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Evaluating transglutaminase crosslinked collagen gel systems for hard and soft tissue repair

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Evaluating Transglutaminase Crosslinked Collagen Gel Systems for Hard and Soft Tissue Repair

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Thesis Summary

Tissue Transglutaminase (TG2) and FXIIIa, members of the transglutaminase (TG) family, catalyses a transamidating reaction and form covalent bond between or within proteins. In bone development, both enzymes expressions correlate with the initial of the mineralisation process by osteoblasts and chondrocytes. Exogenous TG2 also promotes maturation of chondrocytes and mineralisation in pre-osteoblasts.

To understand the role of endogenous TG2 in osteoblast mineralisation, the TG2 expression was examined during the human osteoblast (HOB) mineralisation. The expression of the endogenous TG2 increased during the mineralisation, yet, its expression was not essential for mineral deposition due to the compensation effect by other members in the TG family. The extracellular transamidating activity of HOBs was found increased during mineralisation and a shift from FXIIIa dominant- to TG2-dominant crosslinking activity was suggested after differentiation. However, the transamidating activity of both TG2 and FXIIIa were not critical for cell mineralisation.

On the other hand, Exogenous TG2 was found to enhance wild type HOB and TG2 knockdown HOB mineral deposition. The transamidating activity of TG2 was not required but most likely a close conformation was essential for this enhancement. Results also demonstrated that exogenous TG2 may activate the β-catenin pathway through LRP5 receptor thus contribute in cell mineralisation. This enhancement could be abolished by addition of β-catenin inhibitors.

Finally, using of TG2 crosslinked collagen gel for bone and cornea repair was evaluated. Crosslinked collagen gel showed promising results in improving HOB mineralisation, human corneal fibroblast (hCF) proliferation and migration. These effects might be resulted from the trapped TG2 within the collagen matrix and the alteration of matrix topography by TG2.

Key words: FXIIIa, close conformation, LRP5, β-catenin, human corneal fibroblast

Dedication

To my beloved family and most of all my

wonderful grandfather Kuang Hua Yeh

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Chapter I General Introduction

Chapter II Materials and Methods

List of Abbreviations

ALP	Alkaline phosphatase
AM	Amniotic membrane
ApoE	Apolipoprotein E
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
cFXIIIa	Cellular factor XIIIA
CK1α	Casein kinase 1α
Dkk-1	Dickkopf-1
Dvl or Dsh	Dishevelled
ECM	Extracellular matrix
FACIT	Fibril-associated collagen with interrupted triple-helices
FGF	Fibroblast growth factor
FN	Fibronectin
FRAT	Frequently rearranged in advanced T-cell lymphomas
FXIIIa	Factor XIIIa
gpTG2	Guinea pig liver transglutaminase
GRE	Glucocorticoid response element
GSK-3β	Glycogen synthase kinase 3 β
HA	Hydroyapatite
hCECs	Human corneal epithelial cells
hCFs	Human corneal fibroblasts
HOBs	Human osteoblasts
HRE	Hypoxia response element
HSPG	Heparan sulfate proteoglycan
IL-6	Interleukin-6
Krm	Kremen 1/2
LEF	Lymphoid enhancing factor
Lm	Laminin
LRP5	Lipoprotein related-protein 5
MMP	Matrix metalloproteinase
MPs	Microparticles
MSCs	Mesenchymal stem cells
mTG	Microbial transglutaminase

MTI-MMP	Membrane type I-matrix metalloproteinase
MVs	Matrix vesicles
NCPs	Noncollagenous matrix proteins
NF-1	Nuclear factor-1
NF-κB	Nuclear factor κB
NPP	Nucleotide pyrophosphatase
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
PDGF	Platelet-derived growth-factor
Pi	Phosphate
PMCA1	Plasma membrane Ca^{2+} transport ATPase 1
pNPP	p-Nitrophenyl phosphate
PPi	Pyrophosphate
RA	Retinoic acid
RANK	Receptor activator of nuclear factor κB
RAR	Retinoic acid receptors
RRE	Retinoic acid response elements
RWD	Relative wound density
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
sFRP	Secreted frizzled-related protein
Sp-1	Specificity protein 1
TCF	T-cell factor
TG2	Transglutaminase 2
TGF- β1	Tumour growth factor-β1
TGs`	Transglutaminases
TIMP-1	Tissue inhibitor of metalloproteinase-1
TNKS	Tankyrase
TNSALP	Tissue non-specific alkaline phosphatase
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells
WIF-1	Wnt inhibitory factor-1
β-TrCP	β-transducin repeat-containing protein
β–GP	β-glycerophosphate

Chapter I

General Introduction

1. Transglutaminase

Transglutaminases (TGs) are a group of enzymes that catalyse the post-translational modification of proteins at glutamine residues through an acyl-transfer reaction. The enzymes are widely distributed from bacteria (Ando *et al.*, 1989, Nonaka *et al.*, 1989, Kanaji *et al.*, 1993) to plants (Serafini-Fracassini *et al.*, 1995, Serafini-Fracassini *et al.*, 2002) and animals (Clarke *et al.*, 1959, Chung, 1972, Aeschlimann *et al.*, 1995, Kim *et al.*, 1995, Zhang and Masui, 1997). In the mammalian transglutaminase (EC 2.3.2.13) family, the availability of calcium is essential for enzymes to form a covalent bond between the γ-carboxamide group of peptide-bound glutamine and the ε-amino group of a peptide-bound lysine/ a primary amine. The first documented mammalian transglutaminase was derived from the soluble fraction of guinea pig liver by Clarke and co-workers in 1959. Fifty more years later, there are 9 members reported in higher vertebrates, TG1-7, factor XIIIa (FXIIIa) and band 4.2, and each of them have been characterised to some degree (reviewed by Griffin *et al.*, 2002, Lorand and Graham, 2003, Esposito and Caputo, 2005, Mehta, 2005). The key characteristics of these mammalian TGs are summarised in Table 1.1. This thesis focuses on two well studied TGs, TG2 and FXIIIa, in particular their roles in the mineralisation process of bone formation as well as their application in biomedical sciences.

1.1. Transglutaminase 2 (TG2)

TG2, also known as tissue transglutaminase (tTG), cytosolic transglutaminase (TGc), erythrocyte transglutaminase and endothelial transglutaminase, is ubiquitously expressed in all kinds of tissue. Its transamidating activity is regulated by calcium and GTP/GDP binding in which binding of calcium results in active TG2 and binding of GTP/GDP results in inactive TG2 (Smethurst and Griffin, 1996). Besides its primary function as a transamidase,

TG2 also has GTPase/ATPase activity (Takeuchi *et al.*, 1994), protein disulfide isomerase activity (Hasegawa *et al.*, 2003) and may even function as a protein kinase (Mishra and Murphy, 2004).

1.1.1. Transamidating and deaminating function

The transamidation activity in tissue had been first revealed in 1959 by Clarke and co-workers, and till the mid-1980s, the transamidation and deamination reaction of TG2 was well studied (Folk, 1983). The biochemical mechanism underlying the enzyme action involves two major steps. First, the rate-limiting step is formation of thioester bond between TG2 active site cysteine (C277) and the substrate. In this step, the sulfur of the active site C277 performs a nucleophilic attack on the γ-carboxamide group of a glutamine residue, and releases an ammonia molecule as a by-product (Fig 1.1A). At the second step, the acyl intermediate is attacked by the nucleophilic substrate (acyl-acceptor). In this stage, if the attacking group is a primary amine, either a small biological amine or a ε-amino group of a peptide-bound lysine residue, the reaction is called transamidation (Fig. 1.1B, C, D). However, if a water molecule acts as a nucleophile, it is called deamination (Fig. 1.1E).

Two major outcomes result from TG2 transamidation: modification of the substrate protein with small amines or formation of an isopeptide bond between acyl-acceptor and acyl-donor proteins. When the nucleophilic substrate is a small primary amine (Fig 1C), the result of transamidation reaction will be addition of a small biological amine to the substrate protein which can subsequently change its biological properties. On the other hand, the isopeptide bond can form intermolecularly between proteins (Fig. 1.1C), or sometimes crosslinking happens between two proteins via a N,N-bis(γ-glutamyl)polyamine

Table 1.1 Summary of mammalian TGs family (adapted from Griffin *et al.*, 2002, Lorand and Graham, 2003, Esposito and Caputo, 2005, Mehta, 2005)

Protein	Alternative name	Tissue expression	Molecular Mass (kDa)/ Human	Activity and regulation	Localisation (and function/ disease)
FXIIIa subunit	Fibrin-stabilising factor, Laki-Lorand factor, pro-fibrinoligase, plasma pro-TG	Platelets, astrocytes, dermal dendritic cells, chondrocytes, placenta, plasma, synovial fluid	83	Thrombin activated; Ca^{2+} activated, reducing agent required	Cytosolic, extracellular; (blood coagulation, bone growth, wound healing, ECM stabilisation)
TG1	TG_k, keratinocyte TG, particulate TG, membrane-bound TG, TG-B	Keratinocytes and brain	90	Protease activated; Ca^{2+} activated, reducing agent required	Membrane, cytosolic; (cell-envelope formation in keratinocytes differentiation)
TG2	TGc, tissue TG, liver TG, endothelial TG, erythrocyte TG, G_h. $G_{h\alpha}$, tTG,	Ubiquitous	78	Yes; Ca^{2+} activated, reducing agent required	Cytosolic, nuclear, membrane, cell surface, extracellular (cell death, cell signalling ,cell differentiation, matrix stabilisation, adhesion protein)
TG3	TG_E, epidermal TG, callus TG, hair follicle TG, bovine snout TG	Squamous epithelium, brain and hair follicle	77	Latent (protease activated); Ca^{2+} activated, reducing agent required	Cytosolic; (cell-envelope formation during terminal differentiation of kertainocytes, hair shaft formation)
TG4	TG_P, prostate TG, major androgen-regulated prostate secretory protein	Prostate	77	Yes; Ca^{2+} activated, reducing agent required	Extracellular; (semen coagulation)
TG5	TG_X	Ubiquitous except for the CNS and lymphatic system	81	Yes; Ca^{2+} activated	Cytosolic (Cornified cell formation during keratinocytes differentiation)
TG6	TG_Y	Testis and lung	70	Yes (?)	Unknown; (biomarker for developing neurological disease in gluten sensitive patients?)
TG7	TG_Z	Ubiquitous but predominantly in testis and lung	80	Yes (?)	Unknown; (unknown)
Band 4.2	B4.2, ATP-binding erythrocyte membrane protein band 4.2	Red blood cells, bone marrow, foetal liver and spleen	72	No enzymatic activity	Membrane; (structural protein, membrane skeletal component in erythrocyte)

bridge (Fig. 1.1D). There are more than a hundred proteins which have been recognised as TG2 substrates and some of them are summarised in Table 1.2.

The deaminating reaction results in the conversion of an acyl-donor glutamine residue to a glutamate residue (Fig.1 E). The deaminating reaction was first believed to occur under certain conditions, such as limited primary amines as acyl-acceptor, thus water acted as an attacking group. It was also reported that deamination was favoured to happen in low pH pathological environments (Fleckenstein *et al.*, 2002). However, recent studies suggested that besides being pH-dependent, the deaminating process could also be dependent on structure. Crosslinking of small heat-shock proteins (sHsps) by TG2 revealed that only one glutamine residue of protein underwent deamination while other glutamine residues underwent transamidation (Boros *et al.*, 2006). Growing evidence also demonstrated the propensity for deamination by TG2 is both dependent on structure and influenced by pathological conditions (Stamnaes *et al.*, 2008).

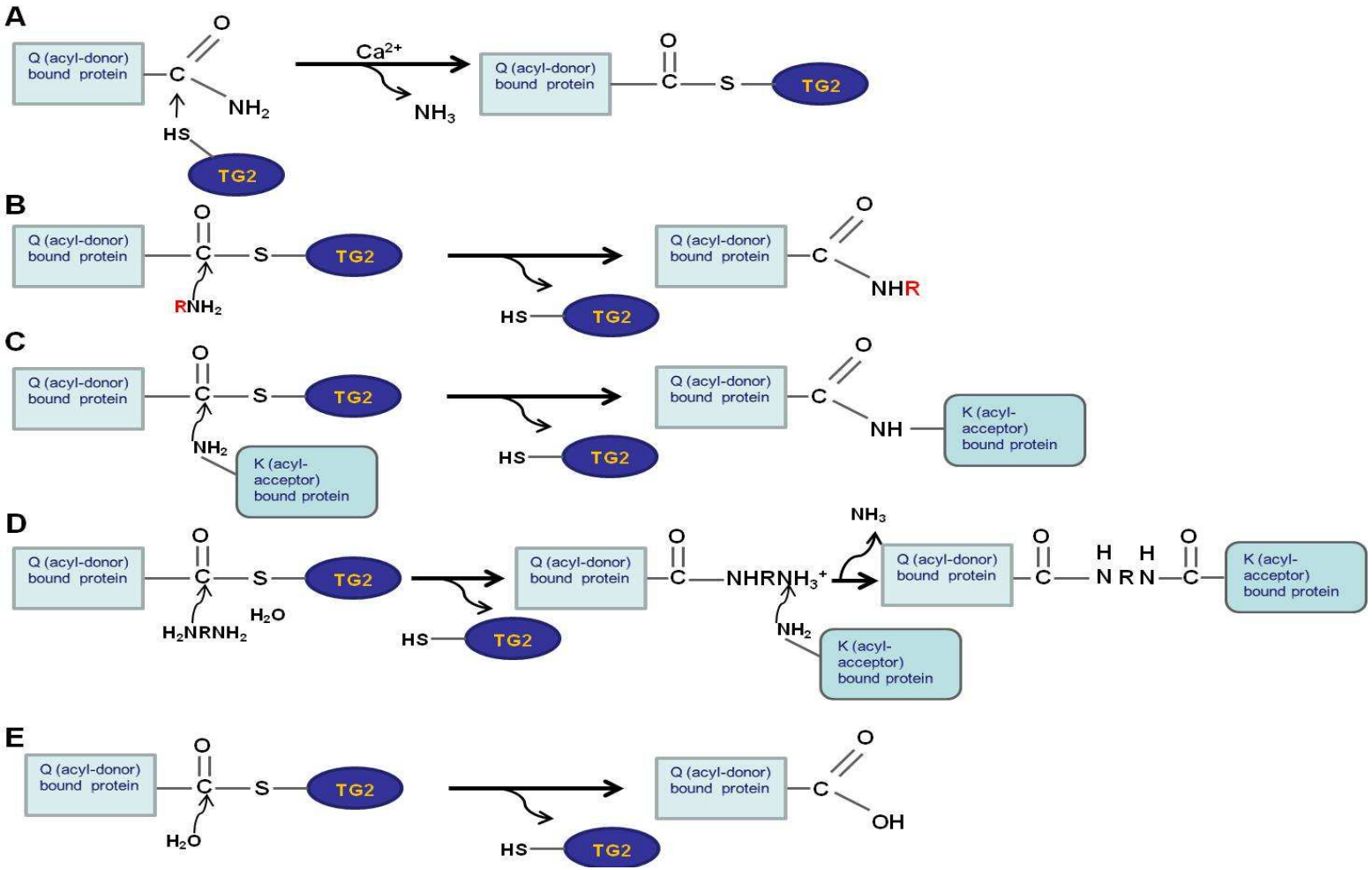

Figure1.1 Post-translational reactions catalysed by transamidase activity of TG2. (A) Formation of a γ-glutamylthiolester intermediate as a common first step for following reactions. (B) The incorporation of amine (H2NR) into the glutamine residue (C) An isopeptide bond forms between a peptide bound lysine residue and a peptide bound glutamine residue resulting crosslinking of two proteins (D) A primary amine acts as a bridge between two proteins by crosslinking activity (E) Water molecule is used as an acyl-acceptor to deaminate a peptide-bound glutamine residue to a glutamate residue.

Table 1.2 Known substrates of the transamidase function of TG2 (adapted from Griffin *et al.*, 2002 and Esposito and Caputo, 2005)

ECM and cell surface	Cytosol	Organelle Proteins	Nucleus	Others
Collagen	Actin	Histone H2B	Core histones	Wheat gliadin
Fibronectin	Aldolase A	α-oxoglutarate dehydrogenase	Importin-α3	Whey proteins
Fibronogen	β -crystallin	Acetycholine esterase	pRB	Soy Protein
Vitronectin	β -tubulin	CD38	Calbindin	Pea legumin
Osteopontin	C-CAM	Cytochromes		Candida albicans surface proteins
Osteonectin	GADPH	Erythrocyte band III		HIV envelope glycoproteins gp120 and gp41
Osteocalcin	GST			HIV aspartyl proteinase
Laminin	Glucagon			Hepatitis C virus core protein
LTBP-1	Lipocortin I			
β-casein	Melittin			
IGFBP-1	Myosin			
Substance P	Phosphorylase kinase			
Phospholipase A2	RhoA			
Midkine	Secretory vesicle IV			
	Tau protein			
	Thymosin β			

1.1.2 GTPase/ ATPase function

Binding and hydrolysis of GTP by TG2 was documented as early as 1980s (Achyuthan and Greenberg, 1987, Lee *et al.*, 1989). It was reported that binding of calcium and binding of GTP to TG2 competitively regulated the transamidating activity of the enzyme as well as proteolysis of TG2 by trypsin. The independence of GTPase activity of TG2 from its crosslinking activity could be further confirmed by Cys 277 mutant TG2 where mutation of the crosslinking active site of TG2 only affected transamidating activity but not GTPase activity (Lee *et al.*, 1993) The GTPase of TG2 was proposed to be the same as Gα(h) protein, a functional α-subunit of a G-protein associated with α1 adrenergic receptor signalling,that participates in activation of phospholipase C enzyme (Im and Graham, 1990, Nakaoka *et al.*, 1994, Im *et al.*, 1997). It was also suggested that oxytocin receptor (Baek *et al.*, 1996) and TPα thromboxane A2 receptor (Vezza *et al.*, 1999) exploited TG2 as a G-protein.

Unlike binding of GTP to TG2, which suppressed crosslinking activity of TG2, binding of ATP to TG2 only affected GTPase activity but not transamidation activity of TG2 (Lai *et al.*, 1998). The authors also suggested that the binding sites for GTP and ATP were distinct due to binding of ATP greatly inhibiting GTP binding, whereas binding of GTP only reduced ATP hydrolysis by 20%. However, the binding sites of GTP and ATP were demonstrated to be located within a 5.5 kDa (47 amino acid) region at the start of the core domain (Iismaa *et al.*, 1997). Later on, the publication of the crystal structure of TG2 in complex with GTP (Liu *et al.*, 2002) and ATP (Han *et al.*, 2010) indicated that ATP bound in the GTP/GDP binding pocket but with different hydrogen bonds and ionic interactions with TG2.

The function of the ATPase activity of TG2 was first suggested as an intrinsic kinase by Mishra and Murphy (2004). In several *in vitro* studies, TG2 was recognised as a kinase

protein for insulin-like growth factor-binding protein-3 (IGFBP-3), histones, p53 and retinoblastoma protein which might participate in cancer cell biology and cell apoptosis (Mishra and Murphy, 2004, Mishra *et al.*, 2006, Mishra and Murphy, 2006, Mishra *et al.*, 2007). Besides its intracellular function, recent research also proposed a functional role of extracellular TG2 in tissue mineralisation. As demonstrated by Nakano *et al.* in 2007 that addition of ATP, instead of β-glycerophosphate, to ascorbic acid treated pre-osteoblast cells was sufficient enough to induce cell mineralisation. And ATPase activity of TG2 was found to play a major role in this ATP-mediated mineralisation by serving as a source of phosphate groups. Further study from the same group also indicated that MT1-MMP was the key regulator of the pro-mineralization function of TG2 in extracellular matrix (Nakano *et al.*, 2010).

1.1.3 Structure and conformation of TG2

The TG2 structure comprises four distinct domains: an N-terminal β-sandwich domain, the catalytic core and two C-terminal β-barrel domains (Fig. 1.2). Some, but not all, critical active and substrates binding sites were revealed after years of studies. A catalytic triad similar to cysteine proteases, but for transamidating activity has been identified, which consists of cysteine 277 (C277), histidine 335 (H335) and aspartate 358 (D358) (Liu *et al.*, 2002). Mutating cysteine to serine at residue 277 (C277A) has been used to completely knock out transamidating and GTP/GDP binding ability of TG2. Another two conserved tryptophan residues, W241 and W332, were also identified as critical residues for TG2 crosslinking activity. These two residues were believed to stabilize the enzyme-thiol intermediate which formed at the first step of catalysis (Murthy *et al.*, 2002). Tryptophan to alanine mutation at residue 241 (W241A) knocked out transamidating activity without altering GTPase activity; whereas, replacing tryptophan at residue 332 with phenylalanine (W377F) resulted in loss of GTP/GDP binding ability. In addition, the tyrosine residue at

position 516 (Y516) within the catalytic pocket was also found to be important for crosslinking activity. As suggested by Pinkas and co-workers (2007), in inactive TG2, a hydrogen bond formed between C277 and Y516 which was believed to favour the inactive state of the enzyme. Point mutation of this residue to phenylalanine (Y516F) disturbed the inactive state of TG2 by greatly changing the conformation of protein. The same authors also suggested a change in enzyme preference for deamination over transamidation by mutation of T360A. Although the details remained unclear, the threonine residue 360 (T360) was shown to be important for the second step of the catalysis by controlling the entry of the acyl-acceptors to the catalytic site.

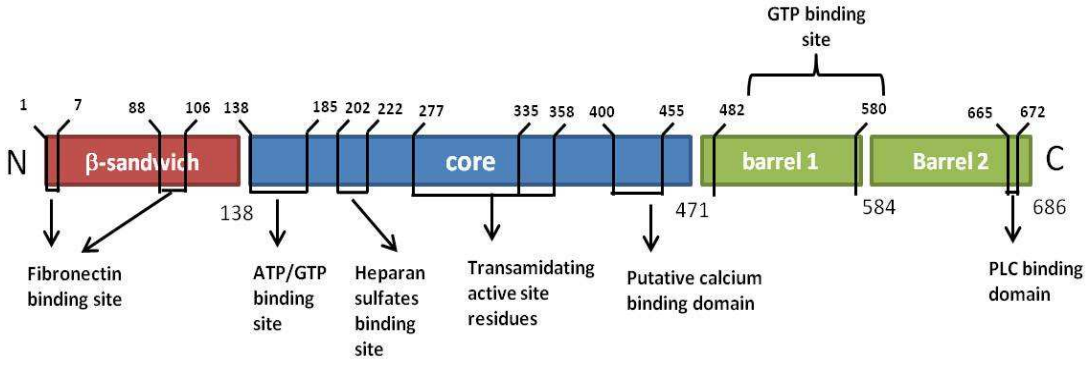

Figure 1.2 Structure of TG2 with putative substrate binding sites. Tg2 was composed of 4 major domains including N-terminal β-sandwich domain (aa 1–137), the catalytic core (aa 138–470) and two C-terminal β-barrel domains (aa 471–583 and 584–686). The fibronectin binding site was identified by Jeong *et al*. (1995) and Hang *et al.* (2005). The postulate binding sites for GTP and ATP was proposed by Iismaa *et al*. (1997) and Han *et al.* (2010). The haparan sulphates binding site was identified by Wang *et al*. (2012). The catalytic triad was identified by Liu *et al.* (2002). The calcium binding site was predicted by Fox *et al.* (1999) and the PLC binding site was recognised by Hwang *et al.* (1995)

The major GTP/ATP binding site was found to be located at the first 47 residues in the core domain (Iismaa *et al.*, 1997). Yet, later research done by Han *et al.* (2010) has suggested that less hydrogen bonding occurred between ATP and the binding pocket when comparing to the GTP complex structure. Also, there are 2 more residues, S482 and R580, which were revealed to specifically participate in GTP binding (Han *et al.*, 2010). Point mutation of R580A resulted in loss of GTP/GDP binding activity and GTP-mediated

inhibition of transamidase activity. This effect was suggested to be related to the alteration of enzyme affinity for GTP and GDP (Begg *et al.*, 2006).

While several studies used point mutated TG2 to evaluate the relevance of the crosslinking activity of TG2 in biological function, one key property of TG2 often overlooked was its conformation. Before being characterised by x-ray crystallography, the presence of multiple conformations of TG2 had been suggested. First, proteolysis studies indicated that GTP-bound TG2 showed higher resistance to calpain and trypsin proteolysis when compared to TG2 in the presence of calcium (Zhang *et al.*, 1998, Begg *et al.*, 2006). Also, the presence of different sub-populations of TG2 was also found, since different anti-TG2 antibodies preferentially bound to a distinct population of the enzyme (Fésüs and Laki, 1977, Monsonego *et al.*, 1998). In 2007, the two major conformations of TG2 were demonstrated using X-ray crystallography (Fig. 1.3): one was GDP-bound (Liu *et al.*, 2002) compact form (inactive) and the other was an active site covalent inhibitor bound (Pinkas *et al.*, 2007) open form (active). In the GDP-bound crystal structure (compact form or closing form, Fig. 1.3A), the catalytic core domain was inaccessible due to the 2 β-barrels domains, which effectively prevent binding of substrate to enzyme. On the other hand,

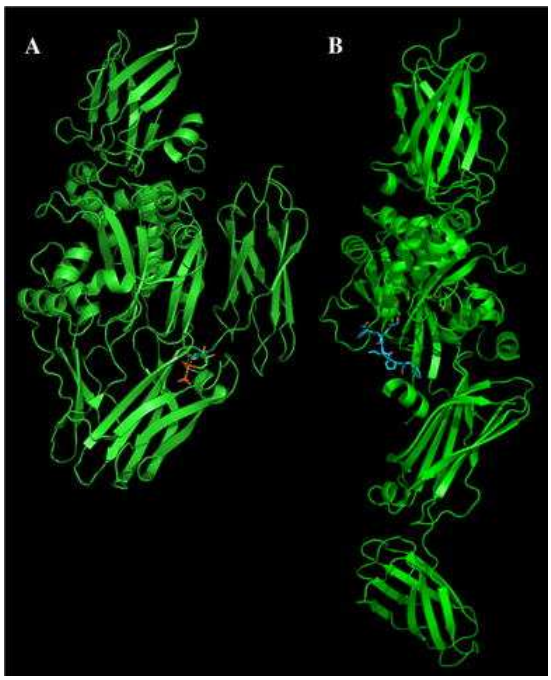

Figure 1.3 Structures of TG2 derived by X-ray crystallography (adapted from Collighan and Griffin, 2009). (A) GDP-bound human TG2 and (B) irreversible peptide inhibitor-bound human TG2. The structures of TG2 are presented with its β-sandwich domain at the top of the figure.

the irreversible inhibitor (Ac–P(DON)LPF-NH_2) reacted TG2 resulted in a stable extended conformation (open form, Fig. 3B). In this conformation, the transamidating active sites were exposed to substrates and it was assumed that a vicinal disulphide bond between Cys370 and Cys371 was the key to stabilise this extended form. These findings further urge future studies to clarify the biological significance of each TG2 conformation.

1.1.4 Regulation of TG2 gene expression

The amino acid and cDNA sequences of TG2 in guinea pig liver (Ikura *et al.*, 1988), bovine vascular endothelial cells (Nakanishi *et al.*, 1991), mouse macrophages and human endothelial cells (Gentile *et al.*, 1991) and chicken erythrocytes (Weraarchakul-Boonmark *et al.*, 1992) indicated that TG2 is highly conserved between species. It is a monomer protein consisting of 685-691 amino acids with a molecular weight around 77-85kDa. Comparing mouse, guinea pig and human TG2, there is approximately 80% homology between these amino acid sequences with 49 of the 51 residues in the active site region identical (Gentile *et al.*, 1991). The expression of human tTG gene (TGM2) was thought to be controlled by various activators. As illustrated in Fig.1.4, there are 4 identified Sp1 binding sites within TG2 and several activator regulating sites including: retinoic acid response elements (RRE-1 and RRE-2), glucocorticoid response element, nuclear factor κB response element, interleukin-6 response element and tumor growth factor-β1 response element. Retinoic acid (RA) is one of the most studied activators of TG2. These retinoid response elements are located around 1.7 kb upstream of the transcription start site (Fig. 5, Nagy *et al.*, 1996). This activation is a tripartite response: first, ligand activates either retinoic acid receptors (RAR)/ retinoid X receptor (RXR) heterodimers or RXR/RXR homodimers. And then RRE, through three hexanucleotide half-sites (two canonical and one non-canonical), binds to RAR/RXR heterodimers or RXR/RXR homodimers. Finally, the complex binds to the Sp1 binding site which is possibly through other co-activators.

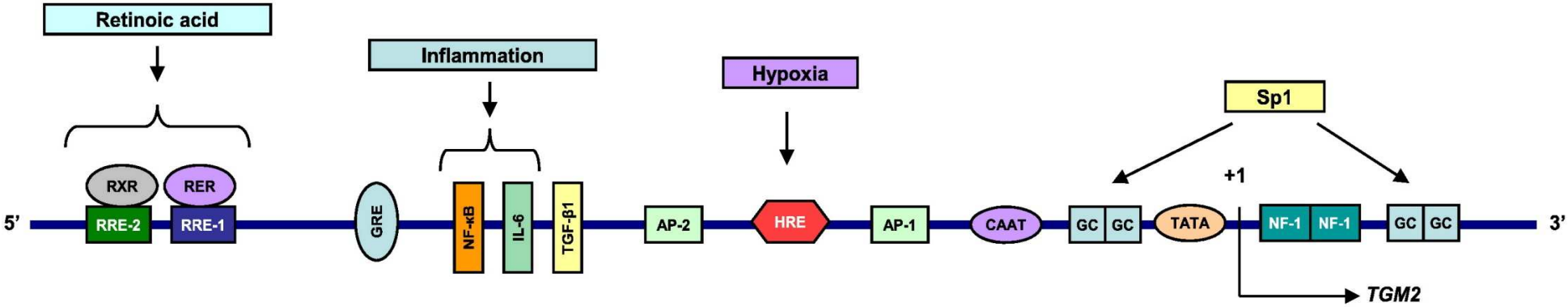

Figure 1.4 Schematic illustration of the tissue transglutaminase promoter (adapted from Gundemir *et al.*, 2012). TGM2 expression is regulated by mainly retinoic acid, inflammation factors and stress-related factors. Retinoic acid response elements (RRE-1, -1731 and RRE-2, -1720), glucocorticoid response element (GRE, -1399), nuclear factor κB response element (NF-κB, -1338), interleukin-6 response element (IL-6, -1190), tumor growth factor-β1 (TGF-β1, -900), activator protein-2 (AP-2, -634), hypoxia response element (HRE, -367), activator protein-1 (AP-1, -183), CAAT box (-96), GC box (Sp1 binding motifs, -54, -43, +59, +65), TATA box (29), nuclear factor-1 (NF-1, +4, +12), retinoid X receptor (RXR) and retinoic acid receptors (RAR).

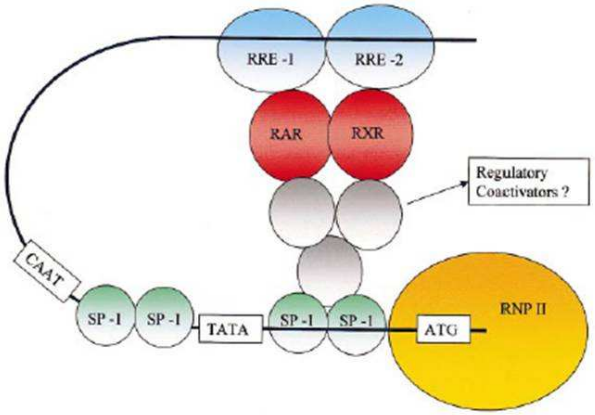

Figure 1.5 Putative model of tTG and of its promoter (adapted from Griffin *et al.*, 2002). This model demonstrates the interaction among retinoic acid response elements (RRE-1 and RRE-2), retinoid X receptor (RXR)/ retinoic acid receptors (RAR) and specificity protein 1 (Sp-1) binding site upon RA induction.

1.1.5 Involvement of TG2 in cell adhesion/ signalling

The biological role of TG2 is highly related to the location of TG2. The majority of cellular TG2 is predominantly distributed in the cytosol (Bruce and Peters, 1983) and it is also found on the plasma membrane (Im and Graham, 1990, Iismaa *et al.*, 2000, Begg *et al.*, 2006), mitochondria (Piacentini *et al.*, 2002, Rodolfo *et al.*, 2004) and nucleus (Bruce and Peters, 1983, Singh *et al.*, 1995, Lesort *et al.*, 1998). There are also accumulating results which show the presence of TG2 and crosslinking activity in extracellular matrix (ECM) (Zemskov *et al.*, 2006). Generally, endothelial cells, fibroblasts and smooth muscle cells express constitutive levels of TG2; however, TG2 is often considered as a stress-related protein as its expression undergoes dramatic change upon stress or injury.

It has been well studied that TG2 can promote cell-ECM adhesion, cell migration and organisation of extracellular matrix protein through two different pathways: syndecan-4–TG2 complexes and Integrin–TG2 interaction (reviewed by Belkin, 2011). TG2 was found to associate with a membrane protein, heparan sulfate proteoglycan (HSPG) syndecan-4, to promote cell-ECM adhesion in an RGD-ligand independent manner (Telci *et al.*, 2008). This adhesion pathway was proposed to be critical for cell survival during tissue injury and/or remodelling since TG2/fibronectin (FN)/syndecan-4 complexes activated PKCα leading to activation of β1 integrin even when the integrins were blocked by RGD peptide. The formation of FN/TG2/syndecan-4 complexes could also activate another HSPG family protein, syndecan-2, through PKCα (Wang *et al.*, 2010). Besides functioning as a scaffold protein, this complex could also mediate fibronectin fibril formation, which was independent of its transamidating activity and occurred in the presence of cell adhesion inhibitors (RGD peptides).

TG2 was also found to non-covalently interact with β1, β3 and β5 integrin subunits. Also, the binding sites for TG2 and integrin did not overlap on fibronectin protein. Therefore, the affinities of TG2 with these two proteins on the cell surface could significantly enhance the interaction of cells with fibronectin and further promote cells adhesion and ECM-triggered signalling (Akimov *et al.*, 2000, Akimov and Belkin, 2001a, Zemskov *et al.*, 2006). Studies also demonstrated that cell surface TG2 could induce integrin clustering in the absence of integrin-ligand interactions (Janiak *et al.*, 2006). These TG2/ integrins complexes were found to down-regulate the Src-p190RhoGAP regulatory pathway thus amplifying the activation levels of RhoA GTPase and its downstream signalling target, ROCK. Overall, the association of TG2 with cell membrane integrins and extracellular matrix protein could be considered as a general amplifier of outside-in adhesion signalling.

1.1.6 Involvement of TG2 in wound healing

The involvement TG2 in wound healing has been well described and summarised recently (reviewed by Verderio *et al.*, 2005, Telci and Griffin, 2006a). As illustrated in Fig. 1.6, TG2 participates in different phases of wound healing process via transamidation-dependent and independent mechanisms. Secretion of TG2 into ECM was observed soon after either chemical or mechanical injury of fibroblasts and the released TG2 was found co-localised with extracellular matrix proteins (Upchurch *et al.*, 1991). The TG2 crosslinking activity was detected in all layers of skin as demonstrated by a wounded rat dermal skin model (Bowness *et al.*, 1988). Indeed, upregulation of TG2 expression and activity in erythrocytes and vascular endothelial cells within wound areas were suggested to support and amplify FXIIIa-mediated blood clot formation (Murthy *et al.*, 1991, Barsigian *et al.*, 1991a, Auld *et al.*, 2001).

It has been postulated that TG2-promoted cell adhesion was especially important in the wound healing process where TG2/FN complexes could interact with cell membrane syndecan receptors and enhance cell adhesion and survival when cells underwent anoikis (Telci *et al.*, 2008, Wang *et al.*, 2010, Wang *et al.*, 2011). In addition, increase in TG2 mRNA expression was also related to several collagen producing phenotypes at matrix remodelling stages (Schnabel *et al.*, 2004, Klingberg *et al.*, 2012, Walter *et al.*, 2005, Ikee *et al.*, 2007). It has been also postulated that TG2-promoted cell adhesion has the ability to facilitate and to prevent apoptosis of cells (reviewed by Fésüs and Szondy, 2005). These two opposite activities may occur distinctly depending on different cell types, the kind of stimuli, the intracellular localization of the enzyme and the type of activity of TG2. However, failure of myofibroblasts to undergo apoptosis has been related to abnormal scar tissue formation at the end of wound healing (Sayah *et al.*, 1999, Aarabi *et al.*, 2007a, Aarabi *et al.*, 2007b). Therefore, the balance between TG2 pro-apoptotic and anti-apoptotic abilities may be critical for normal wound healing process or abnormal scar tissue formation.

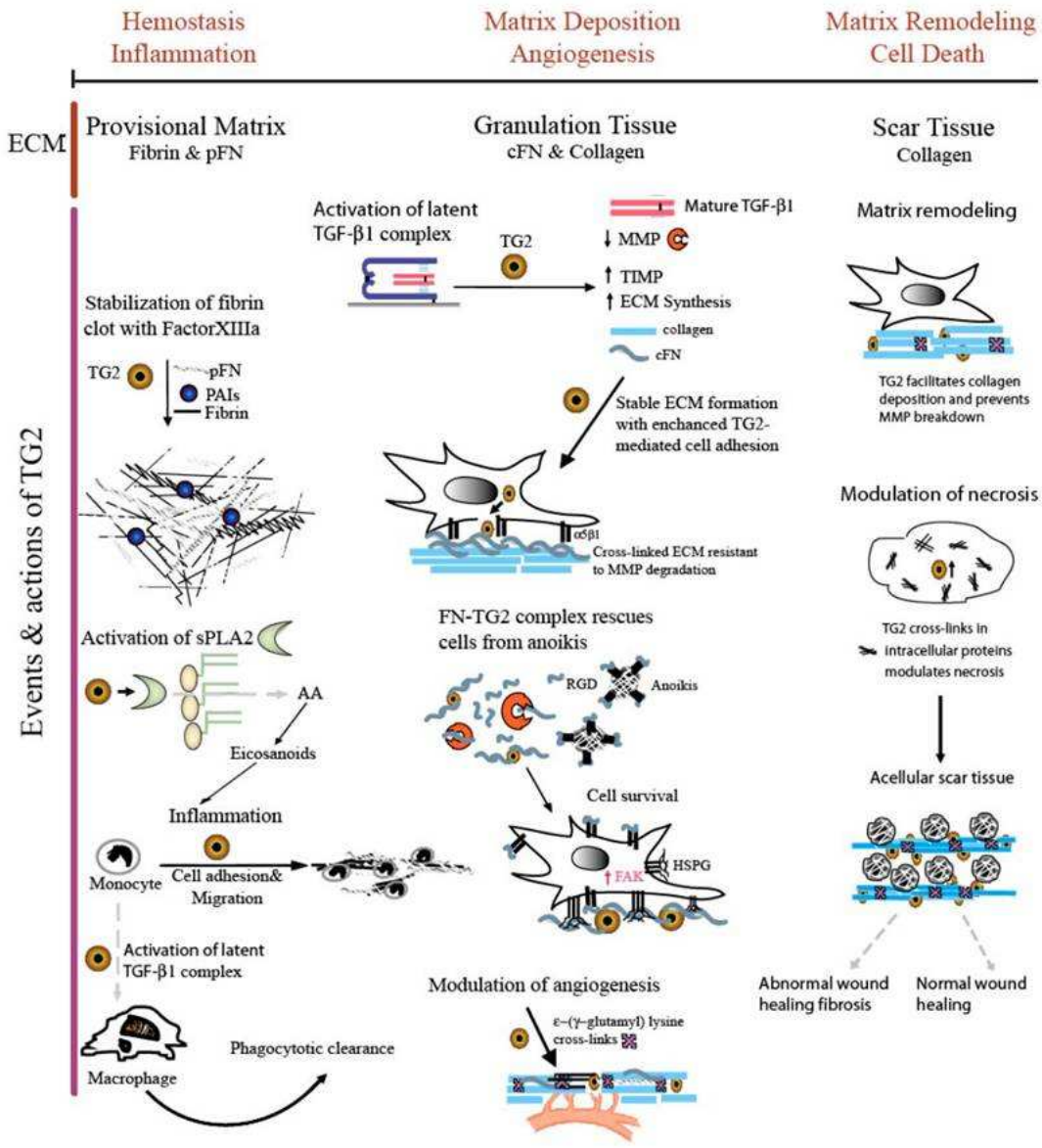

Figure 1.6 Roles of TG2 in the wound healing process (adapted from Telci and Griffin, 2006a). Proposed roles of TG2 in different phases of wound healing were illustrated. It was believed that TG2 could stabilise extracellular matrix, promote cell survival and participate in matrix remodelling during wound closure. pFN, plasma fibronectin; cFN, cellular fibronectin; PAIs, plasminogen activator inhibitors; sPLA2, secretory phospholipase A2 enzyme; TGF-beta1, transforming growth factor beta 1; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; RGD, peptide fragments containing Arg-Gly-Asp recognition sequence for integrins; HSPG, heparan sulfate proteoglycans; FAK, focal adhesion kinase.

1.1.7 Involvement of TG2 in pathological conditions

Cancer

The involvement of TG2 in cancer progression is complicated and seems to be cell type-dependent. Nevertheless, intracellular TG2 has been suggested to have both pro- and anti-apoptotic ability depended on its localisations and activity state (Milakovic *et al.*, 2004). As reviewed by Milakovic and colleagues, once intracellular TG2 was activated by stress or trauma-induced intracellular calcium homeostasis, it could perform cross-linking of cellular proteins and result in apoptotic death. However, on the other hand, studies have indicated that cytosolic TG2 showed an anti-apoptotic effect via activation of the NF-κB pathway in pancreatic cancer cells and neuroblastoma cells (Mann *et al.*, 2006, Condello *et al.*, 2008). The increase in TG2 expression has also been related to increased drug resistance and poor patient survival rate in specific cancers including pancreatic cancer (Verma and Mehta, 2007, Verma *et al.*, 2006), breast cancer (Mangala *et al.*, 2006, Mehta *et al.*, 2004), ovarian (Satpathy *et al.*, 2007, Hwang *et al.*, 2008), melanoma (Fok *et al.*, 2006) and lung (Park *et al.*, 2010b). It was also demonstrated by Gundemir and Johnson (2009) that translocation of TG2 into the nucleus had a protective effect against apoptosis. Even though there are many conflicting results concerning TG2 expression in cancer cells, interestingly, many studies have indicated down regulation of TG2 expression in primary tumours and increased TG2 expression as a key factor for secondary tumours or chemoresistant cancer cells (Mehta *et al.*, 2004, Herman *et al.*, 2006, Verma and Mehta, 2007, Cao *et al.*, 2008, Mehta *et al.*, 2010).

Extracellular TG2, on the other hand, was proposed to be involved in tumour cell survival and metastasis. It has been already demonstrated that extracellular TG2 can promote cell growth, survival and migration in normal cells and these mechanisms were believed also

operating in cancer cells (reviewed by Mehta *et al.*, 2010). It was shown in T lymphocytes that TG2 was important for T cell transendothelial migration (Mohan *et al.*, 2003). Also, the increased expression of cell surface TG2 in TGF-β treated retinal pigment epithelial (RPE) cells was linked to enhancement of cell adhesion and migration (Priglinger *et al.*, 2004). In fact, inhibition of endogenous TG2 using small interfering RNA (siRNA) resulted in the reversal of drug resistance and the invasive phenotype, whereas overexpression of TG2 improved cancer cell invasion and attachment to fibronectin (reviewed by Collighan and Griffin, 2009). Overall, these data implied that the precise role and function of TG2 in cancer cell biology may be highly dependent on the cell type, stage of cancer cells and the localisation of the TG2.

Fibrosis and scarring

TG2 has been long related to abnormal wound healing processes which involves chronic inflammatory response and excessive matrix deposition. Excess ECM crosslinking by TG2 has been established in several fibrotic disease models including pulmonary (Griffin *et al.*, 1979, Richards *et al.*, 1991), renal fibrosis (Johnson *et al.*, 1997, Johnson *et al.*, 1999, Johnson *et al.*, 2007) and liver fibrosis (Skill *et al.*, 2001, Grenard *et al.*, 2001). In development of tissue fibrosis, chronic inflammation and constant activation of TGF-β signalling were observed. TG2 was related to this chronic stress by having a role in storing a large pool of latent TGF-β1 binding protein-1 (LTBP-1) in the ECM via crosslinking. Crosslinking ECM also stabilised ECM proteins and rendered the extracellular matrix resistant to degradation. As seen in a recent study, TG2 knockout mice were protected from the development of fibrotic lesions in obstructive nephropathy as a result of decreasing macrophage and myofibroblast infiltration and TGF-β related ECM over-expression (Shweke *et al.*, 2008b). In addition, using a membrane permeable TG inhibitor,

R283 (1,3-dimethyl-2[(oxopropyl)thio]-imidazolium), and a membrane-impermeable TG inhibitor, R281 (N-benzyloxycarbonyl-l-phenylalanyl-6-dimethylsulphonium-5-oxo-l-norleucine) both caused a reduction in glomerular and interstitial scarring in a rat subtotal nephrectomy model of chronic renal fibrosis (Johnson *et al.*, 2007). As briefly mentioned above, the abnormal hypertrophic scarring, which was characterised by prolonged action of myofibroblasts and excessive collagen deposition, also involved TG2 activation (Linge *et al.*, 2005). Using TG2 crosslinking activity inhibitors, therefore, as a potential treatment for preventing fibrosis and scar formation was evaluated by several groups and a certain level of success was reported. Putrescine (Fibrostat), a general TGs inhibitor, was shown to reduce hypertrophic scar formation in an early stage of clinical trial (Dolynchuk *et al.*, 1996).

Vascular calcification

Vascular smooth muscle cells (VSMCs) have been shown to lose their contractile phenotype and switch to a synthetic phenotype in culture (VanBavel and Bakker, 2008) and during vascular injury and repair. Yet, abnormal vascular remodelling may result in many diseases including hypertension and vascular calcification. Atherosclerosis and aging are common factors for developing calcification in the arterial wall. Although the mechanism of vascular calcification is a subject of ongoing work, induction of an osteochondrogenic phenotype in the VSMCs is generally observed in calcified vasculature. *In vitro*, there are several factors known to induce the osteochondrogenic phenotype which are bone morphogenetic protein-2 (BMP-2) (Wang *et al.*, 1993, Roark and Greer, 1994, Rickard *et al.*, 1994), a member of the transforming growth factor-β superfamily, and inorganic phosphate (Pi) (Beck, 2003, Rickard *et al.*, 1994, Beck Jr *et al.*, 2003). Till very recently, the role of TG2 in vascular stiffness was first pointed out by Johnson *et al* in 2008 . In their research, the involvement of TG2 in calcification process was demonstrated

that TG2 -/- VSMCs failed to develop an osteochondrogenic phenotype following high P_i induction. They further showed that extrinsic wild type TG2, FXIIIa and GTP-binding site mutant TG2 (K173L) but not catalytic site mutant TG2 (C277S) could restore the calcification process in knock out cells. The authors finally concluded that TG2 and its activity could negatively affect remodelling in artery wall. Another study of apolipoprotein E (apoE)/ TG2 double knockout mouse model in atherosclerosis suggested that TG2 did not influence plaque composition or calcification (Williams *et al.*, 2010); however, the researchers also indicated that one or more members of the transglutaminase family may have compensated for the deficiency in TG2 expression.

Transamidating activity may be important for TG2-induced calcification, however, Faverman and colleagues (Faverman *et al.*, 2008) proposed the presence of another TG2 transamidating independent pathway in developing calcified matrix. In their study, activation of β-catenin pathway was observed following interaction between extracellular TG2 and low density lipoprotein related-protein 5 (LRP5) membrane receptors in VSMCs. There is growing evidence that TG2 may promote vascular calcification by activating the β-catenin signalling pathway. A study done by Beazley *et al.* (2012) further confirmed the importance of TG2/β-catenin axis in vascular calcification. Even though the later study suggested that TG2 activity was necessary for preventing warfarin-regulated calcification. Overall, these results identified a crucial role of TG2 in vascular stiffness and targeting TG2 or its downstream proteins could develop a novel therapeutic for the prevention of vascular calcification.

1.2 Factor XIIIa subunit (FXIIIa)

FXIIIa protein is one of the most studied members of the TGs and its physiological role in blood coagulation cascade has been firmly established. FXIII is expressed by a wide variety of cell types including platelets, monocytes, their bone marrow precursors, monocyte-derived macrophages, dendritic cells, chondrocytes, osteoblasts, and osteocytes. The 166kDa cellular Factor XIIIA (cFXIIIa) is a homodimer of two A-subunits; each of which contain 730 amino acid residues. In plasma (plasma FXIII, pFXIII), FXIII is usually presented in a 320kDa tetramer (A2B2) composing of two non-covalently associated catalytic A subunits (A2) and two non-catalytic B subunits (B2) (Schwartz *et al.*, 1973). FXIII protein is usually in a latent form and is activated by either enzymatic cleavage of each A subunit at the Arg37-Gly38 peptide bond by thrombin or at very high, unphysiological Ca^{2+} concentrations (> 50mM).

In Fig.1.7, possible activation forms of FXIIIa are summarised. In physiological conditions, FXIII is activated by the concerted action of thrombin and Ca^{2+}. Thrombin cleaves each A subunit 37 amino acid residues from the N-terminus although the enzyme still remains inactive without the presence of Ca^{2+}. When Ca^{2+} is available at plasma concentrations, the B subunits then dissociate from the A subunits and the active site cysteine of the A subunits become accessible for the substrate or for alkylating agents. However, a thrombin-independent pathway of zymogen activation was suggested under high calcium concentration environment that Ca^{2+} induces the dissociation of FXIIIb, also activates the FXIIIa dimer without involvement of proteolysis (Credo *et al.*, 1978). Similarly, the cFXIII can be activated via thrombin cleavage or via a non-proteolytic pathway. In this case, a lower concentration (2mM with the presence of chaotropic agents) of Ca^{2+} is sufficient to bring about the active configuration of FXIIIa dimer (Polgar *et al.*, 1990).

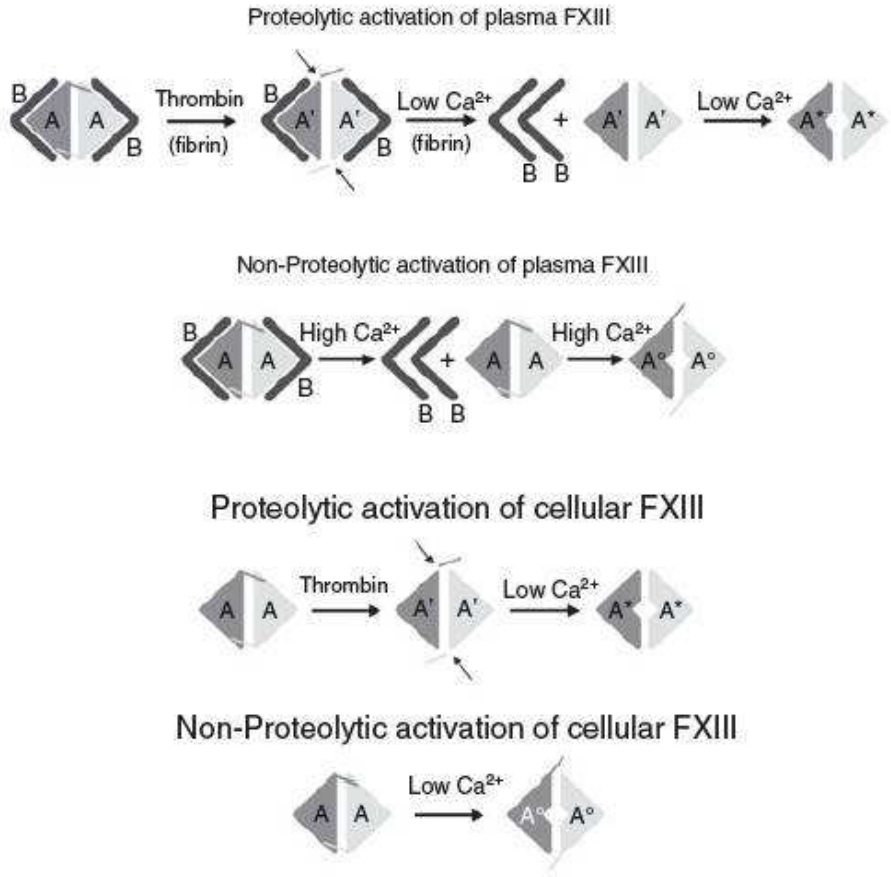

Figure 1.7 Illustration of the plasma and cellular factor XIII activation pathways (adapted from Muszbek et al., 2007). White triangle indicates exposure of the active site cysteine. Inactive proteolytically cleaved intermediate form is presented with a (′). Proteolitically cleaved active form is presented with a (*). Enzymatic form without cleavage by thrombin is presented with a (°)

1.2.1 Structure and Substrate of FXIIIa

The three dimensional structure of FXIIIa was first revealed by x-ray crystallography by Yee *et al.* in 1994 (Fig. 1.8). Similar to TG2, FXIIIa consists of 4 domains including a β-sandwich domain (a.a. 38-184), the catalytic core (a.a. 185-515) and two C-terminal β-barrel domains (a.a. 516-628 and a.a. 629-731). The three residues (Fig.1.9A): Cys314, His373 and Asp 396 are recognised as the proteinase-like catalytic triad of FXIIIa (Pedersen *et al.*, 1994). The calcium binding site (Fig 1.9B) has also been identified with the carboxylate group of Asp438, Glu485, and Glu490 side chains, the carbonyl O atom of Asn436, as well as the backbone carbonyl O atom of the Ala457 residue all involved in calcium binding (Fox *et al.*, 1999).

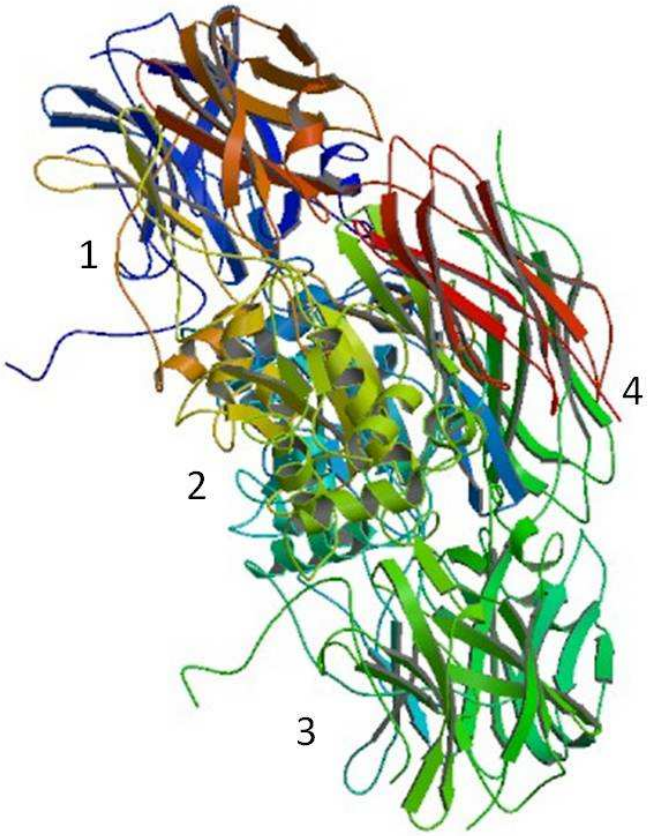

Figure 1.8 Structure of human FXIIIa (DOI:10.2210/pdb1ggt/pdb). The four main domains are (1) a β-sandwich domain (2) the catalytic core C-terminal (3) β-barrel 1 and (4) β-barrel 2.

