), invertebrates (Mehta *et al.*, 1990 and 1992; Singh and Mehta, 1994), plants (Serafini-Fracassini *et al*., 1995) and micro-organisms (Chung, 1972; Folk and Finlayson, 1977; Kanaji *et al.*, 1993). Orthologues of the human TGs have been identified within some of these organisms using phylogenetic analysis (Lorand and Graham, 2003; Grenard *et al*., 2001; Fig 1.1), and suggests two branches arising from a common ancestral gene which underwent an early gene duplication event; with one lineage connecting FXIII, TG1, invertebrate TGs and TG4, and the other connecting genes for TG2, TG3, TG5, TG6, TG7 and erythrocyte band 4.2. Of further interest, a superfamily of archaeal, bacterial, and eukaryotic proteins homologous to animal TGs have also been identified using profiles generated by the PSI-BLAST program (Makarova *et al.*, 1999).

The encoded TG genes have a high degree of sequence similarity (Grenard *et al*., 2001), however gene mapping has revealed distinct chromosomal locations for each one (Table 1.1). Interestingly, alternative splice variants have also been identified for many TGs, including TG1 and TG2 (Citron *et al*., 2001; Shevchenko *et al*., 2000; Kim *et al*., 1992). The mammalian TGs also share similar intron and splice-site distribution (Grenard *et al.*, 2001), with TG2, TG3, TG5, and Band 4.2 containing 13 exons, while TG1 and fXIIIA contain 15. This extra exon within the conserved exon 10 of the TG family, together with a non-homologous N-terminal extension, account for the two extra coding sequences in fXIII A subunit and TG1.

The pathologies associated with mutations in the different TG gene products reinforce the absence of redundancy in this gene family, and further support the importance of protein cross-linking (Ichinose *et al*., 1986; Mikkola and Palotie, 1996; Huber *et al*., 1995; Russell *et al*., 1996; Board *et al*., 1993). In addition to genetic diversity, TGs themselves undergo a number of post-translational modifications such as phosphorylation, fatty acylation, and proteolytic cleavage, as a means of regulating enzymatic activity and subcellular localisation in varying biological situations (Mishra and Murphy 2006; Rufini *et al*., 2004; Aeschlimann and Paulsson, 1994).

Fig 1.1 Phylogenetic tree

The TG family members can all be distinguished from one another based on their physical properties, tissue distribution, localisation, mechanisms of activation, and substrate specificities (Table 1.1 and 1.2). There are, however a number of similar features that they share. Perhaps the most enigmatic of these is that all TGs, including those which are secreted such as TG2 and fXIII, lack the amino-terminal hydrophobic leader sequence typical of secreted proteins (Gaudry *et al*., 1999). Most TGs are post-translationally modified by the removal of the initiator methionine residue followed by *N*-acetylation. Furthermore, common to all TG members is their lack of carbohydrate modificiation and disulphide bonds despite the presence of several potential sites for *N*-linked glycosylation, and many Cys residues. And finally most TGs require Ca^{2+} for activation, except in the still questionable instances of plant and bacterial TGs (Lorand and Graham, 2003).

1.1.2. Enzyme Reaction Mechanisms

More specifically, the TG family of enzymes catalyse the post-translational modification of proteins and is denominated by the Enzyme Commission as the "R-glutaminyl-peptide-amine-γ-glutamyl transferase" reaction (EC 2.3.2.13), although the commonly used term "transglutaminase" remains (Lorand and Conrad, 1984). A myriad of aminolytic and hydrolytic biological reactions are catalysed by TGs (Fig. 1.2), however their protein cross-linking function has attracted the greatest interest to date.

Usually, oligomeric and polymeric protein associations involve secondary chemical forces such as non-covalent hydrogen bonds, ionic bonds and Van der Waal's forces; where the subunits of protein assembly and disassembly are not covalently linked and are regulated by phosphorylation and de-phosphorylation during post-translational modifications. The enzymatic action of TG serves as an alternative to this scenario where supramolecular protein assemblies are generated using Ca^{2+} and covalent bonding (Pisano *et al*., 1968). The biochemical mechanism of the TG enzyme involves an initial rate limiting step where the γ-carboxamide group of a glutamine residue is transamidated to form a γ-glutamylthiolester intermediate (Curtis *et al*., 1974) with the TG active site cysteine (Cys^{277}) which results in the release of

Fig 1.2 TG Reaction Mechanisms

ammonia (Fig. 1.2). Conventionally, this reaction results in the transfer of this acyl intermediate to a nucleophilic substrate that attacks the intermediate thiolester bond, usually the ε-amino group of a peptide-bound lysine residue (Fig. 1.2, A), forming an ε-(γ-glutamyl)lysine isopeptide bond or cross-link (Folk and Chung, 1973). The TG enzyme is released and the active site Cys^{277} is re-established to original form, enabling participation in further catalysis cycles.

At this stage, it is also possible for the monomeric protein units themselves to become cross-linked internally (Porta *et al*., 1991). Formation of these covalent cross-links are extremely stable, cannot be cleaved by any known mammalian protease, and are resistant to tested mechanical and chemical disruption. However, despite being described for a long time as irreversible (Lorand and Conrad, 1984), the hydrolysis of the ε-(γ-glutamyl)lysine isopeptide bond is possible by TG itself (Fig. 1.2, E) as demonstrated by Parameswaran *et al.*, (1997); and by Lorand *et al.*, (1996) where enzymes from the secretory products of the medicinal leech were shown to hydrolyse the ε(γ-glutamyl)lysine isopeptide bond in blood clots.

The TGs show exacting specificities for both their glutamine (Murthy *et al*., 1994) and lysine substrates (Lorand *et al*., 1992), and will react only with the γ-carboxamide of select endo-glutamine residues in some proteins and peptides depending on the conformation of the TG protein (Aeschlimann and Paulsson, 1994). In the cross-linking reaction described above (Fig 1.2, A), a peptide bound glutamine residue acts as an acyl acceptor and a peptide-bound lysine as the acyl donor, however a whole variety of suitable primary amine-groups can also function as the acyl donor (Folk and Chung, 1973). Post-translational modification of proteins by TG using amine incorporation (Fig. 1.2, B), acylation (Fig. 1.2, C), and deamidation (Fig. 1.2, D) are also very significant to the equilibrium of biological processes and can alter conformation, stability, molecular interactions and enzymatic activities of the target proteins (Lorand and Graham, 2003; Fesus and Piacentini, 2002; Griffin *et al*., 2002; Aeschlimann and Thomazy, 2000; Chen and Mehta, 1999).

In transamidation, both amine incorporation (Fig. 1.2, B) and acylation (Fig. 1.2, C) compete with the cross-linking reaction (Fig.1.2, A) and this ‘competition’ is still the

main method of assaying TG activity biochemically. Numerous primary amines are incorporated by TG into proteins through the formation of N'(γ-glutamyl)amine bonds, using molecules such as histamine, putrescine, spermine, and spermidine (to name a few) as acyl donors (Folk and Finlayson, 1977; Lorand and Conrad, 1984). Interestingly, this scenario can become more complicated when a free amine on the acyl donor group can also be linked to another γ-glutamyl group on a second protein, forming an N',N'-bis (γ-glutamyl) polyamine linkage. Post translational modification of proteins by this polyamidation may result in altered biological activity, antigenicity, and turnover rate (Aeschlimann and Paulson, 1994). With regard to TG-catalysed deamidation, even the replacement of a single neutral glutamine residue with a negatively charged glutamic acid side chain (Fig 1.2, D) may have profound biological effects (Lorand and Graham, 2003). Although previously unrecognised, esterification can now also be added to the list of TG-catalysed post-translational modifications of proteins through the linking of long chain fatty acids (ceramides) using an ester bond, found in the terminal differentiation of keratinocytes (Nemes *et al*., 1999).

1.1.3. Protein Structure and Conformation

The catalytic mechanisms of the TGs have been solved based on both biochemical data and X-ray crystallographic tertiary structures. In 1994, the first high resolution structure of any member of the TG family was published (Yee *et al*., 1994), and most importantly, these enzymes were clearly defined as members of the superfamily of papain-like cysteine proteases, which includes papain, calpain, foot and mouth virus protease, de-ubiquitylating enzymes, and *N*-acetyl transferases (Murzin *et al*., 1995).

High sequence conservation and preservation of residue secondary structure (Grenard *et al*., 2001) suggests that all TG family members may share a four domain tertiary structure similar to those already determined for fXIII, sea bream TG, TG3, and TG2 (Yee *et al*., 1994; Noguchi *et al*., 2001; Ahvazi *et al*., 2002, Liu *et al*., 2002, respectively). This four-sequential globular domain arrangement (see Fig. 1.3 and Fig. 1.4 for the example of TG2) consists of: i) an NH_2-terminal β-sandwich; ii) a core which contains a Ca^{2+}-binding site and 'active' transamidation site (that has α

Fig 1.3 TG2 structure

Fig 1.4 TG2 domains

helices and β sheets in equal amounts); iii and iv) two COOH-terminal β-barrel domains.

It is thought that glutamyl substrates approach the enzymes from the direction of the two β-barrels, whereas lysyl substrates might approach the enzyme from the direction of the active site (Lorand and Graham, 2003). The catalytic centre of TG possesses a triad comprised of Cys^{277}, His^{335} and Asp^{358} (where His is histidine and Asp is aspartic acid), with a crucial tryptophan (Trp) residue located 36 residues upsteam from the active-Cys centre (Lorand and Graham, 2003). This catalytic triad is similar to that of thiol proteases and endows high reactivity to Cys^{277} in the formation of thiol-ester intermediates (Liu *et al*., 2002).

The relative positions of residues in the substrate-binding region are highly conserved in TGs (see Fig. 1.3 and Fig 1.4), notably sharing a common sequence for the active site with the amino acid sequence [Y-G-Q-C-W-V] (Ikura *et al.*, 1988; Gentile *et al.*, 1991; Greenberg *et al.*, 1991). Despite sequence similarity however, the charge distributions differ amongst the various isoenzymes. These differences may explain the various substrate specificities and therefore the specialised functions of each isoenzyme.

1.1.4 Transglutaminase Substrates

The complete molecular mechanism governing the differential acceptor-/donor-substrate specificities (and recognition of target amino acids), for TG proteins is still unknown. Nonetheless, a number of biochemical and conformational studies of the individual enzymes exist, within the context of their preferred physiological locations, which shed some light on this aspect. Due to the innumerable substrate possibilities and evidence existing already, a TRANSIT database was recently created (http://crisceb.unina2.it/ASC/) listing protein sequences that function as TG substrates (Facchiano *et al*., 2003).

Firstly, glutamine (Q-acceptors) specificity shows that the spacing between the targeted glutamine and neighbouring residues is of utmost importance in the

specificity of TGs (Esposito and Caputo, 2005). Intriguingly, N- and C-terminal glutamines are not recognised by TGs (Pastor *et al*., 1999). Additionally, it seems essential that glutamine residues be exposed at the surface of the acceptor protein, or located in terminal extensions protruding from the compactly folded domains. It is also thought that the location of the glutamine within the primary structure may not be as important as the secondary or tertiary structure of the acceptor protein for cross-linking to occur (Coussons *et al*., 1992). Two adjacent glutamine residues can act as amine acceptors in a consecutive reaction, eg. βA3-crystallin (Berbers *et al*.; 1984); substance P (Porta *et al*., 1988); oseonectin (Hohenadl *et al*., 1995); and insulin-like growth factor-binding protein (Sakai *et al*., 2001). Furthermore, glutamine residues flanked by positively charged residues prevent the TG reaction, while positively charged residues two or four residues from the glutamine promote the reaction (Esposito and Caputo, 2005). Glycines and asparagines adjacent to the target glutamine favours substrate accessibility (Aeschlimann *et al*., 1992; Coussons *et a*l., 1992), while a glutamine residue in between two proline residues is evidently not recognised as a substrate (Pastor *et al*., 1999), possibly due to the inflexibility that prolines impart to the local protein structure (Kay *et al*., 2000). It is also of significance that alternate TGs can recognise different glutamine residues in the same protein, complicating matters further (McDonagh and Fukue, 1996).

With regard to the amine donors or lysine residues (K-donors), TGs are much less selective. It has been demonstrated, however that the nature of the amino acids directly preceding the lysine may influence reactivity (Grootjans *et al*., 1995). As described above, donor proteins containing aspartic acid, glycine, proline and histidine residues reduce reactivity, whereas uncharged, basic polar and small aliphatic residues favour reactivity (Groenen *et al*., 1994). In addition, steric hindrance between TG and potential substrates can prevent recognition of lysine residues, even in lysine-rich peptides/proteins, for eg. only three lysyl residues out of twenty six is recognised in glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Orru *et al*., 2002); one of five lysine residues acts as amine donors in β-endorphin (Pucci *et al*., 1988); and one of ten in αB-crystallin (Groenen *et al*., 1992). Appropriate conformational changes in the amine-donor proteins are therefore necessary before explaining complete reactivity.

Using both structural and functional proteomics, lists of protein substrates for TG1, TG2, TG3, TG5 and fXIII can be seen in Table 1.2. To date, many more substrates have been found for TG2 than for the other TG isoforms. Unfortunately TG2-mediated modification of substrate proteins cannot be consistently linked to a defined function. In the future, it is thought that the identification of as many TG2-substrates as possible, coupled with a combined structural and functional proteomics approach will lead to the verification of the numerous *in vivo* TG functions. The potential of this research is quite profound when the numerous TG substrates that may be utilised as novel drug targets and/or diagnostic markers for the various TG-related diseases (see section 1.3.7) are considered. If a peptide or recombinant protein can be shown to inhibit a TG, it may potentially represent a therapeutic/prophylactic agent for the future.

1.1.5. Turning a Transglutaminase on

In latent TG conformation, accessibility to the active centre is reduced by inter-domain interaction between the catalytic domain 2 and domains 3 and 4. The binding of Ca^{2+} to the catalytic domain of TG2 alters conformation of the protein by stretching the domains 3 and 4 further apart from the catalytic domain, thus opening access to the transamidation site (Monsonego *et al*., 1998; Liu *et al*., 2002).

Latent TG can be activated inside cells by increasing the concentration of intracellular Ca^{2+} (Clarke *et al*., 1959; Smethurst and Griffin, 1996). This is the usual *in vitro* scenario (Bungay *et al*., 1984a; Lorand and Conrad, 1984). *In vivo*, Ca^{2+} may be released into the cytosol from intracellular stores or it might be brought in from outside the cell (Lorand and Graham, 2003). Given the relatively high concentration of Ca^{2+} that is required for the activation of the different TGs, it can further be assumed that TG activation occurs mainly during extreme situations when the internal Ca^{2+} storage capacity of the cell becomes overwhelmed or when the outward-directed Ca^{2+} pumps fail to keep up with the influx of cations (Gomis *et al*., 1984).

Table 1.2 TG Substrates

It is worth noting here that TG2 and TG3 possess a site that binds and hydrolyses GTP even though the site lacks any obvious sequence similarity with canonical GTP-binding proteins (Liu *et al*., 2002, Ahvazi *et al*., 2004a). Additionally, the primary sequence of TG5 contains a similar GTP-binding pocket, and TG5 transamidating activity is shown to be inhibited by GTP (Candi *et al*., 2004). In the case of TG2, intracellular GTPase activity occurs independently of cross-linking activity, but both activities are regulated by the protein's reciprocal binding to Ca^{2+} or GTP (see figures 1.3). The inhibitory effect of GTP on TG2 transamidating activity is mediated by GTP binding and hydrolysis involving Ser^{171} and Lys^{173} residues of domain 2 (Iisma *et al*., 2000).

1.2. Mammalian Transglutaminases

1.2.1 Plasma Factor XIIIA

One of the most extensively characterised members of the TG family of enzymes is factor XIII (fXIII). Its physiological role has been firmly established as a homodimer (166-kDa) of two 730 amino acid A-subunits, which acts as a pro-enzyme that requires cleavage by the serine protease thrombin during the final stage of the blood coagulation cascade (Schwartz *et al.*, 1973). This TG enzyme is externalised into the plasma and circulates as a 320-kDa heterotetramer composed of a catalytic dimer of two A-chains (A_2) and two non-catalytic B-chains (B_2) which are non-covalently associated (Schwartz *et al.*, 1973).

The human factor XIII A-chain (fXIIIA) gene is localised in chromosome 6p24-25, consists of 15 exons and the mature protein has a molecular weight of 83-kDa (see Table 1.1, Ichinose *et al.*, 1990; Greenberg *et al.*, 1991). The thrombin cleavage site is located between exons 2 and 12; the potential calcium binding sites in exons 6 and 11; and the active centre sequence in exon 7 (Ichinose *et al.*, 1990; Hettasch and Greenburg, 1994). X-ray crystallography of placental and recombinant fXIIIA revealed that the protein is folded in 5 distinct domains: an activation peptide; an N-terminus β-sandwich domain; a core domain; and two C-terminus β-barrels (Yee *et*

al., 1994). The same study revealed that the common TG family catalytic triad (Cys^{314} – His^{373} – Asp^{396}) is present in the active site of fXIIIA (Yee *et al.*, 1994).

The fXIIIA enzyme is expressed in a wide variety of cells including macrophages, megakaryocytes and monocyte cell lines; while tissue distribution is in the placenta, uterus, prostate and liver (Carrell *et al.*, 1989; Takagi and Doolittle, 1974; Schwartz *et al.*, 1973). It is the last enzyme to be activated in the blood coagulation cascade (Lorand and Conrad, 1984), and its activation leads to the cross-linking of various proteins in the plasma. The strength of blood clots are increased by fXIIIa through catalysing transamidation reactions between fibrin molecules via the formation of intermolecular ε(γ-glutamyl)-lysine cross-links (Shainoff *et al.*, 1991; Lorand and Conrad, 1984; Chen and Doolittle, 1971). This enzyme cross-links: i) fibrin to fibronectin and thrombospondin in order to anchor the blood clot to the site of injury (Bale and Mosher, 1986; Hansen, 1984); ii) fibrin to α_2-antiplasmin so as to increase the resistance of the clot to plasmin degradation (Reed *et al.*, 1991; Tamaki and Aoki, 1981); and iii) fibrin to other coagulation substrates such as factor V, platelet actin and von Willebrand factor (Hada *et al.*, 1986). In addition to being a critical component of the blood coagulation system, fXIIIa also cross-links ECM proteins such as fibronectin (Mosher and Schad, 1979), vitronectin (Sane *et al.*, 1988), certain collagens (Akagi *et al.*, 2002; Mosher and Schad, 1979), α-2 macroglobulin (Mortensen *et al.*, 1981), plasminogen activator inhibitor (Jensen *et al.*, 1993), myosin (Cohen *et al.*, 1981) and lipoproteins (Romanic *et al.*, 1998; Borth *et al.*, 1991).

Individuals who are congenitally deficient in fXIIIA experience a lifelong bleeding disorder with delayed wound healing, spontaneous abortion, and male sterility (Kitchens and Newcomb, 1979). Other diseases associated with abnormal levels of fXIIIA include heart disease (Kloczko *et al.*, 1985 and 1986, Reiner *et al.*, 2001), rheumatoid arthritis (Weinberg *et al.*, 1991), sclerosis (Penneys *et al.*, 1991), gastrointestinal disorders (Lorenz *et al.*, 1991), and acute leukaemia (Ballerini *et al.*, 1985). The pathogenic actions of angiotensin II have also been associated with the covalent cross-linking of angiotensin AT1 receptors by intracellular fXIIIA, resulting in stable receptor dimers with enhanced signalling properties (AbdAlla *et al.*, 2004).

The anti-hyperglycaemic drug, metformin, which reduces the risk of cardiovascular disease in type 2 diabetes, has been reported to reduce fibrin clots and inhibit the thrombin-induced cleavage of the fXIII activation peptide (Standeven *et al*., 2002).

1.2.2 Erythrocyte Band 4.2

Band 4.2 is expressed in high levels in erythroid cells and is an important structural component of the cytoskeletal network at the inner face of the red blood cell membrane (Cohen *et al.*, 1993). This erythrocyte membrane TG protein has a molecular weight of 77-kDa, is encoded into the gene map locus at 15q15.2, and is the only catalytically inactive member of the TG family of enzymes (see Table 1.1, Aeschlimann and Paulsson, 1994). Although it shares a close sequence homology with other TG family members (Grenard *et al*., 2001), this TG possesses an alanine in place of the active site cysteine (Korsgren *et al.*, 1990). Band 4.2 is also an ATP binding protein (like TG2) but the precise function of this property remains uncertain (Azim *et al*., 1996). It is believed to interact and bind to anion exchanger band 3, spectrin, ankyrin and band 4.1 cytoskeletal proteins (Cohen *et al*., 1993). Myristylation of an N-terminal glycine residue (similar to TG1) associates band 4.2 to the plasma membrane where it accounts for approximately 5% of the erythrocyte membrane protein mass (Risinger *et al.*, 1992; Rybicki *et al.*, 1994). Other than its involvement in normal red blood cell cation transport (Peters *et al.*, 1999), the lack of band 4.2 by targeted disruption or mutation causes spherocytosis (Kanzaki *et al*., 1997) and a hereditary deficiency resulting in the fragility of erythrocytes through a phenotype of haemolytic anaemia found to be common in Japan (Hayette *et al*., 1995a; 1995b). Isoforms of band 4.2 exist in many non-erythroid cells involved in haematopoiesis such as foetal spleen, foetal liver and bone marrow (Grenard *et al*., 2001) and is believed to provide essential structural stability, however full functional characterisation in these tissues remains incomplete.

1.2.3 Keratinocyte Transglutaminase

Keratinocyte TG (TG1) exists as multiple soluble forms, either intact or proteolytically processed at the conserved sites, which themselves have varying specific activities, and are inferred to have differing functions in keratinocytes (Kim *et*

al., 1995; Rice and Green, 1977). The gene encoding TG1 has been localised to chromosome 14q11.2-13 and contains 15 exons spliced by 14 introns that have a conserved position in comparison to the other main members of the TG family (see Table 1.1, Kim *et al.*, 1992). The TG1 enzyme is located at the inner face of the keratinocyte plasma membrane, is an intracellular/membrane-bound enzyme with a length of 813-824 amino acids, and is anchored in the membrane through fatty acid acylation of cysteine residues linked to palmitic or myristic acid at the N-terminal (Chakravarty and Rice, 1989; Rice *et al.*, 1990). Active TG1 is thought to be released into the cytoplasm in a soluble form by post-translational proteolytic modification of the enzyme, which targets a cluster of 5 cysteine residues present at the protein's N-terminus through fatty acid acylation with palmitate and myristate (Thacher and Rice, 1985; Kim *et al.*, 1995). This was confirmed when a mutation of the cluster of cysteine residues from the N-terminus of TG1 was shown to prevent the enzyme from associating with the plasma membrane (Phillips *et al.*, 1990). Although TG1 is primarily membrane-bound, 5-35% in proliferating or terminally differentiating keratinocytes is usually found in a soluble state in the cytosol (Kim *et al.*, 1995). Cytosolic TG1 exists as a latent full-length form of 106-kDa, with two proteolytic cleavage products (67-kDa, 67/33-kDa) shown to have 5-10 fold higher specific activities. The membrane bound form of TG1 exists as either a full-length zymogen or a highly active complex of 67/33/10-kDa form and accounts for the remaining 65-95% of the enzyme expressed in epidermal keratinocytes (Steinert *et al.*, 1996).

During the terminal differentiation of keratinocytes, TG1 is induced and is capable of cross-linking specific intracellular proteins that contribute to the formation of the cornified envelope. In the granular layer of the epidermis its major function appears to be the cross-linking of keratins (Yaffe *et al.*, 1992), involucrin (Simon and Green, 1988), loricrin (Hohl, 1993), cornifin (Marvin *et al.*, 1992), filaggrin (Steinert and Marekov, 1995), cystatin-α (Takahashi and Tezuka, 1999), elafin (Nonomura *et al.*, 1994), and enzyme cross-link mediated cathepsin-D activation (Egberts *et al*., 2007). Additionally, it has recently been proposed that TG1 can further catalyse the formation of ester bonds between glutaminyl residues in involucrin and ω-hydroxyceramides, which may in turn facilitate anchoring of cornified envelopes to the lipid envelope of the differentiating keratinocytes (Nemes *et al.*, 1999).

The expression and activity of TG1 has also been investigated in other cell types, such as the junctional fractions of mouse lung, liver and kidney epithelial tissue and is reported to be a tyrosine phosphorylated protein (Hiiragi *et al.*, 1999). Furthermore, these authors showed that TG1 cross-linking was co-localised with E-cadherin at cadherin-based adherin and intercellular junctions of cultured simple epithelial cells, and suggests a further role for TG1 in a mechanism of structural cellular integrity. It has also been hypothesised that the cross-linking activity of intercellular TG1 may play an important role in the stabilisation of the vascular endothelium barrier (Baumgartner *et al.*, 2004). The major substrate protein associated with this enzyme was β-actin, suggesting that tissue-specific stabilisation of the cortical actin filament network by TG1 activity may be important in controlling the barrier properties of these endothelial monolayers (Baumgartner and Weth, 2007).

In conditions such as lamellar ichthyosis, hereditary deficiency in TG1 is believed to be the cause of hyperkeratinisation (Huber *et al.*, 1995; Hennies *et al.*, 1998). Inappropriate TG1 expression in the stratum basalis of the corneal and conjuctival epithelium with pathological keratinisation reminiscent of that seen in Stevens-Johnson syndrome has also been described by Nishida *et al.* (1999). Furthermore, TG1 knockout mice (Matsuki *et al.*, 1998) have demonstrated the crucial aspect of abnormal keratinisation which resulted in the reduction of skin barrier formation that has been accredited to the early death of these mice 4-5 hours after birth.

1.2.4 Epidermal Transglutaminase

Epidermal TG (TG3) is an intracellular enzyme, originally purified from human and bovine stratum corneum over 30 years ago (Buxman and Wuepper, 1976; Owaga and Goldsmith; 1976). This enzyme is predominantly involved in the formation of the cornified cell envelope where it cross-links various structural proteins in the terminal differentiation of keratinocytes and inner hair sheath cells (Martinet *et al*., 1988; Lee *et al*., 1993). Furthermore, it is thought that TG3 might be acting in concert with TG1 in the cornified envelope maturation process (Tarcsa *et al*., 1998; Candi *et al*., 1995).

In addition to the epidermis, TG3 mRNA was recently found to be expressed in the brain, stomach, spleen, small intestine, testis, and skeletal muscle (Hitomi *et al*., 2001). Other researchers have suggested that TG3 may also play a role in the hair shaft formation (Lee *et al.*, 1993). The human gene for TG3 has been localised to chromosome 20q11.12 (see Table 1.1, Wang *et al*., 1994).

Like fXIII and TG1, the TG3 protein requires proteolytic cleavage to facilitate activation. The TG3 latent pro-enzyme exhibits low specific activities and has a molecular mass of 77-kDa, which is cleaved to produce a catalytically active 50-kDa fragment and a 27-kDa carboxy-terminal peptide (Kim *et al*., 1990). The proteases responsible for the activation of TG3 remains unknown, however the bacterial enzyme dispase has been routinely used *in vitro* to proteolyse the 77-kDa inactive TG3 zymogen form, after accidental discovery during a tissue disaggregation procedure using this protease (Kim *et al*., 1990; Hitomi *et al*., 2003). It has been hypothesised that the protease calpain is responsible for the generation of the two proteolytic digestion products *in vivo* (Aeschlimann and Paulsson, 1994), and it is also thought that proteolytic post-translational modifications of TG3 in response to the cell's physiological state during epidermal cell differentiation are responsible for the activation and transfer of the enzyme from the cytosol to the membrane bound compartment (Kim *et al.*, 1994).

The TG3 zymogen shares a common structure with most other TGs, which consists of four folded domains and a mechanism of activation that is clearly defined (Ahvazi *et al.*, 2002; 2004a). The TG3 active site is buried in a narrow cleft formed by two β-sheets of the catalytic core and the barrel 1 C-terminus, which comprise the active site triad with Cys_{272} on the catalytic core α-helix, and both His_{330} and Asp_{353} on the adjacent β-sheet (Ahvazi *et al*., 2004a; 2002). Cleavage at Ser_{469} (where Ser is serine) within this loop converts the TG3 zymogen to a form that can be activated by Ca^{2+}. The TG3 zymogen contains one calcium ion per protein monomer, which is retained and thought to be required for stability. For activation, however binding of two additional calcium ions is required, with the third calcium ion producing a marked change in protein conformation and opening a channel that extends inward to the catalytic triad (Ahvazi *et al*., 2003; 2004a). Once proteolytic cleavage of the TG3

zymogen occurs, followed by the binding of Ca^{2+} at three separate locations, cross-linking ensues (Ahvazi *et al*., 2003; Ahvazi and Steinert, 2003). TG3 activity is also regulated by guanine nucleotides (Ahvazi *et al*., 2004b) where the binding of GTP results in a conformational change that closes the active site channel. When this GTP is hydrolysed to GDP, the TG3 enzyme reverts back to its active channel state.

Cross-linking studies using expressed forms of TG3 have revealed that several known cornified envelope proteins such as loricrin, small proline rich proteins 1, 2, and 3, and trichohyalin are substrates for both TG3 and TG1 (Candi *et al.*, 1995 and 1999; Tarcsa *et al.*, 1997 and 1998; Steinert *et al.*, 1999). It is thought that some key substrates are first partially cross-linked by TG3, before being sequestered onto the cornified envelope membrane by TG1 (Tarcsa *et al.*, 1998). Since TG3 is strongly expressed along placental membranes and in the developing brain, any human disease associated with the abnormal expression and activity of this enzyme may have lethal effects. In the skin disease dermatitis herpetiformis, which is commonly associated with coeliac disease, TG3 presents itself strongly with the IgA precipitates in the papillary dermis and is thought to be an auto-antigen in, and a diagnostic marker for the condition (Sárdy *et al*., 2002).

1.2.5 Prostate Transglutaminase

Prostrate TG (TG4) was discovered as an extracellular TG originally found in the seminal fluid of rodents (Wilson and French, 1980). It exists as a 150-kDa homodimer of a 75-kDa protein, consisting of two highly glycosylated and acetylated polypeptide chains that possess a lipid anchor (Wilson and French, 1980). This TG enzyme is responsible for the formation of the copulatory plug by cross-linking seminal vesicle proteins (SVPs) such as SVP-1 and SVP-IV (Ho *et al*., 1992; Seitz *et a*l., 1991).

The human TG4 gene has been mapped to chromosome 3p21.33-p22 (see Table 1.1, Gentile *et al.*, 1995) and encodes a 684 amino acid protein (deduced molecular mass, 77-kDa) that is expressed strictly from luminal epithelial cells of the prostate under the regulation of androgens (Dubbink *et al.*, 1996). Human TG4 has been shown to cross-link a group of androgen-dependent proteins with repeating sequences that are

secreted by the seminal vesicle (Porta *et al.*, 1990; Harris *et al.*, 1990). The concentration and distribution of TG4 in prostatic fluids and tissues varies greatly between individuals, however expression of the human TG4 is reported to be down-regulated in most metastatic prostate cancers (An *et al.*, 1999), whilst up-regulation of TG4 expression has been demonstrated in prostate cancer cell lines with low metastatic potential (Dubbink *et al.*, 1996). It has also been proposed that this TG4 enzyme may play a role in suppressing the immune response elicited by immunocompetent cells in the female genital tract against sperm cells (Paonessa *et al.*, 1984; Esposito *et al.*, 1996). Potential substrates for human TG4 include the SVPs: semenogelin I, and II (Esposito *et al.*, 2005; Peter *et al.*, 1998).

1.2.6 Transglutaminase X,Y,Z

The most recent additions to the TG family have been TG X (TG5, Aeschlimann *et al*., 1998; Grenard *et al*., 2001; Candi *et al*., 2001), TG Y (TG6, Aeschlimann *et al*., 1998), and TG Z (TG7, Grenard *et al.*, 2001). Very little is known about TG6 and TG7 with regards to function. The TG6 gene has been mapped to chromosome 20q11.15, in the vicinity of the region coding for TG2 and TG3 (Aeschlimann *et al.*, 1998). The TG7 protein was discovered in human prostate carcinoma, and the respective gene has been mapped to chromosome 15q15.2 (Grenard *et al*., 2001). A number of TG7 splice variants exist, and this enzyme's expression has been demonstrated in a wide range of tissues, with the highest expression being in the female reproductive organs, foetal tissue, testis and lung (Grenard *et al.*, 2001).

Of these three newly discovered enzymes, a greater amount of functional data is available for TG5. The 35kb TG5 gene was first mapped to chromosome 15q15.2 when isolated from human foreskin keratinocytes (Aeschlimann *et al*., 1998), containing 13 exons and 12 introns. TG5 exists in four different isoforms: one active full-length TG5, one active (delta 11) splice variant, and two inactive (delta 3 and delta 3/11) splice variants (Aeschlimann *et al.*, 1998). Full length TG5 encodes an 84kDa enzyme, whereas the three splice variants encode low molecular weight proteins (Candi *et al*., 2001). TG5 has been significantly implicated in keratinocyte differentiation and cornified envelope assembly (Candi *et al.*, 2001), cross-linking specific epidermal substrates such as loricrin, involucrin, and SPR1 and SPR2

(Esposito and Caputo, 2005; Candi *et al*., 1995), and is localised in the upper epidermal layers of strata spinosum and granulosum (Eckert *et al*., 2005). However, its ability to cross-link non-epidermal substrates such as vimentin, suggests that TG5 may serve other roles beyond skin barrier formation (Candi *et al.*, 2001). A seven-fold increase of TG5 expression in keratinocytes induced using the differentiating agent calcium, suggests that TG5 contributes to the formation of the cornified envelope and could be a marker of terminal keratinocyte differentiation (Aeschlimann *et al.*, 1998). Like TG2 which has an inverse relationship between cross-linking activity and nucleotide-dependent signalling activity (Nakaoka *et al*., 1994), TG5 Ca^{2+}-dependent cross-linking activity is inhibited by GTP and ATP (Candi *et al*., 2004; Ahvazi *et al*., 2004b Iismaa *et al*., 2000). The TG5 enzyme has been associated with numerous pathological skin conditions depending on its expression. For instance, mis-regulation of TG5 results in low expression levels of the enzyme causing Darier's disease (abnormal keratinisation) and lamellar itchthyosis, while abnormal up-regulation of the TG5 protein results in diseases such as psoriasis and itchthyosis vulguris (Candi *et al.*, 2002).

1.3. Tissue Transglutaminase

Tissue transglutaminase (TG2) is the most widely distributed form of the large family of TGs, and is the main focus of this thesis. The structural and functional protein conformations of TG2 and associated substrates have been illustrated above in Figures 1.3; 1.4; 1.5 and Table 1.2. Found in both membrane and cytosolic fractions, TG2 is a ubiquitous enzyme that has been characterised in numerous cell lines and tissues to date (Mishra and Murphy, 2006; Fesus and Piacentini, 2002; Thomazy and Fesus, 1989; Lorand and Sternberg, 1976). The diverse cellular and extracellular physiological roles of TG2 remain to be fully elucidated, and it is not surprising that variations in TG2 activity and expression are suggested to contribute to a wide range of pathologies (Griffin *et al*., 2002; Fesus and Piacentini, 2002; Chen and Mehta, 1999).

1.3.1 Protein Structure of Tissue Transglutaminase

The monomeric TG2 enzyme, consists of 685-691 amino acids and has a molecular weight around 77-85-kDa across different species (see Table 1.1; Aeschlimann and Paulson, 1994). There is high sequence conservation of TG2 between species when considering the complete amino acid sequence for the enzyme from guinea pig liver, mouse macrophages, and human endothelial cells which were deduced from cDNA sequencing (Gentile *et al.*, 1991; Nakanishi *et al.*, 1991; Ikura *et al.*, 1988) and shows approximately 80% homology, with 49 of the 51 residues in the active site region being identical. The amino acid sequence of TG2 contains 17 cysteine residues but no disulfide bonds. It remains to be proven how the enzyme is externalised as it contains no hydrophobic leader sequence, and six potential N-linked glycosylation sites, but is not glycosylated (Ikura *et al.*, 1988), though it is hypothesised that the N-terminal acetylation of the enzyme, following initiator methionine removal (Ikura *et al.*, 1989) may be responsible for its secretion (Muesch *et al.*, 1990).

The three-dimensional structure of TG2 (see Fig. 1.3) has been resolved based on the structures of latent human GDP-bound TG2 dimer (Liu *et al.*, 2002), latent sea bream liver transglutaminase (Noguchi *et al.*, 2001), and is also modelled on the fXIIIa-subunit since almost 75% of the active site sequence is shared (Iismaa *et al.*, 1997). The TG2 protein consists of four distinct regions: an N-terminal β-sandwich, an α/β catalytic core and two C-terminal barrel domains (see Fig. 1.4). The β-sandwich domain commences with a flexible loop, followed by an isolated β-strand (B_1), five anti-parallel strands (B_2-B_6), and terminates with an extra short strand close to B_1. The barrel 1 and barrel 2 domains consists of six β-sheets and a single β turn (barrel 1), or seven anti-parallel β-sheets (barrel 2). The catalytic core domain contains both, α-chains and β-sheets, in equal amounts (Iismaa *et al.*, 1997), and a catalytic triad (Cys^{277}, His^{335}, Asp^{358}) buried at the base of the cavity and bound by the core and barrel 1 domains. A tryptophan residue (Trp^{241}) is thought to stabilise the transition state and is shown to be critical for TG2 catalysis (Murthy *et al.*, 2002). The TG2 protein binds to and hydrolyses GTP, since it contains a GTP-binding site in the hydrophobic pocket between the core and barrel 1 (Liu *et al.*, 2002). Two residues

from the core (Lys^{173}, Leu^{174}) and two from barrel 1 (Tyr^{583}, Ser^{482}) appear to interact with the guanine base (Iismaa *et al.*, 2000).

Of further interest is an inactive form of the TG2 enzyme which has been identified in metastatic cells of both murine and human origin (Knight *et al.*, 1990; Zirvi *et al.*, 1991) expressing a molecular mass of 120-kDa, which can be activated by proteases. Numerous short alternatively spliced isoforms of the TG2 protein lacking the C-terminal have also been found in the tissues and cell lines of rat (Tolentino *et al.*, 2004; Monsonego *et al.*, 1998 and 1997), gerbil (Ientile *et al.*, 2004), human and mouse (Antonyak *et al.*, 2006; Citron *et al.*, 2002 and 2004; Festoff *et al.*, 2002; Fraij *et al.*, 1992).

1.3.2 Post-translational Regulation of the Tissue Transglutaminase Protein

The TG2 protein is regulated either by direct modification of its activity (post-translational regulation) or by modulation of gene expression (transcriptional regulation). Regarding post-translational regulation (which has been mentioned briefly in section 1.1.5), TG2 has a bi-functional role in that it catalyses both Ca^{2+}-dependent protein cross-linking and Ca^{2+}-independent GTP and ATP hydrolysis (see Figure 1.3). The protein cross-linking activity of TG2 is tightly regulated inside the cell by the local concentrations of Ca^{2+} and guanosine nucleotides (Smethurst and Griffin, 1996), while regulation outside the cell is attributed to matrix binding and the redox state of the Cys active site (Cocuzzi and Chung, 1986; Balklava *et al.*, 2002).

Activation of TG2 by Ca^{2+} is critical for transamidating activity as this is thought to promote the assembly of the reactive Cys^{277} active site pocket so that a thiol-ester bond forms with a glutamyl-containing substrate (Casadio *et al.*, 1999), while in the absence of Ca^{2+}, TG2 assumes the latent conformation, whereby the reactivity of Cys^{277} is decreased either by hydrogen bonding with the phenolic hydroxyl group of Tyr^{516} or by formation of a disulphide bond with the neighbouring Cys^{336} (Noguchi *et al.*, 2001). The main Ca^{2+}-binding site is located at the terminal α-helix (H_4) in domain 2, and upon Ca^{2+} binding to H_4, the interactions between the catalytic core domain 2 and domains 3 and 4 are disrupted, forcing the domains to move apart,

hence resulting in the formation of an opening that provides substrate access to the catalytic triad (see Figure 1.3, Murthy *et al*., 2002; Liu *et al.*, 2002; Mariani *et al.*, 2000).

The TG2 enzyme is also thought to act as a GTP-binding protein, where its Ca^{2+}-activation can be counteracted by hetero-allosteric inhibition by GTP and GDP at low Ca^{2+} concentrations, serving as a functional reciprocal switch from protein cross-linking to the GTP hydrolysis mode (Achyuthan and Greenberg, 1987). GTP binding to TG2 has been shown to cause a conformational change in the protein which leads to a reduction in TG2 activation due to reduced affinity for Ca^{2+} (Ichinose *et al.*, 1990; Greenberg *et al.*, 1991; Aeschlimann and Paulsson, 1994). The nucleotide binds to the GTP binding pocket that is located in a β-sheet distant from the active site Cys^{277}, at Lys^{173} and is hydrolysed in a reaction also involving Ser^{171} and Trp^{332} (Murthy *et al.*, 2002). The binding of Ca^{2+}, on the other hand, also reduces the affinity of TG2 for GTP (Bergamini, 1988).

In addition, ATP has been shown to bind to TG2 in a reaction essential for its hydrolysis, where magnesium ions (Mg^{2+}) are a requirement for the hydrolysis of both ATP and GTP (Lee *et al.*, 1993). Furthermore, Lai and co-workers (1998) have since demonstrated that Mg^{2+}-GTP and Mg^{2+}-ATP are the true substrates for TG2. Although the *in vitro* regulation of transamidation has been thoroughly studied, few reports have focused on the physiological regulation of TG2 (Smethurst and Griffin, 1996; Zhang *et al.*, 1998). It is believed that in the absence of GTP/GDP binding, TG2 is an endogenous substrate of calpain, and that calpain may be involved in the regulation of TG2 transamidating activity *in situ* by modulating its intracellular levels (Zhang *et al.* in 1998). Other regulators of TG2 activity include phospholipids and NO donors (Lai *et al.*, 1997; Fesus *et al.*, 1983). In light of reports that implicate TG2 in the activation of phospholipase Cδ during α-adrenergic receptor signalling, and the added observation that this enzyme can bind phospholipids (Fesus *et al*., 1983) localised at the cell surface reveals a potential role for TG2 in surface receptor function. Finally, Ca^{2+}-dependent S-nitrosylation of the 15 out of the total 18 cysteine residues present in TG2 has been shown to inhibit the enzyme's transamidating

activity, an effect that has also been confirmed for fXIII (Catani *et al.*, 1998, Lai *et al.*, 2001).

1.3.3 Transcriptional Regulation of the Tissue Transglutaminase Gene

As indicated above, at the protein level, most regulatory mechanisms serve to inhibit the enzyme. In contrast, at the mRNA level, several signalling molecules and growth factors have been shown to increase expression of TG2. The 32.5 kb human TG2 gene (TGM2) has been mapped on chromosome 20q11-12 (see Table 1.1, Gentile *et al.*, 1995) and consists of 13 exons separated by 12 introns, with the last exon (exon 13) making up almost half of the full-length cDNA (Fraij *et al.*, 1992). A short (2.4-kb) splice variant of TGM2 (sTGM2) of undefined biological significance has also been identified recently in rat astrocytes treated with cytokines such as interleukin-1β (Monsonego *et al*., 1997), as well as in the brains of patients with Alzheimer's disease (Citron *et al.*, 2001).

In many tissues, including endothelium, vascular smooth muscle and lens epithelial cells, TG2 is expressed constitutively and there are high levels of active enzyme (Greenberg *et al*., 1991), while in others (such as monocytes and tissue macrophages) the enzyme is inducible and the basal expression of TG2 is very low (Chiocca *et al*., 1988; Murtaugh *et al*., 1984). Studies of the TG2 gene promoter have given important insights into the pathways regulating TG2 gene expression. The proximal promoter (see Figure 1.5) is comprised of a TATA-box element and two upstream SP1 (CCGCC) binding sites which drives constitutive expression (Lu *et al.*, 1995). Two extra SP1 sites and four NF1 (CGCCAG) sites have been identified in the 5'-untranslated region (5'UTR). The CAAT-box element located upstream of the TATA box has minimal or no effect on transcription. In contrast, the state of methylation of CpG islands located in this proximal promoter provides a mechanism for negative control of the promoter gene family. Differential expression is also regulated by a number of upstream enhancer/silencer elements. Response elements for retinoic acid (Nagy *et al*., 1996; Piacentini *et al*., 1992; Fesus *et al*., 1991; Davies *et al*., 1988; Chiocca *et al*., 1988); and growth factors such as: TGF-β1 (George *et al*., 1990), IL-6 (Ikura *et al*., 1994; Suto *et al*., 1993), TNFα gene family (Kuncio *et al*., 1998), and

Fig 1.5

EGF (Katoh *et al*., 1996) have been identified. A specific retinoid response element (mTGRRE1) has been located 1.7 kb upstream of the transcription start site, containing a triplicate retinoid receptor binding motif (Nagy *et al.*, 1996), which together with a short HR1 sequence is responsible for promoter activation in response to retinoic acid. A potential IL-6 response element as well as transcription factor AP-1 and AP-2 binding sites have also been located (Ikura *et al.*, 1994).

Methylation of the TG promoter is considered to represent another level of gene regulation and may account for tissue specific gene expression (Lu and Davies, 1997). Studies of the TG2 promoter demonstrate that hypo-methylation and de-methylation increase TG2 activity whilst hyper-methylation suppresses TG2 (Lu and Davies, 1997). Transfection studies show this promoter to be functional and to account for constitutive expression in a number of transfected cell lines, though methylation within the main body of the promoter fails to account for changes in gene expression within specific cell-types, suggesting that distal regions of the promoter are required (Lu *et al*., 1995). The flanking region of the human TG2 promoter shares a high degree of homology with both mouse and guinea pig TG2 promoters (Ikura *et al*., 1988; Nanda *et al*., 1999). However, there is little homology between the TG2 promoter and those of other members of the TG family, which reflects their diversity.

1.3.4 Cellular Localisation of Tissue Transglutaminase

The TG2 protein is regarded as a predominantly cytosolic (80%), with 10-15% of the enzyme being localised at the plasma membrane (Griffin *et al.*, 1978; Barnes *et al*., 1985; Slife *et al.*, 1985; Tyrrell *et al.*, 1986; Juprelle-Soret *et al.*, 1988), and about 5% in the nuclear membranes (Singh et al., 1995; Lesort *et al.*, 1998; Peng *et al.*, 1999). It was only in the last two decades that localisation of TG2 antigen and activity within the extracellular matrix has been recognised (Barsigian *et al.*, 1991; Aeschlimann and Paulson, 1994; Martinez *et al.*, 1994; Jones *et al.*, 1997; Johnson *et al.*, 1997, Verderio *et al.*, 1998).

Figure 1.6 Cellular distribution of TG

Subcellular fractionation and electron microscope analysis of both cytoplasmic and particulate/plasma membrane-associated TG2 (see Fig. 1.6) revealed that these localised enzymes shared similar properties, suggesting that the particulate enzyme could be of a specific sub-cellular form (Chang and Chung, 1986). Many cytosolic proteins have been described *in vitro* as good glutamine substrates for TGs, however only a few of them have been verified as physiological substrates of TG2 (see Table 1.2). Among those recognised substrates are: RhoA (Singh *et al*., 2003); β-tubulin (Lesort *et al*., 1998); thymosin β_4 (Huff *et al*., 1999); vimentin (Clement *et al*.,1998); c-CAM (Hunter *et al*., 1998); troponin T and myosin (Gorza *et al*., 1996); actin (Nemes *et al*., 1996); β-crystallin; lipocortin I; PLA_2; glucagons; melittin; and secretory vesicle IV (Esposito and Caputo, 2005; Aeschlimann and Paulson, 1994). RhoA, a member of the Ras superfamily, functions as a constitutively active G-protein, promoting the formation of stress fibres and focal adhesion complexes (Singh *et al*., 2003). The cytoskeletal protein actin has a key physiological role in stabilising cellular morphology by counteracting the release of cytoplasmic material from the cells, and by exerting a morphogenic role in apoptosis (Nemes *et al*., 1996).

Cell surface-associated TG2 (see Fig. 1.6) has been shown to specifically incorporate small molecular weight amines such as dansylcadaverine and histamine into the membrane, generating SDS-insoluble large molecular weight aggregates (Slife *et al.*, 1986), revealing that protein substrates in the plasma membrane are accessible to cell surface-associated TG. These authors have also suggested a role for TG2 in hepatocyte cell adhesion by the generation of covalently protein-cross-linked matrices at the sites of cell-to-cell contact. Cell-surface TG2 also covalently incorporates itself, fibrinogen and fibronectin into high molecular weight aggregates on the extracellular surface of isolated hepatocytes (Barsigian *et al*., 1991). Accumulating evidence suggests that transport of TG2 to the cell surface may involve non-covalent association with β1and β3 integrins during biosynthesis (Akimov *et al.*, 2000). Following further investigation of the functionality of cell-surface TG2 and that of the TG2-FN complex, antisense RNA disruption of TG2 expression in the ECV304 cell line was shown to impair cell spreading and adhesion (Jones *et al.*, 1997), whereas converse cell adhesion experiments (Isobe *et al.*, 1999) indicated that the cell-surface TG2 serves to promote cell spreading and α4β1 integrin-mediated cell adhesion.

Interaction of TG2 with multiple integrins of β1 and β3 subfamilies, and α5β1 integrin in particular, suggested a role for TG2 as an integrin adhesion co-receptor for FN that promotes TGFβ-mediated FN assembly (Akimov *et al.*, 2000; Akimov and Belkin, 2001; Verderio, *et al.*, 2003).

The TG2 enzyme has quite recently been discovered in the nucleus (see Fig. 1.6) of human neuroblastoma SH-SY5Y cells, 6% of which was co-purified with the chromatin associated proteins, and the remaining 1% presiding in the nuclear matrix fraction (Lesort *et al.*, 1998). Within the nucleus, TG acts as a cross-linking enzyme and as a G-protein, showing basal activity comparable to that found in the cytosol (Singh *et al.*, 1995; Lesort *et al.*, 1998). Nuclear substrates for TG2 (see Table 1.2) that have so far been identified and physiologically characterised include: core histones (Shimizu *et al.*, 1997); retinoblastoma protein (Oliverio *et al.*, 1997); transcription factor SP1 (Han and Park, 2000); and importin α–3 nuclear transport protein (Peng *et al.*, 1999). Given the high molecular weight of TG2 (75- to 85-kDa), there is no allowance for passive diffusion, therefore TG2 translocation to the nucleus is believed to be mediated by active nuclear transport through its ability to bind importin-α3 (Peng *et al.*, 1999). This does not rule-out the classical importin-mediated nuclear transport, whereby importin binds to the transported protein in the cytosol prior to dissociation and upon entry into the nucleus. Furthermore, modification of nuclear substrates by polyamination or cross-linking may be relevant to the role of TG2 in cell cycle progression (Mian *et al.*, 1995) and apoptosis (Piredda *et al.*, 1999). In cells undergoing apoptosis, TG2-mediated polymerisation of the retinoblastoma gene product (pRB), a pivotal player in the control of G1/S phase transitions, might represent the key signal for the initiation of programmed cell death (Oliverio *et al.*, 1997). On the other hand, TG2–mediated dimerisation of H2B-H4 in core histones may have a role in the formation of intranuclear inclusions during apoptosis (Ballestar *et al.*, 1996). The discovery that during programmed cell death, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been shown to translocate to the nucleus, coupled with its increased cross-linking to polyglutamine domains by nuclear TG2 (Orru *et al.*, 2002), favours the hypothesis that TG2 may be involved in the nuclear modification of GAPDH during apoptosis.

1.3.5 Extracellular Localisation of Tissue Transglutaminase

The involvement of TG2 in the extracellular matrix (ECM, see Figure 1.6) has been firmly established despite the enzyme's externalisation mechanism being unknown (Fisher *et al*., 2009; Verderio *et al.*, 1998; Jones *et al.*, 1997; Johnson *et al.*, 1997; Aeschlimann and Paulson, 1994; Martinez *et al.*, 1994; Barsigian *et al.*, 1991). The mechanism of secretion is unusual because TG2 lacks a signal peptide and is not secreted by a classical endoplasmic reticulum/Golgi-dependent mechanism (Lorand and Graham, 2003). It has been ascertained that TG2 secretion requires the active state conformation of the enzyme (Balklava *et al.*, 2002) and an intact N-terminal FN binding site (Gaudry *et al.*, 1999). Due to its atypical secretion mechanism TG2 is not frequently released and has been only shown to increase dramatically in situations of tissue damage and cellular stress (Upchurch *et al.*, 1991; Johnson *et al.*, 1999).

Many extra-cellular proteins are known to serve as substrates for TG2 (see Table 1.2). So far it has been reported that TG2 is a potent cross-linker of several ECM proteins such as FN (Turner and Lorand, 1989; Barsigian *et al.*, 1991; Martinez *et al.*, 1994), β-casein (Aeschlimann and Paulson, 1991), laminin-nidogen complex (Aeschlimann and Paulson, 1991), vitronectin (Sane *et al.*, 1988), collagen type III and IV (Juprelle-Soret *et al.*, 1988; Kleman *et al.*, 1995; Esterre *et al.*, 1998), osteopontin (Kaartinen *et al.*, 1997), osteonectin (Aeschlimann *et al.*, 1995), and C1-inhibitor (Hauert *et al.*, 2000). The extracellular environment also provides the high concentration of Ca^{2+} and the low concentration of guanosine nucleotides necessary for the activation of the enzyme. It is therefore possible to expect that TG2 plays a central role in reconstructing or stabilising the fine structure of the ECM. The TG2 protein has been further implicated in the stabilisation of different tissues such as liver, heart, lung, muscle and kidney revealing extracellular distribution of the enzyme with intensive staining in collagen rich connective tissue (Verderio *et al*., 2004), and co-localision with nidogen (Aeschlimann *et al.*, 1991). Other extracellular components such a heparin sulphate proteoglycans are cross-linked in a TG2-mediated manner into high molecular weight polymers and may have a role in anoikis and the cell signalling cascade (Scarpellini *et al*., 2009; Telci *et al*., 2008; Verderio *et al*., 2003; Kinsella and Wight, 1990). Furthermore, an integral component of microfibrillar structures that

plays a critical role in the organisation of elastic fibres in the ECM can form high molecular weight polymers and has been identified as a TG2 substrate (Brown-Augsburger *et al.*, 1994). In the bone, the predominant TG2 substrate in the chondrocyte matrix has been identified *in situ* as osteonectin, a non collagenous protein co-expressed with TG2 in differentiating cartilages (Aeschlimann *et al.*, 1995).

It is has been postulated (Johnson *et al.*, 1999) that instead of directly cross-linking ECM components, TG2 may affect ECM homeostasis indirectly via regulation of the deposition/circulation balance of latent TGF-β precursor into/from the ECM, hence controlling *de novo* ECM protein synthesis (Nunes *et al.*, 1997). TGFβ plays a pivotal role in the ECM synthesis and accumulation and has also been shown to be a multifunctional regulator of cell proliferation and differentiation (Massague, 1990). The storage and subsequent release of TGFβ is crucial to physiological ECM remodelling in wound healing (Border and Ruoslahti, 1992; Nakajima *et al.*, 1997). Interestingly, immunohistochemical studies have revealed that TG2 can incorporate LTBP-1 into the ECM by possibly mediating its cross-linking to FN, while cells expressing increased levels of TG2 also exhibit increased extracellular deposition of LTBP-1 in the ECM (Verderio *et al.*, 1999; Taipale *et al.*, 1994).

1.3.6 Proposed Roles of Tissue Transglutaminase

Given the vast substrate requirements and ubiquitous cellular localisation of TG2, it is not surprising that the enzyme is implicated in a number of essential biological processes which encompasses almost every stage in the life and death of a cell: from regulation of proliferation, control of the cell cycle and signal transduction, to apoptosis. Although the existence of TG2 was first reported over 50 years ago (Sarkar *et al.*, 1957), the true biological role of the enzyme is yet to be fully elucidated. Nonetheless, sequential advances towards understanding the following cellular processes have been made with regard to TG2 function: cell proliferation and cell cycle progression (Mian *et al.*, 1995; Chiocca *et al.*, 1989; Birckbichler and Patterson, 1978); receptor-mediated endocytosis/phagocytosis (Murtaugh *et al.*, 1983; Shroff *et al.*, 1981; Davies *et al.*, 1980); activation of growth factors (Katoh *et al.*, 1996; Eitan and Schwartz, 1993; Kojima *et al.*, 1993); signal transduction

(Scarpellini *et al*., 2009; Feng *et al*., 1996; Nakaoka *et al*., 1994); cell-matrix interactions (Gentile *et al*., 1991; Jones *et al*., 1997); extracellular matrix organisation (Fisher *et al*., 2009; Martinez *et al*., 1994); myofibril assembly (Kang *et al*., 1995); cell differentiation (Aeschlimann *et al*., 1993 and 1996; Gentile *et al*., 1992; Birkbichler *et al*., 1978); tumour growth and metastasis (Mangala and Mehta, 2005; Knight *et al*., 1990; Mehta *et al*., 1990; Hand *et al*., 1987); angiogenesis (Kotsakis and Griffin, 2007; Jones *et al*., 2006); fibrosis (Johnson *et al*., 1997; Mirza *et al*., 1997; Griffin *et al*., 1979); inflammation (Upchurch *et al*., 1991; Bowness *et al*., 1994; Valenzuela *et al*., 1992; Weinberg *et al*., 1991); insulin secretion from the pancreatic β-cell (Porzio *et al*., 2007; Bernassola *et al*., 2002; Bungay *et al*., 1986); apoptosis and necrosis (Lim *et al*., 1998; Knight *et al*., 1991; Fesus *et al*., 1989). A number of these TG-mediated processes are described in further detail below.

1.3.6.1 Transglutaminase 2 in Cell Growth and Differentiation

The theory that TG2 may be a negative regulator of cell growth was proposed by Birckbichler *et al*., (1981) where cells treated with cystamine to inhibit their TG activity were shown to increase proliferation in response to decreased TG2. This is consistent with previous studies in which cells expressing low levels of TG2 showed an undifferentiated phenotype and increased proliferation compared to cells with high TG2 (Birckbichler and Patterson, 1978). The TG2 enzyme may also be involved in the regulation of the cell cycle, as in the instance of hamster fibrosarcoma Met B cells transfected with full length or truncated TG2 cDNA, which demonstrated that inactivating TG2 cross-linking activity delayed progression into the G2/M phase of the cell cycle (Mian *et al*., 1995).

Other lines of evidence have suggested that TG2 may indirectly influence cell growth by influencing growth factors, as in the case of plasmin-mediated activation of the potent growth inhibitor transforming growth factor β (Kojima *et al*., 1993), and dimerization of the growth/differentiation factor midkine which is shown to increase in potency (Kojima *et al*., 1997). It has also been suggested that TG2 negatively regulates the growth signal of epidermal growth factor (EGF) in hepatocytes through down-regulation of its high affinity receptor (Katoh *et al*., 1996). The enzyme has also

been implicated in cell differentiation, where the induction and accumulation of TG2 has been correlated with monocyte and chondrocyte differentiation in the maturation zone of skeletal tissues preceding cartilage calcification in new-born rat paw bones and tracheal cartilage (Aeschlimann *et al.*, 1993). Furthermore, expression of the enzyme was observed in a TG2 transgenic mouse model (containing a mouse TG2 promoter), in both morphologically normal cells and in interdigital mesenchymal cells undergoing differentiation (Nagy *et al.*, 1997).

1.3.6.2 Transglutaminase 2 in Receptor-mediated Endocytosis

The process by which receptor bound proteins are transported into cells via internalisation of clathrin-coated vesicles is termed receptor-mediated endocytosis or phagocytosis. It has been revealed that activated macrophages exhibit higher TG2 activity than their non-stimulated counterparts, resulting in an accumulation of the enzyme (Murtaugh *et al.*, 1983; Leu *et al.*, 1982), which has been linked to an enhanced capacity for phagocytosis (Schroff *et al.*, 1981). Moreover, the TG2 inhibitors cystamine, methylamine and dansylcadaverine have been shown to block Fc-mediated endocytosis (Leu *et al.*, 1982; Fesus *et al.*, 1981), and it is thought that the enzyme may process Fc receptors into a high affinity state promoting phagocytosis (Davis and Murtaugh, 1984). However, the exact mechanism by which this could occur remains unresolved.

1.3.6.3 Transglutaminase 2 as a G-protein in Cell Signalling

Since TG2 binds and hydrolyses GTP, it is classified as part of the heterotrimeric G-protein family that are broadly involved in hormone receptor signalling. This role in receptor signalling, and as a GTPase are distinctly separate from TG2 transamidating function (Chen and Mehta, 1999). The heterotrimeric G proteins consist of a GTP-binding α-subunit (Gα) and regulatory β- and γ-subunits, though the TG2 α-subunit (Gαh) does not possess classical heterotrimeric GTP binding motifs, suggesting that Gαh is a new class of GTP binding protein (Nakaoka *et al.*, 1994). Despite this, the GTPase cycle of Gαh is similar to that of other heterotrimeric G-proteins. TG2 facilitates the signalling response to α1-adrenergic receptor stimulation resulting from the binding of ligands such as catecholamines, norepinephrine and epinephrine

(Im *et al.*, 1997). Additionally, co-purification following ligation of epinephrine to rat liver α1-adrenoreceptors, depicted a novel 74-kDa GTP-binding protein Gαh complexed with epinephrine, the receptor, and Gα (Im *et al.*, 1990). Peptide analysis of Gαh purified from several species has exposed an identical footprinting pattern to that of TG2, and noticeably, monoclonal antibodies against TG2 cross-reacted with Gαh, strongly implying that TG2 and Gαh represent the same molecule (Nakaoka *et al.*, 1994).

