Physiological and therapeutic roles of tissue transglutaminase in tumour growth and angiogenesis

Panayiotis Kotsakis

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

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Declaration

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

This is to certify that the work was carried out by the candidate itself. Due acknowledgement has been made of all assistance received.

Signed.....P Kolst.....
(Candidate)

Signed..... .....
(Director of Studies)

Abstract

This study provides evidence for the potency of the protein crosslinking enzyme tissue transglutaminase (TG2) as a novel inhibitor of tumour growth and angiogenesis. When B16-F1 melanoma cells were injected into TG2 wild-type and knock-out mice a delay in tumour growth rates was observed only in the former. Immunohistochemistry of tumours extracted from TG2 wild-type mice revealed lack of TG2 expression within the tumour body, but increased deposition at the host-tumour interface suggesting that the enzyme is expressed by stromal cells as a host response against tumour development. Conversely, in a syngeneic model, CT26 colon carcinoma tumours demonstrated marked reduction in growth and in certain instances regression following intratumour injection of *gpl*TG2. Application of *gpl*TG2 into CT26 cultures ruled out any cytotoxic, pro-apoptotic, or anti-proliferative effect. Instead, immunohistochemistry of regressing tumours revealed the presence of fibrotic-like tissue rich in applied TG2, collagen and the ε(γ-glutamyl)lysine isopeptide bond. Cross-linking of collagen artificial matrices by TG2 was also shown to reduce the invasive capacity of CT26 tumour cells in an *in vitro* experimental setting of chemotaxis. Relevant to angiogenesis, an observed increase in migration of TG2 knock-out mouse aortic endothelial cells in matrigel suggested that the expression of the enzyme is not needed during neovascularisation. Using *in vitro* co-culture and *ex vivo* aortic models of angiogenesis, repeated application of active TG2 was shown to suppress capillary tube formation without causing cellular toxicity. The applied TG2 led to ECM accumulation, and sequestration of transforming growth factor-β1 into the ECM. Single cell studies demonstrated that TG2-induced matrix accumulation was facilitated by a decreased rate of matrix turnover and an increased resistance of cross-linked matrix to MMP-1. The importance of the transamidating function of TG2 in the blockade of tumour growth was verified when CT26 transfected for stable expression of inactive TG2 constructs failed to elicit tumour regression as opposed to CT26 expressing the active TG2. Both CT26 clones exhibited increased attachment but reduced migration in surfaces coated with fibronectin. Intratumour injection of TG2 creates a reinforced ECM barrier at the tumour/stroma interface that may provide a novel antiangiogenic strategy for use in solid tumour containment.

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Publications

Papers

Jones, R.A., Kotsakis, P., Johnson, T.S., Chau, D., Ali, S., Melino, G. and Griffin, M. (2005). Matrix changes induced by transglutaminase 2 lead to inhibition of angiogenesis and tumor growth. Currently under review by *Cell Death and Differentiation*.

Abstracts

Kotsakis, P., Jones, R.A., Johnson, T.S., Chau, D., Ali, S. and Griffin, M. (2004). Tissue transglutaminase inhibits angiogenesis and delays tumour growth when delivered exogenously in its active form. International Conference on Tumour Progression and Therapeutic Resistance. November 8-9, Philadelphia, PA

Kotsakis, P., Jones, R. and Griffin, M. (2003). Tissue transglutaminase inhibits angiogenesis *in vitro* and *ex vivo* by increasing ECM deposition and down-regulating its processing. Nottingham Trent university Poster day. April 2003, Nottingham. Awarded the 1st prize.

Griffin, M., Jones, R.A., Parry, J., Balklava, Z., Johnson, T.S. and Kotsakis, P. (2002). Tissue transglutaminase in tumour growth and progression: a novel strategy for tumour therapy. *Minerva Biotechnologica*; **14** (2), 213.

List of Abbreviations

AD	Alzheimer's disease
ADP	adenosine-5'-diphospate
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
bFGF	basic fibroblast growth factor
BM	basement membrane
cAMP	adenosine 3',5'-cyclic monophosphate
CD	celiac disease
cDNA	complementary deoxyribonucleic acid
DAB	3',5'-diaminobenzidine
DAG	diacylglycerol
DFMO	α-difluoromethylornithine
DMEM	Dulbeco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
DTT	dithiothreitol
CE	cornified envelope
EC	endothelial cell
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbant assay
EPC	endothelial progenitor cell
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FN	fibronectin
FXIII	factor XIII
G418	geneticin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	gas chromatography
GDP	guanosine-5'-diphosphate
GFP	green fluorescent protein
gplTG2	guinea pig liver tissue transglutaminase
GTP	guanosine-5'-triphosphate
GTPase	guanosine-5'-triphosphatase
HBSS	Hank's balanced salt solution
HD	Huntington's disease

H&E	haematoxylin and eosin
HFDF	human foreskin dermal fibroblasts
HIF	hypoxia induced factor
HRP	horseradish peroxidase
HUVEC	human umbilical large vein endothelial cells
HYP	hydroxyproline
IL	interleukin
IgG	immunoglobulin
IP_3	inositol triphosphate
kDa	kilodaltons
LB	luria bertani
LDH	lactate dehydrogenase
LTBP-1	Latent transforming TGF-1 binding protein1
M	molar
MAb	monoclonal antibody
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
PIP_2	phospatidylinositol diphosphate
PKC	protein kinase C
PLC	phospholipase C
PMSF	Phenyl methyl sulfonyl fluoride
pTG	prostate transglutaminase
RA	retinoic acid
Rb	retinoblastoma
RGD	argine-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
uPA	urokinase plasminogen activator
UV	ultra violet
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
XTT	(2,3-bis[methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide

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Chapter 1:

Introduction

1. Introduction

Supramolecular protein assemblies are held together both by weak non-covalent secondary chemical forces (ionic interactions, van der Waals repulsion, hydrogen bonds) and by covalent bonds, which are 10 to 100 times stronger, with energies up to 400kJ/mol. Although the weakness of non-covalent interactions is essential, for it allows them to be broken and re-formed in a dynamic molecular interplay, covalent linkage of proteins is central to certain physiological processes such as blood clotting, epidermal differentiation and hair shaft formation (Lorand, 1984). One of the bonds involved in the latter processes requires the action of the calcium dependent enzymes transglutaminases (TGs). The term transglutaminase was first introduced by Clarke and co-workers in 1959 to describe the Ca^{2+}-dependent transamidating activity present among other tissues in the liver of the guinea pig (Clarke *et al.*, 1959). TGs can catalyse a wide range of aminolytic and hydrolytic reactions through a double nucleophilic displacement mechanism that mediates a calcium acyl transfer reaction between the γ-carboxamide group of a peptide-bound glutamine residue and the ε-amino group of peptide-bound lysine (Lorand and Conrad, 1984). The resulting bonds are covalent, with a stable double-bond character and were first demonstrated by Pisano *et al.* (1968) in the stabilisation of fibrin monomers. The specificity of TG for peptide-bound glutamine residues distinguishes them from similar enzymes involved in glutamine metabolism. The Enzyme Commission recommends the denomination "R-glutaminyl-peptide-amine-γ-glutamyl transferase" (EC 2.3.2.13), although the commonly used denomination remains "TG" (Lorand and Conrad, 1984).

1.1 TG-mediated post-translational modification of proteins

TG-mediated post-translational modification of proteins can lead to the cross-linking of two proteins through catalysis of a calcium dependent acyl transfer reaction that results in covalent ε(γ-glutamyl)lysine linkage between the proteins (Folk *et al.*, 1973; figure 1.1*A*). In this reaction, peptide bound glutamine residues act as acyl donors and a peptide-bound lysine as the acyl acceptor. However, a whole variety of suitable primary amine-groups can also function as the acyl acceptor (Folk *et al.* 1973). Nucleophilic attack directed by the active site thiol group of the enzyme to the γ-carboxamide group of a glutamine

residue in the substrate protein leads initially to the formation of a γ-glutamylthiolester intermediate, with concomitant release of ammonia (Pedersen *et al.*, 1994). The reaction proceeds driven by the release of ammonia and its subsequent protonation, which occurs readily under physiological conditions. Upon formation of the γ-glutamylthiolester intermediate, the second amine donor substrate binds to the acyl-enzyme complex and attacks the thiolester bond. The acyl group is then transferred to the acyl receptor substrate, resulting in the formation of an isopeptide bond and the release of the enzyme. At this stage, the active site Cys^{277} is re-established in its original form, thereby able to participate in further catalysis cycles. The covalent acyl-enzyme intermediate is believed to be the rate-limiting step in this reaction.

TGs are more selective towards peptide-bound glutamine substrates compared to the amine donor lysine residue (Aeschlimann and Paulsson, 1994), which probably defines their physiological functions (Gorman an Folk, 1984). However, residues preceding accessible amine donor lysine in a native protein may exert their influence on the enzyme's cross-linking potential (Grootjans *et al.*, 1995), as they might have an effect on the tertiary conformation of the protein and consequently hinder the accessibility of the enzyme. Therefore, conformational variation between different TGs results in different affinity/specificity of these enzymes for glutaminyl substrates (Aeschlimann and Paulson, 1994).

The covalent cross-links resulting from TG activity are extremely stable structures which were thought to be irreversible in most situations and resistant to most proteases (Lorand and Conrad, 1984). However, the hydrolysis of the ε(γ-glutamyl)lysine isopeptide by cytosolic TG and Factor XIIIa has now been demonstrated (Parameswaran *et al.*, 1997). Additionally, enzymes from the secretory products of the medicinal leech have been discovered and shown to hydrolyse the ε(γ-glutamyl)lysine isopeptide bond in blood clots (Lorand *et al.*, 1996).

TGs also catalyse the incorporation of primary amines into proteins through the formation of N'(γ-glutamyl)amine bonds, using molecules such as histamine, putrescine, spermine, and spermidine as acyl acceptors (Folk and Finlayson, 1977; Lorand and Conrad, 1984; Figure 1.1*B*). A free amine on the R group can also be linked to another γ-glutamyl group on a second protein, forming a N',N'-bis(γ-glutamyl) polyamine linkage. Post translational modification of proteins by polyamidation may result in altered biological activity, antigenicity, and turnover rate (Aeschlimann and Paulson, 1994).

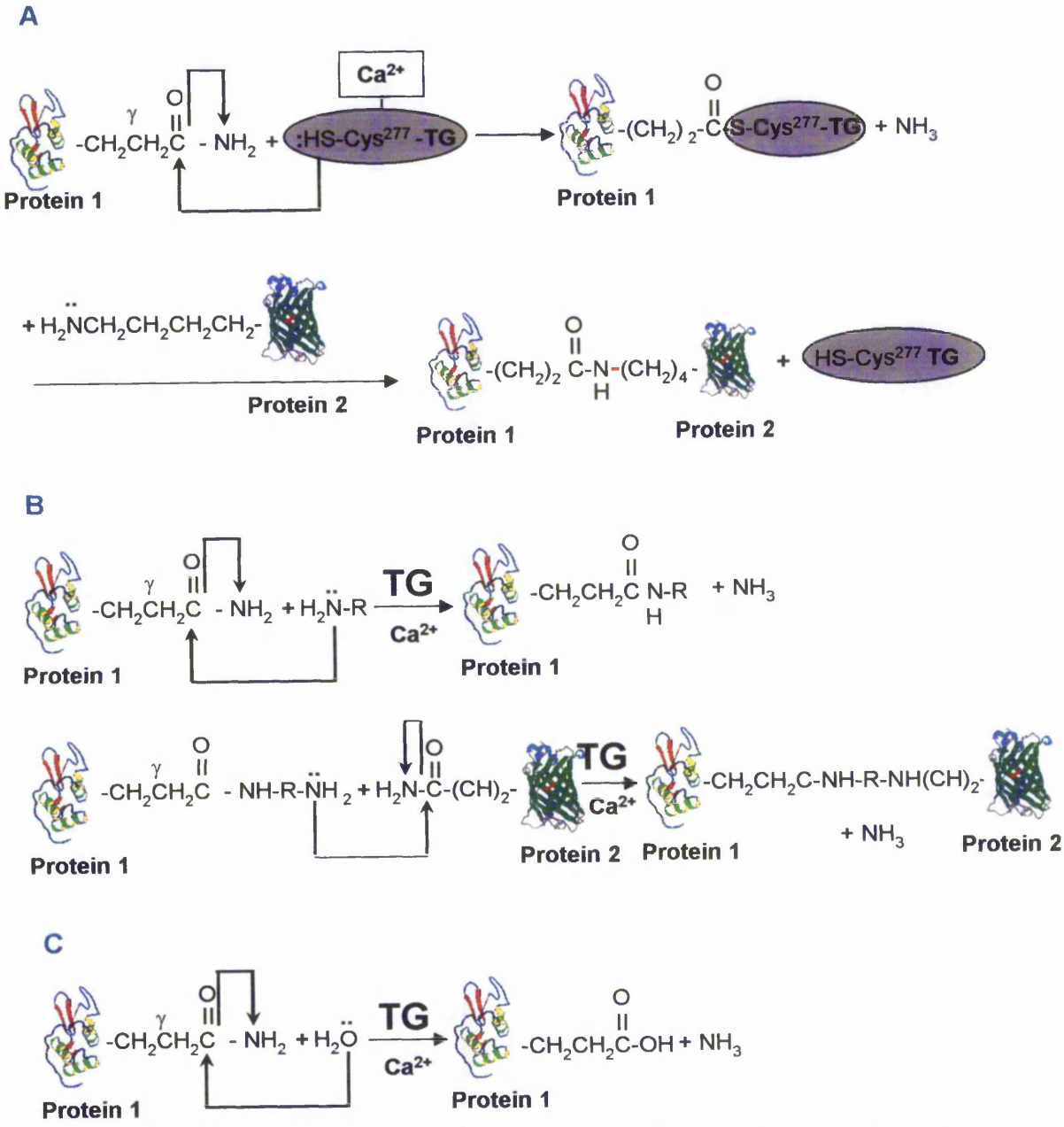


Figure 1.1 Reactions catalysed by transglutaminases. **A**, transglutaminase catalysed transamidation through formation of a ε(γ-glutamyl)lysine bond between the deprotonated lysine donor of one protein and the receptor glutamine residue of another. The reaction involves the formation of a thiolester enzyme intermediate. **B**, the incorporation of an amine into the glutamine residue of an acceptor protein and the formation of an N',N'-bis (γ-glutamyl) polyamine linkage between donor and acceptor proteins when the amine is a diamine. **C**, deamidation of a protein-bound glutamine residue.

However, in the absence of a suitable available amine and under acidic conditions, TGs also catalyse the hydrolysis of protein-bound glutamine to glutamic acid residues, in a deamidation reaction that is normally unfavoured (Mycek and Welsh, 1960; Folk and Finlayson, 1977; figure 1.1*C*).

TGs are also known to be involved in the aminolysis of esters (i.e. p-nitrophenyl-acetate) by insertion of polyamine groups within the ester chain (Folk and Cole, 1966; Folk and Finlayson, 1977). Finally, it has also been proposed that keratinocyte TG (TG_k, type I) can catalyse the ester bond formation between a specific involucrin glutamyl residue and ω-hydroxyceramides, which may be important in epidermal barrier formation by keratinocytes (Nemes *et al.*, 1999).

1.2 Classification of mammalian TGs

TGs have been shown to be expressed in a number of distantly related organisms implying a functional necessity. TG activity has been identified in plants (Serafini-Fracassini *et al.*, 1995), micro-organisms (Chung, 1972; Folk and Finlayson, 1977; Kanaji *et al.*, 1993), invertebrates (Mehta *et al.*, 1990 and 1992; Singh and Mehta, 1994) and mammals (Chung, 1972).

TGs have also been shown to be widely distributed amongst tissues (epithelium, endothelium, stratum corneum, dermis, liver, spleen, bone marrow, CNS) and physiological fluids (platelets, lymphatic system) as shown in table 1.2. RT-PCR analysis of various human cell types has revealed that at least seven different TG enzymes co-exist (fXIIIA subunit, TG1, TG2, TG3, TG4, TG5, and Band4.2), where they perform different functions, most of which are believed to be associated with substrate cross-linking at various body tissues and fluids (Aeschlimann *et al.*, 1998). Certain TG relatives possess inherent (TG2, TG4), some latent (fXIII A subunit, TG1, TG3), and just one (Band 4.2) no catalytic activity, whilst most members of the family require Ca^{2+} for activation (Chen and Mehta, 1999).

Seven of the identified TGs have now been isolated, cloned and sequenced (Ikura *et al.*, 1988; Polakowska *et al.*, 1991; Kim *et al.*, 1993; Ichinose *et al.*, 1988; Seitz *et al.*, 1991; Aeschlimann *et al.*, 1998; Korsgren *et al.*, 1990). Whilst comparison of the gene products revealed a high degree of sequence homology, gene mapping has highlighted different genes with distinct chromosomal localisations. Moreover, different TG family members

Protein	Gene name	Alternative name	Tissue expression	Localisation	Activity	Function
fXIII A-subunit	F13A1	Factor XIIIa, plasma TG, fibrin-stabilising factor, Laki-Lorand factor, fibrinoligase, plasma pro-TG	Platelests, astrocytes, dermal dendritic cells, chondrocytes, placenta, plasma, synovial fluid	Cytosolic extracellular	Latent (thrombin activated)	Blood coagulation, bone growth, ECM stabilisation
TG1	TGM1	TG_K, keratinocyte-, particulate-, membrane-bound TG, TG type 1, TG B	Keratinocytes, brain	Membrane, cytosolic	Latent (protease activated)	Cornified envelope formation by terminally differentiating epidermal keratinocytes
TG2	TGM2	TG_C, tTG, cytosolic-, endothelial-, erythrocyte-, liver-, tissue TG, TG type 2, Gh, Gha	Ubiquitous	Cytosolic, nuclear, membrane, cell surface, extracellular	Yes	ECM stabilisation, formation of cross-linked envelope during cell death, cell signalling
TG3	TGM3	TG_E, epidermal-, snout-, callus-, hair folicle TG, TG type 3	Squamous epithelium, brain	Cytosolic	Latent (protease activated)	Formation of the cornified envelope, hair shaft
TG4	TGM4	TG_P, prostate TG, TG type 4, dorsal prostate protein 1 (DP1), major androgen-regulated secretory protein, Vesiculase	Prostate	Unknown	Yes	semen coagulation in rodents
TG5	TGM5	TG_X, TG type 5	Ubiquitous apart from the central nervous and lymphatic systems	Unknown	Yes	Unknown
TG6	TGM6	TG_Y, TG type 6	Unknown	Unknown	Yes	Unknown
TG7	TGM7	TG_Z, TG type 7	Ubiquitous	Unknown	Yes	Unknown
Band 4.2	EPB42	Erythrocyte protein band 4.2, B4.2	Red blood cells, bone marrow, foetal liver and spleen	Membrane	No	Structural membrane skeletal protein

Table 1.2 TG nomenclature (Adapted from Lorand *et al.*, 2003)

share similar intron and splice site distribution (Grenard *et al.*, 2001a), with TG2, TG3, TG5, and Band4.2 containing 13 exons, while TG1 and fXIIIA contain 15. An extra exon within the conserved exon IX 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.

There is also a certain degree of amino acid sequence homology between the different TGs. Most importantly, they share 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). Recently, a superfamily of archaeal, bacterial, and eukaryotic proteins homologous to animal transglutaminases has also been identified using profiles generated by the PSI-BLAST program (Makarova *et al.*, 1999).

The different primary structures of TGs and the requirement for different external conditions to become optimally active is likely to be responsible for the diverse biological functions that have so far been identified. To date, well characterised TG functions are those relating to fXIIIA and keratinocyte TG (Aeschlimann and Paulsson, 1994).

1.3 Erythrocyte Band 4.2

The erythrocyte membrane protein band is a 77kDa protein encoded by a 20kb gene. Band4.2, a structural component of the cytoskeletal network underlying the red blood cell membrane, is expressed in high levels in erythroid cells (Cohen *et al.*, 1993). It binds to spectrin, integral band/3, ankyrin and band/4.1 cytoskeletal proteins. Myristylation of an N-terminal glycine residue associates band4.2 to the plasma membrane where it accounts for approximately 5% (2000,000 copies per red blood cell) of the erythrocyte membrane protein mass (Risinger *et al.*, 1992; Rybicki *et al.*, 1994). In featuring a substitution of an alanine for the active site cysteine (Korsgren *et al.*, 1990) in an otherwise close sequence homology with other TG family members, the erythrocyte Band 4.2 is the only catalytically inactive member of the TG family of enzymes (Aeschlimann and Paulsson, 1994). In support of the notion that band4.2 is important for red blood cell integrity (Sung *et al.*, 1992) other than its involvement in normal red blood cell cation transport (Peters *et al.*, 1999), comes the finding that hereditary deficiency in this protein is responsible for the fragility of the erythrocytes (Cohen *et al.*, 1993). The epidemiology of

this phenotype is marked by a relatively high occurrence in Japan where erythrocyte fragility is responsible for haemolytic anaemia (Cohen *et al.*, 1993).

1.4 Prostate TG

Although its physiological function has not yet been elucidated, prostate TG (pTG) has been shown to be a 150kDa homodimer consisting of two highly glycosylated and acetylated polypeptide chains that possess a lipid anchor (Wilson and French, 1980). The human pTG gene has been mapped to chromosome 3p21.33-p22 (Gentile *et al.*, 1995) and encodes a 684aa protein that is expressed strictly from luminal epithelial cells of the prostate under the regulation of androgens (Dubbink *et al.*, 1996). Potential substrates for human pTG include the seminal vesicle secreted proteins semenogelin I, and II (Peter *et al.*, 1998). pTG has been shown to circulate in the seminal fluid following secretion by the dorsal prostate and the coagulating gland in rats, where its is believed to be involved in the formation of the copulatory plug by cross-linking seminal vesicle proteins SVP-1 and SVP-IV (Williams-Ashman, 1984; Seitz *et al.*, 1991; Ho *et al.*, 1992). It has also been proposed that it may play a role in suppressing the immune response elicited by immunocompetent cells in the female genital track against the sperm cells (Paonessa *et al.*, 1984; Esposito *et al.*, 1996a). Given that TG activity of pTG has been detected both in the seminal fluids and at the spermatozoa surface, it has been hypothesised that human pTG may play a protective role against immuno-compromisation in humans (Porta *et al.*, 1986). Human pTG has also been shown to cross-link a group of androgen-dependent proteins with repeating sequences that are secreted by the seminal vesicle (Porta *et al.*, 1990b; Harris *et al.*, 1990). Interestingly, expression of the human pTG is reported to be down-regulated in most metastatic prostate cancers (An *et al.*, 1999), whilst up-regulation of pTG expression has been demonstrated in prostate cancer cell lines with low metastatic potential (Dubbink *et al.*, 1996).

1.5 Epidermal TG

Although it has been characterised and purified from human and bovine stratum corneum over 30 years ago (Buxman and Wuepper, 1976; Owaga and Goldsmith; 1976), epidermal TG (eTG, TG3) remains the least understood member of the mammalian TG

family. The human gene for TG3 was localised to chromosome 14 using somatic cell hybrids (Polakowska *et al.*, 1990). TG3 is localised in the cytosol and, like fXIIIA and TG1, exists in a latent, intact, pro-enzyme form (77Kda) that exhibits low specific activities and requires proteolytic cleavage for activation. Following cleavage, conformational changes in the active product facilitate additional Ca^{2+} binding increasing the specific activity of TG3 by two-fold (Kim *et al.*, 1990 and 1995). It has been hypothesised that the protease calpain is responsible for the generation of the two proteolytic digestion products; the catalytically active form (55kDa) of TG3 and a 27kDa C-terminal peptide by-product (Aeschlimann and Paulsson, 1994). It is also thought that proteolytic post-translational modifications of TG3 in response to the cell's physiological state during epidermal cell differentiation is responsible for the activation and transfer of the enzyme from the cytosol to the membrane bound compartment (Kim *et al.*, 1994).

TG3 is believed to be involved in the cross-linking of the cornified envelope (CE) precursor proteins during terminal differentiation as its activity increases as the epidermal cells enter the final stage of keratinocyte differentiation from live, nuclear to dead, anuclear cell envelopes (Buxman and Wuepper, 1975; Rice and Green, 1977). Cross-linking studies using expressed forms of TG3 have revealed that several known CE proteins such as loricrin, small proline rich proteins 1, 2, and 3, and trichohyalin are complete substrates for both TG3 and TG1 (Candi *et al.*, 1995 and 1999; Tarcsa *et al.*, 1997 and 1998; Steinert *et al.*, 1999). Comparison of *in vivo* and *in vitro* residue utilisation (Candi *et al.*, 1995), in conjunction with double *in vitro* cross-linking experiments (Tarcsa *et al.*, 1998), revealed that TG3 might be acting in concert with TG1 in the CE maturation process. It is currently proposed that some key substrates are first partially cross-linked by TG3, before being sequestered onto the CE membrane by TG1 (Tarcsa *et al.*, 1998). Other researchers have also suggested that TG3 may play a role in the hair shaft formation (Lee *et al.*, 1993). Finally, similar to pTG, it is expected that any human disease associated with TG3 could be lethal, as this member of the TG family is strongly expressed along placental membranes and in the developing brain, although its function in these tissues is still unknown.

1.6 Keratinocyte TG

Although maturing keratinocytes are stabilised to a great degree by disulphide bonding, which is particularly prominent in keratins, TG-mediated cross-linking is also important for the integrity of the CE scaffold. Immuno-histochemical evidence has indicated that keratinocyte TG (kTG, TG1) is expressed in minor amounts in the proliferative *stratum basalis*, in moderate amounts in suprabasal cells committed to terminal differentiation (*stratum spinosum*), and in much larger amounts in the *stratum granulosum* as the terminal differentiation proceeds from-bottom-to-top from the dermis to the stratum corneum (Thacher and Rice, 1985; Parenteau *et al.*, 1986; Kim *et al.*, 1995). The gene encoding TG1 has been localised to chromosome 14q11.2-13 and contains 15 exons spliced by 14 introns that have conserved position in comparison to the other main members of the TG family (Kim *et al.*, 1992). TG1 is an intracellular/membrane-bound enzyme of 813-824aa located at the inner face of the keratinocytes plasma membrane where it can be anchored 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). Although the enzyme is primarily membrane-bound, a small fraction (5 -35% of the proliferating or terminally differentiating keratinocytes) is ordinarily found in a soluble state in the cytosol (Kim *et al.*, 1995). Cytosolic TG1 exists as a latent full-length form of 106kDa, and two proteolytic cleavage products (67kDa, 67/33kDa) with 5-10 fold higher specific activities. The membrane bound form exists as either a full-length zymogen or a highly active complex of 67/33/10kDa form and accounts for the remaining 65-95% of TG1 expressed in epidermal keratinocytes (Steinert *et al.*, 1996). The active enzyme can also 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 N-terminus of the enzyme through fatty acid acylation with palmitate and myristate (Thacher and Rice, 1985; Kim *et al.*, 1995). The mutation of the cluster of cysteine residues from the N-terminus of TG1 was indeed shown to prevent the enzyme from associating with the plasma membrane (Phillips *et al.*, 1990).

TG1 is induced during terminal differentiation of keratinocytes and cross-links specific intra-cellular proteins contributing to the formation of the CE. 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 *et al.*, 1993), cornifin (Marvin

et al., 1992), filaggrin (Steinert and Marekov, 1995), cystatin-α (Takahashi and Tezuka, 1999) and elafin (Nonomura *et al.*, 1994). Recently it has been proposed that TG1 can catalyse the ester bond formation between glutaminyl residues in involucrin and ω-hydroxyceramides, which may in turn facilitate anchoring of CEs to the lipid envelop of the differentiating keratinocytes (Nemes *et al.*, 1999). The 15nm highly cross-linked thick callus layer of insoluble proteins on the intracellular surface of the corneocyte plasma membrane is a prime example of the enzyme's involvement in CE structural stability. Recently, it has also been postulated that the crosslinking activity of intercellular TG1 may play an important role in the stabilisation of the vascular endothelium barrier (Baumgartner *et al.*, 2004).

Hereditary deficiency in TG1 is believed to be the cause of hyperkeratinisation, as seen in conditions such as lamellar ichthyosis (Huber *et al.*, 1995; Hennies *et al.*, 1998). TG1 null mice have also demonstrated abnormal keratinisation accompanied by marked reduction in skin barrier formation that led to death 4-5 hours post-birth (Matsuki *et al.*, 1998). Nishida *et al.* (1999) have also correlated inappropriate TG1 expression in the *stratum basalis* of the corneal and conjuctival epithelium with pathological keratinisation reminiscent of that seen in Stevens-Johnson syndrome.

1.7 Plasma factor XIII

Factor XIII (fXIII) is one of the most extensively studied members of the TG family of enzymes. fXIII is expressed by various cell lines (macrophages, megakaryocytes, and monocytes) in several tissues (placenta, uterus, prostate, liver) as a homodimer (166kDa) of two 730aa A-subunits. When externalised into the plasma, it circulates as a 320kDa heterotetramer composed of a catalytic dimer of two A-chains (A_2) and two non-catalytic B-chains (B_2) non-covalently associated (Schwartz *et al.*, 1973). fXIII, like TG1 and TG3, is a pro-enzyme which requires cleavage by the serine protease thrombin at the scissile Arg^{37}-Gly^{38} peptide bond during the final stage of the blood coagulation cascade (Schwartz *et al.*, 1973).

1.7.1 Factor XIII A subunit

Northern blotting analysis has indicated that fXIIIA is expressed mainly in haemopoietic monocytes, macrophages, megakaryocytes, and platelets, as well as in the uterus, the

placenta and the liver (Henriksson *et al.*, 1985; Weisberg *et al.*, 1987; Aeschlimann and Paulson, 1994; Adany, 1996). Immuno-histochemical studies have also demonstrated its presence in heamoglioblastomas (Mizoguchi *et al.*, 1998).

The human factor XIII A-chain gene is localised in chromosome 6p24-25, consists of 15 exons and has a size of 160kb (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 (Ischinose *et al.*, 1990; Hettasch *et al.*, 1994). The primary structure of the human placental factor A-chain was first established by the combination of cDNA cloning and protein sequencing techniques (Ichinose *et al.*, 1986; Grundmann *et al.*, 1986), and was shown to share high homology with the other characterised acyl transferases. 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 mature protein consists of 730-731 amino acids residues with a molecular weight of 83kDa. Although six potential glycosylation sites were identified, no carbohydrate modification was detected in the mature protein (Greenberg *et al.*, 1991). The cDNA sequence reveals the absence of typical hydrophobic leader (NH_2) sequences for secretion (Ichinose *et al.*, 1990) This finding, in conjunction with the observed lack of carbohydrate or disulfide bonds in the protein and the acetylation of the N-terminal serine, suggest the major presence of this molecule is in the cytoplasm. Although the mechanism of its release into the plasma has not yet been elucidated, its co-externalisation with lactate dehydrogenase suggests that this process might be triggered by cell insult (Kaetsu *et al.*, 1996).

1.7.2 Factor XIII B subunit

Early *in vivo* human liver transplantation and *in vitro* metabolic radiolabeling studies pinpointed plasma factor XIII B subunit expression to the hepatocytes (Nagy *et al.*, 1986; Wolpl *et al.*, 1987). The mature glycoprotein contains 8.5% carbohydrate and is thought to have a filamentous structure (Carrell *et al.*, 1989). FXIIIB contains 641 amino acids

and has a molecular weight of 80kDa (Bohn *et al.*, 1972). The 28kb FXIIIB gene is located on chromosome 1q32.32.1 (Bottenus *et al.*, 1990). It consists of 12 exons, the first and last of which encode the leader sequence and the C-terminal portion of the protein respectively, whereas each one of the remaining 10 exons encode the 10 tandem repeats of 60 amino acids and two disulfide bonds often referred to as Sushi domains (Greenberg *et al.*, 1991). Not surprisingly, the ten tandem repeats represent over 95% of the amino acid sequence. With the fXIIIB crystal structure not yet been obtained, it can only be hypothesised by comparison to other proteins that fXIIIB Sushi domains are likely to serve a protein binding role (Aeschlimann and Paulson., 1994).

In order for the FXIII tetramer (A_2B_2) to be formed in the circulation both subunits have to be released from cells. FXIIIA, that lacks a leader sequence, is released by heamopoietic cells and platelets in response to cell injury, whereas FXIIIB is released by hepatocytes through a classical secretory pathway (Kaetsu *et al.*, 1996). The finding that exon1 of FXIIIB subunit codes for a leader sequence, coupled with reports demonstrating that FXIIIB unlike FXIIIA is secreted into the culture medium when expressed in baby hamster kidney cells (Kaetsu *et al.*, 1996), testify for its secretion through a classical pathway.

1.7.3 Factor XIII activation

The activation peptide (Met^1– Arg^{37}) of each A-subunit in an A_2 fXIII homodimer crosses the dimer interface, partially occluding the opening to the active site in the catalytic core of the other subunit. Removal of the activation peptide following thrombin cleavage at the Arg^{37}-Gly^{38} bond leads to fXIII activation *in vivo* (Takagi and Dolittle, 1974; Hornyak and Shafer, 1991). However, fXIIIA crystallography studies have demonstrated that thrombin cleavage and the presence of calcium are not sufficient to force the protein into the conformational changes which are necessary to expose the active site of the enzyme to its substrate. Interactions between the active site and the substrate itself are required for factor XIII to release its cryptic TG activity (Yee *et al.*, 1996). The activation reaction is accelerated by fibrin polymers which provide binding sites for fXIII and thrombin in a Ca^{2+}-independent manner (Greenberg *et al.*, 1987b). Fibrinogen, on the other hand, has been shown to reduce the calcium concentration required for A-chain dissociation to levels that exist in plasma (Credo *et al.*, 1981; Greenberg *et al.*, 1991). Factor XIIIB is thought to stabilise and protect FXIIIA from

proteolytic degradation and to regulate the activation of the zymogen in plasma (Aeschlimann and Paulson, 1994). Calcium ions dissociate the fXIIIB homodimer from the thrombin cleaved fXIIIA homodimer, thus exposing the active-site Cys^{314} (Schwartz *et al.*, 1973). Recently, Weiss *et al.* (1998) have proposed that a cis/trans isomerisation of three peptide bonds among which Arg^{310}–Tyr^{311}, may act as a conformational switch between fXIII zymogen and mature fXIIIA. The fXIIIB subunits released from the tetramer upon thrombin activation are possibly involved in a negative feedback regulatory mechanism of the contact pathway of blood coagulation (Halkier *et al.*, 1988). Activation of the intra-cellular form of fXIII has been reported to occur also in platelets by calpain activation (Devine and Bishop, 1996). This process is thrombin-independent and does not liberate the N-terminus activation peptide.

1.7.4 Factor XIII functions

Homozygous fXIII deficiency has revealed an important role for fXIII in haemostasis, wound healing, and maintenance of pregnancy. During the coagulation process fibrin is generated by the thrombolytic cleavage of fibrinogen (Greenberg *et al.*, 1991). Factor XIII is the last enzyme generated in the pathway of blood coagulation cascade and upon activation catalyses the formation of intermolecular ε(γ-glutamyl)lysine cross-links between selected side chains of fibrin molecules (Lorand and Conrad, 1984), thus mechanically strengthening the blood clots (Doolittle *et al.*, 1979). Additional fXIII-mediated cross-linking of other coagulants (actin, von Willebrand factor, and factor V) reinforces the clots (Cohen *et al*, 1985; Francis *et al.*, 1986; Hada *et al.*, 1986). Factor XIII also cross-links α-2 antiplasmin to fibrin, thus increasing the resistance of clots to plasmin degradation (Tamaki and Aoki, 1981). Moreover, fXIIIA cross-links fibrin to thrombospondin and fibronectin, hence anchoring the blood clot to the targeted site of injury (Bale and Mosher, 1986; Lynch *et al.*, 1987). Other cytoskeletal components that have been shown to serve as substrates for fXIIIA include α-2 macroglobulin (Mortensen *et al.*, 1981), vitronectin (Sane *et al.*, 1988; Skorstengaard *et al.*, 1990), lipoprotein (a) (Borth *et al.*, 1991), plasminogen activator inhibitor (Jensen *et al.*, 1993), myosin (Cohen *et al.*, 1981), vinculin (Asijee *et al.*, 1988) and certain collagens (Mosher and Schad 1979; Akagi *et al.*, 2002).

The association of factor XIIIA with platelets, monocytes aids in the formation of a stable hemostatic plug and facilitates wound healing. Individuals who are congenitally deficient in factor XIIIA 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). A recent report by AbdAlla *et al.* (2004), provided evidence that pathogenic actions of angiotensin II involve covalent crosslinking of angiotensin AT1 receptors by intracellular factor XIIIA, resulting in stable receptor dimers with enhanced signaling properties. Angiotensin II, a small peptide hormone that plays key roles in the regulation of blood pressure, also contributes to inflammatory processes that promote the development of atherosclerosis (AbdAlla *et al.*, 2004; Ogawa and Glass, 2004)

1.8 Transglutaminases X, Y, and Z

Transglutaminases X (TG_X; TG5), Y (TG_Y; TG6), and Z (TG_Z; TG7) have only recently been discovered and their functions, with the exception of TG_X, have not yet been characterised (Griffin *et al.*, 2002).

The 35kb TG5 gene has been mapped to chromosome 15q15.2, and was shown to contain 13 exons and 12 introns. TG5 exists in four different isoforms; active full-length TG5, active splice variant Δ13, and inactive splice variants Δ3 and Δ3Δ13 (Aeschlimann *et al.*, 1998; Candi *et al.*, 2001). Full length TG5 encodes an 84kDa enzyme, whereas the splice variants encode low molecular weight proteins. The ability of TG5 to cross-link specific epidermal substrates such as loricrin, involucrin, and SPR3 as shown by kinetic *in vitro* cross-linking studies, coupled with its localisation at the upper epidermal layers (*strata spinosum* and *granulosum*) inevitably implicated TG5 with keratinocyte differentiation and CE assembly (Candi *et al.*, 2001). A 7-fold increase in TG5 expression in keratinocytes induced to differentiate suggests that TG5 contributes to the formation of the CE and could be a marker of terminal keratinocyte differentiation (Aeschlimann *et al.*, 1998). Its ability to cross-link non epidermal substrates such as vimentin, also suggests that TG5 may serve other roles beyond skin barrier formation (Candi *et al.*,

2001). TG5 expression was also shown to be miss-regulated in certain pathological skin conditions such as Darier's disease (abnormal keratinisation) and lamellar itchthyosis, and up-regulated in others (psoriasis and itchthyosis valguris; Candi *et al.*, 2002).
Currently, little is known about TG6 and TG7. So far, the TG6 gene has been mapped on chromosome 20q11 15, in the vicinity of the region coding for TG2 and TG3 (Grenard *et al.*, 2001). TG7 has been discovered in human prostate carcinoma, and the respective gene has been mapped on chromosome 15q15.2. A number of TG7 splice variants have been so far identified, and TG7 expression has been demonstrated in a number of tissues (Grenard *et al.*, 2001).

1.9 Tissue transglutaminase

Tissue transglutaminase (tTG) also known as type II TG, TG2, TG_C, G_h and G_{ha} and has been characterised in many cells and tissues such as liver, lung, brain, kidney, adrenal glands, testis, pancreas, erythrocyte, macrophage, uterus and muscle (Lorand and Sternberg, 1976; Fesus and Thomazy, 1988, Thomazy and Fesus, 1989). TG2 is a ubiquitous enzyme found in both membrane and cytosolic fractions. Immunohistochemical studies have also demonstrated the presence of TG2 expression in vascular endothelial cells and smooth muscles cells of any origin such as heart muscle, renomedullary interstitial cells, mesangial cells in the kidney and colonic pericryptal fibroblasts (Fesus and Thomazy, 1988). TG2 is the most widely distributed form of the large family of TGs, and is the main focus of this thesis.

1.9.1 The organisation of the human TG2 gene

The 32.5kb human TG2 gene (TGM2) has been mapped on chromosome 20q11-12 (Gentile *et al.*, 1995) and consists of 13 exons separated by 12 introns, with the last exon (exon 13) accounting for almost half of the full-length cDNA (Fraij *et al.*, 1992). A short (2.4kbp) splice variant of TGM2 (sTGM2) of unclear biological significance has also been identified recently in astrocytes treated with cytokines such as interleukin-1β, as well as in the brains of patients with Alzheimer's disease (Citron *et al.*, 2001).
The proximal promoter (figure 1.9.1) is composed of a TATA-box element and two upstream SP1 (CCGCC) binding sites and drives constitutive expression (Lu *et al.*,

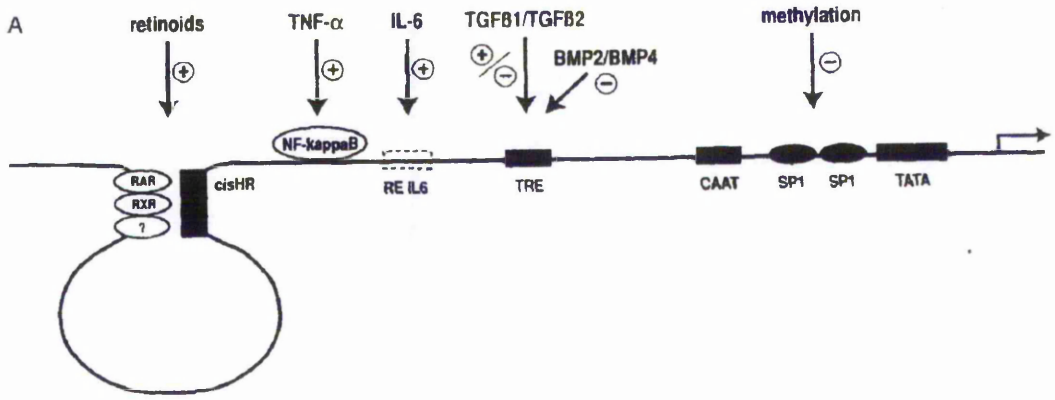


Figure 1.9.1 Schematic of the TGM2 promoter (Aeschlimann and Thomazy, 1999)

The TATA box and the SP1 sites drive constitutive expression. Upstream binding sites for TNFα, retinoids, IL-6 and TGFβ upegulate, whereas binding sites for TGFβ-2, BMP2/BMP4 as well as methylation sites downregulate TGM2 expression.

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 promoter gene family. Differential expression is also regulated by a number of upstream enhancer/silencer elements. Response elements for retinoic acid, members of the TGF-β, and TNFα gene family have been identified (Lu *et al.*, 1995). A specific retinoid response element (mTGRRE1) has been located 1.7kb upstream of the transcription start site, containing a triplicate retinoid receptor binding motif (Nagy *et al.*, 1996b), which together with a short HR1 sequence are responsible for promoter activation in response to retinoic acid. A potential IL-6 response element as well as transcription factor AP1 and AP-2 binding sites have also been located. The later has not been fully characterised yet (Ikura *et al.*, 1994).

1.9.2 Protein structure of TG2

The complete amino acid sequence for TG2 from guinea pig liver (Ikura *et al.*, 1988), bovine aorta (Nakanishi *et al.*, 1991), mouse macrophages and human endothelial cells (Gentile *et al.*, 1991) have been deduced from the cDNA sequences.

The monomeric enzyme, consists of 685-691 amino acids and has a molecular weight around 77-85kDa, which is highly conserved between species (Aeschlimann and Paulson, 1994). There is about 80% homology between the amino acid sequences of TG2 from mouse, guinea pig and human tissue, with 49 of the 51 residues in the active site region being identical. Its deduced amino acid sequence suggests that TG2 contains 17 cysteine residues but no disulfide bonds. It remains to be elucidated how the enzyme is externalised as it contains no hydrophobic leader sequence and, although it contains six potential N-linked glycosylation sites, is not glycosylated (Ikura *et al.*, 1988). It has been suggested that the N-terminal acetylation of the enzyme, following initiator methionine removal (Ikura *et al.*, 1989) might be responsible for its secretion (Muesch *et al.*, 1990).

Recently the three-dimensional structure of TG2 has been solved on latent human GDP-bound TG2 dimer (Liu *et al.*, 2002), on latent sea bream TG2 (Noguchi *et al.*, 2001), and has also been predicted by computer-generated model comparison to the crystal structure of the fXIIIA zymogen (Iismaa *et al.*, 1997) on the basis that as much as 75% of the

active site sequence of the human TG2 is conserved when compared to the factor XIII A-chain. It is composed of four sequential domains: an N-terminal β-sandwich, an α/β catalytic core, and two c-terminal barrel domains (figure 1.9.2). The β-sandwich, domain consists of an initial flexible loop, a short 3_{10} helix, an isolated β-strand (B_1), five anti-parallel strands (B_2-B_6), and an extra short strand close to B_1. Barrel 1 and 2 domains are composed of six β-sheets and one β turn (barrel 1), and seven antiparallel β-sheets (barrel 2), whereas the core domain contains α-chains and β-sheets in equal amounts (Iismaa *et al.*, 1997). This organisation in four domains is highly conserved among the TG family of enzymes (Griffin *et al.*, 2002).

The catalytic triad (Cys^{277}, His^{335}, Asp^{358}) is reminiscent of the Cys-His-Asn triad found in the papain family of cysteine proteases and is buried at the base of the cavity bound by the core and barrel 1 domains. A tryptophan residue (Trp^{241}), thought to be stabilising the transition state and shown to be critical for catalysis, is conserved in all TGs except for the catalytically inactive Band4.2 (Murthy *et al.*, 2002).

TG2 is the only member of the family that binds to and hydrolyses GTP with the exception TG3 and TG5 which can also bind to GTP but with a lower affinity. However, no sequence homology has been identified between TG2 and other GTP-binding proteins. The GTP-binding site of TG2 is located in a 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 (Tyr^{583}, Ser^{482}) appear to interact with the guanine base (Iismaa *et al.*, 2000).

Peptide mapping and site-directed mutagenesis experiments have indicated that multiple regions interact with α_{1B}-adrenergic receptor (K^{547}-I^{561}, R^{564}-D^{581} in barrel 1; Q^{633}-E^{646} in barrel 2). The phospholipase Cδ1 binding/activation site located between the receptor binding sites in barrel 2 at the C-terminal region (V^{665}-K^{672}), is specific for the GTP-bound conformation , and is crucial to the role of TG2 as a GTP-binding effector protein in the transduction of α_1-adrenergic signals (Hwang *et al.*, 1995),

Although equilibrium dialysis experiments have suggested that up to six calcium ions can bind simultaneously to TG2, crystallographic studies of the calcium-bound fXIIIA indicate a sole calcium binding site. The structure of the Ca^{2+}-bound form of TG2 remains unresolved, whereas the putative Ca^{2+}-binding site is distorted in the TG2-GDP structure by the bound nucleotide (Liu *et al.*, 2002). Two regions rich in glutamate residues around amino acids 450 and 470 at the terminal α-helix (H_4) within the core domain have been proposed for the calcium binding site that is crucial for the enzyme's

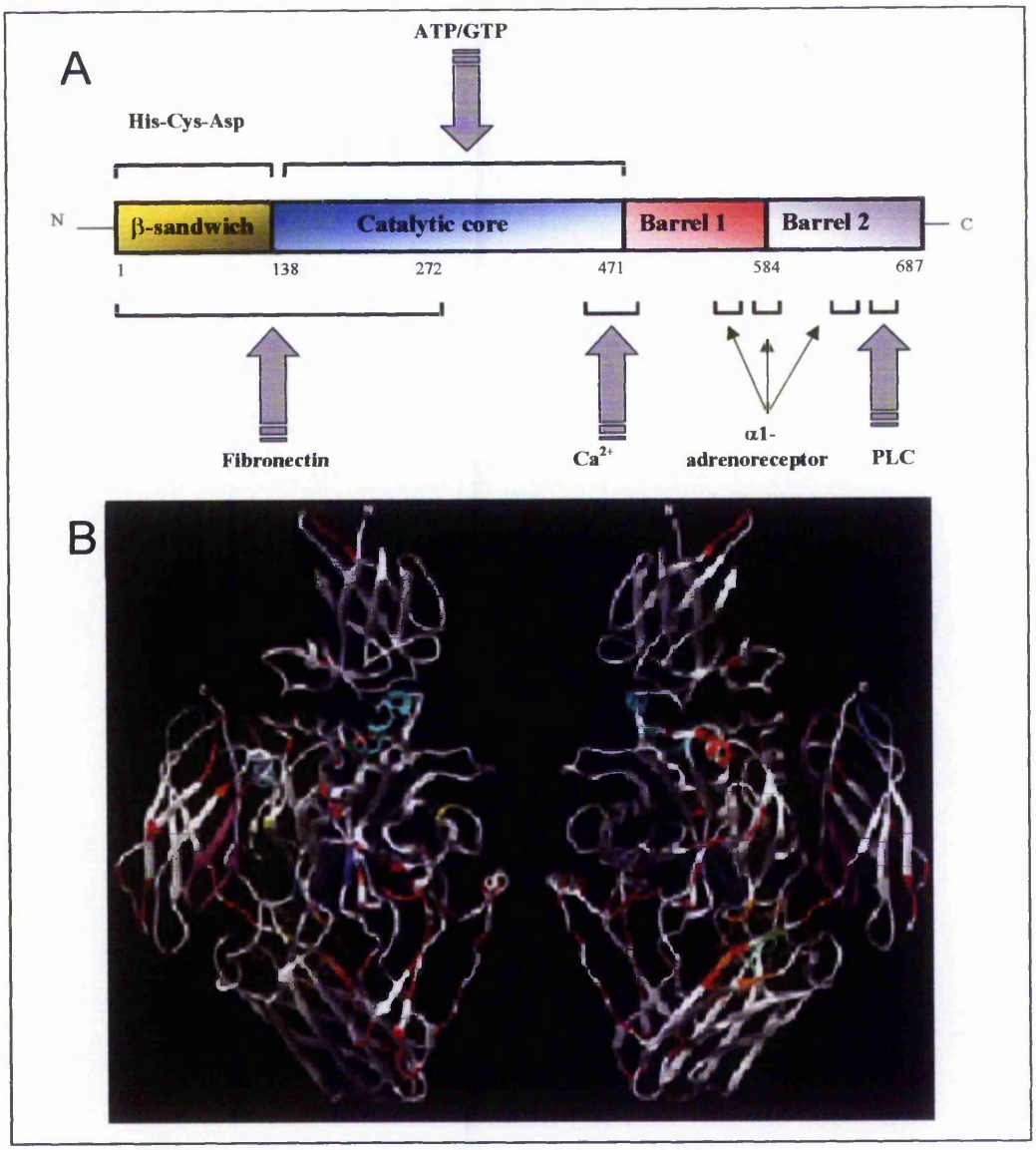


Figure 1.9.2 Structural and functional domains of tTG

A, the proposed structural (β-sandwich, α/β catalytic core, barrel-1 and barrel-2) and functional binding domains (fibronectin, ATP/GTP, Ca^{2+}, α1-adrenoreceptor, phospholipace C) of tTG (adapted from Chen and Metha, 1999). **B**, three-dimensional structure of tTG in complex with GDP (left) and GTPase (right) active sites based on the reported crystal structure. The catalytic triad and the surrounding residues of the transamidating active site as well as amino acids contributing to the GTPase site. The bound GDP molecule is coloured green. Clusters of Glu/Gln and Asp/Asn residues forming putative Ca^{2+}-binding sites are coloured red (Adapted from Fesus and Piacentini 2002).

transamidating activity (Ikura *et al.*, 1988). An inactive form of the TG2 having a molecular mass of 120 kDa, which can be activated by proteases, has also been identified in metastatic cells of both murine and human origin (Knight *et al.*, 1990a; Zirvi *et al.*, 1991).

1.9.3 Regulation of TG2

TG2 has been shown to be regulated either by direct modification of its activity (post-translational regulation) or by modulation of gene expression (transcriptional regulation).

1.9.3.1 Post-translational regulation of TG2 activity

As stated earlier in section 1.9.2, TG2 has a bifunctional role by catalysing both Ca^{2+}-dependent protein crosslinking and Ca^{2+}-independent GTP and ATP hydrolysis. The protein crosslinking activity of TG2 is tightly regulated both inside the cell, by the Ca^{2+} /GTP:GDP ratio and outside the cell by matrix binding and red-ox state of the Cys active site (Cocuzzi and Chung, 1986; Verderio *et al.*, 2003).

The requirement of Ca^{2+} as a regulator of TG2 activation has been reported as early as 1983 (Folk, 1983). Inside the cells, the transamidating TG activity is under the tight control of Ca^{2+} ions and guanosine nucleotides (Smethurst and Griffin, 1996). However, taking into account the low Ca^{2+} and high nucleotide intracellular concentrations as well as the affinity of TG2 for both GTP/GDP and Ca^{2+}, the majority of TG2 activity is expected to remain latent within the cell. Once outside the cell, high Ca^{2+} concentration is expected to activate the enzyme (Smethurst and Griffin, 1996).

Activation by Ca^{2+} is critical for the enzyme's transamidating activity as it has been shown to promote the assembly of the active site pocket so that the reactive Cys^{277} forms a thiolester bond with a glutamyl-containing substrate. 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. 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 (Mariani *et al.*, 2000; Murthy *et al.*, 2002; Liu *et al.*, 2002). *In vitro* kinetic experiments assessing the transglutaminase activation potency of Ca^{2+} have revealed a K_m value of around 20-100μM (Lai *et al.*, 1997). Given that the phosphorylated casein used as an incorporation substrate for ^{14}C-putrescine in these experiments is likely to chelate free Ca^{2+}, K_m values of around 2-3μM estimated in the presence of dephosphorylated casein by other investigators appear closer to the physiological levels (Hand *et al.*, 1985). It has also been demonstrated that the presence of Mg^{2+} may reduce the Ca^{2+} requirement of the enzyme when native (phosphorylated) N', N'-dimethylcasein was used as the protein acceptor substrate (Hand *et al.*, 1985).

TG2 is also thought to act as a GTP-binding protein and that its Ca^{2+} activation can be counteracted by heteroallosteric inhibition by GTP and GDP. This inhibition is reported to be more apparent at low Ca^{2+} concentration and serves as a functional switch from the protein crosslinking mode to the GTP hydrolysis mode (Achyuthan and Greenberg, 1987). GTP binding to TG2 was 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). ATP has also been shown to bind to TG2, in a reaction essential for its hydrolysis. Site-directed mutagenesis studies have indicated a requirement for magnesium ions for the hydrolysis of both ATP and GTP (Lee *et al.*, 1993), whereas Lai and co-workers demonstrated that Mg^{2+}-GTP and Mg^{2+}-ATP are the true substrates for TG2 (Lai *et al.*, 1998). In this particular study it was shown that whilst Mg^{2+}-GTP binding to TG2 inhibits both transamidating and GTPase activities, Mg^{2+}-ATP binding inhibits the GTPase activity, without any effect on the enzyme's transamidating activity (Lai *et al.*, 1998).

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.*, 1998a). Smethurst and Griffin demonstrated that in electropermeabilised TG2-rich human endothelial cells and under physiological Ca^{2+}/GTP/GDP conditions, TG2 exists as an inactive, cryptic enzyme. In a separate study, depletion of intracellular GTP in human neuroblastoma SH-SY5Y cells was shown

to result in an increased *in situ* TG activity (Zhang *et al.*, 1998a). It is also believed that 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 intra-cellular levels (Zhang *et al.* in 1998a).

Other regulators of TG2 activity include phospholipids and NO donors (Fesus *et al.*, 1983; Lai *et al.*, 1997). Lai and co-workers (1997) demonstrated that a membrane lipid, sphingosylphosphocholine can serve as specific cofactor that reduces the Ca^{2+} requirement for activating TG2. In light of reports that implicate TG2 in the activation of phospholipace Cγ during α-adrenergic receptor signalling, the added observation that this enzyme can bind phospholipids (Fesus *et al.*, 1983) and localises 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).

1.9.3.2 Transcriptional regulation of TG2 expression

The expression of TG2 is highly regulated and various factors have been shown to induce its expression in different cell lines and cell types, including intracellular polyamines (McCormack *et al.*, 1994), retinoic acid (Mehta *et al.*, 1985; Davies *et al.*, 1985; Jetten *et al.*, 1986; Chiocca *et al.*, 1988; Defacque *et al.*, 1995; Ando *et al.*, 1996), cyclic AMP (Perry *et al.*, 1995), interleukin-6 (Suto *et al.*, 1993), NF_KB (Mirza *et al.*, 1997), TGF-β1 (George *et al.*, 1990; Ritter and Davies, 1998), TNF-α (Kuncio *et al.*, 1998) and dexamethasone (Johnson *et al.*, 1998). Other agents known to increase expression of TG2 in the cell include sodium butyrate (Byrd and Lichti, 1987), DMSO (Hsu and Friedman, 1983), and teradecanoylphorbol-13-acetate (Mehta *et al.*, 1986; Keogh *et al.*, 1993).

Inhibition of endogenous polyamine synthesis by α-difluoromethylornithine (DFMO) was reported to correlate with a marked reduction in TG activity in rat small intestinal crypt (IEC-6) cells (McCormack *et al.*, 1994), and an increase in TG2 mRNA in human colon carcinoma cells (Wang *et al.*, 1998), suggesting tissue specific regulation of TG2 expression by polyamines.

On the other hand, treatment of several cell lines including normal human myeloid, peritoneal macrophages, and aortic endothelial cells, as well as of malignant hepatoblastoma and neuroblastoma cell lines with retinoids and retinoic acid (RA) in

particular, was shown to regulate the expression of TG2 *in vitro* and *in vivo* by increasing TG2 expression and protein levels (Moore *et al.*, 1984; Nara *et al.*, 1989; Piacentini *et al.*, 1992; Gentile *et al.*, 1992). Although the RA responsive elements have not been identified in guinea pig and human promoters, the receptor binding sites for RA have been located within the proximal 5' end of the mouse TG2 gene. Retinoid-mediated transactivation of the TG2 gene was shown to be regulated by the retinoid response element (mTGRRE1), a region 1.7 kb upstream from the mouse TG gene transcription start site, and by a short DNA segment (HR-1) 1kb upstream of transcription start site (fig. 1.9.2; Nagy *et al.*, 1996b and 1997). Vitamin A-deficient rats were also shown to express reduced levels of TG2 in a number of tissues (Verma *et al.*, 1992). Introduction of RAR or RXR cDNA into HL-60 cells with a non-functional retinoid receptor were also shown to induce a high and moderate increase in TG2 expression respectively. RA induction of TG2 can also be enhanced by other factors such as protein kinase activators, including cholera toxins (Ishii *et al.*, 1994), or reduced by others like pertussis toxins.
Recently it has been proposed that both methylation of the CpG-rich region in the human TG2 promoter and histone acetylation lead to decreased transcriptional activity of this promoter (Lu and Davies, 1997; Struhl, 1998). Acetylation of N-terminal lysine residues of histones is also thought to reduce their affinity for DNA hence creating DNAase I sensitive regions.

1.9.4 Localisation and cellular distribution of TG2

The widespread organ distribution of TG2 is attributed to its constitutive expression in endothelium, smooth muscle and fibroblasts (Thomazy and Fesus, 1989). Other organ-specific cell-types that constitutively express TG2 include thymic sabcapsular epithelial cells, stromal cells of the cycling endometrium, and Z lines of subpericardial and subendocardial myocytes.
Like FXIII A subunit, TG2 does not have any classical hydrophobic leader sequences and therefore exhibits the characteristics of a cytoplasmic protein. Although predominantly a cytosolic protein (80%), there is increasing evidence that 10-15% of the total enzyme is 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 (Lesort *et al.*, 1998; Peng *et al.*, 1999). Recent studies have established the localisation of TG2 antigen and activity within the extracellular matrix (Barsigian *et al.*,

1991; Aeschlimann and Paulson, 1994; Martinez *et al.*, 1994; Jones *et al.*, 1997; Johnson *et al.*, 1997, Verderio *et al.*, 1998). So far numerous cell-surface, intracellular, intranuclear, and extracellular TG2 substrates have been identified (table 1.9.4).

1.9.4.1 Cell surface-associated TG2

Sub-cellular fractionation has indicated that, although most of TG2 is present within the soluble, cytosolic fraction of cell extracts, a smaller fraction remains associated with the membrane fraction (Barnes *et al.*, 1985; Slife *et al.*, 1985; Juprelle-Soret *et al.*, 1988). Electron microscopy studies have confirmed the presence a membrane-associated TG2 form (Barnes *et al.*, 1985; Slife *et al.*, 1985). Further analysis of both the cytoplasmic and the particulate/plasma membrane-associated enzyme revealed that the two enzymes shared similar enzymatic properties indicating that the particulate enzyme could be a specific sub-cellular form of TG2 (Chung and Chang, 1986).

Pulse-chase experiments have indicated that cell surface-associated transglutaminase specifically incorporates 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. The same authors also suggested that TG may participate in the process of hepatocyte cell adhesion by generating covalently protein-crosslinked matrices at the sites of cell-to-cell contact (Slife *et al.*, 1986). Morphological analysis of the membranes by linear sucrose gradient fractionation suggested association of TG2 with SDS-insoluble junctional and filament-bearing membrane structures (Tyrrell *et al.*, 1986, and 1988). The cytoskeleton is a well-documented source of TG2 substrates, which may interact with the enzyme and regulate its sub-cellular distribution (Trejo-Skalli *et al.*, 1995; Chowdhury *et al.*, 1997). More specifically, actin and tropomyosin from rabbit muscle (Derrick *et al.*, 1966), pig brain tubulin (Conrad, 1985), chicken skeletal muscle Z line actin, α-actin and desmin (Gard and Lazarides, 1979) have been shown to be specific substrates for TG2. Immunoblotting and immunofluorescence microscopy experiments have also indicated that one of the SDS-insoluble transglutaminase substrates (SITS) is fibronectin (Tyrrell *et al.*, 1988). Barsigian and co-workers subsequently demonstrated that cell-surface TG2 covalently incorporates itself, fibrinogen and fibronectin into high molecular weight aggregates on

ECM	Blood plasma	Cell-surface	Cytosol	Nucleus
Fibronectin	Fibrin/ Fibrinogen	Erythrocyte membrane	Actin	Core histones
β-casein	Fibronectin	Protein band 3	RhoA	pRB
Nidogen	$\alpha_2$plasmin inhibitor	Spectrin	GST	Importin-α3
Vitronectin	α_2-macroglobulin	Involucrin	β-tubulin	
Collagen type III and IV	C1-inhibitor	Loricrin	β-crystallin	
osteopontin			GAPDH	
Osteonectin			Thymosin β	
Osteocalcin C1-inhibitor			Substance P	
Laminin LTBP-1			C-CAM	
IGFBP-1			Troponin T	
			Myosin	
			PLA_2	
			Glucagon	
			Melittin	
			Secretory vesicle IV	
			Lipocortin I	

Table 1.9.4 Known mammalian tTG substrates (Adapted from Csosz et al., 2002)

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).

1.9.4.2 Cytosolic TG2

Although many cytosolic proteins have been described *in vitro* as good glutamine substrates for transglutaminases only a few of them have been verified as physiological substrates of TG2. Among those recognised substrates are RhoA (Singh *et al.*, 2003), GAPDH- GST (Ikura *et al.*, 1998), β-tubilin (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, β-crystallin, lipocortin I, PLA_2, glucagons, melittin, and secretory vesicle IV (Aeschlimann and Paulson, 1994).

The cytoskeletal protein actin is a physiological TG2 substrate, and has a key role in stabilising cellular morphology by counteracting the release of cytoplasmic material from the cells and exerting a morphogenic role in programmed cell death (Nemes *et al.*, 1996). RhoA, a member of the Ras superfamily, is also a substrate for TG2, functioning as a constitutively active G-protein promoting the formation of stress fibres and focal adhesion complexes (Singh *et al.*, 2003).

1.9.4.3 Nuclear TG2

Recently, sub-cellular fractionation studies revealed that 7% of TG2 is localised to the nucleus of human neuroblastoma SH-SY5Y cells, 6% of which was co-purified with the chromatin associated proteins, with the remaining 1% in the nuclear matrix fraction (Lesort *et al.*, 1998). The same authors have also demonstrated that the basal TG activity of TG2 in the nucleus is comparable to that in the cytosol, and that nuclear TG2 can be activated *in situ* by the endogenous calcium mobilising agent maitotoxin (Lesort *et al.*, 1998). TG2 has been shown to be expressed in the nucleus, both as a cross-linking enzyme and as a G-protein (Singh *et al.*, 1995; Lesort *et al.*, 1998). It has been postulated that TG2 has the capacity of a signal transducing G_H-protein, by a mechanism that potentially involves activation of phospholipase C-δ (Feng *et al.*, 1996). Nuclear substrates for TG2 that have so far been identified and characterised include core histones (Shimizu *et al.*, 1997), the 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 (80kDa) that does not allow for passive diffusion, as well as its ability to bind importin-α3, TG2 translocation to the nucleus is believed to be mediated by active nuclear transport. Peng and colleages have demonstrated co-localisation of TG2 and importin-α3 in the cytosol (Peng *et al.*, 1999), which does not rule-out the classical importin-mediated nuclear transport, whereby importin binds to the transported protein in the cytosol, prior to dissociation upon entry into the nucleus.

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 apoptosis (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 cell-death (Ballestar *et al.*, 1996). Finally, the finding that during cell death, glyceraldehyde-3-phosphate deydrogenase (GAPDH) has been shown to translocate to the nucleus, coupled with its increased crosslinking to polyglutamine domains by nuclear TG2, favours the hypothesis that TG2 may be involved in the nuclear modification of GAPDH during programmed cell death.

1.9.4.4 Extracellular TG2

1.9.4.4.1 Extracellular protein substrates of TG2

Although the TG2 externalisation mechanism has not yet been elucidated, there is increasing evidence for the enzyme's involvement in the extracellular matrix (Barsigian *et al.*, 1991; Aeschlimann and Paulson, 1994; Martinez *et al.*, 1994; Jones *et al.*, 1997; Johnson *et al.*, 1997, Verderio *et al.*, 1998). The mechanism of secretion is unusual because TG2 lacks a signal peptide and is not secreted by a classical endoplasmic reticulum/Golgi-dependent mechanism. It is known 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), but due to its atypical secretion mechanism TG2 is not efficiently released. However, its release dramatically increases in situations of tissue

damage and cellular stress (Upchurch *et al.*, 1991; Johnson *et al.*, 1999; Haroon *et al.*, 1999a), when it accumulates in the ECM initially in complex with fibronectin (FN).

Many extra-cellular proteins are known to serve as substrates for TG2. So far it has been reported that TG2 is a potent cross-linker of several ECM proteins such as fibronectin (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 extra-cellular environment also provides the high concentration of calcium and the low concentration of 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 extra-cellular matrix.

Historically, a report implicating TG in the fertilisation envelope formation, a specialised extra-cellular matrix in sea urchin eggs, was the first suggesting a role for TG in stabilisation of extra-cellular material (Battaglia and Shapiro, 1988). Since that pioneering study, TG2 was further implicated in the stabilisation of different tissues. Studies on TG2 activity on liver, heart, muscle and kidney revealed extra-cellular distribution of the enzyme with intensive staining in collagen rich connective tissue and co-localision with nidogen (Aeschlimann *et al.*, 1991). Collagen V and XI were also identified as specific glutamyl substrates in a rhabdomyosarcoma cell line, suggesting the involvement of TG2 in matrix stabilisation at the early steps of collagen fibrillogenesis (Kleman *et al.*, 1995).

In endothelial cells, other extra-cellular components such a glucuronate-rich dermatan sulfate proteoglycan are cross-linked in a TG2-mediated manner into high molecular weight polymers containing FN (Kinsella and Wight, 1990). On the other hand, an integral component of microfibrillar structures that plays a critical role in the organisation of elastic fibers in the extra-cellular matrix 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 has been recently hypothesised (Johnson *et al.*, 1999) that, rather than directly crosslinking ECM components, TG2 may also 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 central in 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 possibly by mediating its cross-linking to FN, whereas it has also been demonstrated that cells expressing increased levels of TG2 also exhibited increased extracellular deposition of LTBP-1 in the ECM (Taipale *et al.*, 1994; Verderio *et al.*, 1999).

1.9.4.4.2 The TG2-fibronectin association

The FN-TG2 association has been the best characterised among an extensive list of TG2-substrate interactions. As described previously (section 1.9.4.1), cell-surface-associated, TG2-mediated high molecular weight SDS-insoluble complexes were believed to stimulate hepatocytes' cell-cell adhesion (Slife *et al.*, 1986). Immuno-blotting analysis of these membrane SDS-insoluble complexes revealed that one of their main component was fibronectin (Tyrrell *et al.*, 1988). Fesus and co-workers first identified four TG2 sensitive glutamine residues on human plasma fibronectin (Fesus *et al.*, 1986). Subsequently, the possible role of plasma fibronectin as a specific carrier of TG2 was described since the two proteins were shown to form a complex (Lorand *et al.*, 1988; Achyuthan *et al.*, 1995). The first *in situ* confirmation of TG2-FN complexation was provided by Martinez and colleagues who established first that endothelial cell-surface TG2 immobilised and processed fibronectin at the basolateral surface of the endothelial cell in a transamidase-independent manner (Martinez *et al.*, 1994). TG2 co-localisation with fibronectin in the pericellular matrix has since been confirmed by confocal and immunogold electron microscopy (Gaudry *et al.*, 1999).

Anisotropic shifts of fluorescein-labelled TG2 upon addition of FN indicated that the enzyme binds to FN with a stoichiometry of 2:1 (LeMosy *et al.*, 1992). Studies with

human erythrocyte transglutaminase demonstrated that two gelatin or collagen binding fragments of 56 and 46kDa displaying affinity for TG could be isolated after fibronectin proteolysis (Turner and Lorand, 1989; Radek *et al.*, 1993). Rotary shadowing electron microscopy of FN-gplTG2 complexes led to the identification of the fibronectin binding site for TG2 within 5-10nm of its N-terminus (LeMosy *et al.*, 1992). More precisely, overlay analysis of proteolytic fragments of FN generated by controlled digestion with endoproteinase Lys-C, demonstrated that TG2 binds to FN via the first seven amino acids of the enzyme's 28kD N-terminus domain (Jeong *et al.*, 1995). Transient transfections of COS-7 cells with a human TG2 cDNA construct encoding for a truncated TG2 variant lacking the putative FN binding site confirmed that this N-terminal β-sandwich domain is indeed the FN binding site, also suggesting that TG complexation with FN is critical for its sequestration to the cell surface (Gaudry *et al.*, 1999b). A different study by the same authors provided evidence for the colocalisation of TG2 with β1 integrins at cell adhesion points (Gaudry *et al.*, 1999a).

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 lead to impaired cell spreading and adhesion (Jones *et al.*, 1997), whereas *in vitro* cell adhesion experiments indicated that the cell-surface TG2 serves to promote cell adhesion and spreading (Isobe *et al.*, 1999). The same authors also identified that TG2 cell adhesion is mediated by α4β1 integrin (Isobe *et al.*, 1999). 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 fibronectin assembly (Akimov *et al.*, 2000; Akimov and Belkin, 2001; Verderio, *et al.*, 2003).