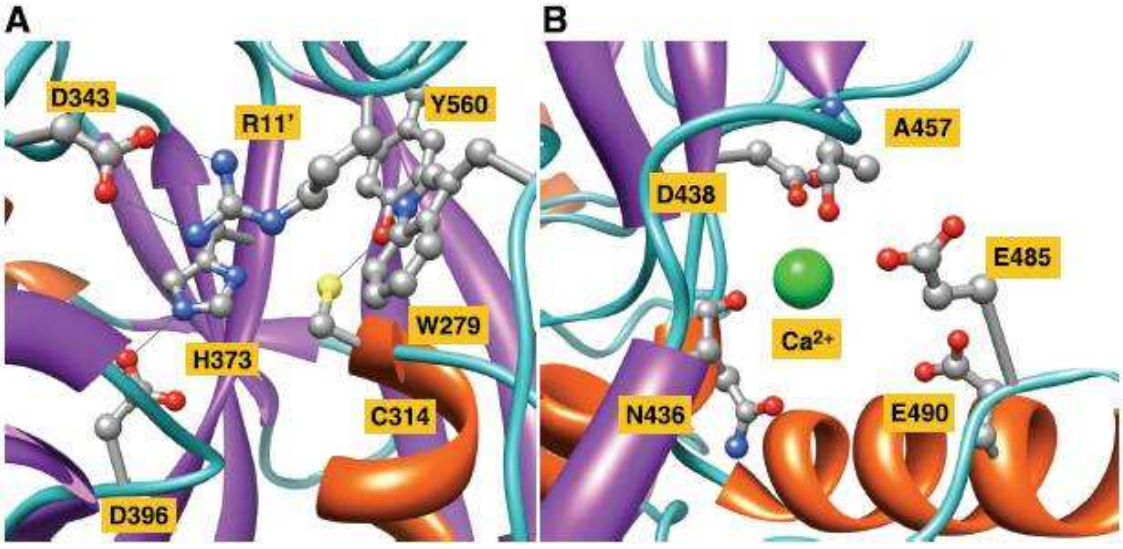

Figure 1.9 (A) Catalytic site of FXIIIa and (B) calcium binding site of FXIIIa. Picture is adapted from Muszbek *et al.,* 2011.

FXIIIa has a more restricted specificity for substrate compared to TG2. In addition to 2 primary physiological substrates of FXIIIa, fibrin (Lorand *et al.*, 1962) and α2-

plasmin inhibitor (Sakata and Aoki, 1980), another 27 proteins have been identified and documented in the TRANSDAB database (http://genomics.dote.hu/wiki). Compared to TG2 which has more than 150 listed substrates in the TRANSDAB database, most of the FXIIIa substrate are substrates for TG2 as well (Table 1.3).

Table1. 3 Summary of substrates for FXIIIa (adapted from Muszbek *et al.,* 2011. and the TRANSDAB database)

Coagulation factors	Fibrinolytic proteins	ECM and Adhesion proteins	Cytoskeletal proteins
Fibrin(ogen) α chain Fibrin(ogen) γ chain Factor V	α2- plasmin inhibitor Lipoprotein A Plasminogen Procarboxypeptidase B/U	Fibronectin Osteopontin Vitronectin Collagen Laminin Thrombospon-din	Actin Myosin Vinculin

1.2.2 Functions of FXIIIa

The transamidase activity of FXIIIa is important for its physiological role in the blood coagulation cascade. It serves as a clot stabiliser at the final step of coagulation by forming γ-glutamyl-Є-lysyl-isopeptide bonds between fibrin molecules (Fig. 1.10). The field of FXIIIa research has been extended in addition to its contribution to haemostasis for the past few decades. The role of FXIII in the wound healing process, angiogenesis as well as its contribution in cartilage and bone development, inhibition of vascular permeability, cardioprotection and maintaining pregnancy are suggested by current studies (reviewed by Muszbek *et al.*, 2011).

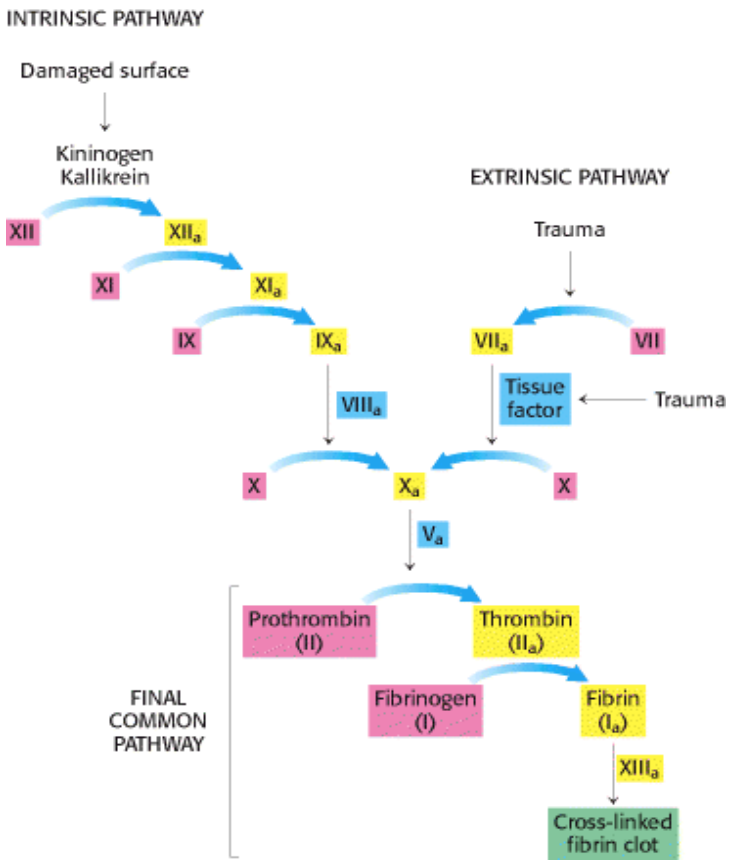

Figure 1.10 The blood coagulation cascade and proteins involved (adapted from http://blogs.scientificamerican.com/the-curious-wavefunction/2012/07/27/gene-duplication-frees-up-enzymes-for-molecular-promiscuity/)

According to clinical data, FXIII-deficient patients suffer not only from severe bleeding complications but also poor wound healing and abnormal scar formation (Board *et al.*, 1993, Anwar *et al.*, 1999, Ivaskevicius *et al.*, 2007). The relation of FXIIIa deficiency and impaired wound healing was also demonstrated in FXIII-deficient mice (Inbal *et al.*, 2005). The impaired wound healing process observed in FXIIIa deficiency models could be due to low proliferation of fibroblasts and failure of collagen fibre production by fibroblasts (Beck *et al.*, 1961). And the impairment of cell function could be restored using supplementation of FXIIIa and FXIIIa crosslinked matrix (Ueyama and Urayama, 1978). Besides proliferation, the migration of fibroblasts was also enhanced on FXIIIa treated fibrin gel (Brown *et al.*, 1993) and fibrin clot (Grinnell *et al.*, 1980). A recent study suggested that the effect of FXIIIa on fibroblasts involved binding of FXIIIa to integrin $\alpha_v\beta_3$

which leads to cJun upregulation and TSP-1 downregulation (Dardik *et al.*, 2007). Another possible cell to be affected by FXIIIa deficiency is the monocyte/macrophage. A similar effect was found in monocytes, that FXIIIa enhanced cell proliferation and migration and inhibited cell apoptosis. This effect was found to be abolished by the addition of antibody against integrin $\alpha_v\beta_3$ and the activity of FXIIIa was essential for this effect on monocytes (Dardik *et al.*, 2007). However, the effect of FXIIIa deficiency on monocyte/macrophage migration still remains questionable since contradictory evidence are reported (Akimov and Belkin, 2001b) and requires further study.

FXIIIa is also a proangiogenic factor and this function is believed to take part in the wound healing process. The effect of FXIIIa in angiogenesis has been well established in several *in vitro* and *in vivo* models. FXIIIa could induce a dose-dependent effect on tube formation of human umbilical endothelial cells (Dardik *et al.*, 2003). The same study also showed that injection of FXIIIa into cornea sub-epithelium resulted in neovascularisation of cornea. Also, the formation of new blood vessels into implants was greatly reduced in FXIIIa-deficient mice (Dardik *et al.*, 2006). The mechanism behind the proangiogenesis mediated by FXIIIa was found to be crosslinking activity dependent (Dardik *et al.*, 2003). This effect also involved its binding to integrin $\alpha_v\beta_3$ which enhanced the formation of vascular endothelial growth factor receptor-2 (VEGFR-2)/β_3 complex and further activating VEGFR-2 signalling (Dardik *et al.*, 2005).

Involvement of TG2 and FXIIIa in physiological bone development

TGs have long been implicated in the promotion of chondrocyte and osteoblast differentiation and matrix mineralization. It is generally believed that one or more members of the TG family are involved in these processes and these effects can be mediated by protein cross-linking activity of TGases, by GTPase activity of TG2 or via non-catalytic signalling effects (reviewed in Nurminskaya and Kaartinen, 2006). The expression patterns of TG2 and FXIIIa in early limb development has been demonstrated in animal models. The immunohistochemistry of embryonic tibia revealed that, during bony collar formation, TG2 and FXIIIa were both present throughout the areas of chondrocyte condensation with higher levels of both proteins in the hypertrophic zone and epiphyseal regions of the long bone, and a slightly lower expression in the zone of chondrocyte proliferation. Later in development, the TGase expression was more restricted, and towards the end of embryonic development, the expressions of TG2 and FXIIIa were only in the superficial layers of cells (Pechak *et al.*, 1986, Nurminskaya and Linsenmayer, 1996). These results suggested an early activation of TG2 and FXIIIa in the mesenchymal condensation phase. And in later development, the expression of these proteins was restricted to proliferative chondroblasts close to the epiphyses, and to the pre-hypertrophic and differentiated hypertrophic chondrocytes.

Study of TGs expression in the perichondrium/ periosteum area also gave a clue of TGs role in osteoblast differentiation (Fig. 1.11). There was an absence of TGs expression in perichondrium (cartilage part) during the mesenchymal condensation phase; however, the expression of TGs coincided with the ossification occurring in periosteum (mineralised part). It suggested that TGs were expressed in cells undergoing differentiation into osteoblasts. Therefore, initiation of TGs synthesis by osteoblasts correlated with deposition of mineral matrix (Pechak *et al.*, 1986, Nurminskaya *et al.*, 2003).

Intriguingly, according to the expression of TGs in long bone development which was located at the borderline of differentiated hypertrophic chondrocytes and differentiated osteoblasts, Nurminskaya *et al.* (2003) hypothesised that chondrocyte-derived transglutaminase could mediate maturation of preosteoblasts in periosteal bone (refer to Fig 1.14 for bone structure). In fact, they showed that co-culture of pre-osteoblasts with hypertrophic chondrocytes significantly increased the mineralisation area and shortened the time course for mineralisation while only limited mineralised matrix was found in pre-osteoblast monoculture. They further demonstrated that addition of exogenous guinea pig TG2 to pre-osteoblast culture could increase mineralisation by 3 to 5- fold over the untreated cultures. Exogenous TG2 also increased gene expressions of bone sialoprotein (BSP) and osteocalcin (OCN), molecular markers that indicated osteoblast maturation.

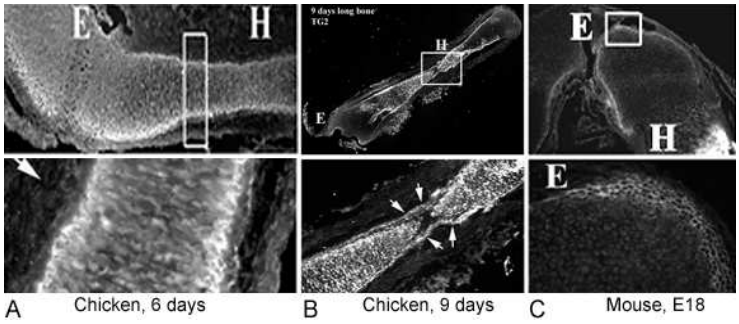

Figure 1.11 Expression of FXIIIa (and TG2, same pattern) analyzed by immunohistochemistry (adapted from Nurminskaya and Kaartinen, 2006). (A) chicken wing, (B) chicken tibia, (C) mouse tibia. Pictures are taken under 10x magnification objective. Boxed areas are shown at higher magnification in the lower panels. Perichondrium/periosteum is indicated by arrow points, zone of hypertrophy is indicated with a H and epiphysis is indicate with a E.

Many studies have demonstrated that transamidating activity of TG2 was important for its role in bone development and TG2 induced mineralisation. Blocking TG2 with general TGs inhibitors, cystamine, resulted in impaired mineralisation in MC3T3-E1 (subclone 14) pre-osteoblast culture (Al-Jallad *et al.*, 2006). Later on, the same group emphasised that transamidating activity of FXIIIa, but not TG2, was important for pre-osteoblast mineralisation (Al-Jallad *et al.*, 2009). Meanwhile, other research groups proposed

different roles that TG2 may play in mineralisation, such as via its ATPase activity (Nakano *et al.*, 2007, Nakano *et al.*, 2010) or via activating LRP/ β-catenin axis (Faverman *et al.*, 2008, Beazley *et al.*, 2012).

1.3.1 Matrix Maturation Hypothesis

TGs are well known as matrix stabilisers and there are several bone matrix proteins, for example collagen I, fibronectin, OPN and BSP that have been identified as TGs substrates (Mosher and Schad, 1979, Kaartinen *et al.*, 1997, Kaartinen *et al.*, 1999, Forsprecher *et al.*, 2011). TGs expression level, especially TG2, has been related to secretion and deposition of ECM proteins. Also, crosslinking of ECM proteins has been demonstrated to improve cell adhesion, proliferation and differentiation in many studies.

Type I collagen comprises 90% of the organic bone matrix and is one of the well studied TG substrates in mineralised tissues. Up regulation of TG2 has been related to increase of collagen I deposition in many pathologically fibrotic conditions (Jones *et al.*, 2005, Shweke *et al.*, 2008a, Telci *et al.*, 2009). On the other hand, additional FXIIIa potentially lowers collagen synthesis in a fibroblast model (Paye *et al.*, 1990, Paye *et al.*, 1989). However, the data remain controversial since lower collagen I expression was found in FXIII-deficient mice that underwent a myocardial repair process (Nahrendorf *et al.*, 2006). It has also been suggested that membrane FXIIIa is crucial for collagen deposition and its expression was regulated by extracellular type I collagen as part of the ECM-feedback loop (Al-Jallad *et al.*, 2011, Piercy-Kotb *et al.*, 2011). For extracellular collagen, polymerisation could occur via intramolecular crosslinking (Chau *et al.*, 2005) or between collagen and other non-collagenous bone matrix proteins (Mosher and Schad, 1979, Mosher, 1984, Aeschlimann *et al.*, 1995, Kaartinen *et al.*, 2005). TGs were suggested to play a role in fibre organisation via their crosslinking activity. Indeed, a well organised fibrillar collagen

network is the foundation of mineralisation and evidence has shown that TGs could promote collagen synthesis and assembly and thus promote cell differentiation (Al-Jallad *et al.*, 2006).

Fibronectin is another important ECM molecule produced by osteoblasts. It is strongly suggested that fibronectin could be involved in the early stage of osteogenesis. Weiss and Reddi (1981) showed the appearance of fibronectin and fibrillar networks of fibronectin during mesenchymal precursor cell proliferation and osteoblast maturation. The mRNA and protein levels of fibronectin were found highly increased in the early stage of bone development with a subsequent increase in type I collagen expression (Stein *et al.*, 1990, Cowles *et al.*, 1998). In fact, increased TG activity has been linked to an increase in extracellular matrix proteins under both physiological and pathological conditions. It has been demonstrated that TG2 over-expression in Swiss fibroblasts led to higher fibronectin deposition and synthesis (Telci *et al.*, 2009). Furthermore, the assembly of fibronectin was colocalised with TG2 expression and could be reduced by inhibition of transamidase activity in glioblastoma cells (Yuan *et al.*, 2006). Fibronectin and collagen I were also found colocalised with crosslinking activity on the osteoblast cell surface (Al-Jallad *et al.*, 2006). It has been demonstrated that crosslinking of fibronectin matrix could fundamentally affect collagen deposition (McDonald *et al.*, 1982, Speranza *et al.*, 1987, Dzamba *et al.*, 1993, Kadler *et al.*, 2008, Shi *et al.*, 2010). Taken together, TGs could participate in fibronectin fibril maturation and thus contribute to collagen matrix formation and osteoblast mineralisation.

In most mineralized tissues, certain non-collagenous proteins, many of which belong to the SIBLING (small Integrin binding ligand N-linked Glycoprotein) protein family, were found also structured at the macromolecular scale via crosslinking. OPN and BSP are two proteins belong to SIBLING family and their presence, both monomeric and oligomeric

forms, in bone have been demonstrated. OPN has been identified as a substrate of TG2 by Prince *et al.* (1991) where the polymerization of OPN occurred in connective tissues. TGs mediated crosslinking of OPN to fibronectin was suggested (Beninati *et al.*, 1994) and altering the conformation of OPN via crosslinking was also reported to increase its binding to collagen (Kaartinen *et al.*, 1999). Furthermore, the co-localisation of oligomerised OPN, TG2 and integrin αvβ3 on the cell surface suggested that high molecular weight OPN may function as a cell attachment molecule (Wozniak *et al.*, 2000). Although very little is known about the biological role of OPN and BSP oligomerisation, it has been shown that surface immobilised and oligomerised OPN and BSP by TGs improved osteoblast adhesion (Forsprecher *et al.*, 2011).

Overall, the putative role of TG2 in osteoblast mineralisation involves promoting matrix protein secretion, deposition and maturation via its crosslinking- mediated modification of ECM which further enhances cell attachment.

1.3.2 Putative transamidating independent pathway

Cell-ECM Adhesion and Signalling

Currently, there are several models of TG2-containing adhesive/signalling pathways which have been proposed. Among all, involvement of TG2 in RGD dependent integrin signalling is one of the well studied pathways. It was first indicated by Akimov *et al.* (2000) that TG2 non-covalently interacted with the β1 and β3 integrins on the cell surface and regulated the interaction between Fn and integrin. In this case, TG2 was proposed as an integrin-associated coreceptor to improve cell adhesion and spreading. Later on, this interaction between extracellular TG2 and integrins was implied to play a role in Fn deposition

(Akimov and Belkin, 2001a, Zemskov *et al.*, 2006), integrin clustering on cell surface (Janiak *et al.*, 2006) and cell migration (Akimov and Belkin, 2001b).

Another important TG2-containing adhesion pathway was also reported by Telci *et al.* (2008) that TG-FN binding to syndecan-4 could associate with integrin β1 signalling through PKCα and trigger an RGD-independent cell adhesion process. These complexes were also found to trigger another downstream signal effector, syndecan-2, which modulated the cytoskeletal organization through the ROCK pathway to maintain the RGD-independent adhesion of fibroblasts (Wang *et al.*, 2010) and osteoblasts (Wang *et al.*, 2011). Overall, increasing Fn interaction with cell surface integrins and syndecans by TG2 and further maturation of the cytoskeleton structure has been shown to improve fibronectin deposition as well as fibronectin fibril formation, cell differentiation and the promineralising effect of biomaterials (Geiger *et al*., 2001, García and Reyes, 2005, Chau *et al.*, 2005, Forsprecher *et al*., 2009, Vagaska *et al.*, 2010).

ATPase activity

TG2 was also proposed to act as ATPase to regulate the level of local phosphate (Pi) concentration within mineral tissue. Local phosphate (Pi)/ pyrophosphate (PPi) ratio plays an active role in regulation of physiological and pathological mineralization (reviewed by Sapir-Koren and Livshits, 2011). PPi is a common by-product of cellular metabolic reactions and is considered as an inhibitory molecule for hydroxyapatite (HA) formation. Hydrolysis of PPi into 2 Pi molecules takes place in the ECM by the tissue-nonspecific alkaline phosphatase (TNSALP) enzyme which is located on the osteoblast cell surface (Murshed *et al*., 2005, Hessle *et al.*, 2002). TNSALP promoted bone mineralization by increasing Pi level, meanwhile, maintaining proper low extracellular PPi concentrations and allowing normal bone mineralization. However, other phosphatases, including plasma

membrane Ca^{2+} transport ATPase 1 (PMCA1) (Stains *et al.*, 2002, Francis *et al.*, 2002, Nakano *et al.*, 2004) and ATPase activity of TG2 (Nakano *et al.*, 2007), might also contribute to mineralisation by locally raising Pi levels. Studies further suggested that cleavage of guinea pig TG2 by membrane type I- matrix metalloproteinase (MTI-MMP, also known as MMP-14) in vitro resulted in approximately a 3-fold increase in the ATPase activity of TG2. The cleaved 56-kDa fragment of TG2 was also demonstrated to have ATP binding ability. Nakano *et al.* (2010) proposed a hypothesis that MT1-MMP functioned as a modulator for extracellular TG2 as part of a regulatory mechanism which could activate the pro-mineralisation function of TG2.

Exogenous TG2 signalling Pathway

Studies in various cell cultures revealed that addition of exogenous TG2 promoted pre-osteoblast differentiation (Nurminskaya *et al.*, 2003), chondrocyte maturation to hypertrophy (Johnson and Terkeltaub, 2005) and calcification of vascular smooth muscle cells (VSMCs) (Faverman *et al.*, 2008). These studies demonstrated a correlation between extracellular TG2 and the mineralising process in a variety of cell lines. The following observations were also suggested by these researches:

First, exogenous TG2 promoted pre-osteoblast differentiation and matrix mineralisation in a non-crosslinking activity dependent mechanism. This hypothesis is supported by the finding that there are no changes in the pattern of protein crosslinking after treatment of pre-osteoblasts with chondrocyte-derived TG2 (Nurminskaya *et al.*, 2003). Chondrocytic cells transfected with mutant inactive TG still reserved the ability to develop hypertrophic differentiation. Meanwhile, extracellular TG2 induced hypertrophy was not affected by using GTP-bound, transamidase inactive TG2 (Johnson and Terkeltaub, 2005).

Secondly, GTP-bound TG2 acted as a molecular switch for hypertrophic differentiation and calcification of the chondrocytes in which its transamidase and GTPase activities were not required. As suggested by (Johnson and Terkeltaub, 2005), this could be due to the nucleotide-bound form of TG2 being in an ideal conformation for triggering type X collagen expression and calcification of chondrocytes in response to specific agonists. The importance of protein conformation in TG2-induced mineralisation also provides critical information that the results should be evaluated carefully when crosslinking inhibitors are used. Since transamidating activity inhibitors are reported to change the conformation of TG2 (reviewed by Pinkas *et al.*, 2007).

Finally, direct binding of TG2 to cell surface protein in pre-osteoblasts, hypertrophic chondrocyte and VSMCs without the formation of cross-links in these proteins has been demonstrated (reviewed by Nurminskaya and Kaartinen, 2006). More recently, a potential cell surface receptor superfamily was identified to interact with TG2 directly. TG2 was first found to interact with low density lipoprotein receptor related-protein (LRP) 1, one of the major endocytic receptors functioning in lipoprotein metabolism (Zemskov *et al.*, 2007). This interaction was suggested to play a role in internalisation and degradation of cell surface TG2 and further regulate cell adhesion and signalling. Later on, another member of this superfamily, LRP5, was also reported to interact with TG2 and was believed to be involved in pathological calcification of vascular smooth muscle. A clear example has been demonstrated in VSMCs that binding of exogenous TG2 to cell membrane LRP5 was revealed using immunoprecipitation (Faverman *et al.*, 2008). LRP5 has been implied in the bone formation process for more than a decade since the discovery that loss-of-function mutants in LRP5 presented a severe osteoporosis phenotype (Gong *et al.*, 2001). Conversely, studies of gain-of-function mutant in LRP5 showed a high bone mass phenotype (Boyden *et al.*, 2002, Little *et al.*, 2002a). Current researches widely agreed

that β-catenin signalling, a major component of the canonical Wnt pathway (Fig 1.12), via the cell-surface molecule LRP5/LRP6 could directly regulate osteoblast maturation or function (reviewed by Ling *et al.*, 2009). Alternatively, a parallel pathway mediated by LRP5 was demonstrated where increased gut-derived serotonin synthesis was found in LRP5-/- mice and specifically deleted LRP5 in duodenum but not osteoblasts resulted in low bone mass (Yadav *et al.*, 2008). It was proposed that the LRP5 functioned indirectly through its effects on serotonin synthesis in the duodenum and to regulate bone mass.

Even though there is a great controversy on whether crosslinking activity is essential for the exogenous TG2 induced mineralisation (Beazley *et al.*, 2012), the interaction between TG2 and cell membrane LRP5, as well as the downstream activation of β-catenin pathway might play a common role in calcified tissue.

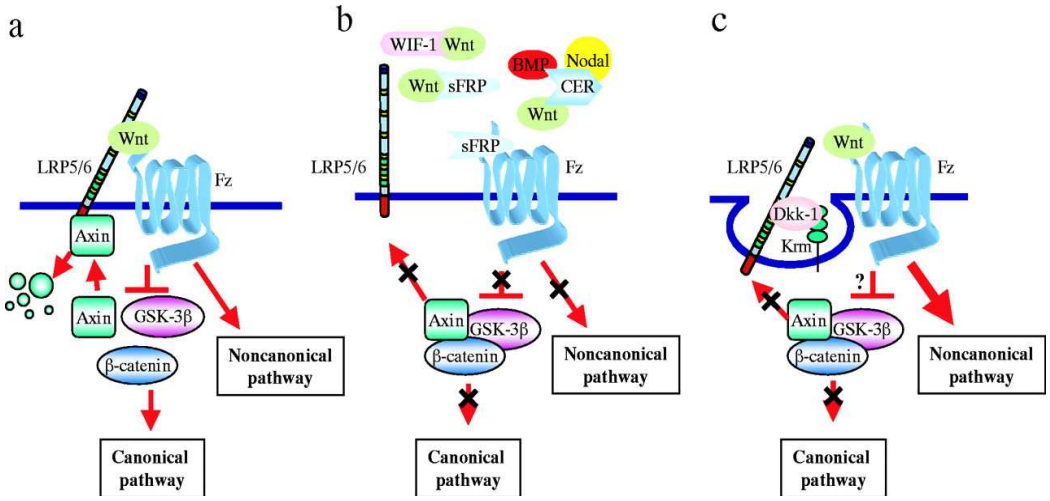

Figure 1.12 Scheme of Wnt signalling and its antagonists (adapted from Kawano and Kypta, 2003)(a) Binding of Wnt to Frizzled and coreceptors, LRP5/6, activates the canonical pathway. It involves recruitment of Axin to LRP5/6 and subsequent degradation of Axin and the disruption of the link between β-catenin and Glycogen synthase kinase 3 beta (GSK-3β). Intracellular β-catenin is stabilised due to free from phosphorylation. (b) Presence of Wnt signalling antagonists, such as secreted Frizzled-related protein (sFRP), Wnt inhibitory factor-1 (WIF-1) and Cerberus (CER), block both the canonical and the noncanonical pathways. Bone morphogenetic protein (BMP) and Nodal are two subsets of transforming growth factor-beta (TGF-β) superfamily. (c) Interaction among antagonist Dickkopf-1 (Dkk-1) and LRP5/6 and the co-receptor Kremen 1/2 (Krm, green) triggers LRP5/6 endocytosis. The canonical pathway is inactive due to absence of LRP5/6–Wnt–Frizzled complex. β-catenin is phosphorylated by GSK-3β thereby undergoes degradation. However, the noncanonical pathway remains active via interaction of Wnt with Fz without LRP5/6..

1.4 Expression of TGs in cornea tissue and ocular diseases

Abundant TGs transamidase activity was first demonstrated by Raghunath *et al.* (1999) that they found TGs activity in all ocular tissues especially in ciliary body, zonular fibers, and blood vessel walls. They also suggested TG2 as the predominant crosslinking enzyme in ocular tissue in comparison with TG1, TG3 and FXIIIa. A later study by Barathi *et al.* (2011) clearly demonstrated that TG1 expression (Fig.1.13) had a remarkable preponderance for the epithelium while TG2 expression was barely found in epithelium but mainly in the corneal subepithelium (CS) and stroma (S). On the other hand, TG3 and TG5 were localised in the entire corneal epithelial, stromal and endothelial layers. Although literatures have shown the involvement of TG in ocular wound healing process and many numerous ocular diseases, such as pterygium (Kim *et al.*, 1998), dry eye (Toshino *et al.*, 2005), cicatricial conjunctivitis (Nakamura *et al.*, 2001)and glaucoma (Priglinger *et al.*, 2006), only a few molecular pathways have been studied. Therefore, the elucidation of molecular mechanisms underlying TGs-related ocular disease is needed.

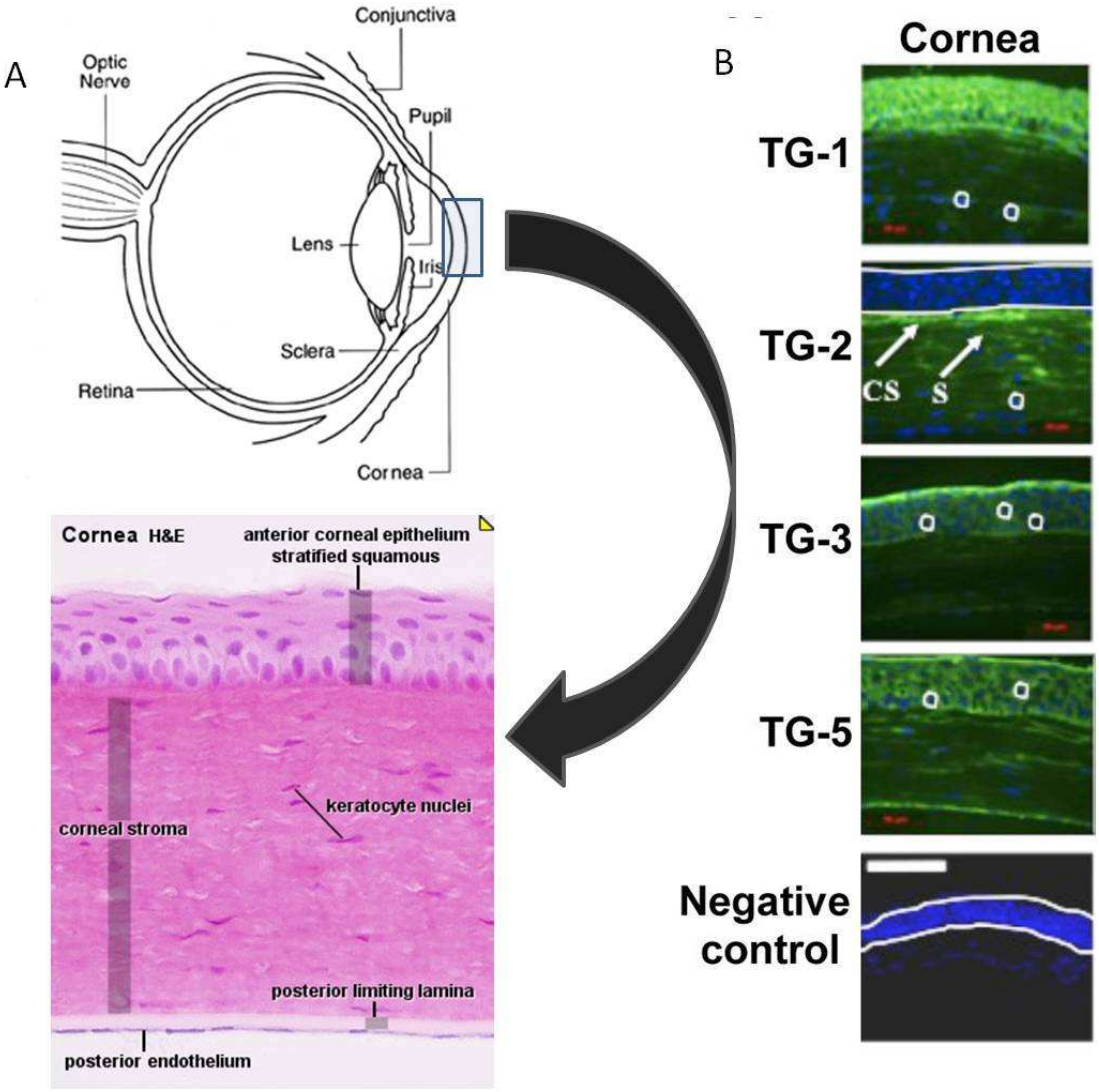

Figure 1.13 (A) Structure of eye and cornea. Picture source: http://www.lab.anhb.uwa.edu.au-/mb140/corepages/eye/eye.htm (B) Localization of transglutaminases (TGs) in mouse eye as demonstrated by immunohistochemistry (adapted from Barathi *et al.*, 2011). The nuclear DAPI staining is shown in blue and the TGs staining is shown in green. The epithelial layer is highlighted with white line and corneal subepithelium and stroma are indicated with CS and S respectively.

2. Biomaterial for Tissue Repair

Since TG2 is widely expressed in a range of tissues and it is involved in several tissue developmental and wound healing processes, it is reasonable to assume that a TG2-based biomaterial would benefit from the different properties of TG2. Here, the potential and advantage of using TG2 crosslinked biomaterial for soft and hard tissue repair are summarised.

2.1 Tissue engineering

The term "tissue engineering" is thought to have been first coined in 1988 at a National Science Foundation (NSF) workshop by Robert Nerem. The concept was further extended and formalized in a review paper in *Science* (Langer and Vacanti, 1993) in which the authors gave a modern definition: "*Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain or improve tissue function*." This concept introduces a great promise and potential for producing engineered replacements as alternative therapies for human disease and injury. Yet, decades of research since the field of tissue engineering was formalized, few successful approaches have been made as determined by current engineered constructs approved for clinical use.

The main progress in this area is first understanding the cell-cell interaction, then selecting appropriate matrices based on cell-matrix interaction and finally supplying with extra biochemical signalling (growth factors). Therefore, cell sources, biomaterials and growth factors are three fundamental elements in tissue engineering. In addition, the importance of vascularisation of engineered tissues has drawn more attention recently especially in reconstructing bioengineered tissue on a larger and more complicated scale (Naderi *et al.*,

2011). Following this classical approach, a certain level of achievement has been made in basic research and in the clinic including skin, cartilage, bone, muscle, heart valves, blood vessels, esophagus, connective tissue, pancreas, liver, peripheral nerves and tracheal constructs (reviewed in Horch, 2006).

Bioengineered bone substitutes are considered as one of the most studied and successful subjects in tissue engineering. Currently, there is high demand for bone substitutes to repair and replace damaged and diseased tissue due to trauma, degenerative disease and cancer. Also, due to the increasing aging population, substitutes to replace, restore, or regenerate bone have become a major clinical need in the fields of orthopaedic, spinal, dental, cranial, and maxillofacial surgery. Even though bone implements are only second to transfused blood products as the most implanted materials, the availability and quality of bone substitutes still fall short of the current requirement. Therefore, developing appropriate bone constructs for clinical use is important. On the other hand, due to regeneration of complex tissues or organs, such as heart, muscle, kidney, liver, and lung, are still a distant milestone in tissue engineering, developing comparably “simple” tissue, such as cornea, is a more promising short term aim (Ruberti and Zieske, 2008). Cornea is a thin and avascular tissue but with a highly organised matrix layer. Again, the quality and quantity of artificial cornea graft is far behind the clinic demand (Aiken-O'Neill and Mannis, 2002, Thompson Jr *et al.*, 2003). Thus, it is beside the academic challenge but also clinical demand in developing a suitable replacement for cornea graft.

In this thesis, evaluating TG2 crosslinked type I collagen as a biomaterial for both bone and cornea reconstruction is one of the aims. The background and detail of the bone and cornea regeneration will be summarised respectively in following sections.

2.2 Bone tissue engineering

According to statistics, the annual medication cost for fractures is $10 billion with over 1.2 million cases in USA and 5% of overall cases showed delayed healing or non-union after surgery. In fact, there are around 4 million operations involving bone grafting or bone substitutes performed around the world each year (reviewed in Brydone *et al.*, 2010). A growing number of joint replacements due to aging societies also highlights the requirement of bioengineered bone tissue. During 2011/2012, there were about 180,000 cases of primary hip and knee replacement procedures entered into the National Joint Registry (NJR) in the UK with around 8.7% of primary joint replacements undergoing revision surgery (NJR, 2011). Therefore, the clinical need for continued advances in bone engineering is urgent. The following sections aim to summarise the bone biology, current options available for bone grafting and recent developments in cell engineering.

2.2.1 Bone biology

Bones are rigid tissues which provide mechanical support for anchoring muscles, facilitating movement and protecting organs. The detail of the bone structure is illustrated in Fig. 1.14. According to the morphology, there are two forms of bone which are cortical (compact) bone and trabecular (cancellous or spongy) bone. The cortical bone is a condensed layer, consisting of densely packed collagen fibrils in concentric lamellae, of all bone tissues with low porosity (5-30%). It accounts for the mechanical property of the skeleton and contributes to 80% of the total bone mass of an adult. The trabecular bone is composed of a porous latticework matrix and mainly functions as a reserve of minerals in body (Standring and Gray, 2008). Bone tissue contains several types of bone cells and bone extracellular matrix. There are four different cell types in bone: osteoblasts, bone lining cells, osteocytes and osteoclasts

- *Osteoblasts/ bone lining cells/ osteocytes*

Osteoblasts arise from osteoprogenitor cells which synthesise new bone matrix composing mainly type I collagen. Flattened bone lining cells are thought to be quiescent osteoblasts which line nonremodeling endosteal bone surfaces. It has been suggested that bone lining cells may regulate bone remodelling signals and they are important in the maintenance of mineral homeostasis (Miller *et al.*, 1989). Osteocytes are star shaped cells that originate from osteoblasts trapped inside the matrix they secret. Osteocytes reside inside lacunae and network to each other, in a similar way to the nervous system, via long cytoplasmic extensions in tiny canals called canaliculi. Although osteocytes have lower synthetic activity, research has shown that they are actively involved in turnover of bony matrix through various mechanosensory mechanisms.

Mesenchymal stem cells (MSCs) are capable of differentiating into lineages of mesenchymal tissue including bone, cartilage, muscle, fat and fibrous connective tissue (Pittenger *et al.*, 1999) and they have been isolated from adult peripheral blood, tooth pulp and bone marrow. The canonical Wnt/β-catenin pathway is considered as one of the master modulators in differentiation of MSCs to osteoprogenitor cells (Logan and Nusse, 2004). In fact, high and low bone mass phenotypes have been associated with activation of LRP5, a co-receptor of Wnt protein receptor (Gong *et al.*, 2001, Boyden *et al.*, 2002, Little *et al.*, 2002a, Little *et al.*, 2002b). Furthermore, multipotential myogenic cells give rise to osteoprogenitor by dedifferentiation under the right circumstances (Doherty *et al.*, 1998). In this case, blood vessel pericytes may undergo dedifferentiation and develop other phenotypes such as osteoblasts, chondrocytes, adipocytes, and fibroblasts.

Figure 1.14 The histology of bone. (A) A human femur (Resource: *The Internet Encyclopedia of Science* http://www.daviddarling.info/encyclopedia/ETEmain.html) (B) Structure of the diaphysis in long bone (C) Enlarged aspect of Haversian systems in compact bone (D) Detail of spongy bone trabculae (Resource: Rutgers University online lecture http://www.rci.rutgers.edu/~uzwiak/AnatPhys/APFallLect8.html)

Osteoblast precursors go through morphology change, from spindle-like to large cuboidal shape, when differentiated into mature osteoblasts. The mature, functioning osteoblasts are usually surrounding by alkaline phosphatase (ALP) positive osteoprogenitor cells in the bone remodelling nodules. Stein *et al.* (1990) proposed that there are three major stages of osteoblast differentiation/ mineralisation which can be summarised in Figure 1.15.

Highly proliferating osteoblastic cells produce abundant ECM protein including fibronectin and type I collagen at the early stage of differentiation. The proliferation rate declines when the early mineralisation marker, ALP, appears and it accompanies the secretion of osteopontin and osteonectin. An increase in osteocalcin expression and mineral deposition, composed of calcium and phosphate, can be observed at the onset of mineralisation while the ALP expression starts to decline.

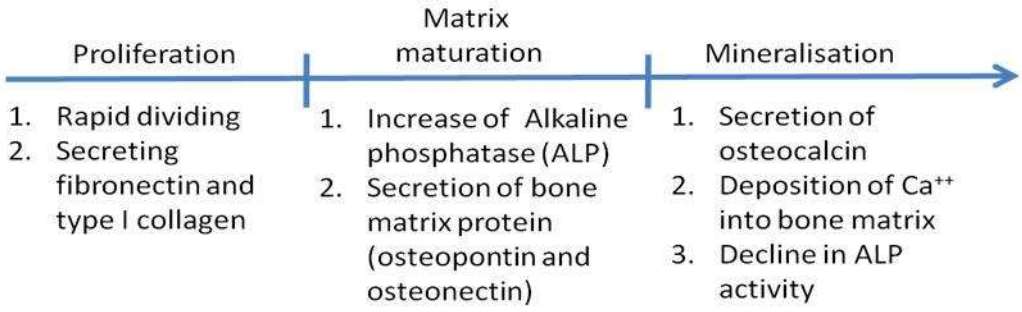

Figure 1.15 Three principle stages of osteoblast differentiation- a) proliferation, b) matrix maturation and c) mineralisation

- *Osteoclasts*

It is generally believed that osteoclasts are derived from mononuclear precursor cells of the monocyte-macrophage lineage in bone marrow (Nijweide *et al.*, 1986). Two cytokines, Receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), have been identified as key regulators in osteoclast formation. Both stromal cells and osteoblasts are required in osteoclastogenesis and this process is

regulated by membrane anchored and soluble RANKL and M-SCF which are secreted by stromal cells and osteoblasts (Teitelbaum and Ross, 2003). RANKL signals through Receptor activator of nuclear factor κB (RANK) on osteoclast precursor cells and mediates osteoclast differentiation, activation and survival during normal bone modelling and remodelling processes and in a variety of pathologic conditions characterized by increased bone turnover. This signalling can be antagonised by osteoprotegerin (OPG) which binds to RANKL and prevents binding of RANKL to RANK (Boyce and Xing, 2007). M-CSF promotes survival, proliferation and differentiation of osteoclast precursors and regulates the cytoskeleton of cells upon bone resorption.

Osteoclasts are the main cells known to resorb bone mineral during bone remodelling. The interaction between osteoclast and bone matrix relies on cell membrane integrins recognising bone matrix peptides. The β1 integrin is the major anchored integrin expressed in osteoclasts which can bind to collagen, fibronectin and laminin. On the other hand, matrix degradation is facilitated by an integrin αvβ3-mediated event that results in polarisation of the cell and secretion of resorptive molecules such as hydrochloric acid and acidic proteases (Ross and Teitelbaum, 2005).

2.2.2 Bone development and healing

Condensation is the initial phase in the development of mesenchymal tissues in which a previously dispersed population of cells gathers together to differentiate into a single cell/tissue type such as cartilage, bone, muscle, tendon, kidney, and lung (Hall and Miyake, 2000). In skeleton development, two ossification mechanisms, endochondral ossification and intramembranous ossification, occur after the process of MSCs condensation. Most of the skeleton, including all long bones, are formed via endochondral ossification while

intramembranous ossification occurs primarily in the development of flat bones including the skull, scapula and mandible.

As illustrated in Fig. 1.16A, endochondral ossification is a process including formation of a cartilage scaffold by direct differentiation of the precursor cells to pre-chondrocytes and chondrocytes and followed by its replacement by mineralised bone matrix. In long bone, the primary ossification usually begins at the centre of this model where chondrocytes stop proliferating, become hypertrophic, mineralise the matrix and secrete vascular endothelial growth factor (VEGF) to attract migration of chondroclasts and promote vascular invasion. Hypertrophic chondrocytes direct adjacent perichondrial cells to become osteoblasts, which secrete collagen I-rich matrix resulting in the formation of a bone collar. Hypertrophic chondrocytes then undergo apoptosis and the cartilage matrix left behind provides a scaffold for osteoblast mineralisation. Secondary ossification centres are established in epiphysis where again, chondrocytes stop proliferating, become hypertrophic and signal the influx of blood vessels and osteoblasts. The lengthening of long bone is facilitated by zones of proliferating chondrocytes (known as the growth-plate) in between primary and secondary ossification centres (reviewed in Kronenberg, 2003).

The development of flat bone and bone widening of long bones occur primarily via intramembranous ossification. This process involves the direct differentiation of mesenchymal cells into pre-osteoblasts and osteoblasts, yet is not well characterized as endochondral ossification. Studies indicate the difference in the composition and structure of the bone matrix formed via endochondral and intramembranous ossification (Scott and Hightower, 1991), however, several common molecular regulators of these processes have been identified by recent study (Abzhanov *et al.*, 2007).

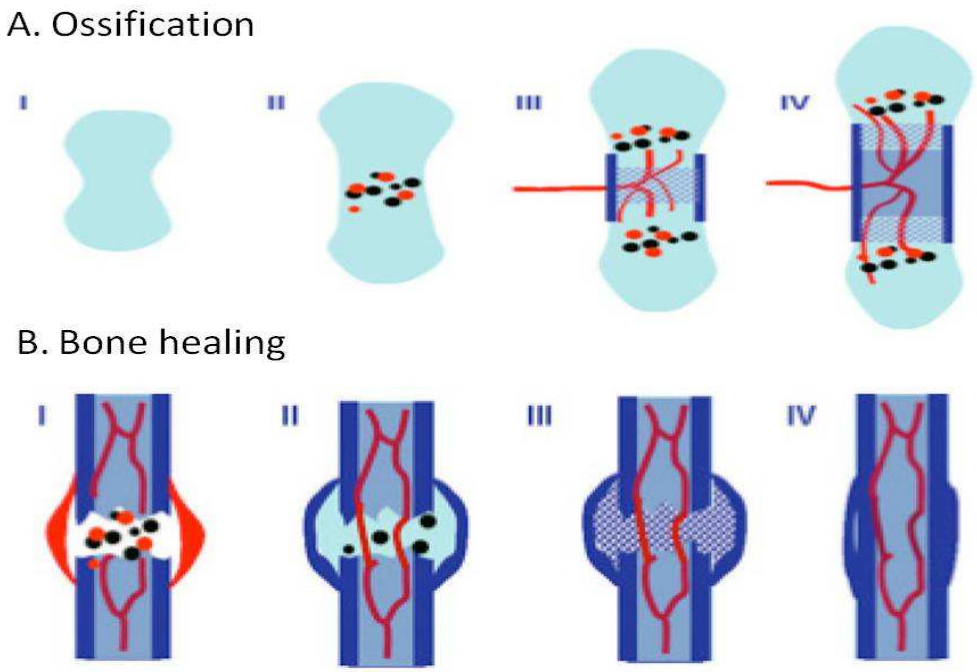

Figure 1.16 Scheme of endochondral ossification and bone healing process (adapted from Frohlich et al., 2008). (A) endochondral ossification process (I) a cartilage mould produced by chondrocytes (II) hypertrophic chondrocytes express both angiogenic (red circles) and osteogenic (black circles) growth factors (III) Vascular invasion and formation of a bone collar (dark blue rectangle) and cartilage is replaced with trabecular bone (IV) lengthening of bone. (B) Bone healing process (I) hematoma formation, (II) fibrocartilaginous callus formation (III) bony callus formation, and (IV) remodelling of the bony callus.

When a bone fracture happens, the bone healing process involves four major stages: hematoma formation, fibrocartilaginous callus formation, bony callus formation, and remodelling of the bony callus (Fig 1.16B). First, formation of a haematoma due to the damage of blood vessels initiates an inflammatory response. This inflammation accompanies the release of signalling molecules involved in new bone formation such as fibroblast growth factors (FGFs), BMPs, platelet-derived growth-factor (PDGF) and VEGF. Intramembranous and endochondral ossification both mediate the bone healing process where bone formation occurs immediately at the cortex and periosteum via intramembranous ossification and the internal (chondrified) callus is formed as an initial platform for endochondral ossification. The internal callus further ossifies via endochondral ossification and toward the end of ossification, the chondroclasts resorb the spongy bone and mechanical continuity is established via remodelling of cortical bone (reviewed in Dimitriou *et al.*, 2005).

2.2.3 Bone matrix protein and bone mineral

Bone is composed of 50-70% mineral, 20-40% bone matrix proteins, 5-10% water and less than 3% lipids. The major bone matrix proteins are collagenous matrix proteins in which type I collagen is most abundant with trace amounts of type III, type IV and fibril-associated collagen with interrupted triple helices (FACIT) collagen. With 85-90% of the bone matrix proteins composed of collagen, there are another 10% noncollagenous matrix proteins (NCPs) and some of them are found exclusively in bone tissue and cells. The list of identified bone matrix proteins and their putative biological functions are summarised in table 1.4.

Type I collagen is the major collagenous protein in bone. The collagen types I, III and V are known as the fibrillar collagens which are abundant and able to form fibres in the ECM. Collagen is composed of a triple helix, which generally consists of two identical α1 chain and α2 chain with slight differences in their chemical composition. Ascorbic acid is required for normal collagen I synthesis because it is a cofactor for lysyl hydroxylase in the synthesis of hydroxyproline and hydroxylysine in collagen. Hydroxyproline stabilises the collagen triple helix and its absence results in an unstable collagen structure which affects synthesis of collagen (Jeffrey and Martin, 1966). FACIT collagens are a group of nonfibrillar collagens which are important for the organisation and stabilisation of ECM by serving as a molecular bridge between fibrils or fibrils and other proteins

Table 1.4 List and function of bone matrix proteins

Protein (Chromosome Location)	Characteristics and Function
Collagen-related proteins	
type I (17q21.23, 7q22.1)	Most abundant bone matrix protein
type X (6q21)	Found in hypertrophic cartilage
type III (2q31)	Trace amounts in bone; may regulate collagen fibril diameter
type V (9q34.2-34.3; 2q24.3-31; 19q13.2)	Trace amounts in bone; may regulate collagen fibril diameter
Serum proteins in bone matrix	
albumin (4q11-13)	Decreases hydroxyapatite crystal growth
a-HS glycoprotein (3q27)	Bovine analog is fetuin
Glycoaminoglycan-containing proteins and leucine-rich repeat proteins	
aggrecan (15q26.1)	Matrix organization, retention of calcium/ phosphorus
versican (5q14.3)	Defines space destined to become bone
decorin (12q21.3)	Regulates collagen fibril diameter; binds TGF-β
biglycan (Xq28)	Binds collagen; binds TGF-β
hyaluronan (multigene complex)	Associates with versican
Glycoproteins	
alkaline phosphatase (1p36.1-p34)	Hydrolyzes mineral deposition inhibitors
osteonectin (5q31.3-32)	Regulates collagen fibril diameter
SIBLING proteins	
osteopontin (4q21)	Inhibits mineralization and remodelling
bone sialoprotein (4q21)	Initiates mineralization
MEPE (4q21.1)	Regulator of phosphate metabolism
RGD-containing glycoproteins	
thrombospondins (15q15, 6q27, 1q21, 5q13, 19p13.1)	Cell attachment
fibronectin (2q34)	Cell attachment
vitronectin (17q11) fibrillin 1 and 2 (15q21.1, 5q23-31)	Cell attachment Regulates elastic fibre formation
γ-Carboxy glutamic acid–containing Proteins	
matrix Gla protein (12p13.1-p12.3)	Inhibits mineralization
osteocalcin (1q25-q31)	Regulates osteoclasts; inhibits mineralisation
protein S (3p11.2)	Unknown

Osteoblasts synthesise several noncollagenous proteins which can be divided into different categories including proteoglygans, glycoproteins, glycosylated proteins with potential cell-attachment activities, and γ-carboxylated (Gla) proteins. Osteocalcin (OCN) is one of the noncollagenous Gla proteins secreted by osteoblasts. Although recent studies suggested that OCN could mainly play a role in regulating energy metabolism (Lee and Karsenty, 2008, Crockett *et al.*, 2011), carboxylation of OCN confers high affinity for minerals. Thus, it was first assumed that osteocalcin, like other Gla proteins, is directly involved in the mineralisation process (Lian *et al.*, 1989, Hauschka *et al.*, 1989, Price, 1989). Yet, a high bone mass phenotype observed in osteocalcin knockout mice suggested that OCN inhibited bone formation (Ducy *et al.*, 1996).

Alkaline phosphatase (ALP) is the main glycoprotein in bone and presents in two forms, anchored form and released form. Anchored ALP is bound to the osteoblast cell surface via a phosphoinositol linkage or within mineralised matrix and the released form is in serum (Raymond *et al.*, 1993). The role of ALP in mineralisation is not yet defined. However, the deficiency of ALP is related to hypophosphatasia, a hypomineralisation phenotype. It is generally believed that ALP is involved in hydrolysis of extracellular pyrophosphate (PPi), a mineralisation-inhibiting byproduct of nucleotide pyrophosphatase (NPP), and maintains the balance of phosphate (Pi)/PPi (Orimo, 2010).

The mineral content of bone is mostly hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) with trace amounts of carbonate, magnesium, and acid phosphate. Bone hydroxyapatite crystals are approximately 200 Å in dimension and are more soluble than geologic hydroxyapatite crystals, thereby allowing them to respond to mineral metabolism. The mineralisation process is associated with the expression of ALP and many other noncollagenous proteins and is facilitated by extracellular matrix vesicles (MVs) which are secreted by osteoblasts and chondrocytes. The initial HA precipitation happens within MVs which contain a

nucleation core. The mineralisation starts with recruiting Ca^{2+} to MVs by calcium-binding molecules, including calcium-binding acidic phospholipids and calcium binding proteins. Meanwhile, levels of local intra- and perivesicular Pi are increased by the enzymatic activity of ALP and nucleoside triphosphate pyrophosphohydrolase that are enriched on the MV membrane. The release of HA crystals from MVs into the extravesicular fluid is facilitated by phospholipases and proteases within MVs and further propagation of mineral into the matrix requires enzymatic activity of MMPs in MVs, which are capable of degrading mineral-inhibiting proteoglycans (reviewed in Anderson, 2003). Interestingly, TG2 is found in MVs secreted by chondrocytes (Aeschlimann *et al.*, 1993, Rosenthal *et al.*, 1997, Rosenthal *et al.*, 2001), in microparticles (MPs) secreted by smooth muscle cells (van den Akker *et al.*, 2012) and its transamidating activity is also found in MVs secreted by pre osteoblasts (Johnson *et al.*, 2000). Although the exactl role of TG2 in these vesicles is unclear, it is suggested that the release of TG2-enriched vesicles could be the unconventional secretion pathway of TG2 or be directly involved in the mineralisation process via a transamidating-dependent or independent pathway.

2.2.4 Biomaterial for bone repair and regeneration

Current options for bone grafting including autografts, allografts and synthesised bone graft substitutes. These options are different in their strength and osteoconductive, osteoinductive, and osteogenic activity which are summarised in table 1.5. In the clinic, autografts are considered as ideal since they incorporate the patient's own osteogenic cells and an osteoconductive mineral matrix. However, the limited amount and the prolonged pain at the harvest site are the major disadvantages of using autografts. For allografts, the decellularisation of tissue is needed to avoid host-versus-graft immune response. In this case, the allograft lacks the osteogenic properties of an autograft.

Table 1.5 Bone graft activity by type (modified from a table by Brydone et al., 2010)

Bone graft	Osteo-conduction	Osteo-induction	Osteogenic	Mechanical properties
Autograft				
Cancellous	++	+	++	+
Cortical	+	+/-	+	++
Allograft				
Cancellous	++	+	-	+
Cortical	+/-	+/-	-	-
Demineralised bone matrix	++	+++	-	++
Bioactive composites / growth factor	+	+	-	+

Traditionally, metals such as stainless steel 316L, cobalt chromium alloy, titanium, or titanium alloy (Ti-6%Al-4%V) are used in orthopaedic and dental implants. Although the high stiffness of the metal material is appreciated, it results in a significant change in the mechanics of the implicated bone and subsequent bone remodelling may decrease the strength of surrounding normal bone tissue. Current strategy of bone graft design is using synthesized or nature structural proteins to recreate the structure and chemical composition of natural bone. Common materials used in bone graft are HA, collagen, calcium sulphate, silicate-substituted calcium phosphate (Si-CaP) and polyethylene (PE) matrix modified with HA. Addition of osteoinductive growth factors is also found to increase osteoinductive property of biomaterial. Two osteoinductive growth factors of the TGF-beta superfamily, rhBMP-7 (OP-1 Implant® /OP-1Putty®, Stryker-Biotec, Hopkington, MA, USA) and rhBMP-2 (INFUSEH/InductOs®, Wyeth, UK), are currently available on the market. Although comparable healing results of OP-1/BMP7 and rhBMP2 with autografts are reported in clinic, in the USA, the Food and Drug Administration (FDA) advisory committee still holds concern of side effects, such as undesired ectopic bone formation, which may result in critical complications particularly in cervical spine surgery. Besides, the high cost (BMP-2 is £3,200 and BMP-7 is £2,450 for 100 μg) and short shelf life are two

major disadvantages of using these products. Therefore, a new generation of bone graft is required for clinical use.