The association of TG2/Gαh with the plasma membrane is shown to be mediated by the presence of a fatty acid anchor on TG2 (Harsfalvi *et al.*, 1987). In common with all G-proteins, the TG2/Gαh subunit is in its inactive form when bound to GDP. Interaction between the receptor and Gαh results in the activation and release of GDP, allowing GTP to bind. This facilitates a conformational change in Gαh and the dissociation of the α- and β-subunits. Once in the GTP bound state, TG2 affects the transmembrane signal by interacting with phospholipase Cδ, via its binding site located on the C-terminal domain, in turn influencing phosphatidyl inositol metabolism (Chen *et al.*, 1996; Hwang *et al.*, 1995). More specifically, Gαh/TG2 is thought to be involved in the activation of a 69-kDa PLC isoform that was later identified as PLCδ1 (Baek *et al.*, 2001; Feng *et al.*, 1996). An 8-amino acid region (Leu^{665}-Lys^{672}) of the α subunit of TG2 has been identified to be involved in the interaction and activation of PLC. Association of TG2 with a 50-kDa protein (Gβh), a calreticulin-like Ca^{2+}-binding protein, generates the Gh holoprotein. Upon ligation with epinephrine, the α1-adrenorceptor binds TG2 and promotes the exchange of TG2-bound GDP with GTP. The GTP-bound TG2 then activates PLCδ1, which in turn can hydrolyse phospatidylinositol diphosphate (PIP_2), thus generating the second messengers: inositol triphosphate (IP_3) and diacylglycerol (DAG). IP3 is a potent inducer of Ca^{2+} release from intracellular pools, whereas DAG can activate protein kinase C (PKC), a serine/threonine kinase involved in a wide spectrum of biological functions (Lee and Severson, 1994).

1.3.6.4 Transglutaminase 2 and the Cytoskeleton

Three main types of fibre maintain the internal architecture of cell: microfilaments, microtubules and intermediate filaments. Many cytoskeletal proteins are known to be TG2 substrates *in vitro*, including actin and myosin (Eligula *et al*., 1998; Kang *et al*., 1995), tubulin (Cohen and Anderson, 1987), α-actinin (Puszkin and Raghuraman, 1985), and spectrin (Harsfalvi *et al*., 1991). It is uncertain whether cross-linking occurs *in vivo*, except in the case of ageing erythrocytes where TG2 was found to cross-link cytoskeletal elements such as band 3, band 4.1, ankyrin and spectrin (Lorand and Conrad, 1984).

With respect to specific components of the cytoskeleton such as the intermediate filament network of dermal fibroblasts, TG2 was found to co-localise with vimentin (Trejo-Skalli *et al*., 1995). This co-localisation was retained when the cells were treated with colchicine, which induces microtubule disassembly and the reorganisation of intermediate filaments into perinuclear aggregates, however microinjection with an anti-TG2 antibody caused a collapse of the vimentin intermediate filament network in live fibroblasts (Trejo-Skalli *et al*., 1995). The association of TG2 with myosin-containing cytoskeletal structures has also been proposed as in the case of maturing chick embryonic myoblasts, where TG2 was found to be co-localised with the cross-striated sarcomeric A band of myosin thick filaments (Kang *et al*., 1995). In the same study, monodansylcadaverine (MDC, a competitive substrate inhibitor of TG2 activity) was found to reversibly inhibit the assembly of myofibrils in developing cells, and a 200-kDa protein thought to be myosin heavy chain was extensively labelled with MDC in developing fibrils, suggesting a role for the cross-linking of myosin molecules by TG2 to be a critical factor in the process of myofibrillogenesis (Kang *et al*., 1995). Another study reported co-localisation of TG2 with stress fibre-associated myosin in human vascular smooth muscle cells and human umbilical vein endothelial cells (Chowdhury *et al*., 1997), where the enzyme was found to co-immunoprecipitate with myosin in high molecular weight complexes, and it was proposed that the enzyme might auto-cross-link itself to stress fibre-associated myosin.

1.3.6.5 Transglutaminase 2 in Wound Healing Processes

TG2 is an important participant in wound healing, and is thought to be involved in all overlapping wound healing phases such as inflammation, tissue formation/stabilisation and tissue remodelling (Telci and Griffin, 2005; Verderio *et al*., 2004). Early studies using rat skin reported an increase in TG2 activity and antigen levels following biopsy punch wounding (Bowness *et al*., 1987 and 1988), and increased enzyme activity was also demonstrated in paraquat damaged rat lung (Griffin *et al*., 1979). The enzyme has also been significantly implicated in the cross-linking of the papillary dermis and the dermo-epidermal junction during the healing of autografts (Raghunath *et al*., 1996), a study which identified collagen VII as a key TG2 substrate; leading the authors to speculate that the topical application of purified TG2 to wounded tissue might accelerate the healing process. The use of TG2 in the experimental repair of rat optic nerves has been reported (Eitan *et al*., 1994) which suggests the enzyme may be of value in treating human injuries. It has been also been reported that the adhesive strength using TG2 was superior to that obtained with a traditional fibrin sealant (Jurgensen *et al*., 1997), which makes it very possible that TG2 may soon be commercially available as a biological glue for cartilage-cartilage interfaces.

In relation to the inflammation process, TG2 antigen was found to be highly expressed in macrophages, adjacent to the re-epithelialisation zone and in the provisional fibrin matrix during rat dermal wound healing (Haroon *et al.*, 1999). This was coupled with an increased expression of TG2 in endothelial cells and macrophages invading the fibrin clot and the new granulation tissue during wound healing (Haroon *et al.*, 1999). It has been recently suggested that macrophages lacking TG2 are unable to efficiently phagocytose dead cells, as has been observed in the thymus of TG2-null mice after induction of apoptosis, and that this was correlated to the impaired activation of TGF-β1, which is specifically released by macrophages on recognition of dead cells and plays an important function in down-regulating the inflammatory response (Szondy *et al.*, 2003). In addition, activity of the secretory isoform of phospholipase A2 (sPLA2), a membrane protein which releases arachidonic acid during inflammation, also appears to be enhanced by TG2 either through the formation of an isopeptide bond

within sPLA2 or through its polyamination (Cordella-Miele *et al.*, 1990 and 1993). Whilst new chimeric peptides derived from proelafin and antinflammins have been shown to inhibit sPLA2, TG2 activity, and TG2-mediated modification of sPLA2, displaying strong *in vivo* anti-inflammatory activity (Sohn *et al*., 2003).

Following inflammation, tissue remodelling occurs through the transition from granulation tissue to scar tissue, in which the balance between the synthesis and degradation of collagen is crucial (Singer and Clark, 1999). Simultaneously, many of the extracellular roles of TG2 which have implications in the early stages of wound repair involves its interaction with FN which provides a provisional matrix prior to collagen deposition and is essential for adhesion, migration and proliferation (Akimov *et al.*, 2000). Cell-matrix interactions can also be affected by TG2, either as an adhesion co-receptor of β1 and β3 integrins, or as an independent cell adhesion protein (Isobe *et al.*, 1999; Gaudry *et al.*, 1999; Belkin *et al.*, 2001; Balklava *et al.*, 2002; Verderio *et al*., 2003). Subsequently, given the regulation of TG2 expression by cytokines such as transforming growth factor β1 (TGF-β1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Kuncio *et al.*, 1998; Ikura *et al.*, 1994; Suto *et al.*, 1993; George *et al.*, 1990), a role for regulated TG2 in the tissue repair mechanism is very likely.

1.3.6.6 Transglutaminase 2 in Cell-Matrix Interactions

The involvement of TG2 in the assembly of the ECM and its role in cell-matrix interactions (see Fig. 1.8) are dependent on the cell-surface expression of the enzyme, which can exercise a pronounced effect on cell proliferation, adhesion, migration and cell survival (Zemskov *et al*., 2006). The matrix assembly role of TG2 mostly relates to its association with FN and its involvement in FN polymerisation (Gaudry *et al*., 1999). The enzyme has a high affinity binding site for FN, localised to the initial seven N-terminal amino acids (Jeong *et al*., 1995), which appears to interact with the 42-kD gelatin binding region of FN (Akimov and Belkin, 2001; Radek *et al*., 1993; Turner and Lorand, 1989). In endothelial cells and fibroblasts, TG2 has been demonstrated to cross-link FN polymers at the cell surface (Balklava *et al*., 2002; Verderio *et al*., 1998; Jones *et al*., 1997; Martinez *et al*., 1994).

Fig. 1.8. Cell-matrix associations

The precise mechanism by which TG2 promotes FN assembly remains unclear. However, the enzyme is known to act as an integrin co-receptor for FN (Akimov *et al*., 2000) and associates with several β1 and β3 integrins, whilst simultaneously binding to FN. The presence of cell-surface TG2 enhances FN-matrix formation mediated by the α5β1 integrin (Akimov and Belkin, 2001). The adhesive function of TG2 is independent of its cross-linking activity but requires the formation of ternary complexes with FN and β1 and β3 integrins (Akimov *et al*., 2000). The formation of such complexes may influence adhesion by causing the enlargement of focal adhesions and amplifying the integrin-mediated activation of focal adhesion kinase (FAK) (Akimov *et al*., 2000). The association of TG2 with matrix-associated FN appears to promote cell adhesion by RGD-independent mechanisms (Verderio *et al*., 2003). RGD mediated cell adhesion plays an important role in cell survival and RGD containing peptides can induce apoptosis in a number of cell types. However, adhesion via TG2-FN has been suggested to promote cell survival in the presence of RGD peptides and probably plays such a role in the response to cell stress (Verderio *et al*., 2003).

The ability of TG2 to form highly stable and proteolytically resistant ε-(γ-glutamyl) lysine cross-links between extracellular proteins of the ECM, has implicated the enzyme in tissue maintenance and matrix stabilisation. The TG2 enzyme has been portrayed to cross-link and stabilise the fibrin clot during wound healing (Auld *et al*., 2001; Haroon *et al*., 1999), together with mediating the formation of FN multimers (Gross *et al*., 2003; Verderio *et al*., 1998; Jones *et al*., 1997; Martinez *et al*., 1994), and the cross-linking of collagen fibrils (Kleman *et al*., 1995) which leads to ECM stabilisation that is resistant to the action of matrix metalloproteinases. Similarly, in opossum proximal tubular cells stimulated to over-express TG2 by elevated glucose an accumulation in collagen and FN matrix is observed, suggested to be due to increased ECM cross-linking (Skill *et al*., 2004). The potential role of TG2 in the stabilisation of the ECM is likely to be important in maintaining tissue integrity by influencing the balance between matrix deposition and breakdown in diseases where altered tissue homeostasis is a characteristic feature, such as wound healing and fibrosis.

1.3.6.7 Transglutaminase 2 in Cell Death

The involvement of TG2 in the mechanisms of programmed cell death or apoptosis, where a cell actively participates in suicide (Wyllie *et al*., 1980), is now widely accepted and the enzyme is thought to play both pro- and anti-apoptotic roles. The onset of apoptosis *in vivo* is often characterised by the induction of the TG2 gene (Amendola *et al.*, 1996; Knight *et al.*, 1993; Piacentini *et al.*, 1992; Fesus *et al.*, 1989). With respect to *in vitro* studies, transfections of mammalian cells with a full length TG2 cDNA revealed a marked increase in the spontaneous cell death rate (Gentile *et al.*, 1992), while conversely, stable transfections with antisense TG2 constructs led to pronounced decreases in spontaneous and induced apoptosis (Melino *et al.*, 1994). Knight *et al.* (1991) hypothesised that cross-linking of intracellular components by TG2 was pivotal in the stabilisation of the apoptotic cells prior to clearance by phagocytosis, as it had the potential to prevent leakage of intracellular, inflammatory components to the extracellular space, thus maintaining tissue integrity. TG2 is also shown to share substrates, such as histone H2B, pRB, actin, troponin with pro-apoptotic caspases (Piacentini *et al.*, 1999), and most interesting was the finding that TG2 accumulates in clusters of apoptotic cells in the interdigital web resulting in the separation of fingers into distinct digits (Thomazy and Davies, 1999). These studies suggest that the TG2-catalysed cross-linking of intracellular proteins may be a very important event in apoptosis, however definitive roles for the enzyme have yet to be firmly established.

The pro-apoptotic role of TG2 is thought to be mediated through both upstream and downstream events in the apoptotic pathway. The induction of TG2 is regulated by a number of factors such as retinoic acid and TGF-β1 that are also able to regulate apoptosis suggesting that TG2 is able to act as an early effector "death" protein (Melino and Piacentini, 1998). Similarly, the GTP-binding ability of TG2 may also contribute to the regulation of apoptosis (Melino and Piacentini, 1998) as GTP availability affects second messengers that are known to inhibit apoptosis such 1,2 diacylglycerol (DAG) (Leszczynski *et al*., 1994; Nakaoka *et al*., 1994). Apoptosis may also be promoted by TG2 through direct interaction with proteins of the apoptotic pathway such as *Bax* where interaction with the BH3 domain of TG2 can cause conformational changes leading to translocation of *Bax* to the mitochondria,

the release of cytochrome c, and cell death (Rodolfo *et al*., 2004). In the downstream stages of apoptosis, the activation of TG2 leads to extensive cross-linking of intracellular proteins and the formation of detergent insoluble protein polymers, which again may serve to stabilise apoptotic cells, preventing leakage of proinflammatory intracellular components prior to clearance by phagocytes (Fesus, 1998; Fesus *et al*., 1987).

The TG2 enzyme may also protect cells from apoptosis via non-classical adhesion dependent mechanisms such as anoikis. Studies on osteoblasts and dermal fibroblasts demonstrate that TG2 is able to form complexes with fibronectin and heparin sulphate leading to the activation of RhoA and stimulation of the cell survival focal adhesion kinase (Verderio *et al*., 2003). Similarly, studies of renal scarring *in vivo* also suggest that TG2 may participate in a novel form of cell death in which epithelial cells die through extensive cross-linking of their intracellular proteins as a result of accumulating levels of TG2 (Johnson *et al*., 1997).

1.3.7 Disease States Associated with Tissue Transglutaminase

The clinical importance of TG2 is becoming increasingly recognised, and in recent years much attention has been focussed on the potential role of the enzyme in the pathogenesis of a number of disease states. Seeing as TGs participate in such varied cellular and extracellular processes, it is no surprise that the variations in the enzymes activity is suggested to contribute to a wide range of pathologies when the loss of Ca^{2+} homeostasis occurs. These range from: Huntington's disease (Lesort *et al.*, 2000; Karpuj *et al.*, 2002); Alzheimer's disease (Tucholski *et al.*, 1999; Zhang *et al.*, 1998; Murthy *et al.*, 1998); coeliac disease, (Schuppan *et al*., 2009; Arentz-Hansen *et al.*, 2000; Molberg *et al.*, 1998; Dieterich *et al.*, 1997); tissue fibrosis (Grenard *et al*., 2001; Johnson *et al.*, 1997; Mirza *et al.*, 1997; Griffin *et al.*, 1979); cancer (Kotsakis and Griffin, 2007; Jones *et al*., 2006; Hand *et al.*, 1988; Birckbichler and Patterson, 1980) and diabetes (Porzio *et al.*, 2007; Bernassola *et al*., 2002; Bungay *et al*., 1986).

1.3.7.1 Transglutaminase 2 in Neurodegenerative Disorders

The physiological role of the brain TG is not known, but some evidence points to the enzymes involvement in neurotransmitter release (Facchiano *et al.*, 1993). Besides participation in the normal functioning of neurons, TG2 may also play a role in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease and Huntington's disease, where it is associated with the presence of insoluble protein complexes in the brain (Lesort *et al.*, 2000; Lorand *et al.*, 1996). Alzheimer's disease is associated with selective damage to the neurons in the neurocortex, hippocampus and amygdale, where extracellular senile plaques containing fibrils are composed of β-amyloid protein (Aβ) and neurofibrillar tangles, comprising an abnormally phosphorylated form of the microtubule-associated protein tau. The TG2 enzyme is thought to be involved in the development of abnormal and insoluble neurofilaments (Selkoe *et al*., 1982 and 2002) in Alzheimer's disease, where several protein components of senile plaques have been shown to be TG2 substrates, including tau (Dudek and Johnson, 1993), amyloid precursor protein (Ho *et al*., 1994), βA4 (Dudek and Johnson, 1994), and a peptide derived from α-synuclein (Jensen *et al*., 1995). However, it has not yet been shown that the βA4-plaques contain isopeptide cross-links, so the involvement of TG2 in amyloidogenesis remains hypothetical.

Huntington's disease is among eight neurodegenerative diseases that have been positively associated with $(CAG)_n$ expansions in the genome and to corresponding polyglutamine (Q_n) expansions in the encoded proteins (Vonsattel and DiFiglia, 1998). Peptides containing Q_n domains are excellent substrates for TG2 (Kahlem *et al.*, 1996) where increased Q_n size may lead to aberrant or increased TG2 activity (Green, 1993) that yields high-molecular weight polymers as favourable substrates for attachment to other Q_n domains (Gentile *et al.*, 1998). The TG2 enzyme has also been shown to cross-link Huntingtin protein and as a consequence may be involved in the formation of nuclear inclusions found in the brain (Karpuj *et al.*, 1999). Subsequent treatment with TG2 inhibition by cystamine in a transgenic mice model revealed a reduction in Huntington's disease-related symptoms and increased survival (Karpuj *et al.*, 2002). Recent reports have also shown that the elevated expression of the TG2 gene, coupled with its conversion into a shorter splice isoform that lacks the GTP

binding site, causes increased TG2 cross-linking activity and may be a key factor in the neuropathological conditions of the brain (Citron *et al.*, 2001 and 2002).

1.3.7.2 Transglutaminase 2 in Coeliac Disease

Perhaps the most widely studied aspect of TG2 in the clinical arena is its possible role in coeliac disease (Dieterich *et al*., 1997). Coeliac disease presents itself as chronic inflammation and damage of the upper small intestine as a result of inappropriate T-cell mediated immune response, in genetically pre-disposed individuals (mostly HLA-DQ2 and –DQ8 positive), against ingested gluten proteins found in wheat, barley and rye (Molberg *et al.*, 2000). It is organ-specific, as only gut-derived T-cells react with modified gliadin peptides (Molberg *et al.*, 1998). Since TG2 has been identified as the dominant autoimmune antigen against immunoglobulin A in this malabsorption syndrome, work has focused on improving diagnosis and understanding the mechanism of disease progression (Bazzigaluppi *et al*., 1999). A current hypothesis suggests that deamidation of certain glutamine residues in gliadin by TG2 may activate T-cells and could be a key factor in the development of gluten intolerance (Molberg *et al*., 1998), and the enzyme is now established as a serologic marker of the disease (Dickey *et al.*, 2001; Hoffenberg *et al.*, 2000; Gillett and Freeman, 2000).

1.3.7.3 Tranglutaminase 2 in Fibrosis

Given the high affinity of TG2 for the ECM, it is not surprising that its accidental release from cells would cause undesirable protein cross-linking. In situations where large-scale tissue damage occurs, the enzyme could contribute to the development of fibrotic conditions. Early work by Griffin *et al*. (1979) implicated TG2 in the progression of paraquat induced pulmonary fibrosis in rats. The enzyme is also thought to be involved in the development of liver cirrhosis, as high amounts of ε-(γ-glutamyl)lysine isopeptide bonds have been detected in the highly insoluble matrix found in Mallory bodies (Zatloukal *et al*., 1992). The TG2 protein has since been linked to the fibrotic phenotype in a number of tissues, such as the kidney (Johnson *et al*., 2009; Fisher *et al*., 2008; Skill *et al.*, 2001; Johnson *et al.*, 1997 and 1999), the

liver (Kuncio *et al.*, 1998; Mirza *et al.*, 1997; Piacentini *et al.*, 1999; Grenard *et al.*, 2001), the heart (Small *et al.*, 1999), and the vasculature (Bowness *et al.*, 1994). Johnson *et al.*, (1997) demonstrated that increasing TG2 activity and cross-link formation mirrored the progression of renal scarring in a subtotal nephrectomy model of kidney fibrosis in rats, and later provided the first line of *in vitro* evidence in support of the notion that TG2 cross-links confer resistance to the ECM against degradation by matrix metalloproteinases (Johnson *et al.*, 1999), findings which were indicative of a role for the enzyme in the mechanisms of stabilisation and accumulation of ECM.

1.3.7.4 Transglutaminase 2 in Cancer

Early work by Yancey and Laki (1972) implicated TGs in tumour growth. These authors proposed that solid tumours required a covalently stabilised fibrin network for proliferative growth. However, TG2 activity and cross-links was later shown to be reduced in neoplastic cells as in the instance of chemically- and virally-transformed cells which contained less TG2 than their normal counterparts (Birckbichler *et al.*, 1978; Birckbichler and Patterson, 1980). This reduction of TG2 activity seemed to be accompanied by a redistribution of the enzyme to the cell surface (Barnes *et al.*, 1985; Hand *et al.*, 1988). Other studies have also noted an inverse correlation between TG2 activity and the metastatic potential of clonal cell lines (Delcros *et al.*, 1986; Knight *et al.*, 1991; Beninati *et al.*, 1993). A link between TG2 activity and tumour metastasis was proposed by Kong *et al.*, (1997), where TG2 was found to stabilise cell contact points of free-floating melanoma cells with the subendothelial matrix of arterioles. This is consistent with a role for cell-surface TG2, and could explain how malignant cells are able to colonise other tissue types. Van Groningen *et al.*, (1995) also noted that TG2 expression was elevated in highly metastatic human melanoma cell lines compared to weakly metastatic ones. In addition, studies using hamster fibrosarcomas showed that the reduction of TG2 was identified as a specific reduction of the cytosolic enzyme (Hand *et al.*, 1988), and was accompanied with the increase in expression of a 120-kDa inactive TG (Knight *et al.*, 1990a), which was thought to be a result of inappropriate gene expression (Knight *et al.*, 1990b).

Further contradictions to the above reports, show that TG2 does not represent a biochemical marker of malignancy in human brain tumours, (Röhn *et al.*, 1992), and that the *in vivo* quantification of TG expression in skin, colon, pancreas, stomach, and lung tumour tissue, TG2 was concluded to not act as a tumour related marker (Takaku *et al.*, 1995). Taken together, the above findings suggest that TG expression and activity may vary between various tumour tissues. Despite these reports, the enzyme is now thought to act in the body's natural defence against tumour formation as increased TG2 expression in the tissue surrounding tumours has been found (Hettasch *et al.*, 1996; Grigoriev *et al*., 2001). More recently, investigations undertaken with experimental animal models and primary tumours by Jones and co-workers (2006), showed that TG2 activity and expression in the tumour body and surrounding matrix decreased with tumour progression, favouring matrix destabilisation, but supporting angiogenesis and tumour invasion (Kotsakis *et al*., 2007; Jones *et al*., 2006). In contrast, studies with secondary metastatic tumours have shown TG2 to be highly expressed, where potential roles in both intracellular and extracellular cell survival may be important (Chhabra *et al*., 2009). Considering the conflicting evidence, it is difficult to delineate a role for TG2 in the development of the malignant phenotype, and the involvement of TG2 in this process is likely to remain controversial.

1.4. Transglutaminase in Pancreatic β-cells and Diabetes

The hypotheses generated in this thesis are centred on the potential importance of TG2 in pancreatic β-cells and the associated pathogenesis of diabetes mellitus. Evidence for the role of TG within pancreatic β-cells is discussed below, with a preceding commentary on the mechanism of insulin release and current therapeutic strategies for diabetes.

1.4.1. Biosynthesis of Insulin and Exocytosis

The discovery of insulin in the 1920s is thought to be one of the most dramatic events in the treatment of disease, and has proved to be the primary therapy used to extend the life of diabetics to this day. The importance of insulin has ensured its presence in many milestones of scientific research; being one of the first proteins to be utilized for medical purposes (admittedly as a crude and impure fraction) in the landmark 1922 paper by Banting and Best (Rosenfeld, 2002; Bliss, 1993), and was consequently one of the first purified proteins to be crystallized (Abel, 1926). The discovery of insulin won Banting and Macleod the Nobel Prize for Physiology and Medicine in 1923, the prize money of which was later shared with Best and Collip. Insulin was also the first protein to be fully sequenced (Sanger and Tuppy, 1951a and 1951b; Sanger and Thompson, 1953a and 1953b; Ryle *et al*., 1955), an achievement which clarified that proteins are made up of a single-stranded un-branching sequence of amino acids, ultimately also resulting in a Nobel Prize in 1958 (Sanger, 1959). While it was artificially synthesised from pre-proinsulin, or by catalyzing disulphide bonds between pre-isolated A and B chains from the late 1950s onwards, these methods were not commercially viable, and it was therefore also the first protein to be mass produced from *E. coli*, ushering in the dawn of biotechnology (Goedell *et al*., 1979).

Human insulin (see Fig 1.9) is a 51 amino acid polypeptide hormone derived from a 110 amino acid (11.98-kDa) precursor called pre-proinsulin (Howell and Bird, 1989), which is formed from a gene on the short arm of chromosome 11 (Owerbach *et al*., 1981). Once synthesized, the hydrophobic signal sequence at the N terminus of the polypeptide promotes association of the molecule with the endoplasmic reticulum (ER) membranes. This hydrophobic sequence is cleaved to produce the proinsulin precursor, which is an 86 amino acid (9.4-kDa) polypeptide whereby rapid proteolysis results in a molecule consisting of two chains (A and B) joined by a connecting C-peptide of 30-35 amino acids (Howell and Bird, 1989). From the ER, proinsulin is transported to the Golgi apparatus in microvesicles; a process that is presumably mediated by small GTP-binding proteins (Balch, 1991).

Figure 1.9

In studies using colchicine to inhibit glucose-stimulated insulin release (Lacy *et al*., 1968), a translocation process was conceived where vesicles containing proinsulin are thought to make their way to the periphery of the cell by interacting with microtubules. This process may be affected by the dynamic nature of microtubule polymerisation, and the accepted interactions between microtubules and microfilaments in the cytosol (Howell, 1984). As well as travelling from the *trans*-Golgi network, the environment containing proinsulin is thought to acidify as a result of a Ca^{2+}/ H^{+} pump within the vesicles, which utilises ATP (Orci *et al*., 1987; Hutton 1982). As the pH drops, proteases (PC1, PC2 and carboxypeptidase-H) within the vesicles are activated to cleave the connecting C-peptide from the insulin molecule (Hutton, 1994). Removal of the C-peptide subsequently reduces the solubility of the insulin molecule, causing it to co-precipitate with Zn^{2+} into dense crystalline granules. Upon secretion, insulin and C-peptide are secreted in equimolar amounts, with 95% of secreted hormone being insulin and 5% in the form of proinsulin (Howell and Bird, 1989). Mature insulin granules contain high concentrations of crystalline insulin together with Zn^{2+}; Ca^{2+}; C-peptide; biogenic amines produced as a result of granule acidity; and a range of bioactive proteins and peptide precursors known as secretogranins (Howell and Bird, 1989). These granules are stored in dense core vesicles (DCVs) that are throught to be clathrin-coated, named for their appearance in electron micrographs (MacDonald and Rorsman, 2007; Orci *et al*., 1994)) and are retained by the β cell until rising secretatgogues such as blood glucose indicates that they are required.

Insulin is produced exclusively by pancreatic β-cells, which while making up to 80% of the cells within the islets of Langerhans (Foster *et al*., 1993), represent less than 1% of the total pancreatic mass (Gammelsaeter *et al*., 2009). The resultant insulin hormone is formed from two peptide chains of similar length, bridged by two disulphide bonds ($Cys^{31\text{-}96}$ and $Cys^{43\text{-}109}$) which are critical for maintaining insulin as a single molecule (see Fig. 1.9). In solution, the insulin molecule tends to dimerise due to interactions between the C-termini of the B chains, except in the presence of zinc where the formation of poorly soluble hexamers is catalysed (De Metys *et al*., 2004). The entire insulin sequence has no unoxidised cysteines, as a third disulphide bond ($Cys^{95\text{-}100}$) is found within the insulin A chain, and is thought to either prevent erroneous bond formation between the other cysteines in the A chain and those in the

B chain, or provide the conformational stability required for inter-chain bonds to be formed (Yuan *et al*., 1999).

Despite its high solubility, significant amounts of insulin C-peptide are also bound up within the insulin granules and secreted when insulin is released into the blood. While for a long time this peptide was considered biologically inert, more recently it has been recognised as having roles in both enhancing glucose disposal and preventing neuropathy and nephropathy (Brandenburg, 2008; Rebsomen *et al*., 2008; Forst *et al*., 2008; Wahren *et al*., 2007), presumably by GPCR-mediated binding (Hills and Brunskill, 2009).

The mature granules are secreted in a regulated way, in what is believed to be a 'sub-plasmalemmal web' of microfilaments and other cytoskeletal components that are involved in the fusion of the granule membrane to the plasma membrane. It is also thought that the dynamic relationship of mature granules with the actin network is likely to account for the existence of different pools of secretory granules which differ in their readiness to undergo exocytosis upon stimulation of secretion (Klenchin and Martin, 2000). The effect of Ca^{2+} and other secondary messengers on the secretion of insulin is usually carried out over a period of several minutes to one hour. Within this time-frame, biosynthesis cannot lead to secretion (Howell and Bird, 1989). Therefore, the effects most commonly measured are those on the translocation of granules to the cell periphery, and exocytosis. The pancreatic β-cell is able to maintain intracellular stores of insulin at a relatively constant level. When insulin is lost from the β-cell by glucose-induced exocytosis, there is a reciprocal rapid upregulation of proinsulin biosynthesis at the level of translation (Goodge and Hutton, 2000). The control of exocytosis and margination by secondary messengers in the β-cell remains an area of prolific study, and involves the changing conformation of proteins by Ca^{2+}-binding and/or protein phosphorylation. A further post-translational modification which may occur in a Ca^{2+}-activated manner at this time, may be the cross-linking by transglutaminase, since it has been reported in rat islets that inhibitors of the enzyme also inhibit insulin release (Bungay *et al*., 1984). This is discussed in further detail later on.

1.4.2. Insulin Secretion

The pancreatic β-cell is a very complexly-regulated system of stimulus-secretion coupling, tailor-made for the purposes of adjusting insulin secretion on a second-by-second basis to the metabolic needs of the individual (for a recent review refer to Rorsman and Renstrom, 2003). This is combined with regulated secretory pathways that allow for almost instantaneous secretion of exactly the desired amount of stored insulin, independent of the rate of synthesis, followed by the replenishment of insulin stores (see Fig. 1.10). A healthy adult has approximately 5 mM glucose in their blood (equivalent to ~ 0.9 mg/ml) (Westwood, 1999). The β-cell is able to monitor circulating glucose concentrations in the physiologically relevant range (4–10 mmol/l) and pathophysiological range (10-20mmol/l) because it expresses the combination of glucose transporters (whether GLUT2 or another) and glucokinase (Newgard and McGarry; 1995), with a carefully constructed balance of metabolic enzymes geared up for generating metabolic secondary signals to regulate its function. Insulin secretion may be divided into two phases, early stage secretion, involving release of these stored granules as a response to the consumption of simple sugars; and late stage secretion involving the release of newly synthesised insulin as a consequence of extended glucose uptake from the digestion of complex sugars (Rorsman and Renstrom, 2003). The regulated release of insulin from pancreatic β-cells is pivotal for the maintenance of serum glucose concentrations within the physiological range.

Glucose itself is the major physiological stimulus for insulin secretion, but a number of hormones and neurotransmitters can also act on β-cells to modulate the secretory process (Ahren, 1999). There is the so-called incretin effect, which is a communication between the gut and endocrine pancreas that induces nutrient-regulated insulin production and secretion. Incretin refers to the peptide hormones: glucose-dependent insulinotropic polypeptide (GIP; also known as gastric inhibitory polypeptide) and especially glucagon-like peptide 1 (GLP-1) (Fehmann *et al*., 1999). GLP-1 receptors are predominately found on β-cells (and some hypothalamic neurons) leading to the specific glucose-regulated GLP-1–mediated regulation of insulin secretion, proinsulin biosynthesis, and β-cell proliferation (Drucker, 2001). Insulin release may also be potentiated by neurotransmitters such as acetylcholine

Figure 1.10 insulin secretion

and pituitary adenylate cyclase activating polypeptide (PACAP) (Ahren, 2000), while conversely other neurotransmitters such as somatostatin and noradrenaline are capable of inhibiting insulin release (Ahren, 2000; Sharp, 1996).

Rises in blood glucose concentration affect the action of the GLUT-2 passive glucose transporter (Ohneda *et al*., 1993) found primarily on the gap-junction microvilli adjacent to other endocrine cells (Orci, *et al*., 1989). Once inside the β-cell, glucose is phosphorylated by glucokinase to gluocose-6-phosphate, which is processed to pyruvate by glycolysis, and subsequently subjected to the Krebs cycle and oxidative phosphorylation, producing ATP. This rise in intracellular ATP causes the β-cell's ATP-sensitive potassium channels (K_{ATP} channels) to close (Rajan *et al*., 1990), an event which may be mediated in part by Crk-associated substrate (Cas), which is tyrosine phosphorylated and associates with Crk as part of the β-cell's response to rising glucose (Lee *et al*., 2004; Konrad *et al*., 2003; Konrad *et al*., 1996). The closure of these channels results in depolarization of the membrane to below the threshold value of -40 mV, which in turn leads to Ca^{2+} influx via voltage-dependent Ca^{2+} channels thus repolarising the cell (Szollosi *et al*., 2007; Henquin and Meissner, 1984). Interestingly, mutations in the gene for the K_{ATP}-channel in humans was found to lead to uncontrolled insulin secretion and disease states known collectively as PHHI (persistent hyperinsulinemic hypoglycemia of infancy) (Aguilar-Bryan *et al*., 2001).

A characteristic of primary islet β-cell metabolism, but not other mammalian cell types, is very low lactate dehydrogenase and plasma membrane mono-carboxylate pyruvate/lactate transporter activities (Ishihara *et al*., 1999), making lactate output in primary β-cells almost undetectable. Consequently, there is a marked increase in mitochondrial metabolic shuttle activities (e.g., the glycerol-3-phosphate shuttle) to reoxidize cytosolic NADH back to NAD^+, a requirement for glycolysis normally provided by lactate dehydrogenase in most eukaryotic cells. In addition, the islet β-cell has several-fold–increased pyruvate carboxylase activity to efficiently direct pyruvate toward mitochondrial tricarboxylic acid cycle and oxidative phosphorylation metabolism for efficient ATP production (Schuit *et al*., 1997). Changes in intracellular ATP production, in turn contributes as the key metabolic stimulus-coupling factor in the β-cell to control insulin release (Deeney *et al*., 2000).

It is not fully understood how the rise in cytosolic Ca^{2+} leads to insulin release, and there is some debate as to whether depolarisation is fully necessary for this process (Gembal *et al*., 1992). This debate is complicated by the fact that in culture different β cells may vary considerably in their response to glucose, while in the pancreas their insulin release threshold is much more uniform (Zhang *et al*., 2003; Misler *et al*., 1992; Rorsman and Trube, 1986; Atwater, *et al*., 1983). This is believed to be due to synchronisation of electrical activity across the gap junctions (De Vries and Sherman, 2000; Kinard *et al*., 1999; Sherman *et al*., 1988). Therefore, intra-islet interactions that result from homotypic β-cell-to-β-cell contact and communication may be necessary for the pulsatile secretion of insulin in response to synchronised increases in cytosolic Ca^{2+}. Several theories have been proposed to explain the co-operative insulin release of neighbouring β-cells in response to these intercellular Ca^{2+} waves, which include: i) direct communication via gap junctions (Moreno *et al*., 2005; Rogers *et al*., 2007); ii) the presence of other endocrine cells (Ishihara *et al*., 2003; Pipeleers *et al*., 1992); as well as the existence of extracellular diffusible secretory products (Squires *et al*., 2002; Hellman *et al*., 2004).

Cells communicate locally via gap junctions that physically connect adjacent cells and permit the free flow of ions and small molecules (Hills *et al*. 2006), or through the release of local paracrine messengers (Squires *et al*. 2002). The biosynthetic and secretory function of the islet depends largely on the architecture of the islet, itself dictated by specialised cell adhesion molecules such as the cell surface adhesion protein epithelial (E)-cadherin (ECAD) and β-catenin (D'Souza-Schorey 2005). The co-localisation of adherens junction proteins to secretory granules (Hodgkin *et al*., 2008) suggests that the adherens junction may play a novel role in β-cell function, both in terms of β-cell proliferation (Carvell *et al*. 2007) and insulin secretion (Hodgkin *et al*., 2008, Rogers *et al*. 2007). Neutralising ECAD-mediated cell adhesion decreases glucose-evoked synchronicity in Ca^{2+} signals between adjacent cells within islets (Rogers *et al*. 2007). Evidence from human epidermal keratinocytes revealed that inactivation of the extracellular Ca^{2+} sensing receptor (CaR) suppressed the assembly of the ECAD–catenin–phosphotidylinositol 3–kinase (PI3K) complex (Tu *et al*. 2008), suggesting that CaR could also influence the

synchronicity of Ca^{2+} activity between β-cells within the islet and thus insulin secretion (Hodgkin *et al*., 2008).

It has been suggested that glucose may activate two simultaneous pathways within the β-cell, each of which might have the potential to lead to insulin secretion (Henquin, 2000), though the Ca^{2+}-dependent mechanism appears to be by far the more powerful of the two (Sato *et al*., 1998). In its absence, the Ca^{2+}-independent mechanism requires activation of protein kinase A and protein kinase C (Komatsu *et al*., 1997) and shows only a poor response to glucose-dosage, but which working together with the Ca^{2+}-dependent mechanism produces an amplified response (Szollosi *et al*., 2007; Henquin, 2000).

The speed with which insulin is secreted has lead to speculation that kinase and phosphatase signalling cascades, which may or may not be driven by Ca^{2+}, calmodulin activated kinases (Jones and Persaud, 1998) or by arachidonic acid simulated phosphatase activity, may play a role in the control of insulin secretion. As already mentioned, in Ca^{2+} depleted conditions both PKA and PKC must be activated in order for the cell to produce insulin (Komatsu *et al*., 1997), however only PKC can be activated by arachidonic acid, and inhibition of it and a range of other arachidonic acid-related kinases have little impact on Ca^{2+}-stimulated insulin release. Therefore, the involvement of arachidonic acid may lie within other aspects of the secretory response (Jones and Persaud, 1993). There is also evidence suggesting that ERK 1/2 (and by extension, the MAPK kinase pathway) may be involved in re-arranging the actin cytoskeleton prior to insulin secretion, a process which may also involve Rho GTPases such as CdC42 (Nevins and Thurmond, 2003). Following secretion, the cytoskeleton is transiently de-polymerised to possibly halt exocytosis of excess insulin (Thurmond *et al*., 2003).

Once released into circulation, insulin has a half life of 4-6 minutes (Duckworth *et al*., 1998) and rapidly binds to insulin receptors on a wide range of tissues (predominantly muscle, adipose and liver tissue). The insulin receptor is dimerised and as a result composed of an extracellular subunit (A) and a transmembrane subunit (B), linked by disulphide bridges (De Meyts *et al*., 2004). Two splice variant isoforms exist for the receptor, with or without exon 11 (a 12 amino-acid C-terminal

addition to the A subunit which doubles the affinity for insulin), the expression ratio of which differs across the tissues in which they are expressed (Sesti, *et al*., 2001a). As it binds and activates its receptor, both insulin and the receptor undergo conformational changes. Insulin undergoes this conformational change by unfolding the alpha helices in the B chain (Hua *et al*., 1993), while the L1 beta helix of the receptor responds to this change (Huang *et al*., 2004). The result of which leads to the autophosphorylation of numerous tyrosines in the B domain, and the subsequent phopshorylation of insulin receptor substrates (IR_{S1} - IR_{S4}), which are then recognised by phosphoinositide 3-kinase (PI3K) via its SH2 domain, setting off a kinase signalling cascade involving recruitment of PKB and ultimately inducing production of glycogen synthase and the translocation of the glucose transporter, GLUT-4 to the plasma membrane for active transport of glucose into the cell (Lizcano and Alessi 2002; Saltiel and Kahn 2001). The insulin receptor meanwhile, is internalised and the hormone degraded by insulin-degrading enzyme (IDE) (Bevan 2001). The role of insulin is not limited to glucose uptake since it also stimulates amino acid and fatty acid uptake, and the synthesis of proteins and lipids; while also inhibiting lipid metabolism and the production of glucose from glycogen (Saltiel and Kahn, 2001).

1.4.3. Diabetes Mellitus

Diabetes mellitus translates to “honey-sweetened urine in excess,” and develops as a result of a failure of the body to effectively utilize or control blood glucose levels. It has been estimated by the International Diabetes Federation (IDF) in 2010 that more than 284 million children and adults suffer from all types of diabetes worldwide making it a crucial health issue in terms of disease incidence, morbidity, mortality, and financial impact research; with type I diabetes resulting in an estimated lifetime cost of $500,000 per patient spent on the management and treatment of diabetes-related complications (http://www.idf.org).

Type 1 diabetes, formerly referred to as juveline-onset diabetes or insulin-dependent diabetes mellitus (IDDM) occurs as a result of the autoimmune destruction of pancreatic β-cells, the body’s only natural source of the polypeptide hormone insulin. When insulin production falls too low, ensuing hyperglycaemia induces an osmotic

diuresis with glucosuria, and concomitant tissue starvation which stimulates an inappropriate metabolic response, leading to severe dehydration and electrolyte depletion, metabolic acidosis, and eventually coma and death. While it is believed to be influenced by genetic factors, the root of this disorder is unknown (Westwood, 1999). There are numerous autoantigens recognised, varying from patient to patient (e.g.: insulin; glutamic acid hydrolase; ICA512; P69; heat shock protein 65; carboxipeptidase H; zinc transporter 8 and Slc30A8) (Auchenbach *et* al., 2009; Nepon, 1995; Christie *et al*., 1994; Aguilar-Diosdado *et al*., 1994; Pietropaulo *et al*., 1993), however few are unique to β-cells (Cohen, 2004). Type I diabetes usually develops during childhood, frequently leading to serious late-onset complications in early adulthood. In time, chronic hyperglycaemia leads to high levels of advanced glycation end-products (AGEs), which are believed to contribute to a pathogenesis manifested as diabetic nephropathy (the leading cause of renal failure), diabetic retinopathy (the leading cause of adult blindness), peripheral neuropathy, diabetic ulcers (the main disease-related cause of amputations) and cardiovascular disease (MacDonald and Rorsman, 2007). Multiple, daily, subcutaneous insulin injections coupled with frequent blood glucose monitoring to prevent insulin-induced hypoglycaemia, ameliorate the acute symptoms of type I diabetes but cannot maintain constant normoglycaemia.

Alternative causes to autoimmunity in type 1 diabetes include mutations in insulin itself, its receptor, or defects in the β-cell or effector cell pathways. For example, a number of single amino acid polymorphisms of insulin have been documented (Yano *et al*., 1992; Barbetti *et al*., 1990; Chan, *et al*., 1987; Sakura *et al*., 1986; Haneda *et al*., 1983, Shoelson *et al*., 1983), the majority of which have significant consequences, appearing to either result in conformational changes preventing insulin binding to receptors, or impair proinsulin production or cleavage within the β-cell. In the case of the latter, mutations which affect processing within the golgi network and beyond result in secretion of complete or partially-cleaved proinsulin into the serum, while mutations which prevent intracellular transport of proinsulin into or from the ER cause a buildup within the β-cell which may lead to apoptosis (Glaser, 2008).

Type 2 diabetes, also known as non-insulin-dependent diabetes mellitus (NIDDM) despite the fact that many of these patients eventually require insulin treatment, is the other major diabetic subtype. Type 2 diabetes typically presents in adult patients, but is being recognized with increasing frequency in the obese pediatric population (Kahn, 1998). It is a much more prevalent disorder than type 1, affecting approximately 90% of the diabetic population in the developed world (Inzucchi and Sherwin, 2005). Type 2 diabetes rarely causes the acute life-threatening complications of type I diabetes, but shares all of the long-term complications associated with chronic hyperglycaemia and AGEs. Unlike type 1 diabetes, patients with type 2 diabetes rarely lose their β-cells at the same rate, and the pancreas frequently continues to produce insulin, at least during the earlier stages of the disease. Patients with type 2 diabetes become insulin-resistant, with muscle and adipose tissue failing to take up glucose from the blood (Westwood, 1999), while glucose production in the liver continues unimpeded, leading to hyperglycaemia and hyperinsulinaemia, which in turn generates a proatherosclerotic and pro-ischaemic environment (see Fig 1.11). Similar to type 1 diabetes, type 2 is likely to be polygenic in origin, though unlike type 1 which may set in rapidly, insulin resistance in type 2 may build up over many years (Campbell and White, 2003), and is followed by impaired insulin secretion. The slow progression of the condition can mean that initial symptoms may present themselves a decade or more before diagnosis (Harris *et al*., 1992), leaving a significant window for patients to be at risk of the cardiovascular and other complaints which are associated with the condition. While obesity, poor diet and lack of excercise are the most widely publicised risk factors for type 2 diabetes, the specific aspects which lead to insulin resistance and the mechanisms by which they do so are yet to be fully elucidated. This form of diabetes is complex with an unclear pathogenesis, and although traditionally thought of as a disorder of end-organ resistance to insulin, it has become clear that sustained hyperglycaemia does not occur without the added effects of defective insulin secretion and β-cell dysfunction (Kahn, 1998). Management of the disease usually involves dietary changes, weight reduction and exercise regimens, but may also include insulin injections, or drugs which reduce hepatic glucose production, stimulate insulin secretion or potentiate its effects (Longmore *et al*., 2004).

Figure 1.11

In type 2 diabetes, the impairment of insulin secretion following insulin resistance is firstly observed in early phase insulin secretion, but over the course of the disease also comes to affect late stage insulin secretion (Burant, 1998). As with insulin resistance, how and why this occurs is not fully understood, but it is believed that chronic hyperglycaemia (glucose toxicity) and the production of an oxidising environment may over-stress the β-cell, leading to breakdown of insulin secretion (Giaccari *et al*., 2009; Leahy, 1996; Rosetti *et al*., 1990). Additionally, these features appear to intensify insulin resistance, furthering the course of the disease (Giaccari *et al*., 2009; Skyler, 1997; Garvey *et al*,. 1985). At the protein-level, downregulation of GLUT-2 is recorded in diabetic animal models (Ohneda *et al*., 1993), while with regard to the insulin receptor, shifts in alternative splicing to favour isoform A have been linked with insulin resistance (Savkur and Cooper, 2001; Sesti *et al*,. 2001a). Dimerisation of the insulin receptor with type-1 IGF receptors is also recorded, (De Metys *et al*., 2004) with the heterodimer losing affinity for insulin in the process. Downstream of the receptor, reductions in IRS-1 expression have also been reported in obesity-linked insulin-resistance and type 2 diabetes (Sesti *et al*., 2001b).

Pancreatic β-cell function can be modulated both long- and short-term by exercise and body weight. A single bout of aerobic exercise usually results acutely in enhanced glucose utilization by insulin-sensitive tissues, which must be balanced by a rapid change in insulin secretion if hypoglycaemia is to be avoided. With regular exercise, an enhancement of insulin sensitivity and an associated reciprocal change in insulin secretion usually occurs. On the other hand, increases in body weight or redistribution of body fat compartments to a more central location require increases in insulin output if glucose tolerance is to remain unchanged. This enhancement of insulin release must occur in response to the reduction in insulin sensitivity that is typically associated with increases in adiposity. In insulin-resistant states such as obesity, an adaptive response requiring elevations in β-cell mass is usually seen, where increases in both the size of the individual β-cell and β-cell mass through numbers helps to lessen the individual β-cell secretory burden (Finegood *et al*., 2001). Furthermore, for reasons that are still not well understood, healthy ageing is associated with a progressive reduction in glucose tolerance where increases in glucose levels appears to be a compensatory response to a yet unidentified physiological need of the ageing process and is achieved by a reduction in insulin

release, despite the fact that insulin resistance also accompanies this process (Kahn *et al*., 1998).

Maturity-onset diabetes of the young (MODY) is a clinically heterogenous group of disorders that account for 2% - 5% of type 2 diabetes. MODY is characterised by nonketotic diabetes mellitus, and autosomal dominant mode of inheritance, an onset usually before 25 years of age, and a primary defect of pancreatic β-cell function (Fajans *et al*., 2001). MODY can result from mutations in any of at least six different genes that encode the glycolytic enzyme glucokinase (MODY2) and five transcription factors: hepatocyte nuclear factor (HNF)-4α (MODY1), HNF-1α (MODY3), insulin promoter factor-1 (IPF-1), HNF-1β, and neurogenic differentiation 1 (NeuroD1); with mutations in the HNF-1α gene being the common cause of MODY in most populations (Fajans *et al*., 2001).

1.4.4. Current Diabetes Therapies

Following on from Banting and Best's discovery of insulin as an applicable hormone therapy for over 80 years, type 1 diabetes has changed from being a fatal disease to a chronic illness that nonetheless requires concerted efforts towards possible curative strategies. While type 1 diabetics are completely insulin dependent, insulin treatment for type 2 is not always pursued. Insulin therapy has many drawbacks that affect the diabetic patient's quality of life, and it is well known that even aggressive insulin therapy will never re-create the real-time variation of blood glucose. The goal of strict metabolic control continues to be a challenge for most individuals, and the availability of a number of new insulin analogues altered by residue substitutions is becoming slightly more feasible (Philips and Scheen, 2006; Chaterjee *et al*., 2006; Chapman *et al*., 2002; Gerich, 2002; Campbell *et al*., 2001). Use of these insulin analogues are not without consequence however, since a significant number of diabetics are unable to use synthetic human or human analogue insulins due to intolerance or hypoglycaemic attacks (Hunter, 2002). Furthermore, in some studies insulin treatments have been claimed to increase the risk of malignancy in diabetics, and recently fears have been raised regarding a higher incidence of malignancies in insulin glargine-treated patients than those taking unmodified insulin (Hemkens *et*

al., 2009), possibly as a result of its longer pharmokinetics (Stammberger *et al*., 2002).

As a result, β-cell replacement is assumed to be the only treatment for type 1 diabetes that re-establishes and maintains long-term glucose homeostasis with near-perfect feedback controls (Ryan, 1998). The endocrine portion of the pancreas (see Fig. 1.12), which includes the insulin-producing β-cells are confined to the islets of Langerhans, which represent approximately 2% of the total pancreatic mass (the majority consisting of exocrine tissue) (Street *et al*., 2004). Since the exocrine pancreas is unaffected in diabetes, transplantation of purified islets has long been thought to be a preferable alternative to whole or segmental organ replacement. However, poor outcome of the numerous attempts to transplant human islets during the past quarter of a century is interpreted to be caused by a host of factors; the main one being allorejection. Immunosuppression that prevents allorejection of kidney, heart, or liver seems to fail with human islets (Halban *et al*., 2004). The complexity of dealing with three different immune attacks, i.e. acute rejection, chronic rejection, and recurrence of autoimmunity from the type 1 diabetes disease state, has drastically hampered clinical islet transplantation. In some cases, failed islet transplants have been reported to be associated with reappearance of GAD65 autoantibodies, a major serological marker for type 1 diabetes (Keymeulen *et al*., 1998). Scientists are working on ways to fend off the immune system from re-attacking β-cells, and the main approach at the moment is developing encapsulated islets, however this method still falls short of the stringent requirements of clinical application. Furthermore, the use of immunosuppressive therapy in the context of islet transplantation has been associated with the development of malignancy (Gruessner *et al*., 1997). Thus, future approaches needed to include immunosuppressive agents that do not adversely affect the intended long-term survival of the diabetic patient.

Islet isolation and transplantation was pioneered by Paul Lacy from the 1960s (Ballinger and Lacy, 1972). The demonstration by the Edmonton group that islet cell transplantation can work was a watershed event in the field (Shapiro *et al*., 2000). It built upon years of effort by others in the area of islet isolation (Ricordi *et al*., 1989b; Scharp *et al*., 1990; Lakey *et al*., 1999) as well as advances in immunology that led to the development of new, more effective and less toxic immunosuppresive drugs.

Figure 1.12

The Edmonton protocol (Shapiro *et al*., 2000) avoids the use of glucocorticoids, which were the mainstay of regimens used for suppressing the rejection process despite producing insulin resistance *de novo* and at the same time decreasing islet endocrine function. According to the Edmonton protocol (Shapiro *et al.*, 2000), isolated human islet transplants can be performed much less invasively with minor, outpatient "surgery" consisting of a transabdominal injection into the portal circulation, either directly into the hepatic portal vein or through the umbilical vein (see Fig 1.12. C). Islet transplantation involves the isolation of islets of Langerhans from the pancreas of cadaver organ donors through complex digestion and purification processes, transplanting them into the recipient's liver. It results in near-perfect, moment-to-moment control of blood glucose, far more effectively than injected insulin (Ryan *et al*., 2001; Shapiro *et al*., 2000). However, in many patients this is not maintained in the long-term, and most patients still require some injection of insulin by one year after transplantation. The hope with islet transplantation is that with tighter glucose control, the long-term complications of diabetes could be avoided.

The Edmonton Protocol has been successfully replicated in more than 50 centers worldwide, with cumulative data from more than 500 patients, representing a significant goal as more patients with type 1 diabetes have now received islet implants in the past 9 years than in the entire former 30-year history of islet transplantation (Miszta-Lane *et al*., 2006). A significant problem with applying the Edmonton protocol to large numbers of patients is the fact that each patient requires islets from two or more pancreases to achieve insulin independence (Shapiro *et al*., 2000), and there remains the case of lifelong immunosuppressive therapy. The reason for inadequate sources of transplantable islets may be related to inefficiencies in islet isolation as well as loss of islets in the immediate post-transplant period (Davalli *et al*., 1996). With tens of thousands of new type I diabetes cases per year and only a few thousand available donor organs, only 0.5% of candidates will actually be able to benefit from islet transplants (Miszta-Lane *et al*., 2006). Therefore, what is now required is an essentially limitless supply of a physiologically competent substitute for primary human islets of Langerhans.

A number of strategies are being explored that could improve the feasibility of minimally invasive and potentially curative β-cell replacement therapies, which fall into two broad categories of research aimed at either maximizing the capacity of existing tissue resources or expanding tissue availability. The former (commonly termed β-cell replacement therapy) consists of efforts to increase graft survival and function in the post-operative phases, thus reducing the number of islets required to achieve insulin-independence, and the latter (commonly termed β-cell gene therapy) focuses on the generation of alternative sources of glucose-responsive, insulin-secreting cells suitable for human transplants (Mineo *et al*., 2009; Ricordi *et al*., 2005).

One potential source of additional pancreatic transplant material is human foetal tissue. Foetal pancreases have a much higher ratio of endocrine to exocrine tissue and are more readily available than adult cadaveric tissue. Although the foetal endocrine pancreas is under-developed, is has been shown that immature endocrine cells can mature into functional β-cells *in vivo* (Beattie *et al*., 1997; Desai *et al*., 1999). However, the use of foetal tissue remains highly controversial from an ethical standpoint. Also, animal studies have found that multiple fetal pancreata are required to reverse experimental diabetes in a single adult animal (Desai *et al*., 1999). Thus, tissue availability continues to be an issue. The question of animals providing a source of β-cells remains an option, however the potential risk of infection by animal endogenous viruses limits the possibility of such xenotransplantation (Halvorsen and Levine, 2001). Another option is that advances in our understanding of β-cell biology and the mechanisms that control cell growth and differentiation are leading to rapid progress in the development of human β-cell lines. As β-cell development and regeneration is increasingly understood, the prospect of regenerating a patient's own β-cells as a form of therapy may actually become a reality. Currently, the derivation of islets from stem cells seems to be the most promising option to overcome the current problems of islet shortages and immune compatibility (Burns *et al.*, 2004). Stem cells are known as clonogenic since they have the ability to self-renew and produce identical daughter cells; or result in multilineage differentiation by producing daughter cells that are fated to differentiate (Watt and Hogan; 2000). Both embryonic (derived from the inner cell mass of blastocysts) and adult stem cells (derived from adult tissues) have shown promising results in secreting insulin *in vitro*

and normalizing hyperglycaemia *in vivo*. Table 1.3 describes the different cell types as a potential source of β-cells.

Embryonic stem cells have the remarkable capability of differentiating into every type of cell (Hubner *et al*., 2003), and are indeed able to differentiate into insulin-producing cells under culture conditions as well (Lumelsky *et al*., 2001), showing the promise of reversing diabetes in animal islet transplantion studies through the continued synthesis and release of insulin *in vivo* (Hori *et al*., 2002). However, studies using embryonic stem cells derived from humans are controversial because it requires the destruction of a human embryo and/or therapeutic cloning. Therapeutic cloning for an unlimited supply of pluripotent embryonic stem cells for transplantation involves insertion of the nucleus from a patient's somatic cells into an enucleated human egg and allowing it to develop into a blastocyst which can be expanded *in vitro* (Burns *et al*., 2004). Furthermore, problems surrounding the control of differentiation and tumorogenecity of embryonic stem cell-derived insulin-producing cells remain to be overcome, due to the high expression of telomerase (Sipione *et al*., 2004; Kim *et al*., 1994) which is responsible for the immortalization of many human embryonic cell lines. Therefore, existing embryonic stem cell lines are not believed to be identical or ideal for generating islets or β-cells and additional embryonic stem cell lines will have to be established in the future (Cowan *et al.*, 2004; Ying *et al*., 2002).

Stem cells also exist in adult tissues, with the usual source being from mesenchymal bone marrow (Jahr and Bretzel, 2003; Ianus *et al*., 2003; Pittenger *et al*., 1999) liver (Zalzman *et al*., 2003) and pancreas (Seaberg *et al*., 2004; Zulewski *et al*., 2001; Bonner-Weir *et al*., 2000; Ramiya *et al*., 2000). Neural progenitor cells isolated from the brain can also be induced to express the insulin gene (Burns *et al*., 2003), which has the added benefit of all the elements of the glucose sensing mechanism of β-cells being present in neurons (Yang *et al*., 1999), making them a good potential source for optimal β-cell functional development (Jones and Persaud, 1998). In humans, adult stem cells have the potential to be transdifferentiated into insulin-producing cells (Ulrich *et al*., 2002). Adult stem cells are already to some degree specialized and have a shorter lifespan than embryonic stem cells, but they have the added potential of recipients being administered their own stem cells. They do have the

Table 1.3

disadvantages however, of not being pluripotent, being difficult to purify, and are they not maintained well outside the body showing low proliferative capability (Miszta-lane *et al*., 2006).

Current strategies for diabetes treatment show that it is difficult to maintain strict metabolic control using insulin regimens that incorporate either multiple daily injections or insulin pump therapy. Achieving this control would mean that exogenous insulin is provided in a manner that mimics both of the critical components of endogenous insulin release ie. basal and stimulated secretion. Even the use of insulin pumps for the treatment of type 1 diabetes, where continuous infusion of insulin at basal levels is possible, the variability of glucose levels in patients still cannot be controlled (Halban *et al*., 2001). The advantage of an engineered β-cell that is capable of releasing insulin in a pulsatile fashion in comparison to the continuous nonpulsatile administration of exogenous insulin, is that administration of excessive insulin not only carries the risk of hypoglycaemia but at the same time results in the downregulation of the insulin receptor, and thus the development of insulin resistance with impaired insulin responsiveness in many tissues (Ward *et al*., 1990).

If genetically engineered insulin-producing cells are to be contenders in the world of diabetes therapy, they need not only deliver basal insulin, but also release insulin on demand, thus bringing glucose levels in line with those seen in healthy subjects (Jones and Persaud; 1998). Regarding basal insulin levels, it would be essential that this component of insulin release be able to vary, as normal life events, such as exercise, infection, and weight gain, all require potentially rapid changes in insulin output. A failure to do so could result in hypoglycemia or hyperglycaemia, something that is not uncommon in the lives of current insulin users, however possible repeated recurrences as a result of gene or cell therapy should ideally be overcome. Regarding physiologically appropriate stimulated insulin secretion as a result of gene or cell therapy, it would be imperative that these cells release insulin in response to nutrient intake, ie. not only to glucose but also to the building blocks of fats and proteins (Ward *et al*., 1984). Additionally, insulin response to oral ingestion as a result of contributions from the gastrointestinal tract in the form of incretins (Nauck, 1999), including GLP-1 and GIP will be necessary. Furthermore, in order to

ensure the best potential starting material for continued characterization it may be necessary to apply differentiation protocols to the numerous embryonic stem cell lines together with the development of centralised 'stem cell banks' to characterise, store and distribute these cells worldwide (Burns *et al*., 2004). The utilization of stem cells in the form of β-cell replacement and gene therapy for the generation of insulin-producing β-cells *in vitro* is still in its infancy, but advances in this field of research may very well lead to the complete and curable treatment of diabetes, which may well be within worldwide reach in the very near future.

1.4.5. Transglutaminase 2 in Diabetes

The TG2 enzyme has been implicated in various intracellular and extracellular biological processes, however its involvement in the regulation of Ca^{2+}-dependent glucose-stimulated insulin release from pancreatic β-cells (Bernassola *et al*., 2002; Driscoll *et al*., 1997; Bungay *et al*., 1986; Sener *et al*., 1985; Gomis *et al*., 1984) and in insulin receptor aggregation, internalisation and intracellular processing by cross-linking receptors in the area of clathrin-coated pits together with receptor-mediated endocytosis (Bernassola *et al*, 2002; Davies *et al*., 1980; Haigler *et al*., 1980; Baldwin *et al*., 1980) is an intriguing function when viewed in the context of diabetes.