1.9.5 Proposed roles of TG2

TG2 has been implicated in a wide spectrum of cellular and tissue functions including cell signalling (Cordella-Miele *et al.*, 1990; Hwang *et al.*, 1995; Feng *et al.*, 1996; Im and Graham, 1990; Nakaoka *et al.*, 1994), insulin secretion (Sener *et al.*, 1985; Bungay *et al.*, 1986), receptor mediated endocytosis/phogocytosis (Schroff *et al.*, 1981; Murtaugh *et al.*, 1983), cell activation and differentiation (Birckbichler *et al.*, 1978; Gentile *et al.*, 1992; Aeschlimann *et al.*, 1993), programmed cell death and necrosis (Fesus *et al.*, 1987 and

1989; Nemes *et al.*, 1996; Knight *et al.*, 1991; Lim *et al.*, 1998), and numerous pathologies such as wound healing (Bowness *et al.*, 1988; Upchurch *et al.*, 1991; Raghunath *et al.*, 1996; Dolynchuk *et al.*, 1996), inflammation (Valenzuela *et al.*, 1992; Bowness *et al.*, 1994; Weinberg *et al.*, 1991), fibrosis (Griffin *et al.*, 1979; Mirza *et al.*, 1997; Johnson *et al.*, 1997), neurodegenerative diseases (Kahlem *et al.*, 1996; Gentile *et al.*, 1998; Johnson *et al.*, 1997), celiac disease (Molberg *et al.*, 1998), and most relevant to this study tumour growth and metastasis (Hand *et al.*, 1987; Knight *et al.*, 1990a).

1.9.5.1 Involvement of TG2 in cell growth and differentiation

Early work from Birckbilcher and co-workers implicated TG2 in cell differentiation and proliferation, since a correlation between TG2 activity of different cell types and their proliferating capacities was established (Birckbichler and Patterson., 1978). The same authors demonstrated that cells expressing low TG2 were shown undifferentiated or rapidly proliferating when compared to similar cells with high TG2 activity (Birckbichler and Patterson., 1978). Similar experiments using human WI-38 lung fibroblasts treated with TG activity inhibitor cystamine revealed a growth promoting effect (Birckbichler *et al.*, 1981). In support of this study, it has also been demonstrated that stable transfection of hamster fibrosarcoma MetB with the full length cDNA of active and inactive (C277S) TG2 led to delayed progression into the G2/M phase of the cell cycle (Mian *et al.*, 1995). Given that no differences were noted between the wild type and mutant TG2 forms, it was postulated that cell cycle progression control was due to the enzyme's G-protein function. In a subsequent study, hamster fibrosarcoma MetB cells overexpressing the human TG2 cDNA exhibited unaltered cell growth patterns compared to their mock-transfected counterparts (Johnson *et al.*, 1994), suggesting that previous findings by Birckbichler and co-workers (Birckbichler *et al.*, 1981) might be attributed to non-specific effects of TG2 inhibitors. However, it cannot be ruled out that a possible control of the cell growth rate by TG2 may be mediated through growth factors such as TGFβ and hepatocyte growth factor (Kojima *et al.*, 1993; Katoh *et al.*, 1996).

Additionally, matrix engagement of specific receptors represents another important control point for the initiation of signalling events that lead to cell cycle progression (Feng *et al.*, 1996). Sechler and Schwarzbauer established that FN matrices with distinct morphologies had opposite effects on cell growth by specifically altering the rate of

G_0/G_1 to S phase progression (Sechler and Schwarzbauer, 1998). Given the requirement for FN fibril formation for these effects, as well as the well-documented complexation of TG2 with FN (section 1.9.4.4), it is conceivable that the cell-surface TG2 portion may play an indirect role in cell cycle progression.

TG2 has also been implicated in cell differentiation. Induction and accumulation of TG2 has been correlated with monocytic cell differentiation, whereas in a more physiological context of differentiation, TG2 was shown to be expressed during 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 and 1996). In support of the role of TG2 in differentiation, expression of the enzyme in a TG2 transgenic mouse animal model (containing the mouse TG2 promoter coupled to the reporter gene β-galactosidase) was observed both in morphologically normal cells and in the interdigital mesenchymal cells undergoing differentiation (Nagy *et al.*, 1997).

1.9.5.2 TG2-mediated cell signalling

Although the classical Ca^{2+}-dependent crosslinking activity is essential for the extracellular function of TG2, the enzyme's GTPase activity is essential for its intracellular function. Inside the cell, TG2 is thought to function as a G-protein in hormone receptor signalling, involved in the transmission of the α1-adrenergic receptor signal to phospholipase C (PLC) -δ1 (Nakaoka *et al.*, 1994; Feng *et al.*; 1996; Chen *et al.*, 1996; Baek *et al.*, 2001). Co-purification following ligation of epinephrine to rat liver α1-adrenoreceptors, revealed a novel 74kDa GTP-binding protein Gαh complexed with epinephrine, the receptor, and Gα (Im *et al.*, 1990). Peptide analysis of Gαh purified from several species revealed identical footprinting pattern to that of TG2, whereas monoclonal antibodies against TG2 cross-reacted with Gαh confirming that TG2 and Gαh represent the same molecule (Nakaoka *et al.*, 1994). It was subsequently shown that Gαh/TG2 is involved in the activation of a 69kDa PLC isoform that was later identified as PLCδ1 (Baek *et al.*, 1993; 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 50kDa protein (Gβh), a calreticulin-like Ca^{2+}-binding protein, generates the Gh holoprotein. Upon ligation with epinephrine, the α1-adrenoreceptor recruits TG2 and promotes the exchange of TG2-bound GDP with

GTP. The GTP-bound TG2 then binds and activates PLCδ1, which in turn can hydrolyse phospatidylinositol diphosphate (PIP_2), thus generating 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).

1.9.5.3 TG2 in receptor-mediated endocytosis

Cell surface receptor bound proteins are transported into the cells via internalisation of clathrin-coated vesicles, in a process often referred to as receptor-mediated endocytosis. Maxfield and colleagues first implicated TGs in this process, as primary alkylamines were found to inhibit ligand-receptor clustering in clathrin-coated pits (Maxfield *et al.*, 1979). The blockade of Ca^{2+}-dependent α-2 macroglobulin polypeptide hormone aggregation prior to internalisation into fibroblasts by several TG inhibitors (sulphonyl urea, alkyl amines, glutamyl and lysyl peptides) was the first line of evidence suggesting an active role for TG2 in receptor-mediated endocytosis (Davies *et al.*, 1980). However, the obligatory role of TGs in this process was soon ruled out as normal endocytic activity was observed in TG-deficient cells (Davies and Murtaugh, 1984).

Despite the dismissal of the involvement of TG2 in receptor-mediated endocytosis, circumstantial evidence suggests a role for the enzyme in receptor mediated phagocytosis. It has been established that activated macrophages accumulate TG2 and exhibit much higher TG activity compared to their non-activated counterparts (Murtaugh *et al.*, 1983). Although the exact mechanism by which the enzyme participates in this process has not yet been elucidated, it has been proposed that TG2 accumulation in macrophages might be due to enhanced phagocytosis (Schroff *et al.*, 1981), as a result of increased Fc receptor affinity (Davies and Murtaugh, 1984).

1.9.5.4 Importance of TG2 in wound healing

Bowness *et al.* (1988) first reported increased TG activity during skin wound healing in rats. A role for TG2 in tissue repair has been recently confirmed as TG2-null mice (Nanda *et al.*, 2001; De Laurenzi and Melino, 2001) exhibited impaired skin wound healing (Mearns *et al.*, 2002). TG2 has been since found to be involved in all overlapping

wound healing phases (inflammation, tissue formation/stabilisation and tissue remodeling), following immunohistochemical detection of its expression and its ε(γ-glutamyl)lysine crosslink product in cultures of rat endothelial cells, macrophages and skeletal muscle cells (Haroon *et al.*, 1999a). Upchurch *et al.* (1991) demonstrated the binding of endogenous TG2 to the ECM following puncture wounding of embryonic human lung fibroblast cell monolayers and enrichment in TG2 around the wound area for many hours afterwards, an observation that has been expanded to 3 days post-wounding following punch biopsy wound studies in rats (Haroon *et al.*, 1999a). Conversely, in an *in vitro* wound scratch assay, following wounding of a monolayer of Swiss 3T3 cells induced to overexpress TG2, a marked increase in TG2 cross-linking activity could be measured at the edge of the wound bed by monitoring the incorporation of a fluorescent amine substrate for TG2 (Nicholas *et al.*, 2003). The same group also reported a slower rate of migration of TG2 transfected Swiss3T3 fibroblasts when induced to overexpress TG2 compared to cells expressing a low background level of TG2 in the same *in vitro* wound closure test (Verderio *et al.*, 2004). Similar observations were made on human skin where TG2 was implicated in the regeneration process of grafts (Raghunath *et al.*, 1996). Since collagen VII was identified as a TG2 substrate it was proposed that the role of TG2 in the healing process of the grafts was the stabilisation of the anchoring fibres of the dermo-epidermal junction (Raghunath *et al.*, 1996). Interestingly, therapeutic approaches employing topical putrescine application on hypertrophic scars resulted in an improvement of the patients' conditions, suggesting the involvement of TG2 in mediated cross-linking of the wound matrix (Dolynchuk *et al.*, 1996).

Relevant to the inflammation process, TG2 antigen was found to be particularly expressed in macrophages, adjacent to the re-epithelialization zone and in the provisional fibrin matrix during rat dermal wound healing (Haroon *et al.*, 1999a). The same authors reported that the increased expression of TG2 found in endothelial cells and macrophages invading the fibrin clot results in the formation of transglutaminase mediated cross-linking in both fibrin and the new granulation tissue during wound healing (Haroon *et al.*, 1999a). This finding is consistent with the observation that human umbilical vein endothelial cells (HUVEC) are a rich in TG2, the synthesis of which is up-regulated by thrombin (Auld *et al.*, 2001). It has been recently suggested that lack of TG2 in macrophages prevents efficient phagocytosis of dead cells as phagocytosis of apoptotic cells was defective in the thymus of TG2-null mice after induction of apoptosis by either a dose of anti-CD3 monoclonal antibody, a dexamethasone-acetate injection, or γ-

irradiation (Szondy *et al.*, 2003) In this study, the authors correlated this defect in clearance of dead cells by macrophages to the impaired activation of TGF-β1, which is specifically released by macrophages on recognition of dead cells and plays an important function in downregulating the inflammatory response (Szondy *et al.*, 2003). TG2 activity was also found to be upregulated in various inflammatory conditions and in diseases which are characterised by mucosal inflammation, such as celiac disease, Crohn's disease, and ulcerative colitis (Lorand and Graham, 2003). The activity of 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). Recently, Sohn and colleagues have shown that new chimeric peptides derived from proelafin and antinflammins can inhibit sPLA2, TG2 activity and TG2-mediated modification of sPLA2 and display strong *in vivo* anti-inflammatory activity (Sohn *et al.*, 2003).

Following inflammation, and during the transition from granulation tissue to scar, tissue remodelling plays a key role and depends on the balance between the synthesis and degradation of collagen (Singer and Clark, 1999). Many of the extracellular roles of TG2 which have implications in the early stages of wound repair involves its interaction with FN. FN participates in the regulation of the wound-repair response by providing a provisional matrix prior to collagen deposition which is essential for adhesion, migration and proliferation (Davis *et al.*, 2000). The cross-linking function of TG2 in the extracellular matrix leading to ECM stabilisation/remodelling has been identified in a number of biological processes important for tissue repair (Aeschliman and Thomazy, 2000). Conversely, cell-surface TG2 modulates and alters FN matrix stability by multimerisation following cell damage (Gross *et al.*, 2003). The same authors have proposed a role for TG2 in the rapid stabilisation of tissue following UVA damage (Gross *et al.*, 2003). Rather than through increased matrix stability TG2 can also affect cell-matrix interactions 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; Akimov *et al.*, 2000; Belkin *et al.*, 2001; Balklava *et al.*, 2002; Verderio *et al.*, 2003).

Finally, given the regulation of TG2 expression by cytokines implicated in the repair process such as transforming growth factor β1 (TGF-β1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) (George *et al.*, 1990; Suto *et al.*, 1993; Ikura *et al.*, 1994;

Kuncio *et al.*, 1998) as discussed in section 1.9.3, a regulated physiological role of TG2 in the tissue repair mechanism is feasible.

1.9.5.5 TG2 mediated-insulin secretion

Transglutaminases have been implicated before in insulin secretion by mediating cross-linking of membrane proteins (Bungay *et al.*, 1984 and 1986). It has been postulated that TG2 could promote stabilisation of the rat islet B cell membrane during membrane recycling and following insulin secretion, since inhibition of its activity by competitive amine substrates [monodansylcadaverine and N-(5-aminopentyl)-2 naphthalenesulphonamide] has been shown to rapidly reduce Ca^{2+}-mediated, glucose-stimulated insulin release from the islets (Bungay *et al.*, 1986). The *in vitro* formation of transglutaminase-crosslinked polymeric aggregates, following incubation of islet homogenates with labelled-methylamine, provided further evidence for the role of islet TG2 in the membrane-mediated events necessary for glucose-stimulated insulin release (Bungay *et al.*, 1986). Sener and colleagues have also proposed that TG participates in the machinery controlling the access of secretory granules to the exocytotic sites in pancreatic islets B cells, as glycine methylester-mediated inhibition of cellular TG activity inhibited insulin release triggered by D-glucose (Sener *et al.*, 1985).

1.9.5.6 TG2 in cell death processes

Increasing evidence has revealed a protective role for TG2 against cell death and proposed that TG2 may act as a survival protein during cellular insult. Researchers within this laboratory have proposed a novel RGD-independent cell adhesion mechanism that promotes cell survival when the anti-apoptotic role mediated by RGD-dependent integrin function is reduced as in tissue injury (Verderio *et al.*, 2003). Prior to this proposal, other investigators have also provided evidence in support of an anti-apoptotic role for TG2. Antonyak *et al.* (2001) demonstrated that, although pretreatment of HL60 and NIH3T3 cells with RA blocks N-(4-hydroxyphenyl) retinamide (HPR)-induced cell death, monodansylcadaverine (MDC) -which binds to the enzyme TG2 transamidase active site-eliminated RA protection against cell death and in fact caused RA to become an apoptotic factor (Antonyak *et al.*, 2001). Other *in vitro* studies revealed that TG2 protects Rb from caspase-induced degradation by virtue of its ability to modify Rb via

transamidation hence providing protection against apoptotic insults during differentiation (Boehm *et al.*, 2002).

1.9.5.6.1 Cell death mechanisms

It is generally accepted that the maintenance of tissue and organ homeostasis is balanced reciprocally by distinct stimuli that trigger cell proliferation, growth, differentiation, and cell death. Cell death may occur either in an apoptotic or necrotic manner.

Apoptosis, or programmed cell death, may occur in single cells in response to a physiologic (and sometimes pathologic stimulus), in an active, non-inflammatory, and genetically controlled manner that invariably ends in phagocytosis of the apoptotic bodies. Apoptosis has been dissociated from necrosis by Kerr and colleagues (Kerr *et al.*, 1972) and has been so far best understood in the nematode *C. Elegans* (Ellis and Horvitz, 1986). The apoptotic cascade has been divided into four phases: initiation, execution, disintegration, and elimination. Initially inter-cellular adhesiveness is lost, leading to cell shrinkage, chromatin condensation (pycnosis) in the nucleus and blebbing at the cell membrane. With the remaining organelles morphologically intact, the nucleus is disrupted into few fragments (karyorrhexis). Following nucleic acid and cytoplasmic condensation, membrane-bound particles (apoptotic bodies) are formed and engulfed by macrophages or neighbouring cells prior to release of their lytic content in a non-inflammatory fashion. Energy and induction of pro-apoptotic gene expression are prerequisites for the apoptotic process (Arends and Wylie, 1991; Fesus *et al.*, 1991). Many vital tissue functions, such as embryogenesis, physiological tissue remodelling, wound healing, termination of inflammatory reactions, and elimination of DNA-damaged cells, depend on apoptosis.

Necrosis, on the other hand, which occurs in response to acute stimuli, targets groups of cells or part of a tissue, mediating a characteristic cell membrane osmotic barrier loss that eventually leads to inflammatory, irregular disintegration and clearance of cell membrane and organelles. Necrosis is initially marked by an increase in intracellular Ca^{2+}, the swelling of the cells (oncosis) and of the nucleus, the early destruction of cell organelles, and clearance following initiation of an inflammatory reaction due to release of cellular proteases (Majno and Jorris, 1995).

1.9.5.6.2. Implication of TG2 in necrosis

Gilad and colleagues were the first to observe the increase of exogenous and endogenous TG2 activity (in the nuclear fraction) during necrotic processes when rat sympathetic nerve ganglions were crushed (Gilad *et al.*, 1985). Hepatectomy-induced necrosis was also shown to lead to an increase of TG2 activity, with concomitant cross-linking of endogenous cell proteins. This suggested that increased proteolysis results in the release or exposure of additional TG2 substrates and protein cross-linking sites (Haddox *et al.*, 1981a and 1981b; Groenen *et al.*, 1993). A role for TG2 in the cross-linking of proteins within the cytoplasmic inclusions of the necrotic cells has also been proposed in alcoholic hepatitis which is characterised by the presence of Mallory bodies (filamentous cytoplasmic inclusions) in hepatocytes, liver cell necrosis, and inflammation (Zatloukal *et al.*, 1992). Interestingly, it has been recently suggested that cross-linking of cellular proteins by TG2 during necrotic cell death provides a mechanism for maintaining tissue integrity (Nicholas *et al.*, 2003). In this study, the demonstration of entrapped DNA within shell structures, which showed limited fragmentation, provided evidence that the high degree of transglutaminase cross-linking results in the prevention of DNA release, which may help to silence any subsequent inflammatory response (Nicholas, *et al.*, 2003).

1.9.5.6.3 TG2 and apoptosis

Fesus and colleagues first implicated TG2 in apoptosis, by demonstrating that, following induction of liver hyperplasia in rats, TG2 antigen and activity climaxed at the time of maximum cellular regression (Fesus *et al.*, 1987). Subsequently, the same authors classified the resulting spherical hepatocytes structures that were enriched in ε(γ-glutmyl)lysine as apoptotic bodies (Fesus *et al.*, 1989). The onset of apoptosis *in vivo* is often characterised by the induction of the TG2 gene (Fesus *et al.*, 1989; Piacentini *et al.*, 1992; Knight *et al.*, 1993; Amendola *et al.*, 1996). Although the occurrence of apoptosis and increased TG2 expression in the *in vitro* hepatocyte model (Fesus *et al.*, 1987), as well as in an erythroleukemic K562 model (Knight *et al.*, 1993) did not always overlap, the association of TG2 with apoptosis was since substantially strengthened in a number of *in vitro* and *in vivo* systems, including neuroblastoma cells (Melino *et al.*, 1994;

Piredda *et al.*, 1999), tracheal epithelium (Zhang *et al.*, 1995), and myeloid leukaemia cells (Nagy *et al.*, 1996a). Nemes and colleagues correlated first elevated TG2 expression and ε(γ-glutamyl)lysine with apoptosis in an *in vivo* apoptotic system (Nemes *et al.*, 1996). The same authors, also demonstrated that induction of TG2 expression correlates with dexamethasone or irradiation-mediated *in vivo* apoptosis in the thymus, although no induction was observed in fas receptor-driven apoptosis (Nemes *et al.*, 1996). Transfections of mammalian cells with a full length TG2 cDNA revealed a marked increase in the spontaneous cell death rate (Gentile *et al.*, 1992). Conversely, stable transfections with antisense TG2 constructs led to pronounced decrease in spontaneous and induced apoptosis (Melino *et al.*, 1994). These two studies suggest that TG2-catalysed crosslinking of intracellular proteins is an important event in apoptosis. However, neither the general caspase inhibitor Z-VAD, nor the apoptosis suppressor bcl-2 could rescue cells from what was initially believed to be a TG2-mediated human neuroblastoma cell apoptosis (Pirreda *et al.*, 1999; Johnson *et al.*, 1998; Melino *et al.*, 1994). Therefore TG2 is believed to play a role downstream of the apoptotic cascade, as part of a 'fail safe' mechanism separate from the cell-death commitment machinery that ensures protection against excessive inflammation (Nicholas *et al.*, 2003). 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 phogocytosis, as it had the potential to prevent leakage of intracellular, inflammatory components to the extracellular space thus maintaining tissue integrity. Interestingly, TG2 was shown to share substrates, such as histone H2B, pRB, actin, troponin with pro-apoptotic caspases (Piacentini *et al.*, 1999). Given that in U937 cells, the polymerisation of the retinoblastoma protein (pRB) which precedes apoptosis, is mediated by TG2, a role for the enzyme upstream of the cell death commitment machinery cannot be ruled out yet (Oliverio *et al.*, 1997). Recent work from Thomazy and Davies revealed TG2 accumulation in clusters of apoptotic cells in the interdigital web resulting in the separation of the fingers (Thomazy and Davies, 1999).

The involvement of Ca^{2+} in cell death either through activation of nitric oxide synthase (NOS), or through activation of endonucleases responsible for DNA fragmentation, or even through the activation of phospholipase A2 with a consequent rise in cysteine proteases (calpain) has been well-documented (McConkey and Orenius; 1996). On this basis, it is conceivable that TG2 might regulate apoptosis by controlling the release of

intracellular Ca^{2+} stores either through its GTP-binding activity or by assisting in the depletion of ATP stores through its ATPase activity. Despite scores of publications, a definitive role of TG2 in apoptosis has not been firmly established.

1.9.5.6.4 A novel TG2-mediated cell death

Recent animal renal fibrosis studies provided evidence for a novel TG2-mediated cell death mechanism that does not fit the classical apoptotic or necrotic cell death (Johnson *et al.*, 1997). In an *in vivo* rat model of renal fibrosis, which is characterised by loss of renal function and tubular cell death, tubular cells demonstrated overexpression of TG2 and excessive ε(γ-glutamyl)lysine content, whilst lacking biological and morphological signs of classical apoptosis (Johnson *et al.*, 1997). In a similar *in vitro* study, glucocorticoid treatment of the TG2-transfected MetB fibrosarcoma cell line led to crosslinking of cellular proteins without causing nuclear fragmentation or activation of ICE protease and CPP32, an effect that was not reversed by overexpression of the apoptosis suppressor bcl-2 (Johnson *et al.*, 1998). These findings have been expanded in hypetrophic chondrocytes, where overexpression of plasma transglutaminase led to loss of membrane integrity and concomitant leakage of cytoplasmic components, without any evidence of typical apoptotic signs (Nurminskaya *et al.*, 1998; Nicholas *et al.*, 2003).

1.9.5.7 Importance of TG2 in cell adhesion and migration

A plethora of evidence suggests that TG2 is externalised from the cytosol and localises on the cell surface and in the ECM where it plays a role in cell adhesion and migration (Barsigian *et al.*, 1991; Aeschlimann and Paulson, 1994; Martinez *et al.*, 1994; Aeschlimann *et al.*, 1995; Jones *et al.*, 1997; Verderio *et al.*, 1998 and 1999; Akimov *et al.*, 2000; Stephens *et al.*, 2004).

Primarily, the ability of anti-TG2 monoclonal antibody Cub7402 to reduce cell attachment and migration in a number of cells lines and in a dose-dependent manner indicates that cell-surface TG2 is an important component in the migration of cells (Jones *et al.*, 1997; Verderio *et al.*, 1998; Heath *et al.*, 2001; Balklava *et al.*, 2002) in a similar manner as described for cells incubated with antibodies directed against cell surface integrin receptors α1 and β5 (Fogerty and Mosher, 1990). Using a different cell system Akimov and Belkin have demonstrated that TG2, which is expressed on the surface of

monocytic cells, is also involved in the adhesion and migration of monocytic cells on a FN matrix (Akimov and Belkin, 2001). Transfection studies also suggested that fibroblasts overexpressing TG2 exhibit increased cell spreading and reduced susceptibility to detachment by trypsin (Gentile *et al.*, 1992), and that reduced expression of TG2 using antisense RNA is associated with decreased cell adhesion and spreading in different cell lines (Jones *et al.*, 1997). In support of these findings, induction of TG2 expression in Swiss 3T3 fibroblasts transfected with the catalytically active and inactive TG2 ($Cys^{277}Ser$) under control of the tet regulatory system is accompanied by a decrease in cell migration on FN when cell motility was assayed by measuring outward cell migration from an agarose droplet on FN (Balklava *et al.*, 2002). This study provided evidence suggesting that TG2-mediated interaction of the cells with the extracellular matrix is independent of the enzyme's crosslinking activity (Balklava *et al.*, 2002), whereas others suggested it is dependent on its association with $\alpha4\beta1$ integrin (Isobe *et al.*, 1999). In this context, TG2 is required for outside-in signal transduction resulting in the activation of protein kinase Ca (PKCa) (Verderio *et al.*, 2003).

Given that a new role for TG2 as an integrin-binding adhesion co-receptor for fibronectin has already been proposed (Akimov *et al.*, 2000), it is expected that the enzyme participates in the RGD-dependent cell adhesion process via complexation with FN. However, a recent study demonstrated that both artificial and physiological matrices of FN in complex with TG2 have a distinctive adhesive role (Verderio *et al.*, 2003). When seeded on TG2-FN, different cell types (fibroblasts, osteoblasts and endothelial-like cells) were rescued from anchorage dependent cell death (anoikis) following inhibition of the classical FN ArgGlyAsp (RGD)-dependent adhesion pathway mediated by $\alpha5\beta1$ integrin receptors. In contrast, binding of purified gpl TG2 to either tissue culture plastic or the gelatin binding domain of FN, which contains the putative TG2 binding site, did not enhance cell adhesion, which requires the specific complexation of TG2 with FN (Verderio *et al.*, 2003). This matrix complex was shown to be sufficient to support the formation of focal contacts in the presence of RGD peptide.

In the context of tumour progression, it has also been demonstrated that the proteolytic degradation of cancer cell-surface TG2 suppressed cell adhesion and migration on fibronectin, but stimulated cell motility on collagen matrices (Belkin *et al.*, 2001).

Collectively, these *in vitro* models are unlikely to represent the complexity of cell migration *in vivo*. However, initial *in vivo* cell migration studies on punch biopsy-

induced skin lesions appear to show a significant delay in wound closure in TG2-deficient mice compared to control wild type counterparts (Mearns *et al.*, 2002).

1.9.6 TG2 in pathological disorders

1.9.6.1 TG2 and neurodegenerative diseases

TG2 was first characterised in brain cells in 1986 as being present in isolated synaptosomes and in astrocytes in culture (Pastuszko *et al.*, 1986; Reichelt and Poulsen, 1992). The physiological role of the brain TG is not known although circumstantial evidence suggests that the enzyme may be involved in neurotransmitter release (Facchiano *et al.*, 1993). Although TG2 may play a part in the normal functioning of the neurons, it may also play a role in the pathogenesis of Alzheimer's disease (AD) and Huntington's disease (HD), two neurodegenerative disorders marked by the presence of insoluble protein complexes in the brain (Lorand *et al.*, 1996; Lesort *et al.*, 2000).

Alzheimer disease is associated with selective damage of neurons in the neurocortex, hippocampus and amygdala, resulting in impaired memory, thinking and behaviour. Extracellular senile plaques, containing fibrils composed of β-amyloid protein (Aβ), and neurofibrillar tangles, comprising an abnormally phosphorylated form of the microtubule-associated protein tau, are the hallmarks of AD.

Selkoe and colleagues hypothesised first that TG2 might be implicated in AD by demonstrating the presence of TG activity in the soluble fraction from AD patients brain (Selkoe, 2002), a finding that was later strengthened by the observation of elevated (3-fold) TG2 antigen levels in AD prefrontal cortex when compared to controls (Johnson *et al.*, 1997). Subsequent to this study, it was demonstrated that neurofilaments and Aβ in particular as well as its precursor (βAPP) are good TG2 substrates (Miller and Anderton, 1986; Ikura *et al.*, 1993; Rasmussen *et al.*, 1994, Ho *et al.*, 1994). Increased presence of TG2 antigen was also observed in senile plaques of AD brains, suggesting a role for TG2 in neurofibrillary tangle formation (Zhang *et al.*, 1998). Further kinetic and biochemical experiments established that tau is also an excellent substrate for TG2 (Dudek and Johnson, 1994; Murthy *et al.*, 1998; Miller and Johnson, 1995; Appelt *et al.*, 1996), and the sites in tau involved in the TG2-mediated cross-linking have since been mapped (Murthy *et al.*, 1998). Immunohistochemical experiments have also indicated that AD neurons have an increased immunoreactivity for TG2, and that tau is co-localised with

TG2 in neurofibrillar tangles of AD patients (Applet *et al.*, 1996; Citron *et al.*, 2001). Recently, Tucholski and co-workers suggested that modification of tau either by cross-linking or by polyamination alters its metabolism leading to AD pathogenesis (Tucholski *et al.*, 1999).

Huntington's disease (HD) is among eight neuro-degenerative diseases that have been positively associated with $(CAG)_n$ expansions in the genome and to corresponding polyglutamine (Q_n) expansions in the encoded proteins (Cooper *et al.*, 1999). In all these disorders, progressive impairment of co-ordination and/or motor neurone degeneration due to polyglutamine protein aggregate accumulation is associated with variable symptoms (Paulson and Fishbeck, 1996; Lunkes and Mandel, 1997, Vonsattel and DiFiglia, 1998).

It is now believed that increased Q_n size may lead to aberrant or increased TG2 activity (Green, 1993). It was later confirmed that peptides containing Q_n domains are excellent substrates for TG2 (Kahlem *et al.*, 1996). TG2-catalysed attachment of naked Q_n domains to polyamines yields high-molecular weight polymers converting them into favourable substrates for attachment to other Q_n domains (Gentile *et al.*, 1998). Importantly, Karpuj and colleagues demonstrated that TG2 can cross-link huntingtin protein and as a consequence may be involved in the formation of nuclear inclusions found in HD brains (Karpuj *et al.*, 1999). In a follow-up transgenic mice HD therapy model, treatment with cystamine (a competitive TG2 inhibitor) was shown to extend survival, and reduce HD-associated symptoms (Karpuj *et al.*, 2002).

Recent evidence further suggests that the elevated expression of TG2 gene in the brain during neuropathological conditions, coupled with its conversion into an alternative, shorter splice isoform that lacks the GTP binding site confers increased TG crosslinking activity to the enzyme and may lead to neuronal loss (Citron *et al.*, 2001 and 2002).

1.9.6.2 TG2 and fibrosis

Griffin and colleagues demonstrated first the possible involvement of TG2 in lung fibrosis in an experimental model of pulmonary fibrosis induced by the herbicide paraquat (Griffin *et al.*, 1979). In this study increased hydroxyproline levels and lung DNA content were accompanied by increased TG activity (Griffin *et al.*, 1979). TG2 has since been linked to the fibrotic phenotype in a number of tissues, such as the kidney (Johnson *et al.*, 1997 and 1999; Skill *et al.*, 2001), the liver (Kuncio *et al.*, 1998; Mizra *et*

al., 1997; Piacentini *et al.*, 1999; Grenard *et al.*, 2001b), the heart (Small *et al.*, 1999), and the vasculature (Bowness *et al.*, 1994).

In the kidney, in a subtotal nephroctomy (SNx) rat model of renal scarring, the product of TG2 crosslinking, ε(γ-glutamyl)lysine isopeptide, was found to be elevated both in the extracellular space and in the cytoplasm of tubular cells during renal scarring (Johnson *et al.*, 1997). This finding was indicative of a role for the enzyme in the stabilisation and accumulation of ECM. In a follow-up study, the same authors demonstrated that induction of fibrosis in an SNx model leads to *de novo* synthesis of TG2 by renal tubular cells with a concomitant increase in the enzyme's translocation to the pericellular environment (Johnson *et al.*, 1999). The same study 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 MMPs (Johnson *et al.*, 1999). In the liver, pathological states such as cirrhosis, alcoholic hepatopathy, and type C hepatitis have also been linked to the increased crosslinking activity of TG2 (Mizra *et al.*, 1997; Grenard *et al.*, 2001b). In the heart, Small and colleagues showed that transgenic mice with enhanced cardiac TG2 expression developed cardiomyopathy (Small *et al.*,1999), whereas in the vasculature, a number of studies by Bowness and colleagues indicated that increased TG found in atherosclerotic plaques correlates with increases in the ε(γ-glutamyl)lysine crosslink (Bowness *et al.*, 1987, 1989 and 1994; Bowness and Tarr, 1990). In these studies, TG2-mediated crosslinking is believed to be responsible for the stabilisation of the collagen III–lipoprotein interface surrounding the sclerotic plaque.

Taken together, these findings accentuate the importance of TG2 as an effector molecule in the disruption of ECM homeostasis that is consistent with fibrogenesis, as the enzyme appears to tip the ECM deposition/degradation balance towards accumulation. However, the possible involvement of TG2 in the activation of TGFβ -a well-established pro-fibrogenic growth factor (Nunes *et al.*, 1997) -as discussed earlier (section 1.9.4.4.1), does not rule out a possible regulatory role for TG2 in *de novo* matrix synthesis.

1.9.6.3 TG2 and coeliac disease

Some of the clinical features of coeliac disease (CD) are chronic inflammation and damage of the upper small intestine that causes denuding of the outer surface of the villi and production of a characteristic flat mucosa with hypertrophic crypts (Trier, 1991; Marsh, 1992). CD etiology lies in the inappropriate T-cell mediated immune response in genetically predisposed individuals (mostly HLA-DQ2 and –DQ8 positive) against ingested gluten proteins (5 gluten peptides have been identified so far), usually found in wheat or similar proteins in barley and rye (Molberg *et al.*, 2000). CD is organ-specific as only gut-derived T-cells react with modified gliadin peptides (Molberg *et al.*, 1998).

Early studies have indicated that although TG2 is expressed at low levels in the submucosa of the healthy small intestine, its expression is markedly increased (3-fold) in jejunal biopsies of CD patients (Bruce *et al.*, 1985). Subsequent *in vitro* studies indicated that gliadin and its proteolytic fragments are capable of acting *in vitro* as substrates for *gpl*TG2 (Porta *et al.*, 1990a), whereas polyglutamine peptides and polyglutamine-rich proteins were also shown to be incorporated by TG2 into large M_r aggregates (Gentile *et al.*, 1998; De Cristofaro *et al.*, 1999). Immunoprecipitation of proteins from CD patients has shown that TG2 is the dominant endomysial autoimmune antigen toward immunoglobulin A that is produced in CD patients, suggesting that TG2 is a pivotal player in the pathogenesis of CD (Dieterich *et al.*, 1997). Following that pioneering study, TG2 has been established as a CD serologic marker (Hoffenberg *et al.*, 2000; Gillett and Freeman, 2000; Dickey *et al.*, 2001; Fabiani and Catassi, 2001). In favour of the hypothesis that TG2 is involved in the pathogenesis of CD, competitive TG2 polyamine substrates (spermine and spermidine) were shown to protect the small intestine from the inflammatory response to gliadin peptides (Auricchio *et al.*, 1990). In keeping with this finding, Molberg and co-workers effectively reduced immune response to deamidated gliadin by administering the TG2 inhibitor cystamine (Molberg *et al.*, 2001).

Although accumulating evidence suggests an indirect role for TG2 in CD pathogenesis, the exact molecular mechanism by which TG2 elicits T and B cell responses in CD has not yet been elucidated. The first potential mechanism is based on the early finding that deamidation of glutamine residues is negligible at physiological pH, but occurs more readily with increased protonation of amines in an acidic environment (Mycek and Waelsch, 1960). Mass spectrophotometric studies demonstrated that TG2 deamidates gliadin peptides and converts their glutamine residues into glutamic acid (Molberg *et al.*, 1998, Van de Wal *et al.*, 1998; Sjostrom *et al.*, 1998, Quarsten *et al.*, 1999; Anderson *et*

al., 2000; Arentz-Hansen *et al.*, 2000), thus introducing negative charges and increasing their otherwise low antigenicity for HLA-DQ2 and HLA-DQ8 molecules (Johansen *et al.*, 1996). In favour of an indirect mechanism, Porta and colleagues first suggested that *in vivo* TG2 auto-crosslinking on the surface of gliadin might act as a hapten-carrier for the mobilisation of autoantibodies against gliadin (Porta *et al.*, 1991), a notion that was later supported by other investigators (Molberg *et al.*, 2000).

1.9.6.4 TG2 and cancer

1.9.6.4.1 The tumour growth and progression cascade

Whilst great advances have been made in tumour therapy in the recent years, cancer remains one of the leading causes of mortality. Tumour progression is a consequence of changes in the expression of a range of gene products that mark the transition from the benign to the malignant phenotype. Pathological analyses of organ sites often reveal lesions that seem to represent intermediate steps in a process through which normal cells evolve progressively via a series of pre-malignant states into invasive cancers (Foulds, 1954). *In vitro* transformation is itself a multi-step process. Rodent cells require a minimum of two introduced changes in their genetic make-up before acquiring tumorigenic competence, whereas human cells are more difficult to transform (Hahn *et al.*, 1999).

Once the malignant phenotype has been established the metastatic cascade proceeds driven by decreased cell-cell contact, cell disengagement from the primary tumour site, and degradation of the basal membrane. Tumour metastasis occurs due to invasion of the surrounding stroma, angiogenesis, and tumour cell entry into the vasculature or lymphatic system (intravasation). These events are followed by rolling and arrest of the malignant cells at a distant site, attachment to the endothelial cell lining, degradation of the basal membrane and, finally, extravasation. Undoubtedly, degradation of the basal membrane followed by cell adhesion, cell migration and invasion as well as angiogenesis are key stages in tumour growth and progression. Angiogenesis or neovascularisation is a prerequisite for neoplastic cells to grow into primary tumours and metastasise since solid tumours cannot expand in size beyond 1-2mm in diameter in the absence of new independent blood supply (Folkman *et al.*, 1990), and this neovascularisation is in itself important for tumour escape into the circulation (Liotta *et al.*, 1974). In addition, changes

in cell adhesion and changes in matrix turnover and subsequent loss of cell-cell contact provide a springboard for cellular motility and subsequent cell migration (Fingleton *et al.*, 1999). Despite previous evidence incriminating solely the malignant cell in the tumour progression cascade in which the stromal cells were believed to have a 'bystander' role, it is now becoming widely accepted that both malignant and mesenchymal cells act in concert to promote tumour invasion within the tumour-stroma interface (Vacarrellio *et al.*, 1999). Not surprisingly, the most promising cellular target for anti-invasion treatment maybe the stromal fibroblast and endothelial cells (Kohn and Liotta, 1995).

1.9.6.4.2 The tumour-stroma microecology

Malignancy is a state that emerges from tumour-host microenvironment in which the host participates in the induction, selection and expansion of the neoplastic cells (Park *et al.*, 2000). Neoplastic cells perpetually stimulate host stromal and vascular cells to conduct physiological invasion. Motility and invasion is a bi-directional process, as within the same tumour microenvironment capillary sprouts migrate and invade towards the tumour mass, while tumour cells migrate outwards in the opposite direction (Kohn and Liotta, 1995). The tumour-host interdependence can be best manifested in that most of the enzymes and inhibitors complexed at the invasion front are contributed by the host cells, not by the invading tumour cells (Nakahara, 1997). Tumour cells produce angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which bind on receptors on stromal vascular cells causing vascular permeability, endothelial proliferation and invasion. On the other hand fibroblast and endothelial cells secrete latent enzymes, among which matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA), which dock on the surface of the carcinoma invadopodia where they become activated, thus degrading the ECM (Liotta and Kohn, 2001). Conversely, ECM degradation releases bound growth factors such as TGFβ and endothelial growth factor (EGF), which in turn bind to their cognate receptors on the malignant cells. In parallel, ECM degradation exposes cryptic RGD sites, which are recognized by integrins (Liotta and Kohn, 2001).

1.9.6.4.3 TG2 expression/activity profile in carcinogenesis

TGs have been implicated in tumour growth and malignancy as early as 1972, when it was postulated that a covalently stabilised fibrin network was a prerequisite for solid tumours proliferative growth (Yancey and Laki, 1972). Subsequent studies indicated decreased TG activity and ε(γ-glutamyl)lysine content in chemically- and virally-transformed cells compared to their normal counterparts (Birckbichler *et al.*, 1976 and 1978; Birckbichler and Patterson, 1980). In agreement with this finding, it was also reported that chemically induced hepatomas and transplantable rat sarcomas exhibited reduced TG activity (Barnes *et al.*, 1985), an observation that was accompanied by redistribution of the enzyme to the particulate fraction (Hand *et al.*, 1988). In a series of hamster fibrosarcomas, the reduction of TG2 was identified as a specific reduction of the cytosolic enzyme (Hand *et al.*, 1988) accompanied with the increase in expression of a 120 kDa inactive TG (Knight *et al.*, 1990a). The inactive TG expressed in tumour tissues was hypothesised to be a result of inappropriate gene expression (Knight *et al.*, 1990b).

Contradictory to the above reports, it has also been reported that TG2 does not represent a biochemical marker of malignancy in human brain tumours, (Rönn *et al.*, 1992). Further quantification of *in vivo* TG expression in a skin, colon, pancreas, stomach, and lung tumour tissue it has been proposed that TG2 is not a tumour related marker (Takaku *et al.*, 1995), a finding that has been expanded *in vitro* to include several malignant cell lines, which have been shown to express high levels of the enzyme (Denk *et al.*, 1984; Mehta, 1994; Takaku *et al.*, 1995). Recently, Grigoriev *et al.* (2001) demonstrated that a mere 15% of the screened breast carcinomas was positive for TG2 antigen as opposed to a 50% of the stromal tissue. In keeping with these findings, TG2 expression in metastatic human melanomas was also shown to be markedly increased when compared to the corresponding normal tissue (Van Groningen *et al.* 1995).

Taken together, the above findings suggest that TG expression and activity may vary between various tumour tissues. Although TG2 might not represent a tumour-related marker in all tested tumours, the enzyme has been implicated in the body's natural defence against tumour formation as increased TG2 expression in the tissue surrounding the tumour has been reported (Hettasch *et al.*, 1996; Grigoriev *et al.*, 2001). To elucidate whether TG2 expression and TG activity are causal or rather an epiphenomenon of the malignant phenotype Johnson and co-workers demonstrated, by stably transfecting the cDNA of the human TG2 into the hamster fibrosarcoma Met B line, that increasing the expression of TG2 in this fibrosarcoma cell line leads to the reduced incidence of primary

tumour growth (Johnson *et al.*, 1994). The same authors also reported unaltered *in vitro* growth rates between TG2- and mock-transfected controls, but also noted a marked increase in TG2-transfected cell adhesion on FN and tissue culture plastic (Johnson *et al.*, 1994).

1.9.6.4.4 Tissue transglutaminase in cancer metastasis

Barnes and colleagues demonstrated first that TG2 activity was markedly reduced in metastatic transplantable rat sarcomas when compared to normal tissues of rat liver, lung and spleen (Barnes *et al.*, 1985). In keeping with this finding, Hand and colleagues established that the decrease in protein-bound putrescine 30 days post implantation coincided with the onset of metastasis in the P_8 sarcoma cell line (Hand *et al.*, 1987). The reduction of TG2 expression and polyamine content in neoplastic tissue has also been inversely correlated in different studies with an increase in the metastatic potential of tumours such as rat fibrosarcomas, rat prostate carcinoma, HSV-2 induced hamster fibrosarcoma and mouse melanoma (Delcros *et al.*, 1986; Hand *et al.*, 1987; Romijn, 1990; Knight *et al.*, 1991; Beninati *et al.*, 1993).

Counter to the above findings, Elsasser and co-workers first indicated partial correlation between cellular differentiation or metastatic behaviour and increases in TG2 content (Elsasser *et al.*, 1993). This work was later supported by further studies on human breast carcinoma cells (Mehta, 1994), human breast tissue (Hettasch *et al.*, 1996), hemangioblastoma of the central nervous system (Mizoguchi *et al.*, 1998) and human melanoma cell lines (van Groningen *et al.*, 1995), where an increase of TG2 expression was shown to correlate positively with the propensity of tumours to metastasise. More recently, proteomic analysis indicated that TG2 expression was significantly up-regulated in highly metastatic cell lines when compared to lower metastatic tumour cell lines (Jiang *et al.*, 2003). A mechanism of action for the enzyme in the metastatic cascade has been proposed by Kong and Korthuis (1997), in a study based on free-floating melanoma cells in isolated arterioles, which demonstrated that TG2 stabilised tumour cell contact points with the subendothelial matrix.

In contrast, following the use of pharmacological agents to increase *in vitro* TG activity in human colorectal carcinoma cells led to unaltered metastatic potentials, thus

dissociating the enzyme's activity from the ability of these tumour cells to metastasise (Keogh *et al.*, 1993).

1.9.6.4.5 The *de novo* neovascularisation process

As discussed earlier (section 1.9.6.4.1), angiogenesis is central to the tumour growth progression and metastatic cascades (Liotta *et al.*, 1974). In several diseases, including cancer (solid and hematologic tumours), cardiovascular diseases, chronic inflammation, diabetes, and psoriasis, excessive angiogenesis is part of the pathology (Griffioen and Molema, 2000). Over the past twenty years, substantial evidence supporting the hypothesis that the growth of solid tumours is dependent upon the ability of the tumour to elicit growth of a blood supply has accumulated (Holash *et al.*, 1999).

Angiogenesis, the sprouting and development of new blood vessels from pre-existing capillaries is a rare event in healthy adults, occurring only during wound healing in tissue remodelling, during the menstrual cycle in the ovary and endometrium, and during embryonic development (Folkman, 1995). Angiogenesis requires proliferation and migration of endothelial cells (ECs) as well as their interaction with subendothelially located smooth muscle cells (SMCs), fibroblasts and pericytes, the basement membrane (BM) and the extracellular matrix (ECM) to form a complex three dimensional structure that contains endothelial monolayers surrounding the lumen of the vessel (Rissau, 1997). Counter to the prevailing dogma that neovascularisation in the adult resulted from division and differentiation of ECs (Folkman, 1997), recent evidence suggests that endothelial progenitor cells (EPCs) also contribute to capillary vessel formation (Rafii and Lyden, 2003). In either case it has been postulated that the stepwise angiogenic process (figure 1.9.6.4.5), is regulated by growth factors (Meyer *at al.*, 1999), proteases (Stetler Stevenson, 1999), the ECM (Preissner *et al.,* 1996), and the expression of cell surface receptors (Bazzoni *et al.*, 1999). Angiogenesis is initiated under hypoxic or ischaemic conditions, in response to vascular endothelial growth factor (VEGF). Under the control of hypoxia inducible factor (HIF), release of VEGF induces vasodilation via endothelial NO production and its endothelial permeabilising effect, whilst promoting EC proliferation (Ziche *et al.*, 1997). Once the EC has been activated, it migrates to the site of vascularisation, following degradation of the BM by MMPs secreted in a latent form by ECs, fibroblasts and epithelial cells (Stetler Stevenson, 1999). Plasminogen activators u-PA and t-PA convert plasminogen to plasmin, which, apart from degrading BM

proteins such as fibronectin and activating certain MMPs, mobilises bFGF from the ECM pool to up-regulate EC proliferation, motility and proteinase activity (Gleizes *et al.*, 1995). Integrins ($\alpha v\beta 3$ and $\alpha v\beta 5$) have also been shown to be pivotal in EC survival during angiogenesis, as they rescue malignant cells from anoikis (Brooks *et al.*, 1994a). In order for the vasculature to reach maturity, EC mediates synthesis of platelet derived growth factor (PDGF), thus ensuring chemoattraction and differentiation of mural precursor cells into SMCs and pericytes. Upon EC-mural cell contact, activation of TGFβ produced by both cell populations in its latent form is thought to contribute to ECM production and maintenance of growth control. Angiopoietins ang-1 and ang-2, on the other hand, serve opposing roles as they promote vascular maturation and destabilisation respectively, through their interaction with their respective tyrosine kinase receptors (tie1 and ties2; Maisonpierre *et al.*, 1997).

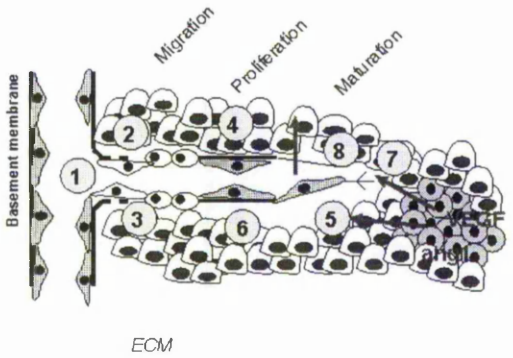


Figure 1.9.6.4.5 Angiogenesis by endothelial sprouting. Overlapping stages in the generation of the new capillary sprout from a pre-existing vessel. The blood vessel and pericytes retract in response to activation of the vascular endothelium lining following angiogenic factor secretion by the tumour cells (1). Proteases degrade the BM beneath ECs (2), rendering them less adherent (3). Fibrin deposition into the tissue is facilitated by an increase in vascular permeability (4). Subsequently, ECs are chemoattracted towards the angiogenic stimulus (5) and enter the cell cycle (6). Circulating endothelial progenitor cells may also contribute to the development of the vessel sprout (7). Once periendothelial structures are formed neovasculatures mature and become established (8), a process that is often defective in tumours. Hypoxic regions of the tumour (shown dark) provide a source for VEGF secretion. VEGF is participating in most of the steps shown, and in certain instances acts in concert with angiopoietin-1 (ang-1) to stimulate vessel sprout formation (Adapted from Sage, 1997)

High vascular density of tumours has now been shown by several groups to be an independent prognostic factor in a number of solid tumour types, including breast, bladder, prostate and head and neck cancer. Angiogenesis is a feature of all solid tumours and as such offers an opportunity for therapeutic intervention. Inhibition of angiogenesis, which involves inhibition of endothelial migration and growth, has the potential to

revolutionise cancer therapy, by starving tumours of their blood supply (O'Reilly *et al.*, 1997). Approaches to controlling angiogenesis have, to date, attempted to inhibit EC growth (i.e. angiostatin, endostatin, vasostatin, restin; O'Reilly *et al.*, 1994 and 1997; Pike *et al.*, 1998), EC cell adhesion and migration (i.e. interferons α and β, anti-αvβ3 antobodies; Sidky and Borden, 1987; Brooks *et al.*, 1994b), or inhibition of metalloproteinase activity (i.e. batimastat, marimastat, prinomastat; Brown and Giavazzi, 1995).

1.9.6.4.6 Tissue transglutaminase in normal and tumour angiogenesis

TG activity has been first identified in the EC by Greenberg and co-workers (Greenberg *et al.*, 1987a), a finding that was later confirmed by various studies (Korner *et al.*, 1989, Auld *et al.*, 2001). More recently, it has been reported that the increased expression of TG2 found in ECs and macrophages invading the fibrin clot results in the formation of transglutaminase-mediated cross-linking in both fibrin and the new granulation tissue during wound healing (Haroon *et al.*, 1999a). This finding is consistent with the observation that HUVEC are a rich source of TG2, the synthesis of which is up-regulated by thrombin (Auld *et al.*, 2001). Additionally, it has been demonstrated that TG2 is expressed in its active form in rat dermal wound healing and angiogenesis, whereas recombinant TG2 enhances blood vessel length density if applied topically in an *in vivo* dorsal skin flap window chamber suggesting the implication of TG2 in promoting angiogenesis and wound repair (Haroon *et al.*, 1999a.)

In the ECs, the crosslinking of ECM proteins by TG2 is thought to play an important physiological role in the stabilisation of the BM (Martinez *et al.* 1994). Interestingly, gene array analysis of differential gene expression during human capillary morphogenesis in 3-D collagen matrices has classified TG2 among the genes which are regulated during this process. TG2 was found to be down-regulated approximately 4-fold at 8 hours and 10-fold at 24 and 48 hours post-culture of endothelial cell cultures in 3-D collagen matrices (Bell *et al.*, 2001), thus confirming that lowered levels of TG2 expression are required during the initial stages of angiogenesis. This finding suggested that TG2 might not be needed during initiation of angiogenesis as it might be inhibitory to the endothelial mobilisation process, possibly by stabilizing the endothelial BM given that that the migration of endothelial cells and the formation of new vessels is affected by

the composition of the endothelial BM. Finally, by modulating matrix storage of latent TGF-β1, TG2 participates in the activation of this important wound healing cytokine (Taipale *et al.*, 1994; Kojima *et al.*, 1993; Nunes *et al.*, 1997; Verderio *et al.*, 1999), possibly regulating the ECM homeostasis not only by virtue of its stabilizing function (through ECM component cross-linking) but also by regulating *de novo* ECM protein synthesis.

1.9.6.4.7 Potential TG2-based antitumour therapies

It has been proposed that six essential alterations in cellular physiology collectively dictate malignant growth (Hanahan and Weinberg; 2000); self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, evasion of apoptosis, sustained angiogenesis, and tissue invasion and metastasis. Understandably, most anti-tumour therapy approaches aim to counteract one or more of these tumour-specific hallmarks.

TG2 has been reportedly involved in the stabilisation of the extracellular matrix (Barsigian *et al.*, 1991), cell adhesion (Verderio *et al.*, 1998) and migration (Balklava *et al.*, 2002), cell death (Piacentini *et al.*, 1999; Autuori *et al.*, 1998), as well as in vascular growth (Haroon *et al.*, 1999a). Therefore, it is conceivable that TG2-based tumour intervention strategies that aim to induce malignant cell pro-apoptosis, disrupt malignant invasion and metastatic cascades, or inhibit angiogenesis could be implemented.

Utilising a pro-apoptotic approach, Johnson and colleagues aimed to induce malignant cell death via induction of TG2 expression through treatment with glucocorticoids (Johnson *et al.*, 1998; Parry J., PhD thesis). Dexamethasone treatment of the MetB, D, and E fibrosarcoma cell lines led to malignant cell death due to crosslinking of cellular proteins without causing nuclear fragmentation or activation of ICE protease and CPP32, an effect that was not reversed by overexpression of the apoptosis suppressor bcl-2 (Johnson *et al.*, 1998).

In an alternative approach, administration of exogenous TG2 to human colon tumour cells deficient in TG (LS174T) inhibited cell spreading, and significantly reduced malignant colony size (Zirvi *et al.*, 1993), suggesting blockade of tumour cell migration

and concomitant colony dissemination. It has also been reported that the epithelial cell adhesion molecule molecule (C-CAM) is a substrate for TG2, raising the possibility that TG2 may exert a negative effect on tumour growth via covalent modification of this molecule (Hunter *et al.*, 1998). Recently, Belkin *et al.* (2001) demonstrated that proteolysis of glioma and fibrosarcoma cell surface TG2 at the leading edge of the motile cells by MT1-MMP regulates cancer cell adhesion and locomotion.

Finally, in a rat dorsal window flap model, topical application of 40μg/ml TG2 post-surgery was found to inhibit both growth of new vasculatures, and tumour growth when a solid piece of the R3230Ac tumour was added to the wound (Haroon *et al.*, 1999b). Tumour growth delay was accompanied by increased collagen deposition, indicating augmented ECM production, hence suggesting hindered malignant and/or EC motility (Haroon *et al.*, 1999b). In contrast, the same authors demonstrated, using a similar window flap model, but without tumour implantation, that three separate doses of active TG2 added on days 0,1, and 2 post-surgery, led to a stimulation of angiogenesis (Haroon *et al.*, 1999a). Given the number of variables present in the two experiments, it is difficult to reconcile these two separate contradictory findings.

1.10 AIMS

Given the contradicting reports on the role of TG2 in tumour growth and angiogenesis the primary aim of the present investigation is to ascertain whether TG2 has a physiological role in tumour growth development and angiogenesis by modulating its expression both *in vitro* in malignant cell cultures and *in vivo* in solid tumours. Consequently, it was also of interest to identify whether TG2 can potently inhibit angiogenesis and tumour growth when delivered exogenously to the tumour-host microenvironment at non-physiological levels. So far, scores of publications accentuate the importance of TG2 in cell adhesion/migration, cell death, and the stabilisation of the ECM, while increasing lines of evidence suggest it is involved in normal wound healing and angiogenesis. The second aim of this study is to elucidate which of the above enzyme functions may be responsible for vascular collapse and tumour growth delay.

A further part of the project seeks to identify the main phenotypic alterations induced by the overexpression of wild-type active and mutant (C277S) inactive TG2 in tumour cells, and the effect these changes might exert on tumour growth and survival.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Materials

2.1.1 General chemicals

Most general chemicals and cell culture products were purchased from the Sigma-Aldrich Chemical Company (Poole, Dorset, UK). Otherwise, chemicals were obtained from the following suppliers:

Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK:

N, N′-Methylene bisacrylamide, rainbow molecular weight markers, Lowry protein assay kit

BDH (Merck), Poole, Dorset, UK:

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

Dako Ltd., High Wycombe, Buckinghamshire, UK:

Hydrophobic pen

Jansen Chemica, Hyde, Cheshire, UK:

Trichloroacetic acid

GibcoBRL,Life Technologies Ltd., Paisley, UK:

Geneticin (G418 sulphate), DH5α competent cells

LKB, Milton Keynes, Bucks, UK:

Optiphase Hi-safe scintillation fluid

Molecular Probes, Oregon, USA:

Biotin cadaverine, Biotin-X-cadaverine, picogreen dsDNA quantitation kit

Vecta Laboratories Inc., Peterborough, UK:

Vecta-Shield fluorescence mounting medium

2.1.2 Radiochemicals

Amersham Pharmacia Biotech, Buckinghamshire, UK:

[1, 4 ^{14}C]-Putrescine (50μCi/ml), [2,3-^{3}H]-Proline and [2,3-^{3}H]-amino acid mixture (50μCi/ml)

DuPont, Stevenage, Hertfordshire, UK:

Deoxycytidine-5'-triphosphate [α-^{32}P] for random primed labeling of cDNA probes (specific activity of 3000 Ci/mmol).

2.1.3 Immunochemicals

Abcam

Anti-TGFβ-1 MAb

BD Biosciences Milton Keysnes, UK

Anti-LTBP-1 MAb

Calbiochem, Nottingham, UK:

Anti-mouse IgG-HRP conjugate

Dako Ltd., High Wycombe, Buckinghamshire, UK:

Anti-mouse IgG-FITC conjugate

Anti-mouse IgG-TRITC conjugate

Neomarkers, Fremont, USA:

CUB7402 anti-TG2 MAb

R & D Systems Europe, Abingdon, UK

Quantikine Human VEGF, bFGF, and TGFβ-1 Immunoassay kits

Santa Cruz, Autogen, Bioclear, Wiltshire, UK

Anti-von Willebrand factor antibody

TCS Cellworks, Botolf Claydon, Bucks, UK:

Anti-von Willebrand factor antibody

2.1.4 Western blotting reagents

SDS-PAGE separated proteins were transferred to nitrocellulose membranes (Gelman Biosciences, Northampton, UK) using the Protean II Biorad wet-blotting equipment. Western blots were revealed on Kodak X-OMAT film using enhanced chemiluminescence (ECL, Amersham, Little Chalfont, Bucks, UK).

2.1.5 Protein reagents

Caspace-3 apoptosis® colorimetric, TGFβ-1 Emax®, and Cytotox 96® non-radioactive cytotoxicity assay systems were all purchased from Promega (Madison, USA). Guinea pig liver TG2 was purified in-house as outlined in section 2.2.4. Extravidin peroxidase, fibronectin (FN), and collagen type I were purchased from Sigma. Molecular weight markers for protein electrophoresis were supplied by Bio-Rad (Rainbow markers). Marvel dried milk powder was purchased from domestic supply outlets.

2.1.6 Expression vectors

The pSG5 constitutive expression vector (Stratagene Inc., La Jolia, USA) containing the full length TG2 cDNA (3.3Kb) ligated into the EcoRI site of the vector (Gentile *et al.*, 1992) and the neomycin resistance plasmid pSV-Neo were kind gifts from P. J. A. Davies (University of Texas Health Centre, USA).
Mutation of the TG2 active site C277 to Ser was achieved by site directed mutagenesis as described previously (Mian *et al.*, 1995) and was a kind gift from Dr S. Mian (Nottingham Trent University, Nottingham, UK). The pSV-βgal plasmid was a gift from Prof. M. Griffin (Nottingham Trent University, Nottingham, UK), and the eGFP-N1 plasmid was purchased from Clontech (Palo Alto, USA).

2.1.7 Molecular biology kits and reagents

Plasmid DNA was purified using the Qiagen midi prep (Qiagen, Dorking, Surrey, UK) and correct kbp size was verified by digestion with either EcoRI or HindII (Promega Ltd., Chilworth Research Centre, Southampton, UK). Electrophoretically separated RNA was Northern transferred to Hybond N membranes (Amersham, Buckinghamshire, UK). Lipofectamine was purchased by Invitrogen (Paisley, UK), whereas Normal dermal fibroblasts Nucleofection kit was purchased by AMAXA

(AMAXA GmbH, Germany). Picogreen *ds*DNA quantitation kit was purchased from Molecular Probes (Oregon, USA).

2.1.8 Bacterial and mammalian cell lines

Plasmid DNA was amplified using the *DH5α* strain of *Escherichia coli* (Gibco, Life Technologies, Paisley, UK). CT26 mouse colon carcinoma was supplied by Prof. R. C. Rees (Nottingham Trent University, Nottingham, UK). Human dermal fibroblasts (HFDF) were isolated from neonatal foreskin as described in section 2.2.1.6. C378 human dermal fibroblasts isolated from human foreskin were kindly provided by Prof. E. J. Woods (Leeds University, Leeds, UK). Human umbilical large vein endothelial cells (HUVEC; *ATCC* no. 1730) were purchased from TCS Cellworks (Botolf Claydon, UK) and B16 F1 mouse melanoma cells (*ATCC* no. 6323) from ATCC. The CT26 mouse colon carcinoma cell line (*ATCC* no. 2638) was a kind gift from Prof. R. Rees (Nottingham Trent University, UK).

2.1.9 Animals

B57/BL6 TG2 wild-type and knock-out mice were a kind gift from Prof. G. Melino (University Tor Vergata, Rome, Italy), and balb/c mice were obtained from Harlan (Shaw's Farm, Blackthorn, Bicester, UK). Animals were bred at the Department of Life Sciences, Nottingham Trent University in accordance with the UK Home Office Animals Act (1986).

2.1.10 Other consumables

All water was deionised using an Elgastat system 2 water purifier (ELGA Ltd., High Wycombe, UK) or Milli-Q water purifier (Millipore/Waters, Watford, UK). General laboratory consumables were obtained from the following suppliers:

Amersham Pharmacia Biotech, Buckinghamshire, UK

Sephadex G50 Nick Columns, Q-sepharose Fast-Flow column.

BDH, Poole, Dorset, UK:

Superfrost Gold, coated glass microscope slides and coverslips

Canberra-Packard, Pangborne, UK:

Scintillation vials

Corning/Bibby-Sterilin, Stone, Staffs, UK:

Tissue culture petri dishes and flasks

Gelman Biosciences, Northampton, UK:

Nitrocellulose membrane

Lab-Tek Brand Products, Naperville (IL), USA:

8-well glass chamber slides

Nalgenenunc International, Rochester (NY), USA:

Cryovials

Non-linear Dynamics, Newcastle upon Tyne, UK:

Phoretix 1D densitometric software

Starstedt Ltd., Leicester, UK:

0.5, 1.5- and 2-ml microcentrifuge tubes, 5 ml scintillation vial inserts, 15 and 50 ml sterile centrifuge tubes, 10 ml sterile pipettes, automatic pipette fillers, 1 ml and 200 µl pipette tips, cell scrapers, 0.22µm filters

Scientific Laboratory Supplies

Tissue culture flasks T25, T7, T150, 10cm and 6cm petri dishes, 6-, 12-, 24-, 48-, and 96- well plates, improved Neubauer haemocytometer.

Whatman Ltd, Maidstone, UK:

Whatmann 1- and Whatmann 3- MM chromatography paper

2.1.11 Equipment

General laboratory instruments were purchased from the following suppliers:

Amaxa, GmbH, Germany:

Nucleofector system, Normal Human Dermal Fibroblast Kit

Beckman Instrument (UK) Ltd, High Wycombe, UK

Spectrophotometer Model DU-7, centrifuges Avanti J-30 I, MSE Centaur 2, GPKR, MSE Microcentaur and Optima TLX Tabletop Ultracetrifuge

Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK:

Mini-protean electrophoresis system, DNA-Sub electrophoresis system and power pack.

B & L Systems , Marseen, The Netherlands:

Atto-minigel protein electrophoresis system

Canberra-Packard, Pangborne, UK:

Tri-Carb 300 Scintillation counter

Corning, Stone, Staffs, UK:

pH meter 130, spectrophotometers

Edwards High Vacuum, Sussex, UK:

Freeze Dryer (Modulyo System)

Flow Laboratories, High Wycombe, UK:

MCC 340 ELISA plate reader, Gelaire BSB 4A laminar flow cabinets

Grant Instruments, Cambridge, UK:

Water baths

Jouan Ltd., Derbyshire, UK:

IG150 tissue culture CO_2 incubators, laminar flow cabinet LC 2.12 and Jouan vacuum concentrator

Leica Lasertechnik, Heidelberg, Germany:

TCSNT confocal laser microscope

MSE, Loughborough, UK:

Soniprep 150 sonicator, Chilspin refrigerated centrifuge

Olympus Optical Company (UK) Ltd., Middlesex, UK:

CK2 and BH2 light inverted microscopes, OM4 Ti 35mm camera, DP10 microscope digital camera

Pharmacia, Milton Keynes, UK:

Electrophoresis power supply, LKB Multiphor II semi-dry blotter

Tecan UK Ltd, Goring-on-Thames:

Spectafluor 96-well ELISA plate reader and XFluor4 software

Thermo-Shandon, Basingstoke, UK:

Shandon Hypercenter II and Tissue-Tek II tissue embedding center.

Carl Zeiss, Germany:

Axioskop epifluorescence microscope

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 General Cell Culture

Human dermal fibroblasts (HFDF), isolated from neonatal foreskin as described in section 2.2.1.6, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat inactivated foetal calf serum, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomysin. C378 human dermal fibroblasts isolated from human foreskin were kindly provided by Prof. E.J. Woods (Leeds University, Leeds, UK) and were cultured in the same medium. Human umbilical large vein endothelial cells (HUVEC; *ATCC* no. 1730) and angiogenesis co-cultures of human dermal fibroblasts and human umbilical vein endothelial cells were obtained from TCS Cellworks (Bucks, UK) and were cultured in large vein endothelial cell growth medium containing endothelial cell growth supplement (ECGS), 2% (v/v) FCS, 2mM L-glutamine, and standard concentrations of antibiotics and antimycotics. B16 F1 mouse melanoma cells (*ATCC* no. 6323) and CT26 mouse colon carcinoma (*ATCC* no. 2638) were grown in DMEM containing 10% (v/v) FCS, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomysin. CT26 cells constitutively expressing wild type, or mutated inactive TG2 (C277S), as well as CT26 transfected with the control vector pSV-Neo were maintained in the same growth media supplemented with 400μg/ml G418. All cells lineages were routinely cultured in a humidified atmosphere at 37°C, 5% (v/v) CO_2, 95% (v/v) air with regular media changes every two or three days.

2.2.1.2 Passage of cells

Cells were passaged at approximately 90% confluency. The cell monolayer was rinsed with PBS prior to incubation for 5 minutes with 0.25% (w/v) trypsin/ 5mM EDTA in

PBS, pH 7.4 at 37°C. Following detachment of cells, trypsin was inactivated by addition of serum supplemented DMEM. A 10µl aliquot of the cell suspension was applied to both chambers of an Improved Neubauer haemocytometer for counting. Cells were transferred into a centrifuge tube and spun in a MSE bench top centrifuge at 300xg for 5 minutes. The cell pellet was then resuspended in the appropriate cell growth medium and cells were reseeded at the desirable density.

2.2.1.3 Cell freezing

Cells were harvested and centrifuged as described previously (section 2.2.12). Cell pellets were resuspended in DMEM 20% (v/v) FCS, 10% (v/v) DMSO to a final concentration of 10^6cells/ml. The suspension was transferred into 1ml cryovials and placed in an Isopropanol-containing cryocontainer at -80°C overnight, before being stored in liquid nitrogen.

2.2.1.4 Thawing Frozen Cells from storage

Cells were removed from storage and placed in a 37°C water-bath until thawed. Immediately after thawing, the cells were transferred into a sterile centrifuge tube and 5ml of cell growth media was added drop-wise, mixing well after each addition. Cells were then centrifuged at 300xg for 5 minutes. The cell pellet was resuspended in the appropriate cell growth media, transferred to the appropriate tissue culture flask and incubated at 37°C as described above (section 2.2.1.1).

2.2.1.5 Coating wells with Fibronectin

Tissue culture plastic surfaces were saturated by addition of the appropriate volume of 5µg/ml fibronectin (FN) in 50mM Tris-HCl, pH 7.4 to each well and subsequent incubation at 4°C overnight. 96-well plates were coated with 100µl whereas with 24-well plates 0.5ml of fibronectin solution was used. Coated surfaces were blocked with

3% (w/v) BSA in the same Tris-HCl buffer for 1 hour at room temperature and washed three times with DMEM prior to the addition of cells.

2.2.1.6 Isolation of TG2-transfected CT26 and human foreskin dermal fibroblasts (HFDF)

Human neonatal foreskin was kindly contributed by the Nottingham City Hospital. Skin was initially incubated for 30 minutes in 15ml DMEM containing 5% (v/v) Dettol for 5 minutes. It was then cut into 5mm square pieces and incubated in DMEM, 100U/ml penicillin and 100μg/ml streptomysin, 0.2% (w/v) dispase (Gibco) at 4°C overnight. Following incubation, the epidermis was peeled off from the dermis and the latter was cut further into 1-2mm pieces using a scalpel in a rocking motion. 2-3 of the resulting pieces were placed in each well of a 6-well plate together with 0.2ml of fully supplemented DMEM containing 10% (v/v) FCS and covered gently with glass coverslips (BDH, Poole, Dorset, UK). A further 1.5ml of growth medium was added to the wells, and after 7-10 days, once fibroblasts have detached from the tissue and reached 40% confluency, they were trypsinised and transferred to a 75cm^2 cell culture flask. Cells were maintained in growth media and passaged up to P20. Isolation of CT26 (transfected with active and inactive TG2 constructs) from balb/c hosts was carried out in a similar fashion. Following three rounds of passaging, isolated tumour cells were cultured in standard cell growth media supplemented with 400μg/ml G418.

2.2.1.7 Viability, apoptosis and cell number assays

2.2.1.7.1 XTT assay

Cell viability was measured by means of orange formazan dye production following cleavage of the yellow tetrazolium salt XTT (2,3-bis[methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide) by metabolic active cells as outlined previously (Scudiero *et al.*, 1988). Cells were seeded in triplicate at a 10^4 cells per 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. 50µl of the XTT reagent mixture (Roche Diagnostics, East Sussex, UK), prepared to a final concentration of 0.3 mg/ml, was added to each well and cultures were incubated for a further 4 hours at 37°C, 5% (v/v) CO_2. Reduction of the yellow tetrazolium salt XTT produced by metabolically active cells was measured by means of absorbance of the orange formazan dye which was read at 492nm using a SpectraFluor 96 well plate reader.