2.2.5 TG2 crosslinked collagen gel for bone healing

Collagen based scaffolds are widely used in a broad range of regenerative medicine application. Using collagen matrix for orthopaedic regeneration offers distinct advantages since type I collagen composes more than 90% of the organic fraction in native bone tissue. Even though collagen scaffold has shown clinical success in a range of soft tissue repair applications, relatively poor mechanical properties remain a limitation for their use in hard tissue regeneration. In fact, only selected researches have showed some degree of success in using collagen scaffolds for bone regeneration (Caiazza *et al.*, 2000, d'Aquino *et al.*, 2009, Keogh *et al.*, 2010) compared to other synthetic materials. The mechanical property problem can be overcome by the addition of a second, stiffer phase such as hydroxylapatite (HA) or crosslinking collagen-based matrices with glutaraldehyde (Weadock *et al.*, 1983), ultraviolet (UV) radiation (Wollensak and Spoerl, 2004), dehydrothermal processing (Yannas and Burke, 1980), carbodiimides (Damink *et al.*, 1996) and enzymatically crosslinking by microbial transglutaminase (mTG) or TG2 methods (Chen *et al.*, 2005, Chau *et al.*, 2005). All of these methods have shown a certain level of success in modifying the degradation rate of collagen matrix, thus, added collagen biomaterial advantages in wider medical applications.

2.3 Cornea tissue engineering

According to WHO global statistics in June 2012, there are about 314 million patients suffering from impaired vision including 45 million who are bilaterally blind. However, about 85% of all impaired vision and 70% of blindness could be avoidable and cured. The epidemiology of blindness shows that corneal damage remains one of the leading causes of blindness (Resnikoff *et al.*, 2004), causing around 2 million new cases of bilateral blindness worldwide in 2002. This number, however, did not include millions of people suffering from impaired and low visual acuity due to corneal opacity. Early research by Negrel and Thylefors in 1998 drew attention to the global prevalence of ocular trauma and ulceration with an estimated 55 million new cases of ocular injuries occurring each year. Of these, 1.6 million people were blind, 2.3 million people had bilaterally impaired vision and another 19 million people were unilaterally blind or had low vision after their injuries.

Corneal abrasion, foreign bodies striking into eyes, chemical burns and corneal ulceration are common eye traumas seen in the clinic. Usually, following injury, cytokines and growth factors secreted by the immune cells will mediate interaction between the healthy tissue and immune cells in order to restore corneal structure and function. Superficial injury usually can heal rapidly within a week. However, severe trauma usually involves stroma damage and loss. In this case, appropriate treatment is needed or the cornea may fail to restore its original structure and function. Consequently, patients may suffer from impaired vision or even blindness.

Ocular trauma is a global problem. However, the prevention of post-wounding blindness can be achieved by better medical treatment. A corneal graft may be highly effective but limited due to the availability of the donor, as well as the prognosis of corneal transplantation is comparably poor in emergency cases. Tissue adhesives are currently

used in ophthalmic surgery even though most of them are used off-label. They can immediately fill the wound area and replace the damaged stroma matrix which is suitable for emergency use.

2.3.1 Cornea structure

The cornea is a transparent tissue which is about 0.5 to 0.6mm thick in the centre and 0.6 to 0.8mm at the periphery. It is composed of five major layers (Fig. 1.17): epithelial layer, Bowman's layer, stromal layer, Descemet's membrane and endothelial layer. Epithelial cells, the main cells in epithelium, form a 5-6 cell layered, stratified tissue to protect the corneal interior from noxious environmental agents. The basal layer is a monolayer of columnar cells. Above the basal layer are another two layers of wing cells and superficial squamous cells with flattened nuclei. The epithelium comprises 10% of the total depth of the cornea and is regenerated by multiplication in the basal layer. Underneath the epithelial layer is a basement membrane where the basal epithelial cells adhere via desmosomes. The components of epithelial basement membrane (EBM) are mainly type IV collagen, type VII collagen, laminin-1, laminin-5, perlecan, fibronectin and entactin/nidogen (Ljubimov *et al.*, 1995).

Bowman's membrane, an acellular layer, consists of irregularly-arranged collagen fibrils. The function of Bowman's membrane is obscure. Early research implied that the strong mechanical strength of Bowman's layer helps to maintain the shape of the cornea. A more recent hypothesis suggested that cytokine-mediated interaction between corneal epithelial cells and stromal keratocytes results in the formation of Bowman's membrane which may involve chemotactic and apoptotic effects on the keratocytes (Wilson and Hong, 2000).

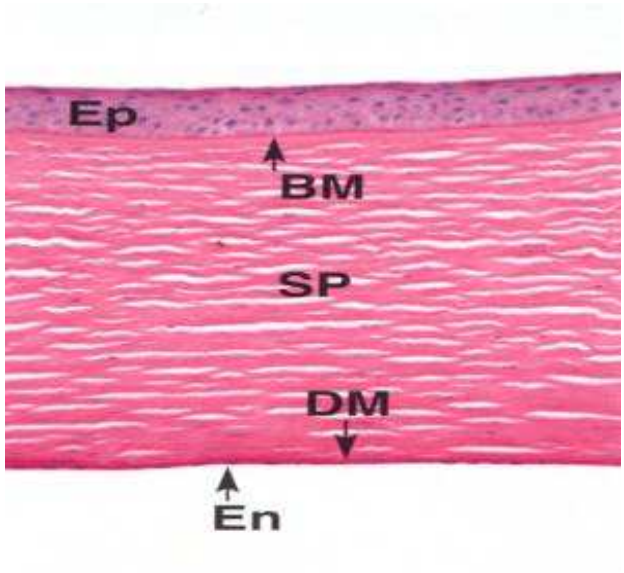

Figure 1.17. Cornea Histology (H&E staining). Ep = epithelium, BM = Bowman's membrane, SP = Substantia Propria or Stroma, DM = Descemet's membrane and En = endothelium. Adapted from *Wheater's Functional Histology, a text and colour atlas*, p. 392, Figure 21.15.

The corneal stroma, which contributes 90% of the total corneal thickness, is composed of regularly-arranged collagen fibrils which are produced by keratocytes. The transparency of cornea depends on the corneal stromal ultrastructure where collagen fibres (corneal lamellae) are obliquely oriented from one layer to another with 49% of lamellae aligned orthogonally (Daxer and Fratzl, 1997). Keratocytes play a critical role in maintaining corneal clarity by synthesizing and organizing stromal constituents. The average density of keratocytes in the central cornea is around 20,500 cells/mm^3 in which most of the cells are populated in the anterior 10% of the stroma (Patel *et al.*, 2001). Normally, the keratocytes are quiescent in healthy cornea. Once injured, keratocytes will transform into repairing phenotypes, fibroblasts and myofibroblasts, to restore the damaged ECM (West-Mays and Dwivedi, 2006).

Descemet's membrane lies between the stromal layer and endothelium and serves as the basement membrane of endothelial cells. Heterogeneity is found vertically across the Descemet's membrane such that type IV collagen alpha 1 and alpha 2 chains and fibronectin are expressed on the stromal face, while alpha 3(IV)-alpha 5(IV) chains, entactin/nidogen, laminin-1, and perlecan appear on the endothelial face (Ljubimov *et al.*,

1995). The bottom layer of cornea is endothelium, a single sheet of squamous cell boundary between the posterior cornea and the anterior aqueous chamber. The main function of the endothelial cells is regulating the hydration of stroma by pumping the fluid in and out of the corneal stromal compartments.

2.3.2 Current treatment for corneal wound

The prevention of post-wounding blindness can be achieved by better medical treatment. A donor corneal graft may be highly effective but limited due to the availability of the donor. Even though with the availability of tissue, donor grafts are typically variable in quality from donor to donor and usually with a chance of immunological rejection or endothelial decompensation resulting in an 18% failure in corneal transplantation (Thompson Jr *et al.*, 2003). Also, the prognosis of corneal transplantation is comparably poor in emergency cases.

Another option of using amniotic membrane (AM) in ophthalmic surgery was proposed by de RÖ (1940) and Sorsby and Symons (1946). In the past few decades, great success has been achieved in the use of AM for repairing severe corneal defects (Kim and Tseng, 1995), and researches reported several advantages of using AM, including reduction of scar formation and inflammatory response in the damaged area (Solomon *et al.*, 2001), enhancement of the wound healing process and re-epithelialisation (reviewed by Choi *et al.*, 2009), as well as anti-microbial and anti-viral properties (Inge *et al.*, 1991). A broad range of applications for its use in ocular surgery have expanded in the past decades, which includes persistent corneal epithelial defects, neurotrophic corneal ulcers, corneal perforations, shield ulcers, infectious keratitis, bullous keratopathy, band keratopathy and chemical injury. A more recent approach also demonstrated the potential of using AM as a carrier for ocular surface epithelial stem cell transplants, corneal endothelial cell and

retinal pigment epithelial substrate (reviewed by Riau *et al.*, 2010). Despite the great benefit brought by using AM, the process of retrieving AM from donor and de-epithelialisation of AM is complicated. There is also possibility of disease transmission which requires pre-screening of the potential donor. Additionally, the quality of AM could be different from donor to donor and subsequent need for storage of AM product is expensive. Furthermore, the clinical indications of AM grafts or patches are generally limited to severe cases of ocular disease/ trauma due to the invasive surgical procedure that is required for this treatment. There is also a great chance of developing a variety of suture-related complications in this procedure (Park and Tseng, 2000).

Alternatively, tissue adhesives are suitable for emergency use in ophthalmic surgery. Current products on the market can be divided into 2 classes, synthetic (e.g., cyanoacrylate and acrylic-based polymers), and biological (e.g., fibrin glue, biodendrimers and riboflavin–fibrinogen compounds) products. The application of tissue adhesive in ophthalmology provides a suture-free option which decreases postoperative discomfort, shortens the healing time, lowers the risk of infection as well as reduces surgical time and scarring. Nevertheless delayed re-epithelialisation, toxic metabolites, possible viral transmission and scar tissue formation are the main disadvantages of these products (Lagoutte *et al.*, 1989, Chan and Boisjoly, 2004).

2.3.3 Biomaterial for corneal wound closure and graft

The properties of an ideal corneal graft include:

- Biodegradable
- Enhance the healing process
- Elastic mechanical properties
- Persist long enough to allow the cornea to regenerate
- Transparency
- Safe to use
- Reasonable price

Biocompatible collagen glue is a prospective solution for treatment of ocular trauma. Ideally, the biodegradable and non-toxic collagen glue can be applied to the wound area and allow cells to infiltrate. Using collagen matrix can avoid the inconsistency of product quality as seen in using AM membrane. Furthermore, by crosslinking collagen with TG2, it is theoretically possible to improve the matrix stability and cell adhesion. Finally, crosslinked collagen gel may be provided to market at a reasonable price due the manufacture process of crosslinked gel being easier and without the need to screen for transmissible diseases.

3. Objectives

The main objectives of this thesis are to examine the expression and possible mechanism of action of TG2 in human osteoblast (HOB) mineralisation, as well as to produce TG2 crosslinked biomaterials with enhanced cell inductive and cell conductive properties for soft and hard tissue repair.

The first approach is to analysis the TG2 expression and extracellular crosslinking activity during the HOB cell mineralisation process. TG2 knock down HOB cell lines will be generated in order to study the relationship of TG2 expression and mineralisation. Furthermore, in order to evaluate the relationship between transamidating activity of endogenous TG2 and cell mineralisation, TG2 crosslinking inhibitors will be used to block TG2 activity. On the other hand, the pro-mineralisation effect of exogenous TG2 will be also examined to determine whether this effect is crosslinking activity dependent or independent and the possible mechanism. Following most exogenous TG2-induced calcification models (Johnson *et al.*, 2008, Faverman *et al.*, 2008), guinea pig liver transglutaminase (gpTG2) will be used to induce HOB mineralisation.

Finally, the advantage of using TG2 crosslinked biomatrix in soft and hard tissue repair will be evaluated. Type I collagen extracted from rat tail and commercial gpTG2 will be used to generate TG2 crosslinked collagen matrix which has been demonstrated in previous work done by Chau *et al.* (2005). HOBs will be used to represent the hard tissue model and human corneal fibroblasts (hCFs) and human corneal epithelial cells (hCECs) will be used as target cells to represent the soft tissue model.

Chapter II

Materials and Methods

1. Materials

1.1. *General Chemicals*

All water used was distilled using a Barnstead Easypure RoDi ultrapure water purification system purchased from Thermo Scientific, Surrey, UK. Sterilisation of stock solutions and chemicals were performed either by filtration through a 0.45µm Millex® Syringe Filter Units purchased from Merck Millipore, Watford, UK or autoclaving at 121°C at 1 bar for 1 hour. Most general chemicals were purchased from Sigma-Aldrich, Poole, UK unless specifically stated:

Guinea pig liver transglutaminase (gpTG2) was purchased from Zedira GmbH, Germany with the activity between 14.7-16 U/mg as stated in certificate of analysis. Recombinant human Factor XIII (His6-rhFXIII) was purchased from Covalab UK Ltd, Cambridge, UK. Fibrogammin P was purchase from CSL Behring UK Ltd, West Sussex, UK. Biotin-cadaverine was purchased from Zedira GmbH, Germany. FITC-cadaverine was purchase from Cambridge Bioscience Limited, Cambridge, UK. VECTASHIELD HardSet mounting medium with DAPI was purchased from Vector Laboratories LTD., Peterborough, UK. Silver (I) nitrate was purchased from Fisher Scientific UK Ltd, Leicestershire, UK. Cetylpyridinium chloride, p-Nitrophenyl Phosphate (pNPP) liquid substrate system, collagenase from *Clostridium histolyticum*, fibronectin from human plasma and laminin from human fibroblasts were purchased from Sigma-Aldrich, Poole, UK. Tankyrase PARP activity inhibitor, XAV939 was purchased from Stratech Scientific Ltd., Suffolk, UK. TG2 activity inhibitors, R283 and R294, were kindly provided from Dr. Russell Collighan.

1.2. *Cell Culture*

Dulbecco's modified Eagle's medium (DMEM) with high Glucose (4,5 g/L), L-glutamine, liquid (200mM), non essential amino acid concentrate (100x) and penicillin/streptomycin (100x) solution were purchased from PAA Laboratories Ltd, Somerset, UK. EpiLife® medium with 60 µM Calcium and human keratinocyte growth supplement (HKGS) were purchased from Invitrogen Ltd, Paisley, UK.

1.3. *Immunochemicals*

Anti-TG2 (TG100) mouse monoclonal antibody was purchased from Thermo Scientific, Surrey, UK. Plasmatic Transglutaminase 13 (FXIII) antibody and HRP-conjugate streptavidin were obtained from Covalab UK Ltd, Cambridge, UK. Anti- N-cadherin (H-4) mouse monoclonal antibody, anti-LRP5 (H-105) rabbit polyclonal antibody, anti-α-tubulin (B152) mouse monoclonal antibody, FITC conjugated donkey anti-mouse IgG antibody and TR-conjugated goat anti-rabbit IgG antibody were purchased from Santa Cruz Biotechnology, Heidelberg, Germany. Anti-β-catenin rabbit polyclonal antibody was purchased from New England Biolabs Ltd, Hertfordshire, UK. HRP-conjugated anti-mouse IgG or rabbit IgG antibodies were purchased from Dako Ltd., High Wycombe, Buckinghamshire. Osteocalcin Human Direct ELISA Kit was purchased from Invitrogen Ltd, Paisley, UK. Pierce Co-Immunoprecipitation Kit was purchased from Thermo Scientific, Surrey, UK

1.4. *Western Blot Chemicals*

Tris-glycine-SDS Buffer (10X Solution) and 40% solution containing 38.67% (w/v) acrylamide and 1.33% (w/v) bis-acrylamide were purchased from Melford Laboratories Ltd., Suffolk, UK. Precision Plus Protein™ Dual Color Standards and blot absorbent filter paper were purchased from Bio-Rad Laboratories Ltd., Hertfordshire, UK. Nitrocellulose membranes were purchased from Gelman Biosciences, Northampton, UK. Enhanced chemiluminescence ECL reagent was obtained from Amersham Pharmacia, Buckinghamshire, UK. Kodak® BioMax™ XAR Film, LX-24 developer solution and FX-40 fixer solution were purchased from Sigma-Diagnostics, Poole, UK.

1.5. *Molecular Biology Kits and Reagents*

GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit was purchased from Sigma-Diagnostics, Poole, UK. NovaBlue Singles™ Competent Cells was purchased from Millipore (U.K.) Limited, Watford, UK. Lipofectamine™ 2000 Transfection Reagent was purchased from Invitrogen Ltd, Paisley, UK. Detergent-compatible colorimetric assay kit was purchased from Bio-Rad Laboratories Ltd., Hertfordshire, UK

2. Methods

2.1. General Tissue Culture

2.1.1 HOB cells culture

Primary Human osteoblast (HOB) cell line, isolated from femoral heads of patients subjected to surgery, was a kind gift from Professor S. Downes and Dr. S. Anderson (School of Biomedical sciences, University of Nottingham). All cells were preserved in 10% dimethylsulfoxide (DMSO)/ 90% foetal calf serum (PAA) and stored in -80^{o}C freezer overnight before transferring into liquid nitrogen. To recover cells from liquid nitrogen, vials of cells were first thawed at 37^{o}C and carefully re-suspended in basic culture medium (see recipe below). The cell suspension was transferred into Falcon tissue culture flasks and cells allowed to attach for at least 6 hours. Medium was discarded and replaced with fresh culture medium after cell attachment. All the cells were incubated at a 37^{o}C, 5% CO_2 incubator with regular change of medium every 4 days.

HOBs were cultured in Dulbecco's modified Eagle medium (DMEM) -high glucose supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2mM L-glutamine, 1% (v/v) non-essential amino acid and 1% (v/v) penicillin-streptomycin antibiotic (complete medium, CM).

2.1.1.1 Differentiation of HOBs

HOBs, ranging from passage 20 to 24, were subjected to differentiation treatment. Cells were seeded at a density of $8\times10^4/cm^2$ in complete medium for 16 hours or until cell cutlure reached more than 90% of confluency and differentiation treatment was initiated by supplementing culture medium with 50 µg/ml ascorbic acid and 10 mM β-

glycerophosphate (differentiation medium, DM). Medium was replaced every 2 days during a 12-day culture for osteoblasts differentiation and mineralisation assays unless otherwise indicated.

2.1.2. *hCECs and hCFs cultures*

Primary human corneal epithelial cells (hCECs) and human corneal keratocytes (hCKs) were kindly supplied from Dr. Naing Tint (Faculty of Medicine & Health Sciences, University of Nottingham). hCECs were maintained in EpiLife® medium with human Keratinocyte Growth Supplement (Invitrogen Ltd, UK). hCKs were differentiated into human corneal fibroblasts (hCFs) and maintained in the fibroblast phenotype using DMEM supplemented with 10% fetal bovine serum (FBS), 1% (v/v) non-essential amino acids and 2mM L-glutamine. Additions of 1% (v/v) penicillin-streptomycin antibiotic and 0.2% (v/v) Normocin™ were used in medium to prevent bacterial, mycoplasma, and fungal contamination. Media were changed routinely every two or three days during this study. All cell lines were cultured in a humidified-atmosphere incubator at 37°C and with 5% (v/v) CO_2.

2.1.3. *Passaging, thawing and storage of cell cultures*

All cells were incubated in a humidified incubator at 37°C with 5% CO_2 and were routinely maintained to ensure the confluency of cell culture were never allowed to reach greater than 85% at any one time. Upon reaching confluency, monolayer of cells were rinsed with phosphate buffered saline (PBS) once and HOBs were detached from the tissue culture flask using adequate amount of pre-warmed 0.5% trypsin (PAA, Pasching, Austria) in PBS containing 2mM ethylenediaminetetraacetic acid (EDTA) for 5 minutes at 37°C. Following detachment of cells which could be observed using a microscope, HOBs were quickly

recovered by addition of a double amount of fresh DMEM with 7% FBS. The HOB cell suspension was centrifuged at 250×g for 5 minutes and the cell pellet was re-suspended in appropriate amount of CM and ready for reseeding into corresponding culture vessels.

For hCEC and hCF cell cultures, a similar passaging protocol was used, except that 0.05% trypsin in PBS containing 0.2mM EDTA and 0.25% trypsin in PBS containing 1mM EDTA were used respectively to detach the cell monolayer. The hCEC and hCF cell suspensions were centrifuged at 180×g for 7 minutes and re-suspended in the growth medium.

Cryogenic preservation was used to maintain backups or reserve of cells. Cells subjected to freezing process were detached by trypsin solution and washed once PBS. The washed cell pellet was carefully re-suspended in adequate amount of 10%(v/v) dimethylsulfoxide (DMSO)/ FBS. The DMSO containing cell suspension was divided into several cryogenic vials and the vials were allow to placed in the -20 ºC freezer for 2 hours and then in the -80 ºC freezer overnight before transferring to a liquid nitrogen freezer for long term storage. To recover the cells from cryogenic storage, the frozen vials were removed from liquid nitrogen and rapidly thawed in 37ºC water bath for 60 to 90 seconds with gentle agitation. Once the cell suspension was completely thawed, the content of the vial was transferred to a T-75 flask containing 12 ml of culture medium and cultured at 37^{o}C with 5% CO_2. Cells were recovered from cryoprotective agent by a medium change once the cells attached to the flask (generally within 6 to 8 hours of thawing).

2.2. Protein expressions, identifications and interactions

2.3.5 Protein Concentration

Lowry protein assay (Bio-Rad RC kit, Life Sciences, Hemel Hempstead, UK) was performed to quantify the obtained protein concentration of cell lysate (Lowry *et al.*, 1951). The whole assay was carried out as per manufacturers' instructions. In brief, 5 µl of samples

(diluted if needed) or bovine serum albumin (BSA) standards (ranging from 0.2-1.5 mg/ml) were added to each well of 96-well plate. Following addition of 25 µl of reagent A and 200µl of reagent B, the mixture were stabilised at room temperature for 10 minutes before reading at 750nm using a SpectraFluor® plate reader. Protein concentrations were determined against a linear standard curve produced by duplicate BSA protein standards.

2.2.2 Collection of Total Cell Lysates and Conditioned Medium

HOBs were initially seeded in 60 mm petri-dishes (Corning) at a density of $8\times10^4/cm^2$. At different time points and treatments, HOB cells were detached using pre-warmed 5mM EDTA in PBS for 5 minutes at 37^{o}C and recovered in complete medium. Cells suspensions were centrifuged at 250×g for 5 minutes and cell pellets were washed in PBS once before re-suspension again in lysis buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol; pH 7.4) supplemented with 0.2mMPMSF and 0.1% Protease Inhibitor Cocktail (Sigma-Aldrich Company Ltd. Dorset, UK). After 5 minutes incubation on ice with periodic mixing, the lysate mixtures were centrifuged at 13,000×g for 10 minutes at 4^{o}C to pellet the cell debris.

For collecting conditioned medium, HOBs cultured in either complete medium or differentiation medium were switched to serum free DMEM medium 24 hours before specified collecting time points. The resultant conditioned medium was collected from the petridish and centrifuged at 250×g for 5 minutes to get rid of cell debris. The conditioned mediums were concentrated using Amicon Ultra-0.5 centrifugal filters with 10kDa cutting point (Millipore Limited, Watford, UK) and subjected to total protein quantification.

A total 50 µg of sample protein or 25 µg of total protein in conditioned medium was add-mixed with 5X Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-

mercaptoethanol, 0.01% bromophenol blue) and adequate distilled water. Sample mixtures were boiled for 5 minutes and placed on ice before loading.

2.2.3 Western Blotting

2.2.3.1 Preparation of Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel

Acrylamide/ bis-acrylamide gels were cast using Mini-PROTEAN Tetra Cell Casting Module (BIO-RAD, Hemel Hempstead, UK) which consisted of a standard 3% (w/v) polyacrylamide stacking gel and a separating gel (5-15% (w/v), depending on the application required). Separating gels were prepared in 4X Tris-SDS stock solution containing 1.5M Tris and 0.4% (w/v) SDS (pH 8.8) and standard 4% (w/v) polyacrylamide stacking gels were prepared using 4X Tris-SDS stock solution (0.5M Tris, 0.4% (w/v) SDS, pH 6.8). Receipts for different concentrations of separating gel were listed in detail as following Table 2.1:

Table 2.1 Recipe for preparing different concentration of separating gel

Stock solutions	Final acrylamide concentration in the separating gel (%) (ml)							
	5	6	7	7.5	8	9	10	12
40% acrylamide/ bis-acrylamide	1.875	2.25	2.625	2.8125	3	3.375	3.75	4.5
4x Tris-HCl/SDS (pH 8.8)	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	9.375	9	8.625	8.4375	8.25	7.875	7.5	6.75
10% ammonium persulphate	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

The mixture of desired concentration was loaded in 80 x 60x 1.5mm module and 500 ml of iso-propan-2-ol was added on the surface to flatten the gel. After 30 minutes of polymerisation at room temperature, the iso-propanol was removed and the surface of the gel was rinsed with distilled water. Another layer of 4% stacking gel (487.5 ml of the 40% (w/v) acrylamide /bisacrylamide solution, 1.25ml of Tris/SDS pH 6.8, 3.23 ml of

distilled water, 25µl 10% (w/v) ammonium persulphate and 5µl of tetramethylethylenediamine (TEMED)) was poured on the top of separating gel. A 10/15 well comb was placed into the mould and allowed to solidify at room temperature for 30 minutes.

2.2.3.2 Gel Electrophoresis and Protein Transferring

Prepared samples were loaded into sample wells and another 3 µl of rainbow marker was loaded at the first lane of each gel. Electrophoresis was performed at first constant 90V for 10 minutes and then 120V for 90 minutes until sample marker dye reached the bottom of the gel or rainbow marker reached desired resolution. SDS-PAGE separated proteins were transferred from gel to polyvinylidene fluoride transfer membrane using Mini Trans-Blot cell system (Biorad, Hertfordshire, UK). Briefly, the gel was removed from the plate module and washed once with transfer buffer contained 48.8mM Tris-HCl, 39mM glycine and 20% (v/v) methanol. The apparatus was set up as manufacturer's instruction with gel placed near cathode side and membrane at anode side. Protein transferring was performed at constant 200mA for 2 hours on ice. The transferred protein on the nitrocellulose membrane was verified by staining with Ponceau Red solution to ensure efficient transfer.

2.2.3.3 Detection of Protein Using Immunoprobing

The polyvinylidene fluoride membrane was blocked with 5% (w/v) Marvel dried milk in Tris-buffered saline – 0.05% Tween (TBS-T, pH 7.6) for 30 minutes, at room temperature. Following blocking step, the membrane was incubated with primary antibodies diluted 1:200 to 1:1000 in blocking buffer at 4ºC overnight. Three times washing with TBS-T washing solution was performed before incubating with secondary antibodies.

Appropriate secondary antibody was diluted 1:2000 in blocking solution and further incubation of membrane in secondary antibody at room temperature for 2 hours. All the dilution factor can be found in Table 2.2 below. Another three- time washing was performed and the membrane was incubated with ECL chemiluminescence substrate (Amersham, Buckinghamshire, UK) according to the manufacturers' instructions. The membrane was covered with cling film and the signal was detected by Kodak X-Omat chemiluminescence detection film (Roche Diagnostics, East Sussex, UK) in a Kodak Biomax exposure cassette. Exposure time was varied depending on the intensity of signal and the resulting film was developed in 20% (v/v) LX-24 developer and fixed in 20% (v/v) FX-40 fixer.

Table 2.2 Dilution of primary and secondary antibodies

Antibodies	Dilution
TG2 (TG100) mouse monoclonal antibody	1:1000
plasmatic Transglutaminase 13 (FXIII) antibody	1:200
N-cadherin (H-4) mouse monoclonal antibody	1:1000
LRP5 (H-105) rabbit polyclonal antibody	1:1000
α-tubulin (B152) mouse monoclonal antibody	1:1000
β-catenin rabbit polyclonal antibody	1:1000
mouse IgG HRP-conjugated antibody	1:2000
rabbit IgG HRP-conjugated antibody	1:2000

2.2.4 Immunohistochemical Staining

HOBs were cultured on Four Well Pattern Microscope Slides (TEKDON, INC., Florida, USA) and differentiated in DM as mentioned before for 4 hours to 48 hours. The samples were washed with PBS (pH 7.4) and fixed with 3.7% (v/v) para-formaldehyde (PFA) /PBS (pH 7.4) at room temperature for 20 minutes. Three times washing with PBS were applied to

samples between each step in the following procedure. To stop the crosslinking of samples by residual PFA, slides were incubated with 30mM Glycine/PBS at room temperature for 10 minutes. Permeabilisation step was only applied to samples which were stained for intracellular protein expression. In order to permeabilise cell membrane, 5% Triton X-100 was added to sample slides and removed after 2 minutes incubation at room temperature. The sample slides were then blocked with 10% goat serum in PBS at room for 30 minutes. Rabbit polyclonal LRP5 (H-105, Santa Cruz biotechnology, Inc., Heidelberg, Germany) antibody or rabbit polyclonal β-catenin (New England Biolabs Ltd, Herts, UK) antibody was used, depending on the experiments, in 1:200 dilution using 0.5% goat serum/TBS-T. Incubation of primary antibody was performed at 37ºC for 1 hour or 4ºC for 16 hours. Following incubation of goat anti-rabbit IgG-TR conjugated antibody (dilute in 1:200, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) was performed at 37ºC for 1 hour or 4ºC for 16 hours. All the samples were co-stained with mouse monoclonal TG2 (TG100, Thermo Scientific, Surrey, UK) antibody and donkey anti-mouse IgG-FITC conjugated antibody (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) at 1:200 dilution. Finally, the samples were washed with PBS for three times and with distilled water for extra 2 times. After washing, the slides were allowed to dry against the tissue carefully from the edge without damaging the samples. Samples were mounted in VECTASHIELD® HardSet™ Mounting Medium with DAPI (Vector Laboratories LTD, Peterborough, UK) and pictures were acquired using Leica TCS MP5 multiphoton microscope.

2.2.5 *Protein Complex Immunoprecipitation (Co-IP)*

The interaction between TG2 and LRP5 receptor was studied using immunoprecipitation technique. The Pierce® Co-Immunoprecipitation kit (Thermo Scientific, Surrey, UK) was used in following experiments to avoid contamination by the IP antibody. The following optimised protocol was used for this study.

2.2.5.1 Lysis of Cell Monolayer Cell Cultures

Cultured and differentiated HOBs cell lysates were sampled at 4 hours, 1 day and 2 days post exogenous 1µg/ml TG2 treatment. Monolayer of cells was washed once with PBS (pH7.4) and lifted up using 5mM EDTA/PBS. Cell suspension was centrifuged at 250×g for 5 minutes to pellet the cells and adequate IP Lysis/Wash buffer provided from the kit was added to resuspend the cell pellet. After 5 minutes incubation with periodic mixing on ice, the cell lysate was centrifuged at 13,000×g for 10 minutes to pellet the cell debris. The resultant supernatant was transferred to a new microcentrifuge tube and subjected for quantification of protein concentration.

2.2.5.2 Pre-clear Cell Lysate Using Control Agarose Resin

For clearing, cell lysate contained total 1mg of protein, the sample clearing column was constructed with 80µL of the control agarose resin slurry. Washing the clearing column with 1X coupling buffer provided from the kit, the columns were ready to be loaded with cell lysates from previous step. The lysates were incubated with clearing resin at 4^{o}C for 1 hour on the orbital shaker with end-to-end mixing. The columns containing samples were centrifuged at 1,000×g for 1 minute to obtain the cleared cell lysates.

2.2.5.3 Immobilisation of Antibody

To prepare the binding column, 25µl of the AminoLink Plus coupling resin slurry was added to the each column and washed it twice with 1X coupling buffer. A total 2 µg of antibody, rabbit polyclonal LRP5 antibody or mouse monoclonal TG2 antibody, was adjusted to final volume at 200µL and added to pre-packed column. After addition of sodium cyanoborohydride solution, columns with antibody/resin mixtures were incubated at room temperature for 90 minutes with gentle shaking. Once finished coupling, the antibody bound resin was washed once with coupling buffer and further incubated with Quenching buffer provided from the kit for another 15 minutes at room temperature with gentle shaking. 2 times washing with 1X Coupling Buffer plus 6 times washing with Wash solution were applied to antibody-coupled resin and the columns were ready to perform Co-IP.

2.2.5.4 Co-IP

The prepared columns from previous step were washed with IP Lysis/Wash buffer for two times and a total 800µg of whole cell lysates was added to each column. The columns with cell lysate were incubated with gentle rocking at 4^{o}C overnight allowing the desired protein to be caught by the resin-bound antibody. After incubation, the protein bound resin was washed with IP Lysis/Wash buffer for 3 times or more if needed.

2.2.5.5 Elution of Co-IP and Evaluating Protein Interaction by Western Blotting

The columns were placed in new collection tubes and 10µl of elution buffer was added to each column. The flow through was collected by centrifuging the column at 1000×g for 1 minute, and another 40µl of elution buffer was added to each column. With 5 minutes

incubation at room temperature without shaking or mixing, the total elution was collected by centrifuging at 1000×g for 1 minute. 5X Laemmli sample buffer was added to each sample to make a 1X final sample solution and the samples were subjected to Western blotting analysis. TG2/LRP5 binding was confirmed by reciprocal Western blot analyses using mouse monoclonal TG2 antibody or rabbit polyclonal LRP5 antibody.

*2.2.6 **ELISA***

2.2.6.1 Sample Collection

HOBs were seeded in 6-cm petri dish the day before differentiation. Once cells were ready, cell layer was washed with PBS (pH 7.4) once before any treatment. HOBs were subjected to different treatments and were collected at specified time points. 24 hours prior collecting samples, the medium were switched to 1.5 ml of serum-free medium. On the day of sample collection, conditioned medium was carefully collected by pipette and cell debris removed by centrifugation at 250×g for 5 minutes. Samples were either used immediately or stored at -80^{o}C for future use. The remaining cell layer was lifted up from tissue culture dish using 5mM EDTA/PBS solution, lysised with RIPA buffer (0.1% SDS, 1% NP-40, 1% sodium Deoxycholate, 5mM EDTA, 150mM NaCl in 10mM Tris buffer, pH 7.2) and total protein concentration was determined using Lowry assay described previously.

2.2.6.2 Quantification of OCN in Conditioned Medium using ELISA assay

The osteocalcin level in conditioned medium was determined using Novex® Osteocalcin Human Direct ELISA Kit (Invitrogen, Paisley, UK). As per manufacture's instructions, standards and controls were reconstructed with distilled water and both samples and microtiter plates were thawed at room temperature before assay. 25 µl of each standard, control or sample was added to appropriate wells and the addition of 100 µl of working

anti-OST-HRP conjugate to each testing well was performed before 2-hour incubation at room temperature. The reaction mix was discarded after incubation and the microtiter plate was washed with the washing solution for 3 times, each time for 30 seconds. Following the washing step, 100 µl of chromogen solution was added to each well and the plate was incubated at room temperature in the dark until the colour development had finished. The reaction was terminated by adding 100 µl of stop solution and the optical density of the sample was read at 450nm using a SpectraFluor® plate reader. The concentration of each sample was further normalised against the total protein extracted from the whole cell layer.

*2.2.7 **Zymography***

Collagenase and gelatinase expression was detected using zymography technique adapted from Herron and colleagues work (1986). The recipes for the resolving gel and stacking gel are summarised in Table 2.3 below.

7.5 % Separating gel		***Stacking gel***	
40% acrylamide/ bis-acrylamide	*3 ml*	40% acrylamide/ bis-acrylamide	*0.639 ml*
1.5M Tris-HCl pH 8.8	*4 ml*	0.5M Tris-HCl pH 8.8	*1.71 ml*
10X gelatine or collagen solution (8mg/ml)	*1.6 ml*	*Sucrose solution (50%)*	*1.71 ml*
Sucrose solution (50%)	*3.44 ml*	*Water*	*2.78 ml*
Water	*3.96 ml*	*10% Ammonium persulfate*	*80 µl*
10% Ammonium persulfate	*60 µl*	*TEMED*	*18 µl*
TEMED	*10 µl*		

Table 2.3 Recipe for preparing separating gel and stacking gel for collagen or gelatine zymography

The conditioned medium collected on day 8 from HOBs treated with CM, DM, or different concentration of inhibitors was mixed with 5X Laemmli buffer to make up final 1X sample solution and loaded to the prepared gel. Electrophoresis was performed at constant 120 V

on the ice in a chilled-cabinet (4°C) to avoiding overheating. After electrophoresis, the gels were washed with enzyme renaturing buffer (see Table 2.4 for recipe) for 2 times, each time 30 minutes. Renatured gels were transferred to digesting buffer (see Table 2.4 for recipe) and incubated at 37ºC for 48 hours. Once finished digestion, gels were washed with distilled water once and stained with PageBlue Protein Staining Solution (Thermo Scientific, Surrey, UK) for 2 hours at room temperature. Destaining was performed using distilled water for at least 4 times until the gel reached desired condition.

Digesting Buffer (500ml, pH 7.5)	
Tris base (Final Conc. 100mM)	6.055 g
NaCl (Final Conc. 200mM)	5.845 g
$ZnCl_2$ (Final Conc. 2.6µM)	0.35 mg
$CaCl_2 \cdot 2H_2O$ (Final Conc. 5mM)	0.37 g
NaN_3 (0.02%)	0.1 g
Renaturing Buffer	
2.5% Triton X-100 in digesting buffer	

Table 2.4 Recipe for preparing digesting buffer and renaturing buffer

2.3 Determination and Inhibition of Transglutaminase Activity

2.3.1 Crosslinking activities of TG2 and FXIIIa

In order to distinguish TG2 enzymatic activity from FXIIIa, a non-specific, cell permeable inhibitor- R283 and TG2-specific, non-permeable inhibitor- R294 were used in following experiments. The inhibitory effect of the inhibitors was first examined at protein level before *in vitro* testing. A modified protocol to evaluate the transglutaminase was used by adapting the procedure mentioned by Slaughter *et al.,* (1992)

2.3.1.1 Sample preparation

Guinea pig liver Transglutaminase (gpTG2) purchased from Zedira, human recombined FXIII purchased from Covalab and Fibrogammin® P manufactured by CSL Behring UK Ltd were used in this 96-well plate based assay. 2U/ml of thrombin treatment to activate FXIII

for and pre-incubation of enzyme with inhibitors were needed before assay. The detail of setting up was summarised in the Table 2.5 below:

	2U/ml Thrombin	Inhibitor R283	Inhibitor R294
TG2 (100ng/well)	-	-	-
	-	+	-
	-	-	+
TG2 (100ng/well)	+	-	-
	+	+	-
	+	-	+
FXIII (100ng/well)	+	-	-
	+	+	-
	+	-	+
Fibrogammin P (13.6µg/well)	+	-	-
	+	+	-
	+	-	+

Table 2.5 Preparation of sample in summary. All sample were diluted in 5mM DTT and 5mM $CaCl_2$ in 10mM Tris buffer (pH 7.4) and adjusted the volume to 50µl per well. Thrombin treatment was performed on ice for 15 minutes. After activation of enzyme, different concentrations of inhibitors (100µM, 250µM and 500µM) were added to pre-activated enzyme and the mixtures were incubated on ice for another 15 minutes.

2.3.1.2 Biotin Cadaverine Incorporation into N,N'-dimethylcasein Assay

A 96-well plate was coated with 200µl of 100mg/ml N,N'-dimethylcasein in 100mM Tris-HCl (pH 8.5) overnight at 4°C. Two-time washing with PBS, 0.05 % (v/v) Tween20 was applied to the coated plate following with another two-time washing with distilled water. The plate was then blocked with 250µl of 3% (w/v) BSA in 100mM Tris-HCl (pH 8.5) for 30 minutes at room temperature. Plate was once again washed 3 times with PBS, 0.05 % (v/v) Tween20 and once more with 100mM Tris-HCl (pH 8.5). The reaction was initiated by addition of 50µl of pre-treated samples and 150µl of 0.1mg/ml biotin-cadaverine in 5mM DTT and 5mM $CaCl_2$ in 10mM Tris buffer (pH 7.4). The reaction was performed at 37°C for 30 minutes and terminated with 10mM EDTA. A series washing with PBS, 0.05 % (v/v) Tween20 and once more with 100mM Tris-HCl (pH 8.5) was applied to the plate. Incorporated biotin-cadaverine into N,N'-dimethyl was detected by incubation with

extravidin peroxidase conjugate in 1:2000 dilution for 1 hour at 37^{o}C. Development of the reaction was performed using SIGMAFAST™ OPD (o-Phenylenediamine dihydrochloride) tablets (P9187, each tablet set dissolved in 20ml dH2O yields a ready-to-use buffered solution containing OPD and urea hydrogen peroxide). The colour development was terminated by addition of 50µl of 2.5M H_2SO_4 and the absorbance was read at 450nm using a plate reader.

2.3.2 Cell Surface Crosslinking Activity Assay

2.3.2.1 Cell Culture and Sample Preparation

HOBs were cultured and differentiated for 2 days, 6 days and 12 days. To measure the cell surface transglutaminase activity, HOBs, at different time points, were detached from the tissue culture flask using 5mM EDTA/PBS. Obtained cells were centrifuged down, washed once with PBS and resuspended in serum free medium. A final 1×10^5 HOBs were added to each well of 96-well plate. A parallel set of samples was set up with addition of 2U/ml of thrombin to activate FXIIIa crosslinking activity. To further distinguish the crosslinking activity between tissue transglutaminase and FXIIIa, a final concentration of 250µM of R283 or R294 was also added to cell suspension. 50ng/well of gpTG2 and 50ng/well of human FXIIIa were used as positive controls.

2.3.2.2 Biotin Cadaverine Incorporation into Fibronectin Assay

Transglutaminase activity on cell surface was determined by the incorporation of biotin cadaverine into fibronectin coated microtiter plate. 96-well plate was coated with 50µl of 5µg/ml fibronectin solution in 50mM Tris-HCl pH 7.4 overnight at 4ºC. Before use, the plate was washed with 50mM Tris-HCl (pH 7.4) once and preserved in serum free cell culture medium. Samples prepared as described above were added to 96 well plate and

the assay was initiated by addition of reaction buffer which contained 0.1mM biotin-cadaverine and 1mM DTT in serum free medium. The reaction was performed at 37°C for 3 hours and terminated using PBS (pH7.4) containing 2mM EDTA. A detergent solution (100µl) consisting of 0.1% (w/v) deoxycholate in 2mM EDTA /PBS (pH 7.4) was then added to each well and the mixture incubated with gentle shaking for 10 minutes at room temperature. Further washing of fibronectin layer with 50mM Tris-HCl (pH7.4) was performed 3 times before blocking with 3% (w/v) BSA in 50mM Tris-HCl buffer for 30 minutes at 37ºC. The incorporated biotin cadaverine was revealed with a 1/2,000 dilution of Extravidin peroxidase conjugate (Sigma-Aldrich Co. Ltd) which was incubated for 1 hour at 37°C. After incubation, a series of washes with 50mM Tris-HCl, pH 7.4 were performed as above. Colour development was achieved using SIGMAFAST™ OPD (o-Phenylenediamine dihydrochloride) tablets and terminated by 2.5N H_2SO_4 and the resultant absorbance was read at 450nm in a SpectraFluor® plate reader.

2.3.3 Cell Mediated Incorporation of FITC-Cadaverine into Extracellular Matrix

HOBs were cultured on Four Well Pattern Microscope Slides (TEKDON, INC., Florida, USA) and differentiated in DM as mentioned before for 2, 6 and 12 days. At different time points, CM and DM were switched to serum free DMEM containing 0.1mM FITC labelled Cadaverine with or without either R283 or R294. Cells were allowed to crosslink FITC-cadaverine into extracellular matrix at 37ºC for 24 hours. After incubation, medium was removed and cells were gently washed with PBS (pH 7.4) for three times. Ice cold 100% methanol (-20 ºC) was used to fix the cells for 15 minutes at -20 ºC. In order to remove non-crosslinked FITC-cadaverine, fixed samples were carefully washed with 70% ethanol for another three times. Finally, another 3-time washing with PBS (pH7.4) was applied to

sample slides before being mounted in VECTASHIELD® HardSet™ Mounting Medium with DAPI (Vector Laboratories LTD, Peterborough, UK). Sample slides were viewed and pictures were acquired using Leica TCS MP5 multiphoton microscope.

*2.3.4 **Knockdown TG2 Expression in HOBs***

2.3.4.1 Plasmid Preparation

- *Plasmid Transformation*

NovaBlue Singles™ Competent Cells (Millipore Limited, Watford, UK) were used for transformation of TG2 MISSION® shRNA Plasmid DNA, shRNA 239, 240, 241, 243 and MISSION® pLKO.1-puro Non-Mammalian shRNA Control Plasmid DNA (Sigma-Aldrich Company Ltd. Dorset, UK), to *E. coli*. As described in manufacturer's instruction, microcentrifuge tubes were pre-chilled on ice before aliquot of 20µl competent cells each tube. 1µl of shRNA plasmid DNA was added directly to competent cells with gently mix. Mixtures were placed on ice for 5 minutes before heat shocking at 42^{o}C water bath for exactly 30 seconds. 2-minute incubation on ice, 140µl of room temperature Luria Broth (LB, 1% tryptone, 0.5% yeast extract and 1% NaCl) was added to each tube. Cell suspensions were allowed to grow at 37^{o}C incubator with shaking at speed 250rpm for 1 hour. 50µl of cell suspension was spread on Luria Agar (LA, 1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar) containing 100mg/L of Ampicillin. Cells were incubated at 37^{o}C incubator overnight and transformed colonies were selected the next day.

- *Plasmid DNA Isolation*

GenElute HP Plasmid Maxiprep Kit (Sigma-Aldrich Company Ltd. Dorset, UK) was used to purify plasmid DNA from transformed *E. coli*. In brief, selected single cell colonies from each group were cultured in 3ml LB medium containing ampicillin at 37°C for

approximately 8 hours while shaking at 250 rpm. These starting cultures were further diluted 1:500 in 200ml of LB medium with ampicillin and incubated at 37°C for 16 hours with shaking at 250 rpm. 150ml of overnight cell culture suspensions were pelleted by centrifuging at 5000×g for 10 minutes. Supernatant was discarded and resultant cell pellet was resupended in Resuspension/RNase A Solution. Resuspended cells were lysed by additional of lysis buffer with gently inverting 6 to 8 times and allowed to be chill on ice for 3 minutes. Within 5 minutes, cell lysates were neutralised with chill neutralization Solution. Resultant cell lysates were first filtered through filter column to get rid of cell debris and binding solution was added to filtered lysates with gently mixing. Mixtures were transferred to prepared binding column and allowed to pass through the binding columns. After a series washing with wash solution, the DNA bound columns were dried for at least 10 minutes by applying vacuum to the columns. Once the columns were dried, DNA plasmids were recovered by adding 1.2 ml of Endotoxin-free water and collected by centrifuging down at 1000 x g for 5 minutes. DNA concentration of samples was determined using NanoDrop 3300 Micro-Volume Full-Spectrum Fluorospectrometer.

2.3.4.2 Generation of Stable Transfected HOBs cell line

- *Determination of Puromycin Working Concentration*

In order to generate stable cell lines expressing the shRNA of interest, it is necessary to determine adequate concentration of puromycin in order to select transfected cells. In short, 1×10^4/well of HOBs were seeded in a 96-well plate and cultured in a 37°C, CO_2 incubator the day before carrying out the assay. A series dilutions of puromycin, 0, 0.125ng/ml, 25ng/ml, 50ng/ml, 125ng/ml, 250ng/ml, 500ng/ml, 1μg/ml, 2μg/ml and 4μg/ml, were prepared in antibiotics-free DMEM and added freshly to HOBs cell culture. After 48 hours incubation with puromycin, the cytotoxicity of puromycin was assayed by

using Cell Proliferation Kit II (Roche Diagnostics Ltd., West Sussex, UK) as mentioned previously in section 2.5.1. The lowest concentration that killed 100% of the cells was chosen as initial working concentration for stable cell line selection.

- *shRNA Transfection and Stable Cell Line Selection*

Stable transfected HOBs were generated using Lipofectamine® 2000 Reagent (Life Technologies Ltd, Paisley, UK). According to manufacturer's instruction, initial 5×10^5/well of HOBs were seeded in 6-well plate and grown in normal DMEM culture medium without antibiotics for 24 hours. On the day of transfection, well layer was washed with 1× PBS once and replaced with 2ml fresh DMEM without antibiotics. Desired amount of lipofectamine reagent was add mixed with DMEM medium without antibiotics and plasmid DNA, shRNA 239, 240, 241, 243 and SHC002 was diluted in antibiotics-free DMEM. Transfection reagent and diluted DNA were allowed to incubate at room temperature for 5 minutes separately. After 5 minutes incubation, lipofectamine and diluted DNA were mixed gently in 1:1 ratio and the mixture was further incubated for 20 minutes at room temperature. Once incubation had finished, the lipofectamine/DNA complex was added to each well containing cells and medium immediately and HOBs were incubated at 37°C in a CO_2 incubator for 6 hours. Transfection was terminated by switching lipofectamine/DNA containing medium to fresh antibiotics-free medium after 6 hours. HOBs cell culture was allowed to grown at 37°C, CO_2 incubator for another 48 hours before selection. To start the selection, post-transfected HOBs cells were cultured in DMEM supplemented with 500ng/ml of puromycin until shRNA transfected HOBs were selected. Medium was changed every 2 days if needed. Once stable transfected cells had been selected, the knockdown cell lines were cultured in DMEM supplemented with 250ng/ml of puromycin for all the following experiments. The protein expressions of TG2 and FXIIIa

in each stable knocked down cells lines were analysis using Western blotting to confirm the knock down efficiency.

2.3.5 Inactivation of gpTG2 Crosslinking Activity Using Inhibitors

In order to prepare inactive gpTG2, crosslinking inhibitors, R283 an R294 were used to block the crosslinking activity. The concentration of gpTG2 stock solution was first adjusted to 0.995µg/ml containing 5mM of $CaCl_2$ and 5mM of DTT and the active TG2 mixture was allowed to be incubated for 5 minutes on ice. 500mM R283/R294 stock solution was used to minimise the volume change and the calcium bound active gpTG2 was inhibited with a final concentration of 500µM of R283/R294 on ice for another 15 minutes. To avoid introducing excess inhibitor to the cell culture, inhibitor-treated gpTG2 was underwent dialysis to remove unbound inhibitor. Mini dialysis kit with 1kDa cut out point was used to clean up the inactive gpTG2. According to the instruction, samples were loaded into conical bottom of the tubes. The capped tubes were inserted into floats and then with the membrane down, the tubes were placed into a stirred beaker containing 50mM Tris-HCl buffer (pH 7.4) dialysis solution. Samples were allowed to be dialysed against Tris buffer for 2 hours at 4^{o}C and then recovered from the dialysis device by centrifuging.

2.4 Cell Biological Behaviours

2.4.1 Cell Proliferation- XTT Reduction

The cell proliferations of HOBs, HCFs and HCECs with or without different treatments were measured at different time points by using Cell Proliferation Kit II (Roche Diagnostics Ltd., West Sussex, UK). In brief, XTT labelling reagent and electron coupling reagent were mixed as manual instruction and 40µl of mixture was added to each well of 96- well plate.

Cells and XTT mixture were allowed to be cultured at 37°C in a CO_2 incubator for 4 hours. Once incubation had finished, 100 µl of culture supernatant from each well was transferred to a new microplate and the resultant orange formazan solution was spectrophotometrically quantified using ELISA plate reader (absorbance wavelength: 450nm and reference wavelength: 750nm).

2.4.2 Mineralisation of HOB Cultures

HOBs were subjected to different treatments for a 12-day period with regular change of medium every 2 days. On day 12, Mineralised bone matrix, composed of hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$), could be visualised using von Kossa staining where the silver ions replace calcium and form precipitation complex with phosphate group (Von Kossa, 1901). A modified protocol was used to determine the mineralisation of HOBs at day 12 post differentiation. The samples were washed, fixed in 3.7% Paraformaldehyde for 30 minutes and further dehydrated in 70% ethanol for 1 hour. Dehydrated samples were quickly washed with distilled water once. After additional of 2% silver nitrate, samples were exposed to UV light for at least 20 minutes, washed with distilled water for 5 times and then fixed with 5% sodium thiosulfate for 3 minutes. Another washing with distilled water, samples were viewed at x40 magnification using a Nikon CK2 microscope. At least 12 fixed-size, non-overlapping, random fields from each sample were photographed with an Olympus DP10 digital camera. The images were converted into negative images and the mineralised area was quantified by using Image J software.

2.4.3 *Evaluating ALP Hydrolysis Activity During HOBs Mineralisation*

HOB cells cultured directly on tissue culture plate or seeded on collagen matrices were allowed to attach to the surface for at least 24 hours before switching to differentiation medium (DM). DM was changed every 2 days, and 24 hours prior sample collection, the medium was changed to serum-free DM. The cultured medium collected at different time points were used to determine the release of ALP in the medium and the remained cell layer was reserved for testing anchored ALP activity.

ALP activity was quantified by hydrolysis of *p*-nitrophenyl phosphate (pNPP, Sigma–Aldrich) reaction mixture as described in Chapter III. For analysis of released ALP activity, 50µl of cell culture medium collected from each group was directly admixed with pNPP substrate. For analysis of anchored form ALP, the cell layer was first washed with PBS, replaced with 50µl of fresh medium and directly interacted with pNPP substrate solution. The change in absorbance at 405nm was monitored over 20 minutes at 37°C. The ALP activity was expressed as ΔA/min or mU (1 U reflects 1 µmole of product formed/minute)/cm^2 culture area.

2.4.4 *Determination of Cell Number in 3D Collagen Gel Using Multiphoton Microscope*

hCFs were seeded on collagen matrices at the density 3,000 cells/well in a 96-well plate format. The culture medium was changed once at 48 hour and after total 72-hour incubation, the cell containing collagen gels were washed with PBS (pH 7.4) twice and fixed with 3.7% PFA/PBS (pH 7.4) for 15 minutes. After fixation, the samples were carefully lift from the 96- well plate, placed on the glass slide and a drop of VECTASHIELD® HardSet™ Mounting Medium with DAPI (Vector Laboratories LTD, Peterborough, UK) was added directly to the samples.

Samples were viewed and the images were acquired using Leica TCS MP5 multiphoton microscope. The two photon excitation wavelength used was 785nm and the emission bandwidth was set between 395nm to 498 nm. At least 3 areas were chosen in each sample which was repeated in triplicate and an 880μm size depth was scanned at a z-step size of 4μm. 3D projection and cross-sectional images of each series of scanning were generated using the LAS AF software. The projected images were further analysed in ImageJ for the average cell density on each matrix.

2.4.5 Migration of hCFs on 3D Collagen Gel

Cell migration was determined using Boyden assay system. A transwell protocol adapted from Marshall (2011) used modified and used in this study. As illustrated in Fig.2.1, the polycarbonate membrane transwell inserts with 8μm pore size in 24-well plate format were coated with NC or TG2 gel at approximately 1.5mm in thickness. Gels were allowed to settle overnight at 37 ^{o}C and the hCFs were seeded at 156 cells/mm^2 initially. In the upper chamber, the serum free DMEM culture medium was used and, in order to generate chemo-attraction, 10% FBS/DMEM was added into lower chamber. hCFs were allowed to migrate in transwell system for 48 hours. After incubation, the serum free medium and the matrices were carefully lifted from transwell without disturbing the membrane. The cells remained on the upper chamber side of membrane were removed using cotton swab and the cells attached to the lower chamber side of membrane were visualised using modified Romanowsky staining (Horobin and Walter, 1987). A commercialised product, Diff-Quik® Stain Set (Siemens Healthcare Diagnostics, Surrey, UK), was used and in short, the membrane was dipped into fixative solution (Fast green in methanol) for 30 seconds, then transferred to Stain solution I (Eosin G in phosphate buffer) for 30 seconds and finally stained with Stain solution II (Thiazine dye in phosphate buffer) for 30 seconds. The membranes were allowed to drain and washed with distilled water once in between

staining. The stained membranes were cut out from the insert and placed to glass slide with the cells side facing up. The slides were viewed using a Nikon CK2 microscope. At least 7 fixed-size, non-overlapping, random fields from each sample were photographed with an Olympus DP10 digital camera and the cell count was performed using Image J software.

