Role of Transglutaminases in Signalling that Regulates Epithelial Responses in Wound Healing.

Thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy, Cardiff University

September 2010

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"Science never solved a problem without creating ten more"

George Bernard Shaw.
1856-1950.

Role of Transglutaminases in signalling that regulates epithelial responses in wound healing.

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Acknowledgements

First and foremost, I have to thanks my supervisors Professor Daniel Aeschlimann and Dr Vera Knauper. Secondly, I would like to thanks my friends Dr Lorna Fiedler, and Dr Sally Rosser-Davies for your proof reading and patience! Dr Mathew Locke, Mr Martin Langley, Mrs Pascale Aeschlimann and anyone else in the Dental School who has answered my questions about techniques and equipment, swapped ideas, sympathy and motivation when things have not worked out quite right!

Thank you to my family, for their support both financially and otherwise through what has been a lengthy process increased by illness!

I would also like to thank my fiancé Chris, for listening and supporting me every day.

Finally, this study would not have been possible without the funding from the Dental School.

Summary

Wound healing is a complex process broken down into five main stages fibrin clot formation, re-epithelialisation, granulation tissue formation and wound contraction, and finally angiogenesis. In the second stage, re-epithelialisation over fibroblast-derived matrix occurs which is necessary for wound closure. During this process, TG2, a multi-functional enzyme with both cross linking and GTP signalling ability is involved in both stabilisation of the extracellular matrix by cross linking and by allowing downstream signalling events to occur which lead to the wound closing. This thesis has investigated the mechanisms by which re-epithelialisation occurs with regard to TG2. In the first stage of wound healing, an influx of growth factors and metalloproteinases occurs, that through the initial interaction of TG2 are able to stabilise the matrix and stimulate keratinocyte cells to migrate and proliferate to close the wound. Experiments have indicated that TG2 is able to stimulate proliferation and migration of keratinocyte cells both directly and indirectly by modulating metalloproteinase signalling, leading to the activation of the EGFR by EGF ligands liberated from the ECM. Furthermore, through the course of altering TG2's conformation and activity experiments have determined that TG2 must be in an open and active conformation in order for it to affect keratinocyte signalling leading to proliferation and migration. Finally, a G protein coupled receptor has been investigated as to whether it may be involved in TG2 driven proliferation and migration in keratinocytes. Previous work by Xu et al., 2006 had shown TG2 to be a binding partner of GPR56 and this GPCR has been shown to be involved in proliferation and migration of cells located in the brain and heart. Although investigations of GPR56 are at this time inconclusive regarding keratinocyte proliferation and migration this thesis confirms the presence of GPR56 in keratinocytes. Therefore GPR56 may be involved in keratinocyte migration and proliferation.

Abbreviations

ADAM	A Disintegrin And Metalloproteinase
ADAM-TS	A disintegrin and metalloproteinase with thrombospondin motifs
AREG	Amphiregullin
AS (ECM)	Antisense/ TG2 null extracellular matrix
BCA	Bicinchoninic acid
BMP	Bone morphogenic Protein
BSA	Bovine Serum albumin
BTC	Betacellulin
CBL	Covalently bound lipid
CE	Cornified Envelope
CTGF	Connective tissue growth factor
DEJ	Dermal Epidermal Junction
DMEM	Dulbecco's Modified Eagle Medium
DSFM	Defined serum free medium-keratinocytes
DTT	Dithiothretol
DMSO	Dimethyl sulfoxide
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth factor Receptor
ErBB	Epidermal growth factor receptor 1
EbB1	Alternative name for epidermal growth factor receptor 1
ErbB2 -4	Alternative name for epidermal growth factor receptor 2-4
EPGN	Epigen
EREG	Epiregulin
ERK	MAPK/ERK pathway Activated Ras activates the protein kinase activity of RAF kinase. (MAPK).
FAK	Focal Adhesion Kinase
FAM	Fluorencin
FCS	Foetal Calf Serum
FGF	Fibroblast Growth factor receptor

FITC	Fluorescein
FPLC	Fast protein liquid chromatography
FN	Fibronectin
GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	G protein coupled receptor
GPCR56	G protein coupled receptor 56
GPS	GPCR protein cleavage site
GST	Glutathione S-transferase
HEPES	N-2-hydroxyethlpiperazine-N'-2-ethane sulphonic acid monosodium salt
Her 1-4	Epidermal growth factor receptors (alternative names 1-4)
HB-EGF	Heparin Bound-Epidermal Growth Factor
IGFR	Insulin like Growth factor receptor
IL-	Interleukin
IPTG	Isopropyl β D-thiogalactoside
K	Keratin
LI	Lamellar ichthyosis
LPA	Lysophosphalidic acid
M/Mk (ECM)	Mock (endogenous) extracellular matrix
MDC	Monodansyl cadaverine
MMP	Matrix Metalloproteinase (numbered)
MT-MMP	Membrane Type Matrix Metalloproteinases(numbered)
MAPK	Mitogen-activated protein kinase
NRG 1-4	Neuregulin
PBS	Phosphate buffered saline
PDGFR	Platelet derived growth factor receptor
PMSF	Phenylmethyl sulphonylfluoride
PNGase	Peptide N-Glycosidase
PSG	Penicillin/Streptomycin/Glutamine
P13K	Phosphatidylinositol 3 Kinase
QPCR	Quantitative real time polymerase chain reaction
RGD	Arginine Glycine Aspartate

RT	Room Temperature
SDS	Sodium dodecyl sulphate
SE	Skin Equivalent
SiRNA	Small interfering RNA.
S (ECM)	Sense (overexpressed) extracellular matrix.
SPR	Small proline rich proteins
SVMP	Snake venom metalloproteinases
TAE buffer	Tris/acetate/EDTA buffer
TAMRA	Tetramethylrhodamine
TBS	Tris buffered saline
TBE	Tris-Borate EDTA
TCA	Trichloroacetic acid
TG	Transglutaminase (1-7)
TGF	Transforming Growth Factor (alpha/beta)
TIG3	Tarazotene induced Gene 3
TIMP	Tissue inhibitors of metalloproteinases
TNF	Tumour necrosis factor
VEGF	Vascular Endothelial Growth Factor Receptor
UV	Ultra Violet

Chapter 1:Introduction

Contents. **Page**

1.0 Transglutaminases General introduction

Transglutaminase was introduced as a term in 1957 by Clarke et al, 1957 to describe the transamidation (cross linking ability) seen in guinea pig liver. Further study indicated that transamidating enzymes were able to stabilize fibrin monomers during blood clotting through a cross- linking acyl transfer reaction (Pisano et al., 1986). Since these discoveries were made transglutaminase activity has been seen in many organisms from microorganisms and plants up to higher invertebrates and vertebrates. Transglutaminases are multi-functional; they are able to act as both a transamidating enzyme and as a GTPase through activation via calcium (Ca^{2+}) binding. Transamidation allows transglutaminase to modify glutaminyl side chains in protein substrates. Post-translational modification of glutamine residues occurs by protein crosslinking by the formation of N-γ-glutamyl ε-lysine isopeptide bonds between the donor lysine residue of one polypeptide and the acceptor glutamine residue of another polypeptide. This leads to the formation of covalently cross-linked protein homo and hetero polymers. Alternatively, primary amines can be incorporated at selective peptide bound glutamine residues where the incorporation of either a negatively charged glutamic acid residue or positively charged amine group can have biological consequences. It is however more complicated than the incorporation of single molecules. After the glutamine containing the first substrate (or acceptor) is bound to the enzyme a γ-glutamylthiolester is formed with the active centre cysteine residue of the transglutaminase (acyl-enzyme intermediate). This is accompanied by a release of ammonia or amine. The second substrate (the donor in the transamidation reaction) then binds to the enzyme acyl-enzyme intermediate and attacks the thiolester bond. This allows

the active centre cysteine residue of the enzyme to return to its original form allowing another cycle of catalysis to occur. It is the covalent acyl-enzyme intermediate which is the rate limiting step of the reaction. This is determined by the ability of the transglutaminase to bind the second substrate of the acyl-enzyme intermediate coupled with the active site Cys-His-Asp catalytic triad and a crucial tryptophan residue located 36 residues upstream of the active centre cysteine (Folk and Finlayson 1977, Lorand and Conrad 1984; Lorand and Graham, 2003).

Nine transglutaminase (TG) genes have been identified, eight of which code for functioning enzymes. The TG family comprises of intracellular TG1, TG3 and TG5 isoforms which are expressed predominantly in epithelial tissue, TG2 which is expressed in various tissue types and occurs both intra and extracellularly, TG4 which is found in the prostate gland, factor XIII (FXIII) which is expressed in haematopoietic cells and TG6 and 7 which are not yet tissue assigned. The final TG gene encodes a protein called band 4.2, which is a component protein of the membrane that has lost its enzymatic activity and serves to maintain erythrocyte membrane integrity (Lorand and Graham 2003). There is a high degree of sequence homology between transglutaminases and domain conservation and these proteins are thought to have evolved from cysteine proteases occurring early in evolution (Pedersen et al., 1994). TG genes have been discovered clustered on five different chromosomes and are thought to have evolved by successive duplications (Grenard et al., 2001). The active site of the TG family comprises a catalytic triad of cysteine (Cys), histidine (His) and aspartate (Asp) that is functional in all but one (Pedersen et al., 1994); band 4.2,

which has a Cys to Ala substitution resulting in deficient crosslinking (Korgren et al., 1990).

Transglutaminase activity results in changes in physical and chemical properties of the cross-linked proteins which in turn lead to changes in biological activity and function. This manifests by the formation of protein polymers resulting in increased protein stability and resistance to degradation, be it mechanical, chemical or physical, and is therefore highly important in extracellular matrix (ECM) functionality. If the amine is incorporated into the acceptor protein via glutamine transamidation or by glutamine undergoing deamidation this results in the conversion of glutamine to glutamic acid. This can alter stability, conformation, molecular interaction or the enzymatic activity of the target protein.

TG enzymes have thus been shown to be involved in a wide range of physiological processes. These include fibrin clot formation (Pisano et al., 1986, Chen and Doolittle, 1971, Shainoff et al., 1991), semen coagulation (Williams-Ashman, 1984), wound healing (Raghunath et al., 1996; Haroon et al 1999, Stephens et al., 2004) and generation of cornified envelopes in keratinocyte differentiation (Steinert and Marekov, 1995, 1997, Candi et al., 1999). Transglutaminase family members have also been shown to fulfil a specific function where their expression is limited. For example Band 4.2 has a specific structural role in the cytoskeleton of hematopoietic cells (Aeschlimann et al., 2001). In contrast transglutaminases 2 and 5 can be found ubiquitously expressed (Thomazy and Fesus 1989; Grenard et al., 2001; Candi et al., 2004, Esposito and

Caputo, 2009). In addition, more than one transglutaminase is expressed within a tissue, therefore it has been suggested that there is redundancy within the family. In support of this, the TG2 knock out mouse has no overt phenotype (De Laurenzi and Melino 2000).

1.1Transglutaminase Family Structure.

Early structural studies of transglutaminases were performed using high resolution crystallography. The first to be identified was enzyme FXIII (Yee et al., 1994, 1996, Weiss et al., 1998). This showed that each factor XIII subunit was composed of 4 domains (Figure 1). An N-terminal β sandwich, combined with a largely α-helical active domain 2, forms a 450 amino acid residue core domain containing both the catalytic and regulatory sites. The C terminal β barrel domains 1 and 2 are linked to the catalytic domain by a flexible loop, which is susceptible to proteolytic cleavage. Studies showed that two monomers assemble into the native dimer through surfaces in domains 1 and 2 in opposite orientation. This organisation into the four domains is highly conserved amongst the TG family where minor variations in additional N or C terminal sequences incorporate the functionality of each enzyme, which, in turn impacts on substrate selection (Greenberg et al., 1991). For example it has been suggested that the acyl donor approaches from the C terminal β barrel region ensuring a larger degree of enzyme specificity while the acyl acceptor is believed to dock from the catalytic domain (Lorand and Graham, 2003). It has also been proposed that non-proline cis peptide bonds present adjacent to the active site may be involved in transglutaminase activation (Weiss et al., 1998). This is dependent on the binding of Ca^{2+} and/or substrates that trigger a conformational change from cis to trans isomerisation of these peptide bonds (Weiss et al., 1998). Furthermore, Trp^{241} plays an essential role in activating TG2. This is suggested to occur by stabilising the transition state of the enzyme. It has been shown that Trp^{241} is conserved throughout the entire TG family with the exception of band 4.2 which is catalytically inactive (Murthy et al., 2002, Iismaa et al., 2009). Studies have

shown that different TGs may interact with the same substrate with different affinity, and may target different residues. This has been attributed to structural and charge properties of flanking residues of the active site (Esposito and Caputo, 2004).

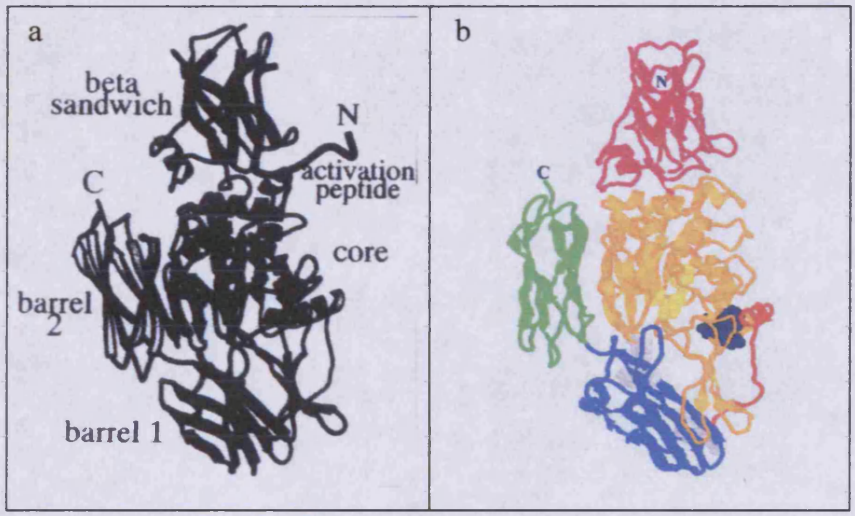

Figure 1. The secondary structure of transglutaminase enzymes is conserved: The domain structure between isoenzymes are observed to conserve the N-terminal sandwich domain, catalytic domain and two C-terminal β-barrel domains as demonstrated by the backbone structure of a) the FXIII monomer (Yee *et al.*, 1994) and b) TG2, where the domains are coloured magenta, orange, blue and green respectively. The flexible loop connecting the catalytic domain and the 1st β-barrel domain is coloured red and the amino acids involved in the active site (Cys^{277}, His^{335} and Asp^{358}), Ca^{2+} binding (Ser^{449}, Pro^{446}, Glu^{451} and Glu^{452}) and interaction with GTP (Ser^{171}, Lys^{173}, Arg^{478}, Val^{479} and Arg^{580}) are coloured yellow, black and grey respectively (Griffin *et al.*, 2002).

1.1.1 Transglutaminase Regulation by Nucleotide Binding.

In order for cell communication to occur there must be a system of signal recogniser, mediator and acceptor. The guanine nucleotide binding proteins (G Proteins) are signal mediators that transfer receptor signals to acceptors. They are effector enzymes that produce biologically active molecules or second messengers (Im et al., 1997). There are over a thousand types of G protein, which share a common GTP binding motif, and which are split mainly into 3 classes. These are heterodimeric G Proteins, Ras like G proteins and small molecular weight G proteins. TG2, 3, 4 and 5 have been shown to be regulated by GTP binding and are capable of hydrolysing these molecules (Iismaa et al., 2009). It has been shown that although the amino acid sequences binding GTP are not conserved between TG family members, a hydrophobic pocket forms the GTP binding site for all isoforms. This GTP binding site is located in the cleft between the catalytic core and β-barrel domain close to the dimerization interface although the specific amino acid sequence involved differs depending on the TG (Liu et al., 2002; Ahvazi et al., 2004,). Isoforms TG2, 3, 4 and 5 can bind GTP but only TG2 is able to utilise it for GTPase signalling activity (Ahvazi et al., 2004). X ray crystallography studies undertaken by Liu at al., indicated that when TG2 is bound to GDP the transamidation site is obstructed by two loops within the β barrel domain and the active Cys reside from the catalytic triad is hydrogen bonded to a Tyr residue (Liu et al., 2002). This inhibition is reversed by the binding of Ca^{2+} causing a conformational change to an open configuration and resulting in an active enzyme (Figure 2). This Ca^{2+} dependence for functionality may explain the nature of TG2 and its activity depending on its location. In the cytoplasm high concentrations of GTPs can be found

accompanied with a low Ca^{2+} concentration favouring GTP cycling and GTPase activity of TG2 whereas in surrounding matrices a high Ca^{2+} concentration may support transamidation activity of TG2. Furthermore it has been suggested that local concentrations of Ca^{2+} and nucleotides influences TG2 regulation (Haroon et al., 1999). This also suggests there may be a direct link between nucleotide and Ca^{2+} levels and the regulation of the other isoforms of transglutaminase. *In vitro* TG5 has a lower sensitivity for Ca^{2+}-mediated activation and GTP inhibition than TG2 (Candi et al., 2004). Therefore at physiological levels of Ca^{2+} TG5 retains 25% of its maximal transamidation activity compared with 75% for TG2 while at physiological GTP levels, up to 55% of TG5 enzyme activity is lost compared to 90% of TG2 activity (Candi et al., 2004).

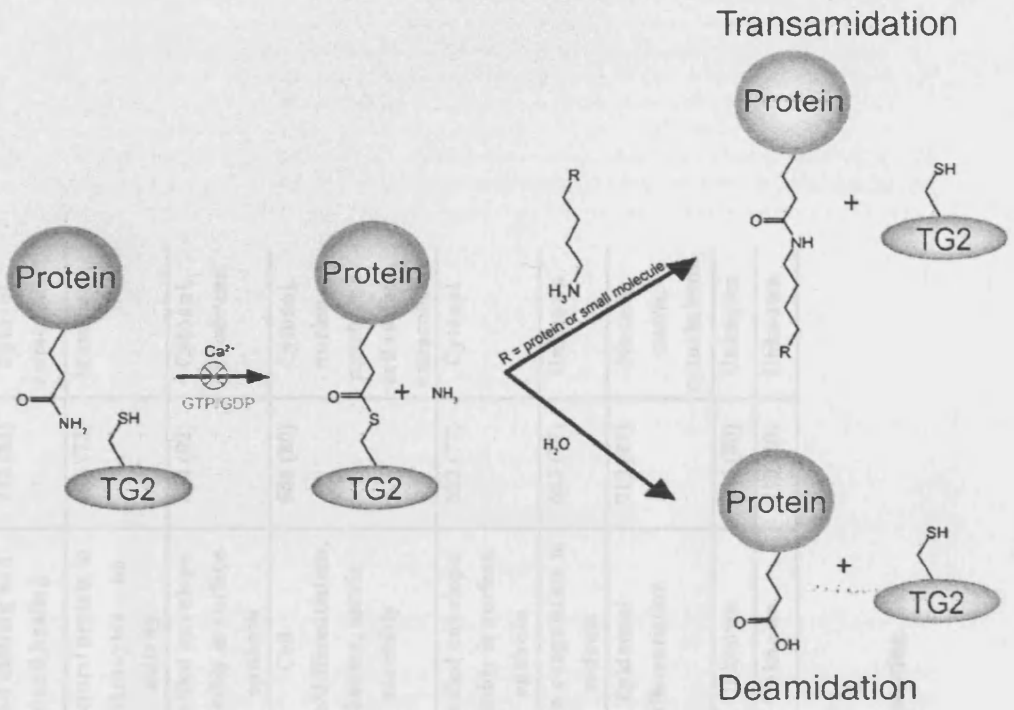

Figure 2: Transglutaminases are capable of catalysing various post-translational modifications: In the presence of Ca^{2+} the TG2 active site cysteine (Cys ^{277}) thiol attacks the γ-glutaminyl side chain of a protein or peptide bound glutamine residue forming a thioester intermediate with the release of ammonia. In transamidation a primary amine nucleophile attacks the thioester carbonyl displacing the TG2 thiol and resulting in an isopeptide crosslink between glutamine side chain and the primary amine. In deamidation, water acts as the thiol-displacing nucleophile resulting in the net conversion of glutamine to glutamate. The presence of GTP or GDP inhibits transglutaminase transamidation activity.

TG	Alternative designations	Chromosome location	Gene	Function	Size aa (kDa)	Location
Factor XIII	Fibrin stabilising factor, plasma TG	6p24-25	F13A1	Blood clotting and wound healing	732 (83)	Cytosol, extracellular
Band 4.2	Erythrocyte membrane protein	15q15.2	EPB42	Structural protein in erythrocytes – no activity	690 (72)	Membrane
1	Keratinocyte TG, particulate TG	14q11.2	TGM1	Cornified envelope assembly in surface epithelia	814 (92)	Cytosol, Membrane
2	Tissue TG, liver TG	20q11-12	TGM2	Cell death/differentiation, adhesion, matrix assembly	686 (80)	Cytosol, nucleus, membrane, cell surface, extracellular
3	Epidermal TG	20q11-12	TGM3	Cornified envelope assembly in surface epithelia	692 (77)	Cytosol
4	Prostate TG	3q21-22	TGM4	Semen coagulation in rodents	683 (77)	Unknown
5	TGx	15q15.2	TGM5	Epidermal differentiation	719 (81)	Nuclear matrix, cytoskeleton
6	TGY	20q11	TGM6	Unknown	706 (80)	Unknown
7	TGZ	15q15.2	TGM7	Unknown	710 (80)	Unknown

Table 1: Summary of the nine isoforms.
The nine TGs are summarised here to briefly illustrate their position, function, size and location.

```
    TG2    ------------------------------------------------------------    D1
    B4.2   ------------------------------------------------------------
    TG3    ------------------------------------------------------------
    TG6    ------------------------------------------------------------
    TG5    ------------------------------------------------------------
    TG7    ------------------------------------------------------------
    FXIII  ---SETSRTAFGGRRAVPPNNS-------------------------------NAAEDDL 26
    TG1    MDGPRSDVGRWGGNPLQPPTTPSPEPEPEPDGRSRRGGGRSFWARCCGCCSCRNAADDDW 60
    TG4    ------------------------------------------------------------
                                          1

    TG2    ----------------------------------------
D1
    B4.2   ----------------------------------------
    TG3    ----------------------------------------
    TG6    ----------------------------------------
    TG5    ----------------------------------------
    TG7    ----------------------------------------
    FXIII  PT--------------------VELQGVVPRG--VN-----                    38
    TG1    GPEPSDSRGRGSSSGTRRPGSRGSDSRRPVSRGSGVNAAGDG
103
    TG4    ----------------------------------------

    TG2    ---AEELVLERCDLELET---NGRDHHTADLCREKLVVRRGQPFWLTLHFEG--RNYEAS 52
D2
    B4.2   ---GQALGIKSCDFQAAR---NNEEHHTKALSSRRLFVRRGQPFTIILYFRAPVRAFLPA 54
    TG3    ----AALGVQSINWQTAF---NRQAHHTDKFSSQELILRRGQNFQVLMIMN---KGLGSN 50
    TG6    ----AGIRVTKVDWQRSR---NGAAHHTQEYPCPELVVRRGQSFSLTLELS---RALDCE 50
    TG5    ---AQGLEVALTDLQSSR---NNVRHHTEEITVDHLLVRRGQAFNLTLYFRN--RSFQPG 52
    TG7    -DQVATLRLESVDLQSSR---NNKEHHTQEMGVKRLTVRRGQPFYLRLSFS---RPFQSQ 51
    FXIII  --LQEFLNVTSVHLFKERWDTNKVDHHTDKYENNKLIVRRGQSFYVQIDLS---RPYDPR 95
    TG1    TIREGMLVVNGVDLLSSRSDQNRREHHTDEYEYDELIVRRGQPFHMLLLLS---RTYESS
160
    TG4    MDASKELQVLHIDFLNQ---DNAVSHHTWEFQTSSPVFRRGQVFHLRLVLN---QPLQSY 54
                 : :   .        *   ***         .**** * : : :    :

    TG2    VDSLTFSVVTGPAPSQEAGTKARFPLRDAVEEGDWTATVVDQQDCTLSLQLTTPANAPIG
112 D2
    B4.2   LKKVALTAQTGEQPSKINRTQATFPISSLGDRKWWSAVVEERDAQSWTISVTTPADAVIG
114
    TG3    -ERLEFIVSTGPYPSESAMTKAVFPLSNGSSG-GWSAVLQASNGNTLTISISSPASAPIG
109
    TG6    -EILIFTVETGPRASEALHTKAVFQTSELERGEGWTAAREAQMEKTLTVSLASPPSAVIG
109
    TG5    LDNIIFVVETGPLSDLALGTRAVFSLARHHSPSPWIAWLETNGATSTEVSLCAPPTAAVG
112
    TG7    NDHITFVAETGPKPSELLGTRATFFLTRVQPGNVWSASDFTIDSNSLQVSLFTPANAVIG
113
    FXIII  RDLFRVEYVIGRYPQENKGTYIPVPIVSELQSGKWGAKIVMREDRSVRLSIQSSPKCIVG
155
    TG1    -DRITLELLIGNNPEVGKGTHVIIPVG-KGGSGGWKAQVVKASGQNLNLRVHTSPNAIIG
217
    TG4    -HQLKLEFSTGPNPSIAKHTLVVLDPRTPSDHYNWQATLQNESGKEVTVAVTSSPNAILG
113
            . . .    *  ..    *   .          * *           : : :.. . :*

    TG2    LYRLSLEAST---GYQGSSFVLGHFILLF
138 D2
    B4.2   HYSLLLQVSG---RKQ---LLLGQFTLLF
137
    TG3    RYTMALQIFS---QGGISSVKLGTFILLF
134
    TG6    RYLLSIRLSS---HRKHSNRRLGEFVLLF
135
    TG5    RYLLKIHIDSF--QGSVTAYQLGEFILLF
139
    TG7    HYTLKIEISQG--QGHSVTYPLGTFILLF
140
```

```
   FXIII KFRMYVAVWTPYGVLRTSRNPETDTYILF
184
   TG1   KFQFTVRTQSDAGEFQLPFDPRNEIYILF
246
   TG4   KYQLNVKTGN-----HILKSEENILYLLF
137
          : : :                    :**

   TG2   NAWCPADAVYLDSEEERQEYVLTQQGFIYQGSAKFIKNIPWNFGQFEDGILDICLILLDV
198 D3
   B4.2  NPWNREDAVFLKNEAQRMEYLLNQNGLIYLGTADCIQAESWDFGQFEGDVIDLSLRLLSK
197
   TG3   NPWLNVDSVFMGNHAEREEYVQEDAGIIFVGSTNRIGMIGWNFGQFEEDILSICLSILDR
194
   TG6   NPWCAEDDVFLASEEERQEYVLSDSGIIFRGVEKHIRAQGWNYGQFEEDILNICLSILDR
195
   TG5   NPWCPEDAVYLDSEPQRQEYVMNDYGFIYQGSKNWIRPCPWNYGQFEDKIIDICLKLLDK
199
   TG7   NPWSPEDDVYLPSEILLQEYIMRDYGFVYKGHERFITSWPWNYGQFEEDIIDICFEILNK
200
   FXIII NPWCEDDAVYLDNEKEREEYVLNDIGVIFYGEVNDIKTRSWSYGQFEDGILDTCLYVMDR
244
   TG1   NPWCPEDIVYVDHEDWRQEYVLNESGRIYYGTEAQIGERTWNYGQFDHGVLDACLYILDR
306
   TG4   NPWCKEDMVFMPDEDERKEYILNDTGCHYVGAARSIKCKPWNFGQFEKNVLDCCISLLTE
197
         *.*   * *:: .    **: : * : *    *   *.:***: ::. .: ::

   TG2   NPKFLKNAGRDCSRRSSPVYVGRVVSGMVNCNDDQGVLLGRWDNNYGDGVSPMSWIGSVD
258 D3
   B4.2  D--------KQVEKWSQPVHVARVLGALLHFLKEQRVLPTPQTQATQEGALLNKRRGSVP
249
   TG3   SLNFRRDAATDVASRNDPKYVGRVLSAMINSNDDNGVLAGNWSGTYTGGRDPRSWNGSVE
254
   TG6   SPGHQNNPATDVSCRHNPIYVTRVISAMVNSNNDRGVVQGQWQGKYGGGTSPLHWRGSVA
255
   TG5   SLHFQTDPATDCALRGSPVYVSRVVCAMINSNDDNGVLNGNWSENYTDGANPAEWTGSVA
259
   TG7   SLYHLKNPAKDCSQRNDVVYVCRVVSAMINSNDDNGVLQGNWGEDYSKGVSPLEWKGSVA
260
   FXIII A-------QMDLSGRGNPIKVSRVGSAMVNAKDDEGVLVGSWDNIYAYGVPPSAWTGSVD
297
   TG1   R-------GMPYGGRGDPVNVSRVISAMVNSLDDNGVLIGNWSGDYSRGTNPSAWVGSVE
359
   TG4   S-------SLKPTDRRDPVLVCRAMCAMMSFEKGQGVLIGNWTGDYEGGTAPYKWTGSAP
250
                      .   * *.  .::   . . *:          *          **.

   TG2   ILRRWKNHGCQRVKYGQCWVFAAVACTVLRCLGIPTRVVTNYNSAHDQNSNLLIEYFRNE
318 D3
   B4.2  ILRQWLTGRGRPVYDGQAWVLAAVACTVLRCLGIPARVVTTFASAQGTGGRLLIDEYYNE
309
   TG3   ILKNWKKSGFSPVRYGQCWVFAGTLNTALRSLGIPSRVITNFNSAHDTDRNLSVDVYYDP
314
   TG6   ILQKWLKGRYKPVKYGQCWVFAGVLCTVLRCLGIATRVVSNFNSAHDTDQNLSVDKYVDS
315
   TG5   ILKQWNATGCQPVRYGQCWVFAAVMCTVMRCLGIPTRVITNFDSGHDTDGNLIIDEYYDN
319
   TG7   ILQQWSARGGQPVKYGQCWVFASVMCTVMRCLGVPTRVVSNFRSAHNVDRNLTIDTYYDR
320
   FXIII ILLEYRSSE-NPVRYGQCWVFAGVFNTFLRCLGIPARIVTNYFSAHDNDANLQMDIFLEE
356
   TG1   ILLSYLRTG-YSVPYGQCWVFAGVTTTVLRCLGLATRTVTNFNSAHDTDTSLTMDIYFDE
418
   TG4   ILQQYYNTK-QAVCFGQCWVFAGILTTVLRALGIPARSVTGFDSAHDTERNLTVDTYVNE
309
         **  :       *  **.**:*.   * :*.**:.:* :: : *.:.    * :: : :
```

```
   TG2    FGEIQGD-KSEMIWNFHCWVESWMTRPDLQPGYEGWQALDPTPQEKSEGTYCCGPVPVRA
377 D3
   B4.2   EGLQNGEGQRGRIWIFQTSTECWMTRPALPQGYDGWQILDPSAPNGGGVLGSCDLVPVRA
369
   TG3    MGNPLD-KGSDSVWNFHVWNEGWFVRSDLGPSYGGWQVLDATPQERSQGVFQCGPASVIG
373
   TG6    FGRTLEDLTEDSMWNFHVWNESWFARQDLGPSYNGWQVLDATPQEESEGVFRCGPASVTA
375
   TG5    TGRILGNKKKDTIWNFHVWNECWMARKDLPPAYGGWQVLDATPQEMSNGVYCCGPASVRA
379
   TG7    NAEMLSTQKRDKIWNFHVWNECWMIRKDLPPGYNGWQVLDPTPQQTSSGLFCCGPASVKA
380
   FXIII  DGNVNSKLTKDSVWNYHCWNEAWMTRPDLPVGFGGWQAVDSTPQENSDGMYRCGPASVQA
416
   TG1    NMKPLEHLNHDSVWNFHVWNDCWMKRPDLPSGFDGWQVVDATPQETSSGIFCCGPCSVES
478
   TG4    NGEKITSMTHDSVWNFHVWTDAWMKRPDLPKGYDGWQAVDATPQERSQGVFCCGPSPLTA
369
                     :* ::   : *: *  *  .: *** :*.:. : .     *.  .: .

   TG2    IKEGDLSTKYDAPFVFAEVNADVVDWIQQDDG---SVHKSINRSLIVGLKISTKSVGRDE
434 D3
   B4.2   VKEGTVGLTPAVSDLFAAINASCVVWKCCEDG---TLELTDSNTKYVGNNISTKGVGSDR
426
   TG3    VREGDVQLNFDMPFIFAEVNADRITWLYDNTTG--KQWKNSVNSHTIGRYISTKAVGSNA
431
   TG6    IREGDVHLAHDGPFVFAEVNADYITWLWHEDES--RERVYS-NTKKIGRCISTKAVGSDS
432
   TG5    IKEGEVDLNYDTPFVFSMVNADCMSWLVQGGK---EQK-LHQDTSSVGNFISTKSIQSDE
435
   TG7    IREGDVHLAYDTPFVYAEVNADEVIWLLGDGQ---AQEILAHNTSSIGKEISTKMVGSDQ
437
   FXIII  IKHGHVCFQFDAPFVFAEVNSDLIYITAKKDG---THVVENVDATHIGKLIVTKQIGGDG
473
   TG1    IKNGLVYMKYDTPFIFAEVNSDKVYWQRQDDG---SFKIVYVEEKAIGTLIVTKAISSNM
535
   TG4    IRKGDIFIVYDTRFVFSEVNGDRLIWLVKMVNGQEELHVISMETTSIGKNISTKAVGQDR
429
          ::.* :        ::: :*.. :                      :*  * ** :  :

   TG2    REDITHTYKYPEGSSEEREAFTRANHLNKL-----------------AEKEE--------
469 D3
   B4.2   CEDITQNYKYPEGSLQEKEVLERVEKEKME-----------------REKDNGIRP----
465
   TG3    RMDVTDKYKYPEGSDQERQVFQKALGKLKP--------------NTPFAATSSMG------
472
   TG6    RVDITDLYKYPEGSRKERQVYSKAVNRLFG--------------VEASGRRIWIRRAGGR
478
   TG5    RDDITENYKYEEGSLQERQVFLKALQKLKARSFHGSQRGAELQPSRPTSLSQDSPRS----
492
   TG7    RQSITSSYKYPEGSPEERAVFMKASRKMLG----------------PQRASLPFLDL----
478
   FXIII  MMDITDTYKFQEGQEEERLALETALMYGAKKP-------------LNTEGVMKSRS-----
516
   TG1    REDITYLYKHPEGSDAERKAVETAAAHGSKP---------------NVYANRGSAE-----
576
   TG4    RRDITYEYKYPEGSSEERQVMDHAFLLLSSE---------------REHRRPVKEN-----
470
            .:*  **. **.  *: .   .                                  2

   TG2    -----------------
469 D3
   B4.2   ----------PSLETA--
471
   TG3    ------LETEEQEPS--
681
   TG6    CLWRDDLLEPATKPS--
493
```

```
   TG5    ------LHTPSLRPSDV
503
   TG7    ------LESGGLRDQ--
487
   FXIII ----------------
516
   TG1    ----------------
576
   TG4    ----------------
470


   TG2    TGMAMRIRVGQSMNMGSDFDVFAHITNNTAEEYV-----CRLLLCARTVSYNGILGPECG
524 D4
   B4.2   SPLYLLLKAPSSLPLRGDAQISVTLVNHSEQEKA-----VQLAIGVQAVHYNGVLAAKLW
526
   TG3    --IIGKLKVAGMLAVGKEVNLVLLLKNLSRDTKT-----VTVNMTAWTIIYNGTLVHEVW
534
   TG6    --IAGKFKVLEPPMLGHDLRLALCLANLTSRAQR-----VRVNLSGATILYTRKPVAEIL
546
   TG5    VQVSLKFKLLDPPNMGQDICFVLLALNMSSQFK-----DLKVNLSAQSLLHDGSPLSPFW
558
   TG7    -PAQLQLHLARIPEWGQDLQLLLRIQRVPDSTHPRGPIGLVVRFCAQALLHGGGTQKPFW
546
   FXIII --NVDMDFEVENAVLGKDFKLSITFRNNSHNRYT-----ITAYLSANITFYTGVPKAEFK
569
   TG1    --DVAMQVEAQDAVMGQDLMVSVMLINHSSSRRT-----VKLHLYLSVTFYTGVSGTIFK
629
   TG4    --FLHMSVQSDDVLLGNSVNFTVILKRKTAALQN-----VNILGSFELQLYTGKKMAKLC
523
                           .  .     . .                      :

   TG2    TKYLLNLNLEPFSEKSVPLCILYEKYRD---CLTESNLIKVRALLVEPVINSYLLAERDL
581 D4
   B4.2   RKKLH-LTLSANLEKIITIGLFFSNFER---NPPENTFLRLTAMATHSESNLSCFAQEDI
582
   TG3    KDSAT-MSLDPEEEAEHPIKISYAQYEK---YLKSDNMIRITAVCKVPD-ESEVVVERDI
589
   TG6    HESHA-VRLGPQEEKRIPITISYSKYKE---DLTEDKKILLAAMCLVTK-GEKLLVEKDI
601
   TG5    QDTAF-ITLSPKEAKTYPCKISYSQYSQ---YLSTDKLIRISALGEEKSSPEKILVNKII
614
   TG7    RHTVR-MNLDFGKETQWPLLLPYSNYRN---KLTDEKLIRVSGIAEVEETGRSMLVLKDI
602
   FXIII KETFD-VTLEPLSFKKEAVLIQAGEYMG---QLLEQASLHFFVTARINETRDVLAKQKST
625
   TG1    ETKKE-VELAPGASDRVTMPVAYKEYRP---HLVDQGAMLLNVSGHVKESGQVLAKQHTF
685
   TG4    DLNKT-SQIQG-QVSEVTLTLDSKTYINSLAILDDEPVIRGFIIAEIVESKEIMASEVFT
581
                  :        .  :    :         .  :

   TG2    YLENPEIKIRILGEPKQKRKLVAEVSLQNPL
612 D4
   B4.2   AICRPHLAIKMPEKAEQYQPLTASVSLQNSL
613
   TG3    ILDNPTLTLEVLNEARVRKPVNVQMLFSNPL
620
   TG6    TLED-FITIKVLGPAMVGVAVTVEVTVVNPL
631
   TG5    TLSYPSITINVLGAAVVNQPLSIQVIFSNPL
645
   TG7    CLEPPHLSIEVSERAEVGKALRVHVTLTNTL
633
   FXIII VLTIPEIIIKVRGTQVVGSDMTVTVQFTNPL
656
   TG1    RLRTPDLSLTLLGAAVVGQECEVQIVFKNPL
716
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```
   TG4    SFQYPEFSIELPNTGRIGQLLVCNCIFKNTL
612
          :    : : :               . *.*

   TG2    PVALEGCTFTVEGAGLTEEQKTVEIPDPVEAGEEVKVRMDLLPLHMGLHKLVVNFESDKL
672 D5
   B4.2   DAPMEDCVISILGRGLIHRERSYRFRS-VWPENTMCAKFQFTPTHVGLQRLTVEVDCNMF
672
   TG3    DEPVRDCVLMVEGSGLLLGNLKIDVPT-LGPKEGSRVRFDILPSRSGTKQLLADFSCNKF
679
   TG6    IERVKDCALMVEGSGLLQEQLSIDVPT-LEPQERASVQFDITPSKSGPRQLQVDLVSPHF
690
   TG5    SEQVEDCVLTVEGSGLFKKQQKVFLGV-LKPQHQASIILETVPFKSGQRQIQANMRSNKF
704
   TG7    MVALSSCTMVLEGSGLINGQIAKDLGT-LVAGHTLQIQLDLYPTKAGPRQLQVLISSNEV
692
   FXIII  KETLRNVWVHLDGPGVTRP-----------------MKKMFREIRPN-----STVQWEEV
694
   TG1    PVTLTNVVFRLEGSGLQRPKILNVGDI--GGNETVTLRQSFVPVRPGPRQLIASLDSPQL
774
   TG4    AIPLTDVKFSLESLGISSLQTSDHGTV--QPGETIQSQIKCTPIKTGPKKFIVKLSSKQV
670
        : .  . : . *:                              : .       .

   TG2    KAVKGFRNVIIGPA---------------------------
686 D5
   B4.2   QNLTNYKSVTVVAPELSA-----------------------
690
   TG3    PAIKAMLSIDVAE-----------------------------
692
   TG6    PDIKGFVIVHVATAK--------------------------
705
   TG5    KDIKGYRNVYVDFAL--------------------------
719
   TG7    KEIKGYKDIFVTVAGAP------------------------
709
   FXIII  CRPWVELDVQIQRRPSM------------------------
711
   TG1    SQVHGVIQVDVAPAPGDGGFFSDAGGDSHLGETIPMASRGGA
816
   TG4    KEINAQKIVLITK-----------------------------
683
                 : :
```

Figure 3: Multiple alignment of the nine human transglutaminase sequences: An alignment of the nine characterised TG human gene products, TG2 (Gentile et al., 1991), band 4.2 (Korsgren et al., 1990), TG3 (Kim et al., 1993), TG6 long form (Thomas, H., Thesis, 2004), TG5 (Aeschlimann et al., 1998), TG7 (Grenard et al., 2001), factor XIII a-subunit (Grundmann et al., 1986; Takahashi et al., 1986), TG1 (Phillips et al., 1990; Kim et al., 1991) and TG4 (Grant et al., 1994) are shown. *Dashes* indicate gaps inserted for optimal sequence alignments. Residues conserved in all sequences are designated "*", those demonstrating conserved substitutions are designated ":" and semi-conserved substitutions are marked ".". The sequences are arranged to reflect the transglutaminase domain conservation based on the crystal structure of factor XIII a-subunit (Yee et al., 1994): N-terminal propeptide domain (D1), β-sandwich domain (D2), catalytic core domain (D3), and β-barrel domains 1 (D4) and 2 (D5). The active cysteine residue, required for transamidation reactions is shown in blue. Identified cleavage sites are indicated with arrowheads (blue); (1) sites identified in FXIII and TG1 (2) sites identified in FXIII, TG1 and TG3.

1.2 Summary of Nine Isoforms.

The nine TG isoforms are summarised in table 1 and multiple alignments of the TG family are shown in figure 3 however the following sections summarise function and activity of each of the nine isoforms.

1.2.1 Factor XIII

Factor XIII can be found in several tissues and cells with a molecular weight of 320 kDa. Factor XIII is a tetramer composed of two alpha and 2 beta chains which are non-covalently associated (Lorand, 1986). Inactive Factor XIII (FXIII) is converted into an active transglutaminase (FXIIIa) by thrombin and Ca^{2+} in the terminal phase of the clotting cascade. Activation of Factor XIII occurs by proteolysis of the serine protease thrombin in the presence of Ca^{2+}. Thrombin cleaves a scissile peptide bond between Arg^{37} and Gly^{38} near the amino terminus of the alpha (α) chain (Lorand, 1986). The activation peptide (residues 1-37) is then thought to dissociate from the protein thus activating it (Weiss et al., 1998). It is believed to circulate as a heterotetrameric ensemble made up of plasma coagulation factor XIII, PFXIII-α2β2 or fibrin stabilising factor where the α subunit belongs to the transglutaminase family and the β subunit is related to the small consensus (sushi) repeat family (Lehtinen et al., 2004). The α subunit is protected in the circulation by the richly sialylated β subunit. The α chain mature protein consists of 730-31 amino acid residues with a molecular weight of approximately 83 kDa. The typical N-terminal hydrophobic linker sequence for secretion is absent in this isoform, which may account for its presence in the cytoplasm. The β chain contains 641 amino acids and has a molecular weight of approximately 80 kDa after addition of carbohydrate (Weiss et al., 1998).

Factor XIII plays an important role in haemostasis, wound healing, and pregnancy. By cross-linking fibrin chains and α (2) plasmin inhibitor to fibrin, FXIIIa mechanically stabilizes fibrin and protects it from fibrinolysis. Showing the importance of this function, severe deficiency of the potentially active A subunit (FXIII-A) results in a rare but severe hemorrhagic diathesis (Karimi et al., 2009). The main role of Factor XIII is to catalyse the formation of isopeptide bonds between the side chains of glutamine and lysine residues, thus stabilising the fibrin soft clot and rendering it resistant to fibrolysis either by crosslinking fibrin itself or crosslinking α2 antiplasmin, a potent inhibitor of the protease plasmin, into the fibrin clot (Muszbek 1996, Uchino et al., 1991, Sakata, 1980). In addition, it protects clots from plasminolysis by covalently linking α2 antiplasmin and α-monoglobulin and the α chain of fibrin (Mortensen, 1981). The pro-coagulate protein Von Willebrand factor is anchored via TG mediated crosslinking of serotonin on the surface of activated platelets thereby forming coat platelets which can be found either when there is haemostatic need or in thrombotic plaques (Esposito and Caputo, 2004). Factor XIII can however, also have a negative effect if a fibrin clot is established and causes thrombosis – therefore inhibitors that specifically target Factor XIII are in development.

In wound healing, crosslinking of fibrin, fibronectin and collagen at the site of injury may facilitate wound closure by providing a scaffold for fibroblasts to proliferate and spread (Akagi et al., 2002). Deficiencies in factor XIII can be acquired or inherited. They lead to prolonged coagulation time and can lead to an increased occurrence of late bleeding. Furthermore, wound healing deficiencies have been linked with deficiencies of Factor XIII (Seitz 1996). Vitronectin,

PAI-2, lipoprotein (a), Von Willebrand factor and platelet vinculin have also been reported to be substrates for factor XIII however the role of this modification is not clear (Esposito and Caputo, 2004).

1.2.2. Band 4.2.

Band 4.2 was characterised by analysis of cDNA which revealed a protein of mass 77 kDa and 691 amino acids (Korsgren et al., 1990). A Cys to Ala substitution at residue 268 produces a crosslinking deficient isoform. Post-translational modifications include the cleavage of the terminal Met and myristylation of the penultimate Gly (Cohen et al., 1993). Cohen et al also suggested that band 4.2 is stabilised by phosphorylation (Cohen et al., 1993). Band 4.2 was the first member of the TG family where alternative splicing was identified; a 30 amino acid insertion following Gln^3 has been observed in some forms of the enzyme and has been designated B4.2L (Cohen et al., 1993). The loss of transamidating activity apparent in Band 4.2 highlights the potential structural function of TG enzymes. Band 4.2 is associated with the face of erythrocyte membranes forming part of the cytoskeleton (Lorand and Conrad, 1984; White et al., 1992; Cohen et al., 1993). Mutations in this gene are accompanied by abnormally shaped blood cells and anaemia. As yet no functional difference has been seen between the two splice variants, however it has been suggested that post translational modifications take place within the additional N- terminal sequence as observed with TG1 (Aeschlimann and Paulsson,1994).

1.2.3 TG1

Kim et al., identified TG1, otherwise known as keratinocyte transglutaminase, by preparing a synthetic oligonucleotide encoding the consensual active site sequence of known transglutaminase sequences and comparing that with the newly seen TG (Kim et al., 1991). A pro enzyme with a predicted size of 92 kDa was identified, made up of 814 amino acids. When this was compared with the corresponding rat sequence, a homology of 92% was observed (Aeschlimann and Paulsson, 1994). The TG1 protein has proven difficult to purify (Thacher, 1989) therefore studies have been conducted using a recombinant form of the enzyme (Kim et al., 1994; 1995a; Nemes et al., 1999a, Hitomi et al., 2000). TG1 is post-translationally modified within its N terminus to incorporate the fatty acid palmitate or myristate via a thioester linkage (Phillips et al., 1993). Treatment with protein synthesis inhibitors suggested that myristylation occurs co translationally while palmitate labelling is a post-translational process (Steinert et al., 1996b). A number of Ser residues in the N terminus are also phosphorylated however there is no corresponding alteration in activity indicating a function in substrate interactions (Rice et al., 1996). A 106 kDa form of TG1 has also been detected, suggesting that the enzyme may undergo further modifications (Kim et al., 1995a). The presence of a fatty acid anchor localises 95% of TG1 to the membrane fraction of the cell (Steinert et al., 1996a) and it has been suggested that this anchorage of the enzyme promotes further enzyme processing (Kim et al., 1995a). Studies have indicated approximately 50% of the membrane bound enzyme is present in its zymogen form while the remaining enzyme undergoes cleavage to produce 3 fragments with calculated masses of 10 kDa (N-terminal membrane anchoring region), 33 kDa (C terminal β-barrels) and 67 kDa (active

domain) (Steinert et al., 1996a). Edman degradation sequencing identified these cleavage sites as residing between Arg^{573}-Gly^{574} and Arg^{93}-Gly^{94} (Kim et al., 1995a; Steinert et al., 1996a). Alignment with other human TG sequences demonstrated the first of these sites correlates with the thrombin activation site of FXIII and the second with the inactivating cleavage of the same enzyme. However the second site also aligns with the cleavage site of TG3 by an unknown enzyme to generate the active form (Kim et al., 1995a). Studies with antibodies distinguishing the N terminus of the two TG1 fragments suggested cleavage first occurs between residues 573-574 and then between residues 93-94. It has also been suggested separate enzymes may be involved or that this may be the result of differential control of a single protease (Iizuka et al., 2003). A smaller pool of soluble TG1 has also been studied identifying both the full length enzyme and cleavage products. The ratio of cleavage products compared with full length enzyme increased in keratinocytes committed to differentiation, suggesting this process of activation is regulated by differentiation signals (Kim et al., 1995a, b, Steinert et al., 1996a, b). Complexes between the 67 kDa with either the 33 kDa fragment or full length enzyme have been obtained by co-elution from Mono Q fast protein liquid chromatography (FPLC) or co-immunoprecipitation followed by calculating the specific activities of each component. The full length enzyme is active; however when the 67 kDa and 33 kDa fragments are associated, an increase from 5 to tenfold activity is seen. The 67 kDa fragment can also be negatively regulated by binding of the full length enzyme which reduces enzyme activity (Kim et al., 1995a). The processed forms of TG1 have a significantly shortened half-life of 7 hours in comparison to 20 hours for the full length fragment. The enzyme responsible for cleavage and

activation of TG1 is likely to be cathepsin D as studies using cathepsin D knockout mice revealed TG1 activity within the epidermis was severely reduced when compared with wild type controls (Egberts et al., 2004). Furthermore, this study also indicated the addition of exogenous cathepsin D increases TG1 activity in cultured keratinocytes (Egberts et al., 2004).

Studies carried out by Baumgarter et al., suggested TG1 has a function in controlling the barrier properties of microvasular monolayers via its crosslinking activity within the intercellular junctions of myocardium endothelial cells (Baumgarter et al., 2004). Nevertheless it is the role of TG1 in N-terminal differentiation of squamous epithelia that is best characterised (Simon and Green, 1985; Kim et al., 1995a; 1995b; Steinert et al., 1996a). Furthermore, TG1 is expressed in the differentiated layers of the epidermis and its expression is associated with transglutaminase activation and cornified envelope (CE) formation (Eckert et al., 2009). *In vitro* studies indicate TG1 is capable of crosslinking several proteins expressed during terminal differentiation including involucrin, loricrin and small proline rich (SPR) proteins (Candi et al., 1999; 2001). These proteins are cross linked to form a shell like macromolecule cornified envelope which contributes to an effective barrier against water loss and infection. A recent study also indicated that an additional protein named tazarotene-induced gene 3 (TIG3) is associated with TG1 activation (Candi et al., 1999). In addition to its role in CE formation TG1 appears to have a unique function within the TG family. Within an *in vitro* vesicle system TG1 has been demonstrated to catalyse formation of ester linkages between a ω-hydroxyceramide analogue and a number of CE precursors including involucrin

(Figure 4) (Nemes et al., 1999). Similar linkages involving involucrin have also been isolated from *ex vivo* samples as well as envoplankin and periplankin (envelope precursors) (Marekov and Steinert 1998). In conjunction with CE formation the sequestering of covalently bound lipids (CBLs) to the surface of differentiating keratinocytes is an important step in the transition of cells into a hydrophobic environment. TG1 ablation produces a lethal phenotype in mice due to their defective skin barrier formation, resulting in death within hours of birth, indicating the key role of TG1 in skin homeostasis (Matsuki et al., 1998).

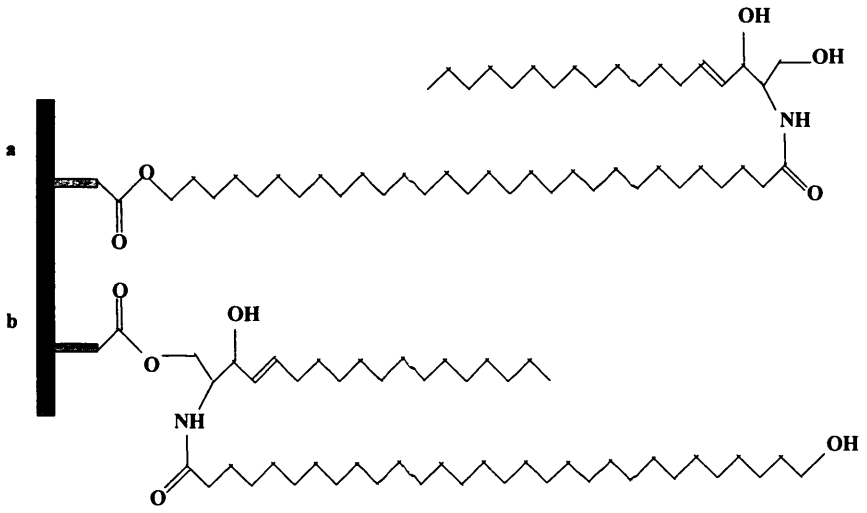

Figure 4. Schematic representation of lipids covalently bound to the envelope precursor involucrin: It was determined that a number of lipids within the stratum corneum are attached to (CE) via ester linkages. Primarily these cross-links occur with involucrin molecules (grey rectangle) and the reaction is thought to be catalysed by TG1. Two examples shown here include (a) a ω-hydroxyl linkage (b) a sphingosine-1-hydroxyl ester linkage (Swartzendruber et al., 1987)

1.2.4 TG2.

TG2 has been identified and cloned from a number of mammals including humans, mice, guinea pigs and cows. TG2 is approximately 80 kDa and contains between 685 and 691 amino acids (Ikura et al., 1988; Gentile et al., 1991). Some conservation was seen between species (65-88% when compared with the human sequence) (Aeschlimann and Paulsson, 1994). TG2 is expressed as an active enzyme that is predominantly localised to the cytosol (80%), but is also localised in the nucleus (5%) or membrane associated (15%). The latter is likely mediated by fatty acid linkages (Harsfalvi et al., 1987). There is no evidence for glycosylation or inclusion of disulfide bonds (Ikura et al., 1988) and the N terminus of this enzyme can be blocked by the removal of the initiator Met and the acylation of the flanking Ala residue. Furthermore, TG2 can be externalised, localising at the cell surface or in association with ECM components in a number of tissues (Aeschlimann and Paulsson; 1991; Barsigian et al., 1991; Martinez et al., 1994; Aeschlimann et al., 1995; Gaudry et al., 1999). As with FXIII, a hydrophobic leader sequence is absent and it has been suggested that the N^{α}-Acetyl group may target the enzyme to an alternative secretory pathway (Muesch et al., 1990). Alternative methods of secretion of this enzyme include specialised pores within the plasma membrane or passive diffusion following transient stress-induced membrane ruptures (Kuchler and Thorner, 1990; Steinhardt et al., 1994; Elliot and O'Hare, 1997). It has been hypothesised that the presence of cis peptide bonds conserved within the TG family may be essential for the secretion of TG2 into the ECM (Balklava et al., 2002). TG2 mutations of the active Cys residue ($Cys^{277}Ser$) or targeting the proposed cis bond ($Tyr^{274}Ala$) have demonstrated that only the active form is retrieved in culture medium or detected

in the ECM, and both mutant forms are able to localise to the plasma membrane (Balklava et al., 2002). This indicates that transamidation activity and/or the tertiary conformation of the active site is required for complete secretion.

TG2 is ubiquitously and constitutively expressed at high levels in both endothelial and smooth muscle cells (Thomazy and Fesus, 1989). It is regulated at the translational level and/or at the rate of protein turnover indicated by discrepancies between relative mRNA (high levels in lung, heart, kidney and blood vessels) and protein quantities (higher in liver and spleen) (Clarke et al., 1959; Aeschlimann and Paulsson, 1994). Two alternative spliced variants of TG2 have been identified within exons VI and X (Fraij and Gonzales, 1997). Studies of the neurodegenerative condition progressive supranuclear palsy have detected significantly raised levels of mRNA encoding a short form of TG2 implicating a functional role for this splice variant (Zemaitaitis et al., 2002). This shortened form lacks the nucleotide binding cleft and in the absence of negative regulation, demonstrates high levels of crosslinking activity. TG2 was the first TG family member identified capable of NTP cycling, specifically ATP and GTP (Achyuthan and Greenberg, 1987). The inactive GDP bound form has been analysed by X ray crystallography (Liu et al., 2002; Pinkas et al., 2007). Protein dynamics simulation indicates that binding of this divalent ion produces major conformational changes moving apart domains 2 and 3 between which the active site is situated (Casadio et al., 1999). Despite being the first member of the TG family to be identified (Clark et al., 1959) its physiological function is still not completely understood. This is a consequence of its ubiquitous expression and the necessary delinearisation of its opposing activities as both a transamidation

enzyme and a G protein. TG2 has been implicated in signal transduction (Nakaoka et al., 1994), cell adhesion, spreading and differentiation (Gentile et al., 1992; Aeschlimann et al., 1993; Jones et al., 1997 Stephens et al., 2004) wound healing (Bowness et al., 1988; Haroon et al., 1999) and apoptosis although conflicting reports on the role of TG2 in this process exist since cytosolic transamidation activity can be pro-apoptotic corresponding to raised Ca^{2+} levels in late stage apoptosis whereas nuclear GTP cycling proves to be anti-apoptotic (Jeong et al., 2009)

1.2.4.1 Intracellular TG2 Function.

1.Cytosol.

TG2 is thought to be capable of targeting a number of cellular proteins, as loss of its Ca^{2+} regulation in 3T3 fibroblasts results in large insoluble protein shells analogous to those formed in keratinocyte differentiation (Nicholas et al., 2003). These structures may play a role *in vivo* by stabilising cells prior to clearance by phagocytosis, and limiting harmful release of cellular components into the surrounding tissue. The absence of this activity has led to reports of inflammatory and autoimmune responses (Pireda et al., 1997). Experiments involving over-expressed TG2 in fibroblasts and neuroblastoma cells have indicated an increase in both spontaneous and induced apoptosis (Gentile et al., 1992; Melino et al., 1994; Piredda et al., 1997). Conversely antisense silencing of TG2 in neuroblastoma and human pro-monocytes resulted in a decreased susceptibility to retinoic acid induced apoptosis (Oliverio et al., 1999).

Further to its function in apoptosis, TG2 has been implicated in cell adhesion, spreading and migration. TG2 has been implicated in vimentin recruitment to

stress fibres via retinoic acid induced transamidation of RhoA. This occurs via ROCK-2 and is accompanied by increased cell adhesion (Singh et al., 2001). Stephens et al., compared fibroblasts stably transfected with antisense, over-expressed and a crosslinking deficient mutant TG2 revealing normal cell attachment in TG2 deficient cells although spreading of cells was delayed (Stephens et al., 2004). TG2 deficient fibroblasts also displayed defects in motility which was not attributed to its crosslinking function. Blocking antibody experiments failed to induce similar defects in the wild type fibroblast indicating the involvement of intracellular TG2 (Stephens et al., 2004). Further experiments revealed these TG2 deficient cells had defective- focal adhesion turnover and stress fibre formation, accompanied by alterations in phosphorylation of focal adhesion kinase (FAK) and failure to activate protein kinase C α, a key enzyme involved in cell spreading (Stephens et al., 2004).

2. Membrane.

As a membrane associated G protein, TG2 couples α_{1b}- and α_{1d}- adrenoreceptors, and thromboxane and oxytocin receptors to phospholipase C, thus mediating inositol phosphate production in response to agonist activation (Nakaoka et al., 1994; Feng et al., 1996).

3. Nucleus.

Peng et al., suggested the nuclear pool of TG2 may be transported with the help of importin-α3 (Peng et al., 1999). The ability of TG2 to cross link histones (Ballestar et al., 1996; 2001), retinoblastoma (Oliverio et al., 1997) and Sp1 proteins has also led to the hypothesis that this enzyme may have a direct role in chromatin modifications and/or gene expression regulation.