Supporting this association, impaired TG2 function was first observed in lymphocytes from type 2 diabetic patients (Berntorp *et al*., 1987). Subsequent studies revealed that targeted disruption of the TG2 gene in mice ($TG2^{-/-}$) was not related to gross defects in multiple tissues (De Laurenzi and Melino, 2001; Nanda *et al*, 2001), but was found to exert a more selective effect which lead to a gradual decline in glucose homeostasis culminating in the development of a type 2 diabetes phenotype (Porzio *et al*., 2007; Bernassola *et al*., 2002). These authors showed that $TG2^{-/-}$ mice exhibited glucose intolerance after intraperitoneal glucose loading, and manifested a tendency to develop hypoglycaemia after administration of exogenous insulin as a consequence of enhanced insulin receptor substrate 2 (IRS-2) phosphorylation (Bernassola *et al*., 2002). These findings were associated with a reduction in circulating insulin levels consistent with either a reduction in β-cell mass or alterations in stimulus secretion coupling at the level of the $TG2^{-/-}$ mouse β-cell.

Furthermore, it was shown that mutations in the active site of TG2 have been found in several patients exhibiting a familial form of impaired glucose tolerance known as maturity-onset diabetes of the young (MODY), with these mutations co-segregating along with the condition, and the $TG2^{-/-}$ mice phenotype resembling that of MODY patients (Porzio *et al*., 2007; Bernassola *et al.*, 2002). During screening for the human TG2 gene in Italian subjects diagnosed with the clinical features of MODY, missense mutations (N333S) in the active site of the enzyme were found (Bernassola *et al*., 2002). In addition to this mutation, two more novel heterozygous mutations (M330R and I331N) in the TG2 gene were consequently detected in a larger screening set of patients with MODY or early-onset type 2 diabetes, where all mutations were found in residues located close to the catalytic site of the enzyme and impaired transamidating activity when assessed *in vitro* in transient transfection studies (Porzio *et al*., 2007) These findings show a possible significance when considered within the context of a range of studies implicating an unidentified susceptibility gene for Type 2 diabetes, which has been mapped to chromosome 20 (20q 12-q 13.1); the region that encompasses the TG2 gene (Permutt *et al.*, 2002; Mori *et al*., 2002; Fossey *et al*., 2001). This suggests that the unidentified gene may play a critical role in the maintenance of β-cell function, and it is tempting to speculate that TG2 could represent this elusive diabetes susceptibility gene.

The association of another TG family member was also studied in type 2 diabetes, where the thrombin-induced cleavage of fXIII activation peptide was inhibited, and fibrin clots reduced in the presence of metformin (Standeven *et al*., 2002). Strikingly, type-I diabetics display an increased risk of developing coeliac disease, and vice versa (Franzeze *et al*., 2007), reinforcing the potential link between diabetes and TG. This suggests either involvement of the gut in the pathogenesis of type-I diabetes or that TG is a secondary autoantigen resulting from β-cell destruction.

1.4.6. Transglutaminase in Pancreatic Islets

The enzymatic activity of TG, which catalyses the cross-linking of proteins, has been the focus of much attention within pancreatic islets of Langerhans to date. Following the discovery of TG as a Ca^{2+}-responsive enzyme (Clarke *et al.*, 1959; Lorand and

Conrad, 1984) it was proposed that TG may be a target for the increase in the cytosolic concentration of Ca^{2+}, which has since been established as the primary mediator of stimulus secretion coupling during glucose-stimulated insulin release in pancreatic β-cells (Wollheim and Sharp, 1981).

The very first piece of evidence that supported the presence of TG activity in pancreatic islets was documented in 1982 by Bungay *et al*., and later confirmed by Gomis *et al*., (1983). In the original published works Gomis *et al*., (1983) showed that a range of primary amines could be incorporated into N'N-dimethylcasein substrate using the TG present from rat islets in a variation of the standard *in vitro* assay devised by Lorand *et al.* (1972). This was followed by Bungay *et al*. (1984a) who reported that rat islets contained a TG activity that was both Ca^{2+}-dependent and thiol-dependent, the latter being demonstrated by a requirement of dithiothreitol (a thiol reducing agent) for optimal enzyme activity. These findings set the foundation for subsequent investigations using the rat islet as a β-cell model, where competitive concentrations of primary amines were added to the physiological medium bathing the islets, with the result of optimal glucose-stimulated (16.8mM) insulin release being inhibited (Bungay *et al*., 1984b). Several reports have shown that incubating islets with various other competitive substrate inhibitors of TG activity similarly leads to the inhibition of glucose-stimulated insulin release (Sener *et al*., 1985, Bungay *et al*., 1986), and that in turn TG activity seems to increase in homogenates of glucose-stimulated islets (Gomis *et al*., 1986a; 1989). These proposals however, remain to be fully clarified on many levels.

Past attempts to identify TG substrates in homogenised pancreatic islets have suggested that the major substrate is a high molecular weight polymer which is unable to traverse a 3 % (w/v) polyacrylamide gel (Bungay *et al*., 1986; Gomis *et al*., 1989). Furthermore, Owen *et al.* (1988) reported the formation of a high molecular weight phosphoprotein polymer during glucose stimulation in islets that was shown to be reduced in expression by competitive TG inhibitors. While all these initial reports may be showing evidence of the same TG substrate, contention over these studies remains because the authors have shown slightly different subcellular distribution of TG in their respective studies. The function of the TG-cross-linked high molecular

weight polymeric aggregates described above is still unresolved, but is thought to be important in the stabilisation of the rat islet β-cell membrane during membrane recycling and following the membrane-associated events of insulin secretion. In further support of this, it was also proposed (Sener *et al.*, 1985) that TG may participate in the machinery controlling the access of secretory granules to the exocytotic sites in pancreatic islet β-cells during insulin release.

1.4.7. The Inhibition of Transglutaminase Activity in Pancreatic Islets

Although several competitive inhibitors of TG activity including alkylamines, monodansylcadaverine, *N*-*p*-tosylglycine, bacitracin, glycine alkylesters and hypoglycaemic sulphonylureas have all been investigated for their effect upon islet function, the results of these studies have failed to provide conclusive evidence that would fully explain the role of TG in the process of insulin release (see Table 1.4). In the case of the alkylamines, this has been due largely to the lack of specificity of these TG inhibitors. Methylamines were seen to have caused lysomotropic effects ie. the intracellular accumulation of ligand-receptor complexes due to the loss of normal modes of ligand-induced receptor processing (Gomis *et al*., 1984), since they rapidly accumulated in the islet cells (Gomis *et al*., 1983), and could be accounted for by the sequestration of the compound within the insulin secretory granules (Hutton *et al*., 1982) which could in turn account for the delayed conversion of pro-insulin and the resulting altered release of insulin. In the absence of glucose as a primary nutrient secretagogue, non-nutrient secretagogues such as gliclazide showed significant inhibition of insulin release as a result of TG inhibition induced by methylamine (Gomis *et al*., 1983). These authors also showed that the presence of methylamine decreased the insulin release provoked by high glucose concentrations in combination with theophylline and cytochalasin B, which suggested that the presence of methylamine interfered with a distal event in the insulin secretory process. Since the treatment of islets with cytochalasin B facilitates insulin release by causing the contraction of the microfilamentous cell web (Somers *et al*., 1979; Malaisse and Orci, 1979), which normally controls the access of secretory granules to the exocytotic site, it was concluded that TG may play a critical role in the mechanical events involved in the translocation of secretory granules to their exocytotic site and

Table 1.4.

the fusion of membranes at such a site. A role for TG in membrane fusion was also compatible with the finding that there was delayed pro-insulin conversion in methylamine treated islets (Gomis *et al*., 1986b). The fact that trimethylamine did not affect TG activity but inhibited insulin release (Gomis *et al*., 1983) also suggested that great caution is required in the consideration of possible cause-to-effect links between inhibited TG activity and altered insulin release (Lebrun *et al*., 1984).

Furthermore, poor penetration into the islet cells as well as undesirable damaging effects was seen with monodansylcadaverine and *N-p*-tosylglycine (Gomis *et al*., 1983). The inhibitor bacitracin was also shown to cause interference with the immunoassay of secreted insulin, since the compound itself was able to bind insulin (Gomis *et al*., 1983). Hypoglycaemic sulphonylureas were reported by Davies *et al*., (1980) to inhibit TG activity. However these studies were hampered by their dramatic effects upon the cationic fluxes in the islet cell, and it has since been established that the insulinotropic (stimulating or affecting the production and activity of insulin) action of these inhibitors is attributed to the stimulation of Ca^{2+} entry into the islet cells (Malaisse *et al*., 1983).

Gomis *et a*l, (1984b) further investigated the effects of the hypoglycaemic sulphonylureas; tolbutamide, gliclazide, glisoxepide, glipizide, glibenclamide and diazoxide under conditions where the cationic response to these compounds was suppressed by quinine. They found that while these compounds bind to the plasma membrane and do not penetrate into the islet cells (Kaubisch *et al*., 1982), both TG activity and insulin secretion were inhibited as described previously with these anti-diabetic agents (Nowak *et al*., 1983), suggesting the extracellular role of TG. In addition, investigations carried out by Sener *et al*. (1985), showed that the TG inhibitor glycine methylester failed to affect D-glucose oxidation, supporting the view that TG participates in a late event in the insulin secretory sequence in pancreatic β-cells. Besides the rapid inhibition of insulin release by glycine methylesters, the process was also reported to be rapidly reversible with a rebound in the secretory insulin rate being observed when the administration of glycine methylester was halted (Sener *et al*., 1985). This phenomenon suggests that the

cross-linking of proteins and the reversal of the insulin secretory rate proceed in a dynamic equilibrium in islet cells stimulated for insulin release. It has been proposed that cross-linking of proteins by TG may play a role in the mechanical events involving the β-cell cytoskeleton and leads to the translocation and exocytosis of insulin secretory granules at the cell membrane (Bungay *et al*., 1986). It was further documented in subcellular fractionation studies that, in addition to its postulated role in the control of intracellular motile events, TG also participates in surface protein interactions at the site of cell-to-cell contact (Gomis *et al*., 1989).

While the results using glycine methylester described so far indicate a possible role of TG in insulin release, the translocation of secretory granules to the exocytotic site does not represent the sole functional process dependent on the integrity of motile events in the islet cells. The conversion of proinsulin to insulin also depends on the oriented translocation of microvesicles from the rough endoplasmic reticulum to the Golgi complex. With this in mind, glycine methylester was found to slow down the rate of conversion of proinsulin to insulin and thereby increase the half-life time of the conversion process (Gomis *et al*., 1986b). TG activity in intact islets could also be increased as a result of a rise in cytosolic Ca^{2+} activity (Gomis *et al*., 1986a), while the mechanism of glucose within β-cells may also increase TG enzymatic activity as a result of *de novo* synthesis of the enzyme or alternatively the induction of a more reduced state, with subsequent changes in the thiol-disulphide balance (Gomis *et al*., 1986a). In support of the latter hypothesis by these authors, TG is known to operate by a cysteine thiol active centre mechanism so that the disulphide exchange reaction may cause reversible inactivation of the enzyme (Lorand and Conrad, 1984) and glucose is known to increase the content of sulphydryl groups within islets (Malaisse *et al*., 1985). However, these findings still do not rule out the participation of regulatory factors other than Ca^{2+} and redox state in the overall control of TG activity within islet cells.

1.5. Hypothesis and Aims

The principal aim of this investigation will be to characterise the expression and activity of TG2 in pancreatic β-cells, with a focus on possible roles for this enzyme in insulin secretion function and β-cell survival under diabetic investigative strategies. The experimental design incorporating pancreatic β-cells will centre on the primary biochemical roles attributed to TG2 as a:

i) Ca^{2+}-dependent transamidating enzyme that catalyses irreversible ε-(γ-glutamyl)-lysine isopeptide cross-links between proteins in concert with cellular post-translational modification;

ii) GTP-binding protein, where its Ca^{2+} activation can be counteracted by hetero-allosteric inhibition by GTP and GDP at low Ca^{2+} concentrations;

iii) cell-surface or extracellular matrix-associated protein which catalytically affects matrix protein accumulation, or acts in a non-enzymatic capacity as an adhesion co-receptor initiating cell signalling.

The initial studies in this thesis will therefore aim to test the hypothesis that TG2 catalytic cross-linking activity plays a crucial role in pancreatic β-cell function and survival, by using a rat pancreatic insulinoma clonal β-cell line (BRIN-BD11). The investigation will then proceed to develop a TG expression profile in clonal β-cells and islets of Langerhans across human and rodent species. The ultimate aim will be to test the maintenance of these β-cells on an extracellular support matrix currently used in a curative diabetic strategy towards intra-hepatic islet transplantation.

Chapter Two

Thesis Rationale and Aims

Lewis Wolpert from *The Unnatural Nature of Science*

A surprising feature of motion is that the most natural state for an object is movement at constant speed - not, as most of us think, being stationary. A body in motion will continue to move forever unless there is a force that stops it. This was a revolutionary idea first proposed by Galileo in the early seventeenth century and was quite different from Aristotle's more common-sense view, from the fourth century BC, that the motion of an object required the continuous action of a force.

Galileo's argument is as follows. Imagine a perfectly flat plane and a perfectly round ball. If the plane is slightly inclined the ball will roll down it and go on and on and on. But a ball going up a slope with a slight incline will have its velocity retarded. From this it follows that motion along a horizontal plane is perpetual, 'for if the velocity be uniform, it cannot be diminished or slackened, much less destroyed.' So, on a flat slope, with no resistance, an initial impetus will keep the ball moving forever, even though there is no force. Thus, the natural state of a physical object is motion along a straight line at constant speed, and this has come to be known as Newton's first law of motion. That a real ball will in fact stop is due to the opposing force provided by friction between a real ball and a real plane.

The enormous conceptual change that the thinking of Galileo required shows that science is not just about accounting for the 'unfamiliar' in terms of the familiar. Quite contrary: science often explains the familiar in terms of the unfamiliar.

Wolpert, Lewis (1929-), Developmental biologist and writer. *The Unnatural Nature of Science*, Faber and Faber, 1992. Quoted from *The Oxford Book of Modern Science Writing* by Richard Dawkins, Oxford University Press, 2008.

Chapter Two:

Thesis Rationale and Aims

2.1. Rationale and Aims

Most of the evidence for a role of TG function in pancreatic β-cells points to the involvement of a predominant TG2 enzyme operating within a Ca^{2+}-dependent cysteine thiol active centre mechanism, which is thought to be involved in the distal events of insulin secretion function (Driscoll *et al*., 1997; Bungay *et al*., 1986; Sener *et al*., 1985; Gomis *et al*., 1984). Recently, mutations in the active site of TG2 were found in patients with a familial form of impaired glucose tolerance; while targeted disruption of the TG2 gene in mice ($TG^{-/-}$) exhibited a diabetes type 2 phenotype (Porzio *et al*., 2007; Bernassola *et al*., 2002).

This line of investigation was pursued in the present thesis by characterising TG activity and expression using novel approaches in two β-cell models:

i. clonal pancreatic insulinoma β-cell lines (BRIN-BD11 and MIN-6);
ii. freshly isolated islets of Langerhans from the pancreases of human, rat, $TG2^{-/-}$ knockout mice lacking TG2 activity, and their $TG2^{+/+}$ wild-type counterparts (De Laurenzi *et al*., 2001).

The principal aims of this study were to determine:

1) What factors associated with insulin secretion function affect the regulation, inhibition, and over-expression of TG enzyme activity in pancreatic β-cells?

BRIN-BD11 pancreatic β-cells will be treated with various post-translational regulators *in vitro* to study their effects on TG expression and activity, using various

substrate specific assays. Cell culture models of hyperglycaemia, oxidative stress and lipotoxicity will be used to assess the effect of diabetic cell stress on TG expression and activity. The effect of site-specific TG inhibition and targeted TG2 gene inactivation will be assessed for the role of TG activity in insulin secretion function.

2) Whether TG2 protein and mRNA expression remains conserved *in vitro* with clonal pancreatic insulinoma β-cell lines; and *ex vivo* from human, rat, and TG2$^{-/-}$ knockout mouse (De Laurenzi *et al*., 2001) pancreatic islets.

β-cell lines and isolated islets of Langerhans across rodent and human species will be characterised for glucose-stimulated insulin antigen, TG antigen, GTP-binding potential of the β-cell TG antigen, and TG mRNA expression.

3) If the inhibition of TG2 antigen expression and catalytic activity in a support matrix changes associated extracellular matrix protein accumulation, would this modified extracellular matrix affect pancreatic β-cell maintenance and survival *in vitro*?

A human urinary bladder carcinoma cell line (5637) will be characterised as a model that is rich in TG2 expression, and will be optimised for use as a β-cell support matrix. Pre-conditioned 5637 matrices will then be prepared *in vitro*, using active-site directed TG inhibitors and TG2 gene silencing siRNA, while extracellular matrix protein levels will be assessed together with a TG2 enzyme profile. Finally, BRIN-BD11 β-cell viability, adherence, spreading, insulin content and aggregation will be assessed on these 5637 pre-conditioned matrices.

Chapter Three:

Materials and Methods

Ernst Mayr from *The Growth of Biological Thought*

"Generalisations in biology are almost invariably of a probabilistic nature. As one wit has formulated it, there is only one universal law in biology: 'All biological laws have exceptions.' This probabilistic conceptualisation contrasts strikingly with the view during the early part of the scientific revolution that causation in nature is regulated by laws that can be stated in mathematical terms. It has remained the dominant idea, particularly in the physical sciences, up to the present day.

With Plato it gave rise to essentialism, with Galileo to a mechanistic world picture, and with Descartes to the deductive method. All three philosophies had a fundamental impact on biology…Constancy and discontinuity are the points of special emphasis for the essentialists. Variation is attributed to the imperfect manifestation of the underlying essences…Darwin, one of the first thinkers to reject essentialism (at least in part), was not at all understood by the contemporary philosophers (all of whom were essentialists), and his concept of evolution through natural selection was therefore found unacceptable.

It was not until the nineteenth century that a new and different way of thinking about nature began to spread, so called population thinking. Population thinkers stress the uniqueness of everything in the organic world. What is important for them is the individual, not the type. There is no 'typical' individual, and mean values are abstractions. Much of what in the past has been designated in biology as 'classes' are populations consisting of unique individuals.

The statistics of the essentialist are quite different from those of the populationist…Essentialistic statistics attempts to arrive at true values in order to overcome the confusing effects of variation. Variation was nothing but 'errors' around the mean values…The most interesting parameters in the statistics of natural populations is the actual variation, its amount and its nature. The amount of variation is different from character to character and from species to species."

Meyr, Ernst. (1904-2005), Evolutionary Biologist. *The Growth of Biological Thought*, Harvard University Press, 1982. Quoted from *The Oxford Book of Modern Science Writing* by Richard Dawkins, Oxford University Press, 2008.

Chapter Three:

Materials and Methods

3.1. Materials

3.1.1. General Chemicals

All water used was deionised using an Elgastat System 2 water purifier (ELGA Ltd., Buckinghamshire, UK) or Milli-Q water purifier (Millipore Waters, Watford, UK). Stock solutions and chemicals were re-suspended, where necessary, to appropriate concentrations in this distilled water which was autoclaved at 121°C at 1 bar for 1 hour and then filtered through a 0.22 μm Whatman sterile filter. General chemicals and sterile cell culture products were mostly purchased from the Sigma-Aldrich Chemical Company, Poole Dorset, UK, unless otherwise stated below.

Glacial acetic acid, sulphuric acid, acrylamide, N, N′-dimethylcasein, glycine, hydrochloric acid, calcium chloride, dimethyl sulphoxide, hydrogen peroxide, sodium dodecyl sulphate, sodium acetate and sodium hydroxide were purchased from BDH (Merck), Poole, Dorset, UK. Trichloroacetic acid was obtained from Jansen Chemica, Hyde, Cheshire, UK. N, N′-Methylene bisacrylamide, rainbow molecular weight markers and Lowry protein assay kit were supplied by Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK. Marvel dried milk powder was purchased from domestic supply outlets. Biotin-cadaverine, FITC-cadaverine and CM-Dil (Cell Tracker, C7001) derivatives for long-term cellular labelling was purchased from Molecular Probes, Oregon, USA. Fluorsave fluorescent mounting reagent; control RAD peptide (GRADSP), MMP inhibitor GM6001 (Galardin; 364205) and negative control (364210) were supplied by Calbiochem, Nottingham, UK. Glucose-free RPMI 1640 cell culture medium was purchased from Cambrex Bioscience, Berkshire, UK.

Optiphase Hi-safe scintillation fluid was obtained from LKB, Milton Keynes, Bucks, UK. Vecta-Shield fluorescence mounting medium with and without propidium iodide present was purchased from Vecta Laboratories Inc., Peterborough, UK. TG2 inhibitor peptide (Boc-DON-Gln-Ile-Val-OMe) was obtained from N-Zyme Biotec, Darmstadt, Germany. Purified human plasma fibronectin and laminin, dithizone stain, GTP-γ-S, all *trans* retinoic acid, FITC-labelled phalloidin, synthetic RGD specific peptides (GRGDTP and GRGDSP) and GTP-agarose was purchased from Sigma-Aldrich Chemical Company, Poole, Dorset, UK. Guinea pig liver TG2 used as standards or treatments, was purified in-house (Leblanc *et al*., 1999) and kindly supplied by Dr. P. Kotsakis and Dr. D.Y.S. Chau (Nottingham Trent University), or else purchased from Sigma-Aldrich Chemical Company, Poole, Dorset, UK.

3.1.2. Human Islets and Human Pancreas Cryosections

The use of human islets was approved by Aston University Ethical Committee (Aston University, Birmingham, UK), and obtained under the supervision of Prof. M. Griffin from the UK Tissue Bank (UKTB, The Innovation Centre, Leicester, UK). Human type-1 diabetic and control pancreas cryosections were a kind gift from Prof. N.G. Morgan (Peninsula Medical School, Plymouth, UK).

3.1.3. Animals

Wild type C57/BL6J strain mice, which fully express TG2, and TG2 knockout C57 mice ($TG2^{-/-}$) that have a deletion of most of exons 5 and 6 of the TG2 gene (containing the TG2 active site) were used in the isolation of primary islets of Langerhans. $TG2^{-/-}$ mice were originally generated by De Laurenzi and Melino using homologous recombination techniques (De Laurenzi and Melino, 2001) and housed for this study at Nottingham Trent University after obtaining them from Prof. G. Melino (University Tor Vergata, Rome, Italy).

Male Wistar rats were obtained from Harlan (Shaw's Farm, Blackthorn, Bicester, UK), or initially provided as a kind gift from Prof. N.G. Morgan (Peninsula Medical School, Plymouth, UK). Animals were bred at the Department of Life Sciences,

Nottingham Trent University in accordance with the UK Home Office Animals Act (1986).

3.1.4. Mammalian Cell Lines

The clonal pancreatic insulinoma β-cell lines: BRIN-BD11, MIN-6 and INS1E were a kind gift from Prof. N.G. Morgan (Peninsula Medical School, Plymouth, UK). The 5637 human urinary bladder carcinoma cell line and ECV-304 (human umbilical vein endothelial cells derived from T24) was purchased from the American Type Culture Collection (ATCC).

3.1.5. Radiochemicals

[1,4-^{14}C]-Putrescine (50 μCi/ml) was purchased from Amersham Pharmacia Biotech, Buckinghamshire, UK. Deoxycytidine-5'-triphosphate [α-^{32}P] for random primed labelling of cDNA probes (specific activity of 3000 Ci/mmol) was obtained from DuPont, Stevenage, Hertfordshire, UK. ^{125}I-Insulin (5 μCi) was supplied by Linco, Biogenesis, Poole, UK.

3.1.6. Western Blotting Reagents

SDS-PAGE separated proteins were transferred to nitrocellulose membranes purchased from Gelman Biosciences, Northampton, UK. Blot absorbent filter paper and gel loading tips were supplied by Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK. Western blots were developed and fixed on Kodak X-OMAT film using enhanced chemiluminescence (ECL) reagents obtained from Amersham, Little Chalfont, Bucks, UK; or developed with the same ECL reagents and captured digitally on the AIDA LAS 3000 Image Reader.

3.1.7. Immunochemicals

CUB7402 TG2 anti-mouse monoclonal antibody (mAb) was supplied by Neomarkers, Fremont, USA. Guinea pig anti-swine insulin antibody, polyclonal anti-rabbit laminin 1 antibody, anti-rabbit IgG-FITC conjugate, anti-rabbit IgG-TRITC conjugate, anti-mouse IgG-FITC conjugate, anti-mouse IgG-TRITC conjugate and

anti-mouse IgG-HRP conjugate were purchased from DakoCytomation., Ely, Cambridgeshire, UK. Anti-rabbit IgG-HRP, polyclonal anti-rabbit fibronectin antibody, anti-rabbit total FAK antibody, anti-mouse β-tubulin antibody and polyclonal anti-mouse collagen IV antibody was purchased from Sigma, Poole Dorset, UK. Monoclonal mouse anti-fluorescein antibody was obtained from Roche Diagostics, East Sussex, UK. Anti-rabbit cleaved caspase-3 (Asp 175) antibody was purchased from Cell Signalling Technology, UK, and anti-rabbit Tyr (p)^{397}-FAK antibody was obtained from Upstate Biotechnology, UK. Anti-mouse ID-10 antibody was a kind gift from Prof. M. Griffin (Aston University, Birmingham, UK). Anti-rabbit N-terminal and C-terminal TG1 and TG3 antibodies (Santa Cruz Biotechnology, USA) were kind gifts from Dr. A. Hargreaves and Dr. J. Cortez (Nottingham Trent University).

3.1.8. Northern Blotting Reagents

Electrophoretically separated RNA was transferred to Hybond N membranes purchased from Amersham, Buckinghamshire, UK, which also supplied the Sephadex G50 Nick columns™. ExpressHyb™ hybridisation solution was purchased from Clontech UK Ltd., Hampshire, UK. RNA molecular weight markers were purchased from Promega, Southampton, UK. Kodak AR/LS detection film was obtained from Roche Diagnostic, East Sussex, UK. 3MM paper wick was purchased from Whatman, Kent, UK.

3.1.9. Molecular Biology Kits and Reagents

Low melting point agarose was purchased from Bioline, London, UK. SV Total RNA isolation system, DNA and RNA standard markers, RQ1 DNase, Wizard SV Gel and PCR Clean-up system, Go Taq flexi DNA polymerase, PCR Nucleotide Mix were purchased from Promega, Southampton, UK. Trizol was purchased from Invitrogen, Paisley, UK. RNEasy Mini kit, Sensiscript Reverse Transcription kit (< 50 ng RNA) and QIAquick DNA gel extraction kit was obtained from Qiagen, West Sussex, UK. Trizol, lipofectamine 2000, custom designed stealth siRNA duplex oligoribonucleotides, and a high GC siRNA negative control, were purchased from Invitrogen, Paisley, UK. TG2 cDNA was a kind gift from Peter Davies (Houston,

USA), while TG1 and TG3 cDNA were a kind gift from Kiyotaka Hitomi (Nagoya, Japan).

3.1.10. Other Consumables

Superfrost Gold coated glass and standard (uncoated and untreated) microscope slides and coverslips were purchased from BDH, Poole, Dorset, UK. Scintillation vials were supplied by Canberra-Packard, Pangborne, UK. Tissue culture, petri dishes and flasks were purchased from Corning/Bibby-Sterilin, Stone, Staffs, UK. 0.5, 1.5- and 2-ml microcentrifuge tubes, 5 ml scintillation vial inserts, 15 and 50ml sterile centrifuge tubes, 10ml sterile pipettes, automatic pipette fillers, 1ml and 200µl pipette tips, cell scrapers, Nunc maxisorp 96-well plates, 0.22 µm filters and NUNC/Nalgene cryovials were all purchased from Starstedt Ltd., Leicester, UK. Tissue culture flasks T25, T7, T150, 10-cm and 6-cm Petri dishes, 6-, 12-, 24-, 48- and 96- well plates, improved Neubauer haemocytometer and parafilm were purchased from Scientific Laboratory Supplies, Nottingham, UK. Whatman no.1, no.42 and 3 MM chromatography/filter paper were purchased from Whatman Ltd, Maidstone, UK. Sterile 8-well glass chamber slides were purchased from Lab-Tek Brand Products, Naperville (IL), USA.

3.1.11. Equipment

Amersham UV cross-linker was supplied by Amersham, Little Chalfont, UK. Spectrophotometer Model DU-7, centrifuges Avanti J-30 I, MSE Centaur 2, GPKR and MSE Microcentaur and Optima TLX Tabletop Ultracentrifuge were supplied by Beckman Instrument (UK) Ltd, High Wycombe, UK. Atto-minigel protein electrophoresis system was supplied by B & L Systems, Marseen, Netherlands. Mini-protean electrophoresis system, DNA-Sub electrophoresis system, Protean II Cell System, Protean II isoelectric focussing (IEF) system, Dodeka cooler unit and power pack were purchased from Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK. Electrophoresis power supply and LKB Multiphor II semi-dry blotter were obtained from Pharmacia, Milton Keynes. Tri-Carb 300 Scintillation counter were supplied by Canberra-Packard, Pangborne, UK. Cecil 1010 spectrophotometer was supplied by Cecil Instruments Ltd., Cambridge, UK. pH meter 130 was purchased

from Corning, Stone, Staffs, UK. MCC 340 ELISA plate reader and Gelaire BSB 4A laminar flow cabinets were obtained from Flow Laboratories, High Wycombe, UK. Water baths were supplied by Grant Instruments, Cambridge, UK. Techne Hybridser HB-1D dryer oven was supplied by Jencons-PLS, East Sussex, UK. IG150 tissue culture CO_2 incubators, laminar flow cabinet LC 2.12 and Jouan vacuum concentrator were obtained from Jouan Ltd., Derbyshire, UK. Soniprep 150 sonicator and Chilspin refrigerated centrifuge were supplied by MSE, Loughborough, UK. Ultra Turrax homogeniser was purchased from Merck, UK. Nikon CK2 and BH2 light inverted microscopes, OM4 Ti 35mm camera and DP10 microscope digital camera were obtained from Olympus Optical Company (UK) Ltd., Middlesex, UK. Spectafluor 96-well ELISA plate reader and XFluor4 software were purchased from Tecan UK Ltd, Goring-on-Thames, UK. TCSNT confocal laser microscope was purchased from Leica Lasertechnik, Heidelberg, Germany.

3.2. METHODS

3.2.1. General Cell Culture

3.2.1.1. Clonal pancreatic insulinoma β-cell lines

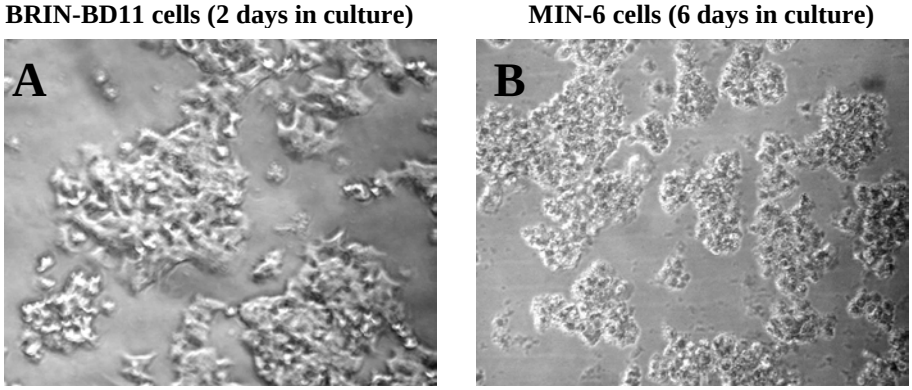

Figure 3.1. Morphology of clonal pancreatic insulinoma β-cell lines: Micrographs of **A)** BRIN-BD11 (from rat) and **B)** MIN-6 (from mouse) cells in culture using a Nikon CK2 inverted light microscope (x 40 magnification).

A novel GLUT-2 expressing glucose-responsive, insulin-secreting cell line, BRIN-BD11 (McClenaghan *et al*., 1996; Rasschaert *et al*., 1996), derived from rat pancreatic insulinoma β-cells, were kindly provided by Prof. N.G. Morgan (Peninsula Medical School, Plymouth), and used as the main experimental model in these studies (see Fig. 3.1.A). The BRIN-BD11 β-cell line was established through the electrofusion of RINm5F cells with New England Deaconess Hospital (NEDH) rat pancreatic islet cells, grows as monolayers with epithelioid characteristics, and maintains stability in tissue culture for over 50 passages with a 5-10 times greater insulin output than the parent RINm5F cell line (McClenaghan *et al*., 1996). BRIN-BD11 cells were cultured in RPMI 1640 medium (10-12 mM D-Glucose, Sigma) supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and only used for experimentation between the passages of 25 to 35. When experiments required lower glucose levels from 0.25 mM to 5 mM glucose, glucose-free RPMI 1640 (Cambrex Bioscience, Berkshire, UK) supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin was used, with the addition of appropriate amounts of sterile/filtered glucose stock solution made up in dH_2O. These cells were routinely cultured in a humidified atmosphere at 37°C, 5% (v/v) CO_2, 95% (v/v) air with regular supplemented media changes every day or no longer than three days depending on confluency and glucose levels remaining within the medium.

An additional mouse pancreatic β-cell line, MIN-6 (Miyazaki *et al.,* 1990; Ainscow *et al*., 2000), was kindly provided by Prof. N.G. Morgan (Peninsula Medical School, Plymouth) and used in additional studies where appropriate (see Fig. 3.1.B). MIN-6 cells are a highly differentiated pancreatic β-cell line established from insulinomas obtained by targeted expression of the simian virus 40 T antigen gene in transgenic mice, and exhibit exclusive expression of the liver-type glucose transporter and glucose-inducible insulin secretion comparable with cultured normal mouse islet β-cells (Miyazaki *et al*., 1990). MIN-6 cells were cultured in DMEM medium (20 mM D-Glucose, Sigma) supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100μg/ml streptomycin, and only used for experimentation between passage numbers of 60 - 68. These cells were routinely

cultured in a humidified atmosphere at 37°C, 5% (v/v) CO_2, 95% (v/v) air with regular supplemented media changes every two or three days. RNA extracted from the rat insulinoma pancreatic β-cell line INS-1E for use in northern blotting was a kind gift from Prof. N.G. Morgan ((Peninsula Medical School, Plymouth).

3.2.1.2. Extracellular Support Matrix

3.2.1.2.1. 5637 cell line

The 5637 cell line (also referred to as HTB-9) is derived from a human urinary bladder carcinoma source and was purchased from the ATCC. 5637 cells have a turnover rate of approximately 24 hours and form an adherent monolayer on tissue culture plastic. These cells are known to produce and release growth factors for granulocytes and monocytes (Welte *et al.*, 1985), and the conditioned medium from 5637 cells is often used as a source for haematopoietic growth factors in order to sustain the continuous growth and proliferation of cytokine-dependent leukaemia cell lines. 5637 cells are the parental strain for 1A6 (PTA-556), which produces between two to ten fold higher amounts of pluripotent hematopoietic colony-stimulating factor than the parental cell line. The 5637 cells were cultured in RPMI 1640 growth medium (Sigma) supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and used for experimentation between the passages of 4 to 10. These cells were routinely cultured in a humidified atmosphere at 37°C, 5% (v/v) CO_2, 95% (v/v) air with regular supplemented media changes every two to three days.

3.2.1.2.2. ECV-304 cell line

The ECV-304 cell line (also referred to as CRL1998) were reported to have started off as a human umbilical vein endothelial cell line, however was later found to be genotypically identical, and derived from a human urinary bladder carcinoma cell line (T24, ATCC: HTB-4) due to contamination (Suda *et al.*, 2001). These authors found that despite this, ECV-304 cells phenotypically show important endothelial characteristics which differ from T24 cells under criteria such as growth behaviour,

cytoarchitecture, tight junction arrangement, transmembrane electrical resistance and TG2 activity (Suda *et al*., 2001). The ECV-304 cells were cultured in DMEM growth medium (Sigma) supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and used for experimentation between the passages 20-30. These cells were routinely cultured in a humidified atmosphere at 37°C, 5% (v/v) CO_2, 95% (v/v) air with regular supplemented media changes every two to three days.

3.2.1.3. Subculturing / Passage of Cells

All cultures were maintained as attached monolayers through exponential cell divisions to approximately 90% confluency and then subcultured to ensure that they were healthy and at the appropriate passage number for experimentation or storage through cryopreservation. Separate stored cultures were maintained using an independent supply of medium and solutions. General ATCC cell line maintenance procedures were followed at all times using aseptic and sterile techniques. All handling and manipulation of cells were confined to double HEPA-filtered laminar flow cabinets under ACGM Containment level 2 regulations. Disposal of cells and other biological wastes were autoclaved and/or disinfected as required by ACGM regulations.

For routine passaging and detachment, a standard trypsinisation protocol was performed. Supplemented growth medium was aspirated, followed by immediate rinsing of the cell monolayer with serum-free solutions of the growth medium pre-warmed to 37 ºC. Cell monolayers were detached from culture flasks or plates using 0.25% (w/v) trypsin/2mM EDTA solution, in PBS (pH 7.4) at 37 ºC in the minimum amount of time for complete monolayer detachment which varied between cell lines. This ranged from 1 minute trypsin exposure in BRIN-BD11 cells to 15 min trypsin exposure for the 5637 cells. Detachment of the monolayer was confirmed visually using an inverted microscope, and the enzymatic action of trypsin was inactivated by the addition of an equal volume of culture growth medium containing 10% (v/v) FCS. This cell suspension was then transferred to a sterile centrifuge tube, and spun down at 1300 rpm (300 x *g*) for 5 minutes in an MSE bench top centrifuge. The cell

pellet was then re-suspended to appropriate volumes of fresh supplemented growth medium or re-seeded at the desired cell density for experimentation following cell counting using 10 µl aliquots of the cell suspension in duplicate chambers of an Improved Neubauer haemocytometer.

3.2.1.4. Cell Freezing and Cryopreservation

Cells were harvested and centrifuged as described above (section 3.2.1.3). Cell pellets were then re-suspended in the appropriate growth medium containing 90% (v/v) FCS and 10% (v/v) DMSO to a final concentration of 10^6 cells/ml. The suspension was transferred to 1 ml cryovials and placed in an isopropanol-containing cryocontainer at -80^{o}C overnight, before being cryopreserved and stored in liquid nitrogen indefinitely.

3.2.1.5. Reviving Frozen Cells from Storage

Cells in cryovials were removed from storage and thawed at 37^{o}C. Immediately after thawing, the cells were transferred into a sterile centrifuge tube and 5 ml of cell growth medium was added drop-wise, mixing gently after each addition. Cells were then centrifuged at 300 x *g* for 5 minutes. The cell pellet was re-suspended in the appropriate cell growth medium and transferred to a tissue culture flask for incubation at 37^{o}C as described above (section 3.2.1.).

3.2.2. Islet Isolation using Collagenase Digestion

Islets of Langerhans were isolated from male Wistar rats, wild type ($TG2^{+/+}$) and knockout ($TG2^{-/-}$) mice (De Laurenzi *et al*., 2001) using collagenase digestion according to the method described by Montague and Taylor (1968). The rats were sacrificed and whole pancreases were dissected out and placed in Gey's Balanced Salt Solution (Sigma) or bicarbonate buffered saline solution (Welters *et al*., 2004) or with a D-Glucose concentration of 5 mM. Two rat pancreases or four mouse pancreases were used for each preparation yielding between 300-1000 islets (see fig. 3.3). The pancreases were distended and injected with syringed needles filled with

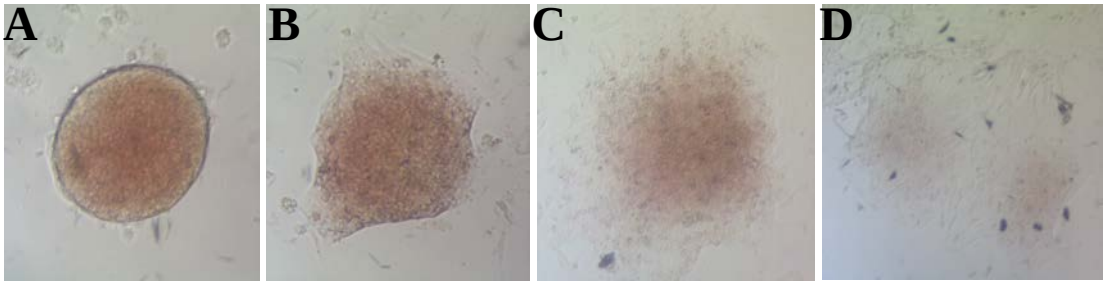

Figure 3.2. Rat islets stained with dithizone to show insulin content: Micrographs showing an example of the morphology and insulin content (red dithizone stain) of rat islets cultured over one week on tissue culture plastic (A: day 1; B: day 2; C: day 3; D: day 7) using a Nikon CK2 inverted light microscope (x 20 magnification).

Gey's Balanced Salt Solution in order to provide oxygenated buffer to the tissue and aid in the chopping and digestion. The pancreases were placed in a small beaker and chopped vigorously with pointed scissors to obtain uniformly small pieces (~1mm^2). collagenase XI (Sigma) or collagenase P (Roche) (6mg per preparation) was then added to the chopped tissue and the flask mixture suspended in a 37ºC water bath, and shaken vigorously with an automated flask shaker for 5 minutes. This was done to aid in the digestion of the exocrine tissue surrounding the islets. The digested tissue was quickly spun down by pulse centrifugation and diluted with Gey's buffer to neutralise the collagenase digestion. Rounded intact islets were then selected by hand with a finely distended glass Pasteur pipette off a blackenend Petri dish using a dissecting microscope. Islets were routinely tested for functional insulin secretion before experimentation (see Fig. 3.2.) using dithizone staining (100 μg/ml in serum free medium) followed by three rinses in PBS (pH 7.4).

3.2.3. Treatment of Cells and Islets

3.2.3.1. Treatment of β-cells with D-glucose

Stock solutions of D-glucose were made up in autoclaved distilled water and filter sterilised before addition to RPMI 1640 medium custom-made without D-Glucose (Cambrex Bioscience). Stimulatory D-glucose parameters ranged from 17 mM to hyperglycaemic levels of 40 mM. Hypoglycaemic conditions were induced with low D-glucose concentrations ranging from 0.25 mM to 5 mM. For concentrations of 10 mM glucose, normal RPMI growth medium (Sigma) was used.

Figure 3.3 Islet Isolation

3.2.3.2. Induction of oxidative stress in β-cells

S-Nitrosoglutathione (GSNO) is a nitric oxide donor *in vivo* and is also reported to be a substrate for γ-glutamyl transpeptidase, which hydrolyzes the γ-glutamyl moiety of glutathione to give glutamate and cysteinylglycine. The compound was made up under sterile conditions in autoclaved dH_2O, and stored in aliquots at -20˚C due to reported rapid decomposition rates in solution at room temperature. Concentrations of 50 µM to 500 µM were made up in serum-free RPMI containing 5 mM glucose for treating BRIN-BD11 cells, usually for 24 hours at 37°C, 5% (v/v) CO_2.

3.2.3.3. Induction of hyperlipidaemia in β-cells

Palmitate (sodium salt) was dissolved in 50% ethanol by heating at 70˚C. The dissolved palmitate was then diluted into a 10% solution of fatty acid free albumin and incubated at 37˚C for 1 hour to allow the palmitate to bind to the albumin. The mixture was then diluted as required into serum-free RPMI containing 5 mM glucose to give final concentrations of 0.5% ethanol and 1% BSA, and stored for short periods at 4˚C. Treatment concentrations ranged from 50 µM to 500 µM, where BRIN-BD11 cells were incubated for 24 hours at 37°C, 5% (v/v) CO_2.

3.2.3.4. Treatment with GTP and $CaCl_2$

The effect of intracellular Ca^{2+} levels on TG activity was tested by the addition of $CaCl_2$ (freshly prepared and diluted to appropriate concentrations in dH_2O) to the reaction mix or culture medium. Concentrations between 0.5 mM to 10 mM $CaCl_2$ were used to facilitate TG catalytic reactions.

GTP-γ-S (Sigma) was diluted to appropriate concentrations (0.5 µM to 1000 µM) in dH_2O and stored at -20°C. To test its effect in TG activity assays, the GTP aliquots were defrosted just before the reactions were initiated by direct addition to the reaction mix.

3.2.3.5. Treatment with Matrix Metalloproteinase Inhibitors

Stock solutions of the matrix metalloproteinase (MMP) broad range inhibitor GM6001 (Galardin; 364205) and the corresponding negative control (364210) (Calbiochem) were prepared according to the manufacturer's instructions. BRIN-BD11 cells were treated with 10 uM Galardin (GM6001, 364205), which is a potent broad-spectrum hydroxamic acid inhibitor of MMPs. Treatments were for 3 hrs at 37°C, 5% (v/v) CO_2. Negative controls included the accompanying 10 µM –MMP solution (364210), and untreated BRIN-BD11 cells.

3.2.3.6. Treatment with Dispase

BRIN-BD11 cells were treated for their susceptibility to dispase and its possible effect on TG, according to the protocol described by Kim *et al*. (1995). Live cells were treated in suspension with 0.01 unit/ml dispase diluted in serum-free RPMI at increasing incubation times (0, 5, 10, 15, 30 minutes) at 37°C, 5% (v/v) CO_2, and thereafter assayed for TG activity.

3.2.3.7. Treatment with Trypsin

BRIN-BD11 cells were treated for their susceptibility to trypsin and its possible effect on TG, according to the protocol described by Rice *et al*. (1990). Live cells were treated in suspension with 0.25% (w/v) trypsin (Sigma) diluted in serum-free RPMI at an incubation time of 15 minutes at 37°C, 5% (v/v) CO_2, and thereafter assayed for TG activity.

3.2.3.8. Treatment with active-site directed TG inhibitors

R283 (1,dimethyl-2[(oxopropyl)thio]imidazolium) and R281(Nbenzyloxycarbonyl-L-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine) were synthesised according to published methods (Griffin *et al*., 2004; Freund *et al*., 1994) by R. Saint and I. Coutts at Nottingham Trent University. The structurally distinct chemical structures of the inhibitors are shown in Chapter 4, where inhibition involves the

irreversible acetonylation of the thiol group contained within the active site cysteine$_{277}$ which results in the thiol group being unreactive towards its glutamyl and amine substrates. The purity of these compounds was determined by NMR and mass spectrometry (Nottingham Trent University). Unless used directly as a negative control or in TG catalysis reactions, efficacy of the inhibitors against pancreatic β-cell TG was determined by the application of inhibitors at 50 μM to 500 μM concentrations for 24 hours at 37°C, 5% (v/v) CO_2, and the TG activity measured as described in Chapter 4 and Chapter 6.

TG2 inhibitor peptide, Boc-DON-Gln-Ile-Val-OMe (MW: 625.7) was obtained from N-Zyme Biotec (Darmstadt, Germany) and was pre-dissolved in DMSO before making a stock solution of 1 mM in sterile dH_2O, which was stored in aliquots at -20°C. This potent active-site directed inhibitor of the TG2 enzyme results in the alkylation of active site cysteine$_{277}$, and was shown to be specific to TG2 since no inactivation of fXIII was seen (N-Zyme Biotec data sheet). Furthermore, inactivated purified TG could not be reactivated suggesting this inhibitor to be either irreversible or slow and tight binding, with an optimal working concentration of 10 μM in cell homogenates for 24 hours at 37°C, 5% (v/v) CO_2.

3.2.3.9. Treatment with Retinoic Acid

All *trans* retinoic acid (Sigma) was solubilised in dH_2O and stored at -20°C, protected from light. To test its effect on TG, these aliquots were defrosted just before the reactions were initiated by direct addition to the culture medium. Treatment concentrations ranged from 0.1 μM to 3μM retinoic acid for 48 hours at 37°C, 5% (v/v) CO_2.

3.2.3.10. Treatment with RGD peptide

BRIN-BD11 cells in suspension (1 x 10^6 cells/ml) were incubated with 50μg/ml RGD (GRGDTP) synthetic peptide (Sigma) or 50μg/ml control RAD (GRADSP) peptide (Calbiochem) for 10 min at 37 °C, 5% (v/v) CO_2 in serum-free RPMI as described previously by Verderio *et al*., (2003). The cells were then seeded onto 96-

well plates pre-coated with fibronectin or fibronectin-TG2 matrices as described in section 3.2.4.1.

3.2.4. Preparation of Support Matrices for β-cells

3.2.4.1. Coating wells with Fibronectin

Tissue culture plastic surfaces were saturated by the addition of the appropriate volume of 5 μg/ml human plasma fibronectin (FN) in 50 mM Tris-HCl (pH 7.4) to each well followed by incubation at 4^{o}C overnight. 96-well plates were coated with 100 μl and 24-well plates with 500 μl of fibronectin solution. Coated surfaces were blocked with 3% (w/v) BSA in the same Tris-HCl (pH 7.4) buffer for 1 hour at room temperature and washed three times with serum-free culture medium prior to the addition of cells.

In the case of FN-TG2 matrices, wells were coated with fibronectin solution as described above, but before the blocking step, 10 – 30 μg/ml guinea pig liver TG2 (*gpl*TG2) diluted in 50 mM Tris-HCl (pH74) was immobilised onto the FN by incubating at 37°C for 1 hour. Following incubation, the wells were washed twice with the same Tris-HCl (pH 7.4) solution and blocked with 3% (w/v) BSA in 50 mM Tris-HCl (pH 7.4) buffer for 1 hour at room temperature and washed three times with serum-free culture medium prior to the addition of cells.

3.2.4.2. Preparation of 5637 lysed matrices

Human urinary bladder carcinoma cells (5637) were seeded at a density of 1 x 10^5 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and allowed to grow to confluency in tissue culture wells for 3 days at 37°C, 5% (v/v) CO_2 as described in section 3.2.1.2.1. The complete medium was then aspirated from the wells, and cell monolayers were washed three times with sterile PBS (pH7.4). The cell monolayers were then lysed as described by Ris *et al*., (2002) with the addition of

Figure 3.4. Preparation of 5637 lysed matrices

sterile dH_2O for 3.5 minutes, followed by three washes in dH_2O, and allowed to air dry. The air dried lysed matrices were used directly for experimentation or else stored at 4°C for no more than 2 days.

3.2.4.2.1. Pre-conditioned 5637 lysed matrices (TG Inhibitors)

In order to prepare 5637 support matrices in the presence of diminished TG activity, the cells were seeded as described in section 3.2.4.2., with 250 μM active site directed inhibitors R283 or R281; or 10 μM Boc-DON-Gln-Ile-Val-OMe TG2 inhibitor peptide, added directly to the growth medium. The medium containing inhibitors was replaced every 24 hours, as the cell monolayers expanded to 3 day confluency. These TG inhibited 5637 support matrices were then lysed as described in section 3.2.4.2.

3.2.4.2.2. Pre-conditioned 5637 lysed matrices (TG2 knockdown with siRNA)

Three day 5637 support matrices were prepared by knocking down TG2 protein expression with the use of siRNA gene silencing. Three non-overlapping custom-made Stealth Select RNAi duplexes (see Table 3.1.), and a high GC negative control siRNA duplex (12935-400) to be used with lipofectamine 2000 were purchased from Invitrogen and used according to the manufacturer's instructions. The lyophilised siRNA duplex oligoribonucleotides were re-suspended in nuclease-free water provided by the manufacturer to a stock concentration of 20 μM and stored at -20°C.

For each transfection sample in a 24-well plate, oligo-lipofectamine 2000 complexes were prepared by diluting 20 pmol of two non-overlapping sequences of Stealth siRNA in separate micro-tubes with 50 μl RPMI 1640 medium supplemented with 5% (v/v) heat-inactivated foetal calf serum and 2 mM L-glutamine. In a separate micro-tube, lipofectamine 2000 was then diluted by adding 1 μl to 50 μl RPMI 1640 medium supplemented as before, and allowed to incubate at room temperature for 5 minutes. The 50 μl diluted siRNA was then combined with the 50 μl diluted lipofectamine 2000 and incubated for a further 20 minutes at room temperature to form a 100 μl oligo-lipofectamine complex. This complex was then added to each well containing freshly seeded cells at 1 x 10^5 cells/ml and 500 μl RPMI 1640

medium supplemented as described above. Fresh RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum and 2 mM L-glutamine was added to the wells after 24 hours transient transfection, and the 3 day 5637 support matrix was lysed as described in section 3.2.4.2. Optimisation of this transient transfection was achieved by testing all three Stealth siRNA duplexes in combination at concentrations ranging from 10 – 50 pmol, and lipofectamine 2000 concentrations ranging from 0.5 to 1.5 μl. Optimum 70% TG2 knockdown as a result of transient transfection was achieved with 1 μl lipofectamine 2000 complexed to 40 pmol of TGM2-HSS110713 and TGM2-HSS110714 in combination.

Table 3.1. Stealth Select siRNA duplexes targeted to Transglutaminase 2

Gene	Duplex RNA Primer Sequences (5` - 3`)
TGM2-HSS110713	AUCACACCUCUCUAAGACCAGCUCC GGAGCUGGUCUUAGAGAGGUGUGAU
TGM2-HSS110714	UUCUUGAUGAACUUGGCCGAGCCCU AGGGCUCGGCCAAGUUCAUCAAGAA
TGM2-HSS110715	UAGGAUCCCAUCUUCAAACUGCCCA UGGGCAGUUUGAAGAUGGGAUCCUA

3.2.4.2.3. Pre-conditioned 5637 lysed matrices (immobilised with gplTG2)

5637 support matrices were prepared in the presence of exogenously supplemented *gpl*TG2 (10 – 30 μg/ml) by direct addition to the growth medium when the cells were seeded as described in section 3.2.4.2. The cell monolayers containing *gpl*TG2 were expanded to 3 day confluency and these 5637 support matrices containing immobilised TG2 were then lysed as described in section 3.2.4.2.

3.2.4.2.4. Pre-conditioned 5637 lysed matrices (cross-linked using gplTG2)

In order to prepare TG2 cross-linked 5637 support matrices, the cells were seeded as described in section 3.2.4.2 and expanded to 3 day confluency. Before lysis, the 5637 matrix was cross-linked in the presence of a reaction mix containing 20 μg/ml *gpl*TG2, 5 mM $CaCl_2$ and 10 mM DTT added to serum free growth medium for 1 hour at 37°C, 5% (v/v) CO_2. The cross-linked 5637 support matrix was then washed three times in PBS and lysed as described in section 3.2.4.2.

3.2.4.3. Preparation of ECV-304 lysed matrices

Human umbilical vein endothelial cells were seeded at a density of 1 x 10^5 cells/ml in DMEM medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and allowed to grow to confluency in tissue culture wells for 3 days at 37°C, 5% (v/v) CO_2 as described in section 3.2.1.2.2. The complete medium was then aspirated from the wells, and cell monolayers were washed three times with sterile PBS (pH7.4). The cell monolayers were then lysed (as described in section 3.2.4.2.) with the addition of sterile dH_2O for 3.5 minutes, followed by three washes in dH_2O, and allowed to air dry. The air dried lysed matrices were used directly for experimentation or else stored at 4°C for no more than 2 days.

3.2.5. Analysis of Proteins

3.2.5.1. Preparation of Cell, Islet and Tissue Homogenates

Attached cells were collected after firstly rinsing with PBS (pH 7.4) followed by the addition of appropriate volumes of homogenisation buffer (0.32 M sucrose, 5 mM Tris-HCl (pH7.4), 2 mM EDTA) containing protease inhibitors phenyl methyl sulfonyl fluoride (PMSF, 20 μg/ml), leupeptin (10 μg/ml), pepstatin (1 μg/ml), and benzamidine (780 μg/ml) and were then collected in 1.5 ml microfuge tubes using a rubber cell scraper. Islets and snap-frozen tissue were made up to 20% (w/v) in the same homogenisation buffer using a mini homogenizer and pestle in 1.5 ml

microfuge tubes. Cell lysates were produced by sonication with a Soniprep 150 sonicator (MSE, UK) on ice for 3 x 5 seconds with 15 second cooling intervals.

3.2.5.2. Protein Content Estimation

Total protein content in cell, islet or tissue homogenates were determined using either the Lowry protein assay (Lowry *et al*., 1951) or bicinchoninic acid (BCA) assay (Brown *et al*., 1989). Choice of the assay was dependent on their compatibility with the reducing agents and detergents contained in the solubilisation buffers.

3.2.5.2.1. Lowry protein assay

The Lowry protein assay was carried out using a kit obtained from Bio-Rad (Watford, Herfortshire, UK). 25 µl of reagent A and 200 µl of reagent B were added sequentially to 5 µl of sample or BSA standards ranging from 0.15 to 10 mg/ml in a 96-well plate format. The plate was incubated for 10 minutes at room temperature and the absorbance was read at 750 nm using a SpectraFluor plate reader.

3.2.5.2.2. BCA protein assay

The BCA assay was used instead of the Lowry assay when protein samples from cell lysates contained considerable concentrations of dithiothreitol (DTT). 5 µl of protein suspension was mixed with 200 µl of BCA reagent. BCA reagent was obtained by mixing solution A [1% (w/v) bicinchoninic acid in sodium salt form, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium tartate, 0.4% (w/v) sodium hydroxide, 0.95% (w/v) sodium hydrogen carbonate, pH 11.5) with solution B [4% (w/v) copper sulphate] at a ratio of 25:1. The values of the protein concentrations were estimated against a parabolic standard curve produced by triplicate BSA protein standards (0.15-10 mg/ml). Colorimetric absorbance readings generated by the addition of BCA assay reagents after incubation at 37°C for 30 minutes was detected using a SpectraFluor plate reader set at 570 nm.

3.2.5.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The method used to separate proteins for analysis was a modification of that described by Laemmli for use with a vertical slab gel apparatus (Laemmli, 1970). Gels were cast in the Atto-minigel system (B & L Systems, Marseen, Netherlands) and consisted of a standard 3% (w/v) polyacrylamide stacking gel and a 6-12% (w/v) resolving gel. The acrylamide stock solution used for all gels consisted of 30% (w/v) acrylamide and 0.8% (w/v) N, N′-methlylene bisacrylamide. Stacking gels were made using a Tris-SDS stock solution, pH 6.8 [0.25 M Tris, 0.2% (w/v) SDS) whereas resolving gels contained a Tris-SDS solution pH 8.8 [0.75 M Tris, 0.2% (w/v) SDS]. Concentrations of polyacrylamide in the resolving gel varied from 6% for separation of proteins with high molecular weight, to 12% for separation of smaller molecular weight proteins. The recipes for different concentrations of acrylamide in the resolving gel are listed in Table 3.2. Resolving gels (80 x 60x 0.75mm) were cast using the Atto-system according to the manufacturer's protocol.

Water-saturated butan-2-ol was poured on the top of the gel to an approximate depth of 5mm to provide the gel with a flat upper surface, and the gel was allowed to polymerise for 40 minutes at room temperature. The upper surface of polymerised resolving gels was washed twice with distilled water to remove residual butan-2-ol, and the edge of the gel was gently blotted dry using filter paper. Stacking gels were prepared by combining 0.65 ml of acrylamide solution, 1.25 ml of Tris/SDS pH 6.8 and 3.05 ml of distilled water. Polymerisation was initiated by the addition of 25 μl 10% (w/v) ammonium persulphate and 5 μl of TEMED with constant mixing. The gel was quickly pipetted between the glass plates and the appropriate comb was inserted to form the sample wells. Polymerisation was allowed to proceed for 45 minutes at room temperature. The sample well comb was gently removed from polymerised stacking gels and wells were washed and filled with Tris-glycine electrode running buffer pH 8.5 [0.025 M Tris, 0.192 M glycine and 0.1% (w/v) SDS]. Sample volumes to be loaded were normalised against protein concentration values, and 5 - 20 μg of protein was combined with the appropriate volume of 2x strength Laemmli loading buffer [125 mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) mercaptoethanol and 0.02 mg/ml bromophenol blue] and pipetted into the sample wells using protein

Table 3.2. Constituents of 2 resolving mini-gels for SDS-PAGE

Stock Solutions	% Acrylamide in separating gel (w/v)		
	6%	**10%**	**12%**
30% acrylamide (w/v)	3 ml	5 ml	6 ml
1.5 M Tris-SDS (pH 8.8)	3.75 ml	3.75 ml	3.75 ml
Distilled H_20	8.25 ml	6.25 ml	5.25 ml
10% ammonium persulphate (w/v)	50 µl	50 µl	50 µl
TEMED	10 µl	10 µl	10 µl

electrophoresis gel-loading tips (Bio-Rad). Electrophoresis was performed at 150 V for approximately 1.5 hours until the bromophenol blue marker dye had reached the bottom of the gel.