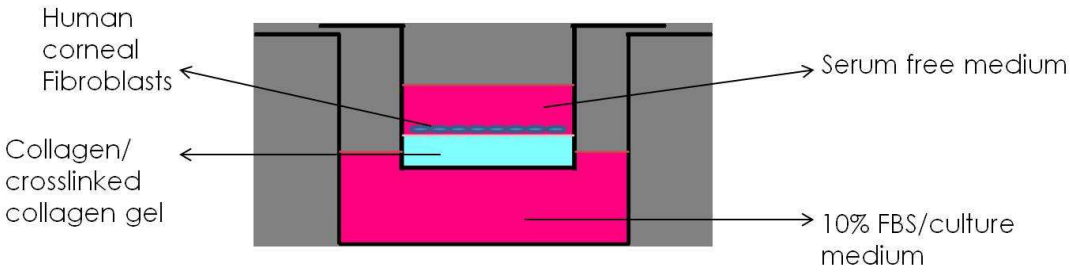

Figure 2.1 Illustration of transwell migration assay. 6.5mm Transwell® with 8.0µm Pore Polycarbonate Membrane Insert (Corning) was inserted into 24-well plate. An extra layer of collagen or crosslinked collagen was added on the top of membrane and hCFs were seeded on the gel. The chemo-attraction generated by the 10% FBS/DMEM would encourage the migration of fibroblasts from upper chamber to lower chamber. By the end of culture, the cell number on the membrane facing the lower chamber was analysed.

2.4.6 CellPlayer™ 96-Well Cell Invasion assay

2.4.6.1 Preparation of collagen coated plate and cell culture

The day before the wound scratching assay, the 96-well plate was first coated with type I collagen. Rat tail collagen I stock solution was diluted into proper concentration using 0.017M acetic acid with stirring at 4°C to make sure the collagen solution was dissolved completely. Total 45µg/cm^2 of collagen I was then added to a 96 well plate and the plate was incubated at 37°C cell incubator for 1 hour. After incubation, the collagen solution was discarded and the plate was washed with PBS (pH7.4) twice and equilibrated in 1X complete culture medium before use. hCFs were trypsinised from the tissue culture flask

and seeded onto collagen coated 96i well plate at the density 8,000 cells/well. Cells were cultured in 10% FBS/DMEM and allowed to form an even monolayer overnight in cell incubator.

2.4.6.2 Scratch wound assay and collagen neutralisation

Collagen gel and TG2 treated collagen gel were prepared as described before and the mixtures were centrifuge for 20 seconds at the top speed using a standard tabletop centrifuge to remove bubbles. The neutralised collagen solutions were kept on ice to avoid polymerisation before use. Once the collagen mixtures were ready, the 96- well plate with 90-100% confluence of hCFs was washed once with PBS (pH7.4) and the wound scratch was performed on cell layer using the 96-well WoundMaker™ (Essen BioScience, Ltd., Hertfordshire, UK). The wounded cell layer was washed with single strength culture medium twice and recovered in culture medium for 5 minutes before the addition of collagen gel. After recovery, the culture medium was carefully pipetted out from each well and 50µg/well of native collagen gel or TG2 crosslinked collagen gel was added on the top of wound cell layer to avoid bubble formation. Polymerisation of collagen gel took at least 2 hour at 37 ^{o}C. Once the gel formed, complete medium was added to each well and the plate was placed into IncuCyte™ (Essen BioScience, Ltd., Hertfordshire, UK).

2.4.6.3 Data collection and analysis

In the IncuCyte™ software, the programme was set to scan 1 image per well, every hour for 24 hours. The invasion of cells was determined by relative wound density (RWD) which was acquired using following equation:

$$\%RWD(t) = 100 \times \frac{W(t) - W(0)}{C(t) - W(0)}$$

w (t) = Density of wound region at time (t)

c (t) = Density of cell region at time (t)

The Relative Wound Density v1.0 algorithm was used to report the data and the average of %RWD from 4 different wells was plotted over the time points to generate a diagram. The real time-lapse movies from each well were also generated using the IncuCyte™ software.

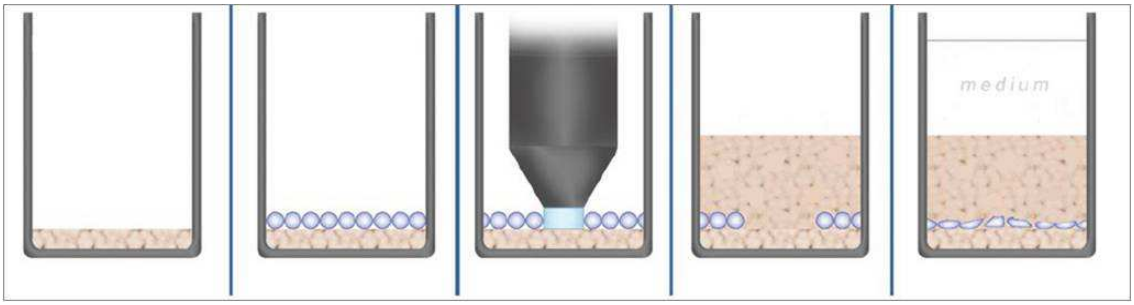

Figure 2.2 Illustration of cell invasion assay. The plate was coated with a thin layer of collagen I protein before seeding cells. Once the cell layer had reached 90-100% confluent, a wound scratch was generated using Essen®96-well WoundMaker™. A layer of native collagen or crosslinked collagen gel was directly added to the wounded cell layer and culture medium was added after polymerisation of matrices. Cells were allowed to incubate and monitored using Essen® IncuCyte™ for at least 24 hours. Figure was adapted from CellPlayer™ cell invasion application note from Essen BioScience, Ltd.

2.5 TG2 Crosslinked Collagen Matrices

2.5.1 Type I Collagen Extraction

Type I collagen was extracted from rat tail tendons following the same procedure used in published paper (Chau *et al.*, 2005). Briefly, tendons were isolated from freshly obtained rat tail, sterilised using 70% (v/v) ethanol for at least 2 hours and allowed to air-dry for 30 minutes. The dried tendons were dissolved in 0.017M acetic acid with stirring at 4^{o}C for 48 hours. Resulted collagen supernatant was centrifuged at 13,000×*g* for 1 hour *at* 4^{o}C to remove debris. The dissolved type I collagen supernatant was collected and neutralised to pH 7.0 using 1M NaOH. Neutralised collagen solution was allowed to be stabilised at 4^{o}C overnight with stirring. After 16 hours incubation, solution was again centrifuged at

3,000×g for 20 minutes at 4°C and resuspended in 0.017M acetic acid. Finally concentration of collagen solution was determined using Lowry method described in Chapter II: section 2. The type I collagen solution was stored and used within 1 month.

2.5.2 Generating and Modifying TG2 Crosslinked Collagen Gel

To fabricate three dimensional collagen scaffolds, collagen solution was mixed with 10X serum free DMEM at ratio 9:1 to make final concentration at 5mg/ml and further neutralised using 1M NaOH to minimise the change of the final volume. All the modification of crosslinked collagen gel happened after neutralisation step. For crosslinked collagen gel and BMP7 incorporated collagen gel, final concentration of 10μg/ml TG2 (unless specific) and/or 2μg/ml of human recombinant BMP7 (Gibco®, Paisley, UK) were added in collagen mixture containing 5mM Dithiothreitol (DTT) and 5mM $CaCl_2$, pH 7.4 upon neutralising and before polymerising of collagen gel. All collagen mixtures were allowed to polymerise at 37°C overnight in cell culture incubator and washed with PBS twice before using.

2.5.3 Determining Trapped TG2 in Crosslinked Matrices

Collagen matrices were prepared for detecting the self incorporation of TG2 into collagen gel. Four different groups were set up in this experiment including 5mg/ml of native collagen gel (NC), TG2 (10 μg/ml) crosslinked collagen gel, R283 pre-inhibited TG2 (10 μg/ml) crosslinked gel and R294 pre-inhibited TG2 (10 μg/ml) crosslinked gel. Inhibitors pre-treated TG2 was prepared. Matrices were allowed to polymerise at 37 °C for 16 hours. On the day of collection, solidified collagen gels were washed with 1X PBS (pH 7.4) three times and directly admixed with 5X Laemmli buffer and boiled in water at 100 °C for 10 minutes until the samples were all dissolved. Electrophoresis on 7.5% SDS-PAGE gel was performed and further transferred to PVDF membrane. Membrane was blocked in 5% dried milk in TBS-T and following mouse monoclonal TG2 (TG100, Thermo Scientific)

antibody was used at 1:1,000 dilution overnight at 4°C. With incubation of secondary anti-mouse HRP conjugated antibody for 2 hours, the signal was visualised with Amersham ECL Western blotting detection reagents (GE Life Sciences).

2.5.4 Analysis of Crosslinked Gel Surface Topography Using Scanning electron microscopy (SEM)

After polymerisation, both native collagen gel (NC) and crosslinked gel (TG2) were first washed with single strength culture medium without serum and fixed with fixative solution which contains 2.5% glutaraldehyde and 1% potassium ferrocyanide in normal strength culture medium for 2 hours at 37°C. Additional wash with serum free medium was performed and samples were freeze dried at a cycle of 50 hours till the matrices were completely dry. The samples then were sputter-coated with carbon and ready for SEM observation. Fibre diameters were measured at the middle of the longest perceptible part of the fibre. Acquired pixels were converted to standard units (μm) of length measurements using SEM image scale bars. At least 15 fibres were measured in each filed and 3 fields were analysed from each sample using Image J software.

2.6 Statistical Analysis

The Kolmogorov–Smirnov test was performed to determine the probability distributions in all individual data sets. For experiments that had one independent variable across 3 or more experimental groups, an one-way ANOVA was used for statistical analysis. For experiments that had one independent variable between two matched experimental groups, a paired t-test was performed for statistical analysis. For experiments that had one independent variable between two sets of experimental groups, data sets were subjected to an unpaired t-test. For experiments that had two independent variables across 3 or more experimental groups, a two-way ANOVA (Turkeys multiple comparisons test) was used for statistical analysis.

Chapter III

Tissue Transglutaminase in Human Osteoblast Mineralisation

1. Introduction

Bone is a highly dynamic tissue which undergoes continuous remodelling processes in response to changes in the mechanical and physiological environment throughout the lifetime (reviewed by SC Marks, 2002). The process of bone remodelling involves two reciprocal activities, resorption of bone matrix by osteoclasts and new bone formation by osteoblasts. Osteoblasts arise from mesenchymal origins and orchestrate bone formation under the systemic regulation of hormones, cytokines and the extracellular microenvironment (Raggatt and Partridge, 2010).

TGs have been implicated in bone development, matrix maturation, calcification and mineralisation in the past decades. Both TG2 and FXIIIa expression were found in cartilage by hypertrophic chondrocytes (Aeschlimann *et al.*, 1993, Aeschlimann *et al.*, 1996, Nurminskaya *et al.*, 1998, Johnson and Terkeltaub, 2005) and in MC3T3 pre-osteoblasts (Al-Jallad *et al.*, 2006) while TG2 showed even wider expression across primary human osteoblasts (HOBs), human osteosarcoma cells HOS and MG-63 (Heath *et al.*, 2001) and rat osteosarcoma cells (Kotsakis and Griffin, 2007). Positive association between increasing TG2 expression and promotion of cell differentiation (Nurminskaya *et al.*, 2003, Johnson and Terkeltaub, 2005) has been described in *in vitro* models. However, the mechanism behind TG-mediated mineral deposition has not been well established. Although most TGs functions are attributed to their enzymatic activity, TG2, as a multifunction protein, may exert its pro-mineralising activity in a transamidase activity-independent way under certain instances (reviewed in Lorand and Graham, 2003, Nurminskaya and Kaartinen, 2006).

The expression of TGs in physiological bone development indicated the appearance of TGs was related to chondrocyte hypertrophy and osteoblast mineralisation (Nurminskaya and

Kaartinen, 2006). The importance of transglutaminase activity in mineralisation has also been demonstrated by Al-Jallad *et al.* (2006) who showed that the mineralisation of mouse pre-osteoblasts was blocked by using transamidase activity inhibitors *in vitro*, even though TG2/FXIIIa double knockouts mice have no abnormality observed in bone formation (Williams *et al.*, 2010). It is reasonable to hypothesise that TG-mediated differentiation could be transamidase activity-dependent since several bone matrix proteins are known to be TGs substrates, for example collagen I, fibronectin, and osteopontin (Mosher and Schad, 1979, Kaartinen *et al.*, 1997, Kaartinen *et al.*, 1999). Studies have indicated that these ECM protein assemblies, in the presence of TG2 could potentially participate in matrix stabilisation and especially cell adhesion processes (Verderio *et al.*, 1998, Chau *et al.*, 2005, Telci *et al.*, 2008, Forsprecher *et al.*, 2009, Wang *et al.*, 2011). Recent work suggests that mineralisation induced by TG crosslinking activity may involve a complex interplay between extracellular matrix proteins and local adjustment of calcium concentration in mineral tissue (reviewed in Nurminskaya and Kaartinen, 2006).

Another question is whether TG2 and/or FXIIIa crosslinking activities are critical for mineralisation. Al-Jallad *et al*. (2006) have shown the importance of TG activity in mineralisation by using TG activity inhibitors to abolish mineralisation in a mouse pre-osteoblast model. Regardless of the expression of TG2 on osteoblasts, it was suggested by Al-Jallad and colleagues (2011) that FXIIIa was the dominant transglutaminase which contributed to cell surface transamidase activity and matrix deposition. However, neither TG2 knockout mice nor FXIIIa knockout mice were reported to have skeletal or dental abnormalities. This suggests that the enzymatic activity of each individual TG is not essential in bone formation or it is very likely that TG2 and FXIIIa can compensate each other in the genetic knockout mice (reviewed by Nurminskaya and Kaartinen, 2006). The

compensation effect of TGs has been demonstrated in TG2 knock-out (TG2-/-) mice where the expression of FXIIIa in femurs of TG2-/- mice was increased (Tarantino *et al.*, 2009). Furthermore, by studying the mRNA transcription levels of different TGs in different tissues from TG2-/- mice, Deasey *et al.* (2013) showed up-regulation of TG1 and induction of TG3 expression within the joint/ ossifying cartilage of TG2-/- mice. TG1 and TG3 compensation for the loss of TG2 expression may also explain the normal skeleton development in TG2/FXIIIa double knock-out mice (Williams *et al.*, 2010).

The aim of this chapter was to evaluate the TG expression and cell surface crosslinking activity during mineralisation by using different approaches. First, the expression pattern of TG2 and FXIIIa in whole cell lysates were evaluated using Western blotting analysis. Secondly, the cell surface TG2 and FXIIIa activities were determined using *in situ* FITC-cadaverine or *in vitro* biotin- cadaverine incorporation assay with or without presence of TG activity inhibitors. Finally, TG2 knockdown expression HOB cell lines were generated to study how TG2 activity is involved in osteoblast mineralisation.

2. Results

2.1 Mineralisation of HOBs

HOBs were cultured in complete medium (CM; 10% fetal bovine serum in Dulbecco's Modified Eagle Medium) or differentiation medium (DM; CM supplied with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate) for 12 days and the mineralisation was determined using von Kossa staining. As shown in Fig. 3.1, HOBs cultured in CM displayed no visible mineral deposition on day 12 while cells cultured in DM showed positive von Kossa staining. It suggested that 12-day differentiation treatment was an appropriate time frame to evaluate osteoblast mineralisation *in vitro* and this model would be used in all following experiment.

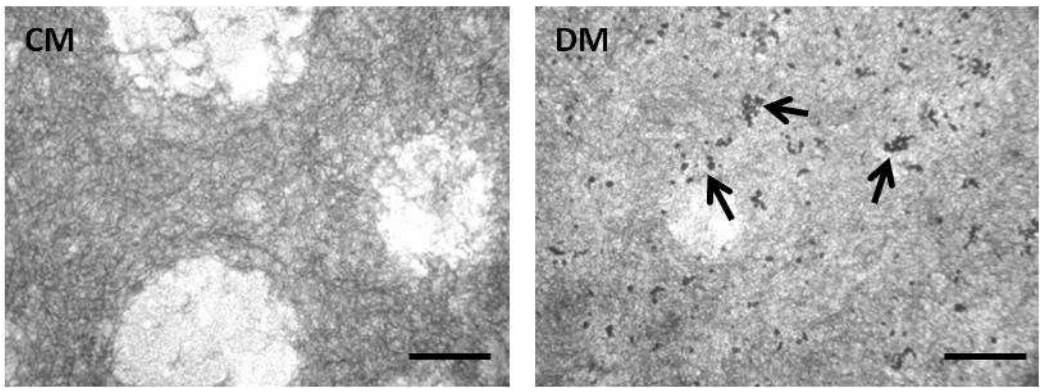

Figure 3.1 Mineralisation of HOBs visualised by von Kossa staining. HOBs cultured with complete medium (CM) or differentiation medium (DM) were subjected to von Kossa staining on day 12. The mineral depositions were stained in black as indicated by arrows. The scale bars represent 300 μm.

2.2 TG2 and FXIII protein expression in HOBs during mineralisation

By monitoring the TG2 expression in whole cell lysates during the mineralisation process, it was found that TG2 levels steadily increased during the whole 12-day culture period (Fig. 3.2A). Although the expression of TG2 in the differentiation medium-treated group was

lower but not significant at the early stage (day 2), it was approximately 1 fold higher than in complete medium at day 12 (Fig 3.2B).

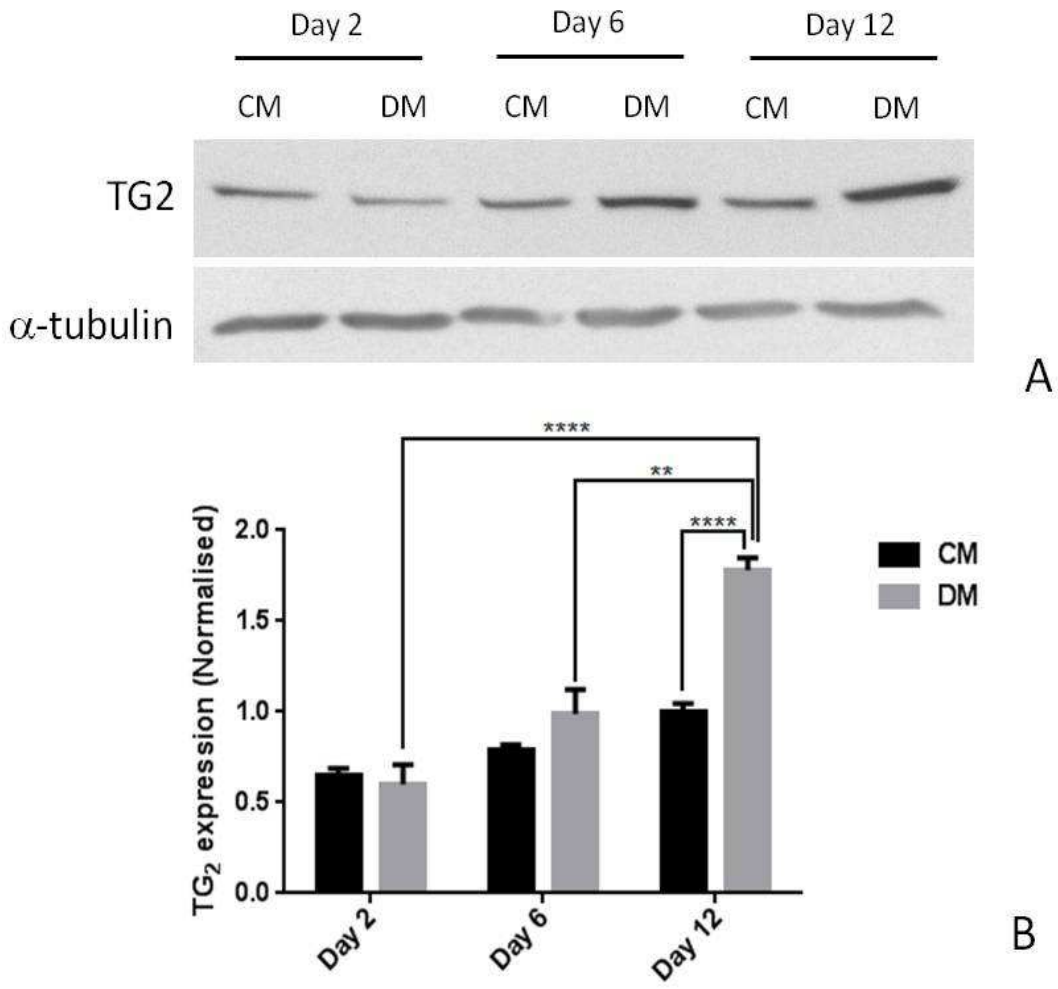

Figure 3.2 Expression of TG2 in whole cell lysate as demonstrated using Western blotting. Whole cell lysates were collected at time point 2 days, 6 days and 12 days post differentiation treatment and Western blotting was performed to analysis TG2 protein expression. A representative western blot result from three individual experiments is shown in (A) α-tubulin was used as loading control and normalised data were showed in chart (B). The results represent mean values +/- SEM. from three individual experiments (n=3). Statistical analysis was carried out using the two-way ANOVA test (Turkeys multiple comparisons test) and the p-values corresponding to p<0.01 was presented with a ** and p<0.0001 was presented with a **.**

Low amounts of ~76kDa and ~51kDa FXIIIa fragments were observed in whole cell lysates during the entire culture period but a short fragment (<25kDa) was most abundant at all time points (Fig. 3.3A). On the other hand, in conditioned medium, ~90kDa and ~51kDa fragments could be detected in HOBs cultured in both CM and DM at all time points and ~76kDa fragments could be detected in conditioned medium from day 6 in DM treated

HOBs (Fig. 3.3B). Also, a low but visible amount of ~80kDa fragment could be observed in DM group on day 12. However, only the <25kDa fragments were majorly found in the biotinylated cell membrane fractions in both groups during mineralisation (Fig 3.3C).

According to literature, full length FXIIIa (80 kDa) is activated by thrombin which cleaves the Arg37-Gly38 peptide bond and releases a 76kDa, transamidase-active subunit, FXIIIa (Chung *et al.*, 1974). Research also has suggested that this cleavage of FXIII mediated by thrombin could produce an enzymatic 51 kDa fragment and inactive 19 kDa fragment (Greenberg *et al.*, 1988) by further proteolysis of FXIIIa. In addition to thrombin, platelet acid protease (Lynch and Pfueller, 1988) and calpain (Ando *et al.*, 1987) have been shown to activate FXIII by cleavage of the Arg37–Gly38 peptide in a similar manner to thrombin. Thus, the ~76kDa and ~51kDa FXIIIa fragments found in this experiment could represent enzyme-cleaved and active FXIIIa fragments. Currently, no study reported ~90kDa and <25kDa FXIIIa fragments and their possible biological functions. The anti-FXIIIa antibody used in this study was in antiserum form, therefore the ~90kDa and <25kDa FXIIIa fragments could be unspecific binding or represent unknown protein bound FXIIIa or cleaved FXIIIa fragments. Although Al-Jallad *et al.* (2011) suggested a large FXIIIa pool could be found in membrane fractions of differentiated mice pre-osteoblast, no full length or active FXIIIa fragments were observed in biotinylated membrane protein fractions. This could be due to different cells types and/or different membrane protein extraction methods used here and in Al-Jallad's study. Therefore, the results in Fig.3.3 showed that possible transglutaminase-active FXIIIa fragments and full length FXIIIa could be found mainly in cells culture medium but not whole cell lysate and membrane fractions during osteoblast mineralisation.

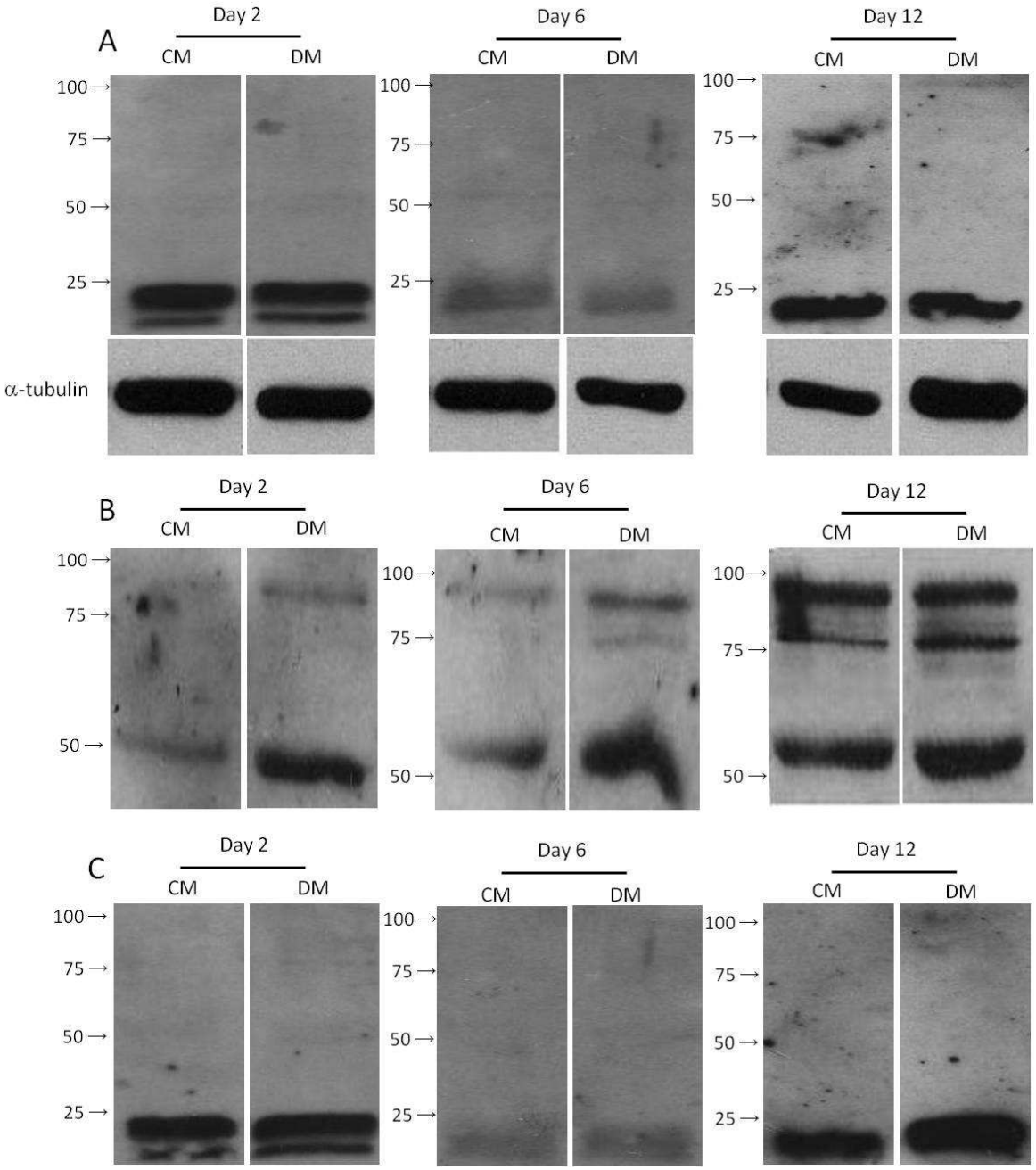

Figure 3.3 Detecting FXIII protein in whole cell lysate and conditioned medium using Western blotting. Western blotting was performed on whole cell lysate (A), conditioned medium (B) and cell membrane protein (C) collected on Day 2, Day 6 and Day 12 post treatment. α-tubulin was used as loading control in whole cell lysate samples.

2.3 Inhibitory effect of TG inhibitors and cell toxicity

In order to distinguish TG2 crosslinking activity from FXIIIa, a TG2 specific inhibitor, R294, and non-specific inhibitor (block both TG2 and FXIIIa), R283 were used in the following assay. Samples were all treated with or without FXIIIa to activate the FXIII latent form. According to the results, R283 was a highly effective inhibitor of TG2 and FXIIIa, at all the concentrations used here (Fig. 3.4A). However, R283 was not able to block the crosslinking activity of Fibrogammin P as efficiently as purified FXIIIa protein. This is possibly because Fibrogammin P is a human plasma-derived product and contains several excipients including human albumin and glucose. This could potentially lower the inhibitory effect. On the other hand, R294 was a TG2-specific inhibitor at lower concentration (<250 μM) without significant inhibitory effect on FXIIIa (Fig. 3.4B).

The cell toxicities of transglutaminase inhibitors were also determined before the using of inhibitors for a 24- hour culture period. As showed in Fig. 3.5, although when R294 used in high dose (500 μM) showed significantly lower XTT reduction rate when compared to the DM group and the R283 (500 μM) at 48 hours, no significant cell toxicity was found at the end of the 96-hour culture period. Therefore, this suggested that both inhibitors showed no significant cell toxicity to the HOBs and could be used during the differentiation process.

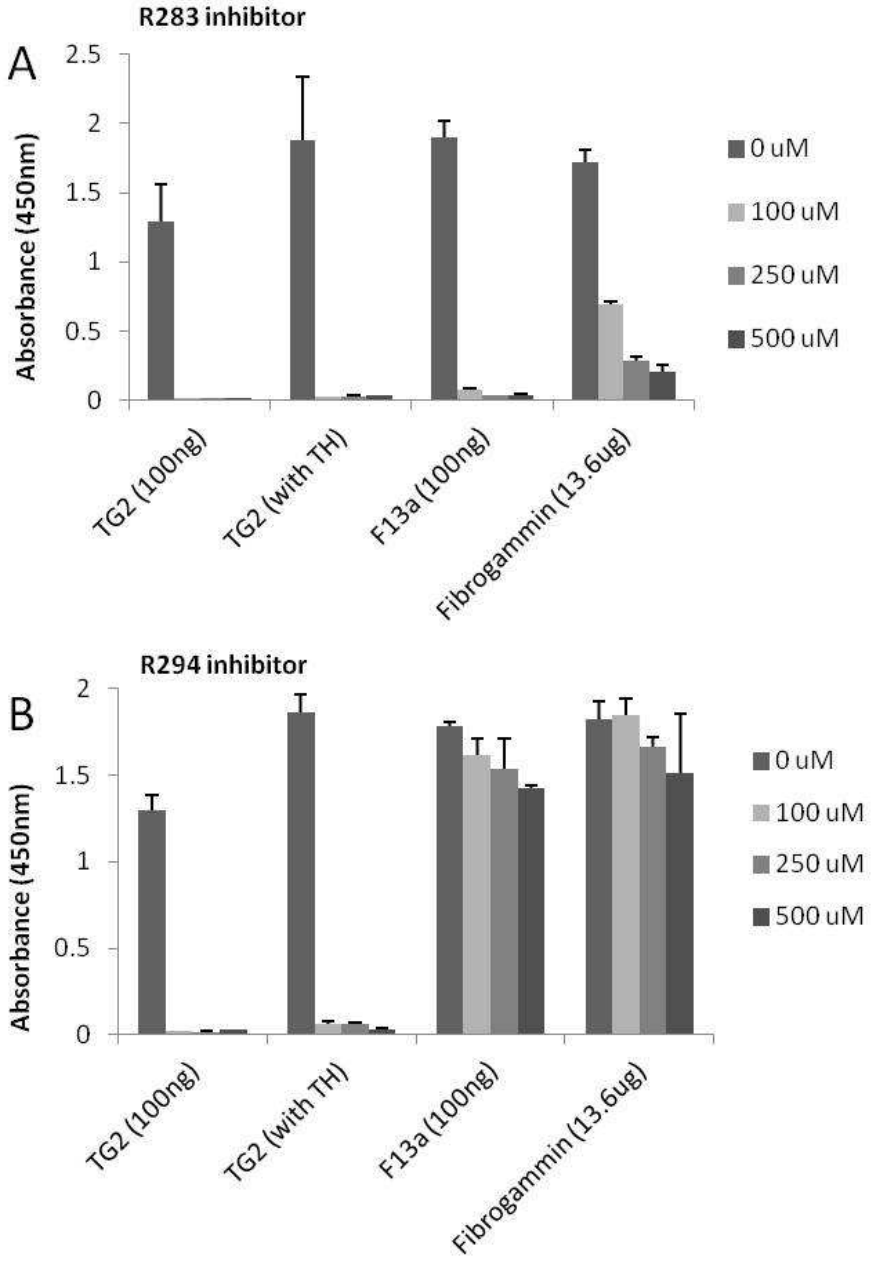

Figure 3.4 Inhibitory assay of inhibitors R283 and R294. Inhibitory effect of (A) R283 and (B) R294 to TG2 (100ng/well) and FXIIIa (100ng/well) determined using biotin cadaverine incorporation into N,N'-dimethylcasein assay. The samples were pre-treated with or without thrombin (TH) as described in Table 2.4 before activity assay. The background absorbance was subtracted from the absorbance value of samples. The results represent mean values +/- S.D where n=3.

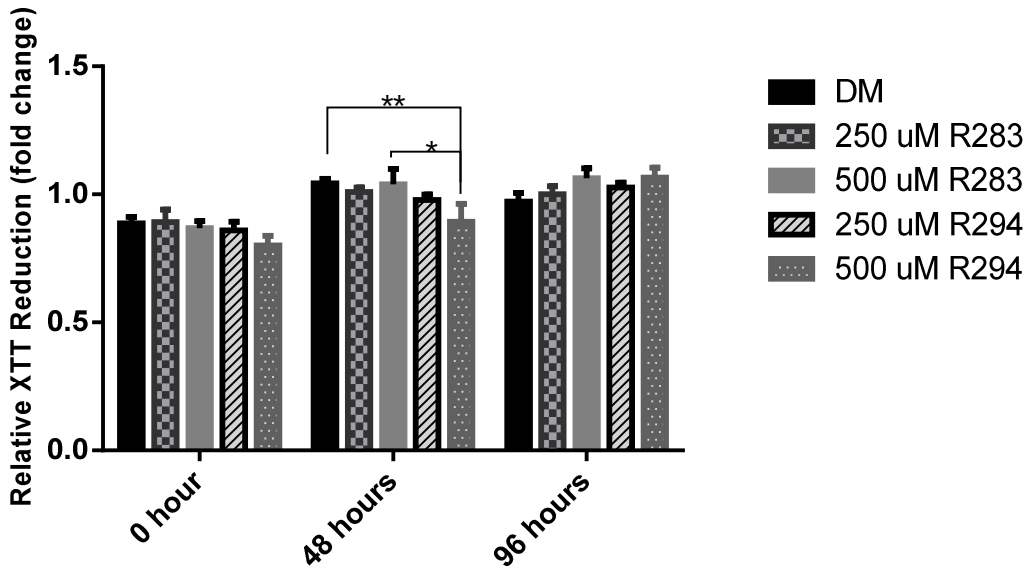

Figure 3.5 Proliferation of HOBs culture treated with crosslinking activity inhibitors. HOBs were seeded at initial number of 24,000/well in a 96-well plate and treated with CM, DM, 250 μM R283 in DM, 500 μM R283 in DM), 250 μM R294 in DM and 500 μM R294 in DM. The proliferation was determined by XTT assay at 0 hour, 48 hours and 96 hours after treatment. The medium was changed every 48 hours. The relative proliferation rate was acquired by normalising the absorbance of experimental groups with the CM group. Data represents mean value +/- SEM from 4 individual experiments (n=4). Statistical analysis using the two-way ANOVA test (Turkey's multiple comparison test) and results showed that besides at 48 hours, no significant difference was found between groups at other time points.

2.4 Cell surface/ECM transglutaminase mediated incorporation of FITC-cadaverine into matrix

In order to see if extracellular TG2 activity increased along with its expression in whole cell lysates during mineralisation process, the crosslinking activity of TGs in differentiated HOBs was determined. An *in situ* method was used to demonstrate cell surface/ECM TG crosslinking activity in HOBs cell culture using FITC- cadaverine. After 2 days (Fig. 3.6), 6 days (Fig. 3.7) or 12 days (Fig. 3.8) differentiation treatment, HOB cell cultures were incubated with FITC-cadaverine with or without R283/R294 for 24 hours in serum free differentiation medium. TG mediated incorporation of FITC-cadaverine into ECM was visualised using a confocal microscope. Incorporation of FITC signal could be detected in both samples treated in complete medium or differentiation medium during the

mineralisation period. Slightly higher but not quantified signal could be found in DM group on day 2 (Fig.3.6) and day 6 (Fig 3.7) when compared to cells cultured in CM. It could suggest that differentiated osteoblasts had higher crosslinking activity in extracellular environment (membrane and/or matrix). Data was not subjected to quantification due to unspecific FITC-cadaverine precipitation on the sample slides which could not be completely removed during the washing step.

On the other hand, partial, but not complete, inhibition of substrate incorporation could be observed while blocking both TG2 and FXIIIa activity with inhibitor R283 and blocking only TG2 activity with R294. Although none of the inhibitors could abolish the crosslinking activity to baseline as in pure protein assay (Fig. 3.4), it might be because of some extracellular TG was potentially masked from inhibitors, for example the enzyme on the cell surface binding to tissue culture/matrix directly could be prevented from binding to inhibitors. Therefore, it suggested that there would be a minimum crosslinking activity presenting in inhibitor-treated *in vitro* system which should be taken into consideration when evaluating data in the future. However, judging from the different inhibition level between R283 and R294, greater inhibitions were found on day 2 cell culture treated with R283 suggesting a greater FXIIIa crosslinking effect was detected. It could imply that the crosslinking activity might shift from FXIIIa dominated at earlier stage toward TG2 dominated at later stage of mineralisation.

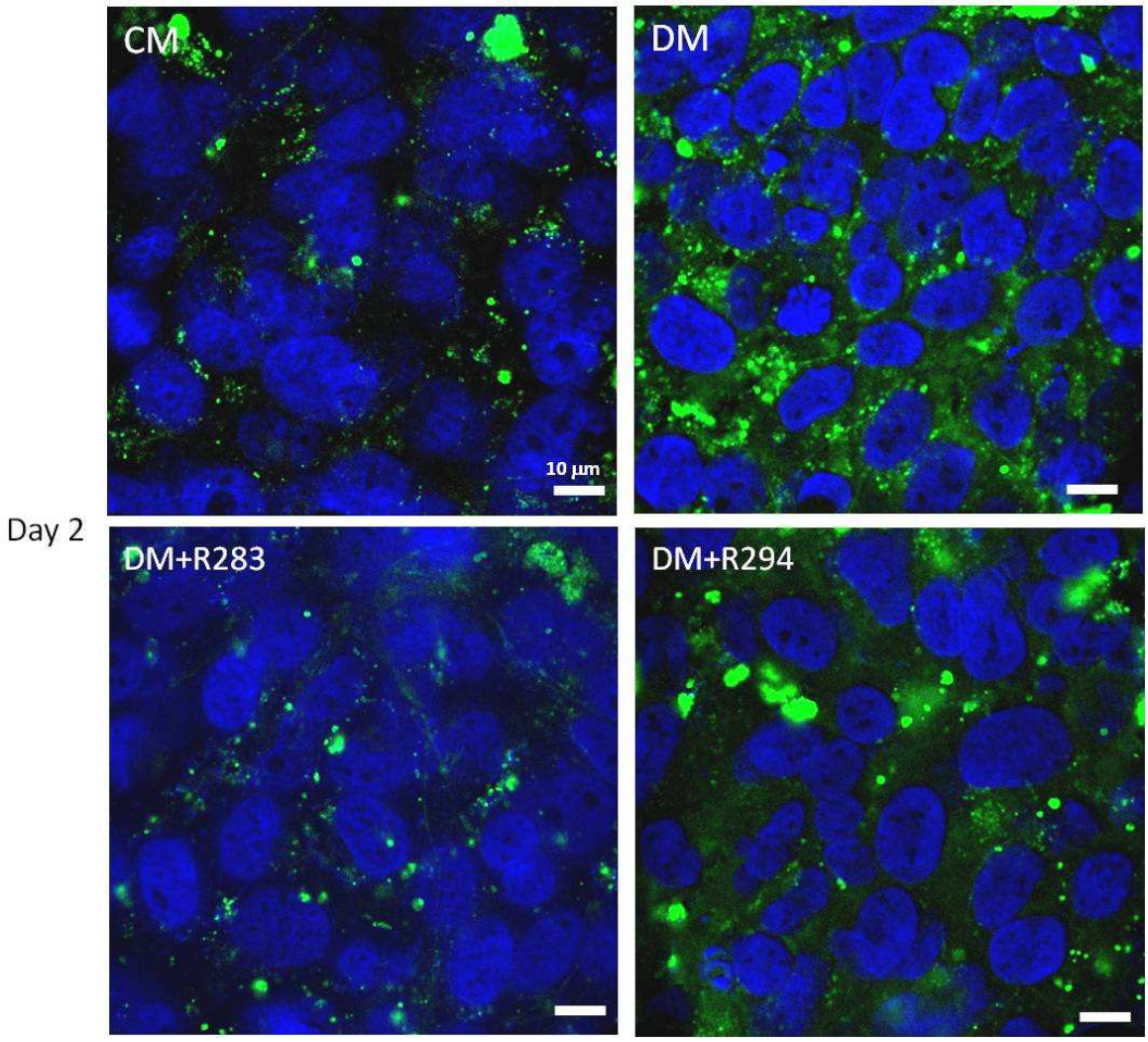

Figure 3.6 Cell surface/ECM crosslinking activity visualised using FITC cadaverine. After 2-day culture in either CM or DM, cells were cultured in 0.1 mM FITC-cadaverine containing serum free complete medium and serum free differentiation medium with or without 250 µM R283 or 250 µM R294 for another 24 hours. The images were acquired by using Leica TCS MP5 multiphoton microscope where the incorporated FITC cadaverine is shown in green and DAPI staining is shown in blue. The scale bar represents 10 µm.

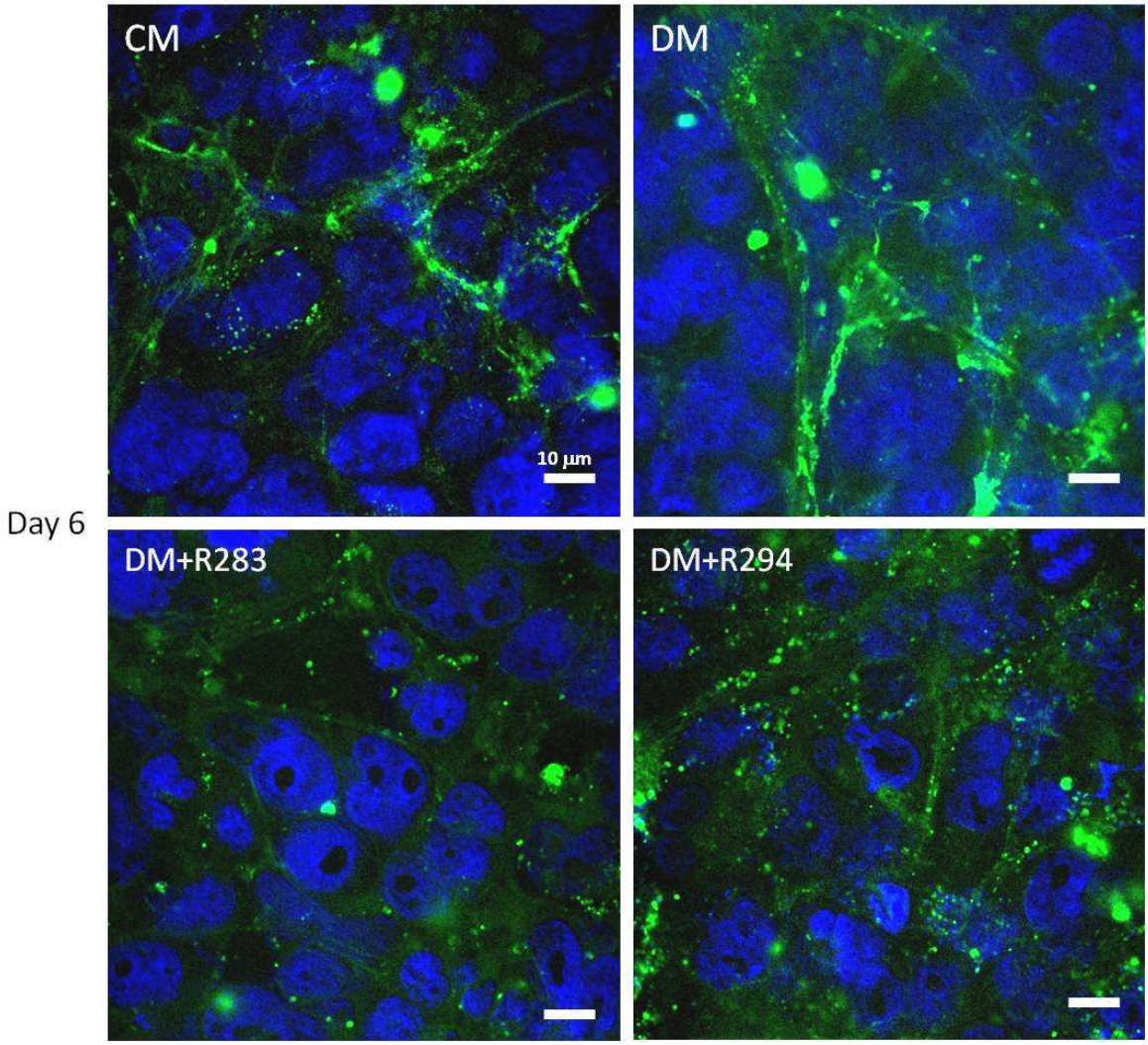

Figure 3.7 Cell surface/ECM crosslinking activity visualised using FITC cadaverine. After 6-day culture in either CM or DM, cells were cultured in 0.1 mM FITC-Cadaverine containing serum free complete medium and serum free differentiation medium with or without 250 µM R283 or 250 µM R294 for another 24 hours. The images were acquired by using Leica TCS MP5 multiphoton microscope where the incorporated FITC-cadaverine is shown in green and DAPI staining is shown in blue. The scale bar represents 10 µm.

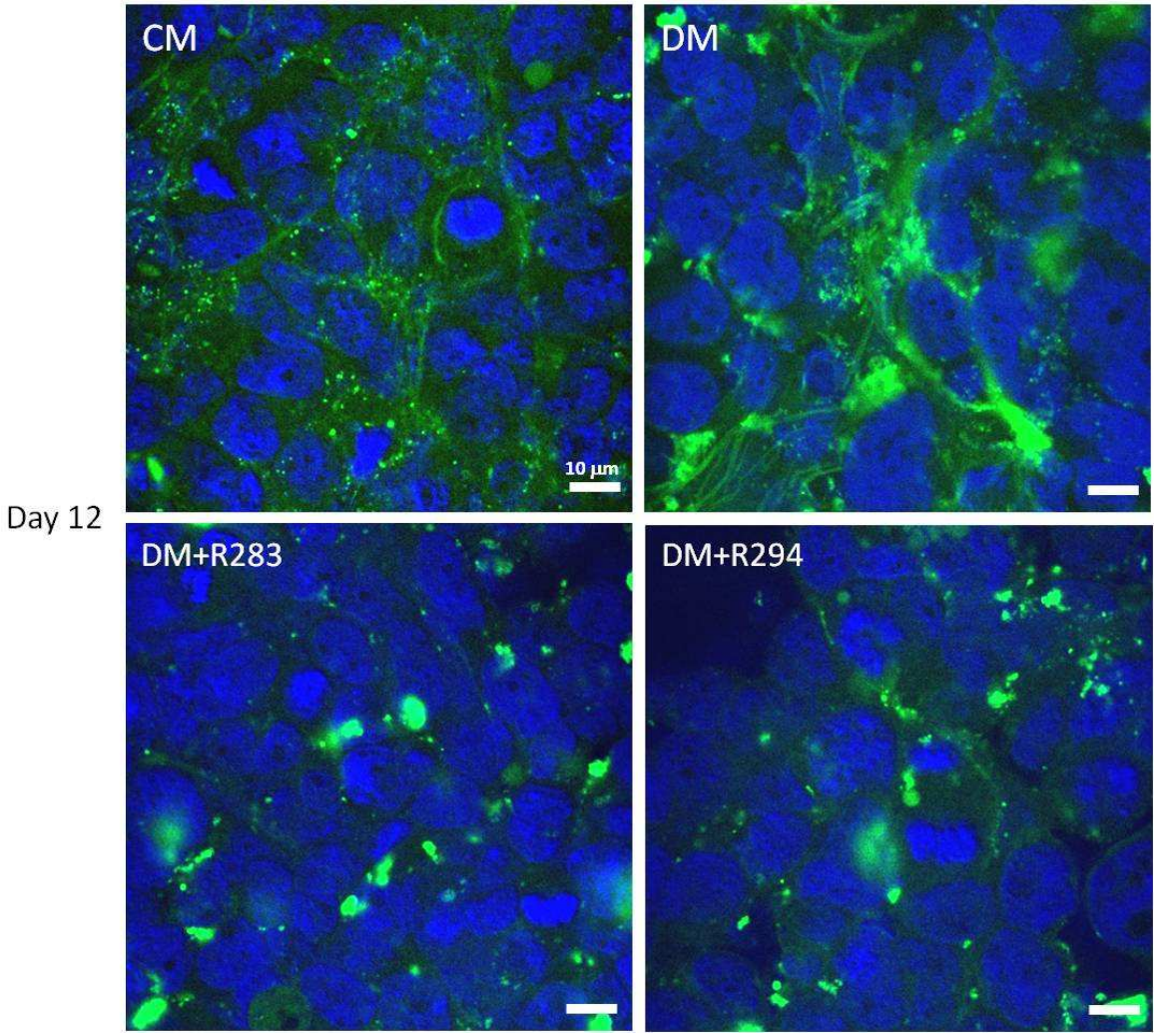

Figure 3.8 Cell surface/ECM crosslinking activity visualised using FITC cadaverine. After 12-day culture in either CM or DM, cells were cultured in 0.1 mM FITC-Cadaverine containing serum free complete medium and differentiation medium with or without 250 µM R283 or 250 µM R294 for another 24 hours. The images were acquired by using Leica TCS MP5 multiphoton microscope where the incorporated FITC cadaverine is shown in green and DAPI staining is shown in blue. The scale bar represents 10 µm.

2.5 Crosslinking activity of the cell surface TG2 and FXIIIa

In order to quantify the crosslinking activity on the cell surface of differentiated cells, another method was used in the following experiment. HOBs cultured in DM for 2 days, 6 days and 12 days were first detached from culture plate, washed and suspended in serum free medium and then subjected to cell surface crosslinking activity assay using the biotin cadaverine incorporation method. This assay was different from the previous *in situ*

system as the cells were detached from the tissue culture plate before assay, therefore, only membrane presenting but not extracellular (cell surface and matrix) TGs activity was assayed. In order to activate the latent FXIII pool on cell surface, samples were pre-activated with thrombin. Thrombin activation of FXIII was first confirmed using human recombinant FXIII protein. As shown in Fig. 3.9A, there was a 5-fold increase in crosslinking activity of FXIII by pre-treatment of thrombin ($p<0.01$) and a slight increase in TG2 activity was also observed after thrombin treatment. This indicated that treating cells with thrombin could amplify the crosslinking effect of latent FXIIIa in osteoblasts. In HOB cell samples, no significant difference in cell surface crosslinking activity was found between HOBs cultured in CM and DM during the 12-day culture. It suggested that the cell surface crosslinking activity was not increased after cell differentiation and the increase in extracellular FITC-cadaverine incorporation (Fig 3.6, 3.7, 3.8) may resulted from trapped TG2 in the matrix.

Thrombin treatment did not significantly alter cell surface TG activity of HOBs cultured in CM. On the other hand, the cell surface TG activity of differentiated cells could be further activated by thrombin treatment at day 2 but not at day 6 and 12. It suggested a presence of FXIII on cell surface at early stage of differentiation. This effect could be further amplified after treating with thrombin such that differentiated HOBs showed higher crosslinking activity when treated with TG2 specific inhibitor (R294) than when treated with non-specific crosslinking inhibitor (R283) at day 2 (Fig. 3.9B). This is consistent with *in situ* FITC-cadaverine incorporation assay that a significantly larger pool of latent FXIIIa appeared when osteoblasts were treated in differentiation medium at an early stage of mineralisation. However, the cell surface crosslinking activity assay was only repeated in twice, further experiments would be necessary to conform this finding.

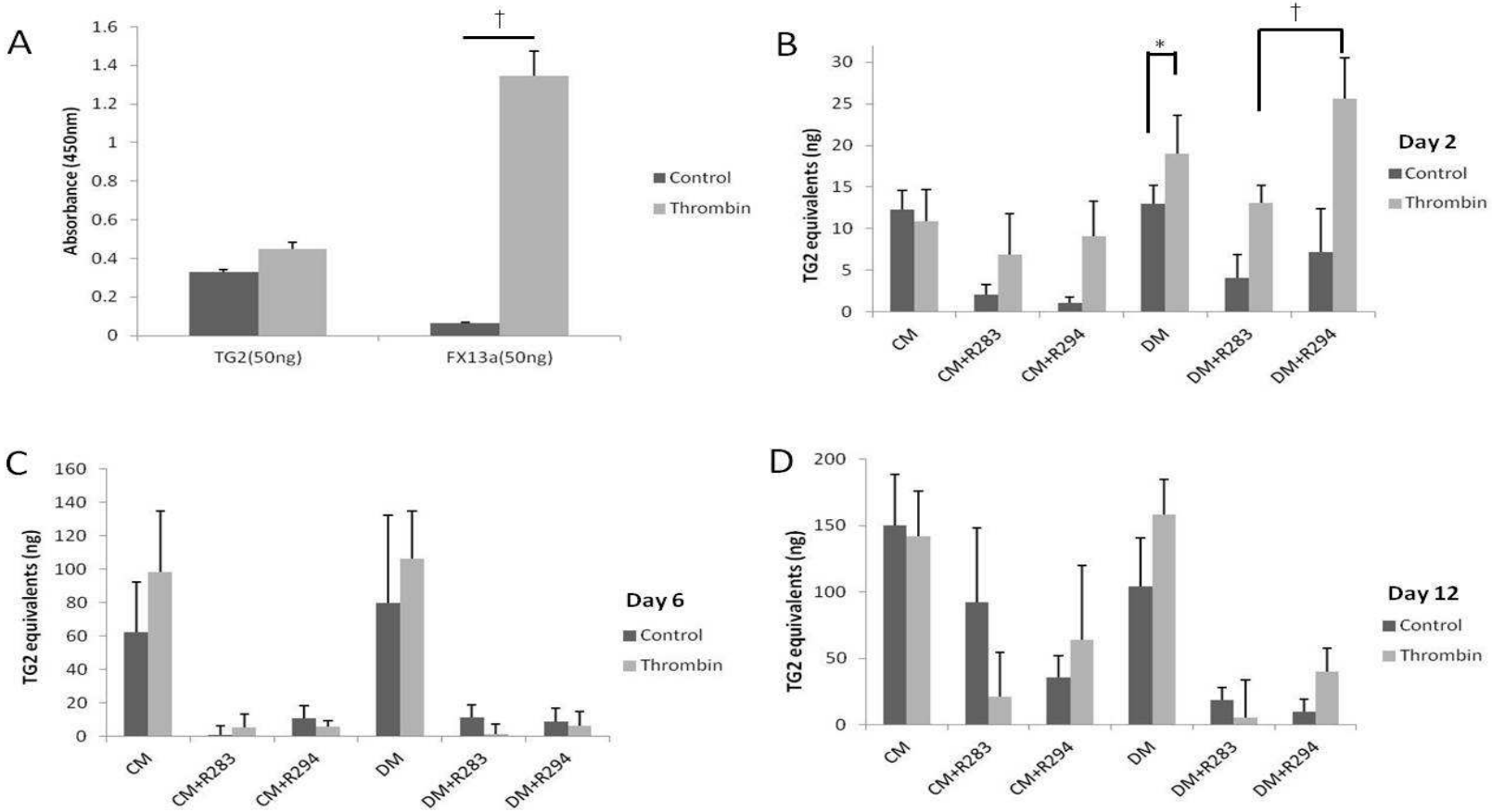

Figure 3.9 Cell surface crosslinking activity determined by biotin cadaverine incorporation assay as described in the Materials and Methods. (A) TG2 and FXIIIa treated with or without thrombin were used as control to validate the assay each time. The data represents mean values +/- S.D. from a representative experiment. HOBs cultured in either CM or DM were harvested on day2 (B), day 6 (C) and day 12 (D) then seeded on fibronectin pre-coated plate with/without crosslinking inhibitors R283 or R294. The crosslinking activity was verified by spectrophotometrically measuring incorporated biotinylated cadaverine. The Y axis stands for equivalent amounts of TG2 (ng) in $1*10^5$ HOBs. Data represents mean values +/- S.D. from a representative experiment with triplicate setting. Statistical analysis was carried out using the one-way ANOVA test (turkey's post test) and the p-values corresponding to P< 0.05 is represented with a * and p<0.01 is represented with a †. Yet, this experiment was repeated twice (n=2) and further experiments would be needed for complete statistical evaluation.

