# The Importance of the Tissue Transglutaminase-Fibronectin Heterocomplex in the RGD-independent Cell Adhesion and Fibronectin Matrix Deposition

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# Thesis Summary

Tissue transglutaminase (TG2) has been reported as a wound response protein. Once over-expressed by cells under stress such as during wound healing or following tissue damage, TG2 can be secreted and deposited into extracellular matrix, where it forms a heterocomplex (TG-FN) with the abundant matrix protein fibronectin (FN). A further cellular response elicited after tissue damage is that of matrix remodelling leading to the release of the Arg-Gly-Asp (RGD) containing matrix fragments by matrix matelloproteinases (MMPs). These peptides are able to block the interaction between integrin cell surface receptors and ECM proteins, leading to the loss of cell adhesion and ultimately Anoikis. This study provides a mechanism for TG2, as a stress-induced matrix protein, in protecting the cells from the RGD-dependent loss of cell adhesion and rescuing the cells from Anoikis. Mouse fibroblasts were used as a major model for this study, including different types of cell surface receptor knockout mouse embryonic fibroblasts (MEFs) (such as syndecan-4, α5, β1 or β3 integrins). In addition specific syndecan-2 targetting siRNAs, β1 integrin and α4β1 integrin functional blocking antibodies, and a specific targeting peptide against α5β1 integrin A5-1 were used to investigate the involvement of these receptors in the RGD-independent cell adhesion on TG-FN. Crucial for TG-FN to compensate the RGD-independent cell adhesion and actin cytoskeleton formation is the direct interaction between the heparan sulfate chains of syndecan-4 and TG2, which elicits the inside-out signalling of α5β1 integrin and the intracellular activation of syndecan-2 by protein kinase C α (PKCα). By using specific inhibitors, a cell-permeable inhibiting peptide and the detection of the phosphorylation sites for protein kinases and/or the translocation of PKCα via Western blotting, the activation of PKCα, focal adhesion kinase (FAK), ERK1/2 and Rho kinase (ROCK) were confirmed as downstream signalling molecules. Importantly, this study also investigated the influence of TG-FN on matrix turnover and demonstrated that TG-FN can restore the RGD-independent FN deposition process via an α5β1 integrin and syndecan-4/2 co-signalling pathway linked by PKCα in a transamidating-independent manner. These data provide a novel function for TG2 in wound healing and matrix turnover which is a key event in a number of both physiological and pathological processes.

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The communication between adhesion-dependent cells and their microenvironment triggers a signal transduction process, which is essential for cells to exert their functions including adhesion, migration, differentiation, proliferation and in some cells matrix remodelling (Hynes, et al., 1999). Among the matrix proteins, fibronectin (FN) is one of the most important matrices for various cell types e.g. fibroblasts and osteoblasts (Mosher, 1984). Via a direct interaction with matrix FN through their surface integrin receptors, cells can anchor themselves onto the matrix and further spread or migrate depending on the environment signals, such as the growth factors and signalling molecule produced by other cells. Cell surface heparan proteoglycans, including syndecans and glypicans (Moyano et al., 1999), can also regulate cell behaviour via mediating actin cytoskeleton organization. Under stress conditions, matrix metalloproteinases (MMPs) can digest matrix proteins leading to the release of the matrix fragments (Chau et al., 2005; Collighan and Griffin, 2009). RGD peptides are one of the most widely reported fragments since they can block the adhesion of cell on matrix proteins containing RGD motifs, resulting in the loss of cell adhesion and ultimately Anoikis (Buckley, et al., 1999; Hadden and Henke, 2000).

Tissue transglutaminase (TG2) was previously known as an enzyme, which can crosslink matrix proteins including FN and collagen, via ε-(γ-glutamyl) lysine bridges (Griffin et al., 2002a), leading to protein stabilization and protection from protease degradation and thereby promoting cell adhesion (Verderio et al., 2004). Recent reports also demonstrate that TG2 can act as a co-receptor for integrins to regulate cell adhesion and migration in a transamidating-independent manner (Akimov et al., 2000a). As a stress-related protein (Ientile et al., 2007), TG2 can be released and deposited into extracellular matrix and function as a matrix protein via its binding to its high affinity binding partner with FN to form the TG-FN hetero-complex (Gaudry et al., 1999a). The function of TG-FN in rescuing the RGD-induced apoptosis was reported by Griffin and colleagues, which is regarded as a novel role for this multifunctional enzyme (Verderio et al., 2003).

## 1.1 Transglutaminases

The posttranslational modification of proteins plays an important role in regulating protein function. Transglutaminases were first reported in 1959 by Mycek and colleagues as an enzyme found in guinea pig liver, which could modify proteins by mediating an acyl-transfer reaction between the γ-carboxamide group of peptide-bound glutamine and a primary amine, which can be either a polyamine or the ε-amino group of peptide-bound lysine, if the latter then protein crosslinking can occur through the formation of ε (γ-glutamyl) lysine bonds. Because of their ability to crosslink proteins together, TGs have been referred to as ‘‘Nature’s Glue’’. Transglutaminases are found widely in nature (Griffin et al., 2002), but in mammals their enzymatic activity is $Ca^{2+}$-dependent, and other factors including GTP/GDP can also affect the activity of some of the mammalian TGs.

Even though the functions of TGs have been under investigation by scientists for more than 5 decades, not all members of the mammalian TGs have been fully characterised. Moreover further novel enzymatic activities of specific mammalian TGs have recently been reported, e.g. the protein disulfide isomerise and protein kinase activities of the tissue transglutaminase (TG2), thus extending the potential cell functions of this diverse group of enzymes.

## 1.2 Members of mammalian transglutaminase family

It has been proved that TGs are widely expressed in the nature, including plants (Serafini-Fracassini et al. 1995), micro-organisms (Chung, 1972, Folk and Finlayson, 1977; Kanaji et al., 1993), invertebrates (Mehta et al., 1990 and 1992; Singh and Mehta, 1994) and mammals. It has been certified that even in the same tissue, the TGs might exert different functions (Griffin et al., 2002). As shown in table 1.1, nine types of TGs have been identified in mammals, of which, apart from erythrocyte band 4.2, require $Ca^{2+}$ for their transamidating activity. These are Factor XIII A, the keratinocyte transglutaminase (TG1), the ubiquitous tissue transglutaminase (TG2), the epidermal transglutaminase (TG3), the prostate transglutaminase (TG4), the non-catalytically active erythrocyte band 4.2, and the recently discovered transglutaminase X, Y, and Z (TG5, TG6 and TG7). They are distributed in various tissues (epithelium, endothelium, stratum corneum, dermis, liver, spleen, bone marrow, central nervous system (CNS), etc.) and physiological fluids (platelets and lymphatic system), and different parts of cells (cytosol, nuclear and membrane), as well as the extracellular matrix.

All transglutaminase enzymes are encoded by a family of closely related genes with a high degree of sequence similarity. All members of the TG gene family share a similar gene organization with remarkable conservation of intron distribution and intron splice types. According to the structure of the individual genes, they can be divided into two subclasses, wherein the genes encoding TG2, TG4, band 4.2 protein and TG5 contain 13 exons, and the genes encoding factor XIII A subunit and TG1 contain 15 exons.

**Table 1.1 Members of Transglutaminase Family**

| Protein | Gene | Gene location | Synonyms | Tissue Expression | Activity | Function |
|---|---|---|---|---|---|---|
| Factor XIIIa subunit | F13A1 | Chromosome 6 | Factor XIIIa, plasma Tg, fibrin-stabilising factor, Laki-Lorand factor, fibrinoligase, plasma pro-TG | Platelets, astrocytes, dermal dendritic cells, chondrocytes, placenta, synovial fluid | Latent (thrombin activated) | Blood coagulation, bone growth, ECM stabilisation |
| TG1 | TGM1 | Chromosome14 | $TG_K$, keratinocyte-, particulate-, membrane-bound TG, Tg type1, TG B | Keratinocytes, brain | Latent (protease activated) | Cornified envelope formation by terminally differentiating epidermal keratinocytes |
| TG2 | TGM2 | Chromosome 20 | $TG_C$, tissue Tg, endothelial-, erythrocyte-, cytosolic-, liver-, tissue TG, Gh, Ghα, TG type 2 | Ubiquitous | Yes | ECM stabilisation, formation of cross-linked envelop during cell death, cell signalling, cell adhesion protein and cell survival. |
| TG3 | TGM3 | Chromosome 20 | $TG_E$, epidermal-, callus-, hair follicle-, bovine snout TG, TG type 3 | Squamous epithelium, brain | Latent (protease activated) | Cornified envelope formation, hair shaft formation |
| TG4 | TGM4 | Chromosome 3 | $TG_P$, prostate TG, Vesiculase, dorsal prostate protein 1 (DP1), major androgen-regulated prostate secretory protein | Prostate | Yes | Semen coagulation in rodents |
| TG5 | TGM5 | Chromosome 15 | $TG_X$, TG type 5 | Ubiquitous expression except for the CNS and lymphatic system | Yes | Unknown |
| TG6 | TGM6 | Chromosome 20 | $TG_Y$, TG type 6 | Brain | Yes | Unkown |
| TG7 | TGM7 | Chromosome 15 | $TG_Z$, TG type 7 | Ubiquitous | Yes | Unkown |
| Band 4.2 | EPB42 | Chromosome 15 | ATP-binding erythrocyte membrane protein band 4.2, Erythrocyte protein band 4.2, B4.2 | Erythrocytes, bone marrow, fetal liver and spleen | No | Structural membrane skeletal component |

## 1.3 Factor XIII

Factor XIII (also known as FXIII, pXIII-A2B2, plasma coagulation factor XIII, or fibrin stabilizing factor) is a heterotetramer composed of a catalytic dimer of two A subunits (FXIII A) and two non-catalytic B subunits (FXIII B) that can protect A subunits in the blood circulation (Schwartz et al., 1973). Plasma FXIII (pFXIII) is the last enzyme in the blood coagulation cascade and in contrast to all other enzymes involved, it is not a serine protease but a transglutaminase, catalyzing the formation of isopeptide bonds between the side chains of peptide bound glutamine and lysine residues. As a zymogen, FXIII requires the cleavage at $Arg^{37}$-$Gly^{38}$ by thrombin in the presence of $Ca^{2+}$ (Schwartz et al., 1973).

### 1.3.1 Factor XIII A subunit

As one of the most widely studied members in the TG family, the structure, amino acid sequence of the catalytic FXIII A and the localization of its genes have been revealed. Localized on human chromosome 6 p24-25, the FXIIIA gene, F13A1, consists of 15 exons and is more than 160kb in size (Ichinose et al., 1990). F13A1 encodes 83kDa FXIII A subunit composed of 731 amino acids (Ichinose et al., 1986a), which has been reported to be regulated by a myeloid-enriched transcription factor (MZF-1-like protein) and two ubiquitous transcription factors NF-1 and SP-1 (Kida et al., 1999). According to the discovery by cDNA cloning (Grundmann et al., 1986) and protein sequencing (Ichinose et al., 1986a), the amino acid sequence of FXIII A was deduced, while the X-ray crystallographs demonstrated the crystal structure of FXIIIA, showing it is composed of 5 domains including an activation peptide, an N-terminal β-sandwich domain, a core domain and two C-terminal β-barrels (Yee et al., 1994). Human FXIII A is expressed mainly in hepatocytes, megakaryocytes, platelets, monocytes and macrophages. At the organ level, FXIII A is mainly located in the uterus, the placenta and the liver (Iismaa et al., 2009). The localization of FXIII A has been identified intracellularly (cytoplasma and nuclei), on the cell surface and extracellularly (extracellular matrix or in serum, secreted through an

unknown non-classic secretion pathway) (Adany and Bardos, 2003). Its mechanism of secretion into the extracellular environment is still unknown.

### 1.3.2 Factor XIII B subunit

Localized at human chromosome 1q31-32.1, the 28kb FXIII B gene— F13B encodes the 80kDa protein containing 641 amino acids (Bohn, 1972; Bottenus et al., 1990). The gene F13B is composed of 12 exons separated by 11 introns, among which the first exons encodes the leader sequence and the 12th encodes the C-terminal portion (Bottenus et al., 1990). Each of the remaining 10 exons is in charge of the expression one tandem repeat known as Sushi domains or GI-I structures (Ichinose et al., 1986b). Synthesized by the liver, even though known as the regulatory/carrier of FXIII A, 50% of FXIII B subunits remain FXIII A free in the plasma (Yorifuji et al., 1988). Unlike FXIII A, Kaetsu and colleagues (1996) reported that, in baby hamster kidney, FXIII B is secreted through a classical endoplasmic reticulum (ER)/Golgi secretory pathway. By using recombinant human FXIII B, recent research identified certain functions of the Sushi domains of FXIII B by Souri and colleagues (Souri et al., 2008). The first Sushi domain is involved in the interaction of FXIII B to the A subunits, while the fourth and ninth domains mediate the formation of the homodimers of FXIII B. Apart from the functions of the individual domains of FXIII B, they also suggested that only the full length or nearly full length FXIII B can protect FXIII A from degradation by covering its surface.

### 1.3.3 Activation of FXIII

Existing as a zymogen, the activation of pXIII-$A_2B_2$ is a multi-step process. Initiated by the disassociation from the B subunits in the presence of fibrin and $Ca^{2+}$, the cleavage of pFXIII by thrombin exposes the catalytic Cys in the active centre. A thrombin-independent activation of FXIII has also been reported, when only a much higher concentration of $Ca^{2+}$ is present in this process. Through $Ca^{2+}$-regulated conformation changes, FXIII A exerts its transglutaminase activity in the final stages of coagulation (Iismaa et al., 2009). Apart from

protecting FXIII A from proteolytic degradation, the importance and the mechanism of FXIII B in regulating the activation of FXIII has been well reported including facilitating the interaction of the zymogen pXIII-$A_2B_2$ with fibrinogen. The FXIII B released from pXIII-$A_2B_2$ can also act as a negative feedback signal to terminate the cleavage of the zymogen by thrombin (Halkier and Magnusson, 1988).

### 1.3.4 Factor XIII functions

Also named as plasma coagulation factor XIII or fibrin stabilizing factor, the main role of FXIII is to stabilize the fibrin soft clots, and to render it less susceptible to fibrinolysis either by cross-linking of fibrin itself or by cross-linking α-antiplasmin, a potent inhibitor of the protease plasmin, into the fibrin clot (Weiss et al., 1998).

The intracellular functions of FXIII have been widely studied, even though there are still conflicting opinions. Like other transglutaminase family members, the cytoskeleton proteins actin (Cohen et al., 1980) and myosin (Cohen et al., 1979) and the focal adhesion protein vinculin (Asijee et al., 1988; Horvath et al., 1992) are substrates for FXIII A, which, if the enzyme is activated by $Ca^{2+}$ (which seems unlikely in the intracellular environment), suggests its possible function in regulating the cytoskeleton organization and remodelling. The impaired capability of phagocytosis in FXIII A deficient monocytes suggested its importance in this process, which could be linked to its function in the cytoskeleton system. Extracellularly, FXIII A is well-defined in maintaining the hemostasis in connective tissues. In the extracellular matrix (ECM), its cross-linking activity is thought crucial in the interaction of ECM proteins, including fibronectin/collagen (Akagi et al., 2002; Mosher, 1984a; Mosher and Schad, 1979) and vitronectin (Sane et al., 1988), also in collagen synthesis (Paye et al., 1989). The impaired wound healing ability in FXIII A deficient patients suggested its importance in this process, which could be due to its function in stabilizing the fibrin/fibronectin network (Corbett et al., 1997), as well as its role in fibroblast cell proliferation and migration (Dardik et al., 2007).

Apart from in vitro studies, the functions of FXIII A have been widely investigated in animal models. In FXIII A deficient animal models, a relative mild bleeding with prolonged tail-tip bleeding times was discovered. Even though fertile, impaired reproduction is significantly observed in FXIII A knockout models, while frequent genital bleeding is found in female animals and leads to fetal abortion (Koseki-Kuno et al., 2003). In tumour metastasis studies, significant decreased lung and liver metastasis was discovered in FXIII A-/- animals, compared to wild type models, which could be due to its inhibiting effect on the functions of natural killer cells or its importance in the formation of tumour emboli (Palumbo et al., 2008). In agreement with in vitro studies and patient case reports, delayed wound healing was discovered in FXIII A-/- animals. Deficiency in extracellular reconstitution, including an attenuated inflammatory response and enhanced ECM degradation, was discovered (Nahrendorf et al., 2006). Recent studies also suggest the role of FXIII A in vascular disease, indicating the cross-linking reaction between β3 integrins and VEGFR-2 by FXIII A can in turn activate VEGFR-2 and its relevant signalling transduction in angiogenesis (Dardik et al., 2005). It has also been reported that FXIII A can catalyse the dimerization of angiotensin II type 1 (AT1) receptor to mediate its signalling pathway (AbdAlla et al., 2004).

The function of FXIII B is still under investigation. But it has been reported that FXIII B deficiency is always accompanied with FXIII A deficiency in patients (Saito et al., 1990). Even though no fetal difference, e.g. survival rates, was discovered in both genders of FXIII B-/- mice compared to the wild type controls, only the male knockout animals developed mild fibrosis with haemosiderin deposits in the heart tissues (Souri et al., 2008). A more recent case report revealed the existence of a specific autoantibody against FXIII B, which caused severe bleeding, while no effect of this antibody on FXIII activation and its activity was found (Ajzner et al., 2009). This finding highlights the importance of FXIII in the coagulation process.

Nowadays, the FXIII A or FXIII B deficient animals have become a very sufficient tool in the study of the functions of these FXIII subunits and it is

promising that in the near future the functions of FXIII A and B will be revealed by using this technique.

## 1.4 Keratinocyte transglutaminase

Keratinocyte TG (TG1) is an 817-residue polypeptide (about 106kDa) and the largest member of the TG family (Kim et al., 1995b). Localized to chromosome 14 q11.2-13, it has been reported that the gene encoding TG1 is composed of 15 exons and 14 introns that have conserved position in comparison to the other TGs (Kim et al., 1992). At the tissue level, the localization of TG1 has been identified in the granular layer at the later stages of differentiation by immunohistological and immunochemical methods (Steinert et al., 1996; Thacher and Rice, 1985), while a specific antibody against the N-terminal of the active fragments of TG1 revealed its existence in suprabasal and spinous layers (Iizuka et al., 2003). Although majorly anchored in the inner plasma membrane by acylated fatty acid, a small fraction of TG1 (5-35%) is normally found in a soluble state in the cytosol (Kim et al., 1995a).

As a specific marker of epidermal differentiation, till now many aspects of the biochemical properties and substrate properties of TG1 have been well understood (Rice et al., 1992). The enzymatic activity of TG1 experiences different stages during keratinocytes differentiation. TG1 exists as a zymogen in the differentiating keratinocytes, while in the terminal differentiation, the proteolysis process results in the formation of a 10/33/67kDa complex of TG1 with a dramatic increase of specific activity (Kim et al., 1995a). The cross-linking function of TG1 plays an important role in the formation and stabilisation of the cellular and tissue structures. TG1 is responsible for the formation and structural stability of the 15nm highly cross-linked thick layer of insoluble protein on the intracellular surface of the plasma membrane. In the granular layer of the epidermis, TG1 catalyzes cross-links of proteins, such as keratins (Yaffe et al., 1992), involucrin (Simon and Green, 1988), cornifin (Marvin et al., 1992), loricrin (Hohl, 1993) and elafin (Simon et al., 1996), so that it can contribute to the formation and stabilisation of the cell envelope. It has been reported that TG1 knockout animals presented defective cell envelope formation and loss of loricrin immunoreactivity in their stratum corneum, which also indicated the irreplaceable role of TG1 in the formation of the cell envelope, even though there

were other TGs, e.g. TG3, expressed in these animals (Matsuki et al., 1998). It has also been reported that intracellular TG1 is also present in the cytoplasm of spinous cells (Ta et al., 1990) and vascular endothelial cells (Baumgartner et al., 2004), suggesting that TG1 might also function in maintaining the intracellular stabilization of cells via its cross-linking activity. As an important protein in the formation of cell envelope, the expression of TG1 can be regulated by different proteins, including ROCK (Kotake-Nara et al., 2007), tazarotene-induced gene 3 (TIG3) (Eckert et al., 2009), βig-h3 and TGFβ1 (Oh et al., 2005), while other proteins, such as cathepsin D, are involved in regulating the enzymatic activity of this transglutaminase (Egberts et al., 2004).

The abnormalities shown in TG1 knockout mice, including degradation of nuclei, definitive cell envelope assembly, and impaired skin barrier function, suggest the importance of TG1 in development. The early neonatal death of the null mice (4-5 hours after birth) indicated that TG1 is also involved in the adaptation to the environment after birth (Matsuki et al., 1998). Yang and colleagues (2001) discovered 3 mutants of TG1 caused lamellar ichthyosis in two different families, which led to the misfolding of the catalytic core domain in TG1 and probably results in the reduced TG1 activity. Their work suggested that TG1 is crucial in the pathological progress in lamellar ichthyosis. In the vitamin A-deficient rats, TG1 activity and expression were significantly increased, leading to the existence of involucrin, loricrin and keratin 10 on the cornea epithelial cells. The above discovery suggests the role of TG1 in the abnormal keratinisation of the cornea (Toshino et al., 2005). A recent study also suggests that TG1 deficiency is the cause of bathing suit ichthyosis (Oji et al., 2006), while inappropriate TG1 expression might be involved in Stevens-Johnson syndrome (Nishida et al., 1999).

## 1.5 Epidermal transglutaminase

Although characterised (Buxman and Wuepper, 1975) and purified (Buxman and Wuepper, 1976) in 1970s, the epidermal TG (TG3) is still the least understood member of TG family. The human gene for TG3 was localised to chromosome 14 (Polakowska et al., 1991). TG3 is a latent, intact proenzyme (77kDa) with low specific activity. After cleavage to two fragments of 47 and 30kDa by certain enzyme/s, the enzyme becomes activated via the non-covalent interaction of the two fragments (Kim et al., 1993). A recent study indicated that cathepsin L can cleave TG3 *in vitro* (Cheng et al., 2006). Like the other TGs, the binding of $Ca^{2+}$ can increase the specific activity of TG3 around two fold (Kim et al., 1990; Kim et al., 1995a).

Until now the cellular and tissue distribution of TG3 has not been well-investigated, but the utilization of polyclonal antibodies indicated that TG3 could be detected in the epidermis in the later stage of differentiation (Lee et al., 1996) and the presence of TG3 in brain, small intestine, the testis and forestomach has been identified (Hitomi et al., 1999). By using a monoclonal antibody raised against the enzyme, Hitomi and colleagues (Hitomi et al., 2003) showed the cytoplasmic distribution of TG3 in the granular and cornified layer, and proposed a role for TG3 in the early phase of cornified cell envelope formation. During epidermal terminal differentiation, activated TG3 can crosslink cornified envelope (CE) proteins, including loricrin, small proline-rich proteins (SPRs) 1, 2, and 3, and trichohyalin, which are also known TG1 substrates (Candi et al., 1995; Steinert et al., 1998; Tarcsa et al., 1997) (Steinert and Marekov, 1995). Unlike TG1, to date no relevance for TG3 mutants in human diseases have been reported (Zocchi et al., 2007). Although pathological roles of TG3 in human diseases have not been completely demonstrated, a recent study indicated that the co-localisation of IgA and TG3 in the healthy skin of coeliac patients, suggesting that TG3 may play a central role in the pathogenesis of Dermatitis herpetiformis (DH) (Cannistraci et al., 2007). By studying autoantibodies against TG3 in serum samples from DH patients on a normal or gluten-free diet via ELISA, Rose and colleagues further confirmed the importance of TG3 in DH and

suggested that anti-TG3 antibodies can serve as a sensitive serological marker for this disease (Rose et al., 2009).

## 1.6 Prostate transglutaminase

Previously named dorsal protein 1, prostate transglutaminase (TG4) has been shown to be a 150kDa dimeric protein (62kDa on SDS-polyacrylamide gels) (Wilson and French, 1980). Human TG4 gene (TGM4) is localized to chromosome 3 p21.33-p22, and the polymerase chain reaction (PCR) technique revealed that TGM4 (approximately 35kb of genomic DNA) consists of 13 exons and 12 introns to encode 684 amino acid TG4 protein (Dubbink et al., 1998; Gentile et al., 1995). Although still unknown at the molecular level, the expression of TG4 by the luminal prostatic epithelial cells in coagulating gland and dorsal prostate is strictly regulated by androgens (Dubbink et al., 1996; Steinhoff et al., 1994; Dubbink et al., 1999). A recent study indicated that the positions between -113 and -61 of TGM4 promoter might be essential in TG4 expression (Dubbink et al., 1999).

Like other TGs, TG4 demonstrates both transamidating and GTPase activities (Spina et al., 1999). By analysing different TG4 mutants, Mariniello and colleagues (2003) demonstrated that TG4 requires its N-terminal end for its catalytic activity, and this domain of TG4 is thought crucial for its interaction with GTP. The crosslinking activity of TG4 secreted into the seminal fluid has been shown to participate in the formation of the copulatory plug in the vagina after coitus (Williams-Ashman, 1984). It has been reported that the polymeric form of TG4 can bind to the epididymal sperm cells and mask their immunogenicity, so that the sperm cells can escape the immune response in the female genital tract (Mukherjee et al., 1983; Paonessa et al., 1984; Porta et al., 1986). As a prostate-specific protein, the relevance between TG4 and prostatic tumours has been studied. Interestingly, there was found to be a down-regulation of human TG4 in most metastatic prostatic cancers, while in prostate tumours with low-metastatic potential TG4 was up-regulated, which indicated that TG4 might be a marker for the invasive potential of prostate cancer cells (Davies et al., 2007; Dubbink et al., 1996). The potential relationship between TG4 and prostate cancer still requires further investigation. However by using prostate cancer cells expressing different level of TG4, recent work suggested that over-expression of

TG4 increased the interaction between prostate cancer cells and endothelial cells, which was inhibited by knocking down TG4 via siRNA treatment (Jiang et al., 2009).

## 1.7 Erythrocyte band 4.2

Human erythrocyte band 4.2 (also known as protein 4.2 and pallidin) is a peripheral membrane protein, which is N-terminally myristoylated and palmitoylated. It has been reported that the erythrocyte protein 4.2 gene *EPB42* is located in bands q15 to q21 of human chromosome 15 (Sung, *et al.*, 1992), which contains 13 exons (20kb) (Cohen *et al.*, 1991; Sung et al., 1992). There are two isoforms of protein 4.2: a protein of 721 amino acids with a molecular weight of 74kDa encoded by the long isoform cDNA (P4.2L) and a protein of 691 amino acids with a molecule weight of 72kDa encoded by the short isoform cDNA (Sung, *et al.*, 1992). Protein 4.2 is a major constituent of the red blood cells (RBCs) membrane skeletal network and comprises approximately 5% of the total membrane protein, which means it is present in about 200,000 copies per RBC (Cohen et al., 1993).

As a member of transglutaminase family, protein 4.2 is the only catalytically inactive one (Aeschlimann and Paulsson, 1994). The exact role of protein 4.2 in RBC has not been elucidated yet, but the binding of protein 4.2 to the cytoplasmic domain of band 3 (Bhattacharyya et al., 1999) and its interacting with spectrin (Colan et al. 1996) and ankyrin (Korsgren et al., 1988) suggest its function in stabilizing linkages between the cytoskeleton and the overlying membrane. Meanwhile the presence of band 3 is also crucial for the stable of protein 4.2 incorporation of protein 4.2 into the RBC membrane. It has been shown that RBCs deficient in band 3 in human (Ribeiro et al., 2000), mouse (Peters et al., 1996) and cow (Inaba et al., 1996) are completely deficient in protein 4.2. Recently, protein 4.2 has been shown to interact with CD47, which probably contributes to the anchoring of the Rh complex to the RBC skeleton (Mouro-Chanteloup et al., 2003). The interaction of band 4.2 with other RBC proteins highlights the importance of protein 4.2 in maintaining the integrity, stability and flexibility of RBCs. Hereditary deficiency in protein 4.2 results in erythrocytes fragility (Cohen et al., 1993). To date, 9 kinds of protein 4.2 mutations have been found associated with herediatary spherocytosis (HS).

## 1.8 Transglutaminases X, Y, and Z

With the further research in TG family, three different types of transglutaminase were discovered, namely transglutaminase X (TG5), transglutaminase Y (TG6), and transglutaminase Z (TG7). Even though the physiological functions of above three TGs have not been well defined, genomic research has revealed the structure and location of their genes (Aeschlimann et al., 1998; Grenard et al., 2001).

The gene encoding human TG5 (TGM5) is localized in the q15.2 region of chromosome 15, composed of 13 exons and 12 introns (about 35 kb) (Grenard *et. al.*, 2001). The enzyme consists of 720 amino acid residues with the molecular weight of approximately 81kDa. Now two isoforms of TG5 have been discovered: a long 720 amino acid form, which contains an insert in the N-terminal region, and a short 638 amino acid form. TG5 is almost ubiquitously expressed (Grenard et al. 2001) and, in particular, is widely expressed in the epidermis (Candi et al. 2002). As a dual-functional enzyme, TG5 exerts both transamidating and GTPase activities. Like TG2 and TG3, the activity of TG5 is inhibited by GTP, and $Ca^{2+}$ is capable of completely reversing this inhibition (Candi, et. al. 2004). Other studies doing in situ measurement demonstrated that TG5 can be activated by phorbol acetate (Rufini et al., 2004). TG5 has been found in keratinocytes of early-staged differenciation and showed high crosslinking activity toward some epidermal proteins, including loricrin, involucrin, and SPR3 (Candi et al., 2001). Recent studies demonstrated that TG5 might be involved in different pathological processes and relevant diseases. Two types of TG5 mutants, T109M and G113C, were discovered in unrelated peeling skin syndrome (PSS) patient families, respectively. Even though no pathological effect of T109M was discovered, G113C, in a position near the catalytic domain of TG5, showed no crosslinking activity. The role of TG5 in mediating the structure of epidermis through its crosslinking activity was also suggested (Gassidy et al., 2005). In Darier's disease, two extreme situations of TG5 expression over-expression or absence, was discovered in different areas of the same lesion (Candi, et al., 2002).

A recent study introduced two types of TG members, TG6 and TG7, located at mouse chromosome 20 q11. By using PCR, Grenard and colleagues revealed TGZ is a protein of 710aa (80kDa) (Grenard et al. 2001). Even though the physiological functions of these TGs still remain unknown, interestingly one recent work by Stamnaes and colleagues (2010) demonstrated the role of TG6, as a target of gluten ataxia (GA) autoantibody, in mediating the autoimmune response in celiac disease (CD).

## 1.9 Tissue transglutaminase

In contrast to the other members of the transglutaminase family, tissue transglutaminase (tTG, TG2, or type II transglutaminase) is a multifunctional enzyme involved in a number of disparate biological processes. Although mainly a cytosol protein, TG2 can also be localized in the plasma membrane, nucleus and nuclear membrane and the mitochondria. Unlike the majority of other secretory proteins, TG2 lacks the hydrophobic leader sequences and cannot be secreted by the classical ER/Golgi-dependent mechanism, but the presence of the enzyme has been detected in cell membrane fractions (Griffin et al., 1978b) and in the ECM (Verderio et al., 1998a). A novel function for TG2 present in the ECM forms the subject of this thesis.

### 1.9.1 Structure of tissue transglutaminase

The cDNA sequence study shows that the TG2 molecule is a monomeric protein of 685-691amino acids with a molecular weight of 75-85kDa. The three-dimensional structure of TG2 has been modelled on that of factor XIIIa and has been predicted to consist of 4 main domains, namely the N- terminal β- sandwich (residues 1-138), α/β catalytic core (residues 139-471), and two C-terminal β-barrel domains (residues 472-584 and 585-686, respectively) (Chen and Mehta, 1999b) (modified in this work, Figure 1.1). Although with 17 cysteine residues and six potential N-linked glycosylation sites, TG2 molecule contains neither disulfide bonds nor glycosylated structures (Ikura et al., 1988a). It has been reported that the protein undergoes a series of post-translational processes, including the removal of its initiator methionine and N-acetylation of the adjacent alanine residue. Even though the transamidation activity of the enzyme is not affected by the acetylation at the N-terminal of guinea pig TG2, this modification may be involved in the protein's secretion (Muesch et al., 1990).

In the N-terminus of the TG2 molecule, there is a fibronectin binding site mediating the interaction between TG2 and FN with high affinity, which has been reported to be involved in regulating cell adhesion (Akimov et al., 2000b),


**Figure 1.1 Schematic structure and functional domains of TG2.**
A) Backbone structure of TG2 (the picture was produced with Rasmol). Domains I-IV are coloured respectively in magenta, orange, blue and green; the regulatory loop between domain II and III is coloured red. The colours yellow, black and light grey represent the amino acids involved in the active site (Cys277, His335 and Asp358), in $Ca^{2+}$ binding (Ser449, Pro446, Glu451 and Glu452) and in interaction with GTP (Ser171, Lys173, Arg478, Val479 and Arg580), respectively. (B) Space-filling presentation of TG2 structure in the absence of ligands. (C) The proposed structural and functional domains of TG2. N-terminal amino acid residue 1-7 and the amino acids 88-106: required for TG2's intereaction with FN; Try241: essential for TG2 cross-linking activity; Cys277 (Cysteine), H335 (Histidine) & D358 (Aspartic Acid): conserved among all enzymatically active members of the TG family; Cys277: crucial for the formation of the thioester bond and secretion of TG2 into the ECM (Chen and Mehta, 1999).

**Figure 1.1**

matrix assembly (Telci and Griffin, 2006) and TG2 externalization (Balklava et al., 2002). The truncation of in the N-terminal of TG2 molecule led to the loss of almost all the ability for its binding to FN, which suggested the importance of the N-terminal sequence in the interaction between TG2 and FN (Gaudry et al., 1999a). Another FN binding site, localized at amino acids 80-106 within the β-sandwich domain of this enzyme, was suggested as the FN binding sites by Belkin and colleagues (Hang et al., 2005). By using a synthetic peptide sequence matching these residues, the interaction between TG2 and FN was significantly inhibited, and the peptide inhibited cell adhesion process on FN mediated by TG2, suggesting this domain is a novel FN binding site in TG2. It has also been reported that the β-sandwich domain is crucial to the transamidating activity of TG2 (Iismaa et al., 1997). The α/β catalytic domain is involved in the GTPase/ ATPase activity of TG2. As a $Ca^{2+}$-dependent enzyme, the transamidating activity of TG2 is regulated by the binding of the enzyme molecule with $Ca^{2+}$ through the $Ca^{2+}$-binding site within the 430-453 amino acids of the core domain (Ikura et al., 1988b). By using mutagenesis of TG2, most recent report demonstrated that there are 5 other possible $Ca^{2+}$ binding sites (around amino acids 228, 395, 305, 149 and 432), the mutations of which led to the decreased transglutaminase activity of TG2 (Kiraly et al., 2009). It has been demonstrated that the GTP-binding site is localised to a 15-residue segment spanning between amino acids 159-173 in the core domain of TG2 and $Trp^{332}$ is also relevant in the regulation by GTP (Murthy et al., 2002). Recently it was reported that $Arg^{580}$ in human TG2 and $Arg^{579}$ in rat TG2 is important in the binding ability of TG2 with GTP. The mutants of these two sites also showed increased *in situ* TG activity compared to the wild type enzymes (Ruan et al., 2008). Within the catalytic domain of TG2, the $Tyr^{241}$ is essential for activity of TG2, while the $Cys^{277}$ forms the thioester bond involved in transamidation (Chen and Mehta, 1999a). Close to the C-terminal of the TG2 molecule, eighteen- amino acid residues, $Val^{655}$-$Lys^{672}$, are crucial for the protein to recognize and activate phospholipase C (PLC), which is important in transmitting the α1B- adrenoceptor (AR) signal (Feng et al., 1999).

### 1.9.2 Regulation of expression

The human TG2 gene (TGM2) has been mapped on chromosome 20q11-12 and is 32.5kb (Gentile et al., 1994). TGM2 is composed of 13 exons separated by 12 introns. Evidence indicates that the expression of TGM2 can be regulated by cytokines and hormones.

Retinoic acid (RA) treatment can induce TG2 expression both at the mRNA and at the protein level *in vitro* and *in vivo* (Defacque et al., 1995; Verma et al., 1992). It has been reported that the TGM2 gene contains regulatory elements for several transcriptional factors, including two tandem retinoid-responsive elements (RREs), which bind the retinoid receptors RAR and RXR. RA promotes the expression of TG2 via its interaction with its receptors to promote the formation of RAR/RXR heterodimers or RXR/RXR homodimers, which triggers the activation of the transcription of TGM2 (Glass, 1994; Mehta et al., 1996). It has been reported that TG2 expression can be regulated by some cytokines including transforming growth factor β1 (TGFβ1), interleukins (e.g., IL-1), and morphogenic protein 4. TGFβ1 can increase the expression of cell surface TG2, which results in the enhanced cell adhesion (Priglinger et al., 2004). On the other hand, TG2 can activate the matrix bound TGFβ via cross-linking of the Large Latent TGFβ-binding protein-1 (LTBP-1). The activation of macrophages, the main "producer" of TGFβ, is characterized by increased TG2 levels, which is related to its phagocytotic capacity and its ability to secrete and activate TGFβ1 (Griffin et al., 2002b; Telci and Griffin, 2006). These results indicate there might be a positive feedback loop between TG2 and TGFβ1 production by cells. Another inflammatory cytokine TNFα can increase the TG2 expression by liver cells through the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)-dependent mechanism, which could be important in the progress of liver fibrosis (Kuncio et al., 1998). Some results also suggest that DNA methylation, together with alterations in the chromatin structure, may play a role in regulating TG2 production (Lu and Davies, 1997; Grunstein, 1997). Blocking endogenous polyamine synthesis with α-difluoromethylornithine (DFMO) decreased TG2 in rat small intestinal crypt (IEC-6) cells, but led to the increase of TG2 in human colon carcinoma (Caco-2) cells, in both transamidating activity and mRNA level

(McCormack et al., 1994; Wang et al., 1998). These data indicated that as a widely-expressed protein, the expression of TG2 is also cell and tissue type-dependent.

### 1.9.3 Regulation of activity

Accumulating research focusing on the regulation of TG2 activity suggests that, as a multifunctional enzyme, TG2 can catalyse both $Ca^{2+}$-dependent transglutaminase activity (transamidation) and $Ca^{2+}$- independent Guanosine-5'-triphosphate (GTP) and Adenosine-5'-triphosphate (ATP) hydrolysis regulated by magnesium (Lee et al., 1989), meanwhile it also can function as a protein disulphide isomerise (PDI) (Hasegawa et al., 2003) and a kinase (Mishra and Murphy, 2004).