3.2.5.4. Western blotting of SDS-PAGE separated proteins

Electrophoretic transfer of SDS-PAGE separated proteins to nitrocellulose was performed as described by Towbin *et al.* (1979) using a Protean II Cell System (Bio-Rad) wet-blot system. SDS was removed from the gel by rinsing in transfer buffer [48.8 mM Tris-HCl, 39 mM glycine, 20% (v/v) methanol] for 10 minutes. The nitrocellulose transfer membrane (Osmonisc, Westerborough, MA) was equilibrated in the same transfer buffer together with the scanting pads and filter paper (Whatman, 27cm). The apparatus was assembled by stacking the scanting pad on the bottom, followed by 3 sheets of filter paper, the gel, the membrane, another three pieces of filter paper and finally another scanting pad. During the assembly bubbles trapped in between the filter papers and the membrane were removed by rolling a glass rod uniformly over the stack. Western blotting was carried out using the Bio-Rad apparatus for 1 hour at 200 mA in pre-chilled transfer buffer. After the proteins were transferred from the gel to the nitrocellulose membrane, the equipment was disassembled and the membrane, separated from the gel, was placed in a plastic tray for further analysis. Transfer of proteins was verified by staining with Ponceau Red solution [0.2% (w/v) Ponceau S, 0.4% (v/v) glacial acetic acid] and subsequent de-staining in distilled water. Washed blots were then blocked by incubation in a

blocking solution containing 5% (w/v) fat-free dried milk powder in PBS (pH 7.4) and 0.05% (v/v) Tween20 for 2 hours at room temperature, before being immuno-probed with the appropriate primary antibody.

3.2.5.5. Immunoprobing of western blots and membrane stripping

Following blocking, the blots were first incubated with the primary antibody in the same blocking buffer for 2 hours at room temperature. Blots were then washed three times in PBS 0.5% (v/v) Tween20, 1% (w/v) milk for approximately 5 minutes and subsequently incubated with a species-specific secondary HRP-conjugated antibody in blocking buffer for a further 1 hour. Another three washes were carried out in order to remove the antibody background followed by a final wash in PBS 0.5% (v/v) Tween 20 to remove residual milk. The HRP component of the secondary antibody was exposed for 90 seconds to enhanced chemiluminescence (ECL) substrate that resulted from the mixture of 2ml of reagent A with 2 ml of reagent B (ECL, Amersham, Bucks, UK). Excess ECL substrate was drained and the membrane was covered with cling film. The resulting light emission was detected by exposure to Kodak X-Omat chemiluminescence detection film (Roche Diagnostics, East Sussex, UK) for 1-20 min, depending on the dilutions of the antibodies and the intensity of the signal, in a dark room. The film was developed using 20% (v/v) LX-24 developer (Sigma), fixed in 20% (v/v) FX-40 fixer (Sigma), and rinsed in tap water before being air dried, and scanned for digital densitometry. When the blots were not developed manually in the dark room, the same ECL reagents as described above were used, and the blot was placed inside an AIDA LAS 3000 ECL Image Reader, where automatic development of the blot was achieved by setting exposure time parameters and the resulting images were captured digitally for further densitometric analysis.

Where further immunoprobing was necessary, the nitrocellulose membrane was made wet again in either PBS and then incubated in stripping buffer [100mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5mM Tris-HCL pH 6.7] at 50°C for 30 minutes with occasional shaking. The membrane was then washed twice in PBS-Tween20 and blocked again in PBS 0.5 % (v/v) Tween20, 1% (w/v) dried milk for 45 minutes.

3.2.5.6.Two-Dimensional (2-D) Gel Electrophoresis

3.2.5.6.1. Sample preparation

BRIN-BD11 cells were grown to 80% confluency in T75 tissue culture flasks, and washed three times in PBS (pH 7.4). Lysis buffer which contained 0.5% (w/v) SDS in PBS (pH 7.4) was pre-heated, and 3 ml added directly to the cells prior to the flask being immersed in boiling water for 3 minutes. The cell lysates were then collected and ultracentrifuged for 20 minutes at 100 000 *x g*, before 100 µl of the supernatant was mixed with 900 µl ice cold acetone and stored at -20 °C overnight. The lysates were then centrifuged at 13 000 x *g* for 20 minutes, the supernatant discarded and the resulting pellet air-dried and re-suspended in 400 µl rehydration buffer which contained 8M Urea, 50mM DTT, 4% CHAPS, 0.0002% (w/v) bromophenol blue, 0.2% bio-lyte ampholyte stock solution in the 4-7 pH range (Bio-Rad), all made up in 50 ml dH_20 (18MΩ). Protein concentrations were corrected to 50 µg in 400 µl and this was loaded in the slot of the rehydration tray in the Bio-Rad Protean II isoelectric focussing (IEF) system. Immobilised pH gradient (IPG) strips in the 4-7 pH range (17 cm, Biorad) were placed in the chamber using the supplied forceps, with the acrylamide strip facing down on top of the sample and passive rehydration was allowed in the Protean IEF system for 1 hour. 4 ml of mineral oil (Bio-Rad) was then applied to the top of the strips, and an automatic progressive voltage program on the Protean IEF system was initiated which took 23 hours to run. Once the run was complete the (IPG) strips were either used directly in SDS-PAGE electrophoresis as follows, or placed in strip trays covered with parafilm and stored at -20°C.

3.2.5.6.2. Two-Dimensional (2-D) SDS-PAGE

Large gel plates were assembled according to the Bio-Rad product manual and 10% acrylamide gels were cast. To prevent leaking during polymerisation of the gel, 2 ml of molten agarose (0.5% (w/v) agarose in TBS pH 8.8) was applied to the bottom of the casting plate. The resolving gel was prepared as described in Table 3.3., and 48 ml of the solution was pipetted in between the plates, and topped with 15 ml of water saturated butan-2-ol and allowed to polymerise for 1 hour at room temperature. The butan-2-ol was washed with dH_2O off the gel top and dried with filter paper. 5 ml of

stacking gel (see Table 3.3) was then added to the top of the gel with a 1.5 mm spacer comb and allowed to polymerise for 30 minutes at room temperature.

The IPG strips which were prepared previously were then equilibrated for use in the two-dimensional SDS-PAGE system (Bio-Rad). The IPG strips (those removed from the -20°C freezer were thawed completely) were then equilibrated in their trays for 10 minutes using a shaker and 20 mg/ml DTT added to basal equilibration buffer which contained 6 M urea, 20% (w/v) SDS, 1.5 M Tris-HCl (pH 8.8), 50% (w/v) glycerol, adjusted to 100 ml of dH_20 (18MΩ). This solution was drained and replaced with 25 mg/ml iodoacetamide in basal equilibration buffer for 10 minutes on a plate shaker.

Table 3.3. Constituents of 2 large resolving gels for 2-D SDS-PAGE

Stock Solutions	**2 x Large 10% Acrylamide Gels**	
	Resolving Gel	**Stacking Gel**
40% acrylamide (w/v)	26.25 ml	1 ml
1.5 M Tris-SDS (pH 8.8)	26.25 ml	2.5 ml
Millipore Distilled H_20	52.5 ml	6.5 ml
10% ammonium persulphate (w/v)	0.3 ml	0.1 ml
TEMED	0.03 ml	0.04 ml
Total volume	105 ml	10 ml

The strips were the washed briefly by immersing them a few times in a 250 ml cylinder containg 1 x SDS-PAGE Tris-glycine electrode running buffer pH 8.5 [0.025 M Tris, 0.192 M glycine and 0.1% (w/v) SDS]. Using clean scissors, a few mm off the acidic (+) end of the IPG strip was trimmed to fit on top of the large gel. Overlay agarose (Readyprep, Bio-Rad) was liquidified in a microwave for 15 seconds, cooled down and then 2 ml was added to the top of the IPG strips. 20 µl of a MW standard (Dual colour, Bio-Rad) was added to the last comb space, and the gels were clipped onto Biorad cells that were pre-cooled using a 15°C Dodeka cooler unit. Running buffer was then added to the middle of the cell, and the 2-D gels were electrophoresed at 48 mA for a whole day, or 15 mA for an overnight run.

3.2.5.6.3. Silver-staining, Mass Spectrometry and Western Blotting

Once the large gels were electrophoresed, the gel plates were disassembled and the gels were moved to porcelain containers with large gel clips for further use. Some gels were stained using the silver stain plus kit (Bio-Rad), or else used for western blotting. Briefly, the silver stain entailed 30 minutes fixation in trichloroacetic acid (TCA), washing in dH_20, 30 minutes sensitizing [ethanol, 25% (w/v) gluteraldehyde, 5% (w/v) thiosulphate, 17g sodium acetate in 250 ml dH_20], washing in dH_20, 20 minute silver reaction [2.5% (w/v) silver nitrate solution, 37% (w/v) formaldehyde in 250 ml dH_20], washing in dH_20, 5 minutes developing [6.25 g sodium carbonate, 37% (w/v) formaldehyde in 250 ml dH_20], stop solution for 10 minutes [EDTA-$Na_2.2H_2O$ in 250 ml dH_20], washing in dH_20, 20 min preserving [ethanol, 87% (w/w) glycerol in 250 ml dH_20].

When gels were silver stained with the intention of picking spots for Mass Spectrometry (Bruker MicroTof, University of Nottingham), formaldehyde was left out of the appropriate silver staining steps. To facilitate spot-picking between the 50-kDa to 80-kDa range, excised gel pieces were reduced with DTT, alkylated with iodoacetamide, and digested with trypsin. The MS/MS spectra were searched against the NCBI Rattus Norvegicus (Rat) database using PeptIdent software (http://ca.expasy.org/cgi-bin/pepdident.pl). The algorithm was set to use trypsin as enzyme, allowing at maximum for one cleavage site and assuming carbamidomethyl as a fixed modification of cysteine, and oxidised methionine and deamidations of asparagines and glutamine as variable. Mass tolerance was set to 4 ppm, and 0.2 Da for MS and MS/MS respectively.

Electrophoretic transfer of 2-D SDS-PAGE separated proteins to nitrocellulose was performed as described in section 3.2.5.4 using a Protean II Cell System (Bio-Rad) wet-blot system. Following the transfer, nitrocellulose membranes were blocked for 2 hours at room temperature in 5% (w/v) fat-free dried milk powder in PBS (pH 7.4) and 0.05% (v/v) Tween20. The blots were then incubated with the primary antibody in the same blocking buffer for 2 hours at room temperature. Blots were then washed three times in PBS 0.5% (v/v) Tween20, 1% (w/v) milk for approximately 5 minutes

and subsequently incubated with an anti-HRP antibody in blocking buffer for a further 1 hour. Another three washes were carried out in order to remove the antibody background followed by a final wash in PBS 0.5% (v/v) Tween 20 to remove residual milk. The HRP component of the secondary antibody was exposed for 90 seconds to ECL substrates as described in section 3.2.5.5. and the resulting light emission was detected by exposure to Kodak X-Omat chemiluminescence detection film (Roche Diagnostics, East Sussex, UK) for 1-5 minutes. The film was fixed, developed and the images captured digitally as described in section 3.2.5.5.

3.2.5.7. Detection of TG protein

3.2.5.7.1. Immunostaining of TG2 antigen using Western Blotting

For the detection of TG antigen, cells or islets were homogenised, sonicated and electrophoresed using SDS-PAGE. Following western blot transfer, nitrocellulose membranes were blocked and immunoprobed with TG antibodies: monoclonal mouse anti-TG2 CUB7402 antibody (1:200) in blocking buffer for 2 hours at room temperature with gentle shaking. Blots were washed, incubated with an anti-mouse-IgG-HRP conjugate diluted 1:1000 in blocking buffer for a further 2 hours, and immunostained TG bands were then revealed by enhanced chemiluminescence as described in section 2.2.5.5.

3.2.5.7.2. Immunoperoxidase detection of TG2 antigen by ELISA

Cells were seeded at a concentration of 1.5×10^4 cells/well in a 96-well plate and allowed to settle overnight. The primary anti-TG2 antibody CUB7402 (Neomarkers) was diluted 1:1000 in cell growth medium and 100µl of the resulting mixture were added to each well prior to incubation at 37°C for 2 hours. Following incubation, medium was removed and the cells were washed three times with PBS (pH 7.4) and then blocked with blocking solution [5% (w/v) fat-free dried milk in PBS, pH 7.4] for 30 minutes. The plate was then washed three times with PBS (pH 7.4) and cells were then fixed with 3.7% (w/v) paraformaldehyde in PBS, pH 7.4 for 15 minutes at room temperature. After fixation, another series of washes with PBS were performed and

the secondary antibody (mouse IgG-HRP) diluted 1: 2000 in blocking buffer was added to each well (100µl/well) prior to an extra 2-hour incubation at room temperature followed by three washes with PBS (pH 7.4) as before. HRP was detected by addition of 100µl/well of tetramethylbenzidine (TMB, Sigma) according to the manufacturer's instructions and incubation for 10 minutes at room temperature. The reaction was stopped at equal times for each reaction by the addition of 2.5 M H_2SO_4 at 50 µ/well, and then read colorimetrically at an absorbance of 450 nm on a SpectraFluor plate reader.

3.2.5.7.3. Immunofluorescent detection of TG2 antigen

Cells were seeded at a concentration of 1 x 10^5 cells/well in 8-well glass chamber slides (Lab-Tek, Naperville, USA) and grown over 24 hours to confluency, prior to the addition of anti-TG2 MAb CUB7402 (Neomarkers) diluted 1 in 200 in fresh growth medium. Cells were incubated in the presence of the antibody for 2.5 hours, washed twice with PBS (pH 7.4) and fixed in 3.7% (w/v) paraformaldehyde in PBS (pH 7.4) while in the chamber wells for 15 minutes at room temperature. In some co-staining experiments and tissue cryosections, CUB 7402 MAb was added post-fixation for 1.5 hours at 37°C. Cell monolayers were then blocked with 3% (w/v) BSA in PBS (pH 7.4) for 30 minutes at 37°C. Following blocking, cells were incubated with either anti-mouse IgG-FITC (DAKO) or anti-mouse IgG-TRITC (DAKO) diluted 1 in 1000 in blocking buffer for 2 hours at 37°C and then washed 3 times with PBS (pH 7.4), prior to mounting with Vectashield mounting medium (Vecta Laboratories) or Fluorsave mounting reagent (Calbiochem). Stained cells were viewed for immunofluorescence using a Leica TCSNT confocal laser microscope system (LEICA, Germany) equipped with an argon krypton laser at 647 nm and 488 nm for rhodamine and fluorescein excitation. Semiquantitation of TG2 protein expression was obtained by using the mean fluorescent emissions per field ($mV/\mu M^2$) from at least 9 non-overlapping 40 x magnification fields.

3.2.5.7.4. GTP-agarose Pull-down Assay for TG2 using Western Blot Analysis

To detect TG2 antigen bound to GTP, a GTP-agarose pull-down assay previously described by Bailey *et al*., (2004) was used. To enrich for GTP-binding proteins, BRIN-BD11 cells (confluent T175 flask) were lysed in a ratio of 1:5 in GTP binding buffer [20 mM Tris-HCl (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$, 0.01% v/v Triton-X 100 containing protease inhibitors phenyl methyl sulfonyl fluoride (PMSF, 20 μg/ml), leupeptin (10 μg/ml), pepstatin (1 μg/ml), and benzamidine (780 μg/ml)]. Cell lysates were produced by sonication with a Soniprep 150 sonicator (MSE, UK) on ice for 3 x 5 seconds with 15 second cooling intervals, followed by ultracentrifugation at 100000 x *g* for 1 hour at 4ºC (Optima TLX Benchtop Ultracentrifuge, Beckman Instrument-UK), and the respective cytosolic and membrane fractions were collected. The protein concentration of each fraction was determined by BCA assay with BSA as the standard. Therafter, 250 μg of lysate protein was incubated with 100 μl of GTP-agarose beads (Sigma-Aldrich, equilibrated in GTP-binding buffer) in a total of 600 μl of GTP-binding buffer for 30 min at 4ºC with gentle shaking. The beads were then centrifuged at 13000 x *g* for 2 min and the supernatant was retained. The GTP-agarose beads were then washed three times with 1 ml GTP-binding buffer and the supernatant was incubated with the beads for a further 30 min as described above. The beads were then washed again, as described above, and incubated with the supernatant overnight at 4ºC with gentle shaking. This was followed by the beads being washed three times with GTP-binding buffer, and bound protein was eluted by boiling in 35 μl of 2x strength Laemmli loading buffer [125 mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) mercaptoethanol and 0.02 mg/ml bromophenol blue]. TG protein that had bound to the GTP-agarose beads was visualised by performing western blot analysis, using TG antibodies as described in section 2.2.5.7.1. Blots were stripped and re-probed for β-tubulin to ensure equal loading.

3.2.6. Measurement of TG activity

3.2.6.1. TG-catalysed incorporation of [^{14}C]-putrescine into N,N'-dimethylcasein

The established filter paper assay (Lorand *et al*., 1972) relies on the ability of TG to catalyse the incorporation of a radioactively labelled primary amine into a protein acceptor substrate (see Fig. 3.5). The acceptor substrate commonly used in this investigation was N,N'-dimethylcasein, however a modification of this assay was developed by simply leaving out the N,N'-dimethylcasein substrate in order to allow the endogenous cell components to act as substrates. Performing the assay involved the addition, at 30 second intervals, of 45 µl sample homogenate (described in section 3.2.5.1) corrected for equal protein which was added to 55 µl reaction mix containing: 10 µl of 50mM Tris-HCl (pH 7.4); 10 µl of 38.5 mM DTT; 10 µl of 12 mM [1,4-^{14}C]-putrescine (Amersham Pharmacia; specific activity 3.97mCi/mmol); 20 µl of 25 mg/ml N,N'-dimethylcasein (Sigma) in 50 mM Tris; and either 5µl of 50 mM $CaCl_2$ or 5 µl of 200 mM EDTA pre-equilibrated at 37°C in a waterbath. At 15 minutes after the initiation of the reaction, triplicate 10 µl aliquots from each sample were removed and spotted onto 10 mm^2 of 3MM filter paper (Whatman) at 30 second intervals. Squares were then placed in 10% (w/v) ice cold TCA for 10 minutes, then washed consecutively three times for 5 minutes in 5% (v/v) TCA, once in acetone: ethanol (1:1, v/v) for 5 minutes, and once in acetone for 5 minutes. A triplicate set of filter papers containing no sample was also washed as a reaction blank. The squares were then air dried for 15 minutes. Once dry, filter papers were placed in scintillation tubes, to which 2 ml Optiphase High Safe liquid scintillation fluid was added, and ^{14}C counts were obtained in a Packard Liquid Tri-Carb LS counter (Packard Biosciences Pangbourne, UK). One unit of transglutaminase activity is defined as 1 nmol of putrescine incorporated per hour per mg of protein.

Figure 3.5. Putrescine radiolabelled TG assay

3.2.6.2. TG-catalysed incorporation of Biotin Cadaverine

Transglutaminase activity associated with the cell surface was measured by the incorporation of 5-biotinamidopentylamine (biotin-cadaverine, BTC) into fibronectin (FN) as described previously by Jones *et al.* (1997). Modifications to this assay were developed and used in this investigation as represented in figure 3.6, where

i) *in situ* TG activity was measured on the cell surface of live cells after 2 hour incubation in the enzyme reaction containing biotin cadaverine and FN-coated sample wells;
ii) total TG activity was measured by the addition of cell homogenates corrected for equal amounts of protein in a 1 hour enzyme reaction containing biotin cadaverine and FN-coated sample wells; and
iii) cells that were allowed to grow and form monolayers were tested for TG activity on the cell surface through the incorporation of biotin cadaverine into endogenous cell substrates by leaving out the initial FN-coating step.

3.2.6.2.1. In situ Cell Surface TG activity

The *in situ* cell surface assay was carried out in 96-well plates which were coated with 5 μg/ml FN in 100 μl of 50 mM Tris-HCl (pH 7.4) (as described in section 3.2.4.1.) and blocked with 3% (w/v) BSA in 50 mM Tris-HCl (pH 7.4) for 30 min at 37°C. Cells were trypsinised, counted and pelleted by centrifugation, before re-suspension at a concentration of 2 x 10^5 cells/ml in serum-free RPMI containing 0.132 mM biotin-cadaverine (Molecular Probes, Oregon, USA). 100 μl of the cell suspension was added to the FN-coated 96-well plates and incubated for 2 hours at 37°C, 5% (v/v) CO_2. Positive and negative control samples were also included, which consisted of a 0-20 ng/well *gpl*TG2 standard curve that was incubated in a reaction mixture consisting of 100 mM Tris buffer (pH 7.4), 10 mM DTT, 0.132 mM biotin-cadaverine and either 10 mM $CaCl_2$ (positive control) or 10 mM EDTA (negative control). After incubation, live cells were then gently washed twice with PBS (pH 7.4) containing 3 mM EDTA to terminate the reaction. Cells were then lifted in 100 μl of 0.1% (w/v) deoxycholate, 2 mM EDTA, in PBS (pH 7.4) for 10 minutes at room

Figure 3.6. Three biotin cadaverine assays

temperature, with gentle shaking. The remaining FN layer was washed three times with Tris-HCl (pH 7.4), and the wells were then blocked with 3% (w/v) BSA in Tris-HCl (pH 7.4) for 30 minutes at 37^{o}C. Biotin-cadaverine incorporation into FN was detected by incubation for 2 hours at 37^{o}C with an extravidin peroxidase conjugate diluted 1:5000 in the same blocking buffer. Colour development was timed for each well (between 1 min to 5 min), in a phosphate-citrate buffer with urea-H_2O_2 (one phosphate-citrate tablet with urea-H_2O_2 in 10ml H_2O) containing 7.5% (v/v) 3,3',5,5'-tetramethylbenzidine (TMB; Sigma). The reaction was stopped by the addition of 2.5 M H_2SO_4 and analysed colorimetrically in a SpectraFluor ELISA plate reader at 450 nm.

3.2.6.2.2. Total TG activity in Cell Homogenates

The total activity present in islets or cell homogenates was carried out in 96-well plates by firstly coating the wells with 5 μg/ml FN in 100 μl of 50 mM Tris-HCl (pH 7.4), then blocking wells with 3% (w/v) BSA in 50 mM Tris-HCl (pH 7.4) for 1 hour at room temperature. The islets or cell homogenates were lysed and sonicated as described above (section 3.2.5.1), before total protein estimations were carried out for each sample which was diluted to a final protein concentration of 20 μg/ml solution ready for immediate addition to the reaction mix. Samples were freshly prepared at the time of the assay and stored on ice until the enzyme reaction was allowed to start at 37°C. 100 μl of cell homogenate was incubated for 2 hours at 37°C (5% (v/v) CO_2) in a reaction mixture consisting of 100 mM Tris buffer (pH 7.4), 10 mM DTT, 0.132 mM biotin-cadaverine and either 10 mM $CaCl_2$ (positive control) or 10 mM EDTA (negative control) together with a *gpl*TG2 standard curve as described in section 3.2.6.2.1. The wells were then washed twice with PBS (pH 7.4) containing 3 mM EDTA to terminate the reaction, followed by a wash with Tris-HCl (pH 7.4), and the wells were then blocked with 3% (w/v) BSA in Tris-HCl for 30 minutes at 37^{o}C. Biotin-cadaverine incorporation into FN was detected using extravidin peroxidase as described in section 3.2.6.2.1., and analysed colorimetrically in a SpectraFluor ELISA plate reader at 450 nm.

3.2.6.2.3. In situ Extracellular TG activity in Cell Monolayers

Cell monolayers were seeded at a density of 2 x 10^5 cells/well and allowed to settle and grow on the tissue culture plastic of 96-well plates between 24 to 48 hours. When ready for the detection of TG activity, serum-free RPMI containing 0.132 mM biotin-cadaverine, 10 mM DTT, and either 10 mM $CaCl_2$ (positive control) or 10 mM EDTA (negative control), was added to the live cells for 2 hours at 37°C (5% (v/v) CO_2). A *gpl*TG2 standard curve was also tested as described in section 3.2.6.2.1., and all wells were then washed twice with PBS (pH 7.4) containing 3 mM EDTA to terminate the reaction. Cells were then lifted in 100 µl of 0.1% (w/v) deoxycholate, 2 mM EDTA, in PBS for 10 minutes at room temperature, with gentle shaking. The remaining extracellular protein layer was washed three times with Tris-HCl (pH 7.4), and the wells were then blocked with 3% (w/v) BSA in Tris-HCl for 30 minutes at 37^{o}C. Biotin-cadaverine incorporation into in the endogenous extracellular cell substrates was detected using extravidin peroxidase as described in section 3.2.6.2.1., and analysed colorimetrically in a SpectraFluor ELISA plate reader at 450 nm.

3.2.6.3. In Situ TG-catalysed incorporation of FITC-cadaverine

Determination of *in situ* TG activity was carried out through the incorporation of fluorescein cadaverine (FITC-cadaverine, Molecular Probes) into live cells or freshly isolated islets of Langerhans. Cells or islets were seeded in 8-well chamber slides at a density of 2 x 10^5 cells/well or 50 islets per well in complete medium, then allowed to settle for 24 hours. During treatments, 0.5 mM FITC-cadaverine was added per well for either 4 or 18 hours at 37°C in 5% (v/v) CO_2. In some experiments, active-site directed inhibitors or EDTA was used to confirm Ca^{2+}-dependent TG-mediated catalytic incorporation of the FITC-cadaverine. Following incubation, cells and islets were gently washed three times in PBS (pH 7.4), followed by fixation and permeabilisation in ice cold (-20°C) methanol for 20 minutes, and two further washes using PBS (pH 7.4). Coverslips were placed on the slides using Vectashield mounting medium containing propidium iodide (Vecta Laboratories), and the slides were viewed for immunofluorescence using a Leica TCSNT confocal laser microscope

system (LEICA, Germany) equipped with an argon krypton laser at 488 nm for fluorescein excitation. Semiquantitation of TG activity was obtained by using the mean fluorescent emissions per field ($mV/\mu M^2$) from at least 9 non-overlapping 40 x magnification fields.

3.2.6.4. Immunostaining of Western blots with anti-FITC monoclonal antibody

For the detection of TG-mediated incorporation of fluorescein cadaverine into cell surface particulate and cytoplasmic proteins, live cells were treated with 0.5 mM FITC-cadaverine for 18 hours then homogenised, sonicated and electrophoresed using SDS-PAGE as described in section 3.2.5.3. Following western blot transfer (as described in section 3.2.5.4.), nitrocellulose membranes were blocked and immunoprobed with a mouse anti-FITC antibody diluted 1:200 in blocking buffer for 2 hours at room temperature with gentle shaking. Blots were washed, and then incubated with an anti-mouse-IgG-HRP conjugate diluted 1:1000 in blocking buffer for a further 2 hours. FITC-labelled protein substrates were then revealed by enhanced chemiluminescence as described in section 3.2.5.5.

3.2.6.5. In vitro peptide cross-linking TG assay

Measurement of the *in vitro* TG-mediated cross-linking of glutamine-rich peptides was performed using a colorimetric assay recently described by Trigwell *et al*., (2004). Biotinylated TVQQEL peptide, and the negative control peptide TVNNEL was a kind gift from Dr. Phil Bonner and Dr. Susan Trigwell (Nottingham Trent University). Briefly, 250 µl of casein [1 mg/ml in 50 mM sodium carbonate (pH 9.8)] was added per well in a 96-well plate format (Nunc Maxisorp plates), covered with parafilm and allowed to coat the well overnight at room temperature. The wells were then rinsed three times with dH_2O and 250 µl of blocking solution [0.1% (w/v) BSA in 50 mM sodium carbonate (pH 9.8)] was added per well followed by incubation at 37°C for 1 hour. The wells were washed three times with dH_2O and 150 µl reaction buffer [100 mM Tris-HCl (pH 8.5) containing 13.3 mM DTT, 5 µM biotinylated

TVQQEL peptide and either 5 mM Ca^{2+} or 5 mM EGTA] was added to each well. Enzyme reactions were initiated by the addition of 50 µl diluted cell homogenates or *gpl*TG2 standard to each well, and incubated for 1 hour at 37°C. To stop the reaction, wells were emptied and washed three times with dH_2O as before. Detection of the biotinylated peptide into casein was possible by the addition of 200 µl probing solution [extravidin peroxidase diluted 1:10 000 in 100 mM Tris-HCl (pH 8.5) containing 1% (w/v) BSA] to each well and incubated for 1 hour at 37°C. Wells were washed three times with dH_2O as before, and extravidin peroxidase binding was revealed with the addition of 200 µl developing solution [100 mM sodium acetate (pH 6) containing 75 µg/ml 3,3`,5,5`-tetramethylbenzidine and 3% (v/v) H_2O_2] per well. The blue colour development was stopped in the well by the addition of 50 µl 10M H_2SO_4. A 0-20 ng/well *gpl*TG2 standard curve that was incubated in the reaction buffer was used to quantify the amount of TG activity. Biotinylated peptide cross-links into casein were determined colorimetrically in a SpectraFluor ELISA plate reader at 450 nm.

3.2.7. Turnover of Extracellular Matrix Proteins

3.2.7.1. Detection of Extracellular Matrix Proteins by ELISA

5637 pre-conditioned matrices were prepared, as described in section 3.4.2., with the cells being seeded at a concentration of 1.5×10^4 cells/well in 96-well plates. The rate of extracellular matrix protein (fibronectin, laminin and collagen) turnover was detected at 24 hour, 48 hour and 72 hour time-points. Following incubation, growth medium was removed and the cells were washed three times with PBS (pH 7.4) and then blocked with blocking solution [5% (w/v) fat-free dried milk in PBS, pH 7.4] for 30 minutes. The cells were then fixed with 3.7% (w/v) paraformaldehyde in PBS, pH 7.4 for 15 minutes at room temperature. Plates were then washed three times with PBS (pH 7.4), and the primary antibodies: anti-rabbit fibronectin (Sigma), anti-rabbit laminin 1 (DAKO) and anti-mouse collagen IV (Sigma) were diluted 1: 1000 respectively in 100µl of the same blocking buffer and incubated at 37°C for 2 hours. This was followed by another series of three washes with PBS (pH 7.4), and the secondary antibody (rabbit IgG-HRP or mouse IgG-HRP respectively) diluted 1:2000

in blocking buffer was added to each well (100µl/well) prior to an extra 2 hour incubation at room temperature followed by three washes with PBS (pH 7.4) as before. HRP was detected by addition of 100µl/well of tetramethylbenzidine (TMB, Sigma) according to the manufacturer's instructions and incubation for 10 minutes at room temperature. The reaction was stopped at equal times for each reaction by the addition of 2.5 M H_2SO_4 at 50 µl/well, and then read colorimetrically at an absorbance of 450 nm on a SpectraFluor plate reader.

3.2.7.2. Detection of Extracellular Matrix Proteins by Immunofluorescence

5637 cells were seeded at a concentration of 1 x 10^5 cells/well in 8-well glass chamber slides (Lab-Tek, Naperville, USA) and prepared over 72 hours as described in section 3.4.2. The cells were fixed at 24 hour, 48 hour and 72 hour time-points in 3.7% (w/v) paraformaldehyde in PBS (pH 7.4) while in the chamber wells for 15 minutes at room temperature, followed by permeabilisation using 0.1% (v/v) Triton-X 100 in PBS (pH 7.4). Cell monolayers were then blocked with 3% (w/v) BSA in PBS (pH 7.4) for 30 minutes at 37°C, prior to the addition of either anti-mouse fibronectin (Sigma) diluted 1:200 or anti-rabbit laminin 1 (DAKO) diluted 1:100 in the same blocking buffer. Cells were incubated in the presence of the antibody for 2 hours and washed twice with PBS (pH 7.4). Following this, cells were incubated with either anti-mouse IgG-TRITC (DAKO) or anti-rabbit IgG-TRITC (DAKO) respectively, diluted 1:1000 in blocking buffer for 2 hours at 37°C and then washed 3 times with PBS (pH 7.4). Slides were mounted with a coverslip using Vectashield mounting medium (Vecta Laboratories) or Fluorsave mounting reagent (Calbiochem). Stained cells were viewed for immunofluorescence using a Leica TCSNT confocal laser microscope system (LEICA, Germany) equipped with an argon krypton laser at 647 nm for rhodamine excitation. Semi-quantitation of fibronectin and laminin protein expression was obtained by using the mean fluorescent emissions per field ($mV/\mu m^2$) from at least 9 non-overlapping 40 x magnification fields.

3.2.8. Cell Viability, Proliferation and Apoptosis Assays

3.2.8.1. Trypan blue exclusion test

Cell counts and viability estimations were performed routinely, using the trypan blue exclusion technique by means of a 0.22μm sterile filtered 0.5% (w/v) trypan blue solution and a haemocytometer. An equal volume of the cell suspension and stain were mixed in a sample well by the pulsing of a pipette - this also ensured that clumps of cells were broken up to allow for a more accurate cell count. Non-viable cells stained blue due to the loss of their membrane integrity and, hence, allowed the passage of dye into the cell. Viable cells remained colourless.

3.2.8.2. Colorimetric MTS Cell Viability / Proliferation

Cell proliferation and viability were also measured using the CellTiter AQ One Solution Cell Proliferation™ assay kit (Promega, Southampton, UK) which is a colorimetric method for determining the number of viable cells. The CellTiter AQ reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability which allows it to be combined with MTS to form a stable solution. This MTS tetrazolium compound (Owen's reagent) is bio-reduced by cells into a coloured formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The assay was performed according to the manufacturer's instructions. Briefly, BRIN-BD11 cells were seeded in quadruplicate at a 1 x 10^5 cells/well density in a 96-well microtiter plate and allowed to grow overnight in fully supplemented media at 37°C, 5% (v/v) CO_2. Assays were then performed after time-point treatments, in reduced lighting, by the addition of 20 μl of CellTiter AQ reagent into the relevant samples of 100μl of culture medium in 96-well plates. These samples were then incubated in a humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2 for between 1 to 4 hours, before the absorbance was read at 492nm using a SpectraFluor plate reader.

3.2.8.3. Colorimetric Caspase-3 activity

Programmed cell death was assessed using the commercially available CaspACE™ Assay system (Promega, Southampton, UK) according to the manufacturer's instructions. Briefly, BRIN-BD11 cells were seeded on the relevant matrices in T25 tissue culture flasks at a density of 2 x 10^6 cells/flask and allowed to proliferate overnight. After daily treatments, each sample was corrected to the amount of total protein present. The apoptotic index of the cell line was measured by the ability of caspase-3 to cleave Ac-DEVD-p-nitroaniline substrate and release a chromophore, p-nitroaniline (pNA). Apoptosis in the positive controls was induced using 1 μM of the protein kinase inhibitor staurosporine, whereas the irreversible and cell-permeable pan-caspase inhibitor, Z-VAD-FMK, was added (50 μM final concentration) to the negative control together with 1 μM staurosporine. The amount of pNA liberated/μg of protein/hour was measured at an absorbance of 405 nm using a SpectraFluor plate reader.

3.2.8.4. Immunofluorescent Detection of Caspase-3 activity

Cells were seeded at a density of 2 x 10^4 cells/well in 8-well glass chamber slides (Lab-Tek, Naperville, USA) and grown over 72 hours to confluency with respective treatments (added fresh every 24 hours) and fixed in 3.7% (w/v) paraformaldehyde in PBS (pH 7.4) while in the chamber wells for 15 minutes at room temperature, followed by 10 minutes permeabilisation using 0.1% (v/v) Triton-X 100 in PBS (pH 7.4). Cell monolayers were then blocked with 3% (w/v) BSA in PBS (pH 7.4) for 30 minutes at 37°C, prior to the addition of anti-rabbit cleaved caspase-3 (Asp 175) antibody (Cell Signalling Technology) diluted 1:200 in the same blocking buffer. Cells were incubated in the presence of the antibody for 2 hours and washed twice with PBS (pH 7.4). Following this, cells were incubated with either anti-rabbit IgG-FITC (DAKO) or anti-anti-rabbit IgG-TRITC (DAKO) diluted 1:1000 in blocking buffer for 2 hours at 37°C and then washed 3 times with PBS (pH 7.4), prior to mounting with Vectashield mounting medium containing propidium iodide (Vecta Laboratories). Stained cells were viewed for immunofluorescence using a Leica

TCSNT confocal laser microscope system (LEICA, Germany) equipped with an argon krypton laser at 647 nm and 488 nm for rhodamine and fluorescein excitation. Semiquantitation of caspase-3 expression was obtained by using the mean fluorescent emissions per field ($mV/\mu M^2$) from at least 9 non-overlapping fields.

3.2.9. β-Cell Attachment, Spreading and Aggregation Assays

3.2.9.1. May Grunwald/Giemsa Stain Assay

BRIN-BD11 cells were seeded at a density of 1 x 10^5 cells/well on relevant matrices (as described in section 3.2.4.2), in the appropriate serum-free medium and allowed to spread and/or attach for 1 hour. The cells were gently washed twice with PBS (pH 7.4) and then fixed by the addition of 3.7% (w/v) paraformaldehyde in PBS (pH 7.4) for 15 minutes at room temperature before being washed twice with PBS (pH 7.4). Following fixation, cells were permeabilised by the addition of 0.1% (v/v) Triton X-100 in PBS (pH 7.4) for 15 minutes at room temperature and washed twice with PBS (pH 7.4). Visualisation of the attached or spread cells required a two-step staining procedure which stained both the cytoplasm and nucleus. Cells were stained for their cytoplasm with 0.25% (w/v) May-Grunwald in methanol, for 15 minutes at room temperature. This stain was then removed and the samples washed three times with PBS (pH 7.4). Nuclear staining was achieved using 0.4% (w/v) Giemsa stain, in methanol (diluted 1:50 with distilled water), which was then added to the cells and incubated for 20 minutes at room temperature in a fume-hood. This stain was removed and the plates washed three times with distilled water and left to air dry. The cells were then viewed at x 20 magnification using a Nikon CK2 inverted light microscope. Nine separate, non-overlapping random fields per triplicate sample were photographed with an Olympus DP10 digital camera and quantified using the Leica QWin Image Analysis Software (Leica Lasertechnik, Heidelberg, Germany). Cell attachment was determined by counting the number of cells present per micrograph field, and cell spreading was assessed by the sum all single cell-spread areas in a micrograph field and represented as total cell spread area (um^2). Spread cells were distinguished and characterised based upon the presence of a clear halo of cytoplasm surrounding their nucleus, following the rearrangement in the actin skeleton, as previously described by Jones *et al*., (1997).

3.2.9.2. Cytoskeletal Staining with FITC-phalloidin

Actin stress fibres in BRIN-BD11 cells were visualised using fluorescein isothiocyanate (FITC)-labelled phalloidin (Sigma). Pre-conditioned 5637 and FN matrices were prepared in 8-well glass chamber slides (Lab-Tek, Naperville, USA) as described previously in section 3.2.4. BRIN-BD11 cells (2×10^5 cells/well) were then seeded onto the matrices and allowed to attach and spread in serum-free RPMI (10 mM glucose) for 18 hours at 37°C, 5% (v/v) CO_2, 95% (v/v) air. The chamber wells were then rinsed gently with PBS (pH 7.4) and fixed in 3.7% (w/v) paraformaldehyde in PBS (pH 7.4) for 15 minutes at room temperature, followed by 10 minutes permeabilisation using 0.1% (v/v) Triton-X 100 in PBS (pH 7.4). The β-cells were then blocked with 3% (w/v) BSA in PBS (pH 7.4) for 30 minutes at 37°C, prior to the addition of 20 µg/ml FITC-phalloidin in the same blocking buffer for 20 minutes at room temperature. Labelled cells were then washed three times with PBS (pH 7.4) and the slides were mounted with a coverslip using Vectashield mounting medium (Vecta Laboratories). Stained cells were viewed for immunofluorescence using a Leica TCSNT confocal laser microscope system (LEICA, Germany) equipped with an argon krypton laser at 488 nm fluorescein excitation.

3.2.9.3. Three Dimensional (3-D) β-Cell Aggregation Assay

Pre-conditioned 5637 matrices grown over 3 days were prepared in 8-well glass chamber slides (Lab-Tek, Naperville, USA) as described previously in section 3.2.4.2., except that before lysis, the cells were labelled with CM-Dil red fluorescent Cell Tracker (Molecular Probes). CM-Dil is a long-chain lipophilic carbocyanine suitable for long-term cellular labelling of intracellular membranes, liposomes and lipoproteins, while being retained in cells through permeabilisation and fixation steps. The CM-Dil reagent was re-suspended in DMSO, according to the manufacturer's instructions and was found to label 5637 cells optimally at a final concentration of 1 µM in RPMI at 37°C for 5 minutes. After labelling, the pre-conditioned 5637 matrices were washed three times with PBS (pH 7.4), and then lysed as described in section 3.2.4.2.

Freshly isolated rat islets (50 islets per well) or BRIN-BD11 cells (2 x 10^5 cells/well) were seeded on respective CM-Dil-labelled lysed 5637 matrices, and allowed to attach and aggregate over 24 hours in complete medium containing 5 mM glucose or 20 mM glucose at 37°C, 5% (v/v) CO_2, 95% (v/v) air. BRIN-BD11 β-cells were then treated with 1 µM CM-Dil reagent in RPMI at 37°C for 5 minutes. The chamber wells were then rinsed gently with PBS (pH 7.4) and fixed in 3.7% (w/v) paraformaldehyde in PBS (pH 7.4) for 15 minutes at room temperature, followed by 10 minutes permeabilisation using 0.1% (v/v) Triton-X 100 in PBS (pH 7.4). Rat islets and β-cells were then blocked with 3% (w/v) BSA in PBS (pH 7.4) for 30 minutes at 37°C, prior to the addition of guinea pig anti-swine insulin antibody (DAKO) diluted 1:200 in the same blocking buffer. Cells were incubated in the presence of the insulin antibody for 2 hours at 37°C and washed twice with PBS (pH 7.4). Following this, cells were incubated with anti-rabbit IgG-FITC (DAKO) diluted 1:1000 in blocking buffer for 2 hours at 37°C and then gently washed 3 times with PBS (pH 7.4), prior to coverslip mounting with Vectashield mounting medium (Vecta Laboratories). Stained cells were viewed for immunofluorescence using a Leica TCSNT confocal laser microscope system (LEICA, Germany) equipped with an argon krypton laser at 647 nm and 488 nm for rhodamine and fluorescein excitation respectively. 3-D images were captured using the x, y, z settings in an area of 62500 μM^2 on the Leica QWin Image Analysis software. Semiquantitation of TG2 protein expression was obtained by using the mean fluorescent emissions per field ($mV/\mu M^2$) from at least 9 non-overlapping fields.

3.2.9.4. Cell Adhesion Assay assessing Focal Adhesion Kinase (FAK)

The BRIN-BD11 cells were tested for FAK-mediated cell adhesion using western blotting. Pre-conditioned 5637 and FN matrices were prepared in 6-well plates as described in section 3.2.4. The BRIN-BD11 cells were trypsinised and seeded onto these wells at a density of 1 x 10^6 cells/ml in serum free RPMI for 3 hours adhesion. The cells were then removed from the matrix-containing wells using a cell scraper, and lysed for SDS-PAGE analysis as described in section 3.2.5.3. Following western blot transfer (as described in section 3.2.5.4.), nitrocellulose membranes were

blocked and immunoprobed with anti-rabbit Tyr (p)^{397}-FAK antibody diluted 1:200 in blocking buffer for 2 hours at room temperature with gentle shaking. Blots were washed, and then incubated with an anti-rabbit-IgG-HRP conjugate diluted 1:1000 in blocking buffer for a further 2 hours. The amount of phosphorylated FAK was then revealed by enhanced chemiluminescence as described in section 3.2.5.5. Blots were then stripped and re-probed with an anti-rabbit total FAK antibody (1:200), and an anti-mouse β-tubulin antibody (1:200) for 2 hours at room temperature with gentle shaking. Blots were washed, and then incubated with anti-rabbit-IgG-HRP conjugate and anti-mouse-IgG-HRP conjugate respectively, and diluted 1:1000 in blocking buffer for a further 2 hours. The amount of total FAK and β-tubulin standards were then revealed by enhanced chemiluminescence as described in section 3.2.5.5. Densitometric analysis of the immunoreactive signals was quantified using the AIDA LAS 3000 software.

The amount of phosphorylated FAK in BRIN-BD11 cells using a similar adhesion assay was also visualised using immunofluorescence. Pre-conditioned 5637 and FN matrices were prepared in 8-well glass chamber slides (Lab-Tek, Naperville, USA) as described in section 3.2.4. BRIN-BD11 cells were trypsinised and seeded onto these wells at a density of 2 x 10^5 cells/ml in serum free RPMI for 3 hours adhesion. The attached β-cells were the rinsed twice with PBS (pH 7.4) and fixed in 3.7% (w/v) paraformaldehyde in PBS (pH 7.4) while in the chamber wells for 15 minutes at room temperature, followed by 10 minutes permeabilisation using 0.1% (v/v) Triton-X 100 in PBS (pH 7.4). Cell monolayers were then blocked with 3% (w/v) BSA in PBS (pH 7.4) for 30 minutes at 37°C, prior to the addition of anti-rabbit Tyr (p)^{397}-FAK antibody diluted 1:200 in the same blocking buffer. Cells were incubated in the presence of the antibody for 2 hours at 37°C and washed twice with PBS (pH 7.4). Following this, cells were incubated with anti-rabbit IgG-FITC diluted 1:1000 in blocking buffer for 2 hours at 37°C and then washed 3 times with PBS (pH 7.4), prior to mounting with Vectashield mounting medium containing propidium iodide (Vecta Laboratories). Stained cells were viewed for immunofluorescence using a Leica TCSNT confocal laser microscope system (LEICA, Germany) equipped with an argon krypton laser at 647 nm and 488 nm for rhodamine and fluorescein excitation.

3.2.10. Measurement of Insulin

3.2.10.1. Acute Glucose Stimulated Insulin Release in BRIN-BD11 Cells

BRIN-BD11 cells were seeded in a 24-well plate at a density of 2.5 x 10^4 cells/well. They were left to settle for 24 hours and then pre treated for 1 hour with 250 μM TG inhibitors (R281 and R283). The medium was then removed and the cells washed once in 500 μl Gey and Gey buffer (see table 3.4), followed by an incubation for 1 hour 30 min at 37°C in 500 μl Gey and Gey buffer (supplemented with 0.1% BSA (w/v) and 0.25 mM D-glucose) in order to stabilise and lower their insulin secreting mechanisms. After incubation the cells were rinsed once in buffer and acute glucose stimulated insulin secretion was carried out in 300 μl Gey and Gey buffer (supplemented with 0.1% BSA (w/v) and a glucose dose curve containing the inhibitor treatments) for 1 hour 30 min at 37°C. After incubation, 250 μl of the cell supernatant was removed and used in the [^{125}I]-insulin radioimmunoassay.

3.2.10.2. Acute Glucose Stimulated Insulin Release in Rat Islets

Gey and Gey buffer (2 mM Glucose, see table 3.4) was used to isolate rat islets. The assay sample buffer was made up with 0.1% BSA in 100 mL of Gey and Gey Buffer (see Table 3.4). Treatments (Control, 500 μM R281, 500 μM R283) were prepared so that 5 μl (1:100) was added per vial containing 500 μl sample buffer. 3 large rounded islets were hand-picked to each vial (6 replicates per treatment) and stimulated to secrete insulin for 1 hour in a 37°C water bath. Treatments used were 2mM Glucose, 20 mM Glucose and 25 mM KCl. Before incubation the vials were gassed with O_2:CO_2 (95%:5%) above the surface of the buffer and capped to maintain the pH. After incubation, 50 μl of the sample buffer was then added to 250 μl Insulin Assay Buffer. Samples were then run in duplicate in an [^{125}I]- insulin radioimmunoassay.

Figure 3.7. Principle of the measurement of acute glucose-stimulated insulin secretion, using [^{125}I]-Insulin Radioimmunoassay.

Table 3.4. Constituents of Buffers for use in Islet Isolations and Insulin Assays

Gey and Gey Buffer (x10)	
NaCl	65 g
$NaHCO_3$	22.7 g
KCl	3.7 g
Make up 1 litre in distilled water.	
Gey and Gey Buffer (x1)	
100ml 10X Gey and Gey Buffer	
895ml distilled water	
Add 1 aliquot M	
Add 1 aliquot P	
Add appropriate amount of glucose	
Gas with CO_2/O_2	
Whilst gassing add 1ml 1M $CaCl_2$ dropwise	
Gas for a total of 10-15 minutes	
M	
$MgCl_2$ $6H_2O$	10.15 g
$MgSO_4$ $7H_2O$	3.7 g
Make up to 100ml with distilled water.	
1 aliquot = 2ml	
P	
Na_2HPO_4	7.1 g
KH_2PO_4	2.0 g
Make up to 100ml with distilled water.	
1 aliquot = 2ml	
Insulin Assay Buffer	
Na_2HPO_4	2.85 g
KH_2PO_4	0.675 g
NaCl	4.5 g
EDTA	1.86 g
BSA	2.5g (add last)
Make up to 500ml with distilled water.	

3.2.10.3. [^{125}I]-Insulin Radioimmunoassay

The radioimmunoassay (RIA) was carried out using 50 µl from six separate replicate samples. Crystalline human insulin (8 ng/ml) was serially diluted in insulin assay buffer (see table 3.4) to create a standard curve (0, 0.25, 0.5, 1, 2, 4, and 8 ng/ml). To each of the samples and the standard curve 50 µl of guinea pig anti-bovine insulin antibody (ICN, final dilution 1: 20 000 in insulin assay buffer) and 50 µl of [^{125}I]-insulin (Linco, 0.5 µCi in 10 ml insulin assay buffer) was added. The tubes were mixed and left to incubate overnight at 4 °C. The following day, 50 µl of Sac-Cel (anti-guinea pig coated cellulose, diluted 1:1 with Insulin Assay Buffer) was added to the tubes and left to incubate at room temperature for 30 min in order to precipitate the insulin bound to the antibody. After incubation, 1 ml of distilled water was added to each tube and the suspension was centrifuged at 1000 x *g* for 5 min. The water was then aspirated and the amount of radioactivity in the remaining pellets was measured using a WALLAC gamma counter. The standard curve was constructed and used to calculate the original insulin content of each sample.

3.2.10.4. Immunofluorescent Detection of Insulin Content

Cells were seeded at a concentration of 1 x 10^5 cells/well in 8-well glass chamber slides (Lab-Tek, Naperville, USA) and grown over 48 hours to confluency with respective treatments and fixed in 3.7% (w/v) paraformaldehyde in PBS (pH 7.4) while in the chamber wells for 15 minutes at room temperature, followed by permeabilisation using 0.1% (v/v) Triton-X 100 in PBS (pH 7.4). Cell monolayers were then blocked with 3% (w/v) BSA in PBS (pH 7.4) for 30 minutes at 37°C, prior to the addition of guinea pig anti-swine insulin antibody (DAKO) diluted 1:200 in the same blocking buffer. Cells were incubated in the presence of the antibody for 2 hours and washed twice with PBS (pH 7.4). Following this, cells were incubated with either anti-rabbit IgG-FITC (DAKO) or anti-rabbit IgG-TRITC (DAKO) diluted 1:1000 in blocking buffer for 2 hours at 37°C and then washed 3 times with PBS (pH 7.4), prior to coverslip mounting with Vectashield mounting medium (Vecta Laboratories) or Fluorsave mounting reagent (Calbiochem). Stained cells were viewed for immunofluorescence using a Leica TCSNT confocal laser microscope

system (LEICA, Germany) equipped with an argon krypton laser at 647 nm and 488 nm for rhodamine and fluorescein excitation. Semi-quantitation of TG2 protein expression was obtained by using the mean fluorescent emissions per field ($mV/\mu M^2$) from at least 9 non-overlapping 40 x magnification fields.

3.2.10. Molecular Biology Techniques

3.2.11.1. RNA Extraction

Total RNA was extracted using the RNEasy minikit (Qiagen) according to the manufacturer's protocol or Trizol™ (Invitrogen, Paisley, UK). For tissue samples in Trizol™, 100-200 mg of snap frozen tissue in liquid nitrogen was crushed using a pestle and mortar in 2 ml of Trizol™ reagent until completely homogenised. For cell samples, a confluent T75 flask of cells that had previously been washed twice with PBS (pH 7.4) was immersed in 2 ml of Trizol™ reagent and the cells lysed by passing through a small gauge needle. Typically, 2 T75 flasks were pooled to gain enough RNA for analysis. RNA was phase separated by the addition of 400 µl of chloroform (200 µl/ml of Trizol™), the tubes capped and shaken vigorously by hand for 15 secs. Samples were incubated at room temperature for 2-3 mins and centrifuged at 12,000 *x g* for 15 mins at 4°C. The upper aqueous layer containing RNA was removed and transferred to a fresh tube. To this was added 1ml of isopropanol (0.5 ml/ml of Trizol™) and samples incubated at 4°C for at least 30 mins or stored overnight at –20°C. Samples were then centrifuged at 12, 000 *x g* for 10 mins at 4°C. The supernatant was discarded and the RNA pellet washed with 2 ml of 70% ethanol with vortexing to loosen the pellet. Samples were centrifuged at 7,500 *x g* for 5 mins at 4°C and the wash procedure repeated. The supernatant was removed and the pellet air dried. RNA pellets were re-suspended in a small volume of DEPC (diethylpyrocarbonate) treated water and incubated for 10 mins at 65°C to ensure that the pellet was completely dissolved. Concentration and purity of the RNA was assessed by spectrophotometry at 260 nm and 280 nm. To prevent ribonuclease contamination of RNA samples, disposable sterile plastic consumables were used whenever possible, working surfaces were wiped in 0.1% (w/v) KOH, and distilled

water used was treated with 0.1% (w/v) diethyl pyrocarbonate (DEPC) for 2 hours and autoclaved prior to use.

3.2.11.2. Determination of RNA concentration and purity

Samples were diluted 1/100 in distilled water, loaded on quartz micro-cuvettes, and readings were recorded at 260 nm and 280 nm. RNA purity was assessed by the ratio of A_{260nm} /A_{280nm}. Taking 1 A_{260} unit that corresponds to 50 μg/ml, the concentration of RNA was determined using the following formula: RNA (μg/ml) = A_{260nm} x 50 x dilution factor. RNA solutions with a A_{260nm} /A_{280nm} ratio ≥1.7 were considered free of phenol, DNA and proteins, and hence suitable for further experimentation.

3.2.11.3. Denaturing agarose gel electrophoresis of total RNA

RNA (20 μg) was electrophoresed on a 1.2% (w/v) ultra pure agarose/1x morpho-linepropane-sulfonic acid (MOPS)/ 2% (v/v) formaldehyde gel. RNA samples were prepared by combining 20 μg of RNA with 25 μl of RNA sample buffer [containing 80% w/v deionised formamide, 8% (v/v) formaldehyde, 50 μl 10x MOPS buffer, 38μg/ml ethidium bromide, 3.5% (v/v) Ficoll, 10 mM EDTA, 0.05% (w/v) bromophenol blue] and heated at 65°C for 15 min. Prepared RNA was pipetted into the sample wells and electrophoresis was performed at 90 V until the bromophenol blue front had migrated approximately ¾ of the length of the gel. The gel was viewed under ultraviolet (UV) light to verify loading and RNA integrity by the presence of intact 28S and 18S ribosomal bands at 4.5 kb and 1.9 kb respectively with an intensity ratio of 2:1.

3.2.11.4. Northern Blotting

Resolved RNA was then transferred to Hybond N nylon membranes (Amersham, Buckinghamshire, UK) by capillary blotting. The Northern transfer apparatus

consisted of a glass plate placed over a plastic tray which served as a reservoir of 20% SCC (3 M NaCl, 0.3 M sodium citrate) pH 7.0. A 3MM paper wick (Whatman, Kent, UK) was constructed and placed on the glass plate so that the overhanging edges were completely immersed in the reservoir below. The RNA gel was inverted and placed onto the wick, and the entire perimeter of the gel was covered in parafilm to avoid short-circuiting. The nylon membrane was applied to the upper surface of the gel ensuring that no air bubbles were trapped between the surfaces, and several sheets of 3 MM paper and approximately 10 cm of paper towels were placed over the membrane. The assembly was completed by the addition of 1kg of weight on top of the paper towels, and transfer was allowed to proceed overnight. Once transfer was complete, the apparatus was disassembled, the membrane was marked to identify the position of the sample wells and the ribosomal bands, and the RNA was fixed to the filter by UV irradiation (70 mJ/cm^2) using a UV cross-linker (Amersham, Little Chalfont, UK).

3.2.11.4.1. Random priming of cDNA probes

Specific random primed DNA probes were labelled from the mouse tissue transglutaminase DNA sequence described by Johnson *et al.*, (1997), refer to Chapter 5. Purified cDNA (12.5 ng) was random-primed with ^{32}P-labeled dCTP (Redivue, Amersham) using the Prime-a-Gene system (Promega) according to the manufacture's protocol. Unincorporated nucleotides were removed using Sephadex G50 Nick Columns™ (Amersham Pharmacia, Buckinghamshire, UK) according to the manufacturer's protocol, and the random primed cDNA probe was denatured by boiling for 3 min and kept on ice to prevent re-annealing prior to addition to the hybridisation solution.

3.2.11.4.2. Northern Hybridisation of cDNA probes and autoradiography

Membranes where pre-hybridised in ExpressHyb hybridisation solution (Clontech) at a probe specific temperature for 1 hour. Hybridisation was performed under the same conditions with the addition of labelled probe to 1x 10^6 cpm/ml for 18 hours.

Depending on the probe, filters were then washed to a stringency of 0.2%/ 0.2 x saline-sodium phosphate-ethylenediaminetetra-acetic acid at temperatures up to 65 °C for 1 hour and then exposed to Kodak XOMAT AR/LS film for up to 7 days in intensifying screens. Loading was corrected by reference to the optical density of ethidium bromide-stained 18S rRNA and the housekeeping gene cyclophillin. Determination of transcript size was carried out by reference to RNA molecular weight markers (Promega, Madison. WI), and was confirmed by visual comparison to the ribosomal RNA subunits. Nylon membranes were routinely stripped in boiling 0.1% (w/v) SDS for up to half an hour.

3.2.11.5. Agarose gel electrophoresis of DNA

Agarose gels were prepared by dissolving 1 g of agarose (Bioline, UK) in 50ml of 1x Tris-Acetate EDTA buffer [TAE: 40mM Tris, 0.114% (v/v) glacial acetic acid and 1mM EDTA]. Agarose was dissolved in TAE by heating the solution twice in a microwave at medium power for 1 min each time. Once cooled, 5µl ethidium bromide was added to the solution to a final concentration of 0.5 mg/ml, and the gels was cast in a Bio-Rad DNA-Sub electrophoresis tray (Bio-Rad, Hampstead, UK) and allowed to set for 50 minutes. DNA samples were supplemented with 10x DNA loading buffer [20% (v/v) ficoll 400, 100 mM EDTA, 1% (w/v) SDS, 0.25% (w/v) bromophenol blue, 0.25% xylene cyanol], mixed and applied into the sample wells. Electrophoresis was performed at 90V for 80 minutes in 1x TAE running buffer. DNA was visualised using a UV transilluminator at 254 nm.

3.2.11.6. RT-PCR Analysis

RNA was isolated and quantified for use in reverse trasncription as described in section 3.2.10.1. For each sample, identical amounts of RNA were reverse-transcribed using a first strand cDNA synthesis kit containing Sensiscript Reverse Transcriptase (Qiagen) as follows: fresh master mix (Qiagen) was prepared on ice containing 2 µl dNTP mix (5 mM), 2 µl 10x buffer RT, 1 µl RNase inhibitor (10

U/μl) and 1 μl sensiscript reverse transcriptase in 11 μl nuclease-free H_2O, to which 1 μl oligo-dT primer (10 μM) (Promega) and 2 μl template RNA (50 ng) was added last. The 20 μl samples were then incubated for 60 minutes at 37 °C for the reverse transcription reaction to proceed. 10 μl of this reaction was then added to successive polymerase chain reaction (PCR) analysis in a total volume of 50 μl.

The resultant cDNA after first strand synthesis was then amplified in a sterile microfuge tube using Go *Taq* DNA polymerase (Promega), which was added last to the master mix (Promega) for each primer pair (see Table 3.5).

Table 3.5. Constituents of the Reverse Transcription PCR reaction

PCR Reaction Mix	**Volume per 50 μl PCR reaction**
10x reaction buffer with 15 mM $MgCl_2$	5 μl
PCR Nucleotide Mix (10mM each dNTP)	1 μl
Upstream primer	0.5 μl
Downstream primer	0.5 μl
***Taq* DNA polymerase**	0.25 μl
Nuclease-free H_2O	32.75 μl
Template cDNA (50 ng)	10 μl

A screening of the expression of TG1, TG2 and TG3 was carried out in rat β-cells using RT-PCR primers that were designed to bind to different exons, thereby eliminating the possibility of chromosomal DNA artefacts as follows:
5`TG1** (CTGTCACCAACTTCAACTC), **3`TG1 (TCATCCAGCAGTCGTTCC),
5`TG2** (CACACCGCAGGAGAAGAG), **3`TG2 (CACGAAGGACGCATCATAC),
5`TG3** (TACAACGGATGGCAGGTG), **3`TG3 (GCATTAACGGAAGCAGGAC).

Positive controls of TG2 cDNA was a kind gift from Peter Davies (Houston, USA), while TG1 and TG3 cDNA positive controls were a kind gift from Kiyotaka Hitomi (Nagoya, Japan).

The shortened alternatively spliced variants of rat TG2 were assessed in BRIN-BD11 cells by designing primers (Sigma, Genosys) according to experiments described by Monsonego *et al*., (1997) and Tolentino *et al*., (2004), where all base pair designations referred to Genebank locus AF106325, rat TG2.

5`TG (ACTTTGACGTGTTTGCCCAC, bp 1470-1489) recognises an upstream homologous sequence in full length TG-2 (TG-L) and truncated TG2 (TG-S) transcripts.

3`TG-L (CAATATCAGTCGGGAACAGGTC, bp 1961-1982) recognises a downstream TG-L mRNA-specific sequence.

3`TG-S (GCTGAGTCTGGGTGAAGGACACAG, bp 1861-1872 and 2083-2093) recognises a downstream TG-S mRNA-specific sequence.