2.2.1.7.2 Lactate Dehydrogenase assay

Lactate dehydrogenase (LDH) release was measured using the Cytotox 96 non-radioactive cytotoxicity assay kit (Promega, Southampton, UK). LDH was measured via a 30 minute coupled enzymatic assay that results in the conversion of a tetrazolium salt into a red formazan product. Lactate and NAD^+ in the presence of lactate dehydrogenase forms pyruvate and NADH. NADH and the tetrazolium salt in the presence of diaphorase forms NAD^+ and formazan (red). The amount of colour is proportional to the number of lysed cells. In brief, cells were seeded at a density of 1 x 10^3 cells/well in a 96-well microtiter plate in two sets of triplicates and allowed to grow overnight at 37°C, 5% (v/v) CO_2. Following incubation, 10µl of lysis buffer [0.9% (v/v) Triton X-100] was added to one set of triplicates. Cells were allowed to lyse for 45 minutes at 37 °C. The plate was then centrifuged for 15 minutes at 500xg and 50µl were removed from each well and placed in a new 96-well plate. 50µl of assay substrate (lactate, NAD and tetrazolium salt) was added to each well and the plate was incubated in the dark for 30 minutes at room temperature. Following incubation the reaction was stopped by addition of 50µl of 1M acetic acid and the colour of the resulting mix was recorded at 492nm on SpectraFluor 96 well plate reader. After subtraction of the background absorbance from the cell-free wells, the viability of cells was expressed as a percentage of LDH released in the cell culture medium.

2.2.1.7.3 Caspase-3 activity assay

Programmed cell death due to exogenous TG2 administration to CT26 cells or endogenous TG2 overexpression in TG2-transfected CT26 clones was assessed using the commercially available Caspace™ Assay system (Promega, Southampton, UK) according to the manufacturer's protocol. CT26 cells were seeded on T25 tissue culture flasks at a density of $2x10^6$ cells/flask and allowed to proliferate overnight. The apoptotic index of the cell line was measured by the ability of caspace-3 to cleave Ac-DEVD-p-nitroaniline substrate and release a chromophore, p-nitroaniline (pNA). Apoptosis in the positive controls was induced using 1μM of the protein kinase inhibitor staurosporine, whereas irreversible and cell-permeable pan-caspace inhibitor Z-VAD-FMK was added to a final concentration of 50μM in the negative control cultures together with 1μM staurosporine.

2.2.1.7.4 Picogreen dsDNA determination

Quantitation of dsDNA was performed using the PicoGreen quantitation reagent kit (Molecular Probes, Eugene, USA) according to the manufacturer's protocol as a value normalisation index. This assay enables detection of as little as 25pg/ml of *ds*DNA. Briefly, a 15μl aliquot was loaded on a microtiter 96-well plate in triplicate together with λDNA standards ranging between 0-1ng/ml. DNA standards were compensated with 15μl of extraction buffer. Measuring DNA concentration was achieved by determination of the Excitation at 480nm and emission at 520nm using a SpectraFluor 96-well plate reader and subsequent reference to the standard curve.

2.2.1.8 Stable Cell transfections

2.2.1.8.1 Killing Curves

CT26 cells were seeded in 6-well tissue culture plates at 10^5 cells/well and maintained in standard cell growth medium containing 10% (v/v) FCS. Once cells reached 60% confluency, they were exposed to a range of G418 concentrations (0, 100, 200, 400,

800, and 1000μg/ml). Cells were observed daily in order to identify the minimum concentration of G418 that induced 100% cell death. Following a 3-4 day incubation cells were washed twice with PBS to remove non-adherent dead cells, trypsinised and counted on a haemocytometer.

2.2.1.8.2 Nucleofections using AMAXA's proprietary technology

Transfection of CT26 mouse colon carcinoma cells with pSV-Neo and pSG5 expression vector containing wild type and mutant ($Cys_{277}Ser$) TG2 cDNA was achieved by co-transfecting cells with 5μg of TG2-containing vector and 0.5μg selection pSV-Neo plasmid (ratio 10:1 respectively) using the AMAXA nucleofector (AMAXA Biosystems) according to the manufacturer's protocol.

Cells (10^6) were seeded in T150 tissue culture flask and allowed to reach 90% confluence just prior to the DNA transfer. Cells were trypsinised, pelleted and resuspended in 100μl of Normal Human Dermal Fibroblasts (NHDF) nucleofector solution at 10^6cells/tranfection. The resulting cell suspension was then mixed with 5μg pSG5-TG2/ 0.5μg pSV-Neo, 5μg pSG5-$TG2_{Cys277Ser}$/0.5μg pSV-Neo or 0.5μg pSV-Neo for the control clones, and transferred to an AMAXA certified cuvette. Nuclear transfer of DNA was achieved by placing the cuvette into the AMAXA nucleofector system and exposing the cells to short electric pulses of different voltage/duration under different pre-set programmes. Cells were then supplemented with 500μl of pre-warmed cell growth media and transferred to 6-well plates also containing 2ml pre-warmed media.

2.2.1.8.2.1 Assessing nucleofection efficiency by confocal microscopy

Transfected clones were trypsinised 16 hours post-transfection, and 10,000 cells were spotted on ringed microscope slides (BDH, Poole, Dorset, UK). Cells were air dried for 15 minutes at room temperature, washed once in PBS, and fixed in 3.7% (w/v)

paraformaldehyde in PBS for 15 minutes. Slides were mounted in Vectashield mounting medium (Vecta Laboratories, Peterborough, UK) and viewed under the reflection (total number of cells), and FITC (GFP expressing cells) filters of a laser TCSNT confocal microscope. Transfection efficiency was estimated as a percentage of the total cells expressing green fluorescent protein.

2..2.1.8.3 Lipofectamine based episomal non-integrating transfections

Cells (0.5 x 10^6) were seeded in 6-cm tissue culture petri dishes and allowed to reach 70% confluence prior to the DNA transfer. Cells were then transfected with 5μg pSG5-TG2/ 0.5μg pSV-Neo, 5μg pSG5-$TG2_{Cys277Ser}$/0.5μg pSV-Neo and 0.5μg pSV-Neo for the control clones complexed with cationic lipid lipofectamine 2000 (Invitrogen) at different w/w ratios (1/3, 1:1, 1:2) according to the manufacturers protocol. DNA was then allowed to integrate episomally into the cells for 6 hours at 37 °C, 5 % CO_2. Cells were screened for transgene expression 48 hours post-transfection.

2.2.1.8.3.1 Assessing lipofectamine transfection efficiency using β-galactosidase assay

Transfected cells were washed twice in PBS, and fixed for 15 minutes in 2ml of 0.25 % (v/v) glutaraldehyde in PBS. Cells were then rinsed gently three times with PBS, making sure that residual glutaraldehyde that could inhibit β-galactosidase activity is removed. β-galactosidase activity was detected by incubation with 1ml 0.2% (w/v) X-gal, 2mM $MgCl_2$, 5mM $K_4Fe(CN)_6$ $3H_2O$, 5mM $K_3Fe(CN)_6$ for 6 hours at 37°C, 5 % (v/v) CO_2. Following incubation, the X-gal solution was removed and the cells were washed in PBS and viewed under a light microscope. Pictures were captured using an Olympus DP10 microscope digital camera. Transfection efficiency was estimated as the percentage of total cells stained blue, due to the expression of X-gal.

2..2.1.8.4 Selection of stably transfected cell lines

Once transfected, cells were maintained in 800µg/ml G418 until clones of cells became visible. After 1-2 weeks, when clones had reached 2-3 mm in diameter, they were washed briefly to remove floating cell debris taking care not to dislodge and hence re-arrange the position of the clones on the tissue culture plate. The clones were then rapidly trypsinised individually by addition of 5µl of 0.5% (w/v) trypsin in PBS, pH 7.4 directly onto the chosen colony for 30 seconds, pipetting the trypsin solution up and down several times. To avoid desiccation this process of washing and trypsinising was carried out separately for each clone. The cell suspension was then transferred into T75 tissue culture flasks, were they were maintained in cell growth medium containing 800µg/ml G418. The clones were grown until sufficient numbers of cells were obtained for liquid nitrogen storage. Once clones were screened for the expression of the transfected DNA the selected clones were routinely cultured in standard growth media supplemented with 400µg/ml G418.

2.2.2 Molecular Biology techniques

2.2.2.1 Transfections of mammalian cells

2.2.2.1.1 Bacterial transformation

100ng of plasmid DNA was mixed with 100µl of DH5a supercompetent Escherichia coli cells (Gibco, Life technologies, Paisley,UK) and stored on ice for 30 minutes. Uptake of the DNA from the cells was induced by subjecting cells to a heat shock at 42°C for 45 seconds prior to storage on ice for 2 minutes. Cells were mixed with 900µl Luria Bertani (LB) pH 7.5 [1% bacto-tryptone, 0.5% (w/v) 0.5% yeast extract and 1% (w/v) NaCl in H_2O] and were incubated for 1 hour at 37°C with shaking at 225 rpm. Cell suspensions were then centrifuged at 1300 rpm for 1 minute, the resulting pellet

was resuspended in 200µl of LB and plated on LB agar plates containing 75µg/ml ampicillin (for pSG5, pSVNeo) or 50µg/ml kanamycin (for eGFPN1). Plates were incubated overnight at 37°C, and the following day a single colony was used to inoculate 50ml of LB containing 75µg/ml ampicillin. Bacterial cells were then allowed to grow exponentially in an incubator/shaker at 37°C/250rpm for up to 16 hours.

2.2.2.1.2 Plasmid preparation

Bacterial cells were harvested by centrifugation at 6,000xg for 15 minutes at 4°C. Plasmid DNA was extracted from centrifuged cells using a Qiagen plasmid midi-PREP kit (Qiagen, West Sussex, UK) according to the manufacturer's protocol.

2.2.2.1.3 Determination of DNA and RNA concentration and purity by UV spectrophotometry

Samples were diluted 1/100 in distilled water, loaded on quartz micro-cuvettes, and readings were recorded at 260 and 280nm. RNA purity was assessed by the ratio of A_{260nm} /A_{280nm}. Bearing in mind that 1 A_{260} unit corresponds to 50µg/ml, the concentration of RNA was determined using the following formula:

DNA] (µg/ml) = A_{260nm} x 50 x dilution factor. DNA solutions with a A_{260nm} /A_{280nm} ratio $\geq$1.7 were deemed free of phenol, and proteins, and hence suitable for transfections.

2.2.2.1.4 DNA enrichment

DNA solutions with a A_{260} /A_{280} ratio $\leq$1.7 were mixed with equal volume of phenol-chloroform-isoamyl alcohol with respective ratios of 25:24:1 (Sigma), and centrifuged at 1300 rpm for 1 minute. The upper DNA layer was then transferred to a fresh tube and mixed with 0.25 volumes of 10M ammonium acetate and 2.5 volumes of ice cold

ethanol, and stored at -20°C overnight. DNA pellet was obtained by centrifugation at 13000rpm for 20 minutes, and then washed in 70% ethanol, air dried, and resuspended in the appropriate volume of 10mM Tris-HCl, pH 7.4, 1mM EDTA (TE).

2.2.2.1.5 Restriction enzyme digestion of DNA

DNA plasmid was digested by mixing 3µg of DNA, 2µl of appropriate restriction enzyme buffer (as recommended by manufacturer), 0.5µl (10U/µl) of restriction enzyme (EcoRI, NotI or Hind III as appropriate), 0.2 µl of 100x BSA made up to a final volume of 20µl with distilled H_2O. EcoRI was used to digest pSV-βgal, pSG5-TG2 and pSG5-$TG2_{Cys277Ser}$ plasmids, whereas EcoRI and HindIII were used for the digestion of pSV-Neo. Digestion was allowed to take place in a water bath at 37°C for 1 hour.

2.2.2.1.6 Agarose gel electrophoresis of DNA

Agarose gels were prepared by dissolving 1g 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 0.5mg/ml, and the gels was cast in a Bio-Rad DNA-Sub electrophoresis tray (Bio-Rad, Watford, Hertfordshire, UK) and allowed to set for 50 minutes. DNA samples were supplemented with 10x DNA loading buffer [20% (v/v) ficoll 400, 100mM 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.

2.2.2.2 Northern analysis of gene expression

2.2.2.2.1 RNA extraction

Cells were seeded on T25 flasks at a concentration of 5 x 10^4 cells/ flask. Cells were treated daily with 50μg/ml of either active (50U/ml) *gpl*TG2, or *gpl*TG2 inactivated by pre-incubation at 4°C and for 6 hours with 500μM of the active site inhibitor NTU283, a (2-[(2-oxopropyl)thio]imidazolium derivative (Freound *et al.*, 1994). Following inactivation, NTU283 was removed from the inactive TG2 solution by dialysis against PBS, 2mM EDTA as outlined in section 2.2.4.5. Cells were trypsinised, counted on a haemocytometer and transferred to 1.5ml eppendorfs where they were washed twice in PBS. Cells were then lysed in 1ml TRI Reagent /5 x 10^6 cells, and total RNA was extracted in the same guanidine thiocynate-based lysis buffer (Sigma) as described before (Chomczynski *et al.*, 1987). 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.

Absorbance of RNA samples at 260nm and 280nm was used to assess the concentration and purity of the RNA extracted in a similar manner as for DNA (section 2.2.2.1.3). Total RNA solutions with an A_{260nm} /A_{280nm} ratio ≥1.7 were deemed free of phenol, DNA and proteins. The RNA yield for C378 fibroblasts following this procedure was estimated at 7μg/10^6 cells.

2.2.2.2.2 Denaturing agarose gel electrophoresis of total RNA

RNA (20μg) were electrophoresed on a 1.2% (w/v) ultra pure agarose/1x morpholinepropane-sulfonic acid (MOPS)/ 2% (v/v) formaldehyde gel. RNA samples were prepared by combining 20μg of RNA with 25μl of RNA sample buffer [containing

80% w/v deionised formamide, 8% (v/v) formaldehyde, 50µl 10x MOPS buffer, 38µg/ml ethidium bromide, 3.5% (v/v) Ficoll, 10mM EDTA, 0.05% (w/v) bromophenol blue] and heated at 65°C for 15 minutes. Prepared RNA was pipetted into the sample wells and electrophoresis was performed at 90V 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 and 1.9kb respectively with an intensity ratio of 2:1.

2.2.2.2.3 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 (3M 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 3MM paper and approximately 10cm 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).

2.2.2.2.4 Random priming of cDNA probes

Specific random primed DNA probes were labeled from the following DNA sequences: collagen (a1) I and rat collagen (a1) III (Virolainen *et al.*, 1995), human collagen (a1)

IV (Kurkinen *et al.*, 1987), rat gelatinase A (MMP2) (Uria *et al.*, 1994), and TIMP1 (Iredale *et al.*, 1996). Each cDNA was kindly supplied in a suitable vector by the relevant author. Purified cDNA (12.5ng) 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 minutes and kept on ice to prevent re-annealing prior to addition to the hybridisation solution.

2.2.2.2.5 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 labeled 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. Northern blots were quantified using Phoretix 1D image analysis software (NonLinear Dynamics).

2.2.3 Analysis of proteins

2.2.3.1 Cell and tissue homogenisation

Cells were harvested and spun down into a pellet prior to resuspending in homogenisation buffer [0.25M sucrose, 5mM Tris-HCl (pH7.4), 2mM EDTA], containing protease inhibitors phenyl methyl sulfonyl fluoride (PMSF, 20μg/ml), leupeptin (10μg/ml), pepstatin (1μg/ml), and benzamidine (780μg/ml). Cells were then sonicated using a Soniprep 150 sonicator (MSE, UK) on ice for 3x5 seconds with 15 second cooling intervals. Tissues were homogenised to 20% (w/v) homogenate in the same homogenisation buffer using a pestle and mortar.

2.2.3.2 Protein content estimation

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

2.2.3.2.1 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). Extracted proteins were precipitated and delipidated at the same time by addition of a mixture of ice cold acetone, tri-n-butyl-phosphate in a 1:1:12 ratio (Mastro *et al.*, 1999), followed by storage at -70°C for 45 minutes, and subsequent centrifugation at 13,000g for 10 minutes. Pellets were sequentially washed in tri-n-butyl-phosphate, acetone, and methanol and reconstituted in 1% (w/v) SDS. 5μl of protein suspension was mixed with 200μl 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 25:1 ratio. The values of the protein concentrations were estimated against a parabolic standard curve produced by triplicate BSA protein standards (0.15-10mg/ml). Absorbance 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 562nm.

2.2.3.2.2 Lowry protein assay

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

2.2.3.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The method employed was a modification of that described by Laemmli for use with vertical slab gel apparatus (Laemmli, 1970). Gels were cast in the Atto-minigel system (B & L Systems, Marseen, The Netherlands) and consisted of a standard 3% (w/v) polyacrylamide stacking gel and a 6-10% (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 [0.25M Tris, 0.2% (w/v) SDS) whereas resolving gels contained a Tris-SDS solution pH 8.8 [0.75M Tris, 0.2% (w/v) SDS]. Concentrations of polyacrylamide in the resolving gel were varied from 6% for separation of proteins with high MW, to 10% for separation of smaller MW proteins. The recipes for different concentrations of acrylamide in the resolving gel are listed in table 2.2.3.3. Resolving gels (80 x 60x 0.75mm) were cast using the Atto-system according to the manufacturer's protocol.

Table 2.2.3.3. Recipes for different concentrations of polyacrylamide separating gels

	% Acrylamide (w/v)		
	6%	7.5%	10%
30% (w/v) acrylamide	3ml	3.75ml	5ml
Tris-SDS, pH 8.8	3.75ml	3.75ml	3.75ml
distilled H_2O	8.25ml	7.75ml	6.25ml
10% (w/v) ammonium persulpha	0.05ml	0.05ml	0.05ml
TEMED	0.1ml	0.01ml	0.01ml

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 0.65ml of acrylamide solution, 1.25ml of Tris/SDS pH 6.8 and 3.05 ml of distilled water. Polymerisation was initiated by the addition of 25µl 10% (w/v) ammonium persulphate and 5µl of TEMED. 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 [0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS]. Samples volumes to be loaded were normalised against protein concentration, values, and 15-20µg of protein was combined with the

appropriate volume of 2x strength Laemmli loading buffer [125mM 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 electrophoresis tips (Bio-Rad). Electrophoresis was performed at 150V for approximately 1.5 hours until the bromophenol blue marker dye had escaped from the bottom of the gel.

2.2.3.4 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 Systems (Biorad, Hempsted, UK) wet-blot system. SDS was removed from the gel by washing in continuous transfer Buffer [48.8mM Tris-HCl, 39mM glycine, 20% (v/v) methanol] for 10 min, and 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 with 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 with a glass vial. Western blotting was carried out using the Biorad apparatus, either overnight at 30mA, or for 1 hour at 200mA in pre-chilled transfer buffer.

Once transfer was completed, the equipment was dissembled and the nitrocellulose membrane was separated from the gel and placed in a plastic tray. 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 destaining in distilled water. The stain was removed during the following blocking step. Washed blots were 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 (Sigma) for 2 hours and at room temperature, before being immunoprobed with the appropriate primary antibody.

2.2.3.5 Immunoprobing of Western blots and membrane stripping

Following blocking, the blots were first incubated with the primary antibody in blocking buffer for 2 hours and at room temperature. Blots were then washed three times in PBS 0.5% (v/v) Tween20, 1% (w/v) milk for approximately five 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 substrate that resulted from the mixture of 2ml of reagent A with 2 ml of reagent B (ECL, Amersham, Bucks, UK). Excess of substrate was blotted against tissue 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 minutes, depending on the dilutions of the antibodies and the intensity of the signal. 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.

Where further immunoprobing was deemed necessary, 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 reblocked in PBS 0.5 % (v/v) Tween20, 1% (w/v) dried milk for 45 minute.

2.2.3.6 Measurement of TG2 antigen

2.2.3.6.1 Detection of cell surface TG2 by ELISA

Cells were seeded at a concentration of 1.5×10^4 cells/well in a 96-well plate and grown overnight. The primary anti-TG2 antibody Cub7402 (Neomarkers) was diluted 1 in 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 in 1000 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 diaminobenzidine (DAB, Sigma) according to the manufacturer's instructions and incubation for 10 minutes at room temperature. The reaction was stopped by washing twice with PBS.

2.2.3.6.2 Detection of cell surface TG2 by Flow cytometry

For flow cytometry analysis, TG2-transfected CT26 mouse colon carcinoma cells were grown to sub-confluence in 6-cm tissue culture petri dishes. Cells were detached from the tissue culture dishes with 2mM EDTA in PBS, pH 7.4 and, following cell number determination, were centrifuged at 300xg and resuspended to a concentration of $2x10^6$ cells/ml in serum free DMEM. Live cells (0.5ml) were stained for cell surface TG2 antigen by addition of 3µg/ml of either an anti-TG2 MAb (Cub7402, Neomarkers) or non-immune mouse IgG 1K (Sigma) with gentle shaking for 1.5 hours at 4°C. Following incubation, the cells were centrifuged at 300xg, washed in serum free medium, and centrifuged again as before. Cell pellets were then resuspended in 0.5ml of serum free DMEM containing 3µg/ml FITC-labelled mouse IgG and incubated for 1 hour at 4°C with gentle shaking. Cells were pelleted once more, washed in serum free DMEM, centrifuged and fixed in 0.5ml 0.5% (w/v) formaldehyde, and analysed for the presence of cell surface TG2 in a DAKO Galaxy flow cytometer (DAKO, UK). Scattering images were further analysed using the WinDMI application.

2.2.3.6.3 Detection of TG2 secreted into the media

TG2-transfected CT26 cells were grown in complete growth media on 6-cm tissue culture petri dishes until they reached 90% confluence. At that point, serum containing medium was removed and circulating TG2 was allowed to accumulate in 2ml serum-free DMEM for 8 hours. Following incubation, the medium was collected, and centrifuged at 300xg to remove any dislodged cells. The protein load of 1.2ml of the medium was then precipitated in 0.3ml ice cold 50% (w/v) TCA (final concentration of 10% TCA) by placing the samples on ice for 30 minutes, followed by centrifugation for 10 minutes at 13000xg. The protein pellets were washed sequentially with 0.5ml of ice cold 10% (w/v) TCA, 0.5ml ethanol-acetone (ratio 1:1) and acetone, and air dried for 20 minutes at room temperature. Protein pellets were finally resuspended in 30µl 2x Laemmli buffer and the presence of TG2 in the pellet was detected by SDS-PAGE followed by Western blotting as described before (section 2.2.3).

2.2.3.6.4 Detection of ECM-deposited TG2 by confocal microscopy

Cells were seeded at a concentration of 10^5 cells/well in 8-well glass chamber slides (Lab-Tek, Naperville, USA) and grown over 48 hour 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 in a coplin jar for 15 minutes at room temperature. Cell monolayers were then blocked in 3% (w/v) BSA in PBS, pH 7.4 for 30 minutes at 37°C. Following blocking, cells were incubated with anti-mouse IgG-FITC (DAKO) diluted 1 in 100 in blocking buffer for 2 hours at 37°C and then washed 3 times with PBS, prior to mounting with Vectashield mounting medium (Vecta Laboratories). Stained cells were viewed under confocal fluorescent microscopy using a Leica TCSNT confocal laser microscope system (LEICA, Germany) equipped with an argon crypton laser at 488nm for fluorescein excitation.

2.2.3.7 Measurement of TG2 activity

2.2.3.7.1 Measurement of TG2 activity by incorporation of [^{14}C]-putrescine into N,N'-dimethylcasein

This assay relies on the ability of the enzyme to catalyse the incorporation of a radioactively labeled primary amine into a protein acceptor substrate (Lorand, 1972). At 30 second intervals, 45µl of sample was added to 45µl reaction mix containing 10µl 50mM Tris-HCl pH 7.4, 10µl 38.5mM DTT, 10µl 12mM [1,4-^{14}C]-putrescine (Amersham Pharmacia; specific activity 3.97mCi/mmol), 20µl 25mg/ml N,N'-dimethylcasein (Sigma) in 50mM Tris and either 5µl 50mM $CaCl_2$ or 5µl 200mM EDTA pre-equilibrated at 37°C in a waterbath. At 15 minutes after the initiation of the reaction triplicate 10µl aliquots from each sample were removed and spotted onto 10mm^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. Filter papers were then air dried for 15 minutes. Once dry, filter papers were placed in scintillation tubes, to which 2ml Optiphase High Safe liquid scintillation fluid was added, and ^{14}C counts were obtained in a Packard Liquid Scintillator (Packard Biosciences).

2.2.3.7.2 Measurement of cell surface TG2 activity by biotin-cadaverine incorporation into fibronectin

Transglutaminase activity associated with the cell surface was measured by the incorporation of biotin-cadaverine into fibronectin (FN) as described previously (Jones *et al.*, 1997). 96-well plates were coated with 5µg/ml FN in 100µl of 50mM Tris-HCl, pH 7.4 and blocked with 3% (w/v) BSA in 50mM Tris-HCl, pH 7.4 for1 hour at room temperature. Cells were trypsinised, counted and pelleted by centrifugation, before

resuspension at a concentration of $2x10^5$ cells/ml in DMEM containing 0.132mM biotin-X-cadaverine (Molecular Probes, Oregon, USA). 100µl of the cell suspension was added to the FN-precoated 96-well plates and incubated for 2hours at 37°C, 5% (v/v) CO_2. Positive and negative control samples were also included, which consisted of 100ng/well *gpl*TG2 that was incubated in a reaction mixture consisting of 0.1M Tris buffer pH 7.4, 10mM DTT, 0.132mM biotin-cadaverine and either 10mM $CaCl_2$ (positive control) or 10mM EDTA (negative control). Cells and enzyme were incubated for 1 hour at 37°C and then washed twice with PBS pH 7.4 containing 3mM EDTA to stop incorporation. Cells were then lifted in 100µl of 0.1% (w/v) deoxycholate, 2mM EDTA, in PBS for 10 minutes at room temperature. 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 for 30 minutes at 37°C. Biotin-cadaverine incorporation into FN was detected by incubation for hour at 37°C with an extravidin peroxidase conjugate diluted 1:000 in blocking buffer. Colour development was achieved 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% 3,3',5,5'-tetramethylbenzidine (TMB; Sigma). The reaction was stopped by the addition of 2.5N H_2SO_4 and the resultant colour was read in an SpectraFluor ELISA plate reader at 450nm.

2.2.3.7.3 Measurement of TG2 activity by biotin-cadaverine incorporation into N,N'-dimethyl casein

96-well plates were coated with 250µl of 100mg/ml N,N'-dimethylcasein in 100mM Tris-HCl pH 8.5 for 16 hours at 4°C.The plates were then washed twice with PBS, 0.05 % (v/v) Tween20 and twice in distilled H_2O, and blocked with 250µl of 3% (w/v) BSA in 100mM Tris-HCl, pH 8.5 for 30 minutes at room temperature. Plates were once again washed 3 times with PBS, 0.05 % (v/v) Tween20 and once with 100mM Tris-HCl pH 8.5. The reaction was initiated by addition of 50µl of transglutaminase-containing solution and 150µl of 0.1mg/ml biotin-cadaverine in 100mM Tris-HCl pH 8.5, 13.3mM DTT, supplemented with 6.67mM $CaCl_2$ (positive control), or with

330μM EDTA (negative control). The plates were then incubated at 37°C for 30 minutes, and the reaction was terminated by washing as described above. Biotin-cadaverine incorporation into N,N'-dimethyl casein was detected by incubation for 1 hour at 37°C with 200μl of an extravidin peroxidase conjugate diluted 1:5000 in blocking buffer. Colour development and quantification was achieved as described previously (section 2.2.3.7.2).

2.2.3.8 Total extracellular matrix and collagen turnover assays

Single C378 and HUVEC cell-type cultures were seeded at a concentration of $5x10^5$ cells/well into 24-well plates in 0.5ml of the appropriate media (section 2.2.1.1) and labelled the following day in the presence of fully supplemented DMEM containing 5μCi/ml of [2,3-^{3}H]-Proline (Amersham, Buckinghamshire, UK) for 48 hours. The radiolabeled medium was then removed and following three washes in PBS cells were treated daily and for two times (t=0 and t=12 hours) with increasing concentrations (10, 20, and 50μg/ml) of active *gpl*TG2 (10, 25 and 50U/ml respectively) in label free media.

Angiogenesis co-cultures (TCS Cellworks), on the other hand, were labelled on day 2 upon arrival of the kit in the presence of fully supplemented HUVEC media containing 5μCi/ml of either [2,3-^{3}H]-Proline or [2,3-^{3}H]-amino acid mixture for collagen or total ECM deposition studies respectively. Following incubation for 48 hours, the radiolabeled medium was then removed and following three washes in PBS cells were treated with four doses of 50μg/ml active (50U/ml) TG2 in fresh label-free media (0.5ml) at 48, 72, 96 and 120 hours.

In both experimental systems, the amount of the incorporated label was measured at 48, 72, 96 and 120 hours after labeling in two separate fractions: the cell medium fraction and the ECM fraction, as described previously (Gross *et al.*, 2003). Following removal of the media and trees washes in PBS, the cellular fraction was obtained by removing the cells with 150μl of 0.1% (w/v) sodium deoxycholate/2mM EDTA for 10 minutes at room temperature. The cellular fraction was used to measure the *ds*DNA content of

each well, as a means of cell number normalisation. Following a further three washes in PBS, the ECM fraction was obtained by digesting the deoxycholate-insoluble matrix with 100µl of 200µg/ml proteinase K (Sigma) in 50mM Tris-HCl, pH 7.4, 10mM EDTA and 10mM NaCl. Initial ECM extraction was followed by further matrix scraping in 100µl of 4% (w/v) SDS and combination of the two matrix-rich aliquots. The incorporated label was measured by addition of 1ml of Ultima Gold™ scintillation liquid (*Packard Bioscience*, Pangbourne,UK) to 100µl of each separate fraction and consequent counting in a Packard Instruments *Tri-carb 300* scintillation counter for 5 minutes.

2.2.4 Tissue transglutaminase purification

Tissue transglutaminase was purified using a protocol described previously (LeBlanc *et al.*, 1999) with a few modification

2.2.4.1 Liver Homogenisation

5 guinea pigs were sacrificed by neck dislocation and extracted livers (200-250g) were homogenised to a 50% (w/v) homogenate in two steps; first on a bench-top blender in 500ml of 5mM Tris-HCl, 2mM EDTA, pH 7.5, 0.25M sucrose, 5mM benzemidine, 1mM PMSF, and then on a homogeniser. Nuclei and large debris were spun out by centrifugation at 30,000g for 1 hour in a Beckman centrifuge (Beckman, High Wycombe, UK). Supernatant was collected and cellular debris and membranes removed by clarification at 100,000g for 1 hour, using multiple runs in the ultracentrifuge and resulting homogenate was then filtered through Whatman no.1 filter paper (Whatman, Maidstone, UK).

2.2.4.2 Anion Exchange Chromatography

Homogenate (~500ml) was loaded on 300ml of Q-sepharose Fast Flow column (Amersham Pharmacia) and gradient was run in two stages:1) 0-36% for 95 minutes with 1 minute fractions, and 2) 36-72% buffer B for 95 minutes. The mobile phase for this step comprised of 5mM Tris-HCl, 2mM EDTA, pH 7.5 (Buffer A) and Tris-HCl, 2mM EDTA, pH 7.5, 1M NaCl (Buffer B). Following loading of the sample, column was washed through with 1*l* of buffer A until UV absorbance dropped to 0.5units. The column was set up to run at 15ml/min from the Econosystem pump. Proteins were eluted with a linear gradient of 0-720Mm NaCl in buffer A over 190 minutes, collecting 15ml fractions. The transglutaminase specific activity profile revealed by the Biotin-Cadaverine TG2 activity assay and the Lowry protein assay, as described in sections 2.2.3.7.1 and 2.2.3.2.2 respectively, were used to identify the TG2-rich elution peaks.

2.2.4.3 Size exclusion chromatography

TG2-rich fractions collected from anion exchange chromatography were pooled, precipitated by gradual addition of ammonium sulphate to 80% (w/v) on ice for 45 minutes, split in four aliquots, and centrifuged in MSE 24 centrifuge (MSE, Cambridge, UK) at 30,000g for 1 hour. Pellets resulting from each aliquot (equivalent to 25% of the total protein) were reconstituted in 10ml of 50mM Tris-Acetate, 1mM EDTA, 0.16M KCl, pH 6.0 and loaded onto a 2.5x100-cm Sephracryl S-200 gel filtration column (Bio-Gel A-0.5m, fine mesh) at a flow rate of 2ml/min in the same buffer. The elution was conducted overnight using 50mM Tris-acetate buffer, pH 6.0, containing 1mM EDTA and 0.16M KCl for 336 min. 5ml fractions were collected between 67 and 246 min. TG2 emerged from the column after a half-column volume has eluted. All fractions relating to high protein concentrations on the chromatogram, judging by their protein content as measured by the Lowry assay, were tested for specific activity. Fractions with high specific activities were pooled and loaded on a GTP-agarose affinity column.

2.2.4.4 GTP-agarose Gel Filtration

SEC fractions were applied onto a 5ml bed volume GTP agarose column (Sigma, Poole, UK) equilibrated in 50mM Tris-Acetate, 1mM EDTA, 1mM DTT, pH 7.5 and run through at 2ml/min using a peristaltic pump. The flow-through was then reloaded to ensure saturation of the binding sites. The column was then washed with 50ml of the same buffer before elution with 5mM GTP in 50mM Tris-HCl, 1mM EDTA, 1mM DTT, pH 7.5 Resulting 1ml fractions were assayed once again for protein content and TG2 activity using the Lowry and Biotin-Cadaverin activity assays respectively. Purity was verified by SDS-PAGE and compared to that of commercially available *gpl*TG2 (Sigma).

2.2.4.5 Dialysis

Following gel filtration GTP contamination was removed by overnight dialysis with three buffer changes at 4°C in PBS/2mM EDTA using dialysis tubing with a 5kDa MW cut-off (*Perbio Science*, Cheshire, UK).

2.2.5 Animal studies

All animal studies were carried out in accordance with the Home Office Animal Act (1986), under project license number PiL 2414.

2.2.5.1 Sub-cutaneous injection of tumour cell lines

The tumour cell suspension was loaded into a syringe, ensuring all air trapped was removed prior to injection. 100µl of cell suspension ($2x10^4$- $8x10^5$ cells/ml) in DMEM was injected subcutaneously in the right flank of 8 week old balb/c mice using 1ml

syringes equipped with 13mm, 25g Microlance needles (BD Biosciences). Upon needle withdrawal, the puncture hole was held for 5 seconds to allow the cell suspension to disperse thus preventing its release. The animals were monitored for signs of distress every two days throughout the experimental period, and sacrificed once the tumour volume approached the $1cm^2$ limit.

2.2.5.2 Tumour injections

Active and inactive TG2 (50µl of 4mg/ml, 10U/inj) was delivered into the tumour as well as into the stroma/neoplasm interface at multiple sites, avoiding overlapping of the injection tracts. Given the high pressure within the tumour body, the needle was withdrawn as the treatment solution was expelled.

2.2.5.3 Harvesting of primary tumour load

Animals were sacrificed by CO_2 asphyxiation and subsequent dislocation of the neck. Tumour masses were removed surgically, snap-frozen in liquid nitrogen and stored at -80°.

2.2.6 Histochemical techniques

2.2.6.1 Organ and tumour tissue fixation

Lungs and livers of knock-out and wild type mice were extracted and washed briefly in PBS. They were then fixed in 3.7% (w/v) PFM for 2 hours, prior to three further washes in PBS, pH 7.4 and storage at 4 °C in PBS, 20% (w/v) sucrose.

2.2.6.2 Sectioning of tissue

2.2.6.2.1 Paraffin wax embedding and microtome sectioning of lung and liver tissue

Lung and liver organs were brought to room temperature and fixed in 4% (v/v) neutral-buffered formalin formalin in PBS, pH 7.4. Sections were then encased into paraffin wax blocks using the tissue embedding centre (Thermo-Shandon, Basingstoke, UK) according the manufacturer's protocol. Sections were obtained at 5-8μm thickness, attached to Superfrost microscope slides (BDH, Dorset, UK), and stored at -80 °C for further histochemical analysis.

2.2.6.2.2 OCT-embedding and cryostat sectioning of tumour tissue

Snap frozen tumour masses were embedded in OCT compound (Raymond Lamb, East Sussex, UK) at -60°C using the cryobar a cryostat (*Thermo-Shandon*, Pittsburgh, US). The embedded tissue was allowed to warm to –14°C and sectioned at 10μm increments. Sections were attached onto BDH Gold microscope slides (*BDH*, Milton Keynes, UK) dried for 10 minutes and stored at -80°C.

2.2.6.3 Histochemical staining of tumour and organ sections

2.2.6.3.1 Haematoxylin and Eosin Staining (H & E)

Cryostat sections of OCT-embedded tumour specimens or microtome sections of wax-embedded organ specimens were fixed in 10% (v/v) neutral buffered formalin at room temperature for 20 seconds. They were then rinsed in tap water for 15 seconds and stained in double strength *Carazzi's* haematoxylin for one minute. Sections were once again rinsed in tap water for 15 seconds, before being stained in 1% (w/v) aqueous eosin for 10 seconds. Following eiosin staining sections were rinsed in tap water for 15 seconds and dehydrated through increasing alcohol concentrations. Slides were then

mounted in DPX mounting medium (Sigma) and allowed to air-dry. After staining, the slides were viewed under a light microscope (Ziess, Axioskop). Following H & E staining the nuclei appear blue, whereas the cytoplasm is stained pink.

2.2.6.3.2 Massons Trichrome stain for total collagens

Collagen fibres were stained using Accustain Trichrome Stain (Sigma Diagnostics) according to the manufacturer's protocol. The procedure carried out as described before (Gomori, 1950) with a few modifications, is a one step system combining the cytoplasmic and connective-fibres stain in a phosphotungstic acid/acetic acid solution. Following staining, the slides were viewed under a light microscope (Ziess, Axioskop). Nuclei appear blue/black, the cytoplasm, muscles and red blood cells appear red, whereas collagen fibres appear blue/green.

2.2.6.4 Immunohistochemical staining of cryostat sections

Endogenous peroxidase of tissue section was quenched with 3% (v/v) H_2O_2 for 5 minutes at room temperature, followed by 3 one-minute washes with PBS. Sections were then ringed using hydrophobic pen (*Dako*), fixed in ice-cold acetone for 15 minutes, before being blocked and permeabilised for 30 minutes at room temperature with 3% (w/v) BSA, 0.05% (v/v) Triton-X in PBS. Sections were then incubated at 37°C for 2 hours with either an mouse anti-TG2 MAb, diluted 1/20 (Cub 7402, Neomarkers), or sheep anti- von Willebrand factor antibody, diluted 1/20 (C-20, Santa Cruz, Autogen, Bioclear, Wiltshire, UK). Staining was revealed by incubation for 2 hours with either anti-mouse TRITC conjugate, diluted 1/50 or an anti-goat IgG-TRITC conjugate diluted 1/50 (Chemicon, Harrow, UK). Negative controls were obtained by omission of the primary antibody and stained sections were mounted in 70% (v/v) glycerol and viewed on a confocal microscope (TCSNT, Leica).

2.2.6.5 Cross-link analysis of tumour homogenates

Tumour specimens were homogenised to yield a 20% (w/v) homogenate in 250mM Sucrose, 2mM EDTA, 5mM Tris-HCl pH 7.4 in the presence of protease inhibitors cocktail (Sigma, Poole Dorset). Samples were then centrifuged at 4,000g at 4 C°, for 15 minutes after the addition of 20% (v/v) trichloroacetic acid (TCA) to a final concentration of 10% (v/v). Protein rich pellets were then washed once in diethyl ether/ethanol (1:1) and another three times with diethyl-ether only and air-dried at room temperature before being resuspended in 50µl of 0.1M $(NH_4)HCO_3$ in the presence of traces of thymol. After resuspension of the pellets by sonication, sequential digestion of proteins was undertaken with a series of proteolytic enzymes (Sigma; 10µg/ml subtlisin, 3x18 hours, at 37°C; 10µg/ml bacterial collagenase, 18 hours, at 37°C; 15µg/ml pronase, 12 hours, at 32°C, 10U leucine aminopeptidase/15U prolidase, 2x10 hours, at 37°C; and 20µg/ml carboxypeptidase, 12 hours, at 30°C), to release the ε(γ-glutamyl) lysine crosslink dipeptide. Samples were freeze-dried and crosslink analysis was carried out by anion exchange chromatography using a LKB 4151 amino acid analyser by a modification of the method previously described using lithium citrate buffers (Griffin and Wilson, 1984). The amount of the ε(γ-glutamyl) lysine crosslink dipeptide was quantified by standard addition using a ε(γ-glutamyl) lysine dipeptide standard as outlined previously.

2.2.9 Statistical analysis

Differences between datasets (expressed as mean ±SD) was determined by the Mann-Whitney or student *t*-test using *Minitab* or *Sigma Stats* packages at a significance level of $p<0.05$.

Chapter 3:

Physiological and therapeutic roles of tissue transglutaminase in solid tumour growth and progression

3.1 Introduction

As described in more detail in the introductory sections 1.9.6.4.1 and 1.9.6.4.2, tumour growth and progression is a cascade of complex, interrelated events. At the early stages, it involves decreased cell-cell contact, cell disengagement from the primary tumour site, degradation of basal membrane through the recruitment of host-cell proteolytic enzymes, and invasion of the surrounding stroma. Changes in cell adhesion and changes in extracellular matrix (ECM) turnover and subsequent loss of cell-cell contact provide a springboard for cellular motility and subsequent cell migration (Fingleton and Matrisisan, 1999). Moreover, proteolytic degradation of the BM is a prerequisite for tumour cell migration and invasion, whereas it also favours host endothelial cell mobilisation during angiogenesis. Critical to the progress of this cascade is also the promotion of new blood vessel formation, through which malignant cells enter into the vasculature system (intravasation), where they roll and arrest at a distant site, prior to extravasation.

Early work by Yancey and Laki (1972) implicated transglutaminases in tumour growth, suggesting that solid tumours require a covalently stabilised fibrin network for proliferative growth. This correlation was disputed later by Birckbichler *et al.* (1976) who reported reduced TG activity in rat hepatomas. For the next three decades subsequent to these initial studies scores of publications have attempted to pinpoint the exact physiological role of tissue transglutaminase in the tumour growth and progression cascades. Several lines of evidence have suggested that TG2 might be a tumour-related marker (Birckbichler and Patterson, 1978 and 1980; Barnes *et al.*, 1985; Knight *et al.*, 1990a; Van Groningen *et al.*, 1995; Hettasch *et al.*, 1996; Jiang *et al.*, 2003). So far, it has been demonstrated that TG2 antigen, TG activity, as well as the number of isopeptide crosslinks catalysed by TGs are reduced in chemically- and virally-transformed neoplastic cells compared to their normal counterparts (Birckbichler and Patterson, 1978 and 1980; Hand *et al.*, 1987; Knight *et al.*, 1990a). Transglutaminase activity in all tested transplantable and certain primary rat sarcomas (Barnes *et al.*, 1985) was also shown to be greatly reduced when compared to the corresponding normal tissue. Reduction of TG activity was accompanied by a redistribution of the enzyme to the particulate fraction

(Hand *et al.*, 1988). It is also worth noting that TG2 has been implicated in the body's natural defense against tumour formation as increased TG2 expression in the tissue surrounding the tumour has been reported (Hettasch *et al.*, 1996). As a continuum to this observation, Grigoriev *et al.* (2001) demonstrated that a mere 15% of the screened breast carcinomas were positive for TG2 antigen as opposed to a 50% of the stromal tissue. Counter to these findings, proteomic analysis indicated that TG2 expression was significantly up-regulated in highly metastatic cell lines when compared to lower metastatic tumour cell lines (Jiang *et al.*, 2003), which is in keeping with the reported increase in TG2 expression in metastatic human melanomas compared to the corresponding normal tissue (Van Groningen *et al.*, 1995).

Contradictory reports have proven it is difficult to establish a role for TG2 in the development of the malignant phenotype. Following quantification of *in vivo* TG expression in a number of tumour tissues (skin, colon, pancreas, stomach, and lung) it has been proposed that TG2 is not a tumour related marker (Takaku *et al.*, 1995), a finding that has been expanded *in vitro* to include several malignant cell lines, which have been shown to express high levels of the enzyme (Denk *et al.*; 1984, Mehta; 1994, Takaku *et al.*; 1995). In agreement with the above findings, Röhn *et al.* (1992) also concluded that TG activity does not represent a biochemical marker of malignancy in human brain tumours following measurement of this activity in a number of adenomas, menigiomas, and gliomas.

In order to clarify the role of TG2 in tumour progression, prior studies within this laboratory sought to overexpress the enzyme in a malignant hamster fibrosarcoma cell line while examining the kinetics of tumour formation when those cells were reintroduced into the animal (Johnson *et al.*, 1994). Following transfection of full length human TG2 cDNA into the Met B cell line, it was reported that TG2 overexpression led to a reduced incidence of primary tumour formation, which was accompanied by an increase in cell adhesion on FN. No differences were noted in the *in vitro* growth rates and cell morphology between TG2-transfected cells and their mock-transfected counterparts. The exact mechanism(s) by which TG2 could bring about loss of tumour growth is still not fully understood. It is postulated that its reported tumouricidal action

might be linked with its involvement in cell death (Piacentini *et al.*, 1999; Autuori *et al.*, 1998), cell adhesion and migration (Verderio *et al.*, 1998), and the stabilisation of the ECM (Barsigian *et al.*, 1991).

In an investigation of the apoptotic capacity of TG2, Johnson *et al.* (1998) demonstrated that induction of TG2 by dexamethasone in the hamster fibrosarcoma cell line BHK-21, led to a dose-dependent increase in TG activity that correlated with transglutaminase-mediated cell death. In keeping with these findings, it has also been demonstrated that overexpression of TG2 in the malignant hamster fibrosarcoma cell line MetB (Johnson *et al.*, 1994) and in the neuroblastoma SK-N-BE(2)-derived cell line (Piacentini *et al.*, 1996) leads to a reduced incidence of tumor development due to TG2-mediated apoptosis.

In favour of a cell-adhesion function for TG2 during tumour development, cells isolated from rat P8 osteosarcomas showed incorporation of labeled primary amine substrates into the ECM, cell surface and BM, suggesting that TG2 is expressed on the cell surface of these tumours, where it encourages cells to attach and commence processing the ECM (Parry, 2003; PhD Thesis). However, when applied exogenously and at non-physiological levels on the human colon carcinoma cell line LS174T, TG was shown to inhibit tumour cell spreading (Zirvi *et al.*, 1993). This observation could be justified as it has also been reported that the tumour suppressor cell adhesion molecule C-CAM is a substrate for TG2, raising the possibility that TG2 may exert a negative effect on tumour growth via covalent modification of this molecule (Hunter *et al.*, 1998).

More convincingly Haroon *et al.* (1999a) demonstrated first that TG2 is expressed as a host response to tumour cell invasion and inhibits tumour growth by stabilising the ECM, through extensive crosslinking of ECM proteins. The same authors reported increased expression of the enzyme in the tumour-host interface and increased levels of cross-links within the tumour host micoenvironment and within the tumour body. Exogenous application of active *r*TG2 in a dorsal skin flap window chamber was also shown to delay tumour growth, whilst increased amounts of collagen were also reported. Previous studies have demonstrated that *in vitro* TG2-mediated crosslinking of collagen and collagen/fibronectin mixtures leads to an increased resistance to their breakdown by

MMP-1 (Johnson *et al.*, 1999). Therefore, it can been hypothesised that the reduction in TG2 expression within the tumour mass (Hand *et al.*, 1987; Knight *et al.*, 1990) leaves the tumour-host ECM more susceptible to degradation by matrix metalloproteinases, thus facilitating tumour cell migration and consequently escape.

The aim of this study was to identify a physiological role for TG2 in the host tissue with respect to its effect on tumour growth. This was undertaken using mice where TG2 had been knocked out ($TG2^{-/-}$) and the respective wild type controls (De Laurenzi and Melino, 2001). Inoculation of B16-F1 mouse melanoma cells in the transgenic mice provides an avenue by which to determine whether lack of endogenous TG2 may lead to altered patterns of tumour growth and animal survival. Moreover, although this cell line is not reported to colonise primary metastatic targets as readily as the B16-F10 variant, it may still prove a useful model by which to study early signs of metastasis in a TG2-free tumour host environment. The secondary aim was to underpin whether TG2 may play a therapeutic role in solid tumours, when delivered into the tumour-host microenvironment at non-physiological levels, and gather evidence in support of an explanatory mechanism. To address this, the CT26 mouse colon carcinoma model was used (Geng *et al.*, 1998), whereby balb/c mice are challenged by subcutaneous CT26 cell injection, prior to intratumour treatment with exogenous *gpl*TG2.

3.2 Methods

3.2.1 Fluorescent cell invasion assay

3.2.1.1 Preparation of artificial collagen-fibronectin matrices

Type I Collagen (*Sigma*) was solubilised overnight at 4°C in 0.2% (v/v) acetic acid, pH 3.0, to a final concentration of 6mg/ml. Fibrillogenesis was initiated by neutralisation of the collagen mixture through addition of 10x DMEM with sodium bicarbonate, pH 7.4 and 0.2M HEPES buffer to final concentrations of 1x and 0.02M respectively. Addition of $CaCl_2$ and DTT to a final concentration of 5mM was made immediately after neutralisation and prior to the addition of *gpl*TG2. The resulting mix was supplemented with 100μg/ml fibronectin and either 50μg/ml active (50U/ml) purified TG2, or 50μg/ml TG2, inactivated by pre-incubation with 500μM of the active site inhibitor NTU283, a (2-[(2-oxopropyl)thio]imidazolium derivative (Freound *et al.*, 1994), for 6 hours at 4°C. Each matrix suspension (100μl) containing approximately 2mg/ml type I collagen was applied per $1cm^2$ surface area of 6μm-pore Fluroblock filters (BD Biosciences, UK) and incubated at 37 °C for 2 hours to polymerise and produce 1mm thick uniform collagen-based gel layers.

3.2.1.2 Fluorescent measurement of cellular invasion through matrix-coated micropore filters

Matrix-coated Fluoroblock membranes (BD Biosciences) were rehydrated in pre-warmed basal culture media for 15 minutes at 37 °C in a CO_2 environment. Medium was then replaced with 10^6 CT26 cells/ml, in DMEM. Culture medium (750μl) containing 10% (v/v) FCS as a chemoattractant was then added to each of the bottom wells. The Fluoroblock insert plates were incubated for 72 hours at 37 °C, in 5% (v/v) CO_2 atmosphere. At the end of the assay inserts were transferred to 24-well glass-bottom

plates (BD Biosciences) containing 4μg/ml calcein AM (Molecular Probes, Eugene, USA) in Hanks Balanced Salt Solution (HBSS; Sigma) and incubated at 37°C at 5% (v/v) CO_2 for 90 minutes. Fluorescence was quantified at 485nm/530nm (excitation/emission) from-bottom-to-top using a SpectraFluor plate reader.

3.2.2 Quantification of OH-proline and total amino acid content by Gas Chromatography

Tumour specimens were homogenised in 250mM Sucrose, 2mM EDTA, 5mM Tris-HCl in the presence of protease inhibitors (1μg/ml pepstatin, 1μg/ml leupeptin, 1mM phenylmethylsulphonyl fluoride) to a final concentration of 200mg/ml (w/v) and sonicated. Following hydrolysis of 50μl homogenates in 6M HCl at 100°C and for 16 hours, and evaporation of remaining liquid from the homogenates at 50°C for 48 hours, pellets were resuspended in 500μl of 50mM Tris pH 8. Amino acids were separated following a solid-phase extraction/derivatisation protocol using the *EZ-Faast* amino acid analysis kit (Phenomenex, UK) according to the manufacturer's instructions. The amino acid and OH-proline content of control and TG2-treated CT26 tumours was measured against internal amino acid standards (5-20nmoles) by Gas Chromatography using a 9890N Network GC system (Agilent Technologies, US). OH-proline content was expressed as percentage of total amino acids.

3.3 Results

3.3.1 B16-F1 melanoma model using TG2 wild-type and knock-out C57BL/6 mice

3.3.1.1 Tumour growth rates and percentage animal survival of B16F1-bearing $TG2^{-/-}$ and $TG2^{+/+}$ mice

B16-F1 melanoma tumour growth rates were compared subsequent to subcutaneous implantation of 10^6 cells/ml into TG2 wild type and knock-out C57BL/6 mice. Animals were monitored for 32 days following tumour cell challenge and sacrificed when tumours approached the $1cm^2$ size limit. The tumour growth rate of B16-F1 cells was found to be significantly higher in the TG2 knock-out animals when compared to their wild-type counterparts (figure 3.3.1.1). Following an initial 4-day growth rate parallel to that of the knock-out animal tumours, TG2 wild-type animals bearing B16-F1 tumours exhibited attenuated growth marked by a characteristic 8-day lag phase. When percentage animal survival for the two tumour-bearing animal populations was assessed, the 8-day tumour growth lag phase observed in wild-type mice was translated into a delay in mortality (figure 3.3.1.1).

3.3.1.2 Characterisation of B16F1-C57BL/6 tumour-host morphology by H & E staining

Having established that tumours growing in TG2 knock-out mice demonstrate accelerated growth rates, we next sought to identify the morphological features within the C57BL/6 tumour-host microenvironment. B16-F1 melanoma tumours were harvested from $TG2^{+/+}$ nice and sectioned prior to staining with Haematoxylin and Eosin (H &E) as described in the Methods (section 2.2.6.3.1). B16-F1 cells exhibited most atypias characteristic of the malignant phenotype, such as enlarged cell size, irregular shape, multipolar mitosis, as well as loss of contact inhibition -indicated by tumour cells growing on multiple layers (figure 3.3.1.2.1). Having identified the histological features of B16-F1 tumour-host interface (nuclei, tumour capsule, muscle fibres, red blood cells) in the wild-type host-tumour interface, we next sought to identify disparate pathological

Figure 3.3.1.1 B16-F1 tumour growth rates in TG2 $^{-/-}$ and TG2 $^{+/+}$ C57BL/6 mice and percentage animal survival

Exponentially growing B16-F1 were harvested and resuspended to a final concentration of 10^6 cells/ml in DMEM, prior to subcutaneous implantation of 100µl cell suspensions into 7-9 weeks old TG2 wild-type and knock-out C57BL/6 mice as outlined in the Methods (section 2.2.5.1). Following injections, animals were monitored every two days for tumour development. **A,** average tumour growth rate of animals, expressed as mean tumour size (cm^2) over time, was measured up to 32 days post-injection. Results represent mean value ±SD (n=8). **B**, percentage animal survival following detection of B16-F1 melanoma tumours. Animals were sacrificed before tumours reached the $1cm^2$ limit. Squares, TG2 $^{+/+}$; triangles, TG2 $^{-/-}$. * Indicates significantly different ($p<0.05$) from the control.

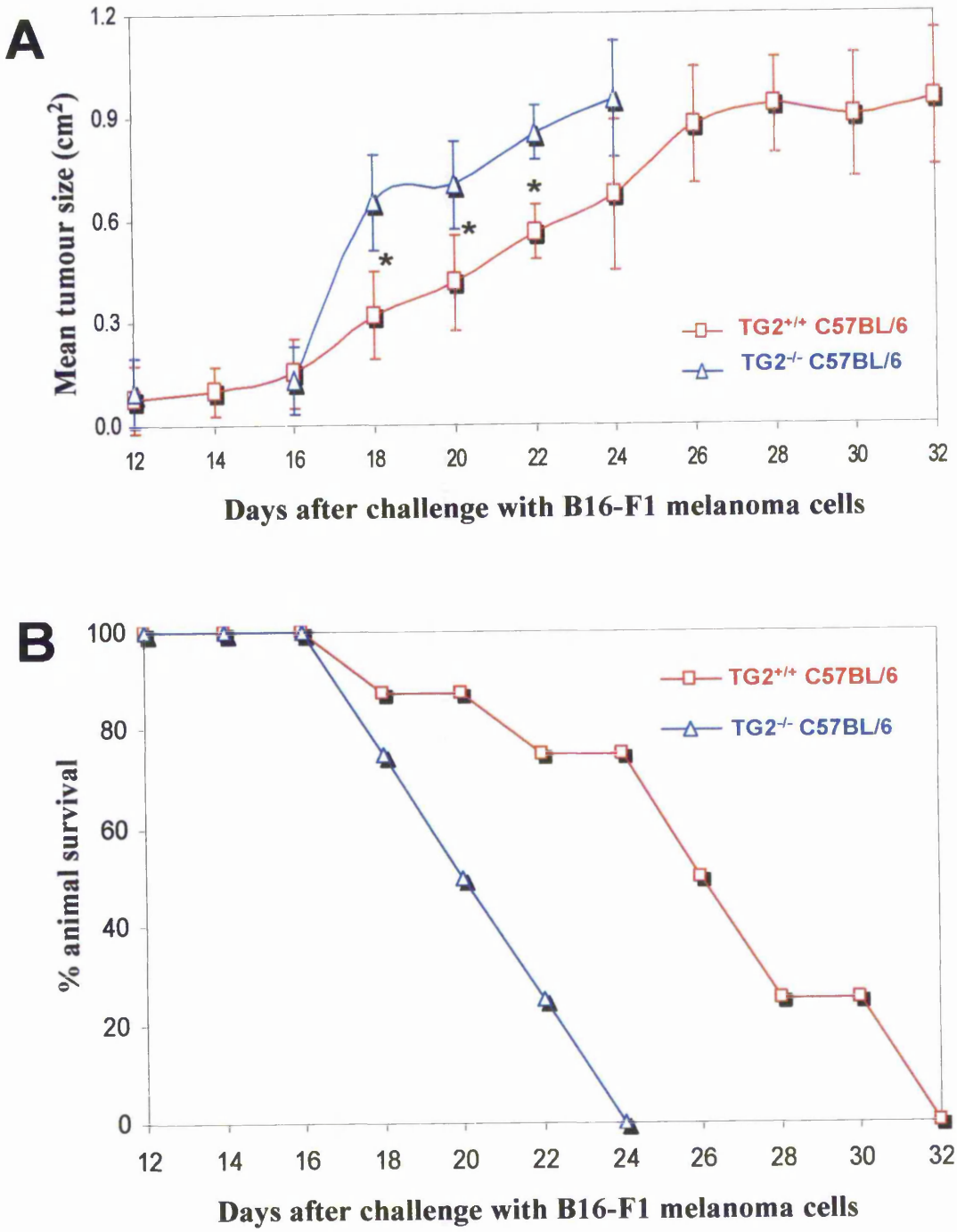


Figure 3.3.1.1 B16-F1 tumour growth rates in TG2 $^{-/-}$ and TG2 $^{+/+}$ C57BL/6 mice and percentage animal survival

Figure 3.3.1.2.1 Characterisation of B16F1-C57BL/6 tumour-host morphology by Haematoxylin and Eosin staining

B16-F1 melanoma tumours were harvested, mounted on OCT and cryostat-sectioned prior to fixation in ice cold 70% (v/v) ethanol and subsequent blocking in 3% BSA in PBS, as described in the Methods (section 2.2.6.2.2). Sections were then stained with Haematoxylin and Eosin (H & E) as outlined in the Methods (section 2.2.6.3.1), prior to mounting in 70% (v/v) glycerol. Images were captured using an *Olympus* digital camera attached to a *Zeiss* light microscope. **A**, B16-F1 tumour-host interface in the $TG2^{+/+}$host depicting major histological features: **B-E,** enlarged images of tissue regions depicting stromal cell nuclei (**B**), stromal red blood cells (**C**), stromal muscle fibres (**D**), and tumour pseudocapsule (**E**). B16-F1 cells exhibit atypical characteristics of malignant cells such as enlarged size, irregular shape, loss of contact inhibition, and multi-polar mitosis (arrow). **T**, neoplastic tissue; **N**, normal connective stromal tissue. Images are representative of three non-overlapping fields (n=6). White bar = 50μm; Black bar = 20 μm.

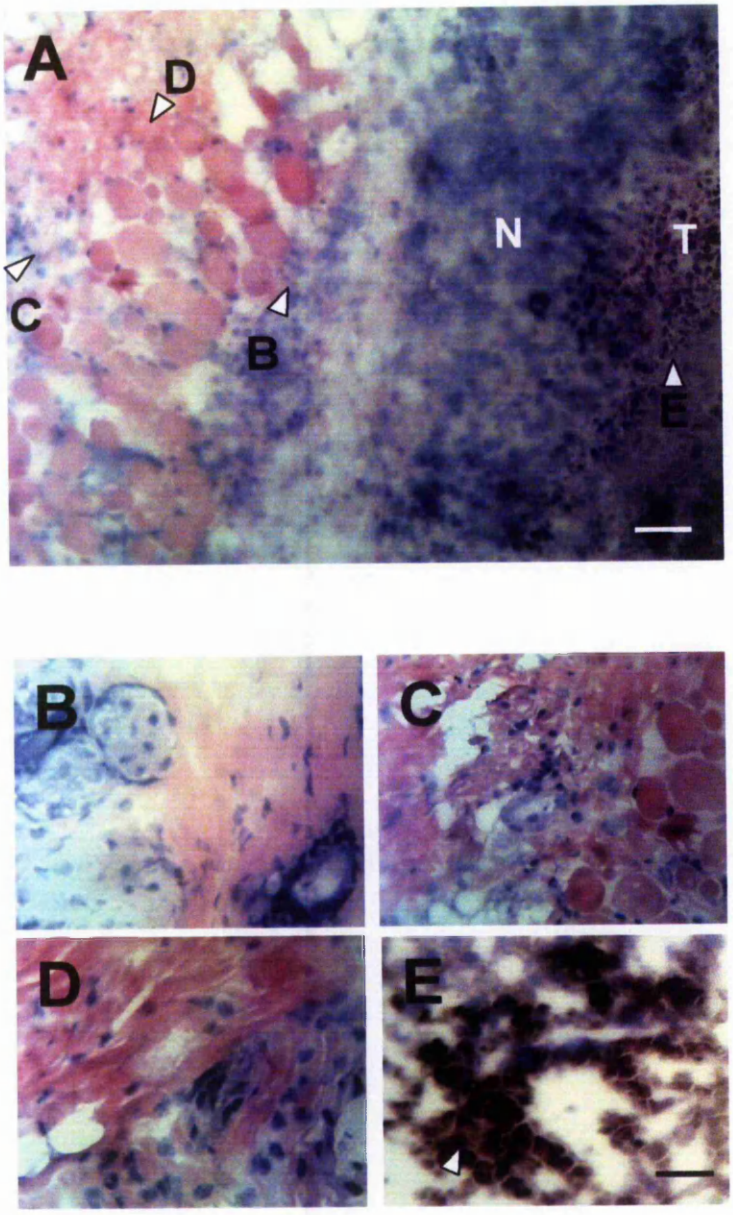


Figure 3.3.1.2.1 Characterisation of B16F1-C57BL/6 tumour-host morphology by Haematoxylin and Eosin staining

features between the stromal tissue of the two animal populations. As shown in figure 3.3.1.2.2, tumours growing on knock-out animals show increased invasive characteristics as measured by their ability to colonise the surrounding host stroma. Histochemical staining of $TG2^{-/-}$ stromal tissue revealed that a larger population of melanogenic malignant cells invade through the fibrous pseudocapsule coat of the tumour core, compared to the $TG2^{+/+}$ tissue.

3.3.1.3 Measurement of TG2 antigen and activity in B16-F1 tumours

In order to establish a direct correlation between tumour growth acceleration and lack of TG2 expression in the animal host, it had to be confirmed that B16-F1 tumour cells do not express the enzyme in abundance. Therefore, TG2 antigen and total TG activity of TG2 wild-type and knock-out tumours were measured. Immunofluorescence staining of tumour sections with a mouse anti-TG2 MAb (Cub7402), which was subsequently coupled to an anti-mouse-FITC conjugate, revealed that the enzyme is solely expressed by the $TG2^{+/+}$ tumour host support tissue, outside the tumour pseudocapsule (figure 3.3.1.3.1). This is in agreement with the increased TG2 expression in the tissue surrounding the tumour as previously reported (Hettasch *et al.*, 1996; Haroon *et al.*, 1999). In contrast, no TG2 antigen was detected either within the tumour mass or in the surrounding $TG2^{-/-}$ host connective tissue. This finding is also in keeping with a recent report demonstrating that a mere 15% of the screened breast carcinomas was positive for TG2 antigen as opposed to a 50% of the stromal tissue (Grigoriev *et al.*, 2001), as well as with numerous publications demonstrating a reduction in TG2 expression and activity in malignant cells (Birckbichler *et al.*, 1976 and 1978; Birckbichler and Patterson, 1980; Barnes *et al.*, 1985). Immunoprobing of western-blotted tumour homogenates originating from TG2 wild-type and knock-out C57BL/6 mice with an anti-TG2 antibody provided further evidence for the observed lack of TG2 expression within the tumour body (figure 3.3.1.3.1). When tumour homogenates were analysed for their ability to incorporate [^{14}C]-putrescine –a well-known TG2 substrate– into N'-N'-dimethylcasein, no TG activity was detected (figure 3.3.1.3.2). Taken together, these findings confirm that any effect that endogenous TG2 might have on tumour growth in this C57BL/6 model, is

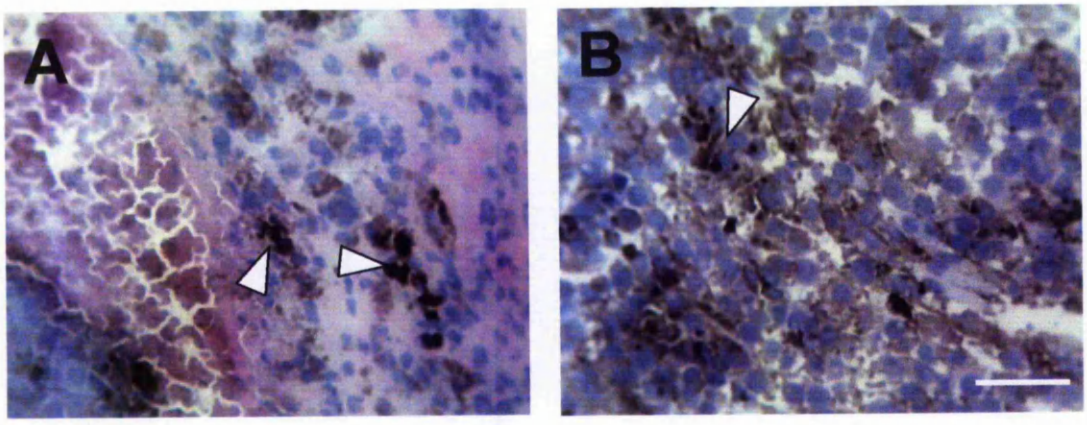
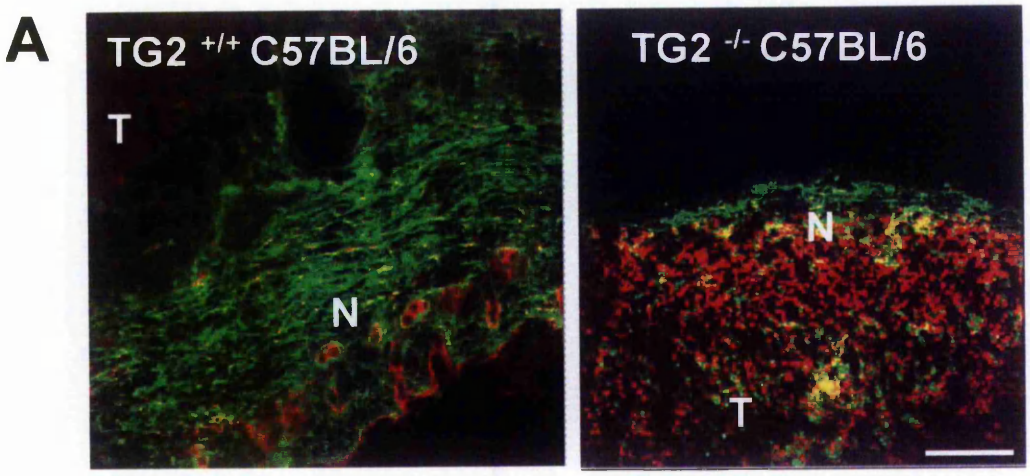


Figure 3.3.1.2.2 Monitoring B16-F1 tumour cell escape from the tumour capsule by H & E staining

B16-F1 melanoma tumours were harvested and sectioned as described in the Methods (section 2.2.6.2.2). Following fixation in 70% (v/v) ethanol and subsequent blocking in 3% (w/v) BSA, sections were stained with H & E prior to imaging using an *Olympus* digital camera attached to a *Zeiss* light microscope. **A**, $TG2^{+/+}$ C57BL/6. **B**, $TG2^{-/-}$ C57BL/6. Arrows indicate tri- and tetra-polar mitosis of melanoma cells infiltrating and colonising the surrounding host stroma. Images are representative of three non-overlapping views (n=4). Bar= 50μm.

Figure 3.3.1.3.1 Measurement of TG2 antigen in B16-F1 tumours by immunofluorescence and western blotting

A, TG2 antigen staining of B16-F1 melanoma tumour sections originating from $TG2^{+/+}$ and $TG2^{-/-}$ C57BL/6 mice. Sections were stained with a mouse anti-TG2 MAb (Cub7402), which was subsequently coupled to a secondary anti-mouse-FITC conjugate as described in the Methods (section 2.2.6.4). Nuclei were counterstained by mounting slides in a propidium iodide-containing medium. TG2 antigen and cellular distribution were viewed using a TCSNT laser confocal microscope under the FITC (green) and TRITC (red) filters respectively. Images are representative of three non-overlapping views (n=6). **N**; normal stromal tissue; **T**, neoplastic tissue. Bar=100μm. **B**, western blot analysis of B16-F1 tumour homogenates. Tumour specimens were denuded of the surrounding connective tissue, homogenised and sonicated in the presence of protease inhibitors as outlined in the Methods (section 2.2.3.1). The total protein content of the homogenates was determined by the Lowry assay, and was resolved on a 10% SDS-PAGE polyacrylamide gel prior to transfer to a nitrocellulose membrane and immunoblotting with a mouse anti-TG2 MAb (Cub7402), followed by conjugation to an anti-mouse-HRP secondary antibody as outlined in the Methods (section 2.2.3.5). Lane 1, 100ng *gpl*TG2; lanes 2-4, tumour homogenates from $TG2^{+/+}$ C57BL/6 mice; lanes 5-7, tumour homogenates from $TG2^{-/-}$ C57BL/6 mice (n=3).