In the intracellular environment, recent reports demonstrate that the activity of TG2 can be modulated both by $Ca^{2+}$ and GTP/GDP levels. A large increase in the intracellular $Ca^{2+}$ can lead to an enhanced transglutaminase activity of TG2 resulting in the crosslinking of a variety of proteins, including signalling molecules, enzymes and the cytoskeleton (Griffin et al., 2002b; Verderio et al., 2004). Unlike other members of the transglutaminase family, TG2 is capable of both binding and hydrolyzing GTP (as a Gunoaine-5'-triphosphatase (GTPase)) (Lorand and Graham, 2003). Due to the presence of high concentrations of GTP and low levels of $Ca^{2+}$ intracellularly, it is now generally believed that intracellular TG2 mainly functions as GTPase instead of a Tgase (Ruan et al., 2008; Smethurst and Griffin, 1996b). The regulatory effect of GTP on TG2 activity has been very well demonstrated from *in vitro* studies. The binding of GTP to TG2 leads to changes in the conformation of the TG2 molecule and results in its low affinity for $Ca^{2+}$ (Kiraly et al., 2009; Ruan et al., 2008; Smethurst and Griffin, 1996a). Meanwhile the ability to bind and hydrolyze GTP links intracellular TG2 to a major signalling pathway, in which TG2 functions as a signal transduction GTP-binding protein, $G\alpha_h$. In this pathway, TG2 transmits outside signals from the membrane (e.g. α1B and α1D adrenoceptor) to

downstream cytoplasmic targets such as phospholipase Cδ (PLCδ) to regulate cellular behaviour, e.g. cell migration (Murthy et al., 1999); (Feng et al., 1999; Kang et al., 2004)). It was demonstrated that, as a complex with TG2, PLC δ1 remain inactive, while the binding of TG2 to GTP can release PLC from the complex and activate this protein (Murthy et al., 1999). Recent *in vivo* study further supports the regulatory effect of GTP on TG2 activity by demonstrating that in human SY5Y neuroblastoma cells, the depletion of intracellular GTP leads to increased *in situ* transamindating activity of TG2 (Zhang et al., 1998). Magnesium ions are also essential for the hydrolysis of both GTP and ATP (Lee et al., 1993). $Mg^{2+}$-ATP induces a conformational change in TG2 that inhibits GTPase but does not interfere with the cross-linking activity, while $Mg^{2+}$-GTP binding induces a different conformation that inhibits cross-linking activity without affecting the ATPase activity of TG2 (Lai et al., 1998). There are also some reports suggesting that nitric oxide (NO) is a potent inhibitor of TG2 activity, through a $Ca^{2+}$-sensitive nitrosylation of multiple cysteine residues (Lai et al., 2001; Telci et al., 2009).

To sum up these findings the binding of GTP or $Ca^{2+}$ cause opposite conformational changes in the TG2 molecule and inhibit, respectively, the transamidation or GTPase functions of TG2. Meanwhile, other molecules, e.g. $Mg^{2+}$ and NO, are also involved in regulating the TG2 activity.

Recent studies proposed a novel function for TG2 as PDI, which can convert the completely reduced/ denatured inactive RNase, a molecule to the native active enzyme. Unlike the other enzymatic activities of TG2, the PDI activity depends neither on the $Ca^{2+}$ nor on the binding of GTP (Hasegawa et al., 2003). A most recent reported demonstrated that the PDI activity of TG2 is important in maintaining the correct assembly of mitochondrial ADP/ATP transporter adenine nucleotide translocator 1 (ANT1) and its potential involvement in mitochondria-related apoptosis (Malorni et al., 2009). Mishra and Murphy also discovered an intrinsic kinase activity for TG2 (Mishra and Murphy, 2004). TG2 present in human breast cancer cell membranes can phosphorylate the insulin-like growth factor-binding protein-3 (IGFBP-3) and enhance its binding affinity to IGF-I to

modulate the pro-apoptotic anti-proliferation effect of IGFBP-3 in cancer cells. This IGFBP-3 kinase activity of TG2 can be inhibited by an increase in $Ca^{2+}$ concentration.

### 1.9.4 Localisation and cellular distribution of TG2

Unlike the majority of other secretory proteins, TG2 lacks the hydrophobic leader sequences and it cannot be secreted by the classical endoplasmic reticulum/Golgi-dependent mechanism, but the presence of the enzyme has been detected in cell membrane fractions (Griffin et al., 1978c) and in the extracellular matrix (Verderio et al., 1998b); (Stephens et al., 2004). Although predominantly a cytosolic protein (80%), TG2 can translocate to the nucleus with the help of importin-α-3 protein or to the membranes in association with integrins (Akimov and Belkin, 2001a; Isobe et al., 1999; Mehta et al., 2006a; Takahashi et al., 2000). In addition, a current study showed TG2 is localised at the outer mitochondrial membrane, which has been suggested to be crucial in maintaining mitochondria function by regulating the ANT1 activity (Malorni et al., 2009).

### 1.9.5 Physiological functions of TG2

As a multi-functional protein and a widely -expressed member of the TG family, and despite attempts to demonstrate a defined role for TG2, the precise physiological functions of TG2 still remain a puzzle to researchers. Accumulating evidence demonstrates that TG2 is involved in various biological and physiological processes, including matrix stabilization (Aeschlimann et al., 1995; Aeschlimann and Thomazy, 2000; Collighan and Griffin, 2009; Verderio et al., 1998b), cell growth and differentiation (Gentile et al., 1992), programmed cell death and necrosis (Fesus et al., 1987) (Nicholas et al., 2003), $Ca^{2+}$ mediated stimulus secretion coupling (e.g. insulin secretion) (Bungay et al., 1986; Sener et al., 1985), receptor mediated endocytosis/ phogocytosis (Schroff et al., 1981), cell adhesion and migration (Akimov and Belkin, 2001b; Akimov et al., 2000a; Balklava et al., 2002; Verderio et al., 2004; Verderio et al., 2003; Zemskov et al., 2006).

### 1.9.5.1 Importance of TG2 in cell growth and differentiation

The involvement of TG2 in cell differentiation and proliferation was initially reported in the 1970s by Birckbichler and colleagues, based on the finding that cells with lower TG2 activity proliferated more rapidly than those with higher enzymatic activity (Birckbichler and Patterson, 1978). By using cystamine to inhibit the transglutaminase in WI-38 human lung fibroblasts, these authors demonstrated that with the decreased intracellular ε-(γ-glutamyl) lysine, some proliferation markers were enhanced, which could not be achieved by using other amines such as methylamine and histamine (Birckbichler et al., 1981). More focused serious work from Griffin and co-worker demonstrated the role of TG2 in regulating cell proliferation by using the highly metastatic hamster fibrosarcoma cell line MetB. Flow cytometry revealed an over-lap between the high TG2 activity and the high apoptotic cell cycle phase at mid-S-phase, while the quantity of the enzyme remained unchanged in the other phases of the cell cycle (el Alaoui et al., 1992). Further investigations were performed to study the activity of TG2 in the progression of the cell cycle. Both wild type and C277S mutant cDNA (in which the transamidation function was multigenized but the enzyme still showed GTPase activity) were transfected into the MetB cells, and a delayed progression from S phase to G2/M was discovered via flow cytometry, which indicated that the regulation of TG2 in cell progression is not dependent on its transamidating activity (Mian et al., 1995). Meanwhile the role of TG2 in modifying fibronectin (FN) was also suggested to be involved in cell progression control, since it was reported that FN with various 3-D structures affected the rate of G0/G1 to S phase progression (Sechler and Schwarzbauer, 1998).

The role of TG2 in cell differentiation has been well documented. Via immuno-fluorescence staining and transamidation assays, the presence of TG2 was studied in different immune cells with various differentiation stages. In induced macrophages more TG2 signal was detected compared to normal peritoneal washout macrophages, suggesting TG2 as a new marker for macrophages in certain differentiation stages (Schroff et al., 1981). In MC3T3-E1 osteoblasts, the role of active TG2 in cell differentiation was reported based on the fact that the

inhibition of TG2 activity resulted in the arrested stage of osteoblasts differentiation, while the transamidating activity of TG2 in moderating the FN-collagen matrices network formation was also suggested to involve the above process and lead to matrix mineralization (Al-Jallad et al., 2006). By using tissue-engineered human skin, the inhibition of TG2 by site directed-transglutaminase inhibitors, NTU283 and NTU285, was reported to block the differentiation of keratinocytes and led to the hyperproliferation in the epidermis (Harrison et al., 2007). Unlike its role in cell differentiation, the GTP-bound form of TG2, but not its transamidating activity, was suggested as a molecular switch in leading the maturation of chondrocytes to hypertrophy (Johnson and Terkeltaub, 2005). The above function of TG2 in chondrocyte maturation was also reported in a study about guinea pig osteoarthritis (OA) by Huebner and colleagues (Huebner et al., 2009). In their work, the role of this protein in regulating the maturation of chondrocytes to hepertrophy suggested TG2 as a biomarker in evaluating the progression levels of OA.

#### 1.9.5.2 TG2 in $Ca^{2+}$-mediated stimulus secretion coupling

The involvement of TG2 in $Ca^{2+}$ mediated insulin secretion was first raised in 1984 by Griffin and colleagues, who discovered that incubation of rat islets with TG2 competitive substrates can inhibit the glucose-stimulated insulin release (Bungay et al., 1984). The role of TG2 in this process was further supported by the fact that monodansylcadaverine, a TG2 competitive amine substrate that can inhibit crosslinking, can decrease the glucose-stimulated insulin secretion significantly (Bungay et al., 1986; Sener et al., 1985). TG2 -/- mice showed decreased glucose tolerance after intraperitoneal glucose loading and a tendency of hypoglycemia induced by the increase of the phosphorylation of insulin receptor substrate 2 (IRS-2) (Bernassola et al., 2002). They also reported that the presence of one of TG2 mutants— N333S, in Italian subjects with the clinical features of maturity-onset diabetes of the young (MODY) (Bernassola et al., 2002). A recent study reported the presence of TG2 mutants located close to the transamidation catalytic site of TG2, including M330, I331 and N333, which may be involved in early-onset type 2 diabetes (Porzio et al., 2007).

### 1.9.5.3 TG2 and receptor mediated endocytosis

Based on the finding that receptor-mediated endocytosis of α2-macroglobulin can be inhibited by TG competitive primary amine substrates, the involvement of TG2 in endocytosis was first demonstrated. It was suggested that TG2 exerted its function by stabilising the membrane for the receptor aggregation or cross-linking the ligand with its receptor (Davies et al., 1980). The role of TG2 in endocytosis was also studied in antigen presenting cells (APC) by using similar monoamines competitive substrates. High TG2 activity was found in lymphocyte accessory cells, peritoneal exudate and spleen adherent cells, meanwhile the TG2 competitive primary amine substrate monodansylcadaverine inhibited the receptor-dependent endocytosis of immune complexes at 100μM, significantly less than the inhibiting effect of methylamine (over 20mM) (Teshigawara et al., 1985). Clear proof of increased TG2 activity and enhanced phagocytosis in macrophages was demonstrated (Murtaugh et al., 1983). In vivo study in TG2 -/- mice indicated that the absence of TG2 led to the prevention of active TGFβ1 production by macrophages exposed to apoptotic cells, which in turn caused the inefficient phagocytosis of apoptotic cells (Szondy et al., 2003) (Boisvert et al., 2006).

### 1.9.5.4 Tissue transglutaminase and cell death

The involvement of TG2 in cell death, including apoptosis and necrosis, has been widely studied. Apoptosis means 'dropping off' in Greek, which was first proposed by Kerr and Searle after discovering a type of cell death that happened in individual liver cells after severe-weak portal vein branch ligation in 1970s (Kerr, 1965; Kerr and Searle, 1972) (Kohn et al., 1987). Apoptosis has been reported important in determining cell termination (removing of damaged cells), maintaining normal tissue homeostasis by balancing cell mitosis, tissue development and lymphocyte-mediated immune response (Gerschenson and Rotello, 1992; Thompson, 1995; Werlen et al., 2003). The role of TG2 in apoptosis varies depending on the activation of its enzymatic activity, GTP binding, or the presence of intracellular $Ca^{2+}$, GTP or polyamines (Melino and

Piacentini, 1998). TG2 can prevent the occurrence of inflammation by promoting the irreversible formation of polymers of intracellular proteins, including actin, annexin, fibronectin, vinculin, retinoblastoma protein, and troponin (Ballestar et al., 1996; Gorza et al., 1997; Nemes et al., 1997; Oliverio et al., 1997), in the dying cells via its cross-linking activity. This effect limits the release of the intracellular proteins and protects the cells from autophagy until they are cleared by immune cells (Piredda et al., 1997). This enzymatic activity of TG2 suggests its role as a downstream effector in cell apoptosis, while recent research demonstrated an opposite effect of TG2 cross-linking activity. In thapsgargin-treated HCT116 colon cancer cells, TG2 can promote the formation of two species of caspase 3, p40 and p64, via its cross-linking activity, thereby TG2 blocked the activation of Bax-dependent pathway mediated by caspase to protect the cells from THG-induced apoptosis (Yamaguchi and Wang, 2006). Antonyak and collagues indicated that GTP-bound TG2, as an intracellular signalling molecule, can protect cells from the synthetic retinoid *N*-(hydroxyphenyl) retinamide (HPR)-induced apoptosis via activating intracellular Ras-ERK pathway in fibroblasts (Antonyak et al., 2001) (Antonyak et al., 2003). In epithelial ovarian cancer cells, TG2 can protect the cells from cisplatin-induced apoptosis by activating the NF-κB survival pathway (Cao et al., 2008). It has also been shown that in fibroblasts and osteoblasts, matrix TG2 can rescue cells from RGD-induced apoptosis, named anoikis (Verderio et al., 2003).

In contract to apoptosis, necrosis is a fatal process under external stimuli including infection, inflammation, injury, and cancer. Griffin and colleagues demonstrated that TG2 can stabilize intracellular proteins by mediating the formation of the cross-linked proteinaceous shells to block the release of the DNA and in turn preventing the cells from the classical apoptotic pathway mediated by Bcl2, $Ca^{2+}$-activated and other cellular proteases and caspase 3. Their data indicated the protecting role of TG2 on necrosis after the loss of $Ca^{2+}$ homeostasis (Johnson et al., 1998; Nicholas et al., 2003). By detecting TG2 in both hepatitis C virus (HCV)-infected patients and carbon tetrachloride ($CCl_4$)-induced liver injury mice, Nardacci *et al.* (Nardacci et al., 2003) discovered increased level of TG2 in the early stage of liver injury mostly localized in

hepatocytes facing periprotal infiltrate, while in the stage of prominent ECM deposition, TG2 was predominantly present in the ECM. This strict presence of TG2 during different stages of liver damage indicated the protective role of TG2 in maintaining liver architecture against the inflammatory stimuli.

The opposite effects of TG2 on determining cell fate, protecting or inducing, apoptosis or necrosis, may be cell type- and stimuli-dependent, and also can be regulated by the intracellular concentration of $Ca^{2+}$ and GTP, as well as the localization of the protein (Fesus and Szondy, 2005).

### 1.9.6 TG2 and human diseases

During the recent decades, the potential role of TG2 in the development of human diseases has become the major interest of scientists. The contribution of its cross-linking activity, GTPase activity and its non-enzymatic function as a signalling protein has been reported in different pathological processes, such as celiac disease, neurodegenerative diseases, fibrosis and cancer in humans.

#### 1.9.6.1 TG2 in Celiac Disease

Celiac disease (CD) is a chronic autoimmune disorder caused by a dysregulated T cell mucosal immune response to gluten from wheat or similar structural cereals such as rye and barley, which is relevant to the genes that encode HLA-II antigens, including HLA-DQ2 and DQ8 (Sollid, 2000). The pathological study of CD suggested that the specific immunogenic peptides within the gluten protein can survive from the digestion of gastric and pancreative enzymes and can active specific $CD4^+$ T-cell line to trigger the CD (Shan et al., 2002). Once activated by $CD4^+$ T-cell, type I helper T cells can produce cytokines (e.g. intereferon-γ (IFN-γ)), leading to the subsequent immune and inflammatory responses and the intestinal lesion, meanwhile the clonal expansion of B cells results in the production of antibodies (Rodrigo, 2006). In serum sample of CD patients, specific antibodies against gliadin, endomysium, reticulin, jejwnum can be detected. The high level of specific anti-TG2 serum antibody found in celiac

patients exposed to gluten suggested the involvement of the enzyme in this disease (Sollid and Scott, 1998). Apart from the anti-TG2 antibody, other evidence suggests the potential role of TG2 in CD: (1) there is higher expression of TG2 in CD patients; (2) the increased TG2 in CD patients is localized to the small intestinal mucosa and in the extracellular matrix, while in normal subjects, TG2 can be found in all layers of the small intestinal wall; (3) the enzymatic activity of TG2 in intestinal mucosa is increased in CD patients compared to controls (Caputo et al., 2004).

It has been well accepted that TG2 acts as a master regulator of CD. TG2 can regulate the progress of CD in several ways. It has been proven that the specific glutamine residues with gluten is an ideal substrate of TG2, and the deamination reaction mediated by TG2 is crucial for HLA-DQ2 binding and subsequent T cell activation (Schuppan and Hahn, 2002). Meanwhile the formation of gliadin-TG2 complexes supports the production of anti-TG2 antibody which can in turn modify the activity of the enzyme and some enzymatic-independent functions of protein on the cell surface (Caputo et al., 2004; Molberg et al., 2000). Other studies indicate that TG2 can activate TGFβ1 by promoting its maturation so that TGFβ1 can increase the activation of mucosal Th1 cells and the differentiation of intestinal epithelium (Hansson et al., 2002). Moreover, the role of TG2 in regulating the humoral response may also contribute to the T cell response in CD patients' small intestine (Molberg et al., 1998). In addition to being a sensitive marker of CD in diagnosis, the potential application of TG2 in CD therapy has also been suggested, via the endocytosis of digestive-resistant oral vaccination with immunogenic peptide mediated by the protein. Meanwhile the T cell immune response to the gluten peptide could be inhibited by blocking the enzymatic activity of the enzyme (Reif and Lerner, 2004).

#### 1.9.6.2 TG2 in neurodegenerative diseases

The physiological and pathological functions of TG2 in the neuronal system have been widely studied. It has been reported that TG2 is involved in the development, regeneration and functioning of neurons. Transient increases of

TG2 activity have been found in rat superior cervical ganglia after nerve injury and in the vagus nerve after crush injury (Ando et al., 1993; Tetzlaff et al., 1988). The increase of TG2 activity has been reported during development (Perry and Haynes, 1993). By introducing wild type TG2 into neuroblastoma cells, Lesort and colleagues (2000) discovered the formation of extensive neurites, but not in the cells transfected with inactive TG2. Midkine, an in vitro TG2 substrate, can be cross-linked by this enzyme to form a stable dimer, which is important in improving the neurite out-growth and neuronal survival (Iwasaki et al., 1997; Kojima et al., 1997; Mahoney et al., 1996). TG2 can also support regeneration of injured rat optic axons by stabilizing the dimers of interlukin-2 via its cross-linking activity (Eitan et al., 1994). The direct interaction of core hisone 2B and TG2 was found in apoptotic SK-N-BE cells (Piredda et al., 1999). The above studies suggest the importance of TG2 in mediating neurite outgrowth and neuron survival and apoptosis.

Based on its importance in neuronal development, regeneration, cell survival and apoptosis, the role of TG2 in different neurodegenerative diseases has been further demonstrated. Alzheimer's disease is one of the most common age-related neurodegenerative diseases, caused by selective damage of brain regions and neural circuits, which can lead to impaired memory, thinking and amygdala (Kim et al., 2002). Selkoe and colleague (Selkoe et al., 1982) first suggested the involvement of TG2 in the pathology of Alzheimer's disease and accumulating evidence further proved its important role in the pathological progression of this disease due to its biochemical properties (Selkoe et al., 1982). Amyloid β-protein (Aβ), is an element of Alzheimer's disease, can disrupt the calcium homeostasis in cultured neurons, which can activate the cross-linking activity of intracellular TG2 and might in turn promote the formation of Aβ dimers, trimers and tetramers and high-order oligomers. These oligomers of Aβ resemble those presented in Alzheimer's disease brain samples (Mattson et al., 1992); (Dudek and Johnson, 1993; Ho et al., 1994). Oxidative stress and free radical production may also contribute the pathological damage of Alzheimer's disease by promoting the oxidation of proteins, which are ideal substrates of TG2 (Groenen et al., 1993; Mattson, 1995). On the other hand, some regulators in TG2

expression, including NF-κB, TNF-α, interleukin-6, have been reported to be up-regulated in Alzheimer's disease brain (Mirza et al., 1997). Tau, a microtubule-associated protein- a component of neurofibrillary tangles, has been shown to be an excellent substrate of TG2 (Dudek and Johnson, 1993), and the co-localization of these two proteins was found in the neurofibrillary tangles of Alzheimer's disease's brain (Appelt et al., 1996). Apart from its cross-linking activity, the involvement of TG2 in apoptosis was also suggested to contribute to the pathological progression of Alzheimer's disease (Lesort et al., 2000).

Huntington's disease is another neurodegenerative disease that has been reported to be relevant to TG2. Huntington's disease is a progressive neurodegenerative motor and psychiatric disorders, with chorea, cognitive decline, incoordination, and behavioural difficulties. Huntington's disease has been identified as an autosomal dominant disease and the presence of the mutated gene product, huntingtin, has been reported, although its function remains unknown (the Huntington's Disease Collaborative Research Group, 1993). Expansion of CAG repeats in huntingtin mutations led to the stretches of polyglutamines of greater than 39 contiguous glutamine residues (Gusella et al., 1993). This increase of polyglutamines, compared to the normal range of 10-25 residues, cannot only serve as an excellent substrate for TG2, but also increases the binding affinity with this enzyme (Gentile et al., 1998; Kahlem et al., 1996; Karpuj et al., 1999). As a distinguishing characteristic of Huntington's disease, aggregates has been found both *in vitro* and *in vivo* samples. Kim *et al.* (2002) also demonstrated that TG2 inhibitors treatment can inhibit the formation of the aggregate and improve the survival rates of the animals carrying the mutant gene (Kim et al., 2002). Other studies also suggested the potential mechanisms that can promote the activation of TG2, including the release of cytokines TNFα, IL-2 and IFN-γ induced by oxidative stress and the production free redicals caused by the damaged mitochondria (Kim et al., 2002). A recent report demonstrated that in striatal cells transfected with wild type huntingtin, as a response to thapsigargin-induced, intracellular over-expressed TG2 showed more activity than in the cells with the mutant huntingtin, which led to the mitochondrial membrane

depolarization and the sensitivity of the cells to thasigargin-induced apoptosis (Ruan et al., 2008).

Although a certain amount of in vitro and in vivo study has been devoted to discovering the role of TG2 in neurodegenerative diseases, there are still questions which remained unaddressed and await further investigation.

### 1.9.6.3 TG2 in fibrosis

Fibrosis and scarring is the result of chronic progressive wounding healing resulting from continuous insults, including that of diabetes, infections, hypertension, toxic chemicals, etc. Failure of the termination of the wound response, will eventually lead to the dysfunction and failure of organs. The cross-linking activity of TG2 was a focus in the formation of fibrosis in a number of different organs, including lung (Griffin et al., 1978a), liver (Kuncio et al., 1998) (Piacentini et al., 1999), heart (Small et al., 1999), vasculature (Dolynchuk et al., 1994), and kidney (Johnson et al., 2003; Johnson et al., 1997b; Skill et al., 2001).

The hypothesis that TG2 is involved in the renal scarring is based on the facts: (1) tubular cells can release TG2 into the extracellular environment with high $Ca^{2+}$, which can activate the transamidating activity of TG2 to promote matrix cross-linking (Johnson et al., 1997a); (2) TG2 can cross-link the LTBP-1 to ECM proteins to regulate the matrix storage and activation of TGFβ1 (Nunes et al., 1997; Verderio et al., 1999). Griffin and colleagues (Johnson et al., 2003) confirmed the importance of the enzymatic activity of this enzyme and its expression in renal scarring by using biopsy samples from patients at different stages of renal scarring process (Johnson et al., 2003). The cross-linking of cytoplasmic proteins by intracellular TG2 has also been suggested to result in cell death in the tubular compartment (Johnson et al., 1997a). Recent studies by using TG2 null mice demonstrated that less fibrillar collagen was found in the KO animals and the decrease of collagen I at both the mRNA and protein levels was thought due to the decreased TG2 mediated TGFβ1 activation (Shweke et al., 2008).

In mice liver injury and fibrosis models induced by $CCl_4$ treatment, Mirza *et al*. reported the increase of TG2 at both the gene level and in enzymatic activity, which might be regulated by the increased binding to NF-κB to the TG2 promoter (Mirza et al., 1997). Studies performed later investigated the role of TG2 in liver fibrosis in different liver fibrogenesis models. In the TG2 null animal treated with $CCl_4$, a progressive accumulation of ECM and a derangement of the hepatic lobular architecture were discovered, which led to animals being more sensitive to the treatment resulting in an increased death rate in the KO animals compared to the wild type control animal. This study indicated that TG2 can act in a protective role in liver fibrosis (Nardacci et al., 2003). Schnabel and colleagues discovered the upregulation of TG2 in both mRNA and protein levels during the transdiffrentiation of hepatic stellate cells to collagen producing myofibroblasts, a principle process of liver fibrogenesis (Schnabel et al., 2004). The similar increased level of TG2 was found in the liver samples from HCV infected patients (Nardacci et al., 2003). In ethanol-induced hepatic injury study, it was reported that inhibition of TG2 activity by pinocembrin can protect the liver from thioacetamide (TAA)-induced cirrhosis, which is regulated by NF-κB-dependent TG2 activation induced by ethanol treatment (Chen et al., 2008). Increased cross-linking activity of TG2 was also found in atherosclerotic plaques, hence it can stabilize collagen III in those areas (Bowness et al., 1989). Later studies by Small and colleagues demonstrated that enhanced cardiac TG2 in the transgenic mouse can induce cardiomyopathy (Small et al., 1999). Even though there are still conflicting ideas about the role of TG2 in the processes of fibrosis, the involvement of this enzyme has been widely proven and it could be a potential target for fibrosis treatment.

#### 1.9.6.4 TG2 in cancer

Accumulating studies have focused on the relevance of TG2 in cancer progression. A huge amount of work based on human samples identified that that TG2 is one of the selectively amplified proteins in metastatic human lung carcinoma (Jiang et al., 2003), while the TG2 gene was also revealed as one of the most differentially expressed genes in pancreatic tumours (Iacobuzio-

Donahue et al., 2003). Further work on the locations of TG2 has suggested different roles for TG2 in tumour behaviour while more recent research has been devoted to discovering the intracellular molecules that are involved in TG2-related tumour behaviour.

Intracellular TG2 was reported as an anti-apoptotic effector in different cell types, including breast cancer, pancreatic cancer and melanoma cells (Mann et al., 2006). In their work, Mann and colleagues discovered the activation of TG2 by the calcium ionophore A23187 can promote the activity of NF-κB, while inhibition of the enzymatic activity of TG2 by A23187 and siRNA treatment blocked the activation of this protein. The potential mechanism was suggested that TG2 modifies the IκBα (a good in vitro substrate to TG2) into high molecular weight polymers and modulates the affinity of IκBα for p65/p50, so that this enzyme can maintain the constitutive activation of NF-κB (Mann et al., 2006). The similar anti-apoptotic effect of TG2 in regulating the activity of NF-κB was also suggested in neuroblastoma cells (Condello et al., 2008). The opposite effect of TG2 was reported in highly metastatic malignant melanoma cells, suggesting that TG2 can inhibit tumour progression via its direct interaction with its binding partner GPR56 (Xu et al., 2006).

High affinity of cell surface TG2 with β1 and β3 integrin receptors in fibroblasts and monocytes (Akimov and Belkin, 2001b), while in human breast cancer cell, the direct interaction of TG2 and β1, β4 and β5 integrins was observed, which was suggested to regulate the cancer adhesion, invasion and migration (Mangala et al., 2007). In glioma and fibrosarcoma cells, overexpression of membrane type 1-metalloproteinase (MT1-MMP) can increase the degradation of cell surface TG2, which led to the inhibition of cell adhesion mediated by its interaction with integrin receptors and in turn promoted the cell migration process on FN (Belkin et al., 2001). A novel role of ECM TG2 in RGD-independent cell adhesion process has been reported, which can rescue the cell from anoikis caused by loss of integrin-mediated cell adhesion (Verderio et al., 2003). The involvement of matrix TG2 in regulating tumour cell behaviour was further studied by Griffin and colleagues (Jones et al., 2006). Reduced tumour growth and increased

survival rate was observed following intratumour injection of TG2 in animals and this effect of TG2 was relevant to its enzymatic activity, which can cross-link matrix proteins and protect them from protease digestion and thereby produce an effective barrier to prevent the tumour growth and metastasis (Jones et al., 2006; Kotsakis and Griffin, 2007). In a different study, the similar increased deposition of stroma TG2 in human breast cancer samples was reported and it was suggested as a natural host defence mechanism (Grigoriev et al., 2001; Hettasch et al., 1996; Kotsakis and Griffin, 2007; Mangala et al., 2007).

In order to investigate the mechanism of TG2 in regulating tumour cell behaviour, different intracellular signalling molecules were studied. The activation of focal adhesion kinase (FAK) and its downstream signal pathway phosphoinositide 3-kinases (PI3K)/ Akt was reported to be involved in TG2-mediated cell-ECM interactions (Herman et al., 2006; Verma et al., 2006). In pancreatic cancer cells, PKCδ/ TG2 signalling was demonstrated in inhibiting autophagy in those cells (Ozpolat et al., 2007). Even though more and more studies have been trying to demonstrate the relationship between TG2 and cancer, the effect of this enzyme on tumour behaviours still remains unknown and conflicting. The role of TG2 in cancer biology might be due the types, locations and progressions of the cancers, as well as the presence of TG2 (Mehta, 2009).

### 1.9.7 Fibronectin and its relevant signalling pathways in cell adhesion process

#### 1.9.7.1 Fibronectin

The extracellular matrix (ECM) is crucial for adhesion-dependent cells to adhere, migrate, differentiate, proliferate and exert their physiological functions. As one of the most important extracellular matrix proteins, fibronectin (FN) and its relevant signal transduction pathways have been widely studied. FN is a high-molecular-mass adhesive glycoprotein present in the pericellular matrix and body fluids. FN could exist as both soluble (in blood, amniotic fluid, joint fluid, and cerebrospinal fluid (Mosher, 1984b) or insoluble (in the ECM or basement

membranes) forms in body. There are two types of sources of FN: (1) hepatocytes, the source of plasma FN found mainly in blood; (2) fibroblasts and other cells are the source of cellular FN (Hynes, 1990). The importance of FN in maintaining the cell morphology, adhesion, migration, proliferation, differentiation has been very-well studied (Kaspar et al., 2006).

The molecular structure of FN has been well-documented, as shown in Figure-1.2. The FN subunits, line into dimers and polymers joined by disulfide bonds in physiological conditions, consist of highly structural domains separated by flexible polypeptide segments. Each subunit of FN is composed of three different components, named type I, II and III modules. These modules compose various functional domains, and from the N-terminal, there are heparin and fibrin-binding domain (30kDa), collagen-binding domain (40kDa), fibrin-binding domain (20kDa), cell-binding domain (57kDa), heparin-binding site (35kDa) and fibrin-binding site (30kDa). The domains are relatively resistant to proteases and contain the binding sites for macromolecules such as collagen, fibrinogen, fibrin, and proteoglycans, as well as cells (cell surface receptors, e. g. integrins and syndecans) (Fesus et al., 1986; Hynes, 1990). Among the domains within FN molecules, there are several sites that are closely relevant to cells and their surface receptors. The amino acid sequence Arg-Gly-Asp (Wollenberg et al., 2002), a widely occurring cell adhesive motif originally discovered in fibronectin (Pierschbacher and Ruoslahti, 1984), is located in the domain of III9-10. The RGD motif in fibronectin and other cell adhesion proteins is the most important recognition site for about half of all known integrins, such as α3β1, α5β1, α8β1, αvβ1, αvβ3 and αvβ6 integrins (Johansson et al., 1997; Leiss et al., 2008). This cell adhesion process can be inhibited by a competitive synthetic peptide— RGD peptide leading to apoptosis (named anoikis) (Buckley, et al., 1999; Hadden and Henke, 2000). Another amino acid sequence PHSRN (Pro-His-Ser-Arg-Asn), which acts in synergy with the RGD site, has been located in III3 within the FN molecule. Studies using synthetic peptides containing the RGD motif, which induces apoptosis in many cell types, demonstrated the importance of the RGD


**Figure 1.2. Domain model of fibronectin.**
The fibronectin protein is a dimer of two subunits which are identical except for the inclusion of the V-segment in one of the chains and are covalently connected close to the C-termini by two disulfide bridges. Three types of homologous structural units called I, II, and III make up most of the protein (represented by small ovals, triangles, and squares, respectively). Integrin binding sites are indicated in one of the subunits by arrows. The fibronectin found in blood plasma is a major form of the protein, but other splice variants are present at other locations and at specific developmental stages. The alternatively spliced exons (IIIA, IIIB, and V) are marked in green and may be present in one or both of the subunits. The known variants of the splicing reactions are illustrated (Johansson, et al., 1997).

**Figure 1.2**

cell-binding domain in cell mediated adhesion, by acting as competitive inhibitors of FN-integrin interaction and activators of caspase 3 (Buckley et al., 1999; Hadden and Henke, 2000). Of the integrins α5β1is probably the major cell surface integrin that interacts with the RGD cell binding site on FN, initiating the cell adhesion process by association with the PHSRN binding site (Ochsenhirt et al., 2006). The binding of αvβ3 integrin to the RGD domain can also support cell adhesion on FN (Danen and Sonnenberg, 2003). The domains located in $III_{12\text{-}14}$ of FN are the heparin-binding site, which bind the heparan sulfate chains of other cell surface receptors- heparan sulfate proteoglycans (HSPGs) (Woods et al., 2000). Recently a novel cell binding site, which mediated RGD-independent cell adhesion via interacting with α4β1/α4β7 integrin, was located in the alternatively spliced V region of FN (Sechler et al., 2000).

It is well-known that many cells produce, secrete and deposit FN into the ECM, known as cell-mediated FN deposition, which is dependent on the presence of cell surface α5β1 integrins and RGD cell binding domain within FN molecules (Fogerty et al., 1990). Initiated by the interaction between soluble FN and cell surface α5β1 integrin, the FN fibril formation process is composed of three steps, through which integrins play an important role in the initiating stage of FN fibril formation, which is acting as the link between the intracellular actin cytoskeleton network and the extracellular soluble FN dimers. The binding of FN dimers with integrins promotes the clustering of these receptors and their interaction with the actin cytoskeleton (Wu et al., 1995). The stretched FN molecules and further interaction between each other are mediated by the relocation of FN-bound α5β1 integrin from focal adhesion to fibrillar adhesion along the actin stress fibres (Ohashi et al., 2002). Other cell surface receptors, including syndecan-2 and syndecan-4, and their downstream signalling molecules (RhoA and PKC) have also been suggested in participating in the above process (Lin et al., 2002; Xian et al., 2010; Zhong et al., 1998). In the extension stage, with the exposure of the binding sites within soluble FN molecules, soluble FN in a compact conformation further interacts with each other and forms the insoluble fibrils until the irreversible formation of a FN fibril network occurs. The further stretching and cell movements contribute to detach the FN fibrils from the cell

surface. Meanwhile other ECM components also participate in regulating FN deposition. It has been reported that in FN null fibroblasts, cells seeded on lamin can still lay down exogenous FN, while vitronectin inhibited the deposition of FN matrix, which could be caused by the relevant signaling pathways mediated by different ECM proteins (Mao and Schwarzbauer, 2005).

### 1.9.7.2 Cell surface receptors in cell adhesion on FN

Among the cell surface receptors interacting with FN, integrins and its co-receptor syndecans deserve a special mention. Integrins are type I transmembrane glycoproteins that mediate the adhesion of cells to the ECM and to other cells (cell-cell adhesion) and play important roles in different aspects of various biological processes, including tissue remodelling, leukocyte migration, organogenesis (Howe et al., 1998). The integrins are non-covalent heterodimers composed of α (120-180kD) and β (90-110kD) subunits. To date, 18 α and 8 β subunits have been described and these combine to form over 20 different receptors (Wegener and Campbell, 2008). These receptors recognize their extracellular ligands, e.g. FN, vitronectin, collagen, with varied specificity (Jokinen et al., 2004).

The syndecans are a four-member family (syndecan1-4) of transmembrane cell surface proteoglycans (PGs) that bear heparan sulfate glycosaminoglycan (GAG) chains. The syndecan family of transmembrane PGs is the major source of cell surface heparan sulfate (HS). It is divided into 2 subfamilies Syndecan-1 and -3, and Syndecan-2 and 4, according to their similarity in core protein size and sequence (Yamagata et al., 1993). Syndecans are characterized by highly conserved transmembrane and cytoplasmic domains. The intracellular part of the syndecans is composed of two constant regions (C1 and C2) separated by a variable region (V), which can be phosphorylated and bind a variety of intracellular ligands. The extracellular domains of the syndecans contain a site close to the plasma membrane (juxtamembrane domain, JMD) and a cell binding domain (CBD). At the distal end of the ectodomains are the GAG-attachment sites, to which the heparan sulfate chains are attached (Elenius et al., 2004). The syndecans are expressed on virtually all cell types throughout development and

adulthood, and their expression can be altered under certain pathophysiological conditions, including the process of tumour onset, progression and metastasis (Sanderson et al., 2004). The heparan sulphate chains bind to "heparin" – binding sites present in matrix ligands, as well as numerous "heparin"– binding growth factors and morphogens, including fibronectin, vitronectin, laminins and the fibrillar collagens (Bernfield et al., 1999). Among the syndecan family, syndecan-4 is widely expressed, in contrast to the other three syndecans, which are thought to exhibit a more tissue-specific distribution (Kim et al., 1994).

### 1.9.7.3 FN-involved signalling pathway(s)

There are two categories of integrin signalling that are widely recognized, namely “outside-in” and “inside-out” signalling pathways (Figure 1.3). The study of lymphocytes indicated the existence of integrin inside-out signalling pathway, which led to un-stimulated non-adherent lymphocytes adhering to other cells or the matrix within seconds in response to outside stimuli (Kinashi, 2005). The inside-out signalling pathway ultimately increases the affinity of the receptors for their matrix ligand, which leads to a change in conformation of the integrin extracellular domain (known as affinity regulation) or the extent to which integrins diffuse and cluster on the cell surface (Calderwood, 2004). Integrins are also subject to "outside-in" signalling in which the ligand-bound receptor initiates intracellular signalling through association of the β subunit cytoplasmic domain with a myriad of intracellular effectors including focal adhesion and Src family kinases (Datta, *et al.,* 2001), and cytoskeletal components, such as α-actin (Brakebusch and Fassler, 2003). Talin has been reported as a crucial protein in integrin activation, especially to β1 and 3 integrins (Tadokoro et al., 2003). The integrin ligation to ECM components initiates the signal transduction from ECM to cells, which regulates many fundamental cellular processes, including cell migration, proliferation, and differentiation (Schwartz et al., 1995). Accumulating evidence indicates that the Ras/ERK1/2 pathway is one potential intracellular signalling pathway involved in the integrin-related cell adhesion processes. The activation of focal adhesion kinase (FAK) by interaction between integrins and ECM proteins triggers the ERK1/2 signal transduction. In


**Figure 1.3 Integrin signalling mechanisms.**
The "inside-out" signalling is initiated by intracellular events, which might involve cytoskeleton reorganization mediated by Rho or the phosphorylation of integrin cytoplamic domain or its associated proteins. The "outside-in" signalling is triggered by the binding between integrin and its extracellular ligands and RhoA-mediated integrin clustering, followed by the activation of intracellular signalling pathways, in which FAK and MAP kinase are engaged (Jones and Walker, 1999).

**Figure 1.3**

fibroblasts, the binding of integrin with its ligands enhances the activation and autophosphorylation of FAK at $Tyr^{397}$, which activates the Ras and leads to the translocation of Raf-1 to the cell membrane. Raf-1 phosphorylates and activates MEK (MAP kinase), which subsequently phosphorylates ERK1/2 (Yee et al., 2008). Recent evidence also supports the involvement of the Rho family of small GTPases. Ligation of integrins activates Rho, and the activation of Rho stimulates the phosphorylation of FAK and regulates the actin cytoskeleton dynamics and adhesive behaviour (Midwood et al., 2006).

Although integrins are the main cell surface receptors responsible for cell-ECM interactions, in some cases, integrin engagement is not sufficient for a complete adhesion signalling process (Bloom et al., 1999; Woods and Couchman, 2001). Syndecan-4 can bind the HepII heparin-binding domain of FN with high affinity, which leads to the formation of focal adhesions necessary for cell adhesion (Woods et al., 2000). The binding of syndecan-4 to the heparin binding domain within FN provides a second signal that is required for formation of stress fibres and maturation of focal adhesions (Couchman and Woods, 1999).