3`TG-S primer bridges the junction created by the absence of bp 1873-2082 (a sequence present exclusively in TG-L). The 3`TG-S sequence will hybridise to both the TG-L and TG-S-specific sequences, while the full length primer will hybridise only to the TG-S mRNA sequence. The predicted fragment of 5`TG-3`TG-L was 512bp for TG-L only, and of 5`TG-3`TGS it was 410bp for TG-S only.

All base pair designations for GAPDH-specific primers refer to Genebank locus AF106860. The upstream primer is designated:

5`GPD (GGCTGCCTTCTCTTGTGAC, bp 903-921),

and the downstream primer is designated:

3'GPD (GGCCGCCTGCTTCACCAC, bp 1624-1641).

The PCR reaction for rat TG1, TG2 and TG3 screening was performed with conditions of one denaturation cycle of 95 °C (2 min), 35 annealing cycles each of 95 °C (30 sec), 60 °C (30 s), 72 °C (30 sec), and finally one extension cycle of 72 °C (7 min) using an ABI Prism 7700 Sequence Detection System. The PCR reaction for

TG-L and TG-S recognition was carried out using conditions of one denaturation cycle of 95 °C (3 min), 30 annealing cycles each of 95 °C (1 min), 60 °C (1 min), 72 °C (1.5 min), and finally one extension cycle of 72 °C (7 min).

PCR products and a 1 kb DNA ladder (Promega) were then resolved by horizontal agarose gel electrophoresis as described in section 3.2.10.5., on a 2% agarose gel prepared in 1 x TAE + 0.5 μg/ml ethidium bromide. Samples were diluted in 6 x DNA loading buffer prior to application in sample wells. Electrophoresed DNA was visualised and images were captured digitally, using a UV transilluminator. In some instances, the DNA was isolated and purified using the QIAquick DNA gel extraction kit (Qiagen) or Wizard SV Gel and PCR Clean-up system (Promega) according to the manufacturer's protocol. Sequences were confirmed by dideoxy terminator sequencing (Cogenics, Saffron Walden, UK).

3.2.12. Statistical Analysis

For simple paired comparisons, the Mann-Whitney or Student's *t*-test, using the *Minitab* or *Sigma Stats* software packages, were utilised and expressed as mean ±SD. Whenever the statistical significance between control and treated samples were evaluated at a 95% confidence level ($p < 0.05$), the data set would be considered to be statistically significant and represented with * on the bars. Where $p < 0.001$, statistical significance was denoted with ** on the bars.

Chapter Four:

Characterisation of Transglutaminase Activity in Pancreatic β-Cells

Peter Atkins from *Creation Revisited*

"All change, I shall argue, arises from an underlying collapse into chaos. The deep structure of change is decay. What decays is not the quantity but the quality of the energy. High-quality, useful energy, is localised energy. Low quality, wasted energy, is chaotically diffuse energy. Things can get done when energy is localised; but energy loses its potency to motivate change when it has become dispersed…The tendency of energy to chaos is transformed into love or war through the agency of chemical reactions. All actions are chains of reactions. From thinking to doing, in simply thinking, or in responding, the mechanism in action is chemical reaction. When it is precipitate it destroys. When it is geared through chains of events it can produce civilisations.

At its most rudimentary, a chemical reaction is a rearrangement of atoms. Atoms in one arrangement constitute one species of molecule, and atoms in another, perhaps with additions or deletions, constitute another. In some reactions a molecule merely changes its shape; in some, a molecule adopts the atoms provided by another, incorporates them, and attains a more complex structure. In others, a complex molecule is itself eaten, either wholly or in part, and becomes the source of atoms for another. Molecules have no inclination to react, and none to remain unreacted. Why, then, do reactions occur? A reaction tends to occur if in the process energy is degraded into a more dispersed, more chaotic form.

The frailty of molecules, though raises questions. Why has the universe not already collapsed into unreactive slime? If molecules were free to react each time they touched a neighbour, the potential of the world for change would have been realised a long time ago. Events would have taken place so haphazardly and rapidly that the rich attributes of the world, like life and its own awareness, would not have time to grow. The emergence of consciousness, like the unfolding of a leaf, relies upon restraint. Richness, the richness of the perceived world and the richness of the imagined worlds of literature and art - the human spirit - is the consequence of controlled, not precipitate, collapse."

Atkins, Peter (1940-), Chemist and writer. *Creation Revisited*, Penguin, 1994.
Quoted from *The Oxford Book of Modern Science Writing* by Richard Dawkins, Oxford University Press, 2008.

Chapter Four:

Characterisation of Transglutaminase Activity in Pancreatic β-cells

4.1. Introduction

Insulin is the body's main blood lowering-glucose hormone, and is secreted by the pancreatic β-cell in a regulated multistage progression that involves the trafficking of insulin granules within vesicles to the plasma membrane (Lang, 1999). While an understanding of this mechanism is fundamental to current therapeutic intervention for diabetes, many protein components in this process have yet to be fully characterised.

It is well established that increases in β-cell cytosolic Ca^{2+} concentration is a key mediator in glucose-stimulated insulin secretion coupling (Rorsman and Renstrom, 2003) and that Ca^{2+} regulates TG cross-linking activity (Casadio *et al*., 1999). Some studies have demonstrated a link between TG cross-linking activity and the regulation of glucose-stimulated insulin release from pancreatic β-cells (Porzio *et al*., 2007; Bernassola *et al*., 2002; Driscoll *et al*., 1997; Bungay *et al*., 1986; Sener *et al*., 1985; Gomis *et al*., 1984), where it was thought that TG may play a critical role in the mechanical events involved in the translocation and membrane fusion of secretory granules to the β-cell exocytotic site (Gomis *et al*., 1983). However, the factors responsible for precise regulation, inhibition or over-expression of TG cross-linking, specific to the β-cell, warrants further investigation.

The enzymatic role of TG in cells is usually assessed by standardised and comparable biochemical assays involving a reaction mechanism carried out under

repeatable *in vitro* conditions where the exogenous addition of TG-compatible amine acceptors, incorporated into specific glutamine-donor substrates, are used as a measure of TG enzymatic activity (refer to section 1.1.2). Rat islets of Langerhans have been the primary β-cell model used to optimise biochemical TG cross-linking assays, where enzyme reactions were initially shown to require dithiothreitol (a thiol reducing agent) and Ca^{2+} (Bungay *et al*., 1984). Although freshly isolated islets have the advantage of a close resemblance to the *in vivo* scenario, the use of these models to study the biochemical aspects of insulin secretion is limited in efficiency by a number of factors; including availability of pancreatic endocrine tissue, difficulty in preparing large numbers of viable islets, maintaining cellular heterogeneity, and the rapid decline of insulin production in tissue culture (Hohmeier *et al*., 1997). Despite these drawbacks, the use of rat islets in previous TG research (refer to section 1.4.7.) would have been partly due to the inaccessibility of stable and immortalised insulin secreting clonal β-cells at the time.

Many researchers have attempted to establish cell lines that retain normal regulation of insulin secretion, but few attempts have been successful due to the difficulty of finding a compromise between the highly differentiated state of normal insulin-secreting β-cells, and the ability to proliferate in tissue culture (McClenaghan *et al*., 1999; Efrat, 1999; Newgard *et al*., 1997). Currently, the most widely used insulin-secreting cell lines are RINm5F (Gazdar *et al*., 1980), HIT-T15 (Santerre *et al*., 1981), INS-1 (Asfari *et al*., 1992), β-TC (Efrat *et al*., 1988), and more recently MIN-6 (Miyazaki *et al*., 1990) and BRIN-BD11 (McClenaghan *et al*., 1996).

Studies of TG2 expression in numerous non-pancreatic cell-types have demonstrated that TG2 is subject to regulation at the post-translational level by Ca^{2+} (Casadio *et al*., 1999; Smethurst and Griffin, 1996) or GTP-binding (Liu *et al*., 2002); and at the transcriptional level by growth factors such as TGFβ-1, Il-6, TNF-α (Ikura *et al*., 1994; George *et al*., 1990), glucocorticoids (Johnson *et al*., 1998) and retinoids (Chiocca *et al*., 1988). To date, only a small number of studies involving TG and clonal β-cell models have been published. Studies with HIT-T15 cells revealed that TG may be involved in β-cell apoptosis as a result of GTP-depletion (Huo *et al*., 2003). Another study demonstrated that TG may be involved in the mediation of

retinoid-induced changes in RINm5F and INS-1 clonal β-cell insulin secretion function (Driscoll *et al*., 1997).

To address the lack of a fully characterised clonal β-cell line which could potentially be used in future studies, the model of choice for this investigation was the BRIN-BD11 rat pancreatic insulinoma β-cell line. This was mainly due to the high proliferative capacity of BRIN-BD11 cells, an established record of having the highest insulin content compared to other rat β-cell lines, and the best step-wise insulin secretory response to increasing glucose concentrations, closely mimicking that of rat islets (Hamid *et al*., 2002). The BRIN-BD11 cells possess GLUT2 and glucokinase expression which are key elements of the β-cell glucose sensing mechanism, in addition to expressing the two component K_{ATP} channel complex (KIR 6.2 and SURI), furnishing these cells with the ability to couple metabolic and electrical events of the stimulus secretion coupling pathway, as in the case of normal pancreatic β-cells (McClenaghan *et al*., 1999).

The use of competitive substrate inhibition by alkylamines, monodansylcadaverine, *N*-*p*-tosylglycine, bacitracin, glycine alkylesters and hypoglycaemic sulphonylureas, has been the most effective way of estimating the formation of isopeptide ε-(γ-glutamyl)-lysine TG cross-links in rat islets biochemically (refer to section 1.4.7). However, these results have failed to provide conclusive evidence (refer to Table 1.4), due to the non-specific effects of these competitive amines on other biological processes or enzymes (Hutton *et al*., 1982; Gomis *et al*., 1984; Lebrun *et al*., 1984), poor penetration into the islet cell (Gomis *et al*., 1983), and the unforeseen effect some inhibitors had on cationic fluxes causing increased Ca^{2+} entry into the β-cells (Malaisse *et al*., 1983). A revised *in vitro* approach, addressing the specificity of this TG inhibition within a monolayer of homogenously penetrable clonal β-cells would therefore be advantageous in providing confirmatory evidence for the role of TG2 in insulin-secreting pancreatic β-cells.

To account for this, irreversible TG active-site directed inhibitors, developed in-house (Griffin *et al*., 2004): R283 (1, dimethyl-2[oxopropyl) thio] imidazolium) and R281 (a synthetic CBZ-glutaminyl glycine analogue; N-benzyloxycarbonyl-L-

phenylalanyl-6-dimethylsulphonium-5-oxo-Lnorleucine), were used in this investigation. The commercially available BOC-DON-Gln-Ile-Val-OMe TG2-specific peptide (N-Zyme, Germany), a large molecule similar in size to R281, was also tested for active-site inhibition of the β-cell TG protein. These compounds represent a novel set of irreversible TG inhibitors that act through alkylation of the active-site Cys residue (refer to section 1.1.3), and have not been previously tested on pancreatic β-cells. However, these site-specific inhibitors still have the potential to act on a broad range of the TG family. In order to assess inhibition of TG2-specific effects, subsequent studies will focus on the *ex vivo* state of TG2 inhibition in islets that have been isolated from TG2 knockout ($TG2^{-/-}$) mice (De Laurenzi and Melino, 2001). In these animals, the targeted deletion of 1,200 base pairs of the TG2 gene from exon 5 to intron 6 by homologous recombination results in the abolition of the active site and associated protein cross-linking activity (De Laurenzi and Melino, 2001), which is reported to have created a Type 2 diabetes phenotype in mice with associated decline in glucose homeostasis (Porzio *et al*., 2007; Bernassola *et al*., 2002).

BRIN-BD11 cells have been previously studied in the context of some diabetic disease states, such as: hyperglycaemia (Davies *et al*., 2001; Wilson *et al*., 1997), nitric oxide-induced β-cell death (Welters *et al*., 2004a; Gao *et al*., 2003; Stickings and Cunningham, 2002), and apoptosis induced by the lipotoxic effector palmitate (Dhayal *et al*., 2008; Diakogiannaki *et al*., 2008; Morgan *et al*., 2007; Welters *et al*., 2006; 2004a and 2004b; Sener and Malaisse, 2002). In addition to transcriptional regulation, TG2 can also be modulated post-transcriptionally during apoptosis (Melino *et al*., 1997), where TG2 activation leads to the assembly of intracellular cross-linked protein polymers, which stabilise the integrity of the apoptotic cell, preventing the release of harmful intracellular components into the extracellular space. Characterising the TG activity of BRIN-BD11 cells within these simulated diabetic stress states, will enable the assessment of a possible cell survival role conferred by TG2.

In the present chapter, a clonal rat pancreatic insulinoma β-cell line (BRIN-BD11) will be characterised for the effect of TG activity upon glucose-stimulated insulin

secretion. Regulation of TG activity by Ca^{2+}, GTP, retinoic acid, membrane proteases and glucose will be assessed in these β-cells, using TG activity assays chosen for optimal substrate-specificity of the β-cell-expressed TG. Following regulation of the β-cell TG enzyme, inhibition studies will be carried out using competitive amine TG inhibitors, novel irreversible active-site directed TG inhibitors, and a $TG2^{-/-}$ mouse islet model shown to exhibit a type 2 diabetes phenotype (De Laurenzi and Melino, 2001). Cytotoxicity studies of BRIN-BD11 cells in response to the diabetic stress conditions of hyperglycaemia, oxidative stress, and lipotoxicity will be initiated using TG enzyme inhibitor treatments. The chapter will be concluded with quantitative [^{125}I]-insulin radioimmunoassay analysis of insulin secretion function, as a result of targeted inhibition of TG cross-linking, comparing BRIN-BD11 cells to rat islets.

4.2. Results

4.2.1. Post-translational and Transcriptional Regulation of TG Activity

4.2.1.1. Regulation of β-cell TG with Ca^{2+} and GTP

The presence of TG activity in BRIN-BD11 cells was confirmed in this investigation through the optimisation of various substrate-specific assays. However the TG catalysed incorporation of biotin cadaverine into fibronectin (FN) proved to be the most sensitive. As a starting point it was necessary to confirm whether the enzyme present in homogenates from BRIN-BD11 cells demonstrated any classical TG2 characteristics. This was firstly addressed by means of a dose-dependent increase in enzyme activity, on account of the incorporation of biotin cadaverine into FN, that was both thiol (10 mM DTT) and Ca^{2+}-dependent (**fig. 4.1A**), establishing an optimum concentration of 5 mM Ca^{2+} which was utilised in all subsequent assays. The addition of 10 mM EDTA to the reaction mixture produced complete inhibition of TG activity (data not shown), and was consistently subtracted as a blank background measurement for each data point in subsequent experiments. The usefulness of this assay in screening for inhibitors was assessed at the outset with the addition of GTP, which acts as a reversible non-competitive inhibitor for TG2,

where inhibition would be expected to partially reverse due to saturating Ca^{2+} concentrations. The TG present in these β-cells at concentrations of 5 mM Ca^{2+} and 10 mM DTT produced a concentration-dependent decrease in activity as a result of the addition of GTP (10 μM – 1000 μM), suggesting potential GTP-binding capabilities for this enzyme (**fig 4.1B**).

4.2.1.2. Regulation of β-cell TG2 with Retinoic Acid

To determine if a well-recognised transcriptional regulator of TG2 was able to induce β-cell TG activity, BRIN-BD11 cells were treated for 48 hours with either 300 nM or 3 μM of all *trans* retinoic acid (**fig. 4.2**). Measurement of TG activity was performed on the cell-surface of BRIN-BD11 monolayers themselves, through the incorporation of biotin cadaverine into endogenous β-cell substrates formed over 48 hours. Treatment with retinoic acid (**fig. 4.2**) was shown to have a potent stimulatory effect on TG activity, with 300 nM showing a 2-fold increase in enzyme activity compared to untreated cells ($p < 0.001$), while the higher concentration of 3 μM resulted in only a modest increase in enzyme activity.

4.2.1.3. Reduction in Cell Surface TG activity by Membrane Proteolysis

Routine subculturing of BRIN-BD11 cells with trypsin revealed that these cells exhibited a high sensitivity to the proteolytic action of this enzyme, with only a minute's exposure resulting in complete detachment of the β-cell monolayers. Both trypsin and dispase have been implicated in the proteolytic release of TG1 from its membrane-bound form in human keratinocytes, where the enzyme is solubilised and subsequently released into the cytosol (Kim *et al*., 1995; Rice *et al*., 1990). To test if the membrane-associated β-cell TG enzyme would be susceptible to the proteolytic action of trypsin or dispase, BRIN-BD11 cells were treated in suspension with 0.25% (w/v) trypsin solution or 0.01 unit/ml dispase solubilised in serum-free RPMI respectively for 15 minutes, prior to seeding on FN-coated 96-well plates (**fig 4.3**). The amount of *in situ* cell-surface TG catalysis was revealed by the amount of biotin cadaverine cross-linked into FN after 2 hours in reaction mix, with trypsin pre-treatment showing a significant 4-fold decrease ($p < 0.001$); and dispase pre-

Figure 4.1. Calcium and GTP

Figure 4.2. and fig 4.3. Retinoic acid/trypsin/dispase

treatment also revealing a significant 2-fold decrease ($p < 0.001$) in cell-surface related TG activity (**fig 4.3**).

4.2.1.4. Glucose-stimulated TG activity in β-cells

The usual method of choice for the precise and comparable measurement of specific TG activity in cell homogenates is the assay by Lorand *et al.* (1972), as described in section 3.2.6.1, where radiolabelled [^{14}C]-putrescine is incorporated into N,N'-dimethylcasein substrate and precipitated onto filter paper discs. However, the results obtained from this assay (**Table 4.1**) revealed that the TG enzyme present in BRIN-BD11 β-cells was almost 10-fold lower in activity compared to freshly isolated rat islet homogenates, which in turn caused any possible changes in glucose-induced TG activity to be indistinguishable.

Subsequent TG assays using biotin cadaverine cross-linking showed significant trends in response to glucose (**Table 4.2**), suggesting that these results were presumably due to the radiolabelled assay being developed for sensitivity with high basal TG concentrations, or perhaps due to an incompatibility of the BRIN-BD11 β-cell TG with the N,N'-dimethylcasein substrate. The β-cell models were significantly lower in their values of specific TG activity when compared to the classical high TG2-expressing model of human urinary bladder carcinoma cells which were approximately 36-fold higher in basal enzyme activity (**Table 4.1**).

A colorimetric assay developed at NTU (Trigwell *et al.*, 2004) to measure the ε-(γ-glutamyl) lysine crosslink using glutamine-rich peptides showed a similar trend, where in spite of total protein standardisation, the amount of TG cross-links from BRIN-BD11 cell homogenates were at least 3-fold lower in comparison to the homogenates from 5637 human urinary bladder carcinoma cells when casein was used as a substrate (**fig. 4.4**).

In order to induce and measure *in situ* increases in cell-surface TG activity, live BRIN-BD11 β-cells were seeded in serum-free RPMI onto FN-coated wells and stimulated for 2 hours with increasing glucose concentrations (5 mM to 40 mM).

Table 4.1

Figure 4.4 peptide cross-link assay

Fig. 4.5 and Fig. 4.6. Glucose and BRIN cells

Cell-surface TG activity (**fig. 4.5**) was measured colorimetrically through the incorporation of biotinylated cadaverine into deoxycholate-insoluble FN (and other possible β-cell derived extracellular protein substrates) after cell lysis, as described in section 3.2.6.2.1.

In the presence of serum-free culture medium only, there was a significant increase ($p < 0.05$) in cell-surface TG activity upon stimulation at glucotoxic levels of 40 mM glucose (**fig. 4.5**). This activity was further increased by the addition of 5 mM Ca^{2+} and 10 mM DTT (in order to maximise reduction of –SH active sites of TG) to the culture medium, where concentrations of 20 mM ($p < 0.05$) to 40 mM ($p < 0.001$) glucose exhibited significant enzyme cross-linking. When irreversible active-site directed inhibitor R283 was added to the culture medium at a concentration of 250 μM for 2 hours, TG activity was found to be significantly suppressed at all concentrations above 5 mM glucose (**fig. 4.5**).

Subsequently, the extracellular TG activity induced by the presence of glucose treatment was assessed using the incorporation of biotin cadaverine into endogenous substrates for 24 hours in live BRIN-BD11 cells (**fig 4.6**). These results showed that at concentrations of 20 mM glucose which would be optimal for insulin secretion coupling in BRIN-BD11 cells (McClenaghan *et al*., 1998), TG activity was significantly higher ($p < 0.001$), whereas at concentrations of 40 mM glucose which induces glucotoxicity, extracellular TG activity was similar to the 5 mM glucose controls.

4.2.1.5. Visualisation of β-cell TG Activity using FITC-cadaverine

It was possible to visualise and quantify TG activity through the addition of fluorescein cadaverine (FITC-cadaverine) into the culture medium resulting in the primary amine's incorporation by the enzyme into living cells (Verderio *et al*., 1998). The advantage of employing this method with the BRIN-BD11 cells was the direct intracellular and extracellular visual detection of TG catalysis as a result of glucose stimulation that was noted in previous assays. Even more significant was the

Fig. 4.7. FITC- cadaverine micrographs

Figure 4.7. Fluorescent visualisation of TG activity in BRIN-BD11 cells using *in situ* FITC-cadaverine incorporation. BRIN-BD11 β-cells were seeded onto 8-well chamber slides and allowed to settle for 24 hours, prior to further 24 hours treatment with glucose, GSNO and active-site inhibitor R283. FITC-cadaverine was added to the normal growth medium with treatments for 18 hours, and enzymatic activity was detected through TG-catalysed incorporation of the molecule into endogenous cellular substrates as described in section 3.2.6.3 Following fixation and permeabilisation of the cells in methanol, slides were mounted for fluorescent visualisation and quantification using a Leica TCSNT confocal laser microscope. **Panel A**, TG active-site inhibition with 250 μM R283. **Panels B to D**, Glucose curve with 5 mM, 20 mM and 40 mM treatments. **Panels E to F**, Treatments used to induce oxidative stress at concentrations of 50 μM and 150 μM GSNO in 5 mM glucose. Images are representative of at least three separate experiments, where 9 non-overlapping fields were captured digitally. Arrows show the localisation of TG activity in the extracellular processes of these cells. Bar equals 20 μm.

Figure 4.8. TG-catalysed FITC-cadaverine incorporation into BRIN-BD11 cells displayed through A) semi-quantitation of confocal fluorescence intensity from fig. 4.7, and B) immunostained TG protein substrates using western blotting. A) The images shown in fig. 4.7 were quantified using the Leica TCSNT confocal software, where 9 non-overlapping images per treatment were examined for their mean fluorescence intensity in a set area of 62500 μm^2. Data represents ±SD of three separate experiments, where ** $p < 0.001$ comparing the TG activity at 5 mM glucose to other treatments. **B)** The cellular substrates, into which the β-cell TG enzyme cross-linked FITC-cadaverine, were analysed based on their molecular weights using western blotting as described in section 3.2.6.4. Blots were immunoprobed with a mouse anti-FITC antibody, which was subsequently bound to an anti-mouse-IgG-HRP conjugate that was visualised using enhanced chemiluminescence. Four predominant immunoreactive bands were present at approximate molecular weights of 150-kDa, 100-kDa, 65-kDa and 20-kDa. This blot is representative of three separate experiments showing similar staining.

Figure 4.8. fitc-cadaverine blot

observed affinity for these β-cells to crosslink FITC-cadaverine into their endogenous substrates (**fig. 4.7, fig. 4.8A and B**).

Firstly, at 5 mM glucose-stimulation over 24 hours in the culture medium, minimal TG activity was visualised (**fig. 4.7 panel B and fig. 4.8A**). Glucose concentrations of 17 mM, known to be optimal for the induction of insulin-secretion, showed significant ($p < 0.001$) increases in FITC-cadaverine incorporation (**fig. 4.8A**) that was localised intracellularly and along the cell membrane with a large amount of signal observed when cells were aggregated (**fig. 4.7 panel C**). A negative control was set up where BRIN-BD11 cells were pre-incubated for 24 hours with 250 μM of the active-site directed TG inhibitor R283, showing a decrease in the quantified fluorescence intensity of cross-linked FITC-cadaverine at 17 mM glucose (**fig. 4.7 panel A and fig. 4.8A**). This inhibition of TG activity as a result of R283 treatment confirmed that the amine incorporation observed was indeed TG-specific. When glucotoxicity was induced at concentrations of 40 mM glucose, TG-mediated incorporation of FITC-cadaverine centred in the nucleus of cells, suggesting morphological cell stress (**fig. 4.7 panel D**), and confirming the lack of extracellular TG activity observed in previous assays (**fig 4.6**), despite significantly higher levels ($p < 0.001$) of total enzyme activity (**fig 4.8A**).

FITC-cadaverine incorporation was also used to detect the molecular weights of specific *in vitro* protein substrates cross-linked by TG, through SDS-PAGE and western blotting. Preliminary experiments using BRIN-BD11 cells have shown that a predominant substrate protein cross-linked to FITC-cadaverine by β-cell TG displays a predominant immunoreactive band at 65-kDa, with three additional substrate bands present at molecular weights of approximately 150-kDa, 100-kDa and 20-kDa (**fig 4.8B**).

4.2.2. Inhibition of TG cross-linking activity in β-cells

The TG inhibitors, R281 and R283 were solubilised from a lyophilised powder into appropriate concentrations as described in section 3.2.3.8, and the different batches were routinely tested for inhibition of TG activity in the presence of purified TG2

from guinea pig liver (*gpl*TG2). Seeing as the R281 and R283 compounds could potentially exert inhibitory effects on other TG's besides TG2, a *gpl*TG2 activity curve (**fig. 4.9**) was used to standardise approximate amounts of TG2 activity in the BRIN-BD11 cells, and to confirm the effectiveness of the TG inhibitors due to batch variation, when biotin cadaverine was incorporated into FN.

To determine the effectiveness of active-site directed inhibition on BRIN-BD11 cell monolayers themselves, extracellular TG activity after 24 hours pre-incubation with R283 and R281 was tested (**fig 4.10A**). A range of inhibitor concentrations from 0 μM to 500 μM was assessed, with 250 μM concentrations for both inhibitors proving optimal in these clonal β-cells (data not shown). Enzyme activity through the incorporation of biotin cadaverine into endogenous β-cell substrates revealed that 250 μM R283 inhibitor was more effective ($p < 0.05$) at blocking the amount of cross-linked biotin cadaverine compared to 250 μM R281 after 24 hours of treatment (**fig. 4.10A**). Furthermore, analysis of BRIN-BD11 cell viability using MTS reagent, was performed after the addition of both R281 and R283 inhibitors at a concentration of 250 μM for 24 hours, confirming that there were no significant differences in cell viability as a result of these inhibitor treatments (**fig. 4.10B**).

The comparative effect of TG inhibitors, R283 and R281, were then assessed in total BRIN-BD11 homogenates (which consisted of both cytosolic and membrane fractions), since the R281 molecule is thought to be too large to reach the cytosol of these β-cells (**fig. 4.11**). BRIN-BD11 cells were pre-incubated for 24 hours with increasing concentrations of R281 and R283, and the respective cell homogenates were then assayed for TG activity in an enzyme reaction that incorporated biotin cadaverine into FN. These dose-response experiments revealed a 50% lower level of TG activity in treated BRIN-BD11 cells compared to untreated controls ($p < 0.001$) when 250μM TG inhibitor R281 was used, and a 60% decrease in activity compared to the control ($p < 0.001$) when 250μM TG inhibitor R283 was used (**fig. 4.11**).

A similar 24 hour pre-incubation assay (**fig 4.12**) as described in fig 4.11 showed a comparative 10% decrease ($p < 0.05$) in BRIN-BD11 homogenate TG activity when 10 μM of the commercially available TG2-specific BOC-DON-Gln-Ile-Val-OMe

Fig. 4.9 and fig. 4.10

Fig 4.11 and fig. 4.12

Table 4.2.

(BOC) peptide was tested. Classical enzyme inhibition studies using the competitive amines, cystamine and putrescine revealed an approximate 50% decrease in enzyme activity at concentrations of 1 mM cystamine ($p < 0.05$) and 10 mM putrescine ($p < 0.001$) respectively.

A summary of the approximate TG2 activity in BRIN-BD11 cells relative to their corresponding *gpl*TG2 standard curves is summarised in **Table 4.2**., where three different modifications to the colorimetric assay, originally described by Jones *et al*. (1997), have been developed using biotin cadaverine. The values in **Table 4.2** were taken from assays presented in this chapter, and show the relative applicability of measuring i) the *in situ* cross-linking activity of cell surface TG enzyme secreted by live cells over 2 hours; ii) the cross-linking activity of TG enzyme present in cytosolic and membrane homogenates; and iii) the *in situ* enzyme activity from aggregated cell monolayers, using endogenous β-cell substrates instead of purified FN (**see fig. 2.4**).

4.2.3. TG Activity in $TG2^{+/+}$ and $TG2^{-/-}$ mouse islets

The investigation thus far has characterised the effects of site-specific irreversible TG inhibitors (R281, R283 and BOC-peptide), however the possibility of other TG enzymes besides TG2 being irreversibly extinguished cannot be fully ruled out using the biochemical assays that have been developed. In order to assess possible TG2-specific effects within β-cells, this section will focus on the *ex vivo* state of TG2 inhibition in islets that have been isolated from TG2 knockout ($TG2^{-/-}$) mice (De Laurenzi and Melino, 2001) (**fig. 4.13, fig 4.14, fig 4.15**).

TG cross-linking activity was visualised and quantified through the enzyme-mediated incorporation of FITC-cadaverine into freshly isolated live TG2 knockout ($TG2^{-/-}$) and wildtype ($TG2^{+/+}$) mouse islets (**fig 4.13 and fig 4.14**), which were treated with 5 mM and 20 mM glucose concentrations over 24 hours. The $TG2^{+/+}$ mouse islets displayed a higher overall amount of FITC-cadaverine signal under low (**fig. 4.13 panel A**) and high (**fig. 4.13 panel C**) glucose treatments, compared to the $TG2^{-/-}$ islets (**fig 4.13 panels B and D**), with 20mM glucose-stimulated $TG2^{+/+}$ islets showing the highest quantifiable fluorescence (**fig 4.14**). Although these results

Figure 4.13. knockout islet fitc-cadaverine

Figure 4.14

Figure 4.15

show the possibility of FITC-cadaverine being used a measure of TG activity in mouse islets, the complete permeability of the fluorescent molecule into the whole islet remained a challenge.

$TG2^{-/-}$ and $TG2^{+/+}$ mouse islets were analysed for specific TG activity through the incorporation of radiolabelled [^{14}C]-putrescine into N, N`-dimethylcasein (DMC) substrate (**fig. 4.15**). The enzyme activity in $TG2^{+/+}$ mouse islet homogenates was 4-fold higher ($p < 0.05$), than in that of $TG2^{-/-}$ islet homogenates or BRIN-BD11 cells (**fig. 4.15, red bars**). In order to determine whether the β-cell TG activity was best reflected with a DMC substrate, a variation of this assay was performed where the radiolabelled [^{14}C]-putrescine was allowed to cross-link to β-cell endogenous substrates instead of DMC. In the latter experiment (**fig. 4.15, blue bars**), specific TG activity was consistently higher for all comparative treatments in the absence of DMC.

4.2.4. The Effect of TG Inhibition and Diabetic Stressors on β-cell Survival

Conditions of hyperglycaemia (D-glucose treatment), oxidative stress (S-nitrosoglutathione/GSNO treatment) and hyperlipidaemia (palmitate treatment) were simulated for a diabetic-like status in BRIN-BD11 β-cells *in vitro* (**fig.4.7 panels D, E and F; fig 4.16; fig. 4.17; fig. 4.18**). TG activity was visualised morphologically using FITC-cadaverine incorporation, where 40 mM glucose induced cellular stress that was evident through the localisation of intense enzyme cross-linking in the nuclei of these β-cells (**fig.4.7 panel D, arrows**). When these β-cells were maintained at 5 mM glucose, and treated with concentrations of 50μM and 150 μM GSNO, TG2 activation lead to the assembly of excessive intracellular cross-linked protein polymers in the nuclei, cytoplasm and extracellular processes (**fig.4.7 panels E and F, arrows**).

BRIN-BD11 cell viability under diabetic stress treatment (**fig. 4.16**) was measured through the mitochondrial metabolism of MTS tetrazolium compound. Under conditions of hyperglycaemia, concentrations between 30 mM to 40 mM glucose were accompanied by a slight but significant ($p < 0.05$) increase in mitochondrial

Figure 4.16. MTS assays

Figure 4.17

Figure 4.18

Figure 4.19

metabolism after 24 hours (**fig 4.16A**). BRIN-BD11 cells appeared to generally sustain high levels of glucose in the medium at concentrations up to 40mM glucose for 24 hours, however with the addition of R281 active site-directed cell surface TG inhibitor (data not shown for corresponding R283 inhibitor treatments due to insignificant changes), cell viability appeared significantly ($p < 0.001$) reduced (**fig. 4.16A**).

Oxidative stress at concentrations between 50μM and 500μM GSNO over 24 hours resulted in no apparent decreases of tetrazolium-measured cell viability (**fig. 4.16B**), however with the addition of R281 TG inhibition at these same concentrations, the BRIN-BD11 cells appeared to be significantly ($p < 0.001$) more susceptible to oxidative stress. The TG inhibitor R283, in corresponding experiments, did not appear to affect cell viability under these conditions (data not shown).

Under conditions of hyperlipidaemia, palmitate was found to be very effective as a lipotoxic agent (**fig. 4.16C**), causing extensive cell death with an LD50 of ~ 150μM after 24 hours treatment, when assessed using MTS reagent. The addition of R281 and R283 (data not shown) did not adversely affect β-cell survival. Due to the high level of cell death as a result of palmitate treatments, optimal values to induce apoptosis were set at 50 μM so that caspase-3 activity could be more measurable.

To assess whether the observed decreases in BRIN-BD11 cell viability were as a result of caspase-3 mediated apoptosis, concentrations of 40 mM glucose, 50 μM GSNO and 50 μM palmitate were assessed after 72 hours treatment, using a colorimetric caspase-3 activity assay (**fig. 4.17**) and immunofluorescent visualisation of a cleaved caspase-3 antibody (**fig. 4.18**). The colorimetric caspase-3 activity assay measured the apoptotic index by the ability of caspase-3 within β-cells to cleave Ac-DEVD-p-nitroaniline substrate and release the p-nitroaniline (pNA) chromophore (**fig 4.17**). Caspase-3 activity was evident in BRIN-BD11 cells after 72 hours treatment under conditions of 40 mM glucose, 50 μM GSNO and 50 μM palmitate ($p < 0.001$). Hyperglycaemia at 40 mM glucose was then expanded to assess the effects of TG inhibition on caspase-3 mediated apoptosis, through the addition of the large cell surface TG inhibitors (250 μM R281 and 10 μM BOC peptide), but no

significant changes were seen when compared to the glucose treatments (**fig. 4.17**). However, when the smaller R283 TG inhibitor (which is believed to enter live β-cells and also act on their cell surface) was added to the hyperglycaemic cells, the amount of caspase-3 specific activity was significantly lowered ($p < 0.001$) to levels nearing that of the caspase inhibitor (50 μM Z-VAD-FMK) which was used as a routine negative control (**fig. 4.17**). These results were confirmed immunofluoresently using a cleaved caspase-3 (Asp175) antibody, where ~70% induction of caspase activity was observed after 72 hours treatment under all 3 simulated diabetic conditions (**fig. 4.18 F, G, H**) compared to the 10mM glucose control (**fig 4.18 E**).

Cleaved caspase-3 antibody staining was also visualised in freshly isolated TG2 knockout ($TG2^{-/-}$) and wildtype ($TG2^{+/+}$) mouse islets (**fig 4.19**), which were treated with 5 mM and 20 mM glucose concentrations (serum free RPMI) over 24 hours. Although it was possible to detect β-cell apoptosis occurring within the islets, insufficient permeability of the antibody and differences in islet size made it difficult to draw quantifiable conclusions.

4.2.5. The Effect of TG Inhibition on β-cell Insulin Secretion Function

It was important to confirm the effect of β-cell TG activity on insulin stimulus secretion coupling, using active-site directed inhibition of the enzyme with R281 and R283, since non-specific competitive amine inhibition studies have been the main source of information in past research (refer to section 1.4.7). Insulin secretion function was therefore analysed quantitatively in both BRIN-BD11 cells (**fig. 4.20**) and freshly isolated rat islets (**fig. 4.21**) by [^{125}I]-insulin radioimmunoassay, after acute glucose stimulation and pre-treatments with active site-specific TG inhibitors R281 and R283.

In clonal rat pancreatic BRIN-BD11 β-cells, there was an almost 3-fold increase in insulin secretion between 0.25 mM glucose and 20 mM glucose after 1 hour 30 minutes ($p < 0.001$), confirming that these cells were glucose-responsive and secreted high amounts of insulin (**fig. 4.20**). The larger irreversible active-site

Figure 4.20

Figure 4.21

directed TG inhibitor R281, brought about significantly ($p < 0.05$) lower insulin secretion at 20mM glucose compared to the normal controls, while R283 which is predicted to inhibit TG activity both intracellularly and extracellularly showed the most marked decrease ($p < 0.001$) in insulin secretion overall (**fig. 4.20**). In this experiment, KCl was used as a positive control to depolarise the K^+ channels in the β-cell membranes and cause insulin release, with the intention of ruling out any possible effects the TG inhibitors themselves may have on depolarising the cells. However, TG inhibition also seemed to affect the complete depolarisation effect induced by KCl, which suggests another possible role for TG activity in this mechanism that could be related to K^+ channels on the cell surface (**fig. 4.20**).

A similar experiment was undertaken using isolated whole rat islets, except that the inhibitors R281 and R283 were introduced at the same time as acute glucose stimulation, and the insulin secretion measured was for a shorter period of 1 hour (**fig. 4.21**). There was a significant ($p < 0.001$) 5-fold increase in rat islet insulin secretion between 2 mM and 20 mM glucose stimulation, and whilst the larger R281 molecule had minimal effects on insulin secretion after 1 hour, the R283 inhibitor lowered ($p < 0.05$) rat islet insulin secretion function at the same time point (**fig. 4.21**), with the inhibitory effect of R281 and R283 on insulin release following KCl membrane depolarisation also being observed in these rat islets.

4.3. Discussion

The precise role of TG2 in pancreatic β-cell function and survival still remains largely unknown. A lasting reflection from early research in this field is that TG cross-linking activity seems to be present in the membrane fractions of pancreatic islets, where the enzyme catalyses the access of secretory granules to the exocytotic sites of islet β-cells during insulin release, and was also found to participate in the machinery controlling the conversion of pro-insulin to insulin (Bungay *et al*., 1984 and 1986; Gomis *et al*., 1983, 1984 and 1989; Sener *et al*., 1985; Owen *et al*., 1988). More recently, targeted disruption of the TG2 gene in mice ($TG2^{-/-}$) lead to a gradual decline in glucose homeostasis and the development of a type 2 diabetes phenotype (Porzio *et al*., 2007; Bernassola *et al*., 2002; De Laurenzi *et al*. 2001). In this chapter,

studies into the catalytic profile of the BRIN-BD11 β-cell TG enzyme were determined using regulatory factors such as glucose, Ca^{2+}, GTP, retinoic acid, membrane proteases and active-site directed inhibition of TG protein activity. Diabetic stressors in the form of hyperglycaemia, oxidative stress and hyperlipidaemia were used to assess the action of the TG protein in β-cell survival, while the $TG2^{-/-}$ mouse model created by De Laurenzi *et al.* (2001) was bred for further characterisation of islet TG dynamics. The insulin secretion function of the BRIN-BD11 cells in comparison to rat islets were then quantified using an [^{125}I]-insulin radioimmunoassay.

In this investigation, the BRIN-BD11 clonal rat β-cells were used as a model to study TG expression and activity for the first time. Clonal β-cells that demonstrate a high level of differentiation such as MIN-6 usually have a slow turnover rate and clustered aggregation of the β-cells during culture in the form of pseudo-islets for a high insulin response (Miyazaki *et al*., 1990). The less differentiated β-cell lines such as BRIN-BD11 have the advantage of a daily turnover rate, while still retaining a high insulin secretion response, making them more conducive as a model for numerous high-throughput biochemical assays. Several substrate-specific TG activity assays were assessed using either *in situ* cell-secreted or extracted β-cell TG from a source of BRIN-BD11 rat pancreatic insulinoma clonal cells. The assays presented in this chapter were modifications to: i) the catalysed TG-mediated incorporation of [^{14}C]-putrescine into N'N-dimethylcasein (DMC) or endogenous substrates (Lorand *et al*., 1972); ii) the cross-linking of biotin cadaverine into deoxycholate-insoluble fibronectin or endogenous β-cell substrates (Slaughter *et al*., 1992; Jones *et al*., 1997); iii) the *in situ* cross-linking of FITC-cadaverine into endogenous β-cell substrates (Lajemi *et al*., 1997; Verderio *et al*., 1998); iv) or the cross-linking of glutamine-rich (TVQQEL) biotinylated peptides into casein (Trigwell *et al*., 2004). Each assay was selected in turn for the optimal expression of controlled or regulated TG activity and site-specific inhibition according to the necessary enzyme reaction specifications. The results showed a high degree of BRIN-BD11 substrate specificity, where the use of biotin cadaverine and FITC-cadaverine proved to be the optimal amines of choice (see fig. 4.22 for chemical structures), when cross-linked into endogenous physiological β-cell substrates. A limitation that currently remains with these established *in vitro* assays is

Fig 4.22.

that the β-cell specific TG activity could represent other low level TG's that may be present in the homogenates in addition to TG2.

Pioneering studies into the TG activity of rat islets (Gomis *et al*., 1983) revealed that a range of primary amines could be incorporated into N'N'-dimethylcasein (DMC) substrate using a variation of the standard *in vitro* assay devised by Lorand *et al.* (1972). This investigation confirmed that homogenised TG extracts from both rat and mouse islets also contained high affinity for the TG-mediated cross-linking of radiolabelled [^{14}C]-putrescine into DMC substrate, with noticeably higher activities in the presence of endogenous islet substrates compared to DMC. However, when the BRIN-BD11 cell extracts were assayed at similar total protein concentrations to the islets, TG activity levels were ~10-fold lower compared to the freshly isolated islets. Nonetheless, the colorimetric biotin cadaverine assays appeared most appropriate to study quantifiable trends in these BRIN-BD11 cells, where low levels of homogenate- and surface-associated β-cell enzyme were still evident. This led to speculation that the clonal β-cells could possess lower overall potential for enzyme activity as is the case with the TG expressed by some immortalised or undifferentiated cell lines (Birkbichler *et al*., 1978; 1980), or that this difference was a reflection of the islets possessing an additional endothelial microcapillary source of TG (exhibiting high substrate specificity for DMC), which the homogenous colonies of BRIN-BD11 lacked. Methods of extracting pure β-cell fractions from intact islets such as immunofluorescent β-cell sorting using flow cytometry (Suzuki *et al*., 2004) could prove useful in determining the true localised subcellular activity of the TG activity in islets.

Additionally, assays that rely on the amine incorporating activity of the TG enzyme, such as the biotin cadaverine incorporation assay and the radiometric [^{14}C]-putrescine incorporation assay may be compromised by Ca^{2+}-dependent and Ca^{2+}-independent incorporation of polyamines into casein substrates by other enzymes in cell extracts, such as diamine oxidases (Carpene *et al*., 1995) and thioredoxin-family protein disulphide isomerases (Chandrashekar *et al*., 1998). In contrast, cross-linking of proteins and peptides via ε-(-γ-glutamyl) lysine linkages is TG-specific (Griffin *et al*., 2002), as was demonstrated previously using the incorporation of a biotinylated hexapeptide (biotin-TVQQEL) into casein substrate (Trigwell *et al*., 2004). In this investigation, the β-cell TG catalysed low levels of ε-(-γ-glutamyl) lysine isopeptide

bonds between the glutamine rich peptide and casein showing similar trends in activity when DMC was used, suggesting that this may be due to substrate specificity or low overall activity of the BRIN-BD11-expressing TG isoform.

Amine incorporation into protein substrates in the TG-catalysed reaction has been shown with purified proteins or with cell lysates in previous studies, but it is essential to determine whether these proteins are truly physiological substrates in β-cells (Bungay *et al*., 1986; Owen *et al*., 1988; Gomis *et al*., 1989). The use of fluorescent derivatives of polyamines as probes was preferred in the current experimental design since simple observation of the cells by direct fluorescence microscopy would permit detection of the substrates accessible to active TG, while the methanol fixative eliminated free FITC-cadaverine and preserved a lattice of covalently labelled fluorescent structures (Lajemi *et al*., 1998; 1997; Verderio *et al*., 1998). In the BRIN-BD11 cells, TG-catalysed incorporation of FITC-cadaverine evidently increased upon normal glucose stimulation, with excessive protein cross-linking and obvious nuclear localisation of the enzyme under conditions of hyperglycaemic and oxidative cell stress, demonstrating a possible switch from normal TG-mediated insulin secretion function to TG-associated cell survival function. Furthermore, $TG2^{-/-}$ mice, which were expected to manifest a type 2 diabetes phenotype, seemed to contain high levels of islet β-cell mass, but revealed decreases in the amount of amine incorporation into endogenous β-cell substrates, confirming the mechanism for TG2-deficient insulin circulating levels reported previously in this model (Bernassola *et al*., 2002). However, the reduced amine cross-linking observed in the islet structure of $TG2^{-/-}$ mice could also possibly be the result of deficiencies in TG2 expression within the islet endothelial microvasculature, and not just the insulin secreting β-cells. This would be a very important point to clarify, before the precise role of the enzyme within $TG2^{-/-}$ islets can be taken further.

Antibodies against the fluorescein moieties made it possible to detect labelled TG2 substrates after western blotting (Lajemi *et al*., 1998; 1997), thus permitting further characterisation of the β-cell protein substrates at molecular weights of approximately 150-, 100-, 65-, and 20-kDa. Interestingly, high molecular weight polymer aggregates that were unable to traverse a 3 % (w/v) polyacrylamide gel

were found in previous reports using rat islets, and were reduced in the presence of competitive TG inhibitors during glucose stimulation (Bungay *et al*., 1986; Owen *et al*., 1988; Gomis *et al*., 1989). These polymer aggregates were thought to be important in the stabilisation of the rat islet β-cell membrane during membrane recycling and following the membrane-associated events of insulin secretion. Seeing as the less differentiated BRIN-BD11 β-cells showed less complex FITC-cadaverine cross-linked substrates, it may be worth pursuing more intensive investigations under non-reducing SDS-PAGE conditions in order to isolate these high molecular weight phosphoprotein polymers, as they could also possibly be a source of large extracellular matrix proteins associated with the endothelial fraction of pancreatic islets. Furthermore, the use of TG inhibition in these β-cells, prior to such substrate analysis could potentially reveal insights into the interaction of TG cross-linking to specific protein substrates during the insulin release mechanism.

BRIN-BD11 β-cell-specific TG exhibited classical reciprocal mechanisms of the functional conformational change necessary for switching between Ca^{2+}- and GTP-binding, that was also thiol-dependent, as described to some extent by Bungay *et al*. (1984a) in rat islets. This was evident through increases in BRIN-D11 TG activity in response to increases in Ca^{2+} concentration, and the non-competitive inhibition of this β-cell specific TG in the presence of GTP-γ-S (see fig. 4.23. for chemical structure). It is possible that the interaction of GTP with β-cell TG induces structural changes that block the accessibility of the Ca^{2+}-binding and active-sites, thereby decreasing cross-linking activity (Achyuthun and Greenberg, 1987; Smethurst and Griffin, 1996; Monsonego *et al*., 1998). These results are interesting in the context of a G-protein function being attributed to a possible β-cell specific TG2/Gαh, seeing as heterotrimeric and low molecular weight GTP-binding proteins have already both been involved in regulating glucose-triggered exocytosis of insulin from pancreatic β-cells (Kowluru *et al*., 1996). The possibility of GTP-dependant inhibition of the β-cell TG may also lend clues about the mechanism of activi 173 enzyme exhibits in response to the Ca^{2+} influx via voltage-dependant Ca^{2+} channels that is necessary during insulin release (Rorsman and Renstrom, 2003). It also confirms the possibility that the β-cell TG could only be either TG2, TG3 or TG5

Figure 4.23. Inhibitors

since GTP-binding is only possible with these types (Liu *et al*., 2002; Ahvazi *et al*., 2004a; Candi *et al*., 2004).

The transcriptional regulation of increases in TG induced by retinoic acid was evident through higher amounts of cross-linking in BRIN-BD11 cells. Retinoic acid has been shown to up-regulate TG2 in both murine peritoneal macrophages, promyelocytic leukemia cells, INS-1E and RINm5F pancreatic β-cells (Murtaugh *et al*. 1983; Chiocca *et al*. 1989; Driscoll *et al*., 1997). Retinoic acid is also known to regulate TG2 expression via the binding of nuclear receptors to specific response elements located within the TG2 gene promoter (Chiocca *et al*. 1988; Lu *et al*. 1995). Previous studies suggest that increased expression of retinoid receptors is critical for an increase in TG2 gene expression (Johnson *et al*. 1998), suggesting that the BRIN-BD11 cells used in this study may possess similar retinoid receptors.

The importance of cell-surface TG activity in the membrane-associated insulin secreting machinery was conceivable due to reductions in cross-linking as a result of broad range serine protease trypsin and dispase treatments. It would be interesting to further investigate β-cell TG proteolysis using western blot analysis of the resulting protein products, since it is possible that proteolysed membrane-associated TG may have been released from the particulate fraction into the cytosolic fraction (Rice *et al*., 1990; Knight *et al*., 1991a). Cell-surface β-cell TG investigations were then carried out using glucose stimulation of the BRIN-BD11 cells, where an apparent increase in surface-related or total homogenate TG activity was evident only in the presence of a Ca^{2+}- and thiol-dependant enzyme reaction, suggesting that if any membrane-associated TG was present, it was not being actively secreted out of the β-cell. It is thought that the mechanism of glucose stimulation within β-cells may increase TG enzymic activity as a result of *de novo* synthesis of the enzyme or alternatively the induction of a more reduced state as a result of a rise in cytosolic Ca^{2+} activity, with subsequent changes in the thiol-disulphide balance due to increases in the content of sulphydryl groups (Gomis *et al*., 1986b). The precise molecular mechanisms by which glucose might stimulate TG activity, and the possibility of β-cell TG being released upon glucose-stimulated insulin secretion still remains to be fully clarified.

Competitive substrate inhibition as a result of alkylamines, monodansylcadaverine, *N-p*-tosylglycine, bacitracin, glycine alkylesters and hypoglycaemic sulphonylureas confirmed the importance of TG cross-linking during insulin secretion function in rat islets (Hutton *et al*., 1982; Malaisse et al., 1983; Gomis *et al*., 1983 and 1984; Lebrun *et al*., 1984), however some of these competitive amines showed non-specificity, poor penetration into the islet cell and effects on cationic fluxes which caused increased Ca^{2+} entry into the β-cells. In this chapter, the specific properties of two inhibitors of TG activity: R283 (1, dimethyl-2[oxopropyl) thio] imidazolium) and R281 (N-benzyloxycarbonyl-L-phenylalanyl-6-dimethylsulphonium-5-oxo-Lnorleucine) have been characterised for the first time in terms of their action against β-cell TG (see figure 4.23. for chemical structures). Both inhibitors contain a dimethylsulfonium group that has previously been shown to interact with cysteine residues including Cys_{277} within the active site via an acetonylation reaction (see fig. 4.24.) leading to non-competitive and irreversible inhibition of the enzyme (Freund, 1994).

Data obtained from pilot studies for the use of these inhibitors in the current cell system demonstrated that both inhibitors were highly effective at reducing β-cell TG activity when applied to BRIN-BD11 homogenates or live cells. The action of both R281 and R283 at optimal concentrations did not affect mitochondrial metabolism through the determination of cell viability under normal culture conditions of the BRIN-BD11 cells. The inhibitors seemed to show slight differences in their ability to cross the cell membrane when TG activity was tested on live β-cells. R283 was found to be highly soluble and was concluded to readily enter the intracellular compartment, while exerting its action on the cell-surface as well. In contrast, R281 was much less able to transfer across the cell membrane due to its large molecular weight (Griffin *et al*., 2004) and its action seemed to be limited to the extracellular compartment, showing slightly lower levels of TG activity. The BOC-peptide TG inhibitor is a large molecule, and showed a mechanism of inhibition similar to the action of R281. Such differences were important in determining whether total (intracellular and extracellular) inhibition or extracellular TG inhibition alone was sufficient to impede the action of TG2 in BRIN-BD11 cells. When these irreversible active-site directed inhibitors were tested on BRIN-BD11 cell homogenates, the level of inhibited TG activity was similar to trends seen with the non-specific

Figure 4.24. Inhibitor mechanism

competitive substrate inhibition of cystamine and putrescine commonly used in TG assays.

The relatively equal effectiveness of R281 and R283 on the insulin secretion function of β-cells indicates that their principle inhibitory actions are extracellular. It could be said that the poor cell solubility expected of R281 could limit any potential non-specific effects on other intracellular TG or thiol containing enzymes, however this inhibitor is not specific to TG2 since it has been shown previously to inhibit the extracellular activity of TG1 in myocardium microvascular endothelial cells (Baumgartner *et al.*, 2004). Furthermore, the chances of R283 affecting intracellular targets apart from TG2 cannot be dismissed given its known ability to inhibit other TG isotypes (Freund *et al*., 1994). Interestingly, the mechanism of R283 inhibition appeared to mimic caspase inhibitor levels in BRIN-BD11 cells suggesting that the role of intracellular TG in caspase-mediated apoptosis may be more complicated than originally expected. There remains the possibility that the R283 molecule could possibly be acting on caspase-3 directly, suggesting questions for the sole specificity of this molecule on TG enzymes within a complex cell system.

Caspases, a family of aspartate-specific cysteine proteases, play an essential role in both initiation and execution of apoptosis (Green and Amarante-Mendes, 1998; Budihardjo *et al*., 1999). Typical morphological changes in pancreatic β-cells (HIT-T15 and INS-1E) and rat islets, associated with apoptosis, include cytoplasmic shrinkage, membrane protuberances, chromatin condensation and DNA fragmentation (Li *et al*., 1998, Huo *et al.*, 2002 and 2003). Many studies have suggested that TG2 may participate in the activation of apoptosis, where the enzyme was found to accumulate and its activity increased in these apoptotic cells (Fesus and Thomazy, 1988; Fesus, 1998; Melino and Piacentini, 1998, Autuori *et al*., 1998). During apoptosis TG2 modifies several proteins including histone protein (Ballestar *et al*., 1996), actin (Nemes *et al*,. 1997), troponin (Gorza *et al*., 1996), and retinoblastoma protein (Oliverio *et al*., 1997). The cross-linking of proteins by TG may therefore play an important role in the morphological changes during apoptosis. Cytoskeletal proteins such as actin, microtubules and intermediate filaments may act as anchorage sites or substrates for TG in cells undergoing apoptosis (Melino and Piacentini, 1998; Trejo-Skalli *et al*., 1995; Piredda *et al.*, 1999, Huo *et al*., 2002 and 2003). These

morphological changes in TG activity as a result of FITC-cadaverine incorporation were evident in BRIN-BD11 cells under conditions of hyperglycaemia and oxidative stress induced by GSNO.

Furthermore, under conditions of oxidative stress the cell-surface associated TG activity, which is thought to be the target of the R281 molecule, was shown to be crucial for the extended viability of BRIN-BD11 cells. Interestingly, increased generation of reactive oxygen species as a result of oxidative stress and subsequent alteration in cell redox state is known to stimulate TG2 expression (Melino *et al*., 1997; Bernassola *et al*., 1999), as was also observed morphologically in this study with FITC-cadaverine cross-linked into BRIN-BD11 cells. Therefore induction of TG2 may be part of a broader biochemical response to oxidative stress, in pancreatic β-cells also. These results may suggest that in a similar *in vivo* situation, alterations in TG expression could render β-cells susceptible to the toxic effects of free radical generation seen during the development of Type 2 diabetes.

Given the complexity with which TG2 interacts with numerous proteins, the role of TG2 in apoptosis is not yet understood (Melino and Piacentini 1998). Furthermore, many of the TG2-targeted proteins are also substrates of caspases, so there is a possibility for an association between TG2 and caspases (Autuori *et al*., 1998). In HIT-T15 insulin secreting β-cells, GTP depletion resulted in increases of TG2 activity (Huo *et al*., 2002 and 2003). However, these authors found that the pan-caspase inhibitor (Z-VAD-FMK) entirely prevented apoptosis induced by mycophenolic acid, but did not block the enhanced TG activity, indicating that GTP-depletion can induce apoptosis and activate TG either independently or part of a cascade of events involving caspases. Interestingly, TG2 is a caspase-3 substrate and is cleaved during apoptosis in lymphoid cells (Fabbi *et al.*, 1999). Cleavage of TG2 causes loss of its cross-linking function, and this event is regarded as a valuable biochemical marker of caspase-3 activation during the late execution phase of apoptosis. This may explain reductions in TG2 activity at the late stages following caspase-3 activation in mycophenolic treated HIT-T15 cells (Huo *et al*., 2003). Alternatively, the reduction in TG2 activity might be due to activation of other proteases, since it was reported in SH-SY5Y cells that depletion of GTP increases TG2 degradation by calpain after elevation of intracellular Ca^{2+} levels (Zhang *et al*.,

1998). This phenomenon may also be the case in pancreatic β-cells. Of further consequence is the finding that increases in TG2 activity was not observed in some cases, such as treatment with dexamethasone (Johnson *et al*., 1998), peroxinitrate (Virag and Szabo, 2000), or by Fas receptor stimulation (Szondy *et al*., 1997). Apoptosis induced by various agents was not affected in $TG2^{-/-}$ mice (De Laurenzi *et al*., 2001), as was also evident in this study using caspase-3 antibody staining. The known cytotoxic action of palmitate and hyperglycaemia (Davies *et al*., 2001; Welters *et al*., 2004 and 2006; Dhayal *et al*., 2008; Sener and Malaisse, 2002) in BRIN-BD11 cells also did not seem to differ significantly under conditions of inhibited TG activity. These studies taken together imply that the notion of TG2 being a crucial component of the main pathway of early apoptotic programs remains to be fully substantiated, in pancreatic β-cells as well.

The use of specific active-site directed TG inhibition made it possible to confirm the precise role played by this enzyme in the insulin secretion function of BRIN-BD11 cells. The TG inhibitors, R281 and R283 caused significant reductions in the insulin levels released by BRIN-BD11 cells and rat islets when measured with the I^{125}]-insulin RIA. Seeing as the secretion of insulin is usually carried out over a period of several minutes to one hour, within this time-frame biosynthesis cannot lead to secretion (Howell and Bird, 1989), suggesting the importance of TG catalysis in the exocytotic machinery necessary to immediately translocate insulin granules out of the β-cell. The contraction of the microfilamentous cell web that controls the access of secretory granules to the exocytotic site was previously found to reduce stimulus secretion coupling of insulin when TG was inhibited (Gomis *et al*., 1983a). Taken together with the number of cytoskeletal proteins which are known to act as substrates for TG (Esposito and Caputo, 2005) there is great potential for understanding this β-cell exocytotic process in future studies. It would also be interesting to clarify any effect TG may have on the molecular mechanisms involved in the biosynthesis of insulin, since most secretion studies reflect trends from the distal stages of insulin release, as a result of the nature of measuring insulin release over the first hour of stimulation. The possibility that TG activity may also be crucial for the conversion of pro-insulin into mature granules produced in response to stimulatory glucose concentrations (Gomis *et al*., 1986b) warrants further clarification. Additionally, these investigations showed that the total insulin content contained in the BRIN-BD11 cells and rat islets seemed to be

reduced in the presence of TG inhibition when KCl was used to release this insulin by depolarising the β-cell membrane. It is entirely plausible that cell-surface TG could also be exerting an effect on the β-cell ATP-sensitive K_{ATP} channels during membrane depolarisation.

The data presented in this chapter revealed that the TG activity specific to BRIN-BD11 β-cells may be regulated by a number of factors within the insulin secreting mechanism unique to this cell system. TG activity appeared to increase in response to elevated glucose sensing mechanisms, and transcriptional regulation by retinoic acid also appeared to over-express the level of enzyme activity in BRIN-BD11 cells. The regulated increase of BRIN-BD11 TG activity in response to increased Ca^{2+} concentrations, and inhibition of this cross-linking activity in the presence of GTP, together with the effective inhibition by active site directed R281 and R283 inhibition, suggests classical thiol-dependent reactivity mechanisms in β-cell TG function. Furthermore, the importance of cell-surface TG cross-linking activity has been highlighted in these insulin-secreting BRIN-BD11 cells. The impaired cross-linking activity observed in $TG2^{-/-}$ mouse islets compared to their $TG2^{+/+}$ counterparts suggests that the TG present within this cell system may be of the TG2 variety, and supports the potential for inhibited TG2 activity being a direct cause of the diabetes type 2 phenotype present in these mice. Cellular stress factors such as oxidative stress and hyperglycaemia could also be potential regulators of TG activity in β-cells. Such data may be important in developing experimental and therapeutic strategies aimed at controlling the expression and activity of TG within β-cells.

Chapter Five:

Characterisation of Transglutaminase Expression in Pancreatic β-Cells

Arthur Eddington from *The Expanding Universe*

"Now I have told you 'everything right as it fell out.'
How much of the story are we to believe?