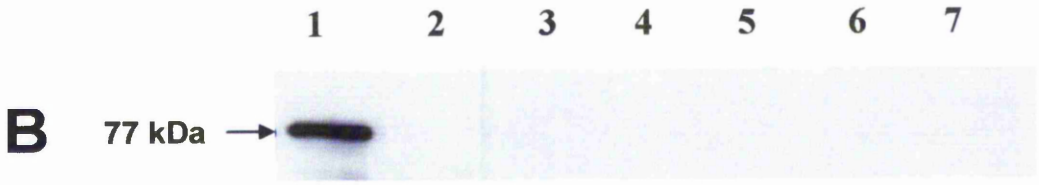


Figure 3.3.1.3.1 Measurement of TG2 antigen in B16-F1 tumours by immunofluorescence and western blotting

Tissue	Activity (U/mg protein)
$TG2^{+/+}$ tumour	0.89 ± 0.5
$TG2^{-/-}$ tumour	0.93 ± 0.7

Figure 3.3.1.3.2 Measurement TG activity in B16-F1 tumour homogenates

Total TG activity of B16-F1 tumour homogenates was assessed by the enzyme's ability to incorporate [^{14}C]-putrescine into N'-N'-dimethylcasein as described in the Methods (section 2.2.3.7.1). Results represent mean value ±SD, from 2 separate experiments, undertaken in triplicate. Unit of activity equals 1nmol of putrescine incorporated per hour, and were normalised against the amount of protein in the homogenates, measured by the Lowry method as described in the Methods (section 2.2.3.2.2).

purely physiological and due to its expression within the non-neoplastic host tissue that supports the tumour mass.

3.3.1.4 Monitoring of B16-F1 metastasis in TG2 $^{+/+}$ and TG2 $^{+/+}$ mice

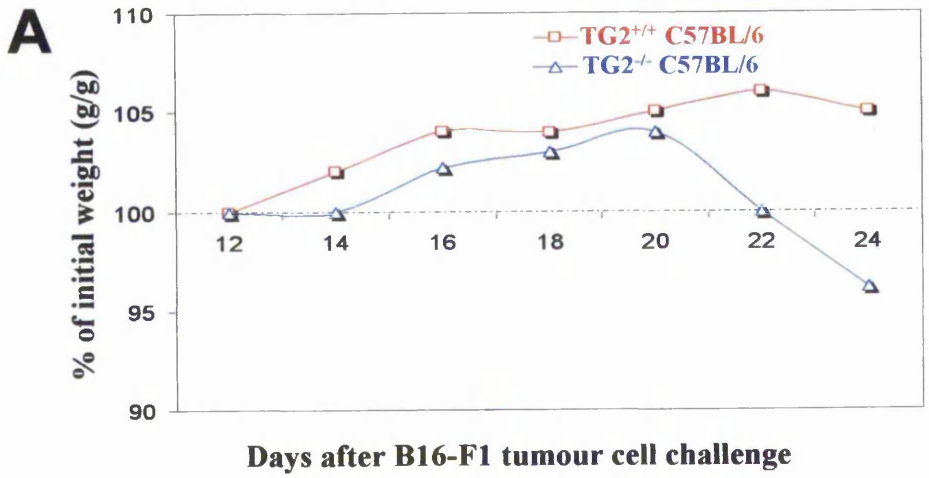
Having established a correlation between disrupted physiological expression of TG2 and accelerated tumour growth, it was of interest to examine whether this finding could be expanded to justify increased metastatic potential within a TG2-free host. Therefore, the metastatic capacity of B16-F1 tumours grown on TG2 knock-out and wild-type mice was evaluated by means of body weight monitoring, through assessment of visual pigmentation signs in a primary site of extravasation (liver), as well as by histological studies (H & E staining) of the sections obtained from both livers and lungs. Body weight monitoring of both mice populations revealed increased loss of weight in the knock-out mice population 32 days post tumour cell challenge, indicating possible metastasis into primary metastatic organ targets (figure 3.3.1.4). However, visual assessment of the primary metastatic organs (livers and lungs) revealed no distinctive brown pigmentations indicative of melanin-containing foci. H & E staining of the sections also revealed no pathological features associated with tumour metastasis. Further immunohistochemical staining with a pan-carcinoma antibody was considered unnecessary as the absence of metastatic signs was confirmatory. It was therefore concluded that, unless an *in vivo* model of metastasis based on a more invasive cell line such as the B16-F10 variant was employed, no evidence for the physiological role of TG2 in tumour metastasis could be collected.

3.3.2.1 TG2 purification from guinea pig livers

Having ascertained that TG2 bears a physiological importance in tumour growth, as lack of the enzyme is responsible for accelerated tumour growth, the next logical step would be to examine the effects the exogenous enzyme might elicit when administered into the

Figure 3.3.1.4 Monitoring of lungs and livers extracted from B16F1-bearing TG2 $^{+/+}$ and TG2 $^{+/+}$ mice for signs of metastasis

A, once tumours were detectable (2mm^2), both animal populations were monitored for body weight loss as a late indicator of tumour metastasis. A 10% decrease in weight within the TG2$^{-/-}$ mice population was detected compared to TG2 $^{+/+}$ mice 32 days after melanoma cell challenge. Squares, TG2 $^{+/+}$; triangles, TG2 $^{-/-}$. **B**, livers harvested from TG2$^{+/+}$ and TG2$^{-/-}$ mice 32 days post tumour cell challenge were fixed in 3.7% (w/v) paraformaldehyde in PBS and preserved in 20% (w/v) sucrose prior to imaging using an *Olympus* digital camera. No visible metastatic foci could be detected at the surface of these primary metastatic targets. Ruler=2cm. **C**, H & E staining of wax-embedded, microtome-sectioned lungs and livers extracted from TG2ase knock-out and wild-type mice was undertaken as described in the Methods (section 2.2.6.3.1). No pulmonary glandular foci in the periphery of the lung or in the lobar bronci (arrow) could be detected, whereas the hepatocyte population from a single lobule extracted from the right liver lobes appeared normal (arrow). Images are representative of 3 non-overlapping fields (n=6). Bar =100μm.



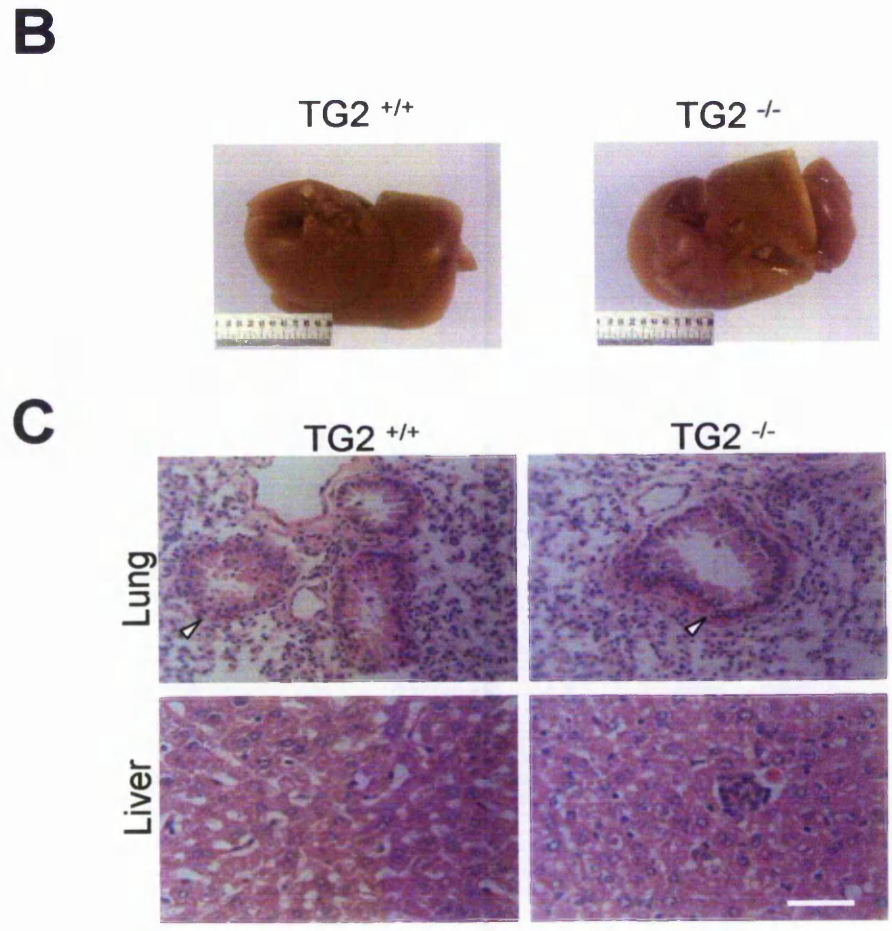


Figure 3.3.1.4 Monitoring of lungs and livers extracted from B16F1-bearing TG2 $^{+/+}$ and TG2 $^{+/+}$ mice for signs of metastasis

tumour mass. To address this, a well-characterised chemically-induced, undifferentiated mouse colon carcinoma tumour model (CT26) was employed (Geng *et al.*, 1998), whereby CT26 tumours are injected with exogenous *gpl*TG2. However, given the reported low purity of the commercially available TG2 (Collighan R., unpublished data), the only way to rule out the possibility that any effect on tumour growth won't be purely due to the enzyme's action, TG2 purification was undertaken in-house. 5 guinea pig livers were homogenised as described in the Methods (section 2.2.4.1), prior to the initial separation of resulting protein supernatants on an anion exchange chromatography (IEC) column as outlined in section 2.2.4.2. TG2-rich IEC fractions were identified by the ability of the enzyme to incorporate biotin-cadaverine into N'-N'-dimethylcasein (figure 3.3.2.1.1). Selected fractions (117-162) were pooled and their protein content precipitated in 80% (w/v) ammonium sulphate prior to centrifugation, as described in the Methods (section 2.2.4.3). Resulting pellets were resolubilised, prior to separation through a size exclusion chromatography (SEC) column as outlined in Methods (section 2.2.4.3). Once again, TG2-rich fractions were identified by means of biotin-cadaverine incorporation into N'-N'- dimethylcasein, pooled, and separated through a GTP-agarose affinity column as outlined in the Methods (section 2.2.4.4). At this stage of the purification the only GTP-binding proteins are TG2, TG3 and TG5, and selected elutants were therefore screened for protein content in order to identify the TG2-rich fractions (figure 3.3.2.1.2). Further analysis of these fractions by SDS-PAGE and western blotting indicated a high degree of TG2 purity (figure 3.3.2.1.3). Finally, transglutaminase activity of the purified enzyme, measured by means of [^{14}C]-putrescine incorporation into N'-N'-dimethylcasein, was shown to be approximately 12,000 U/mg of protein. Pre-incubation of the enzyme with the site-directed inhibitor NTU283 (500μM) for 6 hours and at 4°C resulted in complete and irreversible enzyme activity inhibition (figure 3.3.2.1.3).

Figure 3.3.2.1.1 Ion exchange and size exclusion chromatographic separation of TG2 from guinea pig liver homogenates

5 guinea pig livers (*gpl*) were homogenised in 500ml of 5mM Tris-HCl, 2mM EDTA, pH 7.5, 0.25M sucrose, in the presence of protease inhibitors. Nuclei and large debris were spun out by centrifugation at 30,000g for 1 hour and homogenates were ultracentrifuged and clarified by sieving through no. 4 Whatman filter paper, as described in the Methods (section 2.2.4.1). Resulting protein supernatants were initially separated on an ion exchange chromatography (IEC) Q-sepharose Fast Flow column as outlined in the Methods (section 2.2.4.2). **A**, total protein and TG specific activity profiles, revealed by Lowry total protein and Biotin-Cadaverine incorporation TG activity assays, as described in sections 2.2.3.2.2 and 2.2.3.7.3 respectively, were used to identify the TG2-rich elution peaks from IEC. IEC fractions 117-162 were pooled and their protein content was precipitated in 80% (w/v) ammonium sulphate prior to centrifugation at 1,000xg for 1 hour, as described in the Methods (section 2.2.4.3). The four resulting pellets were reconstituted in 10ml of 10mM Tris-Acetate, 1mM EDTA, 0.16M KCl, pH 6.0, prior to separation through a size exclusion chromatography (SEC) column as outlined in Methods (section 2.2.4.3). **B**, representative protein and specific TG activity profile of SEC elutants from a single representative pellet. High TG2 specific activity fractions 30-60 were chosen for further purification.

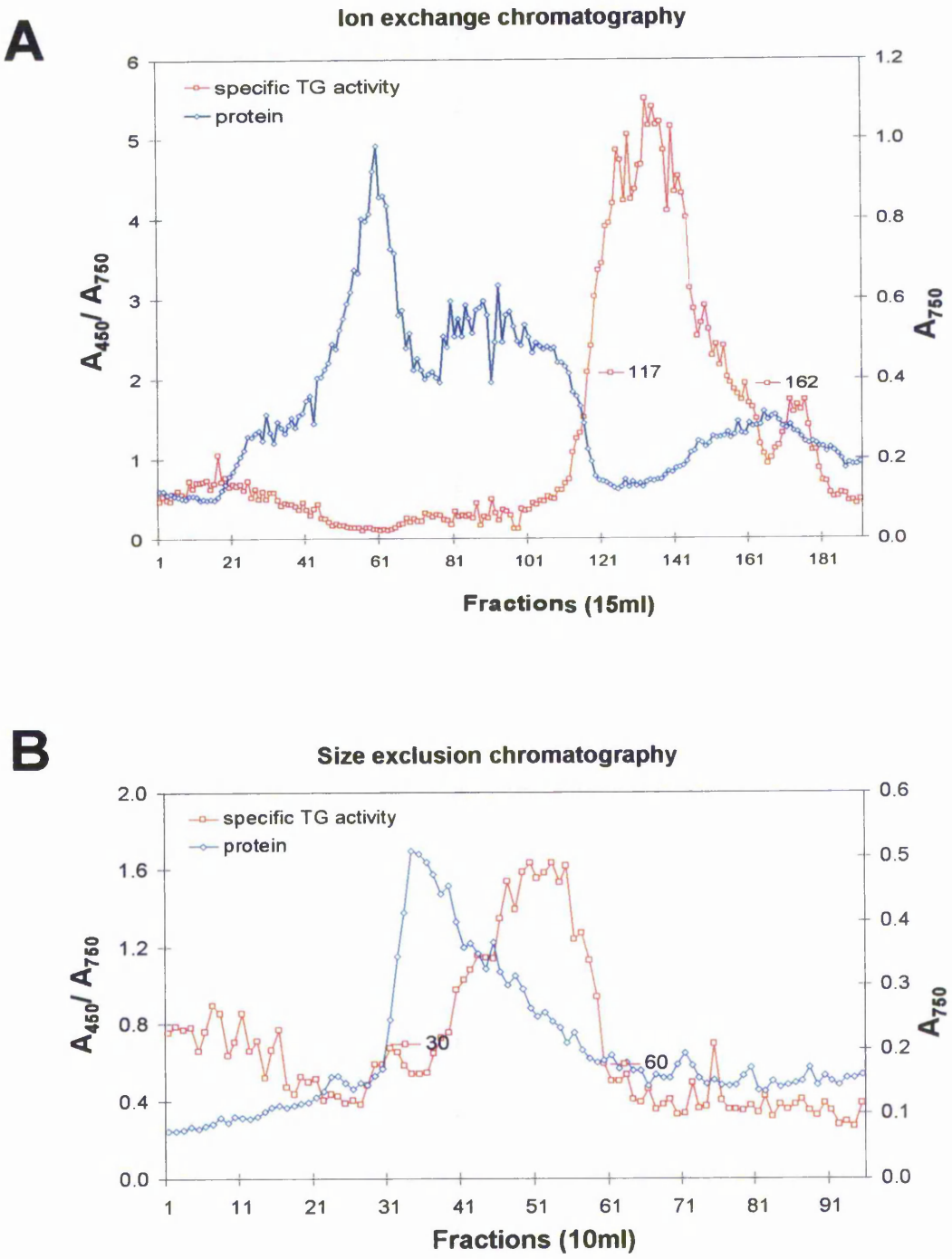


Figure 3.3.2.1.1 Ion exchange and size exclusion chromatographic separation of TG2 from guinea pig liver homogenates

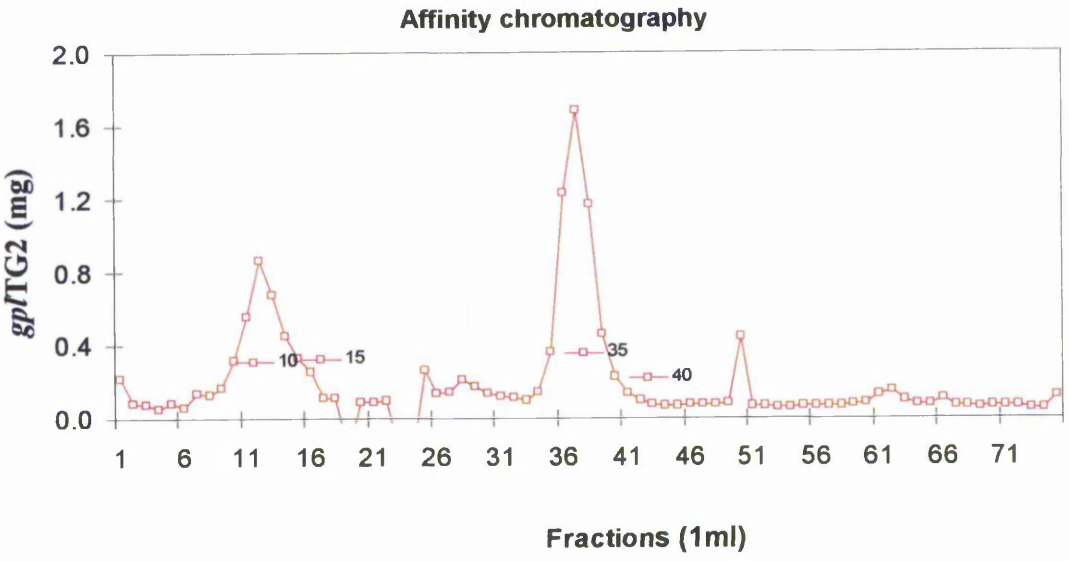


Figure 3.3.2.1.2 GTP-agarose affinity chromatography separation of TG2 from *gpl* homogenates

High TG specific activity fractions 30-60 resulting from the gel filtration step were pooled in 4 x 10ml aliquots and ran separately through an affinity chromatography (AC) GTP-agarose column prior to elution in GTP as outlined in the Methods (section 2.2.4.4). Fractions were assayed for protein content using the Lowry method and mg of protein/fraction were calculated by reference to a BSA protein standard curve. Results depict a representative protein profile of GTP-agarose eluents from a single ammonium sulphate pellet. High protein content fractions 10-15 (2mg of protein) and 35-40 (10mg of protein) were chosen for further analysis by SDS-PAGE and western blotting.

Figure 3.3.2.1.3 Confirmation of TG2 presence in the selected AC fractions by SDS-PAGE, western blotting and TG activity assay

A, 6μg of selected protein fractions (10-15, and 35-40) from AC were electrophoresed on a 10% SDS-PAGE gel. Resolved proteins were either stained with 0.1% (w/v) Coomassie Brilliant blue in 40% (v/v) methanol, 10% (v/v) acetic acid for 1 hour, or transferred to a nitrocellulose membrane and immunoprobed for TG2 as outlined in the Methods (section 2.2.3.4). **A**, coomassie stained SDS-PAGE gel. Lanes 1 and 9, broad range molecular weight makers; lane 2; 2μg of *gpl*TG2 standard; lane 3-4, fractions 10-15 and 35-40 from AC of first ammonium sulphate pellet; lane 5-6, fractions 10-15 and 35-40 from AC of second ammonium sulphate pellet; lane 7-8, fractions 10-15 and 35-40 from AC of third ammonium sulphate pellet. **B**, western blot immunoprobed with a mouse anti-TG2 MAb (Cub7402), which was subsequently conjugated to an anti-mouse-HRP secondary antibody. TG2 antigen was revealed by enhanced chemiluminescence (ECL). Lane 1, 100ng *gpl*TG2; lanes 2-3, representative fractions 10-15 and 35-4 respectively from AC of first ammonium sulphate pellet. **C**, TG2 from pooled AC protein fractions was inactivated with the site-directed inhibitor NTU283 (500μM) for 6 hours at 4°C, prior to overnight dialysis in 3L of PBS, 2mM EDTA at 4°C. [^{14}-C]-Putrescine assay was undertaken on both active or inactive (80μg/ml) TG2 as outlined before in the Methods (section 2.2.3.7.1). Active TG2, black bar; inactivated TG2, open bar.

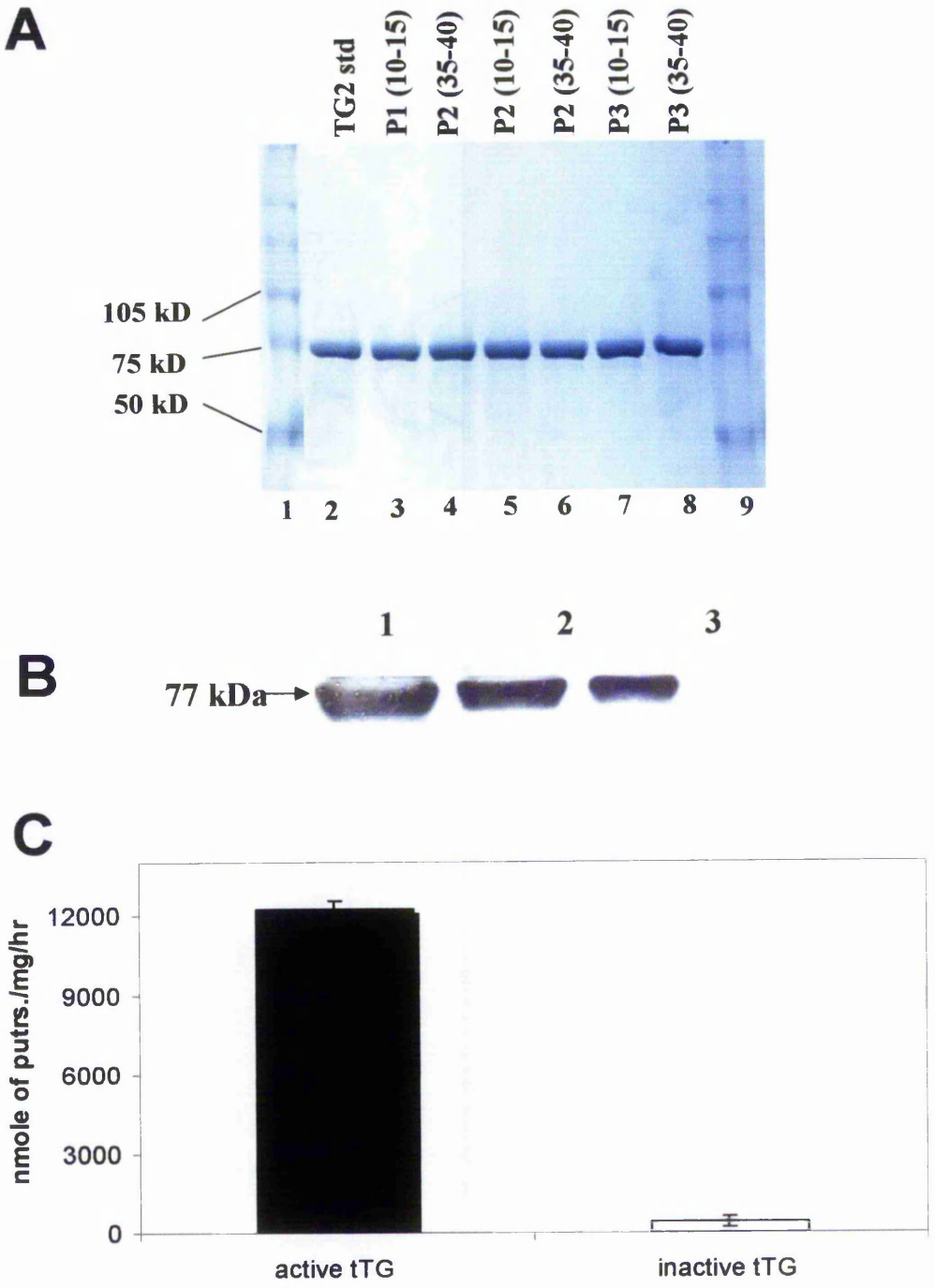


Figure 3.3.2.1.3 Confirmation of TG2 presence in the selected AC fractions by SDS-PAGE, western blotting and TG activity assay

3.3.2.2 CT26 viability, proliferation and apoptosis following exogenous TG2 treatment

Before assessing the effect of exogenous TG2 on CT26 tumour growth *in vivo*, the effect of the enzyme when administered exogenously into CT26 cultures *in vitro* had to be examined. Birckbichler *et al.* (1978) demonstrated that TG activity modulates the differentiation/proliferation cell cycle of different cell types, whereas other studies have more precisely attributed this effect to the ability of the G-protein function of TG2 to delay progression into the G2/M phase (Mian *et al.*, 1995). Therefore, following two daily applications of 50μg/ml active (50U/ml) *gpl*TG2, viability and proliferation of the CT26 cells were measured by an XTT metabolism-based assay (section 2.2.1.7.1) and a *ds*DNA quantitation assay (section 2.2.1.7.4) respectively. As shown in figure 3.3.2.2, both *ds*DNA content, and the ability of viable TG2-treated cells to metabolise XTT is comparable to the untreated and inactive TG2-treated controls. Conversely, caspace-3 activity measurement of the CT26 cell lysates (section 2.2.1.7.3) subsequent to the same TG2-treatment regime, revealed similar apoptotic indices between TG2 treated and mock-treated controls (figure 3.3.2.2). These findings contradict previous studies where transfections of mammalian cells with a full length TG2 cDNA or antisense TG2 constructs revealed a marked increase or decrease in the spontaneous cell death rate respectively (Gentile *et al.*, 1992; Melino *et al.*, 1994). This could be attributed to the insensitivity of tumour cells to anti-growth signals, their limitless replicative potential as well as their ability to evade apoptosis (Hanahan and Weinberg, 2000).

3.3.2.3 Effect of exogenous TG2 on CT26 invasion

At this stage it was of interest to establish an explanatory mechanism for the observed increase in tumour cell escape into the TG2 knock out stromal tissue. Belkin *et al.* (2001) demonstrated that proteolytic degradation of cell surface TG2 stimulated malignant cell motility on collagen matrices. The same authors had demonstrated that anti-TG2 antibodies markedly decrease migration of monocytic cells on fibronectin (Akimov and Belkin, 2001). To assess the role of exogenous TG2 in malignant cell invasion, a model

that introduces a TG2-crosslinked artificial ECM protein barrier to migrating CT26 mouse colon carcinoma was chosen. Orban and colleagues have recently demonstrated that TG-mediated reaction successfully strengthens collagen gels while remaining benign towards cells (Orban *et al.*, 2004). Taken together, collagen and fibronectin (FN) account for a large portion of the total extracellular matrix protein content (van der Rest and Garrone, 1991). However, adhesion of CT26 cells to FN has been shown to be higher than their adhesion to collagen I, collagen IV, or laminin (Geng *et al.*, 1998). To explore whether collagen-fibronectin matrices reinforced by TG2-mediated crosslinking of fibrils could pose a tougher barrier for CT26 cells to penetrate, fibrillogenesis was allowed to take place in the presence of fibronectin facilitated by addition of 50μg/ml active (50U/ml) *gpl*TG2 as described before (section 3.2.1.1). TG2 was also added in its inactive form, following pre-incubation with 500μM of the active site directed inhibitor NTU283 for 6 hours at 4°C. The prepared matrices were applied on micropore (6μm) filters, and exposed to CT26 cells that were encouraged to migrate through them and towards a lower FCS-containing chamber as described before (section 3.2.1.2). Fluorescence in the bottom cell chamber was solely due to the ability of viable CT26 cells that have invaded through the matrix to hydrolyse calcein AM. As shown in figure 3.3.2.3, a statistically significant decrease ($p<0.05$) in the malignant cell ability to invade through TG2-crosslinked matrix was observed. Both inactivation of the enzyme with TG2 inhibitor NTU283 and cell-surface function-blocking with an anti-TG2 MAb (Cub7402) prior to the application to the collagen-fibronectin mixture resulted in a more permeable barrier to the malignant cells, confirming that these effects were due to the enzyme's activity.

Figure 3.3.2.2 CT26 *in vitro* viability, proliferation and apoptosis following exogenous TG2 treatments

A, proliferation of CT26 was measured by quantification of *ds*DNA as described in the Methods (section 2.2.1.7.4). CT26 cells were seeded at 10^4 cells/well in 96-well plates and were administered twice at daily intervals with 50μg/ml exogenous, active (50U/ml) or inactive *gpl*TG2. Results represent values from two independent experiments, and are expressed as percentage of untreated control (*ds*DNA content) ±SD (n=6). **B**, measurement of cell viability by the XTT assay undertaken as outlined in the Methods (section 2.2.1.7.1). Cell viability is expressed as A490nm values ±SD, following 2 daily administrations of 50μg/ml active (50U/ml) or inactive *gpl*TG2 in standard cell growth media (n=6). **C**, CT26 cells were seeded at 2 x 10^6 cells in 25cm^2 flasks and were administered twice at 8 hour intervals with 50μg/ml active (50U/ml) or inactive *gpl*TG2. Programmed cell death, was measured by Caspase-3 activity as described in the Methods (section 2.2.1.7.3), and was expressed as pmole of pNA liberated per hour by reference to a pNA standard curve. Positive control cultures were treated with 1μM of the general protein kinase inhibitor staurosporine for 16 hours, whereas negative controls were obtained by parallel incubation with 1μM staurosporine and 50μM Z-VAD-FMK, a pan-caspace inhibitor. Results represent mean value ±SD from 2 independent experiments, each undertaken in triplicate.

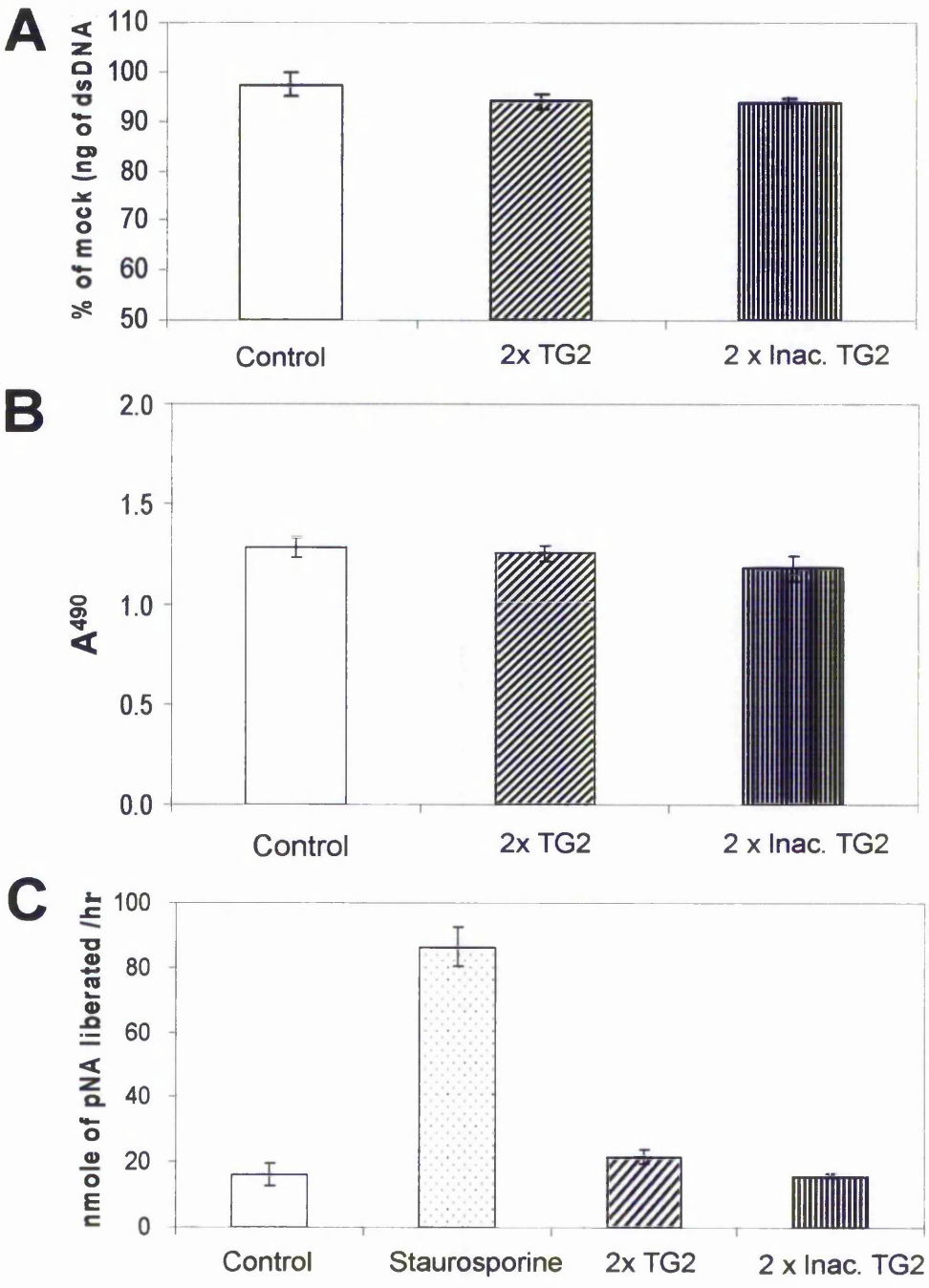


Figure 3.3.2.2 CT26 *in vitro* viability, proliferation and apoptosis following exogenous TG2 treatments

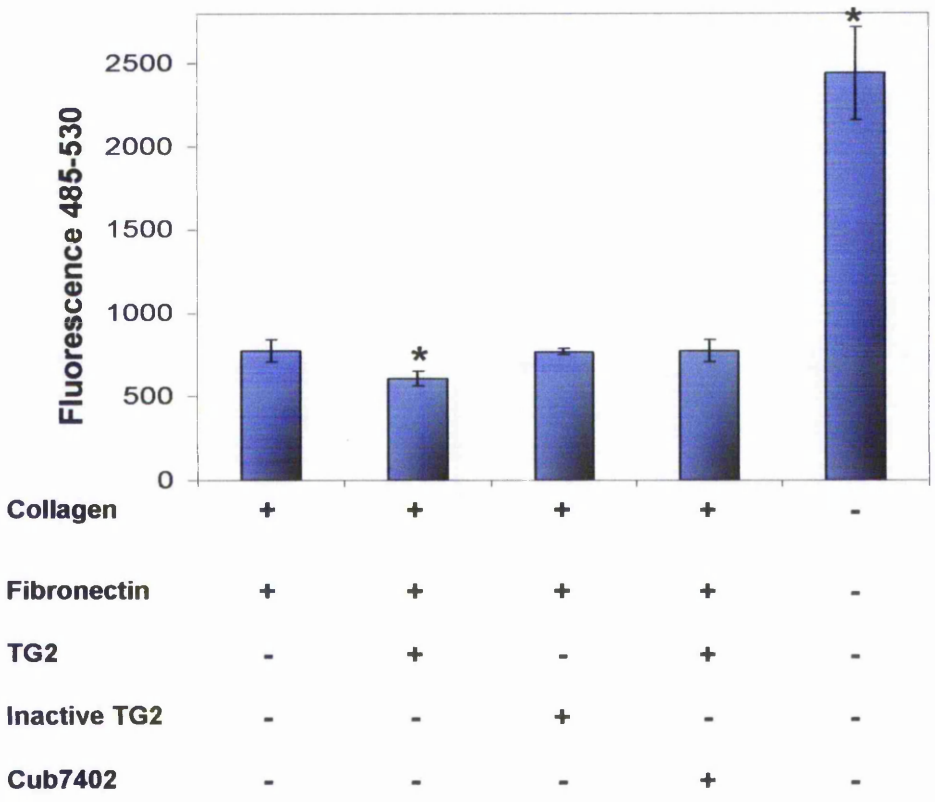


Figure 3.3.2.3 CT26 cell invasion through TG2-crosslinked collagen/fibronectin artificial matrices

10^6 CT26 cells/ml were seeded in DMEM, on Fluoroblock filters coated with collagen type I/fibronectin matrices that had been crosslinked by addition of exogenous TG2 (50μgml; 50U/ml). Cells were allowed to migrate to a lower chamber in response to chemoattraction to 10% (v/v) FCS for 72 hours at 37°C as described before (section 3.2.1). Inserts were transferred to 24-well glass-bottom plates containing 4μg/ml Calcein AM in HBSS and incubated at 37 °C for 90 minutes. Fluorescence due to the ester hydrolysis by live cells that have migrated to the bottom side of the filter was read at 485nm/530nm (excitation/emission) from-bottom-to-top using a SpectraFluor plate reader. Results represent mean values ±SD from two separate experiments, each undertaken in quadruplicate.* Indicates significantly different ($p<0.05$) from the control.

3.3.2.4 CT26 tumour therapy dose response following injections of active *gpl*TG2

So far in this study, it has been established that TG2-mediated crosslinking of artificial collagen-fibronectin slows down malignant cell invasion. Given that exogenous TG2 does not appear to alter either the viability or the proliferation rate of CT26 cells, it would be conceivable that exogenous TG2 may lead to tumour growth retardation *in vivo* as a result of increased ECM protein crosslinking. Thus, the effect of TG2 when delivered exogenously into the CT26 tumour mass of balb/c animals was examined. Initially, tumour growth was monitored in response to 5 daily intratumour injections of TG2 at various specific activities (2, 10, and 20U/injection). As shown in figure 3.3.2.4.1, 10U/injection was the optimum specific activity for tumour growth attenuation, whereas administration of the enzyme at higher (20U/injection) and lower (2U/injection) specific activities did not incur any significant effect on tumour growth. When the enzyme was delivered at an even higher specific activity (100U/injection), no significant effect on tumour growth was observed either (figure 3.3.2.4.2). Given the high affinity of TG2 for a number of ECM-associated substrates, it was hypothesized at this stage that the enzyme's activity is crucial for the homogeneous diffusion of the enzyme through the tumour. Low TG activity might not suffice for complete saturation of the ECM substrates, whereas high enzyme activity might result in the enzyme been sequestered in the vicinity of the injection site, where it could rapidly cross-link ECM proteins.

In support of the notion that TG activity is central to the anti-invasive capacity of the enzyme, inhibition of the exogenous enzyme by incubation with the active site directed inhibitor NTU283 prior to intratumour injection reversed the enzyme's anti-tumour capacity. Surprisingly, inhibition of endogenous TG activity by intratumour treatment with 500μM NTU283 led to tumour growth rates comparable to the PBS controls (figure 3.3.2.4.2).

Figure 3.3.2.4.1 CT26 tumour therapy dose response following injections of active *gpl*TG2 at various specific activities

Exponentially growing CT26 mouse colon carcinoma cells were harvested and resuspended to a final concentration of $5x10^5$ cells/ml in DMEM, prior to subcutaneous implantation of 100µl cell suspensions into 6-8 weeks old balb/c mice. Following tumour cell challenge, animals were monitored every two days for tumour development. Once tumours reached $2mm^2$ animals were divided in groups of 7. Each treatment group received 5 intratumour injections (days 10, 12, 14, 17, and 19) of 50µl active *gpl*TG2 (0.4, 4, 8mg/ml) in PBS containing 3mM DTT/ 5mM Ca^{2+} at varying specific activities (2, 10, and 20U/injection respectively), whereas control group received 50µl of PBS, 3mM DTT, 5mM Ca^{2+}. Results represent mean value ±SD (n=7). **A,** average tumour growth rate of animals, expressed as mean tumour size (mm^2) over time, was measured up to 21 days post-injection. **B**, percentage animal survival following detection of CT26 tumours. Animals were sacrificed before tumours reached the $1cm^2$ limit. Squares, PBS; triangles, 2U/injection TG2; diamonds, 10U/injection TG2; circles, 20U/injection TG2. * Indicates significantly different ($p<0.05$) from the control.

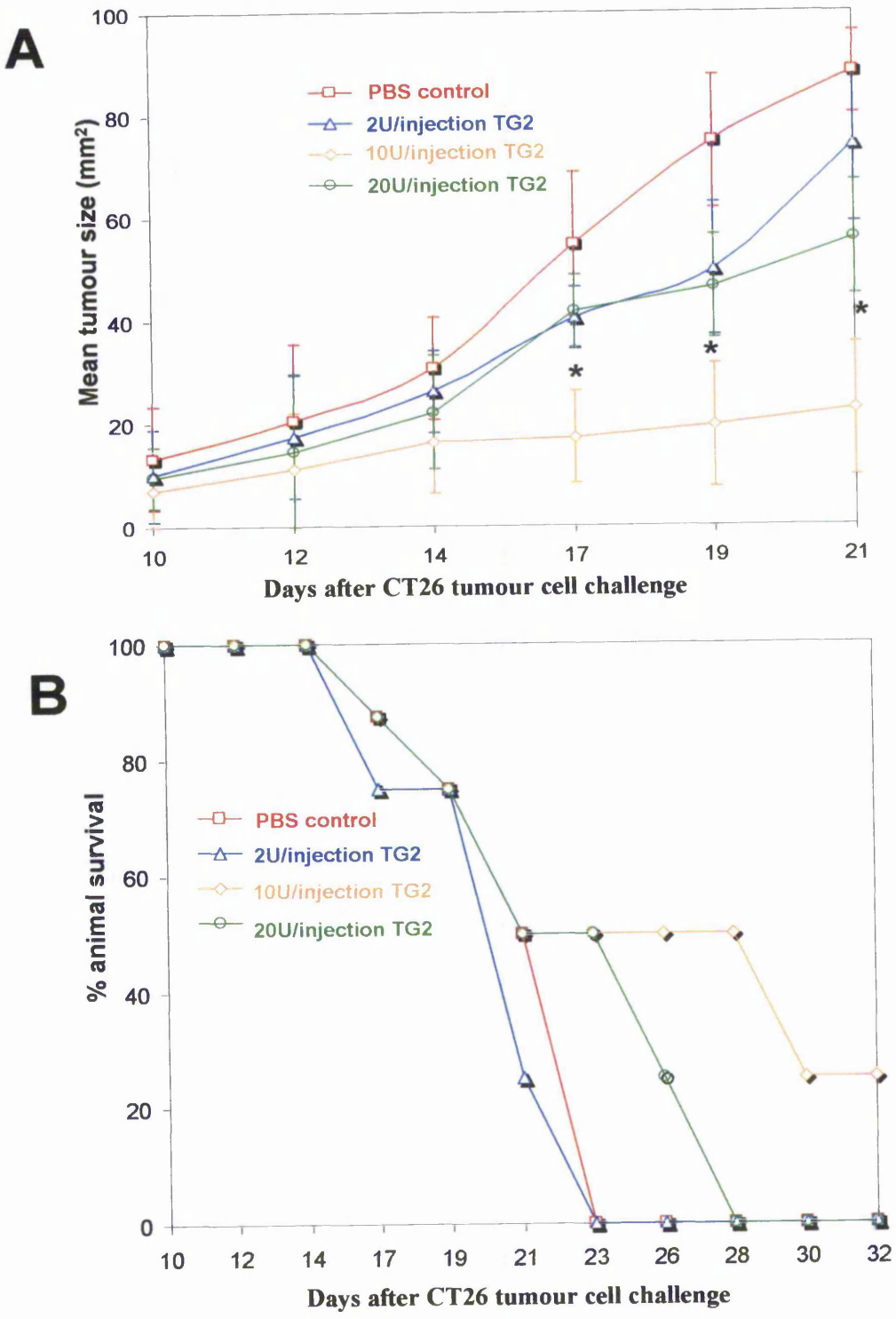


Figure 3.3.2.4.1 CT26 tumour therapy dose response following injections of active *gpl*TG2 at various specific activities

Figure 3.3.2.4.2 Balb/c tumour therapy following injections of high activity (12U/injection) *gpl*TG2, inactive *gpl*TG2, and NTU283

CT26 mouse colon carcinoma cell suspensions (100µl) were implanted subcutaneously into 6-8 weeks old balb/c mice at 10^6cells/ml. Following injections, animals were monitored every two days for tumour development. Once tumours reached $2mm^2$, animals were divided in groups of 7. Groups received 5 intratumour injections (days 12, 14, 17, and 22) of either active *gpl*TG2 in PBS containing 3mM DTT, 5mM Ca2+ (10 and 100U/injection) , or inactive *gpl*TG2 in PBS containing 3mM DTT, 5mM Ca^{2+}), whereas control group received 50µl of PBS, containing 3mM DTT, 5mM Ca^{2+}. An extra group was also administered with 0.5mM of the active site directed TG2 inhibitor NTU283. Results represent mean value (n=7). Average tumour growth rate of animals, expressed as mean tumour size (mm^2) over time, was measured up to 28 days post-injection. **A,** squares, PBS; triangles, 10U/injection TG2; diamonds, 100U/injection TG2. **B** squares, PBS; triangles, inactive TG2; diamonds, NTU283. * Indicates significantly different from the control.

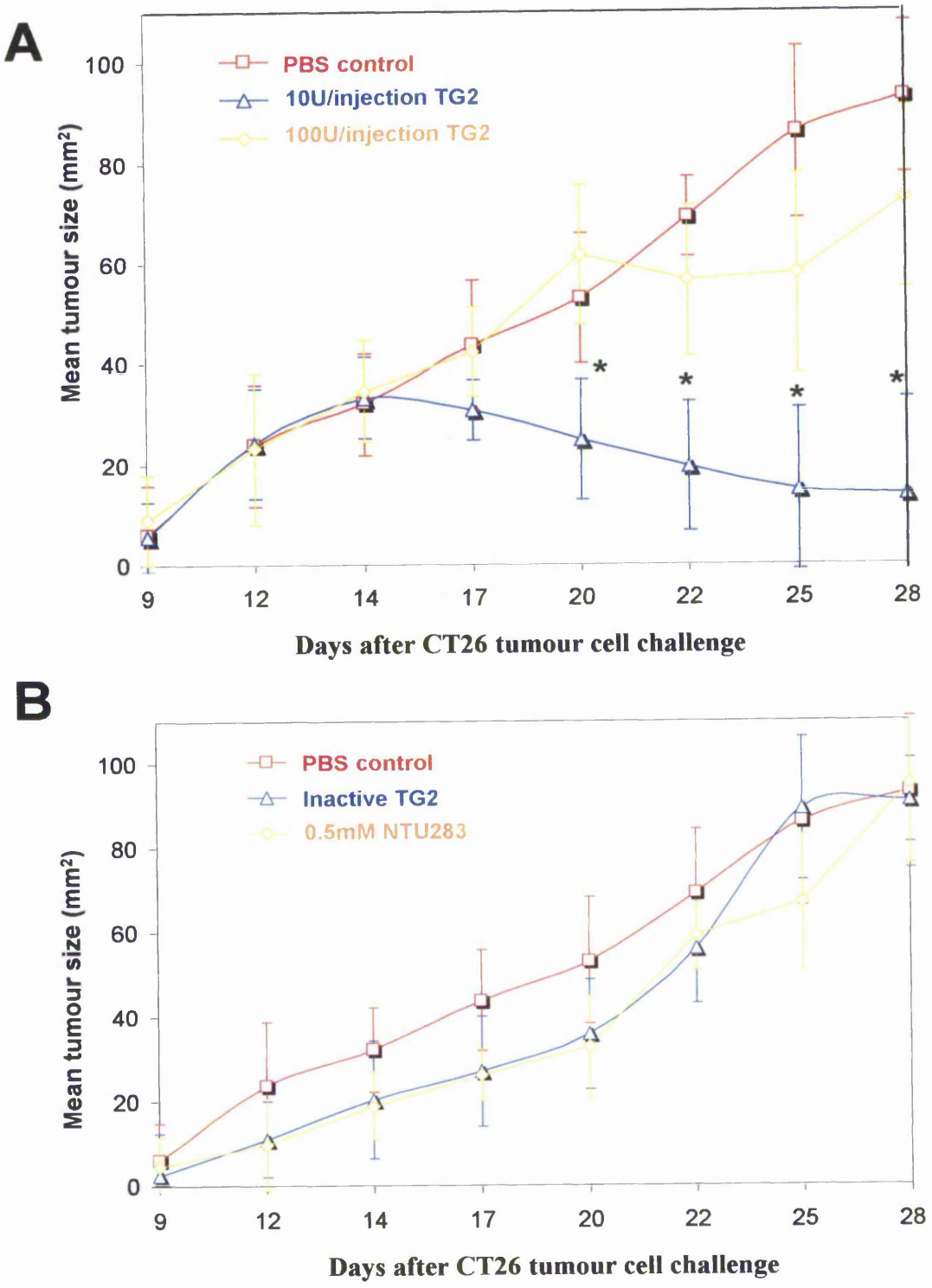


Figure 3.3.2.4.2 Balb/c tumour therapy following injections of high activity (12U/injection) *gpl*TG2, inactive *gpl*TG2, and NTU283

3.3.2.5 Confirmation of gplTG2 delivery into the tumour body

In order to localise the exogenously delivered enzyme into the CT26 tumour body, sections obtained from TG2- and control-treated tumours extracted from the balb/c host were stained with an anti-TG2 MAb (Cub7402), which was subsequently coupled to either an anti-mouse-FITC or an anti-mouse-HRP conjugate. Confocal microscopy of indirect immunofluorescent stained sections revealed an abundance of enzyme within the dermis of the balb/c tumour host, in the vicinity of the tumour body. In the PBS treated tumour, normal host tissue surrounding the tumour was found to be rich in TG2 antigen, whereas the tumour body is devoid of the enzyme. TG2-treated (10U/inj) tumour section staining revealed, apart from TG2 expression in host tissue, homogeneous diffusion of the exogenous TG2 into the tumour body, thus confirming the delivery of the enzyme (figure 3.3.2.5.1). On the other hand, tumours receiving *gpl*TG2 of higher activity (100U/inj) revealed that the exogenous enzyme is immobilised sporadically leading to the formation of high molecular weight aggregates. Western blot analysis of tumour homogenates (figure 3.3.2.5.1), also suggested that TG2 (10U/injection) is delivered within the tumour mass, whilst confirming the absence of intrinsic TG2 antigen within the untreated tumour in agreement with previous findings with the B16-F1 tumour cell line (section 3.3.1.3). Immunoperoxidase staining of tumour sections receiving 10U/inj *gpl*TG2 confirmed deposition of the enzyme on the ECM of CT26 tumour bodies, and lack of endogenous TG2 antigen within the tumour capsule of PBS controls (figure 3.3.2.5.2). Immunoperoxidase staining of tumours treated with higher (100U/inj) enzyme activities was deemed unnecessary as the immunohistochemical findings seemed conclusive.

3.3.2.6 Measurement of ε(γ-glutamyl)lysine crosslink and total collagen of TG2-treated tumours

Quantification of the ε(γ-glutamyl)lysine isopeptide bond levels in the tumour homogenates provided further evidence that the enzyme is deposited and active in the

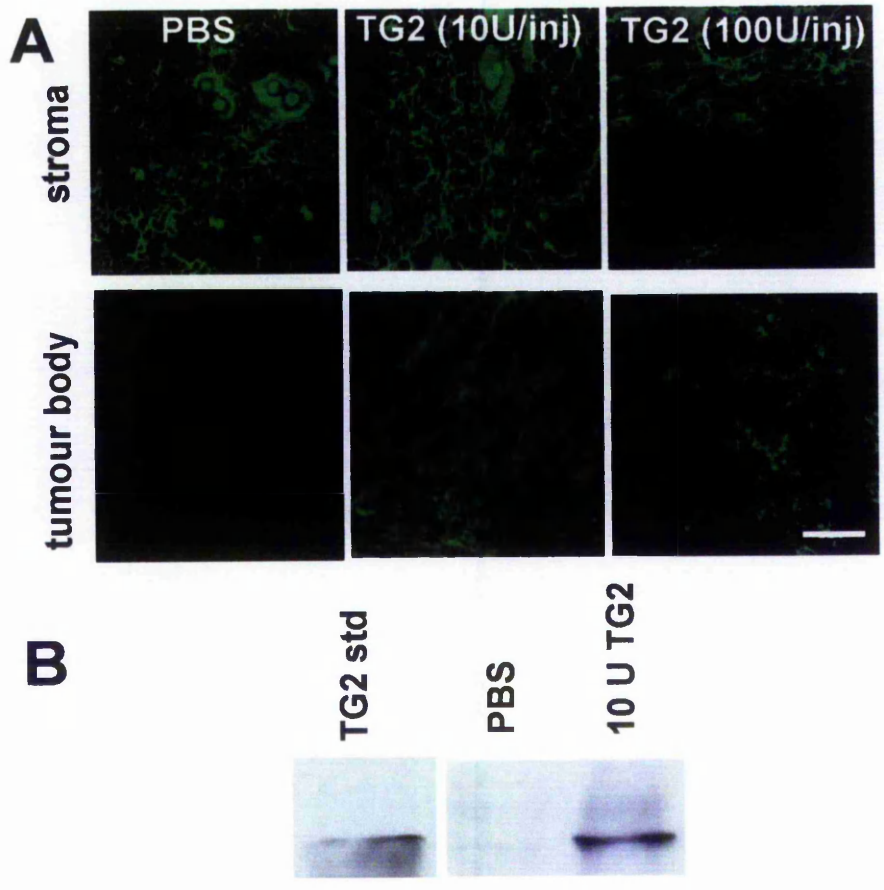


Figure 3.3.2.5.1 Confirmation of *gpl*TG2 delivery into the tumour body by immunofluorescence staining

A, confocal microscopy images of TG2-stained CT26 tumour sections. Control and TG2-treated CT26 solid tumours were harvested, mounted on OCT and sectioned on a cryostat prior to fixation in 70% (v/v) ice cold ethanol and blocking in 3% (w/v) BSA in PBS, as described in the Methods (sections 2.2.6.2.2 and 2.2.6.4). TG2 antigen was recognised by an anti-TG2 MAb (Cub7402) which was subsequently coupled to an anti-mouse-FITC conjugate prior to mounting in 70% (v/v) glycerol and imaging under the FITC filter of a confocal TCSNT microscope. Images are representative of three non-overlapping fields (n=6). Bar = 50μm. **B**, western blot analysis of CT26 tumour homogenates was undertaken in triplicate.TG2 std, 100ng *gpl*TG2.

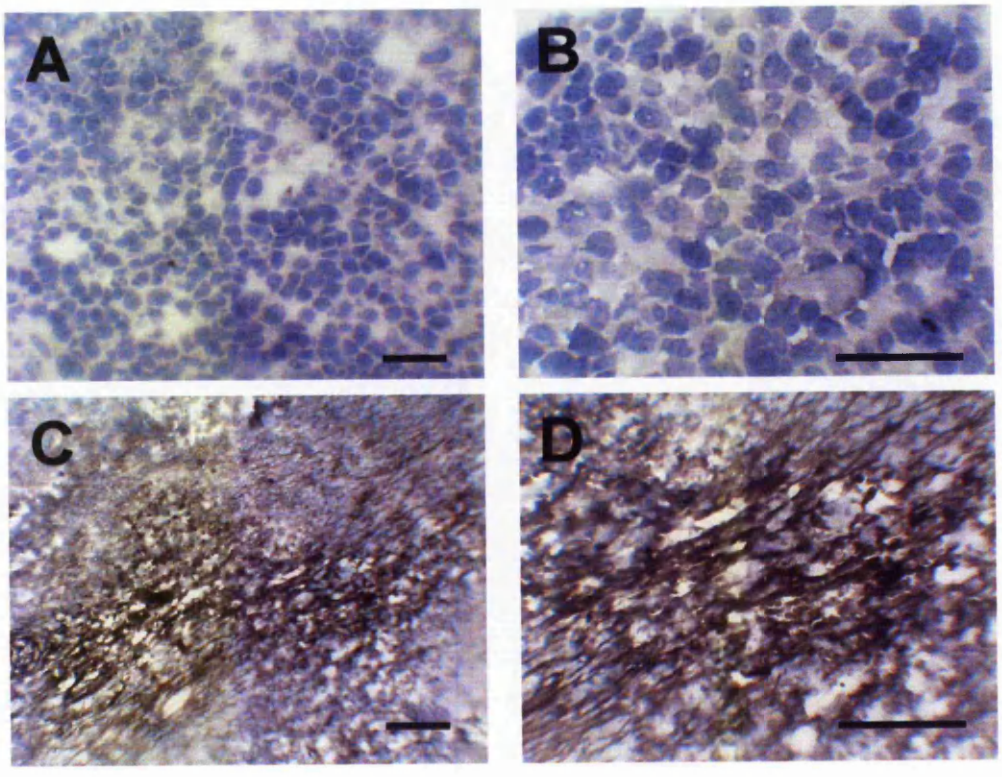


Figure 3.3.2.5.2 Confirmation of *gpl*TG2 delivery (10U/inj) into the tumour body by immunoperoxidase staining

CT26 solid tumours were harvested, mounted on OCT and sectioned on a cryostat prior to fixation and blocking in 3% BSA in PBS, as described in the Methods (sections 2.2.6.2.2 and 2.2.6.4). TG2 antigen was recognised by an anti-TG2 MAb (Cub7402) which was then coupled to an anti-mouse-HRP conjugate. Staining was revealed in DAB/Urea and cells were counterstained with H & E prior to imaging using a *Zeiss* light microscope attached to a digital camera. **A-B**, PBS treated tumour. **C-D**, TG2-treated tumour (10U/inj) staining reveals increased deposition of the exogenously delivered enzyme (brown) into the extracellular space of the tumour cells (blue). Images are representative of three non-overlapping fields (n=6). Bars = 50μm.

ECM of the TG2-treated malignant cells, where it catalyses increased ECM protein crosslinking. Tumours treated with TG2 (200μg/ml; 10U/inj) were shown to contain more crosslinks than the inactive TG2-treated or mock controls (figure 3.3.2.6.1). This finding coupled with the reported decrease in CT26 cell invasion through TG2-crosslinked artificial matrices, provides a mechanistic explanation for the observed tumour growth regression in the CT26 tumour therapy model.

Recently, microbial TG (mTG) has been reported to promote shark collagen fibrillogenesis (Nomura *et al.*, 2001). Further studies within this project have also indicated that TG2 itself may up-regulate collagen deposition (section 5.3.1.1), whilst rendering it less susceptible to degradation by matrix metalloproteinases (section 5.3.3). Masson's Trichrome staining of CT26 tumour treated with TG2 (200μg/ml; 10U/inj) revealed increased collagen deposition, where fibrous collagen structures appear to seal off the tumour pseudocapsule (figure 3.3.2.6.2). Collagen staining was largely absent from the mock treated tumours. This finding was also confirmed by increased OH-proline levels within the TG2-treated tumours as measured by Gas Chromatography (figure 3.3.2.6.3) It is therefore believed that the TG transamidating function is responsible for reinforcing the ECM of CT26 cells via generation of intermolecular isopeptide bonds between the fibrils one of its primary components, collagen.

3.3.3 Tumour take in the presence of TG2

Given that administration of the enzyme into the tumour required multiple injections into the tumour mass, it was of interest to investigate the effect of TG2 on CT26 tumour growth when the enzyme is injected in synergy with the tumour cells. The distinct advantage of this model is that TG2 is expected to incur its cross-linking effect from the outset of the tumour growth, and in the locality of the primary tumour load within the ECM of connective stromal tissue. As shown in figure 3.3.3, inoculation of CT26 cells into balb/c mice in the presence of 50μg/ml active (2.5U/injection) *gpl*TG2, resulted in attenuated tumour growth, leading to TG2-treated animal population having an increased percentage of survival of approximately 25%.

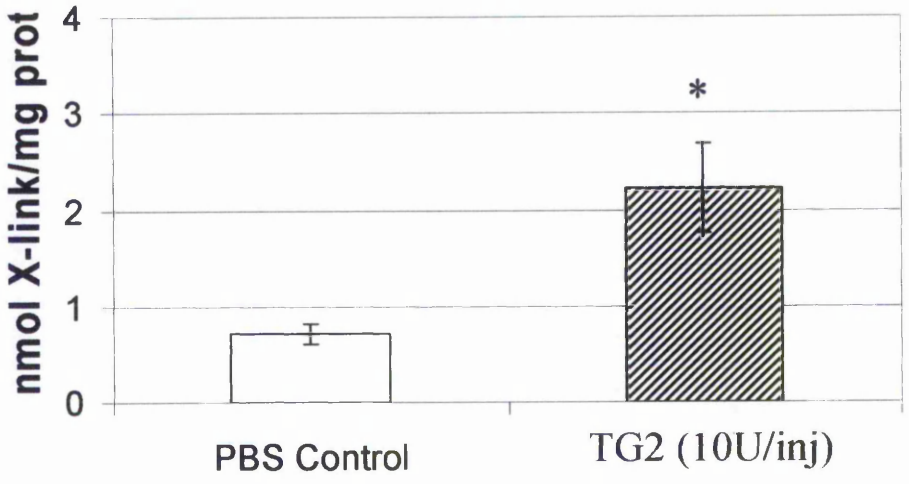


Figure 3.3.2.6.1 Crosslink analysis of control and regressed CT26 tumours

Following treatments, tumour specimens were harvested and homogenised to a 20% (w/v) homogenate as described in section 2.2.3.1 , prior to precipitation in 20% (v/v) TCA and proteolytic processing as described in the Methods (section 2.2.6.5). Following sequential washing in diethyl ether/ ethanol (1:1), and diethyl-ether only, protein rich pellets were air-dried before being resuspended in 50µl of 0.1M $(NH_4)HCO_3$ in the presence of traces of thymol. Cell homogenates were then subjected to a series of sequential proteolytic digestions, the samples were freeze-dried and cross-link analysis was carried out by anion exchange chromatography using a LKB 4151 amino acid analyser with lithium citrate buffers as outlined in section 2.2.6.5. The surface areas of the resulting cross-link peaks were measured and the amount of the crosslink dipeptide was quantified by reference to a ε(γ-glutamyl)lysine dipeptide standard curve prior to normalisation against the protein content of the proteolytic digests. Results represent mean values ±SD, from 4 specimens.* Indicates significantly different ($p<0.05$) from the control.

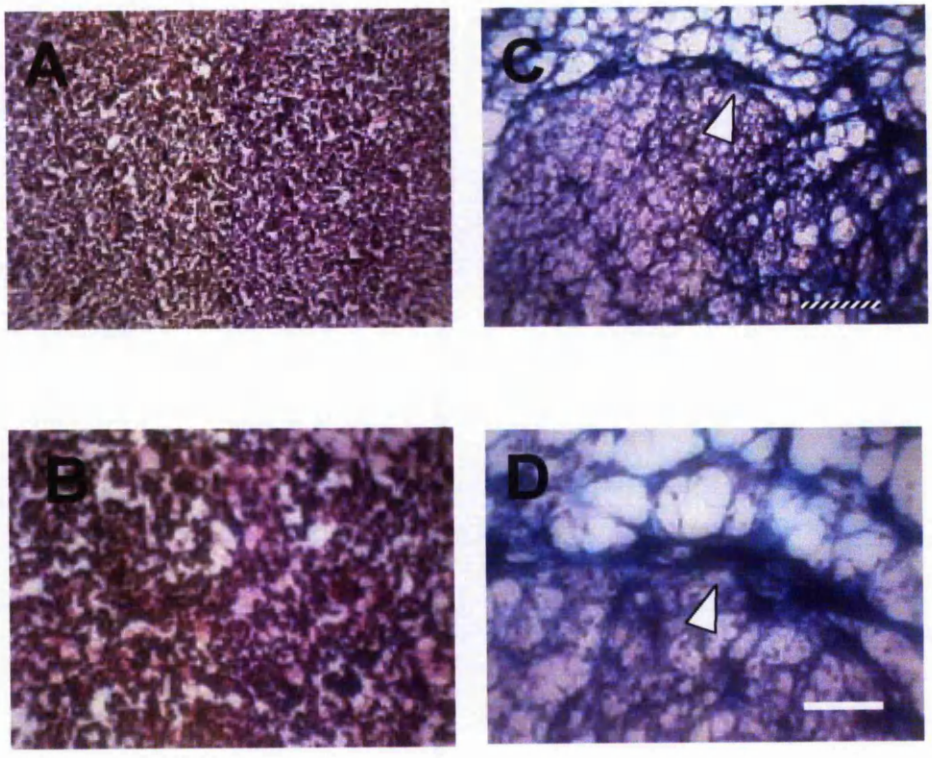


Figure 3.3.2.6.2 Total collagen deposition in control and regressed CT26 tumours receiving 10U/inj TG2

PBS and TG2-treated (10U/inj) CT26 solid tumours were harvested and sectioned as described in Methods (sections 2.2.5.3 and 2.2.6). Sections were then stained with Masson's Trichrome according to the manufacturer's protocol prior to imaging using a *Zeiss* light microscope attached to an *Olympus* digital camera. **A-B**, low and high magnification images of PBS treated tumour staining where collagen structures are largely absent. **C-D**, low and high magnification images of TG2-treated tumour staining where network of collagen fibres (dark blue) appears to encapsulate the tumour cell mass (purple) as indicated by the arrows. Images are representative of three non-overlapping fields (n=6). Striped bar = 100μm; white bar = 50μm.

Figure 3.3.2.6.3 OH-proline content of TG2-treated (10U/inj) and control CT26 tumours

Control and regressing tumour specimens (10U/inj TG2) were homogenised as described in section 2.2.3.1 to yield a 20% homogenate and, following hydrolysis in 6M HCl at 100°C and for 16 hours, amino acids were separated following a solid-phase extraction/derivatisation protocol using the *EZ-Faast* amino acid analysis kit as described in the Methods (section 3.2.2). The total amino acid and OH-proline (HYP) content of control and TG2-treated CT26 tumours was measured against internal amino acid standards by Gas Chromatography using a 9890N Network GC system. **A**, representative amino acid profile (in nmoles) of PBS-treated tumours. **B**, representative amino acid profile (in nmoles) of TG2-treated tumours. **C**, collagen (OH-proline content) is expressed as a percentage of total amino acids (nmoles). Results represent mean values ±SD, from 4 specimens.* Indicates significantly different ($p<0.05$) from the control.

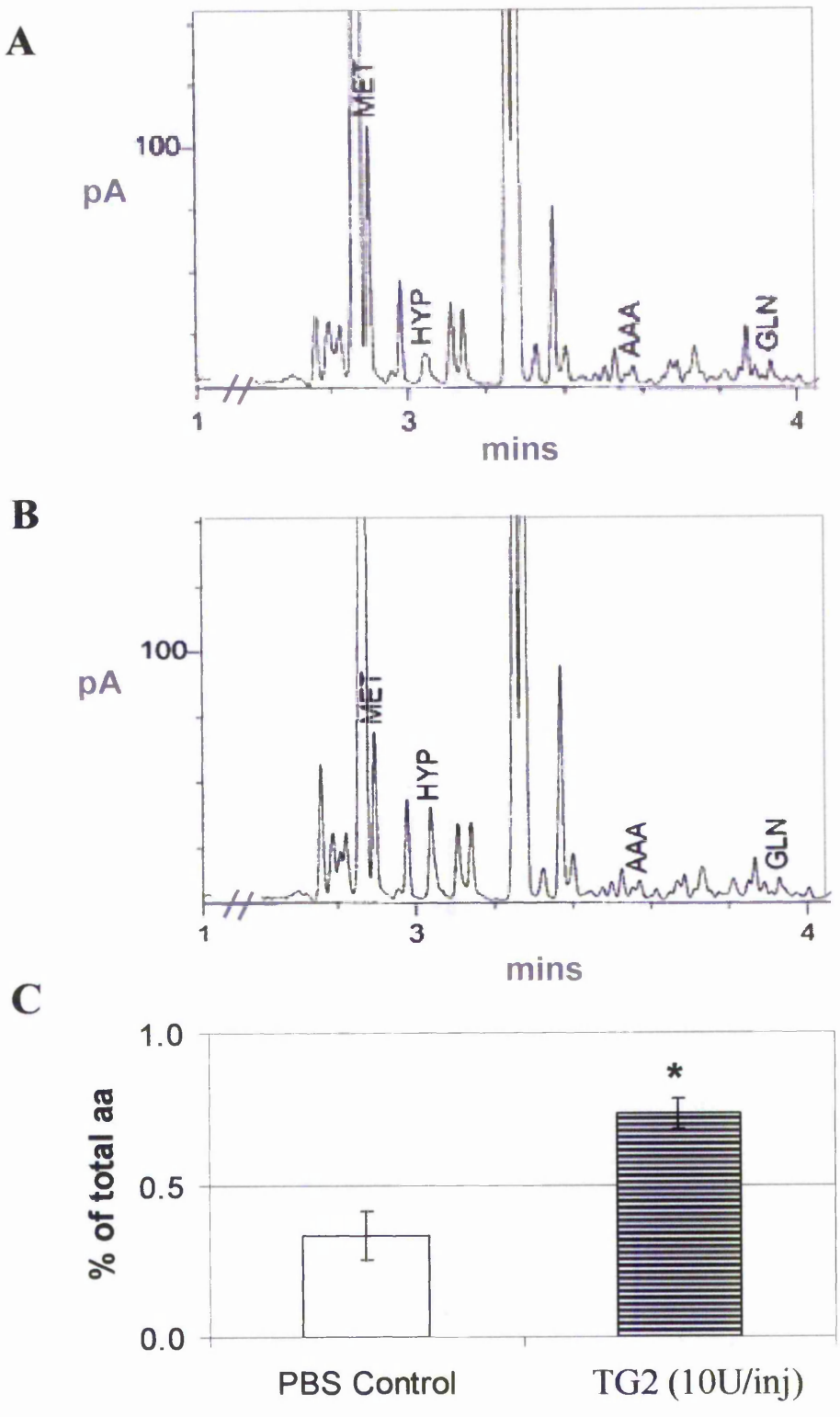


Figure 3.3.6.3 OH-proline content of TG2-treated CT26 tumours

Figure 3.3.3 CT26 tumour take in the presence of active *gpl*TG2

Exponentially growing CT26 were harvested and resuspended to a final concentration of 10^6 cells/ml in DMEM, prior to subcutaneous implantation of 100µl cell suspensions either in either 3mM DTT/5mM $Ca2^+$ or 50µg/ml active (2.5U/injection) *gpl*TG2 containing 3mM DTT/5mM $Ca2^+$ into 7-9 weeks old balb/c mice. Following injections, animals were monitored every two days for tumour development. **A,** average tumour growth rate of animals, expressed as mean tumour size (mm^2) over time, was measured up to 41 days post-injection. **B,** percentage animal survival following injection of the CT26 cells. Animals were sacrificed once tumours reached approximately the $1cm^2$ limit. Results represent mean value ±SD (n=8). Squares, CT26; triangles, CT26-TG2. * Indicates significantly different ($p<0.05$) from the control.