4. Extracellular TG2.

It has been shown that TG2 may be involved in cell adhesion (Stephens et al., 2004). Fibroblasts over-expressing TG2 have been reported to demonstrate a decreased susceptibility to trypsin treatment (Gentile et al., 1992; Verderio et al., 1998). Furthermore, the use of antisense silencing techniques in endothelial cells resulted in a reduction in adhesion and spreading (Jones et al., 1997). It was first thought that the transamidating activity of TG2 and its remodelling of the pericellular matrix may be responsible for this function (Jones et al., 1997). Studies have demonstrated TG2 is capable of binding fibronectin with high affinity, with the recognition sites for this glycoprotein residing within the N-terminus (Jeong et al., 1995). TG2 co-localises with pericellular fibronectin (FN) whilst truncated TG2 lacking the fibronectin binding site is not sequestered to this region (Gaudry et al., 1999). More recent studies have discovered the existence of cell surface TG2/β integrin co-receptors for fibronectin (Figure 5). The predominant complex forms with α5β1 integrin, but despite TG2 functioning within this complex it cannot substitute for the action of these integrins in fibronectin assembly (Akimov and Belkin, 2001). The TG2/β integrin receptors are calculated to be a 1:1 ratio and dependent on cell type; up to 40% of β1 integrins may complex in this way (Akimov et al., 2000). Further, these co-receptors have been found to facilitate cell adhesion and spreading (Isobe et al., 1999) and motility (Balklava et al., 2002). Much of this work has been carried out on fibronectin and these co-receptors may play an important role on this substratum. This could explain disparities with studies established on alternative ECM components or tissue grade plastic (Stephens et al., 2004). Despite the ability of TG2 to cross link fibronectin (Barsigian et al., 1991; Martinez et al.,

1994; Jones et al., 1997) these functions are independent of transamidation activity (Akimov et al., 2000; Balklava et al., 2002) indicating a structural role for TG2. *In vitro* studies carried out using transgenic mice also indicated a role for cell surface TG2 in fibroblast migration (De Laurenzi et al., 2001; Verderio et al., 2005). Fibroblasts isolated from $TG2^{-/-}$ mice were found to repopulate wounds at a slower rate than their wild type controls. However this could be partly counteracted by the addition of exogenous purified guinea pig liver TG2. Furthermore this was found to improve the stability of the cell sheets and shifted the pattern of healing toward the control phenotype (De Laurenzi and Melino, 2001).

Although original sequence data suggested TG2 was a cytosolic protein, significant quantities of TG2 have been detected associated with ECM of certain tissues. TG2 found here has been implicated in wound healing, angiogenesis, remodelling and stabilisation (Upchaurch et al., 1991; Haroon et al., 1999; Aeschlimann and Thomazy, 2000). TG2 and its role in wound healing will be discussed in detail in section 1.4.5 however its ability to remodel ECM tissue has been attributed to its crosslinking activity. This has been demonstrated *in vitro* by its ability to contract floating collagen lattices where crosslinking deficient forms of the enzyme produced by a substitution of Cys^{277} to Ser residue demonstrates a reduced rate of contraction accompanied by reduced levels of MT1-MMP and active MMP2 (Stephens et al., 2004). TG2 substrates within the ECM vary; fibronectin (Jones et al., 1997), vitronectin (Sane et al., 1988), collagen (Kleman et al., 1995), osteonectin (Aeschlimann et al., 1995), osteopontin (Kaartinen et al., 1997) and nidogen (Aeschlimann and Paulsson 1991) have all been found to

be TG2 substrates. Furthermore TG2 has demonstrated a high affinity for several basement membrane components leading to its proposed stabilisation of the dermo-epidermal junction (DEJ). TG2 may also affect matrix deposition indirectly; the secreted enzyme is found to impact on the activation of TGF-β possibly through covalent modification of activating factors (Kojima et al., 1993; Nunes et al., 1997). This produces a positive feedback mechanism for TG2 expression and that of several other ECM genes (Ritter et al., 1998; Akimov and Belkin, 2001)

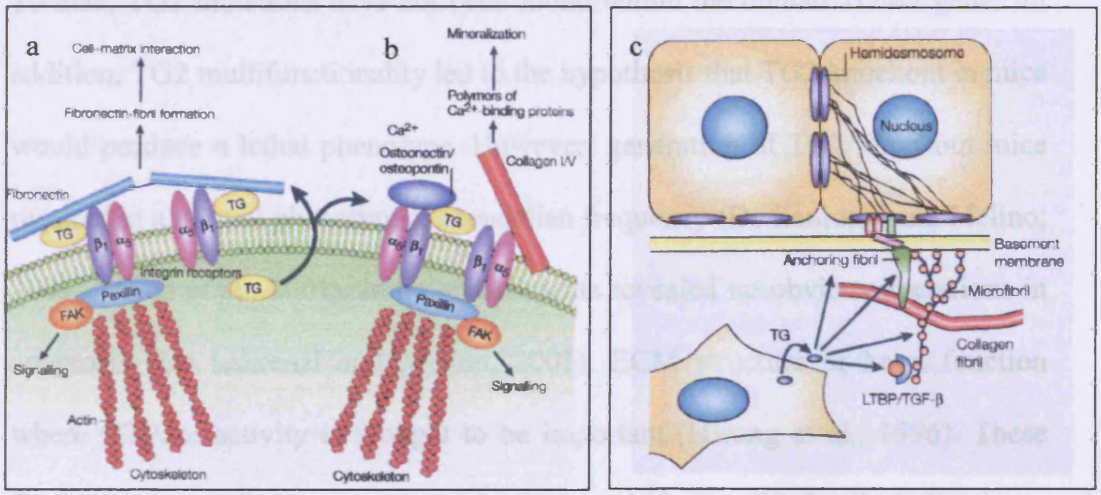

Figure 5. Schematic representation of the role of extracellular TG2: Transglutaminase 2 (TG2) acts as an integrin co-receptor and binds fibronectin with a high affinity, thereby aiding the organisation of the extracellular matrix (ECM). Through interactions with adhesion components such as paxillin and FAK, the α5β1 integrin receptor can influence intracellular signalling and the cytoskeleton. Unlike the more reversible processes of cell-matrix interactions (a) that are dependent only on non-covalent associations with TG2, irreversible mineralisation (b) requires the covalent cross-linking of connective-tissue substrates (osteonectin/osteopontin and collagens) by the Ca^{2+}-activated enzyme (Figure courtesy of D. Aeschlimann, University of Wales, Cardiff, UK). (c) TG2 also stabilises the dermo-epidermal junction. This enzyme catalyses the covalent attachment of TGFβ, through its latent TGFβ protein (LTBP) subunit, to the microfibrils. As such, large stores of this growth factor can accumulate in the connective tissue from where it can be liberated by the action of proteases. Hemidesmosome-mediated attachment of cells to the basement membrane and the underlying connective tissue proteins (anchoring fibrils, microfibrils and collagen fibrils) – all of which are substrates for TG2 – are shown. (Lorand and Graham, 2003).

1.2.4.2 Murine $TG2^{-/-}$ model.

To date, TG2 mutations have not been found within the human TGM2 gene. In addition, TG2 multifunctionality led to the hypothesis that TG2 knockout in mice would produce a lethal phenotype. However, generation of TG2 knockout mice resulted in a normal phenotype at mendelian frequency (De Laurenzi and Melino, 2001; Nanda et al., 2001). Initial experiments revealed no obvious alterations in apoptosis (De Laurenzi and Melino, 2001), ECM structure or heart function where GTPase activity is thought to be important (Hwang et al., 1996). These findings suggested there may be redundancy within the TG family indicating a compensatory mechanism, however only FXIII is localised to the ECM and this is not capable of GTP cycling. Further investigation of these mice identified a decrease in primary fibroblast adhesion (Nanda et al., 2001) and impaired wound healing (Mearns et al., 2002). Furthermore, following dexamethasone induction of apoptosis, phagocytic clearance by macrophages is defective within the thymus and the liver (Nanda et al., 2001). This concurs with previous studies indicating TG2 crosslinking is important in stabilising apoptotic cells prior to clearance (Piredda et al., 1997). Finally knockout mice have also demonstrated glucose intolerance as a result of reduced insulin secretion that correlates strongly with maturity onset diabetes in humans (Bernassola et al., 2002).

1.2.5 TG3

TG3 is a virtually inactive zymogen of 77 kDa and 692 amino acids that localises to the cell cytosol (Kim et al., 1993; Hitomo et al., 2003). Activation of the enzyme is a result of cleavage at Ser^{469} to produce a 50 and 27 kDa fragment (Kim et al., 1990; 1993). The 50 kDa cleavage products which consists of the N-terminal sandwich and catalytic domains is capable of catalysing transamidation reactions (Chung and Folk, 1972; Ogawa and Goldsmith, 1976) however when it is complexed with the 27 kDa C-terminal β barrel domain this activity is increased and it is thought that the two fragments stay associated (Kim et al., 1990). The enzyme responsible for this cleavage *in vivo* has not yet been identified but *in vitro* studies have utilised the bacterial protease dispase to produce correlating cleavage products (Kim et al., 1993). The cleavage site is a unique sequence of 12 polar amino acid residues residing in the flexible loop connecting the catalytic and β barrel domain 1 (Kim et al., 1993) and correlates strongly with cleavage sites found in TG1 and FXIII (Thibaut et al., 2009). Conservation of TG3 between species ranges from 50-75% indicating this enzyme is still undergoing rapid evolution (Kim et al., 1993; Aeschlimann and Paulsson 1994). The protease cleavage site has particularly low homology and may be evident of species evolving alternate activation mechanisms (Kim et al., 1993). Resolution of several TG3 crystallographic structures has indicated the conformational changes which accompany activation. A total of three Ca^{2+} binding sites have been identified within the enzyme (Ahvazi et al., 2002). The first (Asn^{224}-Asn^{229}) demonstrates constitutive binding (Kd 0.3μM) and has a role in enzyme stabilisation, the second (Asn^{430}-Asn^{448}) and third (Asn^{393}-, Glu^{443} and Glu^{448}) Ca^{2+} binding sites occur after zymogen cleavage and cooperate to

produce a movement of the β-strand (Gly^{322}-Ser^{325}). This conformational change opens a channel through the enzyme and exposes two (Trp) residues near to the active site (Figure 6). This is thought to stabilise the transition state of the enzyme. The conformational change also makes Asp^{324} accessible for coordination to the Ca^{2+} ion at site 3. Ca^{2+} ions at sites 2 and 3 can be substituted for lanthamides and for site 3 Mg^{2+}. Despite this, Ca^{2+} binding at site 3 is required to activate the enzyme. Therefore it has been suggested that the Mg^{2+} binding of the cleaved zymogen provides a mechanism to stop aberrant crosslinking activity in the absence of increased Ca^{2+} concentration (Ahvazi et al., 2003). Biochemical and crystallographic evidence has been shown to indicate that TG3 can undergo GTPase cycling; however unlike TG2 this enzyme does not target ATP (Ahvazi et al., 2004, Thibaut et al., 2009). In order for GTP/GDP binding to occur there must be a substitution of Ca^{2+} ion at site 3 for Mg^{2+} accompanied by regional conformational changes producing movement of the ^{320}DKGSDS^{325} sequence motif. This indicates therefore this structural alteration targets the same sites involved in the activation of the enzyme and indicates the negative regulatory role of nucleotides.

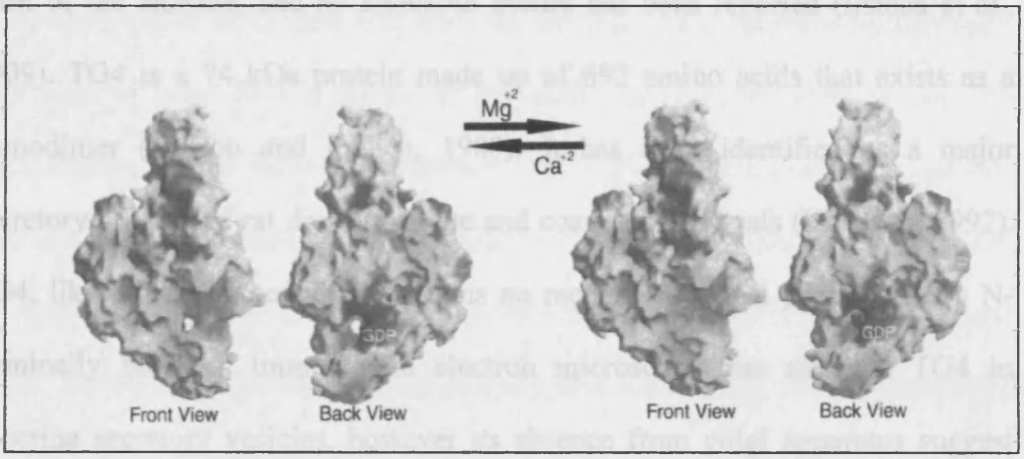

Figure. 6 The electrostatic surface potential comparison of the TG3-GDP complex when bound to Ca^{2+} or Mg^{2+} at site 3: The front and back view represent images rotated 180° with respect to each other. The acid and basic regions are coloured red and blue respectively. An open channel is clearly evident in the Ca^{2+} bound form and is lost following Mg^{2+} substitution (Ahvazi et al., 2004).

1.2.6 TG4

Currently, the most extensive studies carried out with TG4 have included the rat form of the enzyme, and no knockout mouse has been reported (Iismaa et al., 2009). TG4 is a 74 kDa protein made up of 692 amino acids that exists as a homodimer (Wilson and French, 1980). It has been identified as a major secretory product of rat dorsal prostate and coagulating glands (Ho et al., 1992). TG4, like other secreted TGs, contains no recognised signal peptide and is N-terminally blocked. Immunogold electron microscopy has detected TG4 in apocrine secretory vesicles, however its absence from golgi apparatus suggest direct entry from the cytoplasm (Seitz et al., 1990; 1991). TG4 has been shown to be mannosyl linked and has a phophotidyl anchor post modification (Seitz et al., 1991) however its best characterised function is its rapid catalysis of polyamines to produce seminal plugs (Williams-Ashmann 1984).

1.2.7 TG5

TG5 has been shown to be ubiquitously expressed in low levels in human tissues with the exception of both the lymphatic and central nervous system (Aeschlimann et al., 1998; Candi et al., 2004). TG5 is an 81 kDa protein made up of 720 amino acids, is expressed in its active form and has been shown to be N-terminal acetylated (Rufini et al., 2004). TG5 mRNA isolated from human keratinocytes indicates 3 alternative splice products in addition to the full length enzyme, in which exons III, XI or III or XI are absent. *In vitro* studies have determined that exon III is required for enzyme activity (Candi et al., 2001). Splice products lacking exon XI produce a frame shift mutation resulting in a novel sequence of 25 amino acids followed by premature termination (Candi et

al., 2001). Due to its slow expression several TG5 studies have utilised keratinocytes that over-express the protein; such studies have shown this enzyme to be resistant to extraction and retrieval has only been possible by treatment with SDS or urea. This is similar to the profile of insoluble proteins and cell fractionation studies have demonstrated that TG5 associates with the nuclear matrix and cytoskeleton. Like TG2, TG5 is capable of binding ATP and GTP, which has been demonstrated to inhibit the enzymes crosslinking ability (Candi et al., 2004) although its GTPase activity and subsequent affect on cellular function is not yet known. TG5 expression is increased several fold in induced differentiating cultured keratinocytes, leading to its investigation in CE formation and consequent binding to CE components such as involucrin and loricrin (Candi et al., 1995).

1.2.8 TG6.

TG6 has remained elusive for study due to its insolubility, despite extreme measures. Consequently, little data has been published on TG6 except that concerning its role in gluten sensitivity in celiac disease (Hadjivassiliou et al., 2010). TG6 is calculated to be an 80 kDa protein consisting of 708 amino acids. TG6 has been shown to undergo alternative splicing of exon XII, the absence of which produces a frame shift and premature termination within exon XIII. TG6 was first amplified from the small lung carcinoma cell line H69 (H Thomas PhD thesis, 2004) and subsequent northern blot analysis identified widespread expression at low levels in human tissues. High expression levels have been determined in gluten sensitivity sufferers in brain tissue (Hadjivassiliou et al.,

2010) and in addition, murine in situ data suggests that this isoform is also localised to the epidermis (Hadjivassiliou et al., 2010).

1.2.9 TG7

TG7 is the most recently discovered member of the TG family. It was first isolated from a prostate carcinoma cell line, and is an 80 kDa 710 amino acid protein. Northern blot analysis has indicated this TG7 is expressed at low levels in many human tissues with the highest concentrations found in testis and lung tissue (Grenard et al., 2001). TG7 has been amplified from a number of cell lines by RT-PCR including dermal fibroblasts (TJ6F and HCA2), primary keratinocytes and mammary epithelium (Grenard et al., 2001). Although physiological functional data of this enzyme is limited and there is no knock out mouse currently reported, data concerning neurodegeneration has linked mutation in this enzyme with activated microglia leading to severe brain vacuolation and neurodegeneration (Kercher et al., 2007).

Metalloproteinases are cited in literature as being responsibly for extensive changes in tissues caused by proteolytic activity and remodelling. Therefore the following section details the metalloproteinase family and their potential role in mediating Transglutaminase affects on re-epithelisation

1.3 Metalloproteinases General introduction.

Proteinases are implicated in many processes in tissue remodelling; cell motility, morphogenesis and cell and organ growth and development, through the degradation and processing of matrix components. This mechanism allows tissue and organ turnover through removal and replacement of cellular or matrix components (Fowlkes and Winkler, 2002).

The Metzincin superfamily is a diverse and expansive group of zinc peptidases, from both eukaryote and prokaryote families. Of the four sub-families the Astacins (crayfish collagenolytic enzyme and bone morphogenic protein 1 (BMP-1), Serralysins (bacterial proteinases), Adamalysins, (snake venom proteinases and a disintegrin- and metalloproteinase (ADAMS) and Matrix Metalloproteinases (MMPs) (Stocker and Bode, 1995) only the metalloproteinases, MMPs and ADAMS, will be discussed in this review due to the volume of research available on these four subfamilies and the relevance to this study.

The metalloproteinase family share several characteristics. They display a high level of sequence homology and all but the membrane type (MT)-MMPs are secreted from cells in a non active state known as a zymogen and termed a pro-MMP. They are activated by the removal of the propeptide sequence and they can be secreted as soluble proteinases or inserted into the cell membrane (MT-MMPs). Their activity is highly dependent on both zinc and Ca^{2+} ions, and once activated, MP activity is controlled by the tissue inhibitor of metalloproteinases (TIMPs) of which there are four family members (Nagase et al., 1999). Since

metalloproteinase activity is highly dependent on both zinc and Ca^{2+} ions, they can also be readily inhibited chemically, by chelating agents such as EDTA.

1.3.1 Structure of Metalloproteinase Family.

Classification of proteases is generally based on whether they cleave terminal or internal sites within peptides or proteins and are therefore referred to as exopeptidases or endopeptideases respectively. Further classification involves examining their catalytic mechanism and/or their specific inhibitor sensitivities. They can then be further divided into serine, cysteine, aspartic or metalloproteinases (Stocker and Bode, 1995). A typical metalloproteinase consists of a propeptide of around 80 amino acids, a catalytic metalloproteinase domain of around 170 amino acids, a linker peptide of variable length (also referred to as the hinge region) and a hemopexin (Hpx) domain of around 200 amino acids. The zinc binding motif HEXXHXXGXXH in the catalytic domain and the cysteine switch motif PRCGXPD in the propeptide are common structural signatures where the three histidines in the zinc binding motif, and the cysteine in the propeptide co-ordinate with the catalytic zinc ion (Bode et al., 1993). This Cys-Zn^{2+} coordination inhibit metalloproteinase activity by preventing a water molecule essential for catalysis from binding to the zinc ion. The catalytic domain also contains a conserved methionine which forms a Met turn eight residues from the zinc binding motif, providing a base to support the structure surrounding the catalytic zinc (Bode et al., 1993). The zinc binding motif and the Met turn are also conserved in members of the ADAMs family, ADAMTS sub family, the Astacins and the Serralysins and while these

subfamilies have little homology in their primary structures, the overall protein folds are similar (Gormis-Ruth, 2003).

1.3.2 Metalloproteinase Family and Domain Structure.

In 1962, Gross and Lapiere first reported vertebrate collagenolytic activity in tadpole tissues (tailfin, skin, intestine and gill) undergoing metamorphosis (Gross and Lapiere, 1962), prompting the investigation of remodelling in further tissues, as the deposition and remodelling of collagen is important in tissue turnover, structure and function. The first human collagenase to be purified was from rheumatoid synovium (Woolley et al., 1975) which has similar properties to tadpole collagenase, cleaving triple helical type I collagen at a single site about three quarters away from the N-terminus (Woolley et al., 1975). It took another 11 years to deduce the primary structure of human collagenase (MMP-1) from fibroblasts by cDNA cloning (Goldberg et al., 1986).

To date, the MMPs now comprise a family of over 20 members which are all highly homologous zinc dependent matrix degrading proteinases. By comparison of the domains and their preferred substrates, metalloproteinases are grouped into collagenases, gelatinases, stromelysins, matrilysins, membrane-type (MT)-MMPs and others. Figure 7 shows the variations in metalloproteinase domain arrangements and Table 2 describes the composition of each member.

The collagenases are active against fibrillar forms of collagen. The collagenases are able to cleave interstitial collagens I, II and III into characteristic ¾ and ¼ fragments but they are also able to digest other ECM molecules and soluble

fragments (Woessneer and Nagase, 2000; Visse and Nagase, 2003). Although MMP2 and MMP14 (MT1-MMP) also have collagenolytic abilities, these are grouped differently due to their domain composition. The gelatinases have high activity against denatured collagens (gelatin), and bind to gelatin and collagen via their 3 fibronectin type II repeats. They are also able to digest collagens IV, V and XI, laminin, aggrecan core protein amongst others (Aimes and Quigley, 1995). MMP 2 is also able to digest collagen I, II and III similarly to the collagenases (Aimes and Quigley, 2005). The stromelysins exhibit activity against a wide range of non collagen components of the ECM; they have a domain arrangement similar to the collagenases but they are able to digest interstitial collagens as well as digestion of some ECM molecules and participation in proMMP activity (Murphy et al., 1993). The Matrilysins lack a hemopexin domain. As well as processing ECM components, MMP 7, a member of this family, is able to process cell surface molecules such as pro-α- defensin and Fas-Ligand (Nagase et al., 2006).

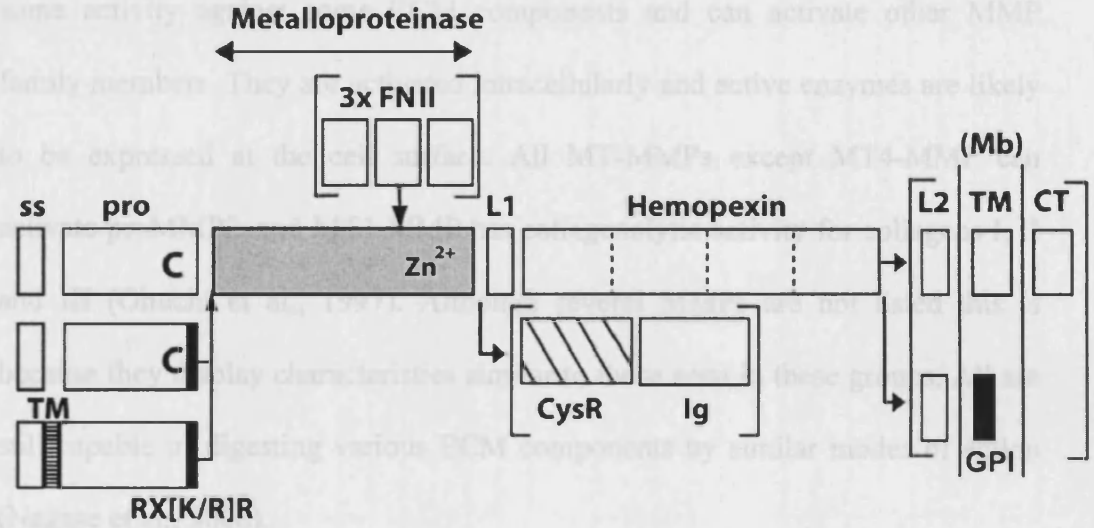

Figure 7. **Domain structures of the metalloproteinase family. ss, signal sequence; pro, pro-domain, FNII, fibronectin type II motif; L1, linker 1; L2, linker 2; Mb, plasma membrane; TM, transmembrane domain; CT, cytoplamic tail; CysR, cysteine rich; Ig, immunoglobulin domain; GPI, glycosylphosphatidylinositol anchor; C, cysteine. Taken from Murphy and Nagase. 2008.**

The MT-MMPs all contain a furin recognition sequence RX[r/k]R at the C terminus of the propeptide. MT-MMPs are transmembrane MMPs that have some activity against some ECM components and can activate other MMP family members. They are activated intracellularly and active enzymes are likely to be expressed at the cell surface. All MT-MMPs except MT4-MMP can activate proMMP2, and MT1-MMP has collagenolytic activity for collagens I, II and III (Ohuchi et al., 1997). Although several MMPs are not listed this is because they display characteristics similar to those seen in these groups. All are still capable of digesting various ECM components by similar modes of action (Nagase et al., 2006).

Enzyme	MMP	Chromosome location(Human)	SS	Pro	CS	RX[R/K]R	Cat	FN2	LK	Hpx	Lk2	TM	GPI	Cyt	CysR-Ig
Collagenases															
Interstitial collagenase; Collagenase 1	MMP-1	11q22-q23	*	*	*		*		*	*					
Neutrophil collagenase; Collagnease 2	MMP-8	11q21-q22	*	*	*		*		*	*					
Collagenase 3	MMP-13	11q22.3	*	*	*		*		*	*					
Collagenase 4 (Xenopus)	MMP-18	Not in humans	*	*	*		*	*	*	*					
Gelatinases															
Gelatinase A	MMP-2	16q13	*	*	*		*	*	*	*					
Gelatinase B	MMP-9	20q11.2-q13.1	*	*	*		*	*	*	*					
Stromelysins															
Stromelysin 1	MMP-3	11q23	*	*	*		*		*	*					
Stromelysin 2	MMP-10	11q22.3-q23	*	*	*		*		*	*					
Matrilysins															
Matrilysin 1	MMP-7	11q21-q22	*	*	*		*								
Matrilysin 2	MMP-26	11p15	*	*	*		*								
Stromelysin 3	MMP-11	22q11.2	(*)	(*)	*	*	*		*	*					
Membrane Type MMP Transmembrane type															
MT1-MMP	MMP-14	14q11-q12	*	*	*	*	*		*	*	*	*		*	
MT2-MMP	MMP-15	15q13-q21	*	*	*	*	*		*	*	*	*		*	
MT3-MMP	MMP-16	8q21	*	*	*	*	*		*	*	*	*		*	
MT5-MMP	MMP-24	20q11.2	*	*	*	*	*		*	*	*	*		*	
(B) GPI-anchored															
MT4-MMP	MMP-17	12q24.3	*	*	*	*	*		*	*	*		*		
MT6-MMP	MMP-25	16p13.3	*	*	*	*	*		*	*	*		*		
Others															
Macrophage elastase	MMP-12	11q22.2-q22.3	*	*	*		*		*	*					
-	MMP-19	12q14	*	*	*		*		*	*					
Enamelysin	MMP-20	11q22.3	*	*	*		*		*	*					
-	MMP-21		*	*	*	*	*		*	*					
CA-MMP	MMP-23	1p36.3	*	*		*	*								*
-	MMP-27	11q24	*	*	*		*		*	*					
Epilysin	MMP-28	17q21.1	*	*	*	*	*		*	*					

Table 2: Metalloproteinases listed with their alternative names, location and alternative splicing arrangements.

1.3.3 Control of Metalloproteinases.

MP activity is tightly regulated at 3 levels; they are expressed at low levels, by transcription control either positively or negatively controlled by cytokines and growth factors, and post transcriptional control where their activity can be restricted. A number of inflammatory cytokines [interleukin-1-β (IL-1β), IL-6], growth factors [platelet derived growth factor (PDGF)], hormones (corticosteroids) and tumour promoters [tumour necrosis factor-α (TNFα)], control the gene expression and the secretion of MMPs (Dollery et al., 1995; Thompson and Parks, 1996).

Inhibition of proteolytic activity of metalloproteinases occurs by endogenous inhibitors of matrix metalloproteinases (TIMPS). The tissue inhibitors of matrix metalloproteinases (TIMPs) are the dominant inhibitors of MMPs (detailed in section 1.3.4), they are a family of 4 proteinase inhibitors which bind to MMPs rendering them inactive while α_2-macroglobulin and tissue-factor pathway inhibitor-2 inhibit MMPs to a lesser extent. TIMPs bind either active or latent forms of MMPs in a molecular 1:1 ratio (Visse and Nagase, 2003). The balance of relative concentrations of active MMPs and their inhibitors determines the net proteolytic activity (Knox et al., 1997).

MMPs are synthesised as latent pro-enzymes that require activation by disruption of cysteine-zinc interaction of the cysteine switch and the removal of the pro-peptide for full activity (Visse and Nagase, 2003). The signal peptide is removed during translation and the proMMP is generated. Activation of the zymogen (proMMP) is an important regulating step. Thirteen of the MMPs are secreted as

proMMPs. Within the propeptide there is a susceptible bait region which allows tissue and plasma proteinases to activate the proMMP. The removal of the bait region cleaves only a part of the propeptide; complete removal often occurs in trans by the action of a MMP intermediate or by another active MMP. This process has been termed Stepwise Activation (Nagase et al., 1990). Ten more of the proMMPs possess a furin-like proprotein recognition site RX[K/R]R at the end of the propeptide and are activated intracellularly before secretion or association with the cell surface. The activity of these MMPs is regulated by their tissue position and inactivation by either endogenous inhibitors such as a TIMP or by proteolysis (Nagase et al., 2006) MT1-MMP is unique in that it becomes rapidly endocytosed and partially recycled to the cell surface (Stawowy et al., 2005). Furthermore, MMPs can be readily activated by treatment with mercurial compounds, Sh reagents and chaotropic agents, thought to be due to perturbation of the molecule. Alternatively oxidants such as HOC1 and ONOO- can activate proMMPs by reacting with the cysteine (cys) of the cys switch in the propeptide; this mode of activation may therefore occur under inflammatory conditions (Peppin and Weiss 1986; Gu et al., 2002).

1.3.4 Endogenous Regulation of MMPs (including TIMPS).

MMPs are regulated endogenously in two ways; firstly they can be regulated by α2-macroglobulin; a plasma glycoprotein of 725 kDa containing four identical subunits of 180 kDa each. α2-macroglobulin inhibits proteinases by entrapping the proteinase within the macroglobulin and the complex is rapidly cleared by a low density lipoprotein receptor related protein 1 which endocytoses the complex (Strickland et al., 1990). This process usually occurs for MMPs which are active

in the fluid phase. Secondly, TIMPs can also inactivate MMPs. TIMPs are proteinase inhibitors usually consisting of 184-194 amino acids and are subdivided into an N-terminal and a C terminal subdomain. Each domain contains three conserved disulphide bonds and the N-terminal domain folds as an independent unit with MMP inhibitory activity. TIMPS have been shown to inhibit all MMPs thus far, however, some are more effective against specific MMPs than others. For example TIMP-1 is a poor inhibitor of MT1, 3 and 5 - MMP, but is effective against the other MMPs and ADAM10 (Nagase et al., 2006). The mechanism of TIMP inhibition of MMPs has been determined based on crystal structures of the TIMP-MMP complex (Gomis-Ruth et al., 1997). The overall shape of the TIMP molecule is 'wedge-like' which allows the N terminus four residues Cys^{1}-Thr-Cys-Val^{4} and the residues Glu^{67}-Ser-Val-Cys^{70} in the case of TIMP1, to be slotted into the active site of the MMP via a disulphide contiguous bridge. This region accounts for 75% of the protein-protein interaction in the case of the complex of the catalytic domain of MMP3 and TIMP1. The catalytic zinc atom is bidentately chelated by the N-terminal amino group and the carbonyl group of Cys1 which expels the water molecule bound to the zinc atom and therefore inactivates the MMP (Nagase et al., 2006). Furthermore a mutation of the position 2 residue (Thr in TIMP-1) can greatly affect the affinity of the TIMP for the MMP. A substitution to glycine in this position inactivates TIMP1 in MMP inhibition (Meng et al., 1999). Further amino acid changes can affect the four TIMPs' affinity to regulate the MMPs and depending on the substitution can render them ineffective as a means of MMP control.

1.3.5 ADAMS Sub Family.

ADAMS (a disintegrin and metalloproteinase) are membrane bound enzymes that make up a further sub family of the metzincin superfamily, and whose function is closely related to that of the snake venom metalloproteinases. Their structure consists of multidomains; proproteinase, metalloproteinase, disintegrin, cysteine-rich, epidermal growth factor–like, and cytoplasmic domains. Mammalian ADAMs are involved in regulation of cell-cell fusion, adhesion, and intracellular signalling as well as in sperm egg binding and fusion (Van Goor et al., 2009). To date, 34 ADAM proteins have been identified in different species and the human family contains 23 members (Zhong et al., 2008). Approximately half have a consensus metalloproteinase catalytic sequence, rendering them proteolytically active; indeed, several ADAM family members have been found to release cytokines, growth factors, receptors, adhesion molecules, and other membrane proteins from the cell surface, a process termed ectodomain shedding. The remainder are likely to primarily have roles in cell adhesion, through their interaction with integrins via the disintegrin/cysteine–rich domains (Zhong et al., 2008).

1.3.6 ADAMS Structure.

An ADAM is a single span transmembrane protein that comprises a pro domain, a zinc metalloproteinase domain which faces extracellularly, a disintegrin domain, a cysteine rich region, an EGF like sequence, a transmembrane region and a cytoplasmic tail (see Figure 8). The N terminus of ADAMs contains a signal sequence

that directs ADAMs into the secretory pathway and a prodomain that functions in maturation. Primarily however the purpose of the prodomain is to maintain the metalloproteinase site of ADAMs in an inactive conformation, through a cysteine switch (Van Wartand Birkedal-Hansen 1990; Becker et al. 1995). A conserved cysteine residue within the prodomain preferentially coordinates the required active site zinc atom, which sequesters the metalloproteinase domain in an inactive conformation. The second main function of the prodomain is to chaperone the proper folding of ADAMs, in particular the metalloproteinase domain. This has been suggested by studies showing that the removal of the prodomain of ADAM17 generates a protease-inactive protein (Milla et al. 1999). The crystallization of several metalloproteinase domains of metzincin family members, including ADAM17, has allowed the mechanism of proteolytic activity to be elucidated (Maskos et al., 1998). Similarly to matrix metalloproteinases, the active site of the ADAM contains zinc and water atoms that are necessary for hydrolytic processing of protein substrates. This is coordinated by three conserved histidine residues and a downstream methionine. The methionine lies in a Met turn motif that loops around to face the consensus HExxHxxGxxH site. This region is highly conserved amongst the various Metzincins. However, individual proteins can be distinguished by other structural features that may impart specificity for substrates and protease inhibitors (Stocker et al., 1995). Not all ADAMS are enzymatically active, as some individual ADAMs lack the characteristic catalytic zinc binding signature.

The disintegrin domain of ADAMs proteins is around 90 amino acids long. Structurally, there is little known about the disintegrin domain of ADAMs,

although a comparison with structural studies of Snake venom metalloproteinases (SVMP) crystals and other integrin receptor ligands may allude to its function (Gomis-Ruth et al., 1994). The disintegrin domains of SVMPs mimic the ligand site of matrix proteins like fibronectin for integrin receptors. Like fibronectin, many have an RGD consensus sequence within a 13 amino acid stretch called the disintegrin loop, which projects from the surface of the protein and confers binding to αIIbβ3 and αvβ3 integrin receptors (Blobel et al., 1992). The cysteine-rich and EGF-like domains have been described as the black box of the ADAM (Seals and Courtneidge, 2003). What is known of these domains does not appear to indicate a functional theme that would characterize these domains in the same manner as the metalloproteinase and disintegrin domains.

ADAMs 1, 3, 12, and 14 have a motif in their cysteine-rich domain that is very similar to sequences found in viral fusion peptides (Blobel and White, 1992). This, coupled with the observations that ADAMs 1, 3 and 12 participate in cell fusion reactions, led to the proposal that the cysteine-rich domain is involved in membrane fusion, although this hypothesis has not been experimentally tested (Seals and Courtneidge, 2003). It is also postulated that the cysteine-rich domain complements the binding capacity of the disintegrin domain, and potentially confers specificity to disintegrin domain-mediated interactions. Furthermore, another theory suggests a cysteine-rich domain-specific function as a ligand for the cell adhesion molecule syndecan (Iba et al., 1999, 2000).

The cytoplasmic tail of the ADAM is variable both in sequence and length. This domain contains specialized motifs that are thought to be involved in the inside-out regulation of metalloproteinase activity, the outside-in regulation of cell signalling, and/or the control of maturation and subcellular localization. The most common motifs are PxxP binding sites for SH3 domain-containing proteins. Several ADAMs also have potential phosphorylation sites for serine-threonine and/or tyrosine kinases. This may regulate ADAM function directly, as well as the resulting phosphotyrosine residues providing ligands for SH2 domain-containing proteins. Consequently, ADAMs may serve adaptor functions to assemble complexes of proteins at critical sites of functional activity (Seals and Courtneidge, 2003). ADAM structure and types of ADAMs are shown in figure 8 and described in table 3.

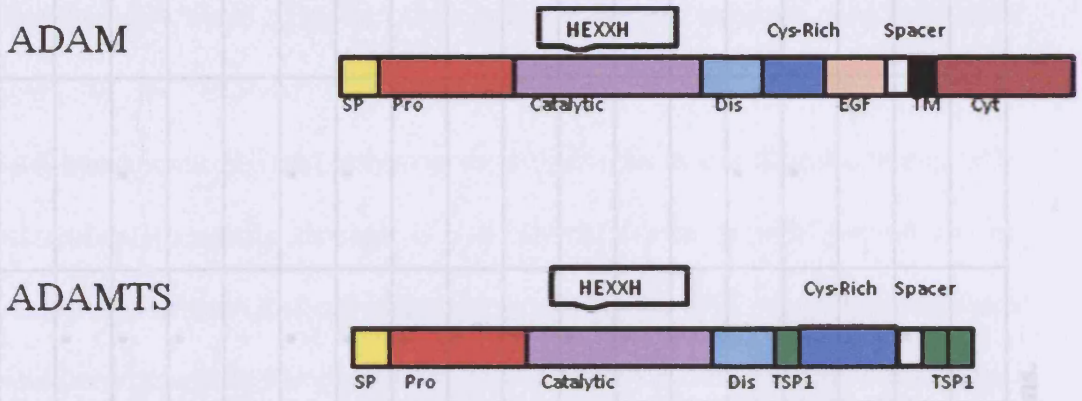

Figure 8. The domain structure of a typical ADAM protein. SP, signal peptide; Pro, Pro-domain, including cysteine switch region; Catalytic, catalytic domain, including the zinc-binding HEXXH consensus sequence; Dis, disintegrin domain; EGF, epidermal growth factor domain; TM, transmembrane domain; Cyt, cytoplasmic domain. In the ADAMTS family, the EGF, transmembrane and cytosolic domains are absent and are replaced by one or more thrombospondin type-1 (TSP1) motifs. The central TSP1 domain is highly conserved among the ADAMTS proteins but the number of C-terminal TSP1 domains can vary from zero (ADAMTS4) to 14 (ADAMTS9 andADAMTS20). Some ADAMTS contain additional C terminal extensions (not shown). The disintegrin-like domain in the ADAMTS proteins lacks the typical arginine–glycine–aspartic acid (RGD) integrin-binding motif of ADAMs proteins and of the snake venom disintegrins (Van Goor et al., 2009).

No.	Common name	Potential functions	Expression	Alternative splicing	MP Active	Integrin Binding	SH3 binding site cytoplasmic tail
2	Fertilin-β, PH-30β	Sperm/egg binding fusion	Testis			*	
7	EAP1		Epididymis				*
8	MS2, CD156		Granulocytes/monocytes		*		
9	Meltrin-γ, MDC9	Sheddase, Cell migration	Somatic	*FLS	*	*	*
10	Kuz, MADM, SUP-17	Sheddase, cell fate determination	Somatic	*L/S	*		*
11	MDC	Putative tumour repressor	Brain	*			
12	Meltrin-α	Sheddase, Myoblast fusion	Somatic	*L/S	*	*	*
15	Metargidin, MDC15	Cell/Cell binding	Somatic		*	*	*
17	TACE	Sheddase	Somatic		*		
18	tMDCIII		Testis		*		
19	Meltrin-β, MADDAM	Sheddase, dendritic cell development	Somatic		*		*
20			Testis		*		
21			Testis		*		
22	MDC2		Brain	*γ,δ,ε			*
23	MDC3	Cell adhesion/Neural development	Brain			*	
28	MDC-L	Immunosurveillance	Epididymis, Lung, Lymphocytes	*M/S	*	*	
29			Testis	*α,β,γ			*
30			Testis	*α,β	*		
33		Genetically linked to asthma	Somatic	*	*		*

Table 3: ADAMS found in humans and their associated position, function and splice variations.

1.3.7 ADAM Family Function.

Unsurprisingly, since ADAMs have a complex domain structure, they have been shown to be involved in several proteolytic driven processes via their metalloproteinase activity, adhesion to integrins via a disintegrin domain, cell-cell fusion potentially through a hydrophobic fusion peptide present in the cysteine rich domain and cell signalling involving the SH3 recognition sequence sometimes present in the cytoplasmic tail (Black et al., 1997, 1998; Killar et al., 1999; Yamamoto et al., 1999; Blobel et al, 2000). Approximately half of the all current known ADAMs have been assigned a function dependent on their catalytic abilities however the catalytically inactive ADAMs are less well understood. It has been suggested they could be involved in as acting as peptide binding receptors to mediate cell signalling (Van Goor et al., 2009). Furthermore the related ADAMTS family also differ from the ADAMs in that they lack the EGF like sequences and transmembrane domains and hence function as secreted proteins. ADAMTS' also retain the metalloproteinase domain as well as the disintegrin like domain however the function of this is not yet understood; potentially the interactions of the disintegrin like domain with integrins seen *in vitro* may yet be deemed physiologically relevant (Van Goor et al., 2009). ADAMS are highly involved in development and therefore have been implicated in spermatogenesis and sperm egg formation as well as neurological development and branching morphogenesis in the lung, kidneys and pancreas (Tousseyn et al., 2006). Furthermore they have also been shown to be ubiquitously expressed in healthy human tissues, although particular ADAMS are restricted to specific organs. Furthermore, their presence appears to prevent diseases such as Alzheimer's and thrombotic thrombocytopenic purpura, both of

which have been linked with defects in ADAMs signalling (Van Goor et al., 2009).

1.3.8 ADAMS as Modulators of the EGF Receptor Signalling.

The epidermal growth factor family (EGF) family have already been mentioned briefly in section 1.3.5 however their regulation by members of the ADAM family warrants further discussion. The EGFR family are type 1 receptor tyrosine kinases that participate in several cellular processes including differentiation, proliferation, migration and cell survival (Holbro et al., 2003). EGFR signalling has been implicated in a wide variety of disease including cancer, inflammation and fibrosis (Melenhorst et al., 2008). The ErbB receptor family is composed of four members; HER1, HER2 (HER2/Neu), HER3 and HER4. All of these are capable of homo or heterodimerizing with one another to form several combinations of functional receptor. Furthermore they are also capable of binding to multiple members of functionally and structurally similar growth factors increasing their activities in diverse cellular function. All EGFR ligands are synthesized as membrane-bound precursors that require metalloproteinase mediated proteolytic cleavage to produce the soluble, active forms. Although paracrine/juxtacrine signalling by transmembrane precursors has been shown to mediate biological effects in some experimental systems (Iwamoto and Mekada, 2000; Miyoshi et al., 1997; Singh et al., 2004; Willmarth and Ethier, 2006), findings from numerous studies strongly suggest that major EGF-like growth factor functions including cell proliferation depend on proteolytic release of soluble EGFR ligands from their membrane-bound precursors (Peschon et al., 1998; Sanderson et al., 2006; Iwamoto et al., 2003).

Most EGFR ligands are shed by ADAMS including Epidermal growth factor - EGF, Transforming growth factor alpha -TGFα, Heparin binding-epidermal growth factor -HB-EGF, amphiregullin AREG, epiregulin EREG, betaceullin BTC, the neuregulins NRG1-4 and epigen EPGN (Harris, 1997, Singh and Harris, 2005,). ADAM17 is the predominant sheddase of EGF ligands as ADAM17 knockout mice display phenotypes similar to both EGF and TGFα knock outs (Sahin et al., 2004). ADAM17 activity can be stimulated externally by G protein coupled receptor activation (GPCRs) to induce shedding of cell surface ligands. When the GPCR is activated signalling takes place via mitogen activated protein kinases and protein kinase C, resulting in ADAM activation and ADAM-mediated shedding of EGFR ligands from the cell membrane, allowing the EGF ligand to bind to the EGFR (Van Goor et al., 2009). This process is called EGFR transactivation. A relatively recent EGFR transactivation process was discovered in the activation of EGFR via angiotensin II activation of the AT1 receptor which promotes vascular remodelling (Ohtsu et al., 2006).

EGFR ligand binding results in conformational changes of the extracellular receptor domains (Jorrisen et al., 2003), initiating signalling mechanisms that regulate multiple cellular responses such as migration, proliferation, differentiation and survival (Citri and Yarden, 2006; Yarden and Sliwkowski, 2001). Downstream of the EGFR a cascade of distinct signal transduction protein becomes phosphorylated upon EGFR activation. These include phosphatidylinositol 3-kinase (P13K), MEK and ERK which regulate gene transcription via transcription factor activation thereby regulating cell growth, proliferation and migration (Holbro et al., 2003). There is substantial evidence

implicating EGF-like growth factor activity in the regulation of cell migration, proliferation, survival and differentiation of normal and malignant epithelial cells (Hashimoto et al., 1994; Piepkorn et al., 1998; Yarden and Sliwkowski, 2001).

Human keratinocytes express multiple EGF-like growth factors including AREG, BTC, EREG, HB-EGF, and TGF-α (Barnard et al., 1994; Coffey et al., 1987; Hashimoto et al., 1994; Piepkorn et al., 1998; Tokumaru et al., 2000). These cells also express substantial levels of EGFR, ErbB2, and ErbB3 but no detectable ErbB4 protein (De Potter et al., 2001; Press et al., 1990; Prigent et al., 1992; Stoll et al., 2001) suggesting that EGF-like growth factor signalling in keratinocytes proceeds through the formation of EGFR homo- or EGFR/ErbB2 and/or EGFR/ErbB3 heterodimers. Although the function of EGFR ligands in human keratinocytes appears to be highly redundant (Barnard et al., 1994; Coffey et al., 1987; Cook et al., 1991; Hashimoto et al., 1994; Shirakata et al.,2000; Strachan et al., 2001), the importance of individual growth factors in specific cellular contexts has not been identified. Animal models and other experimental systems have indicated that EGF-like growth factors have distinct roles in various tissues. For example, HB-EGF has been shown to be important for wound healing (Marikovsky et al., 1993; Stoll et al., 1997; Tokumaru et al., 2000), arteriosclerosis (Nakata et al., 1996), blastocyst implantation (Das et al., 1994), and heart function (Iwamoto et al., 2003; Jackson et al., 2003; Yamazaki et al., 2003), whereas AREG has been implicated in mammary gland development (Sternlicht et al., 2005). Targeted expression of AREG in the epidermis results in a dermatosis with many similarities to psoriasis (Cook et al., 2004; Cook et al., 1997). Both AREG and HB-EGF have been shown to be important for retinoic

acid-induced epidermal hyperproliferation (Rittie et al., 2006; Varani et al., 2001). TGF-α is implicated in hair follicle development and eye formation (Luetteke et al., 1993) whereas EREG appears to be a mediator of dermatitis and lung metastasis (Gupta et al., 2007; Shirasawa et al., 2004; Sternlicht et al., 2005). BTC null mice have no detectable defects (Luetteke et al., 1999) however in transgenic animals it was recently shown that BTC regulates hair follicle development and angiogenesis during wound healing (Schneider et al., 2009).

Acute stimulation of keratinocytes with high concentrations of EGF or other EGFR ligands leads to increased expression of multiple EGF family members including AREG, HB-EGF and TGF-α (Barnard et al., 1994; Shirakata et al., 2000; Stoll and Elder, 1999). Although keratinocytes express multiple EGF-like growth factors, their importance and specific function in different cellular contexts has been incompletely characterized. Stoll et al., carried out an investigation to assess the relative expression of EGF ligands in cultured keratinocytes and normal and organ cultured human skin (Stoll et al., 2010).

Using Q-PCR, they found that proliferating normal human keratinocytes express at least 19 times more AREG mRNA than EPGN, EREG, HB-EGF or TGF-α, and that betacellulin (BTC) mRNA was nearly undetectable (Stoll et al., 2010). This study also demonstrated that the recently discovered EGFR ligand epigen – (EPGN), is also expressed by keratinocytes (Strachan et al., 2001). Similarly, using a multiplex EGFR ligand assay, AREG was the most abundant EGF-like growth factor shed into the culture medium, whereas EREG, TGF-α, and HB-EGF were very close to the lower detection limit. These findings showed that

Amphiregullin (AREG) is the most abundantly expressed and shed EGF-like growth factor in keratinocytes which may largely explain why autocrine keratinocyte growth and ERK phosphorylation were selectively blocked by antibodies against AREG but not by antibodies against four other EGF-like growth factors. Nevertheless AREG has a much lower binding affinity for EGFR than does EGF, due to the lack of a conserved leucine residue necessary for high affinity binding to EGFR (Adam et al., 1995). Thus, it is possible that the strong dependence of keratinocyte proliferation on AREG might be further explained by relatively weak desensitization of ligand-receptor complexes. Findings from earlier studies showing that AREG antibodies block growth of cultured keratinocytes under autocrine conditions (Bhagavathula et al., 2005) was confirmed by Stoll et al, whereas TGF- α antibodies had no effect under these conditions (Pittelkow et al., 1993). In support of the importance of AREG in skin homeostasis, *in vivo*, overexpression of AREG in transgenic mice leads to a hyperproliferative skin phenotype with many similarities to psoriasis (Cook et al., 2004; Cook et al., 1997). Furthermore, a humanized antibody against AREG also markedly blocked the psoriatic phenotype of human skin grafts on immunodeficient mice (Bhagavathula et al., 2005).

Expression of all EGF-like growth factors in cultured keratinocytes from normal skin was very low; however, HB-EGF, EREG, TGF- α and AREG were strongly induced in human skin organ culture (Stoll et al., 2010). This *in vitro* model displays many similarities to cutaneous wound healing (Bhora et al., 1995; Eisen, 1969; Hebda, 1988; Mackie et al., 1988; Reaven and Cox, 1968; Sarkany et al., 1965; Stoll et al., 1997; Stoll et al., 2002). Furthermore, Stoll et al., demonstrated

a sequential regulation of HB-EGF and AREG expression, and suggest that HB-EGF may be important in the earliest phases of wound healing, with AREG increasing later during the process (Stoll et al., 2010). This correlates with the division of wound healing into an early phase during which keratinocytes migrate but do not proliferate and a later phase characterized by vigorous proliferation (Bhora et al., 1995; Hebda, 1988; Marks et al., 1972; Stenn, 1978; Stoll et al., 1997). The importance of AREG for autocrine KC proliferation might explain its increased expression during the later phase of organ culture. Interestingly, increased expression of AREG during wound healing has been reported (Schelfhout et al., 2002). The early expression of HB-EGF in this model and its importance in scratch wound closure; strongly suggest an important function of HB-EGF during the early migration phase of wound healing (Xu et al., 2004). Consistent with this, it has been shown that skin wound closure was markedly impaired in keratinocyte-specific HB-EGF-deficient mice (Shirakata et al., 2005). Stoll et al also confirm earlier findings that keratinocyte migration is sensitive to EGFR, HB-EGF and MP inhibitors (Tokumaru et al., 2000, Stoll et al., 2010). However, in those experiments keratinocyte migration was assessed on tissue culture plates coated with type 1 collagen. Although keratinocyte migration was sensitive to antibodies against several ligands, expression of soluble HB-EGF markedly improved migration even in the presence of MP inhibitors (Tokumaru et al., 2000). In contrast, soluble AREG by itself is not sufficient to promote keratinocyte migration, but instead requires the proteolytic release of one or more additional growth factor(s) (Tokumaru et al., 2000). Lysophosphatidic acid (LPA) is an important constituent of blood and serum and has been implicated in migration, proliferation, cancer and wound healing

(Watterson et al., 2007). The strong activation of EGFR by HB-EGF depends on MP-mediated release of HB-EGF suggesting an important role of HB-EGF during the early phases of wound healing. Metalloproteinase-mediated release of membrane-bound EGF-like growth factors is required for EGFR-dependent autocrine ERK phosphorylation, migration and proliferation of normal human keratinocytes (Stoll et al., 2010). This study indicated that autocrine keratinocyte proliferation and ERK phosphorylation are selectively regulated by MP-dependent release of AREG, whereas proteolytic release of HB-EGF is required for keratinocyte migration as well as LPA-induced ERK phosphorylation. These data suggest important but distinct functions of HB-EGF and AREG during the migratory and proliferative phases of cutaneous wound healing respectively.

1.4 Wound Healing General Introduction.

Wound healing is a complex process driven by molecular events during which damage occurring to skin is repaired by the interplay of several key cell types. Several systems are involved including the inflammatory system, circulatory system and cellular repair which result in the production of "new skin" or wound closure. Over the previous two decades attempts have been made to understand the process of wound healing, often using tissues arising from periodontal surgical procedures (Hakkinen et al., 2000). These tissues represent surgically wounded sites and incorporate a cascade of cellular and molecular events for initiating wound repair. The classical description of wound healing comprises an initial temporary repair characterised by the formation of a clot in wounded tissues. Inflammation follows, caused by inflammatory cells after which fibroblasts and endothelial cells migrate to cover the damaged surfaces. Finally a maturation phase occurs where healing of the tissue matrix is seen alongside contraction of the wound and scarring. As well as several important cell types including those belonging to the inflammatory system such as macrophages and neutrophils, fibroblasts, epithelial cells and endothelial cells release a cascade of factors which ensure these processes follow on from one another. These include the enzymes transglutaminases, cytokines such as decorin and biglycan and growth factors such as TGF beta. However, this is a simplified version of events and more detail is required on the individual processes that occur during wound healing.

1.4.1 The Fibrin Clot and Inflammatory Cells

Any injury to the skin or organ can initially cause injury to blood vessels. This causes a fibrin rich clot to form as a result of blood coagulation and platelet

aggregation which plugs the cut blood vessels and serves to protect at least temporarily, the freshly wounded tissue underneath (Clark, 1996). The clot is formed by platelets enclosed by a network of fibrin fibres. In addition, plasma fibronectin, vitronectin and thrombospondin is also present (Martin, 1997). The clot serves as a provisional matrix for cell migration, and provides a rich source of growth factors and cytokines from degranulated platelets. Initially there is a recruitment of inflammatory cells to the wound site followed by epithelialisation, granulation tissue formation and angiogenesis. Neutrophils and monocytes are recruited into the wound space by signals present within the clot. Neutrophils cleanse the wound of foreign particles, debris and bacteria; this is accomplished by both the release of enzymes and toxic oxygen products (metabolites) (Clark, 1996). When there is a large and increasing number of contaminating bacteria present in the wound, neutrophils can cause additional tissue destruction during the removal of these foreign objects and bacteria. Neutrophils also fulfil another role; they are a source of further pro-inflammatory cytokines which signal to the adjacent fibroblasts to activate them. Keratinocytes then begin the re-epithelialisation process (Hubner et al., 1996). Neutrophil infiltration of the wound ceases after a few days, and they become phagocytosed by either macrophages or fibroblasts (Clark, 1996). Peripheral blood monocytes continue to be recruited into the wound site and differentiate into macrophages upon activation. Fibrin, along with fibronectin in the clot acts as a provisional matrix for the influx of monocytes and fibroblasts (Brown et al., 1993). Macrophages continue the process started by neutrophils and phagocytose bacteria and cellular and matrix debris in the wound. Growth factors and cytokines are continuously synthesised and secreted into the wound environment by macrophages. Thus the

wound repair signals are initiated by degranulating platelets and neutrophils, and are maintained by macrophages.

1.4.2 Re-Epithelialisation.

Using gingival tissue as an example, under normal conditions the basal layer of epithelium is attached to the basal lamina. Keratinocytes use integrins, which are receptors on the cell surface, to bind to a major component of the basal lamina; laminin (Hakkinen et al., 2000). Integrins are a family of cell adhesion receptors that mediate cell surface interactions predominantly with extracellular matrix but sometimes with other cells (Hynes, 1990). The integrin family is made up of 24 heterodimers composed of 18 α subunits and 8 β subunits where each integrin is made up of one alpha and one beta subunit in a non covalent complex (Yamada et al., 1996). A number of combinations of these subunits allow individual integrins to be specific for a particular ligand, further; many integrins are also capable of recognising the same substrate. Table 4 shows the ligands for integrins linked with wound healing.

Migration of cells is potentially dependent on integrin expression and the changes that occur as a result of changes in the wound environment. In normal tissue, keratinocytes use the integrins α6β4 to bind to laminin in the basal lamina, and these integrins have intracellular links with the keratinocyte cytoskeletal network. When migration of the keratinocytes is necessary, the keratinocytes at the wound edge dissolve the hemidesmosome attachment and begin to upregulate integrins needed to stimulate healing in the wound environment (Hakkinen et al., 2000). Migrating keratinocytes express integrins α5β1 and αVβ6 to bind the wound components fibronectin and tenascin, and αVβ5, to bind vitronectin. In

addition integrin α2β1 is reorganised to redistribute collagen receptors (Hakkinen et al., 2000). This activation of integrins allows the keratinocytes to adhere to provisional matrix molecules as well as wound debris. Once the migration of epithelial keratinocytes has begun cells from the basal layer away from the wound edge begin to proliferate providing a further source of basal cells. The mechanism driving epithelial migration is not completely understood.

Integrin	Ligands
α, β; α2β1	Fibrillar collagen, laminin
α3β1	Fibronectin, entactin, epilgrin, laminin, denatured collagen
α4β1	Fibronectin, VCAM-1
α5β1	Fibronectin (RGD)
α6β1; α7β1; α6β4	Laminin
α8β1	Fibronectin, vitronectin
α9β1	Tenascin
αvβ1; αvβ5	Fibronectin; vitronectin
αvβ3; α11bβ3	Vitronectin (RGD); fibronectin, fibrinogen, von Willenbrand factor, thrombospondin, denatured collagen
αvβ6	Fibronectin, tenascin
α4β7	Fibronectin (IIIcs)
αvβ8	Vitronectin
αMβ2	Factor X, fibrinogen
αXβ2	Fibrinogen

Table 4: A list of the substrates of integrins associated with wound healing.

Growth Factor	Source	Effect
Fibroblast growth factors 1,2 and 3	Macrophages, endothelial cells	Fibroblast proliferation and angiogenesis
Transforming growth factor α	Macrophages, Keratinocytes	Re-epithelialisation
Transforming growth factor β1 and β2.	Platelets, macrophages	Fibroblast and macrophage chemotaxis; extracellular matrix synthesis; secretion of protease inhibitors
Epidermal Growth Factor	Platelets	Re-epithelialisation
Platelet derived growth factor (isoforms AA,AB,BB)	Platelets, macrophages, keratinocytes	Fibroblast and macrophage chemotaxis, fibroblast proliferation and matrix synthesis
Keratinocyte growth factor	Dermal Fibroblasts	Keratinocyte proliferation
Insulin-like growth factor	Plasma, platelets	Endothelial and fibroblast proliferation
Vascular endothelial growth factor	Keratinocytes, macrophages	Angiogenesis
Interleukin 1α and β	Neutrophils	Activate growth factor expression in macrophages, keratinocytes and fibroblasts
Tumour necrosis factor-α	Neutrophils	Activate growth factor expression in macrophages, keratinocytes and fibroblasts

Table 5: Common growth factors associated with wound healing

However chemotaxic factors, active contact guidance or an absence of neighbouring cells and cell to cell contact or a combination of these may contribute (Hakkinen et al., 2000). Once re-epithelialisation has been completed the components of basal lamina are deposited starting from the wound edge and the epithelial cells revert to their quiescent phenotype. In addition to cues from the extracellular matrix, these processes are highly dependent on growth factor signalling, summarised in Table 5.