2.6 Generation of TG2 knockdown HOBs cell lines

In order to understand the relationship between TG2 expression and mineralisation, TG2 expression knockdown HOBs were generated for study. *PLKO.1 vector* backbone inserted with non-mammalian target shRNA was used as a control and commercially available TG2 shRNA plasmids,TG2 MISSION® shRNA Plasmid DNA (shRNA 239, 240, 241 and 243, Sigma-Aldrich, Poole, UK), were used to generate stable TG2 knockdown cell lines. PLKO.1 vectors contain puromycin resistance gene for mammalian selection and 500 ng/ml of puromycin was chosen as selective concentration for transfected HOBs according to cell toxicity assay (Fig. 3.10).

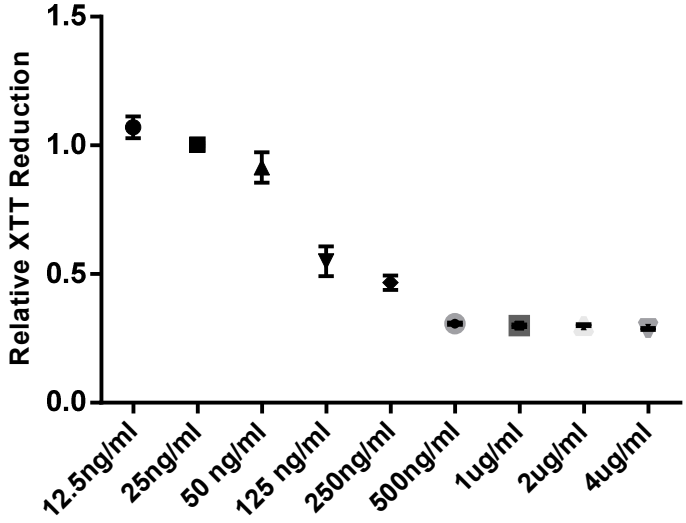

Figure 3.10 Determining cell toxicity of puromycin using XTT assay. HOBs were cultured in 0, 0.125ng/ml, 25ng/ml, 50ng/ml, 125ng/ml, 250ng/ml, 500ng/ml, 1µg/ml, 2µg/ml or 4µg/ml of puromycin /DMEM for 48 hours and XTT assay was performed. Data represents mean value +/- SEM, from 3 experiments (n=3).

HOBs transfected with shRNA 239 (HOB-239), shRNA 240 (HOB-240), shRNA 241 (HOB-241), shRNA 243 (HOB-243) and shRNA Control Plasmid (HOB-SHC) were selected out by treating with 500ng/ml puromycin. The expression of TG2 and FXIII were detected using Western blotting as shown in Fig. 3.11. The TG2 expression was knocked down in HOB-

239, HOB-241 and HOB-243 but not in HOB-240. However, a greater reduction of TG2 expression was found in HOB-SHC cells compared to wild type HOBs. Giving the fact that although TG2 expression decreased in HOB-SHC cell culture, HOB-239 and HOB-241 still presented approximately 60% and 90% of reduction in TG2 expression when compared to HOB-SHC.

Furthermore, the overall FXIII expressions, full length and 76 kDa were found up-regulated in all the transfected cells compared to wild type HOBs (Fig. 3.11 C). It suggested that up-regulation of FXIIIa compensated the loss of TG2 in this model. This is consistent with studies done by Tarantino *et al.* (2009) and Deasey *et al.* (2013) that loss of TG2 was compensated by increased FXIIIa in TG-/- mice. However, the quantification of TG2 and corresponding FXIIIa expression in knockdown HOBs was only done once, further experiments would be needed to validate this finding was statistically significant.

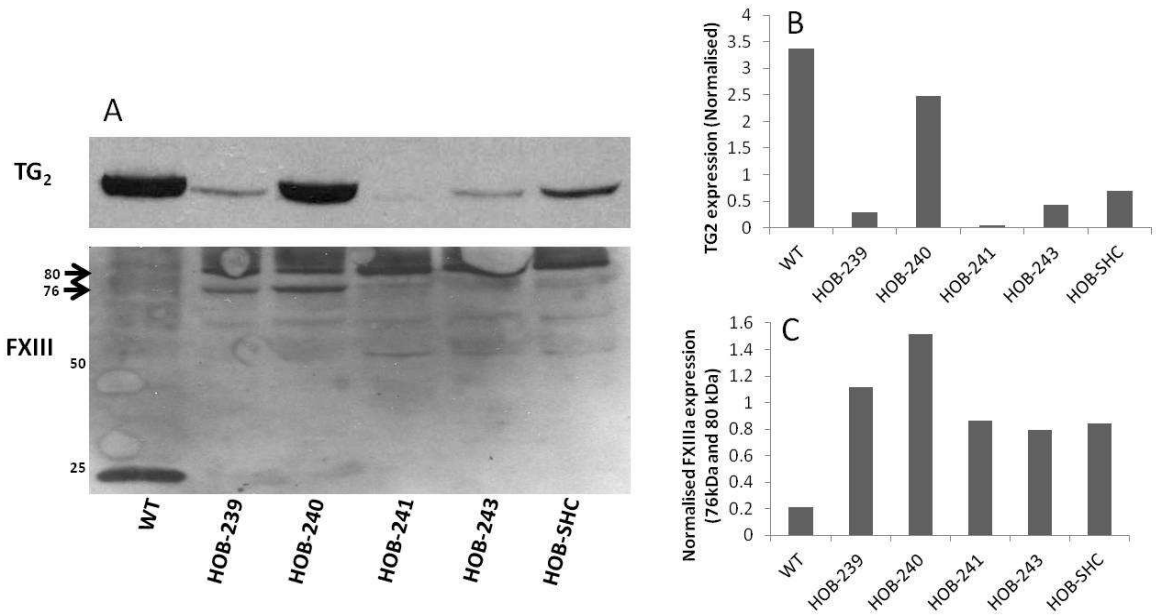

Figure 3.11 Expression of TG2 and FXIII in TG2 knockdown HOB cell lysates as demonstrated using Western blotting. (A) Whole cell lysates were collected from wild type HOBs (WT), shRNA 239 transfected HOBs (HOB-239), shRNA 240 transfected HOBs (HOB-240), shRNA 241 transfected HOBs (HOB-241), shRNA 243 transfected HOBs (HOB-243) and shRNA control plasmid transfected HOBs (HOB-SHC) cell culture and Western blotting was performed to analyse TG2 and FXIIIa protein expression as described in the Materials and methods. α-tubulin was used as a loading control and normalised expression of TG2 and both 80kDa and 76kDa FXIII fragments were shown in chart (B) and chart (C) respectively.

2.7 Mineralisation of TG2 knockdown HOBs

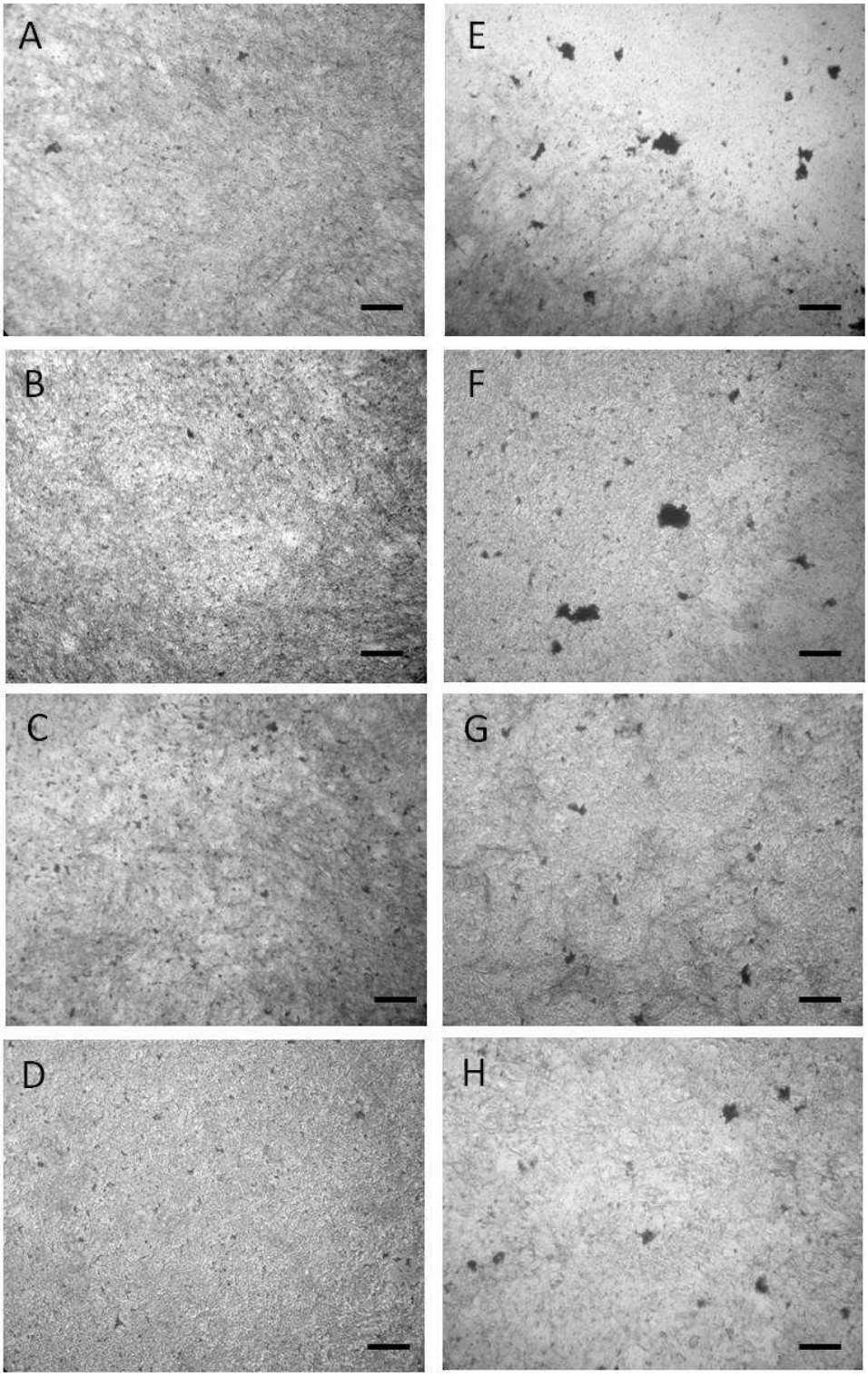

Figure 3.12 Mineralisation of HOBs visualised by von Kossa staining as described in the Materials and Methods. Wild type HOBs (A, E), vector transfected control cell line: HOBs-SHC (D, H) and TG2 knockdown HOBs: HOBs-239 (B, F) and HOBs-241 (C, G) cultured on tissue culture plate were treated with DM (A, B, C, D) or 1μg/ml of gpTG2 in DM (E, F, G, H). After 12 days, samples were fixed and von Kossa staining was performed. Samples were viewed at ×40 magnification using a Nikon CK2 (10× objective) and photographed with an Olympus DP10 digital camera (4× optical zoom). The mineral deposition is stained in black. The scale bars represent 100μm.

The mineralisation of TG2 knock down cells was determined in order to further assay the mineralisation ability of TG2 knockdown cells and test if adding back exogenous gpTG2 could recover the effect of reduced TG2 expression. The TG2 expression knockdown cell lines, HOBs-239 and HOBs -241, cultured with or without exogenous TG2 were subjected to mineralisation assay on day 12. According to the von Kossa staining (Fig. 3.12), mineralisation occurred in all cell lines grown in differentiation medium. It suggested that knockdown of endogenous TG2 did not affect cell mineralisation. Even though no significant association was found between endogenous TG2 levels and mineralisation (Fig. 3.13A), exogenous TG2, surprisingly, improved cell mineralisation in a significant manner. The exogenous TG2-induced mineralisation could be observed in both wild type and TG2 knockdown cells with an approximately 2 to 3 fold increase compared to non-treated one (Fig. 3.13B).

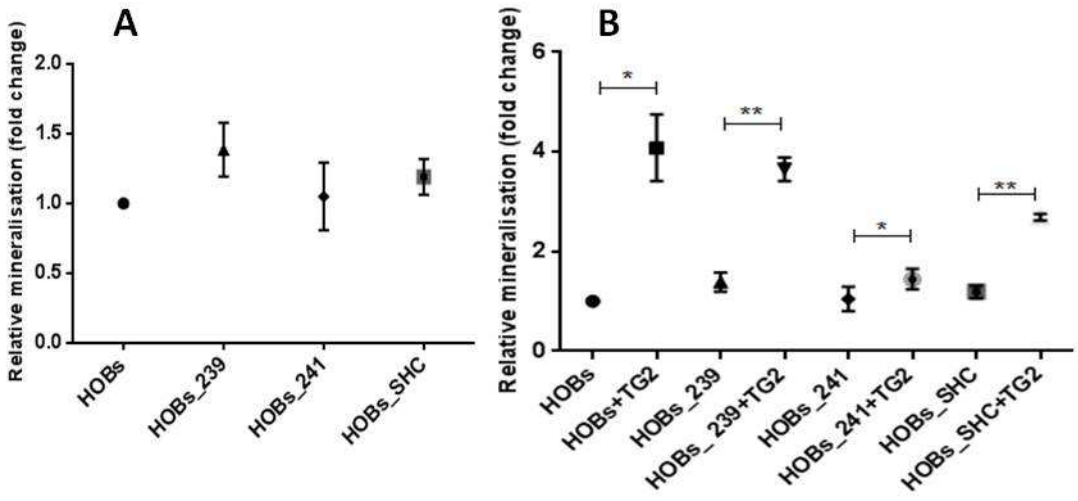

Figure 3.13 Relative mineralisation of HOBs culture. The mineralised area was visualised by using von Kossa staining and the percentage area of positive staining was quantified by image J. The diagram represents the mean values ±SEM of mineralised area where n=3. In diagram (A), the statistic analysis was carried out using the one way ANOVA (Dunnett's multiple comparison test). No significant difference was suggested between wild type HOBs and TG2 knockdown HOBs. In diagram (B), the statistical analysis was carried out using the paired t test. Exogenous TG2 enhanced mineralisation in both wild type and TG2 knockdown HOBs. The p-values <0.05 are presented with a * and the p-values <0.01 are presented with a **.

3. Discussion

The aim of this study was to understand the transglutaminase expression, both TG2 and FXIIIa, during mineralisation and evaluate the relationship between crosslinking activities and expression of enzymes in osteoblast differentiation using several approaches including functional inhibitors and gene silencing techniques.

The expression of full length TG2 analysed by Western blotting was found to steadily increase during mineralisation. This finding was in contrast with previous works which suggested that the TG2 protein expression (Al-Jallad *et al.*, 2006) and mRNA level (Piercy-Kotb *et al.*, 2011) remained constant after differentiating. On the other hand, it was also demonstrated that FXIIIa was detected in HOBs culture during mineralisation and the transglutaminase active fragments of FXIIIa distributed mainly in conditioned medium but not in whole cell lysates and membrane protein factions. However, according to cell surface crosslinking activity, a clear shift from TG2 dominated crosslinking activity to FXIIIa dominated crosslinking activity was observed on osteoblast cell surface 2 days after treating with differentiation medium. This finding supported the model proposed by Al-Jallad *et al.* (2011) that expression of FXIIIa on cell membrane occurred only after inducing differentiation and this membrane FXIIIa was suggested to be involved in collagen I secretory machinery.

Another trend observed in *in situ* crosslinking activity was that differentiating osteoblasts had mainly TG2 oriented activity in later stages of mineralisation. According to cell surface TG activity assay, there was no significant difference in cell surface crosslinking activity of differentiated cells and non-differentiated cells during mineralisation, therefore, the increase in FITC-cadaverine incorporation observed in differentiated cell culture may have resulted from TG2 trapped in the ECM. Many studies have showed that the interaction

between collagen I and fibronectin in the extracellular matrix is important for a more stable matrix formation (McDonald *et al.*, 1982, Dzamba *et al.*, 1993, Shi *et al.*, 2010). This could imply that TGs activity plays a critical role in stabilising the extracellular matrix. Although type I collagen could act as a FXIIIa substrate *in vitro* (Mosher, 1984), no further study suggested that crosslinking of collagen I by FXIIIa was found in cell culture systems (Barry and Mosher, 1989). Hence, it would be reasonable to assume that TG2 dominated crosslinking activity at later stages of mineralisation could directly contribute to matrix stabilisation considering TG2 has a wider range of substrates compared to FXIIIa (Mosher and Schad, 1979, Mosher, 1984, Aeschlimann *et al.*, 1995, Kaartinen *et al.*, 2005).

In this chapter, the correlation between the expression of TG2 and osteoblast mineralisation was also examined using TG2 knockdown cells. Mineralisation assay of TG2 knockdown cell lines showed that, even with lower endogenous TG2 expression, knockdown of TG2 in osteoblasts had no effect on mineralisation. Since the TG2 expression was not completely knocked down in the HOBs in this system, the possibility that minimum TG2 expression and/or crosslinking activity was sufficient for osteoblast mineralisation cannot be ruled out. Furthermore, the notion that expression of one TG isoform could be compensated by other members of the TG family was supported by an up-regulation of FXIIIa expression that was observed in TG2 knockdown osteoblasts. This was also consistent with the data that TG2 or FXIIIa or TG2/FXIIIa knockout mice displayed no skeletal or dental abnormalities, possibly due to the compensation between TG2, FXIIIa and other TGs expression (Nurminskaya and Kaartinen, 2006). Therefore, the knockdown models had their own limitation to study the role of TGs in mineralisation and the development of TG2/FXIIIa double knockout model in the future may not be sufficient enough to understand how TGs participate in bone development and formation.

An unexpected result, however, was that HOBs cultured with exogenous TG2 could enhance mineral deposition in both wild type and TG2 knockdown osteoblasts. Along with increasing TG activity observed *in situ*, these data implied that extracellular TG2 could contribute to cell mineralisation. Several pathways have been proposed for how TG2 mediates osteoblast mineralisation via transamidating dependent and independent mechanisms. First, TG2 is recognised as a matrix stabiliser and TG2 crosslinked matrix is known to increase cell adhesion and HOB proliferation (Chau *et al.*, 2005). Therefore, a matrix stabilised by TG2 could enhance HOB mineralisation by accelerating HOB maturation from the proliferation stage to the mineral deposition stage. Also, extracellular TG2 is proposed to contribute to mineralisation through transamidase activity independent pathways, mostly likely through ATPase activity (Nakano *et al.*, 2007, Nakano *et al.*, 2010) or activation of β-catenin pathway (Faverman *et al.*, 2008).

Thus, taking these results and the current study into account, a proposed model of TGs in cell mineralisation is summarised in Fig. 3.14. An early increase in membrane FXIIIa after treating with DM could be involved in the type I collagen secretion pathway (Al-Jallad *et al.*, 2009). Meanwhile, the matrix organisation and stabilisation process involving both FXIIIa and TG2 activity in which the accumulating TG2 in ECM and increasing active FXIIIa in medium during mineralisation could modify fibronectin and collagen I fibres. In particular, the localised TG2 in the ECM, compared to FXIIIa in medium, plays a major role in crosslinking ECM proteins in later stages of the mineralisation process as demonstrated by the *in situ* FITC incorporation assay. TG2 may also interact with integrins and syndecans which further activate adhesion and proliferation signalling with or without binding of ligand to integrins (Wang *et al.*, 2010). Extracellular TG2 has also been found to interact with LRP5 receptors, which consequently activate β-catenin signalling and the canonical Wnt pathway (Faverman *et al.*, 2008). Finally, TG2 is also proposed to behave as an

ATPase after MMP cleavage and contribute to mineralisation by regulating extracellular Pi concentration (Nakano *et al.*, 2007, Nakano *et al.*, 2010). In the following chapters, the role of extracellular TG2 in mineralisation will be discussed in more detail.

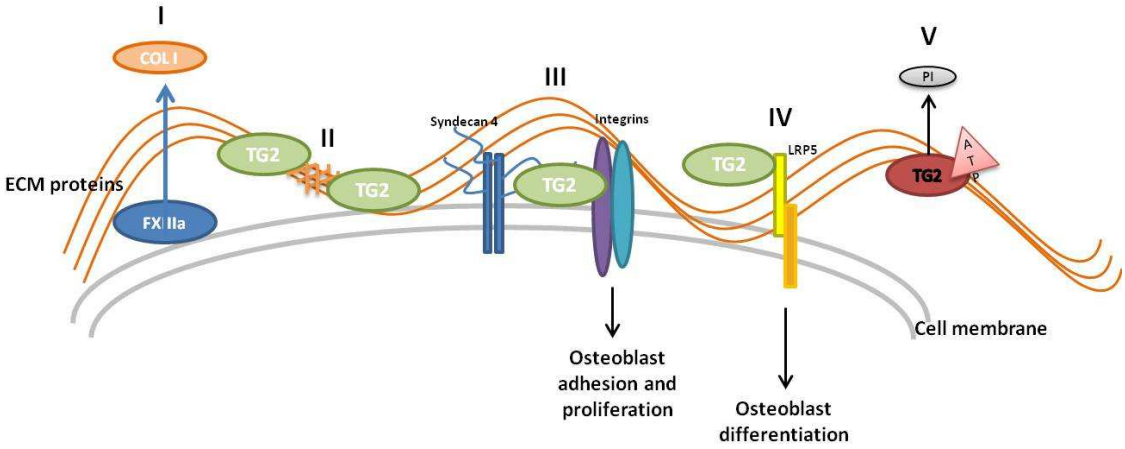

Figure 3.14 A putative scheme of current studies in extracellular TG2 and osteoblasts mineralisation. (I) Presenting of membrane FXIIIa crosslinking activity is stimulated by differentiation medium and it is related to collagen I (COL I) secretion. (II) Membrane and matrix TG2 activity mediates crosslinking of ECM proteins. (III) Extracellular TG2 enhances cell adhesion and proliferation via integrin and syndecans. (IV) Binding of TG2 to LRP5 on cell surface triggers osteogenesis gene transcription. (V) Cleavage of TG2 by MMPs increases ATPase activity of TG2 and further regulates the Pi concentration.

Chapter IV

Tissue Transglutaminase in Human Osteoblast Mineralisation- Crosslinking Activity Independent Pathway

1. Introduction

Immunohistochemistry of mammalian mineralized bone tissue showed that TG2 was highly expressed not only in osteoblasts located in the perichondrium/periosteum area during bone development but also in borderline chondrocytes which are located at the advancing edges of the growth plate (Nurminskaya and Kaartinen, 2006). In fact, increasing data has shown that exogenous TG2 could promote pre-osteoblast differentiation (Nurminskaya *et al.*, 2003), chondrocyte maturation to hypertrophy (Johnson and Terkeltaub, 2005) and calcification of vascular smooth muscle cells (VSMCs) (Faverman *et al.*, 2008).

TG2 is a multifunctional enzyme whose function is regulated by levels of Ca^{2+}/ nucleotides (Lai *et al.*, 1998, Liu *et al.*, 2002) and MMP proteolysis (Belkin *et al.*, 2001, Belkin *et al.*, 2004) in its microenvironment. Although the importance of transamidase activity of TGs was demonstrated by Al-Jallad *et al.* (2006), further studies suggested that it was a FXIIIa dependent pathway rather than a TG2 mediated effect (Al-Jallad *et al.*, 2009, Al-Jallad *et al.*, 2011). Given the fact that TG2 enzymatic activity may not play a dominant role in mineralisation and its activity is transient in non-reducing microenvironments, it is reasonable to assume that TG2 also regulates bone mineralisation in a non-transamidating dependent manner. In this chapter, two hypotheses were examined: that TG2/ATPase regulates mineralisation and that TG2 stimulates the LRP5/ Wnt/ β-catenin pathway.

ATPase activity of TG2

Local phosphate (Pi)/ pyrophosphate (PPi) ratio is suggested to play an active role in regulation of physiological and pathological mineralization. Several enzymes are recognised to regulate Pi and PPi concentration extracellularly including tissue non-specific

alkaline phosphatase (TNSALP) and plasma membrane Ca^{2+} ATPase1 (PMCA1). The concept of TG2 involved in regulating Pi levels via its ATPase activity was first raised by Nakano *et al.* (2007). It was suggested that ATP could induce mineral deposition in a similar way to conventional β-GP treatment by serving as an alternative source of phosphate groups. This ATP-induced mineralisation was not affected by TNSALP inhibitor but was affected by PMCA inhibitor and TG2 crosslinking inhibitor. Yet the release of Pi from ATP was not suppressed by PMCA inhibitor but was by TG2 inhibitor as demonstrated in the same study. Therefore, Nakano *et al.* proposed that PMCA1 could contribute to mineralisation by pumping calcium to ossified sites and TG2 could act as an ATPase in the ECM. This hypothesis was further expanded by the same group in which they showed that MTI-MMP may act as a modulator to activate the pro-mineralization function of TG2 (Nakano *et al.*, 2010).

LRP5/ Wnt/ β-catenin pathway

Canonical Wnt signalling (Fig 3.1) is suggested as a major pathway to control osteoblast differentiation and bone formation in differentiated osteoblasts. The Wnt/β-catenin signalling pathway controlled by LRP5 in bone mass regulation has been widely investigated. Loss-of-function and gain-of-function mutants of LRP5 showed osteoporosis and high bone mass phenotypes, respectively, in human diseases (Gong *et al.*, 2001, Boyden *et al.*, 2002, Little *et al.*, 2002b). As seen in Fig. 4.1, under resting conditions, a degradation complex forms which comprises two scaffold proteins: Axin and adenomatous polyposis coli (APC) and two degradation complex kinases: glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α). This complex mediates the phosphorylation of β-catenin and results in ubiquitination of β-catenin by β-transducin repeat-containing protein (β-TrCP). The ubiquitinated β-catenin is subsequently degraded by the 26S proteosome. Upon Wnt binding to Frizzled and co-receptors LRP5/6, the Dishevelled (Dvl

or Dsh) protein is recruited to the plasma membrane and it further aggregates axin to the LRP5/6 cytoplasmic tail. Sequestration of axin by LRP receptors leads to inhibition of β-catenin degradation complex and thus increases the accumulation of β-catenin and its translocation to the nucleus. Once β-catenin is translocated to the nucleus, it competes with Groucho family protein, a transcriptional repressor, from binding to lymphoid enhancing factor (LEF) and T-cell factor (TCF) and acts as a transcriptional co-activator to modulate downstream gene expression (reviewed by Angers and Moon, 2009).

Wnt canonical signalling is tightly regulated by Wnt proteins and/or Wnt signalling partners. Several endogenous and synthetic inhibitors targeting different components in the Wnt canonical pathway are well studied, including Dickkopf (Dkk), secreted Frizzled-related protein (sFRP), Wnt inhibitory factor (WIF), XAV939 compound, IWR compound and pyrvinium. XAV939 is a synthetic small molecule which can inhibit tankyrase (TNKS). The inhibition of TNKS activity results in prolonging the half life of axin and stabilisation of the axin/ APC/ GSK3α/β complex which consequently increases β-catenin degradation.

The interaction of TG2 and LRP5 was first reported by Faverman and co-workers (2008). They demonstrated binding of exogenous TG2 to cell membrane LRP5 and subsequent activation of β-catenin signalling including translocation of β-catenin to the nucleus. Another cell membrane receptor, N-Cadherin, was also related to osteoblast mineralisation and β-catenin signalling. It was suggested that N-cadherin sequestered β-catenin at the cytoplasmic membrane by direct protein interaction thus inhibiting WNT signalling. Also, it was recently found that N-cadherin could negatively regulate β-catenin signalling via interaction with the LRP5 receptor (Marie, 2009).

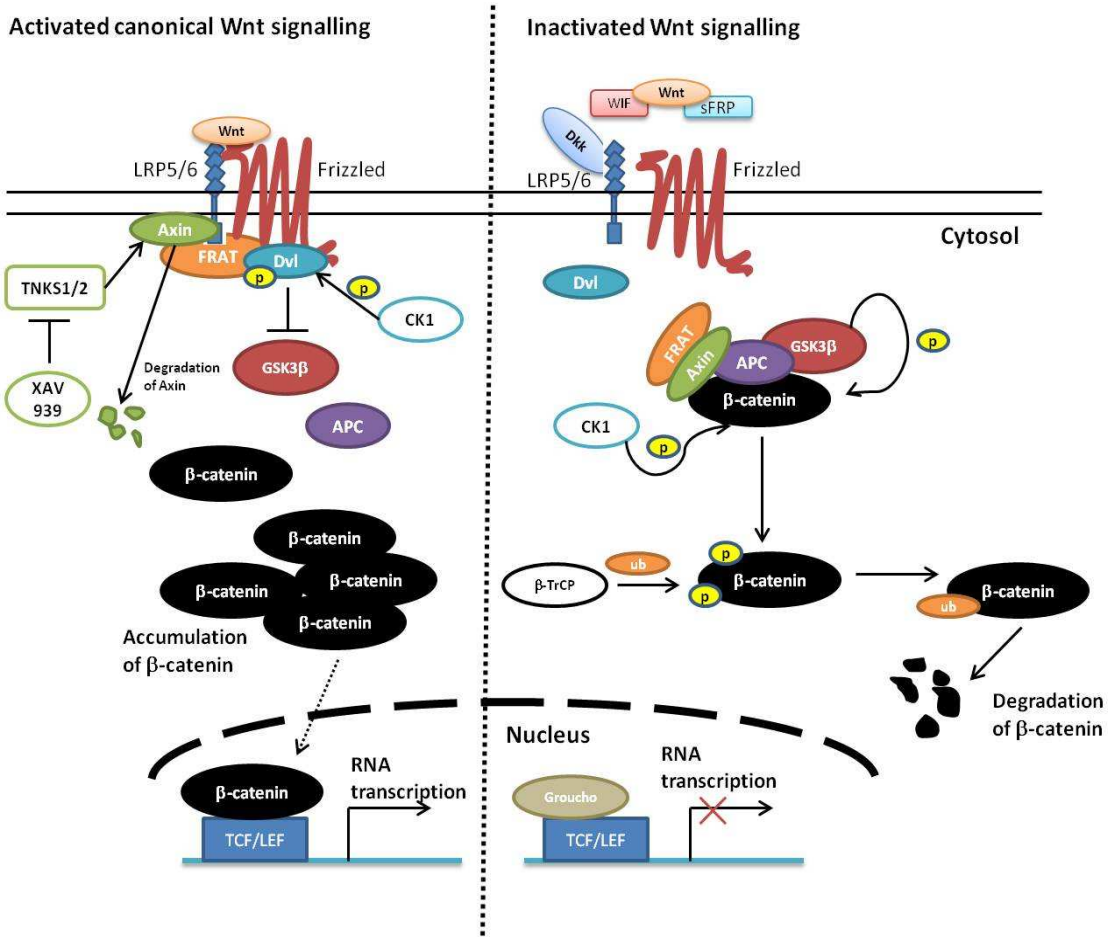

Figure 4.1 The current model of the Wnt canonical pathway implies that binding of Wnt to LRP5 and Frizzled leads to recruitment of axin to LRP5, inhibition of GSK-3β, decrease in phosphorylation of β-catenin and subsequent translocation of β-catenin into the nucleus where it activates TCF/LEF transcription factors and target genes. In the absence of Wnt-signal, β-catenin is targeted for degradation mediates by formation of the APC/Axin/GSK-3β-complex. Phosphorylation of β-catenin by CK1 and GSK-3β leads to its ubiquitination and following proteasomal degradation. β-TrCP- β-transducin repeat-containing protein, CK1- Casein kinase 1, Dvl (or Dsh) - Dishevelled-1, FRAT- Frequently rearranged in advanced T-cell lymphomas, LEF- Lymphoid enhancing factor, TCF- T-cell factor and ub- ubiquitin.

Therefore, the aim of this chapter was to examine current hypotheses of how extracellular TG2 contributes to mineralisation. To address this goal, first, the involvement of TG2 crosslinking activity in HOB mineralisation was determined. Secondly, the possibility of enzyme-mediated TG2 cleavage in osteoblast cell culture and its relationship with mineralisation were examined and collagenase and gelatinase profiles of differentiated HOBs was also analysed. Finally, the expression and interaction of TG2 and LRP5 were examined and the activation of downstream pathway was further evaluated using immunohistochemical and β-catenin inhibitor, XAV939.

2. Results

2.1 Mineral deposition of HOBs treated with crosslinking inhibitors

The expression of endogenous TG2 as well as extracellular crosslinking activity increased during the mineralisation were demonstrated in previous chapter, however, TG2 knockdown HOBs showed no effect on mineralisation and it was plausible that up regulation of FXIIIa expression compensated for the loss of TG2. In order to understand whether the crosslinking activity of endogenous TG2 was essential for mineralisation, a TG2 specific inhibitor (R294) and a TG2/FXIIIa inhibitor (R283) were used to block the endogenous crosslinking activity during mineralisation. Since both inhibitors showed no cell toxicity at working concentrations, HOBs were cultured with R283 or R294 supplemented DM for 12 days and the mineralised area of HOBs was evaluated at the end point. It appeared in Figure 4.2 that no mineral deposition was detected by von Kossa staining in negative control while there was positive staining in DM group. Surprisingly, cells treated with inhibitors still presented positive von Kossa staining after 12 days culture. Further analysis of the mineralised area from each group (Fig. 4.3) suggested that mineralisation was not inhibited when treated with non-specific inhibitor (R283) and TG2-specific inhibitor R294. This result indicated that crosslinking activity of transglutaminase, both FXIIIa and TG2, may not play a dominant role in HOBs mineralisation. Nevertheless, the possibility that residual activity present in the cell/ECM was sufficient for mineralisation could not be ruled out.

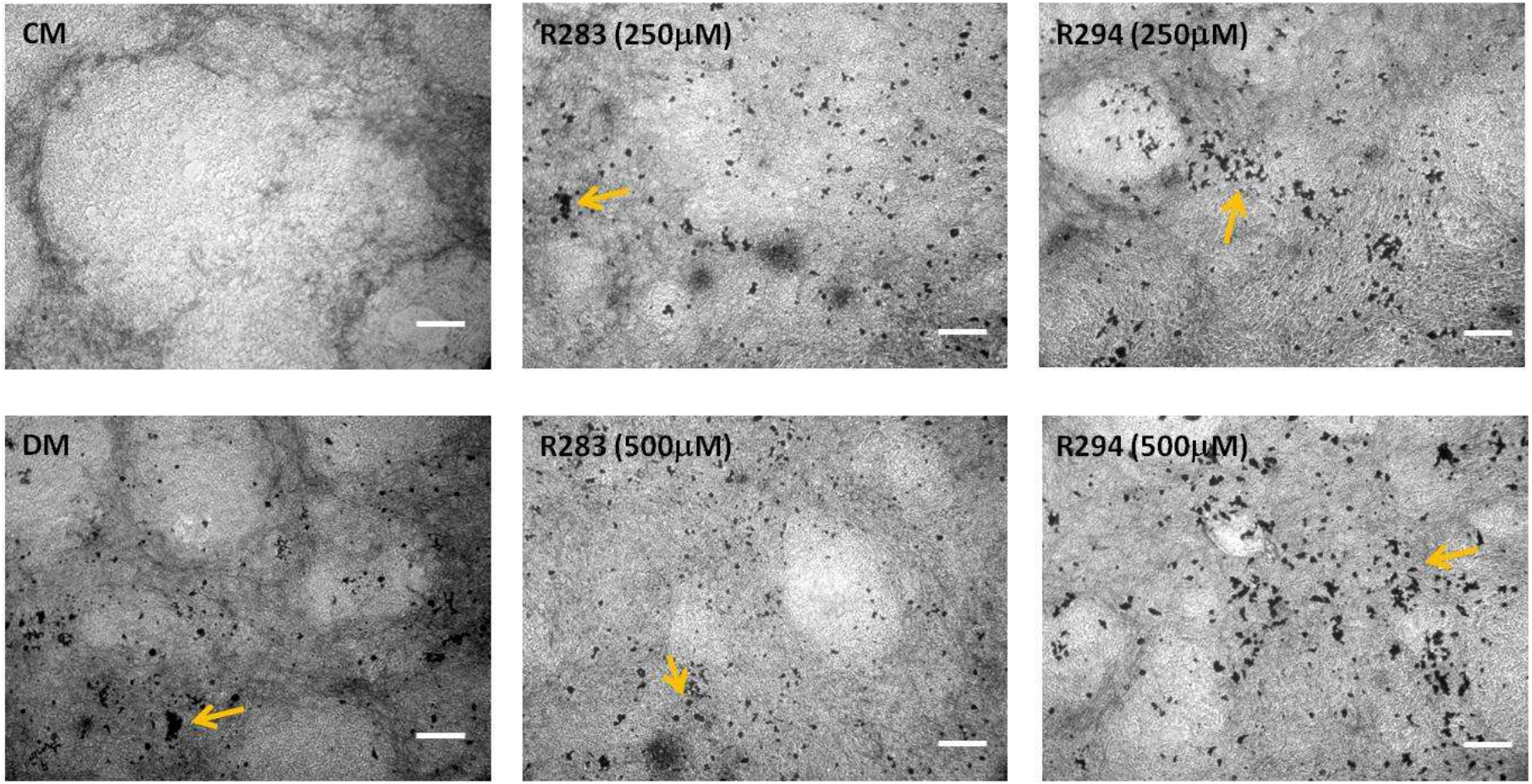

Figure 4.2 Mineralisation of HOBs visualised by von Kossa staining. HOBs cultured on tissue culture plate were treated with CM, DM, 250 μM R283/DM, 500 μM R283/DM, 250 μM R294/DM or 500 μM R294/DM. After 12 days culture, samples were fixed and stained with silver nitrate to assay the mineralisation of HOBs on day 12. Samples were viewed at x40 magnification using a Nikon CK2 and photographed with an Olympus DP10 digital camera. The mineral deposition was stained in black as indicated by arrow. The scale bars represent 150μm.

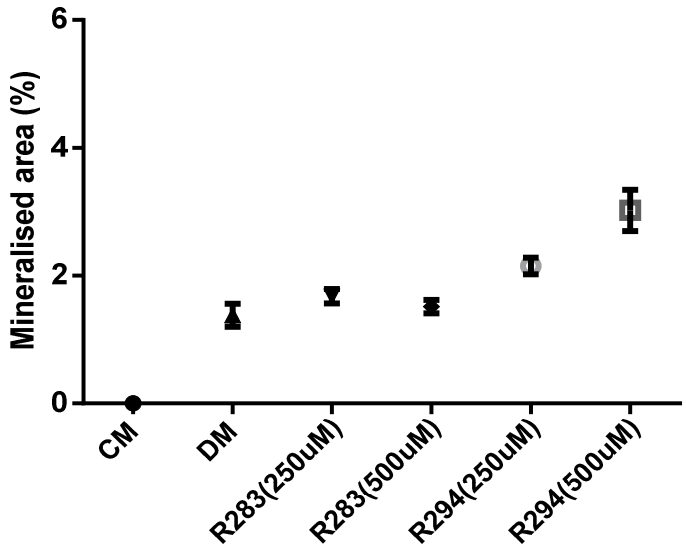

Figure 4.3 Mineralisation of HOBs culture measured by Von Kossa staining. The percentage area of positive staining was quantified by using image J. The diagram represents the mean values ±SEM of mineralised area from one experiment with 3 replicates. This experiment has been repeated twice where further experiments are needed to confirm this finding is statistically significant.

2.2 Existence of small TG2 fragments in ECM in HOBs cultured with inhibitors at the later stage of mineralisation

The mineralisation of HOBs was not inhibited by blocking of endogenous TGs activity, in contrast, the mineralised area could be potentially higher in R294 inhibitor treated group and it requires further experiments to conform this increase was statistically significant. Base on this observation, in the following experiments, the possibility of endogenous TG2 contributing to cell mineralisation via its ATPase activity was examined.

As suggested by Nakano *et al.* (2007), ATP could be a resource of phosphate groups for cell mineralisation and TG2 might contribute in regulating the release of phosphates via its ATPase activity. Several MMPs, especially membrane type-I MMP (MT1-MMP, MP14), were found to modulate the function of extracellular TG2 as part of a regulatory mechanism where the pro-mineralization function of TG2 is activated (Nakano *et al.*, 2010). In this study, the TG2 expression in whole cell lysate and extracellular matrix was determined using Western blotting. According to the results (Fig. 4.4), smaller TG2

fragments were observed in day 9 and day 13 cell/ECM lysate when cells were differentiated. Interestingly, the TG2 fragments appeared earlier and in higher amounts when inhibitors, both R283 and R294, were added to cell culture. There were three major fragments found between 50 to 75 kDa, which were only detected in differentiated cells and a higher molecular weight TG2 fragment (~90 kDa) was detected in all groups on day 9 and mainly in CM groups on day 13. It suggested that the possibility of enzyme mediated cleavage of TG2 was found in differentiated cells and when cells were treated with inhibitors. There was no direct evidence to demonstrate that these TG2 fragments had ATPase activity. However, accompanying the mineralisation results found in Fig. 4.2 that cells treated with transamidase inhibitors had higher mineral deposition, it is highly plausible that these TG2 fragments found in whole cell/ECM lysate could account for the increase in mineralised area found in inhibitor treated cells.

To further confirm if these fragments were present in the conditioned medium (Fig. 4.5) and cell membrane protein (Fig. 4.6) fractions were collected and analysed using Western blotting. As shown in Fig 3.5, faint but detectable small TG2 fragments were found in all DM treated groups from day 5 (Fig.4.5A, indicated by arrow head). On day 9, TG2 fragments, but not full length TG2, could be observed in all groups (Fig. 4.5B, indicated by arrow head) and ~75kDa TG2 could be detected in conditioned medium on day 13 in all groups (Fig. 4.5C, indicated by arrow). On the other hand, full length TG2 was observed in differentiated osteoblast membrane at all time points (Fig.4.6, indicated by arrows) and the TG2 fragments could be detected as early as day 5 in inhibitor treated groups (Fig 4.6A, indicated by arrow head) and in all groups on day 13 (Fig 4.6C, indicated by arrow head).

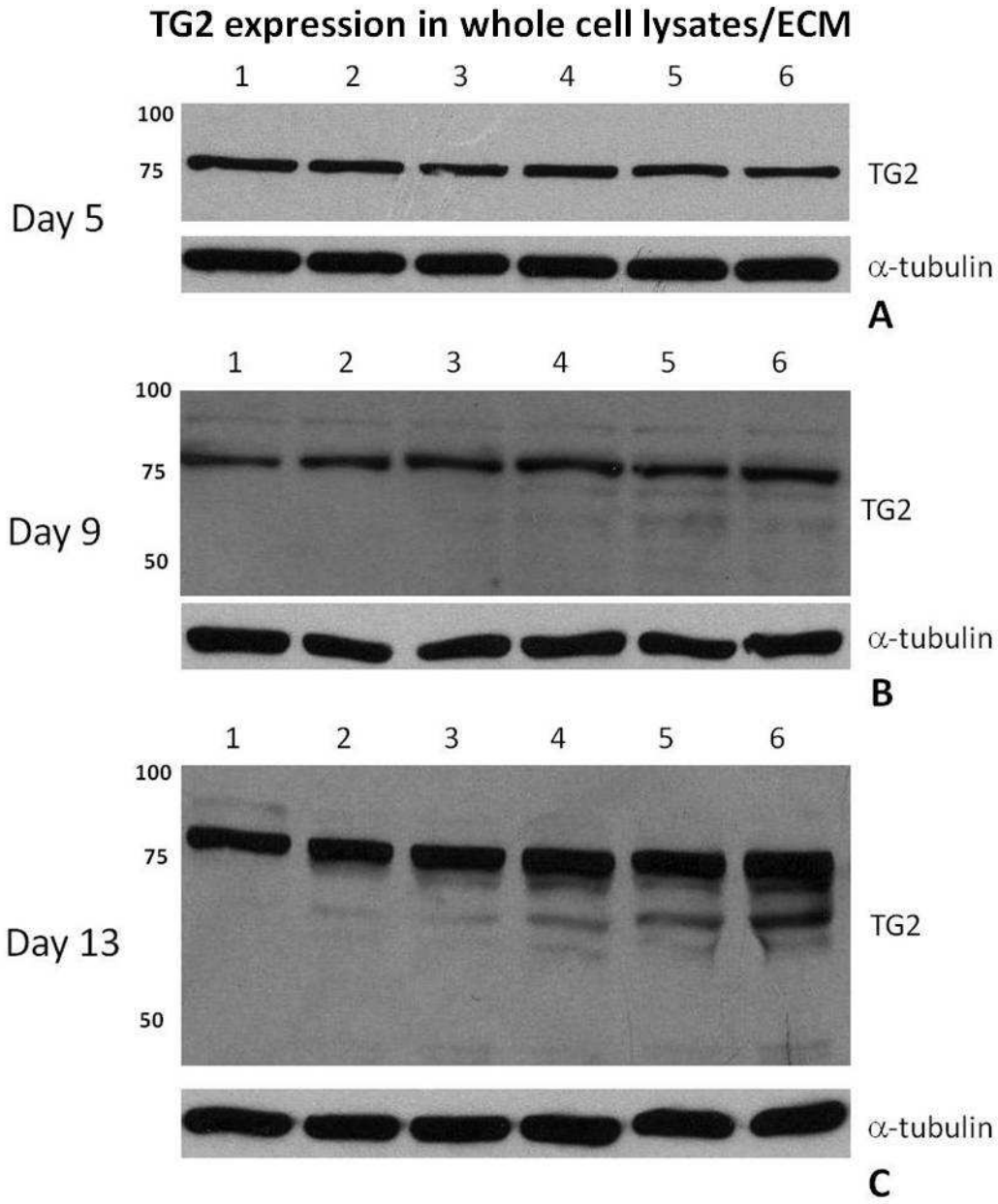

Figure 4.4 TG2 expression in whole cell/ECM lysate during mineralisation. HOBs were cultured with CM (1), DM (2), 250 μM R283/DM (3), 500 μM R283/DM (4), 250 μM R294/DM (5) or 500 μM R294/DM (6). Samples were collected 5 days (A), 9 days (B) or 13 days (C) post-treatment and Western blot analyses were performed. α-tubulin was used as loading control.

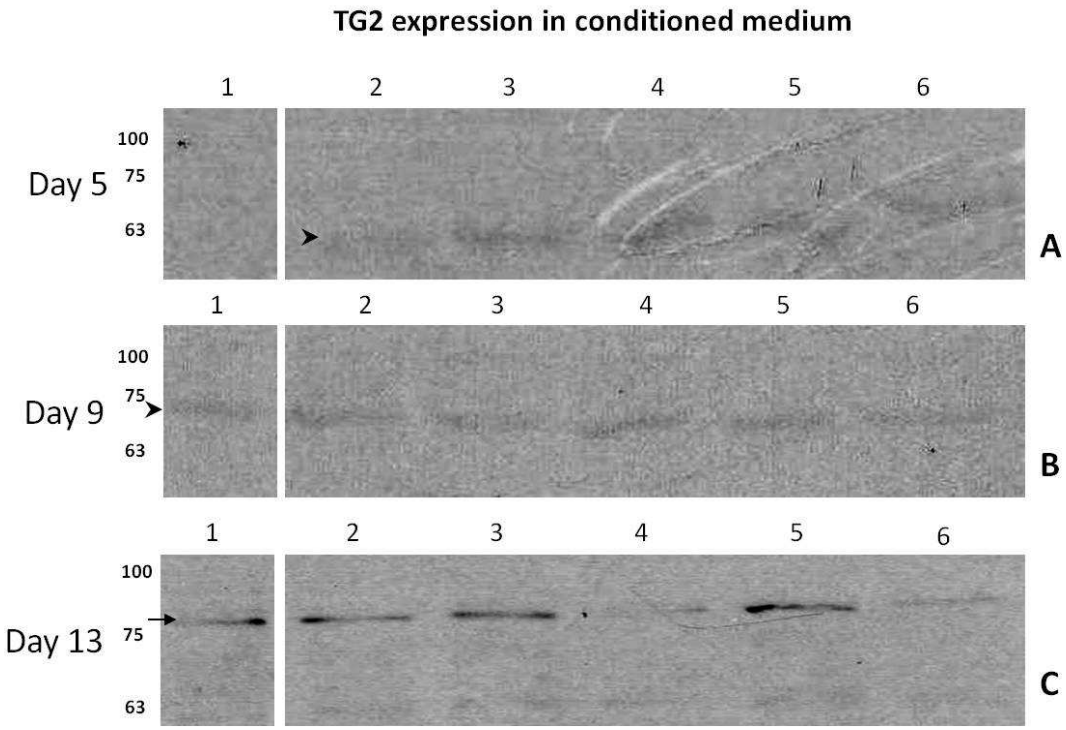

Figure 4.5 Secreted TG2 in conditioned medium during mineralisation. HOBs were cultured with CM (1), DM (2), 250 μM R283/DM (3), 500 μM R283/DM (4), 250 μM R294/DM (5) or 500 μM R294/DM (6). Culture medium was replaced by serum free culture medium 24 hours before specific collecting time points which were 5 days (A), 9 days (B) or 13 days (C) post-treatment and Western blot analyses were performed. The arrows indicate full length TG2 and the arrow heads indicate small TG2 fragments.

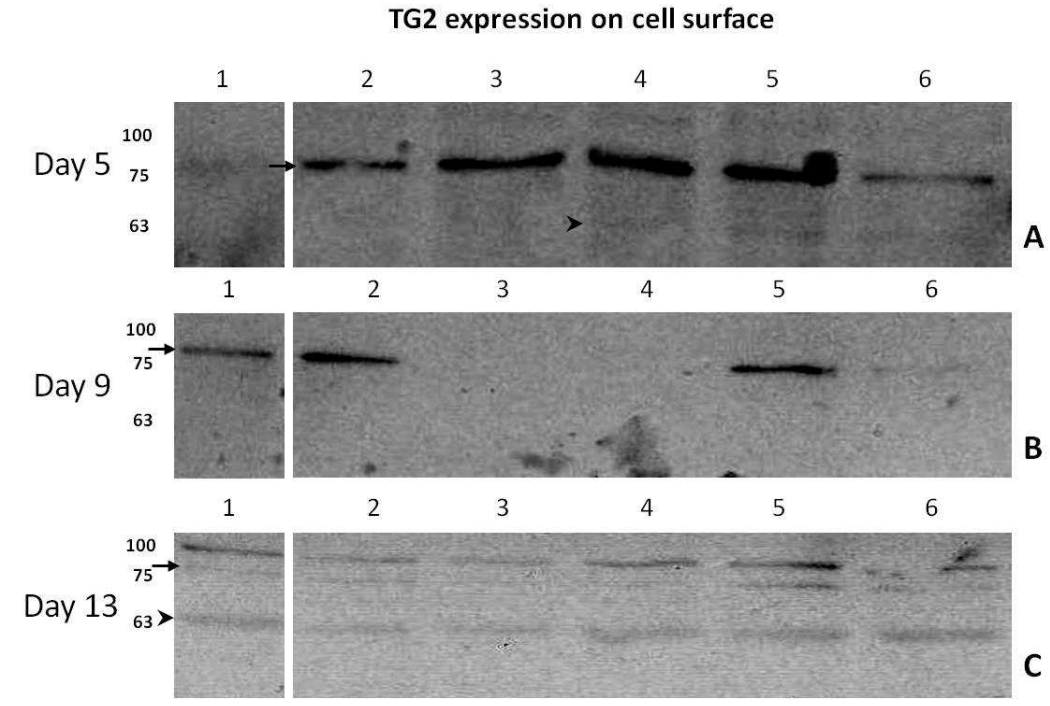

Figure 4.6 Membrane presented TG2 during mineralisation. HOBs were treated with CM (1), DM (2), 250 μM R283/DM (3), 500 μM R283/DM (4), 250 μM R294/DM (5) or 500 μM R294/DM (6). Cell membrane protein fractions were collected on 5 days (A), 9 days (B) or 13 days (C) post-treatment using biotin labelled method and Western blot analyses were performed. The arrows indicate full length TG2 and the arrow heads indicate small TG2 fragments.

2.3 Collagenase and gelatinase expression at the later stage of mineralisation

As suggested by Nakano *et al.* (2010), MT1-MMP is one of the major enzymes to regulate the ATPase activity of TG2. Therefore, to gain a general view on the collagenase and gelatinase profile of HOBs after differentiation, day 10 conditioned medium was subjected to collagen and gelatin zymography. Fig 4.7A showed active collagenase fragments (approx. 42kDa) could be found in CM groups and inhibitor groups but not in the DM group. According to the literature, these fragments could represent stable active MMP-1 fragments cleaved by trypsin (Wilhelm *et al.*, 1984, Grant *et al.*, 1987). On the other hand, the gelatin zymography (Fig 4.7 B) demonstrated the existence of active gelatinase fragments, again in CM groups and inhibitor groups but not DM groups. The major gelatinase fragment was approximately 82 kDa with another minor 130 kDa fragment. It is widely known that MMP-9 is secreted as a glycosylated 92 kDa precursor (Wilhelm *et al.*, 1989) and proteolytic cleavage of the zymogen by MMP-3 yields an active 82 kDa MMP-9 enzyme (Ogata *et al.*, 1992). It has also been shown that MMP-9 exists as a monomer, homodimer, or as a complex with lipocalin/tissue inhibitor of metalloproteinase-1 (TIMP-1) in neutrophils (Kolkenbrock *et al.*, 1996). All these forms showed different levels of enzymatic activity after activation with thrombin (Kolkenbrock *et al.*, 1996). Here, the ~82kDa and ~130kDa fragments observed could be active MMP-9 monomer and MMP-9 (82kDa)/lipocalin (23kDa) /TIMP-1 (23kDa) complex respectively.

TG2 mediated down regulation of gelatinase, for example MMP-9, has been reported in several cell lines after retinoic acid treatment, including monocytes, myoblasts and breast cancer cell lines. This effect could be preserved in the presence of TG2 crosslinking inhibitors (Ahn *et al.*, 2008). Increasing TG2 expression in differentiated cells at later stages of mineralisation was previously shown in Chapter II. Taking the collagenase and gelatinase profile and TG2 expression together, here a TG2 mediated change of enzyme

profile in differentiated osteoblasts was demonstrated. However, there were no bands corresponding to either MMP-2 or MT1-MMP detected in day 10 conditioned medium which could be due to MT1-MMP/MMP-2 complex tending to tether on plasma membrane microenvironment (Toth *et al.*, 2003).