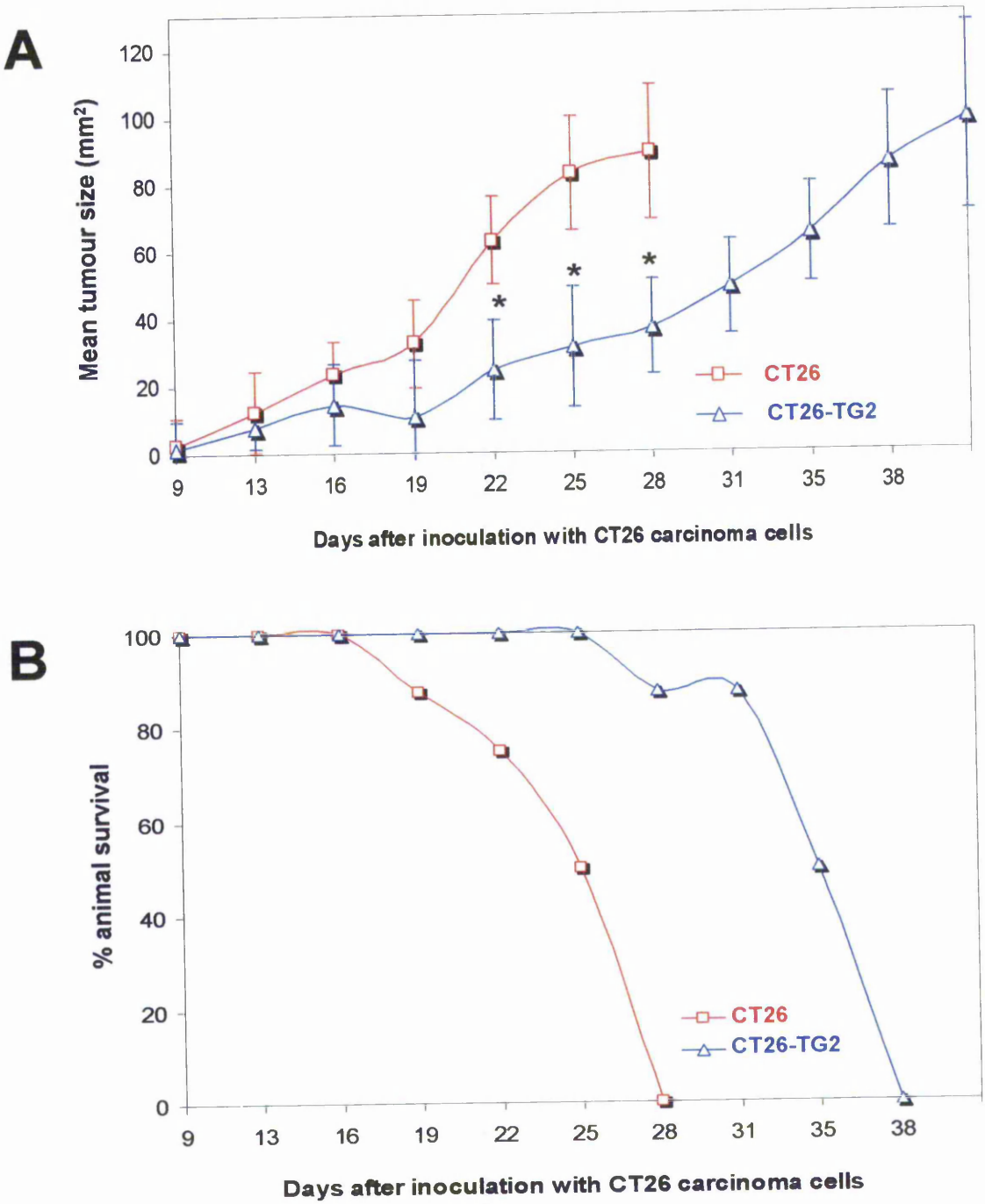


Figure 3.3.3 Tumour take in the presence of active *gpl*TG2

3.4 Discussion

Central to this study was the aim of establishing whether TG2 is of physiological importance within the host tissue during solid tumour growth and progression. To address this, a tumour model in which the C57BL/6 mouse host had its TG2 gene disrupted was employed (De Laurenzi and Melino, 2001). When such TG2 transgenic animals were challenged subcutaneously with B16-F1 mouse melanoma tumour cells, the resulting tumours were shown to grow faster in TG2-null mice, compared to their wild-type counterparts, which survived for longer following tumour cell challenge. Although no major developmental or organ abnormalities have been reported in the knock-out animals so far (DeLaurenzi and Melino, 2001; Nanda *et al.*, 2001), histological staining of the tumour-host microenvironment revealed that B16-F1 tumour cells had an increased ability to infiltrate and colonise the connective stromal tissue of the knock-out animals. When the tumour-host interface was analysed by indirect immunofluorescence, TG2 antigen appeared to be limited to the healthy host tissue of the wild-type animals, where it is believed to reinforce the ECM, thus posing a better barrier to tumour cell invasion (Hettasch *et al.*, 1996; Haroon *et al.*, 1999b). Western blotting of tumour homogenates confirmed that TG2 is not expressed by B16-F1 cells, whereas activity profiling of the same homogenates using a radioactive [^{14}C]-putrescine assay revealed residual TG activity within the tumour. Taken together, these findings accentuate the importance of TG2 as a stress response protein expressed within the tumour host, suggesting that although certain tumour cell lines are incapable of producing TG2, the ability of the host tissue to do so partially hinders tumour growth invasion. Given that TG2 is now recognised as a wound response enzyme (Verderio *et al.*, 2004), these findings suggest that the enzyme may have a role in the host's defense mechanism against tumour growth. Evidence suggests that the host tries to elicit a wound response against the tumour by encapsulating it at the tumour edges (Hettasch *et al.*, 1996). Unfortunately, this model proved inappropriate for the study of experimental metastasis as no macroscopic melanogenic foci were identified in the parenchymal tissue of the major metastatic targets (liver, lungs) 32 days post tumour cell injection.

Following confirmation that stromal TG2 plays a protective physiological role within the tumour-host interface, further experiments were designed to determine whether exogenous TG2 may have a therapeutic role when delivered intratumourally. Exogenous administration of active (10U/injection) TG2 into a tumour mass of CT26 origin was shown to result in slowed tumour growth, with 25% of the tumours showing regression. Interestingly, attenuation of tumour growth appeared to be dose specific, and was not achieved when TG2 of higher (100U/injection) or lower activity (2U/injection) was applied to the tumours. In light of immunohistochemical evidence indicating homogeneous diffusion of the enzyme only in tumours receiving 'medium' TG activity dose of 10U/injection, it is believed that the dosing activity of an enzyme with such a high affinity for many ECM proteins is critical for its homogeneous perfusion throughout the tissue. Similarly, when TG2 of lower activity is administered to the tissue, ECM cross-linking and accumulation may be insufficient for the development of the fibrotic phenotype.

Using a different approach, we also assessed the possible effect of exogenous TG2 on tumour take. When the CT26 carcinoma cells were inoculated in the presence of TG2 in balb/c hosts, a delay in tumour growth and increased animal survival was observed compared to the relative controls. The antitumour function of TG2 is attributed solely to its transamidating activity as application of NTU283-inactivated TG2 had no significant effect on tumour growth. This comes in agreement with previous reports where tumour growth delay due to application of recombinant TG2 in a dorsal skin flap window chamber was not reproduced following application of the inactive enzyme (Haroon *et al.*, 1999a). Intratumour treatment with the active site directed inhibitor NTU283 (500μM) did not have any effect on tumour growth, possibly due to the reported lack of intrinsic activity within the tumour.

At this stage it was also of interest to explore whether the observed tumour growth delay due to exogenous TG2 application was due to a direct effect on the tumour cell population. Our findings were incongruent with previous studies suggesting that TG2-mediated increase in cellular proliferation (Birckbichler *et al.*, 1978; Mian *et al.*, 1995) or cell death (Piacentini *et al.*, 1999; Piacentini *et al.*, 1995) is responsible for tumour

regression, as we report that *in vitro* CT26 malignant cell proliferation, viability and apoptosis remained unaltered when measured following two daily exogenous TG2 administrations into the cultures (50μg/ml; 50U/ml). Given the high affinity of TG2 for various major ECM components, (section 1.9.4.4.1), it was predicted that its application into tissue would cause excessive protein crosslinking. As expected, chromatographic quantification of the ε(γ-glutamyl)lysine isopeptide bond content of the tumours treated with the enzyme revealed increased number of crosslinks in the tumour core when compared to the controls. Pertinent to this observation, total collagen staining of the tumour ECM, exposed an accumulation of this predominant structural ECM component in TG2-treated tumours, as reported previously (Haroon *et al.*, 1999b). This finding was strengthened by further biochemical evidence, as increased OH-proline content was detected within TG2–treated tumours by Gas Chromatography. Numura *et al.* (2001) reported that exogenous TG2 accelerated shark collagen fibril/gel formation. Once crosslinked into the ECM by TG2, collagen has also been shown *in vitro* to confer resistance to breakdown (Gross *et al.*, 2003). Further studies have also demonstrated that *in vitro* crosslinking of collagen and collagen/fibronectin mixtures leads to an increased resistance to their breakdown by MMP-1 (section 5.3.3).

TG2 has been reported to be important in wound healing and tissue fibrosis where it has been demonstrated to have the ability to stabilise the integrity of tissue by crosslinking of both intracellular and extracellular proteins. Extensive tissue damage, has been so far correlated in numerous studies with the leakage of the enzyme into the ECM environment contributing in the development of fibrosis in various tissues, such as the lung (Griffin *et al.*, 1979), the liver (Mirza *et al.*, 1997; Piacentini *et al.*, 1999; Grenard *et al.*, 2001), the heart (Small *et al.*, 1999), and the kidney (Johnson *et al.*, 1997 and 1999). Utilising the etiology of the fibrotic condition, we aimed to contain tumour cells within the tumour fibrous coat, by inducing '*in vitro* scarring' through multiple TG2 intratumour injections. Indeed, when CT26 cell invasion through artificial collagen-fibronectin matrices, was measured by means of fluorescence, matrices crosslinked by TG2 were shown to pose a better barrier to the invading malignant cells. However, it could not be ruled out that TG2-crosslinked matrices provide further RGD-independent attachment sites for the

CT26 cell line (Verderio *et al.*, 2002). Giancotti and Ruoslahti (Giancoti and Ruoslahti, 1991) reported that overexpression of functionally active α5β1 complexes suppressed the ability of Chinese hamster ovary cells (CHO) to form tumours in nude mice, as a direct result of increased tumour cell attachment. As a consequence, increased CT26 cell adhesion to a TG2-rich tumour-stroma microenvironment might also limit their mobility and consequently their tumourigenicity. However, CT26 invasion through artificial matrices containing TG2 denuded of its activity was comparable to the collagen-fibronectin control matrix, whereas injections of inactive enzyme led to unaltered tumour growth rates when compared to the controls.

Chapter 4:

The role of tissue transglutaminase in angiogenesis

4.1 Introduction

In the previous chapter it was established that TG2 expressed by the tumour host is exerting an inhibitory effect on tumour development and that the application of exogenous enzyme also leads to delayed tumour growth. It was also hypothesized that this is due to increased ECM protein cross-linking at the tumour-stroma interface since a pronounced decrease in the invasion of CT26 tumour cells through TG2-crossliked collagen-fibronectin matrices was demonstrated. As a continuum to this study, and given the added necessity for new blood vessel formation during tumour growth (Liotta et al., 1974), this chapter aims to investigate the effect of endogenous and exogenous TG2 on *in vitro* and *ex vivo* vascular growth.

TG activity was first identified in the endothelium by Greenberg and co-workers in 1987, a finding that was later confirmed by various studies (Korner et al., 1989; Auld et al., 2001). Recently, it has also been demonstrated that TG2 is expressed in its active form in rat dermal wound healing and the accompanying angiogenesis (Haroon et al., 1999a). Previous data based on HUVEC cells as well as the endothelial-like cell line ECV304 had suggested that TG2 was involved in the cross-linking of ECM proteins and that the enzyme's activity contributed to both BM assembly and the retardation of EC adhesion and migration (Greenberg et al., 1987; Martinez et al., 1994; Jones et al., 1997). It is therefore tempting to hypothesise that TG2 activity may be required for endothelial cell tube formation. However, to date, whilst some reports indicate that transglutaminase activity is essential for angiogenesis (Haroon et al., 1999a; Dardik et al., 2003), others claim it is inhibitory (Haroon et al., 1999b). In an in vivo dorsal window flap model, topical application of 40μg/ml TG2 post-surgery was found to inhibit both growth of new vasculatures and tumour development, when a solid piece of the R3230Ac tumour was implanted post-surgery to the wound (Haroon et al., 1999a). In contrast, the same authors demonstrated, using a similar, this time tumour-free window flap model, that three separate doses of active TG2 added on days 0,1, and 2 post-surgery, led to a stimulation of angiogenesis (Haroon et al., 1999b). Such differences are difficult to reconcile given the number of variables present in the two experiments. However, in an in vitro study, it has been recently reported that

addition of exogenous recombinant gpITG2 led to a decrease in vessel density (Jones and Griffin; UK Patent W00211747). On the basis of this finding, it is conceivable that TG2 may serve as a therapeutic agent for inhibiting angiogenesis and consequently attenuating solid tumour development.

The formation of new blood vessels out of pre-existing capillaries is fundamental to many physiological and pathological processes such as cancer, chronic inflammation and ischemic diseases. It is now well-recognised that interference with vasculature formation offers a tool for clinical applications, as stimulation of angiogenesis may prove beneficial in the treatment of diseases such as coronary artery disease and critical limb ischemia in diabetes, whereas inhibition of angiogenesis can prevent others marked by excessive vessel growth such as diabetic retinopathy, arthritis, and most importantly cancer. Approaches to controlling angiogenesis have, to date, attempted to inhibit EC proliferation, migration and invasion (Sidky et al., 1987; Brown et al., 1995). Numerous strategies have been adopted such as gene therapy (Albini et al., 2000), monoclonal antibodies (Corada et al., 2002) and peptides (O'Reilly et al., 1997). However, limitations lie in both the delivery and the reliability of these agents (Joki et al., 2001), particularly with respect to their use as antineoplastic agents (Eisterer et al., 2002).

Angiogenesis is primarily driven by numerous mediators such as NO, HIF, VEGF, uPA, tPA, bFGF, TGFβ, MMPs, TIMPs produced by a number of cells under a variety of conditions as reviewed in section 1.9.6.4.5. Vascular endothelial growth factor (VEGF) is secreted by hypoxic tumour cells, macrophages and other cells of the immune system and constitutes the major signal for the initiation of angiogenic sprouting. VEGF binds to VEGFR-1 and 2 receptors on ECs, leading to their dimerisation. Intracellular signal transduction pathways in ECs mediated through VEGF-2 dimerisation lead to permeability enhancement, EC proliferation, migration and survival (Abedi and Zachary, 1997; Yu and Sato, 1999). In a similar manner, bFGF dimerisation in response to binding of FGF to tyrosine kinase receptors induces EC proliferation, differentiation and migration (Kanda et al., 1997). On the other hand, transforming growth factor-β1 (TGFβ) inhibits the proliferation, migration and assembly of ECs through Smad4 signalling during angiogenesis and stimulates the production of protease inhibitors thus favouring ECM depostion and

accumulation (Schwarte-Waldhoff et al., 1997; Roberts et al., 1986). Interestingly, it has been demonstrated that, by modulating matrix storage of Latent TGFβ (LTBP-1), TG2 participates in the activation of this cytokine (Taipale et al., 1994; Verderio et al., 1999).

One of the aims of this study is to investigate the effect of exogenous TG2 on the release of these signalling molecules (VEGF, bFGF and TGFβ) during in vitro angiogenesis. Using an ex vivo angiogenesis model whereby aortic explants from TG2 wild type and knock-out mice are used, a further aim is to ascertain whether TG2 has a physiological role in neovascularisation. Additionally, the effect of increased amounts of extracellular TG2 on EC sprouting and capillary formation will be assessed with a view to identify whether the mechanism by which TG2 alters EC sprouting lies in its ability to stabilise the ECM via extensive protein crossliking.

4.2 Methods

4.2.1 Angiogenesis models

Numerous in vivo assays have been developed so far to study angiogenesis and involve examination of neovessels in microcirculatory preparations (corneal micropocket, hamster cheek pouch, transparent chambers on flanks and ears, and the chick chorialandoic assay), vascularised biopolymers (subcutaneously implanted matrigel or collagen pugs) and excised experimental tumours (Jain et al., 1997). While these models provide a tool for the study of the complex regulation of angiogenesis in vivo, there are a number of problems associated with their use. Many use non-human tissue, are time-consuming, expensive, and difficult to interpret.

In order to elucidate the physiological importance of TG2 in angiogenesis an ex vivo model based on EC outgrowth from intact TG2 transgenic C57BL/6 mouse aortas (TG2 $^{-/-}$and TG2 $^{+/+}$) was used. The possibility that exogenous TG2 might alter angiogenesis was assessed by administering the enzyme into neovasculatures of either a commercial or an in-house in vitro co-culture model of HUVEC and human foreskin dermal fibroblasts (HFDF). Additionally, in an ex vivo model of angiogenesis, capillaries formed following rat aortic explant embedment into matrigel were treated with exogenous TG2.

4.2.1.1 *In vitro* angiogenesis model based on co-culture of HUVEC and HFDF cells

HUVEC (TCS Cellworks) and HFDF cells (isolated from human foreskin as described in section 2.2.1.6) were co-cultured in 24-well tissue culture plates at a seeding density of 20,000 total cells/well and ratios ranging from 1:2 to 3:1. Cells were allowed to grow in large vein EC growth medium (EGM) containing EC growth supplement, 2% (v/v) FCS, with regular media changes as described previously (Bishop *et al.*, 1999). Following a 10-day incubation, cultures were washed twice with PBS pH 7.4, fixed in ice cold 70% (v/v) ethanol, and non-specific binding sites were blocked in 3% (w/v) BSA in PBS. Endothelial

cells were then probed for 1 hour and at 37°C with sheep anti-human von Willebrand factor (vWf) antisera diluted 1:200 in blocking buffer, and washed three times in PBS, prior to incubation with an anti-sheep HRP secondary antibody diluted 1:400 in blocking buffer for 1hour at 37°C. Following another three washes in PBS, colour development was achieved with the addition of chromogenic HRP substrate diaminobenzidine (FastDAB), supplemented with H_2O_2 and metal enhancer. Finally, capillaries were desiccated for 30 minutes at room temperature, and light microscopy images were obtained using an Olympus digital camera (Olympus, London, UK).

Once the optimum seeding densities and ratios (HUVEC/HFDF) were identified by light microscopy, fresh co-cultures were treated sequentially, 2-days post-seeding and at 2-day intervals, with 50μg/ml active TG2 (50U/ml), or TG2 inactivated prior to addition as described in Methods (section 2.2.2.2.1). Once the dosing regime was completed on 10-day, ECs were probed for vWf as described above.

4.2.1.2 TCS *in vitro* angiogenesis kit

Angiogenesis was also modeled *in vitro* using the TCS Cellworks (*Buckinghamshire*, UK) angiogenesis kit following the manufacturer's protocol (Graham *et al.*, 1999). Briefly, on arrival of the kit, 0.5 ml of fresh media was added to each well of the plate and cells were allowed to grow at 37°C, 5% CO_2 (v/v) in a humidified atmosphere. Following a 2-day incubation cultures were treated daily and for four days with 50μg/ml active TG2 (50U/ml), or TG2 inactivated prior to addition as described in Methods (section 2.2.2.2.1). Once the dosing regime was completed on 10-day after the arrival of the kit, angiogenesis cultures were fixed in ice cold 70% (v/v) ethanol for 30 minutes at room temperature, blocked with 3% (w/v) BSA in PBS and stained for vWF by incubation with a sheep anti-vWF polyclonal antibody (TCS Cellworks) diluted 1:200 in blocking buffer. Following 3 washes in PBS, staining was revealed by incubation with an anti-sheep-HRP (Sigma) conjugate diluted 1:400 in blocking buffer for 1 hour at 37°C, and developed with FastDAB substrate (Sigma) supplemented with H_2O_2 and metal enhancer. Finally, capillaries were desiccated for 30

minutes at room temperature, and light microscopy images were obtained using an Olympus digital camera. Quantitative image analysis was performed using QWin software (Leica).

4.2.1.3 *Ex vivo* ring aorta angiogenesis models

These models are based on the ability of aortic ECs to detach, migrate and rapidly form tubules on a gelatinous mixture of extracellular and basement membrane proteins (matrigel) derived from the mouse Engelbreth-Holm-Swarm (EH) sarcoma as described prevously (Lawley *et al.*, 1989; Nicosia and Ottinetti, 1990). Aortas were obtained from either 4-6 week old rats weighing approximately 200g or 6 week old $TG2^{+/+}$ / $TG2^{-/-}$ mice. The midline saggittal section was cut through the diaphragm exposing the chest cavity including the heart and lungs. The aorta was freed from the back wall of the chest cavity and placed in Hank's Balanced Salt Solution (HBSS; Sigma, Poole, Dorset). Residual blood was removed by washing three times in HBSS, and the fat tissue was peeled off mechanically using a scalpel. The aorta was kept for 20 minutes in HBSS containing 25% (v/v) penicillin/ streptomycin, washed again and cut into 1mm rings using a scalpel. Aortic rings were placed in the center of the wells of a 48-well tissue culture plate which had been pre-coated with 120µl of matrigel (Becton Dickinson, Cedex, France) and sealed in place with an extra 50µl of matrigel as described previously (Nicosia and Ottinetti, 1990). Detaching cells were maintained in large vein EC growth medium containing EC growth supplement (ECGS), 5% (v/v) FCS. Following a 10-day incubation, when EC detachment and concomitant outgrowth from the rat/mouse tissue could be observed, pictures of live cells were captured using an Olympus digital camera attached to a light microscope. In a separate experimental setup, rat aortic rings were also treated sequentially with 50µg/ml exogenous active (50U/ml) or inactive *gpl*TG2 for 4 days at daily intervals. Microvessel formation was monitored and capillaries were fixed and stained for a marker of angiogenesis after 14 days as described before (section 4.2.1.2).

4.2.2 Human TGFβ1, bFGF and VEGF immunoassays

Human TGFβ1, bFGF and VEGF present in the media of human angiogenesis cultures were quantified using Quantikine sandwich enzyme immunoassay kits (R & D Systems Europe, Abingdon, UK) according to the manufacturer's protocol. Briefly, human recombinant TGFβ1, bFGF or VEGF standards and diluted conditioned media collected from *gpl*TG2-treated angiogenesis cultures (section 4.2.1.2) were added to separate microtiter plates pre-coated with monoclonal antibodies specific for TGFβ1, bFGF or VEGF. Following washing of unbound substances, enzyme-linked polyclonal antibodies conjugated to HRP and specific for TGFβ1, bFGF or VEGF were added to the wells, prior to washing and incubation with a chromogenic substrate (DAB) supplemented with H_2O_2 for 20 minutes at room temperature. Subsequent to colour development the reaction was stopped by addition of 2N sulfuric and plates were read using a SpectraFluor plate reader at 450nm.

4.3 Results

4.3.1 Delayed ex-vivo endothelial cell outgrowth from TG2 deficient mouse aortas

Previous studies have demonstrated that TG2 was involved in the cross-linking of ECM proteins and TG activity contributed to both basement membrane assembly and cell adhesion during capillary formation (Greenberg et al., 1987; Martinez et al., 1994). To ascertain whether TG2 is of physiological importance in the EC migation event leading to capillary formation, aortas extracted from TG2 wild-type and knock-out mice were embedded into matrigel as described before (section 4.2.1.3). As shown in figure 4.3.1, ECs TG2 deficient were shown to detach and migrate more readily on this artifical basement membrane matrix over a period of 10 days, when compared to their wild-type counterparts. Taking into account that one of the major protein components of matrigel is collagen, this finding is not contradicting previous reports sugesting that degradation of cell surface TG2 by MMPs leads to increased cell migration on artificial collagen matrices (Belkin et al., 2001).

4.3.2 Inhibition of *ex-vivo* angiogenesis in a rat aorta model following exogenous TG2 application

Having established that intrinsic TG2 expression is having a negative effect on the migration of ECs originating from wild-type $TG2^{+/+}$ mouse aortas, we next sought to examine the effect that TG2 may elicit when present at high, non-physiological levels within the extracellular environment. To address this, an *ex vivo* rat aorta model of angiogenesis was initially employed. Rat aortic rings were embedded in matrigel (as in section 4.3.1) and migrating ECs were maintained in EC media containing EC growth supplement (ECGS) and 5% FCS for 14 days as described previously (section 4.2.1.3). Sequential treatment of explants with 50µg/ml (50U/ml) *gpl*TG2 for 4 consecutive days clearly inhibited microvessel formation

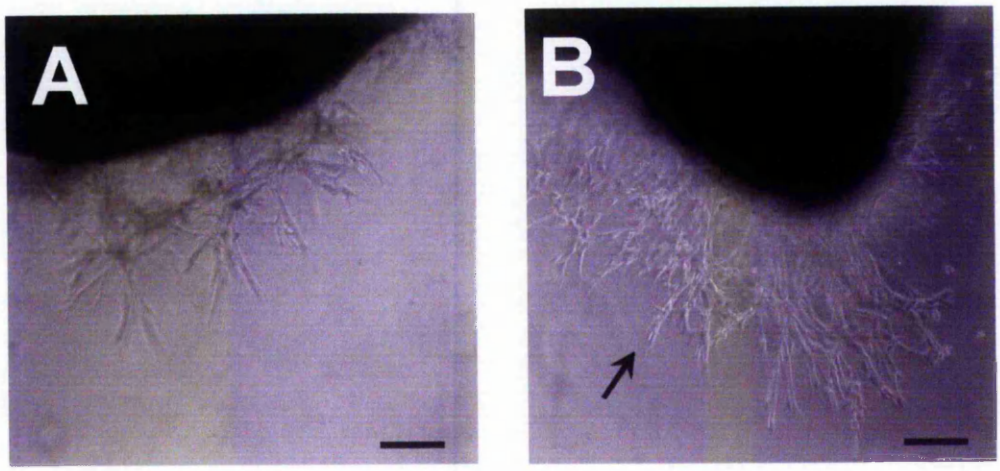


Figure 4.3.1 Endothelial cell outgrowth from $TG2^{+/+}$ and $TG2^{-/-}$ mouse aortas

1-2mm aortic rings were obtained from 6-8 weeks old $TG2^{+/+}$ and $TG2^{-/-}$ C57BL/6 mice as outlined previously (section 4.2.1.3) and embedded in matrigel basement matrix, where endothelial cells were allowed to detach and migrate for 10 days. **A**, phase contrast light microscopy image of live endothelial cell outgrowth arising from the TG2 wild-type mouse aorta on day 10 after explant embedment into matrigel. **B**, phase contrast light microscopy image revealing increased EC migration and sprouting from the intrinsically TG2 deficient aorta 10 days after explant embedment (arrow). Images are representative of 3 non-overlapping fields (n=6), from two independent experiments. (Bars=50μm).

(figure 4.3.2). This is in contrast to the untreated rat aorta endothelial cultures where ECs appeared to align to form mature interconnected capillary tubes in a mesh-like network. Cultures receiving 4 applications of exogenous TG2 revealed undifferentiating ECs with no vascular oganisation (figure 4.3.2).

4.3.3 Inhibition of angiogenesis in an in-vitro HUVEC and HFDF co-culture model

In order to investigate the effect of exogenous *gpl*TG2 on *in vitro* angiogenesis, an in-house quasi-two-dimensional co-culturing model of HUVEC and HFDF cells was also set up and optimized as described previously (Bishop *et al.*,1999). HUVEC and HFDF were co-cultured in 24-well tissue culture plates at a seeding density of 20,000 total cells/well and ratios ranging from 1:2 to 3:1. Cells were allowed to grow and migrate for 10 days and, following staining with an EC marker (vWF), EC structures were visualised and assessed for signs of angiogenesis (figure 4.3.3.1). In the 3/1 (HUVEC/HDF) co-cultures, ECs appear undifferentiated exhibiting lack of the characteristic thread-like structure, possibly due to diminished basement membrane protein deposition by the fibroblast cell population. In the 1/1 (HUVEC/HDF) co-cultures, thread-like structures are more prominent. Co-cultures of HUVEC/ HFDF appear to generate complete anastomising capillary networks when seeded at a 1/3 ratio, as after reaching a certain distance from parent cells, ECs bifurcate and branches tend to incline toward each other and fuse together.

Having established that the 1/3 HUVEC/HFDF ratio was the optimal for capillary tube formation, co-cultures were treated exogenously and at daily intervals with 1, 2, and 4 doses (50μg/ml) of either active (50μg/ml), or *gpl*TG2 that has been inactivated as described previously (section 2.2.2.2.1) or with 1mM of the TG2 active site directed inhibitor NTU283. As shown in figure 4.3.3.2, maximum inhibition of angiogenesis is achieved by 4 doses (50μg/ml) of active (50U/ml) TG2 (figure 4.3.3.2*E*). Interestingly, following 4 daily doses with NTU283 alone, endothelial cells appeared more differentiated into vessels as shown in figure 4.3.3.2*F*.

Figure 4.3.2 The effect of exogenous *gpl*TG2 on *ex vivo* rat aorta angiogenesis

Aortas were obtained from 10 weeks old rats and implanted into matrigel as described previously (section 4.2.1.3). **A**, phase contrast image of endothelial cell outgrowth 8 days after rat aorta explant embedment in matrigel. **B**, light microscopy of tubules stained for the endothelial marker vWF 14 days after explant embedment as described previously (section 4.2.1.2). Arrow indicates the position of the aortic ring explant. Once outwardly migrating endothelial cells were detected on days 8-9 of this *ex vivo* aortic angiogenesis model, cells were treated with four 50μg/ml doses of active (50U/ml) gpl TG2 at daily intervals. **C, D**, live, low (2x) and medium (4x) magnification light microscopy images illustrating capillary formation (arrows) in matrigel, in the absence of exogenous TG2 treatment, 14 days following embedment. **E, F**, live, low and medium magnification respectively of light microscopy images revealing absence of microvessel formation as result of 4 sequential administrations of 50μg/ml (50U/ml) gpl TG2. Images are representative of 3 non-overlapping views per well (n=5), from 3 independent experiments. Bars= 25μm.

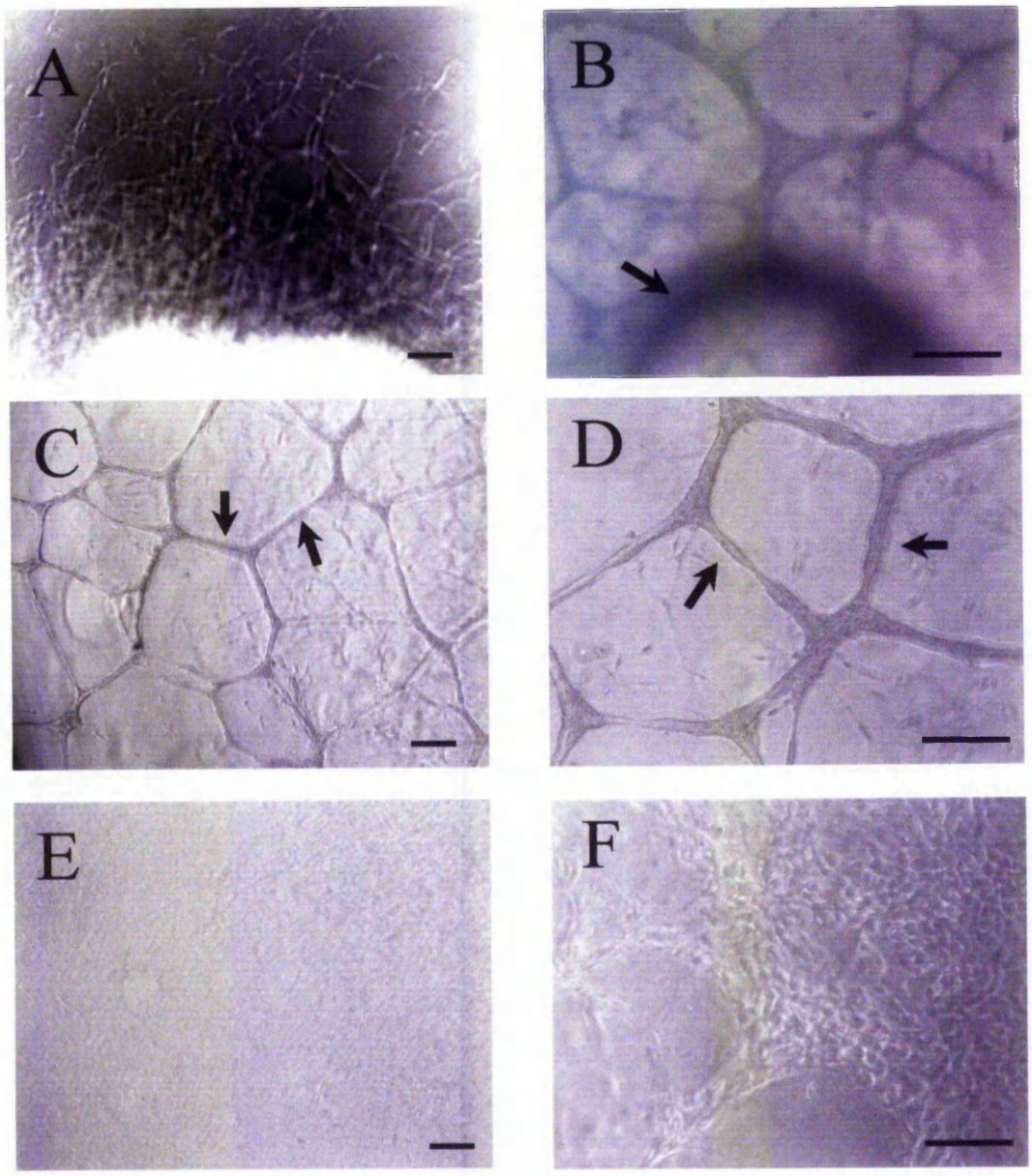


Figure 4.3.2 The effect of exogenous *gpl*TG2 on *ex vivo* rat aorta angiogenesis

Figure 4.3.3.1 Optimisation of an *in vitro* angiogenesis model based on the co-culture of HFDF and HUVEC

HUVEC and HFDF cells were co-cultured in 24-well tissue culture plates at a seeding density of 20,000 total cells/well and ratios ranging from 1:2 to 3:1 as described previously (section 4.2.1.1). Cells were allowed to grow for 10 days with regular media changes. Endothelial cells were then probed with sheep anti-human vWf antisera prior to conjugation to an anti-sheep HRP secondary antibody, and colour development was achieved by addition of DAB supplemented with H_2O_2 and metal enhancer as outlined previously (section 4.2.1.1). **A**, HUVEC/HDFB = 3/1. **B**, HUVEC/HDFB= 2/1. **C**, HUVEC/HDFB= 1/1. **D**, HUVEC/HDFB = 1/2. **E**, HUVEC single cell type control. **F**, HFDF single cell type control. Images are representative of three non-overlapping views (n=3), from 2 independent experiments. Arrows indicate communication networks between EC capillary tubes (anastomosis). Bar=50μm.

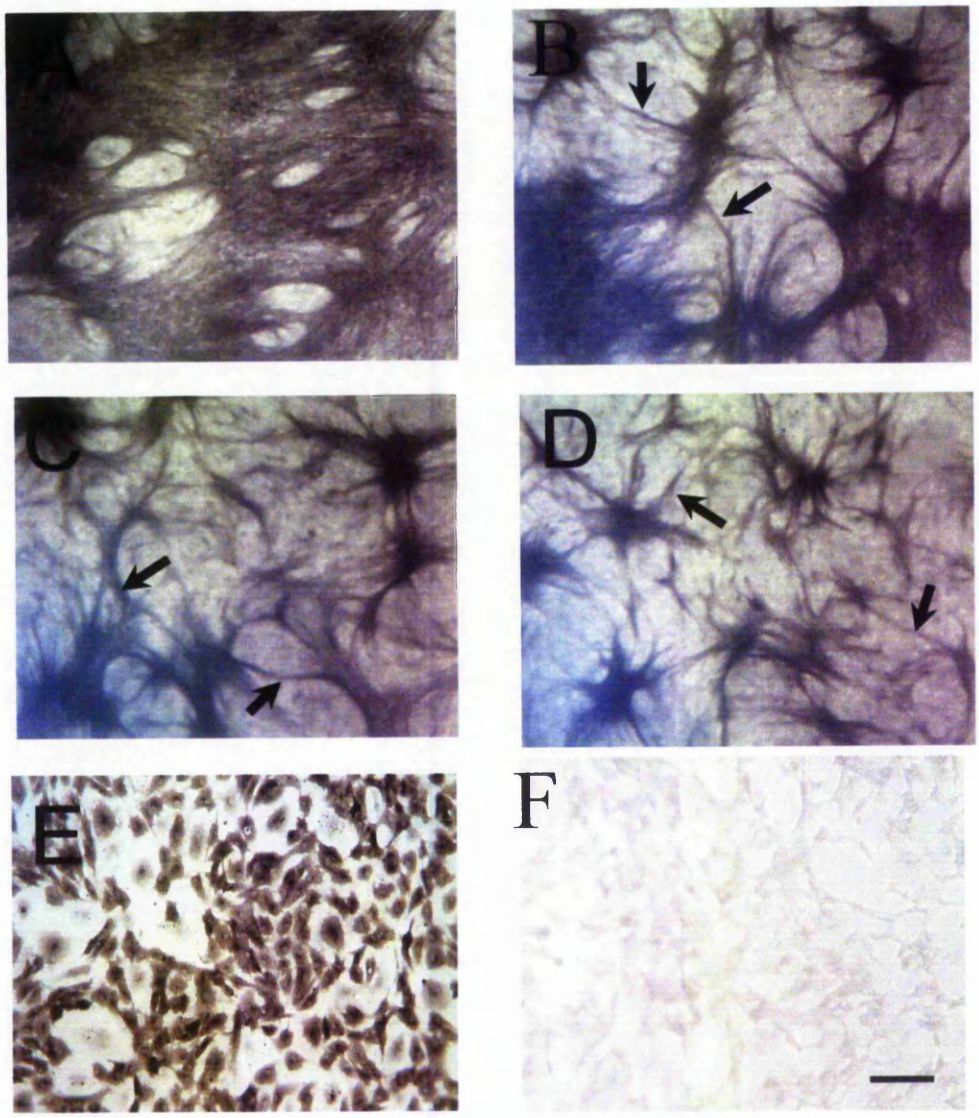


Figure 4.3.3.1 Optimisation of an *in vitro* angiogenesis model based on the co-culture of HFDF and HUVEC

Figure 4.3.3.2 Effect of exogenous *gpl*TG2 on *in vitro* angiogenesis

Human endothelial cells (HUVEC) and fibroblasts (HDFB) were co-cultured at 20,000 total cells/well (1/2) in 24-well tissue culture plates prior to treatment with 1, 2, and 4 daily doses of 50μg/ml of either active (50U/ml) or inactive TG2 inactivated prior to addition as outlined in the Methods (section 2.2.2.2.1). Following fixation and non-specific binding blocking, endothelial cells were then immunostained for vWf as described previously (section 4.2.1.1) and images were obtained using an *Olympus* digital camera attached to a light microscope. **A**, untreated cultures. **B**, cultures treated with 1 dose of 50μg/ml TG2 per well on day 2. **C**, 2 doses of 50μg/ml TG2 per well on days 2 and 4. **D**, 4 doses of 50μg/ml TG2 per well on days 2, 4, 6 and 8. **E**, 4 doses of inactivated TG2 (50μg/ml) on days 2, 4, 6 and 8. **F**, cultures treated with 1mM TG2 active-site inhibitor NTU283. Images are representative of three non-overlapping fields (n=3). Bar=50μm.

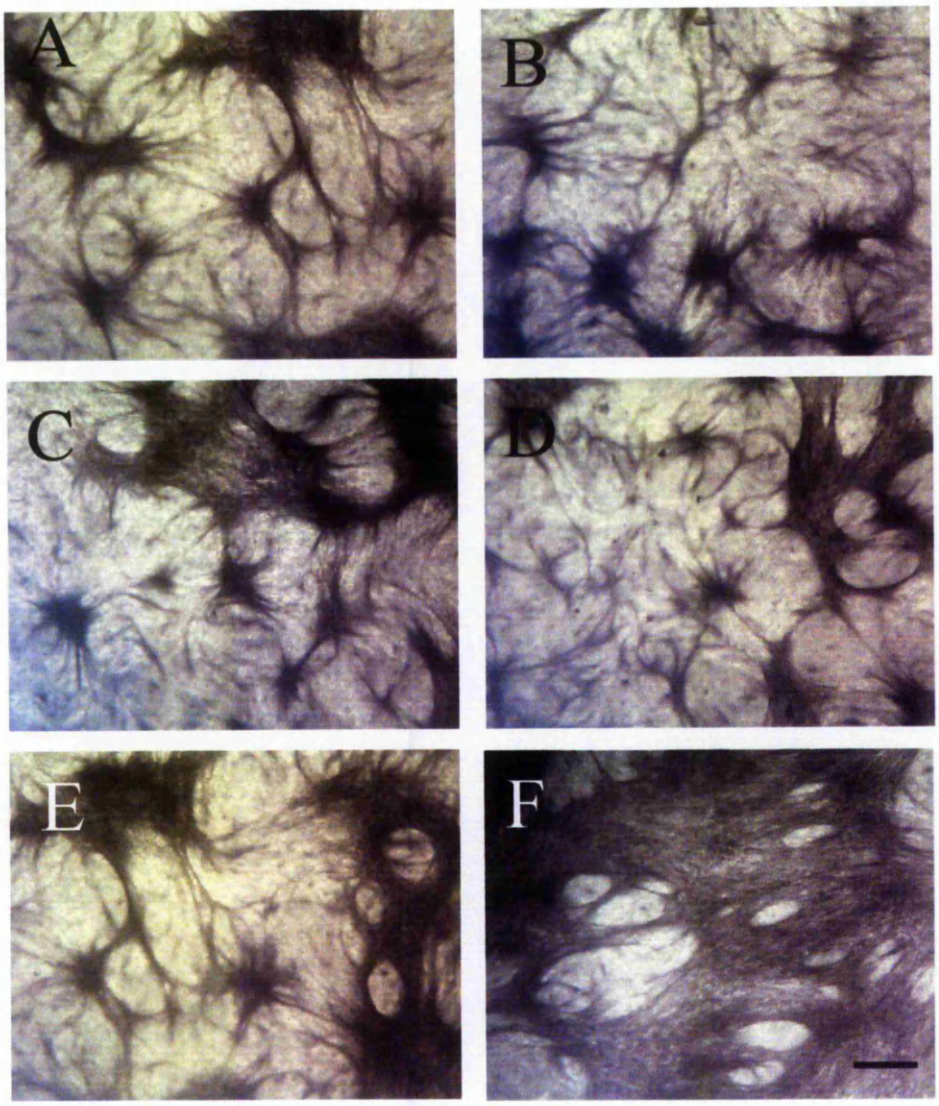


Figure 4.3.3.2 Effect of exogenous gpl TG2 on *in vitro* angiogenesis

4.3.4 Inhibition of angiogenesis in an in-vitro co-culture model following *gpl*TG2 application

To confirm the findings obtained from the in-house co-culture model, commercial *in vitro* human angiogenesis cultures (TCS Cellworks) were also treated with exogenous *gpl*TG2 in a similar dosing regime. By applying 50μg/ml of active *gpl*TG2 (50U/ml) at different stages of development, we observed a dose-dependent inhibition of angiogenesis compared to untreated cultures (figure 4.3.4*A*). Suppression of capillary development was seen after two TG2 treatments and optimal inhibition of angiogenesis was achieved following four applications of enzyme. TG2-treated vessels appeared highly truncated and showed reduced capillary branching. Quantification of vWF staining by image analysis in TG2-treated and untreated angiogenesis cultures was employed to further assess the TG2-mediated inhibition of angiogenesis (figure 4.3.4*B*). Capillary length, number of vessels, number of vessel branching points and percentage area covered by the endothelial tubules were all significantly reduced in a dose-dependent manner in TG2-treated cultures. To establish whether transamidating activity was required to inhibit angiogenesis, cultures were also treated in parallel with TG2, inactivated by pre-incubation for 6 hours at 4°C with the active site-directed inhibitor NTU283 (500μM). Inhibition of the transamidating activity of the applied TG2 reversed the enzymes' anti-angiogenic properties.

4.3.4.1 Application of *gpl*TG2 affects both matrix deposition and turnover

To test whether TG2 affects total ECM protein or collagen deposition and matrix turnover, angiogenesis cultures were pulse-labeled with [2,3-^{3}H]-proline and [2,3-^{3}H]-amino acid mixture respectively (fig. 4.3.4.1.1 and fig. 4.3.4.1.2). Subsequent to the removal of the pulse from the media, the amount of the radiolabel present in the matrix and in the media was then measured. Untreated cultures demonstrated a time-dependent decline in amount of radioactivity present in the ECM.

Figure 4.3.4 Inhibition of *in vitro* capillary formation in a commercial angiogenesis model following exogenous TG2 administration

Co-cultures of human endothelial cells and fibroblasts spontaneously form blood capillaries over 8 days. On days 4-7, angiogenesis co-cultures were treated with 1x, 2x, 3x & 4x daily doses of 50μg/ml active or TG2 inactivated prior to addition as described in the Methods (section 2.2.2.2.1). Co-cultures were maintained for 8 days and stained for the endothelial cell marker vWf as outlined previously (section 4.2.1.2). **A**, light microscopy images indicating neovessel formation at different TG2 dosing regimes. Results are representative of three non-overlapping fields (n=3), from three independent experiments. (Bar=50μm). **B**, Bar graph illustrating reduction of angiogenesis following TG2-treatment (n = 12). Reductions in % area covered by capillaries, number of vessels, vessel length and number of branch points in cultures treated twice (black bars) or four times (stripped bars) with TG2 were calculated using QWin software image analysis and were shown to be significant ($p<0.05$) when compared to the untreated control (white bars). * Indicates significantly different from the untreated control.

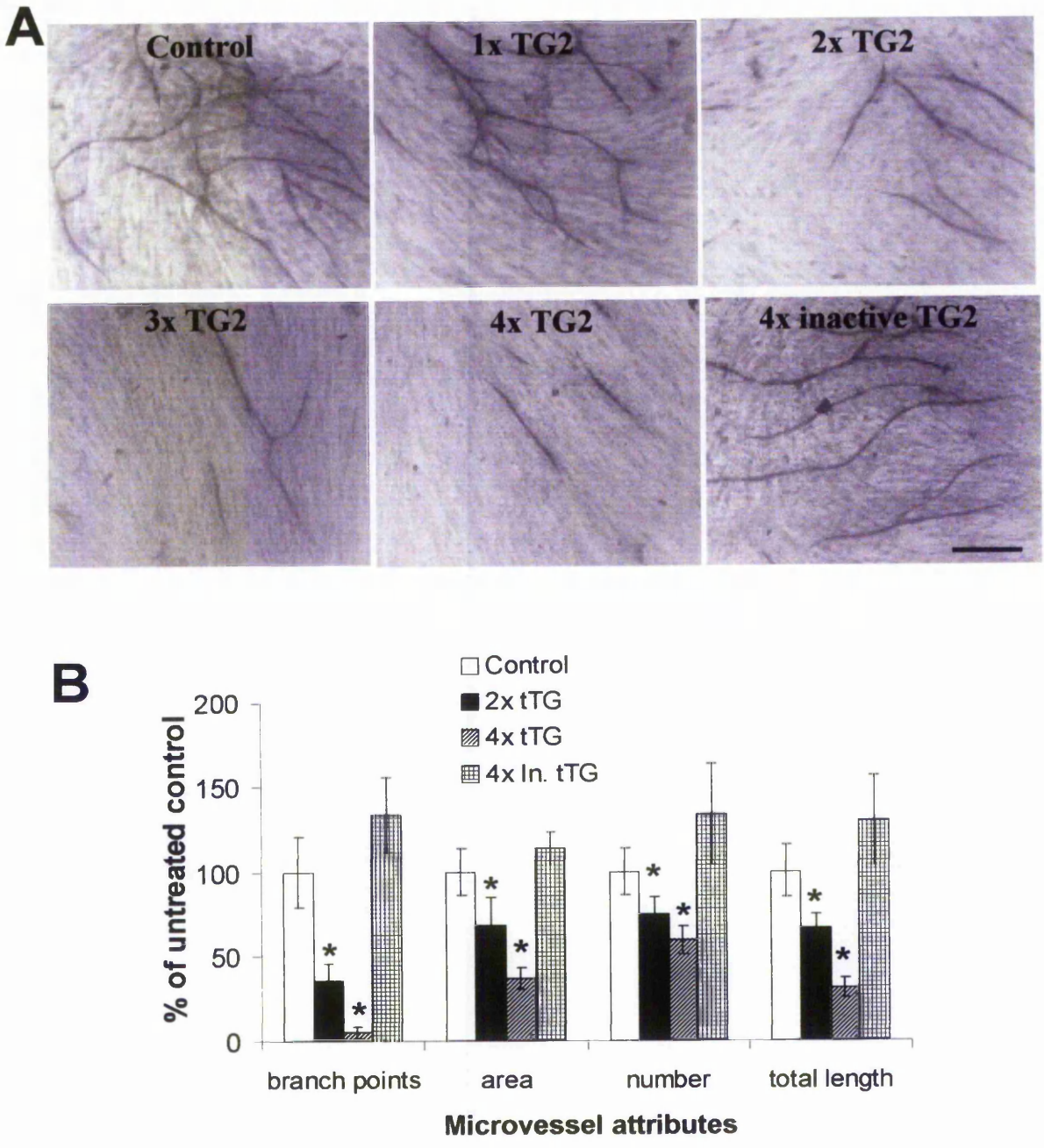


Figure 4.3.4 Inhibition of *in vitro* capillary formation in a commercial angiogenesis model following exogenous TG2 administration

Figure 4.3.4.1.1 Total ECM deposition of human angiogenesis cultures following TG2 application

Human angiogenesis co-cultures were maintained in 24-well plates and allowed to grow overnight prior to labeling with 5μCi/ml [2,3-^{3}H]-amino acid mixture in standard cell growth medium for 48 hours. Following washing in PBS, cells were administered with four doses of 50μg/ml active (50U/ml) TG2 in fresh label-free media at 48, 72, 96 and 120 hours (arrows), and the amount of radiolabel was measured in the media and ECM fractions at 48, 72, 96, 120, and 144 hours as outlined in Methods (section 2.2.3.8). Media scintillation counts revealed that similar amounts of radiolabel were incorporated into the co-cultures after 48 hours. **A**, cell growth media fraction; **B**, ECM fraction. ECM counts are normalised against μg of dsDNA in the cellular fraction, measured as described in the Methods (section 2.2.1.7.4). Results represent mean values ±SD from two independent experiments, each undertaken in duplicate. * Indicates significantly different from the untreated control ($p<0.05$).

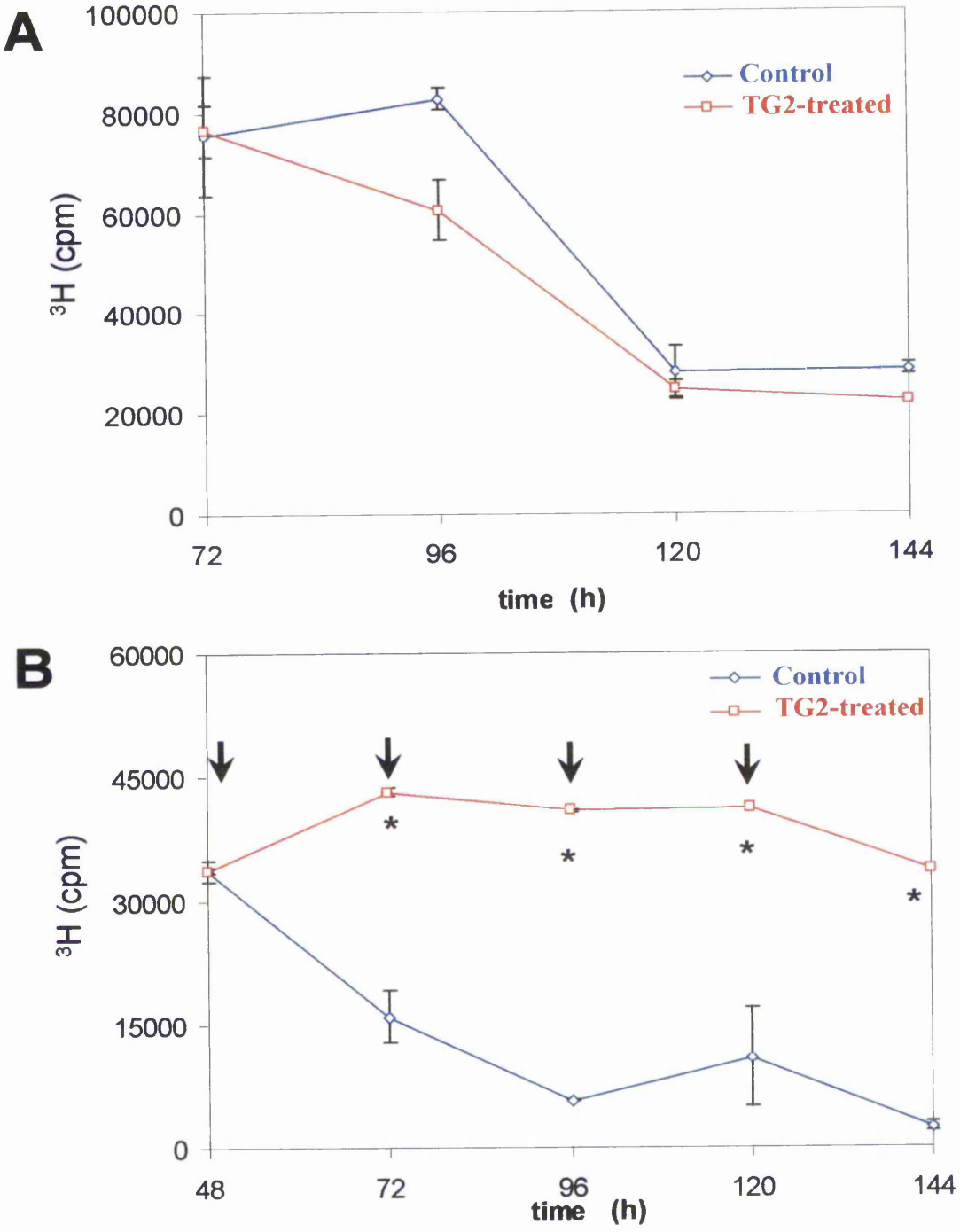


Figure 4.3.4.1.1 Total ECM deposition of human angiogenesis cultures following TG2 application

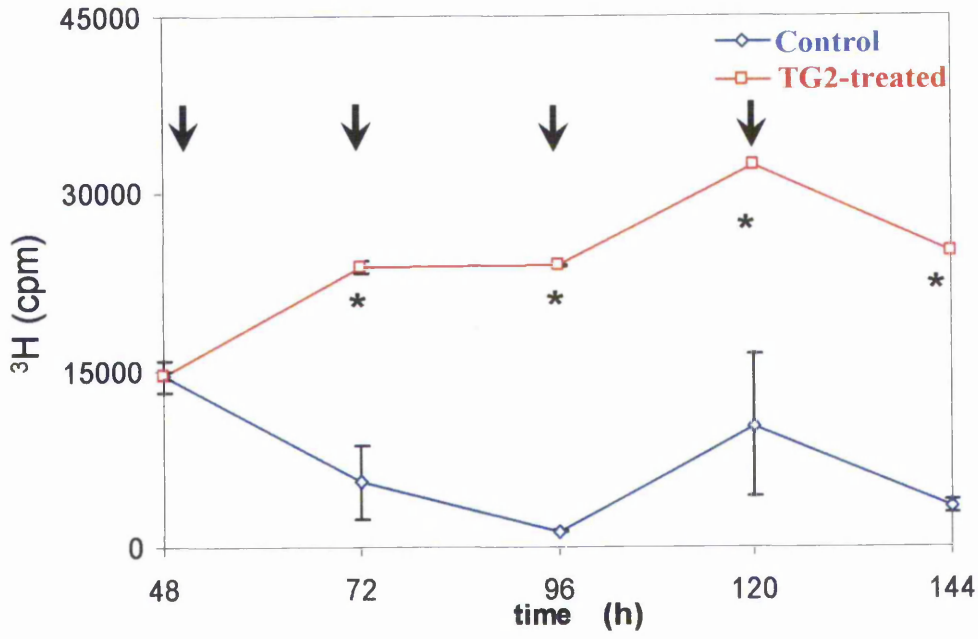


Figure 4.3.4.1.2 Collagen deposition of human angiogenesis cultures following exogenous TG2 application

Human angiogenesis co-cultures were maintained in 24-well plates and allowed to grow overnight prior to labeling with 5μCi/ml [2,3-^{3}H]-proline in standard cell growth medium for 48 hours. Following washing in PBS, cells were administered with four doses of 50μg/ml active (50U/ml) TG2 in fresh label-free media at 48, 72, 96 and 120 hours (arrows), and the amount of radiolabel was measured in the ECM fraction at 48, 72, 96, 120, and 144 hours as outlined in Methods (section 2.2.3.8). ECM counts are normalised against μg of dsDNA in the cellular fraction, measured as described in the Methods (section 2.2.1.7.4). Results represent mean values ±SD from two independent experiments, each undertaken in duplicate. * Indicates significantly different from the untreated control ($p<0.05$).

In contrast, TG2 treatment led to a significant ($p<0.05$) increase in the amount of label found in the ECM indicating that the application of the enzyme initially increased collagen and total matrix deposition, and subsequently reinforced the stability of the ECM, preventing its turnover, since the amount of radiolabel retained in the ECM remained relatively stable.

4.3.4.2 Exogenous TG2 downregulates TGFβ1 release but has no effect on bFGF, or VEGF

Given the pivotal role of serum growth factors such as bFGF, VEGF, TGFβ1 in the angiogenic cascade (Kanda *et al.*, 1997; Schwarte-Waldhoff *et al.*, 1997), it was also of interest to determine whether exogenous administration of TG2 may exert its effect on the circulation of these factors in the periendothelial space. Media collected from the co-culture system at 72, 96, 120, and 144 hours were screened for TGFβ1, bFGF, and VEGF presence as described previously (section 4.2.2). Inherent serum-associated TGFβ1 levels within the cell growth media gave rise to substantial background, despite a 1:100 dilution of the test samples. As shown in figure 4.3.4.2.1, TGFβ1 levels within the media were found to be diminished after the second and fourth exogenous addition of the enzyme. It has to be noted that first and third TG2ase administration were concomitant with a cell growth media change. It is therefore believed that the enzyme is cross-linking endogenous TGFβ1 or its precursor complex -both present in the foetal calf serum- into the matrix rather than inducing increased release of the growth factor from the cell. Further experiments to confirm this finding will be undertaken in the next Chapter (Chapter 5). Similar measurement of bFGF and VEGF availability in the *in vitro* angiogenesis cultures media conditioned for up to 5 days, revealed no significant effect of TG2 on their release levels (figure 4.3.4.2.2).

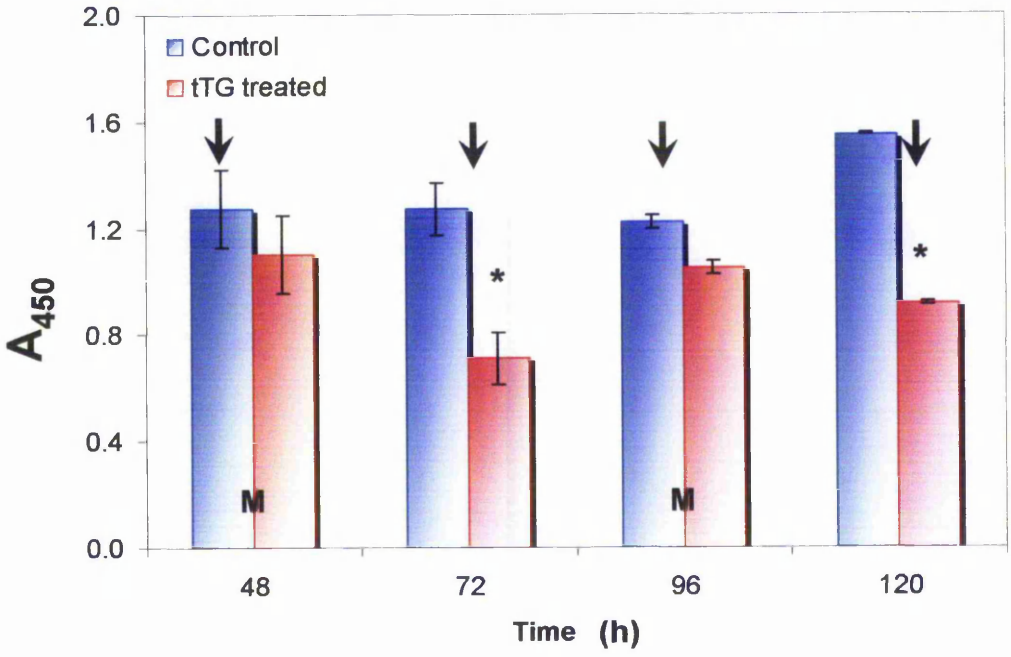


Figure 4.3.4.2.1 The effect of exogenous TG2 treatment on TGF β -1 present in the media of human angiogenesis cultures

Conditioned media from human angiogenesis cultures that were administered with four doses of 50μg/ml active (50U/ml) *gpl*TG2 during the course of the collagen/ECM deposition studies (figures 4.3.4.1.1 and 4.3.4.1.2 respectively) were screened for biologically active TGFβ-1 content using a Quantikine ELISA kit as described previously (section 4.2.2). Arrows indicate the points of exogenous TG2 addition. TGFβ-1 detected in the growth media of human angiogenesis cultures was found to decline in a dose dependent manner after the first (t= 48hours) and third (t= 96 hours) exogenous administration of *gpl*TG2, each undertaken during addition of fresh cell growth media. Results represent mean values ±SD (n=4), from two independent experiments.* Indicates significantly different from the untreated control ($p<0.005$).^{M} denotes time point of cell growth media change.

Figure 4.3.4.2.2 Measurement of VEGF/bFGF present in the media of TG2-treated angiogenesis cultures

Conditioned media from human angiogenesis cultures that were administered with four doses of 50μg/ml active (50U/ml) *gpl*TG2 during the course of the collagen/ECM deposition studies (figures 4.3.4.1.1 and 4.3.4.1.2 respectively) were also screened for bFGF and VEGF content using commercial cytokine-specific ELISAs as described previously (section 4.2.2). Both bFGF (**A**) and VEGF (**B**) cellular release into the growth media of human angiogenesis cultures was shown to remain statistically unaltered following administration of *gpl*TG2 at 48, 72, and 96 hours. Arrows indicate time points of TG2 application. Results represent mean values ±SD (n=4), from two independent experiments.

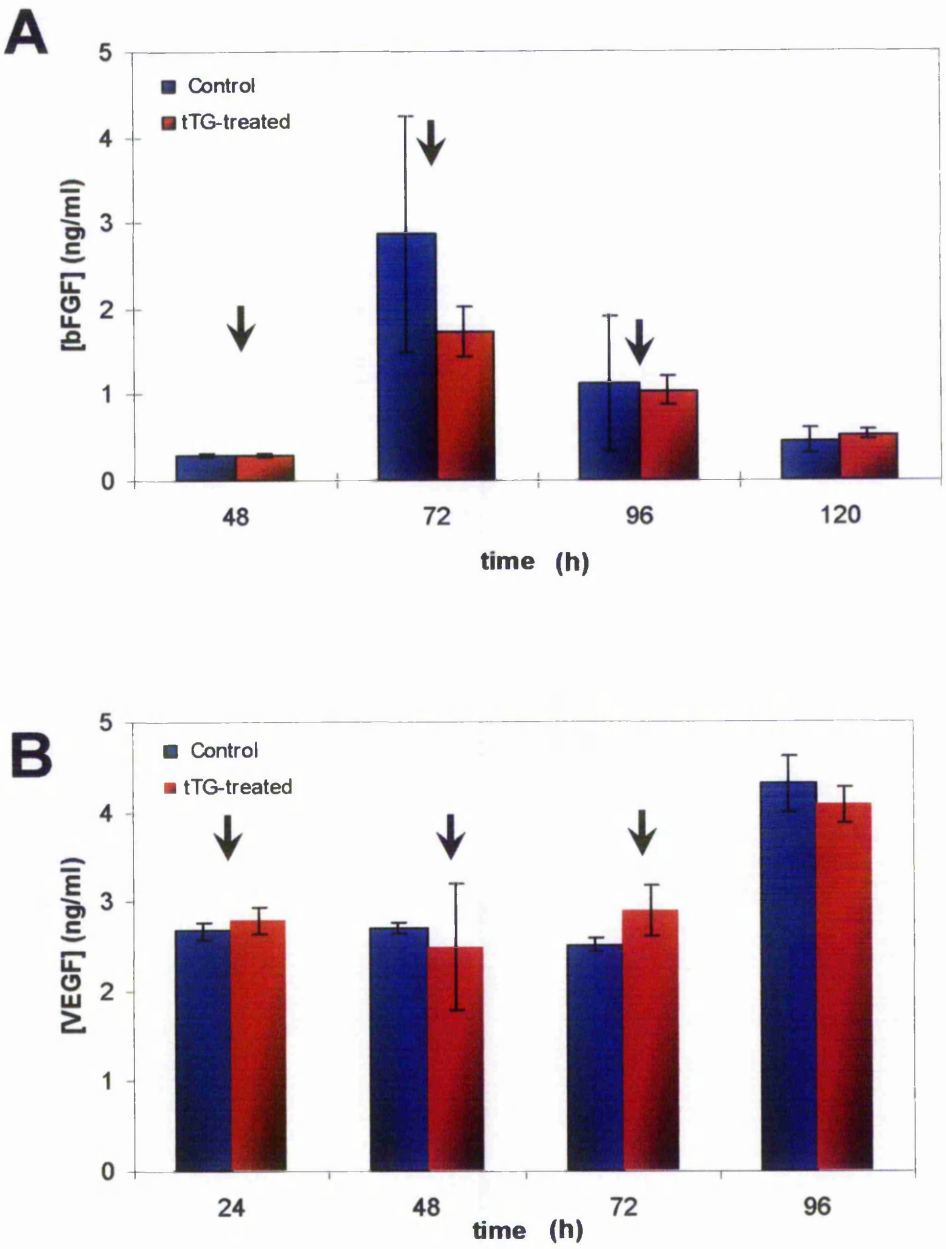


Figure 4.3.4.2.2 Measurement of VEGF/bFGF present in the media of TG2-treated angiogenesis cultures

4.4 Discussion

Central to this study was the physiological and therapeutic importance of TG2 in the events leading to EC tube formation during neovascularisation. Previous data based on HUVEC cells as well as the endothelial-like cell line ECV304 had suggested that TG2 was involved in the cross-linking of ECM proteins and that the enzyme's activity participated in BM assembly and cell adhesion (Greenberg *et al.*, 1987; Martinez *et al.*, 1994; Jones *et al.*, 1997). So far, the necessity of transglutaminase activity during angiogenesis is disputed (Haroon *et al.*, 1999a; Dardik *et al.*, 2003; Haroon *et al.*, 1999b). Conversely, immunostaining of *in vitro* angiogenesis cultures has indicated parallel staining of TG2 with that of the EC marker, vWF (Jones and Griffin, 2001). This staining did not exhibit altered intensity as tube formation progressed contrary to reports using DNA arrays indicating that TG2 expression is down regulated during angiogenesis (Bell *et al.*, 2001). Visualisation of *in situ* TG activity using the incorporation of the fluorescent primary amine substrate fluorescein cadaverine indicated that the enzyme is present on delicate fibrous structures running mainly parallel to the tube axis (Jones and Griffin, 2001). Despite contradictory evidence for the importance of TG2 expression and activity in angiogenesis it is postulated that TG2 activity is not essential for capillary tube formation, given that preliminary experiments within our group have indicated that addition of the specific site directed irreversible inhibitor NTU283 (1,3-dimethyl-2[(oxopropyl)thio] imidazolium) at a concentration of 500μM to angiogenesis cultures results in unaltered capillary tube formation. This is in keeping with the observation so far that TG2 knock-out mice bear no abnormalities in their vasculature (DeLaurenzi and Melino, 2001; Nanda *et al.*, 2001). However, when intact mouse aortas were embedded in matrigel, EC outgrowth from the TG2 knock-out aorta was shown to be significantly more rapid compared to the aorta from the wild type mice. Taking into account that the migration of ECs and consequently the formation of new vessels are affected by the composition of the endothelial BM, this finding suggested that TG2 might not be needed during initiation of angiogenesis as it might be inhibitory to the endothelial mobilisation process, possibly by stabilizing the endothelial BM (Martinez *et al.* 1994). Gene array analysis of differential EC gene expression in 3-D collagen matrices was confirmatory of this observation, as it indicated that TG2 is down-regulated approximately 4- fold at 8 hours and 10-fold at 24 and 48 hours post-culture of EC

cultures in 3-D collagen matrices (Bell *et al.*, 2001), thus confirming that lowered levels of TG2 expression are required during the initial stages of angiogenesis.

Given that intrinsic TG2 expression is not needed during angiogenesis, we next explored whether increasing the amount of TG2 found in the extracellular environment of angiogenesis cultures could affect neovascularisation. TG2 has been reported to be important in wound healing and tissue fibrosis where it has been demonstrated to have the ability to stabilise the integrity of tissue by the cross-linking of both intracellular and extracellular proteins (Nicholas *et al.*, 2003). However, in situations of chronic tissue damage, which might occur from continuous insult such as that occurring in diabetic nephropathy (Floege *et al.*, 1992), the increased cross-linking and stabilisation of matrix proteins by TG2 can lead to massive tissue scarring (Skill *et al.*, 2001). Sequential additions of TG2 to the medium of both commercial and in-house *in vitro* angiogenesis co-cultures led to a dose dependent inhibition of endothelial cell tube formation. The same result was observed when ECs sprouting from rat aortic explants that had been embedded in matrigel were treated with exogenous TG2. The optimum dose regime in the *in vitro* model consisted of four additions of active (50U/ml) TG2 at a concentration of 50μg/ml. Although such a concentration may seem physiologically large, it is expected that the fibronectin present in the serum added to the cell cultures is likely to sequester the added TG2 due to the high affinity binding between those two proteins (Jeong *et al.*, 1995; Gaudry *et al.*, 1999b). Detection of *in situ* TG activity by the incorporation of fluorescein cadaverine following addition of TG2 had previously indicated an organised fibrillar deposition of activity in treated cultures the density of which was found to be dose dependent (Jones & Griffin, UK Patent W00211747). At the maximum *gpl*TG2 dose regime of 4 x 50μg/ml (50U/ml) the cultures demonstrated what appeared to be a total reorganisation of fibrils evolving from a thick and anastomising network to a disorganized structure as the dose regime increased. Evidence that transamidating activity was essential for the inhibition of angiogenesis was provided by the additional observation that the treatment of cultures with TG2, which had been previously inactivated with the active site inhibitor NTU283, reversed the inhibitory effects of TG2.