The studies from fibroblasts indicated that cells attach and spread on the central cell-binding domain of FN via integrin $\alpha_5\beta_1$ but fail to form vinculin-containing focal adhesions unless co-stimulated with the heparin-binding fragment of FN (Bloom et al., 1999). The intracellular syndecan-4 signalling pathway is still under further investigation. But accumulating evidence indicates the involvement of phosphatidylinositol 4, 5-bisphosphate (PtdIns (4, 5) P2) and protein kinase C α (PKCα) in this signal transduction process in cell adhesion and spreading process (Oh, *et al*., 1997a, b, and 1998). Interaction with PtdIns (4, 5) P2 promotes the oligomerization of syndecan-4 cytoplasmic domains (Oh et al., 1997a; Oh et al., 1998) (Figure 1.4), while the oligomerized cytoplasmic domains of syndecan-4 can bind PKCa directly, through the PKCα catalytic domain (Oh et al., 1997b). This results in direct activation of PKCα in the absence of other mediators and superactivation in their presence, which is


**Figure 1.4 A schematic structure of a syndecan molecule.**
The domains of syndecan core protein include the extracellular domain, transmembrane domain, conserved regions C1 and 2, and the variable region V, which are coloured in purple, blue, orange, green and pink, respectively. The extracellular heparan sulfate chains mediate the interaction between extracellular regulating factors, such as the ECM proteins and growth factors; the transmembrane domain is in charge of the self-association process of syndecans; C1 region interacts with the molecules related to actin skeleton organization; the C2 region can interact with the PDZ domain containing proteins such as syntenin and CASK; the structure of the V regions varies between different members of the syndecan family (from Couchman, 2003).

**Figure 1.4**


**Figure 1.5 The function of syndecan-4 on regulating α5β1-related signalling pathway.**
Syndecan-4 functions as a co-receptor for β1 integrin signalling pathway via its interaction with the heparin-binding sites of FN, which triggers the activation of PKCα, a intracellular signalling molecule. Meanwhile the intracellular domain of syndecan-4 core protein can also bind to other signalling molecules including CASK and FAK, as well as actin binding proteins, such as α-actinin to regulate the actin skeleton formation (from Beauvais and Rapraeger, 2004)

**Figure 1.5**

necessary for the cell spreading and focal adhesion formation (De Nichilo and Yamada, 1996; Woods and Couchman, 1992). The cytoplasmic tail also interacts with other structural and signalling proteins including CASK, FAK, paxillin and syndesmos (Beauvais and Rapraeger, 2004). It was also shown that focal adhesion assembly through syndecan-4 clustering is sensitive to inhibition of RhoA by C3 transferase (Saoncella et al., 1999), which indicated a requirement for RhoA signalling downstream of syndecan-4. A recent study demonstrated the existence of the linear pathway involving syndecan-4, PKCα and RhoA for the formation and maintenance of stress fibres in primary rat embryo fibroblasts (Dovas et al., 2006). Figure 1.5 shows the possible signalling transduction pathway mediated by syndecan-4.

### 1.9.7.4 FN and TG2

FN is a well-known substrate for TG2, and the cross-linking action of FN catalyzed by TG2 offers protection to FN from degradation (Kinsella and Wight, 1990). It has also been reported that FN and TG2 secreted into the ECM can interact with each other independently from the enzyme's cross-linking activity (Verderio, *et al.,* 2003). It has been shown that TG2 secreted to the ECM, could bind to the N-terminal portion of FN interacting either with the Type $I_4$-$I_5$ motif (LeMosy et al., 1992) or with a sequence within the gelatin-binding domain of FN ($I_6$-$II_1$-$II_2$-$I_7$-$I_8$-$I_9$) (Lorand et al., 1993).

#### The role of TG2 in FN-mediated cell adhesion

Several studies of TG2, FN structure and the integrin signalling pathway have demonstrated a novel role for TG2 in the cell adhesion process. The structural study for FN showed that the TG2 binding site (42kD gelatin-binding domain) (Lorand et al., 1993) and the integrin binding site (RGD cell-binding site) are located in different parts of the FN molecule, which provides the possibility that TG2 and integrin collaborate rather than compete with each other in the cell adhesion process. Moreover, the binding of TG2 with FN activated FAK and increased the GTP loading of the small GTPase RhoA, which may trigger the


**Figure 1.6 The role of TG2 as an integrin co-receptor for FN.**
Cell surface TG2 binds to the 42kD geletin binding sites of FN, which bridges the cell surface integrins with the matrix FN. This function of TG2 can enhance the cell adhesion via promoting the integrin clustering (from Akimov, et al., 2000).

**Figure 1.6**

intracellular signalling pathway and induce its relevant cell adhesion (Janiak et al., 2006). The studies from different groups indicated that TG2 associates with integrin receptors in a number of different cell types via binding to the extracellular domains of the β1, β3 and β5 integrin subunits (Akimov and Belkin, 2001b; Akimov et al., 2000a; Gaudry et al., 1999b). Unlike FN, integrins do not appear to serve as enzymatic substrates of TG2 or other transglutaminases and the formation of stable non-covalent integrin-TG2 complexes is independent of the transamidating activity of TG2 (Akimov and Belkin, 2001b; Akimov et al., 2000a) (Figure 1.6). Although the binding sites within integrins for TG2 are still unknown, it has been demonstrated that integrin-TG2 complexes have a 1:1 stoichiometry and all the TG2 on the cell surface is bound to integrin receptors in a FN-independent way (Akimov and Belkin, 2001b; Akimov et al., 2000a). Besides the structural interaction evidence, a series of studies led by Mehta and colleagues for tumour cells demonstrated the connection between TG2 and the integrin-relevant downstream signalling molecule-FAK. The culture of TG2-positive breast cancer cells and TG2-transfected fibroblasts on fibronectin-coated surfaces led to the activation of FAK, the downstream signalling molecule of integrins (Mehta et al., 2006b). Conversely, down-regulation of TG2 by small interfering RNA attenuated FN-mediated cell attachment (Herman et al., 2006) and cell survival and FAK phosphorylation (Verma et al., 2006).

As introduced above, TG2 also exists in the ECM as a kind of structural protein (Verderio et al., 1998a). The importance of TG2, as an ECM protein, in RGD-independent cell adhesion was first introduced by Griffin *et al.* (Verderio et al., 2003). The TG2 bound FN matrix compensated the apoptosis (anoikis) induced by RGD synthetic peptides. This process was PKCα and FAK-dependent. A recent study showed that GTP-bound TG2, when present in the ECM, can induce hypertrophic differentiation of chondrocytes through a α5β1 integrin-dependent and FAK-associated cell adhesion processes (Tanaka et al., 2007). The importance and role of the TG-FN mediated and RGD-independent cell adhesion process forms the main topic of this report.

## 1.10 Project Aims

Previous work suggested that TG2 and FN when present in a hetero-complex—TG-FN can compensate the Arg-Gly-Asp (RGD) peptide induced loss of cell adhesion (which is due to the blocking effect of the peptide on the interaction between cell surface integrin receptors and FN). The actual mechanism behind this phenomenon and its physiological influence on cell behaviour remains unknown. The first aim of this project is to determine the involvement of the cell surface receptor(s), including syndecan-4, syndecan-2, α5 integrin, β1 integrin, α4β1 integrin and β3 integrin, in mediating the RGD-independent cell adhesion mediated by TG-FN. The second aim of this project is to further investigate the intracellular signalling pathway(s) that is required by TG-FN to compensate the loss of cell adhesion induced by the RGD peptides following binding of the TG-FN complex to its receptor(s). A further part of this project was to study the role of TG-FN mediated cell adhesion in regulating FN fibril formation and the deposition of FN with emphasis on the role(s) of the different cell surface receptor(s) and their relevant intracellular signalling molecule(s) in the FN deposition process.

# Chapter 2:
# Materials and Methods

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## 2.1 Materials

### 2.2.1 List of Antibodies

| Primary Antibodies | | | |
|---|---|---|---|
| **Company** | **Antigen Name** | **Host Species** | **Clone** |
| Neomarks, Pierce, UK | TG2 | Mouse | Monoclonal |
| Santa Cruz, UK | β1 integrin | Rabbit | Polyclonal |
| Biolegend, UK | β1 integrin | Armenian hamster | Monoclonal |
| Biolegend, UK | Isotype control IgG | Armenian hamster | Monoclonal |
| Cell signalling, USA | β3 integrin | Rabbit | Polyclonal |
| Biolegend, UK | β4 integrin | Armenian hamster | Monoclonal |
| Zymed Invitrogen, UK | Syndecan-2 | Rabbit | Polyclonal |
| Zymed Invitrogen, UK | Syndecan-4 | Rabbit | Polyclonal |
| Upstate Cell signalling | p-Tyr397 FAK | Rabbit | Polyclonal |
| Biosource UK | p-Tyr861 FAK | Rabbit | Polyclonal |
| Santa Cruz, UK | FAK | Mouse | Monoclonal |
| Santa Cruz, UK | PKCα | Mouse | Monoclonal |
| Sigma-Aldrich, UK | α-Tubulin | Mouse | Monoclonal |
| Sigma-Aldrich, UK | Vinculin | Mouse | Monoclonal |
| Sigma-Aldrich, UK | Fibronectin | Rabbit | Polyclonal |
| **Secondary Antibodies** | | | |
| Sigma-Aldrich, UK | Goat anti-mouse HRP conjugated | | |
| Dako, Denmark | Swine anti-rabbit HRP conjugated | | |
| Dako, Denmark | Rabbit anti-mouse FITC conjugated | | |

### 2.2.2 Chemicals

The deionised water ($dH_2O$) used in experiments was obtained form an Eligastat system 2 water purifier or Milli Q water purifier (Millipore/Water, Watford, UK).

The general chemical reagents and cell culture media were purchased from Sigma-Aldrich Company (Poole, Dorset, UK), unless otherwise stated. Other Chemicals and reagents were obtained from the following supplier.

**Zedira, Darmstadt, Germany**

Purified guinea pig liver transglutaminase (gplTG) and biotin-X-cadaverine

**Amersham Parmacia Biotech, UK**

The full range rainbow molecular markers and ECL Chemilunimescence development kit

**Bachem, Merseyside, UK**

The synthetic H-Gly-Arg-Gly-Asp-Thr-Pro-OH (GRGDTP) and H-Gly-Arg-Ala-Asp-Ser-Pro-HO (GRADSP) peptides

**Bio-Rad, Hemel Hempstead, UK**

Bio-Rad protein assay kit

**Calbiochem, Nottingham, UK**

The synthetic The synthetic H-Gly-Arg-Gly-Asp-Thr-Pro-OH (GRGDTP) and H-Gly-Arg-Ala-Asp-Ser-Pro-HO (GRADSP) peptides, PKCα inhibitor Go6976, ERK inhibitor PD98059, Genecticin (G418 sulphate), ROCK inhibitor Y27632, human plasma fibronectin, γ-interferon.

**Vector Laboratories, UK**

Vectashield mounting medium

**Pierce, UK**

Sulfo-NHS-LC-Biotin and resin beads

**Peptide Protein Research, UK**

The GK21 peptide (GENPIYKSAVTTVVNPIYEGK) and the scrambled control peptide (GTAKINEPYSVTVPYGEKNKV) in tandem with the antennapedia third helix sequence (PQIKIWFQNRRMKWKK) and the A5-1 peptide (VILVLF)

**Jackson Immuno Research**

Cy™5-conjugated streptavidin

**Roche, UK**

Sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium-bis (4-methoxy-6-nitor) benzeme sulfonic acid hydrate reagents (XTT reagents)

**Qiagen**

Syndecan-2 targetting siRNAs and HiPerfect Transfection Reagent

**InvivoGen**

Zeocin

**GE Healthcare**

A and G-Sepharose bead slurry

### 2.1.3 Materials

**Amersham Pharmacia Biotech, UK Ltd, Little Calfont, UK**

Electrode paper (grade 1F)

**BDH laboratories suppliers, Milton Keynes, UK**

Coverslips, microscope slides

**Improved Neubauer, Calton, UK**

Haemocytometer

**Sarstedt Ltd., Leicester, UK**

0.5-,1.5- and 2- ml microcentrifuge tubes, 5ml scintillaion vial inserts and tops, 15- and 50- ml sterile centrifuge tubes, 5-, 10- and 25- ml sterile pipettes, pipette tips, and disposable filters (0.2μm pore size)

**Lonza**

Mouse Embryonic Fibroblast nucleofector solution

### 2.1.4 Equipments

**AMAXA Biosystem**

AMAXA nucleofector and AMAXA certified cuvette and pipetts

**Beckman Instrument (UK) Ltd, High Wycombe, UK**

Spectrophotometer Model DU-7, centrifuges Avanti J-30 I, MSE Centaur 2

**Bio-Rad, Hemel Hempstead, UK**

Protein III MInigel Vertical Electrophorisis Apparatus and Western Blot wet transfer system

**Corning, Staffs, UK**

pH meter

**Sanyo, UK**

$CO_2$ incubator MoDel IG150

**Nikon Inc, Badhoevedorp, The Netherlands**

Inverted Phase Microscope model TM-100 and Nikon 500 digital camera

**Carl Zeiss MicroImaging GmbH, Germany**

Zeiss Meta 510 laser confocal microscope

## 2.2 Methods

### 2.2.1 Methods in cell culture

#### 2.2.1.1 Cells and culture conditions

The cells used in this project included Swiss Albino mouse embryo 3T3 fibroblast, primary human osteoblasts (HOB), β1 integrin knock out (ko), add-back, vector control mouse embryo fibroblasts (MEF), syndecan-4 wild type (wt), ko, add-back and vector control MEF cells, wild type and heparan sulphate mutant Chinese hamster ovary cells (CHO), α5 integrin knockout (EA5) and add-back MEF cells (EA5/α5), β3 ko and wt MEF cells. The Swiss-3T3 fibroblasts, HOB, CHO cells, EA5 and EA5/α5, β3 knockout and wt MEF cells were cultured in a humidified atmosphere at 37ºC 5% (v/v) $CO_2$, 95% (v/v) air, while the β1 integrin and syndecan-4 serial MEF cells were kept in a humidified atmosphere at 32ºC 5% (v/v) $CO_2$, 95% (v/v) air.

Swiss 3T3 albino fibroblasts were obtained from American Type Culture Collection (ATCC, USA) and cultured in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2mM L-glutamine, 1% (v/v) nonessential amino acids and penicillin/streptomycin (100U/ml and 100μg/ml, respectively) and used for experiments between passages 10-30.

The β1 integrin and syndecan-4 serial MEF cells were a kind gift by Professor Martin Humphries (University of Manchester, UK) and maintained in complete DMEM medium supplemented with 20U/ml interferon-γ (IFN-γ). The cells above were used between the passages 15-30.

The wild type and heparan sulphate mutant CHO cells were obtained from American Type Culture Collection (ATCC, USA) and cultured in Ham's F12 medium containing 10% fetal bovine serum (FBS), 2mM L-glutamine, 1% (v/v) nonessential amino acids and penicillin/streptomycin (100U/ml and 100μg/ml, respectively) and used for experiments between passage 7-15.

The EA5, EA5/α5, β3 ko and wt MEF cells were kind gift from Professor Eric Danen (Leiden University, Netherland) and cultured as Swiss 3T3 cells.

### 2.2.1.2 Cell passaging

When reaching approximately 90% confluency for immortalised cell lines and 70% confluency for primary cell lines, cells were passaged. The cell monolayer was rinsed once with phophate buffered saline (PBS) pH 7.4 prior to treatment with 0.25% (w/v) trypsin, 5mM EDTA solution in PBS, pH 7.4 at 37 or 32ºC. The detached cells were collected in complete medium or medium with a trypsin inhibitor to inactivate the trypsin and centrifuged at 300×g for 5 min. Cells were resuspended in the growth medium and seeded in tissue culture flasks to the desired confluency.

### 2.2.1.3 Determination of cell number

Cells were trypsinised and centrifuged as described above (Section 2.2.1.2) and resuspended in relevant culture medium. 10μl aliquots of the cell suspension were applied to a haemocytometer. The cells were counted in 4 separate fields using phase contrast microscopy on an inverted microscope. The number obtained from the count was multiplied by $10^4$ to determine the cell number per ml of cell suspension.

### 2.2.1.4 Cell freezing

Cells were trypsinized and counted as described in Section 2.2.1.2 and 2.2.1.3, and resuspended in 10% (v/v) cell culture grade dimethyl sulfoxide (DMSO) in heat inactivated FBS. Cells were then pipetted in 1ml aliquots into cryogenic vials and slowly frozen at -80ºC in a specialised cryogenic container for at least 24 h before transferring to a liquid nitrogen container for a long-term storage.

### 2.2.1.5 Cell defrosting from storage

Cryogenic vials of cells stored in liquid nitrogen were removed and quickly placed in a water bath at 37ºC. The cell suspension was carefully transferred into a sterile Falcon tube and 5ml of supplemented growth medium was added drop wise, with mixing after each addition to ensure slow dilution. The diluted cell suspension was then centrifuged at 300×g to eliminate the traces of DMSO present in the freezing mix. Cells were resuspended in the growth medium and transferred to a tissue culture flask. The media was changed the next 12-24 h and cells were passaged at least once before using them in an experiment.

### 2.2.1.6 siRNA transfection

In order to silence the expression of syndecan-2 in MEF cells, 2 different small interfering RNA (siRNA) sequences targeting mouse syndecan-2 were obtained from Qiagen. The sequences are listed below.

Mm_Sdc2_1 SI01412250: TGGAATTTAATTTGTAGAATA
Mm_Sdc2_2 SI01412264: CAAAGGCAAATTAATGTGTAA

The scrambled negative control siRNAs (listed below) were kindly designed by Dr Russell Collighan, according to the syndecan-2 siRNA sequences, and synthesized by Sigma, UK.

Scrambled siRNA1: GTAAATTATGTAAGTTAGTAT

Scrambled siRNAs: GATAATCAAAGCGAAATGTAT

The universal negative control siRNA was purchased from Qiagen. Both scrambled and the universal negative siRNA were used as the control treatments. 1nmol lyophilized siRNA was dissolved in 100µl of sterile, RNase-free water to obtain a 10µM solution and then stored at -20ºC. The transfection was performed according to the manufacture's protocol. Before transfection, $3 \times 10^5$ cells were seeded into each well of a 6-well plate for 24 h to reach 50-80% confluency. Prior to transfection, cells were washed with PBS, pH 7.4 and the medium was replaced with 2.3ml pre-warmed fresh complete growth medium. 150ng of siRNAs were supplemented with DMEM serum free medium siRNAs to reach the final volume of 100µl of siRNA working solution. The siRNA and HiPerfect Transfection Reagent (Qiagen complex were prepared as directed by the manufacturer by incubating the mixture at room temperature for 5-10 min. The complex was added drop-wise onto the cells with gentle swirling the plate to ensure the uniform distribution of the transfection complex. Following an approximated 30 h siRNA transfection, cells were used in Western blotting or cell adhesion assay.

### 2.2.1.7 Stable cell transfection

#### 2.2.1.7.1 Kill Curves

3,000 cells/well of MEF cells were seeded into 96-well plates and treated with different concentrations of zeocin (0, 100, 200, 400, 600, 800, 1000, 1200 µg/ml, prepectively). After 24, 48 and 72 h incubation with the antibiotic, 30µl of sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitor) benzeme sulfonic acid hydrate (XTT) reagents mixture was added into each well and incubated with the cells for 4 h. The absorbance was read at 490nm and 750nm using a Spectrafluor plate reader. Results were expressed as absorbance at (490nm-750nm). The inhibition of the antibiotic was calculated as below:

$$\text{Inhibition rate \%} = \left(\frac{\text{A in control group} - \text{A in experimental groups}}{\text{A in control group}}\right) \times 100\%$$

### 2.2.1.7.2 Nucleofections using AMAXA's proprietary technology

Transfection of MEF cells with pSV40/Zeo2 vector containing wild type TG2 cDNA was achieved by transfecting cells with 5µg of TG2 containing vector plasmid using the AMAXA nucleofector (Lonza Biosystems) according to the manufacturer's protocol. Cells ($10^6$) were seeded in T150 tissue culture flasks and allowed to reach 90% confluence just prior to the DNA transfer. Cells were trypsinised, pelleted and resuspended in 100µl of Mouse Embryonic Fibroblast nucleofector solution at $10^6$ cells/ transfection. The resulting cell suspension was then mixed with 5µg of relevant vectors, and transferred to an AMAXA certified cuvette. Nuclear transfer of DNA was achieved by placing the cuvette into the AMAXA nucleofector system and exposing the cells to short electric pulses of different voltage/duration under different pre-set programmes. Cells were then supplemented with 500µl of pre-warmed cell growth medium and transferred to 100mm Petri dishes also containing 8ml pre-warmed medium.

### 2.2.1.7.3 Selection of stably transfected cell lines

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

### 2.2.2 Preparation of a physiological extracellular matrix

#### 2.2.2.1 Coating of plates with fibronectin

The wells of a microtitre plate were coated with 5µg/ml FN diluted in 50mM Tris-HCl, pH 7.4 and 50µl/well used to coat the plate for 16 h at 4ºC.

#### 2.2.2.2 Immobilisation of tissue transglutaminase on FN matrix

For immobilisation of guinea pig liver transglutaminase (gplTG) (Zedira, Germany) on FN, wells were washed once in 50mM Tris-HCl, pH 7.4. In some cases, the wells were blocked with 3% (w/v) bovine serum albumin (BSA) in PBS, pH 7.4 for 30 min at 37ºC prior to TG2 immobilisation. The 20µg/ml gplTG solution was prepared by dissolving the 1mg/ml of gplTG in PBS containing 2mM EDTA, pH 7.4 to inhibit $Ca^{2+}$-dependent activity of the enzyme. The gplTG solution was then added to the tissue culture plastic (TCP) coated with FN for 1 h at 37ºC.

#### 2.2.2.3 Detection of relative levels of TG2 in matrices

**Detection of TG2 antigen by ELISA**

The presence of TG2 antigen on different substrates was detected by using a modified enzyme linked immunosorbent assay (ELISA) (Verderio et al., 1999).

After coating the 96-well plate with 50µl of FN as described above (Section 2.2.2.1), the wells were washed once in 50mM Tris-HCl, pH 7.4 and then blocked with 50µl of 3% BSA in PBS, pH 7.4 for 30 min at room temperature. To block the heparin-binding sites within FN, 50µl of 300µg/ml of heparin in 50mM Tris-HCl, pH 7.4 was incubated with FN at room temperature for 30 min. Then the wells were washed three times in 50mM Tris-HCl, pH 7.4. The gplTG solutions at different concentrations (20µg/ml and 30µg/ml) were prepared by dissolving 1mg/ml of gplTG in PBS containing 2mM EDTA, pH 7.4 in order to

inhibit the TG2 activity. The gplTG solutions were then added to the FN matrix with or without heparin. After a 1 h incubation at 37ºC, the gplTG solutions were removed and wells were washed once with 100µl of Tris-HCl, pH 7.4. Prior to the incubation with anti-TG2 monoclonal antibody Cub 7402 (Neomarkers, UK), wells were blocked with 3% BSA in PBS, pH 7.4 (blocking buffer) for 30 min at room temperature. After a 2 h incubation with Cub7402 (diluted 1: 1000 in blocking buffer) at room temperature, the wells were washed 3 times (final wash for 20 min) with blocking buffer. To detect the antigen-antibody complex, the wells were incubated with peroxidase labelled anti-mouse IgG diluted 1:2000 in blocking buffer for 2 h at room temperature. At the end of incubation, the wells were washed three times with blocking buffer and once with PBS, pH 7.4. The wells were then pre-equilibrated with the developing buffer, phosphate-citrate buffer with urea and hydrogen peroxidise (HRP) (Sigma-Aldrich, UK). Development of the reaction was performed by addition of developing buffer containing the substrate 7.5% (w/v) 3,3',5,5'-Tetramethyl benzidine in DMSO (TMB). The colour development was terminated by addition of 50µl of 2.5M $H_2SO_4$ and the absorbance read at 450nm using a Spectrafluor plate reader. Results were expressed as absorbance at 450nm.

### 2.2.3 Cell adhesion assay

#### 2.2.3.1 Inhibition of the RGD-mediated cell adhesion

Exponentially growing cells were trypsinised and collected into complete medium or serum free medium containing trypsin inhibitors. After a 5 min centrifugation at 300×g, the cell pellet was washed twice in serum free medium to remove the traces of serum proteins. In the experiments, cell suspensions of $2.5\times10^5$ cell/ml were prepared in serum free medium, and incubated with G**RGD**TP and G**RAD**SP synthetic peptides (Calbiochem or Bachem, UK) at concentration of 100µg/ml (~150µM) for 20 min at 37ºC in a 5% $CO_2$, 95% (v/v) air atmosphere. Meanwhile coated wells were washed once with 50mM Tri-HCl, pH 7.4 and then pre-equilibrated with serum free medium for 10 min. Cell suspensions (100µl/well) were seeded on wells coated with FN, with and without

immobilised gplTG and allowed to attach in the presence of the synthetic peptides. The incubation time was limited 15-40 min to minimise the secretion of any endogenous proteins.

### 2.2.3.2 Cell fixation and permeablisation

Following cell adhesion and spreading, the medium was carefully removed and wells were gently washed once with PBS, pH 7.4. Cells were then fixed with 100µl of 3.7% (w/v) paraformaldehyde dissolved in PBS, pH 7.4 for 15 min at room temperature and washed twice with PBS, pH 7.4. Following fixation, cells were permeabilized with 100µl of 0.1% (v/v) Triton-X in PBS, pH 7.4 for 15 min at room temperature. The wells were then washed twice with PBS, pH 7.4.

### 2.2.3.3 May-Grunwald and Giemsa co-staining

To visualise the attached cells, a two-step staining process was employed to stain both cytoplasm and nucleus. Following fixation and permeablisation (Section 2.2.4.2), 100µl of May-Grunwald stain (Sigma-Aldrich, UK) was added to the wells to stain the cell cytoplasm for 15 min at room temperature. After the stain was removed, the wells were washed once with PBS, pH 7.4 and then incubated with 100µl of 5% (v/v) Giemsa stain (Sigma-Aldrich, UK) in $dH_2O$ to stain the nucleus for 20 min at room temperature. The plate was finally washed twice with $dH_2O$ and left to dry.

### 2.2.3.4 Quantification of cell adhesion

Digital images of three non-overlapping fields covering the centre of the well were acquired using a video digital camera (Olympus DP10) at 40× magnification. The cell attachment and spreading were quantified using the Scion image analysis programme (http://www.scioncorp.com), which is developed at the National Institute of Health (Washington DC, USA). The number of cells per image was assessed through threshold and particle analysis setting with a minimum particle size of 50 pixels. Spread cells were

discriminated from non-spread cells by their two-colour appearance (dark purple for nucleus and pink for cytoplasm). These were quantified by density slicing and particle analysis setting. The spreading cell particles were selectively highlighted through the adjustment of the Lut intensity. The number of attached cells per well was calculated by summing up the number of cells from the three images of non-overlapping fields. Cell attachment on FN without RGD peptide was considered as the control value for most of the experiments. The mean number of attached cells form three wells was calculated and that of the control was considered as 100%. The mean number of attached cells (cell attachment) for each sample was then expressed as the percentage of cell attachment on FN. The mean percentage of attached cells that are spread (cell spreading) for each sample was determined separately, and the mean percentage of spread cells on FN control was expressed as 100%. The mean percentage of spread cells for each sample was then normalised against that of FN control. Cell spreading was defined as the process whereby a rounded cell is flattened as a consequence of ECM-mediated signal transduction leading to the rearrangement in the actin cytoskeleton.

### 2.2.4 Fluorescence staining of actin stress fibres

Sub-confluent cells were serum-starved for 16 h and harvested. After different treatments, cells were seeded in 8-well glass chamber slides ($8\times10^4$ cells/well) previously coated with FN and TG-FN and allowed to attach and spread for 40 min. Cells were fixed and permeablized as described previously (Section 2.2.3.2). For staining of actin stress fibres, cells were blocked in PBS buffer supplemented with 3% (w/v) BSA and then incubated with FITC-labelled phalloidin (20μg/ml) in blocking buffer. Coverslips were mounted with Vectashield mounting medium and using constant PMT and section depth settings 9 random fields/ sample were captured by using Zeiss LSM510 laser confocal microscope using Zeiss LSM Image Browser.

### 2.2.5 Preparation of total cell lysates

A 6-well plate was coated with 1.5ml of 5µg/ml human plasma FN or heat-inactivated 3% (w/v) BSA in PBS, pH 7.4 by incubation at 4ºC for overnight. The FN matrix was further coated with 3ml of 20µg/ml gplTG as described previously (Section 2.2.2.2). Cells detached by trypsinization were then plated onto wells at a density of $6\times10^5$ cells/well (Section 2.2.3.1). Following cell adhesion and spreading, the medium was removed. Wells were washed once with ice-cold PBS, pH 7.4 and the adherent cells were lysed by addition of 50µl of lysis buffer (1% (v/v) Nonidet, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1mM benzamidine, 1mM NaF, 1mM $Na_3VO_4$, 0.1mM phenylmethylsulfonyl fluoride (PMSF) and 1% (v/v) protein inhibitor cocktail). The non-adherent cells in the medium were collected and centrifuged at 300×g for 5 min at 4ºC and the cell pellets were washed once with ice-cold PBS, pH7.4 and centrifuged at 300×g for 5 min at 4ºC. The plate was then incubated on ice for 30 min and lysates were collected with a scraper to be mixed with the corresponding cell pellet from non-adherent cells. Cell extracts were clarified by further centrifugation at 300×g for 5 min at 4ºC. The samples were stored at -80ºC until use.

### 2.2.6 Determination of protein concentration by Lowry Method

The protein content of cellular extracts was determined using the commercial kit from Bio-Rad based on the Lowry method (Lowry et al., 1951). The determinations were made following the manufacturers instruction using BSA as the standard. Briefly, 5µl of BSA solution ranging from 0.1-1.5mg/ml, and 1µl of cell extract dissolved in 4µl of $dH_2O$, were added to wells of a microtitre plate in triplicate. The wells were then incubated with 25µl of Reagent A, followed by 200µl of Reagent B for 15 minutes at room temperature. The absorbance values were recorded at 750nm using a SpectraFluor plate reader to produce the calibration graph.

### 2.2.7 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

After protein concentration assay (Section 2.2.6), the protein extracts containing certain amounts of total protein were dissolved using 1:1 ratio in 2× strength reducing-Laemmli buffer (125mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (v/v) SDS, 10% (v/v) 2-β-mercaptoethanol and 0.004% bromophenol blue) (Laemmli et.al. 1970), denatured at 95ºC for 5 min, and then stored at -70ºC until use or resolved by the SDS-PAGE method, which was a modification of that described by Laemmli et al. (1970) by using the Atto-minigel system. Some of the supernatants were and then stored at until required for electrophoresis; rest of the cell lysates were stored at -70ºC for protein content estimation. The gels consisted of a 3% (w/v) polyacrylamide stacking gel and a 10% (w/v) resolving gel. The stacking gel was made using 0.2M Tris 0.2% (w/v) SDS stock solution, pH 6.8 and resolving gel contained 0.75M Tris 0.2% (w/v) SDS stock solution, pH 8.8. The polymerisation reaction was initiated by the addition of the indicated volumes of freshly prepared 5, 8 or 12% ammonium persulphate and N,N,N',N'-Tetramethylethylenediamine (TEMED). The stacking and resolving polyacrylamide gels were prepared according to the table below. Resolving gels (80×60×0.75) were cast using the Atto-mini gel system and water-saturated butan-2-ol was poured on the top of the gel to an approximante depth of 5mm to provide the gel with a flat upper surface. The gel was then allowed to polymerise for 1 hour at room temperature.

The upper surface of the polymerised resolving gels was washed three times with distilled water, and the edge of the gel was gently blotted dry using filter paper. Stacking gels were prepared by combing 0.65ml of 30% (w/v) acrylamide stocksolution, 1.25ml of Tris-SDS stock pH 6.8 and 3.05ml of distilled water. Polymerisation was initiated by the addition of 25µl of 10% (w/v) ammonium persulphate and 10µl of TEMED. The gel was quickly pipetted between the glass plates and the 10-well comb that forms the sample wells was inserted. After polymerisation the sample well comb was gently removed from stacking gels and the wells then washed and filled with Tris-glycine electrode running buffer pH 8.5 (25mM Tris, 192mM glycine, and 0.1% (w/v) SDS). A maximum of 50µg of protein was loaded into each well. Electrophoresis was performed at 90V through

the stacking gel, and then at 120V until the Bromophenol Blue tracking dye reached the bottom of the resolving gel.

**Table 2.2 The recipe for the polyacrylamide gels**

| Stock solution | Final % (w/v) acrylamide | | |
|---|---|---|---|
| | **5%** | **8%** | **12%** |
| 30% (w/v) acrylamide/0.8% (w/v) bis-acylamide (ml) | 2.5 | 4.0 | 6.0 |
| Tris-SDS pH 8.8 (ml) | 3.75 | 3.75 | 3.75 |
| Distilled $H_2O$ (ml) | 6.25 | | |
| 10% (w/v) ammonium persulphate (µl) | 50 | 50 | 50 |
| TEMED (µl) | 10 | 10 | 10 |

### 2.2.8 Western blotting of proteins from polyacrylamide gels

Proteins resolved by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes (Gelman Biosceiences, UK) using a Bio-Rad wet blot system following the protocol of Towbin et al. (1979). The fibre pads, electroblotting papers and nitrocellulose sheets were soaked in ice-cold transfer buffer, pH 9.2, (48.8mM Tris, 39mM glycine, 0.0375% (w/v) SDS, and 20% (v/v) methanol). The protein gel was carefully removed from the cast and equilibrated in the transfer buffer for 10 min. The coloured gel holder cassette was opened and the pre-soaked filter pad was placed on the black side of the cassette. Next, two layers of wetted electrode paper were laid on the filter pad and bubbles were removed with a glass roller. The gel was then carefully flattened upon the paper and the soaked nitrocellulose was laid onto the gel. Another two layers of wetted electrode paper were placed on the top of the nitrocellulose membrane followed by the second pre-soaked filter pad, again removing any trapped air bubbles. The blotting cassette was then assembled and inserted into the blotting apparatus so that the membrane side of the assembly

faced the anode electrode. The electroblotting apparatus was filled with 1 l of ice cold transfer and frozen ice container was place inside the tank to prevent the overheating of the system. The electrotransfer of the proteins was then performed at 200mA for 2 h. The transfer of proteins was verified by the transfer of high weight molecular markers on the nitocellulose membrane. The membrane was washed once with TBS-Tween, pH 7.4, and incubated in the blocking solution, 5% (w/v) Marvel in TBS-Tween, pH 7.4, with gentle agitation for 1 h at room temperature to prevent the non-specific binding of antibodies.

### 2.2.9 Immuno-development of Western blots

Following blocking, the membranes were incubated with the appropriate antibody diluted in a blocking buffer with gentle agitation at 4ºC overnight. The blots were then washed four times for 15 min in the blocking buffer with agitation. Incubation with the suitable secondary antibody (conjugated to horseradish peroxidase) with certain dilutions in the blocking buffer, was performed for 2 h at room temperature on a shaker. After incubation with the secondary antibody, another set of washes were performed in TBS-Tween, pH 7.4. Finally membranes were rinsed with PBS, pH 7.4.

The immunodetection of blots were completed using the Amersham enhanced Chemiluminescence (ECL) system kit following the manufacturer's instructions. Briefly, the two reagents A and B were mixed in equal quantities (1ml of each per blot) prior to addition to the nitrocellulose membranes for 1 min. The developing solution was the discarded and the membranes wrapped in a cling film before placing in an autoradiography cassette. Exposure was carried out in dark room with Kodak X-Omat films exposed to the nitrocellulose sheet for varying lengths of time depending on the intensity of the signal. The film was developed and fixed using GBX developer and fixer (Sigma) followed by extensive washing with water before air-drying.

To ensure equal protein loadings, primary and secondary antibodies were removed from membranes with stripping buffer (100mM 2-mercaptoethanol, 2%

(w/v) SDS, 65mM Tris-HCl, pH 6.7). The membrane was submerged in stripping buffer and incubated at 50ºC for 30 min with occasional agitation. After four sets of 15 min washes in TBS-0.5% Tween (v/v), pH 7.4, the membrane was blocked in the blocking buffer for 1 h at room temperature. The immunodetection was then performed as described above using a control mouse monoclonal anti-α-tubulin antibody, diluted 1:1000 in the blocking buffer, followed by peroxidase conjugate rabbit anti-mouse polyclonal secondary antibody (diluted 1:2000 in the blocking buffer).

### 2.2.10 Co-immunoprecipitation

RGD-treated cells were seeded onto TG-FN matrix where the FN was blocked with heparin as described in Section 2.2.2.3. Cells were lysed in cell lysis buffer containing 0.25% (w/v) sodium deoxycholate, 150mM NaCl, 0.1mM PMSF, 1% (v/v) protein inhibitor cocktail, 500μM specific transglutaminase inhibitor R283, and 50mM Tri-HCl, pH 7.4 and put on ice for 30 min with occasional mixing. 200μg of cell extract was then pre-cleared for 1 h at 4ºC with non-specific rabbit or mouse IgG, following by 90 min incubation with 50μl of protein A or G-Sepharose bead slurries on a rocking platform. Precleared cell lysates were then incubated with 0.5μg of appropriate antibody for 90 min at 4ºC. Immune complexes were precipitated with 50μl of protein A or G-Sepharose bead slurries for 2 h at 4ºC, washed with lysis buffer and extracted in Laemmli sample buffer. Samples were resolved by SDS gel electrophoresis, transferred to nitrocellulose membrane and immunoprobed with target antibodies (Sections 2.2.7- 2.2.9).

### 2.2.11 Analysis of protein kinase C α translocation to membrane

The 60mm Petri dishes were sequentially coated with 5μg/ml of FN (in 50mM Tris-HCl, pH 7.4) and with 20μg/ml of gplTG (in 2mM EDTA in PBS, pH 7.4) as described before (Section 2.2.2). The fibroblast cells were grown to 70% confluency and serum starved for 16 h before the experiment. RAD/RGD-treated cell suspensions were prepared as before (Section 2.2.3.1), and the cells were allowed to attach for 30 min. In some cases, cells were treated with 50nM of

PMA (dissolved in DMSO) for 10 min, as a positive control treatment for PKCα activation. The supernatants of non-adherent cells were washed once with PBS, pH 7.4, collected by gentle scraping into 50μl of ice-cold homogenisation buffer (10nM EDTA, 1mM NaF, 1mM $Na_3VO_4$, 50nM okadaic acid, 0.1mM PMSF, 1% (v/v) protein inhibitor cocktail, and 50mM Tris-HCl, pH 7.4) and mixed with the corresponding cell pellet from non-adherent cells. Cells were lysed by sonication three times for 15 sec and centrifuged to pellet the nuclei and unbroken cells at 300×g for 10 min. Protein concentrations were detected as described in Section 2.2.7. Equal amounts of supernatants were collected and further centrifuged at 100,000×g for 60 min to separate cytosolic and membrane fractions. The resulting supernatants were considered to be the cytosolic fraction. The pellet was resuspended in 30μl of the homogenisation buffer and was considered to be the membrane fraction. The cytosolic and membrane fractions were diluted in Laemmli loading buffer and separated by SDS gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane, which is sequentially probed with mouse monoclonal anti-PKCα (Santa-Cruz, UK) and peroxidase conjugated monoclonal anti-mouse IgG (Dako, Denmark) as mentioned in Sections 2.2.8-2.2.10.