Science has its showrooms and its workshops. The public today, I think rightly, is not content to wander round the showrooms where the tested products are exhibited; they demand to see what is going on in the workshops. You are welcome to enter; but do not judge what you see by the standards of the showroom.

We have been going round a workshop in the basement of the building of science. The light is dim, and we stumble sometimes. About us is confusion and mess which there has not been time to sweep away. The workers and their machines are enveloped in murkiness. But I think that something is being shaped here – perhaps something rather big. I do not quite know what it will be when it is completed and polished for the showroom. But we can look at the present designs and the novel tools that are being used in its manufacture; we can contemplate too the little successes which make us hopeful.

A slight reddening of the light of distant galaxies, an adventure of the mathematical imagination in spherical space, reflections on the underlying principles implied in all measurement, natures curious choice of certain numbers such as 137 in her scheme – these and many other scraps have come together and formed a vision. As when the voyager sights a distant shore, we strain our eyes to catch the vision. Later we may more fully resolve its meaning. It changes in the mist; sometimes we seem to focus the substance of it, sometimes it is rather a vista leading on and on till we wonder whether aught can be final."

Eddington, Sir Arthur (1882-1944), Astrophysicist. *The Expanding Universe,* Cambridge Penguin, 1940. Quoted from *The Oxford Book of Modern Science Writing* by Richard Dawkins, Oxford University Press, 2008.

Chapter Five:

Characterisation of Transglutaminase Expression in Pancreatic β-cells

5.1. Introduction

As demonstrated in chapter 4, the TG enzyme specific to β-cells revealed biochemical properties for cross-linking activity through the Ca^{2+}-activated formation of ε-(-γ-glutamyl) isopeptide bonds, which were inhibited in the presence of GTP-γ-S, Cys_{277} active-site directed inhibitors (R281, R283), as well as membrane proteases (dipase and trypsin), but were up-regulated in the presence of all *trans* retinoic acid and glucose stimulation. However, the protein expression profile of this enzyme requires further characterisation in order to establish the functional status of this β-cell TG.

When routine experiments into the protein characterisation of BRIN-BD11 cells were initiated, it was assumed that the pancreatic β-cell TG2 profile would be of the native 75-80-kDa enzyme type. In fact, the first two studies documenting western blot evidence of an ~80-kDa TG2 protein in insulin-secreting HIT-T15 cells (Huo *et al*., 2003) and human islets (Porzio *et al*., 2007) were only published after the start of these investigations. However, the serendipitous discovery of a shorter form of the TG enzyme in pancreatic β-cells resulted in a re-evaluation of the original hypothesis and aims that were set out. The direction of this investigation was then expanded to include the possibility of alternatively spliced variants of the TG2 gene in pancreatic β-cells.

In addition to the two well established roles of reciprocally regulated activities: cross-linking proteins by virtue of transamidating activity, and G-protein function in intracellular signalling (Lorand and Graham, 2003; Griffin *et al*., 2002); TG2 can be viewed as quite a unique member of the TG family, since it is not only ubiquitously expressed in various tissues, but also exhibits a growing amount of multiple enzyme properties. For instance, TG2 has recently been characterised as a protein disulphide isomerise (Hasegawa *et al*., 2003) and a protein kinase (Mishra *et al*., 2007; Mishra and Murphy, 2004). Additionally, TG2 has a wide variety of functions independent of its enzymatic activities (Lorand and Graham, 2003; Griffin *et al*., 2002) such as: functioning as a cell surface adhesion molecule; binding to nitric oxide; serving as a co-receptor for integrins and the G-protein coupled receptors (GPCRs), involvement in signalling functions; promoting cell death, survival and inflammation (see section 1.3.6. for referenced descriptions); as well as being a therapeutic target in neurodegenerative, fibrotic, autoimmune, and cardiovascular disorders (see section 1.3.7. for referenced descriptions). It has therefore remained speculative, how a single TG2 gene product can perform and control such a vast array of activities and functions in so many distinct cellular localisations. There is a growing belief that the many attributes of TG2 function may in fact be a result of post-translational modifications and/or alternative splicing (Fraij and Gonzales; 1997), which could lead to the generation of a subfamily of TG2 molecules, each with their own unique characteristics.

A growing number of shortened versions of the TG2 protein have already been reported in both human and rat tissue (see Table 5.1. for a descriptive summary), in addition to a larger immuoreactive 120-kDa protein seemingly present in some cancerous or immortalised cell-types (Knight *et al*., 1990a; 1990b). Alternative splicing of the TG2 gene was originally observed in human erythroleukaemia (HEL) cells where SDS-PAGE analysis revealed a shorter protein homologue with a molecular weight of 63-kDa (Fraij *et al*., 1992), and an additional even shorter form at 38-kDa in these cells (Fraij and Gonzales, 1996). Another two short-form variants of human TG2 protein were reported recently (Lai *et al*., 2007), exhibiting molecular weights of 70-kDa and 75-kDa respectively, but differing from the preceding two in alternative splicing events (Fraij *et al*., 1992; Fraij and Gonzales, 1996). Furthermore, a short-form of human TG2 was also found in brain tissue from

Table 5.1. Summary of TG2 isoforms, separate from native 80-kDa TG2

Molecular weight	Tissues characterised	Description and Functional attributes	References
120-kDa	Metastasising rat sarcoma, HSV-2-induced hamster fibrosarcomas	Inactive form that is absent in normal tissue; shares common antibody-recognising epitope with native TG2; treatment with trypsin and thrombin resulted in the expression of native 80-kDa TG2	Knight *et al*., (1990a; 1990b)
61.7-kDa	Human erythroleukaemia (HEL) cells	Termed TG-H, **confirmed alternatively spliced human variant**; treatment with retinoic acid revealed transcript, cDNA encodes a shorter polypeptide of 548 amino acids which possesses the native TG2 active site and Ca^{2+}-binding site, possesses a 1.9-kb mRNA transcript	Fraij *et al*., (1992)
38-kDa	Human erythroleukaemia (HEL) cells	Termed TG-H2, **confirmed alternatively spliced human variant**, treatment with retinoic acid revealed transcript, cDNA encodes a shorter polypeptide of 349 amino acids which possesses the TG2 active site and Ca^{2+}-binding site	Fraij and Gonzales, (1996)
36-kDa	Rabbit liver nuclei	GTP-binding TG2 isoform found in the nucleus	Singh *et al*., (1995)
73-kDa	Rat brain astrocytes	Termed S-TGN; **confirmed alternatively spliced rat variant,** treatment with the cytokines IL-1β and TNF-α up-regulated S-TGN transcript levels; cDNA encodes a shorter polypeptide of 652 amino acids; possesses a 2.4-kb mRNA transcript; predicted pI of 4.64; C-terminus lacks a site for GTP-binding resulting in a reduction of Ca^{2+}-dependent transamidating activity, production of aggregated proteins in neurodegeneration, and a switch to apoptotic TG function.	Monsonego *et al*., (1997)
n/a	Middle cerebral and spinal cord injury in rats	S-TGN described by Monseonego et al., (1997) was confirmed by RT-PCR analysis.	Tolentino *et al*., (2004); Festoff *et al*., (2002)
n/a	Human brain (Alzheimers disease patients)	S-TGN described by Monsonego et al., (1997) was confirmed by RT-PCR analysis, however sequencing of the short TG2 DNA revealed a structure similar to the TG-H described by Fraij et al., (1992)	Citron *et al*., (2001)
62-kDa	Mouse central nervous system	Isoform was not due to alternative splicing, but thought to be a product of proteolytic cleavage	Citron *et al*., (2005)
n/a	Mouse Embryonic Fibroblasts (NIH-3T3)	Termed TG2-S; Myc-TG2-S constructs as described by (Fraij *et al*., 1992) were transfected into NIH-3T3 cells; revealed that TG2-S induced apoptosis in NIH 3T3 cells after 12 hours that was not dependent on transamidation activity, but was rather the outcome of its unique capability to undergo high-order aggregation.	Antonyak *et al*., (2006)
75-kDa and 70-kDa respectively	Human leukocytes; Aortic vascular smooth muscle; Human umbilical vein endothelial cells (HUVEC) Neuroblastoma	Termed TG2v1 and TG2v2; **both confirmed as novel alternatively spliced variants of human TG2**; cDNAs encoded 674 and 645 amino acids respectively, that shared 622 identical amino acids with native 80-kDa TG2, with an alternate 52 and 23 amino acids on the C-terminus; C-terminus lacks a site for GTP-binding resulting in a reduction of Ca^{2+}-dependent transamidating activity, however still active in the presence of GTP; retained FN-binding, sensitive to proteolysis by trypsin, did not alter caspase activation suggesting preservation of the anti-apoptotic role of TG2	Lai *et al*., (2007) Lee *et al*., (2010)

Alzheimers disease patients (Citron *et al*., 2001), that was identical to the version discovered earlier in HEL cells by Fraij *et al*., (1992). In rat tissue, pioneering investigations carried out on cytokine-treated rat brain astrocytes (Monsonego *et al*., 1997), middle cerebral artery injury (Tolentino *et al*., 2004) and rat spinal cord injury (Festoff *et al*., 2001) revealed alternative splicing mechanisms similar to those reported in human tissue by Lai *et al*., (2007), where a 73-kDa TG2 protein was observed (Monsonego *et al*., 1997). Taken together, these discoveries present an avenue for an emerging field of TG research that is crucial for a complete interpretation of TG2 biochemical enzyme roles attributed to the specific functions of various cell types.

One characteristic that is evidently shared by all the shorter TG2 isoforms reported to date is that there is a high degree of sequence conservation at the N-terminal end of these proteins where the Cys_{277} active site and Ca^{2+}-binding sites exist, with modifications of the transcripts occurring in the C-terminus, where the main GTP-binding site is found (Fraij *et al*., 1992; Monsonego *et al*., 1997; Liu *et al*., 2007, Antonyak *et al*., 2007; Tee *et al*., 2010). Some of the sophisticated approaches taken in these published studies to isolate and confirm the presence of new TG2 isoforms were initiated by cDNA sequencing, site-directed mutagenesis and other techniques such as stable cell transfection. However, the experimental design adopted in this chapter focussed firstly on the biochemical protein characterisation of β-cell TG in various pancreatic tissues as a starting point, due to the lack of published evidence for this cell system in the literature from which to draw upon. It was already evident from activity studies in the previous chapter that the β-cell TG appeared to catalyse Ca^{2+}-activated cross-linking of various amines into proteins, demonstrating inhibition of enzyme activity when GTP was included in the reaction, and the occurrence of irreversible inhibition of enzyme activity in the presence of Cys_{277} active site directed TG inhibition by R281, R283 and BOC-peptide. These clues led to strategies used in experimental design which focussed on antibody-antigen recognition using the CUB7402 antibody which acts on the Ca^{2+}-binding epitope, further GTP-binding analysis, and the use of molecular primers and probes directed towards the C-terminal end of the β-cell TG molecule.

The current chapter will therefore aim to support the biochemical aspects presented in the previous chapter by characterisation of the TG protein expression profile in clonal pancreatic β-cells and freshly isolated islets of Langerhans. In order to confirm and visualise the morphological expression and molecular weight of TG2 in rat clonal β-cells, glucose-stimulated insulin-secreting BRIN-BD11 cells will be assessed using immunofluorescence and western blotting respectively. Human pancreas cryosections from normal or diabetic donors will then be viewed immunofluorescently for TG2 and insulin content, together with western blot analysis of human islets. Freshly isolated islets from the pancreases of $TG2^{+/+}$ and $TG2^{-/-}$ mice exhibiting a type 2 diabetes phenotype (De Laurenzi and Melino, 2001) will be assessed once again in this chapter for TG expression and insulin content using both immunofluorescence and western blotting. To address the possibility of a unique TG isoform present in pancreatic β-cells, further analysis of BRIN-BD11 cells will be performed by two dimensional SDS-PAGE and western blotting followed by peptide mass fingerprinting using mass spectrometry. The effect of matrix metalloproteinases (MMP) on BRIN-BD11 TG2 and the potential for GTP-binding of this enzyme will then be carried out using western blotting. A simple comparative screening of TG1, TG2, and TG3 in BRIN-BD11 (together with MIN-6 and INS1E) will then be considered by utilising western blotting and RT-PCR analysis. Further molecular characterisation of the BRIN-BD11 TG2 mRNA profile (together with MIN-6 and INS1E) using northern blot analysis will be the next step. The chapter will then be concluded using RT-PCR analysis of BRIN-BD11 RNA by constructing primers directed at the alternatively spliced transcripts reported previously in rat brain (Monsonego *et al*., 1997; Tolentino *et al*., 2004).

5.2. Results

5.2.1. The expression of TG2 protein in pancreatic β-cells

5.2.1.1. BRIN-BD11 rat clonal insulinoma β-cells

As a starting point, it was important to confirm the presence of TG protein reported in chapter 4 by visualising the morphological expression of TG2 antigen in glucose-

Figure 5.1. Immunofluorescence

Figure 5.2

Figure 5.3.

stimulated insulin secreting BRIN-BD11 β-cells. This was achieved using immunofluorescent staining with a well established monoclonal TG2-specific antibody (CUB 7402) originally raised against guinea pig liver TG2. When the BRIN-BD11 cells were stimulated with concentrations of glucose between 5 mM to 40 mM for insulin secretion (**fig 5.1**), TG2 antigen signal appeared to increase intracellularly (**fig. 5.1., panels A, D, G, J**) together with the presence of insulin content (**fig. 5.1, panels B, E, H, K**). Co-localisation of both TG2 and insulin antigen in the extracellular adhesion processes of the glucose-stimulated BRIN-BD11 cells was also evident (**fig 5.1., panels C, F, I, L, arrows**), and confirmed using semi-quantitation of the fluorescence signals, where glucose concentrations of 17 mM proved to result in optimal expression ($p < 0.001$) of both insulin granules and TG2 protein (**fig. 5.2.**). Under conditions of low glucose (5 mM), when the primary insulin secreting function of these β-cells was at basal levels (**fig. 5.1., B; fig. 5.2.**), TG2 antigen expression was also at corresponding low levels (**fig. 5.1., A; fig. 5.2**), suggesting the importance of TG2 in this functional β-cell mechanism.

The molecular weight of the TG2 antigen present in BRIN-BD11 cells was confirmed in an immunoblot, showing a shorter CUB 7402 immuno-reactive protein at 60-kDa, compared to the 75-kDa guinea pig liver TG2 positive control (**fig. 5.3. A**). A second immuno-reactive TG2 band was observed at 120-kDa when whole cells were collected for lysis using a cell scraper (**fig. 5.3A**), which was less noticeable when the cells were collected for lysis using the membrane protease trypsin (**fig. 5.3B**). When BRIN-BD11 cells homogenates were treated with increasing glucose concentrations (10 mM to 30 mM), the TG antigen at 60-kDa appeared to increase in response (**fig. 5.3B**), and was confirmed to show maximal TG2 antigen levels at 17 mM glucose ($p < 0.001$) after densitometric analysis (**fig. 5.3C**).

5.2.1.2. Human Islets

The morphology of human islet structure in diabetic (**fig. 5.4., panels, B, D, F**) and normal (**fig. 5.4., panels A, C, E**) pancreas cryosections was viewed using immunofluorescent labelling with CUB 7402 and insulin antibodies. Islets of Langerhans within normal pancreas exocrine tissue could be delineated using

figure 5.4

Figure 5.5

insulin-positive β-cell staining (**fig. 5.4., C, arrows**). Co-staining for TG2 revealed the presence of this enzyme within the insulin-secreting islet structures, and also amongst the other exocrine tissue (**fig. 5.4., A, E**). The diabetic human tissue showed a lack of insulin-containing islet structures (**fig. 5.4., D, arrows**), however TG2 still seemed to be predominantly present within the surrounding exocrine tissue (**fig. 5.4., B, F**).

The molecular weight of the TG2 protein present within human islets was characterised using western blotting with CUB 7402 antibody, revealing two predominant immuno-reactive bands at the normal size of 85-kDa and an additional shorter protein at 60-kDa (**fig. 5.5**.).

5.2.1.3. Wild type ($TG2^{+/+}$) and Knockout ($TG2^{-/-}$) Mouse Islets

Freshly isolated $TG2^{+/+}$ and $TG2^{-/-}$ mouse islets were stimulated for glucose-mediated insulin secretion at 5 mM and 20 mM concentrations, and visualised immunofluorescently for TG2 expression (CUB 7402) and insulin content (**fig. 5.6**). The wild-type islets ($TG2^{+/+}$) showed insulin responsiveness at both 5 mM (**fig. 5.6., panel A**) and 20 mM (**fig. 5.6., panel G**) glucose concentrations with concomitant increases in fluorescent TG2 expression (**fig. 5.6., panels B, H**) that appeared to co-localise with insulin release (**fig. 5.6., panels C, I**). In comparison to the normal mouse islets, $TG2^{-/-}$ mouse islets showed consistently lower insulin granule staining at 5mM (**fig. 5.6., panel D**) and 20 mM (**fig. 5.6., panel J**) glucose levels. Despite the TG2 gene knockdown in these mice, CUB 7402 staining revealed the presence of low levels of residual TG2 expression at both 5 mM (**fig. 5.6., panel E**) and 20 mM (**fig. 5.6., panel K**) glucose concentrations in the $TG2^{-/-}$ mouse islets, which appeared to co-localise with insulin content (**fig. 5.6., panels F, L**) as in the case of the normal mouse islets.

Immunoblot analysis for the molecular weights of TG2 proteins present in mouse islets revealed the presence of two CUB 7402 immunoreactive short-form proteins in $TG2^{+/+}$ islets at 60-kDa and 35-kDa, compared to the 75-kDa purified guinea pig liver TG2 positive control (**fig. 5.7**.). Surprisingly, despite TG2 gene knockdown, the $TG2^{-/-}$ mouse islets exhibited CUB 7402 immunoreactive proteins at molecular

Figure 5.6 Immunofluoresence

Figure 5.7

weights of 60-kDa and 120-kDa, similar to the BRIN-BD11 rat clonal cells (**fig. 5.7.**).

2-D analysis of TG2 in the BRIN-BD11 proteome

More intensive characterisation of the BRIN-BD11 β-cells was performed by western blotting after 2-D SDS-PAGE analysis (**fig. 5.8**). The total BRIN-BD11 proteome was characterised for the first time by silver staining the 2-D SDS-PAGE gel, with IPG strips in the 4-7 pH range proving optimal for isoelectric focussing (**fig. 5.8.A**). When 2-D SDS-PAGE gels were transferred electrophoretically to nitrocellulose membranes and stained for TG2 proteins using CUB 7402 antibody, one immunoreactive spot was evident at 120-kDa (pI ~5.5), seven predominant spots at ~60-kDa (between pI 4-6), and 3 spots around ~35-kDa (between pI 4-6). Three separate attempts were made to pick immunoreactive spots matching those observed in the immunoblot for peptide mass fingerprinting using mass spectrometry (**Table 5.2.**), however despite a number of proteins being recognised, the method was unsuccessful in confirming a TG protein.

5.2.1.4. MMP proteolysis and GTP-binding potential of BRIN-BD11 TG2

In order to determine whether the 60-kDa short-form immunoreactive TG2 might have been the result of proteolysis by matrix metalloproteases (MMPs), whole BRIN-BD11 β-cells were treated with broad range MMP inhibitors (Galardin, 364205) and resulting western blots were stained using CUB 7402 antibody (**fig. 5.9.**). MMP inhibition resulted in no molecular weight changes of the TG2 short-form proteins originally observed, when compared to negative controls (**fig. 5.9.**).

Following on from the inhibition of TG activity in BRIN-BD11 cells as a result of GTP-γ-S treatment (**fig. 4.1**), it was important to determine whether the 60-kDa β-cell TG protein observed in these investigations might have the potential for this GTP-binding ability. The BRIN-BD11 cells were separated into cytosolic and membrane fractions, and incubated with GTP-agarose beads to reveal predominant GTP-binding by the 60-kDa TG protein in the membrane fraction (**fig. 5.10**).

Figure 5.8. 2-D gel

Table 5.2

Figure 5.9

Figure 5.10

5.2.2. TG1, TG2 and TG3 screening of clonal β-cells

Further analysis of the TG profile in clonal pancreatic β-cells was continued using western blotting. When another TG2 antibody (ID-10) developed in-house and raised against guinea pig liver TG2 was used on the BRIN-BD11 cell lysates, the immunoblot did not pick up the 60-kDa short-form protein previously reported with CUB 7402 (**fig. 5.11A**).

Two additional β-cell lines were tested for TG2 antigen using CUB 7402 antibody, and similar short-form 60-kDa immunoreactive proteins were detected (**fig. 5.11B**) in the more differentiated mouse pancreatic insulinoma β-cell line (MIN-6) and rat pancreatic insulinoma cell line (INS-1E).

A screening for the possibility of other TG proteins besides TG2 in BRIN-BD11 and MIN-6 cells revealed the presence of TG1-positive bands in the molecular weight range of 65- to 75-kDa in both cell lines (**fig. 5.11 C, D**). Western blot analysis using TG3 staining revealed the absence of any immunoreactive bands in both MIN-6 and BRIN-BD11 cells (**fig. 5.11 E, F**).

The mRNA isolated from BRIN-BD11 rat clonal β-cells, freshly isolated rat islets and rat pancreas were used in RT-PCR experiments where conventional probes directed at the TG1, TG2 and TG3 active-site regions were constructed (**fig. 5.12A**). The PCR products were electrophoresed on a 1.5% agarose gel, and ethidium bromide stain was quantified using densitometric analysis (**fig. 5.12B**). Untreated BRIN-BD11 cells (**fig. 5.12A, lane a)**, rat islets (**fig. 5.12A, lane d**) and rat pancreas (**fig. 5.12A, lane e**) possessed high basal levels of TG2, with low levels of TG1 and virtually no signal for TG3 (**fig. 5.12B**) present.

BRIN-BD11 cells treated with 0.1 µM and 3 µM all *trans* retinoic acid for 48 hours (**fig. 5.12A, lane b**), resulted in significant ($p < 0.05$) up-regulation of TG1 and a concomitant down-regulation of TG2, compared to the untreated BRIN-BD11 controls (**fig. 5.12B**).

Figure 5.11.

Figure 5.12

5.2.3. The expression of TG2 mRNA in BRIN-BD11 β-cells

5.2.3.1. Northern blotting

To verify whether the short-form 60-kDa β-cell protein observed using western blotting was possibly sourced from the mRNA level, northern blotting was employed (**fig. 5.13 and fig. 5.14**). BRIN-BD11 cells revealed the presence of a full-length 3.5-kb TG2 transcript (~30% expression), and additional shorter transcripts in the 2.5-kb (~60% expression) and 1-kb range (~10% expression), after a minimum of 5 hrs exposure with a BAM 388 TG2-specific probe directed towards the N-terminus of the TG molecule (**fig. 5.13C**). Exposure of another BRIN-BD11 northern blot for 24 hours with a BAM 1638 TG2-specific probe directed towards the C-terminus of the TG molecule, showed a predominant short-form TG2 transcript in the 2.5-kb range (~90% expression) and minimal expression (~10%) of the full-length 3.5-kb TG2 transcript (**fig. 5.13D**).

Northern blot analysis of the TG2 mRNA profile in other clonal β-cells exhibited only the full-length 3.5-kb TG2 transcript in MIN-6 mouse β-cells when the N-terminus BAM 388 probe (**fig. 5.14A**) was used (5 hours exposure), and no signal evident when the C-terminus BAM 1638 probe (**fig. 5.14B**) was used (24 hours exposure). The other rat clonal β-cell line, INS1-E exhibited a TG2 transcript slighter shorter (~2-kb) than the BRIN-BD11 TG transcript present in the 2.5-kb range, when this northern blot was exposed for 24 hours to the C-terminus BAM 1638 TG2-specific probe (**fig. 5.14B**).

5.2.3.2. RT-PCR analysis

PCR analysis of mRNA derived from the rat clonal BRIN-BD11 β-cells (**fig. 5.15**) was performed using primers constructed specifically to lead to the synthesis of distinct C-terminal fragments, corresponding to either full-length or alternatively spliced short-form TG2 transcripts previously reported in rat brain cells (Monsonego *et al.*, 1997). The BRIN-BD11 rat β-cells exhibited a 512 bp fragment consistent with full-length TG2 and a corresponding 410 bp fragment for short-form TG2 when PCR amplification products were electrophoresed on a 1.5% agarose gel (**fig. 5.15**),

Figure 5.13

Figure 5.14

Figure 5.15

suggesting similarities between the shortened TG2 isoform present in rat brain and rat pancreatic BRIN-BD11 cells.

5.3. Discussion

Investigations into the β-cell specific TG2 present within BRIN-BD11, human and $TG2^{-/-}$ mouse islets were performed in the current chapter using protein and mRNA characterisation techniques. The ~60-kDa short-form CUB 7402-immunoreactive protein in BRIN-BD11 cells was evident from immunoblots at the outset of this investigation, despite numerous attempts at maintaining low temperatures and varying lysis buffers or protease inhibition of the tissue culture samples before western blot analysis. Within the context of a short-form β-cell TG protein being evident through western blot analysis, the question remains about whether the protein may be an alternatively spliced variant, a proteolytically cleaved functional isoform, a possible degradation product, or that the antibody specificity may require further confirmation for the antigen being tested. Nonetheless, the presence of a non-classical truncated TG protein present within these β-cells dictated the route of investigation in the current chapter.

At the outset, membrane proteolysis through routine BRIN-BD11 cell culture trypsinisation procedures resulted in slight changes between the signal strength at 120-kDa and 60-kDa. Interestingly, in studies performed by Knight *et al*., (1990a and b), proteolysis of the 120-kDa TG2 protein by thrombin and trypsin resulted in the expression of an 80-kDa TG2 isoform, suggesting that more extensive proteolytic investigations of the BRIN-BD11 β-cell TG could potentially reveal further information. In the present study, the short-form 60-kDa β-cell TG2 did not seem to be affected by broad range MMP proteolysis. It would be interesting to assess the activation of other proteases such as calpain on the β-cell TG2 during stimulus secretion coupling, since it has been reported in SH-SY5Y cells (Zhang *et al*., 1998) that depletion of GTP increases TG2 degradation by calpain after elevation of intracellular Ca^{2+} levels, which results in a reduction of TG2 activity as well.

Western blot analysis of BRIN-BD11 lysates separated into cytosolic and membrane fractions revealed that the 120-kDa immunoreactive protein was associated mostly with the membrane fraction, and exhibited GTP-agarose binding potential. In addition, the membrane-associated 60-kDa CUB 7402 immunoreactive protein showed the potential to bind GTP-agarose, suggesting that the GTPase function of TG could be attributed to the cytoskeletal exocytotic web present in the particulate fraction of β-cells. The CUB 7402 antibody is monoclonal with an epitope targetting the TG2 Ca^{2+}-binding site region (see fig. 5.16.), suggesting a structural conservation in this region for the short-form β-cell enzyme. This Ca^{2+}-dependent TG activity in β-cells was also evident from the assays demonstrated in chapter 4. The influx of Ca^{2+} during stimulus secretion coupling may therefore be an important contributor to the activation of the TG enzyme in these β-cells. Immunofluorescent visualisation of the TG2 protein in BRIN-BD11 cells using the same CUB7402 antibody confirmed the large extent to which this enzyme was up-regulated upon 17 mM glucose-stimulation, co-localising with the assembly of insulin granules in extracellular secretory processes.

Multiple TG2 CUB 7402-immunoreactive spots were evident in the ~60-kDa molecular weight range between the predicted isoelectric point (pI) 4 to 6, when western blot analysis was performed following 2-D SDS-PAGE separation of BRIN-BD11 proteins. This may be an interpreted as an indication of post-tranlational modifications to the TG2 protein that migrated to ~60-kDa, possibly through phosphorylation (Riederer, 2008). Additional spots were evident at 120-kDa and ~35-kDa from these whole cell lysates, confirming the characterisation of immunoblots described above. The pI from the short-form TG2 characterised by Fraij *et al.,* (1992) was 4.84, while classical human TG2 has a pI of 4.95, guinea pig liver TG2 is 4.92, and human factor XIIIa exhibits a pI of 5.88. In spite of the 147 amino acids missing in the short-form TG2 characterised by Fraij *et al*. (1992), when compared to full-length TG2, the pI's were similar implying that the amino acids at the carboxy-terminal of full length TG2 may not have a drastic effect on the tertiary structure of the protein. Unfortunately, many attempts at protein spot-picking for mass spec analysis did not reveal peptides matching any TG, in the course of these investigations. Nonetheless, a search through the literature revealed that the BRIN-

Fig. 5.16

BD11 proteome has not been published to date, making these investigations a good foundation for the continued characterisation of this particular pancreatic β-cell line.

Despite published reports by Porzio *et al*., (2007) of a single 85-kDa isoform for human islet TG2 when the same CUB 7402 antibody was used, in this investigation, there remained the consistent presence of an ~60-kDa short-form TG2 protein (in addition to the 85-kDa protein already published). The discrepancy between both results could be due to differences in lysate preparation, or half communication by these authors as a result of dismissing any shorter bands as degradation products, as can be the case with western blot analysis. Taking into account the two prominent TG2-postive bands at 60-kDa and 85-kDa in human islets within this investigation, but the lack of an ~85-kDa band in clonal BRIN-BD11 β-cells, it may be tempting to speculate that a microcapillary source of TG2 is always present in freshly isolated islets and could reflect the differences in protein sizes between endothelial- and β-cell sources. DNA and protein sequencing experiments for human islet TG2 would be worth pursuing in the future as a means of substantiating these differences.

Results from RT-PCR analysis of human islet mRNA has been recently published (Porzio *et al*., 2007), revealing high amounts of TG2 expression when primers were directed at the TG2 active site region, which was also evident during preliminary experimentation in this study. The data was not presented here due to the extent of RNA degradation in the three separate batches of cryopreserved human islets obtained from the UK Tissue Bank (UKTB). In the human diabetic pancreas, despite reductions in insulin-producing β-cells or islet-structures compared to normal controls, there were still high levels of TG2 antigen in diabetic exocrine tissue, suggesting additional roles for TG2 that may not be related to insulin function, but rather associated with a cell survival role within the diseased pancreas. However, the result of human pancreas cryosections being viewed by confocal microscopy meant that the TG2 antigen levels could have been over-emphasised, despite background autofluorescence being subtracted, so that it may be worth assessing the presence of TG2 during the stress response of diabetes, using more quantitative biochemical techniques.

Western blot analysis of wild-type mouse islets revealed the presence of a pre-dominant short 60-kDa protein, and an even shorter 35-kDa product, similar to that observed in the 2-D SDS-PAGE BRIN-BD11 immunoblots. Investigations carried out by Fraij and Gonzales (1996) reported the presence of a 38-kDa human TG2 isoform as a result of alternative splicing, which was structurally made up of the N-terminus region of the TG2 molecule, and contained the Cys_{277} active site region. In order to confirm the possibility of shorter functional mouse TG2 isoforms, protein and DNA sequencing would be required. Until then any shorter immunoreactive bands are certainly worth reporting in an attempt towards characterisation for future reference. Immunofluorescent analysis of wild-type mouse islets isolated through collagenase digestion, and stimulated with 20 mM glucose revealed co-localised insulin and TG2 staining, however background autofluorescence within mouse tissue, as in the case with human tissue, remains an issue that needs to be overcome when employing this technique.

Western blot analysis of lysates from both the wild type and $TG2^{-/-}$ mouse islets demonstrated the presence of an immunoreactive band at ~60 kDa corresponding to the short-form protein found in BRIN-BD11 cells. This CUB 7402 immunoreactive band in the $TG2^{-/-}$ mouse islets could be viewed as a contradiction to the original findings of Melino and co-workers (De Laurenzi, *et al*., 2001). The reason for this apparent discrepancy is unclear, although a clue may come from examining the construct used to disrupt the TG2 gene. Generation of the original TG2 knockouts resulted from the replacement of exons 5 and 6 with a targeting vector containing the neomycin resistance gene (fig. 5.17). Whilst this abolished TG2 activity, there could presumably still be transcription to give an inactive protein. It could be that the original recombination resulted in the generation of a stop codon that lead to the production of an inactive and slightly truncated protein. If the truncated region did not incorporate the binding site of the TG2 antibody, then probing would fail to detect a TG2 band in these animals. It could be speculated that the loss of such a stop codon through repeated breeding of the TG2 knockouts would again lead to read through and the generation of the 60-kDa TG2 protein, which appeared prominently in the islets of the $TG2^{+/+}$ wild-type counterparts also. However, it also cannot be ruled out that the original TG2 knockouts might have shown the presence of this 60-kDa TG2 protein in the mouse islets, given that previous western blot

Fig 5.17

analysis was only performed on liver and thymus extracts in the original publication (De Laurenzi *et al*., 2001). In a second TG2-deficient mouse model created by Nanda *et al*. (2001) the absence of a 75-kDa band confirmed the successful generation of a $TG2^{-/-}$ genotype, however three bands at 60-kDa, 50-kDa and 20-kDa were still reported in heart and liver extracts using the same CUB 7402 antibody.

The presence of a TG2 positive 60-kDa band in the $TG2^{-/-}$ islet lysates is likely to represent an inactive form of the enzyme since generation of the knockout results from disruption of the active site of TG2 rather than deletion of the gene. This is supported by the measurement of TG activity in the $TG2^{-/-}$ islets presented in chapter 4 using cross-linked FITC cadaverine and [^{14}C]-putrescine, which showed an extensive decrease of TG activity. However $TG2^{-/-}$ islets still retained about half the TG activity of wild type cells when endogenous β-cell substrate proteins were used. Immunofluorescent analysis of the $TG^{-/-}$ mouse islets upon 20 mM glucose stimulation revealed slight increases in the CUB7402-reactive TG2 protein and associated insulin content, however the levels of expression were much lower compared to the $TG2^{+/+}$ islets. These results are in keeping with studies undertaken by Bernassola *et al*. (2002), where fasting insulin release and glucose-stimulated insulin secretion from $TG2^{-/-}$ islets revealed mild impairment in the β-cell secretory mechanism due to the TG2 gene disruption. Only slight differences were noticeable in islet β-cell mass within the $TG^{-/-}$ islets, compared to wild type controls in this study, corroborating morphological observations made by these authors (Bernassola *et al*., 2002). An additional point worth considering is that the TG2 knockout model is expected to affect TG2 expression in both the β-cells and the endothelial microvasculature of the functional islet, which should in combination contribute towards the diabetes phenotype of these mice.

Not much is known about the expression of other TG enzymes in mouse pancreatic islets apart from TG2 that may account for this enzyme activity. A likely candidate could be TG1 (keratinocyte TG) since this enzyme is expressed in the major organs of the $TG2^{-/-}$ mouse in the absence of TG2 activity (De Laurenzi *et al*., 2001) and is significantly expressed (albeit at low levels) in human islets using RT-PCR analysis (Porzio *et al*., 2007). Taken together with the possibility of other TGs compensating

for the targeted disruption of TG2 gene function as well, the amount of TG activity reflected in the $TG2^{-/-}$ model should be viewed more holistically. It would be interesting to analyse the TG2 mRNA profile of the $TG2^{-/-}$ mice using northern blot analysis and RT-PCR, in order to verify transcription and splicing as a result of intron manipulation in these mice.

Screening for the presence of TG1, TG2 and TG3 in β-cells was performed on the protein level using western blotting, and extended to mRNA analysis using RT-PCR. The ID-10 antibody was raised against a guinea pig liver TG2 immunogen, which is known to detect an 80-kDa protein with purified human TG2, but more specifically targets plant TG (pers. comm. Dr. Alan Hargreaves). In this instance, the ID10 antibody did not detect the BRIN-BD11 β-cell TG. It would be interesting to understand why there was no immunoreactivity in this case, but seeing that the ID10 antibody was developed in-house and has not been fully characterised for site-specificity, these results cannot be further verified. The amount and distribution of rat pancreatic mRNA using specific primer sets and known cDNA sequences for TG1, TG2 and TG3 were analysed through RT-PCR. The relative amounts of TG transcripts standardised to GAPDH content through densitometry revealed an abundance of TG2 within BRIN-BD11 cells, with a low levels of TG1 and virtually no TG3 present in rat β-cells, which was similar to the trend previously reported in human islets (Porzio *et al*., 2007).

Preliminary immunoblots used to detect the presence of TG1 were expected to give a positive band (if any) at 106-kDa or 92-kDa, however immunoreactive bands were evident in the 60-70-kDa size range in both BRIN-BD11 and MIN-6 cells. The TG1 protein is an inactive zymogen at 106-kDa and 92-kDa (depending on membrane attachment through a 10-kDa adduct), and is then proteolytically cleaved into a 67-kDa; 67-kDa/33-kDa complex; or 67-kDa/33-kDa/10-kDa complex that has respectively increasing transamidating activity (Kim *et al*., 1995; Steinert *et al*., 1996). Further analysis for a possible β-cell specific TG1 would be interesting seeing as lysate preparations using keratinocytes are already known to result in the differential expression of TG1 proteins through western blot analysis and TG activity assays. For instance; trypsin treatment for 15 minutes usually results in a proteolytically cleaved 80-kDa version of the TG1 protein within particulate

fractions (Rice *et al.*, 1990); while the use of dispase treatment on cytosolic fractions produces an activated 67-kDa/33-kDa/10-kDa TG1 form (ie. 106-kDa) which has very high transamidating activity when measured through common TG assays (Kim *et al*., 1995). Membrane-bound TG1 can also be released from particulate fractions so that the 10-kDa lipid adduct is still attached to the protein with the use of Triton-X-100 (Steinert *et al.*, 1996), whereas endogenous proteolytic cleavage is also possible by incubating particulate fractions in the presence of high Ca^{2+} at 37°C for 90 minutes with controlled protease inhibition (Rice *et al*., 1990). Additionally, calpain treatment, retinoic acid and the use of protease inhibitors are known to affect the level of TG1 protein expression in western blot analysis (Michel *et al.*, 1989). Immunoprecipitation experiments using both TG1 and TG2 antibodies for the BRIN-BD11 β-cells would confirm whether both TG proteins play any associative or functional roles, as suggested by these preliminary investigations.

Immunoblot analysis for TG3 revealed the absence of this protein in BRIN-BD11 and MIN-6 cells, as was observed through RT-PCR analysis. Much like TG1, the TG3 protein requires proteolytic cleavage to facilitate activation, where the latent pro-enzyme exhibits a molecular mass of 77-kDa which is cleaved to produce a catalytically active 50-kDa fragment and a 27-kDa carboxy-terminal peptide (Kim *et al*., 1990). The proteases responsible for the activation of TG3 remains unknown, however both dispase and calpain have been used successfully to proteolyse the TG3 zymogen. Perhaps the most significant part of the lack of TG3 in β-cells is the possibility that the GTP-binding potential observed in the BRIN-BD11 cells so far could most likely be the result of TG2 or TG5, seeing as only TG2, TG3, and TG5 have the conformational capacity to bind GTP, compared to the other TGs (Liu *et al*., 2002; Ahvazi *et al*., 2004a, Candi *et al*., 2004).

Retinoids are known to increase TG gene transcription and TG activity in a variety of tissues and cell lines (Davies *et al*., 1988; Goldman, 1987; Suedhoff *et al*., 1990; Rubin *et al*., 1986; Jetten *et al*, 1986; Mehta *et al*., 1987; Nara *et al*., 1989; Michel *et al*., 1989; Denning *et al*., 1991, Driscoll *et al*., 1997). TG activity assays presented in chapter 4 showed a dependence of BRIN-BD11 cells on *all trans* retinoic acid for an almost 2-fold increase in TG activity. Pilot experiments using RT-PCR analysis in this chapter, revealed that under the 48 hours treatment of 0.1 μM retinoic acid, there

was a decrease in the amount of TG2 PCR product but a noticeable upregulation in TG1 transcript compared to untreated cells. These results are intriguing when viewed in the context of studies performed by Driscoll *et al*., (1997) where a 100% increase in retinoic acid-stimulated TG activity was observed within RIN-m5F (the parent cell line of BRIN-BD11), however no classical 3.5-kbTG2 transcript was evident in these cells with or without retinoic acid stimulation, using northern blot analysis of mRNA. Of further interest, these authors also reported that the retinoic acid receptor β (RARβ) mRNA was not evident under northern blot analysis of the RINm5F cells, but was present in the slower growing INS-1E and βTC6-7 cells (Driscoll *et al*., 1997). It would have been worthwhile assessing the effects of retinoic acid on the shortened β-cell TG2 mRNA expression in this investigation using northern blotting, but such experiments were beyond the scope of the current timeframe.

Since the TG activity assay used in chapter 4 does not exclude the action of TG1, increases in catalytic activity within BRIN-BD11 cells may still be due to increases in this enzyme on the transcriptional level. The possibility of retinoic acid stimulation inducing a more differentiated form of BRIN-BD11 cells that results in an upregulation of TG1 seems plausible. Cytoplasmic TG1 is known to be directly involved in the cornified envelope of the epidermis by covalently cross-linking a number of different subplasmalemmal proteins. Recently, TG1 was shown to be expressed in tissues other than the keratinocytes in the epidermis (Eckert *et al.*, 2005) such as the brain hippocampus and cerebral cortex (Ientile *et al*., 2004), liver (De Laurenzi *et al*., 2000), lung and kidney (Hiiragi *et al*, 1999), and more recently myocardium microvascular endothelial cells (Baumgartner *et al.*, 2004 and 2007). However, its functional importance within these tissues is far from being understood, but may be worth noting when considering the large endothelial source of TG protein present within the microvascular structures associated between aggregated β-cells in islets (Nikolova *et al*., 2004).

In a recent study, the cytoplasmic TG1 enzyme was shown to be expressed in murine myocardial microvascular endothelial cells (MyEnd cells) where it gradually accumulates underneath the junctional cell surface during maturation of confluent monolayers (Baumgartner *et al*., 2004). This endothelial barrier is regulated

according to external stimuli via two types of intercellular junctions, tight junctions and adherens junctions (Lampugnani *et al.*, 2007). Treatment of the MyEnd monloyers with monodansylcadaverine, TG inhibitor R281, as well as specific down-regulation of TG1 by gene silencing siRNA negatively affected the sensitivity of the monolayer towards permeability-increasing stimuli such as depletion of extracellular Ca^{2+} and treatment with the actin-fragmenting compound cytochalasin-D (Baumgartner *et al*., 2002; 2004), showing that TG1 is causally involved in the strengthening of endothelial barrier properties. Baumgartner *et al*., (2007) showed that the actin network is stabilised in late confluent MyEnd cells, an effect that can be reversed by TG blocking. Effective immobilisation or clustering of cell-cell adhesion molecules by linkage to the cortical actin filament network dramatically increased intercellular adhesion without changing the extracellular affinity of the adhesion proteins (Baumgartner *et al*., 2004). TG1-induced cross-linking or stabilisation of junction associated actin may serve to immobilise cadherins, thereby stabilising cadherin-mediated intercellular adhesion, which could be very interesting in the context of cell-cell adhesion within islets and the exocytosis of insulin in these aggregated β-cells.

The questionable nature of multiple β-cell tissues possessing a low molecular weight ~60-kDa TG2 protein as presented in this chapter, was addressed with the use of northern blotting probes that were designed to specifically target the TG2 molecule (Johnson *et al*., 1997). The original TG3400 probe was digested with BAMH1 to give a BAM 388 (towards the N-terminal) and BAM 1638 probe (towards the C-terminal) as described in fig. 5.18. The use of these probes confirmed for the first time the presence of a shortened mRNA transcript in pancreatic β-cells. The BRIN-BD11 cells exhibited a full-length 3.5-kb TG2 transcript, and additional truncated transcripts in the 2.5-kb and 1-kb range following exposure to the BAM 388 TG2-specific probe. The BAM 1638 TG2-specific probe highlighted a predominant short-form TG2 transcript in the 2.5-kb range with minimal expression of the full-length 3.5-kb TG2 transcript. The differences in signal strength as a result of these two probes could signify clues into the structural nature of this β-cell TG molecule, since the BAM 1638 would be expected to hybridise towards the C-terminal end, a region expected to be the first to undergo possible deletions as a result of shortened transcripts being produced by the β-cell. More specifically (see fig. 5.18), the

Fig. 5.18

BamHI 388bp probe would be expected to recognise rat TG2 in the region 1267-1655 (and mouse 1244-1632); while the BamHI 1638bp probe should recognise rat TG2 in the region 1656-3298 (mouse 1633-3270), suggesting that the β-cell TG may have variations in the C-terminal end, compared to full length TG2.

When two other clonal β-cells were tested for CUB 7402 immunoreactivity, both the mouse insulinoma MIN-6 cells and rat insulinoma INS1-E cells showed a ~60-kDa TG2 protein similar in size to the BRIN-BD11 cells, suggesting the potential for these additional β-cell models to be used in future characterisation studies of the short-form TG2. Northern blot analysis of these two cell types revealed the presence of a full-length 3.5-kb TG2 transcript in MIN-6 mouse β-cells when the BAM 388 probe was used, with no signal evident as a result of the BAM 1638 probe; whereas the INS1-E rat cells exhibited a TG2 transcript slighter shorter than the BRIN-BD11 TG2 transcript in the ~2.0-kb range, after exposure to the BAM 1638 TG2-specific probe. Both MIN-6 and INS-1E are slower growing and more differentiated β-cell lines, compared to BRIN-BD11 and its parent cell line, RINm5F, so it is not surprising that there may be differences in the TG expression profile of these insulin secreting cell-types. For instance, there was the absence of a 3.5-kb TG2 transcript in RINm5F cells, but evidence of this TG2 transcript in INS-1E cells when northern blotting was performed by Driscoll *et al*. (1997), using a different TG2-specific probe. Variations in mRNA and protein expression are a common occurrence in the regulation of gene expression. Discordance in mRNA abundance between the three β-cell lines (BRIN-BD11, MIN-6 and INS-1E) in this investigation using western blotting compared to northern blotting and that of its translated product may also be due to low transcription rates of the mRNA, rapid degradation or aberrant messaging. Furthermore, northern blot analysis may not reflect transcriptional activity of TG2 in a time or dose-dependent manner, and there remains the possibility that mRNA is actively translated to measurable TG protein levels, and then rapidly degraded.

Scanning the literature for shorter TG2 proteins, or shortened mRNA transcripts led to an area of research that dealt with the discovery of alternatively spliced TG2 genes by a few authors (Fraij *et al*., 1992; Fraij and Gonzales, 1996; Monsonego *et al*., 1997; Citron *et al*., 2001; Antonyak *et al*., 2006; Liu *et al*., 2007; Tee *et al*.,

2010). Alternative splicing was observed earliest in human samples of cultured human erythroleukemia (HEL) cells (Fraij and Gonzales, 1997, 1996; Fraij, 1996; Lee *et al*., 1993; Fraij *et al*., 1992), where cloning and sequencing of the protein revealed a 1.9-kb cDNA transcript, consisting of a 548 amino acid polypeptide, with a molecular weight of 61.7-kDa. The deduced amino acid sequence revealed a 98% homology to the human TG2 sequence, with the cysteine position at 277 and the putative Ca^{2+}-binding site (446-453aa) being conserved (Fraij *et al*., 1992). Furthermore, comparison of the 5`-end (bases 1-1747) of the 1.9-kb short form homologue to the full-length 3.5-kb TG2 protein showed a high degree of similarity, with the divergence point starting at 1748 on the 3` end showing no homology, and correlating with known intron-exon consensus boundaries as indicative of alternative splicing (Fraij *et al*., 1992). Northern blot analysis of the short form TG2 in HEL cells (Fraij *et al*., 1992) revealed the absence of the classical 3` end in the shorter 1.9-kb transcript, and eliminated the possibility that smaller RNA species were due to a degradation of the classical 3.5-kb transcript. When the alternatively spliced products in HEL cells were compared to normal fibroblasts (Fraij *et al*., 1992), the short-form 1.9-kb transcript was expressed at much lower levels, suggesting that the short-form in HEL cells may have been associated with oncogenesis.

Furthermore, a third human homologue of TG2 was described by Fraij and Gonzales (1996), and was characterised by a polypeptide encoding 349 amino acid residues with a molecular weight of 38.7-kDa by SDS-PAGE. The region coding for the first 286 amino acids of the short-form TG2 (63-kDa), which contains the active site, was identical to this third TG2 (38-kDa) homologue (Fraij and Gonzales, 1996). This 38-kDa variant differs from the 63-kDa isoform in that it was reported to be 199 amino acids shorter, and had an alternative 63 amino acid COOH-terminal peptide. It was concluded from these studies that the smaller RNA species encode for truncated proteins with novel carboxyl termini, since both 63-kDa and 38-kDa isoforms produced transcripts that start with the regular coding sequence for TG2 and then fail to splice at specific donor sites, resulting in the use of an alternative exon that contains a stop codon (Fraij and Gonzales, 1996). It has been suggested that the additional amino acids in both these TG2 isoforms generate glycine-rich areas homologous to the consensus GTP-binding regions (Takeuchi *et al*., 1992), and it was confirmed that this short-form 63-kDa TG2 isoform displayed a higher GTP-

binding activity than the native 80-kDa human TG2 enzyme (Fraij, 1996), which may be related to functions in cell signalling.

The short TG2 isoform observed in human AD brain (Citron *et al*., 2001) was identical to that found in the HEL cells. The truncation of the TG2 C-terminal region through alternative splicing results in the loss of a domain required for GTP-binding (Citron *et al*., 2002). However, this is not the 15 amino acid region in the core domain that was identified by others as being required for GTP-binding [^{159}YVLTQQGFIYQGSVK^{173}] (Iismaa *et al.*, 1997; 2000). The shorter TG2 isoform differs from the full-length TG2 with respect to the absence of 30 amino acid residues of the C-terminus (Monsonego *et al.*, 1997). This truncation affects the barrel 2 region of TG2, where GTP binds to the region Lys^{171}. It is thought that the loss of this region produces a much more active enzyme, since the inhibitory effect of GTP is minimised allowing optimal conformation for Ca^{2+}-binding. This short TG2 isoform with higher crosslinking activity is also present in the cytokine treated rat astrocytes in culture (Monsonego *et al.*, 1997), and can be seen with the increased cross-linking observed in affected Alzheimers disease brain regions presenting a likely mechanism underlying the deleterious production of aggregated proteins (Citron *et al*., 2001; Norlund *et al*., 1999). Furthermore, the crosslinking found in these neuronal proteins supports a reciprocal role between cross-linking ('irreversible' aggregation) and proteosome function, where proteosome inefficiency is thought to underlie Alzheimers disease with an emphasis on oxidative stress (Citron *et al*., 2001; Checler *et al*., 2000). The important aspect of alternative splicing observed in TG2 may be the result of a critical transcriptional regulatory process that becomes disrupted at some early stage during the development of neurodegeneration. The identification of a balance between aggregation, proteosome function and apoptosis is highlighted within neurodegeneration as a possible therapeutic target (Citron *et al*., 2002).

Pioneering studies by Monsonego *et al*. (1997) on rat brain astrocytes reported that only low levels of the full-length TG2 transcript (3.7-kb) were expressed, whereas treatments with the cytokines IL-1β and TNF-α upregulated TG2 transcript levels with the induction of an additional 2.4-kb transcript. When this rat astrocyte TG was

cloned, the cDNAs were both almost identical and shared a marked homology with full-length TG2 (encoding an ~77-kDa protein), except that the shorter form (~73-kDa) encoded an enzyme with a different C-terminus (than the longer form) that accounted for lower GTP-dependence and consequently higher Ca^{2+}-dependence (Monsonego *et al*., 1997). A schematic representation of both the alternatively spliced TG2 isoforms present in human (Fraij *et al*., 1992) and rat (Monsonego *et al*., 1997) have been described in figure 5.19, where the predicted C-terminal site-specificity of the northern blotting probes (BAM 388 and BAM 1638) used in this study have also been shown.

An interesting study revealed that the clonal isolation of multipotential precursor cells from the adult pancreas express markers characteristic of both pancreatic and neuronal precursors (Seaberg *et al*., 2004). These authors reported that since these cells were able to generate multiple neuroectodermal and endodermal cell types they were termed pancreas-derived multipotent precursors (PMP's), and upon differentiation, these clonal pancreas colonies generated multiple types of neural progeny (including astrocytes, oligodendrocytes and mature neurons). These cells also had the potential to produce islet endocrine sub-types such as insulin-producing β-cells, α-cells and δ-cells, as well as exocrine acinar cells and pancreatic stellate cells (Seaberg *et al*., 2004), where pancreas colonies also generated a significantly higher proportion of neurons than the adult brain-derived clonal neurospheres. The capacity of PMP's to generate neural and pancreatic progeny may be explained by two alternative hypotheses: i) the pancreas and brain have a common embryological origin from an ectodermal/endodermal precursor population that exists during early embryonic development; or ii) the similarity in gene expression patterns of the brain and pancreas (eg. nestin, Ngn3, Beta2/NeuroD, Pax6) indicate that evolution has re-used the same 'toolbox' of genes in two otherwise unrelated tissues (Seaberg *et al*., 2004). It is notable that neuronal cell bodies lie in close juxtaposition to islet β-cells in the postnatal pancreas (Persson-Sjogren *et al*., 2001) and therefore may a play a role in the co-ordination of insulin release. This relationship could be extended to the possibility that the alternative splicing reported in rat brain (Monsonego *et al*., 1997) may very well be related to the shorter TG2 transcripts observed in the BRIN-BD11 rat clonal pancreatic β-cell observed in this investigation.

Fig. 5.19.

The possible similarity in alternative splicing events between brain and pancreatic β-cells was confirmed in the present study with preliminary RT-PCR experiments using the C-terminal directed TG2-specific primers (refer to fig. 5.19) designed by Monsonego *et al*., (1997). As demonstrated previously with the same primers by Tolentino *et al.* (2002) in rat brain, the BRIN-BD11 cells also exhibited PCR amplification products of a 512 bp fragment consistent with full-length TG2 and a corresponding 410 bp fragment for short-form TG2. According to the primers described by these authors (Monsonego *et al*., 1997; Tolentino *et al*., 2002; Citron *et al*., 2002), the 5`TG (bp 1470-1489) recognized an upstream homologous sequence in full-length TG2 (TG-L) and short-form TG2 transcripts (TG-S). The 3`TG2-L (bp1961-82) recognized a downstream TG2-L mRNA-specific sequence, while the 3`TG2-S (bp 1861-1872 and 2083-93) recognized a downstream TG2-S mRNA-specific sequence. It was predicted that the 3`TG2-S primer bridges the junction created by the absence of bp 1873-2082 (a sequence present exclusively in TG2-L). Half of the 3`TG2-S sequence was expected to hybridize to both TG2-L- and TG2-S-specific sequence, while the full length primer was seen to hybridize only to TG2-S mRNA sequence (Tolentino *et al*., 2002). The possibility of a shortened TG2 isoform in BRIN-BD11 cells, that could be similar in function to the rat brain TG2 isoforms remains to be fully elaborated in the future, however these initial data are promising.

The evidence already existing for shortened, alternatively spliced variants of TG2 have shown deletions and alterations in the C-terminal end of these molecules, where the GTP-binding and phospholipase C regions are expected to be compromised (Fraij *et al*., 1992; Fraij and Gonzales, 1996; Monsonego *et al*., 1997; Citron *et al*., 2001; Antonyak *et al*., 2006; Liu *et al*., 2007; Tee *et al*., 2010). It was recently suggested that the site of ATP and/or GTP hydrolysis in TG2 is located in the N-terminal region between amino acid residues 1 and 185 (Lai *et al*., 1996). In another study, a 36-kDa protein was co-purified with an 80-kDa TG2 from rabbit liver nuclei, where the 36-kDa fragment was identified as the N-terminal region of the enzyme, and consisted of the first domain and part of the core domain, retaining GTPase activity (Singh *et al*., 1995). More recently, GTPase activity was detected following deletion of both the N-terminal β-sandwich domain and the two C-terminal β-barrel domains (Iismaa *et al*., 1997), however it remains to be determined

whether this deletion form is dependent on GTP for its cross-linking activity. In the same study it was suggested that a 47 residue region, and in particular the fragment ^{165}-GFIYQGSVK-^{173}, was involved in GTP/ATP binding and hydrolysis (Iismaa *et al*., 1997). Moreover, the C-terminal region of TG2 is important for its ability to interact with phospholipase C (Hwang *et a*l., 1995; Feng *et al*. 1996) and with receptor molecules (Feng *et al*., 1999). The suggestive deletion of this part of the β-cell TG2 molecule could affect the phospolipase C signal transduction potential of this enzyme, since $G\alpha_h$ (TG2) was originally identified as the signal transducer between epinephrine binding to α1-adrenoreceptor and phospholipase C activation (Im *et al*., 1990; Das *et al*., 1993), specifically phospholipase C-δ1 (Feng *et al*., 1996). Further analysis of the short-form β-cell TG in the context of GTP-binding and possible phospholipase C activation could give important clues for the functional reason behind the TG molecule being modified by this cell system.

The identification and characterisation of any new related mRNA is important for further TG studies, and particularly to what functional role the new protein might perform in the cell. In this chapter, the possibility of an alternatively spliced TG2 variant specific to pancreatic β-cells seems likely. Western blot analysis showed that rat, human and mouse ($TG2^{+/+}$ and $TG2^{-/-}$) pancreatic β-cell TG had a predominant shortened molecular weight of ~60-kDa, with close total net changes as judged from the isoelectric points. To our knowledge, no TG of this size has been reported previously in β-cells. It is likely that the short-form TG described here may represent an isoform of the full-length TG2, since northern blotting has revealed multiple novel short-form mRNA transcripts in BRIN-BD11 cells. This assumption is further strengthened by the β-cell TG showing a conservation of the active site cys^{277} region as demonstrated in activity assays from chapter 4, the potential for GTP-binding, and the presence of a putative Ca^{2+}-binding site immunoreacting with the CUB 7402 antibody. Modifications to the major functions of this short-form β-cell TG enzyme in isopeptide crosslinking and GTP hydrolysis could very well be affected, depending on any conformational changes resulting from a shortened TG2 protein. Additionally, the possibility that TG1 (which may be transcriptionally regulated by retinoic acid in concert with TG2) could also play a role in the functional dynamic of the pancreatic islet should be noted. RT-PCR analysis of the BRIN-BD11 β-cell TG

suggests the absence of amino acids from the carboxyl-terminus similar in structure to rat brain alternative splicing transcripts, which may also account for changes in function compared to full-length TG2.

The precise mechanisms of possible alternative splicing in pancreatic β-cells needs to be characterized further using sequencing and carefully designed site-directed mutagenesis studies. However the evidence presented in this chapter certainly points to the exciting possibility of a new TG2 isoform specific to the pancreas, which could represent only the fourth C-terminal truncated TG2 isoform of its type discovered to date (Fraij *et al*., 1992; Fraij and Gonzales, 1996; Monsonego *et al*., 1997; Lai *et al*., 2007). The characterisation of alternative processing in β-cell TG may also have the potential to impact drug discovery paradigms in the future, where specific site-directed modulation of TG isoform activity could have therapeutic value.

Chapter Six:

Extracellular Matrix-Associated Transglutaminase 2 in Pancreatic β-Cells

Sydney Brenner from *Theoretical Biology in the Third Millenium*

"Our analytical tools have become so powerful that complete descriptions of everything can be attained. In fact, obtaining the DNA sequence of an organism can be viewed as the first step, and we could continue by determining the 3D structure of every protein and the quantitative expression of every gene under all conditions. However, not only will this catalogue be indigestible but it will also be incomplete, because we cannot come to the end of different conditions and especially of combinations and permutations of these. Mere description does not allow computation, and novelty cannot be dealt with…To do this effectively not only must we use the vocabulary of the machine language but we must also pay heed to what may be called the grammar of the biological system.

The signal-transduction machinery, a complicated set of interacting proteins, converts signals (delivered to the outside of the cell and to the inside) into chemical currencies, which are used to control a multitude of cellular functions including growth, movement, division, secretion and differentiation. In multicellular organisms, increased complexity has been achieved not by the invention of new genes but simply by the regulation of gene expression.

Building theoretical models of cells would be based not on genes but on their protein products and on the molecules produced by these proteins. We do not have to wait to solve all the difficult problems of protein structure and function, but can proceed by measuring the properties that we require. The reader may complain that I have said nothing more than 'carry on with the conventional biochemistry and physiology'. I have said precisely that, but I want the new information embedded into biochemistry and physiology in a theoretical framework, where the properties at one level can be produced by computation from the level below. I believe that this is what we should be trying to do in the next century. It will require theoretical biology."

Brenner, Sydney (1927-), Biologist and Nobel laureate. *'Theoretical Biology in the Third Millenium'*, 1999. Quoted from *The Oxford Book of Modern Science Writing* by Richard Dawkins, Oxford University Press, 2008.

Chapter Six:

Extracellular Matrix-Associated Transglutaminase 2 in Pancreatic β-Cells

6.1. Introduction

Evidence presented in the preceding chapters has highlighted the importance of intracellular and cell surface TG-mediated expression and cross-linking in the insulin secretion function of pancreatic β-cells via its regulation by glucose stimulation, Ca^{2+}, GTP, retinoic acid and cell surface proteases; together with a possible role for TG2 in cell survival under conditions resembling the diabetic milieu such as hyperglycaemia, oxidative stress and hyperlipidaemia. Furthermore, molecular characterisation of the β-cell TG presented a possible alternatively spliced short-form variant of the classical TG2 enzyme that may lack a portion of the GTP-binding motif, which could confer higher cross-linking activity in the presence of the high cytosolic Ca^{2+} levels during insulin secretion. A strategy that promotes the fine balance of normal enzymatic function for TG within pancreatic β-cells may therefore be effective in preventing the decline of insulin secretion function or loss of β-cell mass associated with diabetes mellitus, and could potentially represent therapeutic avenues in the context of intra-hepatic islet transplantation.