In an attempt to establish an explanatory mechanism for our observations, *in vitro* angiogenesis cultures were pulse-labeled with either ^{3}H-proline or a mixture of ^{3}H- amino acids. Following pulse labeling, the radiolabel present in the ECM was then measured at different time points and used as an indicator of matrix turnover. Sequential addition of exogenous TG2 to the angiogenesis cultures clearly indicated that matrix turnover was nearly completely halted. Following a single addition of TG2, matrix deposition was increased and the amount of radiolabel present in the matrix was maintained at the initial level, following subsequent TG2 treatments. A possible role for transglutaminases in the metabolism of connective tissue has been suggested by the formation of cross-links between collagen α-chains (Jelenska *et al.*, 1980) and the cross-link of fibronectin to specific sites of collagen types I and III (Mosher and Schad, 1979). Given that TGs have been shown not only to cross-link collagen gels (Orban *et al.*, 2004), but also to accelerate collagen gel/fibril formation (Nomura *et al.*, 2001) it was speculated that TG2-mediated ECM cross-linking confers resistance to the matrix from proteolytic degradation by MMPs. In previous studies it has also been demonstrated that *in vitro* cross-linking of collagen and collagen/fibronectin mixtures leads to an increased resistance to their breakdown by MMP-1 (Johnson *et al.*, 1998). Increased protein polymerisation as a result of progressive TG2 treatment was also confirmed, as western blot analysis of lysates obtained from TG2-treated angiogenesis cultures revealed elevated ε(γ-glutamyl)lysine cross-links as well as high M_r TG2 and fibronectin polymers on the top of the stacking and resolving gels (Jones and Griffin, 2001). At this stage it was of interest to determine whether this was a cumulative effect as increased cross-linking might also lead to immobilisation of growth factors pivotal in the angiogenic cascade. Measurement of major growth factors secreted by angiogenesis cultures such as bFGF, and VEGF by ELISAs, suggested that TG2 application does not incur any quantitative changes in their extracellular pool. However, the amount of TGFβ circulating in the media of the angiogenesis cultures, measured by a sandwich ELISA following TG2 application, appeared to be significantly reduced. Given that TGFβ is present in the angiogenesis culture growth media, it is believed that TG2 is sequestering the FCS-associated cytokine into the ECM rather than down-regulating the release of its precursor complex (LTBP) or modulating LTBP activation.

It is well established that modulation of the ECM is central to many aspects of EC behaviour during angiogenesis and that collagen metabolism in particular may exert an effect on the angiogenic cascade. Ingber *et al.* (1988) reported that the antiangiogenic properties of proline analogues in combination with angiostatic steroids are attributed to their ability to 'switch-off' collagen accumulation. Paradoxically, in this study we have shown that ECM accumulation as a result of increased protein cross-linking, can also lead to compromised tube formation.

Both tumour cell invasion and EC migration during tumour angiogenesis require ECM remodeling. Findings from the first two Results chapters collectively suggest that TG2 can potently inhibit tumour growth by a mechanism involving stabilisation of the tumour stroma ECM (Chapter 3) and as a consequence blockade of tumour cell invasion and EC migration. Whilst considerable advances have been made in our understanding of angiogenesis, the development of effective tumour therapeutic strategies involving its inhibition have been less successful when animal studies have been translated into clinical phase trials. This may be due to the fact that angiogenesis inhibitors, may have the unintended effect of metastatic promotion, by depriving the tumours of oxygen (Steed, 2003). Modulation of ECM turnover as a major control point in angiogenesis may still provide a more rational approach for development of new anti-angiogenic regimens. Our preliminary studies using TG2 as a novel tool for inhibiting tumour growth by the induction of tumour fibrosis is an alternative approach for enhancing the body's wound healing response, which has the potential to both complement and improve existing tumour-related anti -angiogenic strategies used in tumour therapy.

Chapter 5:

The role of tissue transglutaminase in extracellular matrix stabilisation

5.1 Introduction

The extracellular matrix is a complex structural entity surrounding cells and providing them with a mechanical support for adhesion and migration on a variety of different components. The ECM consists of structural proteins such as collagens and elastin; specialised proteins such as fibrillin, fibronectin and laminin; glycoproteins, such as proteoglycans and glycoaminoglycans; and molecules that are bound specifically to the ECM, such as certain growth factors/cytokines, matrix metalloproteinases (MMPs) and processing enzymes such as tissue transglutaminase and procollagen propeptidases (Schuppan et al., 2001). Assembly and degradation of the ECM is a dynamic process that occurs during wound healing, angiogenesis and embryogenesis and its modulation is pivotal in the regulation of cellular migration.

Increasing evidence accentuates the importance of TG2 as a protein cross-linker in the ECM of various cell lines. The majority of TG activity in the cell cytosol is predicted to remain latent (Smethurst and Griffin, 1996) due to tight regulation by Ca^{2+} and GTP. The extracellular environment, however, provides a high concentration of Ca^{2+} and low concentration of nucleotides necessary for the activation of TG2. TG2 substrates include fibronectin (Turner and Lorand, 1989; Barsigian *et al.*, 1991; Martinez *et al.*, 1994), fibrin and fibrinogen (Achyuthan *et al.*, 1988), vitronectin (Sane *et al.*, 1991), osteonectin and osteopontin (Aeschlimann *et al.*, 1995; Kaartinen *et al.*, 1997), laminin-nidogen complex (Aeschlimann and Paulson, 1991), and different types of collagen (Juprelle-Soret *et al.*, 1988; Kleman *et al.*, 1995; Esterre *et al.*, 1998). Fibronectin is processed, and assembled into fibrils via a multi-step process that requires the presence of viable cells (Barry and Mosher, 1988; Fogerty and Mosher, 1990). Fibronectin and fibrinogen, when bound to the surface of hepatocytes and endothelial cells in suspension culture, become cross-linked into the pericellular matrix by TG2 (Barsigian *et al*, 1991). Collagen cross-linking occurs outside the cell and the three-dimensional matrix surrounding fibroblasts forms a microenvironment for the accumulation of processing enzymes and for the remodelling of newly synthesised matrix (Eyre *et al.*, 1984; Adams and Watt, 1993). It has been further demonstrated that transglutaminases can improve the mechanical properties of collagen

matrices, via cross-linking (Orban *et al.*, 2004). Nomura and colleagues have previously established that microbial transglutaminase (mTG2) can accelerate collagen self-assembly, in a dose dependent manner (Nomura *et al.* 2001). These findings were later confirmed for the tissue enzyme (Skill *et al.*, 2004).

TG2-catalysed cross-links make proteins more resistant to chemical, enzymatic and physical disruption (Greenberg *et al.*, 1991; Johnson *et al.*, 1999). The high stability of the ε-(γ-glutamyl)lysine isopeptide bond is also suggested to contribute to the low turnover of the ECM (Barsigian *et al.*, 2000). Since many extracellular proteins are known to serve as substrates for TG2, it is believed that TG2 plays a central role in the dynamic process of ECM stabilisation and remodelling. Following the release of TG2 in the extracellular environment, the enzyme tightly binds to the ECM and cross-links the surrounding matrix due to activation by Ca^{2+} (Upchurch *et al.*, 1991). Increased amounts of the enzyme present in the matrix leads to increased cross-link formation (Johnson *et al.*, 1999). Since TG2 covalently and irreversibly cross-links extracellular matrix proteins, it may prevent or delay remodelling of basement membranes and may stabilise other extracellular components (Schittny *et al.*, 1997). Martinez *et al.* (1994) had earlier shown that covalent cross-linking of surface-coated fibronectin by endothelial cell-associated TG2 conferred resistance to detachment from the matrix by trypsin and stabilised the ECM.

So far, there have been numerous reports linking TG2 to altered matrix turnover in the development of fibrosis in various tissues (Griffin *et al.*, 1979; Johnson *et al.*, 1999; Mizra *et al.*, 1999; Piacentini *et al.*, 1999; Small *et al.*, 1999; Grenard *et al.*, 2001). It has been proposed that the increased expression of TG2 in renal fibrosis may contribute to matrix resistance to breakdown, since the cross-linking of ECM proteins by TG2 and the formation of ε-(γ-glutamyl)lysine cross-links was increased in the extracellular environment of fibrotic tissues (Johnson *et al.*, 1997). Since TG2 has a high affinity for the ECM, in the case of chronic or continuous tissue insult its release from the cells would cause extensive protein cross-linking, which could contribute to the development of fibrosis and tissue scarring.

Relevant to tumour growth and progression, it has been shown that a stable ECM is intrinsically anti-angiogenic and inhibitory on cell proliferation and migration because it is more resistant to protease digestion (Dano *et al.*, 1999; Lochter *et al.*, 1998; Werb *et al.*, 1999). Hence, the formation of a stable ECM may be an effective barrier to growth and metastasis of tumours by restricting infiltration by tumour cells and growth of new blood vessels (Haroon *et al.*, 1999b). The same authors also suggested that the proteolytic degradation of TG2 may provide a method to regulate the duration and extent of the cross-linking reaction and allow tissue remodelling to occur in the granulation tissue (Haroon *et al.*, 1999a).

Increased expression of TG2 and its extracellular products are also known to be associated with inflammation and wound healing, a process that is highly dependent on the stabilisation of the provisional wound matrix. An increase in TG2 expression during dermal wound healing in rats was first shown by Bowness *et al.* (1987 and 1988). Increased expression of TG2 has been found in association with increased TGF-β, TNF-α, and IL-6 production in the wound at sites of neovascularisation in the provisional wound matrix following dermal wounding (Haroon *et al.*, 1999a). Moreover, TG2 appears to play an important role in the cross-linking of the papillary dermis, as well as of the dermo-epidermal junction as shown in skin regenerating from keratinocyte autografts (Raghunath *et al.*, 1996).

It has also been proposed that TG2 might be involved in the deposition and homeostasis of the ECM in an indirect manner through the deposition and crosslinking of matrix bound latent transforming growth factor β-1 binding protein (LTBP-1) leading to an increase of TGF-β1 release from the matrix (Johnson *et al.,* 1999). TGFβ1 has been shown to stimulate the expression of collagen and fibronectin, whilst facilitating their incorporation into the ECM (Ignotz and Massague, 1986; Border and Ruoslahti, 1990; Border and Noble, 1994). LTBP-1 appears to be involved in the matrix accumulation and activation of TGFβ1 (Dallas *et al.*, 1995). Induction of TG2 expression through modulation of a tetracycline controlled promoter in Swiss 3T3 fibroblasts has been shown to result in an increased deposition rate of LTBP-1 (Verderio *et al.*, 1999). Nunes *et al.* (1997) have further demonstrated that LTBP-1 complexes are transglutaminase substrates, suggesting that matrix association of

LTBP-1 is transglutaminase dependent. A requirement for TG2 at the cell surface, in the plasmin-mediated activation of TGFβ in bovine endothelial cells has also been proposed (Kojima *et al.*, 1993).

In order to elucidate the role of TG2 in the ECM stabilisation/reconstruction equilibrium, both direct (ECM stabilisation) and indirect (LTBP-1 deposition) scenarios were examined. Primarily, the effect of exogenously administered TG2 on the collagen deposition/turnover of HUVEC and C378 single cell type cultures was determined. The possibility that TG2 might up-regulate collagen, or MMP/TIMP expression, was also explored. To test whether TG2-mediated ECM stabilisation impinges on cellular migration and invasion, the ability of HUVEC cells to migrate through TG2-cross-linked artificial matrices was assessed. Finally, the indirect effect of exogenous TG2 on ECM protein synthesis, via modulation of LTBP-1 deposition/TGFβ1 release was also investigated.

5.2 Methods

5.2.1 Sequential collagen digestion by MMP-1 and proteinase K

C378 human dermal fibroblasts were seeded at 2 x 10^5 cells/well in 24-well tissue culture plates and labeled the following day with 5μCi/ml [2,3-^{3}H]-Proline (Amersham Pharmacia) for 48 hours. Following removal of the radiolabel and three washes in PBS, cells were treated twice at daily intervals with 50μg/ml of active (50U/ml) or inactive TG2 in label-free growth media. TG2 was inactivated prior to addition by incubation with 500μM NTU283 irreversible active site inhibitor as described in the Methods (section 2.2.2.2.1). Following treatment, cells were washed three times in PBS, and removed by exposure to 0.1% (w/v) deoxycholate/2mM EDTA for 10 minutes at room temperature. The remaining ECM fraction was then washed three times in PBS and exposed initially to 200μl of 100ng/ml activated pro-MMP-1 in 0.1M Tris-HCl pH 7.5, 1 μM $ZnCl_2$ for 16 hours. Pro-MMP-1 (Merck Biosciences, Nottingham, UK) was activated in 1mM p-aminophenylmercuric acetate (APMA; Merck Biosciences, Nottingham, UK), 10mM NaOH for 2 hours at 37 °C as described previously (Stricklin *et al.*, 1983). Digested collagen fragments were collected and the remaining insoluble ECM then washed twice with PBS, prior to further digestion in 100μl of 200μg/ml proteinase K (Sigma) in 50mM Tris-HCl, pH 7.4, 10mM EDTA and 10mM NaCl, followed by scraping and dissolution in 50μl of 4% SDS. The incorporated label present in the ECM was measured by addition of 1ml of Ultima Gold™ scintillation liquid (Packard Bioscience, Pangbourne, UK) to the two collected ECM fractions and subsequent counting in a Packard Instruments Tri-carb 300 scintillation counter for 5 minutes.

5.2.2 Immunofluorescent Latent TGF-β binding protein-1 (LTBP-1) staining

HUVEC cells and C378 mouse dermal fibroblasts were seeded on 8-well glass chamber slides (Lab-tek, Naperville, USA) at 5x10^4 and 2x10^4 cells/well densities respectively and

grown overnight in fully supplemented cell growth medium at 37°C, 5 % (v/v) CO_2. Growth media was then replaced by FCS-free media containing rabbit anti-LTBP-1 (ab39) primary antibody (BD Biosciences, Milton Keynes, UK) diluted 1/100. In situ immunostaining was allowed to take place for 2 hours at 37 °C, 5 % CO_2. The antibody-containing medium was then removed, the chamber slides disassembled and the monolayer washed 3 times with PBS. Cells were then fixed in 3.7 % (w/v) paraformaldehyde in PBS for 15 minutes at room temperature, and non-specific binding was blocked using 3% (w/v) BSA in PBS for 15 minutes at room temperature. Cells were then incubated with secondary anti-rabbit IgG-FITC conjugate (Dako, Glostrop, Denmark) diluted 1:100 in blocking buffer for 2 hours at room temperature. Cells were once again washed 3 times, the chamber slides were mounted with Vectashield (Vecta Laboratories, Peterborough, UK) and staining was viewed under the FITC filter of a laser confocal microscope (TCSNT, Leica).

5.2.3 Detection of matrix-bound TGF-β1 by modified ELISA

C378 cells were seeded on a 96-well plate at a concentration of $2x10^4$ cells/well and allowed to proliferate overnight. Cells were then treated with 50μg/ml of either active (50U/ml) TG2 or TG2 inactivated prior to addition as outlined previously (section 2.2.2.2.1). Cells were washed twice in PBS and the cellular fraction was removed by incubation in 50μl of 0.1% (w/v) sodium deoxycholate/2mM EDTA for 10 minutes at room temperature. Following two additional washes in PBS the deoxycholate-insoluble matrix was blocked in PBS, 3% (w/v) BSA and incubated for 2 hours with a primary mouse anti-TGFβ antibody (ab6472E6; Abcam, Cambridge) diluted 1:1000 in blocking buffer. The primary antibody was washed three times in blocking buffer and the ECM was incubated for 2 hours at 37 °C with a secondary anti-mouse HRP conjugate (Sigma) diluted 1/2000 in blocking buffer. Unbound secondary antibody was removed by 3 washes in PBS and colour development was achieved by the addition of 100μl of 100mM sodium acetate pH 6, 310μM 3,3',5,5'-tetramethylbenzidine (TMB), 0.014% H_2O_2. Colour development was terminated by the addition of 50μl of 2.5M H_2SO_4, and absorbance was read at 450nm using a SpectraFluor plate reader.

5.2.4 Detection of biologically active TGF-β1 by sandwich ELISA

For detection of biologically active TGFβ released in the medium the TGFβ Emax sandwich immunoassay system was used (Promega, Southampton, UK). The wells of a 96-well microtiter plate were first coated with 100μl of TGFβ1 mAb diluted 1:1000 in carbonate buffer (25mM sodium bicarbonate, 25mM sodium carbonate) overnight at 4°C. Non-specific binding sites were then blocked by addition of 270μl of TGFβ1 block buffer prior to incubation at 37°C for 35 minutes. The blocking buffer was then removed and wells were washed once with TBST buffer [20mM Tris-HCl, pH 7.6, 150mM NaCl, 0.05 % (v/v) Tween 20]. Following plate blocking and washing, 100μl of TGFβ standards (5.6-1000pg/ml), were added onto the wells in triplicate. Medium samples originating from exogenous gplTG2-treated HUVEC and C378 cultures were then added to the plate diluted 1:16 for the former and 1:3 for the latter in sample buffer to minimize background due to FCS–associated TGFβ1. Following incubation for 90 minutes at room temperature with shaking (500 ±100rpm), the plate was then washed 5 times with TBST buffer and 100μl of anti-TGFβ1 diluted 1:1000 were added to the wells prior to incubation for 2 hours at room temperature with shaking as before. Wells were then washed 5 times with TBST buffer and probed with 100μl of TGFβ-HRP conjugate diluted 1:100 for 2 hours as before. Following washing in TBST buffer 100μl of TMB One solution was added to each well and incubated at room temperature for 15 minutes. The reaction was stopped by the addition of 100μl of 1N HCl and absorbance was recorded on a SpectraFluor plate reader at 450nm.

5.3 Results

5.3.1 Culture treatment with *gpl*TG2 alters collagen matrix deposition and turnover

Having demonstrated that exogenous active TG2 blocks new capillary tube formation by virtue of its ability to stabilise the ECM of in vitro human angiogenesis cultures, we next sought to confirm these findings on single stromal cell type populations. Given that endothelial and fibroblast cells constitute the core cell population of angiogenesis co-cultures (Bishop et al., 1999), matrix turnover and deposition studies were undertaken separately on human umbilical large vein endothelial cells (HUVEC) and C378 human dermal fibroblasts. HUVEC and C378 cultures were labelled with 5μCi/ml [2,3-^{3}H]-Proline for 48 hours, and following two exogenous additions of 50μg/ml active (50U/ml) TG2 in fresh cell growth medium at 0 and 12 hours, the amount of label present in the medium and ECM fractions was measured as described in the Methods (section 2.2.3.8). As indicated by the amount of radiolabel within the ECM fraction of C378 cells, addition of exogenous TG2 promotes a significant increase ($p<0.05$) in collagen deposition, in response to the first enzyme administration, followed by a significant reduction ($p<0.05$) in collagen breakdown after secondary administration (figure 5.3.1.1B). As shown in figure 5.3.1.2 no significant effect on the collagen deposition of HUVEC was observed. Given that HUVEC cells, unlike fibroblasts, do not deposit large amounts of collagen which is manifested here by the low cpm values, it is hypothesised that TG2-mediated cross-linking of a limited extracellular collagen pool, would not translate into substantial matrix reinforcement.

5.3.2 The effect of exogenous TG2 on the viability of HUVEC and C378 single cell type cultures

Birckbichler et al. (1978) demonstrated that TG activity is able to modulate the differentiation/ proliferation of different cell types, whereas other studies have claimed that this effect might be due to the ability of the G-protein function of TG2 to delay progression into the G2/M phase (Mian et al., 1995). Additionally, microarray analysis revealed that

Figure 5.3.1.1 Collagen deposition and turnover of [^{3}H]-labeled C378 fibroblasts following *gpl*TG2 treatment

C378 fibroblasts were seeded at 2 x 10^5 cells/well in 24-well plates and allowed to grow overnight prior to labeling with 5μCi/ml [2,3-^{3}H]-proline in standard cell growth medium for 48 hours. Following washing in PBS, cells were administered with two doses of 10, 20, or 50μg/ml active (50U/ml) TG2 in fresh label-free media at 0 and 12 hours (arrows), and the amount of radiolabel was measured in the media and ECM fractions at 0, 12, and 24 hours as outlined in the Methods (section 2.2.3.8). **A**, cell growth media fraction; **B**, ECM fraction. Results represent mean values ±SD from two independent experiments, each undertaken in duplicate. * Indicates significantly different from the untreated control ($p<0.05$).

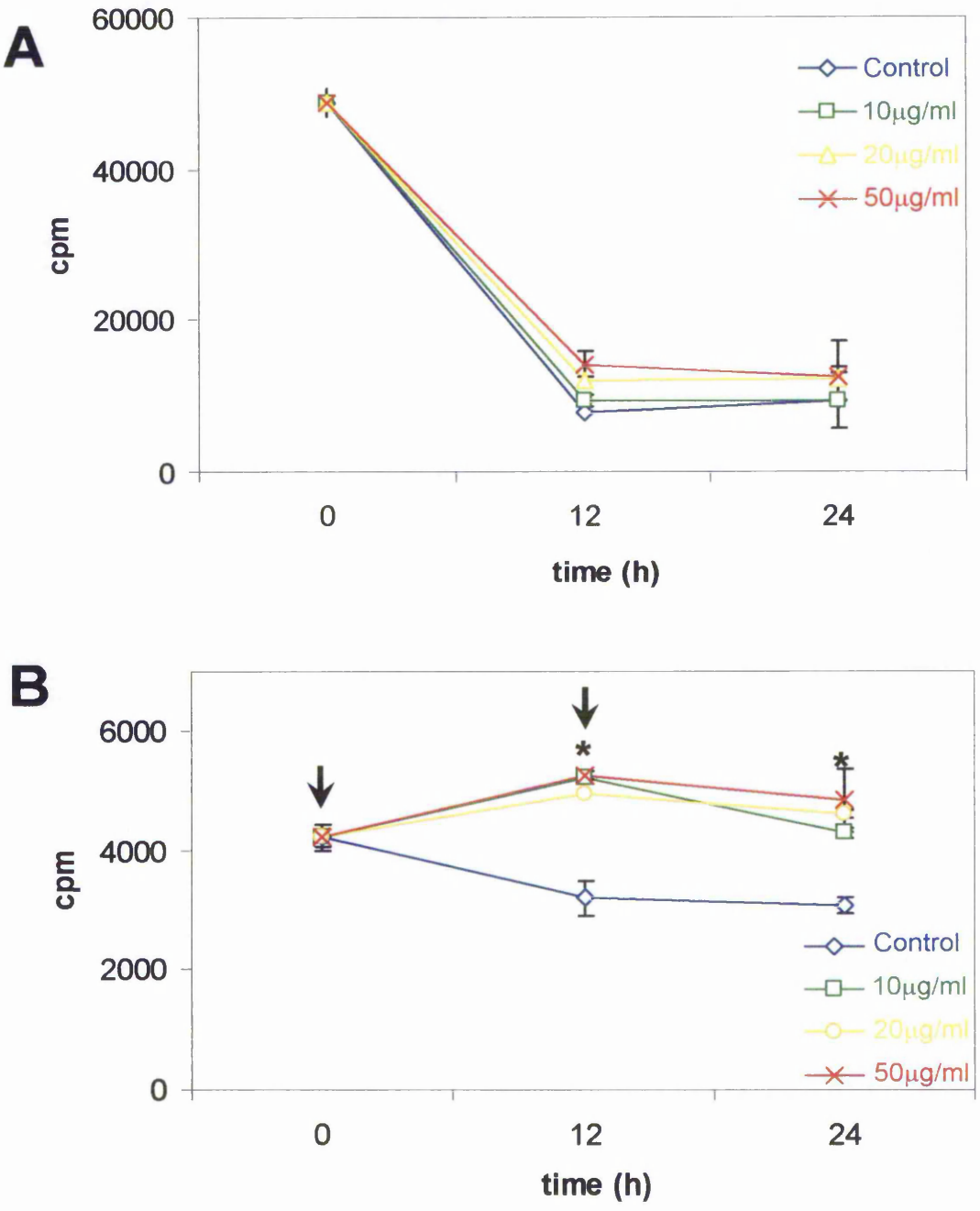


Figure 5.3.1.1 Collagen deposition and turnover of [^{3}H]-labeled C378 fibroblasts following TG2 treatment

Figure 5.3.1.2 Collagen deposition and turnover of [^{3}H]-labeled HUVEC following *gpl*TG2 treatment

HUVEC were seeded at 2 x 10^5 cells/well in 24-well plates and allowed to grow overnight prior to labeling with 5μCi/ml [2,3-^{3}H]-proline in standard cell growth medium for 48 hours. Following washing in PBS, cells were administered with two doses of 10, 20, or 50μg/ml active (50U/ml) TG2 in fresh label-free media at 0 and 12 hours (arrows), and the amount of radiolabel was measured in the media and ECM fractions at 0, 12, and 24 hours. **A**, cell growth media fraction; **B**, ECM fraction. Results represent mean values ±SD from two independent experiments, each undertaken in duplicate.

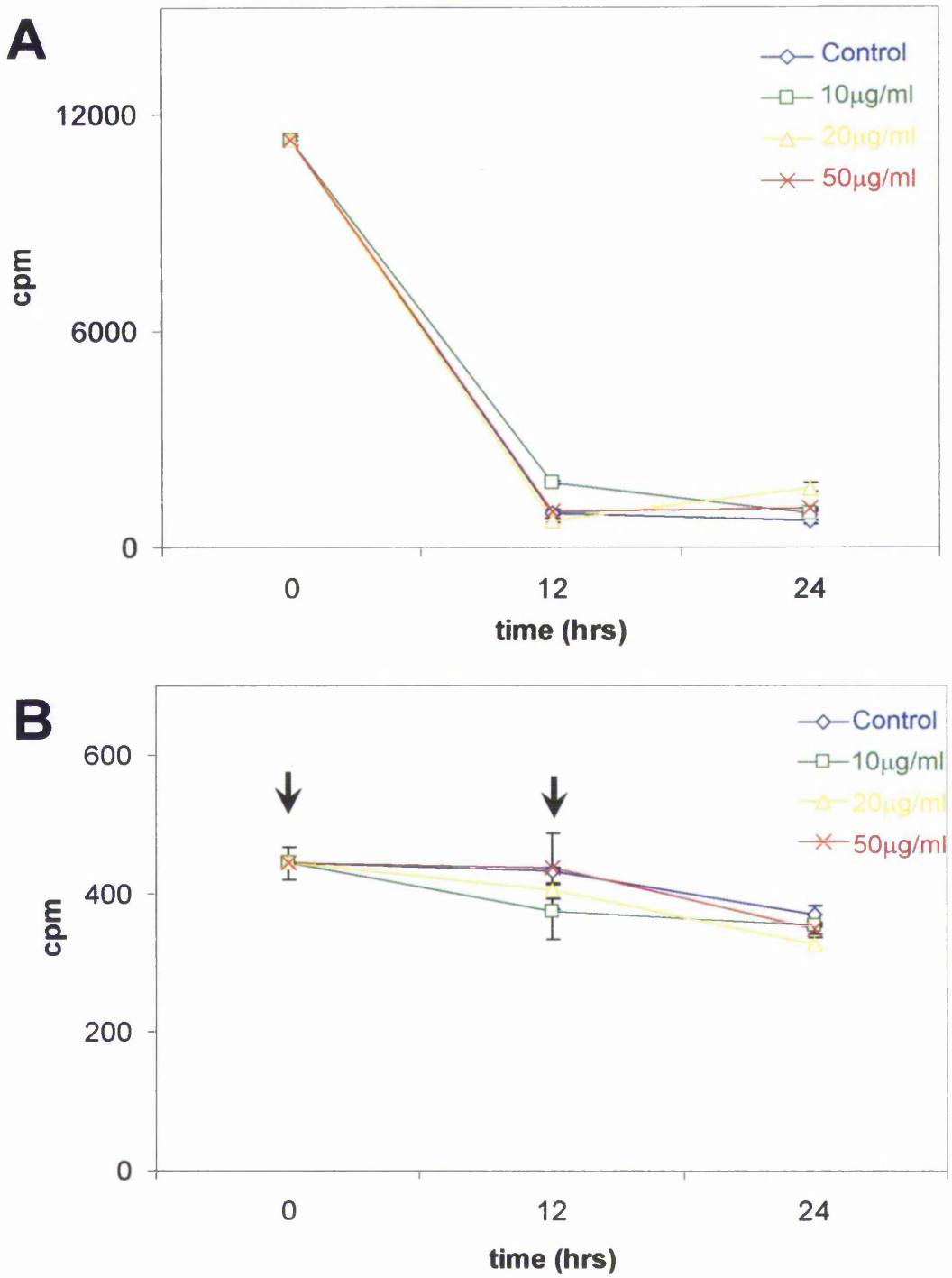


Figure 5.3.1.2 Collagen deposition and turnover of [^{3}H]-labeled HUVEC following *gpl*TG2 treatment

expression of G1/S-specific cyclin D1, which is essential for cell cycle control at the G1/S transition point, is downregulated in Swiss 3T3 fibroblasts induced for overexpression of TG2 (Balklava, 2002; PhD Thesis). Therefore, following two daily applications of 10, 20, and 50μg/ml active (50U/ml) gplTG2, the viability of HUVEC and C378 single cell type cultures was measured by an XTT metabolism-based assay (section 2.2.1.7.1) and a lactate dehydrogenase release (LDH) assay (section 2.2.1.7.2). The XTT metabolism-based assay indicated that endothelial cell viability was unaffected by TG2 treatment, but was slightly elevated in fibroblasts (figure 5.3.2A). Interestingly, LDH release -an indicator of cell permeability following loss of viability- was significantly lower in cultures receiving 50μg/ml TG2 in both HUVEC and C378 cells (figure 5.3.2B). Previous work carried out within this laboratory has indicated that the observed increase in viability following application of exogenous TG2 into C378 cultures does not translate into an increase of cell numbers when the latter was measured by trypan blue staining (Balklava, 2002; PhD Thesis).

5.3.3 Susceptibility of TG2-treated C378 fibroblasts to MMP-1 digestion

Collagen and total ECM turnover experiments within this study have indicated that not only does exogenous TG2 increase the initial deposition of structural proteins into the ECM, but it may also attenuate deposited collagen breakdown due to MMP activity (section 5.3.1). A possible explanatory mechanism for this would be that TG2 reinforces the extracellular fibrillar collagen network through extensive cross-links, thus allowing the matrix to retain partially cleaved collagen fibrils rather than releasing them into the culture media. To confirm this, C378 cultures pulse-labeled with [2,3-^{3} H-proline] were treated with exogenous active *gpl*TG2, and once the cells were removed by treatment with sodium deoxycholate, the remaining ECM was subsequently exposed to pre-activated pro-MMP-1 for 16 hours. The susceptibility of the matrix to active MMP-1 was measured as a

percentage of the total collagen digested (figure 5.3.3). Untreated cultures demonstrated increased susceptibility to MMP-1, with approximately 50% of the total collagen matrix being digested when exposed to the matrix metalloproteinase. In contrast, in the TG2-

Figure 5.3.2 XTT reduction and LDH release of HUVEC and C378 following TG2 treatment

HUVEC and C378 single cell-types were seeded at 10^3 cells/well in a 96-well tissue culture plate and allowed to grow overnight, prior to two doses with 10, 20, and 50μg/ml active (50U/ml) gpl TG2 at 8 hour intervals in standard cell growth media. **A**, measurement of HUVEC (black bars) and C378 (open bars) viability following exogenous treatments of TG2 by the XTT assay as outlined in Methods (section 2.2.1.7.1). Cell viability is expressed as percentage of untreated control (A_{490nm}). Values represent mean ±SD (n=6), from two separate experiments. **B**, measurement of HUVEC (black bars) and C378 (open bars) viability following exogenous treatments of TG2 by means of LDH release into the media was undertaken as described in Methods (section 2.2.1.7.2). Cell viability is expressed as percentage of untreated control (A_{492nm}). Values represent mean ±SD (n=6), from two separate experiments. * Indicates significantly different from the untreated control ($p<0.05$).

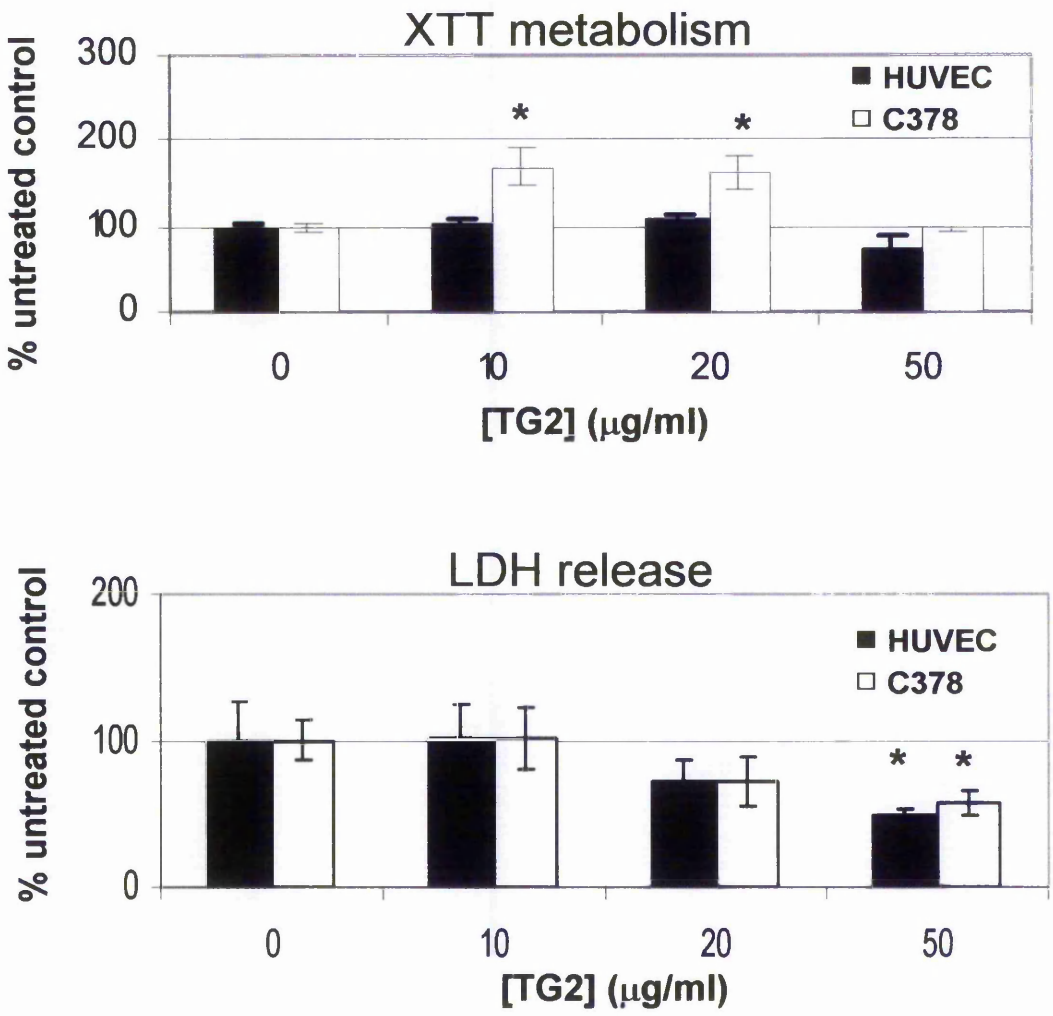


Figure 5.3.2 XTT reduction and LDH release of HUVEC and C378 following TG2 treatment

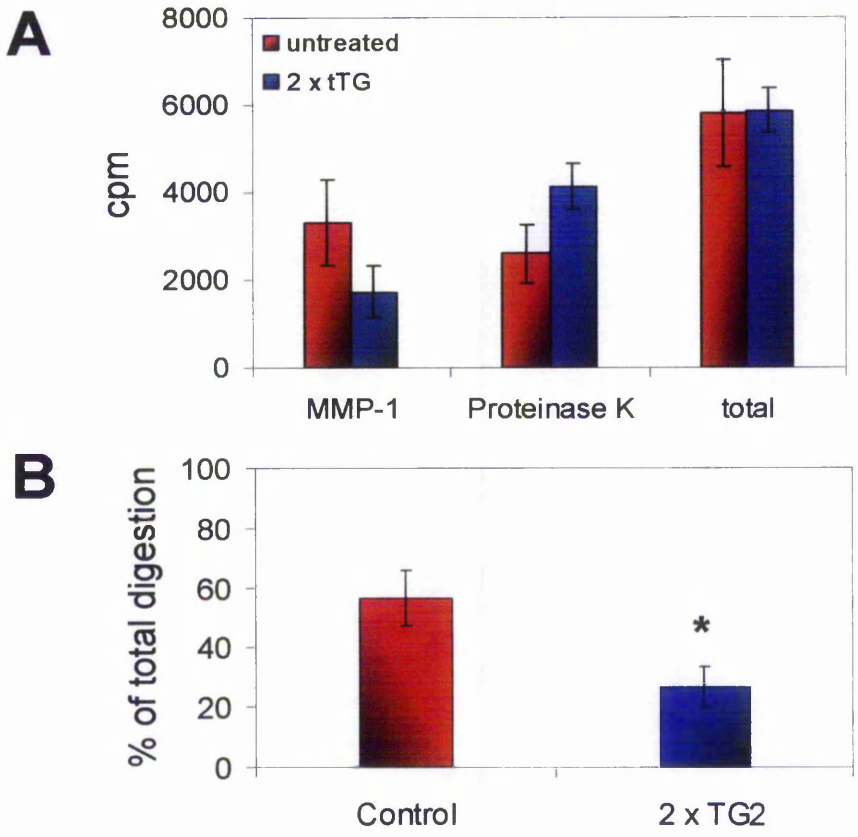


Figure 5.3.3 Susceptibility of TG2-treated C378 to MMP-1 digestion

C378 cells were seeded at 2 x 10^5 cells/well in 24-well plates and allowed to grow overnight prior to pulse-labeling with 5μCi/ml [2,3-^{3} H]-proline in standard cell growth media for 48 hours as outlined before (section 5.2.1). Following 3 washes in PBS, cultures received 2 daily doses of active (50U/ml) gpl TG2 in label-free cell growth media, and once the cells were removed by treatment with sodium deoxycholate, the remaining ECM was subsequently exposed to 100ng/ml MMP-1 for 16 hours. The amount of radiolabel was measured in the MMP1-digest, and the remaining radiolabel was recovered by subsequent digestion with 200μg/ml proteinase K. The susceptibility of the matrix to active MMP-1 was expressed as a percentage of total digestion by MMP-1 and proteinase K. **A**, scintillation counts of each digestion step; **B**, % of total digestion. Values represent mean ±SD (n=6) from two separate experiments. * Indicates significantly different from the untreated control ($p<0.05$).

treated cultures only around 25% of the extracellular collagen matrix was broken down and released under the same conditions.

5.3.4 Collagen I, III, IV and MMP-2 expression of TG2-treated C378 cultures

Given that collagen deposition into the ECM of the angiogenesis cultures increased following TG2 treatment, it was deemed important to explore the possibility that TG2 may up-regulate collagen expression, or alternatively down-regulate MMP expression possibly through TGFβ1 signalling (Ignotz and Massague, 1986; Border et al., 1990; Border and Noble, 1994). Interestingly, exogenous TG2 administration (four daily doses of 50μg/ml; 50U/ml) was shown to correlate with a significant ($p<0.05$) decrease in collagen I, III, and IV expression when cell lysates were analysed by Northern blotting (figure 5.3.4.1). Moreover, expression of gelatinase A (MMP-2) which binds and degrades a number of ECM components (such as elastin, fibronectin, gelatin, and laminin) was significantly ($p<0.001$) up-regulated (figure 5.3.4.2). Unfortunately, the lack of an appropriate in-house cDNA probe for a general tissue inhibitor of matrix metalloproteinases such as TIMP-2, which has been shown to inhibit the activities of several MMPs (MMP-1, MMP-1, MMP3, and MMP-12) did not allow this study to expand on the effect of the fibrotic ECM environment on TIMP expression. However, probing of the membranes with a TIMP-1 cDNA probe revealed a marked increase in the expression of this tissue inhibitor in cells treated with exogenous gplTG2. TIMP1 preferentially binds and inhibits MMP-9, which has been so far associated with invasion, metastasis, and protection of tumour cells from apoptosis (Yu et al., 2000).

5.3.5 HUVEC cell invasion through TG2-cross-linked collagen/fibronectin artificial matrices

Previous experiments within this study have demonstrated that collagen-fibronectin matrices reinforced by TG2-mediated cross-linking of fibrils could pose a tougher barrier for malignant cells to penetrate (section 3.3.2.3).

Figure 5.3.4.1 Collagen a1-I, III, IV mRNA expression in TG2-treated C378 cells

C378 cells were seeded in 75cm^2 flasks at 5 x 10^4 cells/flask and following overnight incubation, treated four times at daily intervals with 50μg/ml active *gpl*TG2 (50U/ml) or TG2 inactivated prior to addition by pre-incubation with 500μM NTU283 at 4°C and for 6 hours. Total RNA was extracted in TRI reagent, and was resolved (20μg/lane) on a denaturing agarose/formaldehyde gel, prior to Northern transfer and hybridisation with collagen I, II, and IV cDNA probes as outlined in Methods (section 2.2.2.2). **A**, Northern blots of collagen I, II, and IV. **B**, agarose/formaldehyde gel electrophoresis of total RNA demonstrating intact 28S and 18S rRNA units. **C**, densitometric quantification of Northern blots using Phoretix 1D image analysis software. Cyclophillin cDNA probing was used for densitometric normalisation. (Cyc) membrane was used for collagens I, and IV, whereas [cyc] was used for collagen III. Densitometric values represent mean ±SD corrected for total RNA loading by normalisation against the (cyc) and [cyc] membrane band densities accordingly (n=3). * Indicates significantly different from the untreated control ($p<0.05$).

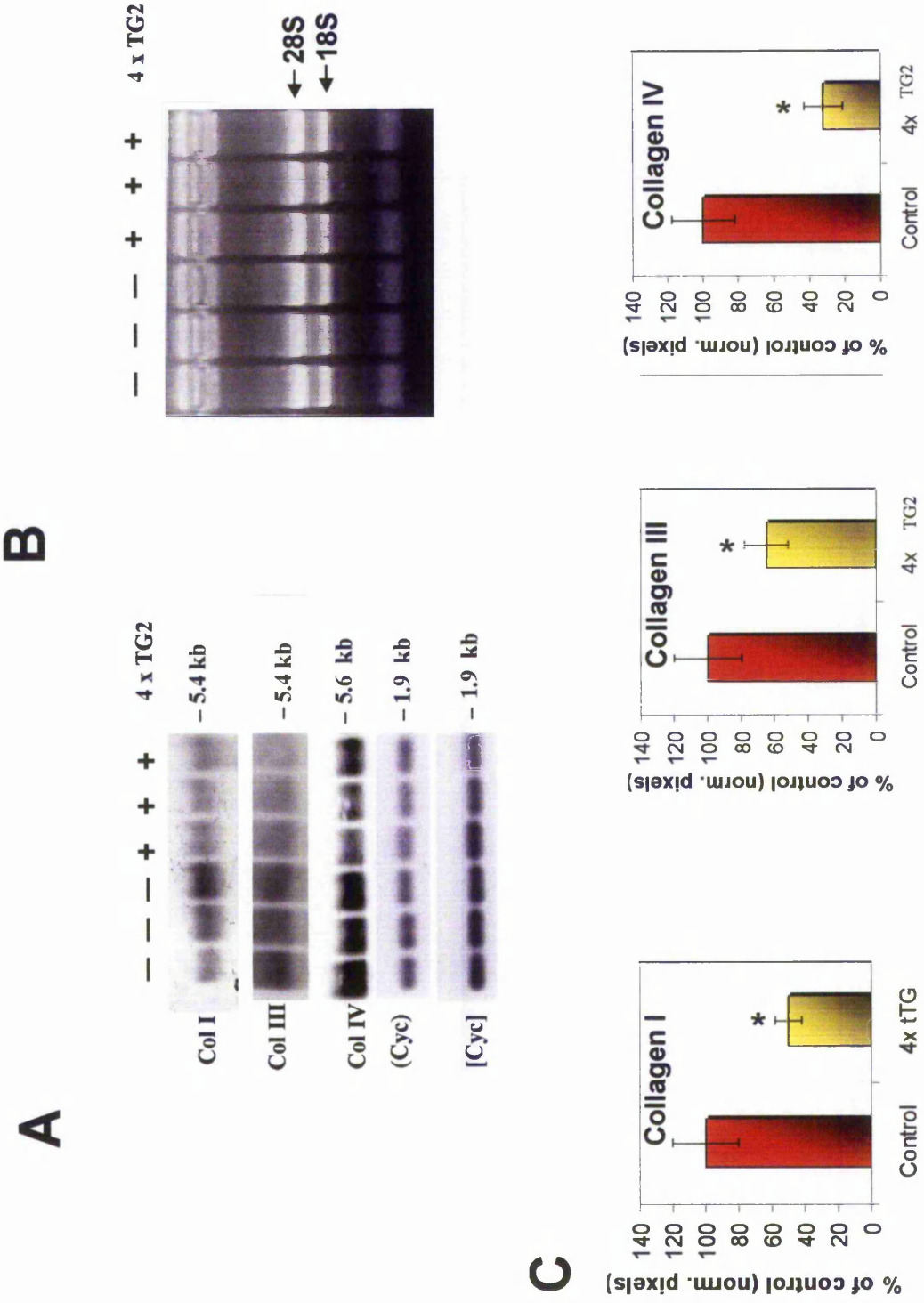


Figure 5.3.4.1 Collagen a1-I, III, IV mRNA expression in TG2-treated C378 cells

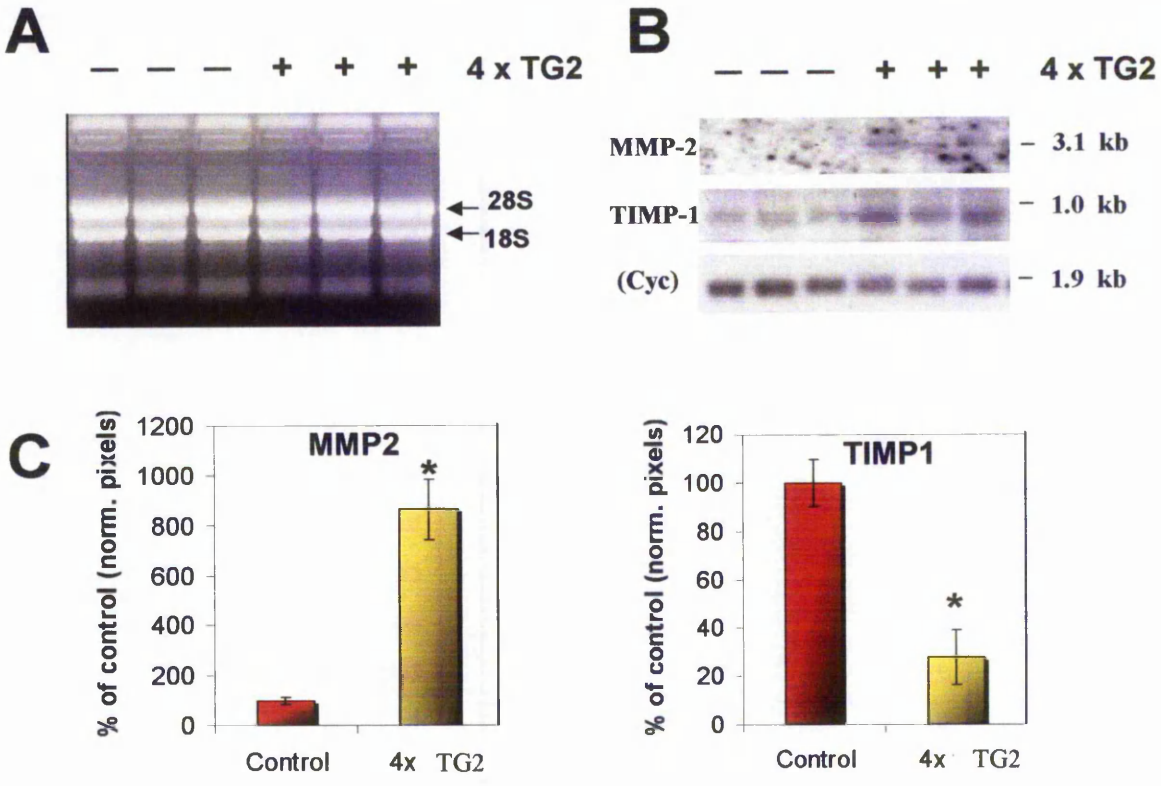


Figure 5.3.4.2 MMP-1 and TIMP-1 expression in TG2-treated C378 fibroblasts

C378 cells were seeded in 75cm^2 tissue culture flasks at 5 x 10^4 cells/flask and cultures were treated four times at daily intervals with 50μg/ml active (50U/ml) or inactive gpl TG2. Total RNA was extracted in TRI reagent, and was resolved (20μg/lane) on a denaturing agarose/formaldehyde gel, prior to Northern transfer and hybridasation with an MMP2 cDNA probe as outlined in Methods (section 2.2.2.2). **A**, Northern blots of MMP2 and TIMP1. **B**, agarose/formaldehyde gel electrophoresis of total RNA demonstrating intact 28S and 18S rRNA units. **C**, densitometric quantification of Northern blots using Phoretix 1D image analysis software. Cyclophillin cDNA probing was used for densitometric normalisation. Densitometric values represent mean ±SD corrected for total RNA loading by normalisation against the (cyc) membrane band densities accordingly (n=3). * Indicates significantly different from the untreated control ($p<0.05$).

To determine whether these findings could be expanded to stromal cells, modified Boyden cellular invasion experiments were undertaken on endothelial cells. Fibrillogenesis was allowed to take place in the presence of fibronectin (100μg/ml) facilitated by addition of 50μg/ml active (50U/ml) *gpl*TG2 in a similar was as described before (section 3.2.1.1). TG2 was also added in its inactive form, following incubation with 500μM of the active site directed inhibitor NTU283 for 6 hours at 4°C. The prepared matrices were applied on micropore (6μm) filters, and exposed to HUVEC that were encouraged to migrate through them and towards a lower FCS-containing chamber as described before (section 3.2.1.2). Fluorescence in the bottom cell chamber was solely due to the ability of viable HUVEC that have invaded through the matrix to hydrolyse calcein AM. High magnification light microscopy images of the matrix-containing side of the filters (filter top) revealed sites of matrix digestion due to HUVEC matrix metalloproteinase activity, whereas low magnification microscopy pictures of the filter bottom confirmed the presence of chemo-attracted HUVEC cells that have migrated through the 6μm filter pores (figure 5.3.5). Following calorimetric quantification of cellular invasion, a statistically significant decrease ($p<0.005$) in HUVEC ability to invade through TG2-cross-linked matrix was observed. Both inactivation of the enzyme with NTU283 active site directed inhibitor and cell-surface function-blocking with an anti-TG2 MAb (Cub7402) prior to exogenous application to the collagen-fibronectin mixture resulted in a more permeable barrier to HUVEC confirming that these effects were due to the enzyme's activity.

5.3.6 The effect of exogenous TG2 treatment on the circulation of TGFβ1 from the ECM

The indirect correlation between TG2 and extracellular matrix stability was assessed by measurement of LTBP-1 deposition into the matrix and biologically active TGFβ-1 release into the media of HUVEC and C378 single cell type cultures. It has been proposed that covalent association between LTBP-1 and extra-cellular matrix is facilitated by a TG2-dependent process (Nunes *et al.*, 1997). Moreover, it has been shown that increased

expression of TG2 in a transfected Swiss 3T3 cell line leads to an increased rate of LTBP-1 deposition into the ECM (Verderio *et al.*, 1999), where once activated, TGF-β1 may

Figure 5.3.5 HUVEC cell invasion through TG2-crosslinked artificial collagen/fibronectin matrices

HUVEC (10^6 cells/ml) were seeded in DMEM, on Fluoroblock filters pre-coated with collagen type I/fibronectin matrices, crosslinked by exogenous active TG2 (50μg/ml, 50U/ml). Cells were allowed to migrate to a lower chamber in response to chemoattraction (10% FCS) for 48 hours at 37°C as outlined in Methods (section 3.2.1). Inserts were then transferred to 24-well glass-bottom plates containing 4μg/ml Calcein AM in Hanks Balanced Salt Solution (HBSS) and incubated at 37°C for 90 minutes. Fluorescence due to the ester hydrolysis (Calcein AM) by live cells that have migrated to the bottom side of the filter was read at 485nm/530nm (excitation/emission) from-bottom-to-top using a SpectraFluor plate reader. **A**, light microscopy images of degraded artificial matrix (filter top) indicating the migration path of cells towards the pores (white) of Fluoroblock filters (blue). Cells that migrated to the bottom chamber (filter bottom) were visualised by staining in 0.1% (w/v) crystal violet. Bars=50μm. **B**, measurement of HUVEC invasion through artificial matrices. Results represent mean values ±SD, from two separate experiments (n=4).* Indicates significantly different from the control ($p<0.05$).

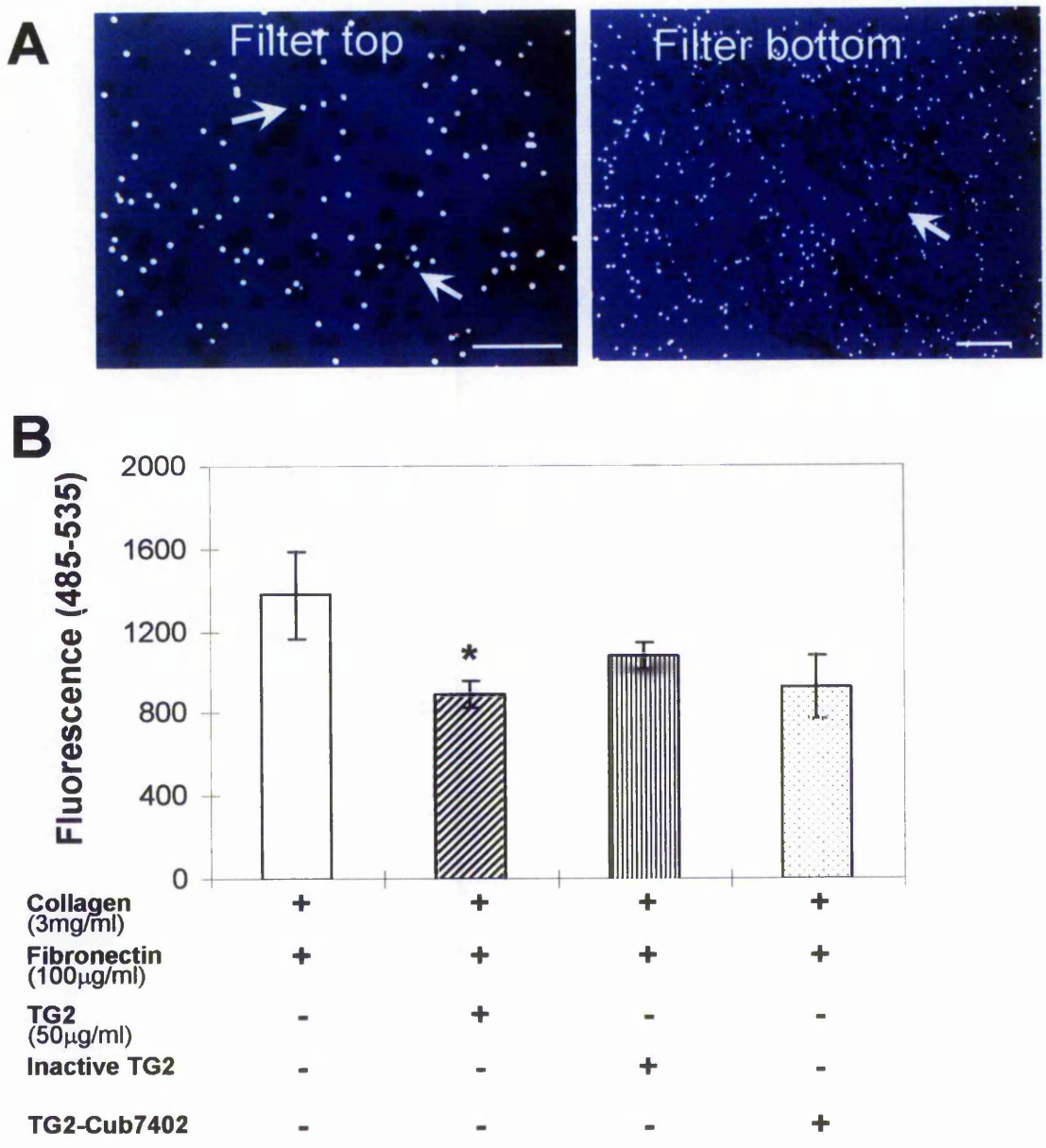


Figure 5.3.5 HUVEC cell invasion through TG2-crosslinked artificial collagen/fibronectin matrices

contribute to an increase in matrix protein synthesis (Ignots and Massague, 1986, Border *et al.*, 1990). However, as shown in figure 5.3.6.1, immunofluorescence staining indicated less pronounced LTBP-1 deposition in TG2-treated C378 fibroblasts in a dose dependent manner. No LTBP-1 could be detected in the ECM of HUVEC cultures. Additionally, administration of increased concentrations of TG2 to HUVEC and C378 cells was followed by decreased levels of biologically active TGFβ in the culture media when measured by TGFβ1 sandwich ELISA (figure 5.3.6.2). This could be attributed to cross-linking of the cytokine or its precursor complex (LTBP-1) into the matrix, rather than decreased release into the media, as suggested by the detection of high M*r* polymers on the top of stacking and resolving gels, when C378 cultures treated with two doses of TG2 were analysed for TGFβ-1 antigen by western blotting (Fig. 5.3.6.3*A*). The presence of the 25kDa band in the low molecular weight region of the gel, confirmed the affinity of the monoclonal mouse anti-TGFβ antibody (ab6472E6) for TGFβ1. A modified TGFβ-1 ELISA (figure 5.3.6.3*B*) undertaken on TG2-treated C378 cultures provided further confirmation for the presence of elevated TGFβ1 antigen within the ECM. In addition, treatment of serum-free and serum-containing media with 50μg/ml active (50U/ml) *gpl*TG2 revealed that the active enzyme facilitates sequestration of FCS–associated TGFβ onto the plastic.

Figure 5.3.6.1 The effect of exogenous TG2 treatment on LTBP-1 deposition of HUVEC and C378 cells

HUVEC and C378 cells were seeded at a density of $5x10^4$ and $2x10^4$ cells/well in 8-well chamber slides and allowed to grow overnight in standard cell growth medium. Cells were then administered with two doses of 10, 20, or 50μg/ml active (50U/ml) gpl TG2 at 8 hour intervals. Cultures were then stained *in situ* with an anti-LTBP-1 antibody, prior to fixation in 3.7% paraformaldehyde in PBS, and subsequent incubation with an FITC-conjugated secondary antibody as described previously (section 5.2.2). Cells were counterstained by mounting slides in a propidium iodide containing medium. LTBP-1 immunofluorescence (green) and cellular distribution (red) were visualised under the FITC and TRITC filters respectively of a TCSNT laser confocal microscope. **A**, LTBP-1 immunofluorescence in HUVEC cultures. **B**, LTBP-1 immunofluorescence in C378 cultures. Images are representative of 3 non-overlapping fields (n=4), from three independent experiments. Bar = 20μm.

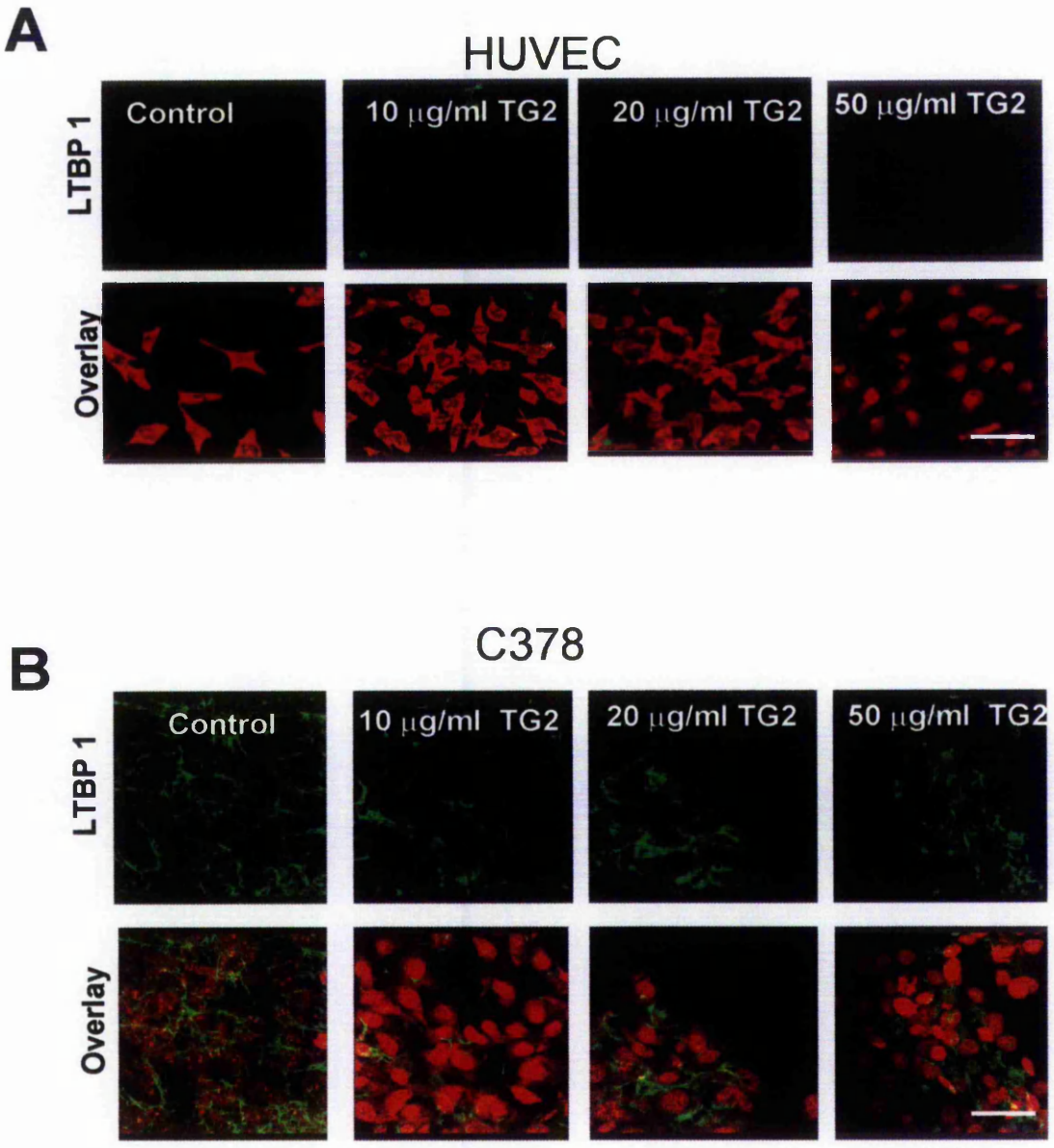


Figure 5.3.6.1 The effect of exogenous TG2 treatment on LTBP-1 deposition of HUVEC and C378 cells

Figure 5.3.6.2 The effect of exogenous TG2 treatment on TGF β -1 release into the media of HUVEC and C378 cultures

Conditioned media from HUVEC and C378 single cell type cultures administered with two doses of 10, 20, or 50μg/ml active (50U/ml) gpl TG2 during the course of the LTBP-1 study (figure 5.3.6.1) were also screened for biologically active TGFβ-1 as outlined previously (section 5.2.4). TGFβ-1 detected in the growth media of C378 cells (**A**) was found to decline in a dose dependent manner following first (t=0hours) and second (t=8 hours) exogenous administration of *gpl*TG2, each undertaken in fresh cell growth media. HUVEC TGFβ1 release levels (**B**) were maintained throughout the first enzyme administration, only to decrease following high enzyme concentration (50μg/ml) dosing. Results represent mean values ±SD (n=4), from three independent experiments.* Indicates significantly different from the untreated control ($p<0.05$).

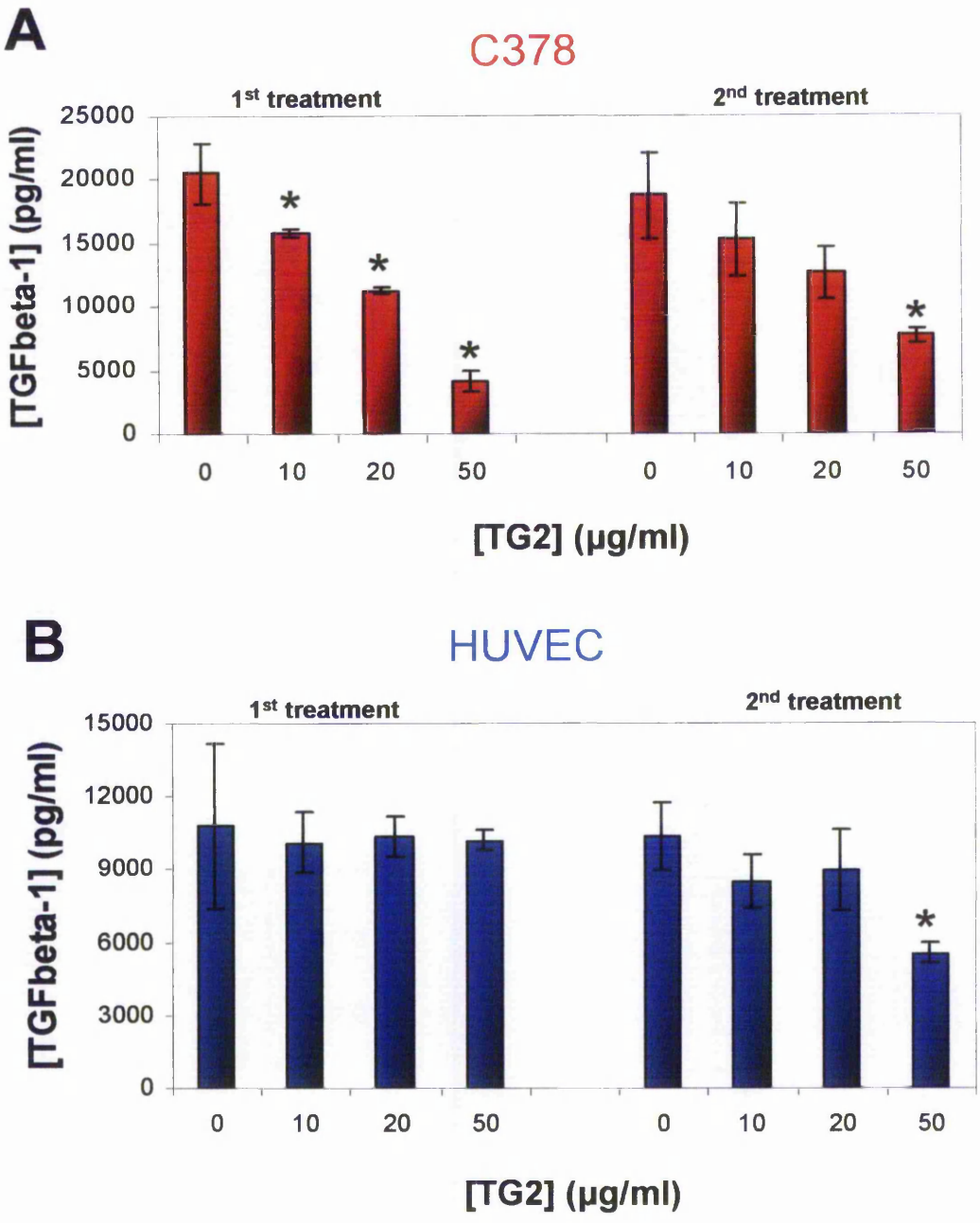


Figure 5.3.6.2 The effect of exogenous TG2 treatment on TGF β -1 release into the media of HUVEC and C378 cultures

Figure 5.3.6.3 Detection of TGFβ1 antigen in C378 cultures treated with *gpl*TG2

C378 cells were seeded at 2 x 10^3 cells/well in 96-well tissue culture plates and allowed to grow overnight, prior to application of exogenous active and inactive *gpl*TG2 as outlined previously (section 5.2.3). Following cell removal the remaining ECM was analysed for TGFβ-1 antigen. **A**, the ECM of C378 cells was solubilised in 2x Laemmli buffer and resolved (20μg/lane) on a 10% SDS-PAGE gel, prior to western transfer to a nitrocellulose membrane. Following incubation with a mouse anti-human TGFβ-1 antibody, and subsequent conjugation to an anti-mouse-HRP secondary antibody, high M*r* TGFβ-1 polymers at the top of stacking (TSG) and resolving gels (TRG), as well as the 25kDa biologically active TGFβ-1 fragment were detected by enhanced chemiluminescence. Lanes 1-3, Untreated control; lanes 4-6, TG2-treated; lanes 7-9, inactive TG2-treated. **B,** the ECM was blocked in 3% (w/v) BSA, and probed with an anti-human TGFβ-1 antibody, prior to conjugation with an anti-mouse-HRP secondary. Detection of matrix-bound TGFβ-1 was achieved colorimetrically by addition of TMB1 substrate, and absorbance values (A450) were obtained using a SpectraFluor plate reader. **C**, serum-free and serum-containing media controls, were also treated with exogenous TG2 in a similar manner to A. Following media removal, and 3 washes in PBS, the tissue culture plastic was blocked in 3% (w/v) BSA, and probed with an anti-human TGFβ-1 antibody, prior to conjugation with an anti-mouse-HRP secondary. Detection of plastic-bound TGFβ-1 was achieved colorimetrically in a similar manner as in B. Results represent mean values ±SD (n=4), from three independent experiments.* Indicates significantly different from the untreated control ($p<0.05$).