### 2.2.12 Detection of intracellular and matrix TG2 via confocal microscopy

Cells were seeded in 8-well glass chamber slides ($2\times10^4$ cells/well) and allowed to grow for 72 h. For the intracellular TG2 staining, cells were fixed and permeablized as described previously (Section 2.2.3.2), blocked in 3% heat-inactivated BSA in PBS, pH 7.4 for 30 min, and then incubated with mouse anti-human TG2 antibody Cub7402 in blocking buffer (1: 100 dilution) at 37ºC for 2 h. For the matrix TG2 staining, the cells were incubated with Cub7402 in complete medium (1:300 dilution) at 37ºC for 2.5 h and then after washing 3 times in PBS, pH 7.4, fixed, permeablized and blocked as described above. The wells were washed 3 times with PBS, pH 7.4 and then incubated with anti-mouse FITC-conjugated secondary antibody in blocking buffer at 37ºC for 2 h. After 3-time washing in PBS, pH 7.4, coverslips were mounted with Vectashield mounting medium and using constant PMT and section depth settings 9 random

fields/sample were captured by using Zeiss LSM510 laser confocal microscopy and the images were analysed by using Zeiss LSM Image Browser.

### 2.2.13 Detection of the cell surface TG2 activity via biotin-cadaverine incorporation into fibronectin

The transglutaminase activity of cell surface TGs were measure via biotin-cadaverine incorporation into FN as introduced previously (Heath et al., 2002). 50µl of 5µg/ml FN in 50mM Tris-HCl, pH7.4 was used to pre-coated 96-well plate at 4ºC for 16 h. After washing 3 times with 50mM Tris-HCl, pH7.4, the wells were blocked with 3% BSA in PBS, pH7.4 (blocking buffer) for 1 h at room temperature. Following trypsinizing and counting, $2\times 10^4$ cells in 100µl of serum free medium were seeded into each well and incubated for 2 h at 37ºC in the presence of 0.132nM biotin-X-cadaverine. 100ng/well of gplTG in serum medium containing 10mM DTT and 0.132nM biotin-X-cadaverine with 10mM $CaCl_2$ (a positive control) or 10mM EDTA (a negative control) was used as the control samples. After incubation, the reaction was terminated by the addition of 100µl of 2mM EDTA in PBS, pH7.4 into each well and the cells were removed by 100µl of 0.1% (w/v) deoxycholate in 2mM EDTA in PBS, pH7.4 for 10 min at room temperature with gentle agitation. After washing 3 times with 50mM Tris-HCl, pH7.4, the wells were blocked with blocking buffer for 30 min at 37ºC. Biotin-cadaverine incorporation into FN was incubated with an extravidin peroxidise conjugate in blocking buffer (1:1000 dilution) at 37ºC for 1 h. After washing 3 times with 50mM Tris-HCl, pH7.4, development of the reaction was performed by addition of developing buffer containing the substrate 7.5% (w/v) 3,3',5,5'-Tetramethyl benzidine in DMSO (TMB). The colour development was terminated by addition of 50µl of 2.5M $H_2SO_4$ and the absorbance read at 450nm using a Spectrafluor plate reader. Results were expressed as absorbance at 450nm.

### 2.2.14 Detection of cell surface proteins via biotinylation

Biotinylation of cell surface protein with EZ-link Sulfo-NHS-Biotin was performed according to manufacturer's protocol. $1\times10^6$ cells were seeded into 60mm Petri dishes. After washed once with ice cold PBS, pH 8.0, the cells were incubated with 0.8μM EZ-link Sulfo-NHS-Biotin dissolved in PBS, pH 8.0 at 4ºC for 20 min. After the removal of biotin solution, the cells were washed 3 times with 50mM Tris-HCl, pH 8.0 and lysed in 1% SDS in PBS, pH 8.0 with Benzonase. Following the incubation on ice for 30 min, protein concentrations were determined as introduced in Section 2.2.6. Cell lysates containing 600μg total protein were centrifuged at 13,000×g for 20 min to pellet the non-broken cells and then incubated with 50μl resin beads at 4ºC on rotating platform for overnight. Following washing three times with PBS, pH 8.0, the biotin-labelled cell surface proteins bound to the resin beads were extracted by boiling in 30μl Laemmli sample buffer at 95ºC for 5 min.

### 2.2.15 Detection of FN deposition

#### 2.2.15.1 Biotinylation of FN

Human plasma fibronectin in PBS, pH 7.4 was incubated with EZ-link Sulfo-NHS-Biotin (Piece) at room temperature for 30 min. The unbound biotin was dialysed in 1 l of dialysis buffer PBS, pH 7.4 at 4ºC for at least 24 h. The dialysis buffer was changed at least 3 times. After the protein concentration assay (Section 2.2.6). The biotin-labelled FN solution was diluted to the concentration of 50μM and then stored at -80ºC until use.

#### 2.2.15.2 Analysis of FN matrix assembly by immunofluorescence

$6\times10^4$ cells /well of wild type, syndecan-4 null, β1 integrin null and EA5, EA5/ α5 cells were seeded onto FN or TG-FN in 8-well glass chamber slides. In some experiments, the cells were pre- treated with RAD or RGD peptide. The cells were incubated for 1 h in serum-free medium and following washing the adherent cells with serum-free medium, the cells were incubated with 50nM exogenous biotinylated FN (prepared according to manufacturer's protocol) in

serum-free medium. After 1 h, 3 h, 6 h and 16 h incubation, fixed cells were blocked with 3% bovine serum albumin (BSA) in PBS, pH7.4 and the cell matrices stained with 1μg/ml Cy5-streptavidin. Slides were mounted with Vectashield mountant and examined via confocal microscopy.

### 2.2.16 Statistical analysis

The differences between data sets in cell adhesion assays were analysed by the Student's t test (two-tailed distribution with equal variance). Statistical significant difference between data sets was defined in the text by a $p<0.05$ (two sides).

# Chapter 3:

# Importance of the syndecan-4/2 and β1 integrin co-signalling pathway in RGD-independent cell adhesion mediated by the TG-FN matrix

# Chapter 3: Importance of the syndecan-4/2 and β1 integrin co-signalling pathway in RGD-independent cell adhesion mediated by the TG-FN matrix

## 3.1 Introduction

The extracellular matrix (ECM) serves as a crucial survival signal for many anchorage dependent cells by providing those cells with a suitable environment to proliferate, differentiate, migrate and transduce signals thus enabling them to exert their biological functions (Hynes, et al., 1999). As one of the major ECM components, fibronectin (FN) can interact with different kinds of cell surface receptors, such as integrins and syndecans and activate their signalling pathways.

The amino acid sequence Arg-Gly-Asp binding site is a widely occurring cell adhesive motif originally discovered in fibronectin (Pierschbacher and Ruoslahti, 1984), and located in $III_{10}$. The RGD motif in fibronectin is one the most important recognition sites for about half of all known integrins, such as α3β1, α5β1, αVβ1, and αVβ3 integrin. When treated with synthetic RGD peptides, the cells can not adhere completely on FN (Pierschbacher and Ruoslahti, 1984), suggesting the importance of this interaction between cells and matrix mediated by integrin receptors. A recent study discovered that, even when seeded on the cell binding domain of FN, the cells could still not accomplish a well-organized actin cytoskeleton, which led to the discovery of another important family of cell surface receptors— heparan sulfate proteoglycans (HSPGs) (Bloom, *et al*., 1999). Only when the heparan sulfate chains interact with heparin-binding sites in FN, can the cell organize mature focal contacts (Woods, et al. 1986). The major group of HSPGs is the syndecan family. The syndecans are a four-member family (syndecan1-4) of transmembrane cell surface proteoglycans (PGs) that bear heparan sulfate glycosaminoglycan (GAG) chains (Bernfield, et al., 1999). The syndecans are expressed on virtually all cell types throughout development and adulthood, and their expression can be altered under certain

pathophysiological conditions, including the processes of tumour onset, progression and metastasis (Sanderson, et al., 2004).

Several studies on TG2, FN structure and the integrin signalling pathway have demonstrated a novel role for TG2 in the cell adhesion process. The structural study for FN showed that the TG2 binding site (the 42kD gelatin-binding domain) (Radek, et al., 1993) and the integrin binding site (the RGD cell-binding site) are located in different parts of the FN molecule, which provides the possibility that TG2 and integrin collaborate rather than compete in the cell adhesion process. Moreover, the binding of TG2 with FN was shown to activate FAK and increase the GTP loading of the small GTPase RhoA, leading to intracellular signalling and induction of cell adhesion (Janiak, et al., 2006).

The studies of Belkin et al. (2000 and 2001) and Gaudry et al. (1999) indicated that TG2 associates with integrin receptors in a number of different cell types via binding to the extracellular domains of the β1, β3 and β5 integrin subunits. Unlike FN, integrins do not appear to serve as enzymatic substrates of TG2 or other transglutaminases and the formation of stable non-covalent integrin-TG2 complexes is independent of the transamidating activity of TG2 (Akimov et al., 2000a). Although the binding sites within integrins for TG2 are still unknown, it has been demonstrated that integrin-TG2 complexes have a 1:1 stoichiometry and all the TG2 on the cell surface is bound to integrin receptors in a FN-independent manner (Akimov et al., 2000a). Besides the structural evidence for interactions, a series of studies led by Mehta and colleagues on tumour cells demonstrated the connection between TG2 and the integrin-relevant downstream signalling molecule-FAK. The culture of TG2-positive breast cancer cells and TG2-transfected fibroblasts on fibronectin-coated surfaces led to the activation of FAK, the downstream signalling molecule of integrins (Mehta, et al., 2006). Conversely, down-regulation of TG2 by small interfering RNA attenuated FN-mediated cell attachment (Herman, et al., 2006) and cell survival and FAK phosphorylation (Verma, et al., 2006). A recent study showed that GTP-bound TG2, present in the ECM, can induce hypertrophic differentiation of

chondrocytes through an α5β1 integrin-dependent and FAK-associated cell adhesion process (Tanaka, et al., 2007).

As introduced above, TG2 also exists in the ECM as a kind of structural protein (Verderio, et al., 1998). The importance of TG2, as an ECM protein, in RGD-independent cell adhesion was first introduced by Verderio et al. (2003). The TG2 bound FN matrix compensated the apoptosis (anoikis) induced by an RGD synthetic peptide. This process was PKCα and FAK-dependent. Treating the cells with heparinase to digest the cell surface heparan sulfate chains abolished the compensatory effect of the TG-FN complex, which indicated the potential involvement of HEPG receptors.

The aim of the work contained in this chapter is to identify the potential involvement of other cell surface receptor(s), including syndecan-4, syndecan-2, β1 integrin, α5 integrin, β3 integrin and α4β1 integrin, and characterise their roles in mediating the RGD-independent cell adhesion and actin cytoskeleton formation on TG-FN. Given the range of cell surface receptors present in fibroblasts, and the availability of receptor mutant cells, mouse embryo fibroblasts (MEF), in which syndecan-4, β1 integrin, α5 integrin, or β3 integrin has been knocked out, were utilized as the major cell model (Gronthos, et al. 1997; Bass and Humphries 2002; Verderio, et al., 2003).

## 3.2 Methods

### 3.2.1 RGD-independent Cell adhesion assay

Cells ($2.5 \times 10^4$ cell/well) pre-treated with RGD or RAD synthetic peptide were seeded on the microtiter plates pre-coated with FN (5μg/ml) or FN with immobilized with gplTG (20μg/ml) and allowed to adhere for 20-40 min (Section 2.2.3.1). Following cell adhesion and spreading, the cells were fixed and stained as mentioned before (Section 2.2.3). The differences between data sets were performed using Student's t test (two-tailed distribution with equal variance).

### 3.2.2 Immunofluorescence staining

To investigate the formation of the actin cytoskeleton in fibroblast with different mutations or with silenced cell surface receptors, FITC-labelled phalloidin (20μg/ml) was used to detect the actin skeleton formation after the RGD-independent cell adhesion on FN or TG-FN matrices in 8-well chambers. The signals were detected by a Zeiss LSM510 laser confocal microscope (Section 2.2.4).

### 3.2.3 siRNA transfection

Syndecan-2 specific targeting siRNA was used to inhibit the expression of syndecan-2 in MEF cells by using the HiPerfect Transfection method (Qiagen). 4 different siRNA sequences targetting mouse syndecan-2 were obtained from Qiagen. The sequences are listed below.

Mm_Sdc2_1 SI01412250 TGGAATTTAATTTGTAGAATA
Mm_Sdc2_2 SI01412264 CAAAGGCAAATTAATGTGTAA

1nM of lyophilized siRNA was dissolved in 100μl of sterile, RNase-free water to obtain a 10μM solution and then stored at -20ºC. The transfection was performed according to the manusfacture's protocol. Before transfection, $3\times10^5$ cells were

seeded into each well of a 6-well plate for 24 h to reach 50-80% confluency. Prior to transfection, cells were washed with PBS, pH 7.4 and the medium was replaced with 2.3ml pre-warmed fresh complete growth medium. 150ng of siRNAs were diluted in DMEM serum free medium siRNAs to reach the final volume of 100µl of siRNA working solution at the final concentration of 5nM. After pre-incubation with 12µl of HiPerfect Transfection Reagent (Qiagen) at room temperature for 5-10 min, the transfection complex was added drop-wise onto the cells with gentle swirling the plate to ensure the uniform distribution of the transfection complex. The following experiments were performed after 30 h siRNA transfection.

### 3.2.4 Detection of the siRNA transfection efficiency

After 30 h incubation of the syndecan-2 targetting siRNA or the negative control siRNA with the MEF cells, cell lysates were collected into 30µl of cell lysis buffer and the protein concentration was detected as introduced in Section 2.2.7. Western blotting was performed to detect the presence of syndecan-2 by using rabbit anti-syndecan-2 polyclonal antibody (1:1000 dilution) and swine anti-rabbit secondary antibody (1:1000 dilution) (Section 2.2.8- 2.2.9). Signals were detected by using enhanced Chemiluminescence (ECL) system kit.

### 3.2.5 The synthesis and detection of the scrambled siRNA against the syndecan-2 specific targeting siRNAs

According to the results from the Western blotting assay for the syndecan-2 siRNAs-treated MEF cells, the scrambled siRNA were designed with the aid of Dr Russell Collighan (Aston University) and synthesized by Sigma-Aldrich. The sequences of the scrambled siRNAs were listed below and the effect of these siRNAs was detected via Western blotting for syndecan-2 antigen as described in Section 4.2.4.

Scrambled siRNA1: GTAAATTATGTAAGTTAGTAT

Scrambled siRNA2: GATAATCAAAGCGAAATGTAT

## 3.3 Results

### 3.3.1 Cell surface TG2 is not sufficient to support RGD-independent cell adhesion.

#### 3.3.1.1 Susceptibility of MEF cells to Zeocin

For the establishment of the stable TG2 transfected MEF cell line, a range of zeocin concentrations (0.1-1.2mg/ml) was used to treat wild type MEF cells to identify the lowest possible concentration that would induce about 80% cell death within 48-72 h as introduced in Section 2.2.1.7.1. The resulting kill curve suggested that at the concentration of 800μg/ml zeocin was the optimal concentration for clone selection (Figure 3.3.1).

#### 3.3.1.2 Establishment of tg2-MEF cell line with stable-transfected TG2

Previous data suggested that cell surface TG2 is a co-receptor in mediating integrin-dependent cell adhesion on FN. In order to distinguish the effects of cell surface TG2 and matrix TG2, MEF cells were stable-transfected with human TG2 as introduced in Section 2.2.1.7 and grown in cell medium supplemented with 800μg/ml zeocin to select the transfected cells. Selection of transformants was undertaken with 400μg/ml of zeocin in the cell culture medium. The effect of transfection was studied via detecting the level of TG2 antigen in both whole cell lysates and biotinylated cell surface protein via Western blotting (Section 2.2.8). As shown in Figure 3.3.2, in wild type MEF cells TG2 was found neither in cell lysate nor on the cell surface, while after transfection, high levels of TG2 antigen was detected on the cell surface. These results prove the successful establishment of a tg2-MEF cell line, which provides an ideal model to investigate the role of cell surface TG2 in the RGD-independent cell adhesion process, since the potential influence of exogenous TG2 is ruled out in the wild type MEF cell.

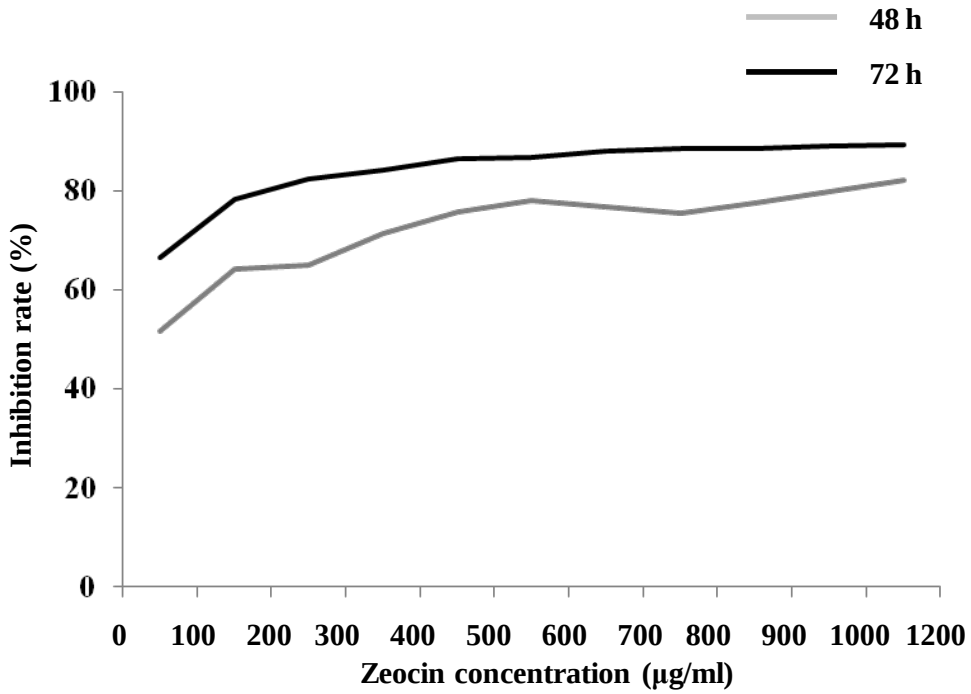



**Figure 3.3.1 The effect of zeocin on MEF cell proliferation via XTT assay.**

MEF cells were treated with different concentrations of zeocin (0, 100, 200, 400, 600, 800, 1000, 1200µg/ml, prepectively). After 48 and 72 h incubation with the antibiotic, XTT assay was performed and the absorbance was read at 490nm and 750nm using a Spectrafluor plate reader (as introduced in Section 2.2.1.7.1).

**Figure 3.3.1**

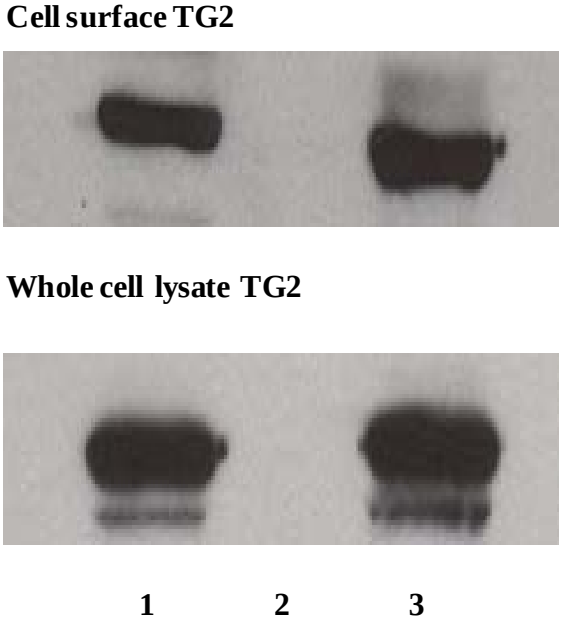



**Figure 3.3.2 Detection of TG2 in wild type and tg2-MEF cells.**

The establishment of tg2-MEF cell line was accomplished as introduced in Section 2.2.1.7. The presence of TG2 antigen in wild type and tg2-MEF cells was detected in both whole cell lysates and biotinylated-cell surface proteins (As described in Section 2.2.14) via Western blotting (As introduced in Section 2.2.8). Lane 1, gplTG marker; Lane 2 wild type MEF cells; Lane3 tg2-MEF cells.

**Figure 3.3.2**

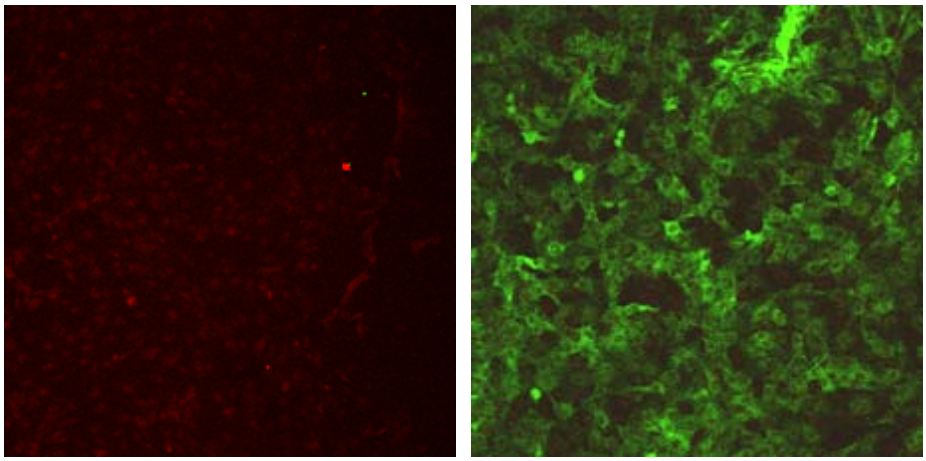
The distribution of the TG2 antigen within the whole population of wild type and tg2-MEF cells, immunofluorescence staining of the intracellular TG2 was applied by using a specific monoclonal antibody Cub7402 against TG2 and the fluorescence signals were detected via confocal microscopy as introduced in Section 2.2.12. No fluorescence signalling was detected in wild type MEF cells, and an equal distribution of TG2 antigen was obtained in tg2-MEF cells, indicating the success of the establishment of tg2-MEF cell line (Figure 3.3.3).

In order to confirm the results from the Western blotting of the presence of the TG2 antigen within the wild type and tg2-MEF cells, cell surface *in situ* TG activity was measured via a biotin-X-cadaverine incorporation assay as described in Section 2.2.13. As shown in Figure 3.3.4, cell surface TG activity was only detected in the tg2-MEF cells, but not in wild type MEF cells, which further confirmed the presence of TG2 on tg2-MEF cell surface.

To further confirm the presence of TG2 antigen and its ability to be deposited into the ECM, the established tg2-MEF cell line was seeded into 8 well chambers and after a 72 h incubation, the matrix TG2 antigen was detected via immuno-fluorescence staining. As shown in figure 3.3.5, in tg2-MEF matrix TG2 could be detected, while no signal was found in the wild type MEF cells, further confirming the very negligible levels of TG2 in the wild type cells and the increased TG2 antigen deposited into the ECM of TG2 transfected cells.

#### 3.3.1.3 Cell surface TG2 could not compensate adhesion to FN in the presence of RGD peptides

The effect of cell surface TG2 (that which is likely to be associated with integrins following its immediate secretion (Akimov et al., 2000b) on RGD-independent cell adhesion was investigated by using wild type and tg2-MEF cells, while wild type MEF cells seeded on FN matrix were used as the control group. As shown in Figure 3.3.6a and b, no significant difference was found between RAD-treated tg2-MEF cells and wild type cells, suggesting that no toxicity was found by over-expression of TG2. Interestingly, over-expression of cell surface TG2 did not



**wt MEF** **tg2-MEF**

**Figure 3.3.3 Detection of intracellular TG2 in wild type and tg2-MEF cells.**

The establishment of tg2-MEF cell line was accomplished as introduced in Section 2.2.1.7. The presence of intracellular TG2 antigen in wild type (wt MEF) and tg2-MEF cells was detected via immunofluorescence staining by using anti-TG2 antibody Cub7402 and the fluorescence signals were detected via confocal microscopy, while the nuclei were staining by using propidium iodide (PI) (As described in Section 2.2.12).

**Figure 3.3.3**

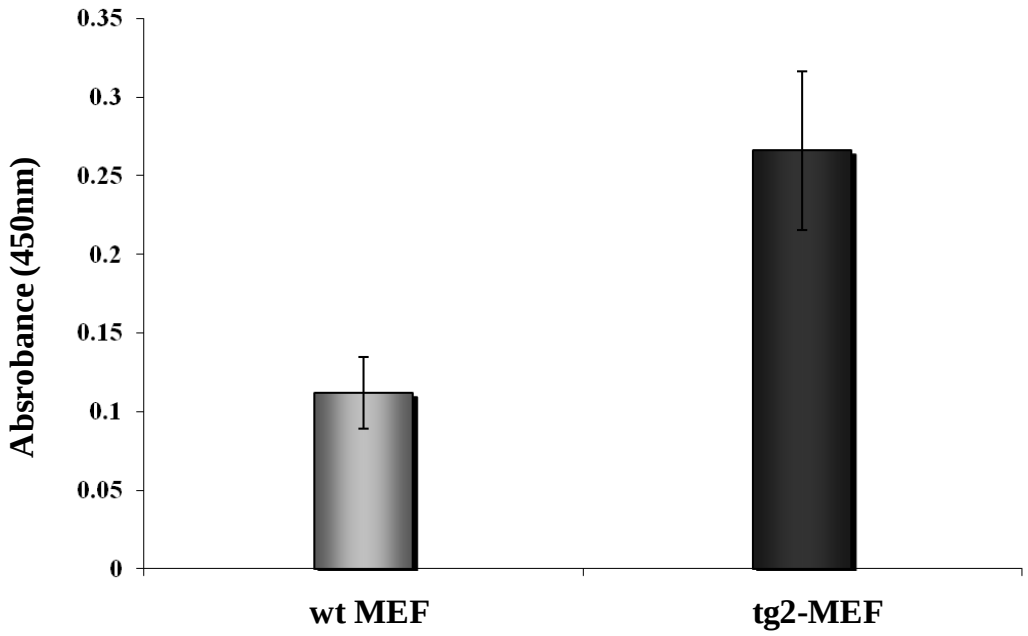
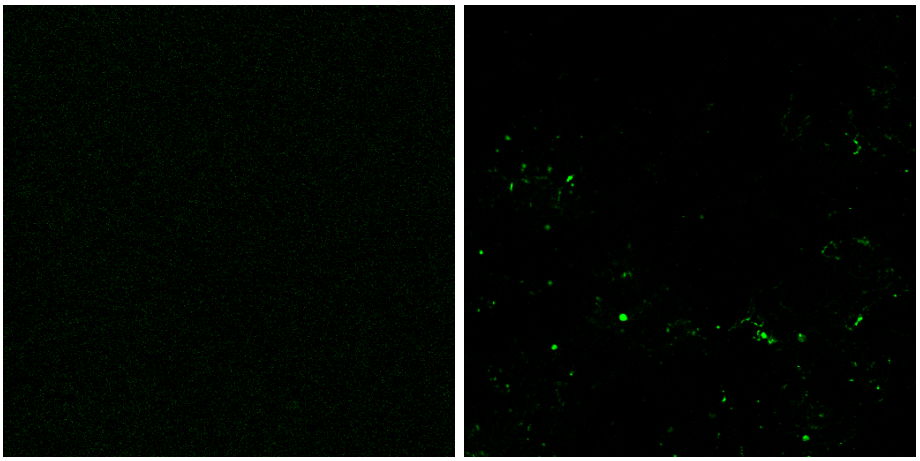



**Figure 3.3.5 Detection of the cell surface TG activity in wild type and tg2-MEF cells.**

The establishment of tg2-MEF cell line was accomplished as introduced in Section 2.2.1.7. The activity of the cell surface TG2 in wild type (wt MEF) and tg2-MEF cells were measured via biotin-cadaverine incorporation into fibronectin (Section 2.2.13).

**Figure 3.3.4**



**wt MEF** **tg2-MEF**

**Figure 3.3.4 Detection of matrix deposited TG2 antigen in wild type and tg2-MEF cells.**

The establishment of tg2-MEF cell line was accomplished as introduced in Section 2.2.1.7. The presence of matrix TG2 antigen in wild type and tg2-MEF cells were detected via immunofluorescence staining as introduced in Section 2.2.12.

**Figure 3.3.5**

improve the cell attachment and spreading process on FN, since no significant differences were observed between wild type and tg2-MEF. RGD peptide treatment led to a 50% reduction of both cell attachment and cell spreading in tg2-MEF, and no significant difference was found when compared to RGD-treated wild type MEF. Only when both sets of cells were seeded on the TG-FN complex were the cells able recover their adhesion capacity (92% in adhesion and 95% in spreading) in the presence of RGD peptide. These results indicated that cell surface TG2 is not sufficient to compensate the effect of the RGD peptides on the cell adhesion and that only when it is deposited into a FN matrix, can TG2 facilitate cell adhesion in the presence of RGD peptides.

### 3.3.2 TG2 is the functional component within the TG-FN complex that exerts the compensatory effect in cell adhesion in the presence of RGD peptides

#### 3.3.2.1 Immobilisation and detection of TG2 on a heparin-treated FN matrix

Although it has been reported that the TG-FN complex can compensate the effect of RGD peptides in inhibiting cell attachment and spreading, the relative contribution of these two proteins in the heterocomplex still remains unknown. In order to distinguish the roles of FN and TG2, soluble heparin, which is natural ligand for FN, was used to block the binding sites for heparan sulphates on FN prior to TG2 immobilisation (Itano et al., 1993). Before performing the cell adhesion assay, a modified ELISA method (Verderio et al., 1999) was utilized to investigate whether heparin-treatment affected the formation of the TG-FN complex. To block the heparin-binding sites completely, a 60-fold excess concentration of heparin (300μg/ml) to FN (5μg/ml) was used. In this experiment, two different concentrations of TG2, 20μg/ml and 30μg/ml, was immobilised on FN following heparin incubation. Previous work has been demonstrated that 20μg/ml of gplTG is the concentration that can saturate 5μg/ml FN coated plastic surface. The relevant levels of TG2 were detected with the mouse monoclonal anti-TG2 antibody Cub7402. The result show that the binding between heparin and FN did not affect interaction between FN and TG2


**Figure 3.3.6 The role of cell surface TG2 in cell adhesion process on FN-TG2 matrix.**

The cell adhesion assay were carried on and the attachment and spreading of wild type (wt) MEF and tg2-MEF cells were analysed as explained in Section 2.2.3. **a**, the cell adhesion of wild type and tg2-MEF cells. The percentage of attached cell (cell attachment) or the percentage of spreading cells (cell spreading) ± S.D. shown is the mean values from three separate experiments performed in triplicate. The mean cell attachment and spreading values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D. The mean number of cell attachment ± S.D. of control taken from 3 experiments was 150 ± 5. The mean percentage spreading value ± S.D on FN was 68±0in the 3 experiments, and normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. **b**, visualization of wild type and tg2-MEF cells on FN and TG-FN matrix in the presence of RGD peptide.

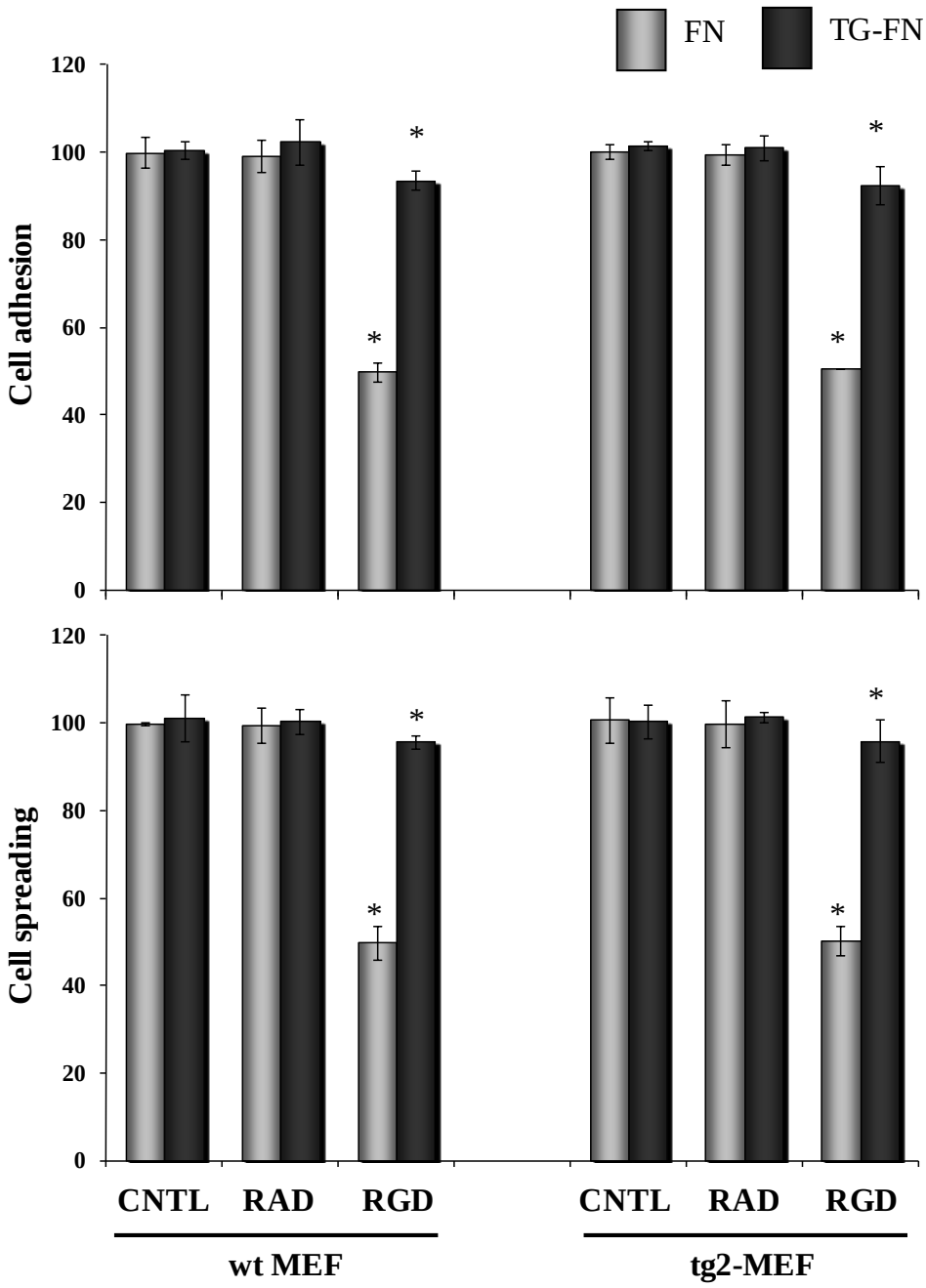

FN
TG-FN
Cell adhesion
Cell spreading
120
100
80
60
40
20
0
*
CNTL
RAD
RGD
CNTL
RAD
RGD
wt MEF
tg2-MEF

**Figure 3.3.6a**

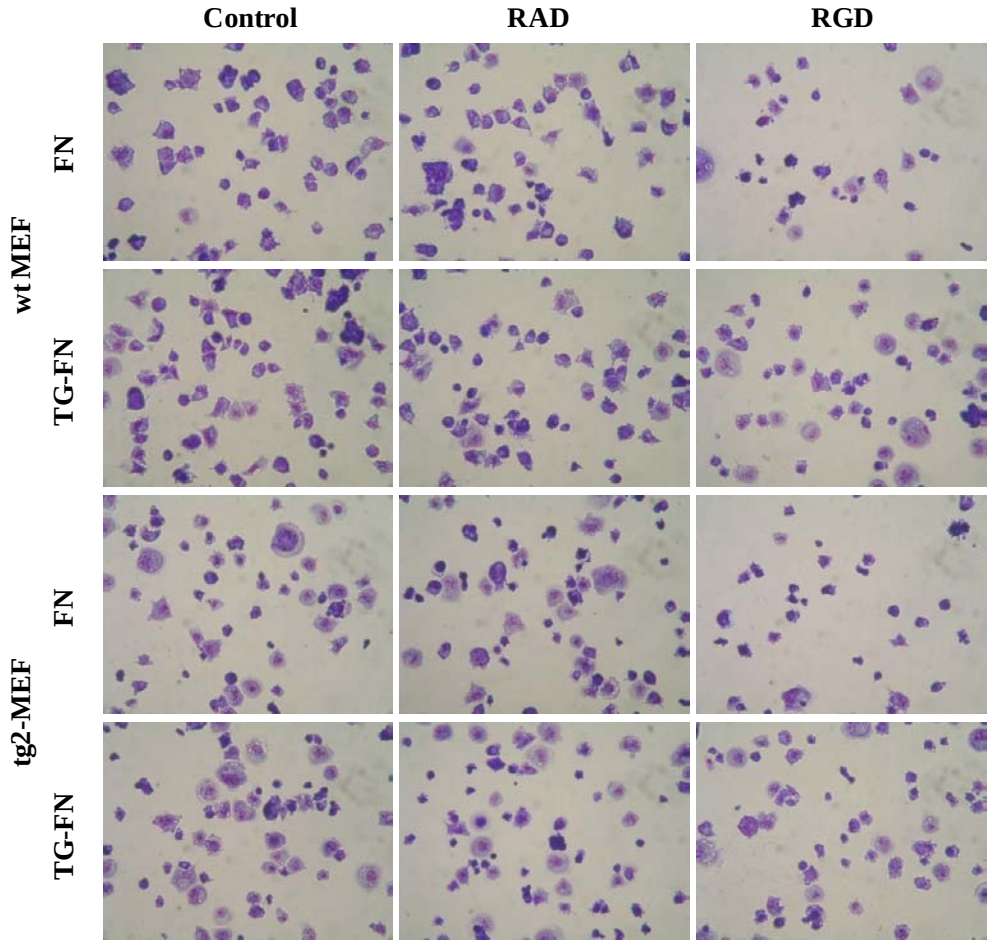



**Figure 3.3.6b**

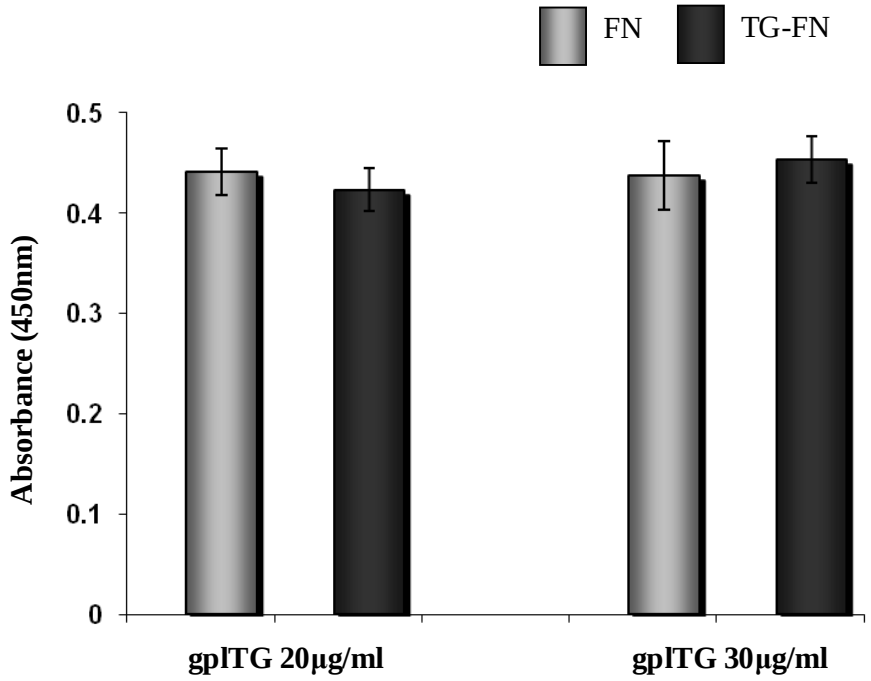



**Figure 3.3.7 Detection of the relative levels of TG2 bound to heparin blocked FN by ELISA.**

The relative levels of TG2 bound to heparin blocked FN immoblized on TCP at different concentrations of TG2 were measured via ELISA. 300µg/ml heparin in 50mM Tris-HCl, pH7.4 was used to block the heparin-binding sites within FN molecule before the immobilization of gplTG onto FN matrix. The presence of gplTG antigen immobilized on FN matrix was detected by using monoclonal antibody Cub7402. Each data point presents the mean absorbance (450nm) values ± S.D of three separate experiments undertaken in quadruplicate. The final values were acquired by substrating the background values obtained from the wells incubated with FN and heparin solution.