Migration of epithelial cells through the fibrin clot is only possible due to the creation of a migrating pathway. This occurs as a result of the dissolution of the fibrin barrier by the enzyme plasmin. Plasmin is derived from the activation of plasminogen within the clot. The two activators tissue type plasminogen activator and urokinase type plasminogen activator along with their respective receptors are up regulated by the migrating keratinocytes. In addition to the activation of plasmin, several other proteases are also expressed to clear the path for migrating cells such as metalloproteinases, which was discussed in section 1.3 and in more detail in section 1.5 later in this chapter.

1.4.2 Granulation Tissue and Wound Contraction.

Granulation tissue is usually formed four days after wounding and is made up of new capillaries, macrophages, fibroblasts and some loose connective tissue. Granulation tissue is a hub of cytokines that drive chemoattractive, mitogenic regulatory reactions (Martin, 1997). Growth factors found in this tissue are derived mainly from macrophages and fibroblasts. Granulation tissue forms in several stages and depending on the stage may drive the dependent cytokine and therefore whether migration, chemotaxis, cell proliferation or phenotypic

expression is supported. During the formation of granulation tissue, macrophages, fibroblasts and new blood vessels invade into the wound space in a coordinated manner. Macrophages can stimulate fibroblasts to synthesise a collagen-rich extracellular matrix by the release of cytokines (Schafer and Werner, 2007). The extracellular matrix supports cell and vascular growth which in turn carries nutrients to sustain cellular function. Fibroplasia is the term applied to the part of granulation tissue made up of fibroblasts and extracellular matrix. Fibroblasts may in turn secrete cytokines to which they can themselves respond. The extracellular matrix and fibroblasts function in a reciprocal manner during wound healing. Fibronectin and collagen facilitate the adhesion and migration of fibroblasts in the granulation tissue, the fibroblasts synthesise and decorate the extracellular matrix and in turn the extracellular matrix regulates gene expression and behaviour of the fibroblasts. The fibroblasts adhere to fibronectin, collagen and vitronectin via various integrins listed in table 3 (Schafer and Werner, 2007). Similar to keratinocytes, fibroblasts change their integrin profiles in preparation for migration. Under normal conditions fibroblasts express primarily collagen-binding integrins as they are usually found embedded in a collagen rich matrix, however, when wounded, fibroblasts surrounding the wound down regulate collagen-binding integrins and up regulate those integrins that adhere to components enriched in the wound, such as fibrin, fibronectin and vitronectin. When fibroblasts are simultaneously challenged by signals from both the provisional matrix (fibrin) and a growth factor such as TGFβ the fibroblasts respond by up regulating receptors for the provisional matrix components. However if challenged by the same growth factors in the presence of a collagen rich matrix the fibroblasts up regulate receptors for

collagen and not the provisional matrix components (Xu and Clark., 1996). Fibroblasts in wounds can also use vitronectin and fibrin directly as substrates for adhesion in migration. This is facilitated by the availability of cell membrane receptors for these matrix proteins. Furthermore, fibroblast migration can be indirectly stimulated by the growth factors themselves such as platelet-derived growth factor (PDGF) or transforming growth factor beta (TGFβ) by up regulating integrins that support migration in the wound environment (Schafer and Werner, 2007).

As wound healing progresses, the provisional matrix becomes replaced by a new collagen rich matrix synthesised by the fibroblasts migrating into the wound. The synthesis of specific extracellular matrix molecules by fibroblasts in the wound is regulated by TGFβ and other growth factors such as those summarised in table 4 (Clark, 1996., Martin, 1997 Schafer and Werner., 2007). Cytokines such as interleukin 4 (IL-4) can also induce expression of collagenous matrices in wounds (Postlethwaite et al., 1992). Once the necessary amount of collagenous matrix is synthesised signals are given to down regulate collagen synthesis. 7 to 10 days after wounding a proportion of the fibroblasts present in the wound become myofibroblasts which express α-smooth muscle actin. This transformation allows the myofibroblasts to generate sufficient contractile forces to contract the wound. This final stage of fibroplasia is followed by the reduction of the number of fibroblasts and myofibroblasts present in the wound as a result of programmed cell death. When embryonic wounds are compared with adult wounds they heal with relatively little contraction or scarring, as there is no transformation of fibroblasts to myofibroblasts and the angiogenic response is

considerably less. In addition, there is less expression of TGFβ and this results in the reduced scarring associated with embryonic healing (Ferguson and O'Kane, 2004).

1.4.3 Angiogenesis.

Angiogenesis is the term used to describe the formation of new blood vessels and is a crucial final step towards the end of wound healing. The large number of new blood vessels cause a red granular appearance in the wound promoting the use of the term granulation tissue. Several growth factors are responsible for inducing angiogenesis in a wound including fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) (Aukhil, 2000). FGF-2 is synthesised by macrophages and damaged endothelial cells while VEGF is induced in the wound edge (Aukhil, 2000). Endothelial cells have to activate specific integrins such as αVβ3 on their surface in order to respond to angiogenic signals. As the endothelial cells migrate into the provisional matrix they form tubes surrounded by provisional matrix, and followed by the formation of a mature basement membrane. Like fibroblasts, endothelial cells involved in angiogenesis undergo programmed cell death during the final maturation of the matrix characterised by the regression of capillaries and complete closure of the wound (Aukhil, 2000).

1.4.4 Mesenchymal-Epidermal Interactions.

Epithelial and mesenchymal interactions have historically been based on their visual appearance and the morphology of the multicellular structures they create (Shook and Keller, 2003). Numerous studies have identified the importance of

epithelial- mesenchymal interactions for epidermal homeostasis and repair including the identification of cross talk between the dermal and epidermal compartments necessary for keratinocyte growth and differentiation (Werner et al., 2007). This signalling pathway is initiated by epithelial interleukin 1 (IL-1) which subsequently stimulates the release of granulocyte macrophage colony stimulating factor (GM-CSF) and keratinocyte growth factor (KGF) (Mann et al., 2001). Keratinocyte migration models utilising cell out-growth over collagen gels demonstrated that migration over fibroblast populated gels could not be replicated in the absence of mesenchymal cells, suggesting mesenchymal cells activate keratinocyte out-growth via a soluble factor, although KGF was eliminated as a stimulant after its exogenous addition or blocking antibody produced no effect on migration (Younai et al., 1994). In addition IL-6 deficient mice indicated mesenchymal requirements for keratinocyte migration as IL-6 deficient mice display significant delays in cutaneous wound healing (Gallucci et al., 2004). Further proteins and tyrosine kinase receptors have also been suggested as being involved in epithelial-mesenchymal interactions such as TGFβ (Werner et al., 2007), and EGFR signalling (Lo et al., 2005) although this is an area under intense investigation and further factors are yet to be identified (Lee et al., 2009). The wound healing process is summarised in Figure 9 where the previously described sections have been categorised into inflammation, proliferation and remodelling phases. Furthermore, the action of transglutaminases in wound healing is also summarised and discussed in section 1.4.5.

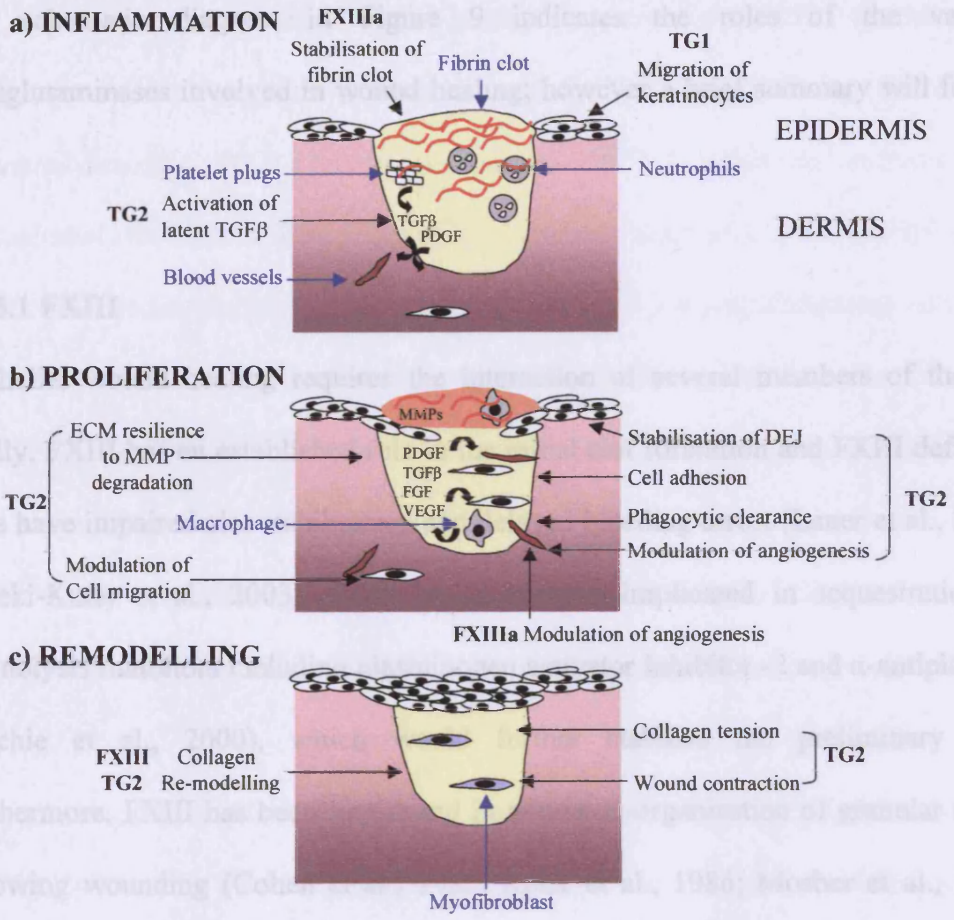

Figure.9: Schematic representation of the three stages of wound healing in relation to the actions of transglutaminases: (a) Following the initial wound event, a blood clot comprising of cross-linked fibrin is rapidly formed. This is followed by invasion of neutrophils succeeded by monocytes and lymphocytes, thereby triggering the inflammatory phase of wound repair. Various cytokines and growth factors secreted by these cells, aggregated platelets and later macrophages (PDGF, TGFβ, FGF, and VEGF) mediate the transition to tissue repair. (b) During re-epithelialisation, keratinocytes undergo extensive alterations, including the dissolution of desmosomes and hemidesmosomal links. This permits cell movement into the wound space, between the collagenase dermis and provisional fibrin matrix. Re-epithelialisation is accompanied by proliferative bursts and migration of dermal fibroblasts from adjacent tissue into the wounded area. These fibroblasts then participate, substituting the temporary matrix with collagenous tissue. MMPs play a key role in matrix remodelling, creating paths for cell migration through their proteolytic activity. Neovascularization occurs to provide oxygen and nutrients required to sustain the proliferation of keratinocytes and fibroblasts and the formation of new tissue. (c) During the transition from granulation to scar tissue, a relatively acellular matrix is generated in which many cells and blood cells are removed by programmed cell death. The multipoint actions of various TG isoforms are indicated. Adapted from (Verderio *et al.*, 2005).

1.4.5 Transglutaminase Role in Wound Healing

The schematic diagram in Figure 9 indicates the roles of the various transglutaminases involved in wound healing; however a brief summary will follow here.

1.4.5.1 FXIII

Epithelial wound healing requires the interaction of several members of the TG family. FXIII has an established role in the initial clot formation and FXIII deficient mice have impaired clot stabilisation and delayed bleeding arrest (Lauer et al., 2002; Koseki-Kuno et al., 2003). FXIII has also been implicated in sequestration of fibrinolysis inhibitors including plasminogen activator inhibitor -2 and α-antiplasmin (Ritchie et al., 2000), which would further stabilise the preliminary clot. Furthermore, FXIII has been implicated in matrix re-organisation of granular tissue following wounding (Cohen et al., 1982; Knox et al., 1986; Mosher et al., 1991; Corbett et al., 1997).

1.4.5.2 TG1.

Increased TG1 levels have been observed in the epidermal edges of wounds in murine models (Inada et al., 2000); these raised levels are evident within hours of wounding and continue until re-epithelialisation is complete (Inada et al., 2000). Increased levels of TG1 were also found in migrating keratinocytes leading to the hypothesis that a premature cornified envelope is formed to provide mechanical strength to migrating cells dissecting the clot or protecting against damage from proteases within the wound space (Tharakan et al., 2010)

1.4.5.3 TG2.

The discovery of γ-glutamyl-ε-lysine isopeptide cross links within the basement membrane region suggested a potential role for TG2 in stabilising the dermo-epidermal junction (DEJ) (Aeschlimann et al., 1995) and this was confirmed by clinical study of human skin grafts. These studies indicated key dermo-epidermal structures including anchoring fibrils are cross linked by transglutaminases and these cross links were observed to increase stability at the tissue interface (Raghunath et al., 1996). The DEJ includes several basement membrane components which have been shown to be TG2 substrates including nidogen/entactin (Aeschlimann et al., 1991; 1992), osteonectin/BM-40/SPARC (Hohenadl et al., 1995) fibronectin (Martinez et al., 1994) and collagen VII (Raghunath et al., 1996).

TG2 involvement is indicated in several stages of wound healing. Models of wound healing employing punch biopsies in rats were investigated to elucidate the distribution of TG2 during this process (Haroon et al., 1999). Haroon et al, reported an up regulation of TG2 that continues up to nine days after wounding and is accompanied by an increase in isopeptide linkages (Haroon et al., 1999). This prolonged increase in TG2 levels may suggest a role for TG2 throughout the wound healing process. TG2 mRNA and protein were detected in migrating epithelial cells, sites of neovascularisation and granulation tissue within a day of wound healing (Haroon et al., 1999). Epithelial expression proved to be transient and was found to return to baseline levels after re-epithelialisation. Conversely, TG2 remained high within the DEJ (Haroon et al., 1999). Furthermore, clinical studies have identified TG2 in catalysing the attachment of the epidermis (Raghunath, 1996). TG2 expression is maintained in endothelial cells, macrophages, and muscle cells

throughout the nine days. These results suggest TG2 involvement in neovascularisation, stabilisation and remodelling of the provisional clot matrix, re-epithelialisation and the migration of cells into the clot. Furthermore several studies have indicated TG2 to be up regulated following wounding in rats (Bowness et al., 1988), binding the ECM after mechanical injury of fibroblast monolayers (Upchurch et al., 1991), increasing the breaking strength of wound tissue (Dolynchuk et al., 1994) and functioning in matrix repair and remodelling (Griffin et al., 2002; Stephens et al., 2004; Zang et al., 2004).

In addition, Verderio et al., identified TG2 in an integrin independent pathway of cell adhesion as well as wound healing (Verderio et al., 2003, 2005). It was reported that extracellular TG2-FN complexes restored lost cell adhesion following the inhibition of integrin co-receptors with exogenous Arg-Gly-Asp peptides (Verderio et al., 2003). Further investigation revealed transamidating activity of TG2 was not required for this function and that the TG2-FN complexes associated with cell surface heparin sulphate (HS). This pathway is capable of ensuring cell adhesion following damage to the ECM, since under these conditions ECM fragments are capable of inhibition of the integrin dependant pathway (Verderio et al., 2003). TG2 is also found at sites of inflammation (Greenberg et al., 1991; Kim, 2006), and is up regulated by a number of acute phase cytokines such as IL-6 (Ikura et al., 1994), TGFβ (Vollberg et al., 1992; Ritter and Davies, 1998) and tumour necrosis factor TNFα (Kuncio et al., 1998).

1.4.5.4 TG2 and Tissue Fibrosis.

TG2 can be further implicated in normal wound healing when the consequences of its misregulation are examined. Fibrosis is the term applied to a condition arising from acute tissue repair transmuting to chronic matrix deposition Excessive TG2 cross linking has been reported in numerous fibrotic conditions such as liver cirrhosis and pulmonary fibrosis (Kunico et al., 1998; Hettasch et al., 1996). Interestingly the TG2 inducer TGFβ is among the inflammatory mediators linked to these diseases (Ziyadeh et al., 2000). The ability of TG2 to cross link the matrix resulting in increased stability and resilience to degradation and its activation of TGFβ and subsequent stimulation of matrix synthesis is still under investigation (Garcia et al., 2008).

1.5 Metalloproteinase Activity in Wound Healing.

Proteinases have long been known to be involved in the breaking down and renewal of matrix components. This mechanism allows tissues and organs that are damaged or impaired to be decomposed and subsequently replaced by new cellular and ECM components. For example, cell matrix and cell-cell interactions may be modified; MMP cleavage of laminin 5 generates a fragment (γ2-chain fragment) which enhances cell motility (Schenk et al., 2003). Furthermore, MMPs can cleave cell surface molecules involved in cell cell interactions such as E-cadherin, and modify cell surface shedding of proteins such as Fas ligand, thus regulating Fas mediated apoptosis (Powell et al., 1999). MMPs may also function to modulate the migration of cells into a given location as has been shown with MMP mediated cleavage of α-1-protease inhibitor, which acts as a bioactive chemoattractant for neutrophils (Banda et al., 1988). Finally, several studies have shown metalloproteinases can

result in the release of growth factors and cytokines, which may affect cellular growth and proliferation (Imai et al., 1997). Members of the four sub families of Metzincins have been identified as being involved in matrix turnover and several *in vivo* models have been developed to elucidate these mechanisms. For example studies involving mice deficient in MMP14 (MT1-MMP) or ADAM17 (TACE) show gross abnormalities in tissue development and growth (Killar et al., 1999; Zhou et al., 2000) indicating the importance of the metalloproteinase family in tissue turnover, growth and wound repair.

1.6 EGF and its Role in Wound Healing.

The complex process of wound healing is executed and regulated by an equally complex signalling network involving numerous growth factors, cytokines and chemokines. Of particular importance is the epidermal growth factor (EGF) family, transforming growth factor beta (TGF-β) family, fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), interleukin (IL) family, and tumor necrosis factor-α (TNF-α) family. As a vast amount of information is available about each family involved in this process a summary of the involvement of EGF and TGF will be discussed here only. EGF is a polypeptide growth factor that binds to EGF receptors, which are members of the ErbB receptor family of type 1 tyrosine kinases (Fontanini et al., 1998). Activation of this receptor has several functions in processes including cell migration, growth and wound repair. The EGF ligand family is composed of 6 members including EGF (Cohen, 1964), TGFα (Derynck et al., 1984) amphiregullin (Shoyab et al., 1989), HB-EGF (Higashiyama et al., 1991), betacellulin (Shing et al.,

1993) and epiregulin (Toyoda et al., 1995). A summary of its regulation in keratinocytes is shown in Figure 10. All 6 members are synthesised as membrane anchored forms that are then processed by proteases such as metalloproteinases to generate bioactive soluble factors. TGFα, Amphiregullin and HB-EGF have all been identified as autocrine growth actors for keratinocytes (Coffey et al., 1987, Cook et al., 1991, Hashimoto et al., 1994) and the transmembrane forms of growth factors are also capable of stimulating the growth of adjacent cells including keratinocytes by cell to cell contacts (juxtacine stimulation) (Brachmann et al., 1989, Higashiyama et al., 1995; Inui et al., 1997). Furthermore it was reported in 1993 by Marikovsky et al that wound fluid from skin also contains EGFR ligands suggesting the regulation of EGFR ligand shedding is an important physiological step in the wound healing process. EGF family members bind to one of four ErbB family receptors which induce homo and heterodimerization (Stoll et al., 2001). Erb1 is universally identified as EGFR and the other family members are identified as ErbB2, ErbB3 and ErbB4 (Pastore et al., 2008). EGFR, ErbB2 and ERbB3 are all expressed in human skin with the predominant type being EGFR. This can be found in the whole dermis of skin however it is more accentuated in the basal cell layer (Nanney et al., 1984; Mascia et al., 2003). EGFR can be activated by several mechanisms under physiological conditions, apart from direct activation by a specific ligand. EGFR activation occurs as a result of G protein coupled receptor (GPCR) signalling. This is a rapid process whereby GPCR induced release of the EGFR ligands induces EGFR transactivation and downstream signalling (Eguchi et al., 1998). As an example, in wound healing angiotensin II stimulates keratinocyte and fibroblast migration through a pathway initiated by the GPCR angiotensin II receptor that leads to HB-EGF shedding and consequently activation of the extracellular signal regulated

kinase (ERK) cascade (Yahata et al., 2006). EGFR knockout mice showed abnormalities such as wavy hair and thin skin when compared with wild type controls however when individual knockouts for EGF ligands have been produced a less striking phenotype is seen (Miettinen et al., 1995; Sibilia and Wagner 1995). For example, the EGF-/- mouse showed no significant abnormalities nor were any abnormalities detected in the TGFα knock out mouse (Luetteke et al., 1993; Mann et al., 1993; Luetteke et al., 1999) suggesting a redundancy of EGF ligands within the family.

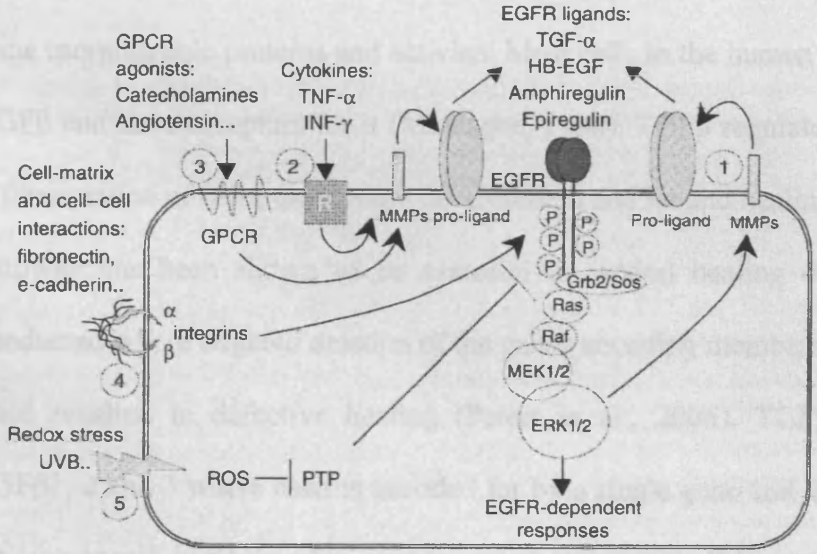

Figure 10: Mechanism of EGFR activation in keratinocytes.
Under unstimulated conditions EGFR contributes to its own activation via an autocrine/ paracrine loop based on denovo synthesis and shedding of mature EGFR ligands. 1. MMP activation can release a variety of G protein coupled receptor (GPCR) ligands. 2. In addition pro-inflammatory cytokines are able to bind to their specific receptors (R). 3. GPCR agonists can cause a promotion of rapid metalloproteinases shedding of EGFR ligands from membrane precursors (via feedback mechanism). 4.In addition after cell-matrix adhesion, integrins associate with the EGFR to trigger downstream signalling from its ligand independent phosphorylation. 5.Inactivation of protein tyrosine phosphatises can occur by intracellular generation of reactive oxidative species in response to pro-oxidative stimuli. . Taken from Pastore et al., 2007.

1.7 TGFβ and its Role in Wound Healing.

TGFβ is a member of a family of dimeric polypeptide growth factors that includes bone morphogenic proteins and activins. Most cells in the human body both produce TGFβ and have receptors for it (Massague, 1998). TGFβ regulates proliferation and differentiation of cells, embryonic development and wound healing. TGFβ signalling pathway has been shown to be essential in wound healing due to experiments conducted where targeted deletion of the genes encoding members of this pathway in mice resulted in defective healing (Peters et al., 2005). TGFβ has 3 isoforms; TGFβ1, 2 and 3 where each is encoded for by a single gene and is expressed in both a tissue specific and developmentally regulated fashion (Massague, 1990). TGFβ 1 and 3 are expressed earlier in endothelial cell development and TGFβ2 is expressed later in mature and differentiating epithelium (Massague, 1990). TGFβ isoforms are synthesised as part of a larger precursor molecule containing a pro peptide (Wrana et al., 1994; Nakao et al., 1997). TGFβ becomes cleaved from the propeptide before the precursor is secreted by the cell but remains attached to the propeptide by noncovalent bonds. After secretion TGFβ is stored in the extracellular matrix as a complex of TGFβ-propeptide and latent TGFβ binding protein which prevents it from binding to its receptors unless a matrix glycoprotein such as thromobospondin 1 changes the conformation of the latent protein to release TGFβ (Wrana et al., 1994; Nakao et al., 1997). TGFβ regulates cellular processes by binding to 3 high affinity cell surface receptors known as types 1,2 and 3 with the third type being the most abundant (Wrana et al., 1994; Nakao et al., 1997). These receptors instigate intracellular signalling via serine threonine protein kinases in their intracellular domains that phosphorylate several transcription factors known as smads. Smad 2 and 3 can be phosphorylated by activated TGFβ1 receptors leading to activation of

smad 4 or smad 2 or 3 which can either induce a smad complex which then translocates to the nucleus where it interacts in a cell specific way to regulate differentiation, deposition of extracellular matrix or apoptosis. Alternatively smad 2/3 can be blocked by smad 6 and 7 to inhibit TGFβ signalling (Wrana et al., 1994; Nakao et al., 1997). TGFβ is a potent regulator of production and deposition of extracellular matrix such as collagen and fibronectin from fibroblasts and other cells (Sonis et al., 1994) and further, TGFβ decreases the production of enzymes that degrade the extracellular matrix including collagenases and heparinase. Dysregulated TGFβ signalling in human disease can be caused for example by over activity of TGFβ leading to fibrosis and progressive cancers while a decreased TGFβ activity can cause developmental defects and artheroschlerosis (Akagi et al., 1996).

1.8 Skin General Introduction.

Skin makes up the largest organ of the body covering it completely. The skin provides protection against chemical and mechanical attack. It prevents ultraviolet light penetration and damage to internal organs as well as microbial attack. Furthermore, as the skin is relatively impermeable unless injured it prevents dehydration of the body. The skin is able to provide sensory detection for the brain as it is rich in receptors for touch, pain, pressure and temperature, and therefore is also important in thermoregulation. Skin is covered by hair which is paramount in the body's response to both heat and cold as well as subcutaneous adipose tissue which insulates against heat loss. Skin also synthesises vitamin D within the epidermis, which is partially able to supplement vitamin D derived from dietary sources. Mammalian skin is made up of two layers; it is formed from a protective epidermis and an underlying collagen rich dermis produced by fibroblasts. These two layers are separated by a basement membrane. Skin tissue regularly undergoes remodelling and rejuvenation both of which are under stringent regulation. Figure 11 shows a cross-section of the skin indicating its main layers of epidermis and dermis. The following sections summarise skins components in more detail.

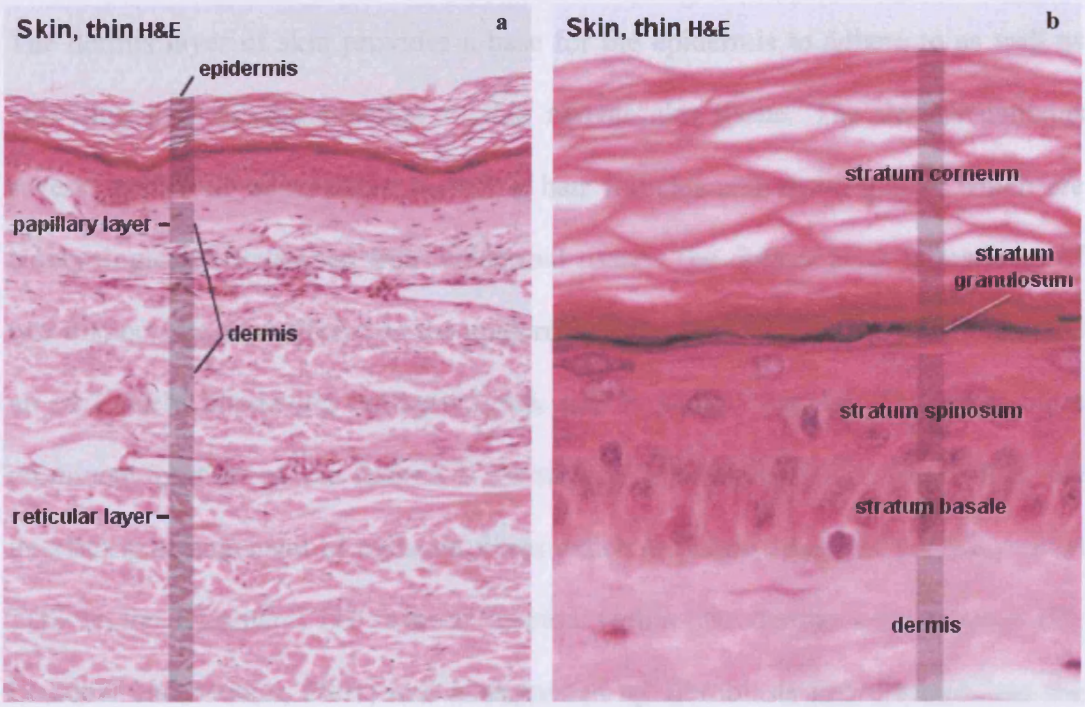

Figure 11. The tissue architecture of skin: Hematoxylin and eosin (H&E) staining reveals three distinct domains (a) the avascular epidermal tissue formed by keratinocytes committed to terminal differentiation. The dermis can be dissected into the papillary and reticular layers. The former of these layers is immediately adjacent to the epidermis and can be distinguished by the greater density of fibroblasts. This region is relatively thin and formed by a fine network of collagen and elastin fibres. The underlying reticular dermis contains coarse collagen and elastic fibres in addition to the larger blood vessels, which feed into the capillary network of the papillary layer. The epidermis can be divided into a further four morphologically distinct layers as seen in a high magnification picture of a region of thin skin (b). The stratum basale is formed from the deepest layer of keratinocytes and demonstrates a cuboidal or columnar morphology. Several layers of polygonal keratinocytes comprise the stratum spinosum. In the case of thin skin regions, the ketohyalin granule-containing stratum granulosum is apparent as a single layer of dark and flattened cells although this can increase to several layers in regions of thick skin. Finally the outermost layers form the stratum corneum, containing anuclear, flattened cells termed corneocytes.
(http://www.lab.anhb.uwa.edu.au/mb/mb140/CorePages/Integumentary/Integum.html#Epidermis)

1.8.1 The Dermis

The dermis layer of skin provides a base for the epidermis to adhere to as well as providing a metabolic function to this nonvascular tissue. The dermis contains several epidermal appendages including hair follicles and sweat glands which are embryologically developed from epidermal tissue. The dermal layer is made up of two distinct zones. Adjacent to the epidermis is the papillary dermis which is made up of loosely interlacing collagen fibres and is highly vascular. The other more prominent portion of the dermis is formed by a reticular layer named after the interlacing arrangement of collagen fibres which are denser than the papillary zone. TG2 is the dominant TG isoform located within the dermal compartment (De Laurenzi and Melino, 2001) and is expressed by fibroblasts and secreted into the extracellular matrix. TG2 has been shown to be linked to regulation of cell adhesion, spreading and motility as well as ECM remodelling and stabilisation. These functions have led to TG2 being confirmed as having an important role in wound healing (Haroon et al., 1999, Stephens et al., 2004). FXIII has also been found in the dermal ECM and has a putative role in wound healing (Cohen et al., 1982; Knox et al., 1986; Mosher et al., 1991; Corbett et al., 1997). mRNA of additional TG isoforms has been amplified from primary fibroblasts including TG1 (Phillips et al., 1993; Stephens et al., 2004), TG5 (Stephens et al., 2004) and TG7 (Grenard et al., 2001; Stephens et al., 2004) although the physiological role of these enzymes within wound healing is still under investigation.

1.8.2 The Epidermis

The epidermis by comparison is constantly undergoing tissue renewal every 28 days (Roop, 1995). The epidermis is comprised of 4 distinct cell layers; the stratum

basale, stratum spinosum, stratum granulosum and stratum corneum. Each of these sub layers has been demonstrated to express a distinct set of marker proteins specific for the cell maturation state (Eckert et al., 1989).

1.8.2.1 Stratum Basale

This is the deepest cell layer made up of keratinocytes with regulated proliferative capabilities (Cotsarelis et al., 1989; Fuchs and Bryne 1994). Keratin present within this layer comprises keratin 5 and 14 (Reichert et al., 1993). Transition from this layer is accompanied by the loss of β1 integrins which are involved in the stabilisation of the DEJ and committing the cells to terminal differentiation.

1.8.2.2 Stratum Spinosum

The stratum spinosum makes up the biggest epidermal cell layer and contains extensive desmosomes. These cell-cell connections contribute to the strength of the tissue and accounts for the spiky appearance of keratinocytes within this layer. At this stage of differentiation the ability of the cells to proliferate is lost (Fuchs and Byrne, 1994; Eckert et al., 1997) and keratin profiles are changed to predominantly keratins 1 and 10 (Fuchs and Green. 1980). These keratins aggregate together to form intermediate filaments which have an important structural function. It is in the more superficial layers of the spinous region that components of the cornified envelope are expressed, including involucrin. The formation of this envelope structure is a key stage in skin barrier formation.

1.8.2.3 Stratum Granulosum

Keratinocytes within this stratum are characterised by keratinisation specific lipid synthesis (Swartendruber et al., 1989; Wertz et al., 1989, Schurer and Elias, 1991; Elias 1996). There is distinct histology of cells within this region as a consequence of the granule enclosed storage of proteins and lipids (Matoltsy and Matoltsy, 1966; Lavker and Matoltsy, 1971; Holbrook and Odland, 1975; Lavker, 1976; Ishisa-Yamamoto et al., 1993). These transient structures contain cornified envelope precursors (Steven et al., 1990) including loricrin (Mehreal et al., 1990) and profilaggrin (Steinert and Marekov 1995) which are both considered markers of late stage differentiation.

1.8.2.4 Transition zone.

During skin formation there is a transition layer marked by extensive remodelling between the granular layer and the stratum corneum including the cornification process. This term describes the process of resorbtion of the cell plasma membrane and its replacement with the extensively cross linked cornified epithelium. This structure comprises of a protein and lipid component to be discussed individually. In addition to this step there is an extrusion of lipids into the inter-cellular space (Landmann, 1986), stabilisation of keratin intermediate filament bundles and the destruction of intracellular organelles by the action of proteases and nucleases. These reactions can cause these cells to be deemed dead however these cells have simply had their metabolism limited to catabolic reactions only (Eckert et al., 2005).

1.8.2.5 Stratum Corneum.

The thickness of this layer of epidermis can vary depending on the body site. It has been shown to be between 4 and 100 cell layers thick (Ya-Xian et al., 1999). Differentiated keratinocytes form plate like structures and are distinguished by the term corneocytes. Similarly the unique cell-cell interactions are termed corneosomes (Allen and Potten 1975; Chapman and Walsh, 1990) and following the breakdown of these structures, cells are lost by the process of desquamation (Ecket et al., 1997). These cells are embedded in lipid lamellae and although the stratum corneum may be dissected into its separate components essentially extensive cross linking creates a continuous macromolecule providing the barrier function of this tissue.

1.9 The Cornified Envelope.

The cornified envelope (CE) is comprised of proteins and lipids accounting for 90% (protein) and 10% (lipid) of the stratum corneum dry weight (Swartzenruber et al., 1988). The protein element makes a 15nm shell that is formed on the cytoplasmic surface of the plasma membrane (Maltoltsy and Balsamo., 1955; Farbman 1966; Hashimoto, 1969). This shell eventually replaces the plasma membrane as the lipid bilayer is penetrated during cornification. The substitution allows the skin to be more robust against mechanical attack (Marks et al., 1983). A 5nm lipid component has also been identified by electron microscopy as a lucent band and is located on the cell surface (Lavker, 1976). This has been characterised as a monolayer of ester linked ω-hydroxyceramides known as covalently bound lipids (CBL) (Wertz and Downing, 1987; Marekov and Steinhert 1998). These lipids are among those that extrude into the extracellular space from keratinocytes in the transition zone, the remaining form unique lipid lamellae with reduced phospholipid content and

increased fatty acids, cholesterol and ceramides (Shurer and Elias, 1991; Elias 1996). The hydrophobic nature of the covalently bound lipids provides an environment in which corneocytes become embedded where they are capable of interdigitating with the surrounding lamellae to enhance the barrier nature of skin (Wertz et al., 1989). CE generation begins in the stratum spinosum, where a scaffold forms in inter-demosomal regions to which further components become sequestered. The resulting structure is highly insoluble and may only be retrieved by boiling in SDS or a reducing agent buffer (Sun and Green, 1976, Manabe et al., 1981). Several methods have been used to investigate the CE. For example, antibodies raised against the isolated CE have been used to identify CE precursors (Kubilus et al., 1987; Michel et al., 1987, Nagae et al., 1987). Further antibodies have also been raised against putative CE components and immunohistochemical studies have indicated staining at the cell periphery of corneocytes (Rice and Green 1979; Lobitz and Buxham, 1982; Zettergren et al., 1984). Sequencing has also been carried out on peptides retrieved following extensive proteolysis of the CE (Candi et all., 1995; Steinhert and Marekov, 1995). Finally the ability of potential precursors to be cross linked by TG enzymes has been assessed *in vitro* (Rice and Green 1979; Simon and Green 1984; Candi et al., 1995, 1999, 2001). The CE has been identified in a range of stratified squamous epithelia such as oral epithelial or hair cuticles, with variation in CE composition between epithelial tissue and body site (Steinhert et al., 1998). This variation is believed to be necessary due to tissue specific requirements (Steinert and Marekov 1995; Steinhert 2000). Interestingly, the protein shell is not a homologous structure (Steinert and Marekov, 1995) suggesting spatial and temporal regulation is involved in its formation.

1.9.1 Transglutaminases and their role in cornified envelope formation.

Early studies indicated the cross links within the CE macromolecule are disulfide bonds (Maltoltsy and Matoltsy, 1970) however further studies also identified γ-glutamyl-ε-lysl (Rice and Green, 1977) and γ-glutamyl-polyamine isopeptides bonds within this structure, with γ-glutamyl-polyamine isopeptides bonds almost exclusively involving spermidine (Piacentini et al., 1988; Martinent et al., 1990). Immunohistochemical studies carried out with antibodies raised against the isopeptide bond indicated that the number of cross links increase as differentiating keratinocytes translocate to the epidermal surface causing a rapid increase in observed staining within the transition zone. This is accompanied by intense staining across the DEJ indicating TG enzymes in the formation of the CE. Of the nine TG isoforms, TG1, 2, 3, 5, 6, and 7 are expressed in the epidermis. TG2 expression is limited to the basal layer of keratinocytes (Aeschlimann et al., 1998; Haroon et al., 1999) where its role is thought to be in the stabilisation of the DEJ. Contrastingly, TG 1,3 and 5 have indicated differentiation specific expression (Kim et al., 1993, 1995a;1995b; Aeschlimann et al., 1998; Candi et al., 2001) and have demonstrated an ability to cross link CE components *in vitro* with high affinity (Candi et al., 1995; 1999; 2001). Furthermore it has been demonstrated that these enzymes target the same Gln and Lys residues involved in cross linking *in vivo* although different isoforms were observed to preferentially cross link distinct residues within the same substrate (Candi et al., 1995; 1999; 2001). Furthermore, the discovery of TG1 mutations being linked to lamellar ichthyosis (LI) a heterogenous group of skin diseases exhibiting defective CE formation and compromised skin barrier formation further indicated TG involvement in CE formation (Huber et al., 1995; Parmentier et al., 1995; Russell et al., 1995). TG1 is the first isoform to be

expressed as low level mRNA has been found in the basal layer (Steinert et al., 1996a) however a rapid increase is observed in the upper spinous layer, terminating within the transition zone (Yamada et al., 1997). Immunohistochemical studies have observed gradient staining with anti-TG5 decorating the spinous and granular layers (Candi et al., 2002). TG3 is expressed in the later stages and is approximate concomitant with the other late stage differentiation markers loricrin and profilaggrin. While components of CE vary greatly between epithelial tissues, several major components have been identified as TG substrates (Eckert et al., 2005) and are summarised below in section 1.9.1.1 onwards and Figure 12 and 13 as to their position in the CE and skin.

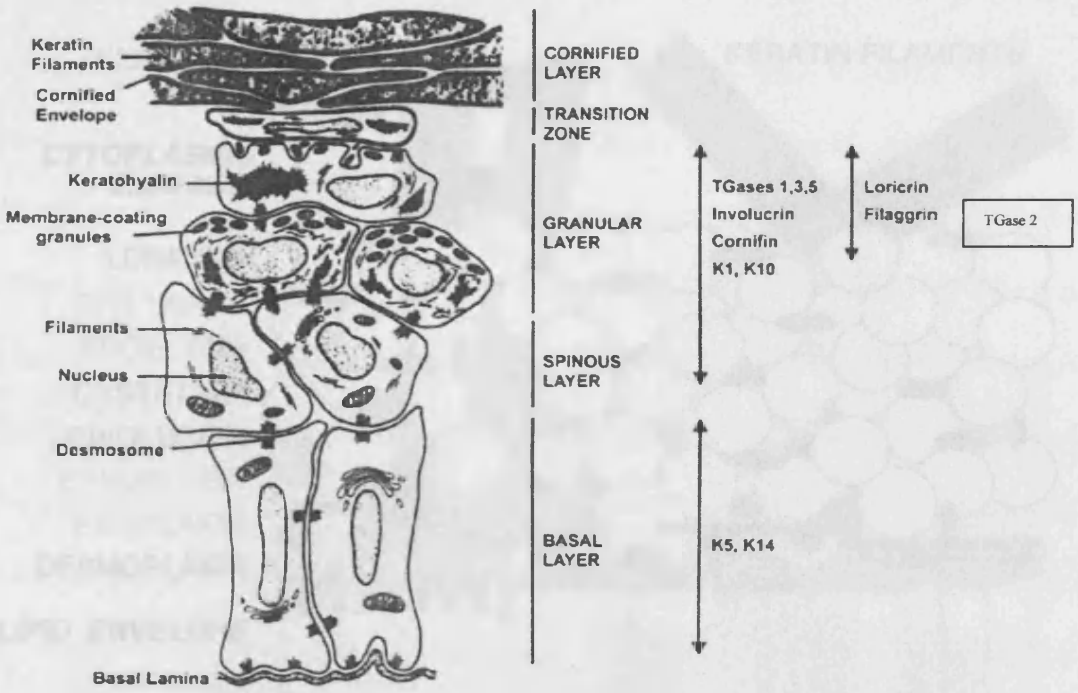

Figure 12 Schematic representation of transglutaminase distribution in skin; Transglutaminase 1 and 3 and 5 are expressed in the spinous and granular layer . Image taken from Eckert et al., 2005.

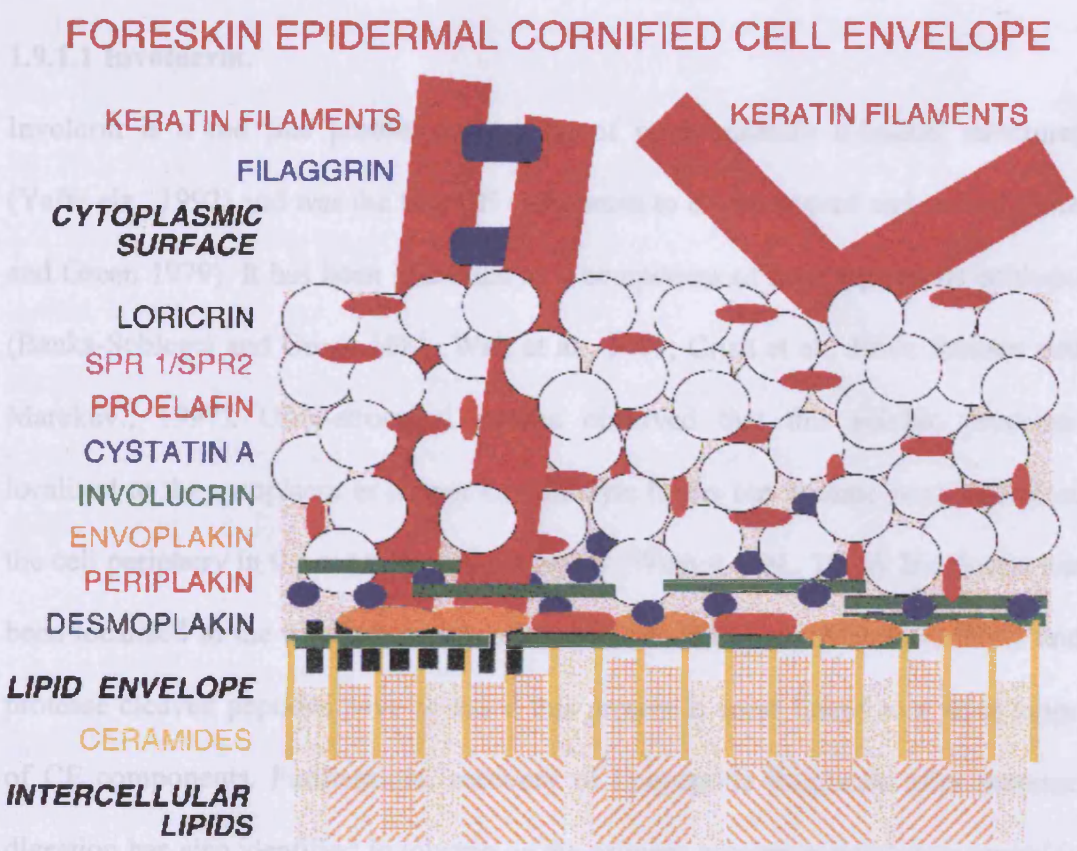

Figure 13: Summary of TG substrates and their position in the cornified envelope.
Indicating the presence of the various TG substrates position through the cornified envelope.
Key. Loricrin is shown in blackSPR1/SPR2 is shown in pinkProelafin is shown in red
Cystaton A is shown in dark bluenvolucrin is shown in greenEnvoplakin is shown in orangePeriplakin is shown in maroonDesmoplakin is shown in dark greenLipid envelope is shown by the crossed linesCeramides are shown by the blocks of yellow neat the bottom of the diagramIntercellular lipids are designated b the diagonal lines. Images taken from Nemes and Steinert 1999.

1.9.1.1 Involucrin.

Involcrin is a rod like protein comprising of predominantly α-helical structures (Yaffe ela., 1992) and was the first CE component to be discovered and cloned (Rice and Green 1979). It has been identified as a component of most squamous epithelia (Banks-Schlegel and Green 1981; Walt et al., 1985; Crish et al., 1993; Steinert and Marekov., 1997). Ultra-structural studies observed that this soluble precursor localised to the cytoplasm in deeper keratinocyte layers but became concentrated at the cell periphery in the more superficial layers (Warhol et al., 1985). Involucrin has been localised to the external region of the CE (Steinhert and Marekov, 1997) and protease cleaved peptides have revealed this protein is cross linked to a wide range of CE components. Furthermore, recovery of lipopeptide fragments after protease digestion has also identified involucrin as the primary precursor coupled to ceramide lipids (Marekov and Steinert, 1998).

1.9.1.2 Loricrin.

Loricrin accounts for more than 75% of the total protein content of the CE (Hohl et al., 1991). Step wise digestion of the CE by proteinase K have localised loricrin to the inner two thirds of the CE at the cytoplasmic face (Steinert and Marekov, 1995) which increases to 95% in the final third demonstrating its late stage recruitment and extent of its incorporation. Loricrin is a highly insoluble protein which has been detected in granules (L granules) within the stratum granulosum (Steven et al., 1990; Ishida-Yamamoto et al., 1993, 1996). This insolubility has been partly attributed to disulfide bonds (Mehrel et la., 1990). Incorporation of loricrin occurs within the transition zone after its release from L granules, although there is speculation surrounding its translocation and crosslinking into the CE is due to its insolubility.

Kalinin et al., suggested that loricrin coupling to highly soluble proline rich proteins may modify its solubility (Kalinin et al., 2002). Loricrin can be cross linked *in vitro* by TG1, 3 and 5. TG1 predominantly catalyses intermolecular cross links whereas TG3 promotes intra molecular bonds between favoured Lys and Gln residues and is unable to form the polymers observed with TG1 action (Candi et al., 2001). Significantly, loricrin has been reported to accumulate in transgenic $TG1^{-/-}$ mice, potentially indicating this enzymes' role in incorporating this protein into the CE (Matsuki et al., 1998).

1.9.1.3 Small Proline Rich Proteins.

The SPR proteins comprise of a 14 member multigene family (Tesfaigizi and Carlson, 1999) comprising several proline rich repeats flanked by N and C terminals rich in Pro, Gln and Lys (Gibbs et al., 1993). The Gln and Lys amino acids present allow the SPR proteins to participate in cross linking and it has been suggested that these proteins function as bridges between CE components (Steinert et al., 1998). Both TG1 and TG3 are capable of using SPR1 as a complete substrate in vitro however TG2 cross links SPR1 poorly (Candi et al., 1999). Nevertheless, different residues are targeted by the isoforms and it would seem that the activity of both enzymes is necessary for the formation of oligomers.

1.9.1.4 Cystatin and Elafin.

These precursors contribute as minor components of the CE structure (Takahashi et al., 1994) although there has been some speculation as to their physiological role. It has been hypothesised that members of the cystatin family and elafin may regulate protease activity required for envelope maturation (Takahashi et al., 1994). Cystatin

A is a protease inhibitor and elafin functions as a potent inhibitor of elastase and proteinase 3. Furthermore, mutations in Cystatin m/E another cystatin family member has been shown to be associated with disturbed cornification and subsequent impaired barrier formation (Zeeuwen et al., 2002; 2004).

1.9.1.5 Filaggrin.

Filaggrin is synthesised as a pro form containing 2 Ca^{2+} binding EF hand motifs (Markova et al., 1993). Synthesis as a pro form is characteristic of other envelope precursors such as S100 proteins (Donato 1999). The processed form of filaggrin has been shown to be involved in both CE formation and in bundling of intermediate keratin filaments (Dale et al., 1978, Mack et al., 1993).

1.9.1.6 Desmoplakin, Envoplakin, Perplakin and Type II Keratins

Keratins are the most abundant protein within the corneocyte, where assembled intermediate filaments are connected to the cell periphery within desmosomal regions (Green and Gaudry, 2000). As the CE scaffold is formed on the cytoplasmic surface of the plasma membrane both desmosomal components such as desmoplakin, envoplakin and periplakin and keratin bundles become incorporated (Steinert and Marekov, 1995; 1997). A Lys residue situated within the N terminus of type II keratins is crucial in the cross linking of this protein by TGs (Candi et al., 1998). Envoplakin and perplakin have been identified as sites of covalently bound lipid (CBL) linkage although not to the extent of involucrin (Marekov and Steinert, 1998).

1.9.1.7 S100 Proteins and Annexin 1.

Both S100A10 and S100A11 have both been found within the CE of normal human keratinocytes (Robinson et al., 1997). They are both members of this Ca^{2+} regulated EF hand motif (helix-loop-helix domain) containing protein family (Donato, 1999). S100A11 has been shown to translocate to the cell periphery following Ca^{2+} stimulation, a process which relies on a tubulin dependent mechanism (Broome and Eckert 2004). S100A11 has been shown to form heterotetramers with annexin 1 including 2 molecules of each component (Rety et al., 2000). Most annexins display Ca^{2+} channel activity *in vitro* (Chen et al., 1993; Benz et al., 1996; Gerke and Moss 2002) although this activity is not seen under normal intracellular conditions. Gerke and Moss suggested it may be possible under oxidising conditions and the more acidic pH observed in epidermal regions that regulates the Ca^{2+} flux (Gerke and Moss 2002).

1.9.2 Regulation of Keratinocyte Differentiation.

Keratinocyte differentiation is still poorly understood, however a combination of signals is believed to trigger keratinocyte differentiation including Ca^{2+} ion concentration and transcription factor activity. Biochemical changes analogous to *in vivo* differentiation can be partly induced in cultured keratinocytes with the phorbal ester 12-0-tetradecanoylphorbol-13-acetate (TPA) or by increasing Ca^{2+} levels in the media (Hennings et al., 1980; Jaken and Yuspa, 1988) although structural changes are only observed when increased levels of Ca^{2+} are applied (Dotto, 2000). A Ca^{2+} gradient is reported to exist *in vivo* where a significant increase in concentration is seen between the mid and upper granular layers (Menon et al., 1992; Forslind et al., 1997). Ca^{2+} can affect the intracellular environment in a number of ways. Ca^{2+}

sensitive receptors may be present in the keratinocyte plasma membrane similar to those seen in parathyroid cells (Herbert and Brown 1995; Bikie et al., 1996). Alternatively increased Ca^{2+} levels may affect cell cell and cell matrix interactions. Ca^{2+} induced human keratinocyte differentiation requires an intracellular Ca^{2+} rise caused by phosphatidylinositol 3-kinase (PI3K)-dependent activation of phospholipase C-γ1 which ultimately results in differentiation (Xie and Bikle, 2007). Furthermore, it has been suggested that differentiation pathways converge to induce p21 expression which arrests the cell cycle resulting in N-terminal differentiation (Wong et al., 2010)

1.9.3 Proposed Mechanism for Cornified Envelope Formation.

The sequence of events leading to CE formation is slowly being determined (Candi et al., 1995; Nemes and Steinert., 1999; Steinert, 2000; Kalinin et al., 2001). Involucrin has been suggested to enrich scaffolds constructed against a background of membrane associated proteins (Eckert et al., 1993; Steinert, 1995; Steinert and Marekov, 1997). Nemes et al (1999b) reported that involucrin spontaneously binds the membrane in a Ca^{2+} dependent manner, and that this initial structure produces deposition sites for other envelope precursors. Involucrin becomes cross linked to envoplakin and periplakin (Marekov and Steinert, 1998) succeeded by the incorporation of SPRs. This amalgen spreads across the cytoplasmic face of the plasma membrane, consequently incorporating desmosomal proteins (Steinert and Marekov, 1995; 1997). Maturation of the scaffold primarily involves the incorporation of loricrin which accounts for the majority of the protein content (Steinert and Marekov, 1995). This proportion increases towards the cytoplasmic face of the envelope indicating its importance in the later stages of the process (Steinert and Marekov, 1995). Studies have indicated that TG3 predominantly

catalyses intra-molecular cross links in contrast to TG1 and its ability to form multimers (Candi et al 1995). Potentially, modifications made by TG3 promote loricrin incorporation by TG1, alternatively TG1 cross links this precursor into the macromolecular structure where it undergoes further modifications by TG3 (Reichert et al., 1993; Eckert et al., 1993; Steinert, 1995). When the TG isoforms are compared for involvement in CE formation it is estimated that loricrin cross links are the result of 65% TG3 activity compared with 35% for TG1 (Candi et al., 1995). Despite the coordinated sequence of events CE generation is both resilient and flexible. If a known precursor is altered there is no overt phenotype seen or one occurs that is quickly compensated for (Yoneda and Steinert 1993, Koch et al., 2000). In the case of loricrin removal in mice, the dry and scaly appearance of neonatal skin is lost within a matter of days. This has led to the "precursor availability" hypothesis suggesting the existence of a compensatory mechanism. Fibroblasts have demonstrated an ability to form pseudo envelopes following the dysregulation of Ca^{2+} However these CE like structures appear to be disordered and non specific (Simon and Green, 1984; Nicholas et al., 2003).

This chapter has introduced the four areas of interest for this thesis. The current knowledge of the structure, function and activity of both the transglutaminase and metalloproteinase families have been discussed and their involvement in the processes of wound healing and the formation of skin. Further chapters indicate investigations into specific members of these families namely transglutaminase 2 and ADAM 17 which may invoke EGFR signalling and consequent cell migration and proliferation.

2.0 Aims of Thesis

1. To establish if and how TG2 regulates keratinocyte motility in a co-culture model of fibroblast and keratinocytes.

2. To ascertain if keratinocyte motility and proliferation is dependent on metalloproteinase activity in a TG2 positive and TG2 null background.

3. To investigate the role of growth factor receptors in keratinocyte motility and proliferation in the presence and absence of TG2.

Chapter 2: Materials and Methods

Contents. **Page.**

2.1 Cell Culture

2.1.1 HCA2 Fibroblast Cell Culture.

Human dermal fibroblasts have been stably transfected with telomerase amphotrophic retrovirus pBABE-hTERT (McSharry et al., 2001) to rescue cells from senescence by expression of the catalytic subunit of human telomerase (hTERT) along with pcDNA3/human TG2 constructs (Bond et al. 1999; Stephens et al., 2004). The resulting HCA2 lines are immortalised but not transformed, are diploid and display characteristic features of normal skin fibroblasts. Transfection with these constructs produced high-level constitutive expression of the transglutaminase TG2 sense mRNA, causing an overexpression of TG2, antisense mRNA which leads to a reduction in TG2 from endogenous expression as well as a TG2 mutant mRNA. The mutant TG2 mRNA allows for a high level of expression of TG2 but it is inactive because the catalytic Cysteine (Cys) residue was replaced by Serine (Ser), generating a cross-linking deficient form of the enzyme (dominant negative for crosslinking function). A clone transfected with an empty vector was also produced as a control (mock-transfected) leading to endogenous TG2 expression from the fibroblasts only. Cultures were seeded from frozen stocks at a density of $1x10^6$ and grown in DMEM supplemented with 10 % FCS, 1% PSG - Containing 10,000 units of penicillin (base), 10,000 μg of streptomycin (base), and 29.2 mg of L-glutamine/ml in 0.85% saline, in a 10 mM citrate buffer and 400 μg/ml Geneticin. Subsequent cell maintenance was carried out through a 1 in four ratio split of the cell culture flask once a week to stop over-confluence and ensure selection of transfected cells. All cells were cultured at 37°C with 5 % CO_2 unless otherwise specified.

2.1.2 Matrix Production.

Fibroblast cells were seeded into a 24 well plate at a density of $1.5x10^5$cells/well (Greiner) and cultured over night in standard HCA2 fibroblast medium for 24 hours. Following two PBS washes fresh HCA2 medium was added without selection agent (geneticin) and matrix secretion stimulated with the addition of ascorbate-2-phosphate (2mM). Cells were allowed to reach hyperconfluence over a period of 48h. Every 48 hours medium was aspirated, cells washed in PBS and fresh HCA2 medium supplemented with ascorbate-2-phosphate (2mM). This was continued for a period of 10 days. Conditioned medium for migration assays was collected on days5, 8 and 10 and frozen. On day 10 medium was removed and matrices were washed twice in PBS. When experiments including live fibroblasts were required matrix was taken immediately for experiment, alternatively if matrix alone was required the matrix was treated by 3 successive freeze thaw cycles. The matrix was then washed with sodium deoxycholate 1% (0.5g sodium deoxycholate) to remove cellular debris and again washed in PBS before experimental setup.

2.1.3 N-Tert Keratinocyte Cell Culture

Immortalised N-Tert human keratinocyte cells (transfected with telomerase reverse transcriptase gene n-tert) were cultured in Solution/Media A containing DMEM/ HAMS F12 (67.5/22.5%v/v) supplemented with 10% v/v FCS, 0.089mM adenine, 5ng/ml insulin, 400ng/ml hydrocortisone, 10ng/ml EGF, 10^{-10}M cholera toxin, 1% antibiotic/antimycotic containing 10,000 units of penicillin (base), 10,000 μg of streptomycin (base), and 25 μg of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulfate, and amphotericin

B as Fungizone® Antimycotic in 0.85% saline. Keratinocyte cultures were seeded from frozen stocks at a density of $1x10^6$ and subsequent cell maintenance was carried out in a 1 in ten ratio split of the cell culture flask weekly to stop overconfluence. Alternatively for migration experiments where inhibitors were added keratinocytes were cultured using defined serum free medium, a patent protected premade medium containing defined growth promoting additives including Human recombinant Epidermal Growth Factor (EGF 1-53) and Bovine Pituitary Extract (BPE) (Invitrogen).