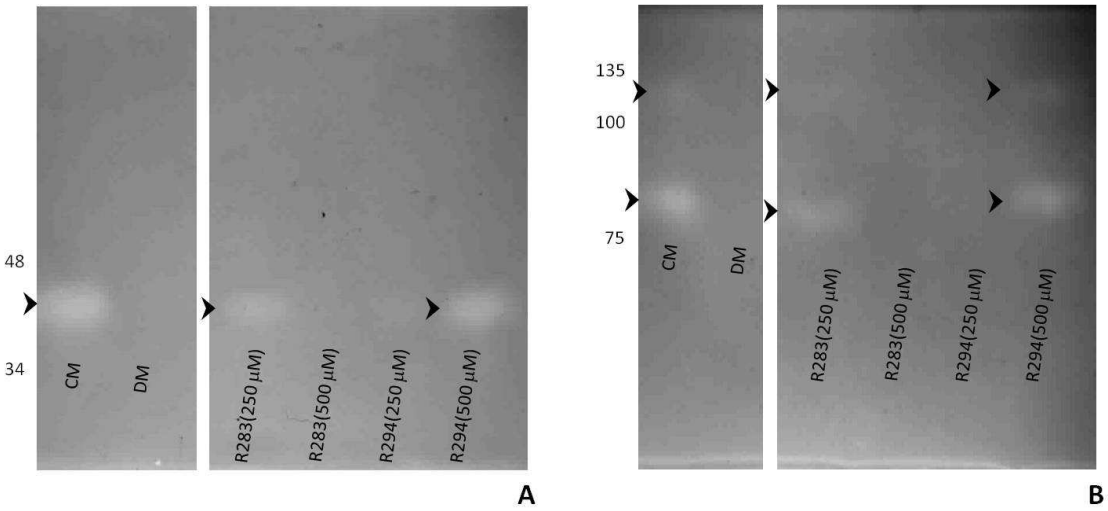

Figure 4.7 MMPs secreted by HOBs treated with inhibitors. Collagen (A) and gelatin (B) zymography of HOBs cell culture supernatants following 10 days culture in CM, DM, 250 μM R283/DM, 500 μM R283/DM, 250 μM R294/DM or 500 μM R294/DM. The enzymatic activity was visualised as clear bands on the gel which could be observed between 34kDa and 48kDa on collagen gel (A) and approximately 82kDa on gelatine gel (B). The digested areas are indicated by arrow heads.

2.4 Effect of ATPase inhibitors on mineralisation

Here the importance of ATPase activity on mineralisation was demonstrated by treating HOBs with a general ATPase inhibitor, ATP-γ-s. As shown in Fig 4.8, the mineralising process was significantly inhibited by ATP-γ-s at concentrations of 10 μM and 400 μM. Released ALP activity was also monitored during the whole mineralisation process. In order to collect the conditioned medium from each group, the HOBs were maintained in inhibitor-containing serum free medium 24 hours before collection. It was found that released ALP in conditioned medium was significantly lower after day 6 in HOBs treated with 200 μM and 400μM ATP-γ-s compared to control group. And this effect was found to be concentration dependent from day 8 (Fig.4.9). These results indicated that ATPase played an important role in mineralisation and possibly through both inhibiting ATPase and down regulating ALP activity of HOBs. However, the possibility that ATP-γ-s in the conditioned medium was inhibiting ALP activity could not be ruled out. In fact, Ciancaglini *et al.*(2010) have suggested that that ATP-γ-s showed non-specific effects on several targets including P2Y1 receptor. The ATP-γ-s treatment greatly inhibited ALP activity at high concentrations. Nevertheless, when used at 10 μM, the maximum inhibiting effect was less than 5% comparing to DM treated cells at the later stage of mineralisation. In this case, 10 μM ATP-γ-s still demonstrated great inhibitory effect on mineral deposition which suggested that inhibiting ATPase activity was sufficient enough to block the mineralisation process. Overall, this preliminary data showed the ATPase activity of HOBs could be essential in mineralisation process, yet, more experiments would be necessary to conform this finding is statistically significant.

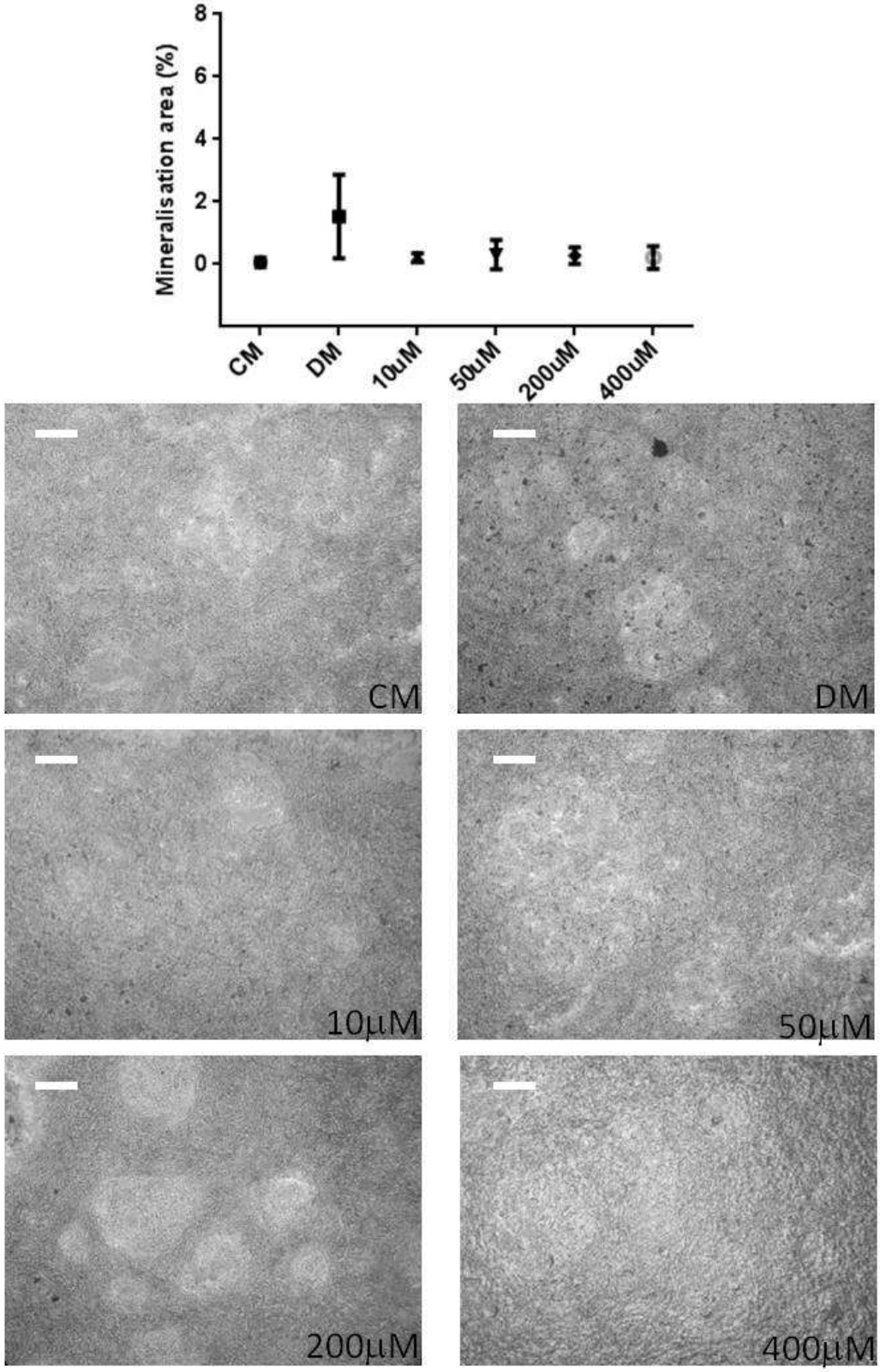

Figure 4.8 Mineralisation of HOBs cultured with ATP-γ-s for 12 days. HOBs were cultured in differentiation medium with different concentration of ATP-γ-s for 12 days. Mineral deposition in extracellular matrix was stained in black using von Kossa staining and the mineralised area was quantified using Image J software. The chart represents the mean values ±SEM of mineralised area from one experiment with 3 replicates. This experiment was repeated twice and further experiments would be needed to confirm that the observed inhibitory effect of ATP--γ-s was statistically significant. The scale bars represent 150μm.

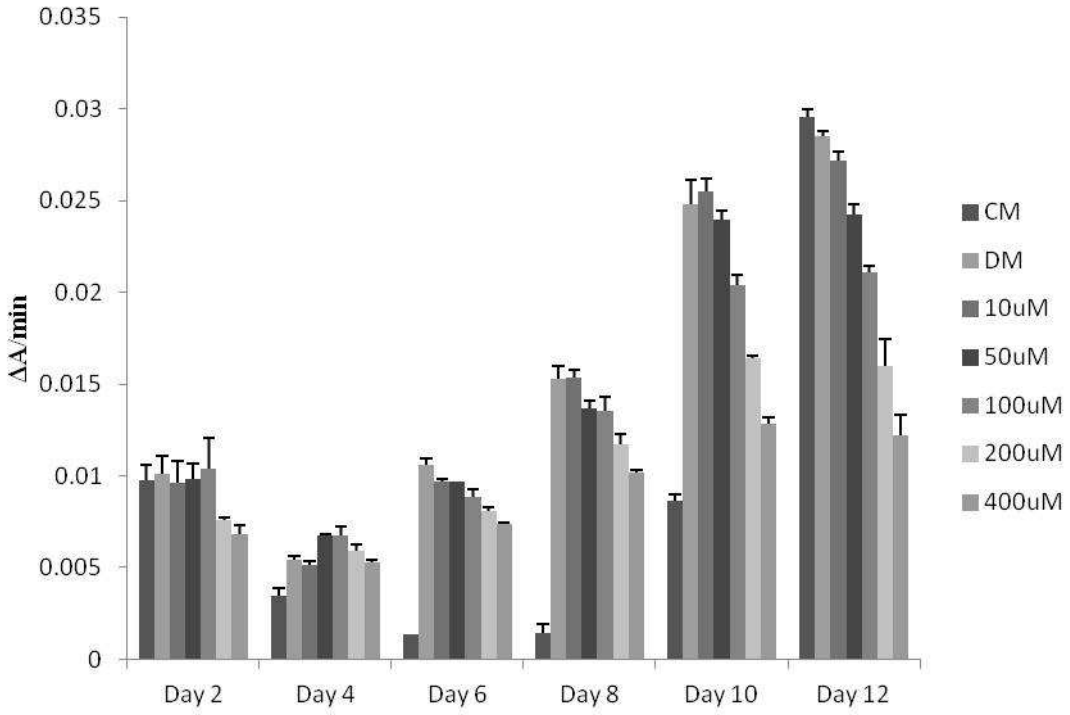

Figure 4.9 Released ALP activity in conditioned medium collected from HOBs culture with ATP-γ-s during mineralisation. Data represents mean values +/- S.D. from a representative experiment with 3 replicates. The experiment was repeated 2 times (n=2) and further experiments would be necessary to confirm the finding was statistically significant.

2.5 Exogenous TG2-induced mineralisation in human osteoblasts cell culture in vitro

Endogenous TG2 activity may not play a dominant role in osteoblast mineralisation, yet, the exogenous TG2 enhanced mineralisation of wild type and TG2 expression knockdown cells *in vitro* was demonstrated in the previous chapter and also a published study (Nurminskaya *et al*., 2003). It suggested that TG2 in the extracellular environment may trigger an outside-in signal for cell mineralisation. Here, the exogenous TG2 dependent effect on HOB mineralisation was demonstrated in Figure 4.10. Cells cultured with 1μg/ml of exogenous TG2 were found to be highly mineralised at day 12 when compared with differentiation medium treated group. An approximately 9 and 3 times increase in mineral area was observed in 1μg/ml of TG2 and 0.5μg/ml of TG2 treated group, respectively.

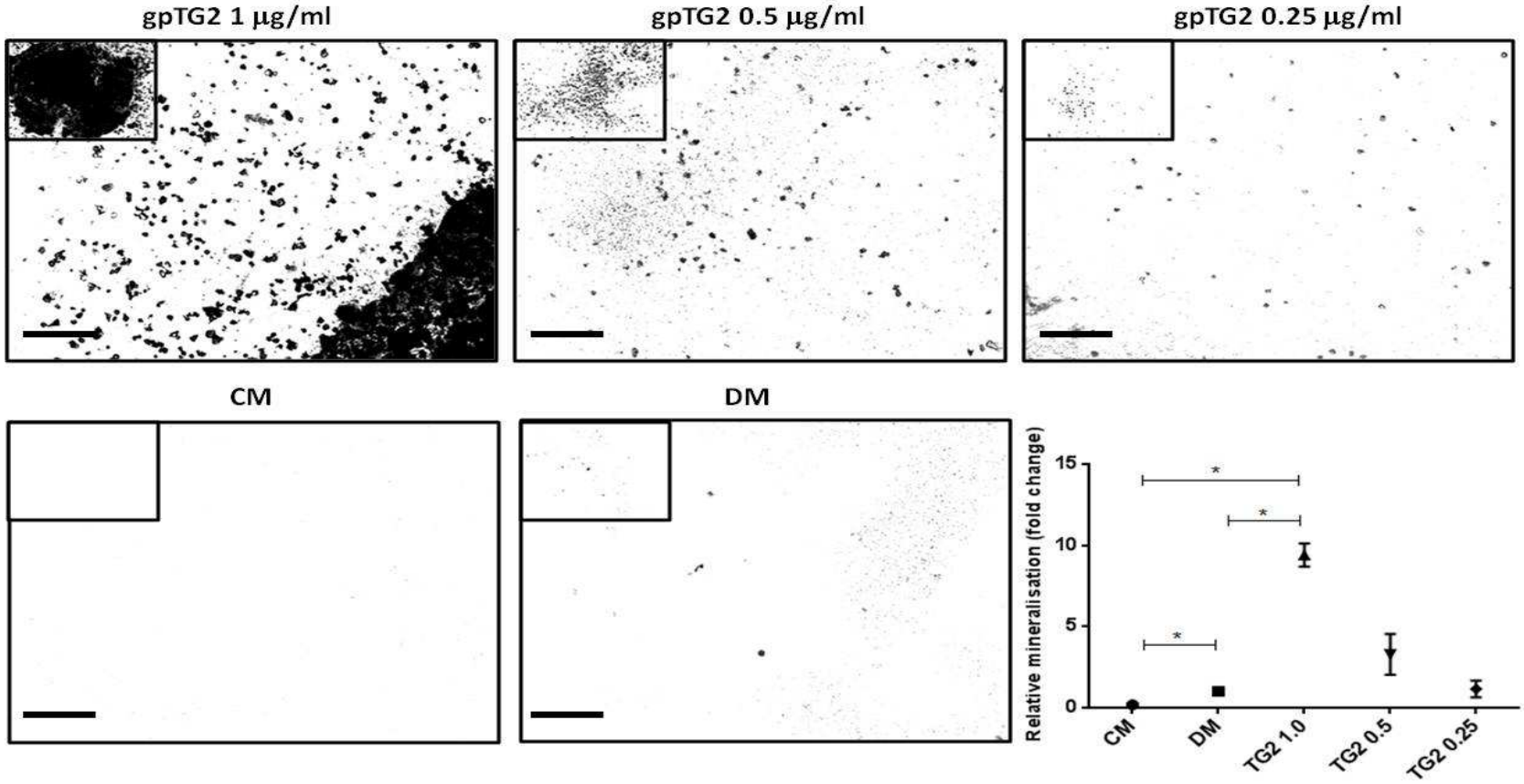

Figure 4.10 Mineral deposition of HOBs cultured with exogenous active TG2 in different concentrations for 12 days. HOBs were cultured in differentiation medium with 1, 0.5 or 0.25µg/ml of gpTG2 (TG 1, TG 0.5 and TG 0.25) for 12 days. Mineralisation of matrix was visualised using von Kossa staining where the mineral deposition was stained in black. The actual mineralisation area was quantified by using image J and the results were further normalised with the DM groups from each individual experiment. The average mineralisation area of the DM group from 3 experiments is 2.28± 0.37%. The diagram represents mean values +/- SEM of fold change from 3 individual experiments (n=3). Statistical analysis was carried out using the one way ANOVA (Turkey's multiple comparison test) and the p-values corresponding to $p<0.05$ is represented with a †. Significant differences were observed in the negative control (CM) group compared with positive control (DM) group, 1µg/ml TG2 group compared with CM group and 1µg/ml TG2 group compared with positive control group (DM), . The scale bars represent 200 µm.

To further understand the exogenous TG2 induced mineralisation, one of the biomarkers, osteocalcin (OCN), was monitored after treatment of cells with exogenous TG2. As shown in Figure 4.11, the TG2 group showed no significant difference in OCN secretion when compared with the DM group on day 2. On the other hand, exogenous TG2 significantly decreased the level of OCN in conditioned medium at day 6 post treatment when compared to the DM group. OCN is a non-collagenous bone matrix specifically secreted by differentiated osteoblasts. Although there is no evidence to show that OCN is a TG2 substrate, it has been suggested that OCN could bind to osteopontin (OPN) and consequently prevent crosslinking of OPN by TG2 (Kaartinen *et al.*, 1997). Here, the decreasing OCN in conditioned medium could result from more binding of OCN to other extracellular matrix proteins, which might be directly or indirectly mediated by crosslinking activity of exogenous TG2.

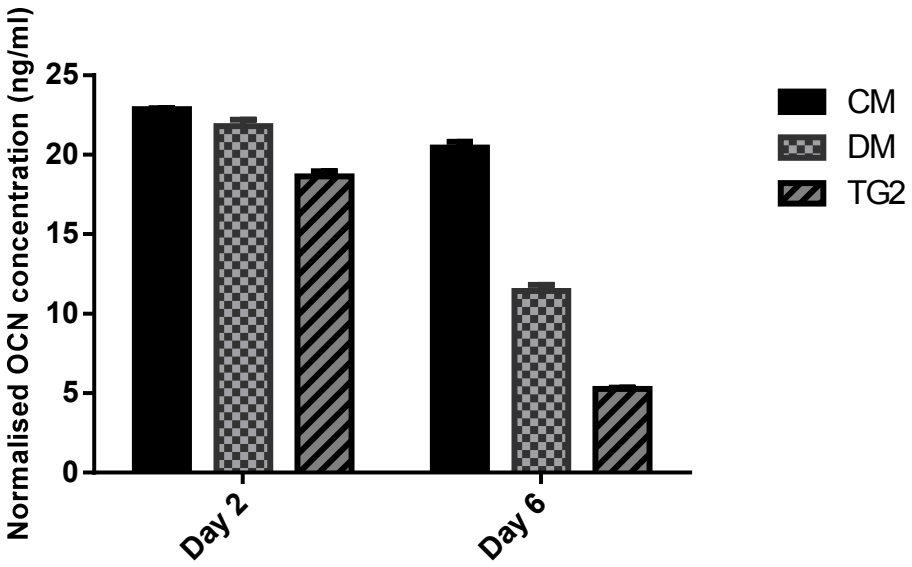

Figure 4.11 Released osteocalcin (OCN) in conditioned medium. The releasing of OCN in the conditioned medium of HOBs cultured in complete medium (CM), differentiation medium (DM) or 1μg/ml of TG2 in DM (TG2) was quantified using ELISA technique. . Samples were collected at 2 days and 6 days after treatment. The acquired OCN concentrations from ELISA kit were further normalised with the total protein (mg) measured from each sample. Data represents mean values ± S.D. from one experiment with 2 replicates. The ELISA has been done twice, yet, further experiment would be necessary to conform the decrease observed was statistically significant.

Alternatively, the decrease in OCN secretion could be directly linked to the increase in mineralisation of TG2 treated HOBs cells since OCN has been demonstrated as a negative regulator of mineralisation, which could be linked to specific inhibition of crystal maturation (Ducy *et al.*, 1996, Boskey *et al.*, 1998). Therefore, it was also possible that exogenous TG2 could down regulate OCN expression in osteoblasts thus increasing cell mineralisation.

In order to distinguish whether this TG2-induced mineralisation was transamidase activity dependent or independent, inactive TG2 was generated by using crosslinking inhibitor R283 or R294. Addition of R283 treated TG2 (TG2/R283) to HOB cell culture induced a significant increase in mineralisation when compared to the DM group. However, the fold change in mineral area was not as high as in the TG2 group (Fig. 4.12). On the other hand, a higher, but not significant, difference in mineral deposition was observed in the TG2/R294 group when compared to the DM group. This result implied that the transamidase activity of TG2 was involved in TG2 induced mineralisation. However, the influence of conformational changes in TG2 when inhibitor is bound must be taken into account. It is clear that multiple conformations of TG2 exist (Liu *et al.*, 2002, Pinkas *et al.*, 2007), but very little is known about the biological relevance of each conformation. Conformational change could potentially mask binding sites within TG2 thus regulating the function of TG2 (reviewed by Park *et al.*, 2010a). Therefore, it was reasonable to suggest that the results obtained here could represent the importance of transamidase activity but also the conformation of the protein in exogenous TG2-induced mineralisation.

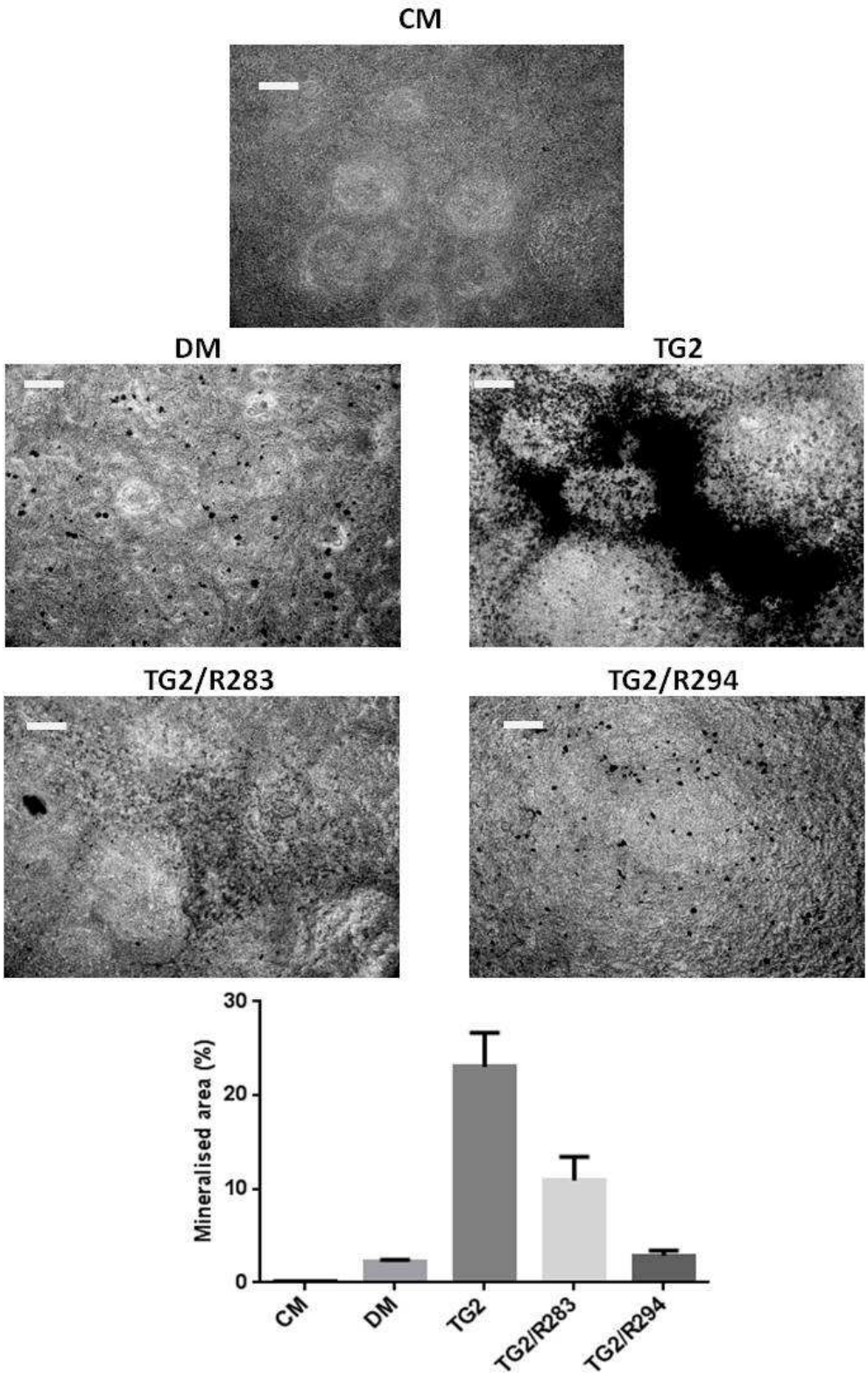

Figure 4.12 Mineralisation of HOBs cultured with inactive TG2 for 12 days. HOBs were cultured in differentiation medium with 1µg/ml of gpTG2 (TG2), R283 pre-treated TG2 (TG2/R283) or R294 pre-treated TG2 (TG2/R294) for 12 days. Mineral deposition in extracellular matrix was stained in black using von Kossa staining and the mineralised area was quantified using Image J software. Data represents mean values +/- SEM from one experiment with 4 replicates. This experiment was repeated in twice, yet, further repeats would be needed to conform the enhancement observed was statistically significant. The scale bars represent 150µm.

2.6 N-Cadherin, β-catenin and LRP5 expression in early differentiation of HOBs

Since the effect of exogenous TG2 induced mineralisation has been established here and in the literature, the possibility that extracellular TG2 serves as an outside-in signal through LRP5 receptors was examined. The expression of related proteins, including LRP5, β-catenin, TG2 and N-cadherin, in HOBs during mineralisation was determined using Western blotting. A 2 to 3 fold decrease in β-catenin and LRP5 expression from the baseline (CM) was observed as early as 4 hours after differentiation (Fig. 4.13). The expression of both β-catenin and LRP5 were lower in differentiated cells at 4 hours post treatment but increased gradually, reaching the highest levels around 48 hours post differentiation and declining after this. On the other hand, the expression of N-cadherin transiently increased in differentiated cells at 4 hours and gradually decreased from 24 hours after differentiation (Fig. 4.14). Interestingly, N-cadherin expression in HOBs showed a reciprocal relationship to the expression of β-catenin, LRP5 and TG2.

A transient upregulation in N-cadherin expression was consistent with current studies in pre-osteoblast or osteosarcoma at early stages of growth factor or anabolic molecular treatment (Haÿ *et al.*, 2000, Debiais *et al.*, 2001, Delannoy *et al.*, 2001). A gradual decrease in N-cadherin expression during osteoblast mineralisation was also supported by recent findings that the mRNA level of N-cadherin decreased during differentiation in chondrocytes (Nurminsky *et al.*, 2011). Furthermore, the relationship found between N-cadherin and β-catenin was also suggested in other research (Hay *et al.*, 2009). There is no clear evidence that TG2 may regulate N-cadherin expression or vice versa; however, elevation in LRP5/β-catenin signalling was observed on day 2, which suggested that an early activation of LRP5/ β-catenin could be important for osteoblast differentiation.

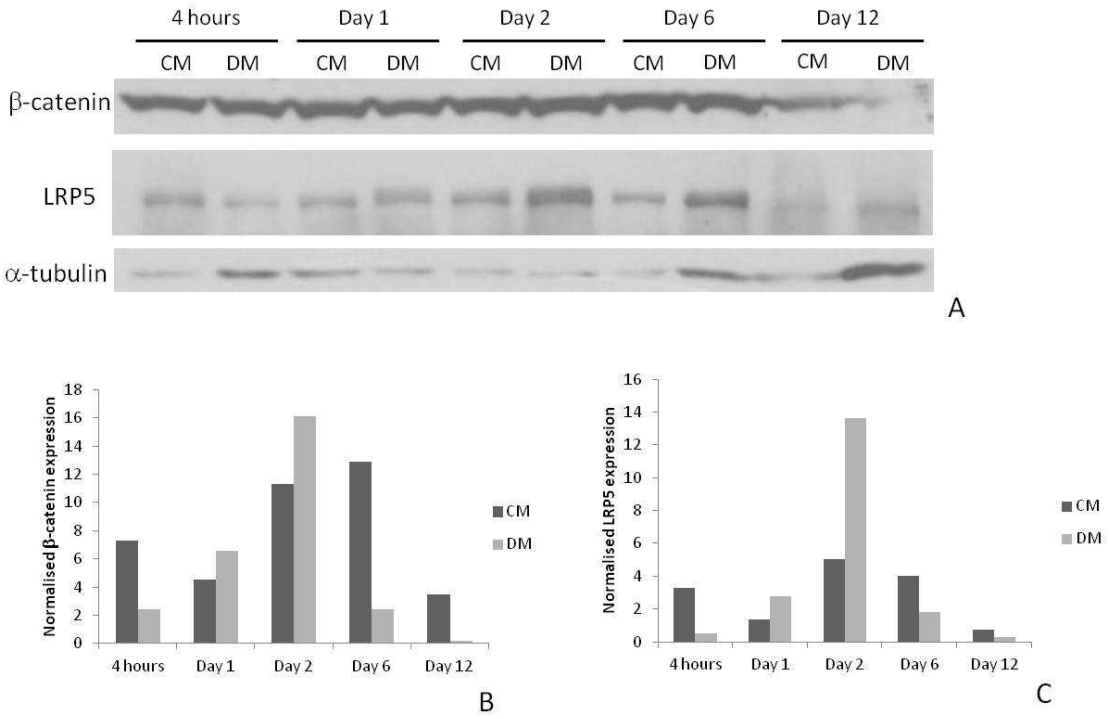

Figure 4.13 (A) Expression of β-catenin and LRP5 in whole cell lysate as demonstrated using Western blotting. Whole cell lysates were collected at time point 4 hours, 1 day, 2 days, 6 days and 12 days post differentiation treatment and Western blotting was performed to analysis β-catenin and LRP5 protein expression. α-tubulin was used as loading control and normalised data of β-catenin (B) and LRP5 (C) were showed in chart.

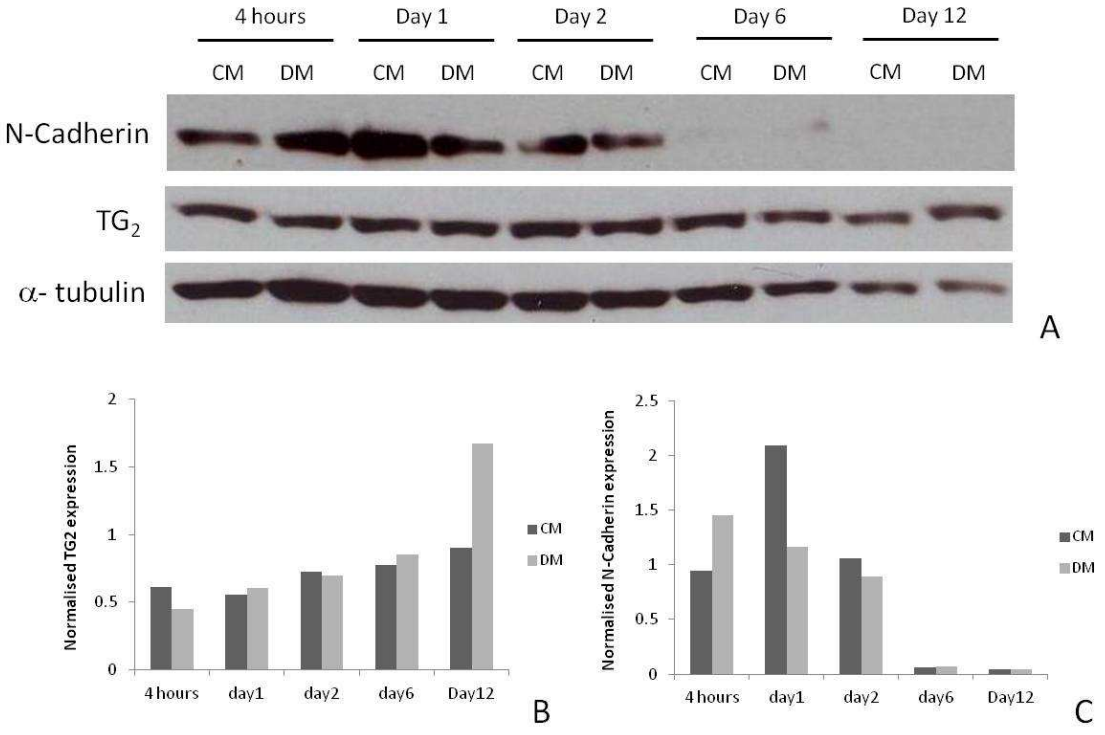

Figure 4.14 (A) Expression of N-Cadherin and TG2 in whole cell lysate as demonstrated using Western blotting. After treating HOBs with DM for 4 hours, 1 day, 2 days, 6 days and 12 days, whole cell lysates were collected and Western blotting was performed to analysis N-cadherin and TG2 protein expressions. α-tubulin was used as loading control and normalised data are shown in chart (B) and (C).

2.7 Binding of TG2 to LRP5 receptor

A possible relationship between TG2 and the LRP5/ β-catenin pathway was proposed by Faverman *et al.* (2008) and the hypothesis of binding of TG2 to LRP5 receptor was tested. As demonstrated by Western blot, the β-catenin pathway related proteins showed a transient increase in early cell differentiation. Therefore, the following experiments focused on determining the presence of early LRP5/ β-catenin signalling in differentiated osteoblasts. The colocalisation of TG2 and LRP5 proteins was examined by double immunohistochemical staining with TG2 and LRP5 antibodies (Fig. 4.15). Colocalisation of these two proteins was found in all groups 24 hours post treatment which suggested a possible interaction between TG2 and LRP5 receptor. However, this data may not represent direct physical contact of TG2 to LRP5. Therefore immunoprecipitation was used to confirm the binding of TG2 to LRP5.

As shown by Faverman and colleagues study (2008), TG2 could bind to LRP5 receptors and trigger the β-catenin pathway in vascular smooth muscle cell*s* (VSMCs). Here, the interaction between exogenous TG2 and LRP5 was demonstrated using Co-IP. Fig. 4.16 shows that binding of TG2 to LRP5 receptor could be observed as early as 4 hours post differentiation with exogenous TG2 treatment but not in HOBs cultured in CM. Whilst the interactions of these two proteins was observed in all groups 24 hours post treatment, the signal decreased at 48 hours with only a weak interaction shown in CM group.

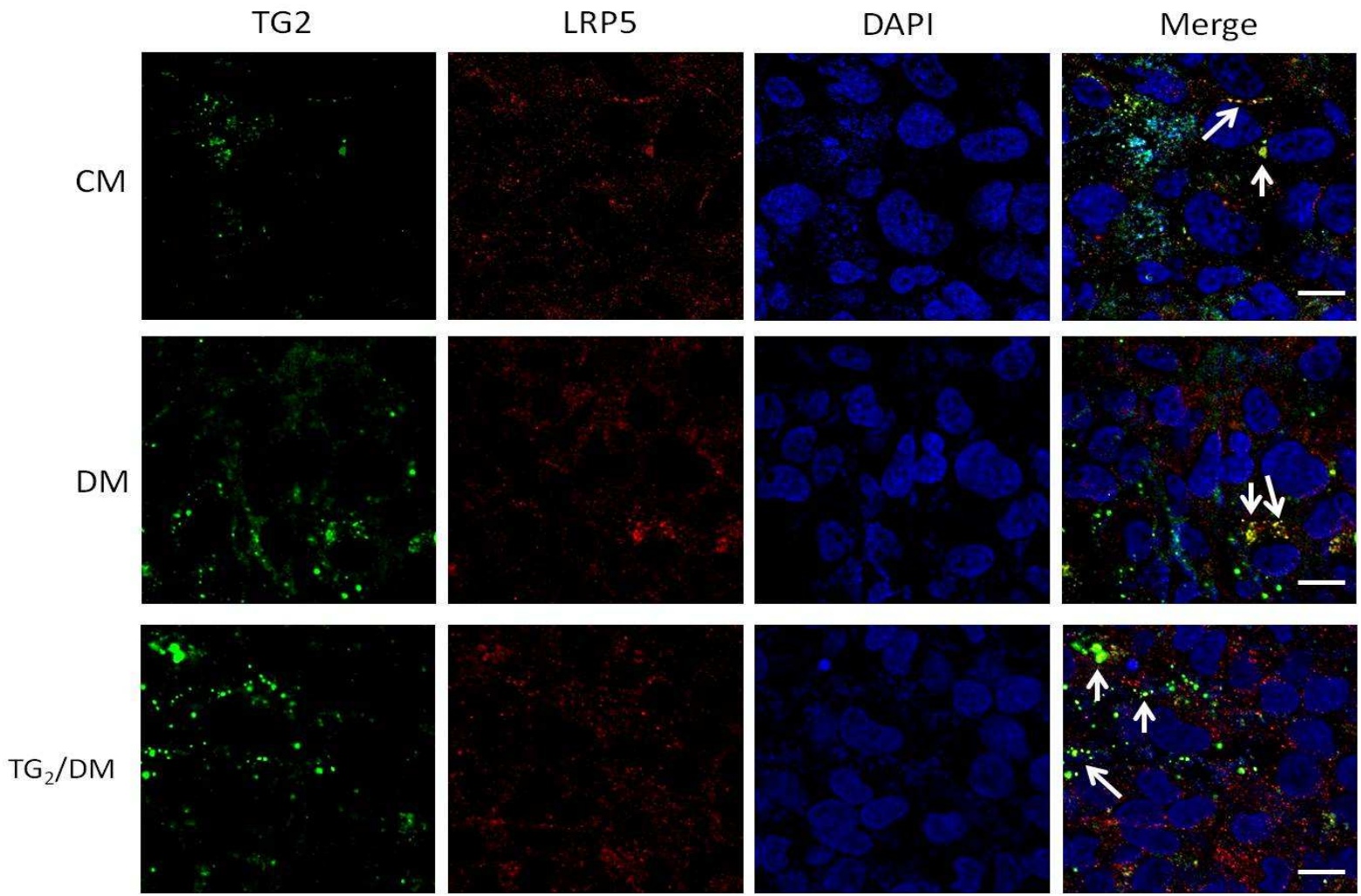

Figure 4.15 Localisation of LRP5 and TG2 proteins on cell surface of HOBs. HOBs were seeded on 4 well microscope slide overnight in complete medium. Following 24 hours incubation in complete medium (CM), differentiation medium (DM) or DM supplemented with 1μg/ml of gpTG2 (TG2/DM), cells were fixed with 3.7% paraformaldehyde without permeabilisation. The sample slides were double stained with anti-LRP5 (red) and anti-TG2 antibodies (green). Colocalisation of LRP5 and TG2 is indicated with a white arrow. The scale bar represents 10μm.

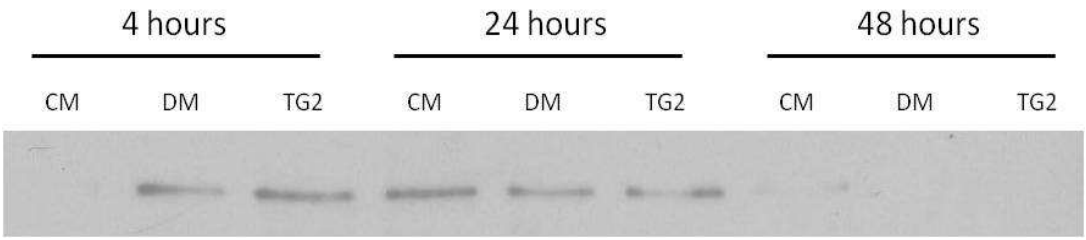

Figure 4.16 Binding of TG2 to LRP5 receptor on human osteoblasts. HOBs were cultured in complete medium (CM), differentiation medium (DM) or 1μg/ml of gpTG2 in DM (TG2) for 4 hours, 24 hours or 48 hours. The Co-immunoprecipitaion was performed with anti-LRP5 antibody (H-105) and the binding of TG2/LRP5 was confirmed using Western blot analysis with anti-TG2 antibody.

Although Co-IP using Anti-LRP5 antibody (H-105) and counter detecting TG2 (TG100 antibody) protein showed that there was direct binding of these two proteins, no LRP5 signal was detected when immunoprecipitation was performed using Anti-TG2 antibody (data not shown). In this study TG100 antibody, which recognises a.a. 447-538 of TG2, failed to precipitate the LRP5/TG2 complex, possibly because the binding site for TG100 antibody on TG2 is masked when TG2 is bound to LRP5. In Faverman's work (2008), Anti-TG2 antiserum recognized full length gpTG2 (multiple epitope sites) and was used to pull down the LRP5/TG2 complex. Also, In Zemskov's paper (2007), mouse anti-TG2 mAb 4G3 which has epitope site 1-165 was used to determine LRP1/TG2 colocalisation *in vitro*. It was suggested that LRP1 might have a similar binding site to LRP5 (Belkin, 2011). Even though there are no current data demonstrating the precise binding site(s) for TG2 on the LRP5 molecule, this result could indicate that a.a. 447-538 of TG2 was involved in binding to LRP5 receptor or masked because of conformation.

2.8 Activation of β-catenin pathway by exogenous TG2

In order to examine whether the β-catenin pathway could be activated via exogenous TG2 binding to LRP5 receptor, HOBs were treated with differentiation medium with or without exogenous TG2 and the β-catenin was stained and visualised using immunohistochemical staining. At 4 hours post treatment, cells grown in complete medium and differentiation medium showed low and mainly cytoplasmic membrane localised signal. Interestingly, cells treated with gpTG2 showed more intense β-catenin staining which had a mainly cytosolic and nuclear localisation (Fig. 4.17). 48 hours after treatment, all groups showed β-catenin positive signal which was mainly cytoplasmic membrane located (Fig. 4.18). There was no significant colocalisation found between TG2 and β-catenin proteins. In contrast, polarisation of these two proteins was found where TG2 mainly distributed away from β-catenin protein

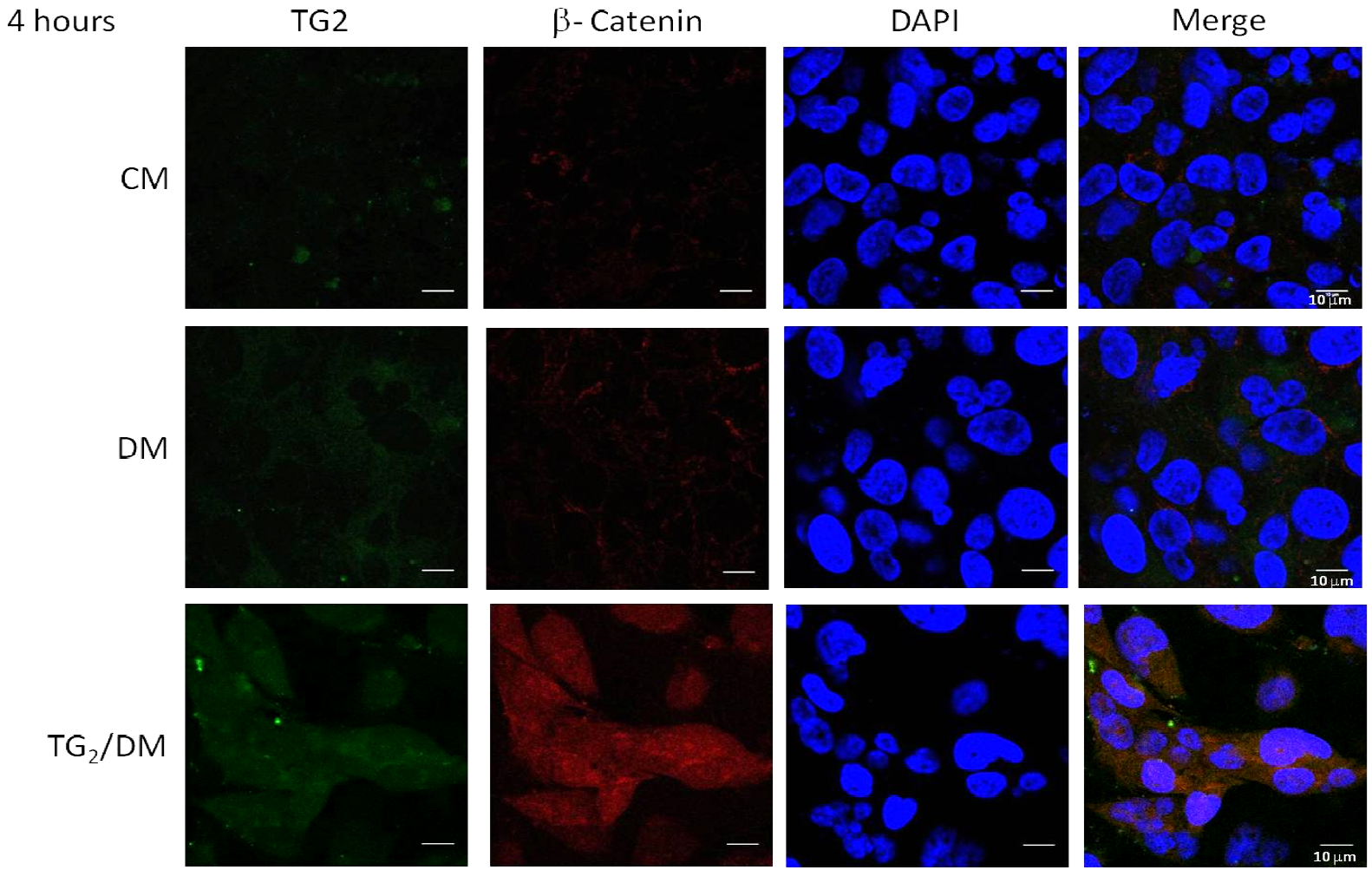

Figure 4.17 Translocation of β-catenin after exogenous TG2 treatment for 4 hours. HOBs were seeded on 4 well microscope slide overnight in complete medium. Following 4 hours incubation in complete medium (CM), differentiation medium (DM) or DM supplemented with 1μg/ml of gpTG2 (TG2/DM), cells were fixed with 3.7% paraformaldehyde and permeabilised with 5% Triton X-100. The sample slides were double stained with anti-β-catenin (red) and anti-TG2 antibodies (green). The scale bar represents 10μm.

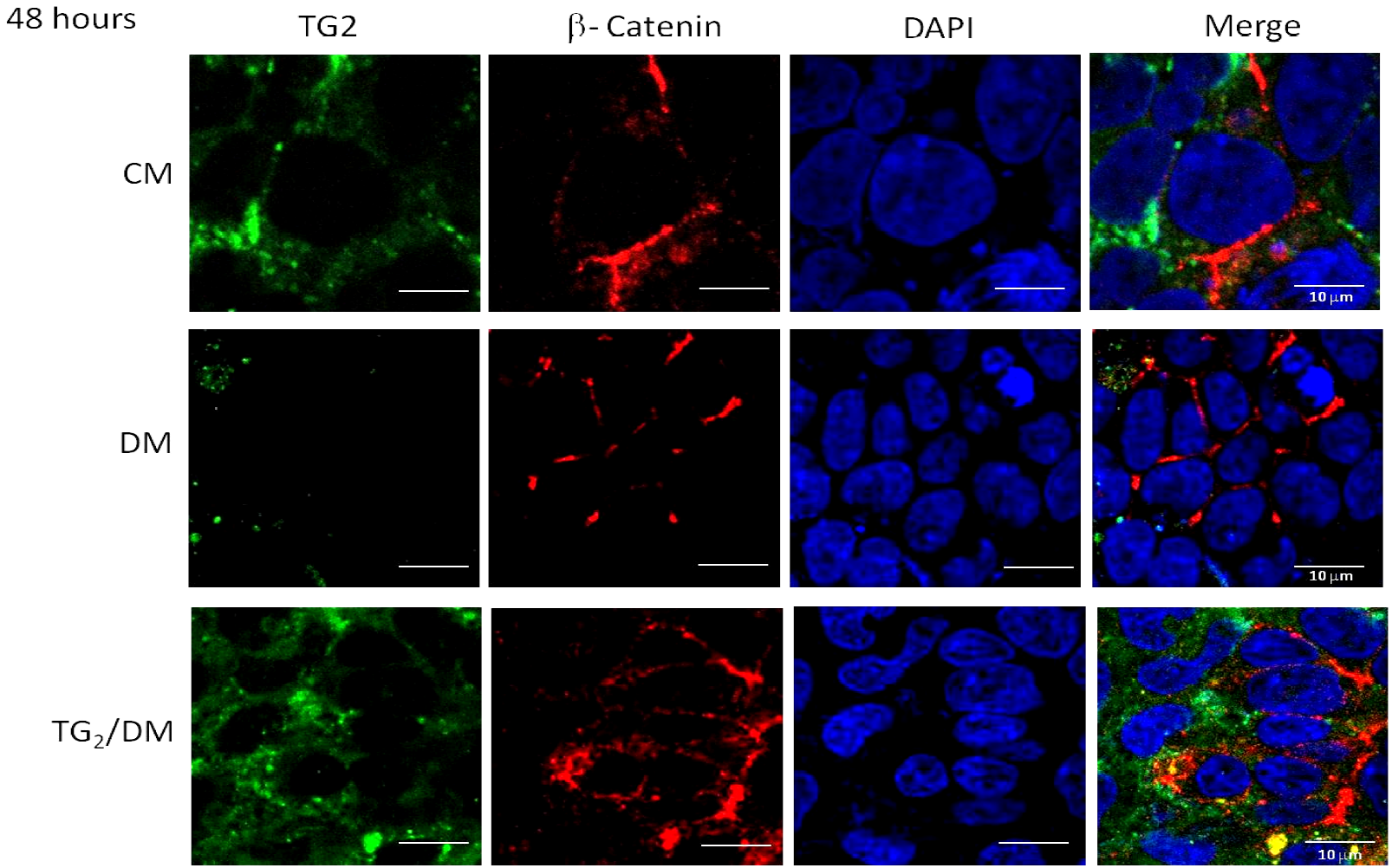

Figure 4.18 Translocation of β-catenin after exogenous TG2 treatment for 48 hours. HOBs were seeded on 4 well microscope slide overnight in complete medium. Following 48 hours incubation in complete medium (CM), differentiation medium (DM) or DM supplemented with 1μg/ml of gpTG2 (TG2/DM), cells were fixed with 3.7% paraformaldehyde and permeabilised with 5% Triton X-100. The sample slides were double stained with anti-β-catenin (red) and anti-TG2 antibodies (green). The scale bar represents 10μm.

2.9 Inhibition of the β-catenin pathway abolishes exogenous TG2 induced mineralisation

Since early stimulation of the β-catenin pathway could be one of the mechanisms behind TG2-induced mineralisation, here a β-catenin pathway inhibitor, XAV 939, was tested to determine the importance of the β-catenin pathway in TG2 induced HOB mineralisation. XAV 939 stimulates β-catenin degradation and axin stabilisation, thus antagonising the Wnt/β-catenin pathway. Cells treated with 0.1μM XAV939/DM for 12 days showed no significant difference in mineralised area compared to cells cultured in DM (Fig. 4.19). This suggested that HOBs could deposit mineral matrix in a Wnt/β-catenin independent manner. However, when treated with exogenous TG2, HOBs treated with β-catenin inhibitor showed a reduced mineralised area comparable to cells treated with DM only. This indicated that TG2-induced mineralisation was Wnt/β-catenin dependent, and blocking β-catenin by forcing protein degradation could abolish this effect. However, this experiment has only be done twice, further experiments would be necessary to validate the importance of β-catenin pathway in TG2 induced HOB mineralisation.

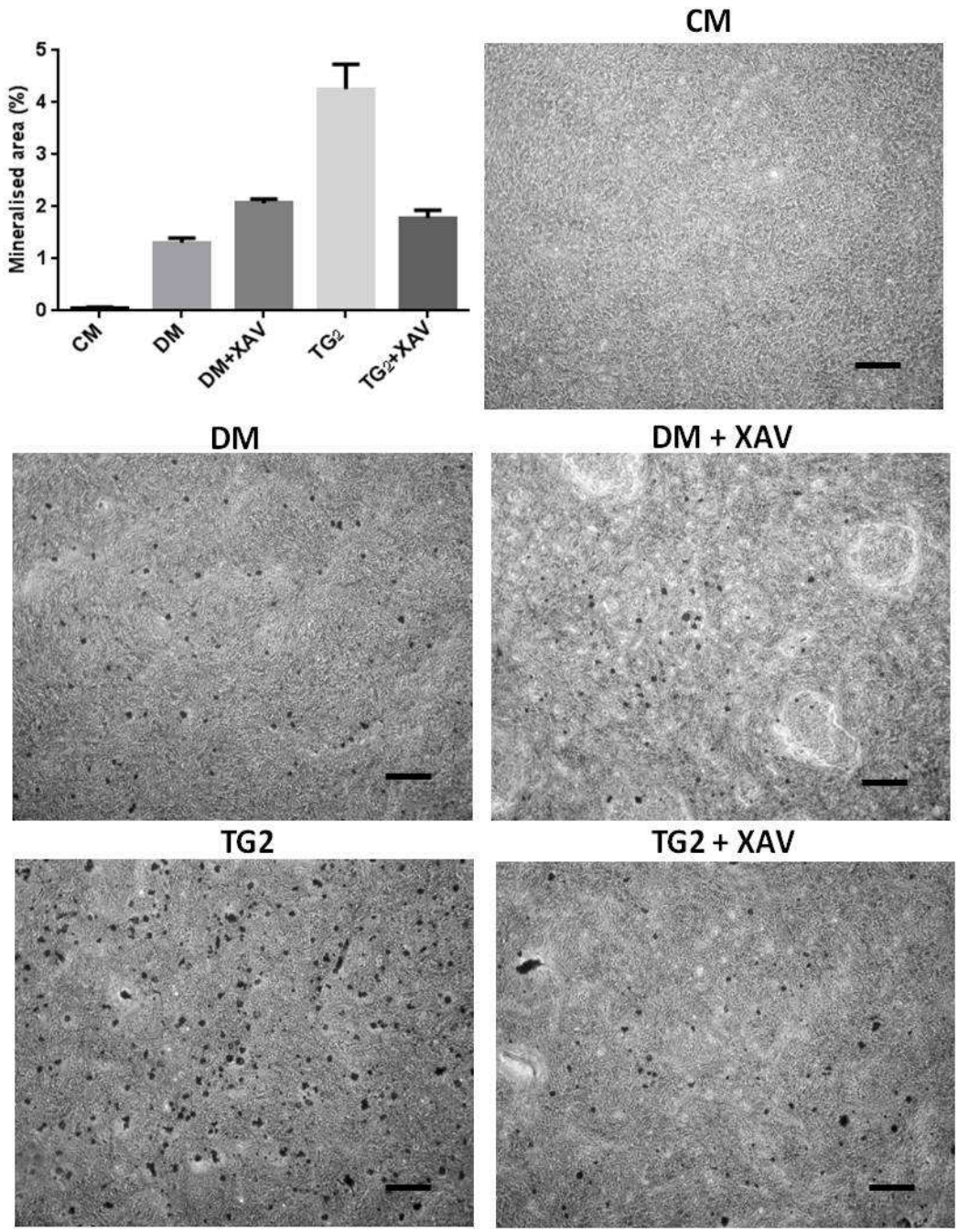

Figure 4.19 Mineralisation of HOBs cultured with β-catenin pathway inhibitor for 12 days. HOBs were cultured in differentiation medium with or without 1μg/ml of gpTG2 (TG2) plus inhibitor XAV939 for 12 days. Mineral deposition in extracellular matrix was determined using von Kossa staining and the mineralised area was quantified using Image J software. The quantified data represent mean values +/- SEM from a representative experiment with triplicate setting. This experiment was repeated two times and further experiments would be necessary to verify the inhibitory effect of XAV939 was statistically significant. The scale bars represent 150μm.

3. Discussion

The object of this chapter was to investigate the possible role of endogenous and exogenous TG2 in human osteoblast differentiation and mineralisation. It was shown in the previous chapter that the expression of endogenous TG2 either did not affect the cell mineralisation process or it could be compensated by upregulation of FXIIIa. In this chapter, it was demonstrated that endogenous TG2 activity was not essential for osteoblast mineralisation, since blocking TG2 activity could not abolish cell mineralisation. Also, TG2/FXIIIa activity inhibitor R283 could not block the mineralisation process, suggesting that both TG2 and FXIIIa activity were not needed in this process or that only minimum crosslinking activity was required for mineralisation.