**Figure 3.3.7**

compared to the control groups and there was no significant difference between the two concentrations of TG2. Therefore this method was used in the following cell adhesion experiments (Figure 3.3.7).

### 3.3.2.2 TG2 can compensate the loss of cell adhesion in the presence of RGD peptides even after blocking the heparin binding sites on FN

Cell adhesion was performed on heparin-treated matrices and control matrices to investigate the ability of TG-FN in RGD-independent cell adhesion by using mouse embryonic fibroblasts. As might be expected, the heparin-blocked FN matrix led to a significant 30% decrease in cell attachment and over 20% decrease in cell spreading (Figure 3.3.8a and b), compared to that of the FN control. Cell attachment on TG-FN was 106% of the control cell attachment on FN in the non-blocked wells. TG-FN matrix compensated the loss of cell adhesion caused by blocking the heparin-binding sites within FN molecules. Similar results were obtained when cells were seeded on FN and TG-FN matrix with or without heparin blocking in the presence of RGD peptide. Once treated with 100μg/ml of RGD peptide, cell attachment was reduced to 50% of the value evaluated for the control on FN, while cell spreading dropped to 18% (Figure 3.3.8a and b) of the control value. The defective cell attachment and spreading due to heparin blocking and the presence of RGD peptide was restored back to 79% and 64%, respectively, when cells were seeded on the TG-FN matrix, This suggests that the RGD-independent cell adhesion mediated by the TG-FN complex is independent of the interaction between cell surface HSPGs and the heparin -binding sites within FN.

## 3.3.3 Cell adhesion in response to the TG-FN matrix is dependent on the interaction between TG and the extracellular heparan sulfate chains.

### 3.3.3.1 Heparan sulfate chains are required for TG-FN to exert its compensatory effect on the RGD peptide-induced loss of cell adhesion


**Figure 3.3.8 The importance of the heparin binding sites within FN in cell adhesion process on FN or FN-TG2 matrix.**

Heparin-treated matrix was prepared as described under Methods (Section 2.2.2) The cell adhesion assay was carried out and the attachment and spreading of Swiss 3T3 fibroblasts were analysed as explained in Section 2.2.3. The percentage of attached cell (cell attachment) or the percentage of spread cells (cell spreading) ± S.D. shown are the mean values from three separate experiments performed in triplicate. The mean cell attachment and spreading values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D. The mean number for cell attachment ± S.D of control taken from 3 experiments was 172 ± 6, 156±11, and 159±4. The mean percentage spreading value ± S.D on FN was 80±1, 90±2, and 87±2 in the 3 experiments, and normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN.

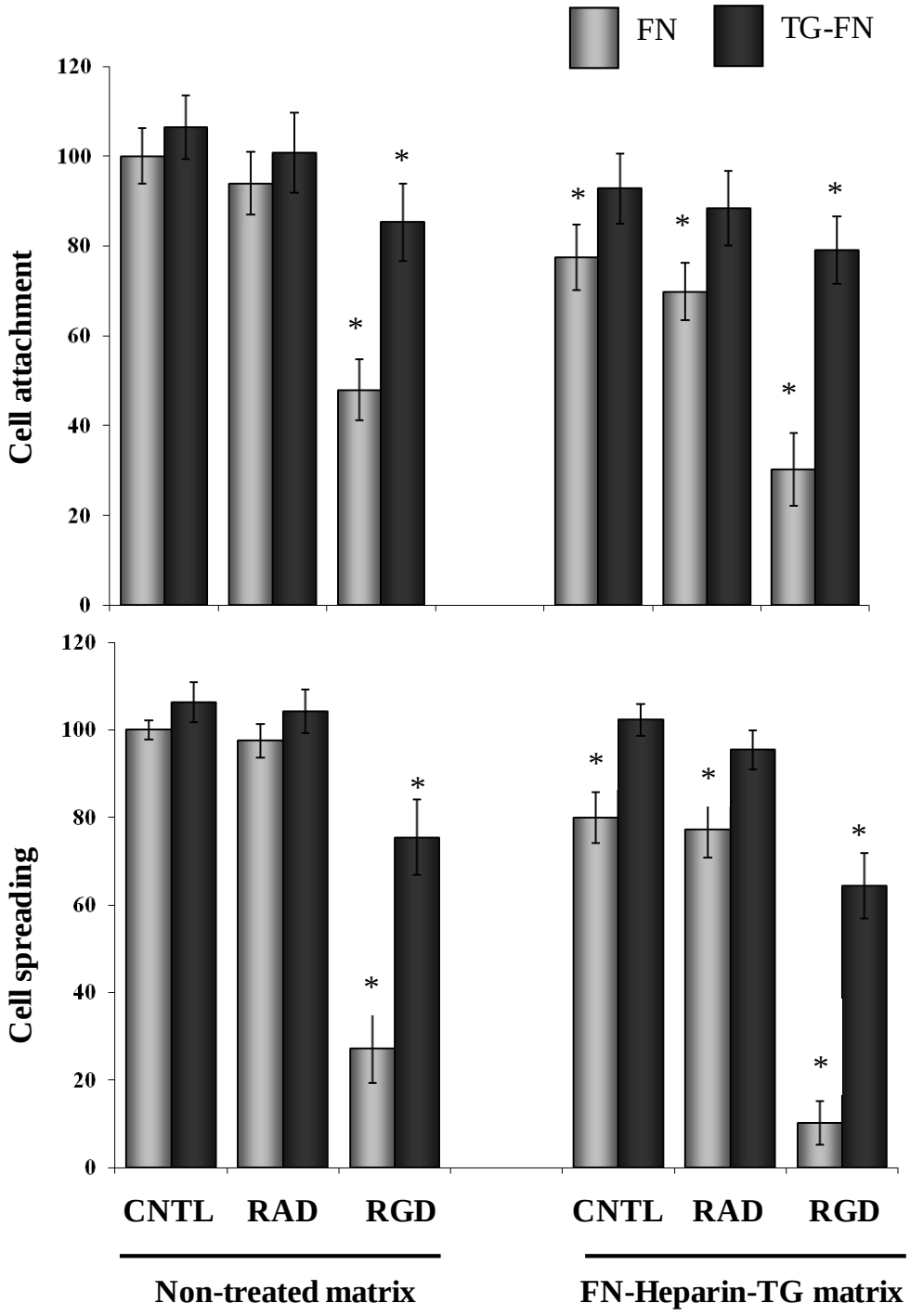

FN
TG-FN
Cell attachment
Cell spreading
0
20
40
60
80
100
120
*
CNTL
RAD
RGD
CNTL
RAD
RGD
Non-treated matrix
FN-Heparin-TG matrix

**Figure 3.3.8**


**Figure 3.3.9 The importance of cell surface heparan sulphate chains in cell adhesion process on FN or TG-FN matrix.**

The cell adhesion assay was carried out and the attachment and spreading of wild type (wt) and heparan sulphate-mutant (hs-m) CHO cells were analysed as explained in Section 2.2.3. **a**, The percentage of attached cell (cell attachment) or the percentage of spreading cells (cell spreading) ± S.D shown are the mean values from three separate experiments performed in triplicate. The mean cell attachment and spreading values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D The mean number of cell attachment ± S.D. of control taken from 3 experiments was 176 ± 5, 181±2, and 144±16. The mean percentage spreading value ± S.D on FN was 83±1, 83±6, and 69±1 in the 3 experiments, and normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups. **b**, visualization of wt and hs-m CHO cells on heparin-treated FN and TG-FN matrix in the presence of RGD peptide.

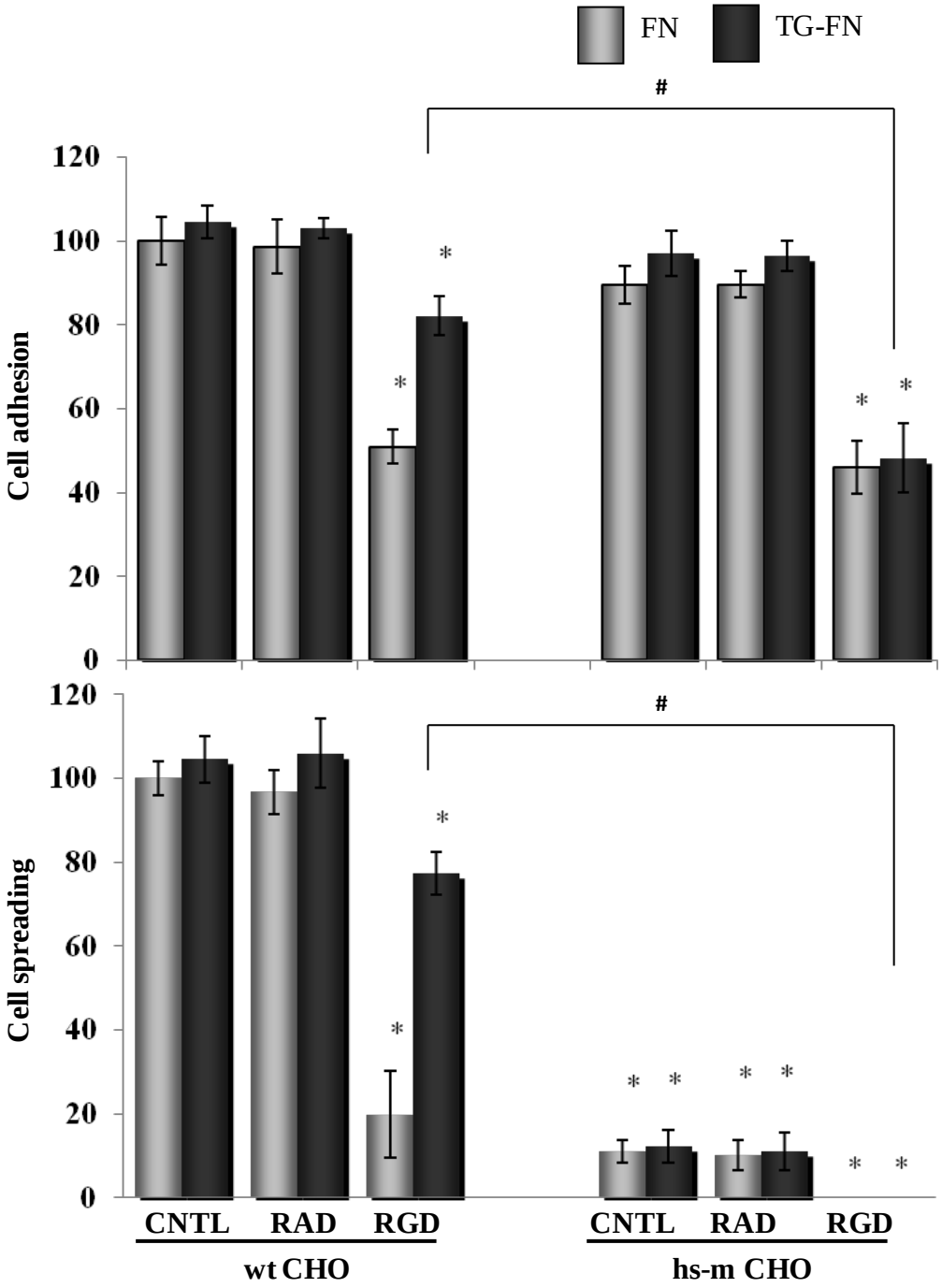

FN
TG-FN
#
Cell adhesion
120
100
80
60
40
20
0
Cell spreading
CNTL
RAD
RGD
wt CHO
hs-m CHO

**Figure 3.3.9a**

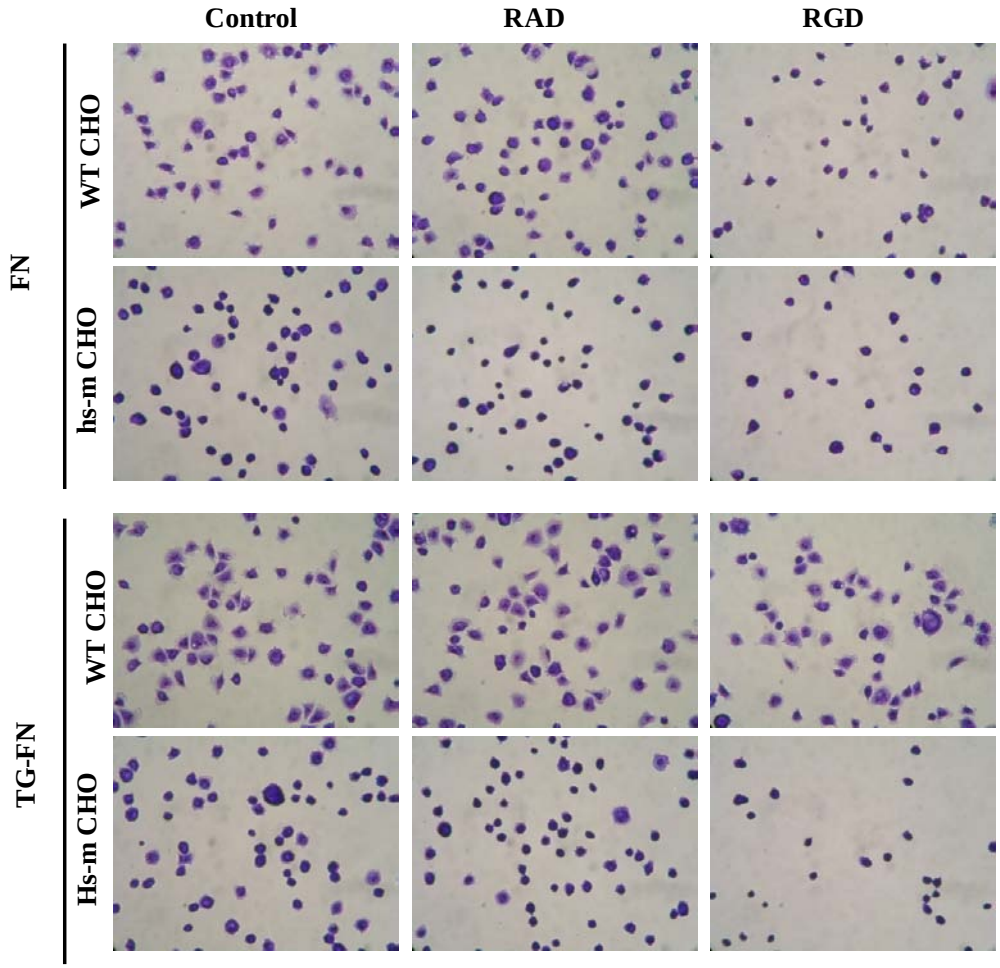



**Figure 3.3.9b**

The experiments above indicated that the compensation effect of TG-FN on the RGD-induced loss of cell attachment and spreading was independent of the interaction between the cell surface heparan sulfate chains and the heparin-binding sites within FN suggesting TG2 to be the functional component in this process. To further investigate whether extracellular heparan sulfate chains are involved in the TG-FN mediated cell adhesion, heparan sulfate deficient Chinese hamster ovary cells (CHO) were used to determine the cell adhesion in the presence of RGD peptide, while wild type CHO cells were used as control cells.
In this experiment, CHO cells incubated with a concentration of 100μg/ml of the RGD peptide considerably decreased cell attachment on FN to approximately 50%, and cell spreading to about 30% (Figure 3.3.9). As expected, cell attachment did not show a reduction when treated with the RAD peptide, which indicated that addition of the exogenous peptides at 100μg/ml did not cause toxicity to the cells. In agreement with the previous experiments, the TG-FN complex restored the cell attachment and spreading back to around 80% of the value of the RGD peptide-treated wild type CHO cells. Although there was only a 10% reduction in cell attachment on FN when heparan sulphate-deficient cells were compared to wild type CHO cells, the cell spreading on FN was dramatically decreased in the heparan sulfate deficient CHO cells to only around 10% of the control values when compared with the non-treated wild type CHO cells (Figure 3.3.9). No spread cells were observed in the heparan sulphate-mutant CHO cells in the presence of the RGD peptides. In contrast to the wild type CHO cells, TG-FN lost its compensatory effect on the cell adhesion caused by the RGD peptides in heparan sulphate-deficient CHO cells. These experiments confirm the importance of the extracellular heparan sulfate chains in the RGD-independent cell adhesion process mediated by the TG-FN complex.

### 3.3.3.2 Heparin treatment of TG2 blocked the RGD-independent cell adhesion mediated by TG-FN.

The high affinity of TG2 for heparin has been reported (Gambetti et al., 2005). Previous work has also demonstrated TG-FN loses its compensatory effect on heparinase-treated cells (Verderio et al., 2003). Heparin was therefore used to


**Figure 3.3.10 The importance of the heparan sulfate binding site(s) of TG2 in RGD-in dependent cell adhesion on TG-FN.**

Cell adhesion was performed on a heparin treated TG-FN matrix after immobilization of gplTG on the FN and the attachment and spreading of MEF cells was then analysed as explained in Section 2.2.3. The percentage of attached cell (cell attachment) or the percentage of spreading cells (cell spreading) ± S.D shown are the mean values from three separate experiments performed in triplicate. The mean cell attachment and spreading values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D The mean number of cell attachment ± S.D of control taken from 3 experiments was 156 ± 5. The mean percentage spreading value ± S.D on FN was 74 ± 0 in the 3 experiments, which was normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups.

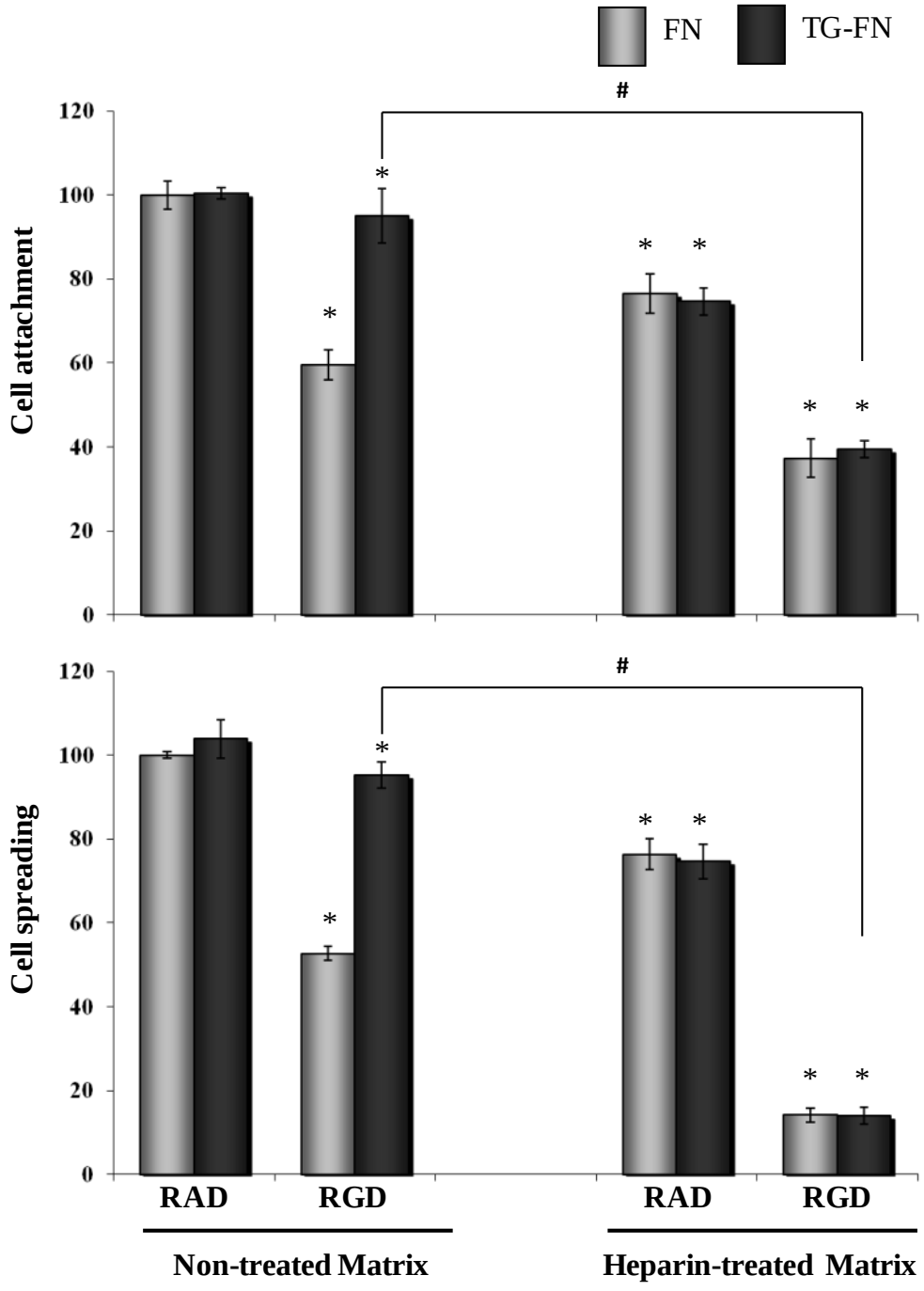

FN
TG-FN
Cell attachment
Cell spreading
120
100
80
60
40
20
0
#
*
RAD
RGD
RAD
RGD
Non-treated Matrix
Heparin-treated Matrix

**Figure 3.3.10**

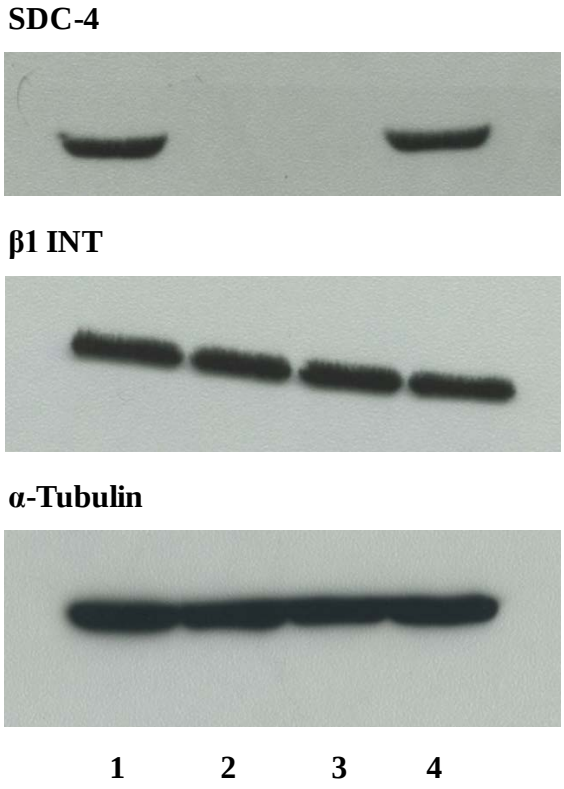



**Figure 3.3.11 Detection of syndecan-4 and β1 integrins in wild type, syndecan-4 knock, syndecan-4 vector control and syndecan-4 addback MEF cells.**

Western blotting was performed to confirm the presence of syndecan-4 and β1 integrins in syndecan-4-relevant MEF cells (Section 2.2.9-2.2.10). The membranes were stripped and re-probed with anti-α-tubulin antibody to ensure the equal loading. Lane 1, wt MEF; Lane 2, syndecan-4 ko MEF; Lane 3, syndecan-4 vec MEF; Lane 4, syndecan-4 ab MEF.

**Figure 3.3.11**

treat the TG-FN complex after the immobilization of gplTG onto FN and the cell adhesion assay was then performed on this heparin-blocked TG-FN matrix. As shown in Figure 3.3.10a and b, in the non-treated and RAD-treated cells, only 70% of cell attachment and 30% of cell spreading was observed on the FN matrix, which could not be compensated by the heparin-treated TG-FN matrix. The RGD peptide treatment reduced the cell attachment to 40% and the spreading to around 12% on both the heparin-blocked FN and TG-FN matrices, compared to 48% of cell attachment on FN matrix and 90% on TG-FN matrix and 35% of cell spreading on FN and 94% on TG-FN, respectively (Figure 3.3.10). This result suggests the importance of the heparin-binding ability of TG2 in mediating the RGD-independent cell adhesion process.

### 3.3.4 Syndecan-4 plays an important role in RGD-independent cell adhesion in response to the TG-FN matrix.

#### 3.3.4.1 Detection of the presence of syndecan-4 antigen in syndecan-4 relevant MEF cells.

In order to confirm the presence or absence of the syndecan-4 antigen, the wild type, syndecan-4 knockout, add-back (ab, syndecan-4 knockout cells transfected with human syndecan-4 cDNA) and empty vector control (vec syndecan-4 knockout cells transfected with vector cDNA) MEF cells were analysed by Western blotting. This was performed by using rabbit polyclonal anti-syndecan-4 antibody (which recognizes both mouse and human syndecan-4), while the expression of another major cell adhesion-related receptor β1 integrins was also detected. The membrane was stripped and re-probed with mouse anti-α-tubulin antibody to ensure the equal loading of the samples. As shown in the top panel of Figure 3.3.11, no syndecan-4 was detected in syndecan-4 knockout and vector-control MEF cells, while the antigen was detected in the wild type and addback MEF cells (Figure 3.3.11 middle panel). The loss of syndecan-4 did not affect the expression of β1 integrins in all of the above cells.


**Figure 3.3.12 The importance of cell surface syndecan-4 in the RGD-independent cell adhesion process on FN and TG-FN matrices.**
**a,** the RGD-independent cell adhesion assay was carried out and the attachment and spreading of syndecan-4 wild type (wt MEF), knock out (SDC4 ko), vector control (SDC4 vet) and add-back (SDC4 ab) MEF cells were analysed as explained in Section 2.2.3. The percentage of attached cell (cell attachment) or the percentage of spreading cells (cell spreading) ± S.D shown are the mean values from three separate experiments performed in triplicate. The mean cell attachment and spreading values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D The mean number of cell attachment ± S.D of control taken from 3 experiments was 181 ± 11, 172±4, and 142±9. The mean percentage spreading value ± S.D. on FN was 68±3, 64±5, and 74±2 in the 3 experiments, and normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups. **b,** visualization of RGD peptide-treated syndecan-4 relevant MEF cells seeded on FN or TG-FN matrices.

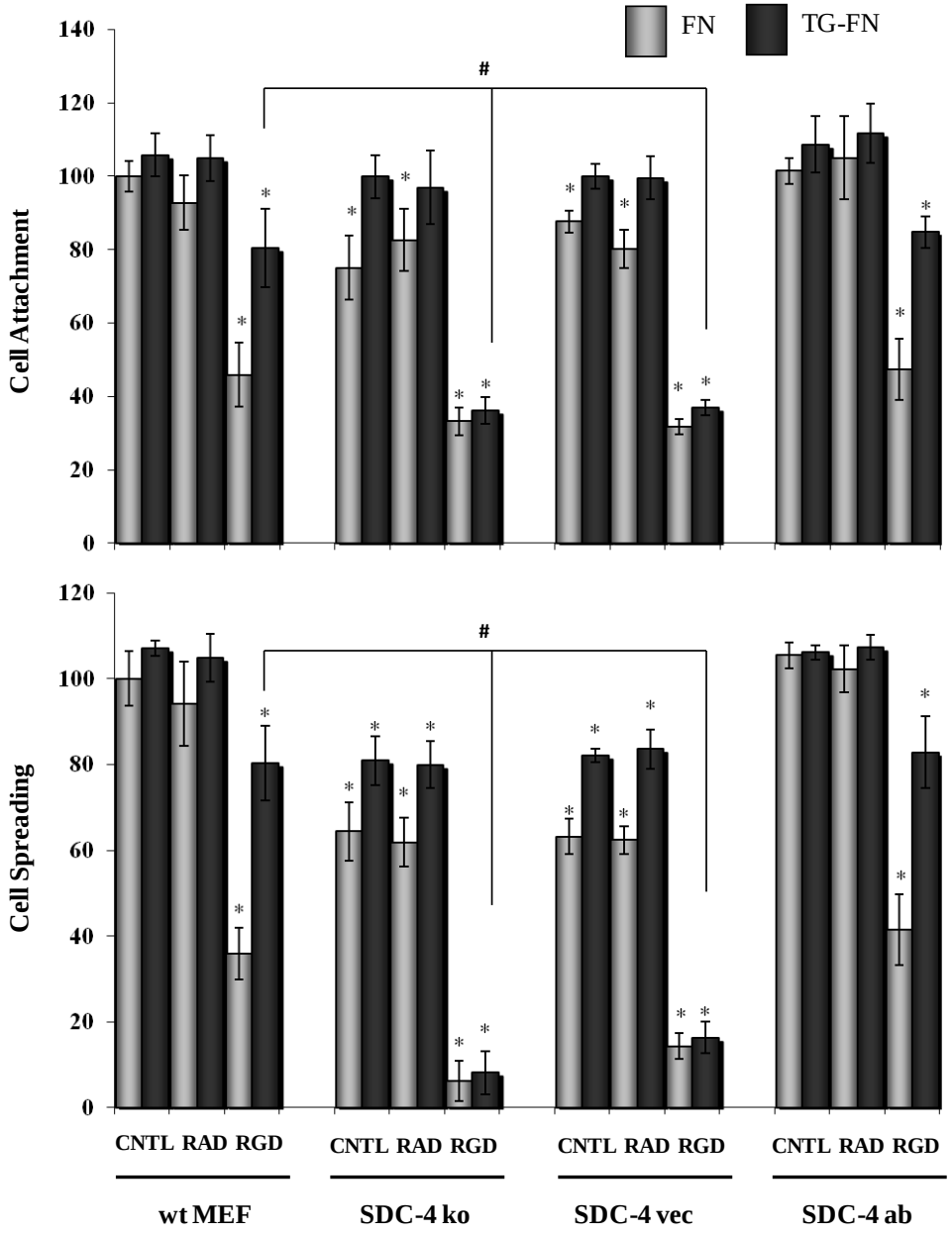



**Figure 3.3.12a**

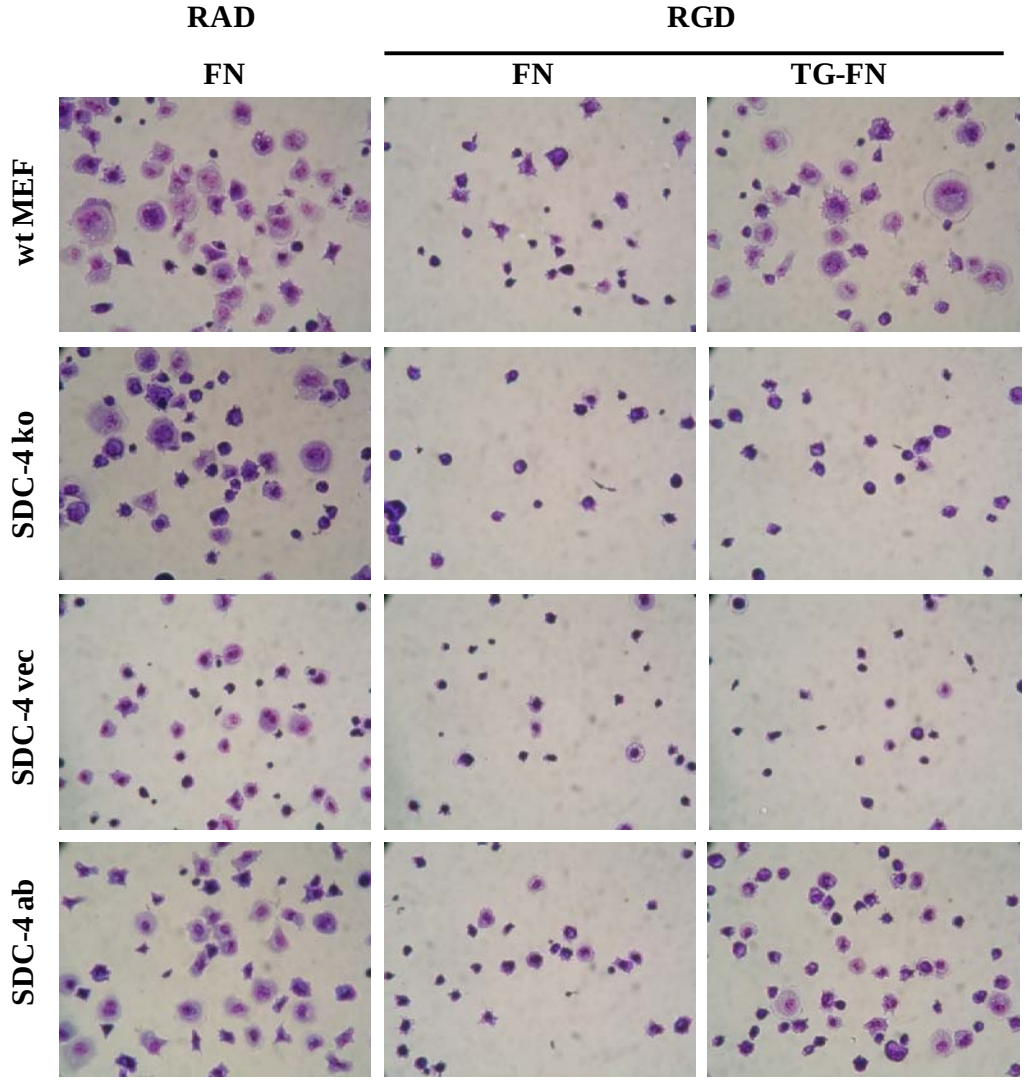



**Figure 3.3.12b**

### 3.3.4.2 TG-FN lost its compensatory effect on RGD-independent cell adhesion in syndecan-4 knock out MEF cells

Previous studies suggested the involvement of cell surface heparan sulfate chains in the RGD-independent cell adhesion process mediated by the TG-FN complex (Verderio, et al., 2003). As the most widely-expressed member of the heparan sulfate proteoglycan (HSPG) family, the potential involvement of syndecan-4 was investigated in the cell adhesion experiments by using syndecan-4 wild type (wt), knockout (ko), add-back (ab, syndecan-4 knockout cells transfected with human syndecan-4 cDNA) and empty vector control (vct syndecan-4 knockout cells transfected with vector cDNA) MEF cells. The cells were seeded on FN and TG-FN matrices following the blocking of the interaction between the integrins and FN via the RGD peptides. The cells were then allowed to attach at 37ºC in a 5% $CO_2$ atmosphere for 30 min.

These results showed that the attachment of these cells on FN was considerably reduced (about 70%), in the presence of 100μg/ml of RGD peptide. Since it has been shown in previous experiments that addition of exogenous peptides at 100μg/ml do not cause cell toxicity in MEF cells (Section 3.3.1.2), this part of the experiment was performed by using this concentration of the RGD peptides. As shown in Figure 3.3.12, the TG-FN complex compensated for the inhibitory effect of RGD peptides on the wild type MEF cells (back to 80% in cell attachment, and 75% in cell spreading, respectively) and in the add-back MEF cells (back to 111% and 82% respectively). In contrast, the cell attachment and spreading on the TG2 immobilised FN matrix was not restored in the RGD-treated knockout and vector control MEF (Figures 3.3.12a and b). In these two cells on both the FN and the TG-FN matrices with the absence of syndecan-4, the levels of cell attachment and spreading remained at around 30% and 10%, respectively, while no significant difference ($p>0.05$) was observed between these two matrices. These results indicated that syndecan-4 plays an important role as the cell receptor in the RGD-independent cell adhesion on the TG-FN matrix.

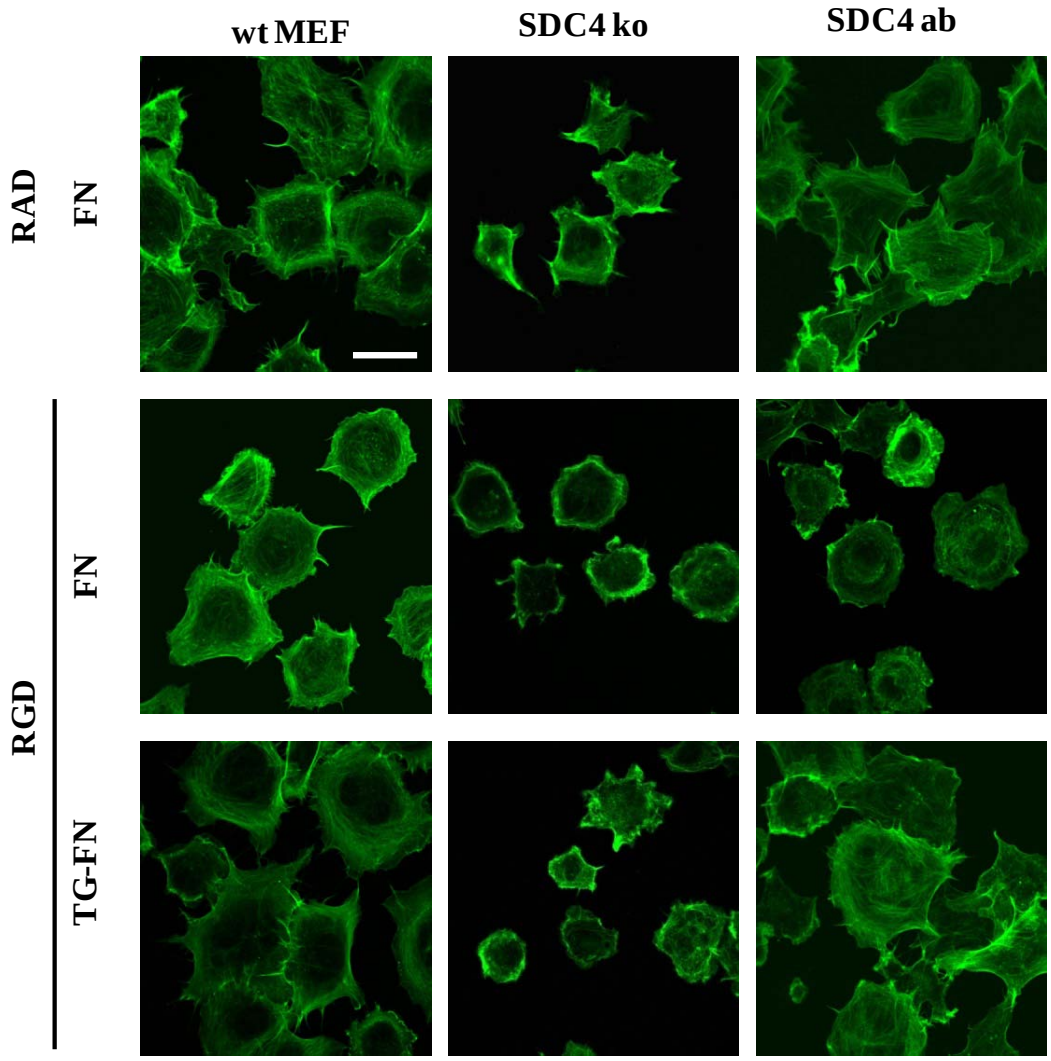



**Figure 3.3.13 The importance of cell surface syndecan-4 in actin stress fibre formation on FN-TG2 matrix.**
The cell adhesion assay was carried out and the attachment and spreading of wild type (wt), knock out (SDC4 ko), and add-back (SDC4 ab) MEF cells was analysed as described in Section 2.2.3. After 20-40 min incubation, the cells were fixed with 3.7% paraformaldehyde in PBS, pH7.4, and the actin stress fibre formation was detected by using FITC-labelled phalloidin (20μg/ml) as introduced in Section 2.2.4. Bar, 20 μm.