2.1.4 Generation of Keratinocyte Spheroids and PKH26 Labelling

Immortalised N-tert keratinocytes cultured in FAD medium (65 % v/v DMEM, 22.5 % v/v HAMS F12, 10 % fetal calf serum (FCS), 400 ng/ml hydrocortisone, 10^{-10} M cholera toxin, 10 ng/ml EGF, 0.089 mM Adenine, 5 ng/ml Insulin and 1% Antibiotic-Antimycotic containing 10,000 units of penicillin (base), 10,000 µg of streptomycin (base), and 25 µg of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulfate, and amphotericin B as Fungizone® Antimycotic in 0.85% saline) were grown to 80% confluence. $1.25x10^5$ keratinocyte cells were labelled using fluorescent PKH26 dye (Invitrogen). PKH26 dye was prepared and staining was achieved by resuspending the $1.25x10^5$ keratinocyte cells into 25µl of Diluent C. 50µl of PKH26 dye was then added to this suspension (10% final volume). Keratinocytes were incubated for five minutes at room temperature before the reaction was stopped with 50µl of FCS. Cells were pelleted by centrifugation (1500g, 5min) before being resuspended in 50µl of normal FAD medium as per manufacturer's protocol. The keratinocytes were then added to 10ml of 30% methylcellulose (100% stock

of methylcellulose solution was prepared by adding 100ml FAD keratinocyte medium pre-warmed to 60°C, to 1.33g methyl cellulose – Sigma). The solution was agitated at room temperature for 1 h using a magnetic stir bar, followed by agitation overnight at 4°C. The solution was cleared by centrifugation at 4,000 x g for 90 min and the supernatant retained as 100% stock solution). Methylcellulose had been prepared by the addition of 10.5mls of 100% methylcellulose being added to 35mls of complete FAD medium. This was sterile filtered through a 0.2μm filter. The keratinocytes were then added to the 30% methylcellulose and mixed gently, this was then placed into a trough for ease of dispensation and a multi-well pipette was used to pipette 100μl of the suspension (2500 cells) per well and then left overnight in a hydrophobic microtitre round bottomed 96 well plate to produce spheroids (Greiner).

2.1.5 CalceinAm Fluorescent Labelling

Previous experiments conducted by Dr Sally Rosser-Davies (PhD thesis-2006) had used CalceinAM green (Molecular Probes) to label keratinocyte spheroids. CalceinAM is converted to green-fluorescent calcein, after acetoxymethyl ester hydrolysis by intracellular esterases. This was abandoned in favour of the PKH26 dye method, as PKH26 had the advantage of overcoming photo bleaching and subsequent reduction in cellular fluorescence seen when hourly photographs were being taken instead of the 24 hourly photographs taken previously. A brief summary of the method follows; FAD medium was supplemented with CalceinAM green at a concentration of 5μM. 1ml of FAD medium was supplemented with 2μl of CalceinAm green and added to 125,000 cells. This

suspension was left for 30 minutes at 37^0C before being added to 30% methylcellulose as seen with the PKH26 dye.

2.1.6 CHO Cell Culture

Chinese Hamster Ovary (CHO) cells stably transfected to overexpress G protein coupled receptor 56 (GPR56) were cultured from frozen stocks donated from Dr Vera Knauper. CHO cells were cultured in HAMS F12 (Invitrogen) supplemented with 10% FCS (Invitrogen), and 100μg/ml Hygromycin (Invitrogen) to ensure selection of transfected cells. CHO cells transfected with L-selectin gene either complete (WT) or delta stalk were used as a control cell line for GPR56 expression experiments and were cultured in the same medium but also containing 100μg/ml zeomycin (Invitrogen).

2.1.7 Primary Human Fibroblast Cell Culture

Human primary oral gingival and periodontal ligament fibroblasts were cultured in DMEM supplemented with 10 % FCS and 1 % Penicillin, Steptomycin, Glutamine_(PSG)- Containing 10,000 units of penicillin (base), 10,000 μg of streptomycin (base), and 29.2 mg of L-glutamine/ml in 0.85% saline, in a 10 mM citrate buffer as previously described for HCA2 cell lines minus the selection agent geneticin.

Primary cells were a kind gift from Dr. Matthew Locke (Dental School, Cardiff University). Cells were derived from patients undergoing tooth extraction or alternative dental procedures and had gone through 2 population doublings.

2.1.8 Primary Murine Fibroblast Extraction and Culture.

TG2 wild type and knockout mice aged 3-9 months were euthanized, shaved and skin removed from their backs by a rectangular incision. The removed skin was cut into 3 pieces (1cm by 2cm) and placed in chlorhexidine mouthwash for 5 minutes. One piece of skin was minced and placed into a T25 flask and allowed to air dry for 5-10 minutes. Prior to mincing the second sample underwent an additional treatment with trypsin (Sigma) for 5 minutes before also being minced and placed into a T25 flask, and allowed to air dry for 5 minutes. The flasks were then filled with primary fibroblast medium (DMEM, 10% FCS, 5ml Antibiotic/antimycrotic; previously described), and care was taken not to disturb attached cells. Gingival primary fibroblasts were also cultured from the mice. These were obtained from the upper gum line hard pallet of the roof of the mouth. This small area of tissue was cut from the mouse and treated by placing it into chlorhexidine mouth wash, minced and placed into a T25 flask and left to air dry. Cultures usually took approximately a week to ten days to show signs of new cell growth. Fibroblasts were used for experiments after 1-2 passages and subsequently removed from culture to avoid senescence.

2.1.9 Cryopreservation and Retrieval of Cells

Following trypsinisation $5x10^5$ cells were pelleted by centrifugation (1500rpm, 5 min) and resuspended in freezing medium (10% DMSO (v/v), 20% FCS (v/v) and 70% (v/v) standard cell medium). Cells were then placed into a quick freezing box and placed in a -80^0C freezer overnight. These cells were then transferred to liquid nitrogen for storage.

2.1.10 Mycoplasma Testing

Cells were seeded (2 x 10^5) onto circular glass cover slips (13mm) within a 24 well plate. Following overnight culture the cells were fixed with two successive methanol washes (500µl each). Hoechst 33258 stain (Sigma) was applied (at a final concentration of 0.05 µg/µl in ddH_20) for a period of 15 minute at 37°C. Following extensive washing with ddH_2O, fluorescence staining was visualised through a DAPI 505 nm filter. This analysis was carried out using a Carl Zeiss Axiocam camera linked to an Axiovert 200M Zeiss microscope. Hoechst staining reveals genomic DNA of both eukaryotic and prokaryotic cells.

2.1.11 Keratinocyte Migration in a Co-Culture Model:

2.1.11.1 Spheroid Migration

Migration assays were carried out by pipetting a 50µl of 30% methylcellulose containing a single spheroid per well onto matrix derived from HCA2 fibroblasts in the presence of FAD medium either alone or mixed 1:1 with conditioned medium or appropriate inhibitor in chapters 3 and 4. Placement of the spheroid in the centre of each well was confirmed manually by phase contrast microscopy. Experimental samples were restricted to spheroids attaching to the centre of each well. Chapters 3 and 4 required FAD or defined serum free medium alone, with DMSO or plus inhibitors (see table 6) due to the previous medium set up interfering with action of the various included inhibitors. The results sections of chapters 3 and 4 will indicate where medium other than standard FAD medium has been used. Time lapse microscopy was carried out for 30 hours with both bright field and fluorescent images being taken every 1 hour. Pictures were taken using a Carl Zeiss Axiocam camera linked to an upright Axiovert 200M Zeiss microscope. Improvision Openlab^{TM} 4.1.2 software controlled the time-lapse microscopy and collected the images.

2.1.11.2 Inhibitors and Antibodies used in Migration Assays.

Inhibitor	Source/Manufacturer	Final Concentration
GM6001	BIOMOL International	50μM in DMSO
TAPI 1	BIOMOL International	10μM in DMSO
GI254023X (ADAM10)	GlaxoSmithKline	10μM in DMSO
GW280264X (ADAM17/10)	GlaxoSmithKline	10μM in DMSO
Cub7402 (Monoclonal)	Neomarkers	1μg/ml in FAD/DSFM.
I15 TG2 Inhibitor	Zedira GmbH	25mM in ddH_20.
TIMP1	Produced by V Knauper Kind Gift.	100nM in ddH_20.
TG2	made in house	0.01-10ug/ml in FAD/DSFM
EGF	Sigma	10μg/ml in ddH_20
AG1478 (EGFR)	Calbiochem	10μM in DMSO
AG1024 (IGFR)	Calbiochem	10μM in DMSO
AG1295 (PDGFR)	Calbiochem	10μM In DMSO
CRM197 (HB-EGF)	Merck/Calbiochem	50μM in ddH_20
Mouse IgG	Jackson	10μg/ml in ddH_20
DMSO	Sigma	(v/v) in FAD/DSFM

Table 6: A list of inhibitors and antibodies used during spheroid migration assay.

2.1.11.3 Analysis of Keratinocyte Migration.

Black and white images were analysed using CTAn.exe software (SKYSCAN©). Following selection of the region of interest, the threshold value was set to mark migrating cells only. This image was then converted to a binary image and examined to see the binary image lined up correctly with the furthermost migrating cells. The same threshold setting was used for all images within an experiment. SkyScan performed algorithmic analysis to generate a quantitative value for cell migration as detailed in chapter 3 figure 15.

2.2 Protein Analysis

2.2.1 Ethanol Precipitation of Protein

Protein solutions were precipitated by the addition of 9 volumes of ethanol (24 h, -20°C). Following pelleting (1500g, 30 minute, 4°C), proteins were resuspended in 8M urea and spun at 1500g for 5 minutes before the urea was removed and substituted for SDS sample loading buffer containing 25 mM Tris (pH8.3), 39 mM EDTA, 4 % w/v SDS, 30 % v/v glycerol, 0.3 % w/v bromophenol blue, supplemented with a 1:50 dilution of β-Mercaptoethanol (Sigma) and boiled for five minutes before analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting.

2.2.2 BCA Assay

To normalise sample loading, protein concentrations were established by bicinchoninic acid (BCA) protein assay (Pierce Chemical Co.) according to the manufacturer's protocols. A bovine serum albumin (BSA) dilution series was

included as a standard. 10µl of sample along with 10µl of control buffer was compared with the BSA dilution series at 540nm. Protein concentration was calculated out to give a 10µg protein in 12µl sample to be run by western blot.

2.2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Each protein sample (10-12µl) was mixed with an equal volume of 1 x SDS sample loading buffer and boiled for 5 minutes prior to loading onto a pre-cast 4-20% Tris-Glycine gel, 1.5 mm x 15 well (Invitrogen). Both reservoirs contained 25 mM Tris HCl (pH 8.3), 192 mM glycine, 0.1 % w/v SDS. Proteins were resolved over a period of 2 h 125 V, 25mAmps.

2.2.4 Western Blotting

Protein was electrophoretically transferred from the SDS page gel to a Protran® nitrocellulose membrane (Schleicher & Schuell) under a current of 125 mA for 2 h in the presence of transfer buffer 25mM Tris (pH8.3), 192 mM glycine, 20% methanol v/v. Protein transfer was assessed using ponceau S staining 5% acetic acid v/v, 0.1% w/v ponceau S. Non-specific protein binding sites were blocked by an overnight incubation (4°C) with 5 % w/v non-fat dry milk powder (Marvel) in Tris Buffered Saline (TBS) 20 mM Tris base, 137 mM NaCl, (pH 7).

All antibodies used to probe Western blots were diluted in TBS with 5 % milk powder (See table 7). All secondary antibodies used were horse radish peroxidase (HRP) conjugated. Unless specified otherwise incubations with primary and secondary antibodies were carried out at room temperature (RT) under agitation for 1 hour. Each incubation step was followed by three 5 minute

washes with TBS containing 0.05 % v/v Tween-20 (Sigma). Protein levels were detected by chemi-luminescence produced following 1 minute incubation with ECL plus Western blotting detection reagent (Amersham Pharmacia) before exposing to Hyperfilm™ ECL™ film (Amersham Pharmacia) for up to 20 minutes.

2.2.4.1Antibodies used in Western Blotting.

Primary Antibody (Monoclonal/Polyclonal- M or P)	Raised In	Dilution/Concentration	Source
TG2 (CUB7402) (M)	Mouse	1:200-1:1000	Neomarkers
ADAM17 (P)	Rabbit	1:200-1:1000	Bioscience
ADAM10 (P)	Rabbit	1:200-1:1000	Bioscience
GPR56 (P)	Sheep (Cross reactive-goat)	0.5-1.0μg/ml	R and D Systems
TGFα (P)	Goat	0.1-0.2μg/ml	R and D Systems
HB-EGF (P)	Goat	0.1-0.2μg/ml	R and D Systems
EGF (M)	Mouse	1-2μg/ml	R and D Systems
V5(M)	Mouse	1:5000-1:10000	Invitrogen
GAPDH (M)	Mouse	1:1000 – 1:5000	Sigma
GPR56 (P)	Rabbit	1:200-1:1000	Abcam
GPR56N-15(P)	Goat	1:200-1:1000	SantaCruz
GPR56N-19(P)	Goat	1:200-1:1000	SantaCruz
Secondary Antibody	Raised in	Dilution/Concentration	Source
Anti-Goat HRP conjugated	Rabbit	1:1000	DAKO
Anti-Mouse HRP conjugated	Rabbit	1:1000	DAKO
Anti-Rabbit HRP conjugated	Mouse	1:1000	DAKO
Anti-Mouse non specific IgG	Mouse	1:1000	Jackson

Table. 7 Summary of antibodies utilised in Western blot Experiments.

2.2.5 Membrane Stripping

In order to remove antibodies from membranes for re-probing, the nitrocellulose was incubated in stripping buffer 1 M Tris HCl (pH 8.3), 10 % w/v SDS, 0.07 % 2-mercaptoethanol for 30 minute at 50°C under gentle agitation. This was followed by three washes with TBS containing 0.05 % v/v Tween-20 and three sequential washes with TBS (5 minute each).

2.2.6 Immunohistochemistry on scratch wounded keratinocytes.

100,000 Keratinocyte cells were plated onto collagen coated sterile cover slips in a 24 well plate and allowed to reach 80% confluence in complete FAD medium overnight. The following day these cells were scratched using a sterile pipette tip 200µl yellow tip (Greiner) followed by a medium change to remove detached cells and their debris. After a 0, 6, 12, 18 or 24 hour incubation period medium was removed from all samples and cells were washed twice with PBS. The cells were then fixed in 4% paraformadehyde in PBS for 10 minutes. Cover slips blocked with 1% fraction 5 BSA (Sigma) took place for 1 hour at room temperature followed immediately by incubation with GPR56 antibody (R and D systems) at a dilution of 1µg/ml at room temperature for one hour. The primary antibody was removed and 3 PBS washes ensured complete removal. The secondary FITC conjugated anti goat antibody (DAKO) which recognised sheep IgG was added at a 1:80 dilution and left for one hour at room temperature followed by a further 3 washes in PBS. Cover slips were then mounted onto slides using fluorescent mounting reagent (DAKO). GPR56 was visualised using the time lapse microscope as previously described in section 2.1.11.3

2.3 Gene Expression and Molecular Biology Methods

2.3.1 Agarose Gel Electrophoresis

DNA samples were mixed with 6X sample buffer 0.05 % w/v bromophenol blue, 6 % v/v glycerol, 12 mM Ethylenediaminetetraacetic acid (EDTA), (pH 8). Fragments were separated through 1-1.2 % agarose (Invitrogen) gels in 1X (TAE) buffer [40 mM Tris acetate, 2 mM Na_2EDTA, (pH 8.5)] supplemented with 0.1 μg/ml ethidium bromide (Sigma). A 300ng 1KB ladder was also separated for calibration of the gel. Electrophoresis was carried out at a constant voltage (100 V) before the DNA was visualised by exposure to an Ultra Violet (UV) light source.

2.3.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse transcription was carried out using Superscript II system (Life technologies). 1μg total RNA was extracted from HCA2 fibroblast cultures using TRIzol (Invitrogen). A total of four 10cm dishes of each cell type were lysed and following the addition of 10 % v/v chloroform, RNA was isolated in the aqueous layer using an Eppendorf Phase Lock Gel^{TM} (12000 x g, 30s). RNA within the aqueous fraction was precipitated in an equal volume of isopropanol and following washing with 75 % ethanol, the pellet was resuspended by heating in 500 μl nuclease free water (65°C) and stored at -80^{0}C. Total RNA was included in a reaction mixture which contained 25ng oligo dT primer (invitrogen), 0.5mM dNTPs, 10nM DTT, and 100 u DNase H Reverse Transcriptase in supplied buffer (250mM Tris-HCl, 375 mM KCl, 15mM $MgCl_2$, [pH8.3]). This reaction took place for 50 minutes at 42^{0}C before the enzyme was inactivated by heating to 90^{0}C. In order to gain maximum yield of cDNA a second transcription was

carried out with a further 100 U of reverse Transcriptase. To confirm TG expression in fibroblast cell lines 1µl of the resulting cDNA was subjected to PCR with specific TG2 primers (table 8). Amplification was carried out in a total reaction volume of 25µl (1 x Taqman buffer A, 2mM $MgCl_2$, 0.2mM dNTP, 0.125 U Taq DNA Polymerase (Promega) and 1µl of each primer. The following conditions were used; 35 cycles of 45 seconds at 95^0C (denaturation), 1 minute at 60^0C (annealing) and 1 minute 30 seconds at 72^0C (extension). The first cycle contained an extra step to activate Taq polymerase (95^0C for 2 minutes) and the final cycle contained an extended extension period 72^0C for seven minutes. Generated PCR products was then resolved through a 1% agarose gel for evaluation.

2.3.3 Restriction Digestion of DNA.

Confirming sequence identity restriction digestion took place using various restriction enzymes. 2µl of PCR product was added to 1µl of appropriate restriction digestion buffer, 1µl of 10xBSA, 0.5µl restriction enzyme and remaining volume made up to 10µl with H_20. Reactions were prepared in eppendorf tubes and left at 37^0C for 90 minutes. Subsequent products were run on a 1% agarose gel, for analysis.

2.3.4 Quantitative Polymerase Chain Reaction (QPCR)

PCR core reagent kit came from Applied Bioscience (Roche, New Jersey). Quantitative PCR (QPCR) probes were synthesised at PE Life Sciences incorporating a 5' 6-carboxyfluorescein (FAM) reporter and a 3' 6-carboxytetramethylrhodamine (TAMRA) quencher. QPCR conditions to assay

TG2 had been optimised in house (Stephens et al., 2004). All assays were carried out in triplicate with a reaction mixture of 25µl (containing 1 x Taqman Buffer A, 2mM $MgCl_2$, 0.2nM dNTP, 0.125U Amplitaq gold DNA polymerase). Primer and probe sequences are summarised below (table 8). Conditions were as follows 40 cycles of 15 seconds at 95^0C (denaturation), 1 minute 60^0C (annealing/extension) with the first cycle containing an extra step to allow activation of the polymerase enzyme (95^0C for 10 minutes). Reactions were carried out in an ABI PrismTM 7000 detection system (Applied Bioscience). The 2-ΔΔCT method was used to calculate relative levels of RNA expression, normalised to housekeeping gene expression (H306B4).

Gene Product	Forward Primer	Probe	Reverse Primer	Concentration (nM) (F,P,R)
TG2	5'ATGAGAAATAC CGTGACTGCCTTA C	5'AGCTACCTGCTG GCTGAGAGGGACC TC	5'CAGCTTGCGT TTCTGCTTGG	300, 150, 300
H306B4	5'AGATGCAGCAG ATCGCAT	5'AGGCTGTGGTGC TGATGGGCAAGAA C	5'ATATGAGGC AGCAGTTTCTC CAG	300,150,300

Table 8: TG2 primers produced for QPCR.

2.3.5 Preparation of Competent E. Coli.

DH5α E.Coli were cultured in LB medium until an OD650 nm of ~0.35 was measured using Beckman Coulter DU® 800 spectrophotometer and then incubated on ice. After 30 minutes the *E.Coli* were collected by centrifugation (1600 x g, 8 minutes, 4^0C) and the pellet resuspended with 10ml ice cold 100µM $MgCl_2$. To induce competency, the cells were collected by centrifugation and incubated in 2mls of 100mM $CaCl_2$ over 16 hours. The *E. Coli* were then flash frozen in 200µl aliquots in a 25% glycerol solution ready for future transformation experiments.

2.3.6 Transformation of E. Coli

Competent E Coli DH5α were thawed on ice and then incubated with 1-5μl of plasmid DNA (TGFβ plasmid (generous gift Dr D Fraser, Nephrology Cardiff University and Luciferase/Renilla Dual Glow Plasmid –Promega) in the presence of 20nM β-mercaptoethanol (Invitrogen) for 30 minutes at 4^0C. The cells were then heat shocked at 42^0C (exactly 30 seconds) before culturing for 1 hour at 37^0C 225 rpm with 800μl SOC medium (Invitrogen) to ensure return of cell integrity. 50 or 100μl of transformation reaction was spread onto LB agar plates containing 50μg/ml ampicillin and left overnight at 37^0C. The following day 3 colonies were picked from each plate and expanded in 3mls of LB medium and 50μg/ml ampicillin overnight at 37^0C while shaking at 225rpm before being prepared via miniprep kit (Qiagen) for experiments.

2.3.7 Preparation of plasmid DNA.

Bacteria from overnight cultures were collected by centrifugation at 5000g. Mini-preps of DNA were prepared using a Qiagen miniprep kit and protocol was carried out as per manufacturer's protocol. DNA was finally diluted with 50μl of ddH_20 and stored at -20^0C.

2.3.8 SiRNA (small interfering RNA) mediated knockdown of GPR56.

2.3.8.1 Dharmacon SiRNA Transfection Protocol.

GPR56 expressing CHO cells or N-Tert immortalised keratinocyte cells were counted and seeded at $5x10^4$ cells per well of a 12 well plate (Greiner) and left overnight in complete growth medium. The following day the cells were transfected. 4μl of silencer select SiRNA (either GPR56 variant 1, variant 2) or

scrambled negative control (Ambion) (Table 8a) was added to 46µl of SiRNA buffer containing 2.2% potassium chloride (Fisher), 1% Hepes free acid (Sigma) 0.02% magnesium chloride $6H_2O$ (Sigma) in RNase free water added to 2.0M potassium hydroxide (Dharmacon) For both SiRNA variants to GPR56 and the scrambled negative control in RNAase free eppendorf tubes. In addition an extra tube to be transfected with either ADAM17 or ADAM10 siRNA (Ali and Knauper, 2007) as a further transfection control was prepared by adding 2µl of ADAM10/17 SiRNA to 48µl of SiRNA buffer. The SiRNA and buffer mixture was added in the presence of 50µl of serum free, antibiotic free medium. In addition a master mix was prepared containing 99µl per sample of serum free and antibiotic free medium with 2µl per sample of Dharmafect transfection reagent (Dharmacon). The buffer/SiRNA/ transfection agent mixture was left for 5 minutes at room temperature. 99µl of the master mix was added into each tube containing siRNA GPR56 variant 1 or 2, scrambled negative control or ADAM17/10. After mixing the SiRNA transfection complex was left for 20 minutes at room temperature and then added drop wise to cells and mixed side to side – by rocking the plate. While the samples were incubating, 500µl complete growth medium was replaced in each well of the 12 well plate (Greiner). The transfected cells were left for 48 hours before the process was repeated. After the second transfection cells were harvested using 400µl of cell lysis buffer containing 1% sucrose/triton by scrapping over ice and BCA assayed to determine protein concentration prior to Western blot analysis.

	Sense	Antisense
Variant 1	AGCCUGGAGUCGAAACUGATT	UCAGUUUCGACUCCAGTCUU
Variant 2	CCGACAUGCUGGGAGAUUATT	UAAUCUCCCAGCAUGUCGGTT

Table 8a: GPR56 Silencer Select SiRNA (Ambion) Sequences for variants 1 and 2 used in both transfection protocols.

2.3.8.2Interferin Polyplus siRNA Transfection Protocol

N-Tert immortalised keratinocyte cells or stably transfected CHO cells overexpressing GPR56 were counted and seeded at $5x10^4$ cells per well of a 12 well plate (Greiner) and left overnight in normal culture medium as per Dharmacon protocol. The following day 4µl of GPR56 silencer select SiRNA GPR56 variant 1, 2 or scrambled negative control (Ambion) (shown in table 8a) were added to 500µl of serum free antibiotic free medium in RNA free eppendorf tubes. 2µl of Interferin reagent was added to each tube and the tube was vortexed immediately for ten seconds; then left to incubate for a further ten minutes at room temperature. While the samples were incubating, medium was replaced in each well of the 12 well plate (Greiner) with 500µl of fresh complete growth medium. Each SiRNA mixture was added to the cells drop wise and the plate swirled. The samples were then left for 48 hours to transfect before being analysed by cell extraction and western blot.

2.3.9 MTT Test for Cell Viability and Proliferation.

An 80% confluent flask of keratinocytes was washed using PBS. Following trypsinisation, 5000 keratinocyte cells were seeded per well of a 24 well plate (Greiner) and left in complete FAD growth medium for 6 hours at 37^0C, 5% CO_2. After 6 hours the medium was removed and the cells washed twice in PBS. This time was determined to be sufficient for cells to adhere and spread.

Depending on the experiment to be performed different growth media was used. Normally a basic keratinocyte medium made up of solution A Solution/Media A containing DMEM/ HAMS F12 (67.5/22.5%v/v) supplemented with 10% v/v FCS, 0.089mM adenine, 400ng/ml hydrocortisone, 10^{-10}M cholera toxin, and 1% antibiotic/antimycotic containing 10,000 units of penicillin (base), 10,000 μg of streptomycin (base), and 25 μg of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulfate, and amphotericin B as Fungizone® Antimycotic in 0.85% saline was added. Proliferation assays were carried out with multiple conditions with either inhibitors or activators and matching controls. Various inhibitors were used to determine the effects of inactivating signalling components implicated in keratinocyte proliferation and migration. Furthermore, the effects of these inhibitors were investigated in the presence of TG2 (10μg/ml) as well as activators such as insulin (5ng/ml) and EGF (up to 50ng/ml) where appropriate in specific proliferation assays. Each inhibitor was added at range of concentrations indicated in table 9. Initially samples of a known cell number were analysed between 0 and $1.2x10^6$ to establish the linear range of the assay and determine the junction correlating cell number with absorbance. When an experiment was set up to determine the effect of an inhibitor cells were analysed at two time points (set of replicate plates), 24 h and 72h, to determine the change in cell number and from this calculate the growth rate. Triplicates of test and appropriate control conditions were induced on the same plate. Growth rate in the presence of vehicle only was then compared to that in the presence of inhibitor only. The effect of conformational arrangement of TG2 was also evaluated by proliferation assay. The variants of TG2 investigated were TG2, TG2 activated by calcium, TG2γs, TG2-GTP, and TG2I-

15. These experiments were set up in the same way, for plate preparation. 5000 keratinocyte cells per well, were seeded and left for 6 hours in complete growth medium. After this time the keratinocytes were washed and the basic 1% keratinocyte medium was added. Previously, proliferation assays had been conducted where inhibitors in the presence or absence of TG2 were added to the basic keratinocyte medium. For TG2 conformational experiments keratinocyte cells were either left in basic 1% keratinocyte medium as a control or a concentration of a particular conformational type of TG2 was added. This allowed the investigation of the effects of the particular TG2 conformation on proliferation.

When the 24 hour time point or 72 hour time point was reached the medium was removed from all plates, and the cells washed twice in PBS. 100µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Invitrogen) was added at 5mg/ml per well and plates left to incubate for 4 hours at 37^0C, 5% CO_2. MTT reagent (Invitrogen) was removed from each well on the plate after this time. 400µl of MTT Lysis buffer containing 20g SDS, 50ml NN-Dimethylformamide, 50ml H_2O plus 2.5% acid mix 2.5ml 1M Hydrochloric acid, 80ml Acetic acid and 17.5ml ddH_2O was added per well and the plate left overnight at room temperature covered with saran wrap. The following day cell extracts from each well was transferred to an eppendorf tube and spun at 1500g for 5 minutes before being diluted 1 to 1 with ddH_20. The mixture was cleared by a further centrifugation step. Absorbance readings were measured using a DU800 Spectrophotometer (Beckman Coulter) at wavelengths of 570 and 650nm for each sample. 650nm is outside of the main absorbance peak of the dye and was used as a means to identify samples with abnormal readings (light scattering).

Data was processed by subtracting the 650nm result from 570nm value, and averages ascertained based on 3 replicates per condition further calculations (detailed p130) indicated change in cell number and standard error of the mean calculated.

2.3.9.1 Inhibitors and antibodies used in MTT Assays.

Inhibitor	Source/Manufacturer	Range of concentration.
GM6001	BioMOL	5.6-50μM in DMSO
TAPI 1	BioMOL	1-30μM in DMSO
GI254023X (ADAM10)	GSK	1-30μM in DMSO
GW280264X (ADAM17/10)	GSK	0.1-30μM in DMSO
I15 TG2 Inhibitor	Zedira GmbH	0.1-10μg/ml in ddh_20
TG2	Made in house	0.01-10μg/ml in ddh_20
EGF	Sigma	10μg/ml in ddh_20
AG1478 (EGFR)	Calbiochem	0-30μM in DMSO
AG1024 (IGFR)	Calbiochem	0.11-10μM in DMSO
AG1295 (PDGFR)	Calbiochem	0.33-30μM in DMSO
CRM197	Merck/Calbiochem	2- 50μM in ddh_20
PD173074(FGF/VEGFR)	Merck/Calbiochem	3.75-30μM in DMSO
DMSO	Sigma	(volume /volume)
Anti TGF (P)	R and D systems	10μg/ml in ddh_20
Anti HB-EGF (P)	R and D systems	10μg/ml in ddh_20
Anti EGF (M)	R and D Systems	10μg/ml in ddh_20

Table 9: A list of inhibitors and antibodies used during MTT proliferation assays.

Proliferation values were calculated as relative growth rates compared to control, where a suitable control of either basic 1% FAD medium minus insulin and EGF was placed with DMSO (v/v), BSA (50μM) or alone to compare growth rates. A calibration graph was constructed to process the 570nm readings from both fibroblasts and keratinocytes to determine an equation to calculate cell number. Cell number was then calculated as follows:

=1208100/ (17.58/A-1) where A is the OD570nm for 24h

=1208100/ (17.58/B-1) where B is the OD570nm for 72h

24h cell number result was subtracted from the 72 hour cell number to determine the change in number of cells over 48 hours. This was then expressed relative to control where the change in cell number sample was divided by the change in cell number of control. Finally the relative proliferation in the control group was set to 1 and the cell number from the testing condition was expressed relative to that.

2.3.9.2 Production of Recombinant Human Transglutaminase and its Variants.

Complementary DNA encoding TG2 was sub cloned into a prokaryotic expression vector PJOE2702. A His_6 tag was added to the native sequence N terminally for purification of the recombinant protein by Ni^{2+}-chelating affinity chromatography. E Coli BL21 transformed with the expression construct was grown in LB broth in baffled flasks at 37^0C and 220rpm to OD_{600} of 0.6 before chilling to 20^0C and induction of transgene expression by addition of rhamnose to a final concentration of 0.5%. After incubation for a further 24 hours at 20^0C, bacteria was collected by centrifugation at 3000g for 20 minutes, resuspended in

buffer A (50mM Na_2HPO_4, (pH8), 300mM NaCl) to obtain a 15% cell suspension, and the expressed protein harvested by lysis of the cells using a French Press (1000psi). The lysate was cleared from insoluble material by centrifugation at 11,500g for 30 minutes and applied to a 1ml His-trap HP column (Amersham Bioscience) equilibrated in buffer A at 4^0C and a flow rate of 0.5ml/minute. The resin was washed initially with buffer A until OD_{280} of less than 0.005 was reached, and then with 100ml of buffer A containing 30mM imidazole before elution of His-tagged protein with buffer A containing 150mM imidazole. Eluted protein was dialysed (Spectra/Por4; Spectrum Laboratories) extensively against buffer B (20mMTris/HCL [pH 7.2]), 1mM EDTA, 100mM NaCl) When required enzyme was purified further by ion exchange chromatography using a HR10/10 column packed with Resource Q10 (Amersham Bioscience) whereby TG2 was eluted as a single sharp peak within a 20 volume gradient of 0.1-0.7M NaCl. Enzyme was dialysed further in buffer B and concentrated to approximately 2mg/ml using centriprep-YM30 (Amicon) concentrators and stored at -20^0C (Hadjivassilious et al., 2008).

For either GTP, GTPγS (nucleotide) or I15 inhibitor treatment 0.5mg TG2 was diluted to a final concentration of 1 mg/ml in PBS containing 5mM $MgCl_2$. For nucleotide treatment TG2 was incubated with a 10 molar excess of either GTP or GTPγS for 30 minutes at 4^0C. 100µl of 2.5mM Inhibitor I15 (Zedira) was added to 0.5mg TG2 and incubated for 30 minutes at 25^0C. After nucleotide/inhibitor treatment unbound nucleotide/inhibitor was removed using a PD10 column (GE Healthcare) and treated TG2 pooled and quantified by absorbance (Rose et al., 2006). Ca2+ loaded TG2 was prepared by the addition of 1mM calcium to 50µg of TG2 before each individual experiment involving this TG2 preparation.

2.3.10 TG2 Activity Assay.

A master mix containing 0.1M Tris, HCl, 5mM CaCl2 (pH8.3), 5mM DTT, 40μM Monodansyl Cadaverine, and 10μg N,N-dimethlcasein/ sample, was prepared. (200μl final volume per reaction). Wild type TG2 or TG2 treated with I15 was added into the mixture at different concentrations (10ug to 0.5μg) and agitated. After vortexing for 10 seconds samples were left at 37^0C for 30 minutes. The reaction was stopped by the addition of Trichloroacetic acid(TCA). Initially a 200μl 50% TCA preparation was added and the samples left for 2 hours at 4^0C. Precipitated proteins were collected by centrifugation at 15000g for 10 minutes. A further 2 final washes using a 1:1 ethanol/ether mix (200μl) by repeated centrifugation were conducted and the resulting pellet was finally resuspended in 15μl of 1:1 sample buffer and 8M Urea and applied for SDS polyacrylamide gel electrophoresis. Proteins were subsequently transferred to nitrocellulose and incorporation of dansylcadavarine into N,N-dimethylcasein revealed by probing with antibodies to the dansyl group diluted 1 in 300 (Aeschlimann et al., 1993).

2.3.11 Statistical Analysis.

Statistical analysis was carried out on both proliferation assays and migration experiments. Migration assays are shown as single representative experiments (3 repeats conducted with similar results) and data presented as the mean +/- SEM of six spheroids. Statistics were calculated using Graph pad Instat for biologists. Repeated measure ANOVA was conducted with either Tukey or Bonferri post statistical tests.

Chapter 3: TG2 and its Role in Re-Epithelialisation.

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3.1 Introduction.

3.1.1 *In Vitro* Models for Re-Epithelialisation and Wound Healing

In order to study the process of wound healing several *in vitro* models have been developed in an attempt to mimic the re-epithelialisation process such as the often used two dimensional scratch wound (reviewed in Rodriguez et al., 2005), or the more complex multiple cell skin equivalent (Nakagawa et al 1989). We have adapted a co culture model developed to investigate endothelial cell differentiation in angiogenesis (Korff and Augustin, 1998) to investigate this process (Rosser-Davies, PhD thesis 2006). As wound healing is a complex process involving multiple cell types and cellular interactions such as an adequate blood supply, fibroblasts, the major cell type within the dermis, epithelial cells, and circulating cells of the immune system it is possible to produce a model incorporating some but not all of these interactions. There must be cell cell and cell matrix interactions that regulate cellular behaviour to re-establish normal architecture and functionality of damaged tissues. Fibroblasts deposit a highly organised and tissue specific specialised extracellular matrix (ECM) which is instrumental in regulating the overlying epithelium (Locke et al., 2007). Previous studies have required keratinocytes to be seeded onto collagen embedded fibroblasts to produce a skin equivalent in which fibroblasts encourage proliferation and differentiation of keratinocytes (El Ghalbzouri et al., 2002). Positive proliferative effects of fibroblasts have been shown in numerous studies (Bell et al, 1981, Prunieras et al., 1983, Asselineau et al., 1986, Xu and Clark., 1996, Florin et al., 2005) indicating fibroblasts are able to stimulate proliferation by the release of interleukins such as IL-6 (Chedid et al., 1994), and growth factors appearing in early stage wound healing such as TGFβ (Wahl et al, 1989) or PDGF (Pierce et al., 1989).

3.1.2 Mesenchymal Control of Keratinocyte Migration.

Fibroblasts have been suggested as being responsible for signals regulating wound healing and therefore keratinocyte migration either by the release of growth factors or the construction of an adequate extracellular matrix (ECM) providing an adhesive and instructive support to migrating cells. Previous studies have concentrated on the release of matrix proteins or soluble factors as contributory to keratinocyte regulation (see Table 10). TGFβ, EGF and MMPs have all been implicated in the regulation of keratinocyte migration. TGFβ suppresses keratinocyte proliferation, but increases their motility in a Smad-dependent manner (TGF β signalling pathway) (Choi et al., 2007). However, conflicting data has been shown that TGFβ in fact increases proliferation of keratinocytes especially during the later stages of wound healing (Zambruno et al., 1995). EGF has also been shown to be up-regulated during early wound healing and can increase keratinocyte migration (Schultz et al., 1991) as well as contributing to the production of fibronectin in fibroblasts (Mimura et al., 2004) therefore aiding stabilisation of the wound and increasing contraction during healing. Furthermore, additional *in vitro* studies have indicated that keratinocytes have an increased ability to bind fibronectin as a result of TGFβ and EGF stimulation of fibronectin production leading to increased keratinocyte motility on this substrate (Takashima and Grinnell 1985., Nickoloff et al., 1988).

Soluble factors may also regulate signalling by γ kinase receptors such as EGFR to increase keratinocyte proliferation and migration in an acute wound (Martin., 1997) or secretion of proteolytic enzymes such as MMP1 (collagenase) and MMP9 (Gelatinase) which remove collagen and other ECM components damaged during injury, and expose cryptic ECM binding sites.

Factor	Reference
Epidermal growth factor	Schultz et al., (1991), Sarret el al (1992), McCawley et al., (1998), Yamasaki et al., (2003) Takahashi et al., (2009)
Fibroblast growth factor 2	Igarashi et al., (1993),Powers et al., (2000),
Fibroblast growth factor 7	Tsuboi et al., (1992)
Fibroblast growth factor 10	Matsumoto et al., (1991), Tsuboi et al., (1992), Cha et al., (1996), Gibbs et al., (2000)
Granulocyte macrophage colony stimulating factor	Barrandon and Green., (1987), Tsuboi et al., (1992), Aragane et al., (1996), Cha et a.,l (1996), Ghahary et al., (1998)
Hepatocyte growth factor	Tsuboi et al., (1992), Sato et al., (1995), McCawley et al., (1998), Liang (1998), Gibbs et al., (2000), Tokumaru (2005)
Insulin like growth factor I	Tsuboi et al., (1992), Sato et al., (1995), McCawley et al., (1998), Haase (2003), Pozzi (2004).
IL-1	Tsuboi et al., (1992), Chen (1995), Weng (1997), Maas-Szabowski (2000), Lian (2008)
Platelet derived growth factor	Robsen (1997), Trengove et al., (2000), Rollman (2003)
Transforming growth factor beta	Kane et al., (1991)., Sarret et al., (1992), Zambruno et al., 1995, Robsen (1997)
Transforming growth factor alpha	Pittelkow (1993), Chen (1995), Cha et al., (1996), Klein et al., (2005)
Heparin binding epidermal growth factor	Marikovsky et al., (1993).,Martin, (1997)., Faull, (2001)., Xu (2004)., Higashiyana (2005)., Shirakata., (2005)

Table 10 indicates common soluble keratinocyte proliferative and/or migratory factors

3.1.3 TG2 Location and Activity in Wound Healing.

Tissue transglutaminase or TG2 is one of five isoforms expressed in human skin. TG2 is found only in basal keratinocytes within the epidermis (Aeschlimann et al., 1998; Haroon et al., 1999) contrasting with other TG isoforms which have keratinocyte differentiation specific expression (Aeschlimann et al., 1998, Candi et al., 2001). TG2 is also expressed in fibroblasts and is the predominant TG in the dermal compartment (Aeschlimann et al., 1998). The mechanism for secretion is elusive, however recent work by Scarpellini et al, indicates heparin sulphate proteoglycans may assist TG2 by cross linking leading to TG2 secretion and extracellular activity (Scarpellini et al., 2009). When TG2 is relocated to the extracellular matrix it has been shown to be involved with extracellular remodelling (Aeschlimann and Thomazy 2000, Stephens et al., 2004) and can function independently of its cross linking ability by acting as an integrin associated co-receptor promoting fibronectin fibril formation in the pericellular matrix and facilitating cell adhesion, spreading and motility (Akimov and Belkin 2001). Tissue TG is found in granulation tissue twenty four hours after wounding (Haroon et al., 1999) and is upregulated by acute phase injury cytokines such as IL-6 (Ikura et al., 1994), TGFβ (Akimov and Belkin 2001) and TNFα (Kuncio et al., 1998). TG2 stabilises the extracellular matrix and promotes granulation tissue remodelling. TG2 has also been shown to be expressed in macrophages, skeletal muscle cells and endothelial cells throughout the wound healing process (Haroon et al., 1999).

3.1.4 Epithelial Migration Model and Aims of Experiments.

Using fibroblasts expressing TG2 at different levels, previous work (Rosser-Davies S, PhD Thesis 2006) has demonstrated that TG2 may influence cross talk between

fibroblasts and keratinocytes to regulate epithelial migration. A co-culture model has been developed in house using fibroblasts and keratinocytes. Our model begins by the production of matrix for ten days allowing the fibroblasts to produce a 3D matrix containing collagen and fibronectin fibrils on which keratinocyte cells can be seeded. Epithelial migration is known to be induced by collagen binding synthesis, in addition to providing a substratum, the matrix contains fibroblast-derived growth factors, cytokines and TG2 which could initiate and support keratinocyte migration. The aim of these experiments was to determine the nature and mechanism of the signals sent and received between keratinocytes, fibroblasts and the extracellular matrix, and the relationship to TG2, using our wound healing model.

3.2 Characterisation of Fibroblast Cell Lines.

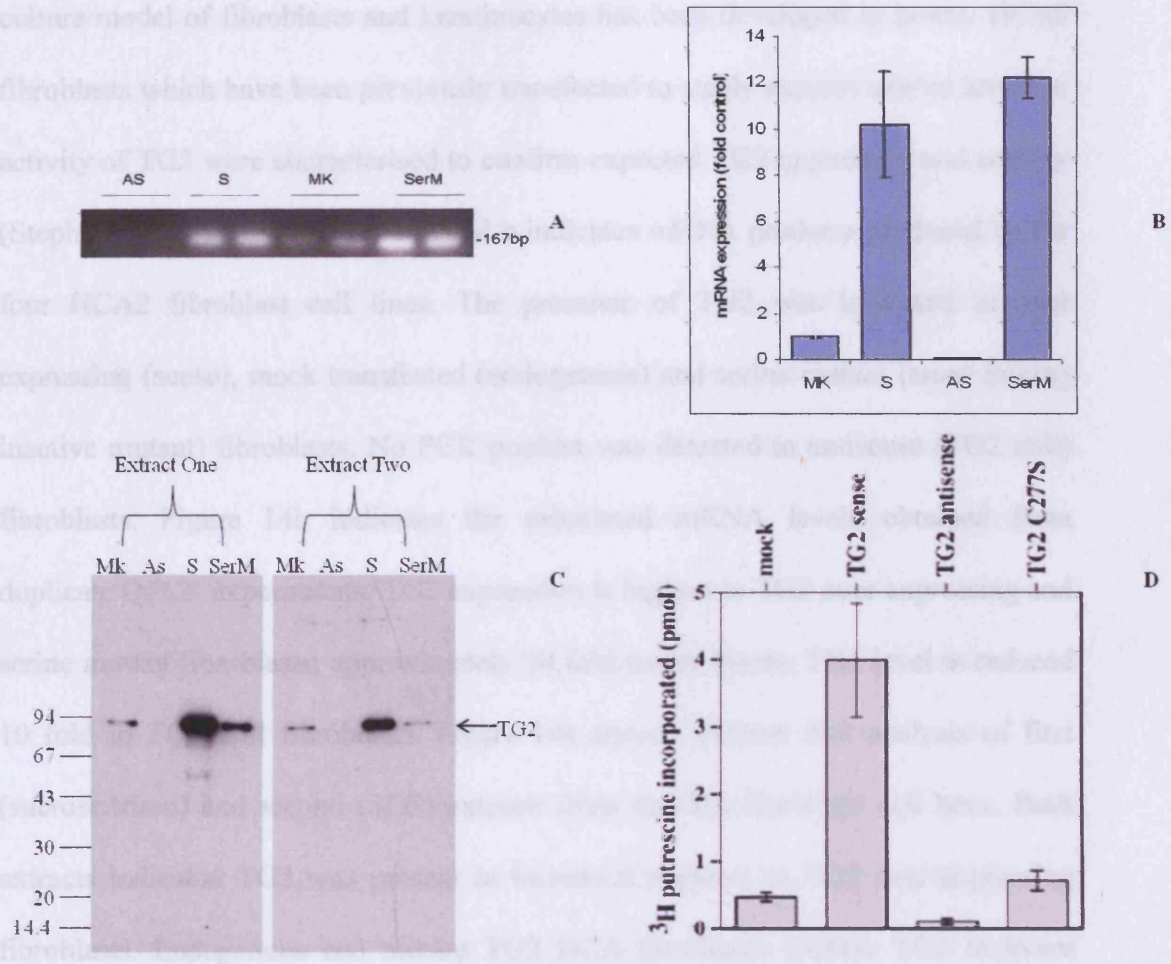


Figure 14: Characterisation of HCA2 Fibroblast cell lines.
(A)mRNA products from the four transfected HCA2 lines (Stephens et al 2004). Antisense (null for TG2), Sense (Over-expressed TG2), Mock (Normal TG2 expression) and Serine Mutant (non cross-linking form of TG2) are shown. (B) Q-PCR results indicating mRNA TG2 expression levels in Mock, Sense, Antisense and Serine Mutant fibroblasts. (C) Western blot of first (sucrose/triton 100x) and second (SDS/ Proteinase inhibitor based) protein extracts from Mock (Normal TG2 expression) Antisense (null for TG2), Sense (Over-expressed TG2), and Serine Mutant (none cross-linking form of TG2) using CUB7402 TG2 Monoclonal Antibody, TG2 detected from 77-85KDa, work carried out by Martin Langley (D)3H putresine incorporation by the fibroblasts indicates the amount of TG2 activity seen (taken from Stephens et al., 2004).

In order to study the process of wound healing in a reductionist environment a co culture model of fibroblasts and keratinocytes has been developed in house. HCA2 fibroblasts which have been previously transfected to stably express altered levels or activity of TG2 were characterised to confirm expected TG2 expression and activity (Stephens et al., 2004). Figure 14a and b indicates mRNA products produced by the four HCA2 fibroblast cell lines. The presence of TG2 was indicated in over expressing (sense), mock transfected (endogenous) and serine mutant (cross linking inactive mutant) fibroblasts. No PCR product was detected in antisense (TG2 null) fibroblasts. Figure 14b indicates the calculated mRNA levels obtained from duplicate QPCR experiments. TG2 expression is highest in TG2 over expressing and serine mutant fibroblasts; approximately 10 fold native levels. TG2 level is reduced 10 fold in TG2 null fibroblasts. Figure 14c shows Western blot analysis of first (sucrose/triton) and second (SDS) extracts from the four fibroblast cell lines. Both extracts indicated TG2 was present in increased amounts in TG2 over-expressing fibroblasts. Endogenous and mutant TG2 HCA fibroblasts express TG2 in lower amounts and the TG2 null fibroblasts do not express TG2 protein Figure 14d is taken from Stephens et al., 2004. This shows TG2 activity seen in the four fibroblast cell lines, determined by 3H putresine incorporation into N,N dimethycasein. TG2 over expressing fibroblasts have eight times more activity than the endogenous control (mock), while the TG2 null (antisense) line has no quantitative TG2 activity. The TG2 mutant fibroblasts have comparable activity to the endogenous control however protein expression is considerably higher.

3.3 Re-Epithelialisation Model and Quantification of Migration Data.

Pilot experiments carried out by Sally Rosser-Davies (PhD Thesis, 2006) indicated keratinocyte spheroids elicited altered migration when placed onto matrices derived from each of the described fibroblast cell lines with altered TG2 activity.

In order to derive quantitative results based on the model from Sally Rosser-Davies preliminary work, the co culture model was optimised with regard to method of matrix preparation, spheroid visualisation and a method for quantification of migration. Figure 15a illustrates the preparation of a migration experiment. Confluent fibroblasts were cultured for a further ten days in the presence of ascorbate-2-phosphate to stimulate matrix production. During this time conditioned medium was collected on days 5, 8 and 10 and frozen. After this time the matrix was either utilised immediately (containing live fibroblasts) or freeze-thawed and de-cellularised with sodium deoxycholate to remove the cell associated proteins. A keratinocyte spheroid was prepared by culturing keratinocytes in a hydrophobic environment (in hydrophobic plates and in the presence of methylcellulose) for 16 hours, to form an aggregated cell "ball" that was transferred onto the matrix. Keratinocytes were labelled to track their migration. The system was modified to ascertain the effects of fibroblasts, TG2 and/or conditioned medium on the migration of the keratinocyte cells over 30 hours.

Migration was initially monitored using a Carl Zeiss Axiocam camera attached to a Carl Zeiss 200 Axiovert Microscope running Improvision Openlab 4.1.3 time lapse software (Figure 15b top). Images were processed into greyscale images that could be interpreted by SkyScan© software and a defined threshold applied to convert the

image into binary images. The boundary of migratory keratinocyte (irregular shaped surface) was defined using a rolling ball algorithm which defined an irregular object the area of which corresponds to the total area covered by cells (figure 15b bottom) Finally, the distance of migration was calculated from a circle of equal area where the area of the spheroid at time of plating was subtracted to give a total migration over the 30 hours (figure 15c). Values were then converted into micrometres.

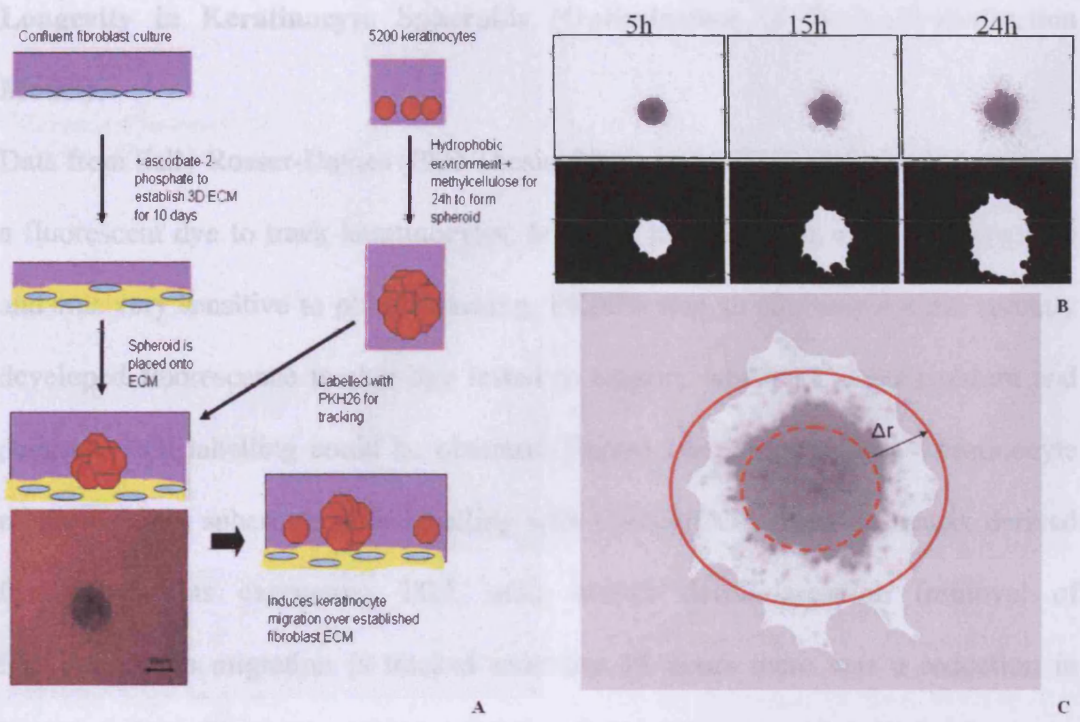


Figure 15: Schematic representation of the co culture model and illustration of method applied for quantification analysis. (A) A schematic diagram showing the preparation of the migration model. A confluent fibroblast layer was incubated in the presence of ascorbate-2-phosphate for ten days and a fluorescently labelled keratinocyte spheroid was placed on top of the matrix and photographed every hour (B top panel) over 30 hours. (B) Shows the SkyScan© computer generated binary images of the spheroids pattern of migration (bottom panel B). (C) Shows the calculated radius calculated by subtracting the original spheroid area from the total area covered by migrating keratinocytes Δr reflects the average distance migrated by the keratinocytes

3.3a Comparison of CalceinAM and PKH26 Fluorescent Dye for Intensity and Longevity in Keratinocyte Spheroids (Optimisation of Re-Epithelialisation Model).

Data from Sally Rosser-Davies (PhD Thesis, 2006) had utilised Calcein AM green as a fluorescent dye to track keratinocytes, however labelling was often heterogenous and was very sensitive to photobleaching. PKH26 was an alternative more recently developed fluorescence tracker dye tested to explore whether more consistent and persistent cell labelling could be obtained. Figure 16a, b and c show keratinocyte migration from spheroids after labelling with Calcein AM green on matrix derived from fibroblasts expressing TG2, after matrix decellularisation (removal of fibroblasts). As migration is tracked over the 48 hours there was a reduction in intensity of labelling and a diffusion of signal caused by photo bleaching. Moreover, this effect was further increased when more photographs were taken over the same time period. In comparison, photographs d, e and f show migrating keratinocytes labelled with PKH26 cell linker dye over the same time period on matrix without live cells. These photographs indicate that there was no loss of signal over the 48 hours or diffusion of dye across the spheroid. Figure 16 indicates PKH26 to be a more stable tracking agent for migrating keratinocytes. In support of this, PKH26 cell tracker dye had been shown to be an effective fluorescent label of live cells over an extended period of time with no apparent toxic effects (Wallace et al., 1993). PKH26 is incorporated into the cell membrane lipid bilayer using aliphatic reporter molecules which do not leak or become transferred, making it a stable fluorescent indicator of cell migration (Horan and Slezak., 1989).

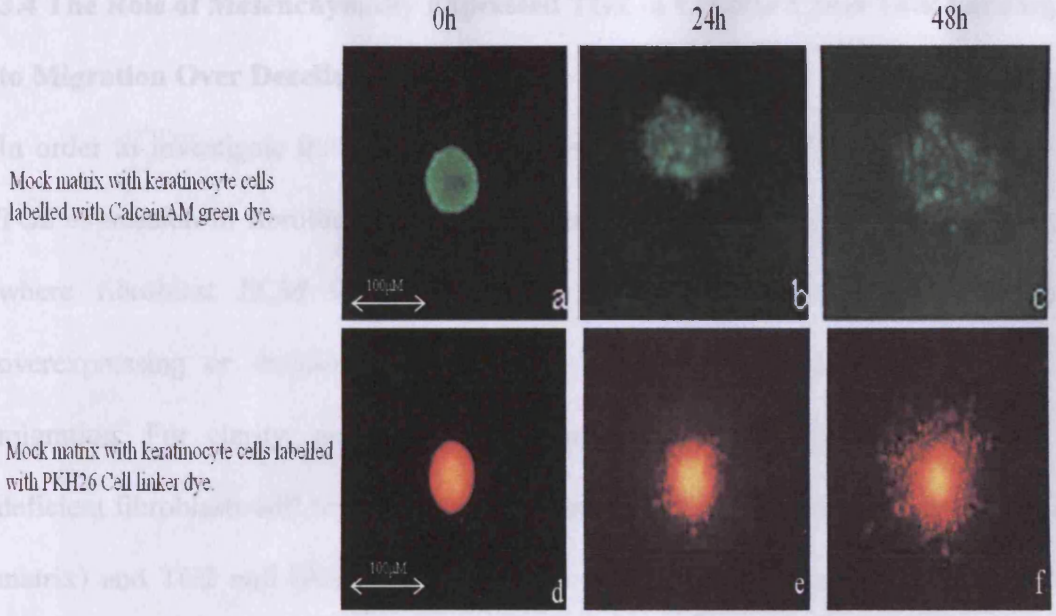


Figure 16: Comparison of CalceinAm Green and PKH26 dyes for keratinocyte tracking. Images A, B and C display migrating keratinocytes over an endogenous (mock) TG2 matrix fibroblast matrix at 2, 24 and 48 hours labelled with Calcein AM green. These images are reproduced with permission of Dr Sally Rosser-Davies. Images D, E, and F show keratinocyte cells also migrating over an endogenous (mock) TG2 matrix mock (normal TG2) matrix labelled with PKH26 dye at 0, 24 and 48 hours. Both matrices have been treated to remove fibroblast cells by freeze thaw and deoxycholate treatment.

3.4 The Role of Mesenchymally Expressed TG2 in Cellular Cross Talk Leading to Migration Over Decellularised Matrix.

In order to investigate the effects of changes in ECM assembly caused by altered TG2 expression in fibroblasts on keratinocyte migration, an experiment was set up where fibroblast ECM from native cells was compared to that from cells overexpressing or deficient in TG2 with regards to modulation of epithelial migration. For clarity, matrix derived from sense, mock transfected and TG2 deficient fibroblasts will be referred to as overexpressed (S matrix), endogenous (Mk matrix) and TG2 null (AS matrix) matrices throughout the document. Figure 17a shows images from a representative spheroid for each of the three conditions. Spheroids were allowed to migrate for 30 hours in normal FAD medium containing 10% FCS, 10μg/ml EGF, and 5ng/ml insulin. Migration on over expressed matrix and endogenous matrix was increased 3 fold and 4 fold respectively when compared to TG2 null matrix (Figure17). In actual distance, migration over endogenous TG2 matrix was 300μm, 220μm for over expressed matrix compared with 75μm for TG2 null matrix. However, the keratinocytes did not respond to the increased level of TG2 present in the over expressed fibroblasts, in fact keratinocytes showed reduced migration compared to endogenous TG2 matrix. This result was consistently seen indicating keratinocytes were either unable to utilise the increased TG2 e.g., endogenous TG2 was sufficient to facilitate matrix assembly or that TG2 increases migration up to a certain level, after which its' increased activity is ineffective at stimulating keratinocyte migration e.g., high levels of matrix crosslinking may impede keratinocyte migration. Furthermore, TG2 may act either directly or indirectly on keratinocytes.