ATPase activity of TG2

TG2 was suggested to act as an ATPase in the extracellular matrix, especially the cleaved TG2 by MT1-MMP, and serve as a source of Pi thus contributing to osteoblast mineralisation (Nakano *et al.*, 2007, Nakano *et al.*, 2010). This study tested the hypothesis that ATPase activity of TG2 plays an important role in the mineralising process by examining TG2 fragments in whole cell/ECM extracts, collagen and gelatin zymography and effect of ATPase inhibitors in mineral matrix deposition. Here, an increase in TG2 fragments from day 8 post treatment with differentiation medium and TG2 inhibitors was demonstrated. In this study, major endogenous TG2 fragments in HOBs culture were found in whole cells/ECM lysis and the protein sizes were between 60 kDa to 70kDa. TG2 inhibitor treated cells showed more TG2 fragments, possibly because inhibitors kept TG2 in an open conformation which favoured MMP degradation. The putative cleavage sites of MT1-MMP on TG2 were at Pro-375, Agr-458 and His-461 while MMP2 was found directly associated with the core enzymatic domain II of TG2 (Belkin *et al.*, 2001, Belkin *et al.*,

2004). As illustrated in Fig. 4.20, R283-bound TG2 is in a semi-open form and R294-bound TG2 is in a fully open form. It is reasonable to assume that R294-bound TG2 is more vulnerable to MMP-mediated degradation due to the cleavage site being exposed in the open conformation. In fact, it has been shown that TG2 is more sensitive to enzyme-mediated degradation when in its calcium bound open form compared to its GTP bound closed form (Zhang *et al.*, 1998, Begg *et al.*, 2006). As future work, the susceptibility of TG2 and inhibitor-bound TG2 to MMP digestion could be demonstrated using purified TG2.

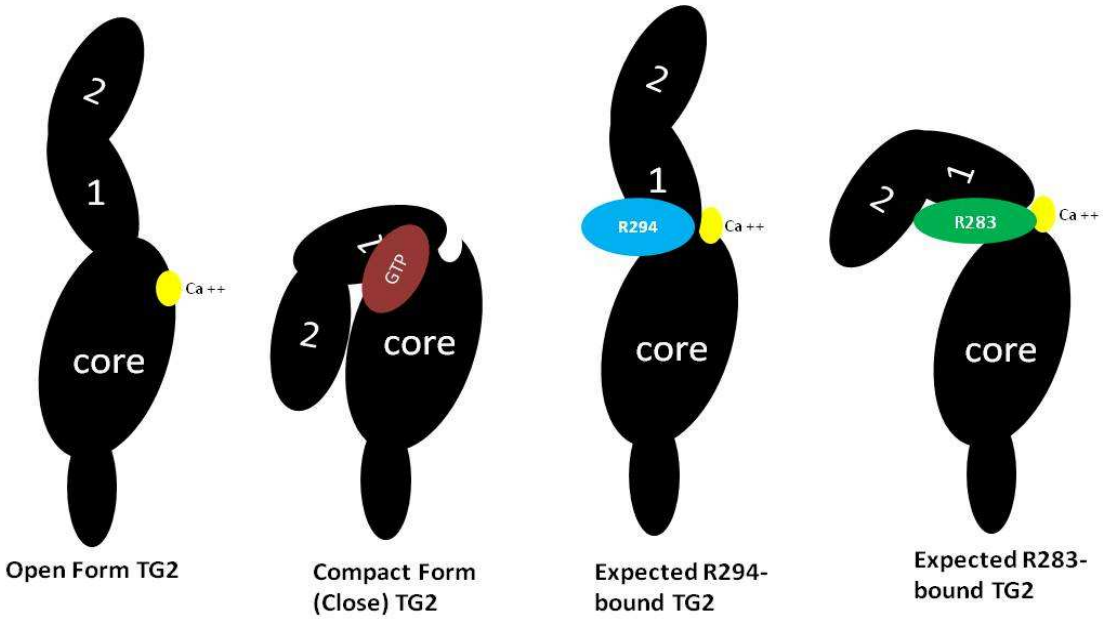

Figure 4.20 Predicted conformation of TG2 with or without inhibitors. The structure of TG2 has been revealed by X-ray crystallography. The active TG2 was suggested to be stable extended form (Pinkas et al., 2007) and GTP/GDP bound TG2 was observed in a compact form (Liu et al., 2002). Inhibitor R294 was predicted to hold TG2 in open form while R283bound TG2 was in putative semi-open form.

It is uncertain if these TG2 fragments retained any biological function, yet, the presence of these small TG2 fragments accompanied an increase in mineralisation area when treating cells with inhibitors. No evidence suggested that these TG2 fragments could bind to LRP5 receptor, since the Co-IP showed only full length TG2 interacting with LRP5. According to Nakano *et al.*(2010), released TG2 in mouse osteoblasts culture medium was cleaved by MT1-MMP into ~69, ~55, ~40, ~30kDa and other minor fragments. Only the ~55 kDa fragment had ATP binding ability as illustrated by its affinity for ATP-agarose. It has been

also suggested that proteolytic cleavage of TG2 by MT1-MMP *in vitro* resulted in increased ATPase activity of TG2. As reported by Belkin and his colleagues, membrane type I MMP (MT-MMP-1, MMP-14) and MMP-2, in tandem, regulate TG2 functionality in cell-surface adhesion/signalling by proteolysis of TG2 at certain cleavage sites (Belkin *et al.*, 2001, Belkin *et al.*, 2004). This study suggests that enzyme mediated degradation of TG2 is also important in the osteoblast mineralisation process and that the proteolysis of TG2 might contribute to mineralisation via its ATPase activity. In order to clarify if MMP-mediated TG2 fragmentation is accountable for the increase in ATPase activity and bone mineralisation, MMP inhibitors can be used in the future to verify the hypothesis.

Collagen and gelatin zymography were performed in this study and the results suggested the possible presence of MMP-1 and MMP-9 in day 10 conditioned medium. Furthermore, unexpected down regulation of collagenase and gelatinase was observed in differentiated HOBs after 10 days of culture. Ahn *et al.* (2008) have demonstrated TG2-induced down regulation of MMP-9 in retinoic acid treated cardiac myoblasts and this effect could be prevented in the presence of TG2 inhibitors. This is consistent with the result found in this study that collagenase and gelatinase were down regulated after stimulation with ascorbic acid and β-GP and addition of TG2 inhibitors could regain the expression of the collagenase and gelatinase. It is hard to judge if the increase in TG2 fragments in the ECM could be directly linked to up regulation of collagenase and/or gelatinase expression in inhibitor treated osteoblasts. So far, there has been no study suggesting TG2 as a substrate for MMP-1 and MMP-9. Yet, the TG2 fragments could be found in whole cell/ ECM in differentiated cells but neither MMP-1 nor MMP-9 was found in conditioned medium of HOBs cultured in DM. This, again, indicated that proteolysis of TG2 was mainly conducted by other MMPs, most likely MT1-MMP and MMP-2. The question then was in which compartment the cleavage of TG2 occurred. It was reported that activation of

MMP-2 was dependent on the trimolecular complex components (MT1-MMP /TIMP-2 /MMP-2) on the cell membrane (Kazes *et al.*, 2000). Therefore, the lack of MMP2 in conditioned medium could result from binding of MMP-2 protein to cell surface MTI-MMP1 via TIMP-2. It is suggested that cleavage of TG2 mainly happens in the cell membrane associated microenvironment, for example on the cell surface and in the extracellular matrix. TG2 fragments could be clearly detected on cell membrane in the later stage of mineralisation. Although it remains to be confirmed, these TG2 fragments potentially had ATPase activity which could mediate the level of Pi in local microenvironment and further contribute to cell mineralisation.

In order to study if the ATPase activity was important for cell mineralisation, a general ATPase inhibitor, ATP-γ-s was used to block the enzymatic activity. The importance of ATPase was demonstrated here that the mineralisation was blocked when 10 μM ATP-γ-s was used which only caused a minimum alteration of ALP activity. According to the literature, both ALP and ATPase activity are presented in matrix vesicles (MVs) where mineralisation happens. It has been suggested that 85% to 90% of ATP hydrolysis is mediated by ALP, the remained 10% to 15% of ATP hydrolysis was not affected by ALP inhibitor on MVs (Majeska and Wuthier, 1975). The presence of another ATPase other than ALP on MVs was also demonstrated in ATP-dependent calcium deposition (Hsu and Clarke Anderson, 1995). Interestingly, TGs activity was found increased in MVs of ALP knockout mice which might suggest that TG2 functions as an ATPase in MVs in extracellular matrix. Unfortunately, there was concern that the negative effect observed in cells cultured with ATP-γ-s may involve a broad range of ATPase related factors including PMCA1, a calcium transport protein on the cell membrane. Although it is widely believed that cell membranes are imperemeable to anions, some researchers suggest cells can uptake extracellular ATP (reviewed by Chaudry, 1982). In this case, uptake of ATP-γ-s

into cytoplasm might inhibit the PMCA1 thus inhibit the output of calcium ion to the calcified site. Therefore, in the future, an indirect approach by using specific MMP inhibitors to abolish TG2 fragments and reverse inhibitors-induced mineralisation in osteoblasts culture would be a more practical way to study biological function of TG2 fragments.

LRP5/ Wnt/ β-catenin pathway

On the other hand, exogenous TG2 induced calcification has been demonstrated in vascular smooth muscle cells (Faverman *et al.*, 2008) where TG2 was shown to up regulate osteoblastic markers in VSMCs. Here in this study, a similar effect of exogenous TG2 could be found in human osteoblasts where the mineral deposition of HOBs was stimulated by extracellular TG2. It has been suggested that the activity of TG2 was important for TG2 induced β-catenin activation where genetically or pharmacologically reduced TG2 activity fail to activate β-catenin in response to warfarin (Beazley *et al.*, 2012). This is consistent with the finding that the inductive effects of R283 inactivated TG2 and R294 inactivated TG2 were lower than wild type TG2. These evidences indicated that TG2 activity was involved in TG2 induced mineralisation.

Nevertheless, It is worth pointing out that the inhibitors have been reported to change the conformation of TG2 (reviewed by Pinkas *et al.*, 2007) and that there is growing evidence for TG2 mediated differentiation dependent on TG2 confirmation but not transamidating activity were suggested. Earlier study by (Johnson and Terkeltaub, 2005) proposed that transamidating activity was not required for TG2 induced matrix calcification as illustrated by the failure of active site point mutant TG2 (C277G, H335A and D358A) to block chondrocyte differentiation and calcification. Moreover, the GTP binding site mutant TG2 (K173L), which is expected to hold TG2 in the open conformation, failed to induce matrix

calcification in chondrocytes. The importance of the closed conformation of TG2 was further emphasised as Mg-GTP treated wild type TG2, which theoretically blocked both GTPase and transamidating activity and kept TG2 in the closed conformation, still retained the capacity to promote matrix mineralisation. Therefore, it was assumed that compact form of TG2 was an ideal conformation for inducing cell mineralisation. As shown in Fig. 4.20, the crosslinking inhibitor R283 could theoretically hold TG2 in a semi-open conformation while R294 tended to keep TG2 in the extended conformation (personal communication with Dr. R Collighan, 2012). Accompanied by the mineralisation results that TG2/R283 has approximately 60% of the inductive effect compared to wild type TG2 and no inductive effect was observed by addition of TG2/R294, this finding was in agreement with previous study indicating the major role of TG2 conformation in TG2 induced mineralisation.

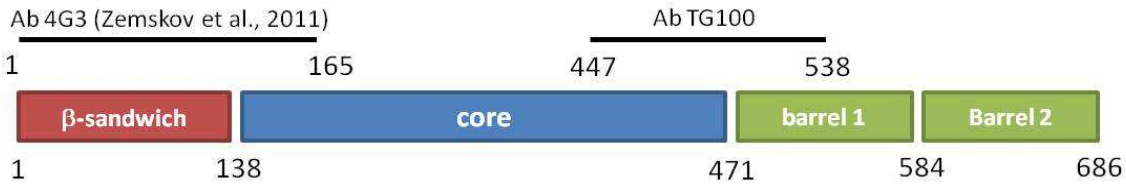

Figure 4.21 Antibodies binding sites in the TG2 enzyme. TG2 contains 4 domains including β-sandwich, core domain, β-barrel 1 and 2. Mouse anti-TG2 mAb 4G3 has epitope site 1-165 (Zemskov et al., 2007) and TG100 antibody binding site occurs at a.a. 447-538. Anti-TG2 antiserum (Faverman et al., 2008), on the other hand, has putatively multiple binding site.

Closed conformation TG2 might also explain the failure of immunoprecipitation of TG2/LRP5 complex using TG100 (anti TG2 antibody). As mentioned before, the TG100 antibody was designed to recognise a.a. 447 to 538 of TG2. As shown in Fig. 4.21, the corresponding epitope site of TG100 is situated at the region which undergoes major conformational change. This again supports the hypothesis that the closed conformation is required for TG2-induced mineralisation. It would be interesting to use W241A mutant TG2 to study the role of TG2 conformation and activity in TG2-induced calcification. W241A mutant lacks crosslinking function without affecting its GTP binding ability,

therefore, it would be a good model to look into the biological functions of different TG2 conformations.

The importance of conformation in TG2 induced mineralisation could give a clue that a certain level of interaction between TG2 and other proteins may take place on the cell membrane and/or in the extracellular matrix. Research has found extracellular TG2 might alter the LRP5/ β-catenin pathway, a master regulatory pathway of bone formation, and contribute to tissue calcification. Therefore, the related protein expression in LRP5/β-catenin pathway was examined in the HOB mineralisation model. The protein expression of β-catenin, LRP5 and N-cadherin in differentiated HOBs revealed a counter pattern between N-cadherin and β-catenin/ LRP5/ TG2 expressions. It is widely believed that N-cadherin is involved in early cell aggregation by enhancing cell-cell adhesion and promoting cell differentiation through activating intracellular signals (Ferrari *et al.*, 2000). However, recent data implied that N-cadherin may participate in another mechanism to negatively regulate osteoblast differentiation and bone formation (Fig. 4.22). The inhibitory effect of N-cadherin could involve interaction with Wnt signalling in which direct binding of β-catenin to cadherin potentially sequestrates β-catenin at the membrane, reduces cytosolic β-catenin pool and inhibits Wnt signalling (Conacci-Sorrell *et al.*, 2002, Wheelock and Johnson, 2003, Nelson and Nusse, 2004). Studies also revealed a novel link between N-cadherin and LRP5 which is independent of β-catenin binding. Interaction of N-cadherin with the intracellular domain of LRP5/ axin resulted in increased β-catenin ubiquitination, decreased β-catenin cytosol levels and subsequent down regulation of nuclear translocation in response to Wnt signalling (Hay *et al.*, 2009, Marie, 2009).

Therefore, the transient increase in N-cadherin of DM treated osteoblasts compared to CM treated cells (4 hours post treatment) could promote a rapid increase in cell

aggregation which was generally observed in differentiating cells. Consistent with this finding, the down regulation in both β-catenin and LRP5 at 4 hours post treatment could indicate the negative effect of N-cadherin mediated inhibition of Wnt/β-catenin signalling via LRP5. Down regulation of N-cadherin during cell mineralisation, on the other hand, could promote the maturation process of osteoblasts.

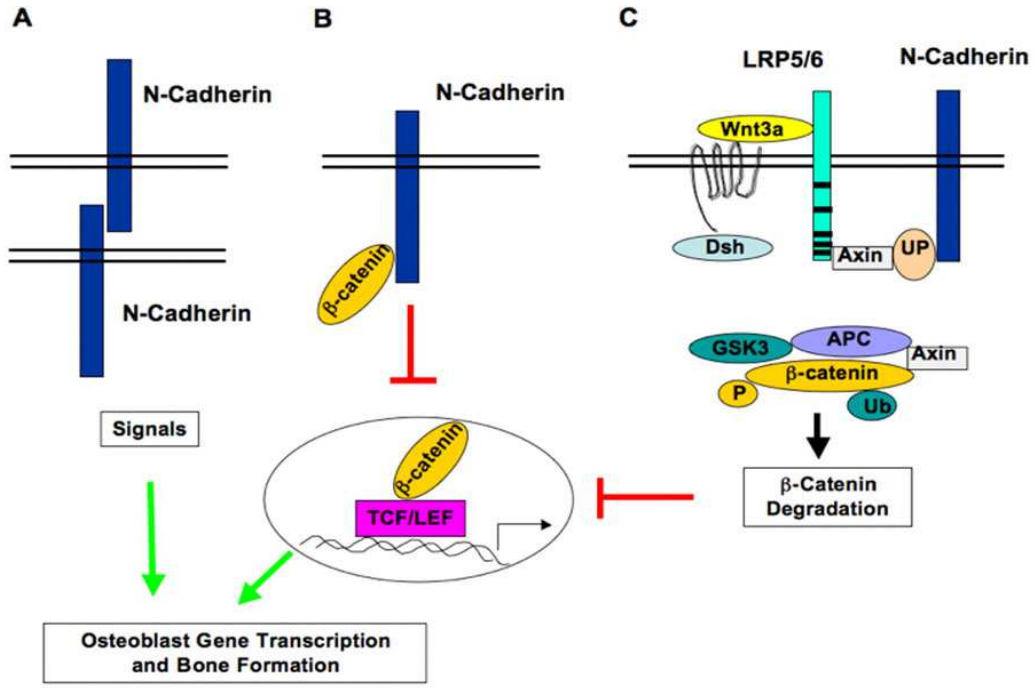

Figure 4.22 Putative mechanism of N-cadherin- mediated osteoblast function (adapted from Marie, 2009). (A) N-cadherin interactions between cells promote adhesion and downstream signals. (B) Direct binding of β-catenin to N-cadherin results in β-catenin sequestration at the membrane and down regulation in Wnt signalling. (C) The interaction between N-cadherin and the LRP5/6 results in increasing β-catenin degradation and inhibition of Wnt/β-catenin signalling. (UP: unknown protein; Ub: ubiquitin)

Many studies have indicated that cell/tissue differentiation requires coordinated regulation of cadherin mediated cell adhesion and the WNT/β-catenin pathway (Westendorf *et al.*, 2004, Nelson and Nusse, 2004, Marie, 2009). Here in this chapter, extracellular TG2 was demonstrated as a possible regulator for this process. Direct binding of TG2 to LRP5 has been demonstrated here in osteoblasts and in VSMCs by (Faverman *et al.*, 2008). Interaction was found in DM treated and exogenous TG2 treated cells as early as 4 hours post treatment and up to 24 hours post treatment as indicated by Co-IP. The β-

catenin activation was also observed in osteoblasts treated with exogenous TG2 for 4 hours by immunohistochemical staining. These evidences support an early activation of the β-catenin pathway through binding of TG2 to LRP5 receptor, especially in a high extracellular TG2 environment. The activation of the β-catenin pathway downstream of TG2/LRP5 binding could be further illustrated where β-catenin inhibitor blocked the TG2 induced mineralisation but not the cell mineralisation in physiological condition.

A regulatory effect on extracellular TG2 triggered β-catenin activation was also suggested in this study. According to Western blot, the expression of LRP5 and β-catenin in differentiated cells was highest on day 2, however, no significant TG2 and LRP5 interaction was shown by co-IP assay. A possible explanation for this observation was the presence of a negative signalling modulator, most likely LRP-1 in this case. LRP-1 is known as a transmembrane modulator of WNT signalling (Zilberberg *et al.*, 2004) and a cell surface TG2 expression regulator (Zemskov *et al.*, 2007). The absence of TG2/LRP5 complex may result from LPR1 competing for binding to TG2 on the cell surface and removal of TG2 to lysosomes for degradation.

Although there is only limited understanding about the TG2 triggered β-catenin pathway, exposure to high levels of extracellular TG2 may counteract the negative effect of N-cadherin in β-catenin pathway, thus enhancing osteoblast mineralisation. In this case, activation of TG2/LRP5 pathway alters the balance of cytosol and nucleus β-catenin levels thus promoting cells to undergo differentiation and mineralisation process. Even though the exogenous TG2 induced mineralisation was suppressed by the β-catenin pathway inhibitor- XAV939, the possibility of non-specific effect of XAV939 could not be ruled out. To further confirm that TG2-induced enhancement in mineralised area requires activation of the β-catenin pathway, setting up a positive control (e.g. Wnt3a treated cell culture) will be necessary in the future work.

Overall, the data in this chapter suggest different possibilities of how extracellular TG2 is involved in mineralisation process. Interestingly, extracellular TG2 has been implicated in the wound healing process in the past decade by promoting repair, matrix stabilization and cell viability in physio-pathological condition (reviewed by Verderio *et al.*, 2005, Telci and Griffin, 2006b). Externalisation of TG2, via an atypical secretion pathway or simply from necrotic cells, during cell stress or tissue damage is commonly observed. Therefore, the TG2 induced osteoblast mineralisation and VSMC calcification could represent a wound healing process mediated by TG2 under pathological conditions. Moreover, in endochondral ossification, both TG2 and FXIII were detected in chondrocytes at the edge of the growth plate and osteoblasts in the perichondrium/periosteum area but were negatively expressed in the area far away from the leading edge of the bony collar as demonstrated in chicken embryonic models (Nurminskaya and Kaartinen, 2006). It has been also indicated that culturing pre-osteoblasts with hypertrophic chondrocytes could increase the expression of several osteoblastic markers in osteoblasts and promote cell mineralisation (Nurminskaya *et al.*, 2003). Taking the accumulating evidences of exogenous TG2 to promote differentiation with the localisation pattern of endogenous TG2 in the embryonic long bones, it suggests TG2 secreted by chondrocytes and/or osteoblasts could act as an initial signal for osteoblast differentiation and mineralisation in bone development.

In conclusion, under physiological conditions, MMPs could act as a switch to convert the function of accumulated TG2 in extracellular matrix or on the cell membrane from transamidase oriented to ATPase oriented, especially in later stages of differentiation and the start of mineralisation. The ATPase activity of TG2 may not play a dominant role in cell mineralisation as it could be compensated by other enzymes such as plasma membrane Ca^{2+} transport ATPase 1 (PMCA1). However, in endochondral ossification and under

pathological condition, a dramatic increase in extracellular TG2 levels may trigger an outside-in signal through binding of LRP5 receptor thus activating the β-catenin pathway which then stimulates osteoblast differentiation and mineralisation. This effect was very likely crosslinking activity independent but the closed conformation of TG2 was required.

Chapter V

Evaluating Transglutaminase Crosslinked Collagen Gel Systems for Hard and Soft Tissue Repair

1. Introduction

Collagen based scaffolds are widely used in a broad range of regenerative medicine applications. Even though collagen scaffolds have shown clinical success in a range of soft tissue repair applications, relatively poor mechanical properties remains as a limitation for their use in hard tissue regeneration. In fact, only selected researches have showed some degree of success in using collagen scaffold for bone regeneration (Caiazza *et al.*, 2000, d'Aquino *et al.*, 2009, Keogh *et al.*, 2010) comparing to other synthetic materials. An ideal biomaterial for bone tissue repair should support osteogenic cells to migrate, proliferate and differentiate, promote new bone formation, and provides mechanical competence during the bone regeneration. In order to address this goal, current strategy focuses on recognising the complex interplay of signals from the extracellular microenvironment to mediate osteogenic cell fates such as adhesion and differentiation and fabricating functional implants for bone repair.

Using collagen matrix for orthopaedic regeneration offers distinct advantages since type I collagen composes more than 90% of the organic fraction in native bone tissue. The mechanical property problem can be overcome by the addition of a second, stiffer phase such as hydroxylapatite (HA) or crosslinked collagen based matrix through glutaraldehyde (Weadock *et al.*, 1983), ultraviolet (UV) radiation (Wollensak and Spoerl, 2004), dehydrothermal processing (Yannas and Burke, 1980), carbodiimides (Damink *et al.*, 1996) and enzymatically crosslinking by microbial transglutaminase (mTG) or TG2 methods (Chau *et al.*, 2005, Chen *et al.*, 2005). All these methods have showed a certain level of success in modifying the degradation rate of collagen matrix, giving collagen biomaterials advantages in wider medical applications.

In the first part of this thesis, TG2 was recognised as a potent pro-osteogenesis factor. As an efficient transamidating agent, TG2 was first reported to play an important role in matrix stabilisation and the cell adhesion process. Later on, it was demonstrated here in this thesis and in other studies (Faverman *et al.*, 2008, Beazley *et al.*, 2012) that TG2 in the extracellular matrix could trigger the β-catenin pathway via binding to low density lipoprotein receptor (LRP) 5 and further contribute to ossification. It also had potential to serve as a source of phosphate via its ATPase activity and this effect was tightly regulated by MMPs activity during mineralisation. Theoretically, crosslinking collagen I gel with TG2 should not only improve its physical and biological stability but also introduce osteoconductive and osteoinductive properties. The advantage of using TG2 treated collagen gel as bone graft was first demonstrated by Chau *et al.* (2005) who showed that TG2 crosslinked collagen matrix enhanced cell attachment, spreading, proliferation and expression of differentiation markers in human osteoblasts. In addition, crosslinked gel also showed better resistance against cell-mediated endogenous protease degradation.

Using collagen-based biomaterial also offers advantages for cornea regeneration since type I collagen composes more than 90% of the cornea. Properties of ideal biomatrix for corneal wound healing include biodegradability, biocompatibility, transparency, sufficient mechanical competence during the cornea regeneration, short wound healing time, easy application, postoperative comfort and cost effectiveness. Using fibrin glue as an original inspiration, here, TG2 crosslinked collagen I gel is proposed as an alternative option for ocular surgery. First of all, endogenous collagen I and TG2 are ubiquitously distributed in the cornea tissue and especially TG2 shows a predominant presence in the corneal subepithelium and stromal layer (Barathi *et al.*, 2011). Secondly, TG2 was found to have a broader range of substrates than FXIIIa. Panengad *et al.* demonstrated abundant TG2 substrates distributed throughout all layers of cornea tissue and a good co-localisation of

TG2 with collagen and fibronectin (Panengad *et al.*, 2011). Finally, it was reported that the adhesive strength and bonding strength of TG2 based adhesive was higher than Tissucol, a commercialised fibrin gel (Jurgensen *et al.*, 1997). The potential of TG2 based glue for ocular applications has not yet been fully evaluated. A recent study by Chau *et al.* (2012) suggested that TG2 treated amniotic membrane (AM) as a novel treatment for ocular surface disease due to its enhanced mechanical strength and stiffness without affecting its transparency. However, so far no data have been published regarding the biocompatibility of TG2 based matrix in cornea tissue.

Here in this chapter, a TG2 modified collagen matrix was analysed for its potential as a graft material for hard and soft tissues. Surface composition and topography were two factors considered in evaluating the properties of TG2 crosslinked collagen gel. The biological properties of crosslinked gel were also assayed including biocompatibility and several cell behaviours. The cells used in this chapter were human osteoblasts (HOBs), human corneal epithelial cells (hCECs) and human corneal fibroblasts (hCFs) in order to represent the hard and soft tissue model.

2. Results

2.1 Self-incorporation of TG2 into type I collagen gel

As reported previously (Shanmugasundaram *et al.*, 2012) ,auto-crosslinking of TG2 into collagen matrix occurred during collagen polymerisation. Here the amount of residual TG2 in crosslinked gel was determined using gel electrophoresis and Western blotting. A modified protocol from Shanmugasundaram's publication was used in this study. Briefly, following overnight incubation with enzyme, treated and non-treated collagen gels were vigorously washed with PBS several times to remove unbound TG2 before boiling with 5X reducing Laemmli sample buffer. Western blotting showed low but detectable TG2 monomer at 78kDa in TG2 crosslinked collagen gel (Fig. 5.1). Interestingly, high molecular weight bands were found in insoluble fragments which were at the very top of the gel (>245kDa). These high molecular weight bands were assumed to be highly polymerised TG2 complex or a crosslinked complex of TG2 and type I collagen. When TG2 activity was inhibited with R283 and R294, the 78kDa TG2 monomer was found to be significantly increased; meanwhile, the highly polymerised TG2 complex band was less intense but still observed. Reduction in the high molecular weight band implied that crosslinking activity of TG2 enhanced the formation of TG2 polymer or TG2/collagen I complex. As for the faint >245kDa band found in Inhibitor-treated TG2 groups may result from residual transamidating activity within the TG2/inhibitor or result from the preparation stage where the gpTG2 was pre-activated with calcium and DTT for 5 minutes on ice before treatment with inhibitors. On the other hand, this study also showed that even without its enzymatic activity, TG2 was still trapped in the collagen gel and could not be removed by washing. These results suggested that trapped TG2 was not negligible and could serve as a source of exogenous TG2 for cells. Consequently, TG2 could incorporate itself into collagen gel via both a crosslinking activity dependent and independent way.

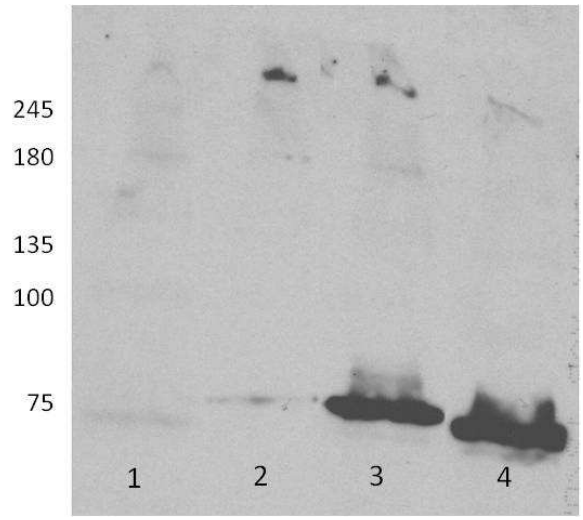

Figure 5.1 Tethering of TG2 into type I collagen gel. Type I collagen gels mixing with active or inhibitors pre-treated (inactive) TG2 were sampled and the bound TG2 determined by Western blotting as described in Materials and Methods. Lane 1: native collagen gel, lane 2: TG2 crosslinked collagen gel, lane 3: R283-inactivated TG2 crosslinked gel and lane 4: R294-inactivated TG2 crosslinked collagen gel.

2.2 Crosslinking by TG2 alters collagen fibre structure

Using electron microscopy, the collagen bundles in native collagen and crosslinked collagen gels showed similar organisation which was highly compact and roughly arranged in isotropic direction (Fig. 5.2). According to image analysis, the average diameter of crosslinked collagen bundles was significantly higher than native collagen fibres which were approximately 2.6 μm and 1.1 μm, respectively (Fig. 5.3). Studies have suggested that the diameters of collagen fibres vary depending on the polymerization temperature and pH (Christiansen *et al.*, 2000, Raub *et al.*, 2007). This study demonstrated that, besides the factors mentioned above, treating matrix with TG2 might also alter the formation of collagen fibres and further affect cell behaviour.

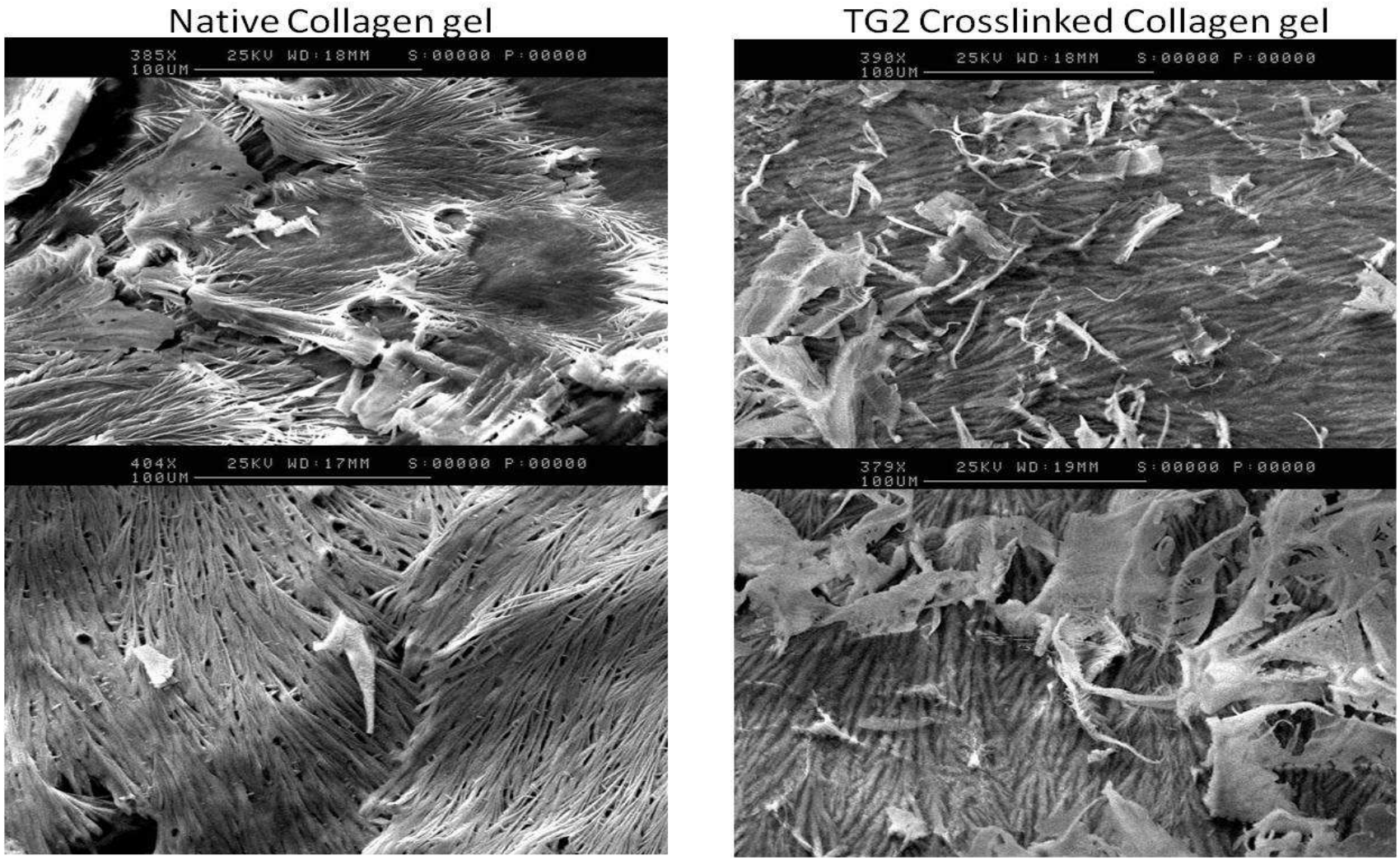

Figure 5.2 SEM images of TG2 crosslinked type I collagen gel. Native collagen gel (left) and TG2 crosslinked gel (right) were then fixed, freeze-dried for 50 hours and sputter coated with gold before taking pictures using SEM. The actual scale bar was stated in each picture.

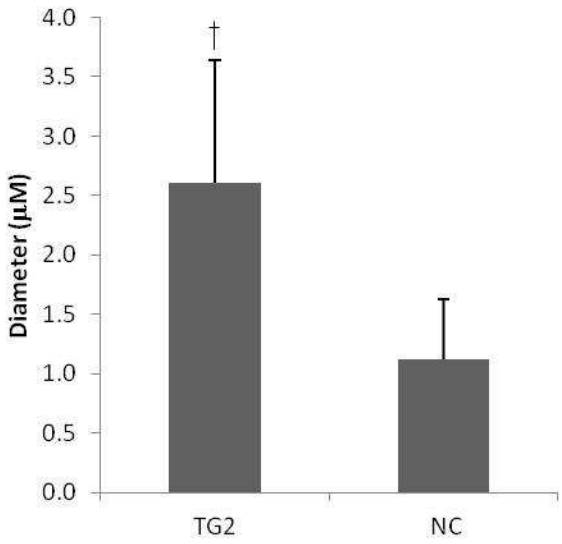

Figure 5.3 Change in diameter of fibres after treatment with TG2. At least 180 fibres were measured in each group from 3 individual experiments using Image J software. Data represents mean values +/- S.D. Statistical analysis was carried out using the one-way ANOVA test and the p-values corresponding to p<0.01 are represented with a †.

2.3 TG2 crosslinked collagen gel promoted HOBs differentiation and mineralisation

It was demonstrated here and elsewhere that exogenous TG2 could enhance calcification of osteoblasts and other cell lines *in vitro*. Evidence has shown that extracellular TG2 may trigger an outside-in signal via LRP5 and LRP6, further activate the β-catenin pathway and consequently promote cell mineralisation. Combining the advantage that crosslinked collagen could improve osteoblast attachment and proliferation notably (Chau *et al.*, 2005), here the differentiation and mineralisation characteristics of crosslinked collagen gel were evaluated.

One of the differentiation markers, ALP, was monitored for its released and anchored forms during the mineralisation process. Native collagen gel add-mixed with 1µg/ml of recombinant human bone morphogenetic protein 7 (BMP7), a known osteoinductive reagent, was used as positive control. 5mg/ml native collagen gel (NC) was used as a

control and 10µg/ml TG2 crosslinked collagen gel (TG2) and TG2 crosslinked BMP7 gel (TG2+BMP7) were evaluated. As shown in Fig. 5.4A, there was no significant change in released ALP between the groups. A very similar trend was found in membrane/ECM bound ALP expression that no significant change was observed between groups in early mineralisation. A small but not significant increase in anchored ALP activity appeared in crosslinked groups (TG2 and TG2+BMP7) on day 10 (Fig.5.4B). This experiment was only done twice, however, further experiments would be needed to conclude this finding.

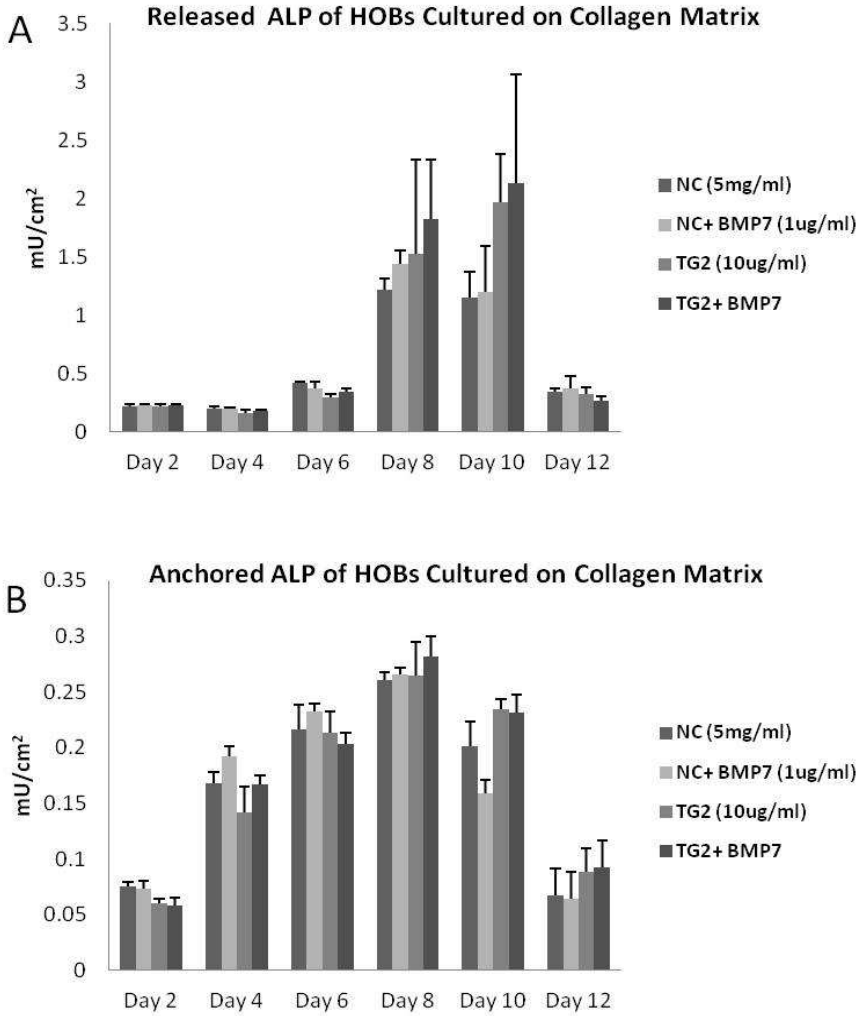

Figure 5.4 Released (A) and anchored (B) ALP activity evaluated by using pNPP substrate system. The change of absorbance per minutes per centimetre square of culture area was converted into milli-unit of enzyme. The data represent mean values +/- SD from a representative experiment with triplicate setting. This experiment was repeated two times and further experiments would be necessary to perform the statistic analysis.

Mineral deposition was visualised by von Kossa staining 12 days post-treatment to evaluate the level of mineralisation of osteoblasts. A more intense positive staining was found in TG2 group comparing to NC group and on day 12 (Fig.5.5). However, admixing BMP7 to collagen gel did not improve cell mineralisation neither did admixing BMP7 to crosslinked collagen gel (TG2+BMP7). According to the supplier, the ED50 of BMP7 used in this study was 0.02–0.04 μg/mL (determined by the concentration response induction of alkaline phosphatase production by murine ATDC5 cells). It was possible that the release of BMP7 from the collagen or crosslinked collagen gel was restricted and was not enough for stimulating osteoblast mineralisation.

Another concern was that crosslinking activity of TG2 may result in unwanted effects on BMP7 activity or vice versa. In the following experiment, the concentration of BMP7 was raised to 2μg/ml and inactive TG2 (TG2+R283) was also used to determine the effect of TG2 crosslinking activity. As shown in Fig 5.6 and Fig 5.7, the mineralised area in the BMP7 group was increased and was significantly higher than the NC group. Meanwhile, the TG2 group demonstrated its osteoinductive characteristic and showed greater mineralised area than NC group and BMP7 group. However, the TG2 + BMP7 group had no synergic effect on cell mineralisation and had a lower but not significant effect on cell mineralisation when compared to the BMP7 group. It was also found that neither inactive TG2 (TG2+R283) showed enhancement on cell mineralisation when compared to the BMP7 group, nor addition of BMP7 increased the mineralisation effect of the TG2 containing biomaterial.

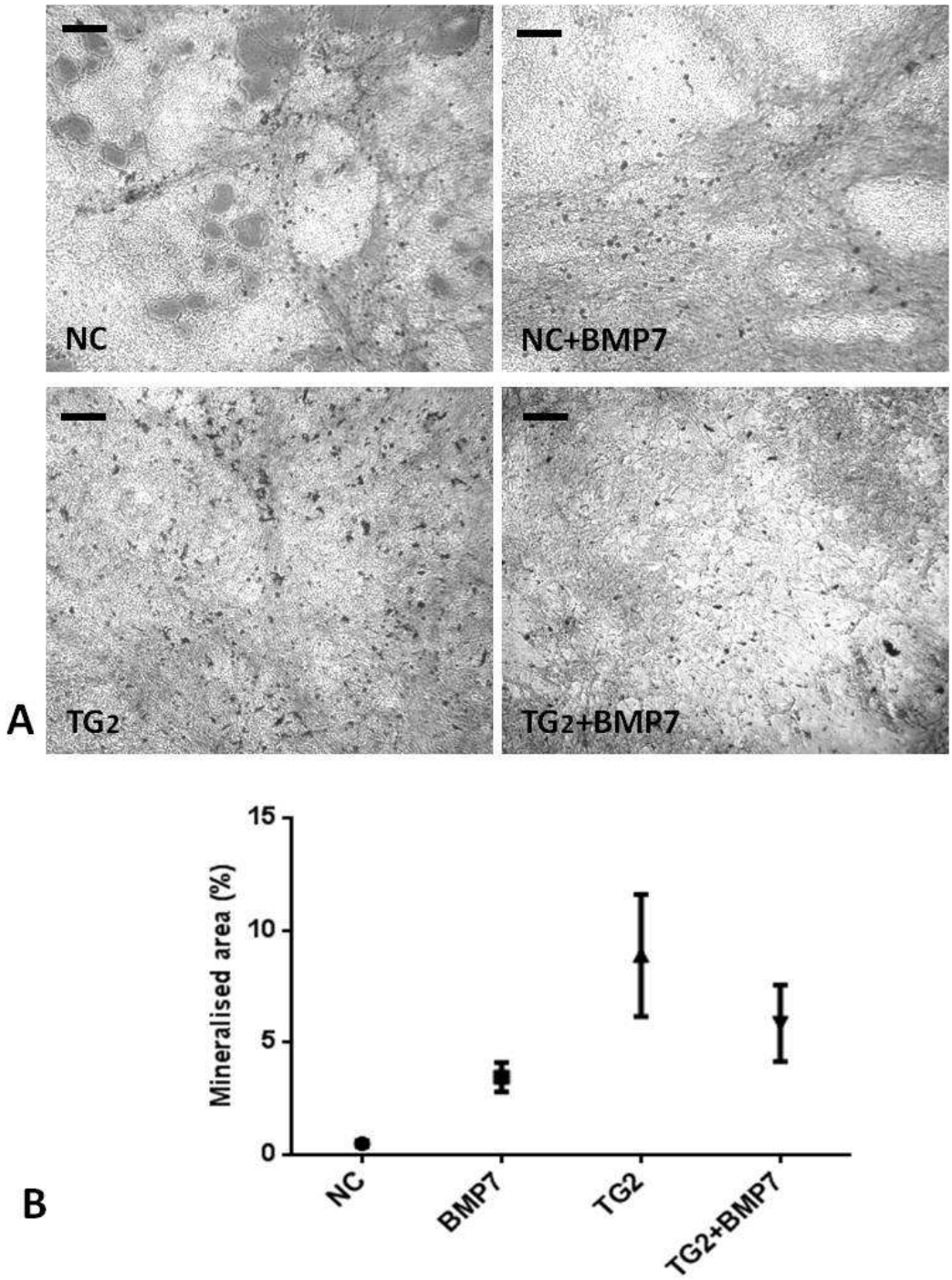

Figure 5.5 Relative mineralisation area of HOBs after seeding on native type I collagen gel (NC), native type I collagen gel admixed with 1μg/ml of BMP7 (BMP7), 10μg/ml of TG2 crosslinked type I collagen gel (TG2) and TG2 crosslinked BMP7 collagen gel (TG2+BMP7) incubated for 12 days. (A) Mineral deposition was visualised by Von Kossa staining in black and (B) the mineralised area was quantified using ImageJ as described in materials and methods. The data represent mean ± SEM value from one representative experiment with triplicate setting. This experiment has been repeated twice where further experiments are needed to confirm this finding is statistically significant.

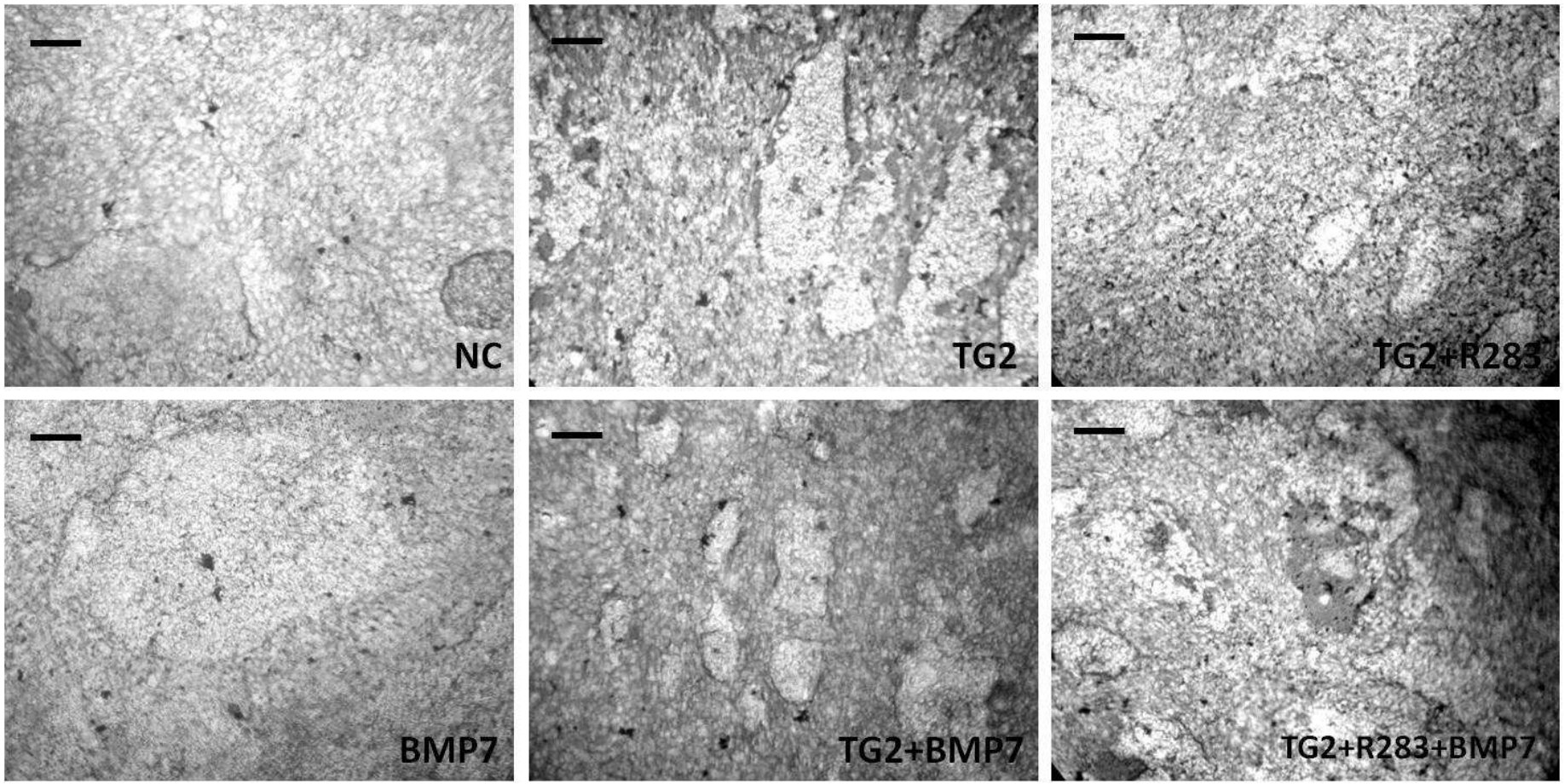

Figure 5.6 Mineralisation of HOBs after seeding on native type I collagen gel (NC), native type I collagen gel admixed with 2μg/ml of BMP7 (BMP7), 10μg/ml of TG2 crosslinked type I collagen gel (TG2), TG2 crosslinked BMP7 collagen gel (TG2+BMP7), R283 inactivated TG2 in collagen gel (TG2+R283) and inactive TG2 admixed BMP7 in collagen gel (TG2+R283+BMP7) for 12 days. Mineral deposition was visualised by Von Kossa staining in black as described in Materials and Methods. The scale bars represent 150μm.

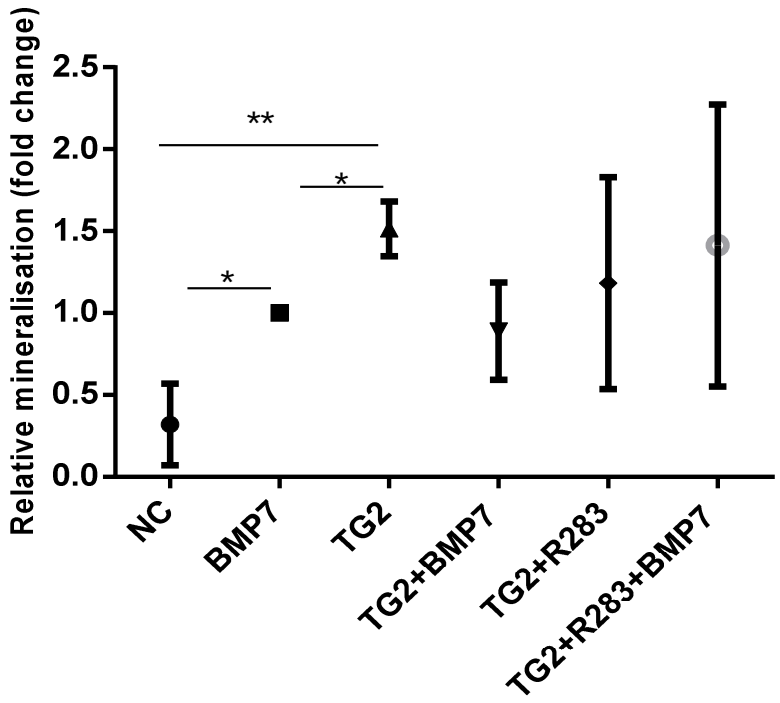

Figure 5.7 Quantification of mineralisation area (black dots) in HOBs cultures by analysing images from each group. All results were normalised with the value of the BMP7 group from each experiment and the average of the BMP7 group is 3.9±3.23% from 3 experiments. The data represent mean value ± SEM, where n=3. Statistical analysis was carried out using the one-way ANOVA test (Turkey's multiple comparison test). The P-values corresponding to $p<0.05$ is represented with a * and $p<0.01$ is represented with a **.

2.4 Proliferation of HCECs and HCFs on collagen matrices determined by XTT assay

The proliferation rate of HCECs grown on different concentrations of collagen gel (Fig. 5.8) was first determined using commercial XTT kit (Cell Proliferation Kit II, Roche Diagnostics Ltd., West Sussex, UK). The result suggested that XTT reduction was lower when cells were grown on collagen gel compared to fibronectin (Fn) coated tissue culture plate (TCP/Fn). There was no significant difference between cells cultured on different concentrations of collagen gel as indicated by XTT assay. Therefore, in the following experiments, 5mg/ml collagen gels were used to obtain the highest mechanical strength.

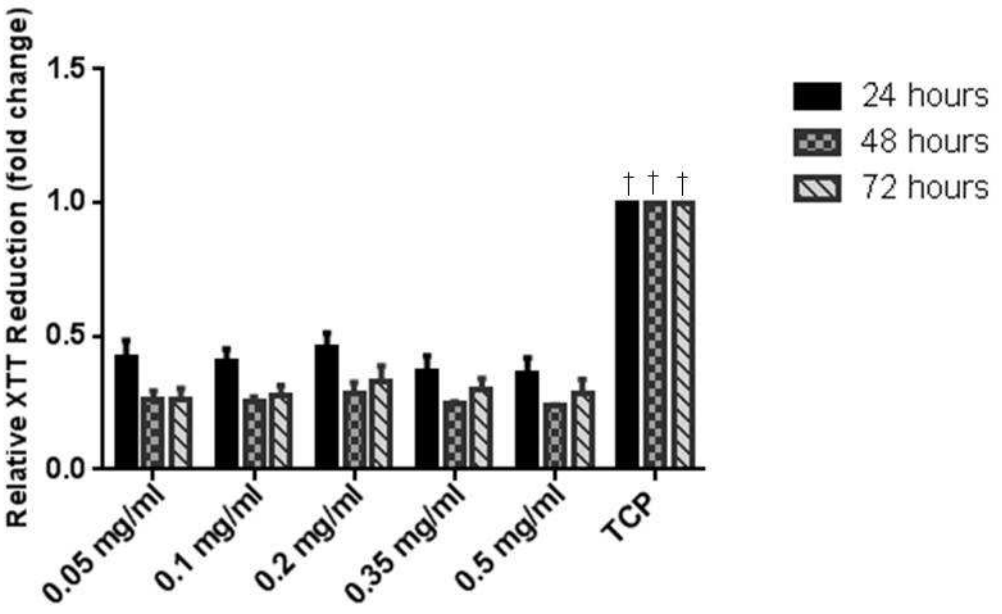

Figure 5.8 Proliferation of HCECs on different concentrations of native collagen gel. Cell proliferation was evaluated using the XTT assay over a 3 day period and the results were normalised with the TCP group. Data represents mean values +/- SEM from 3 different experiments. Statistical analysis was carried out using the two-way ANOVA test and no significant difference was found between groups at the same time points besides when compared to the TCP group. The p value corresponding to p<0.0001 is presented with a †

To further determine the biocompatibility of TG2 crosslinked gel, different concentrations of TG2, from 10μg/ml to 250μg/ml, were used and the XTT reduction rates on these gels were analysed. As showed in Fig. 5.9, the XTT reduction of hCECs was once again higher in the positive control group (Fn/TCP). Although there was a significant difference between 10 μg/ml TG2 treated collagen gel and 250 μg/ml TG2 treated collagen gel at 48 hours, none of the TG2 gels showed a comparable proliferative effect to the positive control (Fn/TCP). This suggested that both native collagen gel and TG2-collagen gel might had poor cell compatibility with hCECs.