**Figure 3.3.13**

### 3.3.4.3 TG-FN failed to restore actin stress fibre formation in syndecan-4 null MEF cells

The importance of syndecan-4 in supporting actin stress fibre formation has been very widely reported (Woods, et al. 1986; Longley, et al. 1999; Woods, et al. 2000). To confirm the importance of syndecan-4 in the TG-FN mediated cell adhesion process in the presence of the RGD peptides, actin cytoskeleton staining was performed by using FITC-labelled phalloidin (Section 2.2.4) in the RAD or RGD peptides treated MEF cells. As shown in Figure 3.3.13, well-organized actin stress fibres were detected in wild type MEF cells seeded on both the FN matrix, while syndecan-4 null MEF cells exhibited a reduction in longitudinal stress fibres and formed stress fibre bundles that were more restricted to the cell periphery compared to the wild type MEF cells. The observed diffused actin cytoskeletal architecture induced by the RGD peptides treatment was only rescued in the wild type MEF seeded on the TG-FN matrix, not on the FN-only matrix, whereas syndecan-4 knockout cells failed to respond to the TG-FN complex. Once the human syndecan-4 molecule was re-introduced into the syndecan-4 knockout cells (in the syndecan-4 addback MEF cells), TG-FN re-obtained its compensatory effect on supporting actin stress fibre formation in the RGD-treated cells. These results indicated the importance of syndecan-4 in regulating actin stress fibre formation on the TG-FN matrix.

### 3.3.4.4 TG2 within the TG-FN complex directly interacts with syndecan-4

To investigate whether there is a direct interaction between matrix TG2 and cell surface syndecan-4, the co-immunoprecipitation assay was performed in the wild type, syndecan-4 knockout and syndecan-4 addback MEF cells. In order to block the cell binding domains within the FN molecules, the cells were seeded on the heparin-treated TG-FN matrix in the presence of the RGD peptides. Thereby only TG2 was left to mediate the potential interaction between the cells and the TG-FN complex. The immunoprecipitation of syndecan-4 by using syndecan-4 antibody followed by immunoblotting for TG2 revealed the presence of TG2 antigen in syndecan-4 wild type and add-back MEF cells, but not in syndecan-4

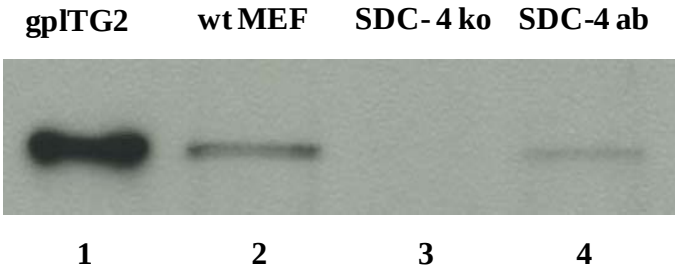



**Figure 3.3.14 Direct interaction between cell surface heparan sulfate and matrix TG2 within TG-FN complex.**
The RGD-independent cell adhesion assay was carried out by using syndecan-4 knockout MEF cells (Sdc4 ko), syndecan-4 add-back (Sdc4 ab) and wild type MEF cells (wt MEF). Cells were seeded on heparin-treated FN-TG2 matrix as explained in Section 2.2.2.3. After incubating the cells for 20-40 min, the cells were lysed in cell lysis buffer and co-immunoprecipitation assay was performed as described in Section 2.2.11 by using syndecan-4 antibody which detects the intracellular domain of syndecan-4 molecule. Western blotting assay was applied to detect TG2 antigen by using TG2 specific antibody Cub7402. Guinea pig liver TG was used as a marker.

**Figure 3.3.14**

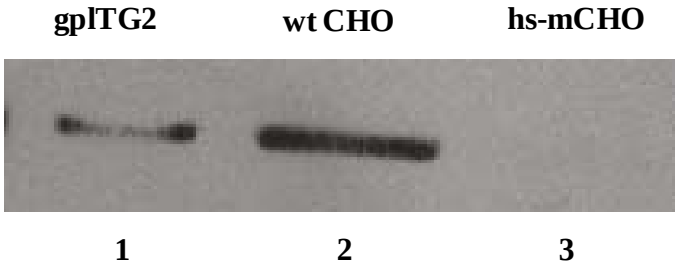



**Figure 3.3.15 The direct interaction between cell surface heparan sulfates of syndecan-4 and matrix TG2 within TG-FN complex.**
The RGD-independent cell adhesion assay was carried out by using heparan sulfate mutant CHO cells (hs-m CHO) and CHO wild type cells (wt CHO). Cells were seeded on heparin-treated FN-TG2 matrix as explained in Section 2.2.2.3. After incubating the cells for 20-40 min, the cells were lysed in cell lysis buffer and co-immunoprecipitation assay was performed by using specific anti-syndecan-4 antibody as introduced in Section 2.2.11. Western blotting assay was applied to detect TG2 antigen by using TG2 specific antibody Cub7402. Guinea pig liver TG was used as a marker.

**Figure 3.3.15**

null MEF cells on the TG-FN matrix (Figure 3.3.14), further confirming that the direct interaction between TG2 within the TG-FN complex and cell surface syndecan-4 .

### 3.3.4.5 The TG-FN matrix mediates RGD-independent cell adhesion by the direct interaction between the cell surface heparan sulfate chains of syndecan-4 and the matrix TG2

To confirm whether there is a direct interaction between the cell surface heparan sulfate chains and the matrix TG2, the co-immunoprecipitation assays were performed by using wild type CHO and heparan sulfate mutant CHO cells after seeding on a heparin pre-treated TG-FN matrix in the presence of the RGD peptides (Section 2.2.11), thus making sure that the heparin binding sites within the FN molecule was blocked by heparin and the interaction between the cell surface integrins and the FN within TG-FN complex was inhibited by the RGD peptides. Anti-syndecan-4 antibody (which detects the intracellular core protein of syndecan-4 molecules) was used in the co-immunoprecipitation assays to pull down the syndecan-4 immuno-complex. Western Blotting using mouse monoclonal anti-TG2 antibody (Cub7402) was applied to detect the presence of TG2 antigen in this complex. As shown in Figure 3.3.15, no TG2 was detected in the heparan sulphate mutant CHO cells seeded on the TG-FN matrix, unlike shown in the wild type CHO cells. These results indicated the importance of the heparan sulfate chains in mediating the interaction between the cells and the matrix bound TG2.

## 3.3.5 TG-FN mediated RGD-independent cell adhesion depends on the activation of the inside-out signalling pathway of β1 integrins

### 3.3.5.1 Detection of the presence of β1 integrin antigen in β1 integrin relevant MEF cells

To further confirm the presence or absence of β1 integrins in the wild type, β1 integrins knockout, add-back (ab, β1 integrins knockout cells transfected with

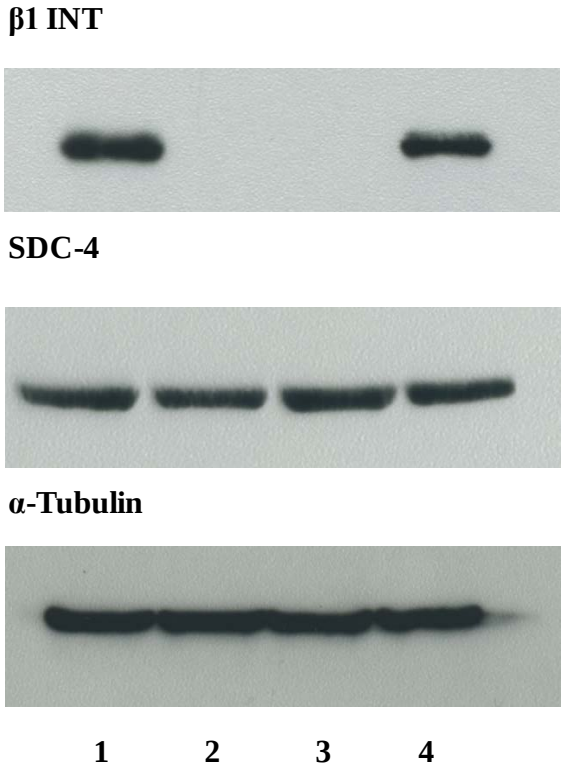



**Figure 3.3.16 Detection of β1 integrins and syndecan-4 in wild type, β1 integrins knock, β1 integrins vector control and β1 integrins addback MEF cells.**

Western blotting was performed to confirm the presence of β1 integrins and syndecan-4 in β1 integrins-relevant MEF cells (Section 2.2.9-2.2.10). The membranes were stripped and re-probed with anti-α-tubulin antibody to ensure the equal loading. Lane 1, wt MEF; Lane 2, β1 integrins ko MEF; Lane 3, β1 integrins vet MEF; Lane 4, β1 integrins ab MEF.

**Figure 3.3.16**


**Figure 3.3.17 The importance of cell surface β1 integrin in the RGD-independent cell adhesion process on TG-FN matrices.**
**a**, the RGD-independent cell adhesion assay was carried out and the attachment and spreading of wild type (wt MEF), knock out (INT1 ko), vector control (INT1 vet) and add-back (INT1 ab) MEF cells were analysed as explained in Section 2.2.3. The percentage of attached cell (cell attachment) or the percentage of spread cells (cell spreading) ± S.D shown are the mean values from three separate experiments performed in triplicate. The mean cell attachment and spreading values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D 147 ± 12, 146±7, and 131±3. The mean percentage spreading value ± S.D on FN was 77±1, 73±1, and 73±1 in the 3 experiments, and normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups. **b**, visualization of RGD peptide-treated β1 integrin relevant MEF cells seeded on FN or TG-FN matrices.

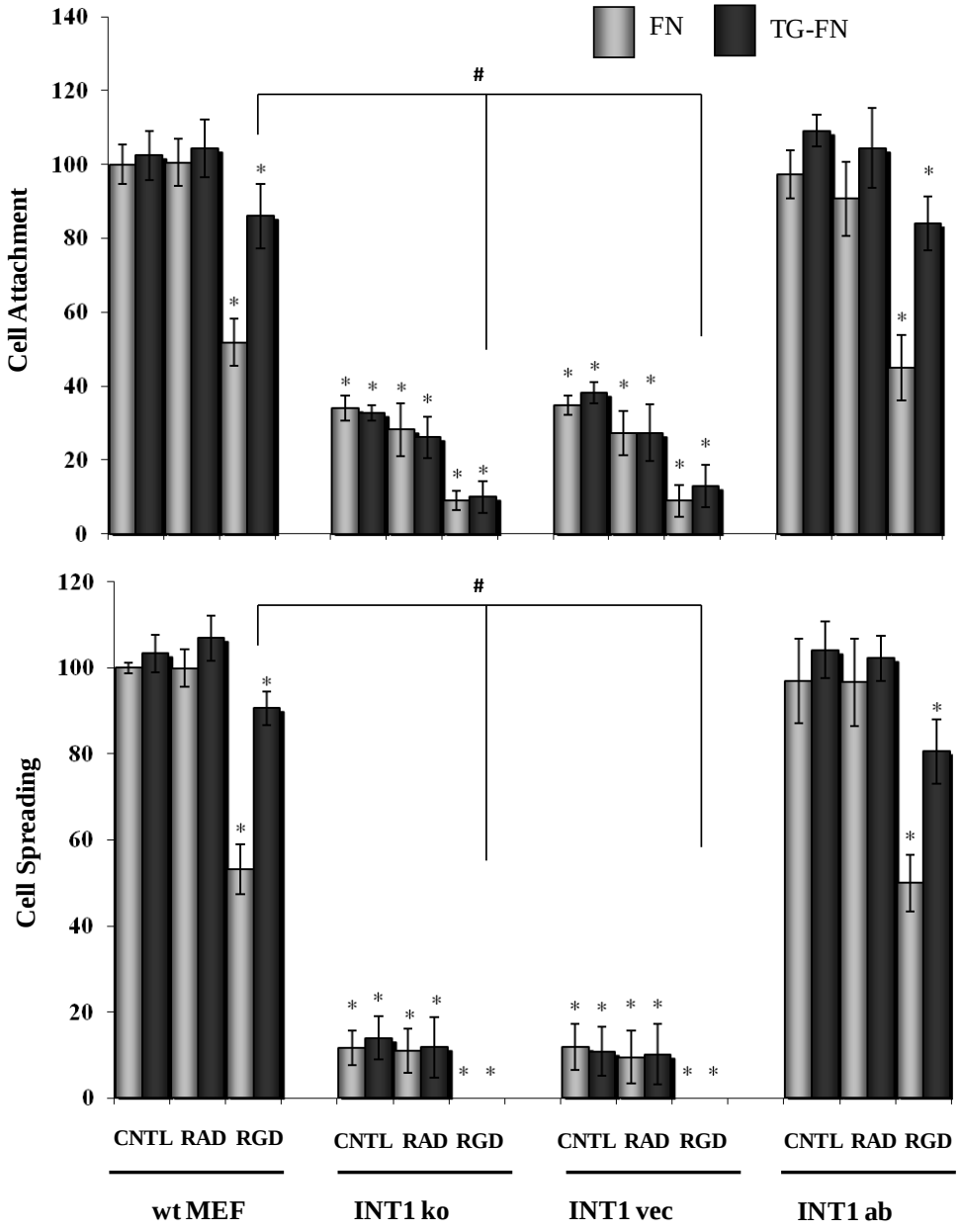



**Figure 3.3.17a**

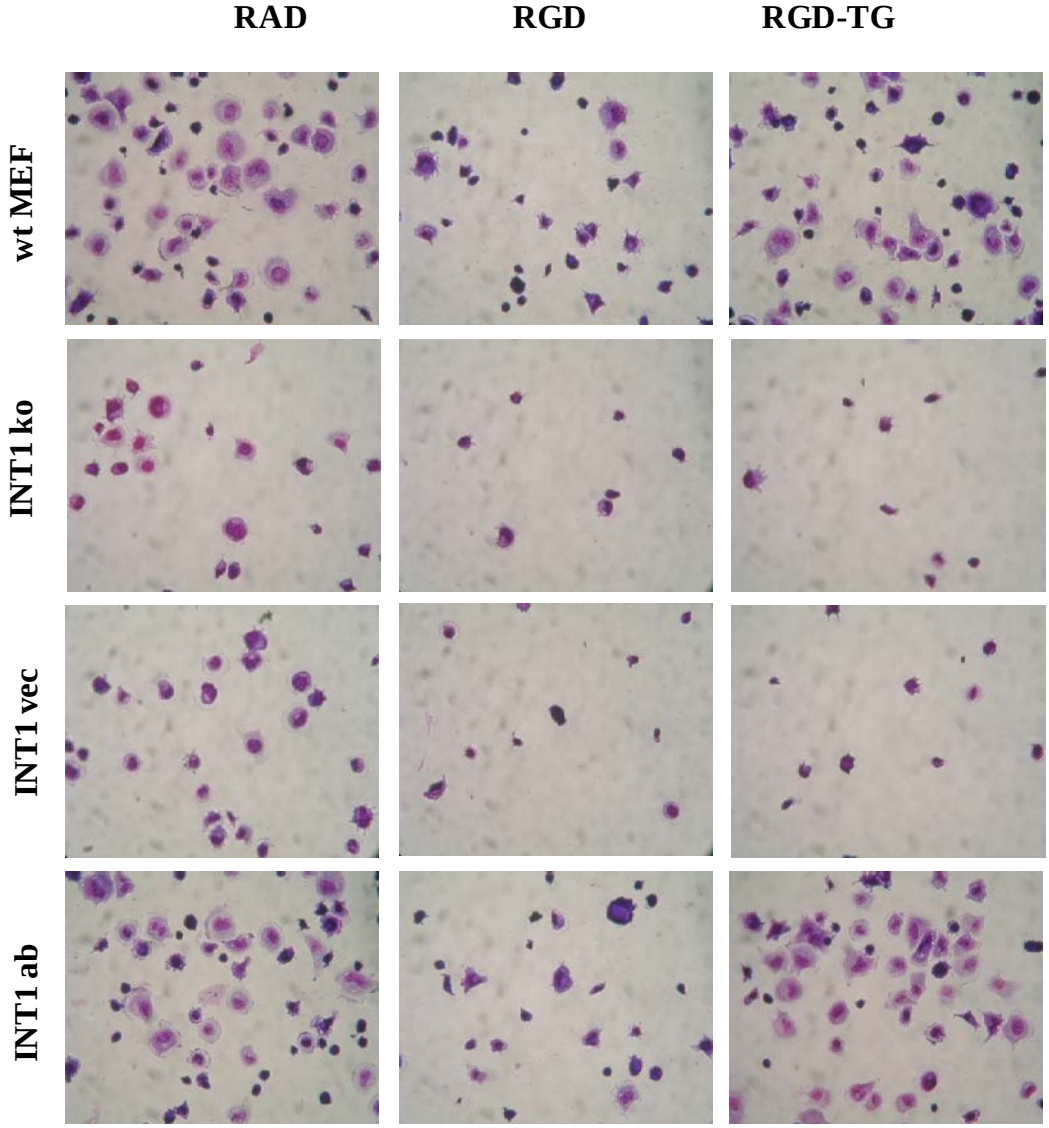



**Figure 3.3.17b**

human β1 integrins cDNA) and empty vector control (β1 integrins knockout cells transfected with empty vector cDNA) MEF cells, Western blotting was performed by using rabbit polyclonal anti-β1 integrins antibody (which recognizes both mouse and human β1 integrins). The expression of another major cell adhesion-related receptor syndecan-4 integrins was also detected using Western blotting. The membrane was stripped and re-probed with mouse anti-α-tubulin antibody to ensure the equal loading of the samples. As shown in Figure 3.3.16, no β1 integrins were detected in β1 integrins knockout and vector-control MEF cells, while the antigen was detected in the wild type and addback MEF cells. The presence of syndecan-4 antigens in all of the cells proved that the loss of β1 integrins did not affect the expression of syndecan-4.

### 3.3.5.2 β1 integrin is crucial for TG-FN to compensate the effect of the RGD peptides

The existence of a syndecan-4 and β1 integrin co-signalling pathway has been very well-reported (Couchman and Woods, 1999). Previous data indicated the role of syndecan-4 in mediating the RGD-independent cell adhesion on a TG-FN matrix. In order to investigate the involvement of β1 integrins in this process, the wild type, β1 integrin null MEF (β1 knockout), and β1 ko MEFs transfected back with the human β1 integrin cDNA (β1 addback MEF) and empty vector (β1 vector control MEF) were used in the cell adhesion assay in the presence of the RGD peptides.

In these cells, no significant difference ($p>0.05$) was found between the RAD peptides-treated and non-treated cells seeded on FN, suggesting that the exogenous peptides at 100μg/ml did not cause cell toxicity in these cell lines. The β1 knockout and vector control MEF cells displayed a 2-fold reduction in cell adhesion on the FN matrix compared to the wild type cells in a short-term adhesion assay (30 min). In the wild type MEF cells, the TG-FN complex restored the inhibition effect of the RGD peptides (100μg/ml) back to 90% on cell attachment, and 66% on cell spreading, respectively. In the β1 integrin add-back MEF cells, the RGD peptides reduced the cell attachment to 20% and the

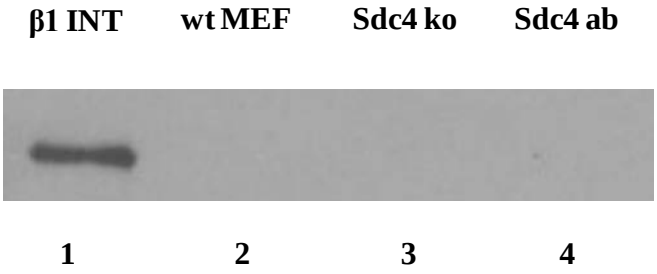



**Figure 3.3.18 No direct interaction between cell surface syndecan-4 and β1 integrins.**
The RGD-independent cell adhesion assay was carried out by using syndecan-4 knockout MEF cells (Sdc4 ko, Lane 3), syndecan-4 add-back (Sdc4 ab, Lane 4) and wild type MEF cells (wt MEF, Lane 1). Cells were seeded on heparin-treated FN-TG2 matrix as explained in Section 2.2.2.3. After incubating the cells for 20-40 min, the cells were lysed in cell lysis buffer and co-immunoprecipitation assay was performed as introduced in Section 2.2.11 by using syndecan-4 antibody which detects the intracellular domain of the syndecan-4 molecule. Western blotting assay was applied to detect β1 integrin antigen by using a specific anti-β1 integrin antigen antibody. Cell lysate with β1 integrins (β1 INT) was used as the standard (Lane 1).

**Figure 3.3.18**

spreading to 58%, which were compensated by the TG-FN complex back to 51% in cell attachment and 92% in cell spreading. In contrast, cell attachment and spreading on the TG2 immobilised FN matrix was not compensated in the β1 integrin ko and vector control MEF cells in the presence of different concentrations of the RGD peptide, which was about 5% in cell attachment, and 0% in cell spreading (Figure 3.3.17). This data strongly suggested that β1 integrins play a crucial role in the RGD-independent adhesion in the response to the TG-FN matrix.

#### 3.3.5.3 No direct interaction between syndecan-4 and β1 integrin was detected in the cells seeded on TG-FN

In order to rule out the possible interaction between cell surface syndecan-4 and β1 integrin during the cell adhesion process on TG-FN in the presence of the RGD peptides, co-immunoprecipitation assays were carried out by using the RGD peptides-treated wt, syndecan-4 knockout and addback MEF cells seeded on heparin-blocked TG-FN matrices. An anti-syndecan-4 antibody that detects the intracellular domain of the syndecan-4 molecule was used in the immunoprecipitation procedure and an anti-β1 integrin antibody was applied to detect the presence of β1 integrins within the immuno-complex. As shown in Figure 3.3.18, western blotting of the precipitates and probing with β1 integrin antibody revealed that no β1 integrins could be detected, suggesting that syndecan-4 does not interact directly with β1 integrin.

### 3.3.6 TG-FN compensates the RGD-induced loss of cell adhesion in an α5 integrin-dependent manner

It has been reported that among the integrin family, α5 integrins present the closest relationship with β1 integrins in FN-mediated cell adhesion. Previous results suggested the crucial role of β1 integrin in RGD-independent cell adhesion mediated by TG-FN. In order to investigate the role of α5 integrins in this process, mouse embryo cell EA5 and its control EA5/α5 (EA5 cells transfected with human α5 integrin cDNA) were used in the cell adhesion assay

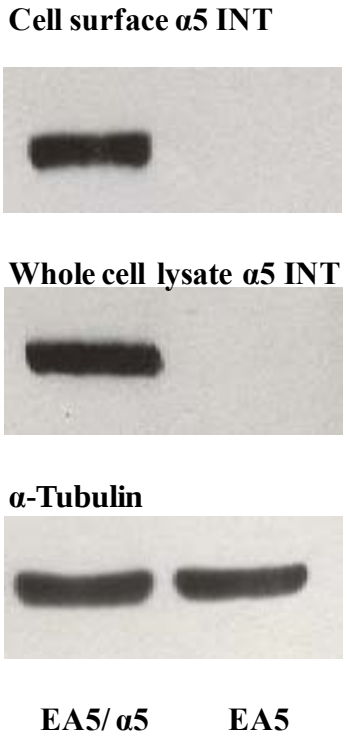



**Figure 3.3.19 Detection of α5 integrins in EA5 and EA5/α mouse embryo cells.**
Western blotting was performed to confirm the presence of α5 integrins in both whole cell lysates (the middle panel) and biotin-labelled cell surface proteins (the top panel) (Section 2.2.14) of EA5/α5 and EA5 mouse embryo cells (Section 2.2.9-2.2.10). The membrane was re-probed with anti-α-Tubulin antibody to ensure the equal loading (the bottom panel).

**Figure 3.3.19**


**Figure 3.3.20 The crucial role of cell surface α5 integrins in RGD-independent cell adhesion process on TG-FN matrices.**
a, the RGD-independent cell adhesion assay was carried out on the attachment and spreading of EA5/α5 and EA5 mouse embryo cells and analysed as explained in Section 2.2.3. The percentage of attached cell (cell attachment) or the percentage of spreading cells (cell spreading) ± S.D. shown are the mean values from three separate experiments performed in triplicate. The mean cell attachment number of values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D. The mean number of cell attachment ± S.D. of control taken from 3 experiments was 155 ± 3. The mean percentage spreading value ± S.D. on FN was 73±2 in the 3 experiments, and normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups. **b**, visualization of RGD peptide-treated α5 integrin relevant MEF cells seeded on FN or TG-FN matrices.

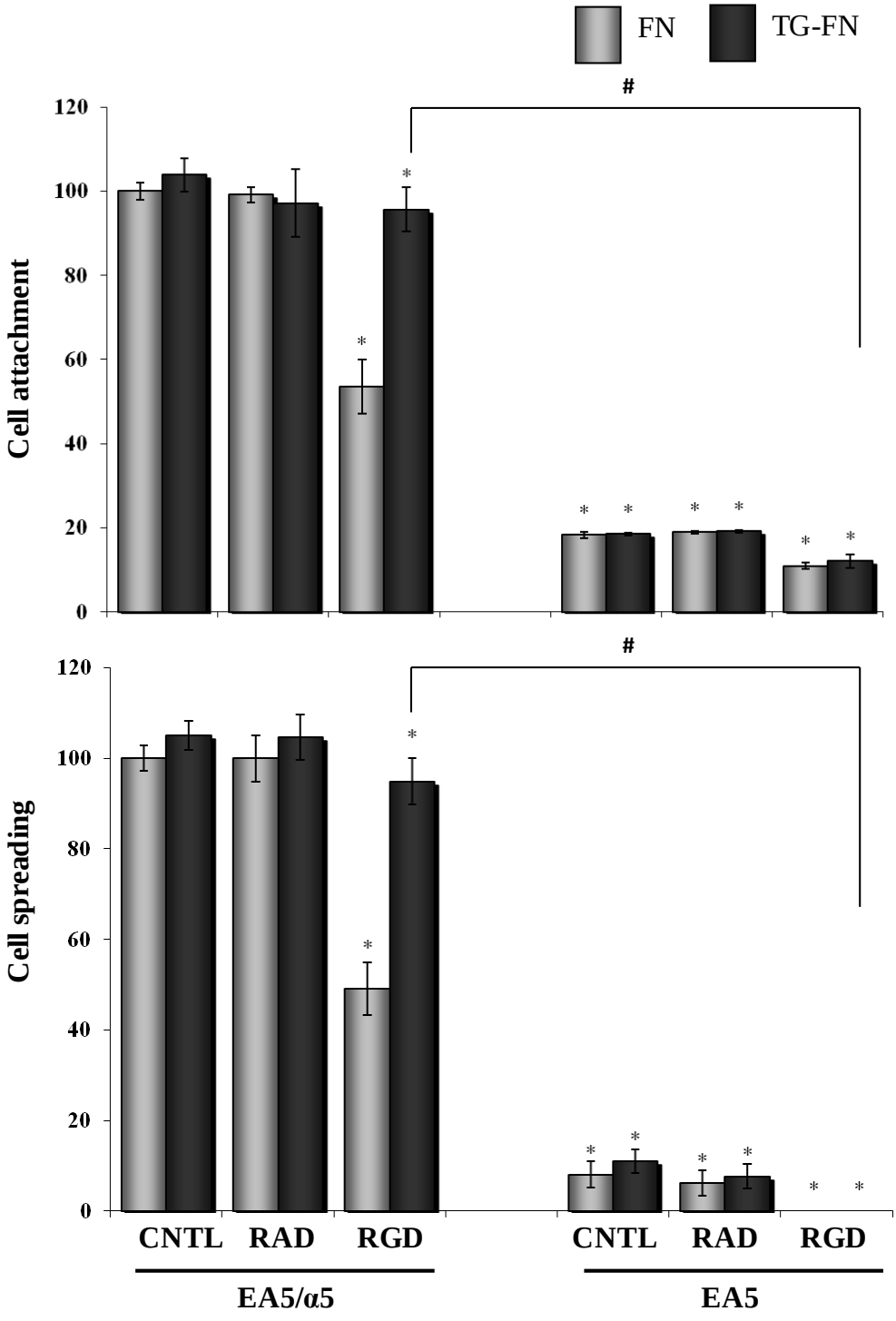



**Figure 3.3.20a**

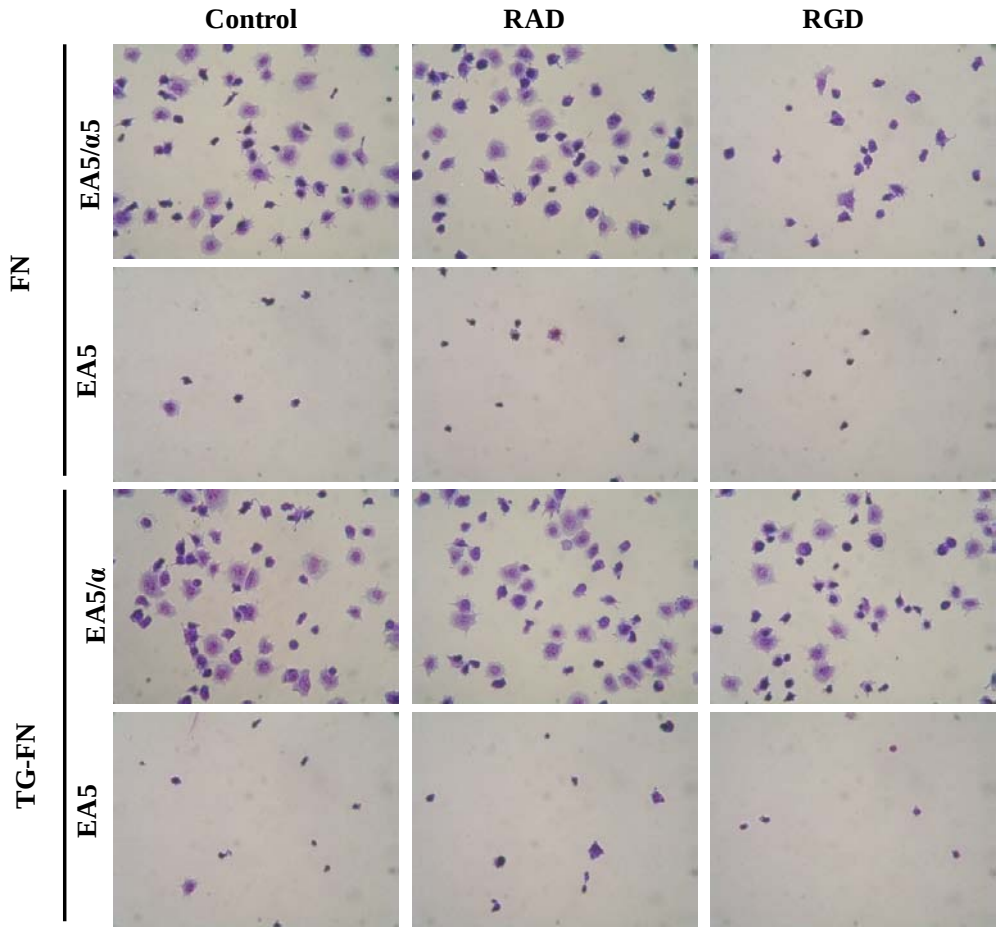



**Figure 3.3.20b**

(Huveneers et al., 2008). To confirm the presence or absence of α5 integrins in EA5 and EA5/α5 cells, Western blotting was performed by using rabbit anti-α5 polyclonal antibody to detect the presence of α5 integrins in whole cell lysates or on cell surface via biotinylation, while α-tubulin was used as the standard of equal loading. As shown in Figure 3.3.19, α5 integrins were only detected in EA5/ α5 cells with re-transfected human α5 integrins, while no α5 integrins could be found in EA5 cells in both cell lysates and cell surface.

Embryo cells are considered fibroblast-like cells, while unlike the EA5/α5 control cells, EA5 cells maintained a round morphology on the FN matrix, which proved the importance of α5 integrins in focal adhesion assembly and in actin stress fibre formation (Huveneers et al., 2008). There is no significant difference ($p>0.05$) observed between non- and RAD-treated (100μg/ml) cells, suggesting that the effect of RGD on cell adhesion is not caused by its toxicity. Like in MEF fibroblasts, TG-FN compensated the RGD-induced loss of the cell attachment and spreading in EA5/α5 cells (53% and 49% on FN matrix, respectively) back to normal level (95% and 94%, respectively). Without α5 integrins, EA5 cells only achieved 18% of cell attachment and 7% of spreading when seeded on FN, compared to EA5/α5 cells. The RGD peptide treatment significantly abolished the cell attachment and spreading and decreased their level to 10% and 0% of the control group (Figure 3.3.20). TG-FN failed to restore these losses and no statistical significance was obtained between the groups of RGD-treated EA5 cells seeded FN and TG-FN matrices, which suggested the cell adhesion process mediated by TG-FN depends on the presence of cell surface α5 integrins.

### 3.3.7 The compensatory effect of TG-FN in cell adhesion in the presence of RGD peptide is blocked by the treatment of the α5β1 integrin inhibiting peptide—A5-1

To further confirm the inhibiting effect of the α5β1 integrin inhibitor, a specific inhibiting peptide, A5-1, against this receptor was applied in the cell adhesion assay. This peptide inhibited the cell adhesion on the FN matrix in a dose-dependent manner, and as the concentration of the peptide increased, its blocking


**Figure 3.3.21 The inhibition of cell surface α5β1 integrins by its specific targetting peptide abolishes the compensatory effect of TG-FN on RGD-independent cell adhesion.**

A range of different concentrations of a specific α5β1 integrin targetting peptide A5-1 was used to block the cell adhesion process mediated by these integrins was used in the cell adhesion assay as explained in Section 2.2.3. The RGD and RAD peptides were used as the control treatments, while DMSO was used as the vehicle control The percentage of attached cell (cell attachment) or the percentage of spread cells (cell spreading) ± S.D shown are the mean values from three separate experiments performed in triplicate. The mean cell attachment and spreading values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D. The mean number of cell attachment ± S.D. of control taken from 3 experiments was 144 ± 7. The mean percentage spreading value ± S.D on FN was 82 ± 1 in the 3 experiments, and normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups.

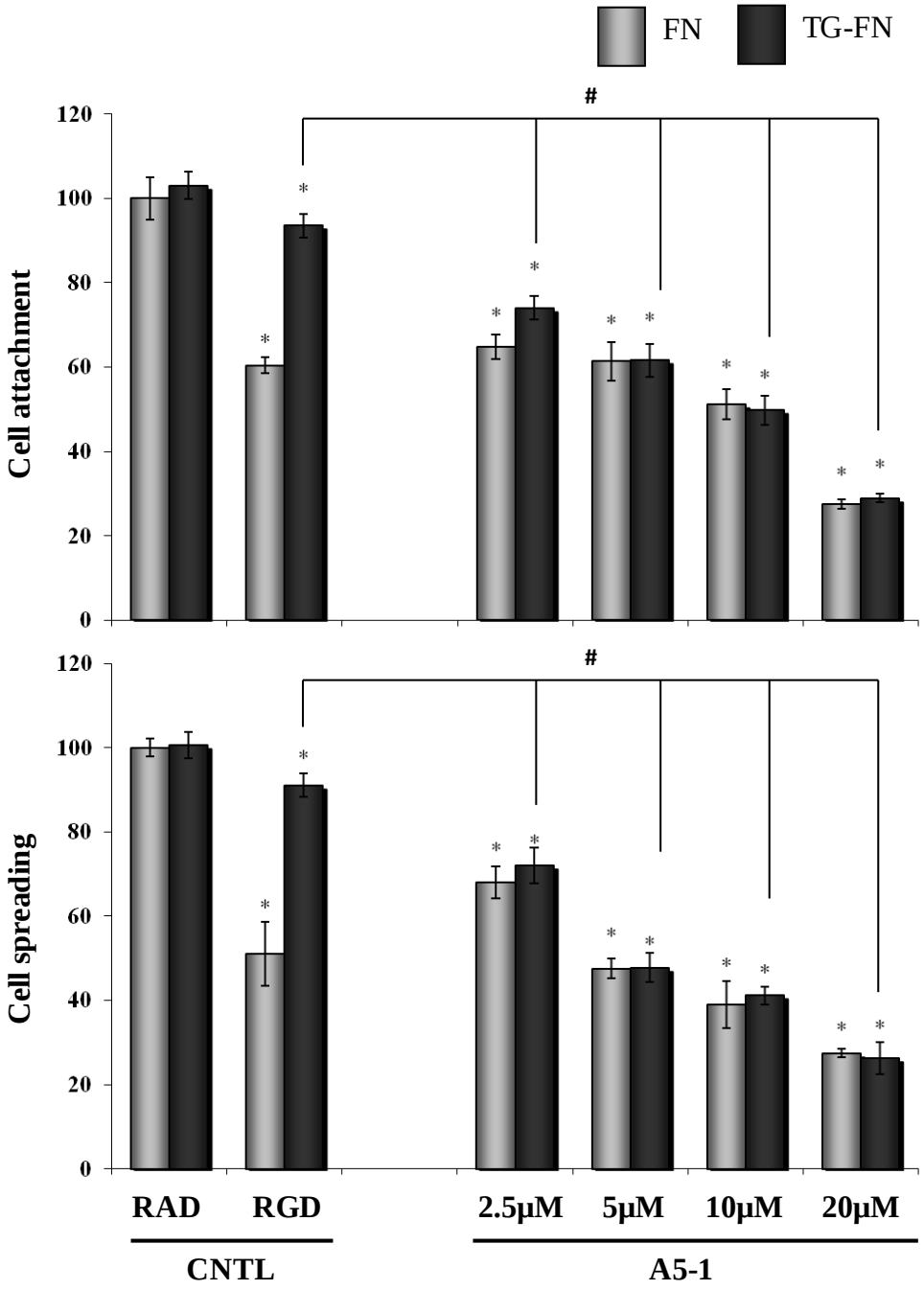

FN
TG-FN
Cell attachment
Cell spreading
#
120
100
80
60
40
20
0
RAD
RGD
2.5µM
5µM
10µM
20µM
CNTL
A5-1

**Figure 3.3.21**

effect on TG-FN mediated cell adhesion was significantly increased. As shown in Figure 3.3.22, at the concentration of 5μM, A5-1 blocked 60% of both cell attachment and spreading on FN, and TG-FN could not restore the loss of cell adhesion, suggesting that the compensatory effect of TG-FN was significantly inhibited by the peptide treatment. This inhibition effect by the A5-1 peptide confirmed the involvement of α5β1 integrin in RGD-independent cell adhesion on the TG-FN matrix.

### 3.3.8 Syndecan-2 is required in the RGD-independent cell adhesion mediated by TG-FN

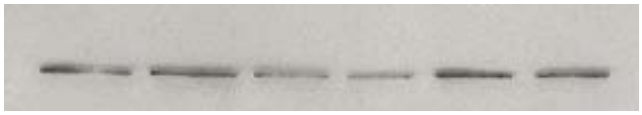
#### 3.3.8.1 The silencing of syndecan-2 by siRNA treatment inhibited the compensatory effect of TG-FN on the RGD-induced loss of cell adhesion

##### 3.3.8.1.1 The inhibition effect of syndecan-2 targetting siRNA on syndecan-2 expression

Syndecan-2 specific targeting siRNAs was used to inhibit the expression of syndecan-2 in MEF cells as introduced in Section 3.2.3. Universal negative control siRNA and scrambled siRNAs designed according to the syndecan-2 specific siRNAs were used as the control treatments. After a 30 h incubation with the siRNAs, the cell lysates were collected into cell lysis buffer and Western blotting was performed to detect the presence of syndecan-2 antigen in the cell lysates (Sections 2.2.6-2.2.10). As shown in Figure 3.3.22, no effect of the control siRNAs, including non-silencing siRNA and the scrambled siRNAs was found on the expression of syndecan-2. The SDC-2 siRNA1 and 2 significantly inhibited the expression of syndecan-2 down to around 50%.