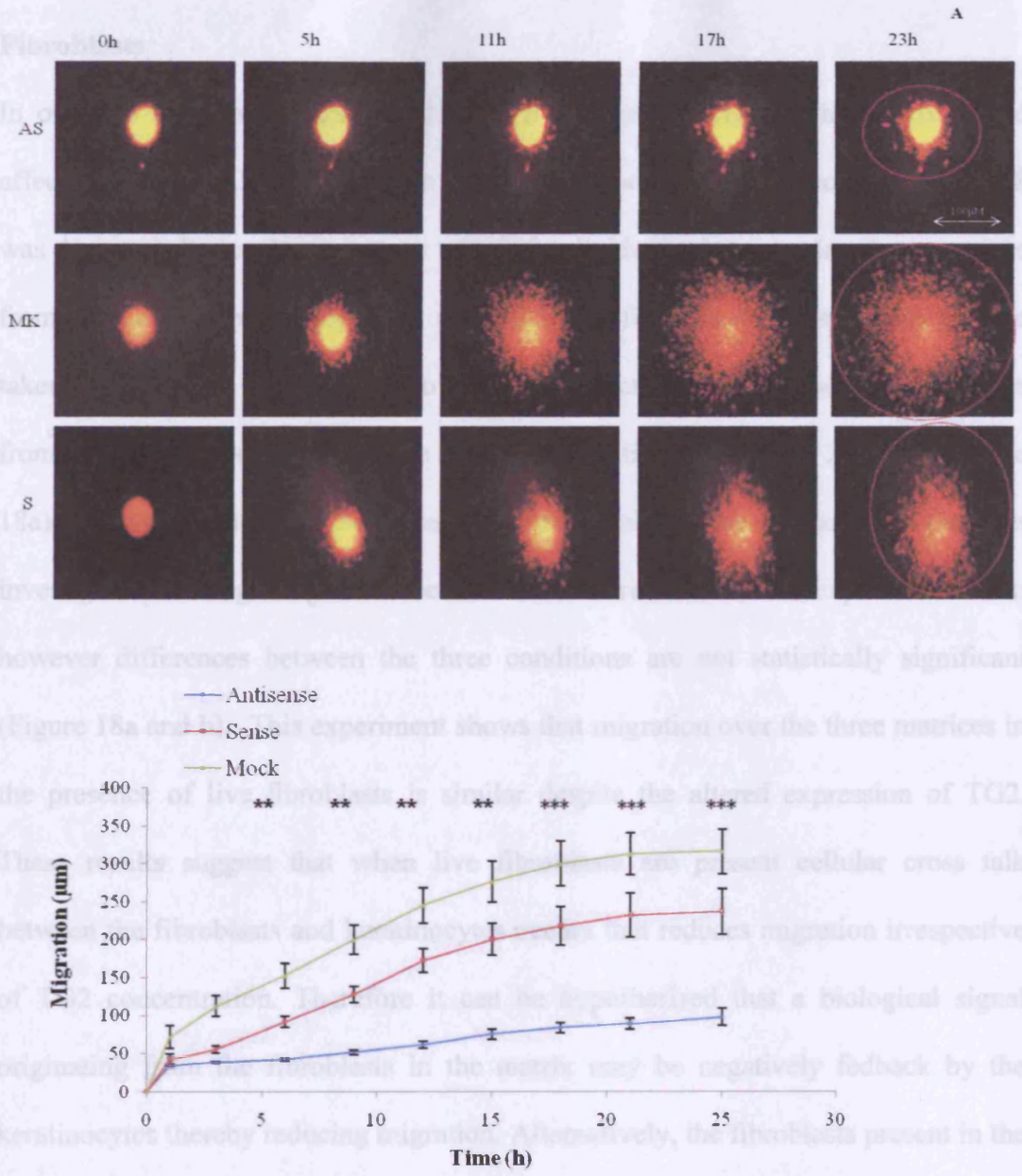


Figure 17: Migration of keratinocyte spheroids on Over expressed, endogenous and TG2 null matrix.(A) Panel of fluorescent images taken using epifluorescence showing keratinocyte spheroid migration over matrix not containing live cells from TG2 null (antisense), overexpressed (sense) and endogenous (mock) fibroblasts. A single representative experiment is shown where the data is presented as the mean +/- SEM (standard error) of six spheroids. Keratinocyte spheroids labelled with PKH26 dye were added to the matrix in FAD medium and the migration photographed over 30 hours by time lapse microscopy. Images taken at 0, 5, 11, 17 and 23 hours are shown. (B) Corresponding graph showing average migration over the three different matrices (n = 6). Repeated measures ANOVA statistical analysis was carried out with a 95% confidence interval. Statistical significance denoted as follows; <*P, 0.0005, <**P, 0.005 compared with control for each point.**

3.4a Keratinocyte Migration on Extracellular Matrix Containing Live Fibroblasts

In order to test whether the presence of live fibroblasts within the matrix would affect migration of keratinocytes from spheroids the experiment shown in figure 18 was designed. Part a shows images taken of individual spheroids placed on matrices from over expressed, endogenous and TG2 null fibroblasts. These matrices were taken straight from their 10 days of matrix production to experiment. Photographs from the three types of matrices are shown for five time points over 23 hours (Figure 18a). Migration patterns in the presence of live fibroblasts are similar for all matrices investigated, although migration seems to be most reduced on overexpressed matrix; however differences between the three conditions are not statistically significant (Figure 18a and b). This experiment shows that migration over the three matrices in the presence of live fibroblasts is similar despite the altered expression of TG2. These results suggest that when live fibroblasts are present cellular cross talk between the fibroblasts and keratinocytes occurs that reduces migration irrespective of TG2 concentration. Therefore it can be hypothesised that a biological signal originating from the fibroblasts in the matrix may be negatively fedback by the keratinocytes thereby reducing migration. Alternatively, the fibroblasts present in the matrix are in some way able to reduce the availability of migration stimulating factors to the keratinocytes.

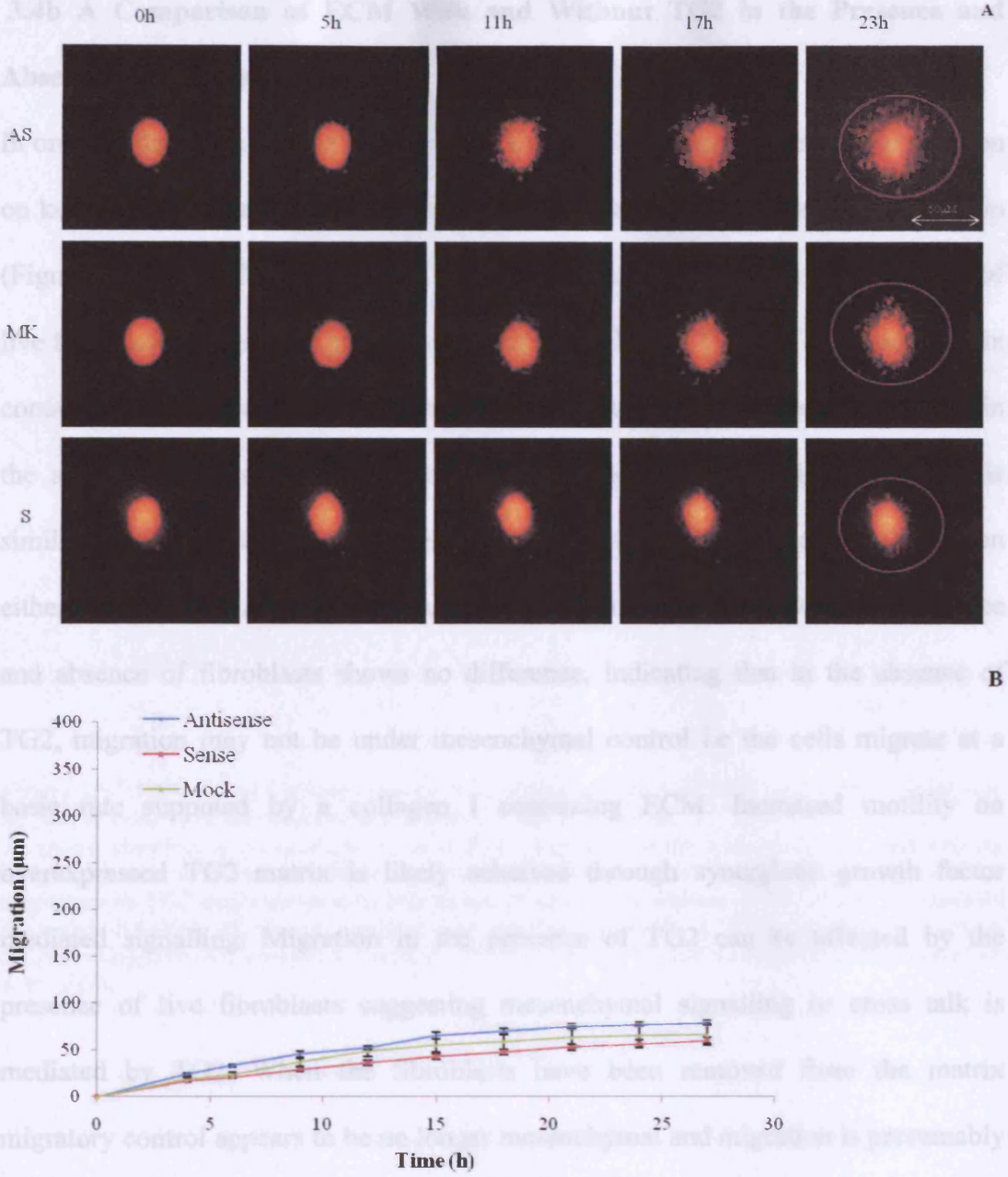


Figure 18: Migration of keratinocyte spheroids over matrix containing live fibroblasts.
(A) Panel of flouresecent images taken using epifluorescence showing keratinocyte spheroid migration over matrix from TG2 null (antisense), over expressed (sense) and endogenous (mock) fibroblasts where the fibroblasts have not been removed by freeze thaw and detergent treatment, therefore still contain live cells. A single representative experiment is shown where the data is presented as the mean +/- SEM of six spheroids. Keratinocyte spheroids labelled with PKH26 dye were added to the matrix in FAD medium and the migration photographed over 30 hours by time lapse microscopy. (B) Corresponding graph showing average migration over the three matrices (n = 6). Repeated measures ANOVA statistical analysis was carried out with a 95% confidence interval.

3.4b A Comparison of ECM With and Without TG2 in the Presence and Absence of Live Fibroblasts.

In order to directly compare the effects of live fibroblasts as well as TG2 expression on keratinocyte migration, a composite graph of the previous experiments is shown (Figure 19). When TG2 null matrix is used regardless of the presence or absence of live fibroblasts a similar migration pattern is seen. When TG2 over expressed matrix containing live fibroblasts is used migration is dramatically reduced compared to in the absence of fibroblasts. Since migration in the absence of live fibroblasts is similar on the two different matrices this suggests that TG2 can promote migration either directly or indirectly. Comparison of TG2 null matrix between the presence and absence of fibroblasts shows no difference, indicating that in the absence of TG2, migration may not be under mesenchymal control i.e the cells migrate at a basic rate suppoted by a collagen I containing ECM. Increased motility on overexpressed TG2 matrix is likely acheived through synergistic growth factor mediated signalling. Migration in the presence of TG2 can be affected by the presence of live fibroblasts suggesting mesenchymal signalling or cross talk is mediated by TG2. When the fibroblasts have been removed from the matrix migratory control appears to be no longer mesenchymal and migration is presumably limited only by the availability of growth promoting factors such as growth factors sequestered within the matrix, integrin binding sites in the matrix itself, or an alternative signalling mechanism.

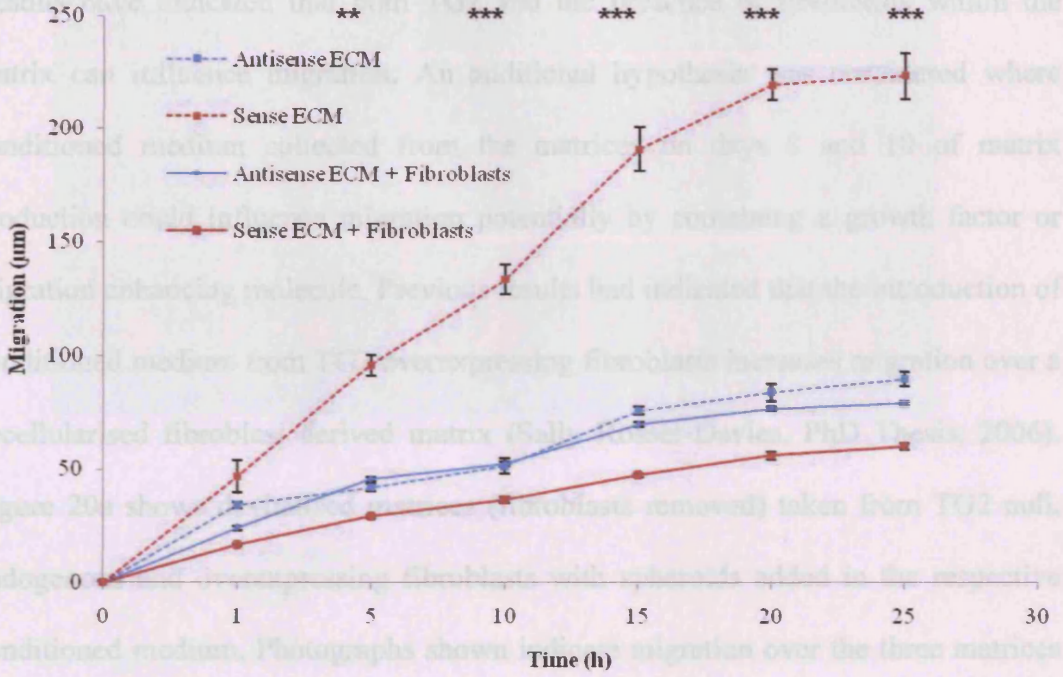
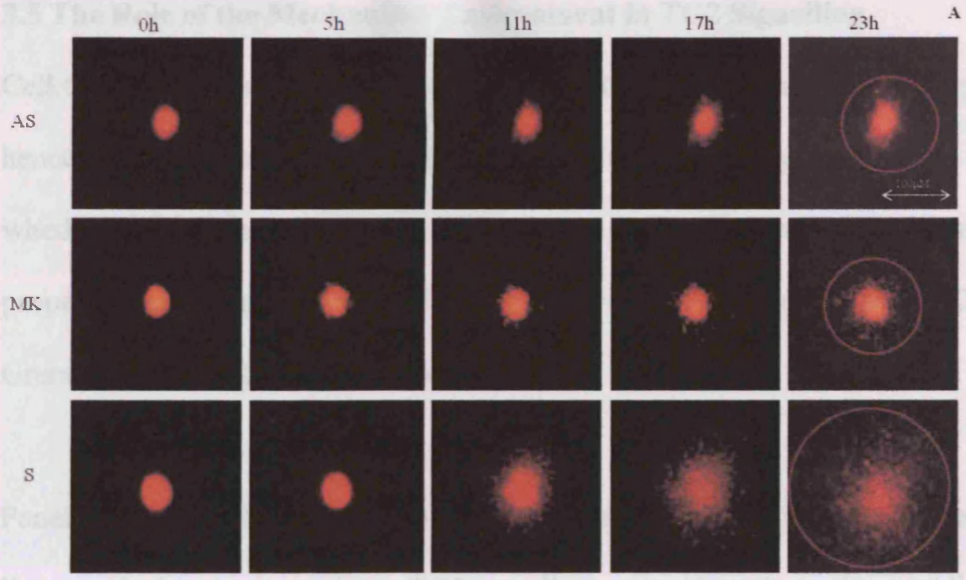


Figure 19: Migration of keratinocytes on TG2 overexpressed and TG2 null matrix (sense and Antisense matrix) in the presence and absence of live fibroblasts.
A graph showing a comparison of averaged migration from both previous experiments. Migration over TG2 null matrix in the presence and absence of fibroblasts is compared with migration on TG2 overexpressed matrix in the presence and absence of fibroblasts.). Repeated measures ANOVA statistical analysis was carried out with a 95% confidence interval. Statistical significance denoted as follows; <*P, 0.0005, <**P, 0.005 compared with control for each point.**

3.4c The Effect of Conditioned Media from Fibroblasts with Modified TG2 Expression in Migration

Results have indicated that both TG2 and the presence of fibroblasts within the matrix can influence migration. An additional hypothesis was considered where conditioned medium collected from the matrices on days 8 and 10 of matrix production could influence migration potentially by containing a growth factor or migration enhancing molecule. Previous results had indicated that the introduction of conditioned medium from TG2 overexpressing fibroblasts increases migration over a decellularised fibroblast derived matrix (Sally Rosser-Davies, PhD Thesis, 2006). Figure 20a shows devitalised matrices (fibroblasts removed) taken from TG2 null, endogenous and overexpressing fibroblasts with spheroids added in the respective conditioned medium. Photographs shown indicate migration over the three matrices at five time points during the 30 hours. Migration was the most extensive on TG2 overexpressed matrix, similar results were obtained for both endogenous matrix/ conditioned medium and TG2 null matrix/ conditioned medium. This suggests that a stimulatory factor may be present in TG2 overexpressed conditioned medium that supports increased migration when compared with endogenous and TG2 null conditioned medium on their respective matrices. Migration in the presence of TG2 null conditioned medium is increased relative to the previous experiment on matrix only shown in figure 18 suggesting that conditioned medium of any type may promote epithelial migration to an extent. The increased motility on overexpressed and endogenous matrix is therefore a consequence of an increase in a promigratory signal either due to enhanced matrix association or gene expression and not an antimigratory signal synthesised by TG2 null cells.



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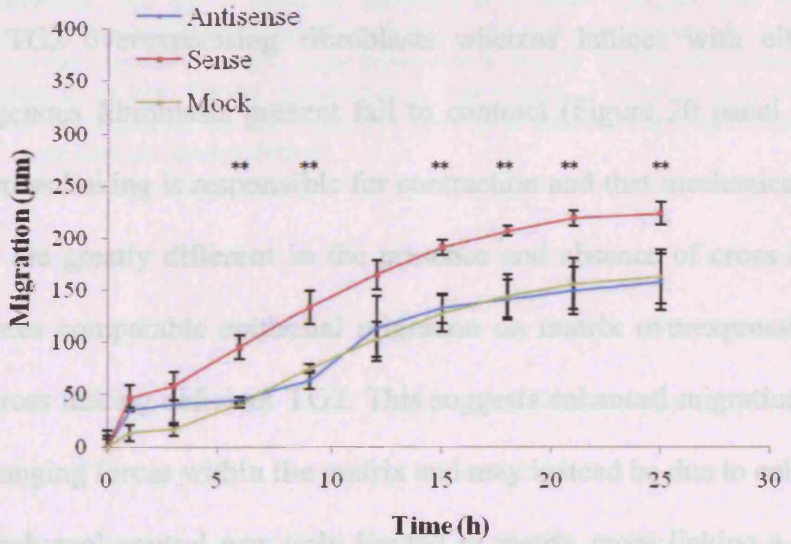


Figure 20: Migration of keratinocyte spheroids over matrix in the presence of relevant conditioned medium.
(A) Panel of fluorescent images taken using epifluorescence showing keratinocyte spheroid migration over matrix from TG2 overexpressed (sense), endogenous (mock) and TG2 null (antisense) matrix in the presence of conditioned medium. A single representative experiment is shown where the data is presented as the mean +/- SEM of six spheroids. Keratinocyte spheroids were added to the matrix in a 1: 1 dilution of FAD fresh medium and conditioned medium of the matching type from days 8 and ten of matrix production and the migration photographed over 30 hours by time lapse microscopy. (B) Corresponding graph showing average migration over the three matrices (n = 6). Repeated measures ANOVA statistical analysis was carried out with a 95% confidence interval. Statistical significance denoted as follows; <*P, 0.05; < P, 0.005; <***P, 0.0005, compared with control for each point.**

3.5 The Role of the Mechanical Environment in TG2 Signalling.

Cell traction is influenced by the resistance of the substratum to the forces generated; hence physical strength of the ECM may influence motility. In order to determine whether the effects seen on keratinocyte migration are the result of mechanical properties of the matrix the following experiment was conducted by Dr Pascale Grenard and Dr Sally Rosser-Davies in house.

Panel A indicates the contraction of collagen lattices seeded with the 3 fibroblast cell lines tracked over seven days. TG2 contributes significantly to the establishment of tension within these matrices as contraction is evident when lattices are populated with TG2 overexpressing fibroblasts whereas lattices with either TG2 null or endogenous fibroblasts present fail to contract (Figure 20 panel A). This suggests that cross linking is responsible for contraction and that mechanical properties of the ECM are greatly different in the presence and absence of cross linking. Panel (B) indicates comparable epithelial migration on matrix overexpressing wildtype TG2 and cross linking deficient TG2. This suggests enhanced migration is not dependent on changing forces within the matrix and may instead be due to cell-cell cross talk. If mesenchymal control was only limited to matrix cross linking a similar pattern of epithelial migration would be expected in the case of both mutant TG2 expressing and TG2 null fibroblasts which is not the case. Therefore this indicates mesenchymal control may be influenced by the availability of soluble factors or additional signalling events.

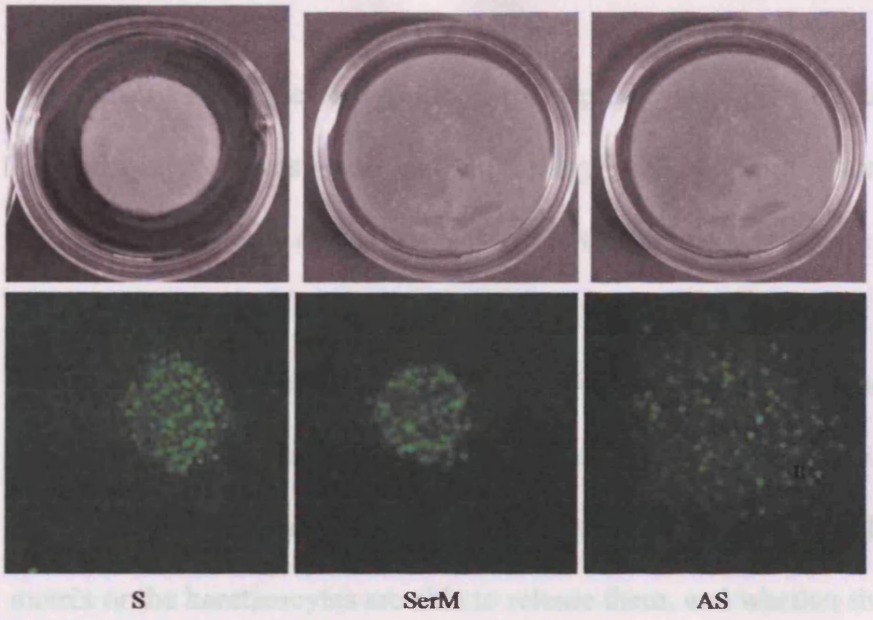


Figure 21: Comparison of collagen lattice contraction and keratinocyte migration over various TG2 matrices. The top 3 pictures indicated 8(A) show collagen gels populated with Over expressed(S), SerineMutant (SerM) and TG2 null (AS) fibroblasts the amount of contraction seen over the course of 7 days, experiment conducted by Dr Pascale Grenard reproduced with permission from Prof Daniel Aeschlimann. The bottom 3 pictures show calcein green labelled epithelial cell migration on the respective fibroblast matrices after 48 hours (B) images from Dr Sally Rosser-Davies, (unpublished).

3.6 Conclusion

Migration of keratinocyte cells on matrices produced by fibroblasts over expressing, endogenously expressing or not expressing TG2 has been examined in both the presence and absence of live fibroblast cells (summarised in Figure 22).

When the matrix is devitalised by freeze thaw and sodium deoxycholate treatment no further cellular cross talk can occur. Stimulatory factors from the matrix may influence migration depending on whether they remain cross linked within the matrix or the keratinocytes are able to release them, and whether they are active only in a soluble form or can signal as a matrix associated form. Keratinocyte migration on matrices produced by fibroblasts either expressing or over expressing TG2 is increased compared to the same matrices containing live cells, suggesting that TG2 is important in promoting keratinocyte migration, which may depend on a factor cross linked to the matrix by TG2 and released by the keratinocytes as they migrate over the matrix. Keratinocyte migration is reduced on devitalised matrix where the fibroblasts have been removed, where TG2 is not expressed indicating that TG2 expression is required to sequester growth factors in the matrix or to cross link matrix constituents in such a way that integrin binding site availability might be modulated, or that the enzyme itself acts as a signalling factor or alters gene expression in fibroblasts and directly enhances synthesis of a promigratory signal. However, if a factor normally sequestered into the ECM were active in a soluble form when released from the matrix, it would be expected that TG2 null media would contain more of this factor and so would be expected to support enhanced motility, which is not the case. This suggests that this factor is only active when

matrix associated, or that TG2 expression alters the properties of the matrix or induced expression of the factor.

Keratinocyte migration across a matrix containing live fibroblasts expressing TG2 is substantially reduced in comparison to matrix with cells removed. The same effect was observed irrespective of the presence of conditioned medium or the level of TG2 expression, indicating that anti-migratory signals are originating from the fibroblasts. This suggests that a pro-migratory factor is present within the matrix triggers signalling in keratinocytes. In the presence of fibroblasts, further signalling occurs that limits migration. Alternatively, mechanical stimuli originating from fibroblasts and transduced through the matrix could alter adhesion and migration of keratinocytes, which is mediated by integrins; (mechanical sensors of the cell surface). *In vitro* studies have previously shown that TG2 affects keratinocyte adhesion, where Taenaka et al., showed that TG2 can, in a dose dependent manner, increase keratinocyte adhesion on a fibronectin substratum (Taenaka et al., 2003). This effect was counteracted by the addition of arginine glycine aspartate (RGD) peptides. The presence of TG2 could therefore modulate integrin-mediated keratinocyte adhesion to fibroblast-derived matrix. A balance between adhesion and de-adhesion is critical for cell migration, thus if adhesion is too strong, keratinocyte migration would be inhibited. Thus, the marginally higher motility on endogenous TG2 matrix as compared to overexpressed TG2 matrix may be a result of increased adhesion to the matrix in this environment. However, overexpression of a cross linking deficient mutant form of TG2 has shown that the signalling pathway regulating enhanced motility is independent of the mechanical environment.

When migration is compared in the presence of conditioned medium from each of the three matrix types when no live fibroblasts are present the level of migration in the presence of mock and antisense conditioned medium is similar, while sense conditioned medium (TG2 over expressed) clearly stimulates migration. This would suggest that the cells respond to stimulatory factors from the conditioned medium such as a growth factor or other soluble factor. The synthesis of which may depend on TG2 and this may be dose dependent. Production of active growth factors such as TGFβ has been shown to be regulated by TG2 and shown to influence cell proliferation (Quan et al., 2005). Alternatively, TG2 may act indirectly through a signalling pathway causing altered gene expression of factors that promote migration. Further, TG2 has been shown to interact with beta chains of the integrin family such as beta 1 and 3, the association of which promotes cellular interactions with the extracellular matrix resulting in an increase in cell migration, proliferation and cell survival (Verma and Mehta 2007). However, our lab has shown that TG2 cannot be detected in conditioned medium even when TG2 is overexpressed; therefore TG2 is unlikely to be solely responsible for increased activity present in conditioned medium.

From the experimental data shown in this chapter there are clear further aims to investigate. Initially, it is of interest to determine the nature of the factor driving keratinocyte migration that is deposited in fibroblast ECM in a TG2 dependent manner. Furthermore, the mechanism by which the keratinocytes are able to mobilise this migration factor must be investigated. Therefore it is necessary to investigate specific factors which could influence migration such as metalloproteinases as well as growth factors such as TGFβ or EGF. It is also crucial to target the associated

receptors that these molecules signal through in order to determine their involvement in migration control. Finally then, it has been shown that TG2 influences factors controlling keratinocyte migration over a fibroblast derived matrix, and subsequent chapters will aim to delineate the signalling cascade involved.

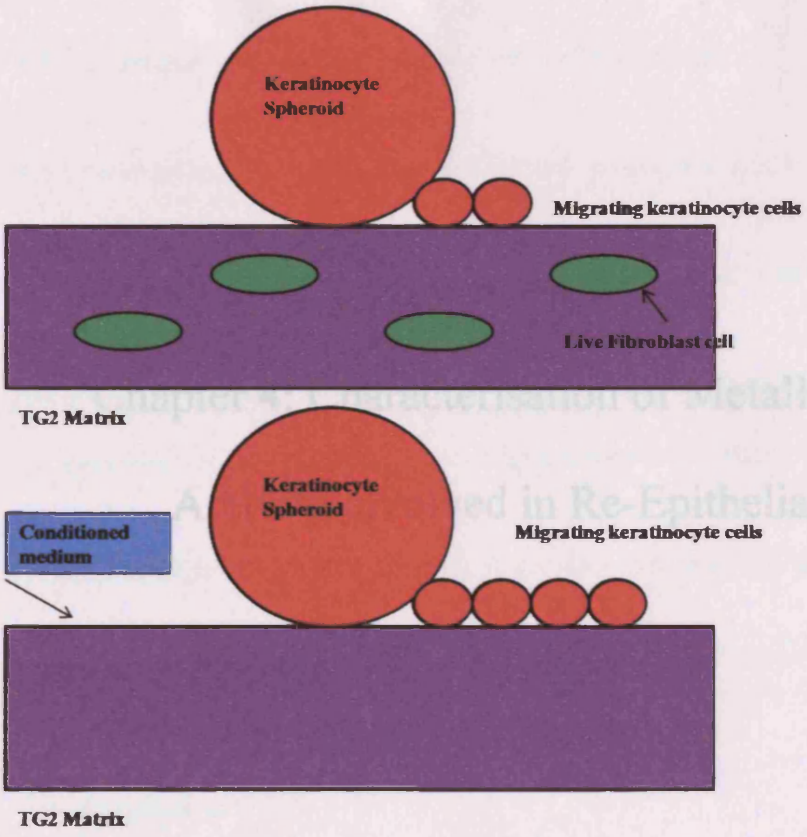


Figure 22: A summary of results from this chapter.

The top half of the figure indicates that in the presence of live fibroblasts keratinocyte migration is limited. It has been shown that keratinocyte responses in wound healing are under the control of the fibroblasts. We expect that in our model system cross talk between fibroblasts and keratinocytes similarly regulate outgrowth of cells from the spheroids. In the absence of live fibroblasts, the epithelial cell can respond to signals from the ECM but no feedback regulation is possible. A basal amount of migration is seen which occurs regardless of removal of fibroblasts from the matrix or the presence of conditioned medium. In contrast ECM of normal fibroblasts or fibroblasts expressing TG2 substantially enhances migration of keratinocytes from the spheroids (lower panel). This suggests that TG2 is part of a signalling pathway that mediates enhanced motility in keratinocyte cells.

Chapter 4: Characterisation of Metalloproteinase Activity involved in Re-Epithelialisation

Contents. **Page**

4.1 Introduction.

The previous chapter indicated that the co culture model of fibroblast matrix and keratinocyte spheroids is potentially regulated both by the presence or absence of fibroblasts and the amount of TG2 present within the matrix. Alternatively, TG2 cross-linking activity could modulate integrin binding site availability or indirectly mediate growth factor signalling. The effect of TG2 can be interpreted in two ways; either the sequestration of TG2 within the matrix allows the keratinocytes to directly respond to TG2, or alternatively, matrix-bound TG2 has cross linked a factor within the matrix which stimulates keratinocyte migration. Either effect of TG2 is a viable hypothesis to test if a factor is mobilised from the ECM by keratinocytes then it is likely that this will require a proteolysis step in order to release a soluble factor and allow binding to its growth receptor. Initially, a candidate molecule or effecter must be identified. The metalloproteinase family, specifically the matrix metalloproteinases, may be involved in proteolytic activity on the cell surface and within the extracellular matrix which leads to cell migration. Matrix metalloproteinase activity can induce changes in proteins which either activate or deactivate a protein or dramatically change its functional properties (Blobel, 2000; Clark et al., 2008). An example is the processing of laminin 5 by either matrix metalloproteinase 2 (MMP2) or membrane type matrix metalloproteinase 1 (MT1-MMP) where cleavage by either MMP results in the exposure of an integrin-binding site that supports cell migration (Koshikawa et al., 2000). Metalloproteinases therefore are candidates for inducing migration in the co culture model. GM6001 is a broad spectrum matrix metalloproteinase inhibitor that has been previously shown to impact on epithelialisation, granulation tissue development and wound contraction

during cutaneous wound repair (Mirastschijski et al., 2004). Investigation of metalloproteinase activity in the co culture model was initiated with GM6001 and TAPI1, an inhibitor of ADAM17 (TACE) as well as metalloproteinases in general. ADAM 17 is a member of the ADAMS (a disintegrin and Metalloproteinase) family, a sub-family of metalloproteinases (Hinkle et al., 2004). ADAMs are membrane anchored metalloproteinases which process and shed the ectodomains of membrane anchored growth factors, cytokines and receptors (Blobel, 2005). This processing activity of cell surface molecules makes ADAMs potential candidates for involvement in keratinocyte migration. For example ADAM 10, (Kuzbanian) has been identified as the main sheddase of the EGFR ligands EGF and betacellulin in mouse embryonic fibroblasts leading to activation of the epidermal growth factor receptor (EGFR) (Sahin et al., 2004). The role of EGFR in promoting keratinocyte migration is well documented in the literature (Blobel, 2005, Xu et al., 2007). The literature has indicated a substantial role for a number of growth factors in cell migration. This chapter investigates the involvement of the three growth factor receptors (EGFR, IGFR, and PDGFR) specifically implicated in regulation of keratinocyte migration over fibroblast-derived matrix.

4.1.1 Aims of experiments.

Our previous work has indicated that TG2 is part of a pathway that promotes keratinocyte motility. The migration model already introduced in chapter 3 will be used to test the effect of various inhibitors of metalloproteinases, ADAMS and growth factor receptors to determine whether they play a role in keratinocyte migration in the presence and absence of TG2. Initial experiments will start with broad acting inhibitors followed by more specific inhibitors to identify in a

systematic way specific components that are part of the pathway requiring TG2, with the aim of identifying pathways important in keratinocyte migration during wound healing. The specific role of TG2 will also be investigated within these experiments to determine whether the pathway is dependent on TG2 activity or protein, and whether altering TG2 levels in the matrix can influence keratinocyte migration or whether the cellular context of matrix assembly is required for this function of TG2.

4.2 A Comparison of Media for Migration Experiments.

A set of inhibitors was selected to inhibit metalloproteinase activity, growth factor receptor activity and growth factor receptor ligand activity. Some of these inhibitors when prepared for experiments were found to be affected by various components of the keratinocyte culture media (FAD medium) which contains additives (detailed in materials and methods) such as EGF, as well as serum e.g. containing protease inhibitors such as α2–macroglobulin and various growth factor which may render inhibitors ineffective, secondly where appropriate 1% serum FAD medium was used where insulin, or EGF or both were removed was used. Nevertheless, there were occasions where this minimal FAD media was unsuitable; therefore an alternative media was sought which could be used. Defined serum free medium (DSFM) distributed by Invitrogen was determined to be a suitable alternative, which has been developed to support keratinocyte growth in the absence of serum. Note however that the growth supplement in this formulation contains high concentrations of various growth factors (details in materials and methods).

An experiment was conducted to test whether the previously observed differences in keratinocyte migration in response to altered TG2 expression could be reproduced when using different culture conditions. Figure 23 shows keratinocyte migration in the presence of either FAD medium or DSFM over TG2 endogenous and TG2 null matrix. Figure 23a shows that migration is comparable over this time period (thirty hours) with either media, both in the presence or absence of TG2, indicating that DFSM media is a suitable substitute. This result also confirms previous findings that showed substantially reduced migration in the absence of TG2 (Chapter 3, Figure 18). Figure 23b further illustrates that total migration (after thirty hours) of keratinocytes from spheroids over TG2 null and endogenous matrix is independent

of the type of media used since total migration is similar for both FAD and DSFM media. For all experiments conducted in media other than standard FAD medium this will be highlighted in the description of the results. To minimise variability, a single batch of serum was selected, tested and then used throughout all experiments in this thesis.

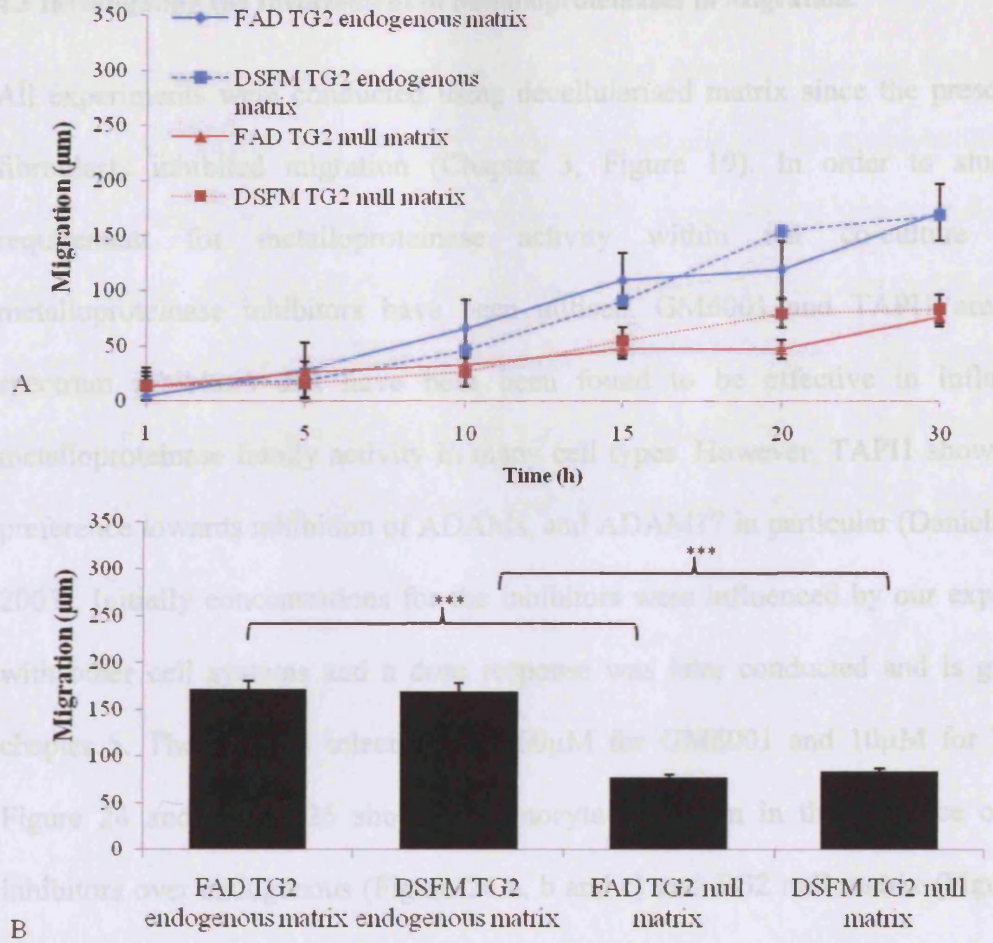


Figure 23: TG2 stimulation of keratinocyte migration occurs in FAD and Defined serum free medium.

Comparison of migration of keratinocytes from spheroids on TG2 endogenous and TG2 null matrix in FAD and defined serum free medium. (A) Migration of keratinocytes over TG2 endogenous matrix in FAD and defined serum free medium is shown in blue, migration over TG2 null matrix in FAD and defined serum free medium is shown in red. A single representative experiment is shown (3 repeats conducted with similar results) and data presented as the mean +/- SEM of six spheroids (n=6). (B): A comparison of total migration over the four conditions at thirty hours. Statistical significance denoted as follows; <* P, 0.005; comparing FAD with defined serum free medium on TG2 endogenous and TG2 null matrix analysed by repeated measures ANOVA.**

4.3 Investigating the Involvement of Metalloproteinases in Migration.

All experiments were conducted using decellularised matrix since the presence of fibroblasts inhibited migration (Chapter 3, Figure 19). In order to study the requirement for metalloproteinase activity within our co-culture model metalloproteinase inhibitors have been utilised. GM6001 and TAPI1 are broad spectrum inhibitors that have both been found to be effective in influencing metalloproteinase family activity in many cell types. However, TAPI1 shows some preference towards inhibition of ADAMs, and ADAM17 in particular (Daniela et al., 2003). Initially concentrations for the inhibitors were influenced by our experience with other cell systems and a dose response was later conducted and is given in chapter 5. The dosages selected were 50μM for GM6001 and 10μM for TAPI1. Figure 24 and Figure 25 shows keratinocyte migration in the presence of these inhibitors over endogenous (Figure 24 a, b and c) and TG2 null matrix (Figure 25). These experiments were performed in DSFM as the presence of serum interferes with cell derived metalloproteinase activity. Both GM6001 and TAPI1 reduced keratinocyte migration over endogenous TG2 matrix compared to vehicle control, DMSO. In addition, DMSO stimulated migration, compared to medium alone (Figure 24). DMSO is known to stimulate migration and proliferation in keratinocytes. Figure 24c further illustrates that total migration is reduced from 270μm (vehicle control, DMSO) to 150μm (GM6001 and TAPI1). These data indicate that metalloproteinase activity is involved in keratinocyte migration in the presence of TG2 (endogenous matrix). In Figure 25 keratinocyte migration over TG2 null matrix in the presence of metalloproteinase inhibitors was compared. Both migration plots shown in Figure 25a and c clearly show that there is no difference between keratinocyte migration across DMSO, GM6001 (50μm) or TAPI1 (10μM)

treatments, which is further illustrated by the images seen in Figure 25b. The migration seen across control and inhibitor treated spheroids was also found to not be statistically different (Figure 25 a and c). Furthermore, keratinocyte migration over TG2 null matrix in the presence of DMSO is comparable to that on endogenous matrix in the presence of GM6001 or TAPI1 suggesting that this corresponds to unstimulated migration based on integrin-ECM interaction. This shows that keratinocyte migration over TG2 null matrix is unaffected by either GM6001 or TAPI1, indicating that metalloproteinases are not involved in TG2 independent migration.

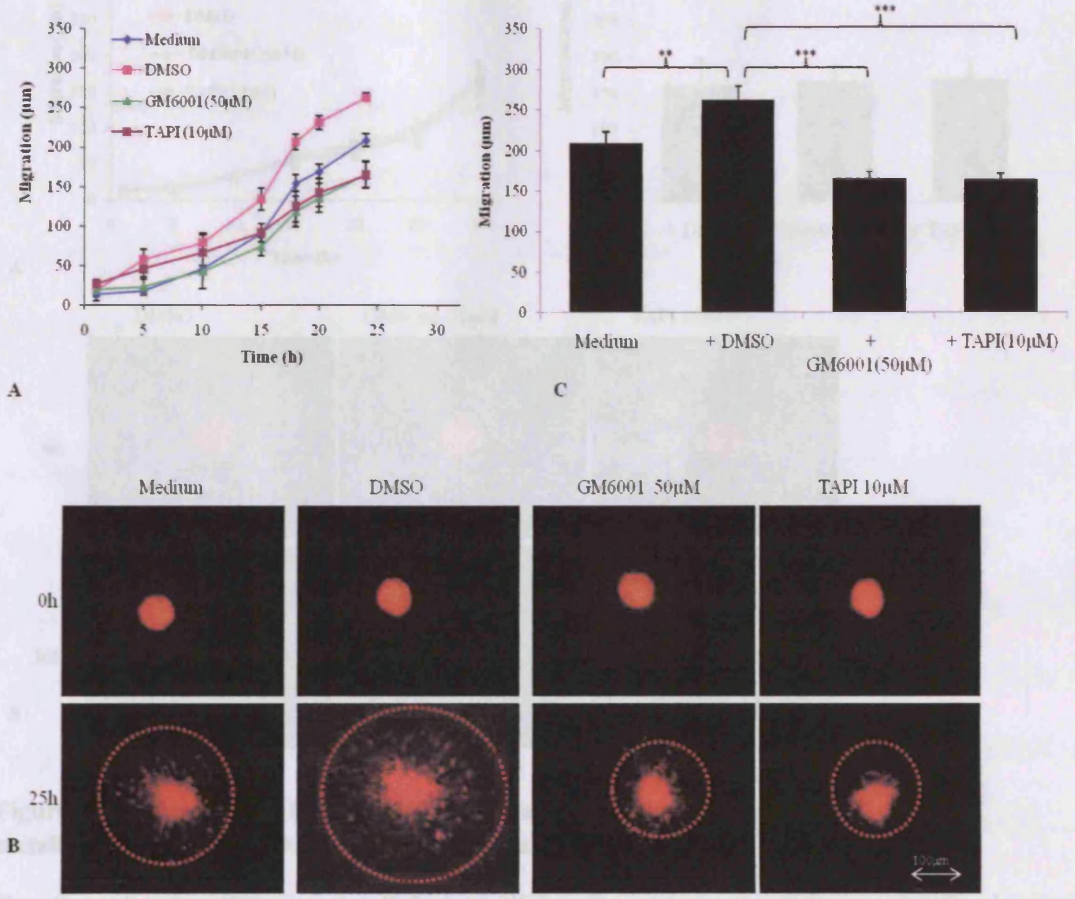


Figure 24: A comparison of migration of keratinocyte cells in the presence of general matrix metalloproteinase inhibitors over TG2 endogenous matrix

Keratinocyte migration seen over decellularised endogenous TG2 matrix in the presence of defined serum, free medium. Migration was recorded in the presence of GM6001 (50μM), TAPI1 (10μM), DMSO (vehicle control for inhibitors) or cells were untreated (Medium). Migration was photographed using epifluorescence illumination and analysed every hour for 30 hours and the results plotted (A) A single representative experiment is shown (3 repeats conducted with similar results) and data presented as the mean +/- SEM of six spheroids (n=6). (B) Shows the corresponding micrographs 0 and 25 hours. The edge of migration of keratinocyte cells from the spheroid is marked by the red circle. (C) Indicates migration from a single time point (hour 25) to compare the total migration under each condition. Statistical significance denoted as follows; ** <P, 0.005; *<P, 0.0005, compared with DMSO control analysed by repeated measures ANOVA.**

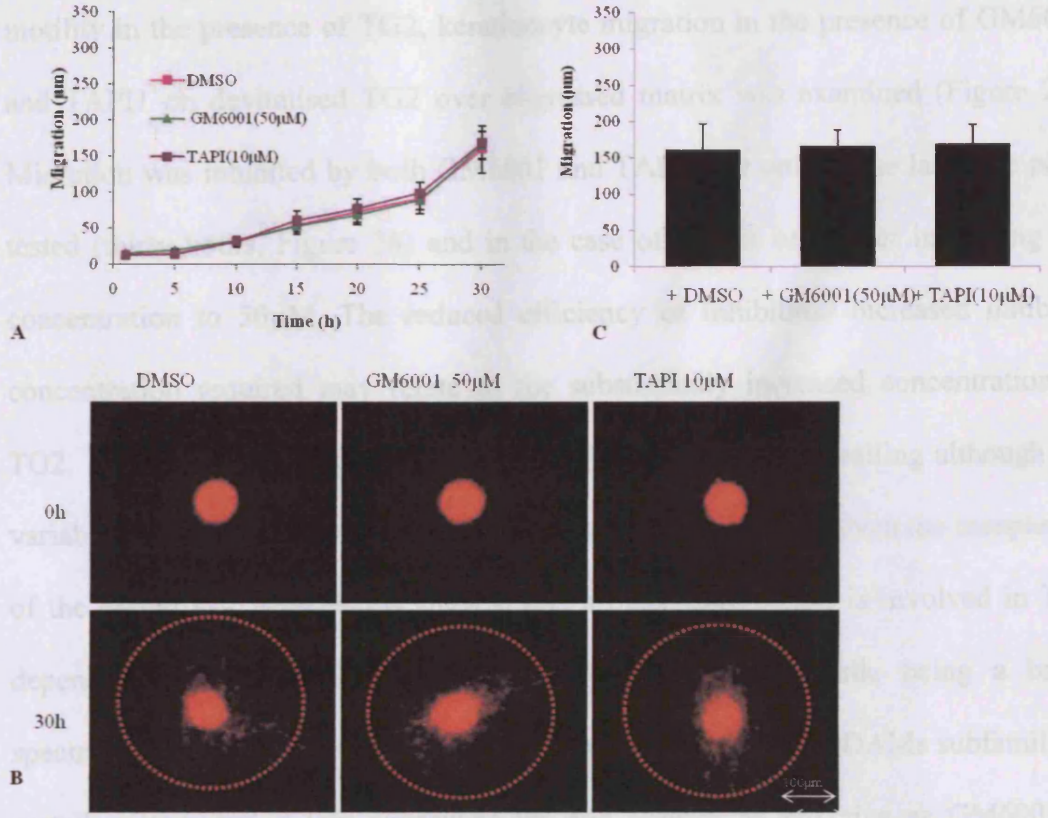


Figure 25 A comparison of migration of keratinocytes in the presence of general matrix metalloproteinases inhibitors over TG2 null matrix.

Keratinocyte migration over decellularised TG2 null matrix in the presence of defined serum free medium. Migration was recorded in the presence of DMSO vehicle control for inhibitors, with GM6001 at 50µM and TAPI1 10µM in defined serum free medium. Migration was photographed using epifluorescence illumination and analysed every hour for 30 hours and the results plotted. (A) A single representative experiment is shown (3 repeats conducted with similar results) and data presented as the mean +/- SEM of six spheroids (n=6). (B) Shows the corresponding micrographs taken at 0 and 30 hours. The edge of migration of keratinocyte cells from the spheroid is marked by the red circle. (C) Indicates migration from a single time point (hour 30) to compare the total migration under each condition. No significant difference was seen between these conditions when compared using repeated measures ANOVA.

To further investigate the requirement for metalloproteinase activity for enhanced motility in the presence of TG2, keratinocyte migration in the presence of GM6001 and TAPI1 on devitalised TG2 over expressed matrix was examined (Figure 26). Migration was inhibited by both GM6001 and TAPI1 but only at the last time point tested (thirty hours, Figure 26) and in the case of TAPI1 only after increasing the concentration to 50μM. The reduced efficiency of inhibition/ increased inhibitor concentration required may relate to the substantially increased concentration of TG2. This may indicate dose dependence of TG2 mediated signalling although the variability in the data was too great to firmly establish that (and given the complexity of the system). These results suggest that a metalloproteinase is involved in TG2 dependent enhanced keratinocyte motility. Since TAPI1, while being a broad spectrum inhibitor, shows specificity towards inhibition of the ADAMs subfamily of metalloproteinases at low concentrations and seemed as effective as GM6001 at inhibiting migration. It is likely that a member of the ADAMs family is involved.

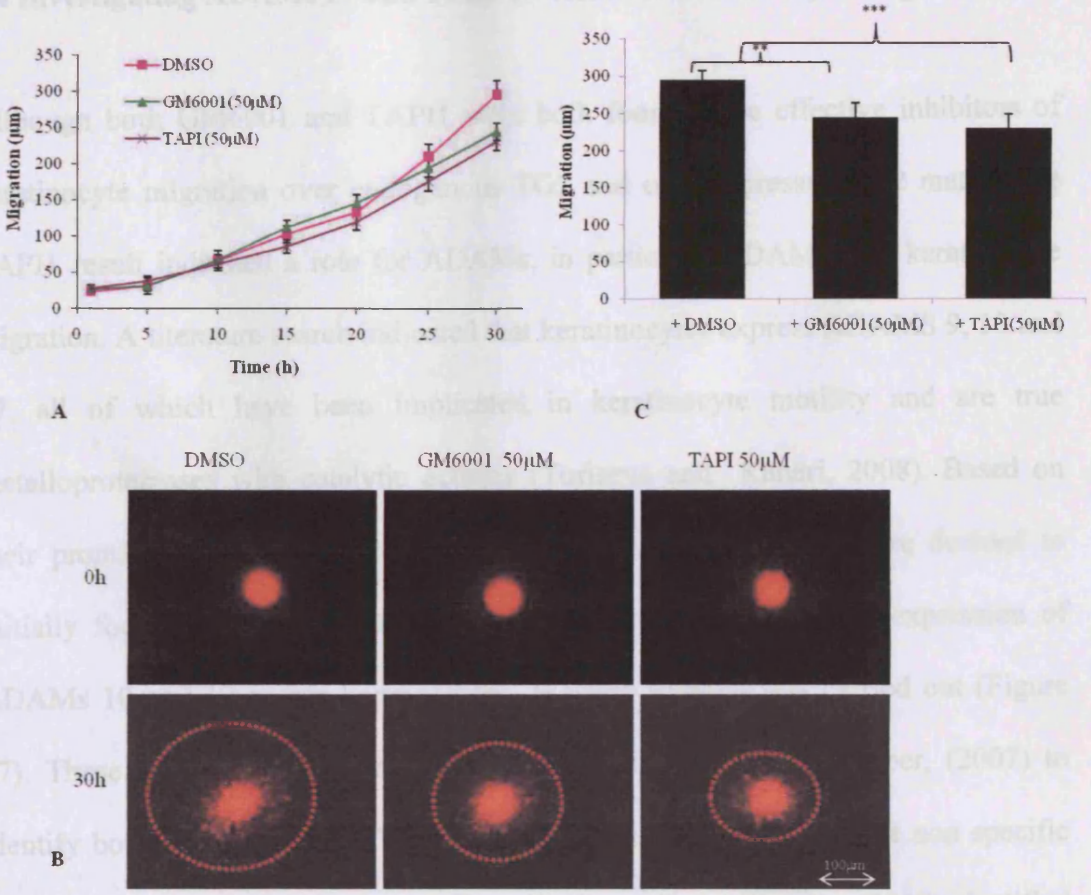


Figure 26 A comparison of migration of keratinocytes in the presence of general matrix metalloproteinase inhibitors over TG2 over-expressed matrix.

Keratinocyte migration over decellularised over expressed TG2 matrix in the presence of GM6001 50µM and TAPI1 50µM and DMSO vehicle control for inhibitors. Migration was photographed using epifluorescence illumination and analysed as previously described, every hour for 30 hours. A single representative experiment is shown (3 repeats conducted with similar results) and data presented as the mean +/- SEM of six spheroids (n=6) (A). (B) Shows the corresponding micrographs taken at 0 and 30 hours indicating the motility of the keratinocytes from the edge of the spheroid indicated by the red circle. (C) Indicates migration from a single time point (hour 30) to compare the maximum migration under each condition. Statistical significance denoted as follows; <*P, 0.0005, <**P, 0.005 compared with DMSO control analysed by repeated measures ANOVA.**

4.4 Investigating ADAMs 17 and 10 Involvement in Keratinocyte Migration.

Although both GM6001 and TAPI1 were both found to be effective inhibitors of keratinocyte migration over endogenous TG2 and over-expressed TG2 matrix, the TAPI1 result indicated a role for ADAMs, in particular ADAM17, in keratinocyte migration. A literature search indicated that keratinocytes express ADAMS 9, 10 and 17, all of which have been implicated in keratinocyte motility and are true metalloproteinases with catalytic activity (Toriseva and Kähäri, 2008). Based on their prominent role in regulating β catenin and EGFR signaling, we decided to initially focus our investigation on ADAM10 and 17. To confirm expression of ADAMs 10 and 17 in our keratinocytes, Western blotting was carried out (Figure 27). These antibodies have previously been used by Ali and Knauper, (2007) to identify both ADAM10 and 17 in other cell types. However multiple non specific interactions have been found with both antibodies. Ali and Knauper (2007) identified the bands corresponding to ADAM10 and 17 by siRNA transfection and the subsequent removal of the ADAM10/17 band when compared with non transfected cells. This identified bands migrating at approximately 97 kDa and 134kDa for ADAM 10 and 17 respectively. Some non specific binding was also seen. This non specific binding has previously been identified with these antibodies in lymph node-derived prostate cancer cells (LNCaPs) and a human embryonic kidney (HEK293) cell line over expressing TMEFF2. Presence of the correct bands for both ADAM17 and 10 were visualised by Ali and Knauper 2007 using siRNA to both ADAM17 and 10 which showed removal of the marked bands. However the presence of the same band in these keratinocyte samples indicated that the keratinocytes express both ADAM10 and 17.

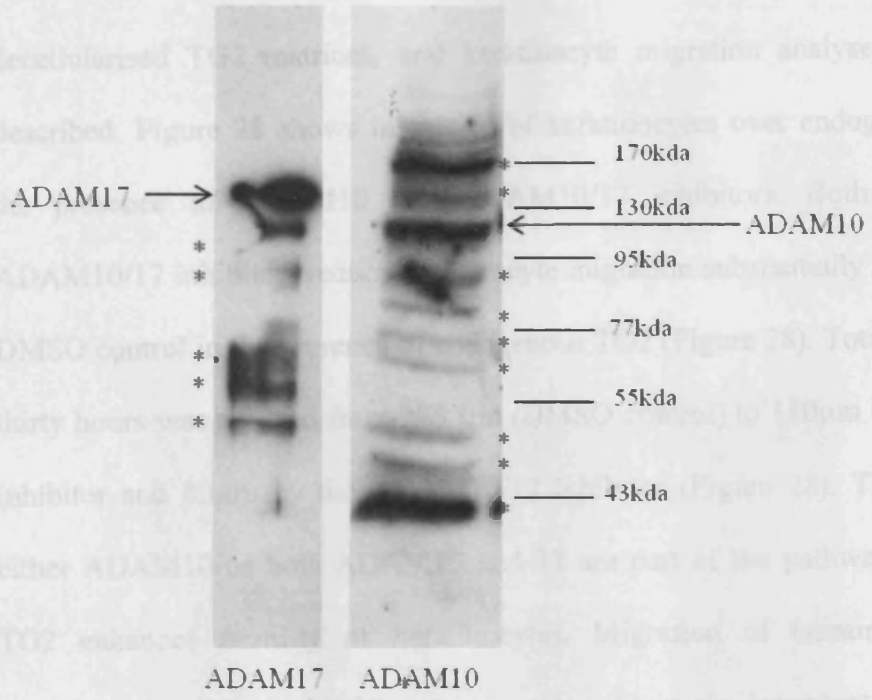


Figure 27: ADAMS10 and 17 protein expression in N-tert keratinocytes in FAD medium.

4.5 x 10^5 keratinocytes were seeded in each well of a 6 well plate and cultured for 24 hours to achieve confluence. At this time cells were washed once with PBS and lysed. Protein content was determined by BCA Assay and lysates analysed by Western blotting (10 µg protein per lane) on a 2-20% reduced SDS-PAGE gel. Membranes were probed using an anti-human ADAM10 antibody (Bioscience) and anti-human ADAM17 (Bioscience) antibody both detected with a HRP-conjugated anti-rabbit secondary antibody. Both ADAM 17 and ADAM 10 are marked on the blot (arrows) as well as * indicating non specific binding of the antibodies.

Proprietary inhibitors were obtained from GSK against ADAM 10 andADAM10/17 (GI254023X and GW280264X). Experiments were set up with the three types of decellularised TG2 matrices, and keratinocyte migration analysed as previously described. Figure 28 shows migration of keratinocytes over endogenous matrix in the presence of ADAM10 and ADAM10/17 inhibitors. Both ADAM10 and ADAM10/17 inhibitors reduce keratinocyte migration substantially in comparison to DMSO control in the presence of endogenous TG2 (Figure 28). Total migration after thirty hours was reduced from 225 μm (DMSO control) to 110μm by the ADAM10 inhibitor and 80μm by the ADAM10/17 inhibitor (Figure 28). This suggests that either ADAM10 or both ADAM10 and 17 are part of the pathway through which TG2 enhances motility of keratinocytes. Migration of keratinocytes on over expressed decellularised TG2 matrix is reduced to a similar extent by the inhibitors to both ADAM 10 and 17 (Figure 29). Migration was reduced from 190μm (DMSO control) to 100μm and 80μm in the presence of the ADAM10 inhibitor and ADAM10/17 inhibitor, respectively (Figure 29). Figure 30 shows the results from an investigation of ADAM17 and 10 involvement in migration over TG2 null matrix. Neither inhibitor has any effect on migration in the absence of TG2. These results suggest that migration in the absence of TG2 is independent of ADAM activity. This is also consistent with the observation that migration in the presence of ADAMS inhibitors on over expressed and endogenous TG2 matrix is comparable to that on TG2 null matrix in the presence of DMSO. These results suggest that TG2 mediated enhanced keratinocyte motility is dependent on both ADAM10 and 17 activity.

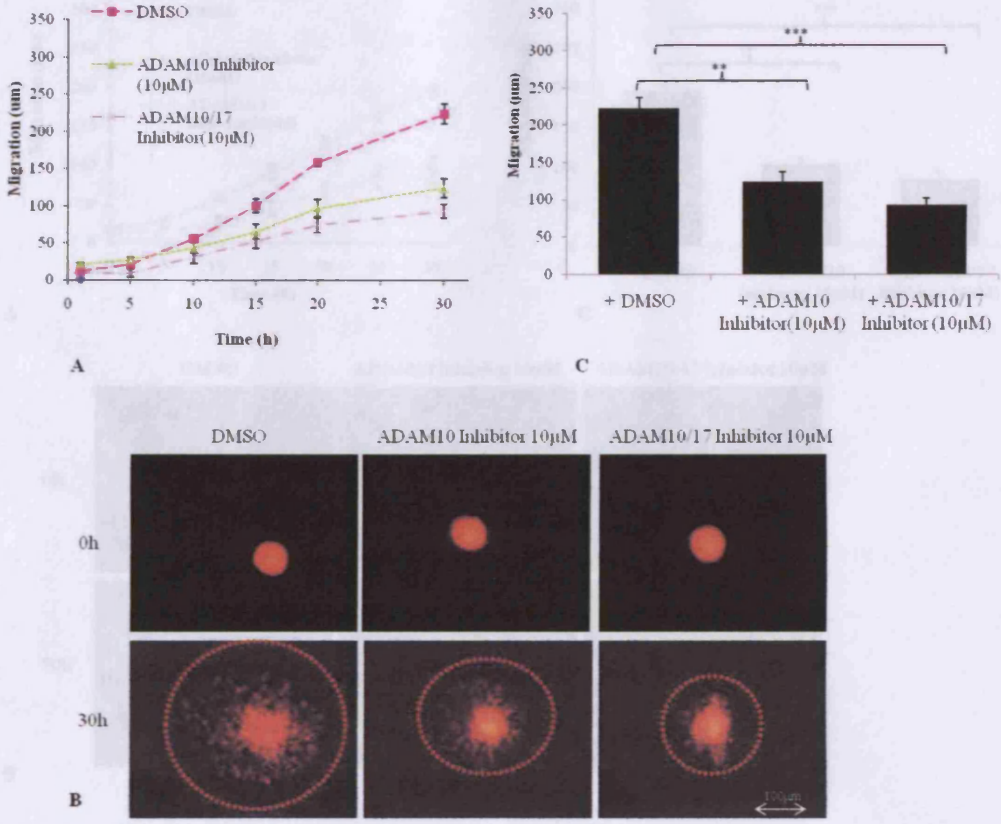


Figure 28 Investigating ADAM10 and ADAM17 involvement in keratinocyte migration using GSK inhibitors GI254023X against ADAM10 and GW280264X against ADAM10/17.