The survival and functional competence of pancreatic β-cells has been shown to depend upon their interaction with the surrounding extracellular matrix (ECM) (Bosco *et al*., 2001). To date, the survival of islet cells in attempts for prolonged culture required the presence of pre-conditioned ECM derived from non-endocrine cell sources, such as endothelial cells or bladder carcinoma cells (Bosco *et al*., 2001; Kaiser *et al*., 1991; Beattie *et al*., 1991; Hulinsky *et al*., 1995; Perfetti *et al*., 1996; Wang and Rosenburg, 1999; Lefebvre *et al*., 1998). Pre-conditioned matrices have

also been used in the culture of isolated β-cells derived from dispersed islets (Hayek *et al*., 1995; Bosco *et al.,* 2000). Further supporting the use of these pre-conditioned matrices, β-cells which spread onto ECM components were found to also maintain optimal insulin secretion in response to glucose stimulation. Pancreatic β-cells may therefore share a commonality with many other anchorage dependent cells, where matrix adhesion is crucial for optimal function and survival. The components of the ECM required for propagating and transducing signals that match respective β-cell surface receptors remains to be fully characterised, especially within the capacity of intracellular survival signals mediated by these interactions. In the present study, a human urinary bladder carcinoma cell line (5637) was used as model to determine the role of ECM-associated TG2 on β-cell survival and function.

Since 1980, 5637 cells have been known to produce and release growth factors for granulocytes and monocytes (Welte *et al.,* 1985). The conditioned medium from this cell line is often used as a source for haematopoietic growth factors in order to sustain the continuous growth and proliferation of cytokine-dependent leukaemia cell lines. These cells are the parental strain for 1A6 (PTA-556), which produces between two- to ten-fold higher amounts of pluripotent hematopoietic colony-stimulating factor (p-CSF) than the parental cell line 5637. The p-CSF is a glycoprotein constitutively produced by human tumor cells.

Table 6.1. Cytokine secretion of 5637 cells measured by ELISA

Cytokine	**pg/ml**
G-CSF	42 000
GM-CSF	2 100
IL1-β	14
IL-3	Negligible
M-CSF	110
SCF	120

IL1-β- Interleukin 1-β; IL3- Interleukin 3; G-CSF- granulocyte-colony-stimulating factor; GM-CSF- granulocyte-macrophage colony-stimulating factor; M-CSF-macrophage colony-stimulating factor ; SCF- stem cell factor. Table adapted from Quentmeier *et al*., (1997).

The 5637 pre-conditioned medium has been used in laboratories to obtain available metaphases, and is shown to improve both mitotic index and morphological index of

cells in leukaemia studies (Gozzetti *et al*., 2004). Alternative applications of the 5637 cells have also been employed successfully as an extracellular matrix for the culture of sorted human β-cells (Ris *et al*., 2002), although here it was a lawn of lysed cells used as a matrix instead of pre-conditioned medium, which has also been used in the culture of whole human islets (Beattie *et al*., 1997). The 5637 cells constitutively produce and secrete several functionally active cytokines (see Table 6.1.; Quentmeier *et al*., 1997).

Numerous secreted constituents of this cell line have been characterised, which are known to induce cell proliferation when used as a support matrix (see Table 6.2), and are described here. Pluripoeitin alpha (Gabrilove *et al*., 1986) is constitutively produced by 5637 cells, and has the capacity to: i) induce the differentiation of the human promyelocytic leukemic cell line HL-60; ii) induce the expression of chemotactic peptide receptors in leukemic cells as well as in normal peripheral blood granulocytes; iii) function as a chemoattractant for neutrophils as well as, under certain conditions, inhibiting their migration; iv) support the growth of eosinophil and granulocyte/macrophage progenitors; and v) support the growth of human mixed pluripotent progenitors and erythroid bursts from normal human bone marrow.

Table 6.2. Other characterised constituents secreted from 5637 cells

Constituent	**Reference**
Pluripoeitin alpha	Gabrilove *et al*., (1986)
Human Interleukin for DA cells (HILDA)/Leukemia Inhibitory Factor (LIF)	Gascan *et al*., (1990)
Hemopoietin 1 (H-1)	Jubinsky *et al*., (1994)
Mononuclear phagocyte specific growth factor (CSF-1)	Jubinsky *et al*., (1994)
Synergistic Factor (SF-1)	McNiece *et al*., (1989)

Leukemia inhibitory factor (LIF) (Gascan *et a*l., 1990) was reported to trigger the proliferation of the DA1.a cell line. Hemopoietin 1 (H-1) and mononuclear phagocyte specific growth factor (CSF-1) (Jubinsky *et al*., 1994) act synergistically on developmentally early bone marrow cells to generate primitive CSF-1 receptor-bearing cells, and synergistic factor (SF-1) McNiece *et al*., (1989), produced by these 5637 cells, stimulates primitive bone marrow progenitor cells, which are

termed high proliferative-potential colony-forming cells (HPP-CFC) in the presence of an optimal dose of macrophage colony stimulating factor (CSF-1).

Cells adhere and spread on an extracellular matrix by virtue of the binding of cell surface integrins to their cognate proteins in the matrix (Lucas-Clerc *et al*., 1993; Wang *et al*., 1999; Aharoni *et al*., 1997; Beattie *et al*., 1991). The identification of a discrete panel of integrins expressed by purified human β-cells was published by Ris *et al*., (2002) to allow for matching their cognate extracellular matrix components with a view to improving attachment and spreading (Ris *et al.*, 2002). The expression of integrin subunits in the human islets, and on the various support matrices by these authors are of interest to this study, and are shown in Table 6.3 and Table 6.4 respectively.

Table 6.3. The expression of select integrin subunits in human islets

	Integrin subunits present in all	**Integrin subunits not present in all**
Islets	α3; α5; α6; αV, β1	β4 - present
β-cells	α3; α5; α6; αV, β1	β4 – not present
Fibroblastoid cells	α3; α5; α6; αV, β1	β4 - present

Table adapted from Ris *et al*., (2002)

The 5637 lysed matrix was the only one of those listed in Table 6.4. that promoted human β-cell attachment, spreading and survival (Ris *et al*., 2002). Nonetheless, survival was still low and only increased by adding anti-apoptotic agents (Z-VAD) and antioxidants (nicotinamide), suggesting that the 5637 cells provide only partial restoration of integrin signalling to purified β-cells (Ris *et al*., 2002). Furthermore, the cell line 804G mentioned in Table 6.4 is a rat bladder carcinoma cell line which showed enhanced attachment and spreading of rat β-cells to this enriched laminin-5-containing matrix (Bosco *et al*., 2000; Kantengwa *et al*., 1997), however it was not suitable for the human β-cells when used by Ris *et al*., (2002).

The 804G matrix was initially considered for use in this investigation, but a patent already existed for this matrix at the time, making it inaccessible. The 5637 cells are considered the human counterpart to these 804G cells and have been used successfully for the culture of monolayers from intact human islets (Quentmeier *et*

al., 1997; Beattie *et al*., 1997; Ris *et al*., 2002). Similar to the laminin-5 present in the 804G cells, the 5637 cell line has also recently (Chakraborty *et al*., 2004) demonstrated G-CSFR-mediated upregulation of β1-integrin-dependent adhesion to fibronectin and laminin. In the current investigation, a lawn of 5637 cells were characterised and modified for TG2 expression and used as a support matrix in the maintenance of BRIN-BD11 rat pancreatic β-cells.

Table 6.4. Cognate integrins present in different matrices

Human β-cell Attachment	**ECM ligand**	**α6β1**	**α3β1**	**α5β1**	**αVβ3**	**αVβ5**	**α1β1**
Yes	Lysed 5637 cells (Human Laminins)	++	++				
No	Lysed 804G cells (Rat Laminins)	++	++				
No	5637 cells conditioned medium (Human Laminins)	++	++				
No	804G cells conditioned medium (Rat Laminins)	++	++				
Yes	Fibronectin		++	++	++	++	
Yes	Vitronectin				++	++	
Yes	Gelatin		++	++			++

++ represents expression of the integrin. Table adapted from Ris *et al*., (2002)

The environment of a cell can be termed a 'niche', which in turn affects its differentiation, survival and proliferation. The term 'vascular niche' has been used to describe the environment of cellular development that happens in close proximity to blood vessels (Palmer *et al*., 2000). Pancreatic β-cells have a high degree of plasticity in that they proliferate *in vivo* to adjust to an increased demand for insulin, but their potential for expansion is severely inhibited *in vitro*, which is usually accompanied by a loss of their endocrine function (Rhodes, 2005). Thus, the therapeutic potential of β-cells can only be fully exploited if the components of their particular 'niche' is better understood (Fuchs *et al*., 2004). Evidence for a vascular niche can be demonstrated by proving that it is not only the blood supply that supports the cells in question.

For pancreatic β-cells this has been shown in co-culture experiments where endothelial cells induced endocrine pancreatic differentiation from embryonic gut epithelium (Dor *et al*., 2004). This idea was supported further by studying the effects of factors predominantly expressed by endothelial cell basement membranes within pancreatic islets such as laminins and collagen IV, which when added *in*

vitro, reproduced increases in insulin secretion and β-cell proliferation that were observed *in vivo* (Nikolova *et al*., 2006; 2007). Most of the cell types that seem to require a vascular niche are also interestingly unable to form their own basement membranes, and it is believed that this limitation may be a result of the plasticity of these cells that interferes with the formation of a stable basement membrane (Nikolova *et al*., 2007). Endothelial cells are remarkable in that they can migrate into every tissue, change their morphology tremendously and simultaneously produce various basement membrane components. These components are found on endothelial cells during angiogenesis and vasculogenesis.

Based on the production of basement membranes, a conserved function of endothelial cells may be to provide the islet β-cell with an ECM (Nikolova *et al*., 2007) that could play an important role during tissue development and homeostasis by (i) facilitating the binding of basement membrane proteins to integrins, which serves to signal on their own and also amplify the cell response to various growth factors; (ii) activation of growth factors such as FGFs and VEGFs by heparin sulphate proteoglycans on the basement membrane; (iii) polarisation of cells by basement membranes enabling symmetric and asymmetric cell divisions that depend on the mitotic cleavage plane; and (iv) provide cells with structural support and facilitate migration.

The basement membrane (basal lamina) is a specialised form of ECM that lies close to the basolateral surface of endothelial cells, epithelial cells, smooth and skeletal muscle cells, peripheral nerve cells and adipocytes (Kalluri *et al*., 2003). The basement membrane provides mechanical stability to cells, serves as a barrier between different cell types and acts as a substrate for cellular interactions. All basement membranes contain laminins, collagen IV, nidogen (entactin) and heparin sulphate proteoglycans. The individual components of the basement membrane have important roles in cell proliferation, differentiation, migration, development and repair. Depending on its specific location and function, the exact composition of the basement membrane in terms of types and isoforms of glycoproteins differs. The formation of the basement membrane is driven by the interesting property of certain laminins (heterotrimeric glycoproteins composed of α, β and γ chains) to

polymerise and to form a scaffold. This scaffold is further stabilised by nidogen and collagen IV. Additional glycoproteins such as heparin sulphate proteoglycans and growth factors bind to this structure (Yurchenco *et al*., 2004). In mature blood vessels, the basement membrane is located between endothelial cells and mural cells.

Islets of Langerhans are like mini endocrine organs in their own right, made up mostly of aggregates of insulin secreting β-cells surrounded by a network of dense vascularisation (Konstantinova and Lammert, 2004). Islets have 5-7 times more capillaries present compared to its surrounding exocrine tissue in the pancreas (Konstantinova and Lammert, 2004), which enables every β-cell to be directly adjacent to a capillary. For many years it was believed that this dense capillary network served the sole purpose of helping secreted insulin gain access to the blood stream (Lammert *et al*., 2003). However it was recently shown (Takahashi *et al*., 2002) that insulin granules are actually released toward the islet interstitium rather than directly into the capillaries. It has now been proposed that one of the main functions of the numerous endothelial cells within the capillary network is actually to provide β-cells with a vascular basement membrane that promotes their endocrine function and proliferative potential (Nikolova *et al*., 2006). It has been shown for the first time by these authors that β-cells do not form their own basement membranes, which has some consequence in the context of ECM-associated TG2.

A characteristic feature of TG2 is that this enzyme can be externalised from cells into the ECM via a non-ER/Golgi route (Gaudry *et al*., 1999). Furthermore, matrix deposition of TG2 occurs in normal physiology, but is increased during cell stress or trauma (Haroon *et al*., 1999; Johnson *et al*., 1999). This in turn leads to the immobilisation of TG2 on matrix proteins such as FN (Verderio *et al*., 1998; Jeong *et al.*, 1995), and subsequent improvements in ECM-adhesion and increased viability in a range of cell types, suggesting that TG2 may propagate survival signals and is therefore an important component of the ECM.

The precise role played by extracellular matrix-associated TG2 in pancreatic β-cells has not been fully established, and the potential for this enzyme as a regulator of β-

cell adhesion and viability has not been investigated to date. In view of a body of evidence that points to the function of TG2 in survival signalling, matrix interactions and a role that supports cell viability, the present chapter explores whether this enzyme may fulfil these roles in pancreatic β-cells also. A human urinary bladder carcinoma matrix (5637), which was previously used as a β-cell support matrix (Ris *et al*., 2001) will firstly be characterised for TG content and cross-linking activity, and optimised for the maintenance of BRIN-BD11 β-cells. The optimised 5637 matrix will then be pre-conditioned during matrix protein accumulation by manipulating the levels of TG2 cross-linking activity with the use of active site directed TG inhibitors, or modified by transient transfection with stealth siRNA oligonucleotides targeted towards the knockdown of TG2 protein levels. The optimised 5637 matrix will then be pre-conditioned by immobilisation of purified guinea pig liver TG2 (*gpl*TG2), or catalytically cross-linked by *gpl*TG2 in the presence of Ca^{2+} and DTT. These pre-conditioned 5637 support matrices will then be characterised for levels of fibronectin, laminin and collagen protein turnover. BRIN-BD11 β-cell and rat islet aggregation on the pre-conditioned 5637 matrices will then be assessed by three dimensional (3-D) morphological visualisation, and quantified for insulin content using immunofluorescence. The adherence and spreading profiles of BRIN-BD11 cells on these pre-conditioned matrices will then be viewed immunofluorescently for actin stress fibre formation and quantified in the presence of RGD peptide, hyperglycaemia, oxidative stress and hyperlipidaemia. The viability and apoptosis levels in BRIN-BD11 cells will then be presented as a result of the 5637 support matrix, using hyperlipidaemia.

6.2. Results

6.2.1. Optimisation of the 5637 Pre-conditioned Support Matrix

6.2.1.1. Characterisation of TG2 in the 5637 matrix

Human urinary bladder carcinoma cells (5637), described previously for optimal integrin-matrix matching with pancreatic β-cells (Ris *et al*., 2001) were selected as a support matrix for the maintenance of BRIN-BD11 cells. The 5637 matrix formed an optimal extracellular support matrix after 72 hours growth, in which very high levels of TG antigen was consistently present when visualised using immunfluorescence with the monoclonal TG2-specific CUB 7402 antibody (**fig. 6.1, panels A, B, C**).

Furthermore, the TG2 antigen present in the 5637 cells was found to be a full-length classical 85-kDa protein when analysed using western blotting with the CUB 7402 antibody (**fig. 6.2**). The TG2 cross-linking activity in 5637 cell homogenates was found to be extremely high, at specific activity levels in excess of 8 nmole radiolabelled [^{14}C]-putrescine incorporated into N'N-dimethylcasein per mg of TG2 protein, after 72 hours 5637 matrix accumulation (**fig. 6.3**).

The 5637 matrices were then optimised and tested routinely using a TG2 ELISA (section 3.2.5.7.2.) after the formation of a 3-day lysed support matrix starting out with 1 x 10^5 cells seeded per ml of RPMI medium (containing 10% foetal calf serum) on day 1, as described in section 3.2.4.2.

6.2.1.2. 5637 Pre-conditioned Matrices Over-expressing TG2

It was possible to modify the untreated 3-day 5637 support matrices before lysis by supplementing 10-20 µg/ml purified guinea pig liver TG2 (gplTG2) in the growth medium of live cells on day 1, which resulted in a 3-fold ($p < 0.05$) increase of immobilised cell-surface TG2 levels, when measured colorimetrically using ELISA with CUB 7402 antibody (**fig. 6.4**). Detection of TG2 activity in these 3-day 5637

Figure 6.1. and figure 6.2

Figure 6.3.

Figure 6.4. and Figure 6.5.

matrices over-expressing TG2 revealed a 50% reduction ($p < 0.05$) in active enzyme through the incorporation of biotin cadaverine into endogenous 5637 matrix proteins (**fig. 6.5**), compared to untreated 3-day 5637 matrices.

Another modified 5637 matrix was created by further cross-linking the 3-day 5637 matrix with the addition of 20 μg/ml gplTG2 in a reaction mix (5 mM $CaCl_2$ and 10 mM DTT added to serum free growth medium for 1 hour at 37°C, 5% (v/v) CO_2) before lysis, which resulted in a 10-fold increase ($p < 0.001$) in matrix TG2 levels when detected with an ELISA using CUB 7402 antibody (**fig. 6.4**). However, despite the high levels of TG2 protein now present on this 3-day 5637 matrix, the levels of cross-linking activity as measured by the incorporation of biotin cadaverine into endogenous 5637 matrix proteins, revealed similar TG2 catalysis to the untreated 3-day 5637 matrix (**fig. 6.5**).

6.2.1.3. 5637 pre-conditioned matrices with inhibited TG2 activity

Manipulation of the 5637 matrix was also possible by the exogenous supplementation of irreversible active-site directed TG inhibitors (R281, R283 and BOC-peptide) in the normal 5637 growth medium on day 1, with a fresh supply of these inhibitors in the growth medium on day 2 and day 3 (see section 3.2.4.2.1). Optimal concentrations for the efficient non-toxic action of R281 and R283 on 5637 cells up until day 3 was measured using MTS viability assays, and was set at 250 μM for both inhibitors (data not shown). Similarly, optimal inhibitory concentrations for the BOC-peptide was set at 10 μM up until day 3 (data not shown). When the level of TG2 activity was measured in these 3-day TG-inhibited 5637 matrices using the incorporation of biotin cadaverine into endogenous 5637 matrix proteins (**fig. 6.5**), the levels of TG activity with both R281 and R283 was found to be reduced by ~50% ($p < 0.05$).

6.2.1.4. 5637 pre-conditioned matrices with transient siRNA TG2 Knockdown

3-day 5637 matrices were also modified by reducing the level of TG2 gene transcription, using transient transfection with TG2 gene silencing technology by

Figure 6.6

Figure 6.7

Figure 6.8

constructing specific short interfering RNA (siRNA) oligonucleotide sequences as described in section 3.2.4.2.2. It was important to optimise the TG2 protein knockdown in these 5637 matrices by determining the most efficient concentrations of stealth siRNA duplex combinations over 3 days (**fig. 6.6**). Concentrations of 40 pmol siRNA oligonucleotide in combination with 1 µl Lipofectamine 2000 over 48 hours in RPMI medium (2% foetal calf serum), with fresh medium (10% foetal calf serum) supplemented on day 3, proved optimal for the transient TG2 gene knockdown in the 5637 cells (data not shown). Western blot analysis of the 5637 cell lysates after 72 hours treatment with the three stealth siRNA duplexes (TG 13, TG14 and TG15) revealed that double combinations of all TG2-specific siRNA complexes knocked down the level of CUB 7402 immunoreactive TG2 at 85-kDa (**fig. 6.6A**), with the combination of 40 pmol TG13/TG14 being most effective by inducing a 50% reduction ($p < 0.05$) in TG2 protein levels compared to untreated 5637 cells (**fig. 6.6B**). Assessment of the specific TG2 cross-linking activity in the siRNA transfected cells was performed by measuring the incorporation of radiolabelled [^{14}C]-putrescine into N'N-dimethylcasein, and all three combinations of stealth siRNA duplexes resulted in a greater than 50% reduction ($p < 0.001$) in TG2 cross-linking activity, with the 40 pmol TG13/TG14 combination being most effective after 3 days (**fig. 6.6.C**).

As a result of these optimisation studies, the 40 pmol TG13/TG14 siRNA duplex combination was then used in all subsequent 3-day siRNA-transfected 5637 matrices. The morphology (**fig. 6.7**) and semi-quantified TG2 activity levels (**fig. 6.8**) of siRNA-transfected 5637 matrix formation over 3 days was visualised fluorescently through the incorporation of FITC-cadaverine, and revealed ~75% reduction ($p < 0.001$) of TG2 activity (**fig. 6.8**) and fewer cell to cell contacts through matrix aggregation as a result of TG13/TG14 siRNA-mediated TG2 gene silencing (**fig. 6.7 panel I, arrows**).

6.2.1.5. 5637 Pre-conditioned Extracellular Matrix Protein Turnover

The current investigative approach of using a support matrix already rich in TG2 protein and cross-linking activity meant that the experimental design focussing on the importance of extracellular matrix-associated TG2 would be achieved by

inhibiting or knocking down TG2 protein levels, and then assessing any functional effects on the β-cell thereof. However, one important consequence that needed consideration as a result of reducing the levels of TG2 protein and/or TG2 activity in the 5637 support matrix is that profound effects on the extracellular matrix protein (fibronectin, laminin, collagen) profile would be expected, resulting in complete re-structuring of the accumulated protein scaffolds, and associated adhesion and signalling receptors or molecules.

As a starting point, it was important to determine the effect of TG2 reduction on extracellular matrix protein turnover in the 3-day 5637 matrices already optimised in this study. This was initiated by visualising and quantifying any changes to the levels of TG2, fibronectin (FN) and laminin (LN) protein expression over the 3 days in which the TG2 siRNA transfected 5637 pre-conditioned matrix was formed using immunofluorescence (**fig. 6.9; fig 6.10**). The result of TG2 gene silencing using siRNA-mediated transient transfection of 5637 matrices, was observed with morphological changes in a reduction of 5637 cell to cell contacts (**fig. 6.9, panels G, H, I, arrows**) and an ~80% reduction ($p < 0.001$) in TG2 (**fig. 6.9, panel G**), FN (**fig. 6.9, panel H**) and LN (**fig. 6.9, panel I**) antibody-mediated fluorescence intensity (**fig. 6.10**), compared to the untreated controls (**fig. 6.9, panels A – F**).

These results were confirmed using an ELISA which measured TG2, FN, LN and collagen IV antigen levels over 72 hours, which showed an ~50% reduction ($p < 0.001$) in all protein levels tested (**fig. 6.11**), when comparing TG2 siRNA-transfected 5637 pre-conditioned matrices to untreated 5637 matrices. The same ELISA experiment was used for 3-day 5637 matrices formed in the presence of irreversible active-site directed TG inhibitors (R281, R283 and BOC peptide), where TG2 antigen levels remained the same for modified matrices as in the untreated 5637 matrices (**fig 6.11**), and ~50% reductions ($p < 0.001$) in FN turnover being evident for all three TG inhibitor treatments. The levels of laminin protein turnover was unchanged in 5637 matrices modified by the larger R281 and BOC-peptide inhibitors which were expected to act only on the cell surface, however the smaller cell permeable R283-inhibited 5637 matrix showed an ~80% reduction ($p < 0.001$) in laminin protein expression compared to the untreated 5637 control (**fig 6.11**). Collagen IV antigen levels remained unchanged as a result of R281 inhibition, with

Figure 6.9. Immunofluorescence

Figure 6.10

Figure 6.11

an ~25% reduction ($p < 0.05$) as result of R283 treatment, and an ~50% reduction ($p < 0.001$) in the 5637 matrices formed in the presence of BOC-peptide (**fig. 6.11**).

6.2.2. β-cell Aggregation and Insulin content on 5637 Pre-conditioned Matrices

The adhesion, maintenance and aggregation of BRIN-BD11 cells and rat islets seeded onto the 3-day 5637 pre-conditioned matrices was assessed immunofluorescently in 8-well chamber slides, using co-staining with insulin antibody and CM-dil cell tracker (**fig. 6.12; 6.13; 6.14; 6.16**). While the usual 2-dimensional (2-D) confocal images (**fig. 6.12, panels C; F and fig. 6.13, panels C; F**) were originally captured to assess and quantify any trends as a result of the various modified matrices, it was not possible to properly assess aggregation in rat islets or BRIN-BD11 cells due to the large size of compacted β-cell structures formed in response to the 5637 support matrices after 24 hours. Extending the Leica confocal software to capture 3-dimensional (3-D) images instead, made it possible to communicate the aggregation trends routinely observed in a larger visual field through the microscope eyepiece.

For instance, rat islets were cultured on untreated glass chamber slides (**fig. 6.12, panels A, B; fig. 6.13, panels A, B**) or 3-day 5637 support matrices (**fig. 6.12, panels D, E; fig. 6.13, panels D, E)** in the presence 5 mM or 20 mM glucose for 24 hours. The rat islets attached and spread on the 5637 matrix more readily, and were also more insulin responsive (**6.12, panel E; 6.13, panel E**), in comparison to no matrix. This was evident through 3-D imaging where, in addition to the x, y function, the 'z function' exposed a measurable 3-fold increase ($p < 0.05$) in the height of rat islets on 5637 matrices under 20 mM glucose (~36 µM; **fig. 6.13, panel D**) compared to 20 mM glucose (~12µM; **fig. 6.13, panel D**) treatments with no matrix. Even, when comparing glucose responsiveness on the 5637 matrices only, there appeared to be an ~40% increase in the height of 20 mM glucose-treated islets (~36 µM; **fig. 6.13, panel D**) compared to the 5 mM glucose-treated controls (~20µM; **fig. 6.12, panel D**).

Seeding BRIN-BD11 cells at a consistent density of 2 x 105 cells/well on 5637 pre-conditioned matrices over 24 hours (**fig. 6.14 and fig 6.16**) made it possible to

Figure 6.12. Immunofluorescent 3-D morphology of rat islets treated with 5 mM glucose on 3-day 5637 support matrices, using CM-Dil cell tracker and insulin antibody. 3-day 5637 pre-conditoned matrices labelled with CM-Dil cell tracker were prepared in 8-well chamber slides, as described in section 3.2.9.3. Rat islets (50 islets/well) were then seeded onto the matrices in serum-containing RPMI growth medium supplemented with 5 mM glucose for 24 hours, and stained *in situ* with CM-Dil reagent for 5 minutes prior to fixation and permeabilisation. Rat islet β-cell insulin content was detected by the addition of anti-swine insulin antibody, revealed through the conjugation of anti-rabbit IgG-FITC, and viewed using a Leica TCSNT confocal laser microscope as described in section 3.2.9.2. **Panels A, B, C,** Confocal micrographs of rat islets on a glass chamber-well surface (no matrix) with 5 mM glucose treatment. A) 3-D image showing islet insulin content (green); B) 3-D image showing islet morphology with cell tracker (red); C) 2-D image of A) showing co-staining with insulin and cell tracker. **Panels D, E, F,** Confocal micrographs of rat islets on a 5637 pre-conditioned matrix with 5 mM glucose treatment. D) 3-D image showing islet insulin content (green); E) 3-D image showing islet morphology with cell tracker (red); F) 2-D image of D) showing co-staining with insulin and cell tracker. Micrographs are representative of at least two separate experiments. Bar equals 40 μm.

Figure 6.13. Immunofluorescent 3-D morphology of rat islets treated with 20 mM glucose on 3-day 5637 support matrices, using CM-Dil cell tracker and insulin antibody. 3-day 5637 pre-conditoned matrices labelled with CM-Dil cell tracker were prepared in 8-well chamber slides, as described in section 3.2.9.3. Rat islets (50 islets/well) were then seeded onto the matrices in serum-containing RPMI growth medium supplemented with 20 mM glucose for 24 hours, and stained *in situ* with CM-Dil reagent for 5 minutes prior to fixation and permeabilisation. Rat islet β-cell insulin content was detected by the addition of anti-swine insulin antibody, revealed through the conjugation of anti-rabbit IgG-FITC, and viewed using a Leica TCSNT confocal laser microscope as described in section 3.2.9.2. **Panels A, B, C,** Confocal micrographs of rat islets on a glass chamber-well surface (no matrix) with 20 mM glucose treatment. A) 3-D image showing islet insulin content (green); B) 3-D image showing islet morphology with cell tracker (red); C) 2-D image of A) showing co-staining with insulin and cell tracker. **Panels D, E, F,** Confocal micrographs of rat islets on a 5637 pre-conditioned matrix with 20 mM glucose treatment. D) 3-D image showing islet insulin content (green); E) 3-D image showing islet morphology with cell tracker (red); F) 2-D image of D) showing co-staining with insulin and cell tracker. Micrographs are representative of at least two separate experiments. Bar equals 40 μm.

Figure 6.12. Immunofluorescence

Figure 6.13. Immunoflourescence

Figure 6.15

Figure 6.14 Immunoflourescence

Figure 6.17

Figure 6.16 Immunofluorescence

quantify the 3-D aggregation status (**fig. 6.17**) and the insulin content (**fig. 6.15**) of these cells using the Leica QWin image analysis software. Visualisation of the BRIN-BD11 aggregration morphology using 3-D image capturing at 5 mM (**fig. 6.14, panels B, D, F, H, J, L**) and 20 mM (**fig.6.16, panels B, D, F, H, J, L)** glucose concentrations, revealed the highest ($p < 0.001$) overall CM-dil cell tracker fluorescent β-cell aggregation on untreated TG2-rich 3-day 5637 matrices (**fig. 6.14, panel D; fig. 6.16., panel D; fig. 6.17**) compared to no matrix or other TG-inhibited 5637 pre-conditioned matrices. The BRIN-BD11 cells appeared to form pseudo-islet aggregated structures on the TG-rich 3-day 5637 matrices under conditions of optimal 20 mM glucose-stimulation (**fig. 6.16, panel D**). When the BRIN-BD11 cells attached and spread on the TG-inhibited 5637 matrices in response to 5 mM glucose, TG2 siRNA-transfected 3-day 5637 matrices showed trends similar to the untreated 5637 matrix (**fig. 6.14, panel D, F; fig. 6.17**), however there was an ~50% reduction (**p** < 0.001) in islet-like aggregation at optimal insulin secreting concentrations of 20 mM glucose (**fig. 6.16, panel D, F; fig. 6.17**) on these matrices, compared to the untreated 3-day 5637 control matrix. Pre-conditioned 5637 matrices formed in the presence of the TG inhibitors: BOC-peptide (**fig. 6.14., panel H; fig. 6.16, panel H**), R281 (**fig. 6.14., panel J; fig. 6.16, panel J**) and R283 (**fig. 6.14., panel L; fig. 6.16, panel L**); showed the most marked reduction of β-cell aggregation, in excess of ~50% ($p < 0.001$) especially under conditions of 20 mM glucose-stimulated insulin release (**fig. 6.16; fig 6.17**), compared to the 5637 untreated control matrices.

The total insulin content BRIN-BD11 cells cultured on the pre-conditioned 5637 matrices was visualised (**fig. 6.14**) and quantified immunofluorescently (**fig. 6.15**), in response to concentrations of 5 mM (**fig. 6.14, panels A, C, E, G, I, K**) and 20 mM (**fig. 6.16, panels A, C, E, G, I, K**) glucose for 24 hours. The fluorescent insulin content of the BRIN-BD11 cells on all 5637 support matrices showed responsive glucose-stimulated insulin secretion at 20mM glucose treatments compared to the lower 5 mM glucose treatments (**fig. 6.15**). The untreated TG2-rich 3-day 5637 matrix proved optimal for β-cell insulin secretion function at 20 mM glucose levels and exhibited pseudo-islet aggregation (**fig. 6.15; fig. 6.16, panel C**). The BRIN-BD11 cells grown on TG2 siRNA-transfected 3-day 5637 matrices showed ~30% reduction in measurable insulin content at both 5 mM and 20 mM (*p*

< 0.001) glucose concentrations, compared to the untreated 3-day 5637 support matrices (**fig. 6.15**). Pre-conditioned 5637 matrices formed in the presence of the TG inhibitors: BOC-peptide (**fig. 6.14., panel G; fig. 6.16, panel G**), R281 (**fig. 6.14., panel I; fig. 6.16, panel I**) and R283 (**fig. 6.14., panel K; fig. 6.16, panel K**); showed the most marked reduction of β-cell islet-like aggregation and fluorescent insulin content, in excess of ~50% reductions (p < 0.001) especially under conditions of 20 mM glucose-stimulated insulin release (**fig. 6.15 ; fig 6.16**), compared to the 3-day 5637 untreated control matrices.

6.2.3. BRIN-BD11 Adhesion and Spreading on 5637 Pre-conditioned Matrices

To determine the level of BRIN-BD11 attachment and spreading in response to TG-modified 5637 support matrices, preliminary experiments assessing actin stress fibre formation (**fig. 6.18**), focal adhesion kinase-mediated cell adhesion (**fig. 6.19**; **fig. 6.20**), and quantification of attachment and spreading profiles using Giemsa/May-Grunwald staining (**fig. 6.21; fig. 6.23; fig. 6.24; fig. 6.25**).

The morphology of BRIN-BD11 cells were assessed fluorescently for actin stress fibre formation using FITC-phalloidin after overnight cell-spreading on a FN matrix (**fig. 6.18, panel A**), FN matrix immobilised with TG2 (**fig. 6.18, panel B**), untreated 3-day 5637 matrix (**fig. 6.18, panel C**), and a 3-day TG2 siRNA-transfected 5637 support matrix (**fig. 618, panel D**). The formation of BRIN-BD11 focal adhesion points was evident in response to the FN-TG2 matrix compared to the FN matrix alone (**fig. 6.18, panel B, arrows**), whereas the spreading profile of these β-cells on the TG2-rich 5637 matrix exhibited intense phalloidin staining of the cell aggregates with more focal adhesion points (**panel 6.18, panel C, arrows**), compared to the TG2-deficient 5637 matrix formed as a result of siRNA treatment.

When the BRIN-BD11 cells were viewed for immunofluorescent phosphorylated FAK staining, enhanced FAK-mediated adhesion was evident in the β-cells attached for 3 hours on TG2-rich 5637 matrices (**fig. 6.19, panel C**) and FN matrices (**fig. 6.19, panel B**), compared to 3-day TG2 siRNA-transfected 5637 support matrix (**fig. 6.19, panel D)** or bovine serum albumin treated surfaces (no matrix) (**fig. 6.19, panel A).** The amount of phosphorylated FAK and total FAK produced as a result of

Figure 6.18 Actin

Figure 6.19. and 6.20 FAK

Figure 6.20 Blue stained cells

3 hours BRIN-BD11 adhesion in response to various 5637 pre-conditioned matrices was quantified after western blot analysis and β-tubulin standardisation for each treatment (**fig. 6.20**). BRIN-BD11 cells attached to TG2-rich untreated 5637 matrix appeared to have the most extensive FAK signal, with an ~50% reduction ($p < 0.001$) in FAK signal as a result of adhesion to TG2-deficient 5637 matrix tranfected with siRNA, and even greater ~75% reduction ($p < 0.001$) in FAK signal when these β-cells were attached to 5637 matrices formed in the presence of active-site directed R281 and R283 TG inhibition (**fig. 6.20**).

It was possible to quantify the effect that these TG-modified pre-conditioned matrices had on BRIN-BD11 attachment and spreading through the quantification of nuclear Giemsa and cytoplasmic May-Grunwald staining (**fig. 6.21**) detected using Leica QWin image analysis, where the number of cells present after 1 hr was taken as a measure of attachment, and the cytoplasmic area (μM^2) of each attached cell was measured per field and added together to give total spread cell area (μM^2). As demonstrated in **fig. 6.21**, quantification values communicated the major differences of the β-cells in response to matrix variation. For instance, there were very low levels of BRIN-BD11 cell attachment in the absence of any support matrix (**fig. 6.21A**), whereas higher cell attachment was evident in response to a FN matrix (**fig. 6.21B**) and TG2-rich 5637 matrix (**fig. 6.21C**), with only spread cell area (μM^2) values being able to express the extensive cell spreading evident on the latter matrix. Some preliminary experiments were attempted with the dissociated β-cells from rat islets seeded on various matrices (**fig. 6.21D** shows rat islet β-cells before dissociation), however these results are not shown due to incomplete data sets.

The BRIN-BD11 cell attachment and spreading profile was assessed at the outset by comparing responses to matrices from various cell-types (data not shown), before the 5637 cells were concluded as being the optimal support matrix for these β-cells. BRIN-BD11 contained low levels of cell-surface TG2 activity ($p < 0.001$), when compared to the TG2-rich human urinary bladder carcinoma 5637 cells or the well characterised human umbilical vein endothelial ECV-304 cells (**fig. 6.22**). The 3-day lysed matrices from both 5637 and ECV-304 TG2-rich cell systems were compared in addition to a FN matrix, where 10 μg/ml gplTG2 was immobilised on all three matrices, before quantification of β-cell attachment and spreading (**fig. 6.23**). There

Figure 6.22 and fig. 6.23

Figure 2.24

Figure 2.25

was an ~50% ($p < 0.001$) increase in BRIN-BD11 attachment and spreading on the 3-day 5637 matrix, compared to FN or ECV-304 matrices (**fig. 6.23**). When the 3-day 5637 matrix was modified to over-express TG2 levels, a 2-fold increase ($p < 0.001$) in the amount of β-cell attachment was observed, despite spread cell area remaining similar to the untreated 5637 matrix, thus reflecting the observation of a higher amount of attached cells that were less spread as a result of exogenous TG2 supplementation. Despite high levels of TG activity (**fig. 6.22**), the 3-day ECV-304 support matrix did not facilitate β-cell spreading as profoundly as the 3-day 5637 matrix (**fig. 6.22**).

Since FN was found to be an important TG2-mediated component of the 3-day 5637 matrix, the interaction of this matrix protein with exogenously supplemented gplTG2 was assessed to reveal slight decreases in the total β-cell spread area, and no significant changes in cell attachment numbers (**fig 6.23 and fig. 6. 24**). When the BRIN-BD11 cells were incubated in the presence of RGD function-blocking peptide (**fig. 6.24**), the number of cells attached and spread was significantly reduced ($p < 0.001$). However, the presence of supplemented TG2 on the same FN matrix resulted in a significant ($p < 0.05$) rescue of β-cell attachment and spreading levels back to normal (**fig. 6.24**).

In order to determine whether the high TG2 levels present in the untreated 3-day 5637 support matrices contributed in any way to the attachment and spreading levels of BRIN-D11 cells under conditions of diabetic stress, β-cell spread area was quantified on 5637 matrices modified for TG2 expression and activity (**fig. 6.25**). Treatments of 40 mM glucose-induced hyperglycaemia (**fig. 6.25A**), 150 µM GSNO-induced oxidative stress (**fig. 6.25B**), and 150 µM palmitate-induced lipotoxicity (**fig. 6.25C**) for 1 hour on the BRIN-BD11 cells resulted in minimal attachment and spreading in the absence of any support matrix ($p < 0.001$), whereas the TG2-rich 3-day 5637 support matrix, and the two gplTG2-modified 5637 matrices over-expressing protein levels, resulted in dramatic 80% increases ($p < 0.001$) of β-cell maintenance assessed through cell spread area. The pre-conditioned 5637 matrices formed in the presence of the TG inhibitors: BOC-peptide, R281 and R283; showed the most marked reduction of attached β-cell spread area, in excess of ~50% reductions ($p < 0.001$) especially under conditions of GSNO-induced

oxidative stress (**fig. 6.25B**) and palmitate-induced hyperlipidaemia (**fig. 6.25C**), compared to the 3-day 5637 untreated control matrices.

6.2.4. BRIN-BD11 survival on 5637 Pre-conditioned Matrices

Preliminary experiments were performed assessing BRIN-BD11 cell viability and apoptosis under diabetic stress treatments was measured through the mitochondrial metabolism of MTS tetrazolium compound (**fig 6.26**) and colorimetric apoptotic index by the ability of caspase-3 within β-cells to cleave Ac-DEVD-p-nitroaniline substrate and release the p-nitroaniline (pNA) chromophore (**fig. 6.27**). When the BRIN-BD11 cells were cultured on untreated TG-rich 5637 support matrices for 24 hours, cell viability was significantly higher ($p < 0.05$) on the 5637 support matrix at normal 10-20 mM glucose-stimulated insulin release concentrations (**fig. 6.26A**) compared to viability without any support matrix. The metabolism of tetrazolium compound was consistently higher ($p < 0.001$) in BRIN-BD11 cells grown on the 3-day 5637 support matrix, under treatments of 50 – 500 µM GSNO-induced oxidative stress conditions (**fig. 6.26B**), compared to no matrix support. Cell viability was also significantly higher in BRIN-BD11 cells attached to the TG2-rich 5637 matrix at concentrations between 50 µM and 150 µM palmitate for 24 hours (**fig. 6.26C**), compared to the absence of matrix support. When diabetic stress treatments were used in 24 hour treatments for BRIN-BD11 cells cultured on the 3-day TG2-rich 5637 matrices, apoptosis induced by palmitate was more noticeable compared to GSNO and high glucose treatments (data not shown), where the 5637 support matrix appeared to reduce the levels of caspase-3 mediated apoptosis by ~50% ($p < 0.05$) at 150 µM and by ~20% ($p < 0.05$) at 250 µM palmitate concentration compared to the β-cells grown in the absence of a support matrix.

Figure 6.26

Figure 6.27

6.3. Discussion

In the current chapter, the optimisation and modification of TG expression and activity in a human urinary bladder carcinoma cell (5637) matrix was attempted so it could be used as a TG-mediated support matrix for the increased maintenance of BRIN-BD11 β-cells, with a view towards current therapeutic strategies described for intra-hepatic islet transplantation. A number of studies have successfully used constitutive TG2 expression systems to study of the role of TG2 in cell matrix interactions (Fisher *et al*., 2009; Telci *et al.,* 2009; Balklava *et al*., 2002; Gaudry *et al*., 1999; Verderio *et al*., 1998; Jones *et al*., 1997; Gentile *et al*., 1992). However, this is the first time that TG2 expression in 5637 cells has been modulated in this way.

A 3-day untreated, lysed 5637 support matrix was prepared as described in a previous study by Ris *et al*., (2001). This matrix was shown to be extremely rich in TG2 expression and activity, and favoured optimal BRIN-BD11 adhesion, cell spreading, islet-like aggregation and viability when compared to other support matrices (data not shown). With the inclusion of this 3-day untreated 5637 matrix, five TG2-modifed variations of the 5637 matrix were used in these investigations. The other four pre-conditioned support matrices included two 5637 matrices exhibiting the over-expression of immobilised guinea pig liver TG2 (*gpl*TG2) protein on the 3-day 5637 matrix, and the over-expression of *gpl*TG2-catalysed extracellular matrix (ECM) cross-links; while the other two 3-day 5637 matrices were down-regulated for TG activity and/or expression by the addition active-site directed inhibitors R281, R283, BOC peptide (described previously in chapter 4), or the addition of siRNA constructed for the silencing of TG2 gene through transient transfection.

Characterisation of these five 3-day lysed matrices revealed the principle intention that: i) the untreated 5637 matrix possessed the highest amount of cell surface TG2 activity and ECM protein accumulation, ii) the *gpl*TG2 immobilised 5637 matrix contained high amounts of inactive cell surface TG2 protein, iii) the *gpl*TG2 cross-linked matrix would exhibit high amounts of immobilised protein with excessively

cross-linked ECM proteins (fibronectin, laminin, collagen, iv) the site-specific R281 and R283 TG inhibited matrix contained inhibited amounts of ECM protein accumulation but high amounts of inactive TG protein, v) the TG2 siRNA-transfected matrix expressed lower levels of TG2 protein resulting in concomitant lowering of ECM protein (fibronectin, laminin, collagen) accumulation.

For the preparation of these five support matrices it was important to keep conditions consistent so that the same number of cells were seeded to reach confluency after 3-days, and screened using ELISA-assessment of the TG2 protein levels. Competitive substrate assays were employed to determine the amount of catalytically active TG2 in the lysed 5637 matrices. The most catalytically active matrix was found to be the *gpl*TG2 cross-linked matrix, since this involved the addition of Ca^{2+} and DTT to create the excessive ECM cross-linking. It was found previously that catalytically cross-linking matrices in this way exposes cryptic sites on the collagen scaffolds that affect cell-matrix interactions (Chau *et al*., 2005). For the preparation of TG-inhibited matrices, it was important to aspirate and replace the treatments containing the irreversible active site-directed inhibitors R281, R283 and BOC inhibitor every 24 hours, in order to maintain a catalytically inactive 5637 matrix after 3 days. Previous studies have shown that the presence of the intact active site Cys^{277} is important for secretion and deposition of the enzyme into the matrix (Balklava *et al*., 2002). Disruption of this site in the R283 inhibited 5637 matrix may have crucial consequences for the export of TG2. Seeing as the active-site TG-inhibited matrices showed consistent detriment towards β-cell maintenance and function, compared to the controls, it could be suggested that this was due to lower overall ECM-associated TG2 levels, however further characterisation remains to be done. In the case of the TG2-modulated 5637 matrix which was transiently transfected using siRNA oligomers, the stealth siRNA duplexes TG13 and TG14 were found to create the most effective matrix for enzyme knockdown.

The principles for characterising the 5637 cells were related to changes in the extracellular expression and activity of TG2. An increase in extracellular TG2 is consistent with *in vivo* models of renal scarring in which an up-regulation of TG2 by tubular cells is associated with elevated levels of TG2 and cross-link in the peritubular space (Huang *et al*., 2010; Fisher *et al*., 2009; Skill *et al*., 2001; Johnson

et al., 1999). Numerous *in vitro* studies have also demonstrated a link between expression of TG2 and extracellular levels of the enzyme. For example, increased intracellular expression of TG2 in human umbilical vein cells (ECV 304) following transfection led to increased externalisation of TG2 (Gaudry *et al*., 1999). Similarly, induction of TG2 in 3T3 fibroblasts by stable transfection resulted in the appearance of an extracellular pool of the enzyme that localised to fibronectin (Verderio *et al*., 1998). In OK tubular cells, glucose stimulated increases in TG2 expression and activity were found to be associated with increased extracellular TG2 in the ECM and culture media (Skill *et al*., 2004). Another study using ECV 304 cells demonstrated that anti-sense transfection-mediated reductions in TG2 expression were accompanied by a decrease in the amount of externalised TG2 enzyme (Jones *et al*., 1997). In this study, characterisation of the ECV-304 cells compared to the 5637 cells as a support matrix showed that the 5637 support matrix was more conducive for the sustenance of BRIN-BD11 cells in particular.

It was evident from the 5 different 5637 support matrices used in this investigation, that manipulation of the amount of active TG2 protein allowed to externalise into the ECM network determined the relative ratios of extracellular TG2 enzyme in combination with fibronectin, laminin, collagen IV, which were differently distributed in each case. Previous trends found in extracellular fibrosis models have shown that fibroblasts stably transfected to over-express TG2 resulted in increased deposition of fibronectin and collagen, along with increases in NFκβ and TGFβ1 activation (Telci *et al.*, 2009; Verderio *et al*., 1999; Verderio *et al*., 1998), while kidney fibrosis models over-expressing TG2 showed similar trends (Huang *et al*., 2010; Fisher *et al*., 2009; Skill *et al*., 2004). Interestingly, application of the irreversible specific R283 inhibitor to these fibrotic models resulted in decreases in the amount of finronectin and collagen deposition leading to an amelioration of the fibrotic condition (Telci *et al*., 2009; Johnson *et al*., 2007; Skill *et al*., 2004). The dynamic nature of these TG2-mediated extracellular protein scaffolds could be quantified further for the 5637 matrices developed here, however this variation was revealed in preliminary protein turnover assays, and should be kept in mind for interpretation of β-cell behaviour on these matrices.

The presence of active extracellular TG2 in 5637 cells had important implications for its effects on the ECM, and β-cell attachment, aggregation and spreading. Whilst both active and inactive forms of the enzyme are capable of being expressed at the cell surface, only active cell surface enzyme is deposited into the ECM from intracellular stores (Balklava *et al*., 2002). It was possible to visualise and quantify the 3 dimensional morphology of rat islets grown on the TG2-rich 5637 matrices using the Leica confocal software. Glucose stimulated (20 mM) rat islets revealed marked increases in the aggregated size of the islets, suggesting that the 5637 matrix supported enhanced functional capabilities following islet-matrix disruption as a result of collagenase digestion. In the case of the 3-D aggregation results developed within this investigation, a pseudo-islet like morphology and high insulin producing content of BRIN-BD11 cells cultured was observed on the untreated TG2-rich 5637 matrices, which may be due to the high amount of TG2 cross-linking activity and preserved ECM protein scaffolds conferred by this matrix. The 5637 matrices inhibited for TG2 activity and expression were incapable of sustaining BRIN-BD11 pseudo-islet aggregation and associated insulin content, suggesting the importance of this enzyme within pancreatic β-cells.

It was proposed previously that matching the laminin-rich 5637 matrix to the integrins present on the β-cells was essential to the successful maintenance by this matrix (Ris *et al*, 2001; Bosco *et al*., 2000). TheTG2 enzyme is known to act as an integrin co-receptor for FN (Akimov *et al*., 2000) and associates with several β1 and β3 integrins, whilst simultaneously binding to FN. The presence of cell-surface TG2 enhances FN-matrix formation mediated by the α5β1 integrin (Akimov and Belkin, 2001). The adhesive function of TG2 is independent of its cross-linking activity but requires the formation of ternary complexes with FN and β1 and β3 integrins (Akimov *et al*., 2000). Another major component of the basement membrane is the laminin-nidogen complex, which extracellular TG2 was found to cross-link through interaction with the nidogen motif (Aeschlimann *et al*., 1992; Aeschlimann and Paulsson, 1991), and is expected to associate with laminin-specific integrins on the cell surface (Akimov *et al*., 2000). An analysis of the integrin profile from the BRIN-BD11 cells in association with the 5637 matrix protein scaffolds could lend further clues into the involvement of TG2 in this cell aggregation. Additionally, the maintenance of cell-to-cell contacts is thought to be important for synchronised

glucose-stimulated insulin secretion, so characterisation of a possible role for TG2 in aggregated β-cell contacts may contribute to a better understanding underlying this mechanism.

The expression of active TG2 in a fibronectin-complex at the cell surface appears to be important for attachment (Gentile *et al*., 1992; Jones *et al*., 1997). This is in keeping with a number of other studies in which modulation of increased extracellular TG2 (both active and inactive), is associated with increases in cell adhesiveness (Balklava *et al*., 2002; Gaudry *et al*., 1999a; Johnson *et al*., 1994; Jones *et al*., 1997; Verderio *et al*., 1998). Preliminary studies into the adhesion profile of BRIN-BD11 cells revealed the presence of increased focal adhesion points through immunofluorescent visualisation of actin on TG2-containing support matrices, and the appearance of enhanced phosphorylated focal adhesion kinase-mediated adhesion in the TG2-rich matrices also. Previous studies on osteoblasts and dermal fibroblasts demonstrated that TG2 is able to form complexes with fibronectin and heparin sulphate proteoglycans (syndecan-2 and syndecan-4) leading to the activation of actin-associated RhoA and stimulation of the cell survival focal adhesion kinase (Wang *et al*., 2011; Scarpellini *et al*., 1999; Telci *et al*., 2008; Verderio *et al*., 2003). The importance of the Rho-ROCK pathway was recently described in β-cell spreading, actin cytoskeleton dynamics, and insulin function (Hammar *et al*., 2009). Pilot studies using RGD-peptide on BRIN-BD11 cells attached to fibronectin matrices, as described previously by Verderio *et al*., (2003) revealed a similar TG2-mediated RGD-independent rescue of β-cells and could be extended as a mechanism that protects cells from apoptosis via non-classical adhesion dependent mechanisms such as anoikis.

In the case of BRIN-BD11 cells, significant increases in attachment and spreading were observed on the TG2-rich 5637 matrices compared to the TG2-inhibited matrices, suggesting the importance of TG2 cross-linking and an appropriate ECM protein scaffold for β-cell maintenance. Under conditions of hyperglycaemia (D-glucose), oxidative stress (GSNO), and lipotoxicity (palmitate), the TG2-rich 5637 support matrix appeared to confer greater β-cell attachment and spreading, suggesting a protective role for this enzyme under diabetic stress conditions. When the same diabetic stressors were assessed for β-cell death using MTS reagent, the

BRIN-BD11 cells attached to TG2-rich 5637 matrices appeared to retain high mitochrondrial viability. In the case of palmitate, caspase-3 mediated apoptosis was seen to decrease as a result of growth on the TG2-rich 5637 matrix. It is tempting to propose a possible protective role for extracellular TG2 through the exogenous addition of TG2 (Jones *et al*., 2006) to the endothelial microcapillary source in islets, having therapeutic potential for islet β-cells in the early stages of diabetes.

The data presented in this chapter demonstrated successful TG2-mediated modifications to a 5637 matrix previously reported for enhanced β-cell maintenance in the context of donor to recipient optimisation techniques preceeding intrahepatic islet transplantation. Such data confirms the importance of ECM-associated TG2 in the survival and insulin-secreting function of β-cells, which in the *in vivo* scenario may have important implications regarding islet β-cell and microvasulature endothelial cell interactions.

Chapter Seven:

General Discussion

S. Chandrasekhar from *Truth and Beauty*

"I am frankly puzzled by the difference that appears to exist in the patterns of creativity among the practitioners in the arts and the practitioners in the sciences: for, in the arts as in the sciences, the quest is after the same elusive quality: beauty. But what is beauty? There is ample evidence that in science, beauty is often the source of delight. One can find many expressions of such delight scattered through the scientific literature. Let me quote a few examples…

David Hilbert (in his memorial address for Hermann Minkowski):

> *Our science, which we loved above everything, had brought us together. It appeared to us as a flowering garden. In this garden there were well-worn paths where one might look around at leisure and enjoy one-self without effort, especially at the side of a congenial companion. But we also liked to seek out hidden trails and discovered many an unexpected view which was pleasing to our eyes; and when one pointed it out to the other, and we admired it together, our joy was complete.*

…indeed everything I have tried to say in this connection has been stated more succinctly in the Latin mottos:

> *Simplex sigillum veri – The simple is the seal of the true.*

and

> *Pulchritudo splendour veritatis – Beauty is the splendour of truth."*

Chandrasekhar, S (1910-1995), Astrophysicist and Nobel Laureate. *Truth and Beauty*, University of Chicago Press, 1987. Quoted from *The Oxford Book of Modern Science Writing* by Richard Dawkins, Oxford University Press, 2008.

Chapter Seven:

General Discussion

7.1. Introduction

The cellular function of the TG2 subfamily has been the focus of extensive research since its first description in 1957 (Sarkar *et al*., 1957). The TG2 enzyme is known for its widespread organ distribution and sub-cellular localisation; its multiple catalytic activities and complex regulation; as well as its implication in diverse cellular functions. This thesis investigates whether TG2 plays a role in diabetic disease and physiological pancreatic β-cell function and survival.

The transamidation reaction is brought about by the Ca^{2+}-dependent catalytic activity of TG enzymes during post-translational modification, which involves protein-protein cross-linking or the incorporation of primary amines into proteins by the formation of isopeptide bonds (Griffin *et al*., 2002). The monomeric TG2 enzyme consists of 685-691 amino acids and has a molecular mass of 77-85-kDa across different species (Griffin *et al*., 2002). The TG2 enzyme has important amino acids involved in the active site (Cys^{277}, His^{335} and Asp^{358}), the Ca^{2+} binding site (Ser^{449}, Pro^{446}, Glu^{451} and Glu^{452}), and the GTP-binding site (Ser^{171}, Lys^{173}, Arg^{478}, Val^{479} and Arg^{580}). In the absence of Ca^{2+}, TG2 assumes the latent conformation, where the reactivity of Cys^{277} is decreased either by hydrogen bonding with the phenolic hydroxyl group of Tyr^{516} or by formation of a disulphide bond with the neighbouring Cys^{336} (Noguchi *et al.*, 2001). The inhibitory effect of GTP on TG2 transamidating activity is mediated by GTP-binding and hydrolysis to GDP involving Ser^{171} and Lys^{173} residues of domain 2 called the GTP-binding pocket (Iismaa *et al*., 2000). The binding and hydrolysis of ATP is also an intracellular function carried out by the TG2 enzyme (Lee *et al*., 1993). The membrane-associated enzyme can bind phospholipids (Fesus *et al*., 1983), and localises at the cell surface for surface receptor function or as an

integrin co-receptor to fibronectin (FN) (Akimov *et al*., 2000). TG2 secretion out of the cells requires the active state conformation of the enzyme (Balklava *et al*., 2002) and an intact N-terminal FN binding site (Gaudry *et al*., 1999), where the TG2 enzyme cross-links to numerous extracellular matrix (ECM) protein scaffolds (Verderio *et al*., 2004).

A number of known mechanisms within the pancreatic β-cell could be extended to incorporate the functioning TG enzyme. A summary of the published literature in this field has been presented in table 7.1. The initial hypothesis for TG2 function in the pancreatic islet linked the Ca^{2+}-responsive transamidating activity of the enzyme (Clarke *et al*., 1959) to a possible catalytic role associated with increased intracellular Ca^{2+} levels which mediates glucose-stimulated insulin secretion coupling (Wollheim and Sharp, 1981). This was first confirmed by the presence of TG activity that was both Ca^{2+}- and thiol-dependent in rat pancreatic islets (Gomis *et al*., 1983a, Bungay *et al*., 1984a), and subsequent findings that TG2 may participate in the conversion of pro-insulin to insulin, as well as being involved in the subplasmalemmal machinery controlling the access of secretory granules to exocytotic sites in rat β-cells during insulin release (Sener *et al*., 1985; Bungay *et al*., 1986; Gomis *et al*., 1989; Owen *et al*., 1988). Studies using the lymphocytes of type-2 diabetics showed impaired TG2 cross-linking activity (Berntorp *et al*., 1989; 1987), while the sera of type-1 diabetics showed TG2 to be an auto-antigen associated with coeliac disease and pancreatic β-cell autoimmunity (Franzeze *et al*., 2007; Lampasona *et al*., 1999). The TG2 enzyme has already been seen to mediate retinoid-induced changes in β-cell insulin secretion (Driscoll *et al*., 1997), and to be involved in GTP depletion-induced caspase-mediated apoptosis in β-cells (Huo *et al*., 2003). There was a decline in insulin secretion and a gradual deterioration of glucose tolerance in $TG2^{(-/-)}$ mice lacking TG2 activity (De Laurenzi and Melino, 2001), which resulted in a type-2 diabetes phenotype (Porzio *et al*., 2007; Bernassola *et al*., 2002). These $TG2^{(-/-)}$ mice manifested a tendency of developing hypoglycaemia after administration of exogenous insulin as a consequence of enhanced insulin receptor substrate 2 (IRS-2) phosphorylation (Bernassola *et al*., 2002). Furthermore, the human TG2 gene in MODY patients revealed 3 missense mutations ($N^{333}S$; $M^{330}R$ and $I^{331}N$) found in residues located close to the catalytic site of the enzyme, which resulted in the

Table 7.1. Beta Cell literature review

loss of transamidating activity (Porzio *et al*., 2007). The most recent development to this field is described by Paulmann *et al*., (2009), where TG2 was found to covalently couple intracellular serotonin (5-HT) to the small GTPases Rab3a and Rab27a during insulin exocytosis.

The aims of this investigation were to characterise the expression and activity of TG2 in the pancreatic β-cell from intracellular insulin secretion and apoptotic survival processes, to cell surface and matrix-associated roles. In this study, various functional roles of TG2 were tested in clonal rat insulinoma BRIN-BD11 β-cells, and islets of Langerhans from human, rat, and TG2 $^{(-/-)}$ mice (De Laurenzi and Melino, 2001). The objective of chapter 4 was to explore the factors that may regulate TG catalytic activity during β-cell insulin secretion function such as post-translational modifications caused by intracellular Ca^{2+}, GTP, glucose and membrane proteases, as well as transcriptional regulation by retinoic acid. Enzyme activity was then inhibited by the use of irreversible active-site directed molecules (R281 and R283) to determine the role of the active enzyme in stimulus secretion coupling. The involvement of TG in β-cell survival under conditions of diabetic pathology such as hyperglycaemia, oxidative stress and lipotoxicity was then assessed. Following this, chapter 5 sought to characterise the expression of a novel short-form β-cell TG2 transcript that was recognised using antigen-antibody immunoreactivity and mRNA probes. In chapter 6, an extracellular support matrix (5637) was modified for TG2 expression and activity, and then used *in vitro* for the adhesion and maintenance of β-cells in culture. A summary of the results in this thesis have been presented in fig. 7.1, where the proposed roles of TG2 have been extended to known mechanisms in the functioning and dying β-cell.