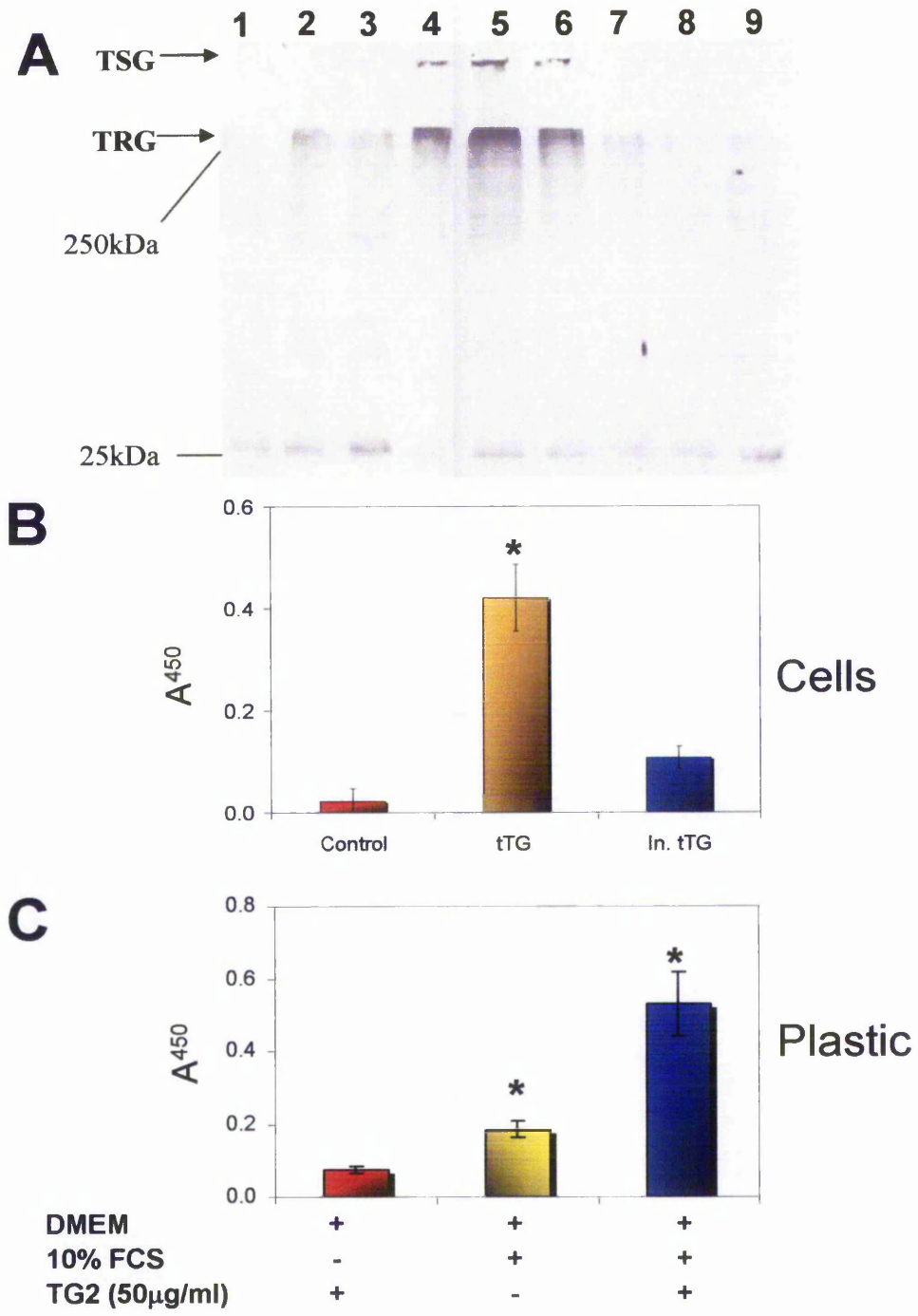


Figure 5.3.6.3 Detection of TGFβ1 antigen in C378 cultures treated with *gpi*TG2

5.4 Discussion

It has been recently proposed that TG2 might be involved in the deposition and homeostasis of the ECM either through a direct, reinforcing mechanism, by virtue of its protein cross-linking function, or in an indirect manner through up-regulation of LTBP-1 deposition and consequent increase of TGF-β activation and concomitant release from the matrix (Johnson *et al.*, 1999).

The experimental system employed in this study involves addition of *gpl*TG2 to cultures. Addition of exogenous TG2 mimics the tissue wounding/fibrotic process, where following physical injury cellular TG2 'leaks' into the surrounding ECM, where it becomes activated and catalyses the cross-linking of proteins (Nicholas *et al.*, 2003). Primarily, C378 and HUVEC cultures were incubated in the presence of increasing concentrations of exogenous *gpl*TG2, in order to examine whether the enzyme addition has any cytotoxic effects. No cytotoxicity was detected when cell viability was measured by the XTT and LDH assays. In fact, augmented cell viability was observed instead. Since no increase in cell number following addition of exogenous TG2 was observed in C378 cultures (Balklava, 2002; PhD Thesis), the reported increase in viability suggests that exogenous TG2 administration might affect cell metabolism, and particularly mitochondrial integrity and activity.

In support of the direct ECM stabilisation mechanism, previous studies from our laboratory have demonstrated that TG2 is present at the cell surface and secreted into the ECM where it remains active as a protein cross-linker (Verderio *et al.*, 1998). The potential direct regulation of ECM stabilisation by TG2 was investigated by labeling C378 and HUVEC single cell type cultures with [2,3-^{3}H]-Proline, hence measuring collagen deposition and turnover. By measuring the amount of radiolabel released by the cells into the media it emerged that similar amounts of radiolabel were incorporated and processed within cultures undergoing different treatment regimes. Initial addition to C378 fibroblast cultures of increasing concentrations of active enzyme, at t=0 after removal of the radiolabel, resulted in a dose dependent increase in the collagen deposition as reflected by the amount of label found in the ECM fraction 12 hours later. This effect may be attributed to the reported ability of active transglutaminases to accelerate collagen gel/fibril formation (Nomura *et al.*,

2001; Orban *et al.*, 2004; Skill *et al.*, 2004). Secondary addition of exogenous enzyme, 12 hours after the labeling process had been completed, resulted in significant reduction of collagen breakdown when compared to the untreated control. This could be the result of an indirect modulation of collagen synthesis, via collagen expression up-regulation, or due to mechanical strengthening of the fibrillar portion of the ECM due to increased collagen cross-linking. An *in vitro* increase in the resistance of cross-linked matrix to proteolytic degradation by matrix metalloproteinase has already been reported (Johnson *et al.*, 1999). Experiments employed in this study also suggest that exposure of radiolabeled C378 collagen matrix treated with *gpl*TG2 to activated interstitial collagenase (MMP1) leads to a diminished susceptibility to degradation by this metalloproteinase. This indicates that exogenous TG2 increases both cell matrix deposition and stability once the matrix is deposited. As the ECM is reinforced by TG2-mediated cross-linking in a fibrotic-like manner, its resistance to degradation by proteases is increased, and the turnover process is attenuated. Moreover, Northern analysis of total RNA extracted from C378, following four exogenous applications of TG2 (50μg/ml, 50U/ml) at daily intervals, revealed that collagen I, III, and IV expression is down-regulated whereas gelatinase A (MMP-2) mRNA was upregulated. This initially suggests that an autocrine cellular response to a highly cross-linked, fibrotic extracellular environment might be responsible for 'shutting down' the collagen deposition pathway and 'switching on' its counterpart digestion pathway. Interestingly TIMP-1 expression was also up-regulated., possibly to restore the imbalance caused by increased metalloproteinase expression.

Having established that TG2 reinforces the ECM stability, and taking into account that the latter is a prerequisite for cell migration and consequently cell invasion to take place, the effect of TG2 on cell invasion potential was also examined. Cell locomotion is a dynamic process, involving cell adhesion to the extracellular matrix, extension of the leading edge of the cell, and retraction of the trailing tail. The role of cell surface TG2 in cell locomotion has been not been yet fully understood. The inhibition of the adhesive function of cell surface TG2 by function-blocking anibodies has been demonstrated to markedly decrease adhesion and migration of endothelilal-like (ECV304) and monocytic cells on fibronectin (Jones *et al.*, 1997; Akimov and Belkin, 2001). It has also been demonstrated that the

proteolytic degradation of cancer cell-surface TG2 suppressed cell adhesion and migration on fibronectin, but stimulated cell motility on collagen matrices (Belkin *et al.*, 2001). Artificial matrices were prepared *in vitro* by cross-linking *gpl*TG2 with collagen and fibronectin. Results suggested that TG2 croslinking of collagen/fibronectin-based gel matrices significantly decreased the invasion potential of HUVEC through these matrices. This could be explained by two disparate mechanisms. The first mechanism implicates TG2 in cell invasion as a cell-surface receptor providing additional cell-matrix contacts. Cell adhesion strength beyond a threshold level and characterised by a fast rate of cellular association to cell adhesion receptors, results in slow cell migration as the cell cannot develop enough traction to migrate and eventually invade through the matrix (Palecek *et al.*, 1997). Alternatively, decreased ability of HUVEC cells to invade through artificial matrices, could be attributed to the strengthening of the matrix and reduced susceptibility to degradation due to TG2-mediated cross-linking. In support of the second mechanism, active site directed inhibition of transglutaminase activity (NTU283), prior to incubation with the collagen/fibronectin mix, restored the endothelial cell ability to digest this artificial barrier.

The indirect mechanism accounting for the ability of TG2 to modulate extracellular matrix deposition/stability was assessed by measurement of LTBP-1 deposition into the matrix and TGFβ1 release into the media. It has been proposed that covalent association between LTBP-1 and extra-cellular matrix is facilitated by a TG2-dependent process (Nunes *et al*, 1997). Moreover, it has been shown that increased expression of TG2 leads to an increased rate of LTBP-1 deposition into the ECM (Verderio *et al.*, 1999), raising the possibility of TG2 contributing to an increase in matrix protein synthesis (Ignots and Massague, 1986, Border *et al.*, 1990). This assumption confers TGFβ1 (and consequently its precursor binding protein LTBP-1) a potentially pivotal role in synthesis and maintenance of the stability of the extracellular matrix. Paradoxically, immunofluorescence indicated less pronounced LTBP-1 deposition in TG2-treated C378 fibrosblasts in a dose dependent manner. Additionally, administration of increased concentrations of TG2 to HUVEC and C378 cells was followed by decreased biologically active TGFβ1 detection in the medium. The reported decrease of TGFβ1 in the medium of TG2-treated C378 cultures could be either due to sequestration of the active cytokine or a decreased deposition of the latent

binding protein complex (LTBP1) into the matrix. The observed decrease in the amount of serum-associated TGFβ suggested that any changes recorded on the circulatory pool of the cytokine are not due to its decreased release in the medium. Western blot analysis of C378 ECM protein revealed high M_r TGFβ polymers at the top of the stacking and resolving gels. Results from a modified TGFβ ELISA, undertaken on C378 ECM proteins or medium-only controls, provided confirmation for the sequestration of TGFβ1 to the ECM/plastic in response to TG2 application. Nunes *et al.* (1997) have already demonstrated that LTBP-1 complexes are transglutaminase substrates, which does not rule out that the TGFβ antibodies used within this study might be recognizing both TGFβ1 and its binding protein complex. These observations inevitably raise questions with respect to the specificity of the monoclonal anti-TGFβ1 used in these methods. It is more reasonable to accept that TG2 facilitates LTBP1 sequestration to the ECM where it can be degraded and its biologically active form released to induce ECM protein synthesis. On the other hand, it was hypothesized that the exogenous enzyme would lock LTBP1 –a known TG substrate- into the ECM. In contrast, the immunofluorescence data obtained using anti-LTBP1 (ab39) revealed a decrease in ECM-associated LTBP1. It remains to be further examined whether the LTBP-1 epitope for ab39 is masked by cross-linking. Despite these TG2-mediated quantitative changes in circulating TGFβ1, the observation that collagen expression is downregulated following culture treatment with exogenous TG2, suggests that TG2 is not exerting its effect via activation of the protein synthesis pathway that is triggered by $TGF\beta_1$. This, however, does not rule out the possibility that initial application of TG2 might up-regulate collagen expression, before autocrine feedback from a highly cross-linked ECM, following multiple TG2 administrations, leads to collagen expression 'shutdown'.

Chapter 6:

Characterisation of CT26 mouse colon carcinoma cells transfected for stable expression of active and inactive tissue transglutaminase

6.1 Introduction

Barnes and co-workers demonstrated first that TG activity was greatly reduced in metastatic transplantable rat sarcomas when compared to normal tissues of rat liver, lung and spleen (Barnes *et al.,* 1985). In the majority of tumours examined *in vivo* and in *vitro*, reduced tissue transglutaminase activity has been shown to be associated with carcinogenesis, tumour progression and malignancy (Hand *et al.*, 1987) leading to decreased numbers of isopeptide cross-links, particularly in proteins associated with the plasma membrane (Knight *et al.*, 1990a). The reduction of TG2 expression and polyamine content in neoplastic tissue has also been correlated in different studies with an increase in the metastatic potential of tumours such as rat fibrosarcomas, rat prostate carcinoma, HSV-2 induced hamster fibrosarcoma and mouse melanoma (Birckbichler *et al.*, 1976; Delcros *et al.*, 1986; Hand *et al.*, 1987; Knight *et al.*, 1991; Beninati *et al.*, 1993). Based on current literature, there are three possible modes of action, by which the enzyme may exert its effect on the tumour-host microenvironment. TG2 has been reported to be involved in cell adhesion and migration (Verderio *et al.*, 1998), cell death (Piacentini *et al.*, 1999; Auturi *et al.*, 1998), and in the stabilisation of the extracellular matrix (ECM) (Barsigian *et al.*, 1991; Johnson *et al.*, 1997).

In support of the first potential mechanism, a plethora of evidence suggests that TG2 plays a role in cell adhesion through its cell surface associated function. Fibroblasts overexpressing TG2 demonstrated increased cell attachment, spreading, and migration as well as reduced susceptibility to detachment by trypsin (Gentile *et al.*, 1992; Balklava *et al.*, 2002). In a different experimental model, reduced expression of TG2 was associated with decreased cell adhesion and spreading in different cell lines (Jones *et al.*, 1997; Verderio *et al.*, 1998). Following these findings a putative role for TG2 as an integrin-binding adhesion co-receptor for fibronectin was proposed (Akimov *et al.*, 2000). More recently, a novel TG2-mediated, RGD-independent cell adhesion mechanism that rescues cells from anoikis has been proposed (Verderio *et al.*, 2003). Also relevant to the adhesive role for TG2 in tumour growth is the finding that the proteolytic degradation of

cancer cell-surface TG2 suppressed cell adhesion and migration on fibronectin, but stimulated cell motility on collagen matrices (Belkin *et al.*, 2001).

Various studies have also explored the possibility that increasing TG2 expression might have an intracellular effect on tumour cell viability and subsequently on tumour growth and progression. Constitutive expression of TG2 in the neuroblastoma cell line SK-N-BE2 was shown to correlate to mitochondrial hyperpolarisation and consequent sensitization to apoptosis, resulting in delayed tumour growth (Melino *et al.*, 1994; Piacentini *et al.*, 2002).

Once externalised from the cell, TG2 has the capacity of inducing qualitative changes to ECM proteins via cross-linking. There have been numerous reports linking TG2 to altered matrix turnover such as in the development of fibrosis in various tissues (Griffin *et al.*, 1979; Johnson *et al.*, 1999; Mirza *et al.*, 1999; Piacentini *et al.*, 1999; Small *et al.*, 1999; Grenard *et al.*, 2001). Previous studies with transfected Swiss 3T3 clone-TG3 have demonstrated increased formation of fibronectin polymers and elevated ε-(γ-glutamyl)lysine cross-links in the cells induced for the overexpression of catalytically active TG2 (Verderio *et al.*, 1998). Preliminary data within this laboratory have shown that overexpression of TG2 by the same Swiss 3T3 clone does not lead to significant differences in matrix deposition (Balklava, 2002; PhD Thesis), although differences were prominent when the enzyme was delivered exogenously to human angiogenesis co-cultures and C378/HUVEC single cell type cultures (sections 4.3.4.1 and 5.3.1 respectively). Given that the increased expression and externalisation of the enzyme leads to more cross-links formed in the ECM, it is conceivable that the matrix would be more resistant to proteolytic degradation, as demonstrated previously (section 5.3.3). Reduced susceptibility to degradation by MMPs could in turn slow down both tumour cell and host endothelial cell migration during angiogenesis and tumour cell invasion respectively. In support of this hypothesis, it has already been established within this study that TG2-mediated cross-linking of artificial collagen/fibronectin matrices confines endothelial cells within these reinforced matrices, which accounts for the reduced migration of endothelial tumour host cells (section 5.3.5). Although endothelial cells constitute a small

portion of the mature tumour body, the observed reduction in the migration of malignant cells- the core of the mature tumour mass- as described previously (section 3.3.2.3), provided a more generic mechanistic explanation for the observed suppression of tumour growth as described in section 3.3.2.4.

Overexpression of TG2 in the hamster malignant fibrosarcoma cell line MetB led to reduced incidence of primary tumour (Johnson *et al.* 1994). In the current study, it has been demonstrated that intratumour injection of exogenous TG2 also leads to tumour regression (section 3.3.2.4). *In vitro* data indicated that this may be attributed to impaired EC migration during the early stages of angiogenesis (section 4.3.3) as a consequence of the protein cross-linking activity of the ECM-associated enzyme (section 4.3.4.1). Moreover, other lines of evidence drawn from *in vivo* experiments carried out in this study suggest that increased protein cross-linking due to the exogenous enzyme stabilises the tumour ECM in a fibrotic manner (section 3.3.2.6), thus blocking tumour cell invasion (section 3.3.2.3) . In order to ascertain whether the antitumour capacity of TG2 is due to its cell-surface adhesion function or instead due to its extracellular transamidating activity we were in search of a model in which tumour cells that possess only the cell-surface TG2 function without transamidating activity could be directly compared to isotypes that also possess extracellular cross-linking activity. In a previous study, Swiss 3T3 fibroblasts were transfected with inactive TG2 constructs (C277S) and shown to lack the ability to deposit the enzyme into the ECM whilst retaining the cell surface adhesion function (Balklava *et al.*, 2002). By employing a similar tumour cell transfection approach with TG2 constructs, a direct interplay between active extracellular enzyme, ECM stabilisation, and consequent attenuation of tumour growth could be demonstrated. To address this, the mouse colon carcinoma cell line CT26, that is almost devoid of TG2 expression, and exhibits minimal transglutaminase activity was transfected for stable expression of both active and inactive (C277S) TG2 prior to implantation into a balb/c host.

6.2 Methods

6.2.1 Cell attachment assay

TG2-transfected CT26 cell pellets were resuspended to 2 x 10^6/ml in DMEM and 100µl/well cell suspension added to 96-well FN-coated plates and incubated for 40 minutes at 37°C, 5% (v/v) CO_2. The medium was removed and cells were gently washed with PBS, pH 7.4. Cells were then stained in 0.5% (w/v) crystal violet in 70% (v/v) ethanol for 15 minutes at room temperature, prior to washing three times with PBS pH 7.4. Cells were then allowed to air dry, visualised under a light microscope and images were captured using an Olympus digital camera. Desicated cells were finally solubilised by addition of 100µl/well 30% (v/v) acetic acid, and absorbance read at 540nm on a Spectrafluor plate reader.

6.2.2 Measurement of MMP1 activity by collagen zymography

The relative amounts of pro-enzyme and active matrix-metalloproteinase (MMP) species released by the TG2-transfected CT26 cells into the cell growth medium was analysed using collagen zymography as described previously (Herron *et al.*, 1986). Resolving gels were prepared by mixing the following components in order: 1ml of type I collagen (Sigma) in 20mM acetic acid, 3.1ml H_2O, 2.5ml 1.5M Tris HCl, pH 8.8, 3.33ml of 29% acrylamide/1 % N,'-methylene biacrylamide, 50µl of 10% (w/v) ammonium persulphate, 10µl N,N,N',N'-tetramethylethylenediamine (TEMED). Stacking gels were prepared as described in Methods (section 2.2.3.3) Conditioned cell growth media (5µl) were diluted 1:1 with loading buffer [1M Tris HCl pH 6.8, 50% (v/v) glycerol, 0.4% (w/v) bromophenol blue] and electrophoresed at 100V in standard Laemmli running buffer (24mM Tris HCl, 192mM glycine, 3.47mM SDS, pH 8.3) for 4-5 hours and at 4°C. Following electrophoresis, SDS was removed and MMP activity was recovered by washing the gels twice in 2.5% Triton X-100 for 60 minutes. MMPs were then activated by overnight incubation in digestion buffer (100mM,Tris HCl, 5mM $CaCl_2$, 0.005 Brij-35,

1μM $ZnCl_2$, 0,001% NaN_3, pH 8) at 37 °C. MMP activity was visualised by staining with 0.2% (w/v) Coomassie Brilliant blue R in 40% (v/v) methanol, 10% (v/v) acetic acid for 1 hour and destained by microwaving for 15 minutes (850W) in 3 changes of deionised H_2O.

6.3 Results

6.3.1 Intrinsic TG2 antigen and activity of parental CT26 mouse carcinoma cells

Prior to transfecting CT26 cells with constructs containing the active and inactive (C277S) TG2 cDNA it was deemed important to assess the cell line's intrinsic TG2 expression and transglutaminase activity levels. Therefore, parental CT26 cells were screened for TG2 antigen expression and cell-surface/total TG activity by means of western blotting and biotin-X-cadaverine incorporation into fibronectin respectively. The human derived cell line ECV304 (intact cells and lysates), which is known to exhibit increased TG2 expression, was used as a positive control. Western blotting revealed that TG2 antigen is expressed at minimal levels by CT26, whereas cell-surface associated activity of fibronectin-anchored CT26 cells and total TG activity of CT26 cell lysates bordered background levels (figure 6.3.1). Taken together, the results indicate that the CT26 mouse colon carcinoma cell line is almost devoid of TG2, which is in keeping with both the B16-F1 melanoma study (section 3.3.1.3) and previous publications reporting absence or reduction of TG2 expression in various tumour cell lines (Birckbichler *et al.*, 1976; Delcros *et al.*, 1986; Hand *et al.*, 1987; Knight *et al.*, 1991).

6.3.2 Susceptibility of CT26 cells to G418

For subsequent transfections a range of G418 concentrations (0.1-1mg/ml) was administered to sub-confluent parental CT26 cultures to identify the lowest possible concentration that would lead to 100% cell death within 4 days as described in Methods (section 2.2.1.8.1). The resulting killing curve indicated that 800μg/ml G418 was the optimal concentration for clone selection (figure 6.3.2).

Figure 6.3.1 Intrinsic TG2 expression and cell-surface/total TG activity of the parental CT26 cell line

A, triplicate CT26 cell lysates were screened for TG2 antigen expression by means of SDS-PAGE, followed by western blotting and immunoprobing with an anti-TG2 MAb (Cub 7402). ECV304 lysates were used as a positive control. Lanes 1-3, CT26 lysates; lanes 4-6, ECV304 lysates. **B**, anchored CT26/ECV304 cells and lysates were screened for cell-surface and total TG activity respectively by means of biotin-X-cadaverine incorporation into fibronectin. Cells were seeded at 4 x 10^4 cells/well on fibronectin-coated 96-well plates and allowed to attach over 4 hours. Cells were then lifted by mild detergent treatment, and the remaining ECM blocked, and immunoprobed as outlined in Methods (section 2.2.3.7.2). In parallel, for measurement of total enzyme activity, cells were homogenised, sonicated, and cell lysates added directly to FN-coated wells prior to incubation for 4 hours at 37°C. Positive controls were obtained by exogenous application of 2μg/ml of either active, or NTU283-inactivated (500μM) *gpl*TG2 to the cells. Results represent values ± SD from two separate experiments (n=6).

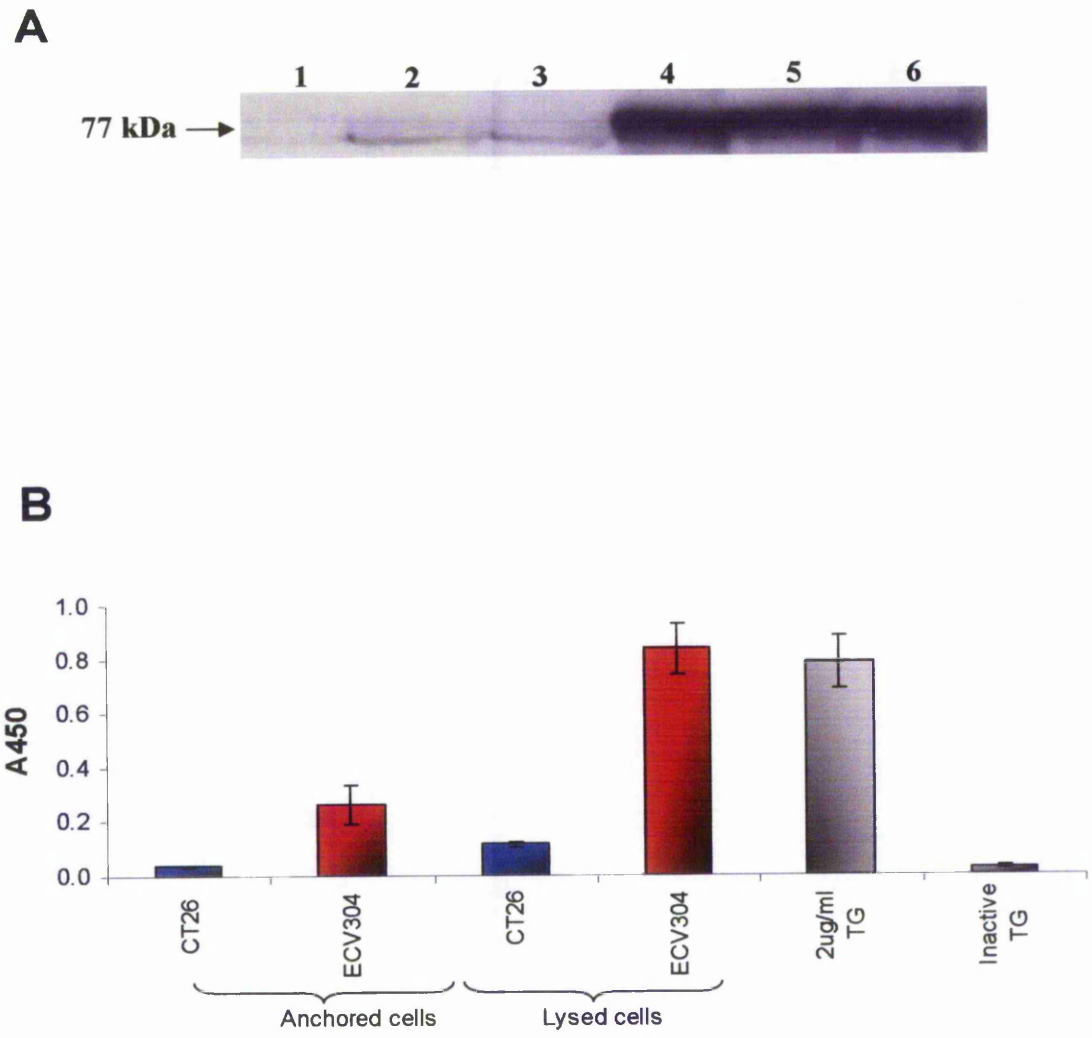


Figure 6.3.1 Intrinsic TG2 expression and cell-surface/total TG activity of the parental CT26 cell line

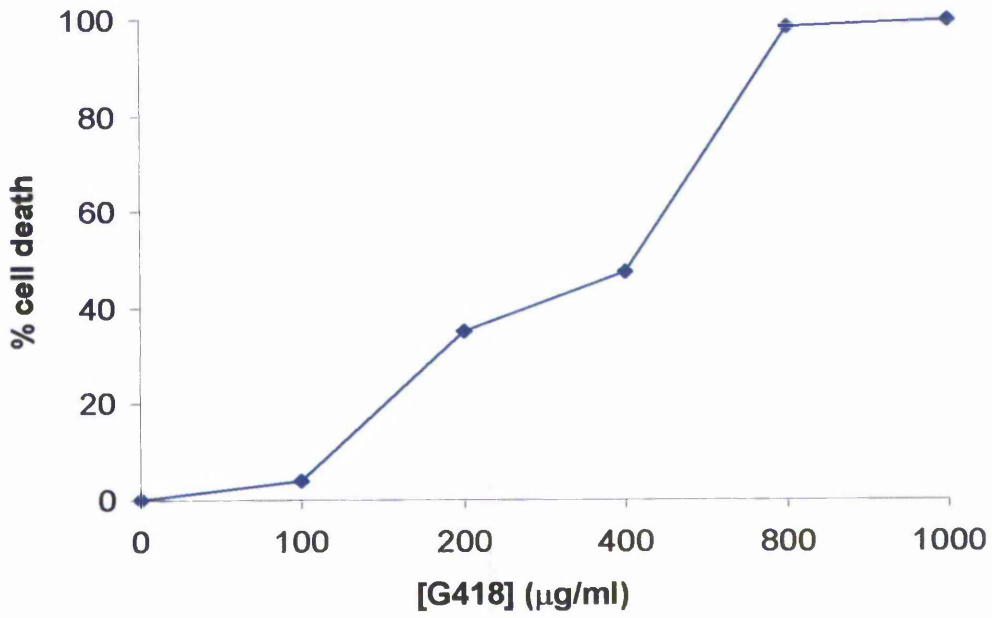


Figure 6.3.2 G418 killing curve of parental CT26

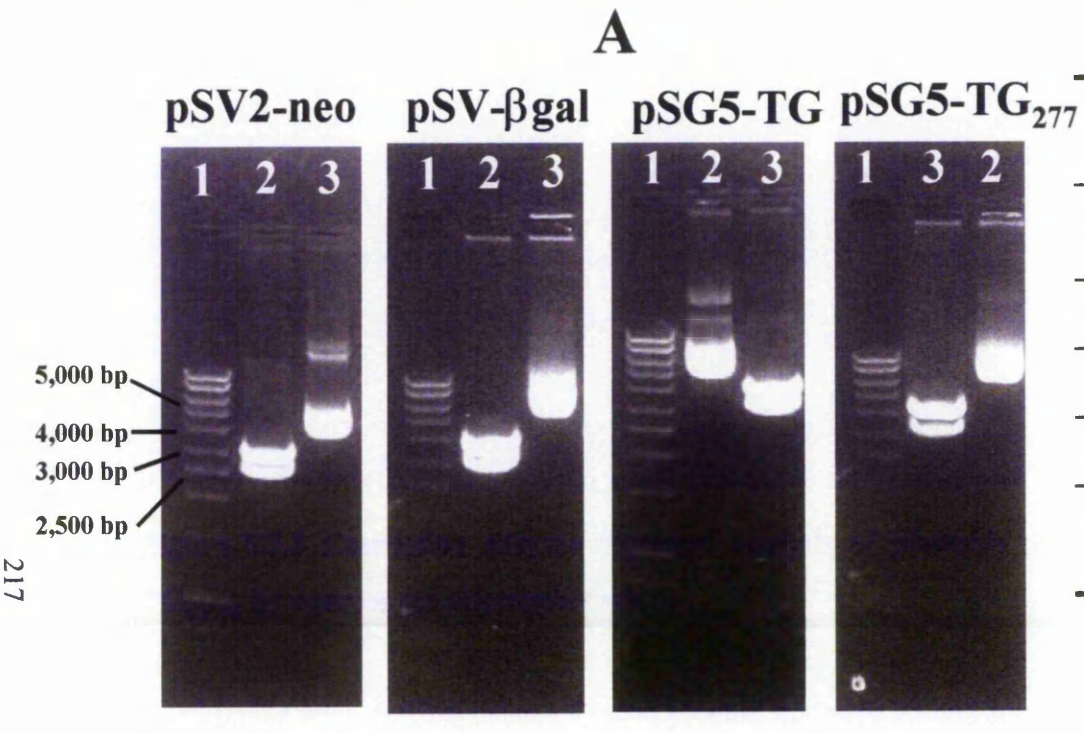
CT26 cells were seeded in duplicate in 6-well plates and allowed to reach 60% confluency overnight. The cells were then treated with a range of G418 concentrations (0.1-1mg/ml) in standard cell growth media. Following incubation for 4 days, it was observed that CT26 treated with 1mg/ml of G418 had all detached from the plastic. At that point dead cells were removed by washing in PBS, and viable cells were trypsinised and counted using an improved Neubaeur haemocytometer. Cell death of CT26 due to G418 was measured as a percentage of untreated control. Results represent mean values (n=3).

6.3.3 Restriction enzyme digestion of TG2-constructs and control plasmids

Plasmids used for the transfections of CT26 mouse colon carcinoma cells were obtained by growing DH5α bacterial cells transformed with the prepared constructs, pSVNeo (control), pSG5-TG and pSG5-TG_{277}, eGFP-N1 or pSVβ-gal (transfection efficiency markers). Plasmid DNA was purified using a Qiagen plasmid midiprep kit, according to the manufacturer's protocol. Plasmid concentration was estimated by measurement of A_{260nm} as described in Methods (section 2.2.2.1.3) and the quality of the plasmid was assessed on a 1% (w/v) agarose gel (figure 6.3.3). The ratio A_{260nm}/A_{280nm} of all plasmids purified was equal to or higher than 1.7.

6.3.4 Comparison of a modified electropermeabilisation method to a conventional cationic lipid transfection system

Transfections were carried out in parallel by employing two separate systems: A cationic lipid-based system (lipofectamine) utilising pSVβ-gal as a transfection efficiency marker (section 2.2.1.8.3), as well as a direct nuclear transfection (electroporation) system provided by AMAXA (AMAXA Biosystems, Germany), utilising eGFP-N1 as a transfection efficiency marker (section 2.2.1.8.2). Transfection efficiencies were optimized by employing confocal microscopy to assess transfer of the enhanced Green Fluorescent Protein-carrying plasmid (eGFP-N1) into the nucleus of CT26 cells and a β-galactosidase assay to evaluate episomal expression of the pSVβ-gal plasmid by the CT26 cells as described in the relevant Methods sections (2.2.1.8.3.1 and 2.2.1.8.2.1 respectively). Transfection efficiencies were estimated as a percentage of total cells expressing the transfection marker in use. Nucleofection efficiency of the eGFP-N1 plasmid was shown to reach 76% when the T20 program was used (figure 6.3.4.1), whereas the episomal transfection efficiency of the pSV-β-gal plasmid was found to be much lower (approximately 3%) as shown in figure 6.3.4.2. Moreover, when increased concentrations of lipofectamine were complexed with the DNA plasmids, augmented



B

Vector	Size of Vector (bp)	Size of Insert (bp)
pSV2Neo	2,600	
pSVβ-gal	6,800	
EGFP-N1	4,700	
pSG5-TG	5,500	3,250
pSG5-TG277	5,500	3,250

Figure 6.3.3 Restriction enzyme digestion analysis of plasmids pSV-Neo, pSVβ-gal, pSG5-TG and pSG5-TG_{C277S} by agarose gel electrophoresis

A, plasmids were purified using a Qiagen plasmid midiprep kit and analysed by 1% (w/v) agarose gel electrophoresis as outlined in the Methods (sections 2.2.2.1.5 and 2.2.2.1.6). Lane 1, 2kbp-50bp λDNA ladder, lane 2 restricted plasmid, lane 3 unrestricted plasmid. pSV2Neo has been restricted by EcoRI and HindIII, pSVβ-gal by EcoRI, pSG5-TG and pSG5-TG_{277} by EcoRI. **B**, expected restriction pattern for all 5 vectors.

Figure 6.3.4.1 Nucleofection efficiency measured by confocal microscopy of GFP expression

CT26 were nucleofected with 5μg eGFP-N1 in AMAXA's Normal Human Dermal fibroblast buffer under different voltage/duration programs (A-23, A-27, T-20, T-27, T-01, T-16) as outlined in the Methods (section 2.2.1.8.2). 16 hours post-transfection, cells were trypsinised and 1,000 cells were spotted on chamber slides, air-dried and viewed under a confocal microscope. Efficiency was measured as a percentage of cells expressing green fluorescent protein. **A**, top panel; green fluorescent cells as viewed under a laser confocal microscope set for FITC excitation; bottom panel, total number of cells as seen under reflection filter. Bar=10μm. **B,** % transfection efficiencies. Images are representative of three non-overlapping fields obtained from three different samples.

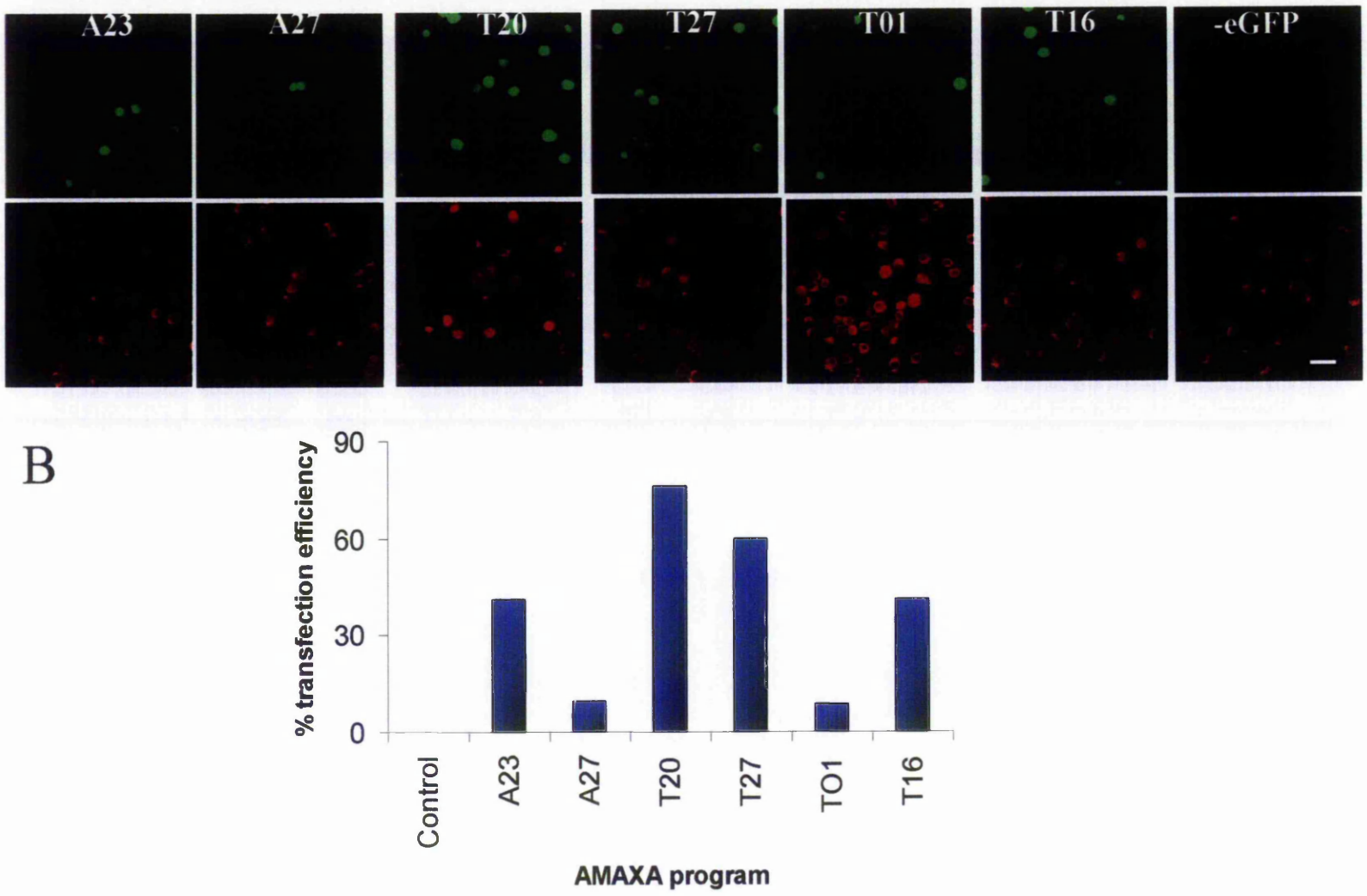


Figure 6.3.4.1 Nucleofection efficiency measured by confocal microscopy of GFP expression

Figure 6.3.4.2 Lipofection efficiency measured by β-galactosidase staining of CT26 clones 48 hours post-transfection

10^6 CT26 cells were seeded on 6-cm tissue culture petri dishes and allowed to reach 80-90% confluency overnight. Cells were then incubated with the pSV-βgal plasmid, pre-complexed, at different ratios and for 20 minutes with lipofectamine, and then incubated at 37°C, 5% (v/v) $C0_2$ for a further 6 hours. Following removal of the DNA-lipid complexes, cells were allowed to grow for 24 hours prior to assaying for transgene expression using a β-galactosidase activity assay as described under Methods (section 2.2.1.8.3.1). **A**, β-galactosidase activity staining (blue) of CT26 clones incubated with 24μg lipofectamine (untransfected), 8μg pSV-βgal complexed with 24μg lipofectamine (DNA/Lipofectamine=1/3), 8μg pSV-βgal in the presence of 8μg lipofectamine (DNA/Lipofectamine= 1/1), and 16μg pSV-gal in the presence of 8μg lipofectamine (DNA/Lipofectamine= 2/1). Images were obtained on a *Zeiss* light microscope and are representative of three non-overlapping fields obtained from three different samples (Bar=50μm). **B**, percentage transfection efficiencies and cell survival following transfections with different DNA/ Lipofectamine complex ratios. Values represent mean ±SD obtained from 3 different samples.

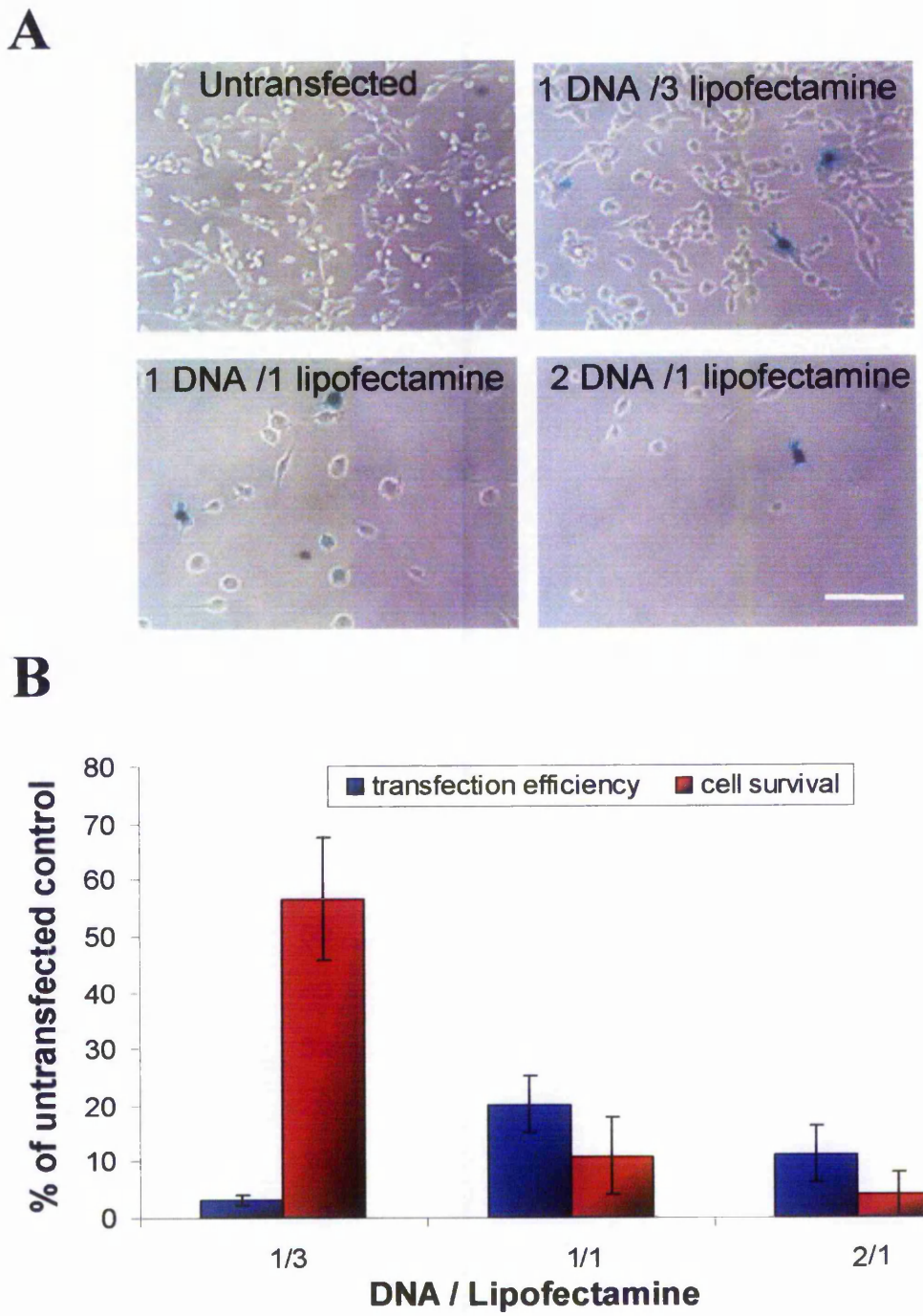


Figure 6.3.4.2 Lipofection efficiency measured by β-galactosidase staining of CT26 clones 48 hours post-transfection

transfection was accompanied by increased cellular toxicity. Therefore, from this point forward, further screening was pursued only on the nucleofected clones.

6.3.5 Screening CT26 clones for total TG2 antigen and activity

Following initial selection from the transfected cultures a total of 60 clones that were resistant to 800μg/ml G418 (12 x pSV-Neo, 24 x pSG5-TG and 24 x pSG5-TG $_{C277S}$) were chosen for sub-culturing. Subsequent cloning-out of the CT26 cells nucleofected with pSG5-TG, pSG5-TG$_{277}$ and pSVneo plasmids generated 40 viable clones. Clones expressing increased amounts of TG2 were identified initially using a high throughput modified ELISA (data not shown) as described in the Methods (section 2.2.3.6.1), followed by SDS-PAGE and western blotting on clones that have been further selected. Cell homogenates from transfected clones were separated by SDS-PAGE, western blotted and immunoprobed for TG2 as described in the Methods (section 2.2.3). Out of the 25 clones selected for screening by western blotting 7 clones exhibiting increased expression of TG2 were detected as shown in figure 6.3.5.1. Clones TG 2, 3, 5, and 10 from the pSG5-TG-transfected CT26, INA12, 13, and 14, from the pSG5-TG$_{277}$-transfected CT26, and Neo1, 2, and 4 from the control vector-transfected CT26 were selected for further screening. To ensure that the obtained TG2 positive clones were expressing the catalytically active form of the enzyme and the inactive TG2 construct did not confer any activity to the CT26 cells, the [^{14}C]-putrescine TG2 activity assay was performed on CT26 clone lysates as described in Methods (section 2.2.3.7.1). As expected, all Neo control- and inactive TG2-transfected clones were found to be negative for TG activity, whilst only clones TG3, 5 and 10, transfected with the active TG2 construct, exhibited detectable TG activity (figure 6.3.5.2).

6.3.6 Detection of cell-surface associated TG2 antigen and activity in CT26 clones

In order to map the cellular distribution of the active and inactive enzyme in TG2-transfected CT26 clones, live cells were initially stained for cell surface-associated TG2

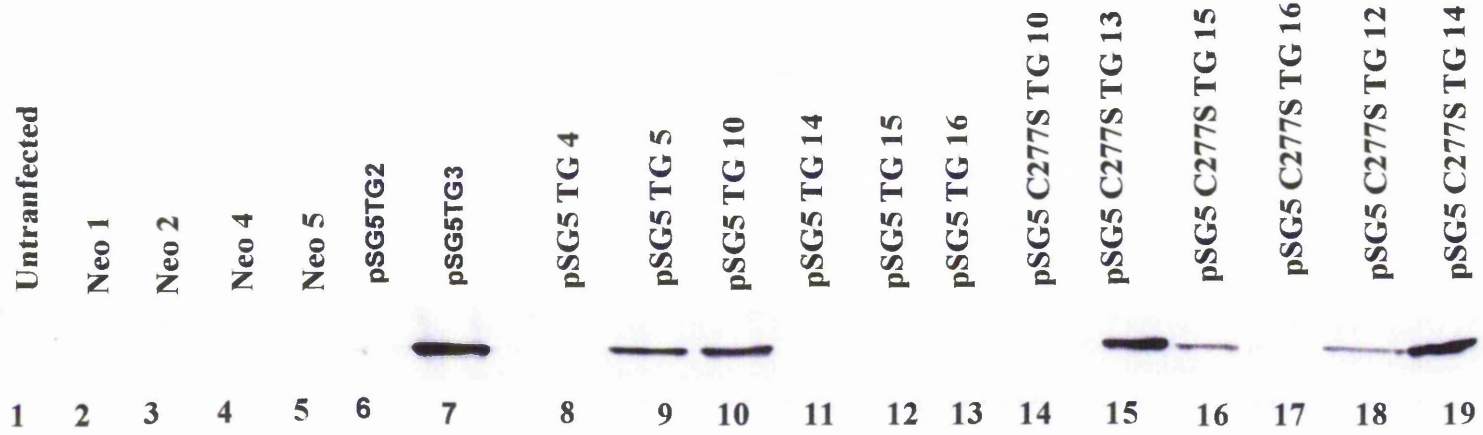


Figure 6.3.5.1 Screening of CT26 clones transfected with TG2 by nucleofection for total cellular TG2 antigen by western blotting

A, western blot indicating expression of the human full-length TG2 cDNA which was inserted into CT26 cells. CT26 clones were harvested by trypsinisation, homogenised in 2x Laemmli buffer and 20μg of protein then loaded on a 10% SDS-polyacrylamide gel. Proteins were then transferred onto a nitrocellulose membrane by western blotting, and TG2 antigen was detected by immunoprobing with an anti-TG2 MAb (Cub 7402), which was recognised by a secondary anti-mouse-HRP conjugate as outlined in the Methods (section 2.2.3.5). Lane 1, parental CT26 cells; lanes 2-5 Neo transfected negative controls; lanes 6-13; cells transfected with active TG2 constructs; lanes 14-19, cells transfected with inactive (Cys277Ser) TG2 constructs.

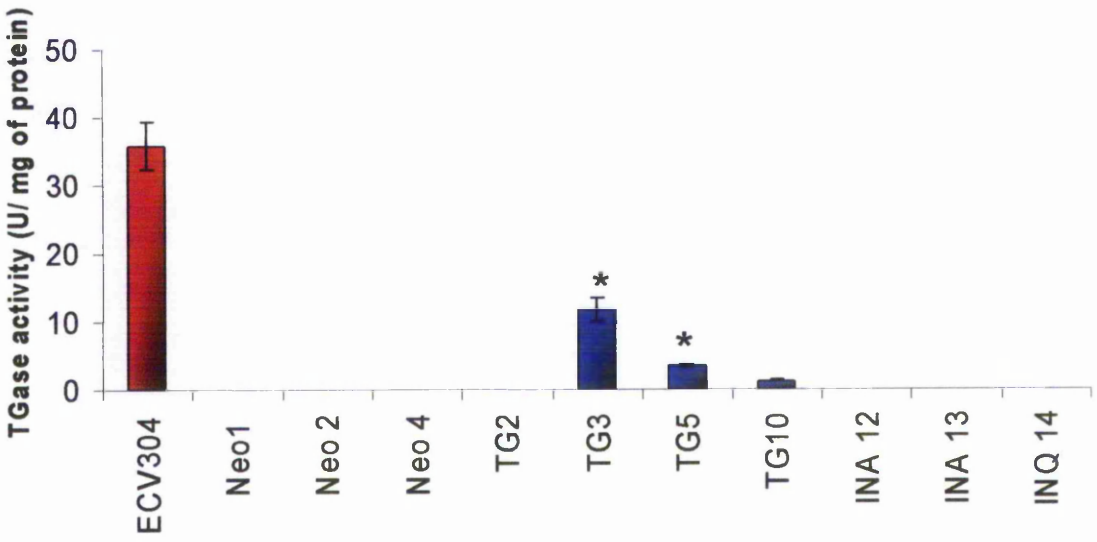


Figure 6.3.5.2 Screening of CT26 clones for total TG activity by means of [^{14}C]-putrescine incorporation into N'-N'-dimethylcasein

Total TG activity of CT26 clones was measured by the [^{14}C]-putrescine incorporation assay as described in Methods (section 2.2.3.7.1). 4 x 10^6 cells were harvested and lysed by sonication in the presence of protease inhibitors. The protein content of cell lysates was determined by the Lowry assay, and TG activity was assessed by the enzyme's ability to incorporate [^{12} C]-putrescine into N'-N'-dimethylcasein. Neo1, 2, and 4, negative control transfected cells; TG2, 3, 5, and 10, active TG2 transfected cells; INA12, 13, and 14 inactive (C277S) transfected cells. Cell lysates from a high TG2-expressing cell-line (ECV304) were used as a positive control. Results represent mean value ±SD, from 2 separate experiments, each undertaken in triplicate. Unit of activity values equal 1nmol of putrescine incorporated per hour. * Indicates significantly different from the Neo-transfected controls.

antigen, by addition of a MAb anti-TG2 antibody (Cub7402), prior to washing and labeling with a secondary anti-mouse FITC secondary antibody and subsequent fixation in 0.5% (v/v) formaldehyde as outlined in Methods (section 2.2.3.6.2).

Flow cytometric analysis of CT26 clones confirmed the presence of TG2 at the cell surface of TG3, TG5, INA13, and INA14 TG2-transfected CT26 clones (figure 6.3.6.1). The relative amount of enzyme expressed at the cell surface of the various clones appeared to be proportional to the total level of TG2 antigen. Cell surface antigen present in clones TG3, INA13 and INA14 was comparable, and was also shown to be lower when compared to clone TG5.

In order to confirm that cell-surface TG activity could only be detected in CT26 cells transfected with the active TG2 construct, cell-surface TG activity in all clones was measured using a biotin-X-cadaverine incorporation assay as described in Methods (section 2.2.3.7.2). Indeed, as shown in figure 6.3.6.2, only clones TG3 and TG5 demonstrated detectable TG activity at the cell surface, which is consistent with the total TG activity observed in these clones as shown in figure 6.3.5.2.

6.3.7 Extracellular TG2 release into the media and deposition into the ECM of CT26 clones

Primarily, the deposition of the enzyme into the ECM of TG2-transfected CT26 clones was assessed by *in situ* staining of matrix-associated TG2 using a mouse anti-TG2 MAb (Cub7402) and a secondary anti-mouse-FITC conjugate as outlined in the Methods (section 2.2.3.6.4). Confocal microscopy analysis of CT26 clones revealed fibrillar staining of the externalised enzyme in clones TG3, and TG5, whereas the ECM of CT26 clones transfected with the inactive TG2 construct appeared to be devoid of deposited enzyme (figure 6.3.7.1). This comes in agreement with the finding that Swiss 3T3 fibroblasts transfected with the inactive form of TG2 (Cys277Ser) fail to deposit the enzyme into the ECM, suggesting that activity is essential for matrix deposition (Balklava *et al.*, 2002). Importantly, these two TG activity isotypes (wild type, and

Figure 6.3.6.1 Screening of CT26 clones for cell-surface TG2 antigen by flow-cytometry

Transfected CT26 mouse colon carcinoma cells were cultured to sub-confluence in 6-cm tissue culture petri dishes. Cells were detached in a protease-free environment (PBS, 2mM EDTA), pelleted by centrifugation and resuspended to a concentration of 2 x 10^6 cells/ml in DMEM. Cell surface TG2 antigen was detected by incubating 0.5ml of live cells with an anti-TG2ase MAb (Cub7402) and revealed with an FITC-labeled mouse IgG secondary, prior to fixation in formaldehyde, as described in Methods (section 2.2.3.6.2). CT26 clones were analysed for the presence of cell surface TG2 in a DAKO Galaxy flow-cytometer. Isotype controls, prepared by incubation of cells with an anti-mouse IgG-1k, were used to gate the resulting peaks. Scattering images were further analysed using the WinMDI application. Values represent percentage of peak shift compared to the isotype control gate. **A-C**, pSV-Neo1, 2, and 4; **D-F**, pSG5-TG3, 5, and 10; **G-I**, pSG5-TG_{C277S}12, 13, and 14.

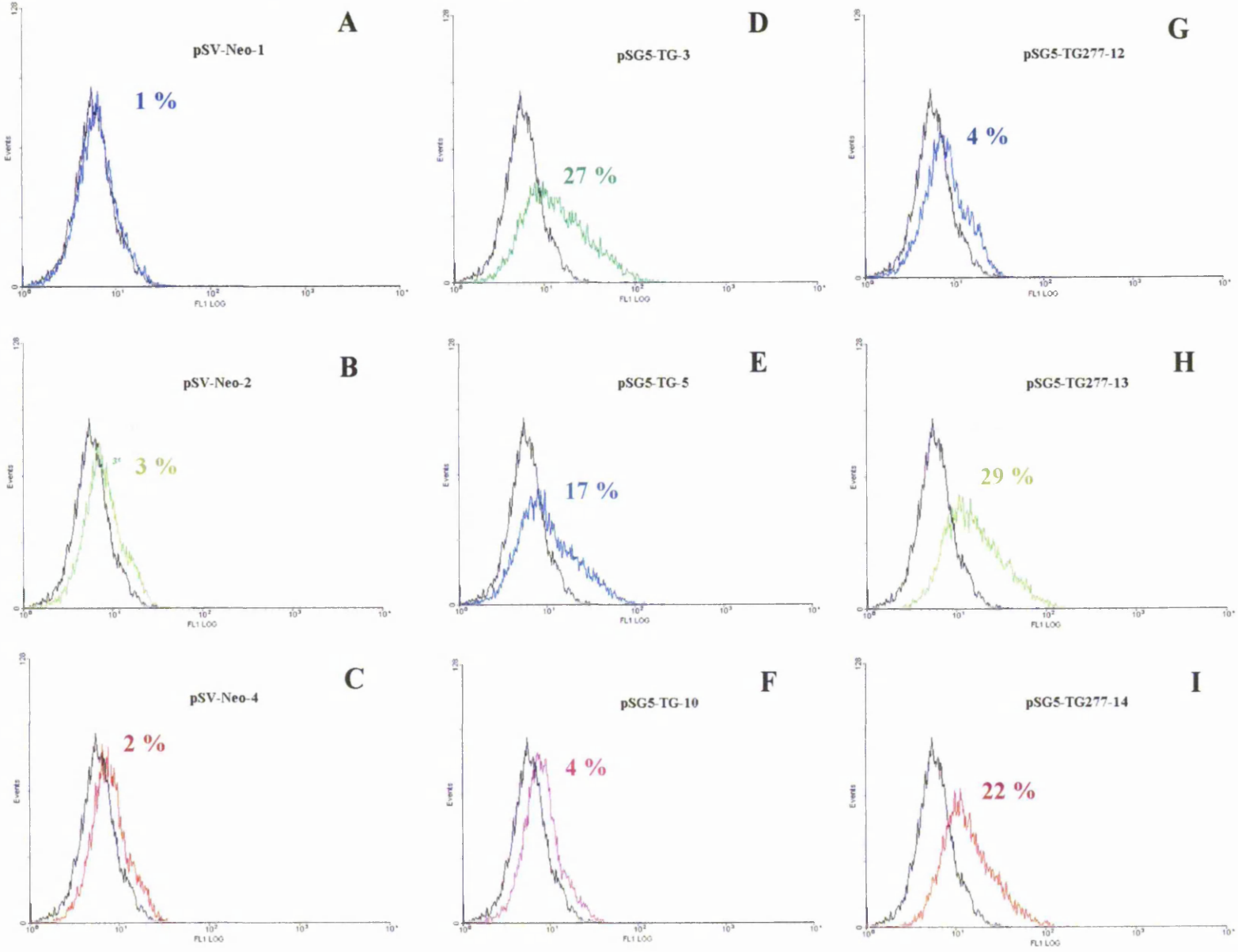


Figure 6.3.6.1 Screening of CT26 clones for cell-surface TG2 antigen by flow-cytometry

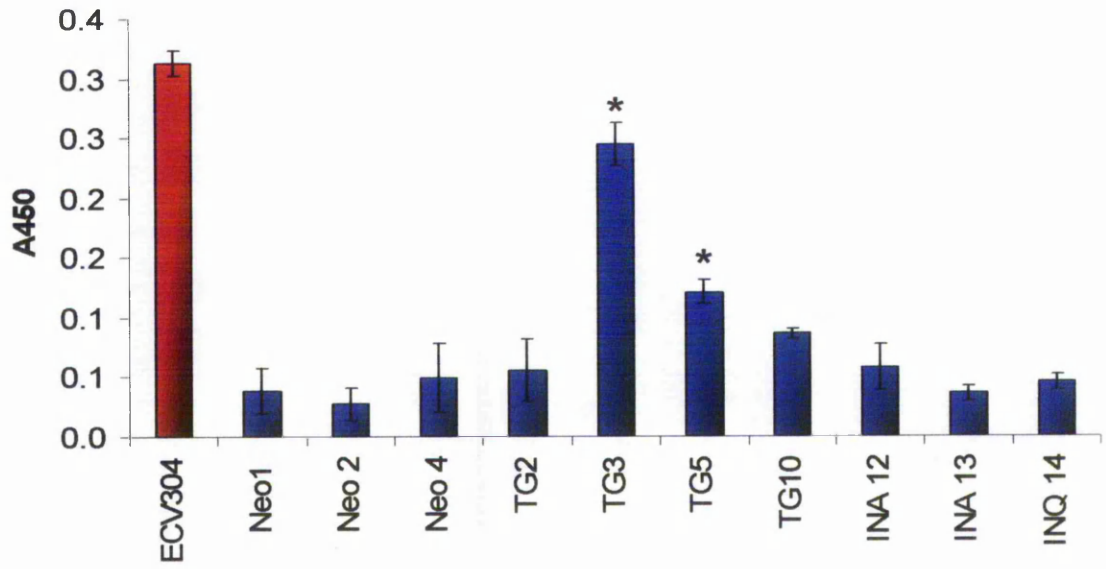


Figure 6.3.6.2 Screening of CT26 clones for cell-surface TG activity by means of biotin-X-cadaverine incorporation into fibronectin

10^6 CT26 cells were seeded in the presence of 0.132mM biotin-X-cadaverine in serum free medium on FN-coated 96-well plates and allowed to attach for 4 hours at 37°C. Negative controls were prepared by addition of cells to the FN-coated wells in the absence of biotin-X-cadaverine. Following incubation cells were lifted by mild non-ionic detergent treatment and incorporated amine was detected by conjugation to extravidin peroxidase, which was subsequently measured by addition of TMB substrate, as described in Methods (section 2.2.3.7.2). Activity of cell-surface TG2 is expressed as absorbance read at 450nm, following termination of the reaction in 2.5M sulphuric acid. Neo1, 2, and 4, negative control transfected cells; TG2, 3, 5, and 10, TG2 transfected cells; INA 12,13, and 14, inactive (C277S) transfected cells. ECV304 cells were used as a positive control. Results represent mean value ±SD, from three independent experiments, where n=6. Data are normalised by subtraction of absorbance values from negative controls. * Indicates significantly different from the Neo-transfected controls.

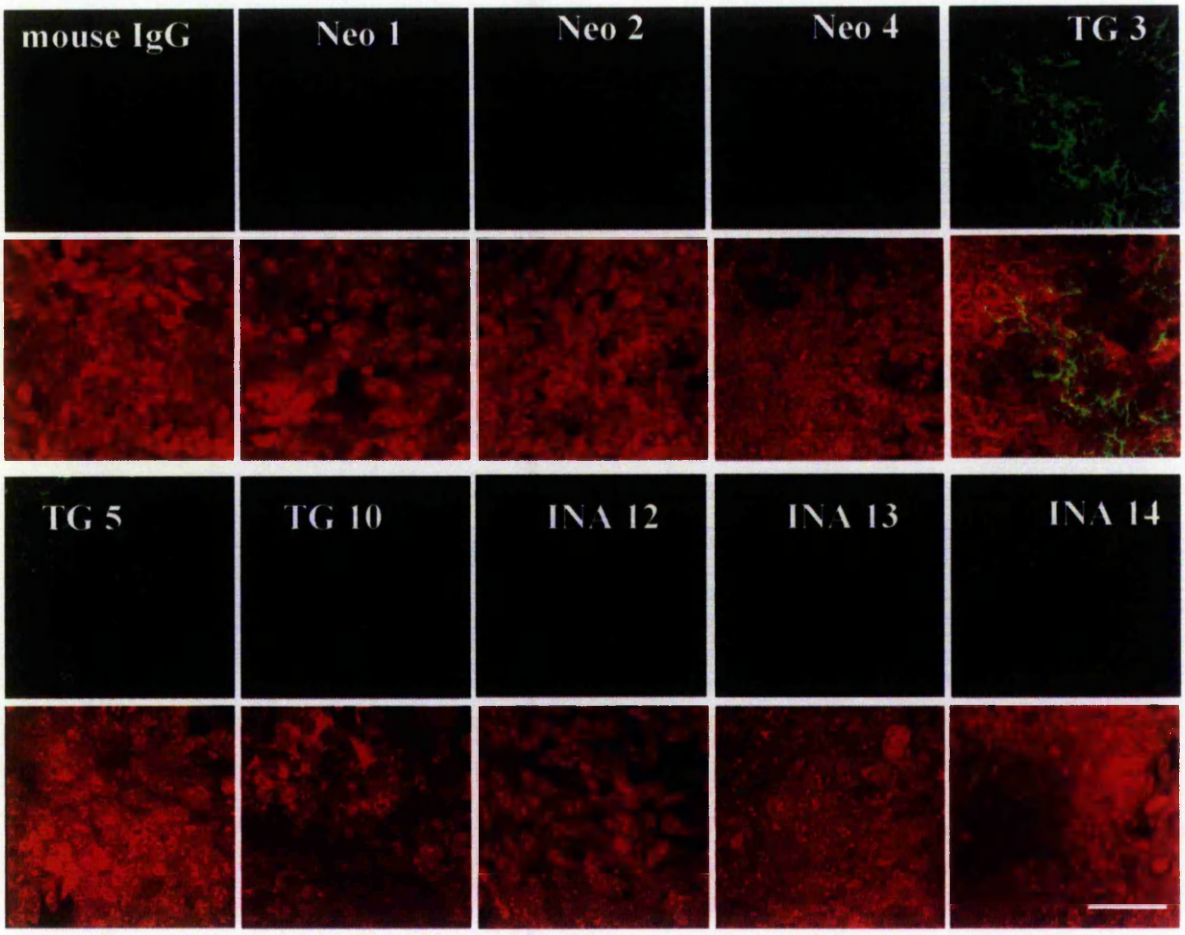


Figure 6.3.7.1. *In situ* immunofluorescence staining of TG2-transfected CT26 for ECM deposited TG2

Deposition of the extracellular TG2 antigen into the ECM of the CT26 clones transfected with active (TG3, 5 and 10) and inactive (INA12, 13, and 14) TG2 was detected by *in situ* staining with the Cub7402 MAb, followed by addition of an FITC-conjugated secondary antibody as described in Methods (section 2.2.3.6.4). Fixed cells were counterstained with propidium iodide. Images of TG2 antigen (green) were captured under the FITC filter (top panels), whereas cells were observed under the TRITC filter (bottom panels) of a Leica TCSNT confocal microscope . A non immune mouse IgG antibody was used as a negative control. Pictures are representative of 3 non-overlapping fields, obtained from 2 separate samples. (Bar=20μm).

mutant C277S) may provide us with a model to ascertain whether cell surface TG2 rather than extracellular TG activity is responsible for any observed tumour growth suppression. At this stage clones TG3, TG5, INA13, INA14, and Neo1 and Neo2 controls were chosen for further characterization. Given that TG2 has been previously implicated in the shaping of cellular morphology (Byrd and Litchi, 1987; Nara *et al.*, 1989), the morphological features of all 6 clones were compared. As shown in figure 6.3.7.2, cultures of CT26 transfected with control, active and inactive TG2-constructs and stained in 0.5% (w/v) crystal violet were characterised by a rod-like body extended in length by outwardly deployed invadopodia. No major morphological differences were observed.

Since the enzyme secreted from TG2–transfected CT26 clones might be released into the cell growth medium rather than the ECM, serum free cell culture media collected from confluent clone cultures cultured for 8 hours were screened for the presence of TG2 antigen by western blotting as described in Methods (section 2.2.3.6.3). Two prominent bands were indicative of the extracellular release of the enzyme from clones TG3, and 5, whereas Neo1- , Neo2-, as well as INA13-, and 14-transfected CT26 cells displayed no enzyme secretion into the medium (figure 6.3.7.3). This provides a confirmation that the inactive form of TG2 is not being released from the cell surface, but is instead retained either within the intracellular pool or at the cell surface.

6.3.8 Viability and apoptotic index of TG2 transfected clones

It is possible that overexpression of TG2 by mammalian cells, coupled with an influx of Ca^{2+} into the intracellular pool due to minor cell insult or cell stress, might result in TG2-dependent cross-linking of intracellular proteins. Indeed, the insertion of foreign TG2 cDNA into mammalian neuroblastoma cells SK-N-BE2 was shown to incur a drastic reduction in their proliferative capacity and rendered cells more susceptible to programmed cell death (Melino *et al.*, 1994). Therefore, the viability of CT26 clones transfected with active and inactive (C277S) constructs was assessed using an XTT metabolism-based assay as described in Methods (section 2.2.1.7.1). In addition, the apoptotic index of these clones was quantified by measuring the capase-3 activity of cell lysates (section 2.2.1.7.3). As shown in figure 6.3.8, cells overexpressing TG2 did not

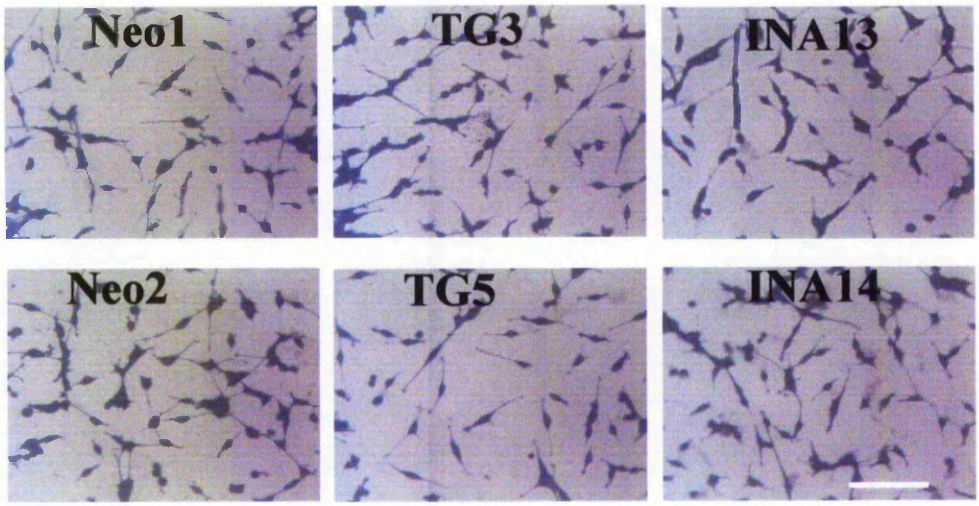


Figure 6.3.7.2 CT26 clone morphology

10^3 CT26 clones were seeded in 96-well plates and allowed to reach 40-50% confluency. Cells were then washed once in PBS, and stained in 0.5% (w/v) crystal violet for 20 minutes at room temperature, prior to washing twice in PBS and once in distilled water. Images were captured using an a Olympus digital camera attached to a light microscope. (Bar=50μm).

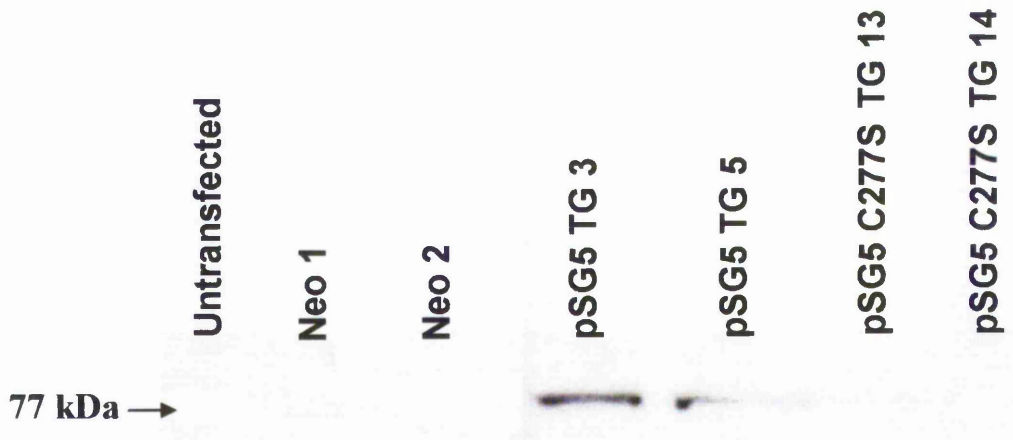


Figure 6.3.7.3 Measurement of TG2 secreted in the culture media of TG2-transfected CT26 cells by western blotting

2 x10^6 CT26 clones were seeded in 25cm^2 tissue culture flasks and allowed to grow overnight at 37°C, 5% (v/v) $C0_2$. Complete cell growth media was then replaced by serum free media, and cells were then cultured for a further 8 hours. Media was then collected, centrifuged, and protein content was precipitated in 10% (w/v) TCA. Following protein content determination using the Lowry method, protein pellets were analysed by SDS-PAGE using equal protein loading, and then western blotted and immunoblotted for the 77kDa TG2 as described in the Methods (section 2.2.3).