Migration of keratinocytes over decellularised TG2 endogenous matrix in the presence of defined serum free medium with DMSO a vehicle control for inhibitors, ADAM10 inhibitor GI254023X at 10μM or ADAM10/17 inhibitor GW28064X 10μM. 6 spheroids were analysed per condition per experiment. A single representative experiment is shown (3 repeats conducted with similar results) and data presented as the mean +/- SEM of six spheroids (n=6) as previously described (A). (B) Shows the corresponding micrographs at 0 and 30 hours. The edge of migration from the spheroid is defined by the red circle. (C) Migration from a single time point (hour 30) is shown to compare the maximum migration under each condition. Statistical significance denoted as follows; < P, 0.005; <***P, 0.0005, compared with DMSO control analysed by repeated measures ANOVA.**

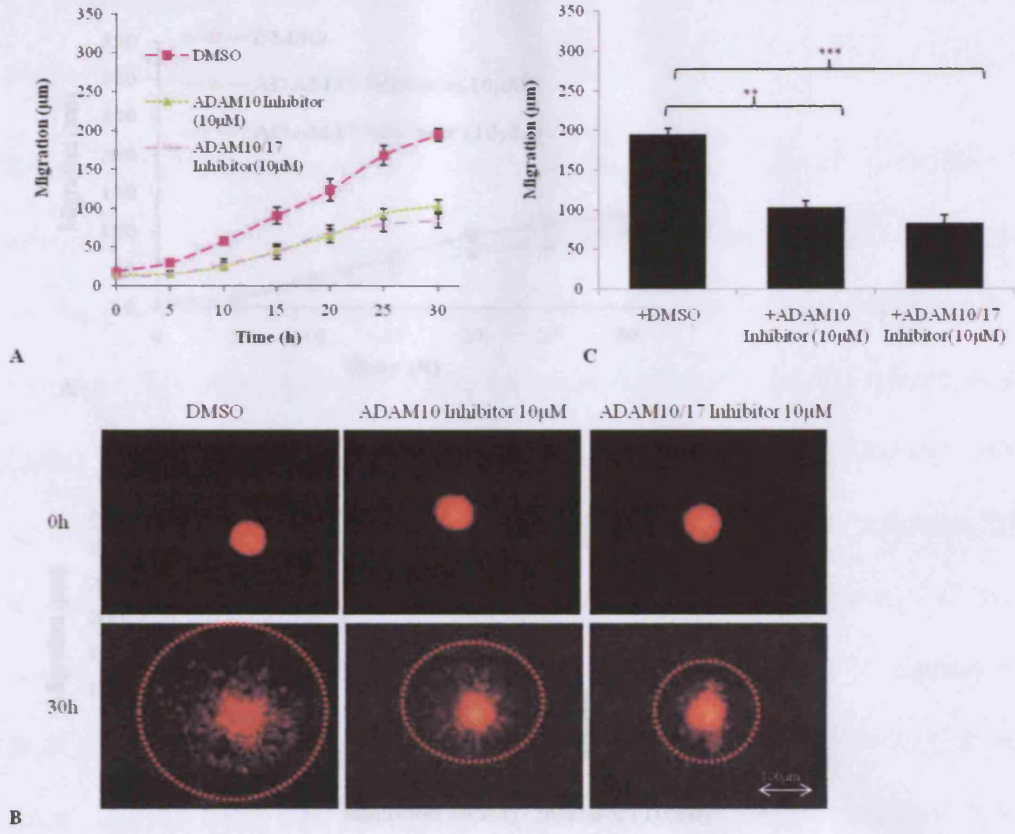
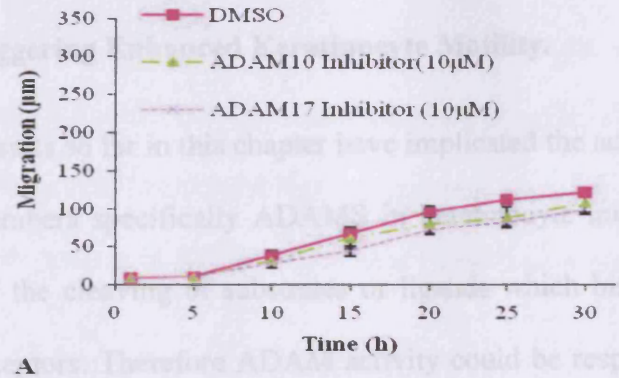


Figure 29 Investigating ADAM10 and ADAM17 involvement in keratinocyte migration using GSK inhibitors GI254023X against ADAM10 and GW280264X against ADAM10/17 on TG2 over expressed matrix.

Migration of keratinocytes over decellularised TG2 over-expressed matrix in the presence of defined serum free medium with DMSO (v/v) as a vehicle control for inhibitors, ADAM10 inhibitor GI254023X at 10µM or ADAM10/17 inhibitor GW28064X 10µM. 6 spheroids were analysed per condition per experiment. A single representative experiment is shown (3 repeats conducted with similar results) and data presented as the mean +/- SEM of six spheroids as previously described (n=6) (A). (B) Shows the corresponding micrographs of DMSO, ADAM10 and ADAM17 inhibition of migration at 0 and thirty hours. The edge of migration is indicated by the red circle. (C) Migration from a single time point (hour 30) to compare the maximum migration under each condition is shown. Statistical significance denoted as follows; < P, 0.005; <***P, 0.0005, compared with DMSO control calculated by repeated measures ANOVA.**



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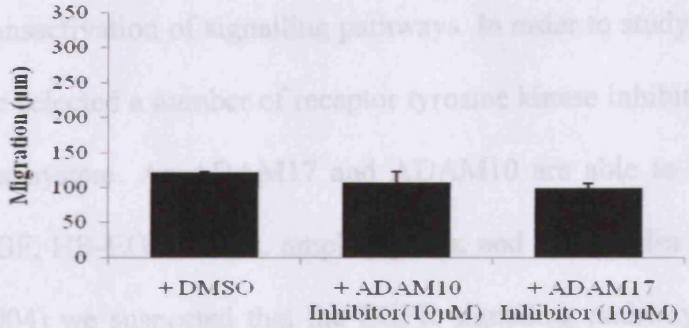

B

Figure 30 Investigating ADAM10 and ADAM17 involvement in keratinocyte migration using GSK inhibitors GI254023X against ADAM10 and GW280264X against ADAM10/17 over TG2 null matrix.

Migration of keratinocytes over a decellularised TG2 null matrix in the presence of defined serum free medium with either DMSO (v/v) as a vehicle control for inhibitors, ADAM10 inhibitor GI254023X at 10μM or ADAM10/17 inhibitor GW28064X 10μM. 6 spheroids were analysed per condition per experiment. A single representative experiment is shown (3 repeats conducted with similar results) and data presented as the mean +/- SEM of six spheroids (n=6) as previously described (A). (B) Shows corresponding micrographs of migration from a single time point (hour 30) to compare the maximum migration under each condition. No statistical significance was seen between conditions and control in these experiments when tested by repeated measures ANOVA.

4.5 Investigating Growth Factor Signalling Pathways Potentially Involved in triggering Enhanced Keratinocyte Motility.

Results so far in this chapter have implicated the activity of metalloproteinase family members specifically ADAMS in keratinocyte migration. ADAMs are responsible for the cleaving of substrates or ligands which bind or interact with growth factor receptors. Therefore ADAM activity could be responsible for the release of growth factors from the ECM or keratinocyte cell surface and thereby stimulate transactivation of signalling pathways. In order to study potential pathways involved we selected a number of receptor tyrosine kinase inhibitors and tested their effects in our system. As ADAM17 and ADAM10 are able to cleave EGF ligands such as EGF, HB-EGF, TGFα, amphiregullin, and neuregullin amongst others (Sahin et al., 2004) we suspected that the EGFR signalling pathway maybe involved. Literature also reiterates the involvement of EGFR signalling in keratinocyte motility and proliferation (Li et al., 2004, Koivisto et al., 2006). In addition IGFR and PDGFR were also investigated as potential signalling receptors for keratinocyte migration. As DSFM has been shown to contain high levels of growth factors including EGF, this medium was substituted for FAD medium with reduced serum (1%) and no EGF for the following experiments. Figure 31 compares keratinocyte migration in the presence of inhibitors to the three growth factor receptors (EGFR, IGFR and PDGFR) on both endogenous and TG2 null matrix. These results indicate that enhanced motility of keratinocytes under these culture conditions is dependent on EGFR signalling, but not on IGFR or PDGFR signalling in a TG2 endogenous background (Figure 31a and b). Keratinocyte migration was reduced from 180μm (DMSO control) to 70μm in the presence of EGFR (AG1478) inhibitor compared

with 150μm and 170μm with inhibitors for IGFR (AG1024) (150μm) and PDGFR (AG1295) (170μm, Figure 31b). These results would suggest that EGFR plays a significant in TG2 dependent enhanced motility of keratinocyte cells. Figure 31 also shows the corresponding experiment with a TG2 null matrix. Migration is independent of EGFR, IGFR, and PDGFR signalling in this case.

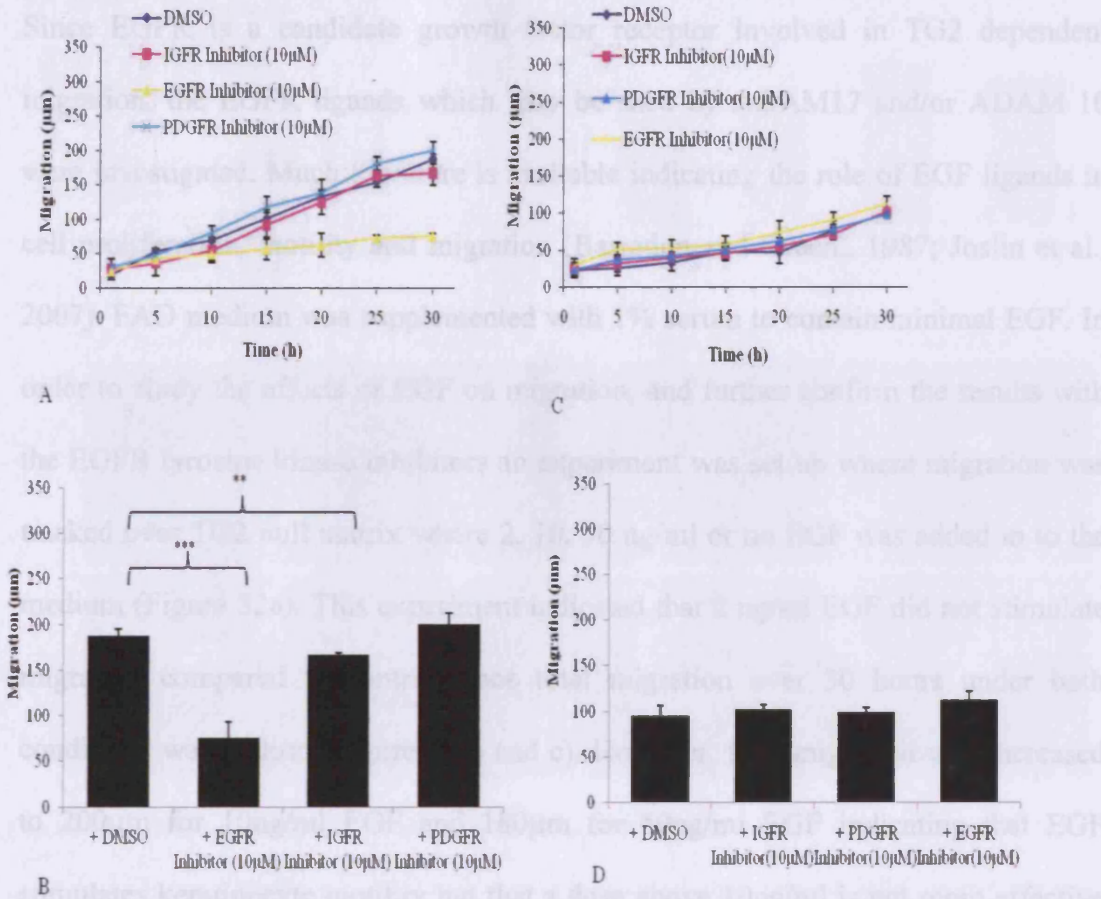

Figure 31. Migration experiment over decellularised endogenous and TG2 null matrix in the presence of growth factor receptor inhibitors for PDGFR, IGFR and EGFR.

Keratinocyte migration was analysed over 30 hours in the presence of growth factor receptor inhibitors. Spheroids were plated onto matrix and inhibitors added (10μM) in FAD media supplemented with 1% FCS and containing no EGF. A representative experiment is shown (1 of 3) and data presented as the mean +/- SEM of six spheroids (n=6) (A). (B) Shows migration from a single time point (hour 30) to compare the maximum migration under each condition. Statistical significance denoted as follows; < P, 0.005; <***P, 0.0005, compared with DMSO control. (C) shows the comparative migration of keratinocyte cells over TG2 null matrix in the presence of the growth factor receptor inhibitors and analysed as described for part A. (D) Migration from a single time point (hour 30) to compare the maximum migration under each condition. No statistical difference between conditions with and without inhibitors was seen analysed by repeated measures ANOVA.**

4.5.1 Investigating the Effects of EGF on Keratinocyte Migration.

Since EGFR is a candidate growth factor receptor involved in TG2 dependent migration, the EGFR ligands which may be shed by ADAM17 and/or ADAM 10 were investigated. Much literature is available indicating the role of EGF ligands in cell proliferation, motility and migration (Barradon and Green., 1987; Joslin et al., 2007). FAD medium was supplemented with 1% serum to contain minimal EGF. In order to study the effects of EGF on migration, and further confirm the results with the EGFR tyrosine kinase inhibitors an experiment was set up where migration was tracked over TG2 null matrix where 2, 10, 50 ng/ml or no EGF was added in to the medium (Figure 32a). This experiment indicated that 2 ng/ml EGF did not stimulate migration compared to control since total migration over 30 hours under both conditions was 140μm (Figure 32 b and c). However, total migration was increased to 200μm for 10ng/ml EGF and 180μm for 50ng/ml EGF indicating that EGF stimulates keratinocyte motility but that a dose above 10ng/ml is not more effective in stimulating migration (Figure 32 b and c). These results show that EGF can stimulate keratinocyte migration independently of TG2. Most importantly, these results show that TG2 null matrix can support enhanced keratinocyte motility if an appropriate signal is present to induce growth factor receptor signalling (in addition to integrin signalling).

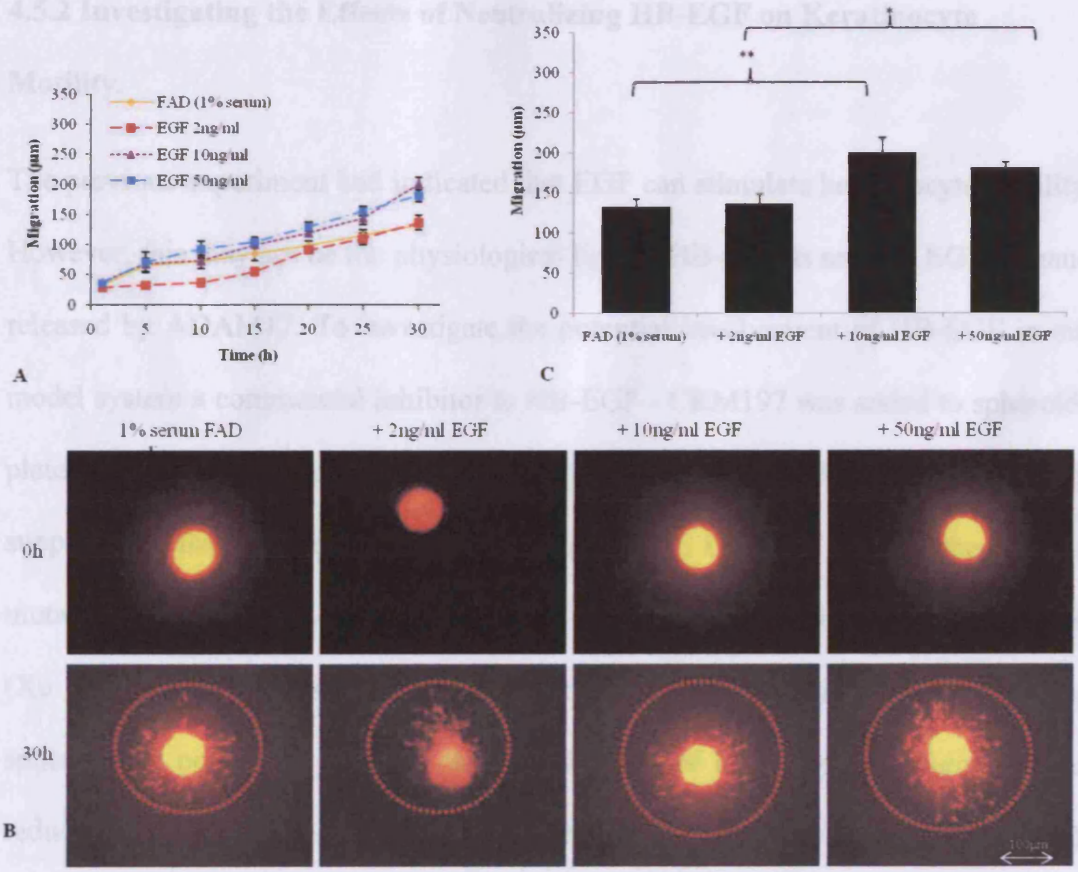

Figure 32: EGF induced enhanced motility on TG2 null matrix.

(A) Spheroids were plated onto TG2 null matrix in FAD medium without EGF containing 1% FCS as described. 2, 10 or 50ng/ml of EGF was added into the medium and migration analysed over 30 hours. This was a single experiment where six spheroids per condition were analysed, the data presented as the mean +/- SEM (n=6). (B) Shows the corresponding fluorescent images of a single spheroid per condition photographed at zero and thirty hours. The edge of migration is defined by the red circle. (C) Migration from a single time point (hour 30) to compare the maximum migration under each condition is shown. Statistical significance denoted as follows; < P, 0.005; compared with growth medium control analysed by repeated measures ANOVA.**

4.5.2 Investigating the Effects of Neutralizing HB-EGF on Keratinocyte Motility.

The previous experiment had indicated that EGF can stimulate keratinocyte motility. However, this may not be the physiological ligand. HB-EGF is another EGFR ligand released by ADAM17. To investigate the potential involvement of HB-EGF in our model system a commercial inhibitor to HB-EGF - CRM197 was added to spheroids plated on TG2 over expressed matrix in FAD media with 1 % serum and growth supplements that did not include EGF (Figure 33). CRM197 is a diptheria toxin mutant that has been shown to bind to HB-EGF with high affinity and sequester it (Xu et al., 2004). CRM197 was added to the matrix overnight prior to plating spheroids to potentially enhance the effectiveness of its inhibition. Migration was reduced in a dose dependent manner over the 3 monitored time points (Figure 33a). Figure 33b illustrates that total migration was inhibited in a dose dependent manner. This result indicated that HB-EGF may be an EGF ligand released by an ADAMs that causes enhanced migration of keratinocytes in the presence of TG2.

It should be noted that repeats conducted in both FAD containing 1% serum medium and DSFM was unable to replicate the same result. It is possible that the concentration of EGF present in the DSFM was able to stimulate migration when HB-EGF was inhibited.

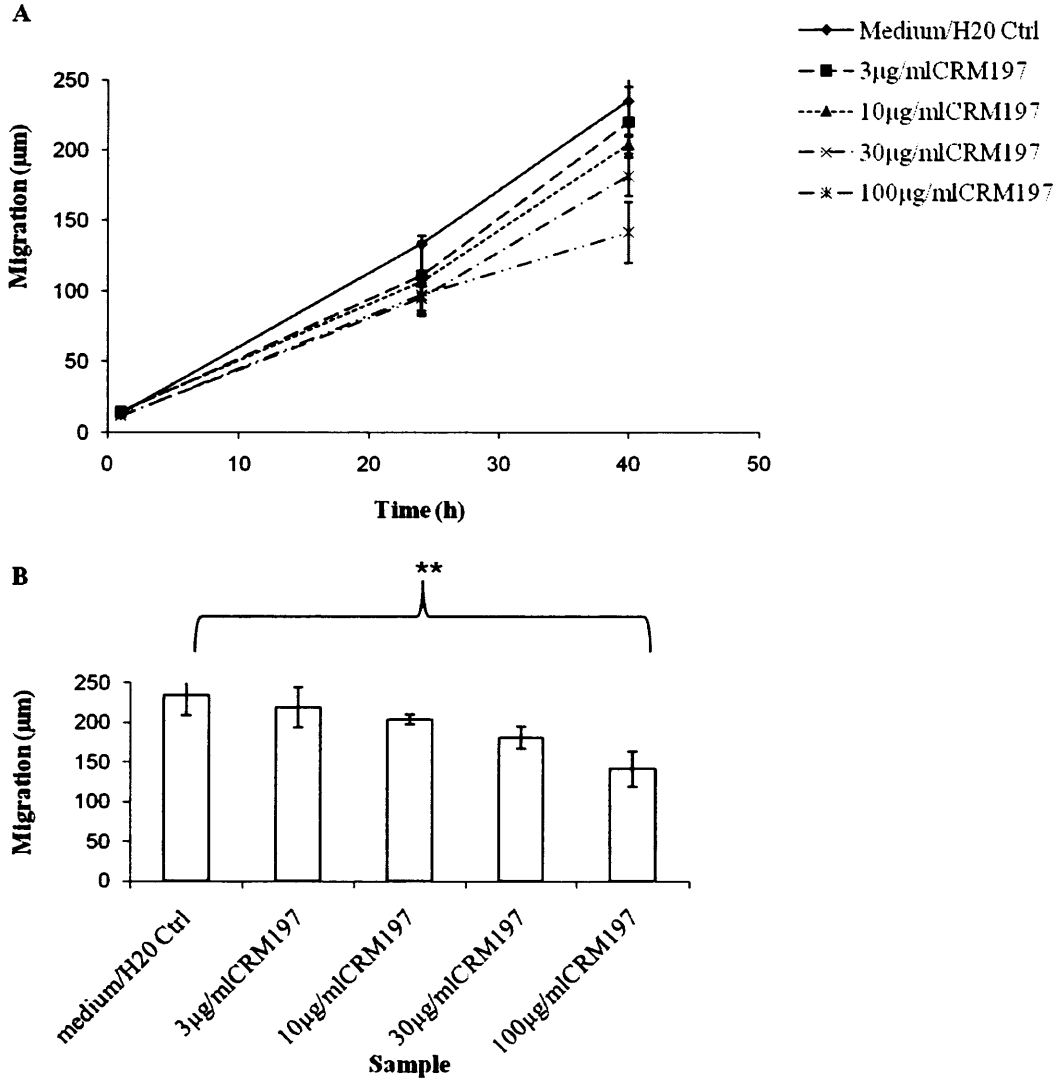

Figure 33: Investigating the role of the EGFR ligand HB-EGF in enhancing keratinocyte motility.

EGFR ligand HB-EGF was investigated in a pilot keratinocyte migration experiment on over-expressed TG2 matrix. FAD containing 1% serum but no EGF was added with no,3, 10, 30 or 100µg concentrations of theCRM197 Note the matrix was pre-incubated with the inhibitor prior to plating for 16 hours. (A) Indicates the migration seen in a single experiment where five spheroids were tested per condition and data given as mean +/-SEM. (B) Shows the maximum migration (at forty hours) seen for the five conditions. Statistical significance denoted as follows; < P, 0.005, compared with FAD containing 1% serum control analysed by repeated measures ANOVA.**

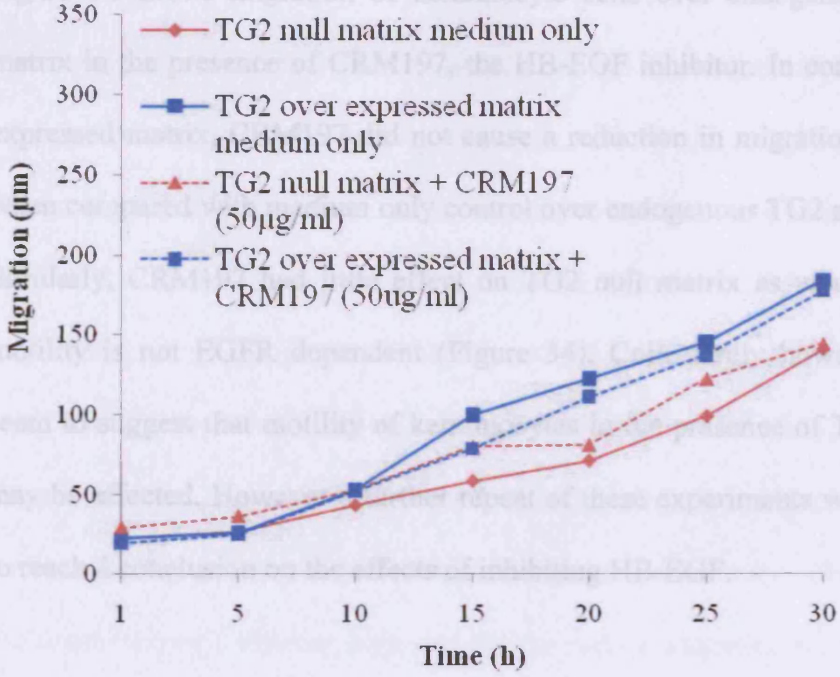

Figure 34: Investigating the role of the EGFR ligand HB-EGF in TG2 dependent and independent keratinocyte migration.

The role of the EGFR ligand HB-EGF was investigated in 2 migration experiments in FAD medium containing 1% serum but no EGF. CRM197 was added at 50µg/ml to TG2 null and TG2 endogenous matrix. Six spheroids were tested per condition and data presented as the mean +/- SEM of six spheroids. No significant difference was found by repeated measures ANOVA.

Figure 34 shows migration of keratinocyte cells over endogenous and TG2 null matrix in the presence of CRM197, the HB-EGF inhibitor. In contrast to TG2 over expressed matrix, CRM197 did not cause a reduction in migration of keratinocytes when compared with medium only control over endogenous TG2 matrix (Figure 34). Similarly, CRM197 had little effect on TG2 null matrix as would be expected if motility is not EGFR dependent (Figure 34). Collectively however, these results seem to suggest that motility of keratinocytes in the presence of TG2 and CRM197 may be affected. However a further repeat of these experiments would be necessary to reach a conclusion on the effects of inhibiting HB-EGF.

4.6 Conclusion.

The experiments reported in this chapter have led to several interesting observations when examining keratinocyte migration over a fibroblast produced matrix both in the presence and absence of TG2. Initial investigations indicated keratinocyte migration is metalloproteinase dependent in the presence of TG2. Both a general metalloproteinase inhibitor GM6001 and ADAM inhibitor TAPI1, which has a preference for ADAM17, were able to reduce the amount of migration seen in the presence of TG2 but not in its absence, suggesting that migration in a TG2 null background is independent of metalloproteinase activity. Further, TIMP1, a metalloproteinase inhibitor, was also able to reduce migration on TG overexpressed matrix (data not shown), confirming an important role for metalloproteinase activity in keratinocyte migration. Indeed, keratinocyte migration has been shown previously to be MMP1 dependent (Pilcher, 1997). Collectively, these data implicate both ADAM and MMP activity in TG2 mediated migration.

When TG2 is present it appears that the inhibition of ADAMs is most effective at reducing migration, more so than the general inhibition of metalloproteinases by GM6001. Therefore this led to considering whether a specific inhibition of ADAM17 would be affective on its own. When migration in the presence of GM6001 and TAPI1 is compared with inhibition using the specific ADAM10 and ADAM10/17 inhibitors over the same endogenous TG2 matrix interestingly, the inhibitor of ADAM17 is more effective than the inhibitor of ADAM10 although both are able to reduce migration. For example migration on over-expressed TG2 matrix in the presence of GM6001 and TAPI1 is reduced from 300μm (DMSO) to 230μm (GM6001, 50μM) and 210μm (TAPI1, 50μM); a reduction of 23 and 30%.

Using the same over-expressed TG2 matrix in the presence of the ADAM10 inhibitor GI254023X (10μM) and ADAM10/17 inhibitor GW280264X (10μM) migration is 190μm (DMSO control) compared with 100μm (ADAM10) and 80μm (ADAM10/17); a decrease in migration of 47 and 58% respectively. These results reflect the relative potency of the inhibitors for 1 or more proteases involved in the pathway that triggers enhanced motility. It also suggests that an inhibition of the ADAMs within this migration pathway causes down-stream events linked to migration not to occur. ADAMs are well known sheddases. It has already been mentioned that ADAM17 and ADAM10 are sheddases of EGFR ligands such as EGF, HB-EGF, TGFα, amphiregullin and neuregullin. Our data has shown that EGFR ligands (EGF and HB-EGF) might be involved in TG2 mediated migration. Taken together this data therefore would suggest that the inhibition of this shedding activity in the presence of TG2 affects the release of EGFR ligands and that TG2 mediated enhanced motility is mediated by EGFR transactivation and downstream signalling.

EGFR signalling has been documented as being involved with keratinocyte migration, nevertheless, two further growth factor receptors which could be involved in cell migration were investigated. (Fang et al., 1999; Pilcher et al., 1999). IGFR has been implicated in migration of murine keratinocytes (Wertheimer et al, 2000), whereas PDGFR has been identified as being responsible for cell migration in wound healing (Kim et al., 1998). Investigation using inhibitors to these growth factor receptors indicated that EGFR was the more likely growth factor receptor active in the keratinocytes of our co culture model. However, it is important to stress

that this again appears to be a TG2 dependent reaction, as all inhibitors had little effect on migration seen in a TG2 null background.

The next step was to try and identify the EGFR ligand binding to the EGFR to stimulate migration. Efforts concentrated on HB-EGF and EGF as a literature search had indicated these as the most likely candidates (Tokumaru et al., 2000; Shirakata et al., 2005). An investigation of EGF as a stimulator of migration in the TG2 null background demonstrated the presence of EGF directly induces enhanced motility further supporting the notion that EGF is potentially part of the TG2 dependent signalling pathway stimulating keratinocyte migration. The experiments showed the optimal dose of EGF in this model was between 2 ng/ml and 10 ng/ml since there was no effect at 2 ng/ml but 50 ng/ml was not able to further increase migration compared to 10 ng/ml. This perhaps indicates a maximum rate of stimulation from EGF, however further experiments would be required to confirm this.

Migration studies involving the HB-EGF inhibitor CRM197 initially indicated that this inhibitor was able to demonstrate a dose dependent reduction in migration of keratinocytes in the presence of TG2 (overexpressed). However, further experiments were unable to replicate this effect. This discrepancy could indicate that CRM197 may not be a suitable inhibitor of HB-EGF activity in the migration model. The keratinocytes are added to the matrix in a spheroid which is essentially a 3D ball of keratinocytes. This arrangement could stop keratinocytes within the centre of the ball of cells being exposed to the CRM197 inhibitor, and result in only the peripheral cells being exposed to a high enough dose of the inhibitor to cause a reduction in migration. Keratinocytes closer to the centre might simply not be exposed for long enough or at a high enough concentration for inhibition to be effective. Alternatively,

HB-EGF may not have been the candidate EGFR ligand involved in keratinocyte migration. That some inhibition was observed however means that its involvement cannot be ruled out.

It is interesting to see the dependency of migration on the EGFR signalling pathway in the presence of TG2. In this model keratinocytes appear to utilise ADAM proteolytic activity to release EGFR ligands to the EGF receptor to allow signalling to take place to increase migration. When TG2 is not present migration is independent of this pathway. Although time does not allow further investigation of this, it would be an interesting next step to investigate what may drive migration in the absence of TG2. It is likely that this relates to ECM mediated integrin activation. However, MMP9 and 13 may be involved in stimulating migration of human keratinocytes since they have been shown to stimulate migration of murine keratinocytes in excision wounds (Hattori et al., 2009). In addition, the fact that GM6001, a general metalloproteinase inhibitor, reduced migration in our model could indicate the involvement of metalloproteinases other than those investigated in this study in TG2 dependent enhanced motility. Particularly the role of MMP1 should be considered given the potent effect of TIMP-1 and its well established role in human keratinocyte motility and epithelial growth in wound healing.

In conclusion, this chapter has shown that TG2 is part of a signalling cascade involving a matrix metalloproteinase, EGFR and EGF ligands to stimulate enhanced motility (summarised in Figure 35). However, migration occurs in a TG2 null background and thus can occur completely independent of TG2; although the migration seen in this situation is greatly reduced when compared with TG2

stimulated migration. This basal migration is therefore under the control of a different mechanism.

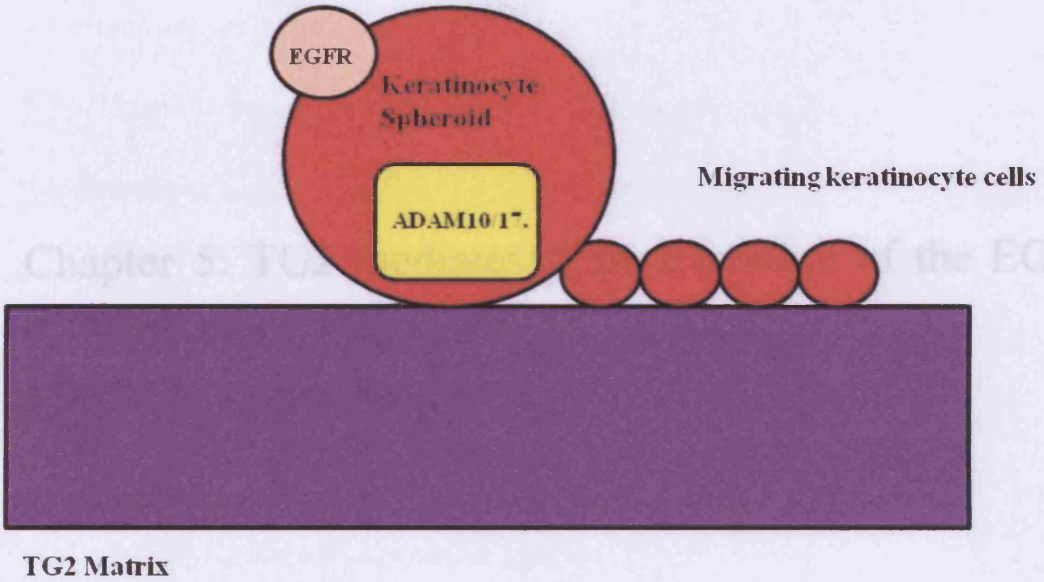

Figure 35:Summary Diagram of the Chapter.

The two results chapters so far have indicated that in the presence of TG2 ADAM 10 or 17 is able to release an EGFR ligand, potentially EGF or HB-EGF which binds to the EGFR and stimulates kerationcyte migration. The linking factor between TG2 and ADAM17 is not yet known and will be the source of investigation in the next chapter.

Chapter 5: TG2 mediates Transactivation of the EGFR pathway in Keratinocytes

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5.1 Introduction

TG2 has been implicated in stimulating keratinocyte cells during migration over a fibroblast derived ECM as shown in chapter 3. TG2 is up regulated during wound healing (Haroon et al., 1999) and has been shown to associate with the ECM following injury (Upchurch et al., 1991; Rhagunath et al., 1996). Therefore an investigation was undertaken to further examine whether the effects of fibroblast ECM were mediated directly by TG2. The conformation of TG2 changes rapidly after deposition into the ECM and this has recently been reported to be associated with a complete loss of catalytic activity (Siegel et al., 2008). TG2 is well known as a crosslinking enzyme of the ECM and catalyzes the transamidation of peptide-bound glutamine residues to lysine residues, as well as its ability to function as a G protein. The two functions use distinct catalytic sites, involve different conformations and are controlled reciprocally by Ca^{2+}/GTP availability and binding (Folk and Chung, 1985, Nakaoka et al., 1994, Griffin et al., 2002, Lorand and Graham., 2003). X ray crystallography studies by Pinkas et al., 2007 on TG2 covalently modified with an inhibitory peptide in its active site revealed a novel conformation where the active site was exposed. Along with structure-based mutagenesis, the new TG2 structure provided mechanistic insights into isopeptide bond formation by TG2 between large substrate proteins and provided proof for the long suspected shifting of the β-barrel domains during catalysis. (Pinkas et al., 2007). An earlier study from Liu and co-workers revealed that when GTP is bound to TG2, TG2 is in a highly compact conformation obstructing access to the active site and making it catalytically inactive (Liu et al., 2002). Most TG2 under physiological conditions will be in a Ca^{2+}-bound active form initially as extracellular

Ca^{2+} concentration is high (about 2mM) but may be inactivated subsequently due to oxidation (Stamnaes et al., 2010). Furthermore, under physiological conditions TG2 release in an active and potentially 'open' conformation may be promoted either by chemical injury, the removal of GTP or integrin signalling or transiently induced in response to innate immune signals such as those from toll like receptors (Pinkas et al., 2007). However, additional experiments conducted independently of this study found that TG2's ability to promote adhesion, spreading, migration, or differentiation in different cell types is independent of catalytic activity (Johnson and Terkeltaub 2005. Zemskov et al., 2006). In addition, a further study has shown that extracellular TG2 complexed with GTP can signal, at least in monocytes (Rose et al., 2006). TG2 therefore does not need to be catalytically active in order to influence keratinocyte responses. Figure 36 illustrates the 4 domain structure of TG2 and the changes that can occur to TG2 by altering its conformation. When TG2 is bound to GTP it is in a closed conformation which does not allow the active site (for crosslinking) to be utilised. However, when the pentapeptide inhibitor Ac-P(DON)LPF-NH_2, was bound in the active site TG2 was found to be in an extended conformation, requiring a 180° shift of the β-barrel domains. Ca^{2+}-binding alone is unlikely to bring this shift about, although no such structure of TG2 is available at this time. Nevertheless, interactions with the peptide backbone of the inhibitor stabilise the open conformation and it is therefore likely that substrate binding is involved in this conformational change.

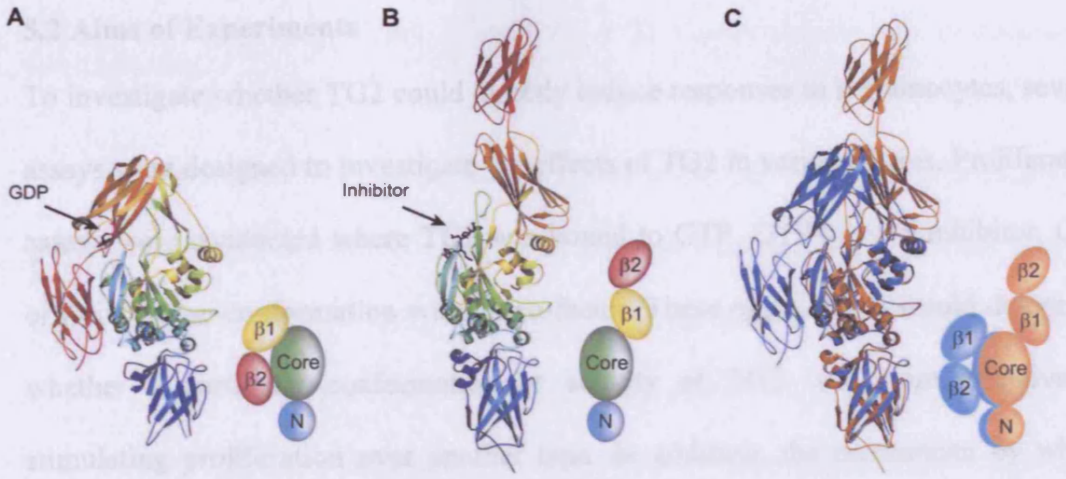

Figure 36. Overall Structures of GTP-Bound and Inhibitor-Bound TG2

Figure taken from Pinkas et al., 2007. The crystal structures are shown as ribbons, and cartoons are included to show the four main domains. In Figure 1 part A and B the N-terminal β-sandwich is shown in blue (N), the catalytic domain (Core) in green, and the C-terminal β-barrels (β1 and β2) in yellow and red, respectively. (A) GTP-bound TG2. (B) TG2 inhibited with the active-site inhibitor Ac-P(DON)LPF-NH2, a synthesized peptide used in Pinkas et al 2007. (C) The N-terminal β-sandwich and catalytic domains of the two structures are superimposed, highlighting the conformational change. The GTP-bound structure is shown in blue and the inhibitor-bound structure in gold. When GTP is bound TG2 is in a closed conformation where access to the active site is not possible. When an inhibitor is bound there is further change in the conformation of TG2 however TG2 has gone into an open structure where part of the active site is bound to the inhibitor causing changes in TG2's transamidation reactions.

5.2 Aims of Experiments

To investigate whether TG2 could directly induce responses in keratinocytes, several assays were designed to investigate the effects of TG2 in various forms. Proliferation assays were conducted where TG2 was bound to GTP, GTP-γS, I15 inhibitor, Ca^{2+} or in its native conformation without co-factor. These experiments would determine whether a particular conformation or activity of TG2 was more effective at stimulating proliferation over another type. In addition, the mechanism by which TG2 promotes proliferation was investigated by the use of various inhibitors to metalloproteinases and growth factor receptors/growth factors to identify whether the same signalling pathway stimulated proliferation and motility.

5.3 An Investigation of the Effects of TG2 Conformation on Keratinocyte Proliferation.

Chapter 4 revealed that TG2 may exert its effect on keratinocyte motility by inducing ADAM-mediated EGFR ligand shedding. However, it is not clear whether TG2 itself induces this reaction or whether expression of TG2 in fibroblasts indirectly affects keratinocyte signalling via altered gene expression or post transcriptional protein modification (altered ECM). TG2 is not present in fibroblast conditioned medium but is a component of the fibroblast ECM and could therefore directly interact with cell surface proteins of migrating keratinocytes. As Figure 36 indicates, depending on the binding of GTP, an allosteric inhibitor, Ca^{2+} can induce a large conformational change in TG2 which can influence the ability of TG2 to interact with other proteins and that regulates its ability to cross link protein substrates. Therefore, TG2 in a specific conformation may be required for an interaction with a keratinocyte cell surface protein. As it was not feasible to investigate this complex problem in our labour intensive migration model, we had to devise an assay that allowed rapid evaluation of a large number of different conditions. EGFR signalling is an important mitogenic signal in keratinocytes and if this was the pathway regulated by TG2, then we expected TG2 to have a strong effect on keratinocyte proliferation. When this is considered it is possible to speculate that conformational shape and cellular location of TG2 may alter its ability to influence proliferation. This chapter therefore investigates whether TG2 itself can directly stimulate keratinocyte proliferation and whether this relates to a specific TG2 conformation.

Initially a proliferation assay was set up to determine the proliferation seen when TG2 was added in various conformational states such as native TG2 (where it is free to undergo conformational changes with activation in a physiological context), TG2 pre-activated with Ca^{2+}, TG2 bound by GTP, and TG2 bound with GTPγS (which is resistant to hydrolysis). The results from a single representative experiment are shown for each TG2 type (Fig. 36). 5000 keratinocyte cells were seeded per well of duplicate 24 well plates and samples were incubated with 0.01, 0.1, 1.0, or 10μg/ml of either TG2, TG2 Ca^{2+}, TG2-GTP or TG2-GTPγS. Cell numbers were determined at 24 and 72 hours after stimulation using an MTT assay as described in materials and methods (section 2.3.9). Results are shown as the relative proliferation compared with a control relevant to the experiment (e.g. medium containing the carrier of activator or inhibitor); this is the change in cell number of a test condition normalised to the change in cell number of the control condition over the same time period. Figure 37 shows that keratinocyte proliferation is substantially increased in the presence of TG2 when compared with control (a value of 1.0, an arbitrary value attributed to the growth rate of the control sample, where every experimental condition is compared to this control).

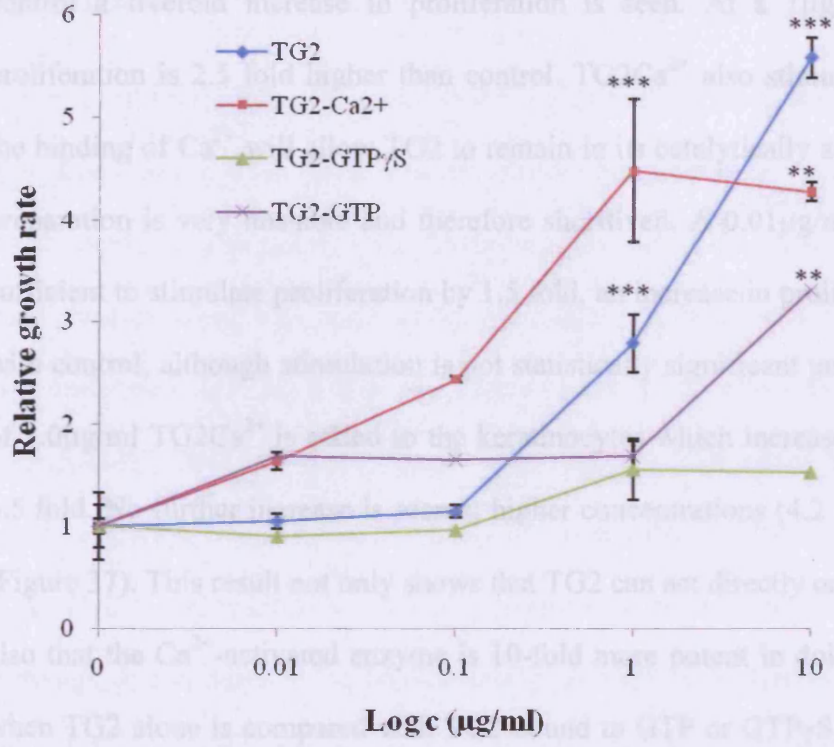

Figure 37: TG2 conformation affects stimulation of keratinocyte proliferation.

Keratinocyte cells were seeded at 5000 cells per well of a 24 well plate. Keratinocytes were allowed to attach for 6 hours before medium was removed. A 1% basic FAD medium was added to each well containing 1% serum, antibiotics, cholera toxin and hydrocortisone only. No EGF or insulin was present in any of the medium used in these experiments unless stated. 1% serum medium with 0.01, 0.1, 1.0 or 10.0µg/ml of standard TG2, TG2Ca^{2+}, TG2-GTPγS and TG2-GTP. Each condition was carried out in triplicate and the experiment repeated 3 times. A representative experiment from each condition is shown. Initial colour development (absorbance) readings were taken 24 hours after medium change to indicate a baseline level of proliferation. Readings were taken at 570 and 650nm to exclude light scattering effects on a Beckmann Coulter Spectrophotometer. After 72 hours a further set of readings were taken to indicate final proliferation values. The 72 hour readings were diluted 1:1 with water to allow the spectrophotometer to analyse the absorbance within its linear range. Subsequently 24 hour readings were subtracted from 72 hour readings and expressed here as relative proliferation calculated as described in materials and methods section 2.3.10 compared with control value. Statistical significance denoted as follows; < P, 0.005; <***P, 0.0005, compared with unsupplemented medium control (not shown; value of one) for each point.**

When proliferation at a higher concentration of TG2 (10ug/ml) is compared to control a fivefold increase in proliferation is seen. At a 1μg/ml dose of TG2 proliferation is 2.5 fold higher than control. TG2Ca^{2+} also stimulates proliferation; the binding of Ca^{2+} will allow TG2 to remain in its catalytically active form but this preparation is very unstable and therefore shortlived. A 0.01μg/ml concentration is sufficient to stimulate proliferation by 1.5 fold, an increase in proliferation compared with control, although stimulation is not statistically significant until a concentration of 1.0μg/ml TG2Ca^{2+} is added to the keratinocytes which increases proliferation by 4.5 fold. No further increase is seen at higher concentrations (4.2 fold at 10.0μg/ml) (Figure 37). This result not only shows that TG2 can act directly on keratinocytes but also that the Ca^{2+}-activated enzyme is 10-fold more potent in doing so. In contrast, when TG2 alone is compared with TG2 bound to GTP or GTPγS, (a GTP analogue which cannot be hydrolysed), interestingly, neither stimulated keratinocyte proliferation, with the exception of the highest dose of TG2-GTP (Figure 37). TG2-GTP can be hydrolysed by TG2 to GMP which is released as affinity for GMP is very low. Under physiological conditions this normal turnover occurs and at least a portion of the population of TG2 will be in the same conformation like native TG2. In accordance with this, the results show that TG2 bound to GTP only stimulates proliferation at the highest concentration – (10μg/ml by 3 fold). In addition, the TG2 bound with the non-hydrolysable GTP analogue GTPγS is not able to stimulate keratinocyte proliferation at any concentration. This suggests that TG2 in its closed conformation is unable to stimulate proliferation and therefore that either a cryptic protein binding site or catalytic activity is required for this function of TG2.

5.3.1 I15 'Open Conformation' TG2 and its Effects on Keratinocyte Proliferation.

Figure 37 showed when TG2 is in its Ca^{2+}-bound active conformation proliferation is stimulated and when in a closed conformation TG2 is unable to stimulate keratinocyte proliferation. To examine whether TG2 activity or the open conformation alone was required for stimulation of keratinocyte proliferation TG2 was incubated with an active site targeting inhibitor similar to that described by Pinkas et al., 2007, shown in Figure 38. Using a peptide based inhibitor developed by Zedira: Boc-DON-QIV-OMe (I15) which covalently attaches to the active site Cys, TG2 is expected to be sterically trapped in the enzymes' open conformation although this remains to be experimentally verified. The trapped TG2 was purified by gel filtration and the degree to which the enzyme had been converted to the inhibited form was estimated by determining the level of monodansylcadaverine incorporation into N,N-dimethylcasein. Figure 38 shows the cross linking products of dimethylcasein as a smear from low to higher Mr complexes, with dimethylcasein itself being visible as doublet around 25 kDa (marked by the arrow at the bottom of the gel). Compared with the starting native TG2, the TG2-I15 preparation was estimated to have less than 10% of its activity remaining (0.5μg TG2-I15 is comparable to 0.05 μg native TG2 (1:20) indicating that >90% has been modified.

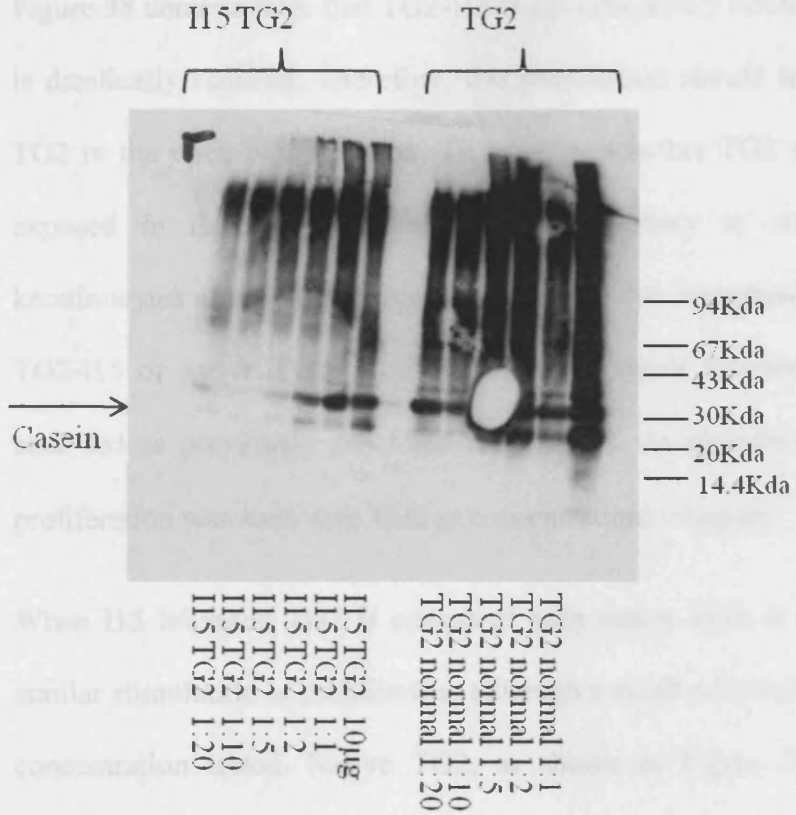

Figure 38: activity of native TG2 and TG2-I15 as compared by incorporation of monodansyl cadaverine.

TG2 and TG2-I15 were compared for activity using a monodansyl cadaverine incorporation assay previously described in (Aeschlimann and Paulsson 1991, and Aeschlimann et al., 1995). TG2 was started at a 5µg/ml concentration for both TG2 and TG2-I15 and a dilution series was prepared for both. Dilutions used were: 5µg/ml, then 1:1,1:2, 1:5, 1:10, 1:20,. After a 30 minute incubation at 37°C, incorporation of the monodansyl cadaverine into N,N-dimethyl casein was revealed by Western blot analysis with an anti-dansyl antibody (diluted 1:300). The dimethylcasein band can be seen as a doublet between the 20 and 30kDa (arrow). Migration of Mr standards is indicated on the right.

Figure 38 demonstrates that TG2-I15 is not completely inhibited however its activity is drastically reduced. Therefore, this preparation should to a large extent contain TG2 in the open conformation. To examine whether TG2 activity or a cryptic site exposed in the open conformation is necessary to stimulate a response in keratinocytes a proliferation assay was carried out. Keratinocytes were cultured with TG2-I15 or native TG2 at different concentrations and the effect on proliferation analysed as previously described (Figure 39). As expected, a strong induction of proliferation was seen with TG2 at concentrations >1μg/ml.

When I15 inhibited TG2 is compared with native TG2, it is clear that there is no similar stimulation of proliferation although a small stimulation is seen at the highest concentration tested. Native TG2, as shown in Figure 37, is able to stimulate proliferation in a dose dependant manner from 1.0μg/ml concentration (2.5 fold) to 10μg/ml to (5.5 fold). When I15 TG2 is compared, no statistically significant stimulation of keratinocyte proliferation is seen, even at the highest dose of 10μg/ml. These results suggest that TG2 must be active in order to stimulate proliferation.

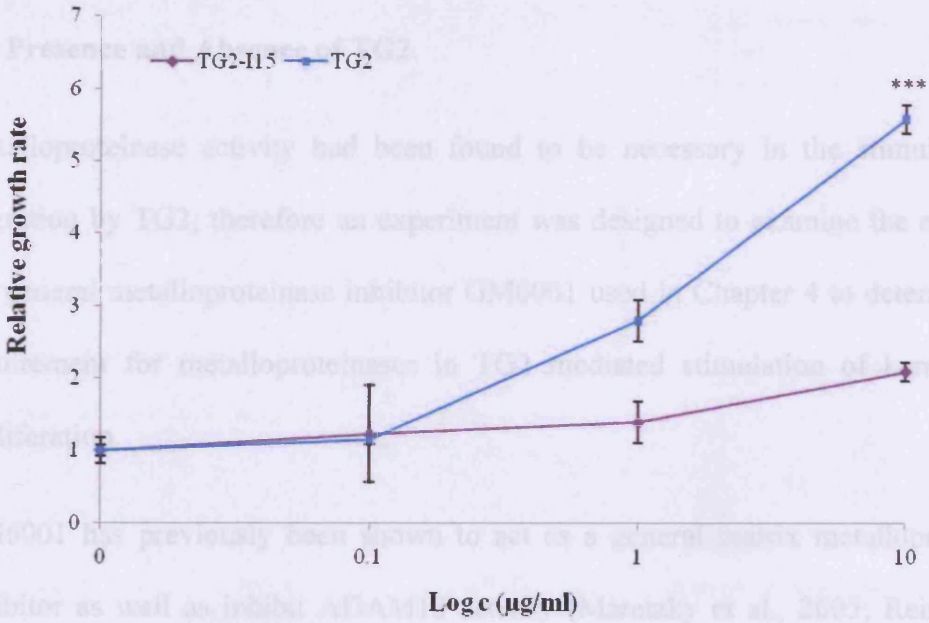

Figure 39: Stimulation of keratinocyte proliferation requires TG2 crosslinking activity.

TG2-I15 was compared with native TG2 to determine whether any stimulation of proliferation of keratinocytes occurred. TG2-I15 and native TG2 were tested at 3 concentrations 0.1, 1.0 and 10µg/ml. Keratinocytes were seeded in 1% serum-containing FAD medium minus EGF and insulin and stimulated with the two preparations of TG2 as indicated after 6h. Proliferation was analysed as has been previously described. 3 replicates per condition of the experiment were carried out and the data is given as the mean +/- SD. A single experiment was conducted. Statistical significance denoted as follows; ***,P<0.0005, compared with medium control for each point.

5.4 Metalloproteinase Inhibition and its Effects on Keratinocyte Proliferation in the Presence and Absence of TG2.

Metalloproteinase activity had been found to be necessary in the stimulation of migration by TG2, therefore an experiment was designed to examine the effects of the general metalloproteinase inhibitor GM6001 used in Chapter 4 to determine the requirement for metalloproteinases in TG2 mediated stimulation of keratinocyte proliferation.

GM6001 has previously been shown to act as a general matrix metalloproteinase inhibitor as well as inhibit ADAM10 activity (Maretzky et al., 2005; Reiss et al., 2006). An experiment was conducted to test at what dose GM6001 was effective at inhibiting proliferation and whether or not TG2 was able to stimulate proliferation in the presence of the inhibitor.

Figure 40 shows the dose dependency curve of GM6001 produced in the presence and absence of TG2 (panel a) and a single dose of GM6001 compared with its DMSO control to indicate the effect of TG2 (panel b). Figure 40 shows that at least 50μM of GM6001 is required to reduce proliferation in both the presence and absence of TG2.

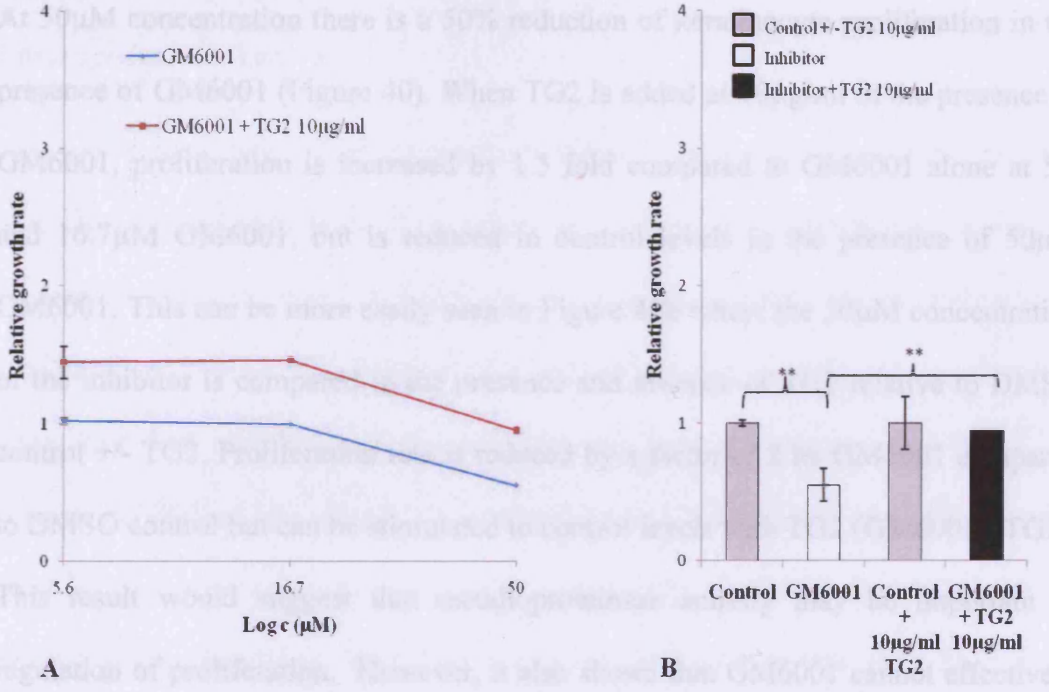

Figure 40: metalloproteinase inhibitor GM6001 is not effective in blocking TG2-mediated stimulation of keratinocyte proliferation.