##### 3.3.8.1.2 The knocking down of syndecan-2 expression did not affect the expression of syndecan-4 and β1 integrins

Before further investigation of the effect of syndecan-2 in the TG-FN mediated cell adhesion, the effect of the SDC-2 siRNAs on the expression of the other two



**1 2 3 4 5 6**

**Figure 3.3.22 Detection of the effect of the syndecan-2 siRNAs on syndecan-2 expression in MEF cells.**

Syndecn-2 siRNAs targetting mouse syndecan-2 were used to inhibit the expression of syndecan-2 in MEF cells (Section 4.2.3). After a 30 h incubation, the cells were collected into cell lysis buffer and Western blotting was performed to detect the presence of the syndecan-2 antigen within the samples by using the rabbit polyclonal anti-syndecan-2 antibody. The universal negative siRNA and the scrambled siRNAs designed according to the sequences of the syndecan-2 siRNAs were used as the negative treatments. Lane 1, non-treated MEFs; Lane 2, non-silencing siRNA-treated MEFs; Lane 3, SDC-2 siRNA1-treated cells; Lane 4, SDC-2 siRNA2-treated cells; Lane5, scrambled siRNA1-treated MEF cells; and Lane 6, scrambled siRNA2-treated MEF cells.

**Figure 3.3.22**

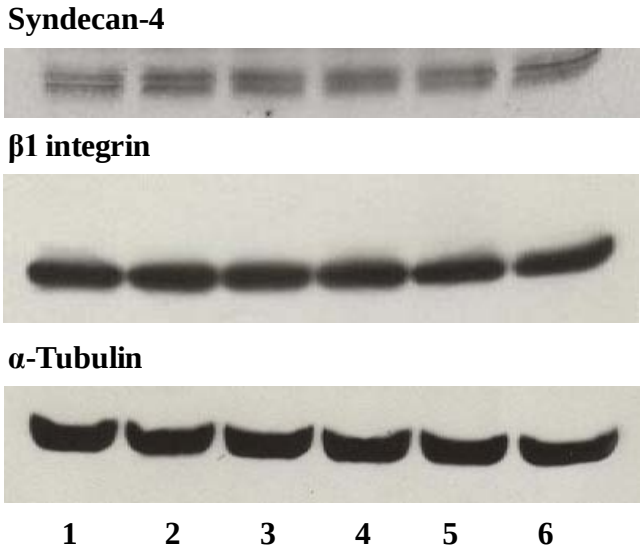



**Figure 3.3.23 Detection of the effect of the syndecan-2 siRNAs on syndecan-4 and β1 integrin expression in MEF cells.**
Syndecan-2 siRNAs targetting mouse syndecan-2 were used to inhibit the expression of syndecan-2 in MEF cells (Section 4.2.3). After a 30 h incubation, the cells were collected into cell lysis buffer and Western blotting was performed to detect the presence of the syndecan-4 and β1 integrin antigens within the samples by using the specific antibodies. The universal negative siRNA and the scrambled siRNAs designed according to the sequences of the syndecan-2 siRNAs were used as the negative treatments. α-Tubulin expression levels in the samples were used as the equal loading standards. Lane 1, non-treated MEFs; Lane 2, non-silencing siRNA-treated MEFs; Lane 3, SDC-2 siRNA1-treated cells; Lane 4, SDC-2 siRNA2-treated cells; Lane5, scrambled siRNA1-treated MEF cells; and Lane 6, scrambled siRNA2-treated MEF cells.

**Figure 3.3.23**

crucial cell surface receptors in this process, syndecan-4 and β1 integrins, was measured by using Western blotting. As shown in Figure 3.3.23, no difference of the expression levels of both syndecan-4 and β1 integrins were detected between the non-treated cells, the control siRNA groups and the syndecan-2 targetting siRNAs treated cells, which confirms the specificity of these SDC-2 siRNAs and provides the possibility for the application of the siRNAs in the cell adhesion assay.

#### 3.3.8.1.3 TG-FN compensates RGD-induced loss of cell adhesion in a syndecan-2-dependent manner

It has been reported that syndecan-2 is the co-receptor in syndecan-4 signalling (Whiteford et al., 2007). In order to investigate the effect of syndecan-2 on the RGD-independent cell adhesion on TG-FN, syndecan-2 targetting siRNAs, SDC-2 siRNA1 and 2, were used to inhibit protein expression and then the cell adhesion assay was performed by using the siRNA-treated cells. The universal negative control siRNA and the scrambled siRNAs were used as controls. As shown in Figure 3.3.24, no significant difference ($p>0.05$) in the cell attachment and spreading was discovered in the negative control siRNAs treated cells in both FN groups, compared to the non-treated cells, suggesting non toxicity of the siRNA treatment in the MEF cells. No further difference was found in the RAD peptide-treated and non-treated cells on FN in the control siRNA-treated cells, which showed no specific toxic effect of RAD peptide to the siRNA-treated cells. These data made sure that the future difference in the cell attachment and spreading will be due the loss of syndecan-2 expression and the RGD peptide-treatment, but not the toxicity from either the siRNA treatment or the peptides TG-FN restored the loss of the cell attachment and spreading caused by the RGD peptides back to over 90% in all of the control groups and no significant difference ($p>0.05$) was found between these groups. With the inhibition of syndecan-2 expression, the cells lost about 25% in cell attachment and 60% of cell spreading on the FN matrix, which could not be compensated by TG-FN. In the presence of RGD peptide, further 30% loss of the cell attachment and 20% of spreading was observed on both the FN and the TG-FN matrices. These results


**Figure 3.3.24 The importance of cell surface syndecan-2 in RGD-independent cell adhesion process on TG-FN matrices.**
**a**, Syndecn-2 siRNAs targetting mouse syndecan-2 treated MEF cells (30 h incubation) (Section 4.2.3)were used in the RGD-independent cell adhesion assay (Section2.2.3). The universal negative siRNA and the scrambled siRNAs designed according to the sequences of the syndecan-2 siRNAs were used as the negative treatments. The mean cell attachment and spreading values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D. The mean number of cell attachment ± S.D. of control taken from 3 experiments was 157 ± 3. The mean percentage spreading value ± S.D on FN was 74 ± 5 in the 3 experiments, and normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups. **b**, visualization of syndecan-2 siRNAs and the control siRNA-treated MEF cells seeded on FN or TG-FN matrices in the presence of RGD peptide.

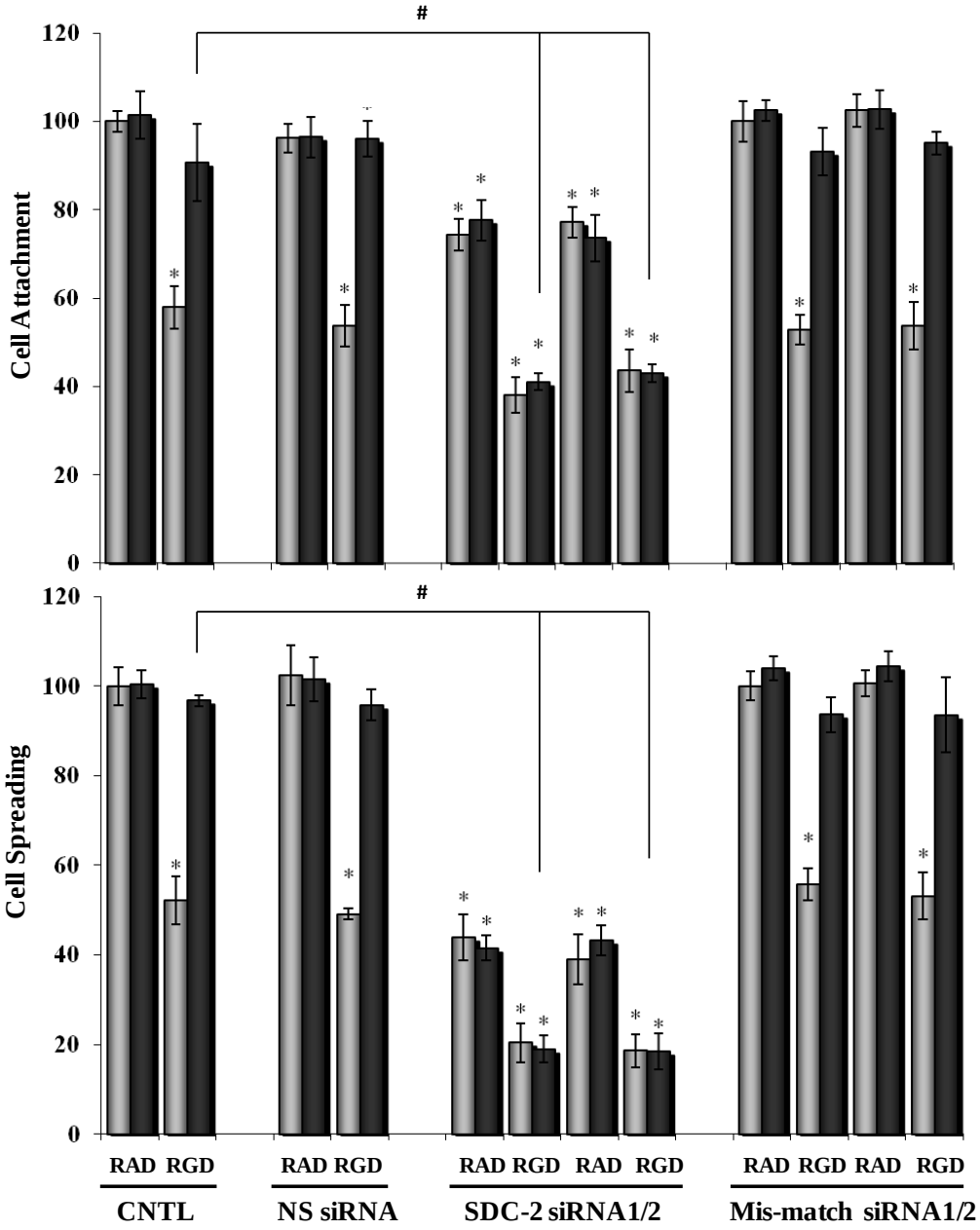



**Figure 3.3.24a**

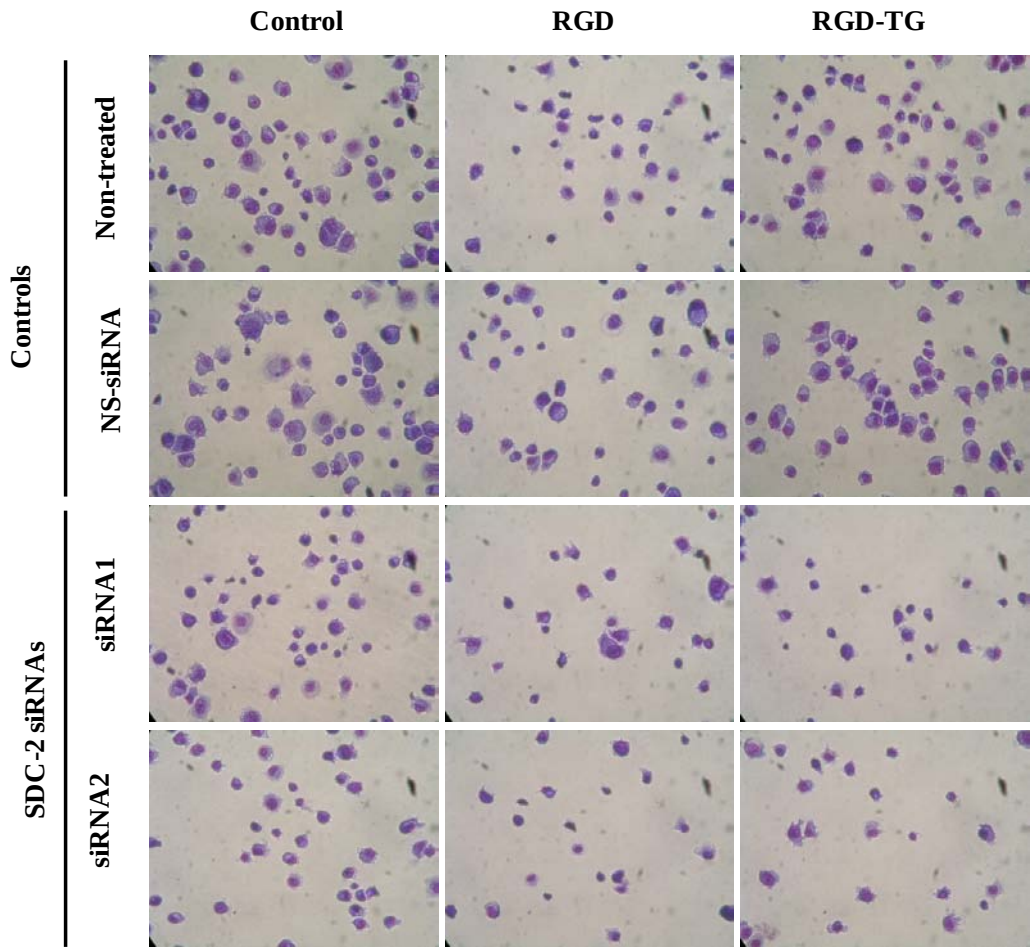



**Figure 3.3.24b**

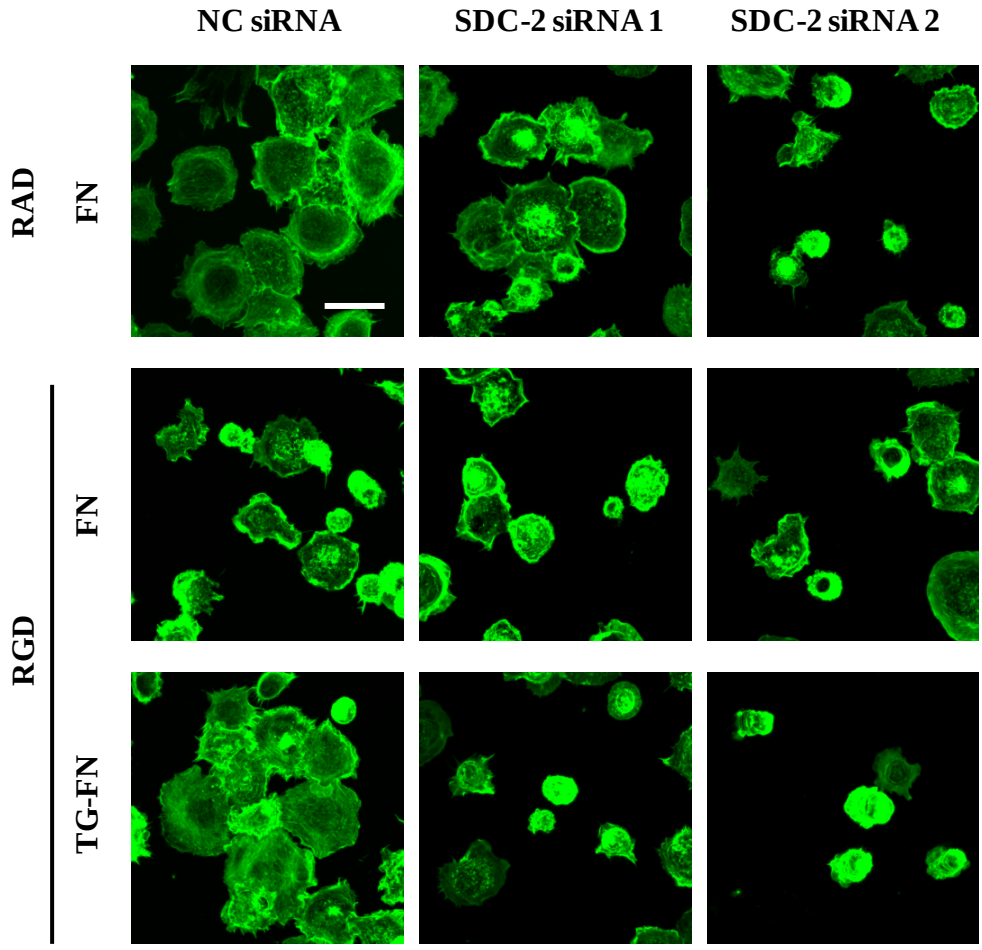



**Figure 3.3.25 The importance of syndecan-2 in actin cytoskeleton formation on TG-FN matrix.**

The cell adhesion assay were carried on by using syndecan-2 targetting siRNA-treated MEF cells (Section 4.2.3) as explained in Section 2.2.3, while the universal negative control (NC) siRNA was used as the control treatment. After 20-40 min incubation, the cells were fixed with 3.7% paraformaldehyde in PBS, pH7.4, and the actin stress fibre formation was performed by using FITC-labelled phalloidin (20μg/ml) as described in Section 2.2.4. Bar, 20 μm.

**Figure 3.3.25**

suggest the involvement of syndecan-2 in the RGD-independent cell adhesion mediated by TG-FN.

### 3.3.8.2 Syndecan-2 is crucial for TG-FN to exert its function in actin cytoskeleton formation

The role of syndecan-2 in actin stress fibre formation has been demonstrated and its involvement in TG-FN signalling pathway was suggested by cell adhesion experiments (Section 3.3.8.2). In order to further investigate the role of syndecan-2 in TG-FN regulated actin cytoskeleton formation, the actin stress fibre was detected via immunofluorescence staining in syndecan-2 targetting siRNA-treated MEF cells, and the non-treated, universal negative control siRNA and the scrambled siRNAs were used as the control groups (Section 3.2.2). As shown in Figure 3.3.25, after 1 h incubation, well-organized actin fibres were detected in the control groups on both FN and TG-FN matrices in the presence of RAD peptide. Once treated with RGD peptide, the cells seeded on FN lose their longitudinal stress fibres, which were restored in the cells plated on TG-FN matrix. The loss of syndecan-2 induced a diffused actin cytoskeletal architecture on FN, and with the reduced expression of syndecan-2, the cells failed to respond to the TG-FN matrix in the presence of the RGD peptides. This work indicates the importance of syndecan-2 in regulating actin cytoskeleton organization, which is crucial for TG-FN to exert its compensation effect on the RGD-induced cell adhesion and actin stress fibre formation.

## 3.3.9 β3 integrins are not required by TG-FN to compensate the effect of RGD peptide on cell adhesion

Even though β1 integrins have been proven to be the major cell surface integrins that mediate cell adhesion on FN, accumulating research indicates that β3 integrins are also involved in this process (Switala-Jelen et al., 2004). In order to study the potential involvement of β3 integrin in the TG-FN matrix model of this


**Figure 3.3.26 The β3 integrins are not required by TG-FN to mediate RGD-independent cell adhesion.**
The RGD-independent cell adhesion assay was carried out and the attachment and spreading of β3 wild type and knockout MEF cells was analysed as explained in Section 2.2.3. The percentage of attached cell (cell attachment) or the percentage of spread cells (cell spreading) ± S.D shown are the mean values from three separate experiments performed in triplicate. The mean cell attachment and spreading values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D. The mean number of cell attachment ± S.D. of control taken from 3 experiments was 158 ± 6. The mean percentage spreading value ± S.D on FN was 80 ± 2 in the 3 experiments, and normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN.

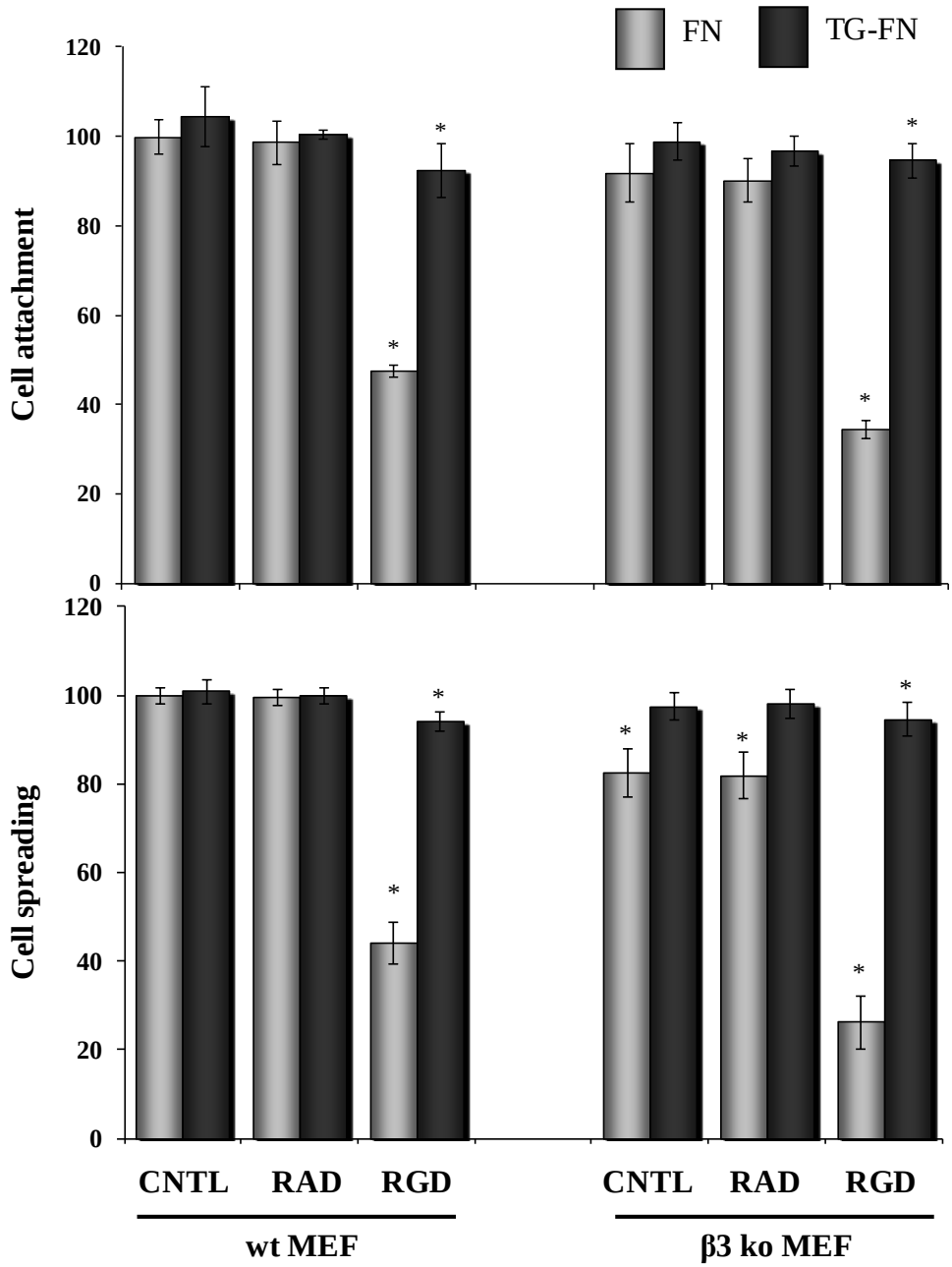

FN
TG-FN
Cell attachment
Cell spreading
120
100
80
60
40
20
0
CNTL
RAD
RGD
CNTL
RAD
RGD
wt MEF
β3 ko MEF

**Figure 3.3.26**

project, β3 integrin knockout (ko) mouse embryo fibroblasts and its wild type control cells were used in cell adhesion assays. As shown in Figure 3.3.26, in wild type MEF cells, RGD treatment led to 50% and 55% loss of the cell attachment and spreading respectively, which was restored to 92% and 94% respectively by the TG-FN complex. During long-term (above 60 min) tissue culture, no significant morphological difference was observed in β3 ko cells compared to the wild type MEF cells. In a short-term incubation on the FN matrix (30 min), even though no significant difference was found in the cell attachment between the wild type and knockout cells, the knockout cells lost ~20% loss of spreading ($p<0.05$) compared to the wild type cells, confirming the involvement of β3 integrins in cell adhesion on FN. No significant difference was observed when both of the cells were seeded onto the TG-FN matrix, when compared to the wild type cells on the FN matrix. In the presence of RGD peptide, β3 ko cells lost 66% and 74% of the cell attachment and spreading, respectively on FN, while TG-FN compensated the effect of the RGD peptides back to 94%, in which no significant difference was found compared to the RGD-treated wild type cells on TG-FN. This suggests that β3 integrins are not required for TG-FN to exert its compensatory effect on the RGD-induced loss of cell adhesion.

### 3.3.10 α4β1 integrins are not involved in the signalling transduction mediated by TG-FN

It has been reported that TG2 can be involved in an α4β1 integrin-related cell adhesion process where it acts as a cell suface corecepto for FN. Hence in order to investigate the role of α4β1 integrin in RGD-independent cell adhesion on TG-FN, rat anti-mouse α4β1 integrin functional blocking antibody-treated MEF cells were used in cell adhesion assays, while its isotype control antibody was used as a negative treatment. In Figure 3.3.21, at the concentration of 25μg/ml, the isotype control antibody did not affect the cell adhesion process on FN, compared to the non-treated cells, suggesting no toxicity of these antibodies to the MEF cells at this concentration. In the experimental groups, no significant difference was found between the functional blocking antibody treated cells on

FN and the isotype control antibody, suggesting that α4β1 integrin is not crucial in the cell adhesion process on FN. No enhanced or reduced cell adhesion and spreading was found in the cells on TG-FN in the absence or presence of RGD peptide, which indicates that α4β1 integrin is not required by TG-FN to exert its compensatory effect on RGD-induced loss of cell adhesion.


**Figure 3.3.27 TG-FN compensates RGD-independent cell adhesion in α4β1 integrin-independent manner.**
The RGD-independent cell adhesion assay was carried out by using rat anti-mouse α4β1 integrin functional blocking antibody (clone 9C10) and its isotype control antibody (Isotype Ab)-treated MEF cells as described in Section 2.2.3. The percentage of attached cell (cell attachment) or the percentage of spread cells (cell spreading) ± S.D. shown are the mean values from three separate experiments performed in triplicate. The mean cell attachment and spreading values were expressed as the percentage of control values of non-treated (control) cell seeded on FN (which represents 100%) ± S.D. The mean number of cell attachment ± S.D. of control taken from 3 experiments was 139 ± 3. The mean percentage spreading value ± S.D. on FN was 80 ± 1 in the 3 experiments, and normalised to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN.

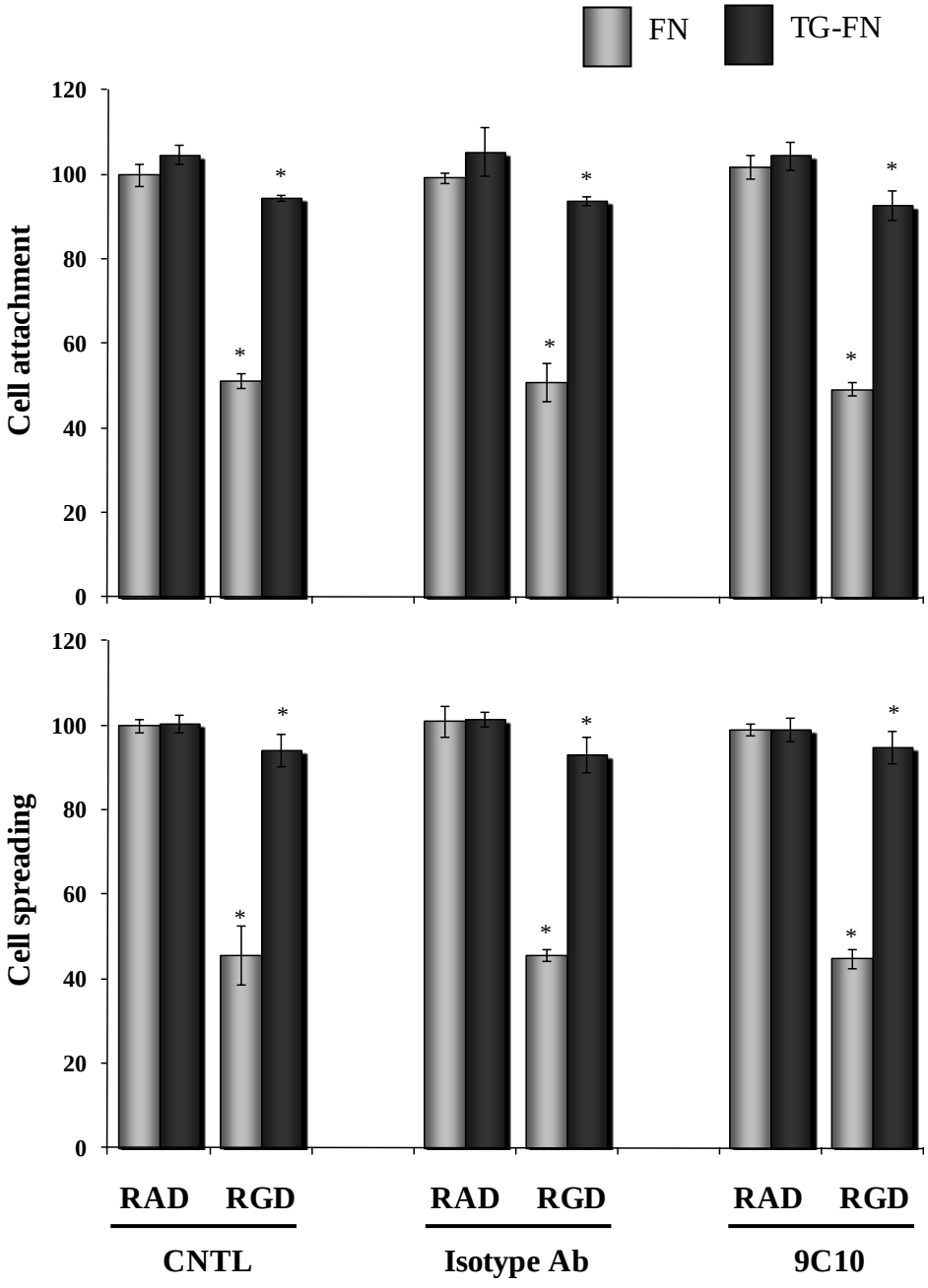

FN
TG-FN
Cell attachment
Cell spreading
120
100
80
60
40
20
0
*
RAD
RGD
CNTL
Isotype Ab
9C10

**Figure 3.3.27**

## 3.4 Discussion

It has been reported that the TG-FN heterocomplex, by acting as a novel cell adhesion matrix, can compensate the RGD peptide-induced loss of cell adhesion (resulting from the blocking of integrin' interactions with the cell binding domains within the FN molecule) (Verderio, et al., 2002). Previous work also demonstrated the involvement of cell surface heparan sulfate chains in this RGD-independent cell adhesion process mediated by TG-FN matrix (Verderio, et al., 2003). The initial aim of this project was to find out which type(s) of cell surface receptor(s) and intracellular molecule(s) are involved in this process.

By using heparin treatment, the heparin binding sites within FN were blocked before TG2 was immobilized on FN, which as shown by ELISA did not affect the formation of the TG-FN complex. The undertaking of the RGD-independent cell adhesion experiments on this heparin-treated matrix indicated that even though FN could not fully mediate cell adhesion through integrins or cell surface proteoglycans on this heparin-treated matrix, TG-FN can still compensate the effect of the RGD peptides, suggesting that TG2 is likely to be the functional component to exert the compensatory effect on the RGD-peptide-induced loss of cell adhesion. The following experiments using heparin to block the potential heparan sulfate binding site(s) within the TG2 molecule completely abolished this function of the TG-FN complex in cell adhesion assays. Also in the heparan sulfate mutant CHO cells, no compensation effect of the RGD peptide was observed in TG-FN groups, supporting the previous data using heparinase-treated cells in which TG-FN lost its compensation (Verderio, et al., 2003). This further indicates that the importance of the cell surface heparan sulfate chains in this novel cell adhesion process.

Heparan sulfate proteoglycans (HSPGs) are the major source of heparan sulfate chains on the cell surface, which are composed of a family of cell adhesion-relevant surface receptors— syndecans. The syndecan family includes four members, syndecan-1-4, among which syndecan-4 is the most widely expressed and has been reported to be involved in focal adhesion assembly and in

supporting the formation of actin stress fibres during the cell adhesion process. The importance of syndecan-4 has been reported in fibroblasts (Woods et al., 2000). By using syndecan-4 knockout MEF cells, the role of syndecan-4 in the RGD-independent cell adhesion on TG-FN was investigated and confirmed. Without the presence of cell surface syndecan-4, TG-FN lost its compensatory effect on the RGD peptides completely in the cell adhesion process, which was due to the loss of the direct interaction between TG2 and syndecan-4, demonstrated by co-immunoprecipitation experiments. The important role of syndecan-4 was also proven in actin stress fibre formation by using syndecan-4 wild type, knock-out and add-back MEF cells, revealing that without the support of syndecan-4, TG-FN could not promote the organization of actin stress fibres in the presence of RGD peptide, while in the wild type and add-back cells, this complex accomplished the restoring of the RGD-independent actin cytoskeleton formation. Since the core protein of syndecan-4 has also been reported in regulating cell adhesion, the heparan sulfate mutant CHO cells were used in co-immunoprecipitation assays by using an anti-syndecan-4 antibody (which detects the intracellular domain of syndecan-4 core protein) to pull the syndecan-4 immuno-complex. The absence of TG2 antigen in immunocomplex of the mutant CHO cells, compared to its presence in the wild type cells, indicated the direct link between TG2 and heparan sulfates and ruled out the involvement of the syndecan-4 core protein in TG-FN mediated cell adhesion.

The involvement of both syndecan-4 and β1 integrins in cell attachment and spreading has been widely demonstrated, suggesting that syndecan-4 is a co-receptor in the β1 integrin signalling pathway in supporting actin stress fibre formation (Bass et al., 2007). Since the involvement of syndecan-4 has been proven in RGD-independent cell adhesion mediated by TG-FN, further studies were performed to investigate whether β1 integrin also participates in this process. In the β1 integrin knockout MEF cells, TG-FN lost its compensatory effect completely, providing the first evidence of the crucial role of β1 integrins in signal transduction mediated by TG-FN. Meanwhile, the activation of β1 integrins by its direct interaction with syndecan-4 was ruled out, based on the discovery that no interaction was detected between these two receptors via co-

immunoprecipitation assays. Among the α integrin family, α5 integrin has been shown to be most important companion to β1 integrin in regulating FN-mediated cell adhesion (Takagi et al., 2003), which led to the hypothesis that α5 integrin could be the crucial partner for β1 integrin to regulate the RGD-independent cell adhesion on TG-FN. The failure of this complex in compensating RGD-independent cell adhesion in α5 integrin knockout cells proved this hypothesis. By using an inhibiting peptide A1-5 designed to target the α5β1 integrin to block its interaction with FN, cell attachment and spreading were inhibited in a dose-dependent manner in the cells seeded on FN. Unlike its compensatory effect on RGD peptide induced loss of cell adhesion, TG-FN failed to rescue the loss of cell adhesion when the inhibitor and the inhibiting peptide was present , further confirming that α5β1 integrin receptor complex is crucial in the cell adhesion process mediated by TG-FN.

Recent work suggested that another member of the syndecan family— syndecan-2 is also involved in regulating actin cytoskeleton formation during cell adhesion (Munesue et al., 2002). Meanwhile it has been reported that syndecan-2 is highly expressed in fibroblasts. To investigate the potential role of syndecan-2 in fibroblast cell adhesion on TG-FN, syndecan-2 specific targeting siRNAs was used to block the expression of syndecan-2 in MEF cells. The success in inhibiting syndecan-2 expression significantly reduced the compensatory effect of TG-FN on the RGD-independent cell adhesion, which gave the first clue of the importance of syndecan-2 in TG-FN mediated signalling transduction. By further investigating the actin cytoskeleton formation, in syndecan-2 siRNA-treated cells it was demonstrated these cells could not organize well-formed actin stress fibres, moreover they failed to respond to the TG-FN matrix in the presence of the RGD peptides which not only confirms the importance of syndecan-2 in actin cytoskeleton formation, but also its involvement in the RGD-independent cell adhesion mediated by TG-FN.

Even though defined as a vitronectin-relevant cell surface receptor, β3 integrin has also been reported to participate in cell adhesion on FN (Switala-Jelen et al., 2004). In β1 integrin knock-out MEF cells, although present in those cells, β3

integrins could not fulfil the role of β1 integrin in RGD-independent cell adhesion on TG-FN, suggesting β3 integrins are not required by TG-FN to compensate the effect of the RGD peptides. In order to confirm this result, β3 integrin knock-out MEF cells were used in the RGD-independent cell adhesion assay. In short-term incubations, the absence of β3 integrin did not affect the cell attachment on FN and only partially inhibited cell spreading (around 20% reduction), confirming the involvement of β3 integrin in the FN-mediated cell adhesion. On the contrary, neither cell attachment nor cell adhesion was affected when the knockout cells were seeded on TG-FN, ruling out the requirement of β3 integrins by TG-FN in mediating RGD-independent cell adhesion.

α4β1 integrin is another cell surface receptor that has been reported involved in cell adhesion on FN, also this integrin is related to TG2 in mediating cell adhesion (Moyano et al., 2003). In α4β1 integrin functional blocking antibody-treated MEF cells, no significant difference was found compared to the Isotype antibody or non-treated cells, suggesting in short-term incubation (30 min), α4β1 integrin is not required by cells to mediate their adhesion on FN. No further reduction or enhancement was discovered in the antibody-treated cells seeded on TG-FN, which ruled out the possibility that α4β1 integrin is essential for TG-FN to exert its compensatory effect on cell adhesion.

To sum up the above work, it was demonstrated that the TG-FN complex can compensate the effect of RGD peptide induced loss of cell adhesion and actin cytoskeleton formation through the syndecan-4/2 and α5β1 integrin co-signalling pathway, which is independent of β3 and α4β1 integrins. This process could be potentially involved in the matrix turnover process important to wound healing and tumour progression and may provide a therapeutic target for regulating the above processes. There are still remaining questions to be answered: (1) What intracellular signalling pathway(s) are involved in this cell adhesion process mediated by TG-FN; (2) What is the relationship between these three cell surface receptors, syndecan-4, syndecan-2 and α5β1 integrin; (3) What is the functional effect of this RGD-independent cell adhesion on physiological or pathological

process(es). The next chapters will explore the intracellular signalling pathway and matrix deposition processes mediated by the TG-FN matrix.

# Chapter 4:
# The characterisation of intracellular signalling molecules in the RGD-independent cell adhesion process mediated by the TG-FN complex

# Chapter 4: The characterisation of intracellular signalling molecules in the RGD-independent cell adhesion process mediated by the TG-FN complex

## 4.1 Introduction

The importance of the ECM protein FN in cell-ECM interaction has been widely investigated and reported (Pankov and Yamada, 2002). There are two major cell surface receptor families that are involved in this process, known as integrins and syndecans. It has been reported that around half of the members from the integrin family can bind to the RGD-cell binding domain of FN, which initiates the cell adhesion process. Integrins, which can interact with FN matrix, including α2β1, α3β1, α5β1, αVβ1, αVβ3, and αVβ5 integrins (Hersel et al., 2003). When integrins bind to the RGD-binding motif of FN, they initiate the cell adhesion process on FN. The involvement of the syndecan family in cell spreading was first reported on the discovery that the interaction of RGD cell binding sites with integrins can only mediate the formation of the nascent focal adhesions and the cells fail to form stress fibres and/or a cytoskeletal network (Bloom et al., 1999; Woods et al., 1984). Within FN there are at least two heparin binding sites mediating the interaction between FN and syndecans. The syndecan family are heparan sulfate proteoglycans (HSPGs) and form the major source of cell surface heparan sulphates. Syndecan-4 is the most ubiquitous member of the syndecan family. Although integrins are the main cell surface receptors responsible for cell-ECM interactions, in some cases, integrin engagement is not sufficient for a complete adhesion signalling process (Bloom et al., 1999; Huhtala, et al., 1995; Izzard, et al., 1986). Syndecan-4 can bind the HepII heparin-binding domain of FN with high affinity, which leads to the formation of focal adhesions necessary for cell adhesion (Woods et al. 2000). The binding of syndecan-4 to the heparin binding domain within FN provides a second signal that is required for formation of stress fibres and maturation of focal adhesions in mesenchymal cells (Woods, and Couchman, 1998). The heparan sulfate chains of syndecan-4 can interact with ECM proteins or growth factors to transduce the outside signals into the cells. The effect of both integrins and syndecans on cell adhesion and spreading

is defined as the β1 integrin and syndecan-4 co-signalling pathway. There are several intracellular signalling molecules that have been reported to be crucial in this signal transduction process, including protein kinase C α (PKCα), focal adhesion kinase (FAK), Rho and Rho kinase (ROCK) and extracellular signal-regulated kinase (ERK).