Figure 6.3.8 XTT reduction and extent of apoptosis in TG2-transfected CT26 cells

A, measurement of TG2-transfected CT26 cell viability by the XTT assay as outlined in Methods (section 2.2.1.7.1). Cell viability is expressed as a percentage of untransfected viable cells ±SD, following a 24 hour incubation in standard cell growth media (n=6). **B**, programmed cell death was measured by caspase-3 activity detected in CT26 clone lysates, 16 hours post-seeding, as described in Methods (section 2.2.1.7.3), and was expressed as pmole of pNA liberated per hour by reference to a pNA standard curve. Positive control cultures were treated with 1μM staurosporine (staur) for 16 hours, whereas negative controls were obtained by parallel incubation with staurosporine and an appropriate apoptosis inhibitor (staur + inhibitor). Results represent mean value ±SD, where n=3.

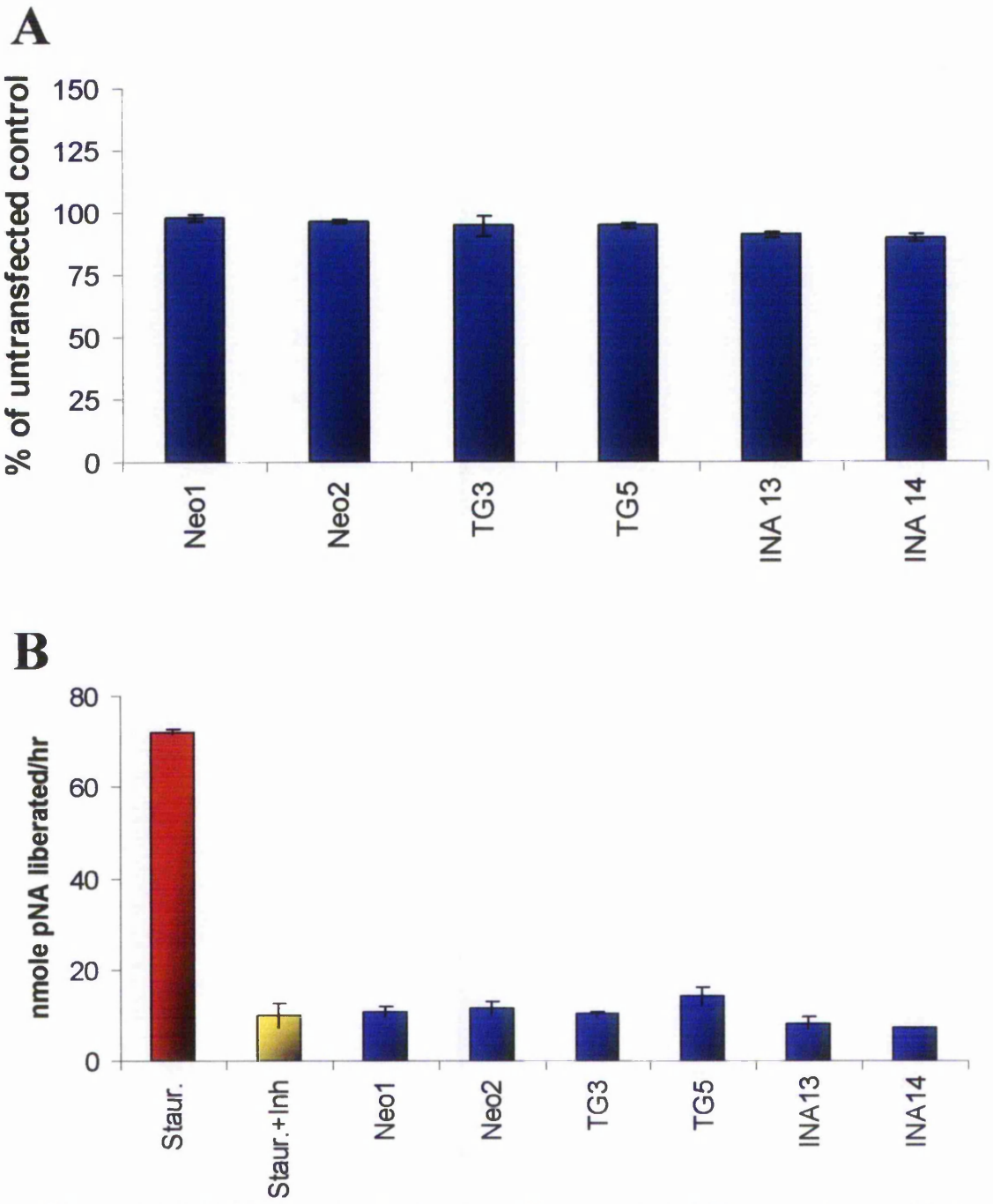


Figure 6.3.8 XTT reduction and extent of apoptosis in TG2-transfected CT26 cells

exhibit reduced viability as measured by their metabolic ability to reduce the yellow tetrazolium salt XTT into an orange formazan dye product. Similarly, Caspase-3 activity levels were also minimal and comparable throughout all the clones.

6.3.9 Differential cell attachment of TG2-transfected CT26 on fibronectin

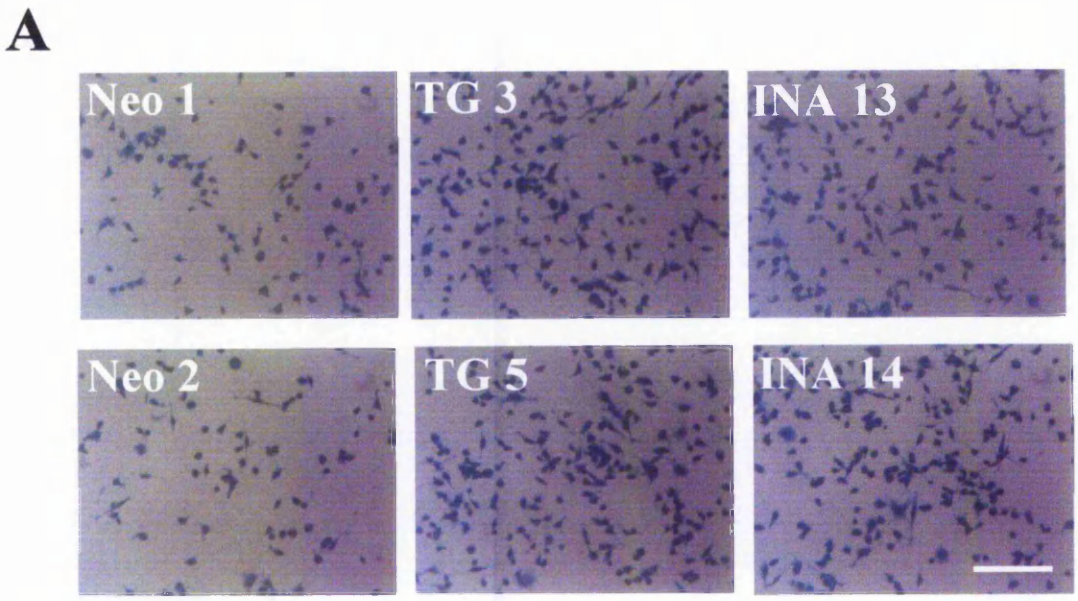
Given the well-documented role of TG2 in both cellular attachment and spreading (Jones *et al.*, 1997; Verderio *et al.*, 1998, Gentile *et al.*, 1992; Balklava, *et al.*, 2002), the possibility that TG2 overexpression may lead to increased cell attachment on fibronectin was explored. In light of recent work suggesting that proteolytic degradation of cell-surface TG2 by MT1-MMP suppresed glioma and fibrosarcoma cell adhesion to fibronectin (Belkin *et al.*, 2001), cells expressing active and inactive (C277S) TG2 were assessed for their ability to attach to fibronectin-coated tissue culture plastic in serum free cell culture media. Attached cells were quantified either via visual counting, following staining in 0.5% (w/v) crystal violet as described before (section 6.2.1), or spectrophotometrically at 540nm, following staining in 0.5% crystal violet and subsequent solubilisation in 30% (v/v) acetic acid. CT26 clones overexpressing the active (TG3 and TG5) and inactive (INA13, INA14) forms of TG2 demonstrated a statistically significant increase in atachment on fibronectin-coated surfaces when compared to the Neo (Neo1 and Neo2) controls, when measured by both methods (figure 6.3.9). This is in keeping with the reported increase in adhesion of MetB fibrosarcoma cells, transfected with active TG2 constructs, to tissue culture plastic and to fibronectin (Johnson *et al.*, 1994). It is believed the cell surface associated-enzyme favours the cell adhesion events of malignant cells through binding to ECM components such as fibronectin, as reported previously (Johnson *et al.*, 1994; Jones *et al* 1997).

6.3.10 Migratory potential of TG2 transfected CT26

Having demonstrated that TG2 is playing a pivotal role in the attachment of CT26 mouse colon carcinoma cells, the next logical step was to examine whether TG2 overexpression can hinder or accelerate cellular migration through artificial matrices, a

Figure 6.3.9 Attachment of CT26 clones on fibronectin coated surfaces

CT26 clones (10^5 cells/ml) were seeded on FN-coated plates and allowed to attach for 40 minutes at 37°C. Following removal of unattached cells by washing three times in PBS, cells were stained in 0.5% (w/v) crystal violet in ethanol. **A,** images of CT26 clones adhered to FN-coated plastic were captured using an Olympus digital camera attached to a light microscope. Images are representative of 3 non-overlapping fields, obtained from four separate samples (Bar=100μm). **B,** stained cells were further solubilised in 30% (v/v) acetic acid for 30 minutes at room temperature and resulting absorbance was recorded at 540nm using a SpectraFluor plate reader. Results are expressed as mean value ±SD from two independent experiments.



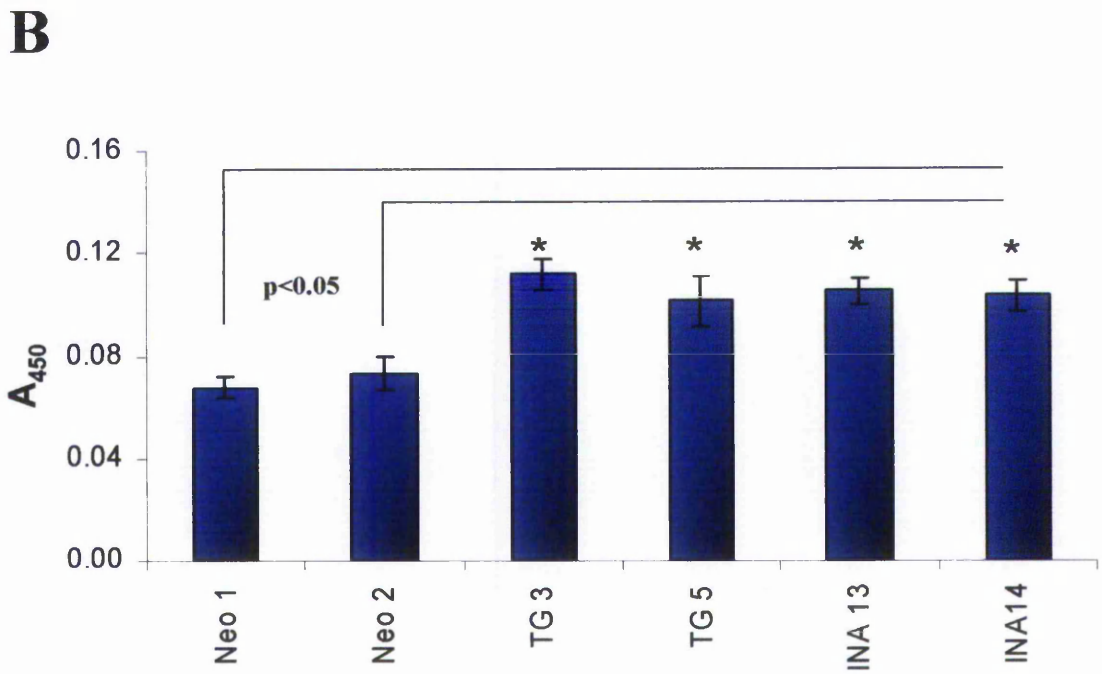


Figure 6.3.9 Attachment of CT26 clones on fibronectin coated surfaces

function of physiological relevance to the invasive nature of tumour cells. To address this, a modified Boyden Chamber method was recruited, as outlined previously (section 3.2.1). As shown in figure 6.3.10, both active (TG3 and TG5) and inactive (INA13 and INA14) TG2-expressing CT26 clones exhibited decreased motility on a fibronectin matrix (panel B). Fibronectin was preferred to collagen as a tumour ECM substrate, as it has been previously shown that the adhesion of CT26 cells to the former matrix component was much higher when compared to the latter (Geng *et al.*, 1998). When the assay was modified so that clones migrate through uncoated filters, TG2-expressing clones demonstrated augmented motility through the filter pores (panel A).

6.3.11 Total ECM turnover of CT26 cells expressing different levels of TG2

To examine whether increased expression of TG2 affects ECM deposition and turnover rate in a malignant cell line, selected CT26 mouse colon carcinoma cells transefected for stable expession of active and inactive (C227S) TG2 (Neo1, TG3, and INA14) were labelled with [2,3-^{3}H]-amino acid mixture and the amount of the incorporated radioactive amino acids was measured in the ECM fraction up to 72 hours from labelling, as outlined in the Methods (section 2.2.3.8). As shown in figure 6.3.11, no significant differences in the ability of these cells to deposit (t=24) and degrade (t=48, t=72) the ECM were observed. This may be attributed to the fact that most malignant cell lines fail to deposit ECM components, as demonstrated by the relatively low cpm counts recorded, so that their mobility is not hindered by adhesion to their microenvironment (Ruoslahti, 1988). To rule out the possibility that any changes in the ability of CT26 expressing relatively high amounts of TG2 to invade through artificial FN matrices is not due to altered MMP activity, the activities of the major collagen-targeted MMP -interstial collagenase (MMP-1)- was assessed by analysing CT26 clone cell growth media conditioned for 24 hours on a collagen/SDS zymogram. As shown in figure 6.3.11.2, comparable MMP-1 activity was detected in the media of CT26 cells transfected with control, active, and inactive TG2 constructs. The MMP-1 precursor (pro-MMP1) was also found to be circulating in the media in abundance.

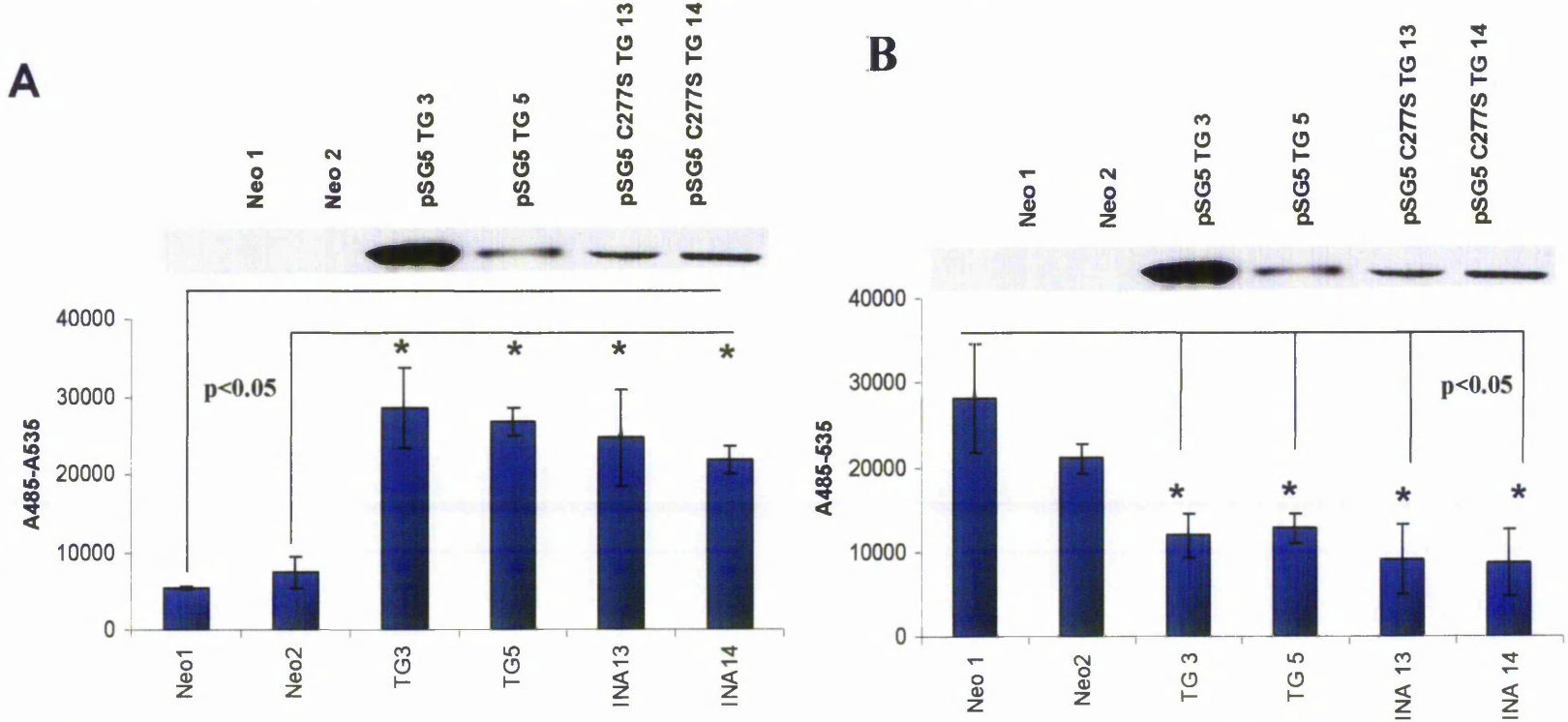


Figure 6.3.10 Migratory potential of TG2-transfected CT26 on uncoated and FN-coated surfaces

10^6 CT26 clones were seeded in DMEM, in uncoated (**A**) and FN-coated (5µg/ml) (**B**) Fluoroblock PET filters and allowed to migrate to a lower chamber in response to chemoattraction (10%) FCS for 24 hours at 37°C as described before (section 3.2.1). Inserts were transferred to 24-well glass-bottom plates containing 4µg/ml Calcein AM in Hanks Balanced Salt Solution and incubated at 37°C for 90 minutes. Fluorescence due to the ester hydrolysis by live cells that have migrated to the bottom side of the filter was read at 485nm/530nm (excitation/emission) from-bottom-to-top using a SpectraFluor plate reader. Results represent mean values ±SD, from two separate experiments.

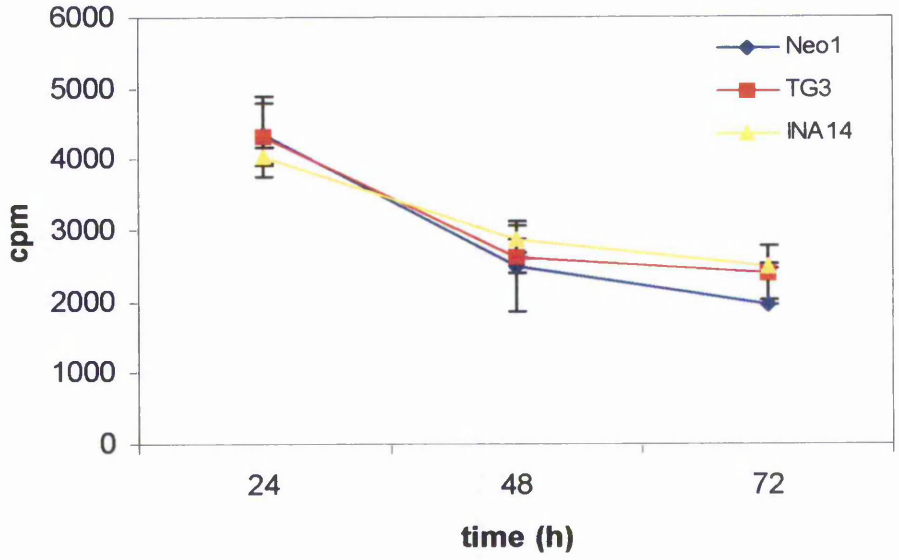


Figure 6.3.11 Total ECM protein deposition and turnover rates of TG2-transfected CT26

Selected CT26 clones (Neo1, TG3, and INA14) were labeled with [2,3-^{3}H-amino acid mixture] for 48 hours in a similar way to section 2.2.3.8. Following removal of label, cells were cultured for a further 4 days, in the presence of label-free cell growth media. The amount of the incorporated radiolabel in the ECM fraction was measured over a 96 hours time period. Results represent mean values ±SD. Counts are normalised against μg of DNA in the cellular fraction.

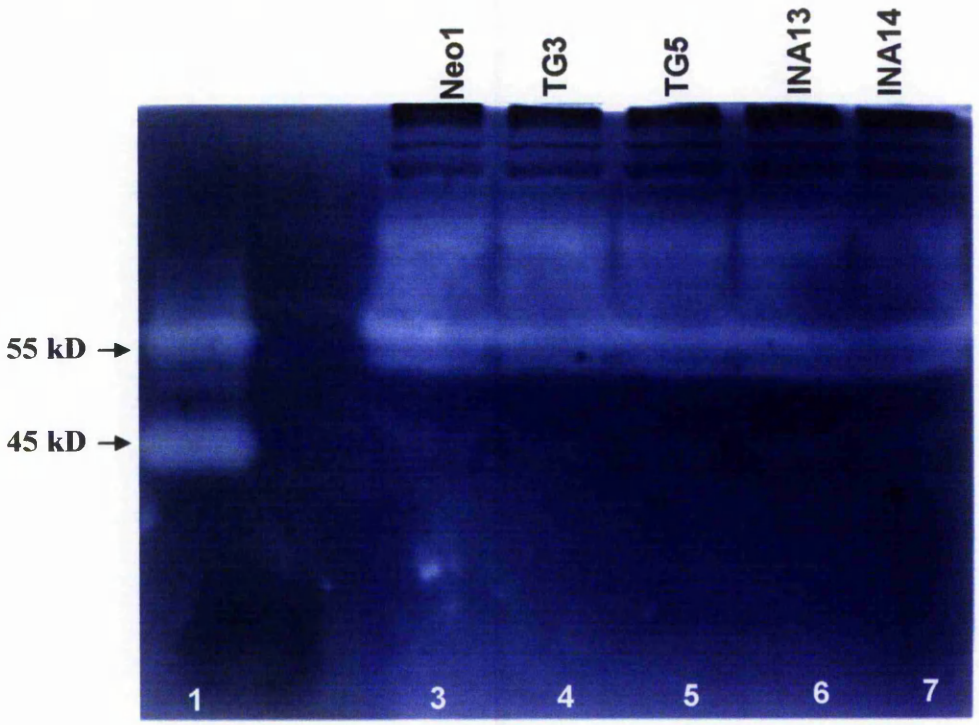


Figure 6.3.11.2 Measurement of active MMP-1 released into the cell growth media of TG2 transfected CT26

The amount of MMP-1 released in the media of the different clones was analysed using collagen zymography as described before (section 6.2.2). Conditioned media (5µl) were electrophoresed through a 7.5% SDS-PAGE gel containing 5mg/ml calf skin collagen type I. MMP-1 activity was visualised by staining with coomassie Brilliant Blue R, and the gel image was captured using a digital camera. Lane 1, pro- and active MMP-1 standard (55 and 45kD respectively); lane 3- neo mock-transfected CT26; lanes 4 and 5, active TG2–transfected CT26 (TG3, TG5); lanes 6 and 7, inactive (C277S) TG2-transfected CT26 (INA13, INA14).

6.3.12 Tumour incidence, growth rates, and animal survival following introduction of CT26 clones into mice

Within the tumour-host microenvironment, tumour cells act in synergy with animal-host cells by recruiting host cell functions through paracrine cross-talk. Having characterised the TG2–transfected CT26 clones *in vitro* and established that overexpression of TG2 leads to increased cell attachment to fibronectin and decreased migration through fibronectin coated filters, tumour cells were introduced into animals to assess their ability to grow *in vivo*. CT26 cells transfected with TG2 and control constructs were injected subcutaneously into 8 weeks old female balb/c mice as described in Methods (section 2.2.5.1). Initially, animals were injected with 10,000 CT26 cells and were monitored every two days for primary tumour incidence, tumour growth rate, and percentage survival. As shown in figure 6.3.12.1, implantation of CT26 clone TG5 was accompanied by decreased tumour growth rate (as measured by mean tumour size) and increased animal survival. Primary tumour growth incidence was comparable in all 6 clones. Surprisingly, CT26 clone TG3, which expressed the enzyme in larger quantities was shown to exhibit unaltered primary tumour incidence and growth rate, and animals bearing this clone had survival percentage comparable to that of the Neo (Neo1, Neo2) control clones. Similar to TG3, clones INA13 and INA14, transfected with inactive (C227S) TG2, were also comparable to the Neo controls, when the same three attributes were measured. Prior work within this laboratory has indicated that the hamster fibrosarcoma MetB clones transfected with the full TG2 cDNA exhibited reduced primary tumour growth incidence when introduced into animals. This difference, however, was only prominent when lower numbers of cells (1,000) were injected. Therefore, given that the TD50 (number of cells required for half of the animal population to develop tumours) for this cell line has been estimated at 1,000 cells (Dr S. Ali, unpublished data), the tumour growth study was repeated with injections of 3,000 CT26 into balb/c animals. As shown in figure 6.3.12.2, animals bearing the CT26 clone TG5, demonstrated decreased primary tumour incidence, reduced tumour growth rate and increased survival compared to their Neo control and inactive TG2 counterparts.

6.3.13 Evaluation of tumour host's ability to retain tTG expression and activity following natural selection

Strong physiological stimuli present only *in vivo*, are likely to be responsible for the different behaviour of transfected cell lines when introduced into the animal. Previous studies (Johnson *et al.*, 1994) have indicated that tumours originating from transglutaminase transfected MetB malignant cells lost the expression of the tTG cDNA, due to unfavourable selection during tumour development in the animal host. In that particular study, it was postulated that cells carrying tTG might have become more susceptible to apoptosis. It is likely that, either because tTG-expressing CT26 cells are adhering faster to physiological substrates, or because they are confined in a reinforced self-assembled extracellular matrix and hence migrate slower, tumour development favours cells that do not express this enzyme. Therefore, CT26 clones Neo1, Neo2, TG3, TG5, INA13, and INA14 were isolated from extracted tumours as described in Methods (section 2.2.1.6). Following three passages, and culturing in standard cell growth media supplemented with 400μg/ml G418, the cells were harvested and lysed, prior to SDS-PAGE analysis and western blotting. Immunoprobing of the nitrocellulose membrane with an anti-tTG MAb (Cub7402) revealed that expression of the inserted cDNA by malignant cells originating form the animal tumours is comparable to that prior to the injection into the animal (figure 6.3.13).

Figure 6.3.12.1 Tumour incidence, growth rates, and animal survival following subcutaneous injection of 10,000 TG2-transfected CT26 cells into balb/c mice

Exponentially growing CT26 clones were harvested and resuspended to a final concentration of 10^5 cells/ml in DMEM, prior to subcutaneous (s.c) implantation of 100µl cell suspensions into 8 weeks old balb/c mice as outlined in Methods (section 2.2.5.1). Following injections, animals were monitored every two days for tumour development. Results represent mean value (n=6). **A**, percentage of animals developing tumours following s.c. injection of CT26 clones. **B,** average tumour growth rate of animals expressed in mm^2 was measured up to 33 days post-injection. **C**, percentage animal survival following injection of the malignant CT26 clones. Animals were sacrificed once tumours reached approximately the $1cm^2$ limit or when animals appeared to be in distress.

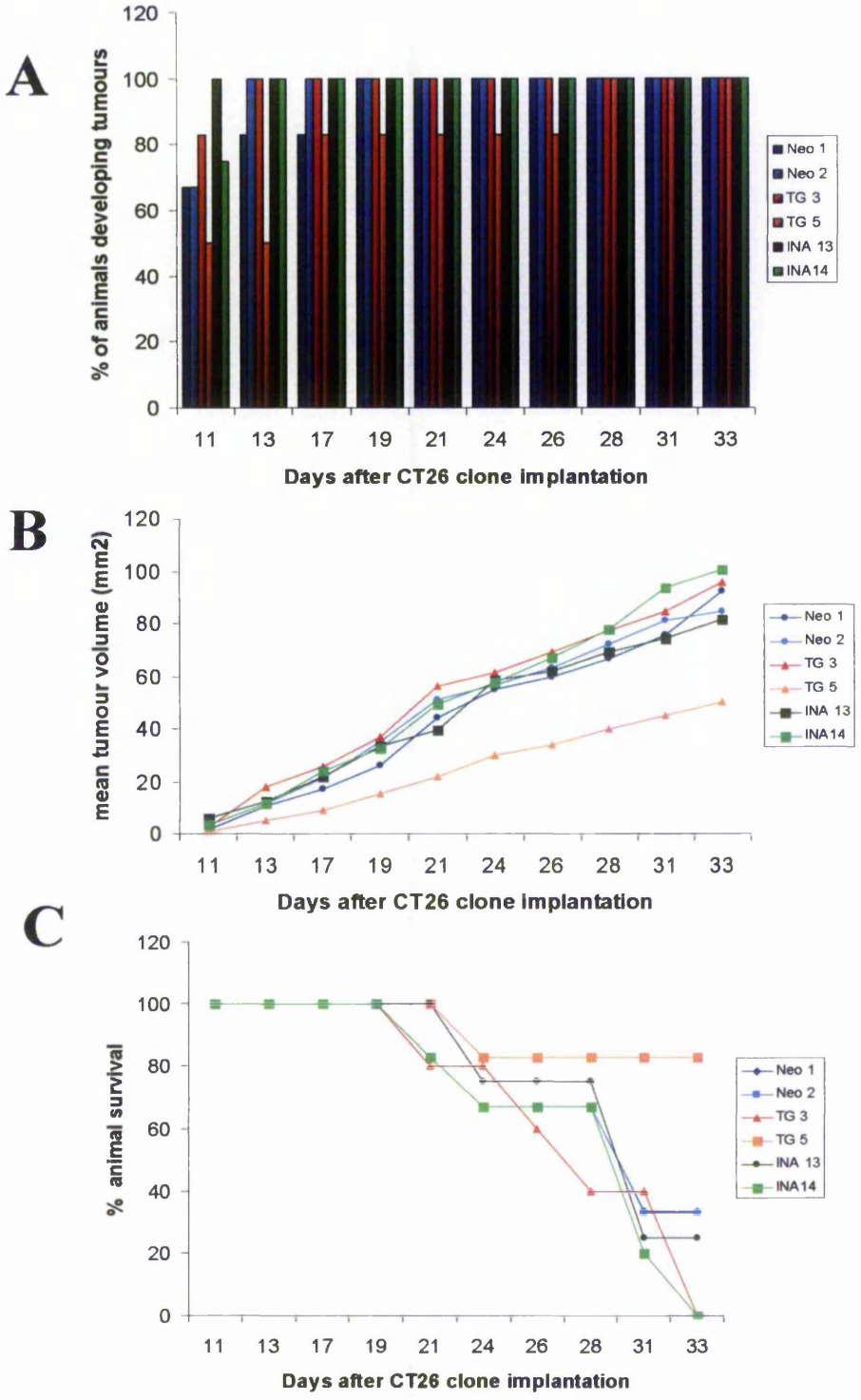


Figure 6.3.12.1 Tumour incidence, growth rates, and animal survival following subcutaneous injection of 10,000 TG2-transfected CT26 cells into balb/c mice

Figure 6.3.12.2 Tumour incidence, growth rates, and animal survival following subcutaneous injection of 3,000 TG2-transfected CT26 cells into balb/c mice

Exponentially growing CT26 clones were harvested and resuspended to a final concentration of 2 x 10^4 cells/ml in DMEM, prior to subcutaneous (s.c) implantation of 100µl cell suspensions into 8 weeks old balb/c mice. Following injections, animals were checked every two days for tumour development. Results represent mean value (n=6). **A**, percentage of animals developing tumours following s.c. injection of CT26 clones. **B,** average tumour growth rate of animals expressed in mm^2 was measured up to 38 days post-injection. **C**, percentage animal survival following injection of the malignant CT26 clones.

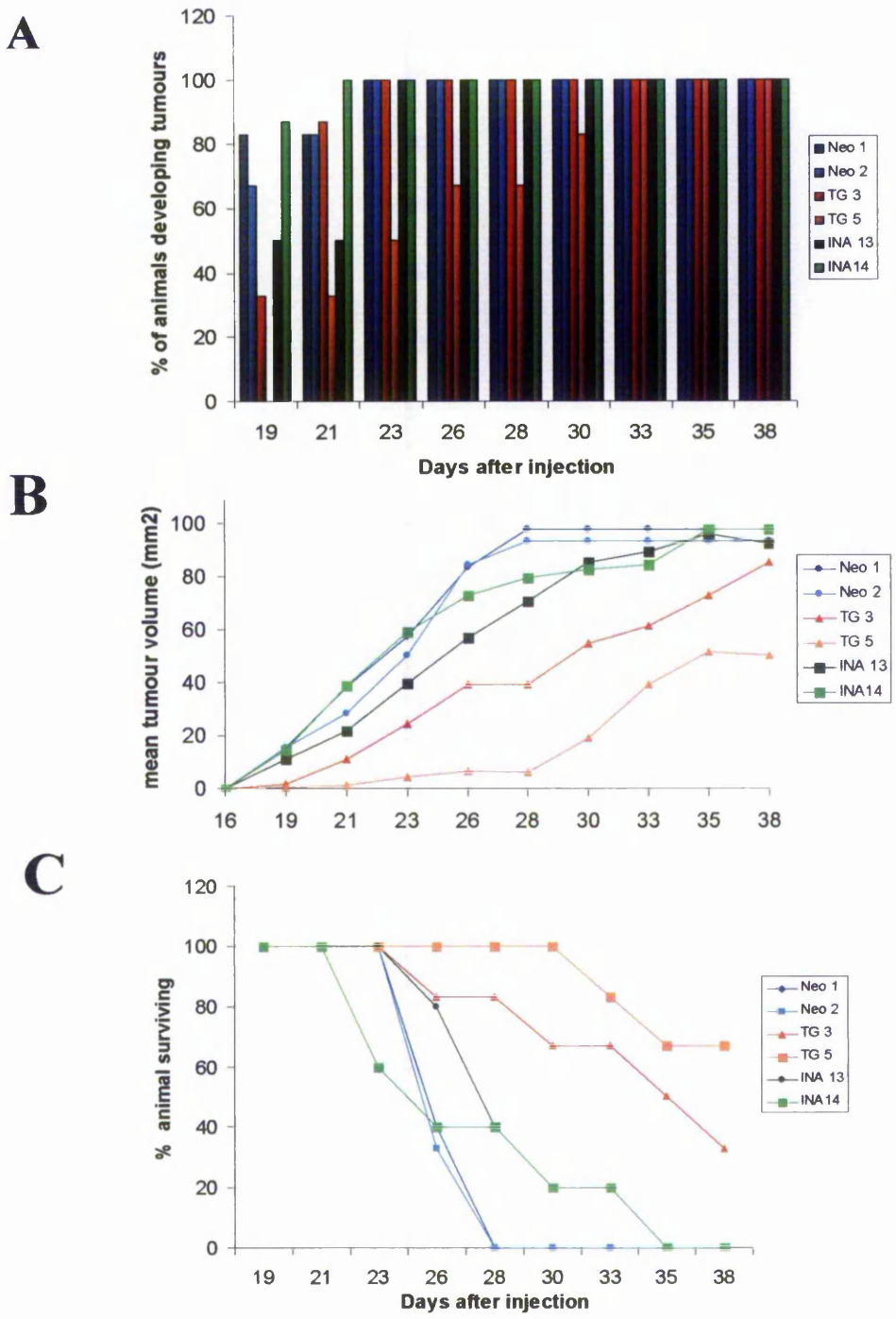


Figure 6.3.12.2 Tumour incidence, growth rates, and animal survival following subcutaneous injection of 3,000 TG2-transfected CT26 cells into balb/c mice

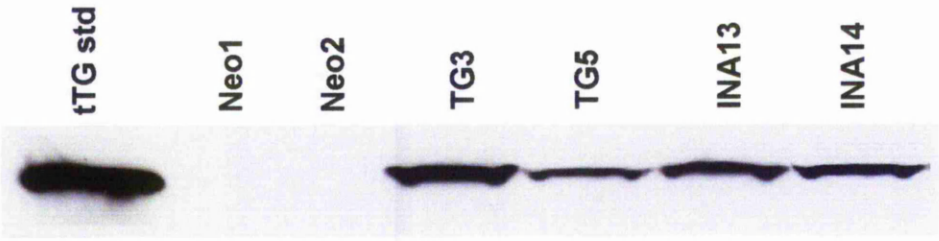


Figure 6.3.13 Evaluation of tumour host's ability to retain tTG expression and activity following natural selection

Neo- and tTG-transfected CT26 tumours were harvested from the host animals once tumour size approached $1cm^2$. Malignant CT26 clones were isolated from the tumour mass as described in Methods (section 2.2.1.6). Cells were initially isolated in standard cell growth media and, once confluent, cultures were enriched in transfectants in cell growth media supplemented with 400μg/ml. CT26 clone cell lysates were analysed for tTG antigen expression by western blotting and subsequent immunoblotting. 100ng of gpl tTG were also electrophoresed as a positive control..

6.4 Discussion

Using a mouse colon carcinoma cell line transfected for stable expression of active and inactive TG2, we have demonstrated that introduction of active TG2-overexpressing clones into balb/c mice led to decreased primary growth incidence, decreased tumour growth and increased survival compared to the inactive TG2- and Neo -transfected controls. The observed differences appeared to be optimal when a relatively small number of cells (3,000), close to the cell line's TD50 index (1,000), were implanted into the animals. This experimental approach was found to suffer from clonal variation, as tumour growth rate attenuation was less prominent in the clone TG3, in which TG expression was found to be at least 40% higher compared to TG5.

So far, it has been reported that separate subcellular localities of TG2 could potentially influence tumour progression through a variety of mechanisms, including regulation of cell growth- intracellular TG2 (Borge *et al.*, 1996), cell death- intracellular TG2 (Knight *et al.*, 1991; Melino *et al.*, 1994; Piacentini *et al.*, 2002), ECM organisation- extracellular TG2 (Griffin *et al.*, 1979; Johnson *et al.*, 1999; Mizra *et al.*, 1999; Piacentini *et al.*, 1999; Small *et al.*, 1999; Grenard *et al.*, 2001), or by acting as a cell surface receptor– cell-surface TG2 (Gaudry *et al.*, 1999b; Belkin *et al.*, 2001).

In vitro characterization of the transfected clones revealed no significant morphological differences in the TG2 overexpressing clones when compared to the Neo controls. Moreover, neither active nor inactive TG2 clones exhibited altered viability/ proliferation as measured by their ability to metabolise XTT. This comes in agreement with previous reports on malignant and normal TG2-overexpressing cells lines (Johnson *et. al.*, 1994; Balklava *et al.*, 2002).

Additionally, no increase in the apoptotic index of the CT26, TG2-overexpressing clones was observed when measured by means of Caspase-3 activity. Previous work within this laboratory has also indicated that, although there is a good correlation of dexamethasone-induced TG2 expression with an increase in SDS-insoluble bodies, no increase in classical apoptosis was observed as determined by DNA fragmentation, Capase-3 activity

and ATP: ADP ratios (Parry, 2002; PhD Thesis). Given that dexamethasone-treated MetB cells have been reported to die in a manner not consistent with either apoptosis or necrosis (Johnson *et al.*, 1998), it was postulated that a new form of cell-death, termed transglutaminase-associated cell death and characterised by increased intracellular TG2-induced cross-links, could be occurring in response to TG2 overexpression. Prerequisite for this form of cell death is external insult, such as trypsinisation, leading to leaky plasma membranes and as a consequence increased Ca^{2+} influx. In view of the fact that in our model such insult is incurred by the cells either via trypsinisation or through shear forces upon cell injection, the possibility that a large number of TG2 expressing cells are lost prior to or following delivery into the animal could not be ruled out. However, given that inactive TG2-expressing CT26 clones did not demonstrate altered tumour incidence and growth rates compared to the Neo controls when implanted on mice, the possibility that intracellular TG2 mediates cell death through extensive cellular component cross-linking should be dismissed.

Having established that neither cell proliferation nor programmed or other cell death pathways were altered following introduction of the TG2 cDNA into CT26 cells, the involvement of cell adhesion and ECM stabilisation functions of the enzyme were examined. Screening of both active and inactive TG2 clones has confirmed as reported previously (Balklava *et al.*, 2002) that only the active TG2 clones externalise the enzyme into the media, thus allowing for its deposition into the ECM. TG2 antigen and activity in the inactive (C277S) TG2 clones was limited to the cell-surface and the intracellular pool. Having employed a system in which certain tumour cell clones contain only intracellular and cell surface-associated TG2 (INA13, INA14), whereas others in addition to the presence of intra- and peri- cellular externalised TG2 also secrete the enzyme into the ECM (TG3, TG5), we aimed to identify which of the two mechanisms is primarily responsible for tumour growth attenuation.

By utilising an *in vitro* approach, clones TG3, TG5, INA13, and INA14 were all shown to adhere more rapidly to fibronectin coated surfaces when compared to Neo controls (Neo1 and Neo2). This observation was conceivable, as both active an inactive TG2-containing clones were shown to express cell-surface associated TG2, which has been

repeatedly associated with increased cell adhesion and spreading (Gentile *et al.*, 1992; Martinez *et al.*, 1994; Jones *et al.*, 1997; Verderio *et al.*, 1998; Akimov *et al.*, 2000; Balklava *et al.*, 2002). However, when seeded on fibronectin-coated filters and encouraged to migrate through 6μm pores, both active and inactive (C277S) CT26 clones demonstrated delayed migration compared to the mock-transfected controls. The fact that TG2 overexpression impedes the ability of the cells to migrate has also been suggested in ECV304 epithelial cells and Swiss 3T3 fibrosblasts (Jones *et al.*, 1997; Balklava *et al.*, 2002). Taken together, these two findings substantiate a possible cell surface-associated mechanism by which TG2 inhibits tumour growth. However, the observation that only the active TG2-expressing clones led to reduced primary tumour growth favours the hypothesis that neither the cell-surface associated function of TG2 nor the intra-cellular TG2 pool alone is responsible for tumour growth attenuation. Thus, although cell surface-TG2 may impinge on the tumour cells ability to migrate through artificial matrices, the tumour regression effect that it elicits should be attributed solely to the reported ECM stabilisation due to the transamidating activity of the externalised TG2. Unfortunately, pulse labeling experiments indicated that ECM deposition and turnover rates of CT26 clones transfected with active and inactive TG2 constructs were comparable to that of the Neo controls. Malignant cells have been shown to deposit reduced amounts of ECM components, thus minimising adhesion to tumour ECM components that would otherwise counteract their ability to migrate (Ruoslahti, 1988). On this basis, this *in vitro* model fails to mimic the *in vivo* tumour-stroma microenvironment where increased expression of TG2 by a malignant cell line is expected to incur qualitative changes to the ECM deposited by stromal cells in the vicinity of the tumour body.

Interestingly, CT26 clone TG3 did not have a significant effect on growth and animal survival when inoculated in balb/c mice, whereas clone TG5 led to diminished tumour growth and increased animal survival. This finding appears to complement findings drawn from the CT26 tumour therapy model based on intratumour injection of exogenous enzyme (section 3.2.2.4). Attenuation of tumour growth following intratumour injections of TG2 (10U/inj) appeared to be dose specific, and was not achieved when TG2 of higher

or lower activity was applied to the tumours. In this instance, it is believed that the constitutive expression levels of an enzyme that is externalised and demonstrates such a high affinity for many ECM proteins are critical for the survival of the TG2-transfected cell against natural selection. It has been previously suggested that the expression of the enzyme in a MetB fibrosarcoma cell line transfected with active TG2 constructs is lost or reduced due to selection, or animal host immune response (Johnson *et al.*, 1994). Overexpression of TG2 beyond a threshold level is likely to induce extensive *in vivo* scarring thus jeopardising the cell's chances for proliferation, migration and survival.

Chapter 7:

General Discussion

8. General Discussion

Since the term transglutaminases was first coined by Clarke and co-workers in 1959 (Clarke *et al.*, 1959), the cellular function of the TG2 subfamily of these enzymes has been the focus of extensive research. However, its widespread organ distribution and sub-cellular localisation, its multiple catalytic activities and its complex regulation, as well as its implication in diverse cellular functions have made it difficult for researchers to pinpoint its exact cellular roles.

So far various models have been used to assess the physiological role of TG2, either via modulation of the enzyme's expression, or through regulation of its activity. However observations drawn from each one of these models had to be carefully interpreted as they presented individual limitations. The use of different inhibitors of TG activity that were initially employed to study the effect of loss of TG activity can lead to non-specific effects on other biologically active molecules including other TG isoforms (Cornwell *et al.*, 1983; Bungay *et al.*, 1984), whereas induction of TG2 expression by agents such as retinoids often induces expression of an array of other genes (Davies *et al.*, 1985; Chiocca *et al.*, 1989; Chen *et al.*, 2001). The use of cell lines stably transfected with the full length TG2 constructs to overexpress the enzyme, together with transfection with 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 to these *in vitro* models, the study of the effect that TG2 overexpression or silencing has on a single cell line in isolation was soon expanded to *in vivo* mouse models where the TG2 gene has been knocked out (De Laurenzi and Melino, 2001; Nanda *et al.*, 2001). In light of recent evidence suggesting that TG2 might provide a novel therapeutic tool during the wound healing process where it is present at increased levels in the ECM (Verderio *et al.*, 2004), part of the work presented in this thesis has also sought to use an exogenous enzyme delivery approach, whereby *gpl*TG2 is administered to cultures and tumour tissue in its active or inactive form. Addition of exogenous TG2 mimics the tissue wounding/fibrotic process, where following physical injury cellular TG2 'leaks' into the surrounding ECM,

where it becomes activated and catalyses the cross-linking of proteins (Nicholas *et al.*, 2003).

Recently, the focus of TG2 research was drawn to its clinical relevance in a number of pathological conditions such as neurodegenerative diseases (Applet *et al.*, 1996; Citron *et al.*, 2001; Karpuj *et al.*, 2002), autoimmunity (Dieterich *et al.*, 1997; Hoffenberg *et al.*, 2000), fibrosis (Griffin *et al.*, 1979; Mizra *et al.*, 1997; Johnson *et al.*, 1997 and 1999; Grenard *et al.*, 2001b), and tumour growth and progression (Barnes *et al.,* 1985; Hettasch *et al.*, 1996; Johnson *et al.*, 1994; Haroon *et al.,* 1999b). Although the enzyme's role in most of these conditions has been established, its involvement in tumour growth and metastasis has been less understood. Following the initial hypothesis that covalently stabilised fibrin network was a prerequisite for a solid tumours proliferative growth (Yancey and Laki, 1972) it was later postulated that cellular transformation is accompanied by a reduction in TG2 expression and activity (Birckbichler *et al.*, 1976 and 1978; Birckbichler and Patterson, 1980). TG2 levels were also shown to be reduced in neoplastic tissue compared to normal tissue (Hand *et al.*, 1987). However, it has also been suggested that TG2 does not represent a biochemical marker of malignancy in human brain, colon, pancreas, stomach, and lung tumour tissue (Rönn *et al.*, 1992; Takaku *et al.*, 1995).

It has been proposed that several essential alterations in cellular physiology collectively dictate malignant growth; insensitivity to anti-growth signals, limitless replicative potential, evasion of apoptosis, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Understandably, most anti-tumour therapy approaches are targeted against one or more of these tumour-specific hallmarks. Taking into account that TG2 has been implicated in a wide range of cellular functions most of which are crucial in the tumour growth process, it is conceivable that the enzyme might provide a tool for designing a successful anti-tumour strategy.

TG2 could potentially influence tumour progression through a variety of mechanisms that correlate to the tumour cell physiology hallmarks such as regulation of cell growth

(Borge *et al.*, 1996), cell death (Knight *et al.*, 1991), as a cell surface receptor (Gaudry *et al.*, 1999b; Belkin *et al.*, 2001), via its involvement in ECM organisation (Barsigian *et al.*, 1991; Nunes *et al.*, 1997), or through its involvement in tumour angiogenesis (Haroon *et al.*, 1999a and 1999b). Given the contradictory reports on whether TG2 is a tumour related marker, and more importantly the lack of a clear role for TG2 in tumour development, this thesis sought to identify whether this enzyme has a physiological or/and a therapeutic role in tumour growth.

Tumours are known to elicit a wound healing response from the host tissues, resulting in formation of granulation tissue at the advancing margins (Dvorak *et al.*, 1986; Senger *et al.*, 1994). Following the observation of increased TG2 expression in the tissue surrounding the tumour, previous reports in this field have hypothesised that TG2 participates in the body's natural defense against tumour formation (Hettasch *et al.*, 1996). In this study, challenge of C57/BL6 TG2 transgenic mice with B16-F1 melanoma cells led to increased tumour growth rates and reduced survival within the TG2 knock-out population when compared to their wild type counterparts. Histochemical analysis of tumour sections obtained from TG2 knock-out mice bearing B16-F1 tumours also revealed increased invasion of these melanoma cells through the tumour pseudocapsule, as opposed to a mild invasion observed in the TG2 wild type mice. Immunohistochemical and western blotting studies, on the other hand, confirmed the lack of TG2 antigen within the tumour body, as reported previously (Bichbichler and Patterson 1976 and 1978, Hand *et al.*, 1987). However, the presence of increased TG2 antigen at the boundary between the tumour body and the stromal tissue in TG2 wild-type mice suggested that the enzyme is possibly stabilising the ECM at the tumour-host interface, hence sealing off the tumour. This is in keeping with a recent report suggesting that TG2 is expressed as a host response to tumour invasion and inhibits tumour growth (Haroon *et al.*, 1999b). In that particular study, increased expression of TG2 and augmented TG activity was observed in the ECM of the host tumour interface of the subcutaneously implanted rat mammary adenocarcinoma R3230Ac (Haroon *et al.*, 1999b). Host response mechanisms include induction of cytokines that promote wound healing, stimulation of the immune system to resist foreign invasion, upregulation of tumour suppressor genes, and ECM accumulation

and stabilisation. *In vitro* experiments undertaken in this study suggested that artificial collagen-fibronectin matrices cross-linked by TG2 present a better barrier to invading CT26 tumour cells. This effect was shown to be due to the enzyme's cross-linking activity as inactivation of TG2 prior to incubation with the collagen/fibronectin mixture did not have any significant effect on tumour cell invasion when compared to the control collagen/fibronectin matrices. A rigid ECM favours tumour growth inhibition as it is more resistant to proteolytic degradation by proteases and mechanical disruption by the expanding tumour mass (Dano *et al.*, 1999; Lochter *et al.*, 1988; Werb *et al.*, 1999). It is therefore conceivable that the increased TG2 antigen present at the tumour edges is stabilizing the ECM of the tumour-host microenvironment, hindering the infiltration by tumour cells and consequently the expansion of the tumour mass.

Within the aims of this thesis was also to determine whether TG2 may have a therapeutic role against tumour growth when delivered by intratumour injections. Using a rat dorsal window flap model, Haroon and colleagues demonstrated that topical application of 40μg/ml active TG2 post-surgery inhibits both growth of new vasculatures, and tumour growth when a solid piece of the R3230Ac tumour was added to the wound (Haroon *et al.*, 1999b). In that study, tumour growth delay was accompanied by increased collagen deposition (measured histochemically), indicating augmented ECM production, and hence suggesting hindered malignant and/or EC motility (Haroon *et al.*, 1999b). However, from a therapeutic consideration, this model is inadequate since the enzyme is delivered to an open tumour surface shortly after tumour implantation, whereas most therapeutic regimes against tumours are delivered well after a growing tumour has been established. The tumour therapy models undertaken for the purpose of this thesis took into consideration these limitations and indicated that exogenous administration of active (10U/injection) TG2 into a growing tumour mass of CT26 origin led to increased survival rates and in some cases it favoured complete regression. Interestingly, attenuation of tumour growth appeared to be dose specific, and was not achieved when TG2 of higher (100U/injection) or lower activity (2U/injection) was applied to the tumours. It is believed that the dosing activity of an enzyme with such a high affinity for many ECM proteins is critical for its homogeneous perfusion throughout the tissue, as demonstrated

by immunofluorescent staining of tumours treated with exogenous TG2 at various specific activities. Similarly, when TG2 of lower activity is administered to the tissue, ECM cross-linking and accumulation may be insufficient for tumour therapy. Histochemical and chromatographic examination of the CT26 tumours treated with TG2 revealed increased collagen deposition as manifested by increased collagen staining and hydroxyproline levels (indicative of the fibrotic phenotype) and accompanied by elevated ε(γ-glutamyl)lysine isopeptide bonds. Having obtained immunohistochemical confirmation of the delivery of the enzyme to the tumour body, the increased presence of the isopeptide product of the transglutaminase reaction provides further evidence for its activity as well as its ability to induce collagen accumulation and stabilisation of the ECM. Both of these findings are in agreement with the reported increase in collagen deposition and ε(γ-glutamyl) lysine isopeptide, when these where measured immunohistochemically following exogenous *gpl*TG2 treatment of R3230Ac tumours implanted on a rat dorsal flap window model (Haroon *et al.*, 1999b). Subcutaneous injection of tumour cells together with TG2 also led to reduced tumour growth and increased survival. This tumour model had the distinct advantage of enzyme delivery from the outset of the tumour growth and within the vicinity of the primary tumour mass, which bears more similarities to the model used by Haroon and colleagues (Haroon *et al.*, 1999b). Given that exogenous *gpl*TG2 was shown to have no effect on CT26 tumour cell viability, proliferation or apoptosis when applied *in vitro* it is believed that the enzyme is exerting its anti-tumour effect on the ECM of the tumour host microenvironment. Unfortunately, since the F1 variant of the B16 melanoma cell line did not generate any organ metastases and given our current Home Office license limitations on intravenous treatments, there was no available in-house model for the study of the role of TG2 in experimental metastasis.

Having established that exogenously delivered TG2 potently inhibits tumour growth, it was also of interest to ascertain whether the enzyme's anti-tumour capacity was due to inhibition of tumour angiogenesis. Although no vascular abnormalities have been detected in TG2 knock-out mice (Nanda *et al.*, 2001; De Laurenzi and Melino, 2001), such TG2-null mice did exhibit impaired skin wound healing (Mearns *et al.*, 2002).

Previous work within our group suggested that application of exogenous *gpl*TG2 within the body of CT26 tumours led to inhibition of *in vivo* neovascularisation, since immunohistochemical staining of tumour vasculatures originating from TG2 treated CT26 tumours appeared disorganised when compared to the untreated controls (Jones and Griffin, UK Patent W00211747). Counter to this observation, other groups have correlated application of exogenous TG2 with stimulation of angiogenesis in a tumour-free rat dorsal flap window model of wound healing (Haroon *et al.*, 1999a). Given that the latter model was designed to study angiogenesis in the context of wound healing, it is possible that the applied enzyme is promoting the formation of a provisional collagen matrix within the granulation tissue as reported therein (Haroon *et al.*, 1999a), thus accelerating the events leading to the angiogenesis process. Experiments undertaken within the course of this study revealed that EC outgrowth is delayed in wild type mouse aortas when implanted on matrigel. Taking into account that the migration of ECs and the formation of new vessels is affected by the composition of the endothelial BM, this finding suggested that TG2 might not be needed during initiation of EC tube formation as it might be inhibitory to the endothelial cell migratory process, possibly by stabilizing the endothelial BM, or by facilitating increased cell adhesion (Martinez *et al.* 1994). Gene array analysis of differential EC gene expression in 3-D collagen matrices was confirmatory of this observation, as it indicated that TG2 is down-regulated approximately 4-fold at 8 hours and 10-fold at 24 and 48 hours post-culture of EC cultures in 3-D collagen matrices, thus confirming that lowered levels of TG2 expression are required during the initial stages of angiogenesis (Bell *et al.*, 2001). When applied to migrating rat aortic endothelial cells in an *ex vivo* model of angiogenesis, the enzyme was shown to block neovascularisation, a finding that was confirmed following treatments of both commercial and in-house *in vitro* angiogenesis co-cultures. The observation that inactive exogenous TG2 did not hinder angiogenesis indicated that the enzyme's anti-angiogenic properties are solely due to its activity and not its cell surface adhesion function, which is independent of its transamidating activity. In light of the previous finding that TG2 reinforces artificial collagen/fibronectin matrices it was not unreasonable to postulate that the reported inhibition of angiogenesis following TG2 application might be due to its ability to strengthen the ECM. Indeed, pulse-labeling

experiments of human angiogenesis co-cultures revealed an initial increase in both total ECM protein and collagen deposition following first exogenous TG2 application, and stabilisation of the deposited matrix following multiple enzyme treatments. Previous work undertaken within this laboratory has indicated that exogenous treatment with *gpl*TG2 has a similar effect on matrix deposition of Swiss 3T3 fibroblasts (Gross *et al.*, 2002). The same finding was also reproduced within the course of this study for single C378 dermal fibroblast cultures, without any cytotoxicity. To this end, a possible role for transglutaminases in the metabolism of connective tissue has been suggested through the formation of cross-links between collagen α-chains (Jelenska *et al.*, 1980) and the cross-linking of fibronectin to specific sites of collagen types I and III (Mosher *et al.*, 1979). Recent turbidity studies have demonstrated that microbial TG can accelerate collagen gel/fibril formation (Nomura *et al.*, 2001), a finding that has been confirmed for TG2 (Skill *et al.*, 2004). These reports account for the initial increase in collagen deposition as reported in this thesis. The possibility that TG2 might indirectly regulate *de novo* collagen synthesis through TGFβ signalling had to be ruled out as the circulating pool of the biologically active cytokine was shown to be reduced, possibly due to its cross-linking by TG2, and therefore unavailable to exert its profibrotic effects following exogenous TG2 application of both angiogenesis and single C378 fibroblast cultures. On the other hand, the circulating pools of VEGF and bFGF, which are crucial for EC proliferation and motility during angiogenesis (Ziche *et al.*, 1997; Gleizes *et al.*, 1995), were found to remain unaffected in response to TG2 application.

Evidence emerging from several experiments within this study collectively suggested that the central mechanism behind TG2-mediated tumour regression and anti-angiogenesis lied in the ability of the enzyme to reinforce the ECM. It still remains to be fully elucidated whether stabilisation of the ECM is achieved directly by mechanical strengthening and protection against proteolytic degradation or indirectly via the tipping of the deposition/degradation balance towards up-regulation of protein synthesis and down-regulation of MMP expression/activity through TGFβ signalling.

TG2 has been shown to crosslink several constituents of the ECM, including fibronectin (Martinez *et al.*, 1994), collagen (Mosher and Schad, 1979), fibrin (Greenberg *et al.*, 1987), fibrinogen (Martinez *et al.*, 1989), vitronectin (Sane *et al.*, 1988), and laminin/nidogen (Aeschlimann and Paulsson, 1991). TG2 can cross-link these matrix proteins with each other to form a protease-resistant barrier that would pose an unsurpassed obstacle to either migrating ECs or invading tumour cells. It has also been demonstrated within this study that EC migration which is crucial for neovessel formation was hindered when HUVEC were seeded on artificial collagen/FN matrices that have been reinforced by TG2. This is in keeping with the hindered tumour cell invasion through similar matrices as reported previously in this thesis and is solely due to TG2 crosslinking. On the other hand, MMP-1 susceptibility experiments undertaken on C378 fibroblasts demonstrated that not only does exogenous TG2 accelerate initial collagen deposition, but also that TG2-mediated ECM cross-linking confers resistance to the matrix from proteolytic degradation by MMPs. An *in vitro* increase in the resistance of cross-linked collagen to proteolytic degradation by matrix metalloproteinase has already been reported (Johnson *et al.*, 1999). MMP-1 digests collagen fragments close to glutamine residues (Nagasse and Fields, 1996), which are the potential residues involved in the TG-mediated cross-linking reaction. At the molecular level, it appears that following collagen accumulation due to exogenous TG2 application cells are shutting down the collagen deposition pathway as expression of collagens I,III, and IV was shown to be down-regulated, whilst MMP-2 expression was up-regulated. Such a process might occur by an outside-in signaling mechanism facilitated by the mechanochemical signaling known to occur as the ECM environment becomes increasingly rigid, due to increased cross-linking (Ingber, 2002).

It has been recently hypothesised that rather than directly crosslinking ECM components, TG2 may also affect ECM homeostasis indirectly, via regulation of the deposition/circulation balance of latent TGF-β precursor (LTBP-1) into/from the ECM, hence controlling *de novo* ECM protein synthesis (Nunes *et al.*, 1997). The same authors have demonstrated that LTBP-1 is a good transglutaminase substrate (Nunes *et al.*, 1997) Other than its purely structural role, LTBP-1 (figure 7.1) is believed to participate in the

matrix storage of the latent TGFβ (Dallas *et al.*, 1995). Immunohistochemical studies within this thesis revealed that LTBP-1 deposition is down-regulated in C378 fibroblasts treated with exogenous *gpl*TG2 in contrast to previous reports demonstrating increased LTBP-1 deposition in the ECM of Swiss 3T3 fibroblasts overexpressing TG2 (Verderio *et al.*, 1999). On the other hand, biologically active TGFβ released in the media of TG2-treated C378 fibroblast cultures is diminished. Subsequent experiments using a modified ELISA method localised the biologically active cytokine in the ECM. Western blots confirmed its presence in high molecular weight bands at the top of stacking gel, which are indicative of its cross-linking by TG2. These observations inevitably raise questions with respect to the specificity of the monoclonal anti-TGFβ1 used in these methods. It is more reasonable to accept that TG2 facilitates LTBP-1 sequestration to the ECM where TGFβ1 can be activated and its biologically active form released to induce ECM protein synthesis. Irrespective to these TG2-mediated quantitative changes in circulating TGFβ, the observation that collagen expression is down-regulated following culture treatment with exogenous TG2, suggests that TG2 is not exerting its effect via activation of the protein synthesis pathway that is triggered by TGFβ. This, however, does not rule out the possibility that initial application of TG2 might up-regulate collagen expression, before feedback from a highly cross-linked ECM, following multiple TG2 administrations, signals for collagen expression 'shutdown'.

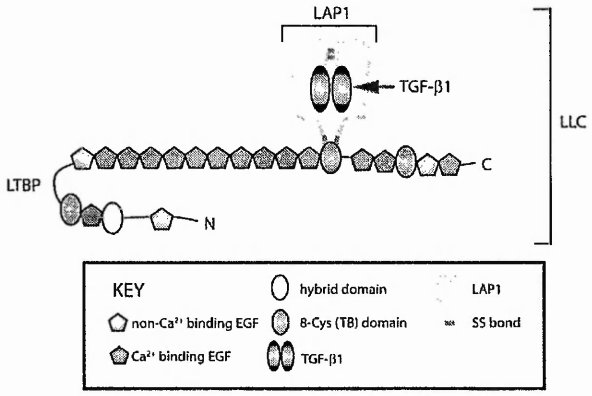


Figure 7.1. Schematic illustration of the large latent complex (LLC). The LLC comprises three proteins: an LTBP, a LAP (latency associated protein) homodimer, and a TGF-β homodimer. Each homodimer is stabilized by covalent disulfide crosslinks. In addition, disulfide bonds form between the LTBP and LAP dimer as shown.

The construction and use of CT26 clones stably transfected for the overexpression of catalytically active and inactive (C277S mutant) TG2 allowed the evaluation of tumour cell TG2 functions, which may or may not be dependent on the cross-linking activity of the enzyme. Active TG2 clones release TG2 and deposit the enzyme on the ECM unlike the inactive mutants, reinforcing the hypothesis that the active site thiol (Cys277) is essential for the correct folding of the protein to achieve the necessary conformation for its secretion (Balklava *et al.* 2002). Having established that morphology, viability and apoptosis were unaltered between the different transfectants, active and inactive (C277S) clones were used to study the role of TG2 in the attachment and migration of colon carcinoma cells, two interrelated processes which are very important in tumour progression (Johnson *et al.*, 1994; Haroon *et al.*, 1999b). Tumour cells overexpressing the active and inactive (C277S) forms of TG2 exhibited increased attachment on fibronectin coated surfaces compared to mock-transfected controls in keeping with previous reports utilising sense and antisense TG constructs (Jones *et al.*, 1997; Johnson *et al.*, 1994; Verderio *et al.*, 1998, Balklava, *et al.*, 2002). Utilising a modified Boyden chamber method, migration of active and inactive TG2 clones through uncoated filters was shown to be more rapid compared to the mock-transfected controls. However migration though FN-coated filters was severely compromised for both the active and inactive TG2 clones. This finding comes in agreement with the observations made on Swiss 3T3 fibroblast transfected for stable expression of the same active and inactive TG2 constructs (Balklava *et al.*, 2002). In this experimental setting, fibronectin was preferred to collagen as a tumour ECM substrate, as it has been previously shown that the adhesion of CT26 cells to the former matrix component was much higher when compared to the latter (Geng *et al.*, 1998). Increasing evidence proposes a role for cell surface TG2 in cell adhesion and migration that is independent of its crosslinking activity (Akimov *et al.*, 2000 and 2001; Belkin *et al.*, 2001). Given that in the former studies, the ability of TG2 antibodies to block both cell attachment and cell migration in a similar manner to antibodies directed against β1 and α5 integrins (Fogerty *et al.*, 1990), it is believed that TG2 provides additional cell-matrix contacts. With FN providing an excellent TG

substrate, the opposing observations between migration through uncoated and FN filters cold be attributed to increased adhesion of CT26 overexpressing TG2.

Introduction of CT26 cells transfected with the active TG2 construct into balb/c mice resulted in reduced primary tumour incidence, reduced growth rates, and increased survival. The observation that the CT26 clones carrying the inactive construct resulted in tumour growth rates almost parallel to the mock-transfected clones indicated that tumour growth attenuation is due to the enzyme's crosslinking activity. Western blot analysis of TG2-transfected CT26 isolated from the animals could not provide a definitive answer as to whether the expression of the enzyme is lost due to unfavorable selection. However, preliminary experiments based on western blotting of tumour lysates suggests that the sustained tTG expression is possibly due to enrichment following re-culture and selection in G418 of tumour clone cells (fig. 7.2). The presence of high M_r tTG-polymers in the lysates of active tTG clones (TG3, TG5) provides evidence for extracellular substrate cross-linking. It is, however, notable that the 77kDa tTG band is absent from this highly tTG-expressing clone (TG3), but present in the low-expressing tTG clone (TG5). Interestingly, clone TG3 did not have a significant effect on growth and animal survival when inoculated in balb/c mice, whereas clone TG5 led to diminished tumour growth and increased animal survival. This indicates that constitutive tTG expression might be lost in clone TG3 due to unfavourable selection possibly from the outset of tumour growth, as the externalised enzyme was incurring increased cross-linking thus impeding on the malignant cell ability to digest and migrate through self-reinforced extracellular spaces. The presence of the 77kDa band in the inactive clones (INA13, INA14) also suggests that lack of externalisation is unlikely to have an effect on the natural selection of this cell line. In order to provide a clearer picture, further experiments such as Southern hybridisation of restriction fragments from TG2 clone DNA and/or RT-PCR amplification could be designed to determine the presence of the TG2 insert in the transfected clones following introduction into the animals. RNA subtraction studies could also confirm the expression of the inserted TG2 gene.

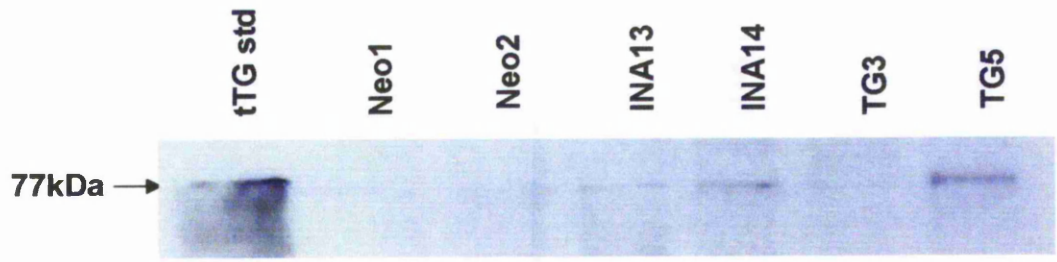


Figure 6.3.12 Evaluation of tumour host's ability to retain tTG expression and activity following natural selection

Tumour specimens were homogenised in 250mM Sucrose, 2mM EDTA, 5mM Tris-HCl in the presence of protease inhibitors to yield a 20% (w/v) final homogenate concentration. 20μg of total protein yielded was analysed for tTG antigen by Western blotting. 100ng of gpl tTG were resolved as a positive control.

Altered tumour cell-ECM interactions are now considered pivotal in the invasion and metastasis of tumour cells (Liotta and Kohn, 2001). The central finding in this thesis is that an excess of exogenous TG2 delivered *in vitro* and *in vivo* can lead to fibrogenesis in agreement with the enzyme's observed pathological role in diabetic nephropathy, lung fibrosis, liver fibrosis and hypertrophic scarring. Importantly, in this instance the enzyme has been used to inhibit tumour growth and angiogenesis by a mechanism involving increased stabilisation of the tumor-host ECM, thus enhancing the host's defense mechanisms against EC and tumour cell migration. Despite considerable advances in the understanding of angiogenesis and tumour growth the development of elegant tumor therapeutic strategies involving its inhibition through antiangiogenesis have been less successful when animal studies have been translated into clinical phase trials. This may also be due to the fact that angiogenesis inhibitors may have the unintended effect of metastatic promotion, by depriving tumors of oxygen (Steed, 2001). Modulation of ECM turnover as a major control point in angiogenesis and tumour growth may still provide a more rational approach for development of new anticancer regimens. Our preliminary studies, which involve intratumour injection of TG2 for modulation of the tumour environment leading to fibrosis provided an alternative therapeutic strategy that 'quarantines' the tumour tissue and cuts off the communication with the neighbouring stromal microenvironment. It is envisaged that TG2 may provide a therapeutic tool for solid tumour growth containment possibly following tumour resection.

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