7.2. Experimental pancreatic β-cell model

In order to assess the physiological role of TG2, various experimental models have been successively developed over time to either modulate the enzyme's expression, or to regulate its activity. These models have been subjected to careful interpretation for use in this investigation with pancreatic β-cells, since each of these individual systems result in their own respective limitations. For instance, numerous inhibitors of TG activity that were initially employed to study

Fig 7.1. Proposed role for TG function in the life and death of the pancreatic β-cell.

the loss of enzymatic activity, had the drawback of leading to non-specific effects on other biologically active molecules, including other TG isoforms (Cornwell *et al*., 1983; Bungay *et al*., 1984). Later, the transcriptional regulation of TG2 expression by agents such as retinoids often induced the expression of an array of other genes (Chen *et al*., 2001; Chiocca *et al.,* 1989; Davies *et al*., 1985). The use of cell lines stably transfected with the full length TG2 constructs to over-express the enzyme, together with transfection of TG2 cDNA in antisense orientation to silence the TG2 gene have proved more resourceful (Gentile *et al*., 1992; Melino *et al*., 1994; Mian *et al*., 1995; Jones *et al*., 1997; Verderio *et al*., 1998; Balklava *et al*., 2002). As a natural progression from these *in vitro* models, the effect that TG2 over-expression or silencing had on a single cell line in isolation was soon expanded to *in vivo* mouse models where the TG2 gene was knocked out (De Laurenzi and Melino, 2001; Nanda *et al*., 2001). The TG2 enzyme might also provide a novel therapeutic tool through the use of an exogenous enzyme delivery approach, in which *gpl*TG2 is administered to cultures in its active or inactive form mimicking the tissue wounding/fibrotic process, where physical injury results in cellular TG2 'leaking' into the surrounding ECM and becoming activated to catalyse the cross-linking of proteins (Balklava *et al*., 2002; Verderio *et al*., 2004; Jones *et al*., 2006).

Clonal BRIN-BD11 β-cells used as an experimental model for TG2 characterisation was investigated for the first time in this study. These cells offered the advantages of consistency, reproducibility, proliferation and propagation of highly uniform cell monolayers, the ability to quantitatively test the effect of various drugs or chemical compounds, manipulation by transfection, and the analysis of endogenous and exogenous protein functions related to cell signalling and transcription events. In biochemical and molecular research, the use of primary islet β-cells is limited by the availability of pancreatic endocrine tissue. Initial islet isolation of individual pancreatic cells, cell purification and the maintenance of native characteristics is a technically demanding process. In previous TG research (Gomis *et al*., 1983a; 1984a; 1989; Bungay *et al*., 1984, 1986, 1989; Sener *et al*., 1985; Lebrun *et al*., 1984), the use of primary rat islets was also limited by cellular and hormonal heterogeneity among different individuals. In this context, the major disadvantage of the BRIN-BD11 cell line

being used as an experimental model is primarily the disruption of islet cell-to-cell interaction which would influence cellular functions with known and still-unknown consequences.

The ability of immortalised β-cell lines to grow without limits is related to their tumour origins and genetic mutations, which could result in abnormal chromosomal content or protein expression, resulting in modified metabolism. Although insulinoma-derived cell lines have the advantage of unlimited growth in tissue culture, many exhibit vast differences in their insulin-secretory responsiveness to glucose compared to normal β-cells (Efrat, 2004). Only a few cell lines show a normal response to glucose concentrations in the physiological range with most transformed β-cell lines manifesting hypersensitivity to glucose (Efrat, 2004). The MIN-6, INS-1 and BRIN-BD11 cell lines used in this study have the advantage of best reflecting physiological conditions since they express appropriate levels of glucokinase compared to hexokinase (Ulrich *et al*., 2002). Most β-cell lines are derived from Simian vacuolating virus 40 (SV-40) T-antigen transfection, however the BRIN-BD11 cells were derived via electrofusion from a parent cell line, RINm5F which was derived by irradiation of a rat insulinoma (McClenaghan and Flatt, 1999; Gazdar *et al*., 1980). Although all these cell lines represent transformed cells, they have retained most of the characteristics of normal β-cells.

Chapters 4 and 5 presented the enzymatic and structural characterisation of TG2 in the β-cell. Unusual results obtained from the immortalised BRIN-BD11 β-cells revealed that the active TG2 protein expressed was ~20-kDa shorter than the classical TG2 isoform, which usually has a molecular weight between 77- to 85-kDa in other tissues (Griffin *et al*., 2002). Whether the shortened TG2 transcript reported in this study is expressed by BRIN-BD11 cells compensating for the immortalisation process by alternative splicing has yet to be fully determined. Nonetheless, this ~60-kDa β-cell specific TG2 appeared in primary islet β-cells as well, demonstrating both Ca^{2+}-activated cross-linking activity and GTP-binding potential. The biochemical results obtained using TG2 enzyme sourced from the BRIN-BD11 cells should therefore be interpreted throughout this study as the ~60-kDa novel TG2 isoform.

7.3. The Regulation of TG activity in pancreatic β-cells

The results from chapter 4 dealt with the potential regulators of TG activity in BRIN-BD11 β-cells using substrate-specific TG assays that quantified cross-linking activity. A consistent observation was that the amount of TG activity present in the clonal β-cell lines from all assays were significantly lower compared to the cross-linking activity found in primary islets of Langerhans standardised to the same total protein concentration. This difference could be attributed to the additional endothelial microcapillary source of TG present in the complex islets but not in the clonal β-cell monolayer. A comparison of the TG2 activity in a total islet fraction compared to a β-cell sorted fraction after mild trypsination may reveal the reason for these large differences in enzyme activity. In a complex cell system such as pancreatic islets, it is possible that the standardised competitive amine assays developed for TG2 cross-link analysis, also reflect the activity of other TG family members such as fXIII and TG1. The non-aggregated, undifferentiated state, and daily turnover potential of the BRIN-BD11 clonal β-cells is also likely to account for this low level of active TG expression (McClenaghan *et al*., 1998; Birkbichler *et al*., 1978; 1980). Physiologically, the turnover of adult β-cells is a slow process, since only about 1% of cells enter into the mitotic phases G1, S, G2 in M from resting G0 phase (Berne, 1998). As the steady-state replication rate is just over 2% per day, the lifespan of a rat β-cell can be estimated as ~58 days (Finegood *et al*., 1995). Adult β-cells are functionally heterogeneous (Pipeleers, 1992), but this heterogeneity has not been correlated to replicative ability or age. It is possible that those β-cells which divide might 'de-differentiate' and transiently lose function as they replicate (Bonner-Weir *et al*., 1989).

Previous assays carried out on rat islets revealed that a range of primary amines could be incorporated into N'N'-dimethylcasein substrate by the TG present in β-cells (Gomis *et al*., 1983, Bungay *et al*., 1984). Kinetic analysis of the competitive substrates methylamine, monodansylcadaverine, propylamine and ethylamine showed that the most effective primary amine was monodansylcadaverine with a

potency that matched their ability to inhibit glucose-stimulated insulin release in rat islets (Bungay *et al.,* 1984a). The incorporation of radiolabelled [^{14}C]-putrescine into N'N'-dimethylcasein used in this study, which is a modification of the radiolabelled filter paper assay by Lorand *et al.* (1972), revealed that the specific TG activity of rat islets was $7.02^{\pm 0.8}$ U/mg protein, while BRIN-BD11 cells were $0.193^{\pm 0.1}$ U/mg protein. In this investigation, optimal quantifiable amine incorporation was achieved using either biotin cadaverine or FITC-cadaverine into endogenous protein substrates. The chemical parameter usually used to assess the inhibition potency of competitive amines is the specificity constant k_{cat}/Km, where k_{cat} is the turnover rate, and *Km* is the Michaelis constant (Siegel and Khosla, 2007). It could be useful to compile a comparison of the inhibition potencies of competitive amines on the β-cell models used in this study, in order to further understand the substrate specificity of TG2 in β-cells.

Experiments into the regulation of the TG2 enzyme showed that it was possible to activate the TG stores in BRIN-BD11 cell homogenates with the addition of exogenous Ca^{2+}, under low glucose conditions. The Ca^{2+}-activated TG stores from these cells were then inhibited in the presence of the reversible TG2 inhibitor, GTP-γ-S, and later bound to GTP-agarose in western blotting experiments, suggesting normal enzymatic functional switches between GTP-binding and Ca^{2+}-binding for the ~60-kDa β-cell TG2 isoform. The thermodynamics of Ca^{2+} and GTP ligand-binding to TG2 occurs via conformational changes which facilitate or interfere with the peptidyl-glutamine substrate interaction, where the intrinsic ability of TG2 for high affinity Ca^{2+}-binding (Kd: 0.15 uM) occurs together with high affinity binding of GTP (Kd: 1 uM) now thought to involve the replacement of GDP normally bound to the protein (Bergamini *et al*., 2010). The physiological levels of GTP in most studied cells correspond to the calculated Ki (90-150 μM) for TG inhibition by GTP, implying that triggering TG activity into cells requires both a decrease of guanine nucleotide levels and major influx of extracellular Ca^{2+}, two events that usually happen in pathological situations like cell senescence, degeneration or apoptosis (Mhaouty-Kodja, 2004). However, the pancreatic β-cell is a special exception that contains a Ca^{2+}-rich environment during stimulus secretion coupling, which may affect the subcellular behaviour of

TG2 in a unique way. For instance, in a previous study with rat insulinoma cells using 30 mM glucose stimulation, intracellular cytosolic Ca^{2+} concentrations increased from 104 to 248 nM in the first 15 minutes (Hoenig and Sharp, 1986). In the resting β-cell (ie. not stimulated by glucose), it could be assumed that the cross-linking function of TG2 would be limited to the cell surface where guanine nucleotides are absent and Ca^{2+} concentrations are in the mM range.

In β-cell research, the TG2 protein may also be known as a G protein called the Ghα subunit which exhibits both GTPase and TG activities (Nakaoka *et al*., 1994). Activation of Gh by G protein coupled receptors (GPCRs) turns off TG activity and shifts Ghα into a signal transducer that regulates downstream effectors. Interaction of Gh with α1-adrenergic receptors switches off TG activity, and directly stimulates PLCδ1, thereby resulting in phosphoinositide hydrolysis and an increase in intracellular concentrations of Ca^{2+} (Nakaoka *et al*., 1994). When the distributional pattern of the numerous phospholipase C (PLC) isozymes were analysed in the rat pancreas, PLCδ1 expression strongly featured in islet tissue, suggesting that the modulation of pancreatic endocrine function occurs through PLC-mediated signal transduction (Kim *et al*., 2001). Ghα can also integrate signals from retinoic acid and nitric oxide. Retinoic acid is a known inducer of Ghα expression and in HeLa cells it was also shown to increase the GTP-binding ability and associates with the plasma membrane inducing phosphoinositide hydrolysis (Singh and Cerione, 1996). Nitric oxide selectively inhibits the cross-linking function of Ghα without altering its GTPase activity (Lai *et al*., 2001). Interestingly, such S-nitrosylation is expected to increase the sensitivity of TG activity to the inhibitory effect of GTP. It would be interesting to determine if the ~60-kDa BRIN-BD11 β-cell TG2 reported here acts in the same capacity as the full-length Ghα signal transducer that stimulates PLCδ1 and causes increases in intracellular Ca^{2+} concentrations as a result of phosphoinositide hydrolysis. Heterotrimeric and low molecular weight GTP-binding proteins have also been involved in regulating glucose-triggered exocytosis of insulin from pancreatic β-cells (Kowluru *et al.*, 1996), where TG2 was found to covalently couple intracellular serotonin (5-HT) to the small GTPases Rab3a and Rab27a during insulin exocytosis (Paulmann *et al*., 2009).

In the presence of retinoic acid which is a known transcriptional regulator that increases TG2 expression (Chiocca *et al*., 1988), BRIN-BD11 TG activity increased, supporting the idea that the usually low level of differentiation and high proliferative potential of this cell line could be a contributing factor towards the low enzyme activity observed (Birkbichler *et al*., 1978; 1980). It was reported previously with INS-1 and RINm5F cells that retinoic acid treatment resulted in increased glucose-stimulated insulin secretion, TG expression and activity (Driscoll *et al.*, 1997). Similarly in this study, the presence of 0.3µM *all trans* retinoic acid for 2 days led to the BRIN-BD11 cells adopting a more differentiated and aggregated morphology compared to untreated cells. Previous studies suggest that increased expression of retinoid receptors is critical for an increase in TG2 gene expression (Johnson *et al*. 1998), suggesting that the BRIN-BD11 cells used in this study may possess similar retinoid receptors. These results for TG2 function in β-cells are promising when viewed in the context of stem-cell based therapies for type-1 diabetes (Ostrom *et al*., 2008), where retinoic acid promoted the generation of endocrine progenitor cells that differentiated into insulin-producing β-cells. The use of retinoid therapy to differentiate cancer cells and downregulate pancreatic cancer could also point to a role for TG2 in this mechanism (Riecken and Rosewicz, 1999). It is worth noting here that TG2 may also have the potential to indirectly influence β-cell growth by the transcriptional regulation of growth factors such as the growth inhibitor TGFβ1 (Kojima *et al*., 1993), IL-6 (Ikura *et al*., 1994; Suto *et al*., 1993), TNFα gene family (Kuncio *et al*., 1998), increase growth/differentiation through the factor midkine (Kojima *et al*., 1997), or negatively regulate the growth signal of EGF through down-regulation of its high affinity receptor (Katoh *et al*., 1996).

When live BRIN-BD11 cells were treated for 15 minutes with the membrane proteases, trypsin and dispase, the β-cell surface-associated TG activity was significantly reduced compared to untreated cells. Proteolysis studies have shown that TG2 is easily proteolysed by calpain and trypsin in the presence of Ca^{2+}, while GTP-binding protects the protein against proteolysis (Zhang *et al*., 1998; Begg *et al*., 2006). The decrease in cell-surface TG activity in BRIN-BD11 β-cells

as a result of membrane proteases supports previous findings that TG2 catalysed the formation of phosphoprotein polymers in the membrane fraction in rat islets (Owen *et al*., 1988). In addition, subcellular fractionation studies revealed that, in addition to its postulated role in the control of intracellular motile events, TG also participated in surface protein interactions at the site of cell-to-cell contact (Gomis *et al*., 1989). Partial proteolysis by trypsin led to the cytosolic release of an activated enzyme from membrane-bound TG2 in metastatic tumours (Knight et al., 1990) and keratinocytes (Rice *et al*., 1990), suggesting that the BRIN-BD11 membranes that were proteolysed with trypsin in this study could have been released intracellularly instead of being active at the cell-surface.

Another regulator of increased TG2 activity in BRIN-BD11 cells was D-glucose. This was shown previously with studies on rat islets where glucose stimulation resulted in the *de novo* synthesis of the enzyme or alternatively the induction of a more reduced state due increased sulphydryl groups, with subsequent changes in the thiol-disulphide balance (Gomis *et al*., 1986b). The use of biotin cadaverine in the formation of amine-isopeptidyl adducts in BRIN-BD11 cells also proved useful for the determination of cell-surface glucose-mediated enzyme activity. The presence of high TG activity at the cell surface of BRIN-BD11 cells when stimulated with glucose (10 mM to 20mM) could potentially contribute to cell adhesion through covalently protein-crosslinked matrices at the sites of cell-to-cell contact. In the case of cell surface TG expression, protein substrates in the plasma membrane are accessible to cell surface-associated TG by small molecular weight amines in the form of SDS-insoluble large molecular weight aggregates (Slife *et al.*, 1986). Cell-surface TG2 was previously shown to covalently incorporate itself, fibrinogen and fibronectin into high molecular weight aggregates on the extracellular surface of isolated hepatocytes (Barsigian *et al*., 1991). Accumulating evidence suggests that transport of TG2 to the cell surface may involve non-covalent association with β1and β3 integrins during biosynthesis (Akimov *et al.*, 2000). Interaction of TG2 with multiple integrins of β1 and β3 subfamilies, and α5β1 integrin in particular, suggests a role for TG2 as an integrin adhesion co-receptor for FN that promotes TGFβ-mediated FN assembly (Akimov *et al.*, 2000; Akimov and Belkin, 2001; Verderio, *et al.*, 2003). The integrin

profile of BRIN-BD11 cells may lend further clues into the observed formation of a TG2-FN complex to enhance cell-to cell contact.

The successful incorporation of FITC-cadaverine into BRIN-BD11 endogenous substrates allowed for the visualisation of cellular expression and activity of the TG2 enzyme switching from normal physiological expression to highly cross-linked stress conditions with hyperglycaemia and oxidative stress. The amount of FITC-cadaverine incorporated into endogenous β-cell substrates by TG2 catalysis appeared to increase in aggregation upon glucose stimulation. The use of antibodies against FITC-cadaverine also made it possible to detect labelled β-cell TG2 substrates by molecular weight after western blotting. The formation of high molecular weight phosphoprotein polymer substrates during glucose stimulation in rat islets have been described previously (Smethurst *et al*., 1993; Lindsay *et al*., 1990; Gomis *et al*., 1989; Owen *et al*. 1988). The conversion of proinsulin to insulin also depends on the oriented translocation of microvesicles from the rough endoplasmic reticulum (ER) to the Golgi complex, in which TG2 activity has been implicated (Gomis *et al*., 1986b). Pro-insulin is produced in the ER by the cleavage of the signal peptide and formation of disulfide bonds, thereafter passing to the Golgi apparatus where it is packed into vesicles. After cleavage of the C peptide, mature insulin is formed in the vesicles and is stored in the form of zinc-containing hexamers or secretory granules until secretion (Koolman and Röhm, 2005). The treatment of islets with cytochalasin B facilitated insulin release by causing the contraction of the microfilamentous cell web (Somers *et al*., 1979; Malaisse and Orci, 1979), which normally controls the access of secretory granules to the exocytotic site. Many cytoskeletal proteins are known to be TG2 substrates *in vitro*, including actin and myosin (Eliqula *et al*., 1998; Nemes *et al*., 1996; Kang *et al*., 1995), β-tubulin (Lesort *et al*., 1998; Cohen and Anderson, 1987), α-actinin (Puszkin and Raghuraman, 1985), and spectrin (Harsfalvi *et al*., 1991). Further characterisation of the endogenous substrates interacting with the ~60-kDa BRIN-BD11 β-cell TG2 would be worth expanding on in order to understand the mechanical events involved in the translocation of secretory granules to their exocytotic site through membrane trafficking and the fusion of membranes at such a site.

7.4. The inhibition of TG activity in pancreatic β-cells

Although many biological functions have been attributed to TG2, the precise *in vivo* function of this protein in pancreatic β-cells still remains a mystery. The *in vitro* functions which occur as a result of TG2 being transfected into the cell, or TG2 being up-regulated with cell-differentiation factors such as retinoic acid, or the enzyme being artificially activated by inducing large intracellular Ca^{2+} fluxes, should be cautiously broached since these unnatural and incompatible cellular environments may never occur in an *in vivo* environment. It is therefore important to attempt to study TG in its natural environment, both in cell culture and *in vivo*. The use of specific, potent irreversible TG inhibitors has the advantage of clarifying a biological function by blocking only a fraction of the TG protein which has been enzymatically activated under physiological conditions. Disadvantages associated with the use of these TG inhibitors are the possibilities of off-target inhibitor binding, or the effect of trapping the inhibited TG2 protein into an unnatural conformation relative to its cellular localisation (Siegel and Khosla, 2007). Conformational constraints placed upon TG2 when inhibited could prevent it from performing its natural biological functions, which could cause additional side-effects. Correlating TG2 conformation to biological function may be a key element in the design of TG2 inhibitors for the future, since these molecules should ideally allow for the conformational flexibility of the TG protein.

Small molecule and peptidomimetic TG inhibition is an important field of study in possible therapeutic targets of various disease states, and in understanding the mechanism of normal TG activity under physiological conditions. TG2 inhibitors can be divided into 3 classes based upon their mechanism of inhibition: competitive amine inhibitors (such as putrescine, biotin cadaverine and fluorescein cadaverine), reversible inhibitors (such as GTP and ATP), and irreversible custom-designed inhibitors. Previous investigations on pancreatic islets using competitive substrate inhibition as a result of alkylamines, monodansylcadaverine, *N*-*p*-tosylglycine, bacitracin, glycine alkylesters and hypoglycaemic sulphonylureas confirmed the importance of TG cross-linking

during insulin secretion function (Hutton *et al*., 1982; Malaisse et al., 1983; Gomis *et al*., 1983 and 1984; Lebrun *et al*., 1984). Hindrances to be overcome with these compounds was non-specificity, poor penetration into the islet cell and effects on cationic fluxes which caused increased Ca^{2+} entry into the β-cells.

The use of custom-designed irreversible active-site inhibitors R283 and R281 have been documented in numerous investigations, with a view towards therapeutic medical application in various pathologies (Kotsakis *et al*., 2010; Caccamo *et al*., 2010; Mastroberardino and Piacentini, 2010; Huang *et al*., 2010 and 2009; Collighan and Griffin, 2009; Griffin *et al*., 2008; Telci *et al*., 2009 and 2008; Garcia *et al*., 2008; Maiuri *et al*., 2008; Siegel and Khosla, 2007; Jones *et al*., 2006; Beck *et al*., 2006; Griffin *et al*., 2004, Griffin et al., 2002; Freund *et al*., 1994). These irreversible TG2 inhibitors prevent enzyme activity by covalently modifying the enzyme, thereby preventing substrate binding. They contain a dimethylsulfonium group that has previously been shown to interact with Cys_{277} within the active site via an acetonylation reaction leading to non-competitive and irreversible inhibition of the enzyme (Freund *et al*., 1994; Griffin *et al*., 2004).

The R281 and R283 inhibitors were applied previously to stably-transfected mouse colon carcinoma CT26 cells expressing a catalytically active (wild type) and a transamidating-inactive TG2 (Cys_{277}Ser) mutant (Kotsakis *et al*., 2010), where active externalised TG2 from the cells resulted in inhibited tumour growth, increases in TGF-β1 levels and matrix-deposited fibronectin, the effect of which was reversed by the use of these TG inhibitors. A role for TG2 in pregnant human myometrial contractility has been established, where application of R283 and R281 attenuated contractility, thus suggesting the importance of TG2 in the regulation of pregnant uterine contractility (Alcock *et al*., 2010). The active-site directed inhibitor, R283 was used to determine the importance of TG activity in the development of a collagen scaffolded dermal rudiment for enhanced wound healing response (Garcia *et al*., 2008). The importance of TG2 activity was demonstrated in differentiated human SH-SY5Y neuroblastoma cells, where the use of inhibitor R283 resulted in enhanced Parkinsonian neurotoxicity (Beck *et al*., 2006). Investigation into cystic fibrosis showed that increased TG2 activity lead to functional sequestration of the anti-inflammatory peroxisome proliferator-

activated receptor (PPARγ) and increases in the classic parameters of inflammation, such as TNF-α, tyrosine phosphorylation, and MAPKs, wherein specific inhibition of TG2 by R283 was able to reinstate normal levels of PPARγ and dampen down inflammation both in cystic fibrosis tissues and transmembrane conductance regulator (CFTR)-defective cells, suggesting potential therapeutic benefits for this targeted inhibitor (Maiuri *et al.*, 2008). The R283 site-directed inhibitor of TG abolished NF-κB and TGFβ1 activation and the subsequent elevation in the synthesis and deposition of extracellular matrix proteins such as collagen, confirming that nitric oxide may play a role in the regulation of extracellular matrix-associated TG2 activity (Telci *et al*., 2008; 2009). TG2 inhibition by R283, achieved via drug treatments was beneficial to the treatment of Huntington's disease in animal models, where inhibition of TG-mediated cross-linking of Huntington protein stopped the progression of pathogenesis (Mastroberardino and Piacentini, 2010). In celiac disease, the inhibition of TG2 by R283 could represent a strategy to prevent the toxic action of gliadin by controlling gliadin-specific T-cell activation (Maiuri *et al*., 2005).

Diabetic nephropathy fibrotic and scarring models using renal proximal tubular epithelial cells showed that both R283 and R281 site-directed inhibitors of TG normalized TG activity and ECM-associated epsilon(gamma-glutamyl)lysine levels per cell, returning them to near control levels with non-transcriptional reductions in deposited collagen and fibronectin (Skill *et al*., 2004). Furthermore, application of the site-directed TG inhibitor R281 caused a 25% reduction in the levels of active TGF-β1 in the kidney of a streptozotocin model of diabetic nephropathy and TG2 overexpressing opossum kidney (OK) proximal tubular epithelial cells, exerting a renoprotective effect by targeting not only direct extracellular matrix collagen deposition but also TGF-β1 activation and recruitment, suggesting a clinical application for the R281 TG inhibitor in the progression of experimental diabetic nephropathy (Huang *et al*., 2009 and 2010).

The R283 and R281 inhibitors were also highly effective at reducing β-cell TG activity in BRIN-BD11 homogenates and live cells within this investigation. These inhibitors caused significant reductions in the insulin levels released by BRIN-BD11 cells and rat islets when measured with the I^{125}]-insulin RIA.

However, the relatively equal effectiveness of R281 and R283 on the insulin secretion function of β-cells indicates that their principle inhibitory actions are extracellular. This cell-surface TG could also be exerting an effect on the β-cell ATP-sensitive K_{ATP} channels during membrane depolarisation. It could be said that the poor cell solubility expected of R281 could limit any potential non-specific effects on other intracellular TG or thiol containing enzymes, however this inhibitor is not specific to TG2 since it has been shown previously to inhibit the extracellular activity of TG1 in myocardium microvascular endothelial cells (Baumgartner *et al.*, 2004). Furthermore, the chances of R283 affecting intracellular targets apart from TG2 cannot be dismissed given its known ability to inhibit other TG isotypes such as fXIII (Freund *et al*., 1994).

TG2 conformation may be a critical factor in the success or failure of enzymatic inhibitors of TG2. It is clear that multiple conformations of TG2 exist. The binding of GTP or irreversible inhibitors to TG2 causes significant shifts in electrophoretic mobility of the protein under native conditions (Murthy *et al*., 1999). Certain anti-TG2 antibodies have a high affinity for one population of TG2, while other antibodies bind preferentially to a distinct population of the enzyme (Fesus and Laki, 1977; Monsonego *et al*., 1998; Maiuri *et al.*, 2005). In the GDP-bound crystal structure of TG2, the two C-terminal β-barrels overlap a significant surface area of the catalytic core domain effectively blocking substrate access to the active site (Liu *et al*., 2002). A crystal structure of TG2 bound to an irreversible inhibitor was recently done (Siegel and Khosla, 2007; Pinkas *et al*., 2007) where the two C-terminal β-barrels were extended away from the catalytic core and twisted 180°, giving the protein a rod-like shape and making the active site easily accessible to substrates. It is not possible to predict the biological consequence of trapping TG2 in certain conformations, due to a lack of data correlating TG2 conformation to biological function (Siegel and Khosla, 2007). It is possible that conformational constraints placed upon TG2 when inhibited prevent it from performing one or more of its natural biological functions, causing potential side effects.

7.5. The Experimental TG2$^{(-/-)}$ Mouse Pancreatic Islet Model

The generation of TG2$^{(-/-)}$ knockout mice was aimed at understanding the physiological role of TG2 (De Laurenzi *et al.*, 2001; Nanda *et al*., 2001; Bernassola *et al*., 2002). However these animal models did not clarify predominant functions for this enzyme, since mild glucose intolerance associated with defective first phase insulin secretion (Bernassola *et al*., 2002) was the main feature observed in the model created by De Laurenzi *et al*., (2001), while altered fibroblast function and impaired wound healing was primarily observed by the model from Nanda *et al*., (2001). The lack of TG2 in the mice generated by De Laurenzi *et al*. (2001) also prevented the production of active TGFβ1 in macrophages exposed to apoptotic cells, affecting the efficient deletion or phagocytosis of CD4+CD8+ apoptotic bodies in the thymus (Szondy *et al*., 2003), and de-regulated the autophagosome maturation process (D'Eletto *et al*., 2009). Taking these observations into account, the TG2$^{(-/-)}$ mice generated by De Laurenzi *et al*. (2001) were bred in-house for this investigation, and the islets of Langerhans from young mice were isolated and characterised further for TG2 expression and activity, apoptotic signals, and insulin secretion function.

These TG2 knockout mice rendered the advantage of inhibiting the expression of a specific full-length TG2 protein, resulting in not only a lack of TG2-mediated cross-linking activity in this mouse tissue, but the absence of the full-length TG2 protein entirely (De Laurenzi *et al*., 2001). Immediate morphological observations while analysing the islets for confocal microscopy was that the C57BL6 wild-type TG2$^{(+/+)}$ mouse islets showed significantly higher specific cross-linking activity through the incorporation of radiolabelled [^{14}C]-putrescine into N'N'-dimethylcasein compared to the TG2$^{(-/-)}$ islets. The use of FITC-cadaverine may have been compromised by the molecule's entry into live, intact islets, however the TG2$^{(+/+)}$ mouse islets still showed high fluorescein incorporation, which was obviously lacking in the TG2$^{(-/-)}$ islets even upon 20 mM glucose stimulation. Given the importance that TG2 cross-linking is claimed to have in the insulin secretion process (Gomis *et al*., 1984; Bungay *et al*., 1984), it was surprising that the transgenic knockdown of the full-length TG2 did not cause severe alterations

in glucose-stimulated insulin secretion compared to the $TG2^{(+/+)}$ mouse islets, as measured in this study with confocal microscopy and through [I^{125}]-insulin RIA previously carried out by Bernassola and co-workers (2002). The authors who created this transgenic mouse, have not characterised the TG protein expressed in the $TG2^{(-/-)}$ islets of Langerhans through western blotting as yet. In this investigation, western blotting experiments revealed that the CUB7402 antibody immunoreacted with a predominant ~60-kDa protein in both the $TG2^{(+/+)}$ and $TG2^{(-/-)}$ islets, suggesting that the animals used in this investigation may have been expressing the shortened β-cell TG2 isoform all along, similar to the short-form BRIN-BD11 rat β-cell TG2 described above. If the presence of a shortened β-cell TG2 isoform in the $TG2^{(+/+)}$ mouse islets can be corroborated, it may explain the reason why the glucose-stimulated insulin secretion in the $TG2^{(-/-)}$ islets was only mildly impaired despite the genetic knockdown of full-length (75-kDa) TG2. It is already accepted that the $TG2^{(-/-)}$ mouse models still express other TG family members (Porzio *et al*., 2007; De Laurenzi *et al*., 2001) such as TG1, however a reason why this model would express a possible alternative spliced shortened β-cell TG2 transcript, recognised by the TG2-specific CUB7402 antibody, may only be properly understood through further understanding of the mechanism of genetic TG2 deletion in the transgenic knockdown (De Laurenzi *et al*., 2001).

Supporting the role of full-length TG2 in diabetes, is that $TG2^{(-/-)}$ mice were found to have low energy levels with a maturity onset diabetes of the young (MODY) phenotype (Porzio *et al*., 2007; Bernassola *et al*., 2002) and a mitochondrial functional defect that lead to alterations in the respiratory chains, which decreased the global production of ATP in tissues (Szondy *et al*., 2006), leading to the conclusion that TG2 acts as a protein disulphide isomerase by contributing to the formation of disulphide bridges in proteins of mitochondrial respiratory complexes (Mastroberardino *et al*., 2006). Bernassola *et al.* (2002) have implicated TG2 in the aggregation, internalisation and intracellular processing of the insulin receptor (IR) in which TG2 is thought to cross-link receptors in the area of clathrin coated pits (Davies *et al*., 1980) through increased insulin-induced tyrosine phosphorylation of IRβ and IRS-2 in skeletal muscle. At the cellular level insulin associates with target tissues through the autophosphorylation of the insulin receptor which possesses intracellular tyrosine

kinase activity (Baldwin *et al.*, 1980). Insulin resistance, which is associated with obesity and type-2 diabetes occurs as a result of defects in the insulin receptor internalisation process, as was seen with the $TG2^{(-/-)}$ mice (Bernassola *et al.* 2002). The interaction of different ECM proteins with cell surface intregins would result in a changed cytoskeletal organisation (polymerised actin networks) that may affect the rate of insulin receptor endocytosis or internalisation. An additional reason why the $TG2^{(-/-)}$ mice may have exhibited the diabetes type-2 phenotype is that insulin receptor internalisation may have been affected by the ECM proteins onto which the cells adhere, since the lack of ECM-associated TG2 in these cells could have re-modelled the poorly cross-linked islet endocrine matrix scaffolds surrounding the β-cells (Verderio *et al*., 1999; Johnson *et al*., 1999).

The results obtained in this study with $TG2^{(-/-)}$ mouse islets support some of the findings in previous publications using the same transgenic animals (Porzio *et al*., 2007; Bernassola *et al*., 2002). However, new evidence was obtained on these islets using [^{14}C]-putrescine and fluorescein cadaverine as competitive amine substrates, and the characterisation of a novel western blotting profile. Preliminary experimentation suggests the satisfactory use of this islet model in order to understand the various roles that TG2 may be playing. Of further interest, is that the MIN-6 cells originate from a transgenic C57BL6 mouse insulinoma expressing an insulin-promoter/T-antigen construct, whereas the $TG2^{(-/-)}$ mice and their wild-type counterparts are also of the same mouse strain. The MIN-6 cells are highly differentiated, forming pseudo-islets in culture, and they express GLUT-2 and glucokinase, responding to glucose within the physiological range in the presence of nicotinamide (Miyazaki *et al*., 1990). The MIN-6 cell line could potentially make a good accompaniment to any future $TG2^{-/-}$ mouse experiments.

7.6. Survival Role for TG2 in Pancreatic β-cell Apoptosis

A role for TG2 in β-cell apoptosis remains to be fully addressed. This investigation showed the potential for excessive TG2 cross-linking being involved in BRIN-BD11 cells under the stress-induced conditions of hyperglycaemia and oxidative stress, through the visualisation of FITC-cadaverine incorporation into endogenous β-cell substrates. The use of R281 as an extracellular irreversible

inhibitor of TG, resulted in exacerbated mitochondrial-associated cell death measured with tetrazolium compound under the conditions of hyperglycaemia and oxidative stress, while the induction of lipotoxicity using palmitate (Welters *et al*., 2004 and 2006) resulted in a sufficient *in vitro* model to assess caspase 3-mediated apoptotic cell death in the BRIN-BD11 cells. The presence of caspase-3 mediated apoptosis was also evident in the freshly isolated $TG2^{(+/+)}$ and $TG2^{(-/-)}$ mouse islets visualised using confocal microscopy, however poor penetration of the antibody into the permeabilised islet prevented quantification of this trend. In HIT-T15 insulin secreting β-cells, GTP depletion resulted in increases of TG2 activity (Huo *et al*., 2002 and 2003), however the induction of apoptosis as part of a cascade of events involved reductions in the enzyme during late-stage caspase activation.

The physiological occurrence of β-cell apoptosis has been shown *in vivo* during the involution of the β-cell mass in the post partum pancreas (Scaglia *et al.*, 1995) and in a remodelling of the endocrine pancreas in the neonatal rat (Scaglia *et al.*, 1997). Elevated glucose levels can have beneficial effects, such as stimulating β-cell proliferation, as well as having detrimental effects, such as cell death. In chronic hyperglycemia, oxidative stress is increased and can lead to apoptosis, but antioxidants prevent the loss of cells (Kaneto *et al*., 1999). The β-cells exposed to hyperglycemia accumulate intracellular proteins modified with *O*-linked monosaccharide *N*-acetylglucosamine (O–Glc–Nac), with a sustained accumulation of such modified proteins suggested to cause the β-cell apoptosis induced by hyperglycemia (Liu *et al.* (2000). Prolonged exposure to increasing levels of hyperglycemia correlates with progressive loss of β-cell differentiation in rats (Jonas *et al.* 1999) as indicated by altered expression of several key islet transcription factors and other islet genes important for normal glucose-stimulated insulin secretion, however despite the increased apoptosis in these animals, there was increased β-cell mass.

The induction of oxidative stress in the BRIN-BD11 cells using S-nitrosoglutathione (GSNO) induced a marked increase in intracellular TG2 cross-linking as a result of the cellular stress response. In keratinocytes, GSNO treatment resulted in inhibited cornified envelope formation, but increased cross-

linking of a natural substrate of TG called locicrin (Rossi *et al*., 2000). The presence of NO modifies proteins through nitrosylation of free cysteine residues, which is important in mediating biologic activity. TG2 is in proximity to sites of NO production, has 18 free cysteine residues, utilizes a cysteine for catalysis, and was found in the presence of Ca^{2+} to result in an inhibition of TG2 activity by NO (Lai *et al*., 2001). Titration of the thiol groups of TGs indicated that nitric oxide (NO) regulated enzymatic activity by chemically modifying a cysteine residue through S-nitrosylation (Rossi *et al.*, 2000). The use of GSNO as a NO donor modulates and decreases apoptosis at an upstream level, by interfering with the ability of AP-1 to induce CD95L expression (Melino *et al*., 2000; Bernassola *et al*., 1999; Melino *et al*., 1997). A role for NO in the regulation of TG2 function in the extracellular environment has also been proposed, where NO donors caused decreases in TG activity, which was paralleled by a reduction in activation of NF-κβ and TGFβ1 production with a subsequent decrease in collagen expression and deposition (Telci *et al*., 2009).

It is now widely accepted that TG2 plays both pro- and anti-apoptotic roles in apoptosis (Wyllie *et al*., 1980). The onset of apoptosis *in vivo* is often characterised by the induction of the TG2 gene (Amendola *et al.*, 1996; Knight *et al.*, 1993; Piacentini *et al.*, 1992; Fesus *et al.*, 1989). The cross-linking of intracellular components by TG2 was found to be pivotal in the stabilisation of the apoptotic cells prior to clearance by phagocytosis (Knight *et al.*, 1991). TG2 is also known to share substrates, such as histone H2B, pRB, actin, troponin with pro-apoptotic caspases (Piacentini *et al.*, 1999). TG2 is thought to be mediated through both upstream and downstream events in the apoptotic pathway. The induction of TG2 is regulated by a number of factors such as retinoic acid and TGF-β1 that are also able to regulate apoptosis suggesting that TG2 is able to act as an early effector “death” protein (Melino and Piacentini, 1998). Similarly, the GTP-binding ability of TG2 may also contribute to the regulation of apoptosis (Melino and Piacentini, 1998) as GTP availability affects second messengers that are known to inhibit apoptosis such 1,2 diacylglycerol (DAG) (Leszczynski *et al*., 1994; Nakaoka *et al*., 1994).

TG2 may also promote apoptosis by direct interaction with proteins of the apoptotic pathway such as *Bax* where interaction with the BH3 domain of TG2 can cause conformational changes leading to translocation of *Bax* to the mitochondria, the release of cytochrome c, and cell death (Rodolfo *et al*., 2004). In the downstream stages of apoptosis, the activation of TG2 leads to extensive cross-linking of intracellular proteins and the formation of detergent insoluble protein polymers, which again may serve to stabilise apoptotic cells, preventing leakage of proinflammatory intracellular components prior to clearance by phagocytes (Fesus, 1998; Fesus *et al*., 1987). TG2 may also protect cells from apoptosis via non-classical adhesion dependent mechanisms such as anoikis. Studies on osteoblasts and dermal fibroblasts demonstrate that TG2 is able to form complexes with fibronectin and heparin sulphate leading to the activation of RhoA and stimulation of the cell survival focal adhesion kinase (Verderio *et al*., 2003). Similarly, studies of renal scarring *in vivo* also suggest that TG2 may participate in a novel form of cell death in which epithelial cells die through extensive crosslinking of their intracellular proteins as a result of accumulating levels of TG2 (Johnson *et al*., 1997). Taking these findings into account, a more intensive investigation into the role of TG2 in pancreatic β-cell apoptosis may prove useful.

7.7. Novel Shortened TG2 Isoform in Pancreatic β-cells?

The evidence presented in chapter 5 described a potentially new TG2 isoform specific to the pancreas (see fig. 7.2. and 7.3. for proposed β-cell TG models), which could represent only the fourth C-terminal truncated TG2 isoform of its type discovered to date (Fraij *et al*., 1992; Fraij and Gonzales, 1996; Monsonego *et al*., 1997; Lai *et al*., 2007). However, these preliminary results still require much confirmation on the molecular level. The presence of a ~60-kDa CUB 7402-immunoreactive protein in pancreatic BRIN-BD11 β-cells using western blotting seems plausible when supported by the demonstration of truncated ~2.5kb and ~1.0kb mRNA transcripts in these β-cells using northern blotting, and similar short-form transcripts present in these cells when using primers directed at a previously described alternatively spliced short-form of TG2 in rat brain (Monsonego *et al*., 1997). This ~60-kDa β-cell TG2 was also present in western

Figure 7.2. proposed beta cell protein

Figure 7.3. proposed beta cell transcript

blots of rat, mouse, and human islets. In the BRIN-BD11 cells, the ~60-kDa protein showed the ability to bind GTP-agarose, however whether this level of GTP-binding might be compromised as a result of functional changes in the truncated isoform was not fully ascertained in these preliminary experiments. In addition to the ~60-kDa short-form TG2, the BRIN-BD11 cells also revealed the presence of an ~120-kDa protein similar that described by Knight *et al*., (1991), and an ~38-kDa protein similar to the alternatively spliced product described by Fraij and Gonzales, (1996) which was structurally made up of the N-terminus region of the TG2 molecule, and contained the Cys_{277} active site region.

The evidence for a truncated form of the β-cell TG in this investigation was supported by the design of the northern blot probes using BAMH1 digestion (Johnson *et al*., 1997), where the BAM1638 probe was directed towards the C-terminal end, with the BAM388 probe directed towards the N-terminal. The differences in the probe specificity revealed changes in the expression level of the ~2.5-kb and ~1.0-kb truncated β-cell TG2 isoforms, suggesting a possible alternative splicing event in the C-terminal end of the transcript. Northern blot analysis of the short form TG2 in HEL cells (Fraij *et al*., 1992) revealed the absence of the classical 3` end in the shorter 1.9-kb transcript, and eliminated the possibility that smaller RNA species were due to a degradation of the classical 3.5-kb transcript. When the alternatively spliced products in HEL cells were compared to normal fibroblasts (Fraij *et al*., 1992), the short-form 1.9-kb transcript was expressed at much lower levels, suggesting that the short-form in HEL cells may have been associated with oncogenesis. It remains to be confirmed whether the short-form transcript reported here in BRIN-BD11 cells may only be the result of cell immortalisation and oncogenesis.

Further molecular studies may confirm whether the shortened mRNA β-cell transcripts revealed through northern blotting in this study are a result of possible genetic mutations or oncogenesis in the immortalised BRIN-BD11 cells by analysing and comparing the mRNA from normal *ex vivo* primary islets of Langerhans in human, rat and mouse. The isolation of non-degraded mRNA from primary islets is a challenge due to the amount of time it takes before intact islets can be isolated free from the remnants of exocrine tissue that remain after the

collagenase digestion process. In this study, the addition of the freshly isolated islets immediately into Trizol was found to be the best way to obtain high quality total RNA for use in northern blotting experiments. It was also possible to obtain intact RNA from rats and mice by snap freezing the islet suspension immediately after isolation in cryovials using liquid N_2. However, these samples were nonetheless transferred to Trizol soon after. In the case of human islet tissue obtained from the UK Tissue Bank, the amount of time most of the tissue was stored after snap freezing in cryovials, and the transportation process meant that the RNA was mostly degraded before extraction with Trizol. If these experimental measures can be optimised, a northern blot of the primary islets from human, rat and mouse could confirm whether the shortened β-cell transcript reported in this investigation is a result of BRIN-BD11 oncogenesis or a novel alternatively spliced TG2 isoform that could be related to the unique β-cell environment where its protein has numerous functions.

Further support for a truncated alternatively spliced TG2 isoform in BRIN-BD11 β-cells was confirmed in the present study with preliminary RT-PCR experiments using the C-terminal directed TG2-specific primers designed by Monsonego *et al*., (1997) in rat brain, where the PCR amplification products of a 512 bp fragment consistent with full-length TG2 and a corresponding 410 bp fragment for short-form TG2 was evident using DNA electrophesis. The possibility of the two shortened rat clonal BRIN-BD11 β-cell TG2 transcripts being similar in structure to the previously reported (Monsonego *et al*., 1998; Fraij *et al*., 1992) alternatively spliced rat brain TG2 isoforms opens a new avenue for future experimental design in order to characterise these novel β-cell-specific TG2 isoforms further.

Western blot characterisation of the TG2 protein in pancreatic β-cells was carried out in this investigation, using the commercially available CUB7402 protein. The BRIN-BD11 rat clonal cells revealed a predominant ~60-kDa protein that was interpreted as a shortened spliced isoform of TG2. Additional immunoreactive proteins in the BRIN-BD11 cells migrated to 120-kDa and 35-kDa. The 120-kDa protein was interpreted as the inactive, oncogenic TG2 reported previously by Knight *et al.*, (1990). In addition all three subtypes of this β-cell TG, were evident

from 2-D gel electrophoresis and western blotting experiments, where the ~60-kDa TG2 protein showed multiple post-translationally modified spots that could be interpreted as phosphorylation. The ~60-kDa protein appeared in lysates from MIN-6 mouse clonal β-cells, $TG2^{(+/+)}$ and $TG2^{(-/-)}$ mouse islets as well. Human islet homogenates exhibited two predominant proteins at 85-Da and ~60-kDa. It would be interesting to fractionate the different subtypes of TG2 reported here to enable fuller protein characterisation in the future.

Shortened mRNA transcripts that have been confirmed as alternatively spliced events of TG2 genes have only been reported by a small number of authors (Fraij *et al*., 1992; Fraij and Gonzales, 1996; Monsonego *et al*., 1997; Citron *et al*., 2001; Antonyak *et al*., 2006; Liu *et al*., 2007; Tee *et al*., 2010). The short-form TG2 proteins reported by Monsonego *et al*., (1997) featured at 73-kDa and 77-kDa, while the short TG2 isoform described by Fraij *et al*., migrated to 61-kDa. A shorter N-terminal homologue of TG2 was described by Fraij and Gonzales (1996), and was characterised by a polypeptide encoding 349 amino acid residues with a molecular weight of 38.7-kDa. It is possible that smaller RNA species may encode for the truncated proteins with novel carboxyl termini, since both 63-kDa and 38-kDa isoforms produced transcripts that start with the regular coding sequence for TG2 and then fail to splice at specific donor sites, resulting in the use of an alternative exon that contains a stop codon (Fraij and Gonzales, 1996). It has been suggested that the additional amino acids in both these TG2 isoforms generate glycine-rich areas homologous to the consensus GTP-binding regions (Takeuchi *et al*., 1992), and it was confirmed that this short-form 63-kDa TG2 isoform displayed a higher GTP-binding activity than the native 80-kDa human TG2 enzyme (Fraij, 1996), which may be related to functions in cell signalling.

The evidence already existing for shortened, alternatively spliced variants of TG2 have shown deletions and alterations in the C-terminal end of these molecules, where the GTP-binding and phospholipase C regions are expected to be compromised (Fraij *et al*., 1992; Fraij and Gonzales, 1996; Monsonego *et al*., 1997; Citron *et al*., 2001; Antonyak *et al*., 2006; Liu *et al*., 2007; Tee *et al*., 2010). However, the site of ATP and/or GTP hydrolysis in TG2 is located in the N-terminal region between amino acid residues 1 and 185 (Lai *et al*., 1996),

suggesting that most of the enzymatic function remains conserved. Given the evidence in this study with BRIN-BD11 cells, where a truncated version of the β-cell TG2 was revealed using protein, mRNA and cDNA analysis, it is plausible that this truncated version may be similar to other alternatively spliced TG2 isoforms with deletions in the C-terminal region (Citron *et al*., 2002). Assigning a role for this shortened TG2 protein in β-cells could be related to its primary function of insulin secretion, in which glucose causes increases in ATP that are accompanied by increases in cytosolic Ca^{2+}, switching TG2 cross-linking activity on. The fine balance between Ca^{2+}, GTP and ATP ligand-binding by TG2 in the Ca^{2+}-rich insulin secreting β-cell may hold the reason behind a possible alternative splicing event in the adaptation of this protein to its unique insulin-producing environment.

7.8. Extracellular Matrix Associated TG2 in pancreatic β-cells

The possible role of TG2 in the extracellular matrix of pancreatic β-cells is a previously undescribed area of research. It would be expected that within an islet the pancreatic β-cells would be closely associated through cell-to-cell contacts, but also exposed to a rich source of extracellular TG2 from the surrounding endothelial microcapillaries. The involvement of TG2 in the extracellular matrix has been firmly established in many tissue-types despite the enzyme's externalisation mechanism being unknown (Fisher *et al*., 2009; Verderio *et al*., 1998; Jones *et al*., 1997; Johnson *et al*., 1997; Aeschlimann and Paulson, 1994; Martinez *et al*., 1994; Barsigian *et al*., 1991). It would be expected in pancreatic β-cells that the mechanism of protein externalisation would be unusual because TG2 lacks a signal peptide and is not secreted by a classical endoplasmic reticulum/Golgi-dependent route (Lorand and Graham, 2003). Many ECM proteins are known substrates of TG2 (Esposito and Caputo, 2005), and the cross-linking of these proteins by endothelial cell TG2 is thought to play a role in the stabilisation of the basement membrane (Martinez *et al*., 1994). Pancreatic β-cells have recently been found to lack a basement membrane, instead using the rich source of endothelial cells from microcapillaries dispersed throughout the islet (Nikolova *et al*., 2006) to compensate for normal cellular function and survival.

There is much potential for ECM-associated TG2 to offer a protective role towards the prolonged maintenance of pancreatic β-cells during diabetic pathogenesis, as a support matrix application for donor islets before intrahepatic islet transplantation and associated stem cell research. In this investigation, five 3-day lysed 5637 matrices were developed so that: i) the untreated 5637 matrix possessed the highest amount of cell surface TG2 activity and ECM protein accumulation, ii) the *gpl*TG2 immobilised 5637 matrix contained high amounts of inactive cell surface TG2 protein, iii) the *gpl*TG2 cross-linked matrix would exhibit high amounts of immobilised protein with excessively cross-linked ECM proteins (fibronectin, laminin, collagen), iv) the site-specific TG inhibited matrix contained inhibited amounts of ECM protein accumulation but high amounts of inactive TG protein, v) the TG2 siRNA-transfected matrix expressed lower levels of TG2 protein resulting in concomitant lowering of ECM protein (fibronectin, laminin, collagen) accumulation.

The use of the 5637 pre-conditioned matrix in order to culture β-cells had the advantage of mimicking the *in vivo* environment of a rich external source of TG2 from the endothelium of the islet microvasculature which enhanced insulin secretion function as a result of integrin-matrix matching (Ris *et al*., 2002) from dispersed β-cells. These TG2-rich matrices appeared to compensate for the loss of cell-cell contact following the protein digestion as a result of islet isolation and also promoted migration and aggregation in the clonal undifferentiated BRIN-BD11 cells into pseudo-islet structures. It is interesting that the external TG2 present on the β-cell surface may be involved in cell migration (Balklava *et al*., 2002), and cell adhesion (Jones *et al*., 1997) by a mechanism that is independent of its transamidating activity (Verderio *et al*., 2003). This was demonstrated in the current study, using inactivated TG2 that was immobilised on the matrix surface, but still supported enhanced β-cell function.

Disadvantages associated with the use of this support matrix for the prolonged culture of β-cells before islet transplantation or other experimentation is the introduction of a whole array of complex changes that may occur in the β-cell as a result of the support matrix, and the further disruption of cell-cell contact in order to re-use the cultured islets. The 5637 urinary bladder carcinoma cell line was

chosen in this investigation, due to previous reports of successful integrin-matrix matching (Bosco *et al*., 2001; Ris *et al*., 2002) with β-cells. Since islet transplantation involves the injection of islets through the hepatic portal vein, to be embedded within the liver (Shapiro *et al*., 2000), perhaps a more conducive model for matrix support should be sourced from the liver. TG2 has already been implicated in the stabilisation of different tissues such as liver, heart, lung, muscle and kidney revealing extracellular distribution of the enzyme with intensive staining in collagen rich connective tissue (Verderio *et al*., 2004), and co-localision with nidogen (Aeschlimann *et al*., 1991). The use of the 5637 support matrices under *in vitro* conditions meant that it was difficult to gauge the concentration of active TG2 once added to the culture medium since much of it was likely to be sequestered by the FN-containing serum. Perhaps the closest physiological equivalent could be equated to situations of chronic tissue damage, where increased extracellular TG2 both secreted and released by virtue of cell damage leads to increased cross-linking, promoting massive tissue scarring and fibrosis (Johnson *et al*., 1997; Grenard *et al*., 2001).

The 5637 matrix which was deficient in TG2 expression and activity could also be viewed as a similar environment to the previously described $TG2^{-/-}$ model (De Laurenzi *et al*., 2001; Bernassola *et al*., 2002; Porzio *et al*., 2007), where it would be expected that the microcapillary source of external TG2 would have been compromised due to gene silencing. The TG2 cross-linked ECM is thought to be composed of TG2 autopolymers, together with heteropolymers of TG2, FN and or other ECM proteins deposited by the pre-conditioned matrix (Jones *et al*., 2006). It has been demonstrated through previous turnover studies that the *in vitro* cross-linking of a conditioned cell-deposited matrix by TG2 leads to an increased resistance of MMP digestion, thus accounting for a slower rate of turnover (Jones *et al*., 2006). The same was observed in the current investigation, where the ECM levels of fibronectin, laminin, and collagen production in the 5637 support matrix were lessened when TG2 expression and activity was blocked. It would be interesting to determine whether the ECM-associated TG2 and scaffold proteins are affected within human pancreatic islets under conditions of diabetic stress.

Preliminary studies into the adhesion profile and cell signalling of BRIN-BD11 cells revealed the presence of increased focal adhesion points through immunofluorescent visualisation of actin on TG2-containing support matrices, and the appearance of enhanced phosphorylated focal adhesion kinase (FAK)-mediated adhesion in the TG2-rich matrices. These results could be potentially applied to the growing understanding FAK-mediated cell signalling mechanisms within the context of β-cells. Previous studies have shown that during tissue injury and/or remodelling, the compensatory effect of the TG-FN complex in the presence of RGD-containing peptides is mediated by TG2 binding to the heparan sulphate chains of the syndecan-4 cell surface receptor (Wang *et al*., 2010 and 2011; Telci *et al*., 2008; Verderio *et al*., 2003). This binding mediates activation of protein kinase Cα (PKCα) and its subsequent interaction with β_1 integrin, where cell signalling by this process leads to the reinforcement of actin-stress fiber organization, the activation of focal adhesion kinase (FAK) and ERK1/2 mitogen-activated protein kinases, and the involvement of the Raf-1 protein (Telci *et al*., 2008). Activation of α5β1 integrin was shown to occur by an inside out signalling mechanism (Wang *et al*., 2010). Unlike syndecan-4, syndecan-2 does not interact directly with TG2 but acts as a downstream effector in regulating actin cytoskeleton organization through the ROCK pathway, in which PKCα is likely to be the important link between syndecan-4 and syndecan-2 signalling (Wang *et al*., 2011). Membrane trafficking of TG2, and hence its extracellular activity is involved in TG2 binding to cell-surface heparin sulphate proteoglycan (HSPG), with fibroblasts deprived of syndecan-4 being unable to effectively externalize TG2 (Scarpellini *et al*., 2009). It is likely that pancreatic β-cells may be following similar mechanisms during the membrane trafficking involved with insulin secretion.

7.9. Summary and Conclusion

A number of diseases result from abnormalities in insulin secretion, including type 1 and type 2 diabetes, persistent hyperinsulinemic hypoglycaemia of infancy (PHHI) and insulinoma. Understanding the role of TG in the mechanisms that regulate insulin secretion may allow for the development of new therapies for these diseases as well as contribute to our ability to engineer insulin-producing

cells for cell replacement therapies of type 1 diabetes. Additionally, the role of TG in regulating insulin secretion could be important for the development of new drugs and to generate substituted insulin-producing cells. There still remains an urgent need to establish a "normal" not cancerogenic β-cell line of human origin either by establishment of a spontaneously transformed stable cell line directly from the healthy pancreas or by the use of stem cells.

Ideas that have developed from this project could lead to promising future research for characterising the role of TGs in the pancreatic β-cell. The clustered aggregation of β-cells in human islets make them a difficult model to manipulate by transfection. Following on from the results with the BRIN-BD11 and MIN-6 clonal β-cells in this investigation, these experimental models could be used for future transfection studies, where up-regulation or down-regulation of various isoforms of TG2 could reveal a better understanding of enzymatic action. The primary role that sets the β-cell apart from other cell types in which TG activity has been involved, is that of a Ca^{2+}-rich intracellular environment during insulin secretion function. The dynamic between Ca^{2+}-, GTP- and ATP-binding of the intracellular TG enzyme would be an important mechanism to unravel biochemically. It would be useful to carry out a more intensive characterisation of the physiological β-cell substrates on which the enzyme acts, in order to understand its precise balance on the membrane trafficking of insulin granules through to exocytotic release. This association could be extensive, considering the high aggregation of phospho-polymers in β-cells that result from TG enzyme activity. The modulation of TG expression using transcriptional growth factors such as retinoids would be worth pursuing, considering the therapeutic potential for β-cell survival during diabetes. The behaviour of TG on the β-cell membrane could shed light on a number of important functions such as cell-cell contacts which are important for a synchronised physiological glucose response.

New developments in the invention of site-specific TG2 inhibitors could prove useful in β-cell research. For instance, fluorescently tagged site-specific TG inhibitors would improve upon the use of non-specific competitive amines. A novel group of site-directed irreversible specific inhibitors for TGs have been developed by Griffin and co-workers (Daneshpour *et al*., 2010; Griffin *et al*.,

2008), targeted to the extracellular matrix, and with some using a liposome-based drug-delivery system for the site-specific delivery of these TG inhibitors into the liver. The inhibited full-length TG2 expression in the $TG2^{(-/-)}$ mice could have important implications in the context of a shortened β-cell TG2 transcript. The presence of alternative processing of TG could have an impact on drug discovery paradigms, where modulation of activity by targeting unique structural elements of the enzyme may have therapeutic value. Furthermore, a protective role for ECM-associated TG2 in β-cells could be extended to current advances in diabetes treatments and curative strategies using intra-hepatic islet transplantation and β-cell stem cell research, where the application of TG2 protein could potentially prolong the maintenance of donor islet tissue.

Interpretation of the significant results presented in this thesis, involves a wholistic view of TG2 function in the pancreatic β-cell, and being a part of an aggregated islet structure that is richly supplied by a microcapillary vasulature containing endothelial cells that have their own extracellular source of TG2 as well (fig. 7.1.). In this investigation, the TG enzymatic activity specific to BRIN-BD11 β-cells was regulated by a number of important factors. The influx of high cytosolic Ca^{2+} concentrations which are associated with a response to elevated glucose levels and insulin secretion, or transcriptional up-regulation by retinoic acid were seen to increase TG cross-linking activity. The reversible inhibition of TG cross-linking activity was observed in the presence of GTP, and the short-form β-cell TG2 protein was seen have GTP-binding capability despite possible deletions in the C-terminal region. Irreversible active site-specific R281 and R283 TG inhibitors caused a significant quantifiable decrease in glucose-stimulated insulin secretion in rat β-cells. Cellular stress factors such as oxidative stress and hyperglycaemia could also be potential regulators of TG activity in β-cells. Such data may be important in developing experimental and therapeutic strategies aimed at controlling the expression and activity of TG within β-cells.

The identification and characterisation of a possibly alternatively spliced shortened TG2 mRNA transcript resulted in a re-assessment of the functional role the new protein might perform in the β-cell. Western blot analysis showed that rat, human and mouse ($TG2^{+/+}$ and $TG2^{-/-}$) pancreatic β-cell TG2 appeared as a predominant

shortened ~60-kDa molecular weight protein with an additional ~120-kd and 35-kDa protein in some cases. The impaired cross-linking activity observed in $TG2^{-/-}$ mouse islets compared to their $TG2^{+/+}$ counterparts supports the potential for inhibited TG2 activity being a direct cause of the diabetes type 2 phenotype, however the short-form immunoreactive TG2 protein that was evident in these mouse islets, suggests a possible functional difference between the full-length (~80-kda) TG2 and the shortened isoform (~60-kDa) in the pancreatic islet. To our knowledge, no TG of this size has been reported previously in β-cells. It is likely that the short-form TG described here represents an isoform of the full-length TG2 since northern blotting revealed multiple novel short-form mRNA transcripts in BRIN-BD11 cells, while RT-PCR analysis of the β-cell TG suggested the absence of amino acids from the carboxyl-terminus similar in structure to rat brain alternative splicing transcripts. This assumption is further strengthened by the β-cell TG showing a conservation of the active site Cys^{277} region as demonstrated in activity assays, the potential for GTP-binding, and the presence of a putative Ca^{2+}-binding site immunoreacting with the CUB 7402 antibody. Additionally, TG1 (which may be transcriptionally regulated by retinoic acid in concert with TG2) could also be playing a role in the functional dynamic of the pancreatic islet.

Investigations into a role for ECM-associated TG2 in pancreatic β-cells lead to the characterisation of five 3-day lysed matrices where: i) the untreated 5637 matrix possessed the highest amount of cell surface TG2 activity and ECM protein accumulation, ii) the *gpl*TG2 immobilised 5637 matrix contained high amounts of inactive cell surface TG2 protein, iii) the *gpl*TG2 cross-linked matrix would exhibit high amounts of immobilised protein with excessively cross-linked ECM proteins (fibronectin, laminin, collagen), iv) the site-specific TG inhibited matrix contained inhibited amounts of ECM protein accumulation but high amounts of inactive TG protein, v) the TG2 siRNA-transfected matrix expressed lower levels of TG2 protein resulting in concomitant lowering of ECM protein (fibronectin, laminin, collagen) accumulation. The TG2-rich matrices which possessed high cross-linking activity proved optimal for maintaining β-cell adhesion, spreading, aggregation and resulting insulin production, a process seen to be mediated by actin filaments and the phosphorylation focal adhesion sites.

In conclusion, the investigations in this thesis have laid the foundation for many avenues of research, that include an intracellular role for the active TG enzyme, an intracellular role for TG with as a G-protein function, a possible cell survival role, and an extracellular matrix-associated role within pancreatic β-cells.

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