GM6001 was used to investigate the effects of inhibiting metalloproteinases in TG2 mediated stimulation of keratinocytes.. Part A shows proliferation in the presence of GM6001 at 3 concentrations 5.6, 16.7 and 50µM in FAD medium containing 1% serum in the presence and absence of 10µg/ml native TG2. A representative experiment is shown. A representative experiment is shown and the results are given as mean +/-SD of 3 replicates. Statistical significance denoted as follows; <P, 0.005, compared with DMSO control. Dose curves were carried out twice and at least one further experiment was conducted at a single effective dose.**

At 50μM concentration there is a 50% reduction of keratinocyte proliferation in the presence of GM6001 (Figure 40). When TG2 is added at 10μg/ml in the presence of GM6001, proliferation is increased by 1.5 fold compared to GM6001 alone at 5.6 and 16.7μM GM6001, but is reduced to control levels in the presence of 50μM GM6001. This can be more easily seen in Figure 40b where the 50μM concentration of the inhibitor is compared in the presence and absence of TG2 relative to DMSO control +/- TG2. Proliferation rate is reduced by a factor of 2 by GM6001 compared to DMSO control but can be stimulated to control levels with TG2 (GM6001 +TG2). This result would suggest that metalloproteinase activity may be important in regulation of proliferation. However, it also shows that GM6001 cannot effectively block TG2-mediated stimulation of keratinocyte proliferation.

TG2 is only able to induce a low level stimulation in the presence of DMSO (compared with that seen in Figure 37). This is due to the fact that DMSO itself promotes keratinocyte proliferation and that proliferation under basal conditions therefore was already high.

5.4.1 ADAM10 and 17 Activities and their Effect on Proliferation in the Presence and Absence of TG2.

As the previous chapter has indicated a potential role of metalloproteinase activity, and more specifically ADAM activity, in TG2-induced keratinocyte migration further experiments were designed to examine the effects on proliferation of inhibiting ADAM10 and ADAM10/17. ADAM10 can be affected by GM6001 suggesting that inhibiting ADAM-10 alone may not be sufficient to block TG2-mediated proliferation. ADAM17 is structurally similar to ADAM10 but unlike ADAM10 is not constitutively active in many cells (with high levels of activity present after induction). Therefore a specific inhibitor to this may be a more promising approach to specifically affect proliferation stimulated by TG2. Figure 41 shows the effects of inhibiting either ADAM10 alone or ADAM17/10 in the presence and absence of TG2. Both inhibitors were tried at several concentrations to determine an effective dose of inhibitor to cause a reduction in proliferation rate.

Figure 41 shows the dose response curve for the ADAM10 inhibitor in the presence and absence of TG2. 3μM of ADAM10 inhibitor is sufficient to inhibit proliferation to 50% of control. Proliferation is dose dependently inhibited up to 10μM (a reduction of 90%) with no further change at 30μM. When 10μg/ml TG2 is added in the presence of inhibitors at the same doses there is a substantial increase in proliferation (by 50% at 1.0μM of ADAM10 inhibitor compared with control) and this level is maintained for concentrations up to 10μM). Interestingly, TG2 is able to stimulate proliferation even at a 30μM inhibitor dose when compared with the inhibitor added alone. Part b shows the difference between ADAM 10 inhibitor at

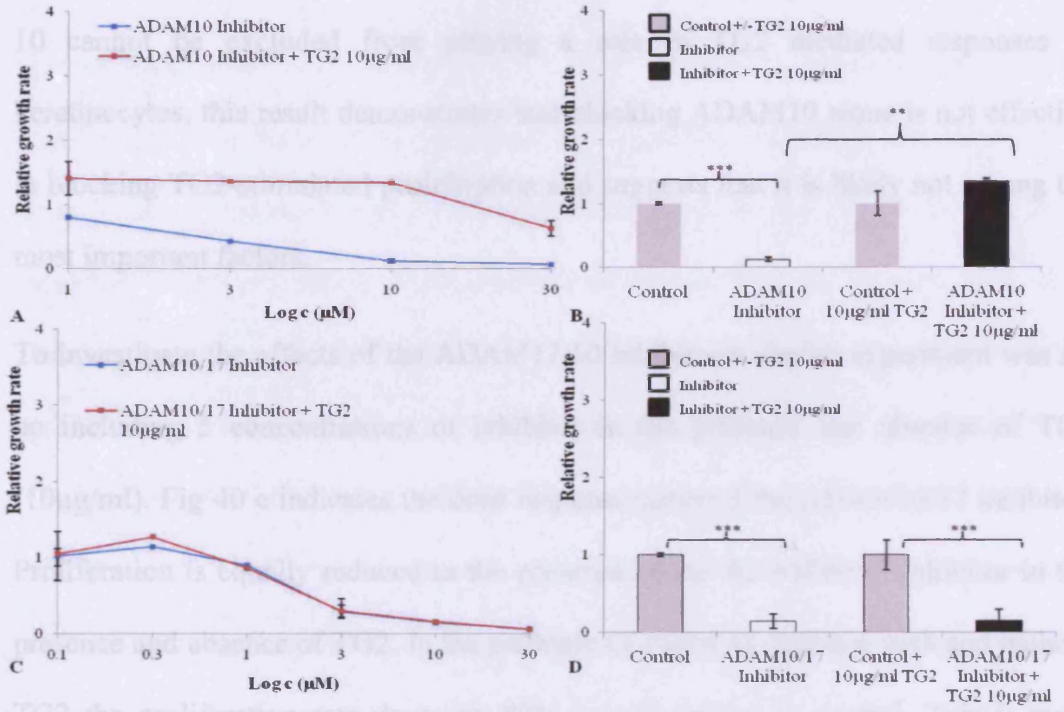

Figure 41: ADAM17 inhibition blocks TG2-mediated keratinocyte proliferation.

ADAM10 and 17 inhibitors were used to investigate the effects of inhibiting ADAM 10 and 17 in TG2 mediated stimulation of proliferation in keratinocytes. Part A shows proliferation of keratinocytes in the presence of ADAM10 inhibitor at several concentrations 1, 3, 10 and 30µM in FAD medium containing 1% serum in the presence and absence of 10µg/ml TG2. Part B shows corresponding proliferation for ADAM 10 inhibitor at a single effective dose of 10 µM dose compared with DMSO control in the presence and absence of TG2. Parts C and D show the corresponding results for the addition of ADAM10/17 inhibitor. Part C indicates the 5 concentrations of ADAM10/17 inhibitor tried and part D shows the effective dose at 10µM compared with DMSO control A representative experiment is shown in each case. The results are given as mean +/-SD of 3 replicates. Statistical significance denoted as follows; <P, 0.005, <***P, 0.0005. Dose curves were carried out twice and at least one further experiment was conducted at a single effective dose per inhibitor.**

10μM with and without TG2 compared with DMSO control. TG2 is able to stimulate proliferation despite the presence of the ADAM10 inhibitor. While ADAM 10 cannot be excluded from playing a role in TG2 mediated responses in keratinocytes, this result demonstrates that blocking ADAM10 alone is not effective in blocking TG2-stimulated proliferation and suggests that it is likely not among the most important factors.

To investigate the effects of the ADAM17/10 inhibitor a similar experiment was set up including 5 concentrations of inhibitor in the presence and absence of TG2 (10μg/ml). Fig 40 c indicates the dose response curve of the ADAM10/17 inhibitor. Proliferation is equally reduced in the presence of the ADAM10/17 inhibitor in the presence and absence of TG2. In the presence of 10μM of inhibitor with and without TG2 the proliferation rate drops by 80% in comparison to control. This is more easily seen at a single concentration of 10μM ADAM10/17 inhibitor in the presence and absence of TG2 10μg/ml shown relative to DMSO control shown in Fig 40d. There is no statistical difference between the proliferation of cells seen in the presence of the inhibitor alone or inhibitor plus TG2. This result indicates that ADAM 17 may be involved in regulation of both basal and TG2 mediated keratinocyte proliferation.

5.5 Growth Factor Receptor Inhibition and the Effects on Keratinocyte Proliferation in the Presence and Absence of TG2.

Having found both TG2 and ADAM 17 to be involved in regulating keratinocyte proliferation, we went on to examine the signalling pathways linked including growth factor receptors. This would determine whether any of the growth factor receptors tested previously were involved in proliferation of keratinocytes. Most importantly, this would further confirm in an independent way whether specifically the EGFR pathway is involved in the keratinocyte response to TG2. Inhibitors to IGFR, EGFR, PDGFR and FGF/VEGFR were tested as these are the major pathways through which proliferation of keratinocytes is regulated. A preliminary experiment was conducted to establish a suitable concentration before testing each inhibitor at a single dose in the presence and absence of TG2. Proliferation assays were carried out as previously described. Figure 42 shows the proliferation seen with IGFR inhibitor in the presence and absence of TG2. Part a shows that the IGFR inhibitor did not reduce proliferation effectively until a 10μM concentration was applied where proliferation dropped to about 50% of control. In the presence of TG2, the inhibitor was again unable to reduce proliferation until a 10μM dose of inhibitor was applied. Importantly, TG2 was able to stimulate proliferation despite the presence of the inhibitor. When the single 10μM dose of IGFR inhibitor is examined in the presence and absence of TG2 compared with a DMSO control in part b; proliferation in the presence of TG2 was >2-times that than with the inhibitor alone. This result suggests that TG2 effects on keratinocyte proliferation are not dependent on IGFR signalling.

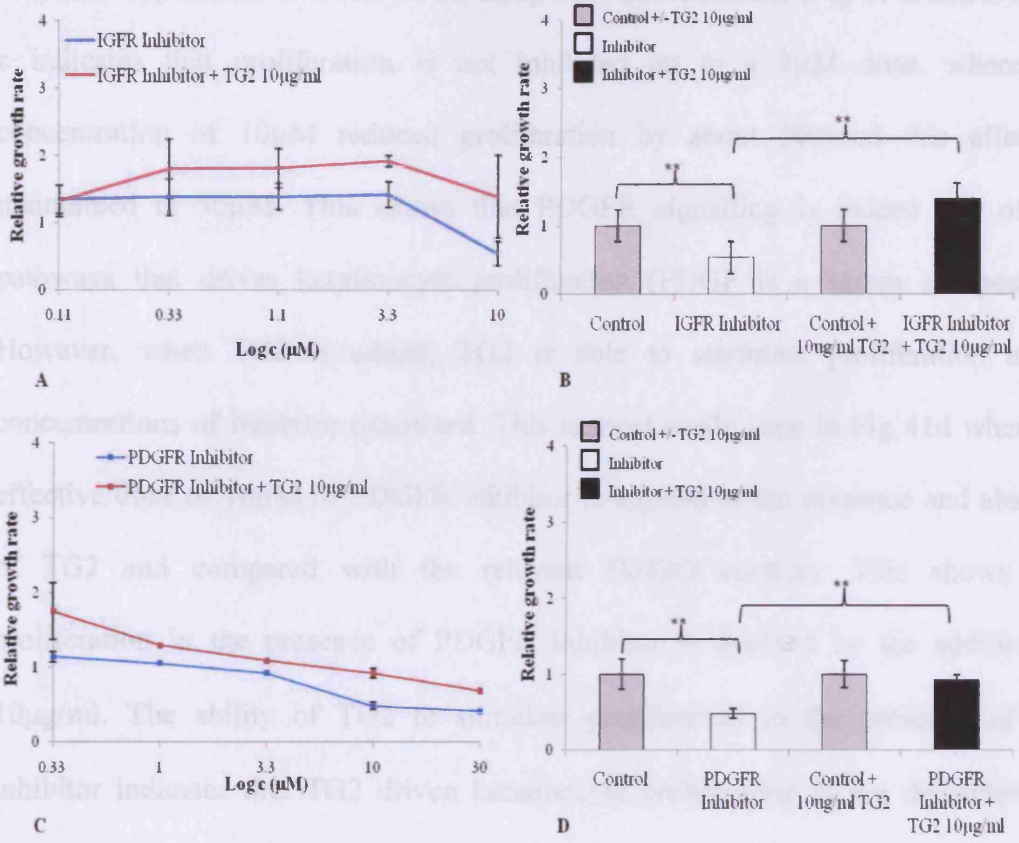

Figure 42: TG2 effects on proliferation are not mediated by IGFR or PDGFR signalling.

IGFR (AG1024) and PDGFR (AG1295) inhibitors were used to investigate the effects of inhibiting these growth factors in TG2-mediated stimulation of proliferation of keratinocytes. Part A shows proliferation of keratinocytes in the presence of IGFR inhibitor at 5 concentrations 0.11, 0.33, 1.1, 3.3 and 10µM in FAD medium containing 1% serum in the presence and absence of TG2 at 10µg/ml. Part B shows corresponding proliferation for IGFR inhibitor at a single dose of 10 µM compared with DMSO control all shown in the presence and absence of TG2. Parts C and D shows the corresponding results for PDGFR inhibitor at 0.33, 1.1, 3.3, 10 and 30µM concentrations in the same medium plus and minus TG2 10µg/ml. Part D shows a single dose of 10µM of the PDGFR inhibitor and the corresponding proliferation compared with DMSO control all shown in the presence and absence of TG2. A representative experiment is shown in each case. The results are given as mean +/-SD of three replicates. Statistical significance denoted as follows; ** p<0.005, Dose curves were carried out twice and at least one further experiment was conducted at a single effective dose per inhibitor.

A similar experiment was conducted using a PDGFR inhibitor (Fig 41 C and D). Part c indicates that proliferation is not inhibited up to a 3μM dose, whereas a concentration of 10μM reduced proliferation by about 50%and this effect is maintained at 30μM. This shows that PDGFR signalling is indeed one of the pathways that drives keratinocyte proliferation (PDGF is a serum component). However, when TG2 is added, TG2 is able to stimulate proliferation at all concentrations of inhibitor examined. This is most easily seen in Fig 41d where an effective dose of 10μM of PDGFR inhibitor is applied in the presence and absence of TG2 and compared with the relevant DMSO controls. This shows that proliferation in the presence of PDGFR inhibitor is doubled by the addition of 10μg/ml. The ability of TG2 to stimulate proliferation in the presence of this inhibitor indicates that TG2 driven keratinocyte proliferation is not dependent on PDGFR.

Figure 43 shows the corresponding dose response curves using inhibitors against EGFR and FGF/VEGFR in the presence and absence of TG2. The effect of these was tested at various doses twice before a single dose was tested again and 3 repeats were conducted for each inhibitor in the presence and absence of TG2. Figure 43A indicates that the inhibitor for EGFR was able to effectively inhibit proliferation both in the presence and absence of TG2 at concentrations of 10 and 30μM. However, there was only a small difference in the growth rate of cells in the presence of different inhibitor concentrations (0.3 to 30μM) without stimulation suggesting that proliferation under these culture conditions (no EGF in medium) is largely EGFR

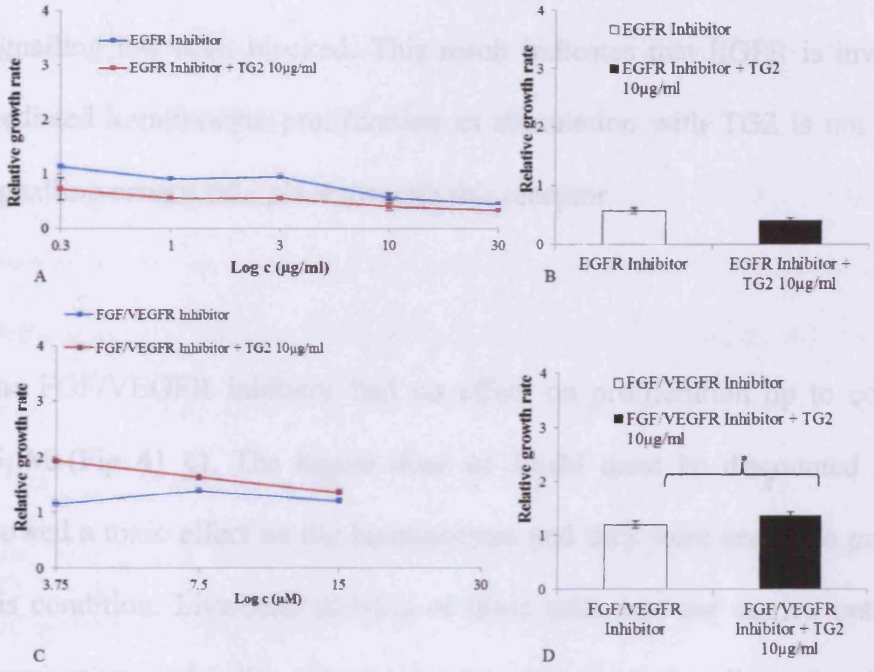

Figure 43: TG2 effects on proliferation are dependent on EGFR signalling.

EGFR (AG1478) and FGF/VEGFR (PD173074) inhibitors were used to investigate the effects of inhibiting these growth factors in TG2 mediated stimulation of proliferation of keratinocytes. Part A shows proliferation of keratinocytes in the presence of EGFR inhibitor at 5 concentrations, 0.33, 1, 3, 10 and 30µM in FAD medium containing 1% serum in the presence and absence of TG2 at 10µg/ml. Part B shows corresponding proliferation for EGFR inhibitor at a single dose of 10 µM compared with DMSO control all shown in the presence and absence of TG2. Part C show the corresponding results for FGF/VEGFR inhibitor at 3.75, 7.5, and 15 in FAD medium containing 1% serum plus and minus TG2 10µg/ml. Part D shows a single dose of 15µM and the corresponding proliferation compared with DMSO control all shown in the presence and absence of TG2. A representative experiment is shown in each case. The results are given as mean +/-SD of three replicates. Statistical significance denoted as follows; <*P, 0.05, compared with DMSO control. Dose curves were carried out twice and a further experiment was conducted at a single effective dose per inhibitor.

signalling independent. Figure 43b shows a single effective dose of 10μM of EGFR inhibitor in the presence and absence of TG2 compared with DMSO control. This shows that proliferation cannot be stimulated with TG2 in cells in which EGFR signalling has been blocked. This result indicates that EGFR is involved in TG2-mediated keratinocyte proliferation as stimulation with TG2 is not possible when signalling cannot take place through this receptor.

The FGF/VEGFR inhibitor had no effect on proliferation up to concentration of 15μM (Fig 41 c). The higher dose of 30μM must be discounted as this clearly showed a toxic effect on the keratinocytes and they were unable to proliferate under this condition. Live/dead staining of these cells was not carried out. However, an examination under the microscope revealed floating cells and a lack of colour development (translucent samples) in the MTT assay indicated the loss of most of the cells. Fig 42d indicates the proliferation seen at the single 15μM dose in the presence and absence of TG2 compared with the respective DMSO controls. This shows that proliferation was not inhibited in the presence of this inhibitor when compared with control, giving a relative proliferation rate of 1.2, and after stimulation with TG2, a rate of 1.4. Therefore, there may be a small degree of stimulation by TG2. However, this could not be ascertained under these conditions where in the presence of this inhibitor and DMSO keratinocytes proliferated at near maximal rate.

Figure 44 provides further evidence for the notion that the response of keratinocytes to TG2 is independent of IGFR. Insulin is a normal component in standard FAD

media, and has been shown to stimulate keratinocyte growth (Fuchs and Green, 1981; Gilchrest et al., 1982; Tsao et al., 1982). Enhanced proliferation in the presence of insulin is mediated via IGFR. We have therefore examined the ability of TG2 to stimulate proliferation in FAD medium (1% serum) in both the presence and absence of insulin. Figure 44 shows proliferation of keratinocytes at 3 doses of TG2 0.1, 1.0 and 10μg/ml in the presence and absence of 5ng/ml insulin, which is the same dose used in standard FAD medium and corresponds to the dose of insulin that yields maximal increase in keratinocyte proliferation. Figure 44 shows that TG2 is able to stimulate proliferation in a dose-dependent manner in the presence and absence of insulin, with a 1.0-10μg/ml dose of TG2 more than doubling the growth rate. This result further indicates that IGFR is not involved in the proliferation pathway we are examining.

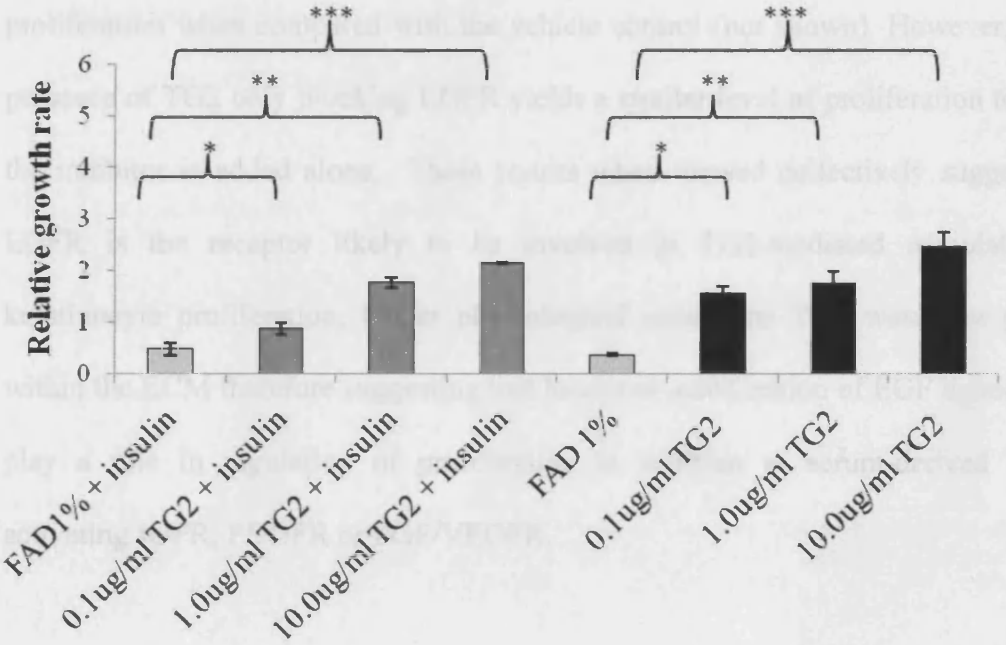

Figure 44: TG2 and insulin-mediated stimulation of keratinocyte proliferation occurs through independent pathways.

5000 cells per well were added to a 24 well plate. Keratinocytes were allowed to settle for 6 hours before medium was removed. A 1% serum-containing basic FAD medium was added to each well containing antibiotics, cholera toxin and hydrocortisone only. Cells were stimulated with 3 concentrations of TG2 in either the presence or absence of 5ng/ml insulin and cell growth was assessed over a 72h period. A representative experiment is shown. The results are given as mean +/-SD of three replicates. Statistical significance denoted as follows; <*P, 0.05, <P, 0.005, <***P, 0.0005. Dose curve was carried out twice. 2 repeats of the experiment were carried out, a representative experiment is shown.**

Figure 45 shows a comparison of the effect on keratinocyte proliferation of all effective doses of inhibitor of various growth factor receptors in the presence and absence of TG2 stimulation. All except FGF/VEGFR inhibitor are able to reduce proliferation when compared with the vehicle control (not shown). However, in the presence of TG2 only blocking EGFR yields a similar level of proliferation to when the inhibitor is added alone. These results when viewed collectively suggest that EGFR is the receptor likely to be involved in TG2-mediated stimulation of keratinocyte proliferation. Under physiological conditions TG2 would be present within the ECM therefore suggesting that localized mobilization of EGF ligands may play a role in regulation of proliferation in addition to serum-derived factors activating IGFR, PDGFR or FGF/VEGFR.

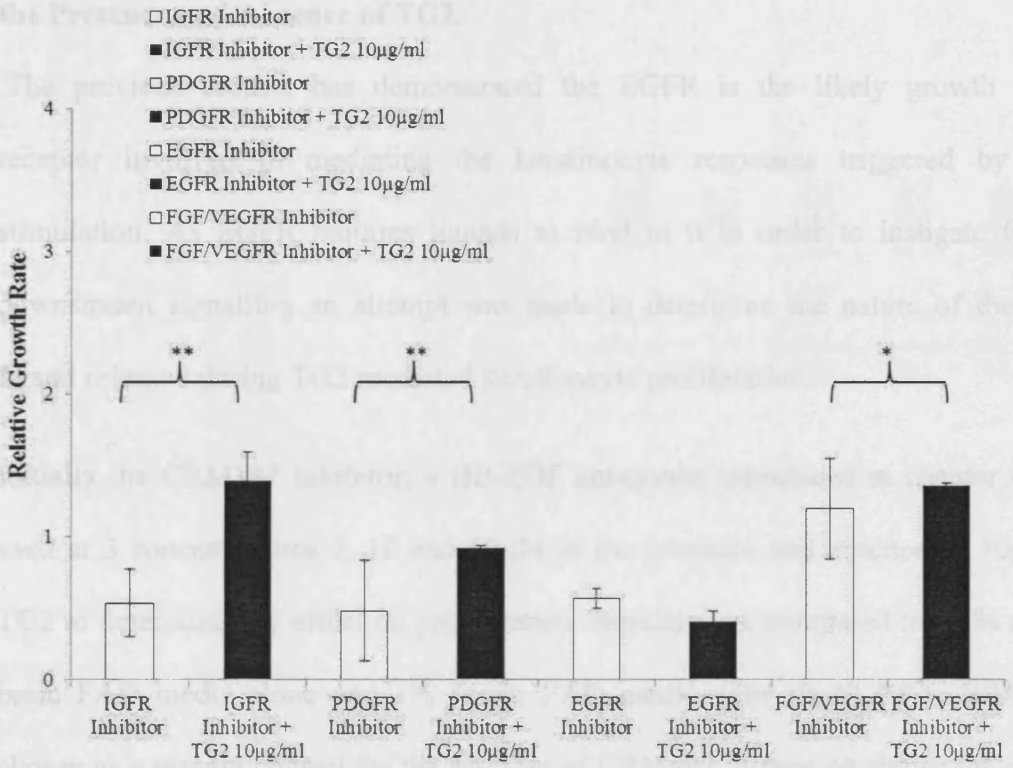

Figure 45: Investigating growth factor receptor pathways involved in keratinocyte proliferation.

EGFR, IGFR, PDGFR and FGF/VEGFR inhibitors were used to investigate the effects of inhibiting these signalling pathways on proliferation of keratinocytes. A direct comparison of a single concentration for all 4 inhibitors is shown taken from the earlier individual experiments. A 10µM dose of IGFR, EGFR and PDGFR is compared to a 15µM dose of FGF/VEGFR inhibitor as the closest comparable dose. Further details are given in Figs. 41 and 42.

5.6 EGFR Ligand Inhibition and the Effects on Keratinocyte Proliferation in the Presence and Absence of TG2.

The previous section has demonstrated the EGFR is the likely growth factor receptor involved in mediating the keratinocyte responses triggered by TG2 stimulation. As EGFR requires ligands to bind to it in order to instigate further downstream signalling an attempt was made to determine the nature of the EGF ligand released during TG2 mediated keratinocyte proliferation.

Initially the CRM197 inhibitor, a HB-EGF antagonist introduced in chapter 4 was used at 3 concentrations 2, 10 and 50μM in the presence and absence of 10μg/ml TG2 to determine any effect on proliferation. Results were compared to a 1% serum basic FAD media alone and 1% serum FAD media with 50μM BSA. BSA was chosen as a protein control for the addition of CRM197. Figure 46 shows the results of a single representative experiment after 2 repeats had been carried out. CRM197 does not reduce proliferation when compared to control, neither in the presence nor absence of TG2. Even at the highest concentration of 50μM HB-EGF antagonist, there was no statistically significant reduction of proliferation. This is a similar result to that seen in the migration studies and potentially indicates that HB-EGF is not the EGFR ligand released in response to TG2 that stimulates proliferation and migration, or that CRM197 is not a suitable (effective) inhibitor of HB-EGF in this system.

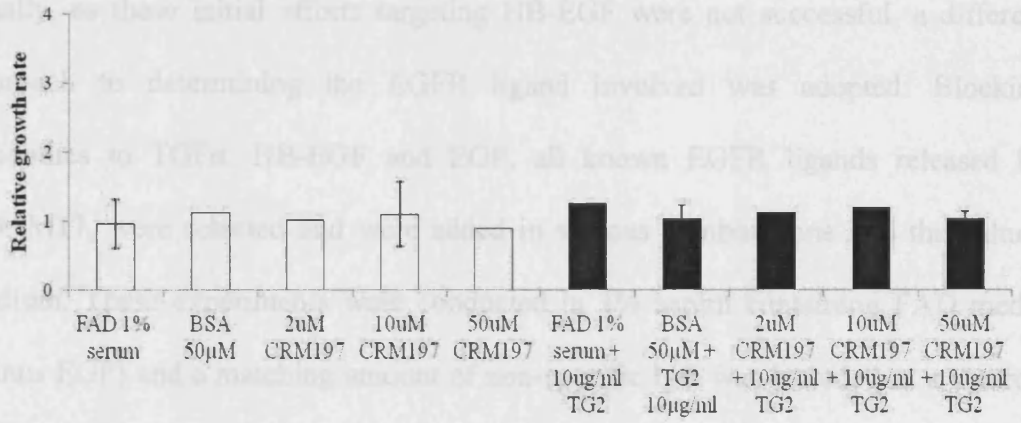

Figure 46: HB-EGF antagonist CRM197 and its effects on keratinocyte proliferation.

Keratinocyte proliferation in the presence and absence of TG2 (10µg/ml) and with inclusion of different concentrations of HB-EGF antagonist CRM197 (3 concentrations, 2, 10 and 50µM) was assessed. Cells were grown in A 1% serum-containing FAD medium minus insulin and EGF A representative experiment is shown and the results are given as mean +/-SD of three replicates. No statistical difference was seen. Dose curves were carried out three times.

Finally, as these initial efforts targeting HB-EGF were not successful, a different approach to determining the EGFR ligand involved was adopted. Blocking antibodies to TGFα, HB-EGF and EGF, all known EGFR ligands released by ADAM17, were selected and were added in various combinations into the culture medium. These experiments were conducted in 1% serum containing FAD media (minus EGF) and a matching amount of non-specific IgG was included as a control. Initially, individual antibodies were tested at different concentrations but the effect was small despite the fact that concentrations were employed (10μg/ml) which were known to completely block the response of ligand at a concentration of up to 50ng/ml. This led us to hypothesize that more than one ligand might be involved and we therefore tested each of the antibodies individually, in the 3 combinations of groups of 2 and finally all 3 antibodies together. The experiments were carried out as previously described, i.e. cell number was determined at 24 and 72 hours and the growth rate derived there from. Growth rates were expressed relative to control. It should be noted, however, that only one experiment included all conditions. However 2 replicate experiments were carried out where all 3 blocking antibodies were added together and or each was tested individually.

Figure 47A shows the reduction in proliferation rate when all 3 blocking antibodies are added together as compared with IgG control. TG2 is able to stimulate proliferation in the presence of control IgG, giving a proliferation rate of 1.5 times that in its absence. However, TG2 was not able to stimulate proliferation in the

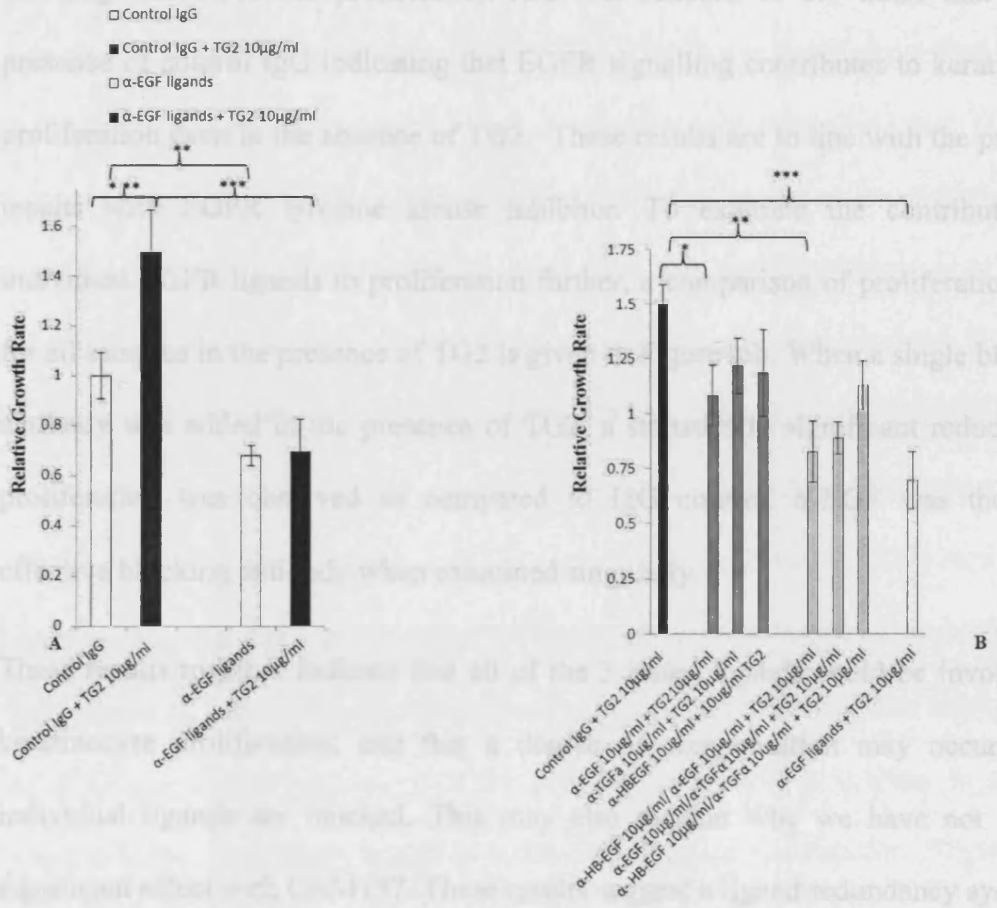

Figure 47: Antibodies to EGFR ligands block TG2-mediated stimulation of keratinocyte proliferation.

Blocking antibodies to three of the known EGFR ligands were tested to investigate the effects of blocking one, two or three of these ligands in combination on proliferation. FAD 1% serum media was prepared as a basis for all conditions. Part A: FAD medium containing 1% serum was added to Jackson Anti-goat IGG antibody control 10µg/ml in the presence and absence of TG2 10µg/ml. This was compared with FAD containing 1% serum added with α-TGFα 10µg/ml α-EGF 10µg/ml and α-HB-EGF 10µg/ml. Part B shows all conditions in the presence of TG2 10µg/ml. Control IgG was compared with single blocking antibodies, α-EGF 10µg/ml, α-TGFα 10µg/ml and α-HB-EGF 10µg/ml. α-HB-EGF 10µg/ml and α-EGF 10µg/ml was compared with α-EGF 10µg/ml and α-TGFα 10µg/ml and α-HB-EGF 10µg/ml with α-TGFα 10µg/ml. Finally all 3 blocking antibodies were compared in the presence of TG2 10µg/ml. A representative experiment is shown in each case. The results are given as mean +/-SD of 3 replicates. Significance denoted as follows; <*P, 0.0005, <**P, 0.005, <*P, 0.05. Dose curves were carried out twice with at least one further experiment was conducted at a single effective dose per blocking antibody.**

presence of all 3 blocking antibodies. Furthermore, in the presence of EGF ligand blocking antibodies the proliferation rate was reduced to 0.7 times that in the presence of control IgG indicating that EGFR signalling contributes to keratinocyte proliferation even in the absence of TG2. These results are in line with the previous results with EGFR tyrosine kinase inhibitor. To examine the contribution of individual EGFR ligands to proliferation further, a comparison of proliferation rates for all samples in the presence of TG2 is given in Figure46b. When a single blocking antibody was added in the presence of TG2, a statistically significant reduction in proliferation was observed as compared to IgG control. α-EGF was the most effective blocking antibody when examined singularly.

These results together indicate that all of the 3 tested ligands could be involved in keratinocyte proliferation, and that a degree of compensation may occur when individual ligands are blocked. This may also explain why we have not seen a significant effect with CRM197. These results suggest a ligand redundancy system is in place where if one ligand is unavailable EGFR signalling continues with a different ligand.

5. 7.Conclusion.

Chapter 5 has shown that proliferation of keratinocyte cells is similarly controlled to keratinocyte migration in the presence of TG2. Firstly, we could show that TG2 by itself is able to stimulate this response in keratinocytes suggesting that TG2 may indeed be the active component in fibroblast ECM that promotes re-epithelialisation in our wound model. Initial experiments to determine what conformation of TG2 is important to the stimulation of proliferation revealed an interesting result. Figure 37 indicated that proliferation in the presence of TG2 is greatest when TG2 is in its Ca^{2+} -activated form, while TG2 in a closed catalytically inactive conformation (TG2-GTPγs and TG2-GTP) was not able to stimulate proliferation. This was further confirmed by the experiment shown in Figure 39. Figure 39 also showed that TG2 stimulation of keratinocyte proliferation is not controlled by the open conformation of TG2 but by its activity since the I15 inhibited TG2 is trapped in its open conformation but is inactive and is unable to stimulate proliferation. Together this data shows that TG2 mediated proliferation may be conformation dependent and that active TG2 is necessary to stimulate proliferation. Hence, crosslinking of keratinocyte cell surface proteins may play a role. However, this is not consistent with the observation in Chapter 3 that ECM produced by fibroblasts expressing crosslinking deficient TG2 promotes epithelial motility similar to native TG2.

TG2 had been shown to stimulate proliferation therefore a series of experiments were undertaken to examine whether TG2 could stimulate proliferation in the presence of various inhibitors against known components of signalling pathways regulating keratinocyte proliferation. Initially the general metalloproteinase inhibitor GM6001 was tested in the presence and absence of TG2 (Figure 40). A 50%

reduction in proliferation was produced by the inhibitor alone. However, TG2 stimulated proliferation in the presence of the inhibitor at 50μM (the effective dose for this inhibitor), therefore this suggests that a different pathway may be active in TG2 control of keratinocyte proliferation. It is however important to note that proliferation in the presence of TG2 in control medium was not stimulated as much as would be expected due to the selected conditions already supporting near maximum proliferation. Therefore, no overt stimulation of proliferation occurred in controls without inhibitor. This result suggests that metalloproteinase activity in general contributes to regulation of keratinocyte proliferation but does not play a major role in the TG2-mediated keratinocyte response as cell proliferation is stimulated despite the presence of the inhibitor,

Given the importance of ADAMS 10 and 17 in migration shown in the previous chapter the next step was to examine the effects of ADAM10 and ADAM17/10 inhibitors on keratinocyte proliferation in the presence and absence of TG2. When the ADAM10 inhibitor was tested, it reduced proliferation substantially (down to 0.1). In contrast, in the presence of TG2 no inhibition of proliferation occurred at its effective dose of 10μM (Figure 41) suggesting that ADAM10 may not be involved in TG2 mediated keratinocyte proliferation. When this was compared with the ADAM10/17 inhibitor a different result was seen. This showed that in the presence of TG2 at its effective dose of 10μM proliferation rate was reduced to approximately 0.2 relative to control in both the presence and absence of TG2 suggesting that ADAM17 is involved in regulating both basal and TG2 mediated keratinocyte proliferation.

Having found both TG2 and ADAM 17 to be involved in regulating keratinocyte proliferation, we went on to examine the major pathways known to regulate keratinocyte proliferation, such as growth factor receptors. Growth factor receptors for IGF, EGF, PDGF, FGF and VEGF were all examined as to their role in regulating keratinocyte proliferation under our culture conditions. Figure 42 showed that in the presence of TG2, proliferation can be stimulated in cells where IGFR has been blocked. Furthermore, Figure 44 showed that TG2 can stimulate proliferation similarly in both the presence and absence of insulin, which if TG2 mediated proliferation was dependent on the IGFR would not be expected in the presence of near 'saturating' concentrations of insulin. Figure 42 showed the PDGFR to not appear be involved in keratinocyte proliferation. When the inhibitor to PDGFR was added alone, keratinocytes proliferation rate reduced to 0.5 that of control at its effective dose of 10μM, however in the presence of TG2 proliferation rate increased to 1.1 suggesting that PDGFR is not involved in TG2-mediated stimulation of keratinocyte proliferation. An inhibitor known to block FGFRs 1&3 as well as VEGFR had no effect on keratinocyte proliferation either in the presence or absence of TG2 suggesting that neither of these pathways plays a major role under these conditions. In contrast, proliferation was found to be inhibited in both the presence and absence of TG2 with EGFR inhibitor where proliferation rate was found to be at 0.5 and 0.4 respectively when compared to control. This showed that TG2 mediated stimulation of proliferation of keratinocytes is dependent on EGFR mediated signalling. This result is in line with the previous finding that EGFR signalling was a downstream effector of TG2 in the pathway regulating re-epithelialisation.

As the data had suggested EGFR was the candidate growth factor receptor involved, attempts were made to identify a potential EGFR ligand that may be released by ADAM17. Figure 46 shows CRM197 the HB-EGF antagonist to be ineffective in reducing proliferation in both the presence and absence of TG2. This observation is similar to that seen in chapter 4 where this inhibitor was unable to reduce keratinocyte migration. Therefore blocking antibodies to HB-EGF, EGF and TGFα were used instead to determine if one of these EGFR ligands was involved in modulating keratinocyte proliferation.

Only the combination of the 3 blocking antibodies together reduced proliferation effectively in both the presence and absence of TG2 suggesting that at all 3 ligands are involved in stimulation of keratinocyte proliferation. Furthermore, blocking all 3 ligands together reduced proliferation rate to a similar level as that observed with EGFR tyrosine kinase inhibitor, thereby lending further support to the notion that these are indeed the major EGFR ligands involved. When added individually, EGF blocking antibodies had the most significant effect (although small). When the blocking antibodies were added in combinations anti-HB-EGF together with anti-EGF was most effective. This suggests that TGFα may be the least effective ligand of the 3 as proliferation rates whenever TGFα is inhibited are slightly higher than when HB-EGF or EGF are inhibited. Thus results for EGF and HB-EGF would suggest a possible preferential use of either of these ligands when compared with TGFα.

The results shown in this chapter have delineated a pathway involved in TG2 mediated enhanced keratinocyte proliferation. The results have shown that proliferation can be stimulated by TG2 when it is in its catalytically active

conformation, further, we have identified ADAM17 and the EGFR as both being necessary components in this signalling pathway. Although a single EGFR ligand could not be identified in this system, the results would suggest ligand redundancy in activation of the receptor, presumably erbB1. It is known that all of these EGFR ligands can bind erbB1 and induce signalling. It would be interesting to extend this investigation to other EGF ligands to see if the redundancy system continues or whether a different ligand would be preferentially used to those tested here. Nevertheless, we have investigated the key EGFR ligands known to be produced by keratinocytes and to be processed by ADAM17 (Sahin et al., 2004). Taken together, these results suggest that TG2 itself mediates EGFR transactivation in keratinocytes.

The proliferation assay was based around keratinocyte monoculture. This is a simple yet effective model to test the role of the various components of a hypothesised proliferation pathway. Nevertheless this could not be considered representative of skin wound healing where multiple cell types may contribute to the pool of soluble signalling factors and different upstream signalling factors may use EGFR signalling as an effector pathway (Pucinilli et al., 2010; Stoll et al., 2010).

Chapter 6 GPR56: a Potential Receptor Linking Extracellular TG2 to Cell Migration and Proliferation.

Contents. **Page.**

6.1 Introduction.

TG2 has been shown in the previous two chapters to be able to influence keratinocyte migration and proliferation through activation of ADAM proteinases to release an EGFR ligand allowing the EGFR to signal. However, how TG2 is able to initiate this signalling process is unknown. TG2 is derived from the fibroblasts in our model, and is therefore found in the ECM. In order for TG2 activity to influence keratinocyte behaviour it must act through a cell surface protein/molecule found on the keratinocytes. Iismaa et al 2006 identified TG2 as interacting with and having involvement in signalling by the super family of heterotrimeric guanine nucleotide–binding protein (G protein) coupled receptors (GPCRs). GPCRs are principle membrane proteins that transmit various extracellular stimuli into intracellular signalling events. When a GPCR is activated by a specific signal or cognate ligand, the GPCR catalyses the exchange of GTP for GDP on the Gα subunit which facilitates the release of Gα from the complex with Gβγ (Hamm, 1998). However, in the case of β-adrenergic receptor, TG2 acts as a G-protein intracellulary by binding with α_1-adrenergic receptor and couples the receptor to phospholipase Cδ (Nakaoka et al., 1994; Aeschlimann and Thomazy, 2000) to mediate downstream signalling. More recently, Richard O' Hynes group identified that TG2 could interact with a different GPCR, GPR56 (Xu et al., 2006). In this case, extracellular TG2 modulated cell adhesion by binding to the GPCR on the cell surface However; it is at present not clear whether TG2 is a receptor agonist or a scaffolding protein in the cell's surface. Therefore, TG2 can potentially interact with GPCRs and trigger cellular responses in one of two ways: (i) through activation of guanosine triphosphate–

binding proteins that activate intracellular effectors, such as phospholipase C; (Nakaoka et al., 1994) or (ii) by direct interaction with GPCRs (but not activation).

GPR56 is a G protein coupled receptor (GPCR) and a member of the adhesion GPCR family, a secretine like family which may maintain the balance between cell adhesion and detachment during cell migration. GPR56 contains 7 transmembrane domains and a mucin-like domain in the N-terminal region. Currently, 7 splice forms have been identified in man from comparison of transcripts in the DNA database with the respective genomic sequence. GPR56 possesses a large extracellular domain and a cysteine box which is located immediately upstream of the first transmembrane domain (Bjarnadottir et al., 2004). GPR56 is autocatalytically processed within this GPS domain during trafficking but the cleaved extracellular portion remains associated with the membrane portion of the receptor. Processing is necessary for targeting to the cell surface.

GPR56 mRNA is selectively expressed in hematopoietic stem cells and neural progenitors suggesting a role in multipotent cell identity and tissue development (Jin et al., 2007). It can also be detected in various mature tissues with highest expression being in the brain and thyroid tissues (Jin et al., 2007). Mutations in GPR56 have been detected especially in the extracellular domain, including frameshift, splicing and point mutations which appear to compromise normal proliferation and migration of cells, particularly in the frontal cortical region of the brain during early development (Kim et al., 2010). A complex splicing and glycosylation pattern for GPR56 has been reported. Kim et al., 2010 observed that Hek293 cell lysates contained GPR56 with molecular weights between 100 and 70kDa, with alternative splice variants around 40-50 kDa. The latter bands are thought to correspond to

truncated C terminal regions. Several prominent bands have also been detected above 100kDa suggesting GPR56 may exist at the membrane in complex with itself or another protein (Kim et al., 2010). GPR56 also contains multiple glycosylation sites such as its amide nitrogen in asparagine (Asn) or at the oxygen in serine (Ser) and threonine (Thr) side chains. Jin et al, 2007 identified a shift in molecular weight of both GPR56 and mutated variant forms where the size of the higher bands >80 and 60kDa were reduced after PNGase (Peptide N-Glycosidase) treatment. In fact, 7 sites for N-linked glycosylation are present and introducing point mutations in any of these sites yielded a shift in Mr suggesting that all 7 sites are functional. Further, GPR56 has also been documented to undergo alternative splicing leading to changes in trafficking of GPR56 resulting in altered cell surface expression and secretion (Jin et al., 2007; Della Chiesa et al., 2010).

Xu et al., 2006 has identified GPR56 as an important G protein coupled receptor in metastatic melanoma cells, where a reduced expression of GPR56 enhanced tumour progression. Furthermore, TG2 was identified as the binding partner of GPR56 in the extracellular matrix and this interaction was suggested to be mediated through the TG2 C- terminal two beta barrel domains (Xu et al., 2006). GPR56 has also been identified as necessary for the regulation of granule cell adhesion seen in rostral cerebellar development, with mutations causing bilateral front parietal polymicrogyria (BFPP) (Bai et al 2009). The latter phenotype was suggested to be a consequence of improper ECM assembly. Taken together, these data suggest that an interaction of extracellular TG2 with GPR56 may control cell behaviour including adhesion, motility and gene expression in specific biological contexts. Hence, this interaction may also initiate the signalling cascade to drive TG2 mediated migration

of keratinocytes, and more specifically, the binding of TG2 to GPR56 may potentially activate ADAM17.

6.1.1Aims of Experiments.

Firstly, it is necessary to establish whether the keratinocytes express GPR56. GPR56 like other GPCRs is likely to be tightly controlled via sequestration by internalization and its activity controlled by restricted cell surface availability. Therefore, we performed an investigation as to where GPR56 can be found, and whether it is upregulated, in keratinocytes upon 'wounding'. This will provide evidence of whether GPR56 is involved in the regulation of migration and proliferation of keratinocytes occurring during epithelial regeneration. Furthermore, in order to test the hypothesis that GPR56 is the GPCR involved in TG2 mediated signalling in keratinocytes a method to inhibit GPR56 function must be established.

6.2 Investigating GPR56 Protein Levels and Processing in GPR56 Transfected CHO Cells using Candidate Antibodies.

GPR56 may be the candidate cell surface receptor in keratinocytes that links TG2 to ADAM17 enabling this signalling cascade. In order to investigate the expression of GPR56 in keratinocytes, initially the specificity and suitability of GPR56 antibodies were evaluated. This was done by western blotting of cell lysates from an in house generated stably transfected GPR56 over-expressing CHO cell line. As a further positive control, HeLa cell lysates were tested, since HeLa cells endogenously express GPR56 (Huang et al., 2008). Two GPR56 antibodies raised against the N and C terminus of the protein (Santa Cruz, clones N15 and N19) failed to identify GPR56. However, an antibody from R& D systems with an epitope in the extracellular domain of GPR56 was able to detect GPR56 in control cell lysates from Chinese Hamster Ovary (CHO) cells over-expressing GPR56 (Figure 48 lanes 1 and 2 HeLa and CHO control samples). To test whether time after seeding (=degree of confluence of culture) can affect GPR56 expression and processing, CHO cell lysates were examined from 6 to 72 hours after seeding on plastic. Figure 48 indicates that several bands appear to be detected by this antibody to GPR56. The broad band detected at ~94kDa is thought to be a glycosylated form of GPR56 as previously identified by Jin et al., 2007 and Kim et al., 2010. A further band is also seen around 76kDa 6 and 12 hours after seeding, which corresponds to the previously published GPS (GPCR protein cleavage site) cleaved GPR56 size (Kim et al., 2010) (corresponding to the N-terminal extracellular domain). Further bands seen at lower molecular weights are likely to be proteolytically processed

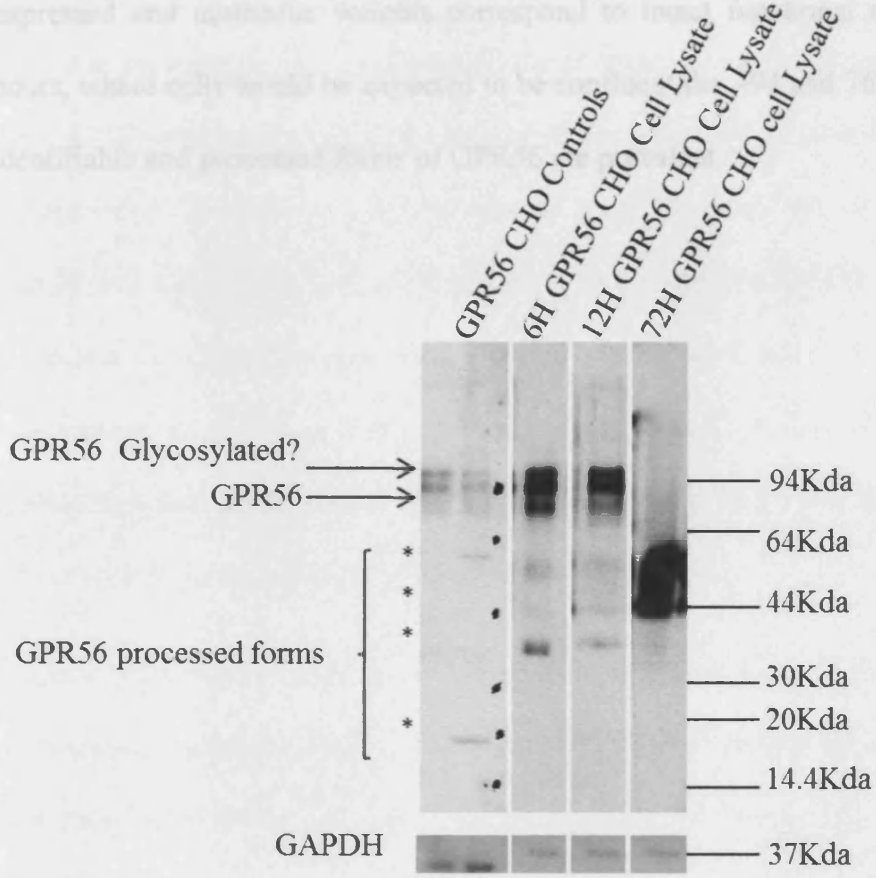

Figure 48: GPR56 Levels in GPR56 Transfected CHO cells.

GPR56 levels in cell lysates from GPR56 CHO cells. Samples were collected at 6, 12, 24 and 72 hours after seeding. Cells were lysed in buffer containing SDS and samples processed for western blotting along with two previously used GPR56 CHO samples to confirm presence of GPR56. Samples were separated in 2-20% SDS-PAGE gel under reducing conditions and transferred to nitrocellulose. All samples were probed with polyclonal R&D GPR56 antibody at 1µg/ml. Migration of Mr standards is indicated on the right. R and D GPR56 antibody identified bands at ~94kDa, 76Kda, labelled by the arrows. It also appears to identify processed forms of GPR56 at lower molecular weight as identified by the stars. The membrane was stripped and re-probed with antibodies to GAPDH to demonstrate equal protein loading.

forms of the protein. This Figure also indicates that GPR56 expression and processing is influenced by cell density. At early time points of 6 hours and 12 hours after seeding, where cells are at lower density and more motile, GPR56 protein is expressed and molecular weights correspond to intact functional receptor. By 72 hours, where cells would be expected to be confluent the ~94 and 76kDa band is not identifiable and processed forms of GPR56 are prevalent.

6.3 Investigating GPR56 Protein Levels in Keratinocyte Cells.

6.3.1 GPR56 Protein Expression of Keratinocytes in Culture at Different Cell Densities.

A similar experiment was conducted on the keratinocytes as had been carried out on the GPR56 over-expressing CHO cells to investigate GPR56 protein expression in keratinocytes. In addition, a 1 hour sample was examined from both a suspension culture and a culture plated on plastic to identify whether GPR56 expression was dependent on cell adhesion. It was expected that trypsinisation carried out in other experiments not involving lysing cells would strip the cell surface from any receptor present and that the presence of mature protein, at this very early time point would reflect GPR56 protein sequestered in intracellular stores.

Keratinocytes express GPR56 protein after 1 hour regardless of whether being in suspension or adherent (Figure 49A). Mature GPR56 is present up to 24 hours, with some processed forms of GPR56 appearing after 12 hours. The 94kDa form of GPR56 is down regulated by 72 hours although some processed forms are still present, a pattern reminiscent of that seen in the GPR56 CHO cells (Figures 47 and 48). Jin et al., 2007 had confirmed that wild type GPR56 could be released into conditioned medium as the large $GPR56^{N}$ (N terminus) subdomain. To investigate whether keratinocytes were releasing GPR56 into the medium, corresponding conditioned medium samples were taken at the same time points and tested. GPR56 is released into conditioned medium by keratinocyte cells after 1 hour with increased levels seen at 6 and 12 hours (results not shown). Only degraded forms of

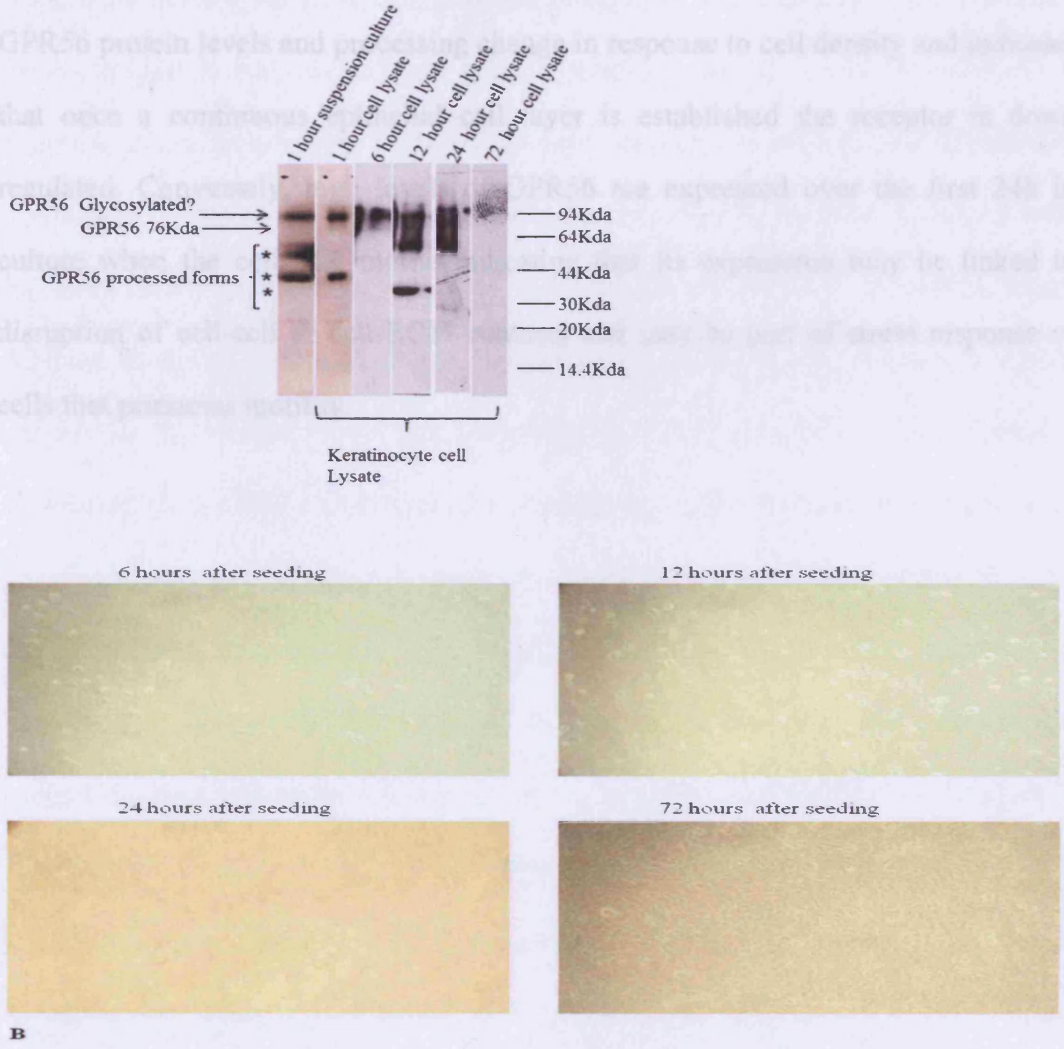

Figure 49: GPR56 expression in keratinocyte cells

Keratinocyte cells were counted and 5000 seeded per well of 6-well plates in normal FAD medium (containing 10% FCS, 10µg/ml EGF) and incubated for 1,6,12,24 and 72 hours. In addition, a one hour samples was prepared, which was left in medium in a cell suspension culture. At the various time points, the medium was removed and kept as conditioned medium samples. The keratinocytes cell layer was lysed using buffer containing SDS and prepared for western blotting as had been done previously for the GPR56 CHO cells. Samples were separated in 2-20% SDS-PAGE gel under reducing conditions and transferred to nitrocellulose. A GPR56 CHO sample was run as a control for GPR56 identification (not shown). The first lane on the left shows the suspension culture. The next five lanes contain all time points of the cell lysate samples (panel A). Samples were probed with R&D GPR56 antibody at 1µg/ml. R and D GPR56 antibody identified intact GPR56 as a broad band at 94kDa labelled by the arrow. It also appears to identify bands of 60kDa and 33kDa. These are likely processed forms of GPR56. Migration of Mr Standards is shown on the right. B: corresponding microscopic phase contrast images of the cultures at 6, 12, 24 and 72 hours are shown.