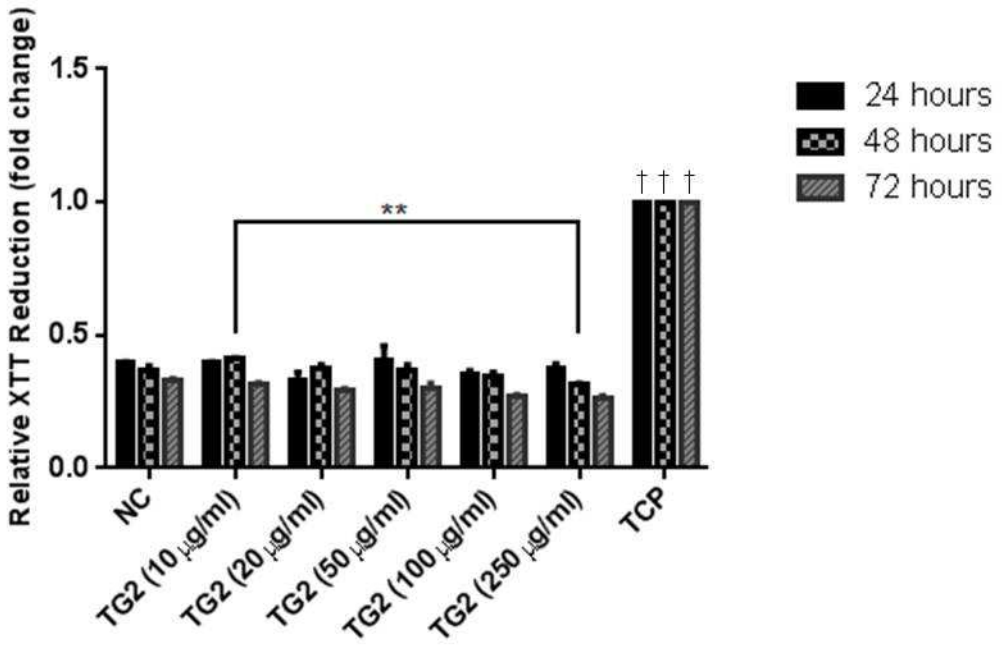

Figure 5.9 Proliferation of HCECs on collagen gel treated with different concentration of TG2. XTT assay was performed every 24 hours post seeding, up to 72 hours and the results were normalised with the TCP/FN group. The results represent the mean values ±SEM from 3 individual experiments. Statistical analysis was carried out using the two-way ANOVA test (Turkey's multiple comparison test) and each group were compared to each other at the same time point. The p-values corresponding to p<0.01 is presented with a ** and p<0.0001 is presented with a †.

In order to improve its biocompatibility, several modifications were made to the surface of crosslinked gel. Laminin was chosen as one of the major proteins in the corneal epithelial basement membrane (Ljubimov *et al.*, 1995) and it has been shown to promote spreading and attachment of corneal epithelial cells in primary culture (Qin *et al.*, 1997). Fibronectin was also used since it is an adhesion molecule which has been demonstrated to improve cell adhesion in a wide range of cell types. Early research using rabbit corneal tissue and corneal epithelial cells suggested that fibronectin (Fujikawa *et al.*, 1981, Nishida *et al.*, 1983) and its fragments (Mooradian *et al.*, 1993) could enhance epithelial cell adhesion, attachment and mobility. After polymerisation of TG2 collagen gel overnight, an extra layer of laminin (Lm) or Fn was coated on the surface. However, a positive effect on proliferation was only detected on Lm (Fig. 5.10A) or Fn (Fig. 5.10B) modified TCP. Again,

hCECs cultured on Lm and Fn modified TG2 collagen showed no significant difference on XTT reduction when compared at the same time point.

On the other hand, the XTT reduction rate of was also monitored over a 168 hours period. As suggested by XTT assay (Fig. 5.11) the crosslinked gel showed an early increase in reduction rate at 24 hours when compared to native collagen gel. But there was no significant difference in XTT reduction rate between TG2 group and NC group at 72 and 168 hours. Overall, the XTT assay offered a general view of cell metabolic activity but not necessarily the actual cell number or proliferation rate. The results might be affected by a change of metabolic rate when cells are attached to different surfaces. Furthermore, cells which had migrated into the gel might not be detected by XTT assay. Therefore, other factors shall be considered when evaluating XTT results.

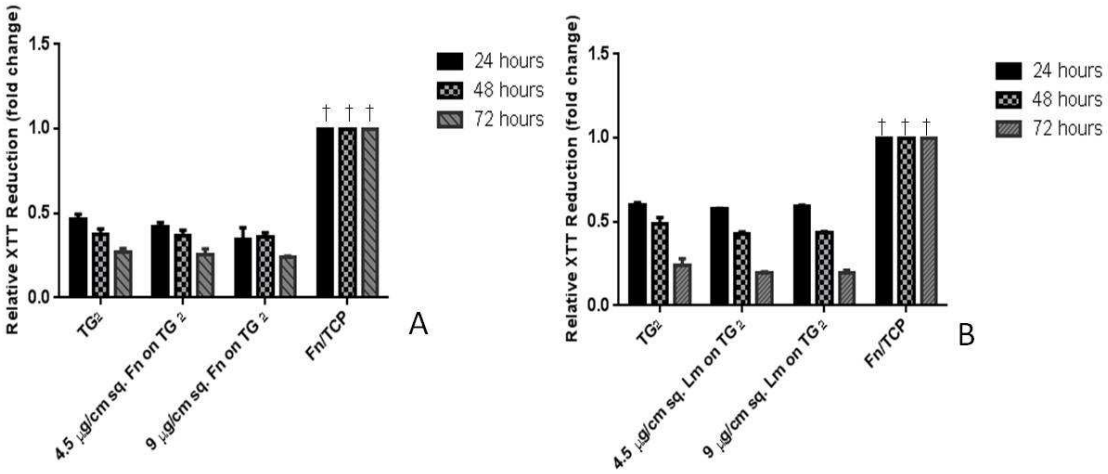

Figure 5.10. Cell proliferation of hCECs on Lm or Fn modified TG2-collagen gel. Cells proliferation when cultured on Lm modified (A) or Fn modified (B) crosslinked gel was analysed every 24 hours as described in Materials and Methods. Data were normalised to the average value of the positive control groups and is presented as mean values +/- SEM from 3 individual experiments (n=3). Statistical analysis was carried out using the two-way ANOVA test (turkey's multiple comparison test) and no significant differences were found between treated groups at some time points besides when compared to the positive control groups which are Lm/TCP and Fn/TCP respectively. The p value corresponding to p<0.0001 is presented with a †

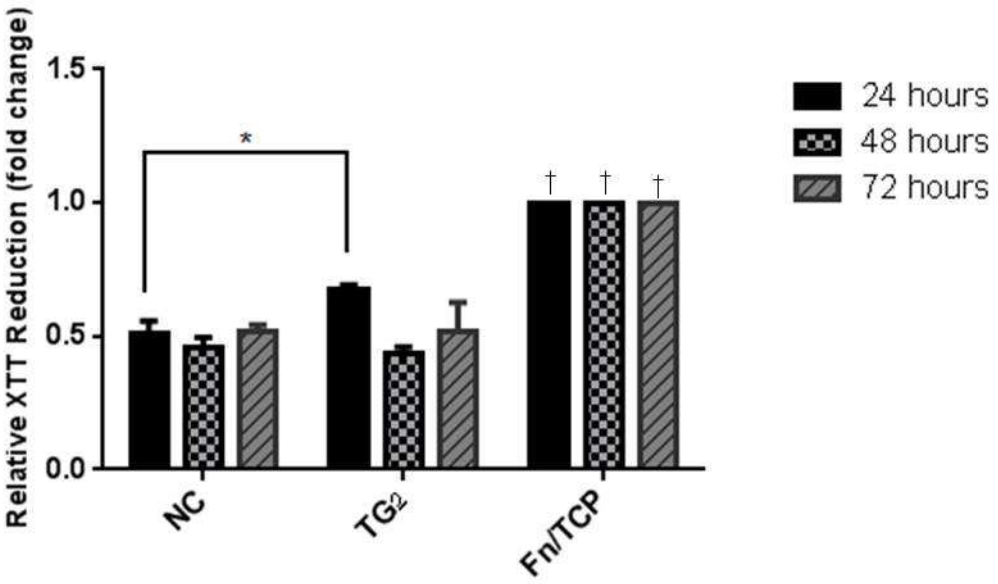

Figure 5.11 Proliferation of hCFs cultured on crosslinked collagen gel. 3,000 hCFs/well were seeded on NC, TG2 gel or TCP/Fn surface (positive control). XTT assay was performed at 24 hours, 72 hours and 168 hours post seeding to determine cell proliferation. The results represent the mean values ±SEM from 3 individual experiments (n=3). Statistical analysis was carried out using the two-way ANOVA test (turkey's multiple comparison test) and each group were compared to each other at the same time point. The p-values corresponding to $p<0.05$ is presented with a * and $p<0.0001$ is presented with a †.

2.5 Cell proliferation and migration of hCFs assayed by multiphoton images

With the assumption that XTT assay could not truly reflect the actual cell number/ proliferation rate, the cell number was determined with another methodology. The multiphoton microscope generated projection images from an 880μm depth z-scanning. Using multiphoton scanning could avoid the hCFs, which migrated into the collagen gel, being masked from previous XTT assay. Fig. 5.12 showed there were more hCFs grown on TG2 gel (Fig 5.12C) than NC gel after 72 hour culture (Fig. 5.12A). Further cell counting (Fig 5.13) suggested that the average cell density on NC was approximately 290 cells/mm^2 while the average cell density on TG2 gel was around 330 cells/mm^2. However, this increased was not statistically significant. Another interesting finding was revealed by the projection image from cross-sectional angle which showed more hCFs distributed into the TG2 collagen gel (Fig.5.12D) while the hCFs on NC (Fig 5.12B) gel were mainly retained on

the matrix surface. It suggested that TG2 collagen gel promoted hCF migration along its collagen fibres and increases cell infiltration into the matrix.

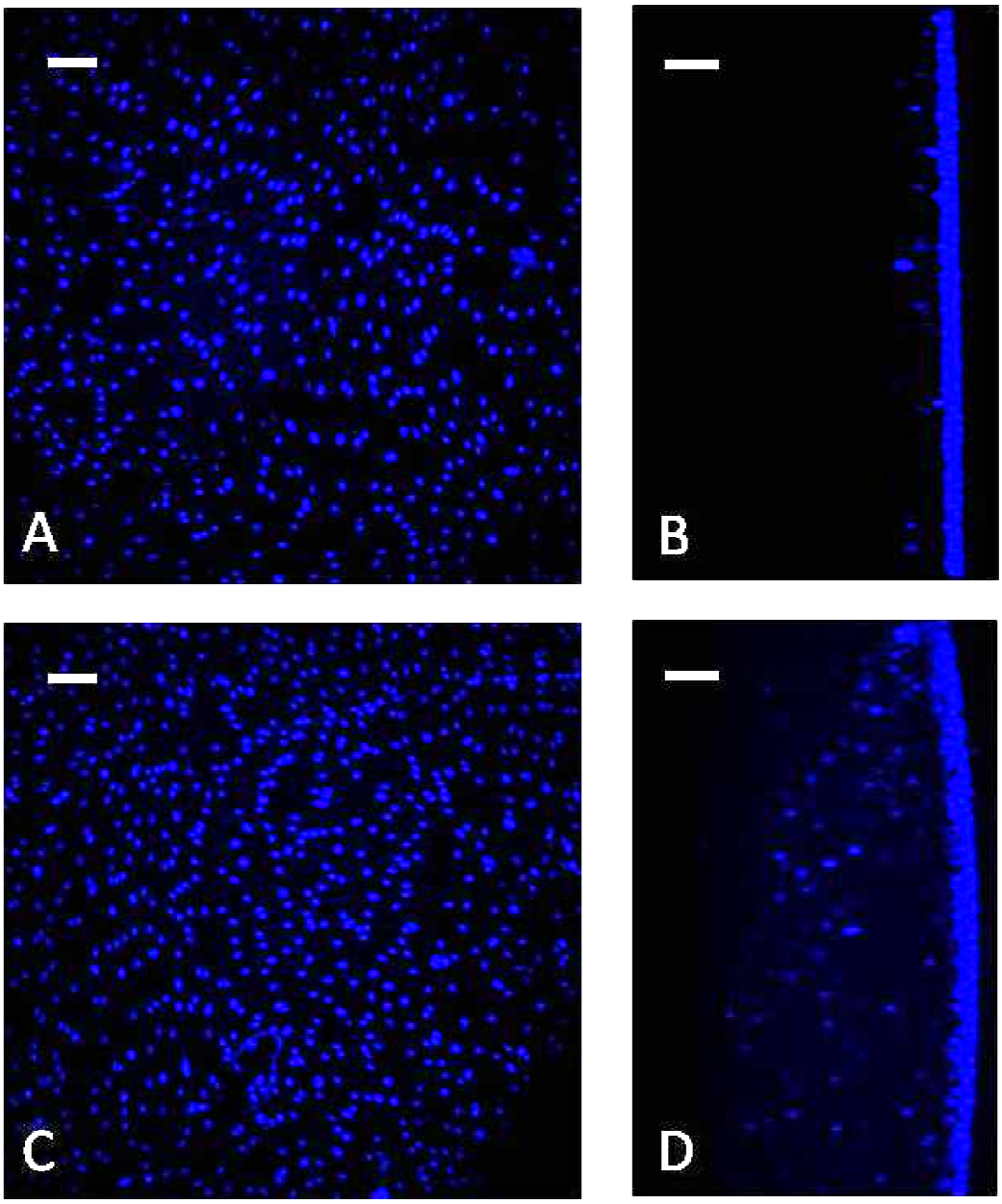

Figure 5.12 hCFs cultured collagen gel images from multiphoton microscope. hCFs were cultured on either NC gel or crosslinked gel for 72 hours. Samples were fixed with 3.7% PFA/PBS (pH 7.4) and the cell nucleuses were stained with DAPI. 3D projection (A,C) and cross-sectional images (B,D) of each series of scanning were combined using the LAS AF software. The DAPI staining is shown in blue colour. Cells grown on NC gel (A,B) were comparably fewer in number and surface located than cells grown on TG2 treated gel (C,D). The scale bar on top left of each picture represents 100μm.

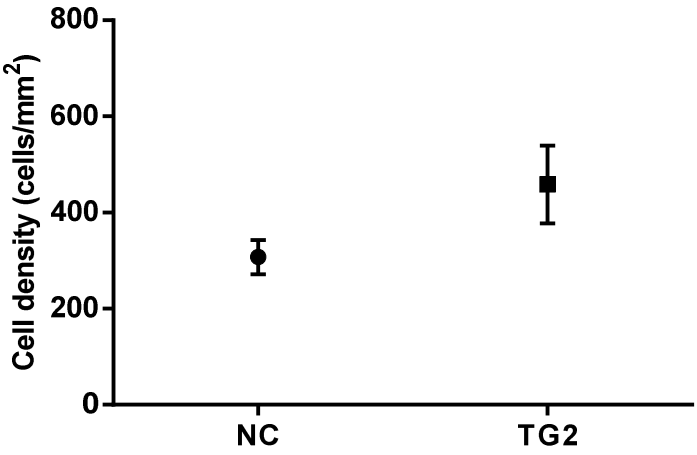

Figure 5.13 The average cell density on collagen gel after 72-hour culture. The initial cell density seeded on each matrix was 91 cells/mm^2. The average cell density of both groups after 72-hour culture period were quantified in ImageJ and analysed by unpaired T test. The results represent the mean values ±SEM where from 3 individual experiments (n=3). Statistical analysis was carried out using the Mann-Whitney test and no significant difference was found between NC and TG2 groups.

2.6 Invasion of hCFs measured by the scratch assay

To set up a model which could represent the cornea wound, hCFs were seeded onto type I collagen pre-coated tissue culture plate at 100% confluence and the confluent cell monolayer was subjected to scratch assay. The cell layer and the wound area were immediately covered by collagen gel or crosslinked collagen gel and the closure of the wound underneath the collagen matrix was monitored. In this model, hCFs would need to degrade native collagen or crosslinked collagen gel first before invasion. As showed in Fig 5.14, the leading front of the cells met at 18 hours post-scratching in the NC group while the cells in the TG2 group had only just begun migrating between 12 to 18 hours post-scratch. The average relative wound density (RWD) obtained from each group was presented over a 24-hour time frame in Fig. 5.15. The wound healing process of hCFs started as early as 6 hours post-scratch. But it was not until 12 hours post-scratch that hCFs in the crosslinked group started to migrate.

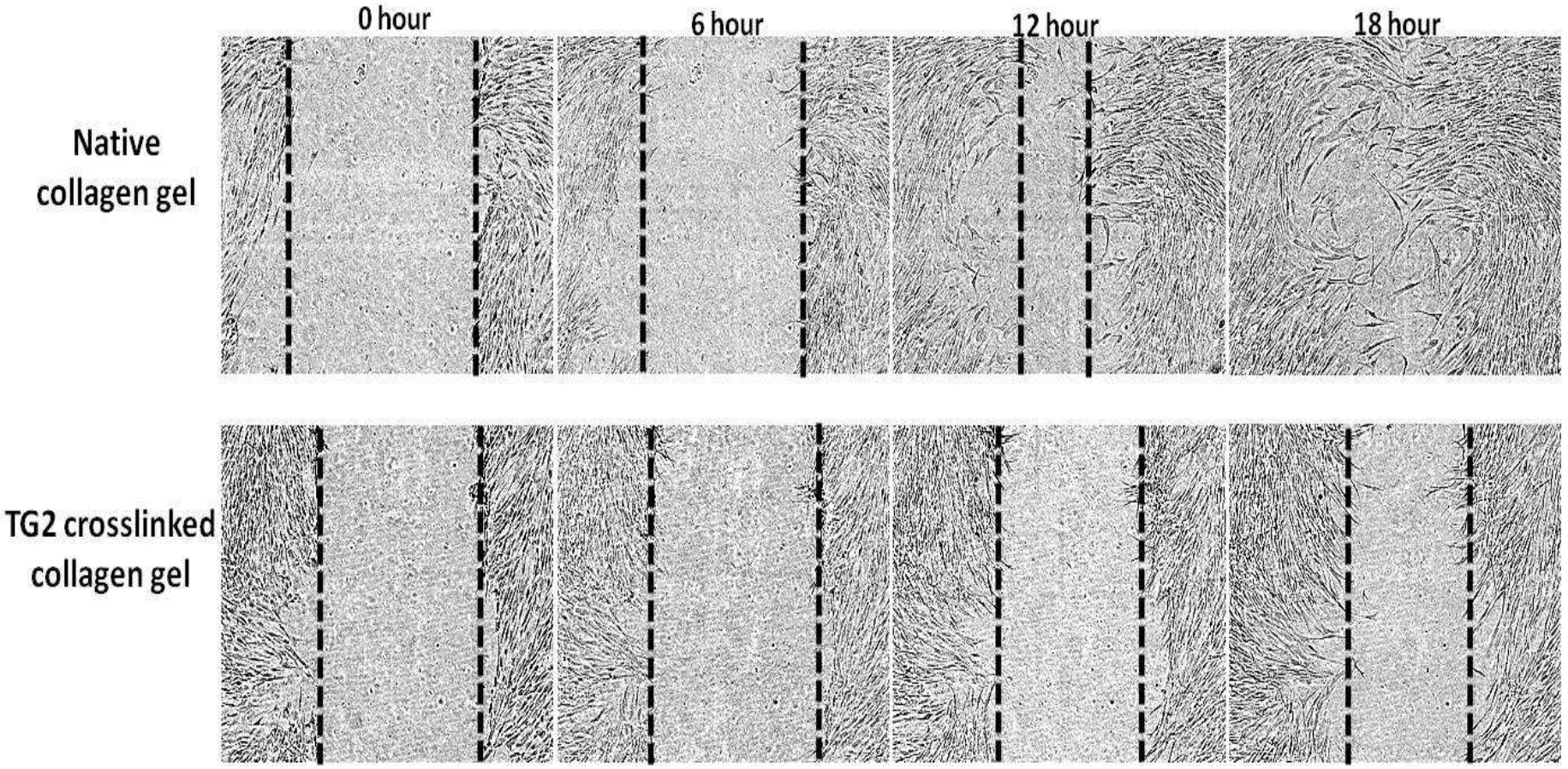

Figure 5.14 Wound closure process of hCFs cultured on collagen gel. The pictures were captured from the real time-lapse movie at 0 hour, 6 hours, 12 hours and 18 hours. Cells in NC group (upper panel) started migrating at 6 hours post wounding while cells in the TG2 group (lower panel) only started migrating after 12 hours post-scratching. The dashed line highlighted the migrating front of the cell.

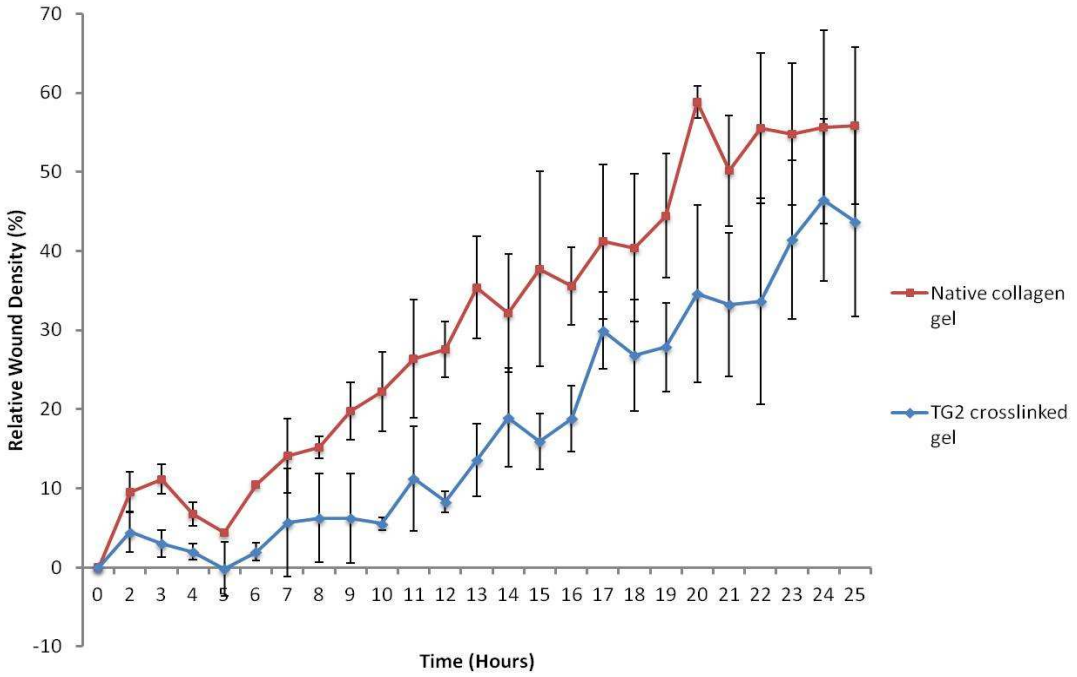

Figure 5.15 Wound closure process of hCFs cultured in collagen gel or crosslinked gel as shown in RWD over time diagram. Results represent the mean values ±SD from a representative experiment with triplicate setting. The experiment was repeated 2 times.

2.7 Migration of hCFs determined by transwell assay

In order to examine the migration of hCFs on crosslinked collagen gel, a modified transwell assay was performed. hCFs which were attracted by the 10% FBS/DMEM in the lower chamber would first migrate across the collagen or crosslinked collagen gel, then squeeze through the membrane pore and finally reach the underside of the filter. In Fig. 5.16, hCFs seeded on TG2 treated gel had higher mobility than cells cultured on NC according to the number of migrating cells after 48 hours. This result is consistent with the previous finding on multiphoton images that TG2 modified collagen gel enhanced hCFs migration. There was a concern that the difference in proliferation of hCFs on the NC and TG2 group during the 48 hours migration time frame might also influence this result. Yet, cells were cultured in serum free medium which minimised the proliferation of HOBs. Therefore, the cells observed at the lower side on the membrane were more likely to be migrating osteoblasts.

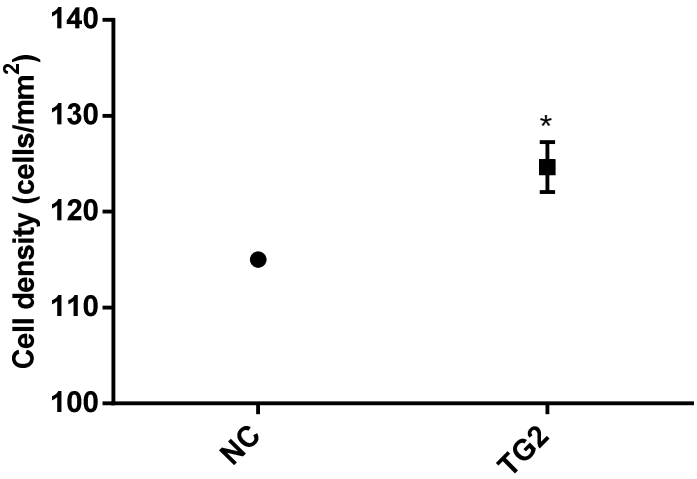

Figure 5.16 Analyse of transwell migration assay. The mobility of hCFs was determined by the number of migrating cells after a 48-hour time frame. The cells reached to the underside of the filter were fixed and stained with Diff-Quik® Stain Set. Results represent the mean values ±SEM from 3 individual experiments (n=3). The unpaired t test was used to analyse the data. The p-value corresponding to $p<0.05$ when compared to the native collagen group (NC) is presented with a *

3. Discussion

Several studies have demonstrated that TG2 treated matrix could increase cell adhesion of a variety of cell lines including osteoblasts, fibroblasts, hepatocytes, smooth muscle cells and endothelial cells (Verderio *et al.*, 2001, Chau *et al.*, 2005, Khew *et al.*, 2008, Forsprecher *et al.*, 2009, Spurlin *et al.*, 2009, Ciardelli *et al.*, 2010). Currently, most studies recognise the importance of matrix alteration resulting from crosslinking in regulating cell behaviour through integrin receptors. However, along with the recent study by Shanmugasundaram *et al*. (2012), the importance of exogenous TG2 in mediating cell behaviour was emphasised in this study. Trapping of TG2 into collagen gel has been demonstrated in this study, although it was not entirely unexpected regarding crosslinking of TG2 to other substrates including β2-macroglobulin, fibronectin and type XI collagen was reported by others (Fesus *et al.*, 1981, Barsigian *et al.*, 1991b, Shanmugasundaram *et al.*, 2012). It has been also demonstrated that TGs was able to bind to collagen (Juprelle-Soret *et al.*, 1988, Upchurch *et al.*, 1991) and the GTP-, GDP- and ATP-bound TG2 had no significant effect on its binding to ECM although there was a 2-3-fold enhancement in binding when TG2 was treated with calcium (Upchurch *et al.*, 1991). Therefore, the trapping of TG2 in the collagen matrix was consistent with current studies and it is feasible to suggest that the presence of TG2 within the matrix could be considered as a source of exogenous TG2.

As demonstrated in previous chapter that exogenous TG2 could induce osteogenesis via binding of LRP5 receptors, there was a possibility that trapped TG2 in the matrix could bind to cell surface LRP5 and further contribute to the increased HOB mineralisation observed on crosslinked collagen gel. Although the question of whether the high molecular weight TG2 complex still has ability to bind to LRP5 remains unknown. Also, increased mobility of cells cultured on TG2-rich cell surface/ extracellular environment has

been demonstrated here and with monocytes (Akimov *et al.*, 2000), breast cancer cells (Mangala *et al.*, 2006) and keratinocytes (Edwards *et al.*, 2006). Several models were suggested to explain the possible role of TG2 in cell migration since it was found to interact with integrin β1 and β3 on cell membranes and in recycling endosomes (reviewed by Belkin, 2011). Zemskov *et al.* (2007) proposed that binding of extracellular TG2 to LRP1 could accelerate the endocytosis process which might also include cell membrane integrin internalisation and recycling. Interestingly, in migrating cells, the localisation of integrin complexes to the leading edge was mediated through endocytic trafficking, suggesting a complex and dynamic control of cell surface adhesion molecules on the migrating cells. Therefore, it was possible that trapped TG2 in the TG2 gel could enhance hCF cell migration by hastening endocytosis of major integrins.

Besides the auto-crosslinking of TG2 into the collagen matrix, a change in matrix surface topography was also observed in this study. It was shown that the average collagen fibre size was increased in crosslinked collagen gel (2.7 ± 0.7 μm) compared to native collagen gel (0.9 ± 0.3 μm) and increased mineral deposition and ALP activity in the later stage of mineralisation were suggested when osteoblasts were cultured on crosslinked collagen gels. The effect of surface topography on cell adhesion, morphology, proliferation and differentiation was demonstrated in many studies (reviewed by Martínez *et al.*, 2009). Despite the fact that the effect of substrate topography on cell behaviours was varied according to differences in cell type, substrate material, feature geometry and parameters measured, several studies in agreement with the finding of this study are summarised as follows. On silicon substrates with nanogrooves from 150nm to 1000nm groove pitches, the expression of bone differentiation genes, including alkaline phosphatase (ALP), osteocalcin (OCN), type I collagen and integrin α1 and β1, were higher when the groove width was wider (Lamers *et al.*, 2010). Also, increased nodule formation and ALP activity

were found when increasing the groove depth (ranging from 0 to 30μm) on titanium (Ti)- and hydroxylapatite (HA)-coated grooved substrata (Perizzolo *et al.*, 2001). Therefore, as illustrated in Fig. 5.17, greater diameter of crosslinked collagen fibres than non-crosslinked collagen fibres may result in deeper grooves between fibres and could be considered as one of the factors that affects osteogenesis.

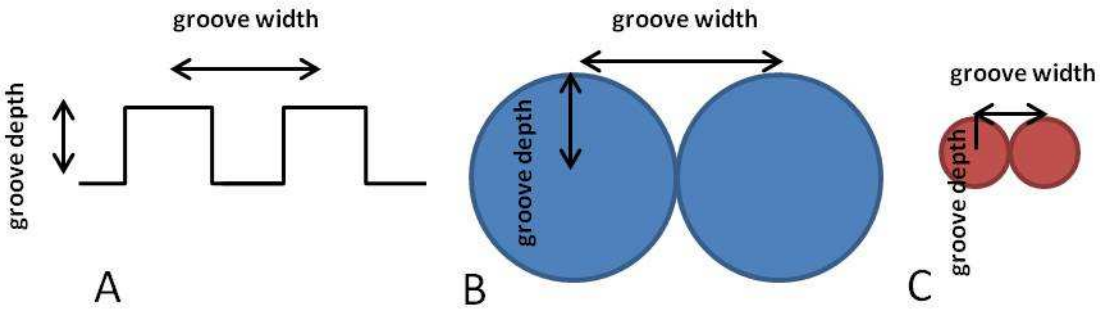

Figure 5.17 Illustration of groove width and depth. (A) The synthetic grooved substrata (B) larger fibres and (C) smaller fibres. Larger fibres result in wider groove width and deeper groove depth.

On the other hand, the average collagen fibre diameter in human cornea is 3.2 ± 0.8 μm according to immunohistochemical analysis (Mencucci *et al.*, 2010). Here, TG2 crosslinked collagen fibres (2.7 ± 0.7 μm) provided a microenvironment which was very similar to the physiological environment of corneal stroma than native collagen gel in terms of diameter of collagen fibres. TG2 crosslinked collagen, therefore, might enhance hCFs proliferation and migration by recreating a similar microenvironment to that of the corneal stromal layer.

The mechanism of how TG2 alters the size of collagen fibres still requires investigation. However, higher magnification SEM images (100,000X) of collagen hydrogels which were fabricated under different temperatures revealed parallel-aligned fibrils within collagen fibres and the difference in size of collagen bundles was due to different number of fibrils per fibre rather than changes in fibril diameter (Raub *et al.*, 2007). Thus, it is reasonable to assume that instead of altering the formation of triple helix polypeptide chains, TG2 may

alter the fibril array by connecting more collagen fibrils to form larger collagen bundles. Binding of TG2 to collagen I could happen with or without its crosslinking activity even though the collagen binding site of TG2 has not yet been determined. It was highly possible that crosslinking and/or binding of TG2 to collagen gel was associated with the alteration of biomaterial topography. Regardless of these unsolved questions, important information was gained that TG2 might mediate the osteogenesis process by changing the size of extracellular collagen fibres. This hypothesis could also suggest the role that TG2 plays in physiological mineralisation. Since the expression of extracellular/cell surface TG2 and crosslinking activity were gradually increased during the mineralising process, these ECM/cell surface TG2 might alter the organisation of collagen fibres extracellularly and enhance cell mineralisation.

Another important factor to evaluate was the cell compatibility of crosslinked collagen gels. Although the Cell Proliferation Kit (Roche Diagnostics Ltd., West Sussex, UK) suggested a low XTT reduction rate of cells when cultured on collagen based matrix, here, a question was raised that whether the XTT assay was a reliable method to evaluate cell growth in this collagen gel system. It was suggested that XTT assay was majorly based on the pyridine nucleotide redox status of cells (Gonzalez and Tarloff, 2001). Therefore, this assay would be greatly influenced by the mitochondrial metabolic activity. Alteration of mitochondrial metabolic activity was found with aging (Ji *et al.*, 1990), chemical treatment (Hernández-Muñoz *et al.*, 1994), cellular hydration state (Haussinger *et al.*, 1994) and proliferating / quiescent stage of cells (DeBerardinis *et al.*, 2008). It was possible that the metabolic activity of cells grown on NC gel, TG2 gel and Lm/Fn coated plate were very different. In this case, cells cultured on Fn coated plate could potentially increase metabolism to a higher rate than cells cultured on collagen based matrices. Therefore, when using XTT value of cells cultured on Fn coated plate as a positive control, the

difference in cell basic metabolism rate on different surface shall be taken into consideration.

The concern of XTT assay should not be directly linked to actual cell proliferation rate was further confirmed in this study. While the XTT assay showed that there was decrease in XTT reduction during the 72-hour culture period, the multiphoton scanned pictures suggested that increasing cell number was found on native collagen and TG2 crosslinked gel. For cells migrated to deeper layer of matrix, obtaining and releasing of XTT products were restricted by the physical barrier and relied on medium diffusion rate. In other words, the metabolic activity of cells inside the collagen gel might be hidden by the matrix. The invasion of hCFs into collagen gel may explain the reason why the XTT value was low in the TG2 group.

hCFs showed increased cell mobility in the TG2 collagen gel system as demonstrated by multiphoton scanning and transwell migration assay. These findings show the potential of applying TG2 collagen gel to corneal wound areas, since it could encourage the repopulation of corneal fibroblasts into the matrix. Infiltration of hCFs into collagen gel within the wounded area hopefully could accelerate the healing process and attract epithelial cells and endothelial cells migration and proliferation via chemoattration.

The invasion model used in this study suggested that a delayed onset of cell invasion might be expected when applying crosslinked collagen gel to corneal wound areas (Fig. 5.18). This delayed onset of invasion could result from specific MMPs secretion being required to initialise the invasion of hCFs into crosslinked collagen gel. The hCFs were cultured on type I collagen for 24 hours before being subjected to invasion assay. It is reasonable to assume that it takes time for hCFs to express and secrete specific MMPs when they are first introduced onto crosslinked collagen gel as it is distinct from the original matrix they

were cultured on. This observation might also suggest that crosslinked collagen gel has higher resistance to hCFs mediated degradation. It was not completely surprising since higher resistance to enzymatic digestion was generally observed in crosslinked gel which has been demonstrated by Chau *et al.* (2005) previously. Interestingly, even though the onset of cell invasion was delayed, the average invasion rate of hCFs on crosslinked collagen gel after start of invasion was comparable and even higher than hCFs on native collagen gel.

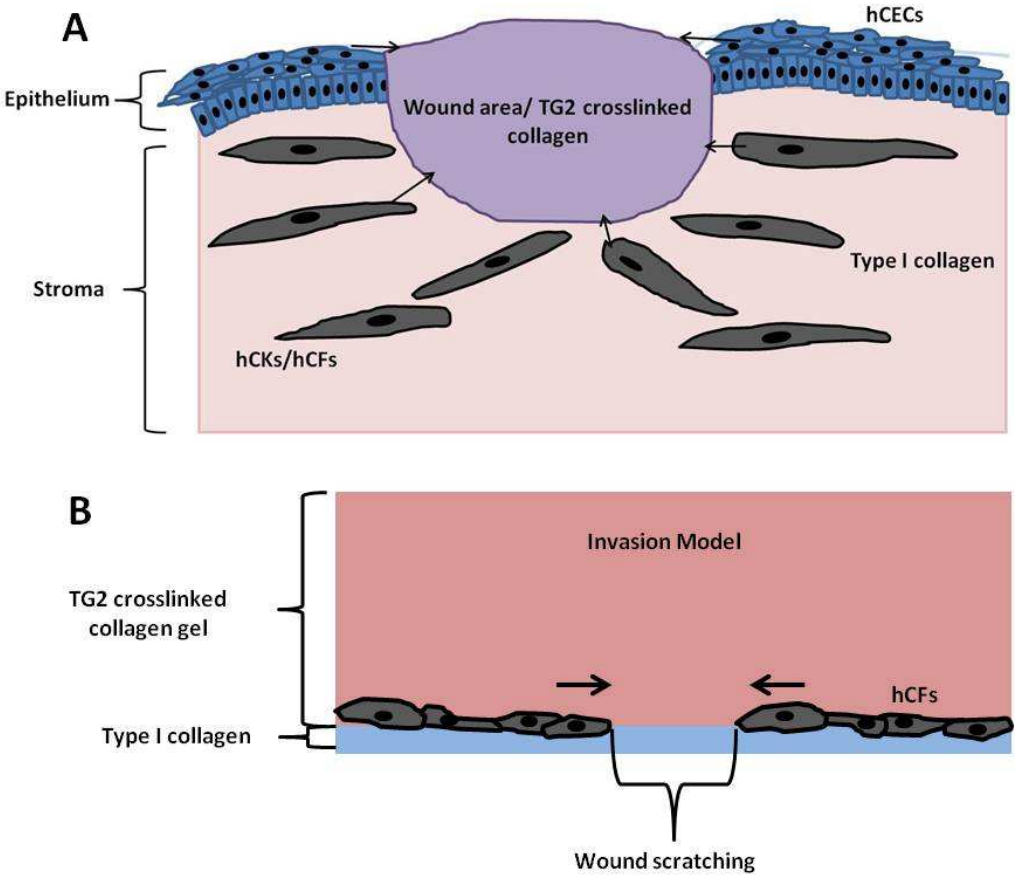

Figure 5.18 (A) A putative models for application of TG2 crosslinked gel to corneal wound area. Quiescent human corneal keratocytes (hCKs) are transformed into human corneal fibroblasts (hCFs) once the injury occurred. Ideally, hCFs and human corneal epithelial cells (hCECs) are encouraged to migrate into or over the crosslinked collagen gel at the wounded area. Therefore, the invasion model (B) used in this study tests the possible scenario of hCFs invasion in the *in vitro* model. Cells are cultured and wound scratching is performed on the cell monolayer. The wound closure happens underneath a layer of the crosslinked collagen gel.

Overall, these results provided a promising point of view in using crosslinked collagen matrix for hard and soft tissue regeneration. Currently there are two BMP bone graft

products approved for clinical use including INFUSE® bone graft which contains BMP-2 for spinal fusion and for treatment of open tibial shaft fractures and Stryker's OP-1 which contains BMP-7 for treating long bone non-union fractures and lumbar spinal fusion. Collagen gel, in these cases, is used as a biodegradable carrier for BMPs instead of having a functional role in promoting osteoconduction, osteoinduction and adhesion or providing microarchitecture for the subsequent formation of new tissue. Here, the TG2 treated collagen gel demonstrated competitive osteoinductivity at later stages of cell culture when compared to BMP-7 containing gel. However, in order to achieve the same osetoinductive effect as BMP-7 containing gel in this study, the prime cost of TG2 crosslinked gel was 20 times less when constructed in the laboratory. In fact, the currently approved BMP-7 product for bone fracture repair, OP-1 Implant, costs $5250 for one unit (3.3mg of BMP7 addmixed with 1g of bovine bone collagen). Here, TG2 crosslinked collagen gel demonstrated a comparable effect on improving osteoblast mineralisation to current treatments for bone fracture and the cost of crosslinked gel was much lower than using BMP containing product. Nevertheless, combined with a previous study conducted by Chau *et al.* (2005), TG2 –treated collagen gel showed great potential to improve cell attachment and mineralisation via its crosslinking and non-crosslinking characteristics without compromising its resistance to enzymatic degradation. Therefore, TG2 crosslinked collagen is a promising biomaterial which meets the current requirements of bone grafts in the clinic. Future work in applying TG2 crosslinked collagen gel to *in vivo* animal models may provide more insight into the mechanism of TG2-collagen induced mineralisation.

This chapter also demonstrated the promising potential of TG2 crosslinked matrix for corneal wound healing. It promotes corneal fibroblast proliferation and cell infiltration which could potentially improve the corneal wound healing process. The major issue

remains to be conquered is the relatively poor proliferation of hCECs on crosslinked collagen gel. According to XTT assays, corneal epithelial cells showed low proliferation even when cultured on TG2-collagen gel with Lm- or Fn- modified surfaces. Developing a hCFs/hCECs co-culture system in the future may improve the poor epithelial cell proliferation on crosslinked collagen gel and advance the use of TG2 crosslinked gels in cornea repair and even the possibility of fabricating artificial substitutes.

Chapter VI

General Discussion

TG2 has been implicated in bone development and mineralisation for years, yet the detailed mechanism still remains unclear due to the complexities of its multifunctional characteristics and compensation effects from other TG family members. In this thesis, the relationship between expression of TG2 and osteoblast mineralisation was established, where the level of TG2 protein increased steadily during cell mineralisation. A strong relationship between TG2 expression and mineralisation has been well demonstrated here and in other literatures (Nurminskaya and Kaartinen, 2006), yet, studying of the mechanism behind is still a great challenge.

There are two main restrictions for the models used for studying biological functions of endogenous TG2 and mineralisation. First, in this thesis, upregulation of FXIIIa was also observed in TG2 knockdown osteoblasts where mineralisation was not affected by low expression of TG2. Therefore, even though the expression of TG2 may be coincident with bone development which suggested TG2 as one of the major regulators in physiological mineralisation, TG2 alone was not essential for bone development and could be compensated by other TG members. It is also feasible that only a minimum level of TG2 expression and/or crosslinking activity are required for mineral deposition. The compensation effects from other TG members is in agreement with current TG2 knockdown and knockout systems which explains the lack of gross skeletal abnormalities in TG2 knockout mice (De Laurenzi and Melino, 2001, Nanda *et al.*, 2001) and TG2/FXIIIa double knockout mice(Williams *et al.*, 2010). The possible upregulated TG family members due to TG2 knockout were demonstrated to be TG1 (De Laurenzi and Melino, 2001, Deasey *et al.*, 2013), TG3 (Deasey *et al.*, 2013) and FXIII (Tarantino *et al.*, 2009).

Secondly, the crosslinking inhibitors may act not only through their inhibitory effect on transamidating activity but also through conformation change and both effects should be considered when evaluating the results. Culturing osteoblasts with synthetic TG2

crosslinking inhibitors, R283 (non-specific) and R294 (TG2-specific), did not block mineralisation of HOBs in this thesis. Although it could be concluded that TG2 activity was not necessary for cell mineralisation, again, it was also feasible that only a minimum level of TG crosslinking activity was required for this process since there was still a low level of crosslinking activity present in the extracellular environment after treating with inhibitors. Interestingly, R294 treated groups showed an even higher mineralised area at the end point. The Western blot results also suggested that inhibitor-treated cells showed more cleaved TG2 fragments and the fragments appeared coincidently with mineral deposition. Here, the increase in TG2 fragments was proposed to be related to the conformational change due to the binding of inhibitors which made TG2 more sensitive to enzymatic digestion. Also, the enhancement of mineralisation and increase in TG2 fragments fitted the hypothesis that TG2 could contribute to mineralisation by acting as ATPase and this function is regulated by enzymatic digestion. Therefore, these results demonstrated that TG2 might contribute to mineralisation through other biological functions and these functions were likely to be related to TG2 conformation. Current study also supports this finding that restricting TG2 in certain conformations has been shown to affect different functions of TG2, for example, TG2 failed to induce differentiation of chondrocytes in its open conformation (Johnson and Terkeltaub, 2005).

Another subject to be addressed was to distinguish the contribution of crosslinking activity from the 2 major TGs in osteoblasts, TG2 and FXIIIa, during mineralisation. Judging from the results gained from ECM and/or cell surface transamidating activity, there was a shift from FXIIIa-dominant crosslinking activity to TG2-dominant crosslinking activity during mineralisation. This was compliant with Al-Jallad *et al.* (2011) studies that an early increase in cell surface FXIIIa was observed in differentiation treated pre-osteoblasts. They also indicated that this increase in FXIIIa activity on the cell membrane was related to

collagen deposition. However, a TG2 dominant crosslinking activity at later stages of differentiation was also observed in this thesis. Therefore, a new postulation was proposed here that an early peak in FXIIIa activity on the differentiated cell surface could be important for collagen matrix deposition and, in the later stage of differentiation,TG2 still played a major role in matrix stabilisation considering TG2 has a broader range of substrates than FXIIIa in ECM.

The exogenous TG2 induced-calcification and downstream activation of β-catenin signalling were observed in the HOB model used here. According to the results, TG2-mediated activation of LRP5/β-catenin pathway occurred transiently at a very early stage of treatment. Furthermore, according to the preliminary data, R283-inactivated TG2 (semi-open form) and R294-inactivated TG2 (open form) might still reserve partial ability to enhance bone mineralisation. And it seems that R283-inactivated TG2 could induce higher levels of mineralisation than R294-inactivated TG2. Another important information gained from Co-IP assay was that TG100 antibody failed to recognise TG2/LRP5 complex on cell surface. This could be due to the binding site for TG100 antibody on TG2 being masked in the TG2/LRP5 complex. It is known that the epitope site of TG100 on TG2 undergoes large conformational change when TG2 is in compact form. All these suggested that certain conformation(s), most likely the closed conformation, of TG2 was required for the binding of TG2 to LRP5. It, again, implies the important of TG2 conformation in mineralisation.

Therefore, a putative model of regulatory signalling for osseous cell fates combined with findings in this thesis was proposed in Fig 6.1. In this model, osteoprogenitor cells differentiated from mesenchymal origins express LRP5 receptors and immature osteoblasts usually undergo apoptosis. The LRP5 was proposed to promote proliferation and/or survival of early osteoblast progenitors but not essential for osteoblast lineage

development from mesenchymal stem cells (reviewed by Westendorf *et al.*, 2004). Exogenous TG2, either secreted from chondrocytes or from apoptosis of osteoblasts, could act through LRP5 receptor and further activate the Wnt/β-catenin pathway thus encouraging cell survival and differentiation. Also, TG2 has been proposed to enhance cell adhesion and proliferation in the extracellular environment through both crosslinking dependent and independent pathways. Later in mature osteoblasts, the Wnt signalling was down regulated by secreted Wnt inhibitors, such as sFRP and WIF, and the binding of Dkk to LRP5 would encourage the endocytosis of cell surface LRP5. The extracellular TG2, during the terminal differentiation and mineralisation, could act as a matrix stabiliser through its crosslinking activity or act as a phosphate regulator through its ATPase activity. At the very end of the process, osteocytes lose the expression of LRP5 and undergo apoptosis. The extracellular TG2, again, could promote cell adhesion and survival through different interactions with integrins and/or syndecans.

Exogenous TG2 is also recognised as a stress or rescue protein in wound healing and may play a role in bone healing. Cell necrosis always accompanies wounding, therefore, the releasing of TG2 from necrotic cells could promote cell survival and accelerate maturation of osteoblasts through binding to LRP5 receptor at the very early stage of bone regeneration. The extracellular TG2 induced β-catenin activation was involved in pathological calcification which has been demonstrated in vascular smooth muscle cells and warfarin induced valve calcification models (Faverman *et al.*, 2008, Beazley *et al.*, 2012). Overall, extracellular TG2 contributed to physiological and pathological calcification processes through both transamidating dependent and independent pathways and the function of TG2 may be regulated by endocytosis or enzymatic digestion at different stages of mineralisation.

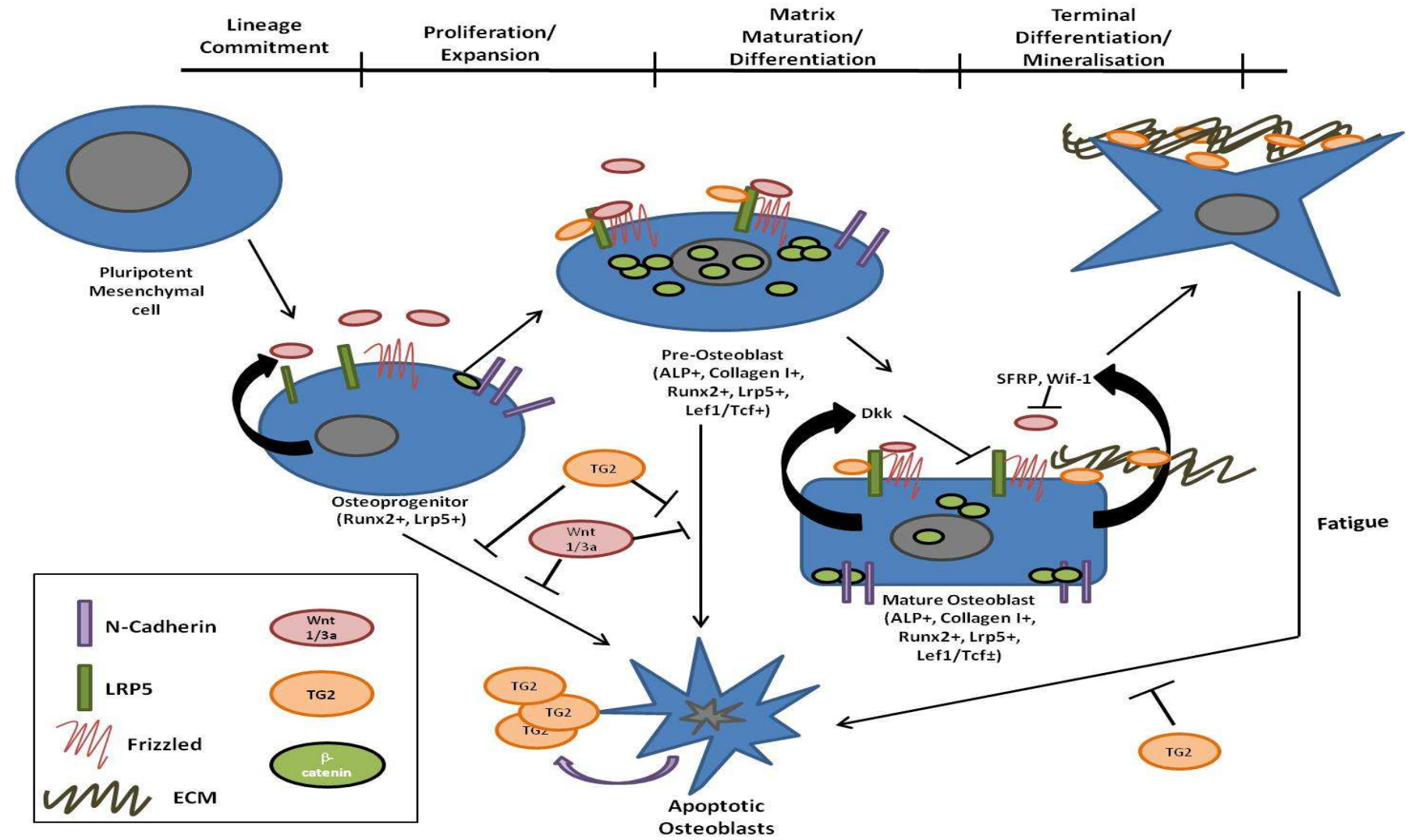

Figure 6.1. Putative model for TG2-related regulation of osseous cell fates.

The advantages of applying TG2 for medical use were also demonstrated in this thesis. Crosslinking collagen gel with TG2 is an effective way to overcome the physical weakness of native collagen gel. A previous study by Chau *et al.* (2005) has shown that TG2 crosslinked collagen not only had improved physical characteristics, but also provided a more favourable environment for cell growth and differentiation. The same study also suggested that the improved biological characteristics of crosslinked gels were the result of alteration of collagen matrix by TG2 transamidating activity. In this thesis, TG2 crosslinked gel elicited greater mineral deposition of HOBs than the positive control, BMP7 contained collagen gel, used in the experiments. This osteoinductive effect of crosslinked collagen gel was suggested to result from alteration of collagen fibre topography and/or trapped TG2 in the matrix. This advanced biomatrix showed a great potential in bone repair and could be produced at a reasonable price when compared to current BMP products on the market. However, BMP7 and TG2 did not have a synergic effect in osteoinduction. A possible explanation was that BMP and TG2 mediated mineralisation had opposite effects in the process. It was proposed in this thesis that trapped TG2 acted as a resource of exogenous TG2 and could further activate Wnt/β-catenin pathway through LRP5. As reviewed in the literature, interaction between BMP and Wnt pathways is particularly complex in bone development and had opposite or cooperative effects depending on the developmental stages (Itasaki and Hoppler, 2010). Therefore, combining BMP7 and TG2 was not an ideal method to improve the osteoinductive property of biomatrix.

Results also demonstrated that TG2 crosslinked collagen was highly compatible for hCF growth and provided a greater environment for hCF repopulation. This effect could be related to alteration of collagen fibres by TG2 which resulted in a similar size of collagen fibres to that observed in human corneal stroma. This thesis also tested the possible

scenario in *in vivo* corneal wound closure. It suggested that a delay in invasion onset time was expected due to a certain amount of time that was needed for cells to secrete the correct MMPs for invasion when first introduced to new matrix. However, higher mobility of cells cultured in TG2 crosslinked group was observed in the transwell assay. Future work will involve adequate modification of the matrix surface for HCECs proliferation and migration in order to advance the use of crosslinked collagen gel in corneal wound dressing.

To summarise, the key contribution of this thesis was to refine current hypotheses of TG2 in osteoblast mineralisation process. The crosslinking activities of extracellular TG2 in cell mineralisation are likely to play a role in matrix maturation and stabilisation in the later stage of differentiation. The extracellular TG2, even after proteolysis, can also contribute to Pi synthesis for mineral deposition via its ATPase activity. The exogenous TG2, on the other hand, induces cell differentiation via binding of LRP5 receptor and activating downstream β-catenin signalling in HOB. This effect is β-catenin signalling dependent and is likely to be an initial differentiation signal for osteoblasts in physiological bone development and the wound healing process. In the future, it would be interesting to examine if TG2 activity and/or conformation are essential for the endogenous TG2-mediated and exogenous TG2-induced mineralisation. Using the wild type TG2, mutant C277S TG2 and mutant W271A TG2 could be a good model to study the effect of TG2 conformation and activity during mineralisation.

The use of TG2 modified matrix also demonstrated a great potential as an advanced biomatrix in bone repair. Combining the finding in previous studies and the results here, TG2 crosslinked collagen gel is a mechanically strong biomatrix and its cell compatibility and pro-mineralisation effects are well established in *in vitro* systems. It is worth studying the TG2 crosslinked collagen gel in animal bone fracture models and ultimately in clinical

trials. Further added value of using TG2 crosslinked collagen gel as a corneal wound dressing was also demonstrated where the matrix shows great cell compatibility for hCFs to proliferate and migrate. The next challenge to overcome in the future is to improve the cell compatibility of TG2 crosslinked gel for hCECs proliferation. One possible way to address this goal is using hCFs/hCECs coculture system, addition of growth factor and modifying with type IV collagen.

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