GPR56 were detected in conditioned medium samples at 24 and 72 hours. After 72 hours the keratinocyte monolayer is confluent (Figure 49B). This result indicates that GPR56 protein levels and processing change in response to cell density and indicates that once a continuous epithelial cell layer is established the receptor is down regulated. Conversely, high levels of GPR56 are expressed over the first 24h in culture when the cells are motile indicating that its expression may be linked to disruption of cell-cell or cell-ECM contacts and may be part of stress response of cells that promotes motility.

6.4 Investigating GPR56 Protein Expression Under Conditions Promoting Keratinocyte Differentiation.

To further investigate GPR56 expression in relation to keratinocyte differentiation, an investigation was carried out under conditions which would be expected to promote cell differentiation. Extracellular calcium a known inducer of differentiation was shifted from a low (0.5mM supporting a proliferative state) to a high (2.0mM inducing differentiation) concentration to investigate this (Xie et al., 2005). Figure 50 (part A) shows the results of the experiment conducted in the presence of 0.5mM calcium in the medium (low concentration). In cell lysates from cells cultured in 0.5mM calcium GPR56 protein can be seen up to 24 hours as previously shown and has disappeared at 72 hours. A small amount of the ~94kDa glycosylated band has appeared under these conditions in conditioned medium samples. This is unlikely to be 'free soluble' protein but probably reflects contamination of the medium with membrane bound particles released by the cells or cell envelopes from dying cells. Morphological analysis of the keratinocytes was also conducted under these conditions. The appearance of the keratinocytes had not altered from that of keratinocytes treated with normal FAD only (results not shown).

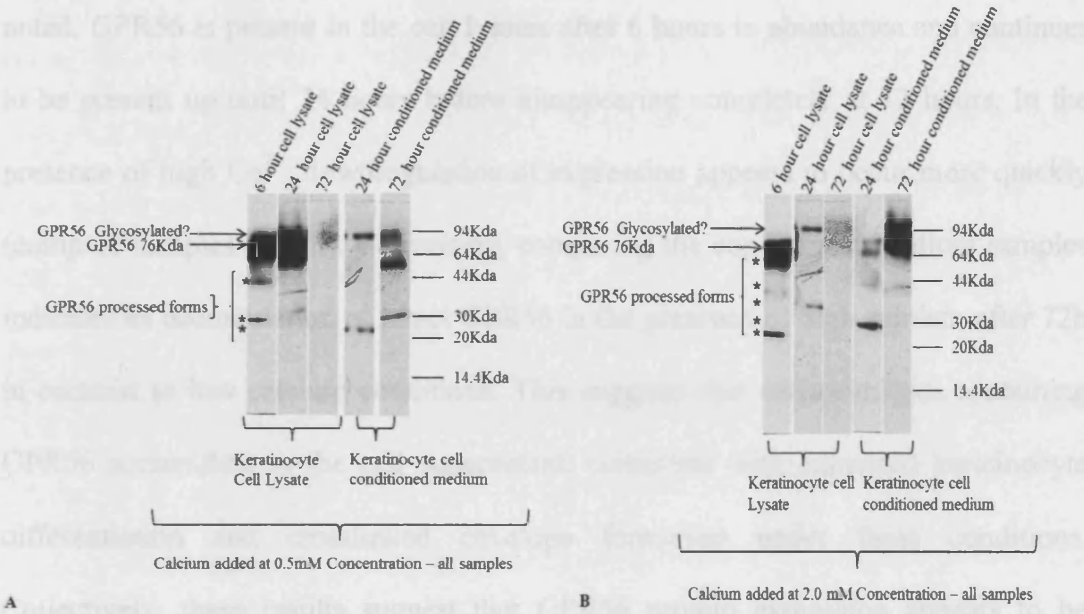

Figure 50: GPR56 expression in keratinocyte cells is unaffected by differentiation.

The same time points 1, 6, 12, 24, and 72 hours were used to investigate GPR56 levels in keratinocyte cells in FAD medium containing either 0.5mM (panel A) or 2.0mM (panel B) calcium. The addition of calcium to the medium induces terminal differentiation. Keratinocyte cells were counted and 5000 seeded per well of 6 well plates in normal FAD medium containing 10% serum and 10µg/ml EGF and incubated for 1,6,12,24 and 72 hours as described for the previous experiment. At the indicated time points, the medium was removed and kept as conditioned medium samples. The keratinocyte cell layer was lysed using buffer containing SDS and prepared for western blotting as had been previously shown. Conditioned medium was spun before analysis at 1500g. Samples were separated in 2-20% SDS-PAGE gel under reducing conditions and transferred to nitrocellulose. Lanes contain 6, 24 and 72 hour cell lysates and 24 and 72 hour conditioned medium samples in either low calcium (panel A) or high calcium (panel B). R and D antibody identified intact GPR56as a broad band at 94kDa labelled by the arrow. It also appears to identify bands of 60kDa and 33kDa. These are likely processed forms of GPR56. Migration of Mr Standards is shown on the right.

When calcium concentration in the medium was raised to 2mM, (Figure 50B) it does not appear to overtly affect GPR56 expression. However, some differences were noted. GPR56 is present in the cell lysates after 6 hours in abundance and continues to be present up until 24 hours before disappearing completely at 72 hours. In the presence of high Ca^{2+}, downregulation of expression appears to occur more quickly (compare samples at 24h). Conversely, comparing the conditioned medium samples indicates an accumulation of intact GPR56 in the presence of high calcium after 72h in contrast to low calcium conditions. This suggests that cell envelopes containing GPR56 accumulate in the cell supernatant, consistent with enhanced keratinocyte differentiation and crosslinked envelope formation under these conditions. Collectively, these results suggest that GPR56 protein expression appears to be largely unaffected by differentiation, and strongly indicate that GPR56 is not upregulated in association with terminal differentiation.

6.5 GPR56 Protein Localisation in Motile, Proliferating Keratinocytes.

Scratch wounding a confluent cell monolayer is a simple model to mimic an injury and investigate the cellular response to wounding. Since the previous experiments have suggested that GPR56 expression may be associated with a motile phenotype of keratinocytes, we decided to use immunocytochemistry to localise GPR56 in this model and investigate whether a correlation between cell activation and GPR56 expression could be seen. In order to compare GPR56 protein localisation and levels from monolayer keratinocytes with wound edge keratinocytes, cells were seeded and left for 24 hours. Keratinocytes were then scratched and left for 6, 12, 24 and 72 hours in FAD medium containing10% FCS,10μg/ml EGF, before being fixed and processed for immunocytochemical analysis. Figure 51 shows GPR56 staining over the 4 time points. There was positive staining for GPR56 at both the wound edge and at the confluent cell layer. However, the expression of GPR56 was low indicated by the fluorescence detected being very faint. GPR56 staining was seen in all samples and was still visible at 72 hours following scratching although the wound is not closed. A secondary antibody only control confirmed that the staining for GPR56 was the result of specific interaction with the primary antibody and not non-specific cell staining or autofluorescence. Figure 51B shows higher magnification images of keratinocytes at the wound edge after 12 hours. GPR56 is found throughout the cells. However, cells migrating from the wound edge appeared brighter when compared with the staining of the confluent keratinocytes. This suggest that GPR56 may be upregulated in motile keratinocytes but a further experiment where GPR56 staining in keratinocyte scratch wounds could be observed by confocal microscopy would be advantageous to determine whether GPR56 staining reduces over time in the scratch

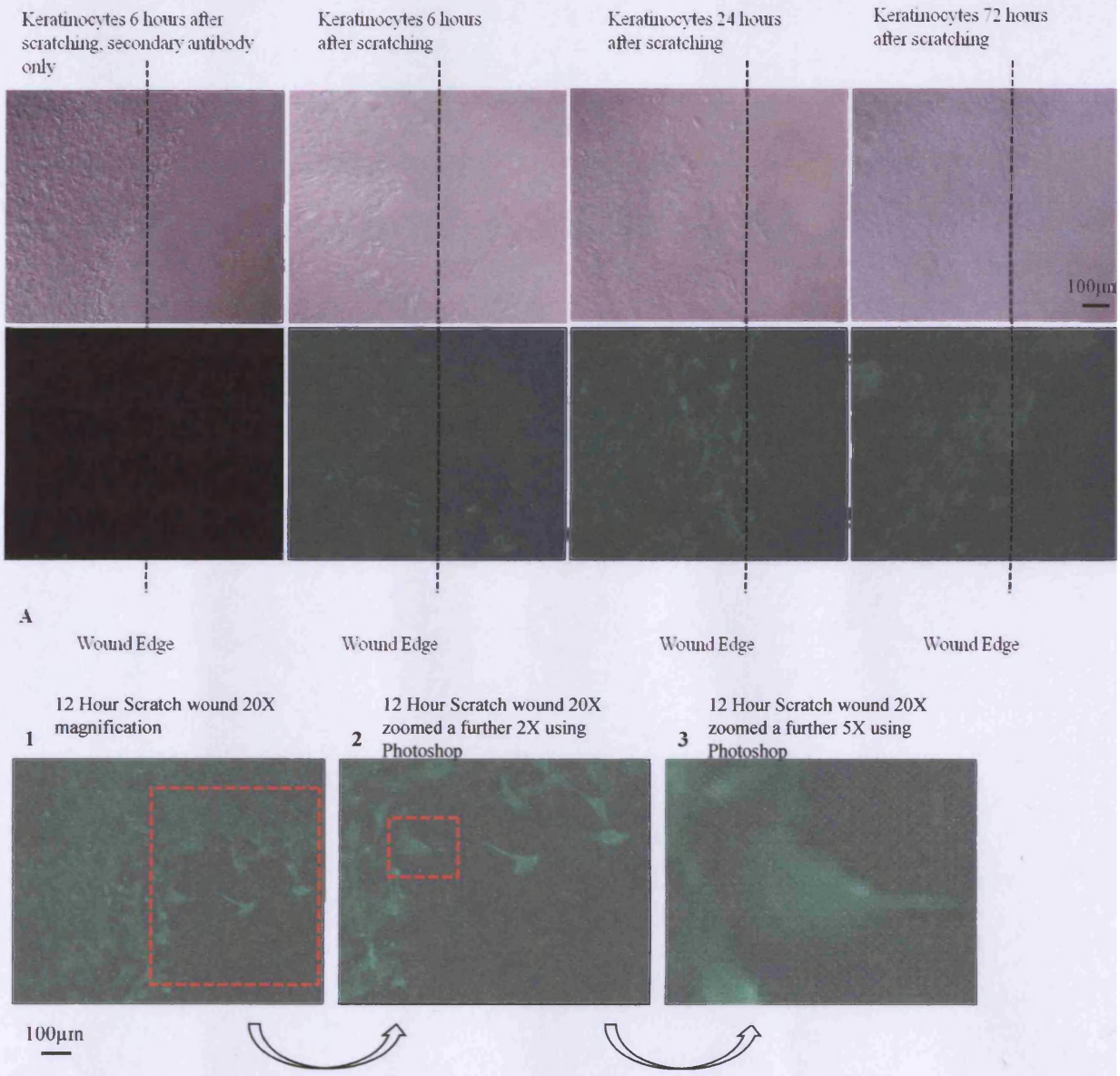

Figure 51: GPR56 is expressed in motile keratinocytes at the wound edge.A: 400,000 keratinocytes were seeded overnight before being scratched with a metal loop the following day. Keratinocytes were left for 6, 12, 24 and 72 hours after scratching in FAD medium containing 10% FCS, 10µg/ml EGF before being fixed with 4% paraformaldehyde in PBS. The cells were labelled with the R&D GPR56 antibody at 1µg/ml and binding visualized using a monoclonal FITC anti-goat IGG. Images were captured on a Carl Zeiss Microscope with a 20x objective, Axiocam 2000 CCD camera and Openlab 4.3.1 software. Phase contrast and fluorescent images are shown from 6, 24 and 72 hour time points. In addition a secondary antibody only control photograph is shown. B: The 12 hour time point picture shown has been zoomed using photoshop zoom, indicated by the red boxes to 2X and 5X the original 20X magnification to indicate the staining of GPR56 on a few cells at 2X and a single cell at 5X original magnification.

wound and to investigate its precise localization within the cells.

6.6 Investigating siRNA Knockdown of GRP56 in GRP56 Over expressing CHO Cells.

To test the hypothesis that TG2 induces cell migration through GPR56, siRNA approaches were tested to block GPR56 expression in GPR56 over-expressing CHO cells as well as keratinocytes. Initially, siRNA in two variants was transfected into stably expressing GPR56 CHO cells to determine whether either variant of siRNA could reduce GPR56 expression levels seen by Western blot. Figure 52 shows that when compared to untransfected cells or cells transfected with scrambled negative control siRNA, variant 1 was the most effective in reducing GPR56 expression.

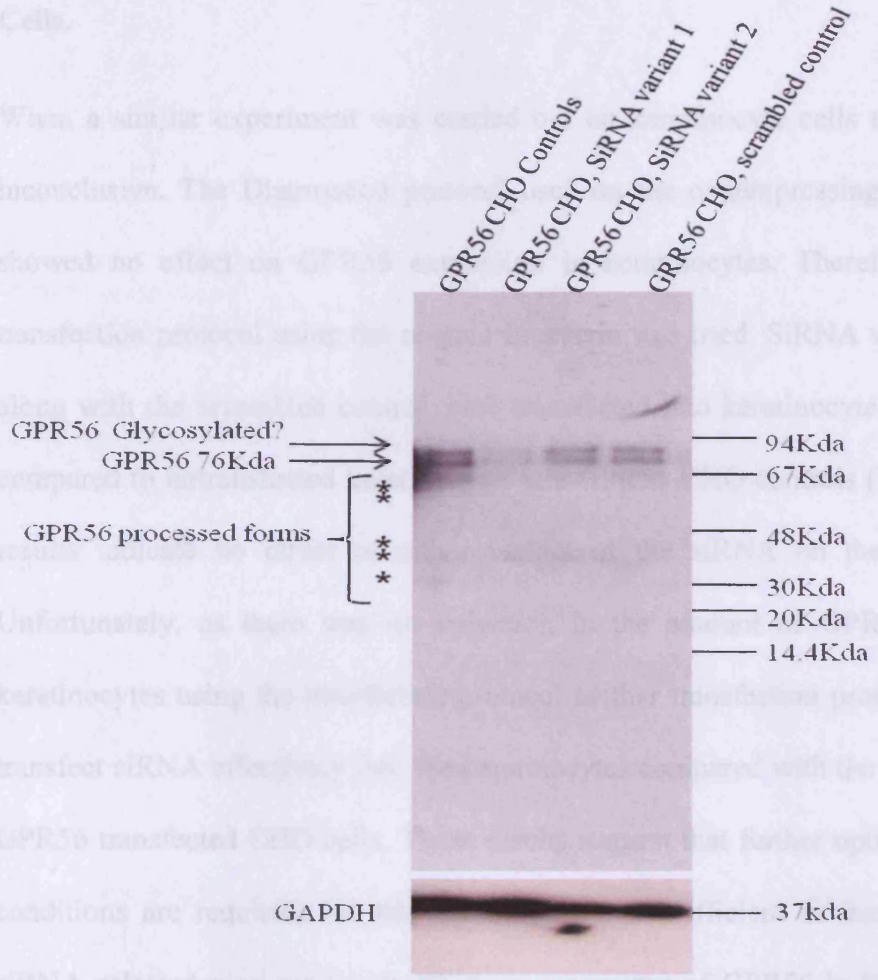

Figure 52: SiRNA knockdown of GPR56 in GPR56 over expressing CHO cells.

A: : Western showing GPR56 CHO cells transfected with GPR56 SiRNA variants 1, 2, as well as untransfected GPR56 CHO cells and a scrambled SiRNA control using the Dharmacon protocol for transfection. Lanes contain: (1) Untransfected GPR56 expressing CHO cells, (2)GPR56 over expressing CHO cells transfected with GPR56 variant 1 SiRNA (Ambion) , (3) GPR56 over expressing CHO cells transfected with GPR56 variant 2 SiRNA (Ambion), (4) GPR56 CHO cells transfected with a scrambled negative control, (Ambion). Samples were separated in 2-20% SDS-PAGE gel under reducing conditions and transferred to nitrocellulose. Primary GPR56 antibody was added at a1μg/ml for one hour at room temperature. Secondary Anti Goat IGG was added at 1:2000 concentration for 1.5 hours at room temperature. Western blot was developed using ECL plus and film exposed for five minutes before developing. A duplicate GAPDH blot is shown in the lower panel indicating equal loading of the samples. Western indicates partial knock-down of GPR56 by GPR56 Variant 1 SiRNA.

6.7 Investigating the Effect of siRNA Knockdown of GRP56 in Keratinocyte Cells.

When a similar experiment was carried out on keratinocyte cells the results were inconclusive. The Dharmacon protocol used on the overexpressing GPR56 CHOs showed no effect on GPR56 expression in keratinocytes. Therefore a different transfection protocol using the reagent Interferin was tried. SiRNA variants 1 and 2 along with the scrambled control were transfected into keratinocyte cells and were compared to untransfected keratinocytes and GPR56 CHO controls (Figure 53). The results indicate no effect of either variant of the siRNA on the keratinocytes. Unfortunately, as there was no reduction in the amount of GPR56 detected in keratinocytes using the interferin© protocol neither transfection protocol worked to transfect siRNA effectively into the keratinocytes compared with the overexpressing GPR56 transfected CHO cells. These results suggest that further optimisation of the conditions are required, i.e. the transfection was inefficient in these cells, or the siRNA selected may not be an effective suppressor of GPR56 in keratinocytes. In addition, as the longevity of siRNA knockdown was not fully tested by these experiments, even if successful in such a short-term experiment, this may not be a suitable method for investigating the effects of GPR56 expression in TG2 dependent signalling in our spheroid migration model due to the length of time it takes to set up and carry out one of these experiments.

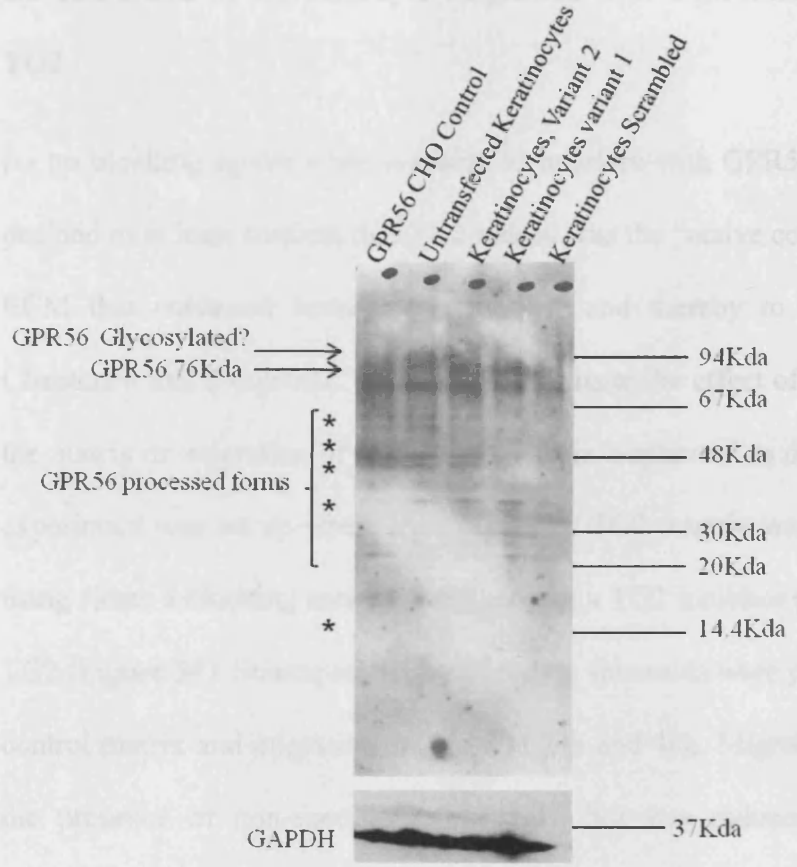

Figure 53: SiRNA knockdown of GPR56 expression in Keratinocytes.

Western showing SiRNA transfection of GPR56 variant 1, 2, and scrambled negative control into keratinocyte cells using Interferin protocol. Samples were separated in 2-20% SDS-PAGE gel under reducing conditions and transferred to nitrocellulose. Lanes contain: (1) GPR56 expressing CHO cells, (GPR56 control) (2)Untransfected keratinocytes (3) Keratinocytes transfected with GPR56 SiRNA variant 2 (Ambion), (4)Keratinocytes transfected with GPR56 SiRNA variant 1 (Ambion) (5) Keratinocytes transfected with scrambled negative control (Ambion). Western indicates little \(weaker band) removal of GPR56 by either SiRNA Variant. Primary GPR56 Antibody was added at a1µg/ml 5% Milk/PBS and left for one hour at room temperature. Secondary Anti Goat IGG was added at 1:2000 concentration for 1.5 hours at room temperature. Western was developed using ECL plus, exposed for five minutes before developing. A GAPDH blot is show in the lower panel indicating equal loading control.

6.8 Inhibition of Keratinocyte Migration over Fibroblast ECM by Blocking TG2.

As no blocking agents were available to interfere with GPR56 on keratinocytes, we decided to at least confirm that TG2 indeed was the "active component" of fibroblast ECM that enhanced keratinocyte motility and thereby to tie the findings from Chapters 4 and 5 together. In order to investigate the effect of inhibiting TG2 within the matrix on migration of keratinocytes from a spheroid as described previously an experiment was set up where over expressed TG2 matrix was pre-treated overnight using either a blocking anti-TG2 antibody or a TG2 inhibitor (I15) which inactivates TG2 (Figure 54). Subsequently, keratinocyte spheroids were plated on pre-treated or control matrix and migration analysed at 24h and 48h. Migration was unaffected by the presence of non-specific mouse IgG, but was reduced with the CUB7402 monoclonal antibody at the same concentration (Fig 53). Furthermore, migration was reduced even more effectively by I15 treatment, which is in line with our observation that TG2-I15 was unable to stimulate keratinocyte proliferation and demonstrates that a specific form of TG2 is required to promote keratinocyte motility.

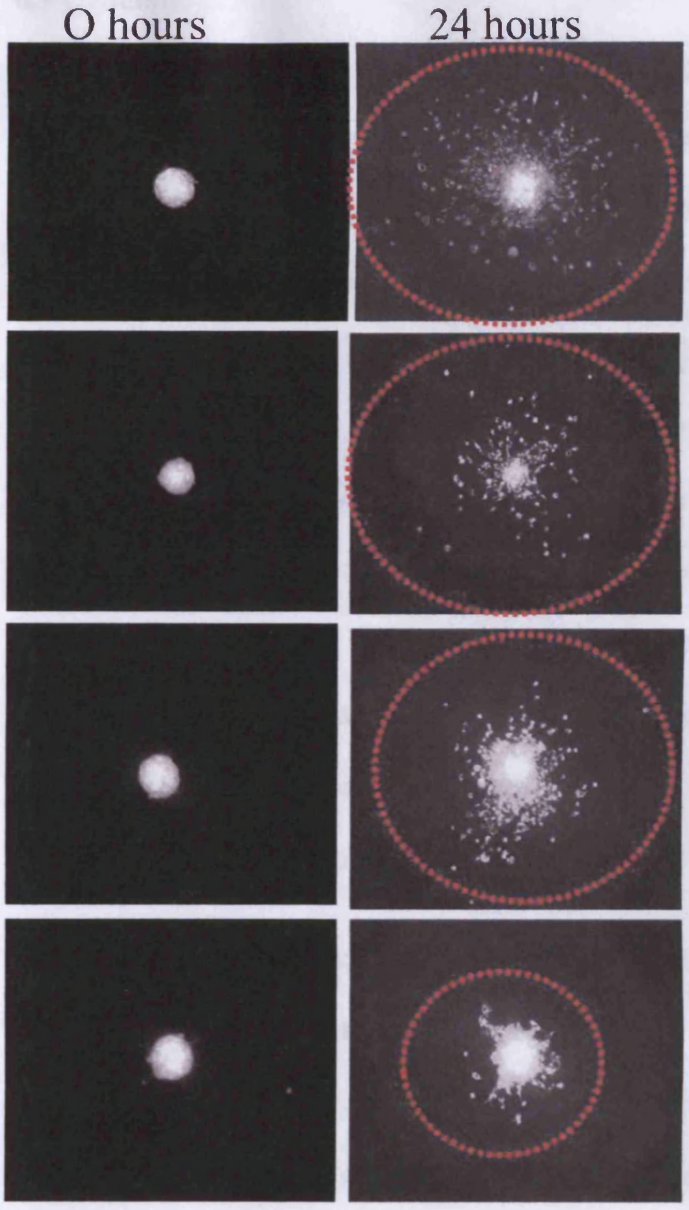

Figure 54: Enhanced keratinocyte motility is dependent on TG2 in fibroblast ECM

Overexpressed TG2 matrix was pre-treated with either standard FAD medium, mouse IgG at 10µg/ml (control for TG2 antibody), TG2 antibody CUB7402 at 10µg/ml, or TG2 inhibitor I15 (25µM) in FAD medium overnight before the addition of keratinocyte spheroids (n=6). The matrix was washed twice in PBS before spheroids were plated on the matrix and keratinocyte migration was observed in standard FAD medium.

6.9 Conclusion.

GPR56 is a relatively novel G protein coupled receptor that is the subject of much recent investigation, especially in brain development and cancer studies. The link between TG2 and GPR56 was first investigated in 2006 by Xu et al., where GPR56 was found to be down regulated in metastatic tumours, and where it's up regulation could suppress tumour growth and further metastasis. Experiments in figures 47 showed that over expressing GPR56 CHO cells did produce mature GPR56 only when cultured at low density. This not only suggests that GPR56 may be associated with a motile cell phenotype but also that post-transcriptional regulation is key in control of protein levels in the cell. Similar results were obtained in keratinocyte cells (Figure 49) and a calcium shift experiment revealed that GPR56 protein levels were unaffected by the presence of high calcium concentration, confirming that its expression is not linked to terminal differentiation of keratinocytes (Xie et al., 2005).

Further experiments indicated that GPR56 could be seen in a scratch wound more abundantly in cells migrating into the wound space, again suggesting that there may be a link to cell motility. However, further analysis using confocal microscopy is needed to indicate more clearly the changes in GPR56 expression and localisation during re-establishment of a continuous cell layer.

Figures 51 and 52 showed that GPR56 could be suppressed in GPR56 over expressing CHO cells but not in keratinocytes by siRNA gene silencing. This could be an effect of the siRNA being isoform/splice variant specific and reflect the different expression of these in different cell types. Furthermore, it is not clear whether this experimental set up could be applied to a migration model due to the time needed to perform the transfection before the production of keratinocyte

spheroids. However, as this experiment indicated GPR56 could be knocked down, it is possible to speculate that maybe a siRNA treated scratch wound of keratinocytes by an alternative transfection reagent or an alternative siRNA, would heal 'less well' due to keratinocytes being unable to respond to TG2 for enhanced migration and proliferation. It would also be interesting to investigate what would happen to migration and proliferation in the absence of TG2 if GPR56 was inhibited. It is important to realise that while TG2 may interact with GPR56 it may not be an agonist or alternatively, there may be much more potent physiological ligands.

This chapter has identified that keratinocytes produce GPR56 and that it might be localised predominantly to the wound edge. GPR56 expression can be suppressed by siRNA knockdown in overexpressing GPR56 CHOs. Further experiments investigating whether TG2 could up-regulate GPR56 production in keratinocytes would be an interesting experiment, as well as adapted migration and proliferation experiments incorporating the siRNA silencing of GPR56 to see if this caused a change in the keratinocytes proliferating and migration behaviour. Results from the Xu et al papers of 2006 and 2007 may suggest that suppression or enhancement of the interaction between TG2 and GPR56 could alter cell behaviour and this may in the future lead to tumour suppression strategies or potentially be targeted to stimulate re-epithelialisation in non-healing wounds. The summary diagram in Figure 55 speculates as to the role of GRP56 in enhancing keratinocyte proliferation and migration. Results in previous chapters have identified that TG2 activates ADAM17 to release an EGFR ligand by shedding (potentially EGF, HB-EGF or TGFα) which binds to the EGFR to cause motility and proliferation. Here we confirm that enhanced keratinocyte motility in response to contact with fibroblast ECM is indeed

mediated by TG2 itself which is assembled into this ECM. GPR56 may potentially be the missing link as a binding partner for TG2 on the keratinocyte cell surface which would allow this signalling cascade to take place, although we have not been able to conclusively proof this last point.

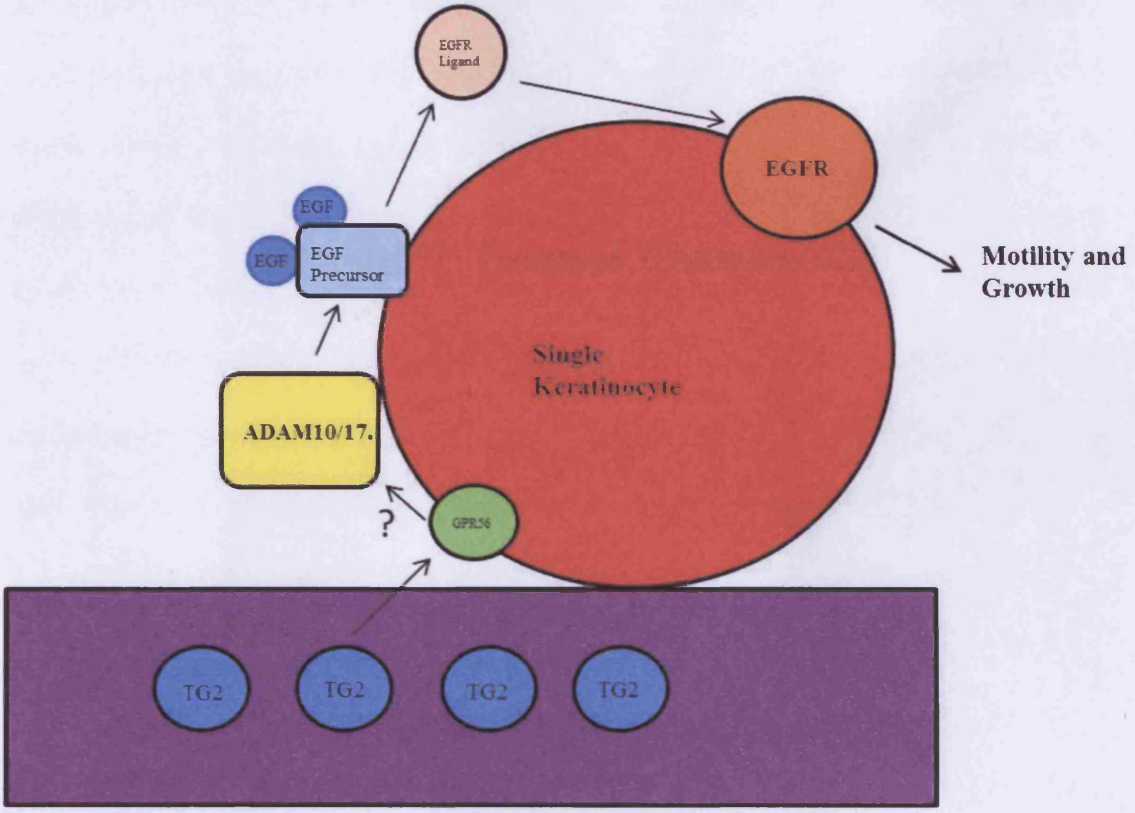

Figure 55:Summary Diagram of the Chapter.

The results chapters so far have indicated that in the presence of TG2 ADAM 10 or 17 is able to release an EGFR ligand, potentially EGF or HB-EGF which binds to the EGFR and stimulates kerationcyte migration. The linking factor between TG2 and ADAM17 is not yet known, however it is possible to speculate that GPR56 present on the cell surface may be the linking factor which promotes kerationcyte motility.

Chapter 7: General Discussion.

The process of wound healing is highly complex, several *in vitro* models have been developed to mimic the re-epithelialisation process such as a two dimensional scratch wound assay (Rodriguez et al., 2005), or the more complex multiple cell skin equivalent (Nakagawa et al 1989). We have adapted a co-culture model developed to investigate angiogenesis by Korff and Augustin, 1998, to investigate the re-epithelialisation process using fibroblasts and keratinocytes (Rosser-Davies, PhD thesis 2006). Fibroblasts have previously been shown to produce ECM which is instrumental in regulating the overlying epithelium as well as encouraging proliferation and differentiation of keratinocytes (El Ghalbzouri et al., 2002; Locke et al., 2007). Positive proliferative effects of fibroblasts have been shown in numerous studies (Bell et al, 1981, Prunieras et al., 1983, Asselineau et al., 1986, Xu and Clark., 1996) indicating fibroblasts are able to stimulate proliferation of keratinocytes for example by regulating the expression of two critical paracrine-acting cytokines, keratinocyte growth factor (KGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Florin et al., 2005).

TG2 is ubiquitously expressed in many tissues. For example work by Van Strein et al., showed that TG2 mediates the enhanced interaction of astrocytes with fibronectin in the extracellular matrix increasing astrocyte adhesion and migration leading to extensive tissue remodelling (Van Strein et al., 2010). Previous work by Rosser Davies, (PhD thesis 2006) had shown that keratinocytes seeded onto an ECM derived from fibroblasts expressing TG2 had increased motility when compared with TG2 null fibroblasts. My work has shown that keratinocyte migration was reduced

on matrix with live fibroblasts, when compared to matrices without fibroblasts present in a TG2 dependent manner.

In order to distinguish between matrix and fibroblast signals in regulating keratinocyte migration, experiments were designed to only measure the contribution of the matrix. For this purpose fibroblasts were killed by freeze thawing and keratinocyte migration analysed over matrices synthesised by fibroblasts positive or negative for TG2. In our co-culture model in the presence of endogenous, over expressed and TG2 null matrices the result showed that keratinocytes migrate further in the presence of endogenous TG2 (220μm), than matrices prepared with TG2 null fibroblasts (130μm). These data suggest that an increase in keratinocyte motility occurred in the presence of TG2, when compared to TG2 null conditions (Figure 17, page 148). However, the increased concentration of TG2 present in fibroblasts overexpressing TG2 yielded a median distance migration and thus did not increase keratinocyte migration further when compared to endogenous TG2. It was not clear at this point whether TG2 deposited into the fibroblast ECM mediated these affects or whether post translational modification or gene expression in these fibroblasts was responsible. This result may therefore suggest that a high level of TG2 present within this matrix increases crosslinking sufficiently to impede keratinocyte migration. Alternatively, depending on whether the keratinocytes respond directly or indirectly to TG2, the increased concentration is in excess of saturation of a keratinocyte signalling pathway or the generation of the signal may be regulated separately from gene expression, e.g., by TG2 adopting a specific conformation.

An experiment to confirm the effects on migration of keratinocytes with endogenous, overexpressed or TG2 null matrix in the presence of live fibroblasts was analysed (Figure 18, page 150). This experiment confirmed earlier work by Dr Sally Rosser Davies that keratinocyte migration decreases in the presence of endogenous or over expressed TG2 matrix in the presence of live fibroblasts. Further, the overexpressed TG2 matrix supported the least amount of keratinocyte migration in the presence of live fibroblasts. This was reduced to the level of migration seen in the presence of TG2 null matrix in the absence of fibroblasts (approximately 75μm). This is thought to be a basal level of migration induced by contact of cells with a collagenous ECM (Grenache et al., 2007). Therefore in the presence of live fibroblasts either an anti-migratory signal is sent by the fibroblasts or cross talk between the fibroblasts and the keratinocytes reduces migration in response to an increased presence of TG2.

The effects of conditioned medium from different fibroblasts was also examined to determine if conditioned medium from TG2 deficient fibroblasts could support an increased amount of keratinocyte migration (Figure 20, page 154). Keratinocyte migration was most extensive on matrix from fibroblasts overexpressing TG2 in the presence of the corresponding conditioned medium. Migration was less in the presence of matrices with endogenous and TG2 null fibroblast conditioned medium. This is thought to be a consequence of a pro-migratory signal in the conditioned medium that is expressed by fibroblasts in a TG2 dependent manner and potentially due to a released growth factor such as EGF or TGFβ. However, it is interesting to note that conditioned medium analysis by Dr Mathew Caley in our lab (2009, unpublished) indicated increased MMP1-protein levels in conditioned medium from

fibroblasts expressing TG2. Therefore this suggests migration in the presence of conditioned medium may be dependent on MMP1 signalling or activity. MMP1 has previously been shown to be an intrinsic part of the wound healing process as inhibition of MMP1 causes a reduction in cell proliferation and migration (Shyu et al., 2007). Furthermore, MMP1 is thought to signal through the PAR1 receptor causing an increase in migration of keratinocyte cells in the presence of conditioned medium from TG2 overexpressing fibroblasts (Dr Mathew Caley, unpublished, 2009; Yang et al., 2009). Nevertheless, these data also showed that the absence of TG2 did not simply lead to an accumulation of a signalling factor in the medium as a consequence of the inability to cross link it into the ECM.

The experiments containing the live fibroblasts together with the effects of conditioned medium indicated the presence of alternative and contrasting signalling pathways and the analysis thereof would be beyond the scope of this thesis. Therefore, it was decided to pursue the effects of ECM synthesised by fibroblasts expressing TG2 in instructing keratinocyte migration and proliferation. This would simplify the model by ablating the anti-migratory signals from the live fibroblasts and would allow us to compare the migration data with subsequent work addressing the regulation of keratinocyte proliferation by TG2 dependent signalling.

Metalloproteinase activity had previously been linked to keratinocyte resurfacing, wound contraction, and granulation tissue organization (Mirastschijski et al, 2004). Therefore experiments were conducted in the presence and absence of TG2 to determine the effects of metalloproteinase inhibition in our keratinocyte migration

model system. Initial experiments utilising general metalloproteinase inhibitors GM6001 and TAPI1 showed keratinocyte migration reduced in the presence of these inhibitors on TG2 positive matrix but not on TG2 null matrix (Figures 24/25, page 171-2). TAPI1, as well as being a general metalloproteinase inhibitor shows some specificity to ADAMs, a sub family of metalloproteinases (Daniela et al., 2003). A literature search had indicated that keratinocytes express ADAMs 9, 10 and 17 all of which have been linked with keratinocyte motility (Toriseva and Kähäri, 2008). Commercially available inhibitors to ADAM 10 and ADAM10/17 were obtained from GSK, and a migration experiment in the presence of these inhibitors was conducted (Figure 28-30, page 178-80). These experiments showed that keratinocyte migration is reduced in the presence of inhibitors to ADAM10 and 17 in the presence of endogenous and overexpressed TG2 matrix to a level seen with TG2 null matrix. Furthermore, migration in the absence of TG2 remained unaffected by the presence of ADAM 10 and 17 inhibitors. This data showed that migration of keratinocytes is dependent on metalloproteinases; specifically either ADAM 10 or both ADAM10 and 17 in the presence of TG2 but not in its absence. Therefore this suggests ADAM 10 and 17 are part of the pathway through which TG2 enhances keratinocyte motility.

A literature search indicated ADAMs 10 and 17 had been linked with the release of EGFR ligands to enhance keratinocyte motility and proliferation (Sunnarbourg et al., 2002; Lee et al., 2003; Li et al., 2004; Iacob et al., 2008). However IGFR and PDGFR have also been previously linked with increased cell motility (Barrientos et al., 2008; Iacob et al., 2008). Therefore an experiment was devised where inhibitors

to EGFR along with IGFR and PDGFR were investigated (Figure 31, page 188). Migration of keratinocytes is significantly reduced in the presence of EGFR inhibitor on TG2 containing matrix, but not in its absence (TG2 null). Migration in the presence of IGFR and PDGFR inhibitors was unaffected in our model system in both the presence and absence of TG2. Therefore my data indicated that TG2 stimulated enhanced motility keratinocytes is dependent on EGFR signalling.

The next step was to determine whether EGFR ligand release could be affected in the migration model. The literature indicated ADAMs 10 and 17 release EGF, HB-EGF, TGFα, amphiregulin and neuregulin (Sahin et al., 2004). In order to examine potential EGFR ligands in our keratinocyte migration model system, EGF and HB-EGF were investigated as both ligands had been linked to cell proliferation, and migration (Barradon and Green 1987, Joslin et al., 2007). Initially, TG2 null matrix was supplemented with EGF in a 2, 10 or 50ng/ml concentration to determine whether EGF could independently of TG2 stimulate keratinocyte migration (Figure 32, page 185) . Interestingly, migration in the presence of EGF in a TG2 null background is stimulated at a 10ng/ml concentration. However, an increase of EGF to 50ng/ml does not increase keratinocyte motility further. This suggests that maximal activation of this pathway occurs at 10ng/ml which is in line with studies in keratinocytes. It has been shown that high doses of EGF-ligand leads to rapid internalisation of ligand bound EGFR which will then be degraded in intracellular compartments. This may explain reduced affect that the high dose tested in this thesis (Wang et al., 2009). Most importantly, this result suggests EGF can support enhanced keratinocyte migration independently of TG2 which is known from the

literature (Wang et al., 2010; Pucinilli et al., 2010). It also shows that TG2 with ECM can support enhanced motility and therefore that the observed differences are likely due to fundamental changes in the matrix causing differences in cell matrix interactions.

The potential contribution of HB-EGF ligand to keratinocyte migration was examined using a TG2 positive matrix and the commercially available inhibitor CRM197, which blocks this ligand specifically. TG2 positive matrix was chosen since EGFR signalling had been shown above to be involved in keratinocyte migration using the kinase inhibitor AG1478. CRM197 was added initially as a pre-treatment to an overexpressed TG2 matrix for 16 hours in FAD medium containing 1% serum before the addition of keratinocyte spheroids. CRM197 was added at a 3, 10, 30 and 100μg/ml concentration (Figure 33, page 187). Inhibition of keratinocyte migration appeared at a 10μg/ml concentration and this effect increased as the dose of inhibitor increased. This initial result suggested that HB-EGF could be inhibited in the co-culture model and that its inhibition by CRM197 reduced keratinocyte motility. However, further experiments using TG2 null and overexpressed TG2 matrices failed to yield the same result in the presence of FAD containing 1% serum.

Results so far, had indicated that in the presence of TG2, an ADAM (either ADAM10 or 17 or both) was able to facilitate EGFR signalling and thereby enhance keratinocyte motility. Further, EGF a known EGFR ligand was able to stimulate migration in the absence of TG2. Although our results are inconclusive regarding HB-EGF, it would appear that HB-EGF may also be involved in TG2 mediated

signalling. For example, Stoll et al., 2010 showed human keratinocytes to express multiple EGFR ligands and amphiregulin and HB-EGF to be strongly induced in human skin culture. Multiple EGFR ligands may similarly be involved in stimulating keratinocyte migration in our model. It has been shown that keratinocyte migration in scratch wound assays was highly metalloproteinase and EGFR dependent, and was markedly inhibited by EGFR ligand antibodies (Stoll et al., 2010). This was confirmed by Rahman et al., 2010 where HB-EGF was found to stimulate the growth and migration of human oesophageal keratinocytes in a dose dependant manner. In this study recombinant HB-EGF was found to stimulate oesophageal epithelial cell migration at a 1 and 10ng/ml concentration.

In parallel with the migration studies, an examination of TG2 dependent keratinocyte proliferation was undertaken using an MTT proliferation assay. This allowed us to independently assess the importance of TG2 in EGFR signalling and to test in detail the role of individual components in a high throughput assay. TG2 concentration and conformation dependence of keratinocyte proliferation was analysed to determine whether direct signalling may occur. Currently, it is unknown which conformation TG2 assumes in the matrix and whether any of those do stimulate keratinocyte migration. Furthermore, it was not known whether TG2 activity was required to stimulate keratinocyte proliferation. Pinkas et al, had determined that depending on the binding of GTP, calcium or substrate the conformation of TG2 was altered (Pinkas et al., 2007). Therefore the present experiments were designed to gain insight into this important question.

The proliferation assay was used to investigate if keratinocytes respond to TG2 and whether this was TG2 conformation dependent (Figure 37, page 203). TG2 was tested in its native form at different concentrations (up to 10μg/ml) and compared with TG2 bound to GTP or GTPγS (a non hydrolysable form) or to TG2-I15 (inhibited form). Additionally, TG2 stimulated with calcium, to ensure it is in the catalytically active conformation was compared. This assay showed that keratinocyte proliferation in response to TG2 is conformation dependent. TG2 added in its native form stimulates a dose dependent increase in proliferation which was enhanced in the presence of TG2 stimulated with calcium. Contrastingly, in the presence of TG2-GTPγS no increase in proliferation is seen. This may be because TG2-GTPγS cannot be hydrolysed and therefore is stably trapped in a closed conformation unable to bind Ca^{2+}. TG2 bound to GTP, undergoes hydrolysis of GTP, causing GDP or GTP to dissociate from TG2, potentially allowing the TG2 conformation to change from closed to active. Indeed, a low level of stimulation of proliferation was seen at the highest dose of 10μg/ml TG2-GTP only. Therefore the results suggest that only TG2 in its open or catalytically active conformation is able to stimulate keratinocyte proliferation.

A further experiment was undertaken to examine whether TG2 cross linking activity as well as an open conformation is needed for stimulation of proliferation (Figure 39, page 208). Native TG2 was compared with TG2 incubated with a peptide based active site targeting inhibitor similar to that described in (Pinkas et al., 2007) and developed by Zedira. The cross-linking defective TG2-I15 is trapped in the open conformation the results clearly show that no stimulation of proliferation occurs in

the presence of TG2-I15 i.e. the small amount of stimulation at higher concentration is likely being caused by a small amount of TG2 not inhibitor bound that is present in the preparation. This result suggests that the cross-linking activity of TG2 may be necessary to stimulate proliferation but likely not a cryptic binding site exposed in this open conformation.

In order to examine other potential signalling mechanisms regulating keratinocyte proliferation, metalloproteinase activity was again examined to determine whether their activity was part of the TG2 dependent proliferation pathway. The general metalloproteinase inhibitor GM6001 was initially tested in the presence and absence of TG2 to determine the effects of inhibiting metalloproteinase activity (Figure 40, page 210). This experiment showed only a single dose of GM6001 of 50μM was able to reduce proliferation in both the presence and absence of TG2, which indicated that metalloproteinase activity could be important in regulating basal but not TG2 stimulated proliferation. However, the rate of proliferation seen in this experiment under control conditions were close to the maximum and further stimulation by TG2 may have been masked. Furthermore the use of DMSO as a solvent control can increase shedding of ligands from the cells which may explain the increased growth rate (Watanabe et al., 1986). An alternative inhibitor of metalloproteinase activity was considered in future experiments outlined below. A further experiment to examine the effects of inhibition of ADAM 10 and ADAM17 revealed that both inhibitors alone could reduce proliferation (Figure 41, page 213). When TG2 was added to the keratinocytes, proliferation was not inhibited in the presence of the ADAM10 inhibitor suggesting ADAM 10 may not be involved in

keratinocyte proliferation. Contrastingly, proliferation in the presence of the ADAM 17 inhibitor was inhibited in both the presence and absence of TG2. This would suggest that ADAM17 may be involved in TG2 stimulated keratinocyte proliferation. These data are in good agreement with literature data showing regulation of EGFR ligand shedding by ADAM17 (Fang et al., 1999; Sahin et al., 2004; Cao et al., 2006; Koivisto et al., 2006; Stoll et al., 2010; Puccinelli et al., 2010).

In order to confirm that EGFR signalling was initiated by TG2 treatment, keratinocytes were seeded with 4 growth factor receptor inhibitors in the presence and absence of TG2 (Figure 42/3 pages 216-218). Proliferation was stimulated by TG2 and only the EGFR inhibitor blocked TG2 dependent enhanced proliferation, while inhibitors for IGFR, PDGFR and receptor FGFR were ineffective. These results collectively indicate that EGFR may also be involved in TG2 dependent signalling regulating keratinocyte proliferation as well as inducing migration as shown earlier. A critical role of EGFR is in line with the current literature (Xu et al., 2004; Koivisto et al., 2006; Mendelson et al., 2010)

So far my data has indicated that ADAM17 releases a ligand to bind to the EGFR to promote keratinocyte proliferation. An attempt was made to determine which EGFR ligand was released by ADAM 17 and regulating proliferation. Blocking antibodies to EGF, HB-EGF and TGFα (another EGFR ligand known to be released by ADAM17) were tested individually, or in combination to investigate whether they could block TG2 mediated enhanced proliferation HB-EGF (Stoll et al., 2010), EGF

(Puccinelli et al., 2010) and TGFα (Poindester et al., 2010) have all been previously linked with enhanced keratinocyte proliferation. Blocking antibodies were used to block the activity of all 3 EGFR ligands at the same time and the data showed proliferation to be reduced to the rate seen in the presence of the EGFR tyrosine kinase inhibitor both in the presence and absence of TG2 (Figure 47 page 227). Inhibition of proliferation under these conditions shows that one or more of these ligands is involved in TG2 dependent keratinocyte proliferation. Antibodies were added in various combinations to determine if proliferation decreased in the presence of particular antibodies. The treatment with blocking antibodies to HB-EGF with EGF showed 50% less proliferation then control IgG in the presence of TG2. Proliferation seen with antibodies to EGF and TGFα combined gave a growth rate 40% less than control IgG. The highest growth rate was seen in the presence of antibodies targeting HB-EGF and TGF-α where a proliferation rate 10% less than control IgG was seen. This suggested that TGFα is the least effective or least abundant ligand used in EGFR signalling in keratinocytes. TGFα has nevertheless been previously linked with keratinocyte proliferation and so cannot be discounted from EGFR signalling in keratinocytes (Stoll et al., 2010; Poindester et al., 2010). Due to the reduction in proliferation seen in the presence of all 3 blocking antibodies this shows that a degree of ligand redundancy was occurring in keratinocytes which preferentially use EGF or HB-EGF for signalling (Rahman et al., 2010; Stoll et al., 2010). This is consistent with the overlap in ligand specificity of the EGFR and also the ability of ADAM17 to process the relevant precursors and release the soluble ligands for binding to EGFR.

In addition to the blocking antibody studies CRM197, a HB EGF inhibitor was tried at different concentrations in the presence and absence of TG2 to determine its effect if any on proliferation (Figure 46, page 225). CRM197 failed to inhibit proliferation, which agrees with the data showing a lack of inhibition in the migration assay. The blocking antibodies showed that three ligands contribute to the increase in proliferation; therefore we can expect that any inhibitory activity of CRM197 alone would be marginal.

So far my work has shown that ADAM17 can release an EGFR ligand to bind to the EGFR to stimulate both keratinocyte proliferation and migration. However, the mechanism regulating activation of ADAM17 in a TG2 dependent manner had not been addressed. In 2006 Richard O'Hynes group identified an orphan G-protein coupled receptor, GPR56, as an extracellular binding partner for TG2 (Xu et al., 2006). There are a number of well characterised cases where a GPCR is responsible for assembling a signalling platform leading to activation of ADAMs. We therefore speculated that the interaction of TG2 with GPR56 may initiate the signalling cascade to drive TG2 mediated migration and proliferation of keratinocytes by potentially activating ADAM17. Therefore an attempt was made to determine whether GPR56 could be identified in keratinocytes (Figure 49, page 245). Western blotting of cell lysates was undertaken to determine if GPR56 was expressed in keratinocytes at 1, 6, 12, 24 and 72 hours after seeding and if its protein expression changed over that time. This experimental setup was chosen as it was likely that a GPCR regulating motility was not constitutively expressed and may not therefore be present in confluent cultures. GPR56 protein appeared to be present in keratinocytes

1 hour after seeding and continued to be seen until 24 hours. By 72 hours protein expression appeared down regulated. Multiple bands were identified by the R and D antibody to GPR56 including a broader band of a larger than published size (for recombinant protein). This was thought to be the result of glycosylation of GPR56 (Kim et al., 2010). Further protein bands seen at lower molecular weights on the blot were thought to be the processed forms of GPR56. Kim et al, 2010 observed that cell lysates from different splicing variants of GPR56 produced different protein banding patterns. However, GPR56 processing in its GPS domain is thought to be important for its trafficking and cell surface expression (Jin et al., 2007; Kim et al., 2010). With the exception of the low Mr band (40kDa), the bands observed likely correspond to receptor with varying extent of glycolysalation and GPS cleaved extracellular domain.

These experiments confirmed that GPR56 can be expressed by keratinocytes. Furthermore, this indicated that it may be present only in activated motile keratinocytes. This was further confirmed by Ca^{2+} shift experiments which showed that induction of keratinocyte differentiation did not induce GPR56 expression (Figure 50, page 248) Therefore, we evaluated the expression of GPR56 after wounding of a keratinocyte monolayer. In order to evaluate whether GPR56 expression was localised to the leading edge of a scratch wound immunolocalisation experiments were performed (Figure 51, page 251). The data showed that GPR56 is expressed at low level in the keratinocyte monolayer but upregulated in cells migrating from the edge of the scratch wound. This may not entirely reflect the result seen by Western blot which suggests GPR56 may be released or degraded by

keratinocytes after reaching confluencency after 72 hours. Therefore further experiments are needed which double label the N and C-terminal of GPR56 to understand receptor upregulation and processing that may occur upon wounding.

In order to evaluate the contribution of GPR56 in keratinocyte migration siRNA transfections were designed to ablate its expression. For this purpose CHO cells expressing GPR56 were transfected with 2 variants of SiRNA to determine if GPR56 could be successfully knocked down. CHO cells transfected with SiRNA to GPR56 showed successful knockdown of GPR56 by variant 1 SiRNA (Figure 52, page 254). Therefore, keratinocytes were transfected with the same SiRNA, but unfortunately this was unsuccessful (Figure 53, page 256). It is notoriously difficult to achieve high levels of transfection of keratinocytes using conventional lipid based transfection reagents. Subsequent transfection of keratinocytes with GPR56 SiRNA using an alternative reagent failed to yield any reduction in GPR56 protein expression in these cells and unfortunately time constraints did not allow me to optimise transfection conditions or use viral delivery systems to ablate GPR56 in keratinocytes.

GPR56 is a member of the adhesion GPCR family, a secretine like protein, which may maintain the balance between cell adhesion and detachment during cell migration (Bjarnadottir et al., 2004; Iguchi et al., 2008). GPR56 expression has been studied in both the heart and brain but transcripts are actually present in most tissues (UNIGENE data, NCBI, NIH) (Iguchi et al., 2008). Mutations within the extracellular as well as intracellular domains of GPR56 have been recently identified in patients with bilateral frontoparietal polymicrogyria (BFPP). These mutations

include frameshift, splicing and point mutations, and they affect neuronal progenitor proliferation and migration to the frontal cortical region of the brain during early human development (Kim et al., 2010). These observations are also apparent in the GPR56 knock out mouse which also displays a severe malformation of the rostral cerebellum (Koirala et al., 2009). These defects are thought to result from defects in basement membrane assembly. TG2 has been shown to be a basement membrane constituent also in the brain (Aeschlimann and Paulsson, 1991). Thus, an interaction between TG2 and GPR56 may not only be important to regulate keratinocyte motility but may also contribute to establishment of a new basement membrane.

Final Words.

This thesis has attempted to determine the relationship of TG2 and various other matrix related proteins to extracellular remodelling and wound healing. It has concentrated on the role that TG2, Metalloproteinases, growth factor ligands and receptors have on the behaviour of keratinocyte cells seeded on a fibroblast derived ECM. This thesis has delineated a mechanism for TG2 mediated stimulation of keratinocyte motility. The release of an EGFR ligand by ADAM 17 allows signalling through the EGFR to take place. Experiments which would further substantiate this pathway would be to establish if phosphorylation of the EGFR occurs and affects downstream signalling. EGFR phosphorylation could be examined by immunoprecipitation of the receptor following TG2 treatment. Alternatively, downstream ERK phosphorylation leading to MAPK activation could be investigated.

The experiments in this thesis were carried out using N-tert immortalised human keratinocytes and TG2 transfected fibroblasts (human skin). While both of these cell lines are good models further experiments to examine the effects of using primary fibroblasts derived from a TG2 knock out mouse would be used to confirm the role of TG2 in this signalling pathway. TG2 null mice have been shown to have defective healing of excision skin wounds and it would be interesting to examine whether this relates to the pathway outlined in this thesis.

GPR56 could also be investigated further. Transfection of SiRNA to inhibit its activity had proven unsuccessful so far in keratinocytes; this transfection could be optimised to cause ablation of GPR56 in the keratinocytes. Unpublished data has indicated that over expression of GPR56 causes an increase in EGFR ligand shedding (Gaweska and Knauper, unpublished, 2010) providing further support of a direct link between GPR56 and EGFR signalling. A successful knockdown could then directly prove GPR56 involvement in TG2 dependent migration and proliferation.

Finally, this thesis has shown that TG2 can stimulate keratinocyte migration and proliferation through activation of the EGFR via EGFR ligand release by ADAM17 shedding. This has indicated a novel function of TG2 in instigating this signalling cascade and may in future contribute to better understanding of the process of re-epithelialisation in wound healing.

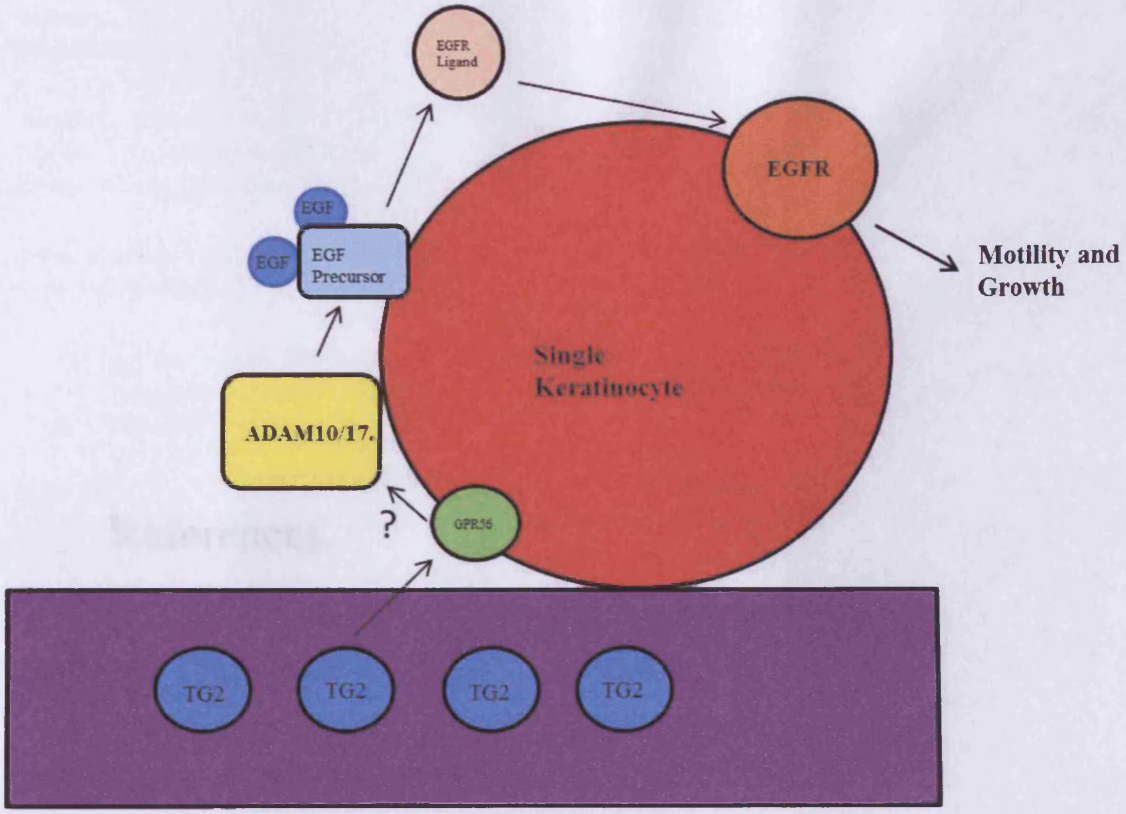

Figure 56:Summary Diagram of the Thesis.
The results chapters have indicated that in the presence of TG2 ADAM 10 or 17 is able to release an EGFR ligand, potentially EGF or HB-EGF which binds to the EGFR and stimulates kerationcyte migration. The linking factor between TG2 and ADAM17 is not yet known, however it is possible to speculate that GPR56 present on the cell surface may be the linking factor which promotes kerationcyte motility. This thesis has demonstrated a novel function of TG2 is controlling this signalling cascade however results in this thesis are by no means exhaustive, further experiments could be conducted which would lead to better understanding of the process of re-epithelialisation in wound healing.

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