Once activated by the outside signals (via the interaction with ECM proteins or the binding with growth factors), the intracellular variable (V) region of syndecan-4 core protein binds directly with the catalytic domain of PKCα (Keum et al., 2004; Lim et al., 2003) in the presence of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) (Simons and Horowitz, 2001; Woods and Couchman, 2001), which triggers the activation of PKCα and its interaction with β1 integrins (Bass and Humphries, 2002). Activation of PKCα facilitates its translocation from the cytosol to the membrane and permits its binding with the cytoplasmatic domain of β1 integrins, which activates the inside-out signalling mechanism for this integrin family (Bass et al., 2007a; Nakashima, 2002). As an important downstream molecule in integrin signalling, FAK has been well studied. The interaction of integrins and their relevant ligands can trigger the phosphorylation of FAK at different Tyr sites, such as Tyrosine 397, 861, 925 etc., so that the FAK signalling complex will be formed. Initiated by the autophosphorylation of FAK at $Tyr^{397}$, FAK binds to the Src family, which in turn promotes the phosphorylation of Tyrosine sites 407, 576, 577, 861 and 925, leading to the maximal activation of FAK function (Parsons, 2003). Interestingly the phosphorylation of $Tyr^{861}$ suggests the activation of a ligand-independent signalling pathway (also known as inside-out signalling) (Shi and Boettiger, 2003). Once activated FAK can also trigger the cell survival signalling pathway mediated by ERK1/2 (Yee et al., 2008).

It has been reported that the Rho small GTPase family, composed of Rho, Rac and Cdc42, is involved in cell adhesion and migration, especially Rho and its downstream molecule ROCK in syndecan signalling transduction (Whiteford et al., 2007). By using syndecan-4 activating antibody, Saoncella and colleagues (1999) accomplished the full cell spreading on the cell binding domain of FN

(cell adhesion process mediated by β1 integrins), which was blocked by Rho inhibitor C3 exotransferase (Saoncella et al., 1999). This discovery demonstrated the role of Rho as a downstream signalling molecule in the syndecan-4 pathway. Other researchers suggested that Rho and ROCK is also involved in actin skeleton formation mediate by syndecan-2 (Whiteford et al., 2007). Even though there are still conflicting opinions, the importance of Rho and ROCK in cell spreading and actin stress fibre organization has been accepted widely.

TG2 is also regarded as a cell adhesion mediator. Interacting with the gelatin-binding domain of FN, cell surface TG2 can facilitate integrin-regulated cell adhesion (Radek et al., 1993) via its non-covalent interaction with β1 and β3 integrins (1:1 stoichiometry), which is independent of its transamidating activity (Akimov and Belkin, 2001; Akimov et al., 2000). Moreover, the binding of TG2 with FN activates FAK and increases the GTP loading of the small GTPase RhoA, which may trigger the intracellular signalling pathway and induce its relevant cell adhesion (Janiak et al., 2006). Mehta and colleagues also demonstrated that FAK is the downstream signalling molecule in TG2-related tumour cell adhesion and survival (Mehta et al., 2006), which can be attenuated by TG2 siRNA treatment (Herman et al., 2006; Verma et al., 2006). The role of matrix TG2 in RGD-independent cell adhesion was initially reported by Griffin and colleagues. Once deposited into the ECM, TG2 can form a hetero-complex with FN, named TG-FN, which can function as a structural protein (Verderio et al., 1998). The novel function of TG2, as an ECM protein, in RGD-independent cell adhesion was firstly introduced in 2003 (Verderio et al., 2003). Even though the function of this matrix complex has been proven, the actual intracellular signalling pathway in this RGD-independent cell adhesion process on TG-FN has not been completely revealed. In order to pursue earlier work about cell surface receptors and investigate the involvement of the intracellular signalling pathway that mediates RGD-independent cell adhesion on TG-FN, the intracellular signalling molecules, including PKCα, FAK, ERK1/2, ROCK, were studied.

The aim of this chapter is to continue the investigation of the signalling pathway in the RGD-independent cell adhesion mediated by TG-FN. Previous work from

Chapter 3 suggested the involvement of syndecan-4/2 and α5β1 integrin co-signalling pathway in the above process. In order to further confirm the discoveries and study the intracellular signalling, the downstream signalling molecules in this pathway were studied, including PKCα, FAK, ERK1/2 and ROCK by using specific inhibitors and the detection of the phosphorylation or translocation status of certain kinases.

## 4.2 Methods

### 4.2.1 Inhibition of the signalling transduction processes by specific treatments in the RGD independent cell adhesion

Before detachment by trypsin, Swiss 3T3 or mouse embryonic fibroblast (MEF) cells were pre-treated with different treatments to block the activity or function of certain signalling molecules or receptors, including PKCα inhibitor Go6976 (5μM dissolved in DMSO, 30 min), GK21 peptide (8μM dissolved in DMSO, 1 h), ERK1/2 inhibitor PD98059 (10μM dissolved in DMSO, 1 h), ROCK inhibitor Y27632 (10μM dissolved in DMSO, 30 min), or 25μg/ml of β1 integrin functional blocking antibody HMβ1-1 or its Isotype control antibody for 1 h. In some cases, DMSO (0.1%) was used as the vehicle control to rule out its toxic effect on the cells. Following the removal of the treatments, RGD-independent cell adhesion was performed as introduced in Section 2.2.3.

### 4.2.2 Detection of the PKCα translocation from cytosol to membrane

Following PKCα inhibitor treatment (Section 4.2.1), RGD-independent cell adhesion was performed in Swiss 3T3 cells. The cells were allowed to adhere for around 30 min in the 60mm Petri dishes were previously sequentially coated with 5μg/ml of FN (in 50mM Tris-HCl, pH 7.4) and with 20μg/ml of gplTG (in 2mM EDTA in PBS, pH 7.4) as described before (Section 2.2.2). 50nM of PMA in DMSO was used in the positive control treatment. Non-adherent cells were collected and washed with ice-cold PBS, pH 7.4 and gently mixed with ice-cold homogenisation buffer (Section 2.2.12), while adherent cells were harvested in the same buffer and mixed with the corresponding samples. The cells were lysed via sonication and the cell membrane and cytosol fractions isolated followed the procedures introduced in Section 2.2.12. The presence of PKCα antigen in the different cell fractions was detected via Western blotting by using specific anti-PKCα antibody (Section 2.2.8-10).

### 4.2.3 Detection the interaction between PKCα and syndecan-2 via co-immunoprecipitation

Co-immunoprecipitation assays were performed to detect interaction between syndecan-2 and PKCα by using wild type, syndecan-4 knockout and Y188L syndecan-4 mutant MEF cells as introduced in Section 2.2.11. Briefly rabbit anti-syndecan-2 polyclonal antibody and protein A beads were used to pull down the syndecan-2 immunecomplex in the pre-cleared cell lysates samples. Western blotting (Section 2.2.9) was used to detect the presence of PKCα antigen in this immune-complex by using mouse anti-PKCα monoclonal antibody.

## 4.3 Results

### 4.3.1 TG-FN compensates the effect of RGD peptide on cell adhesion in a PKCα-dependent signalling pathway.

#### 4.3.1.1 PKCα inhibitor Go6976 inhibits the compensatory effect of TG-FN on the RGD-independent cell adhesion process

As an important intracellular molecule in the syndecan-4 signalling pathway, the role of PKCα was investigated by using its specific inhibitor Go6976 in treated Swiss 3T3 fibroblasts. DMSO was used as the vehicle control. As shown in Figure 4.3.1, in the presence of RAD peptide, no significant difference of both cell attachment and spreading was found compared to non-treated cells, suggesting no toxicity of the RAD peptide (100μg/ml). As shown before, TG-FN restored the loss of cell attachment and spreading caused by RGD peptide treatment back to 93.8% and 94.5% respectively. When treated with PKCα inhibitor Go6976, a reduction of 33.2% of cell attachment and 26.7% of spreading was obtained on the FN matrix in non-treated cells, while in the RAD groups, 31.8% and 24.4% of cell attachment and spreading was blocked by Go6979, respectively. RGD peptide-treated cells on FN matrix could only reach 31.4% of cell attachment and 21.5% of cell spreading, compared to the control cells. In the vehicle control group, TG-FN restored the loss of cell attachment and spreading induced by the RGD peptide back to normal and accomplished around 95% compensation, while in the inhibitor groups, TG-FN failed to recover the effect of the inhibitor in RGD treatments. These results suggest the involvemen t o f PKCα in TG-FN signal transduction in Swiss 3T3 fibroblast cells.

#### 4.3.1.2 The importance of the interaction of PKCα with the syndecan-4 intracellular domain.

It has been reported that PKCα is an important downstream molecule of the syndecan-4 signalling pathway. To investigate the potential importance of the


**Figure 4.3.1 The involvement of PKCα in RGD-independent cell adhesion to FN and TG-FN matrices.**
The Swiss 3T3 fibroblasts were treated with PKCα inhibitor Go6976 and cell adhesion was performed as described previously (Section 4.2.1). The attachment and spreading of Swiss 3T3 cells was analysed as introduced in the Methods (Section 2.2.3). Each data point represents the mean percentage of attached cell (cell attachment) or the mean percentage of spreading cells (cell spreading) ± S.D. of three separate experiments performed in triplicate. The ordinates in the top graphs represent the mean cell attachment expressed as mean percentage of DMSO-treated Swiss 3T3 cells (control) attachment to FN ± S.D., presenting 100%. The mean number of cell attachment ± S.D. of control taken from 3 experiments was 139 ± 1. The ordinates in the lower graphs designate the mean percentage of spreading cell, expressed as mean percentage of spreading on FN ± S.D. The mean percentage of control spreading on FN ± S.D was assessed as 75 ± 4, and normalized to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups.

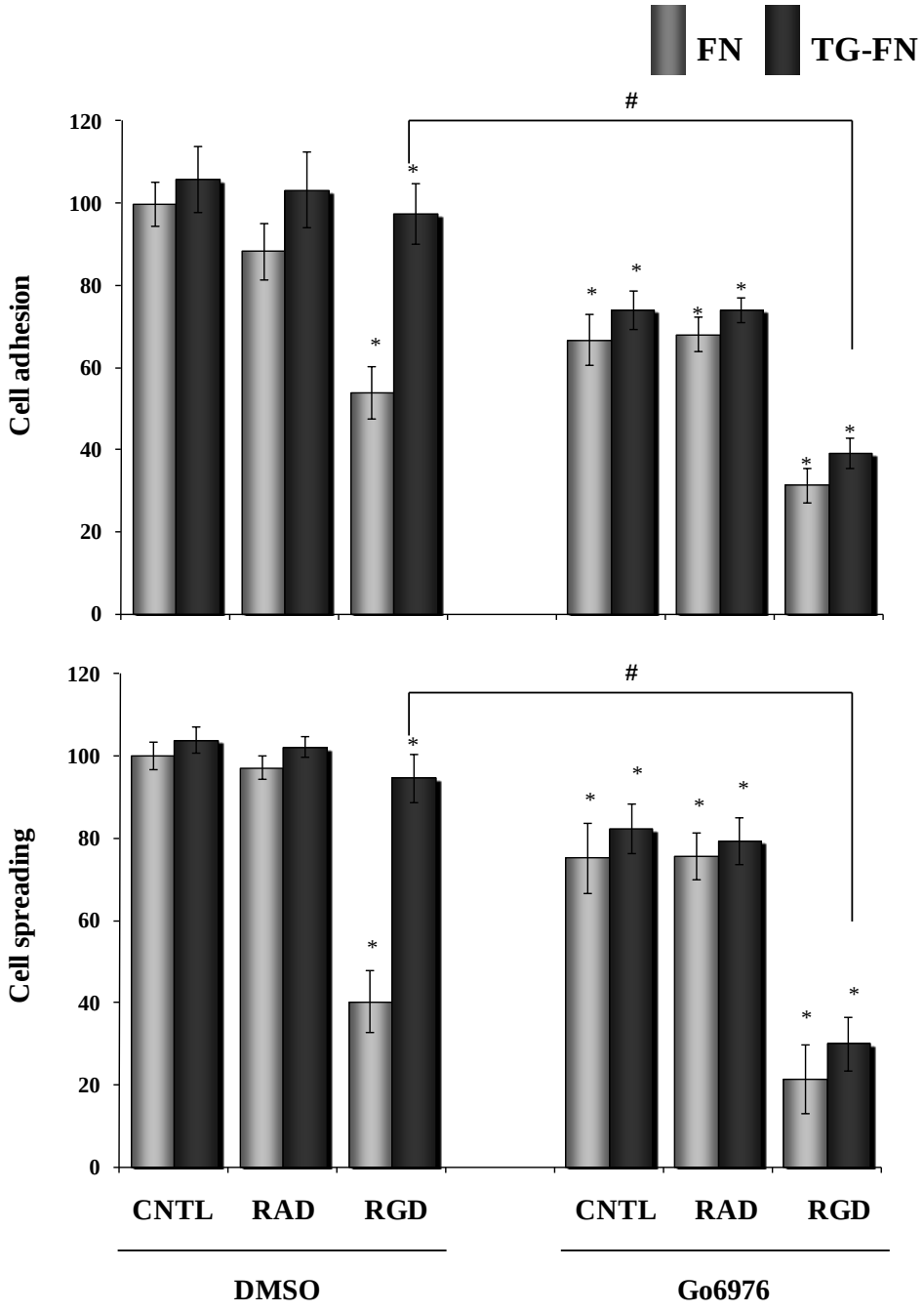

FN
TG-FN
Cell adhesion
Cell spreading
120
100
80
60
40
20
0
#
*
CNTL
RAD
RGD
DMSO
Go6976

**Figure 4.3.1**


**Figure 4.3.2 The involvement of the interaction between PKCα and syndecan-4 in RGD-independent cell adhesion to FN and TG-FN matrices.**
The Y188L mutant MEF cells and wild type MEF cells were used in cell adhesion assays performed as described previously (Section 2.2.3). The attachment and spreading of MEF cells was analysed as introduced in the Methods (Section 2.2.3). Each data point represents the mean percentage of attached cells (cell attachment) or the mean percentage of spreading cells (cell spreading) ± S.D. of three separate experiments performed in triplicate. The ordinates in the top graphs represent the mean cell attachment expressed as mean percentage of wild type MEF cells (control) attachment to FN ± S.D., presenting 100%. The mean attachment value ± S.D. was 126 ± 1. The ordinates in the lower graphs designate the mean percentage of spreading cell, expressed as mean percentage of spreading on FN ± S.D. The mean percentage of control spreading on FN ± S.D was assessed as 82 ± 4, and normalized to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups.

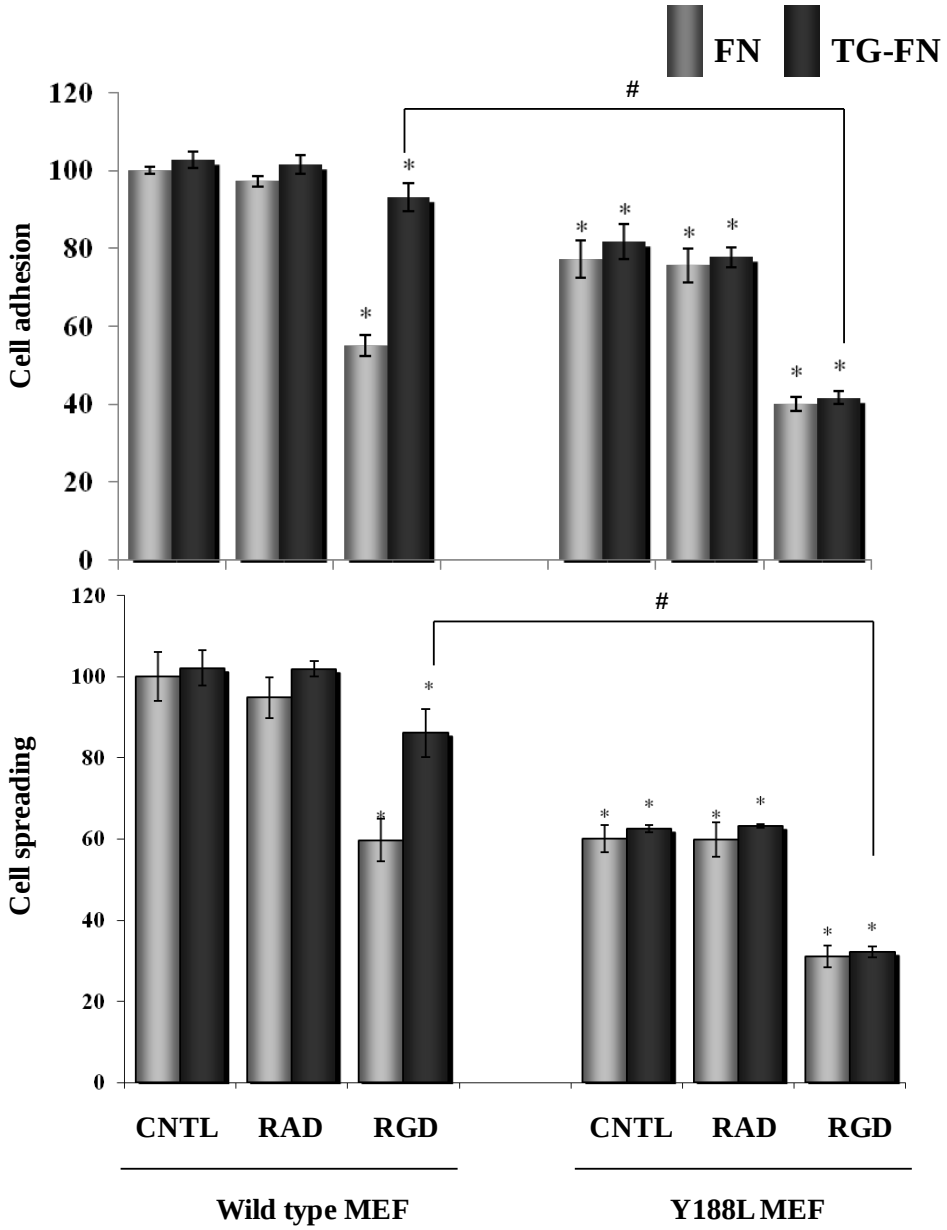

FN
TG-FN
Cell adhesion
Cell spreading
120
100
80
60
40
20
0
#
*
CNTL
RAD
RGD
Wild type MEF
Y188L MEF

**Figure 4.3.2**

interaction between PKCα and syndecan-4, cell adhesion was performed by using syndecan-4 mutant MEF cells Y188L (syndecan-4 null MEF cells re-transfected with human syndecan-4 cDNA with the mutation of the PKCα binding site within its cytoplastic domain) (Bass et al., 2007) and wild type MEF cells were used as the control group. As shown in Figure 4.3.2, the RAD treatment did not affect either the cell attachment or spreading process in wt MEF cells and Y188L MEF cells on FN, compared to non-treated cells on FN, suggesting at the concentration of 100μg/ml, no toxicity of RAD or RGD peptide was found in both cell types. In wt MEF, RGD treatment reduced 45% of cell attachment and 40% of spreading on FN, which was restored back to 93% and 86% by TG-FN, respectively, suggesting the compensatory effect of TG-FN on the RGD-induced loss of cell adhesion. While in the Y188L mutant cells, 23% and 40% loss of cell attachment and spreading, respectively, were observed on the FN matrix only, compared to the wild type control group. The RGD peptide further reduced 37% of cell attachment and 29% of cell spreading on the FN matrix, which could not be compensated by the TG-FN complex, since similar level of cell attachment and spreading were found in both FN and TG-FN matrices ($p>0.05$). The above results confirm the importance of the interaction between PKCα and syndecan-4 in cell adhesion on FN. Importantly the involvement of this binding is also required in mediating the TG-FN signalling transduction process.

#### 4.3.1.3 TG-FN compensates RGD-independent cell adhesion mediated by the binding of PKCα with β1 integrins.

It has been reported that the GK21 peptide mimics the intracellular domain of β1 integrin which mediates its interaction with PKCα (Parsons et al., 2002). As such, this peptide can be used to inhibit cell adhesion on FN. To investigate the role this interaction in cell adhesion on TG-FN, cell adhesion was performed by using GK21 and its scrambled peptide in Swiss 3T3 cells. Cells treated with DMSO and RAD peptide seeded on FN were used as one vehicle control group, while the scrambled peptide was used as a negative control. As shown in figure


**Figure 4.3.3 The importance of PKCα and β1 integrin interaction in RGD-independent cell adhesion to FN and TG-FN matrices.**
The Swiss 3T3 fibroblasts were treated with GK21 peptide (which blocks the interaction between the intracellular domain of β1 integrin and PKCα) and its scrambled control, while DMSO was used as the vehicle control. The attachment and spreading of Swiss 3T3 cell was analysed as introduced in the Methods (Section 2.2.3). Each data point represents the mean percentage of attached cells (cell attachment) or the mean percentage of spreading cells (cell spreading) ± S.D. of three separate experiments performed in triplicate. The ordinates in the top graphs represent the mean cell attachment expressed as mean percentage of DMSO-treated Swiss 3T3 cells (control) attachment to FN ± S.D., representing 100% in the presence of RAD peptide. The mean attachment value ± S.D. was 139 ± 1. The ordinates in the lower graphs designate the mean percentage of spreading cells, expressed as mean percentage of spreading on FN ± S.D. The mean percentage of control spreading on FN ± S.D was assessed as 75 ± 4, and normalized to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups.

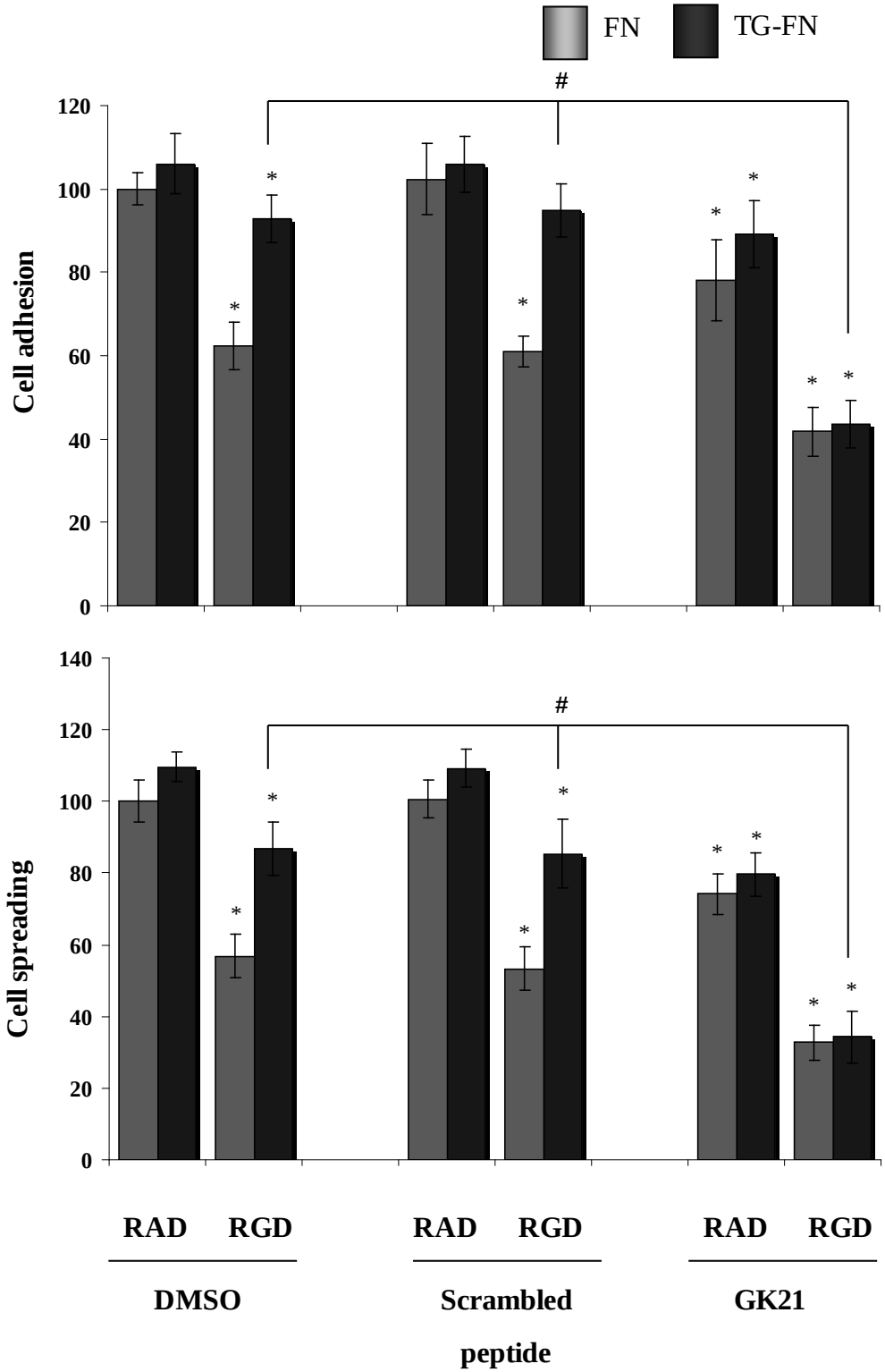

FN
TG-FN
#
Cell adhesion
120
100
80
60
40
20
0
Cell spreading
140
120
100
80
60
40
20
0
RAD
RGD
RAD
RGD
RAD
RGD
DMSO
Scrambled peptide
GK21

**Figure 4.3.3**


**Figure 4.3.4 Detection of the translocation of PKCα in the RGD-independent cell adhesion in Swiss 3T3 cells.**
RGD-independent cell adhesion assay was performed as introduced before in PKCα inhibitor Go6976-pre-treated (5μM, 1 h) Swiss 3T3 cells (Section 2.2.3 and Section 4.2.1). 50nM PMA was used as a positive control treatment for PKCα activation, while DMSO was used the vehicle control treatment. Cell lysates were collected into membrane fraction collection buffer. Membrane and cytosol fractions were isolated from total 200μg protein/sample as introduced in Section 2.2.12. The presence of PKCα antigen was detected by using mouse monoclonal anti-PKCα antibody (1:1000 dilution) via Western blotting. The densitometric values obtained for the bands are represented in Lane 1, PMA-treated cells seeded on FN; Lane 2, DMSO-treated cell seeded on FN in the presence of RAD; Lane 3, DMSO-treated cells on FN or TG-FN (Lane 4) in the presence of RGD; Lane 5, Go6976-treated cells FN in the presence of RAD; Lane 6, Go6976-treated cells on FN or FN-TG (Lane 7) in the presence of RGD.

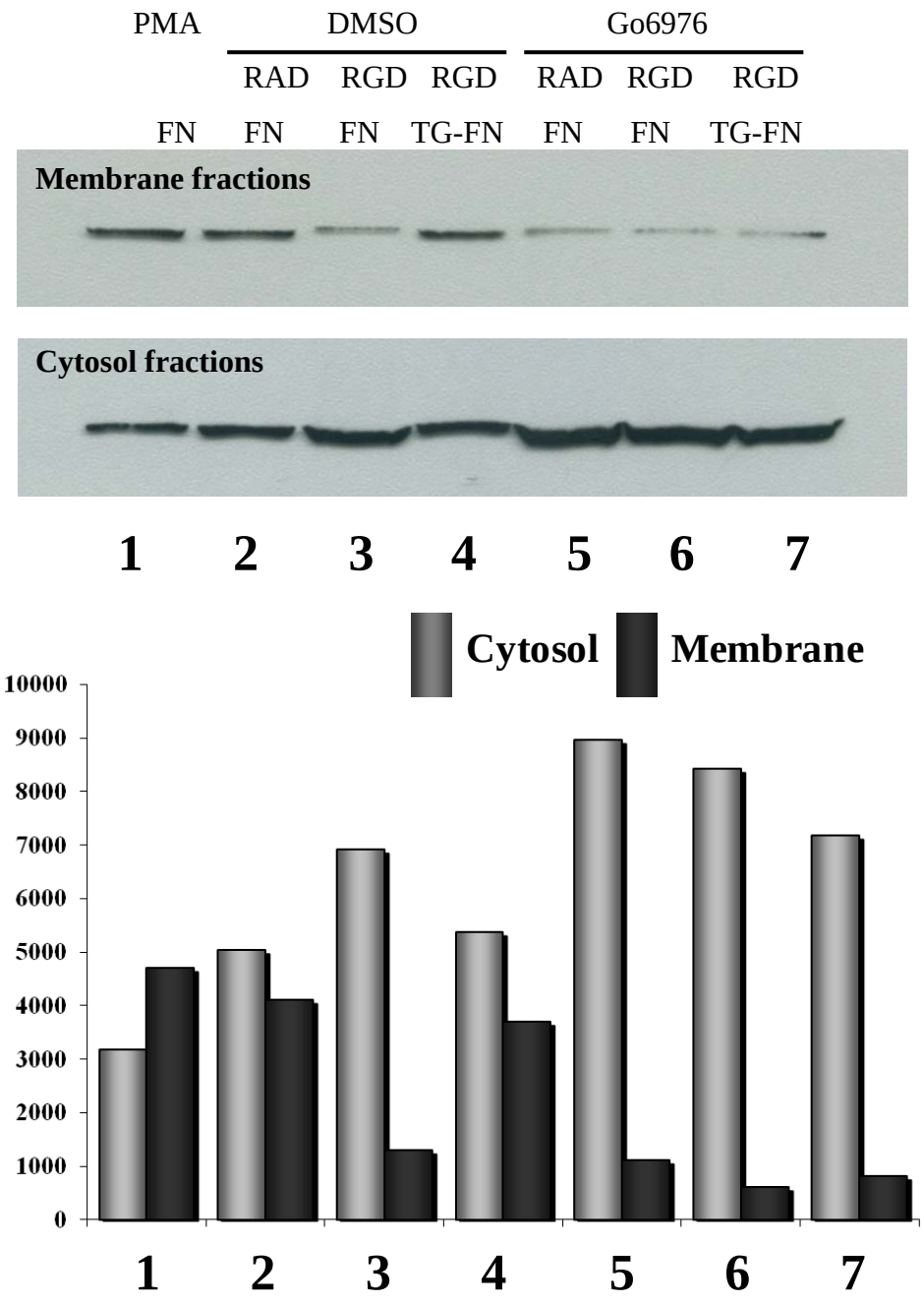

PMA
DMSO
Go6976
RAD
RGD
RGD
RAD
RGD
RGD
FN
FN
FN
TG-FN
FN
FN
TG-FN
Membrane fractions
Cytosol fractions
1 2 3 4 5 6 7
Cytosol
Membrane
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
1 2 3 4 5 6 7

**Figure 4.3.4**

4.3.3, nosignificant difference of cell attachment and spreading was found between DMSO vehicle control cells and scrambled peptide-treated cells in the presence of RAD peptide in both Swiss 3T3 cells, indicating no toxicity of these two peptides. RGD peptide led to 37.6% and 43.3% loss of cell attachment and spreading, respectively, in Swiss 3T3 cells on FN, which was restored back to 92.8% and 86.7% by the TG-FN complex, respectively. In the presence of RAD peptide, GK21 peptide at the concentration of 8μM led to 21.9% and 25.8% loss of cell attachment and spreading in Swiss 3T3 cells seeded on FN, which could not be restored by the TG-FN complex. When treated with RGD peptide, only 41.8% of cell attachment and 32.8% cell spreading were found in these fibroblasts on FN, only 43.5% in cell attachment and 34.3% in cell spreading on TG-FN, and no significant difference was found between the matrices, suggesting TG-FN could not compensate the loss caused by the GK21 peptide. The above data indicates that TG-FN mediated cell adhesion in the presence of the RGD peptide is dependent on the interaction between PKCα and β1 integrins.

#### 4.3.1.4 TG-FN promotes the translocation of PKCα.

The translocation of PKCα indicates its activation in supporting cell adhesion. To investigate whether the translocation of PKCα occurs in cell adhesion on TG-FN, the fractions of cell cytosol and membranes were isolated and Western blotting was performed to detect the presence of PKCα antigen in both fractions. Serum-starved Swiss 3T3 cells were pre-treated with 5μM PKCα inhibitor Go6976 or DMSO (vehicle control) for 1 h, and then cells were trypsinized and seeded on FN or TG-FN matrices in the presence of RAD or RGD peptide. After around 1 h incubation, cells were collected in membrane isolation buffer and membrane and cytosol fractions were isolated as introduced in Materials and Methods (Section 2.2.12 and Section 4.2.2). RAD-treated cells on FN were used as control group, while cells treated with 50nM PMA were used as positive control in the presence of RAD peptide. Increased PKCα in the membrane fraction was found in PMA control groups compared to the non-DMSO treated cells seeded on FN. The RGD treatment led to the decrease of PKCα translocation from cytosol to

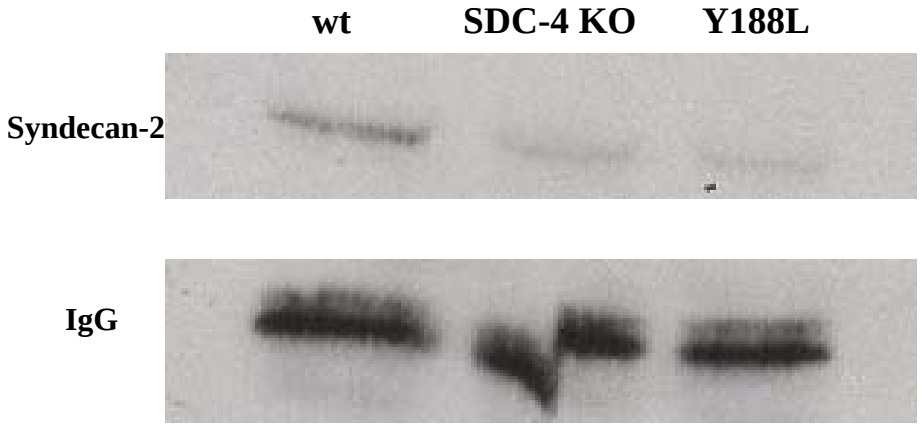



**Figure 4.3.5 Detection of the interaction between PKCα and syndecan-2.**
Wild type, syndecan-4 knockout, Y188L syndecan-4 mutant MEF cells were used in co-immunoprecipitation experiments as introduced in Section 4.2.3. After collection into cell lysis buffer (Section 2.2.11) and pre-cleared by using rabbit IgG (0.5μg/sample) and protein A beads, rabbit anti-syndecan-2 polyclonal antibody (0.5μg/sample) and protein A beads were used to pull down the syndecan-2 immuno-complex and Western blotting was then used to detected the presence of PKCα antigen (top image) (1:1000 dilution) within this complex from the different cell samples (Section 2.2.9). The IgG bands (bottom image) were used to represent equal protein loadings.

**Figure 4.3.5**


**Figure 4.3.6 Detection of the phosphorylation of FAK Tyr$^{397}$ in the RGD-independent cell adhesion in Swiss 3T3 cells.**
The RGD-independent cell adhesion assay was performed as introduced before (Section 2.2.3). 50µg/sample of total protein collected in cell lysis buffer was used to detect the presence of specific antigens by using specific antibodies, including rabbit polyclonal anti-p-Tyr397-FAK (1:1000 dilution) (a, top image) and mouse monoclonal anti-FAK (0.6µg/blot) (b, top image). The membranes were stripped and re-probed with mouse monoclonal anti-α-tubulin antibody to ensure the equal loading (a and b, bottom images). The densitometric values obtained for the bands was normalised against the values for the tubulin bands and represented as the mean net area value (relative FAK-397, or relative FAK) ± S.D. from three separate experiments. Each data point was expressed as a percentage of the control FN level, which represents 100%. The * symbol indicates the statistically different values when compared to the non-treated cells seeded on FN. Lane 1, non-treated cells seeded on BSA; Lane 2, non-treated cell seeded on FN and TG-FN (Lane 3); Lane 4, RAD-treated cells FN and FN-TG2 (Lane 5); Lane 6, RGD-treated cells on FN and TG-FN (Lane 7).

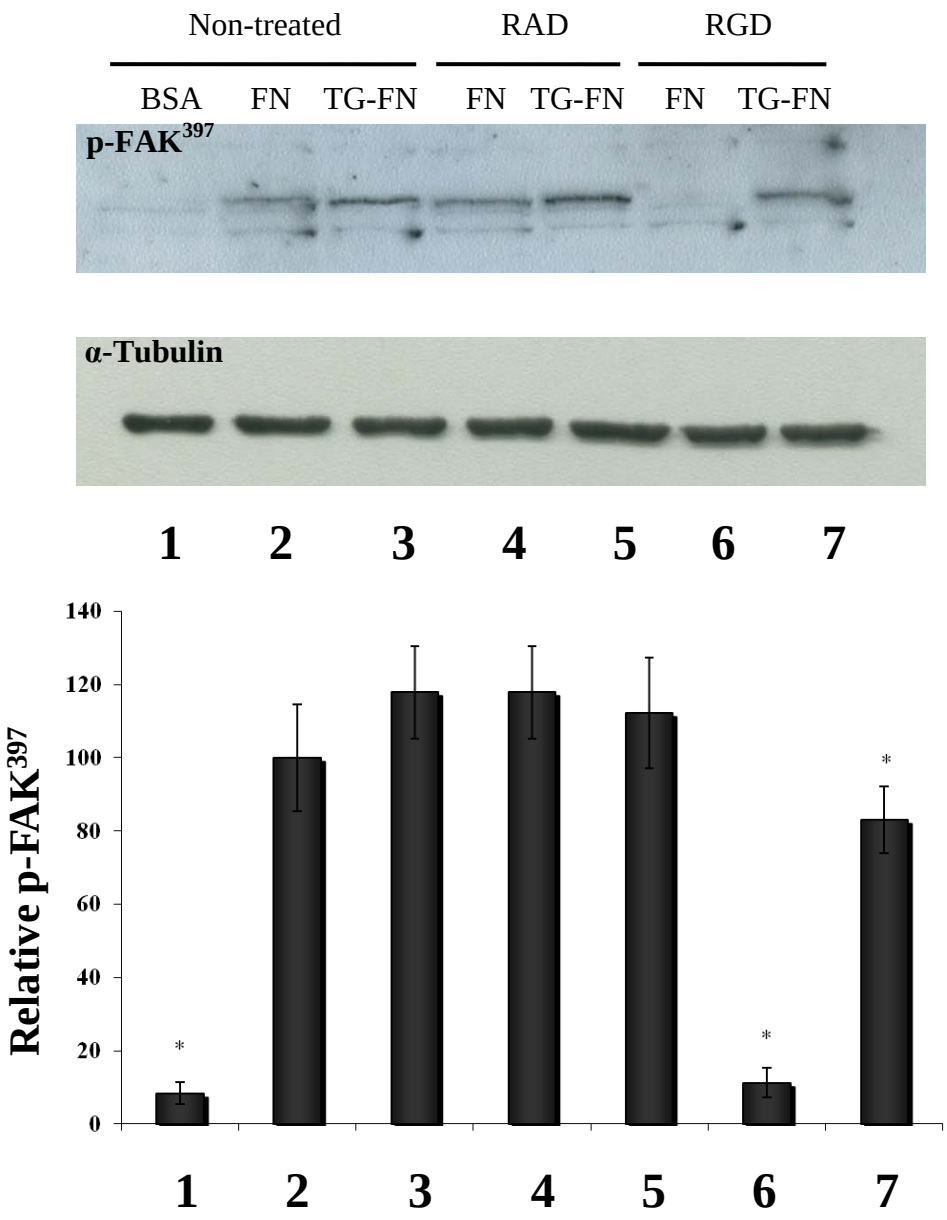

Non-treated
RAD
RGD
BSA
FN
TG-FN
FN
TG-FN
FN
TG-FN
p-FAK397
α-Tubulin
1 2 3 4 5 6 7
Relative p-FAK397
140
120
100
80
60
40
20
0
*
*
*
1 2 3 4 5 6 7

**Figure 4.3.6a**

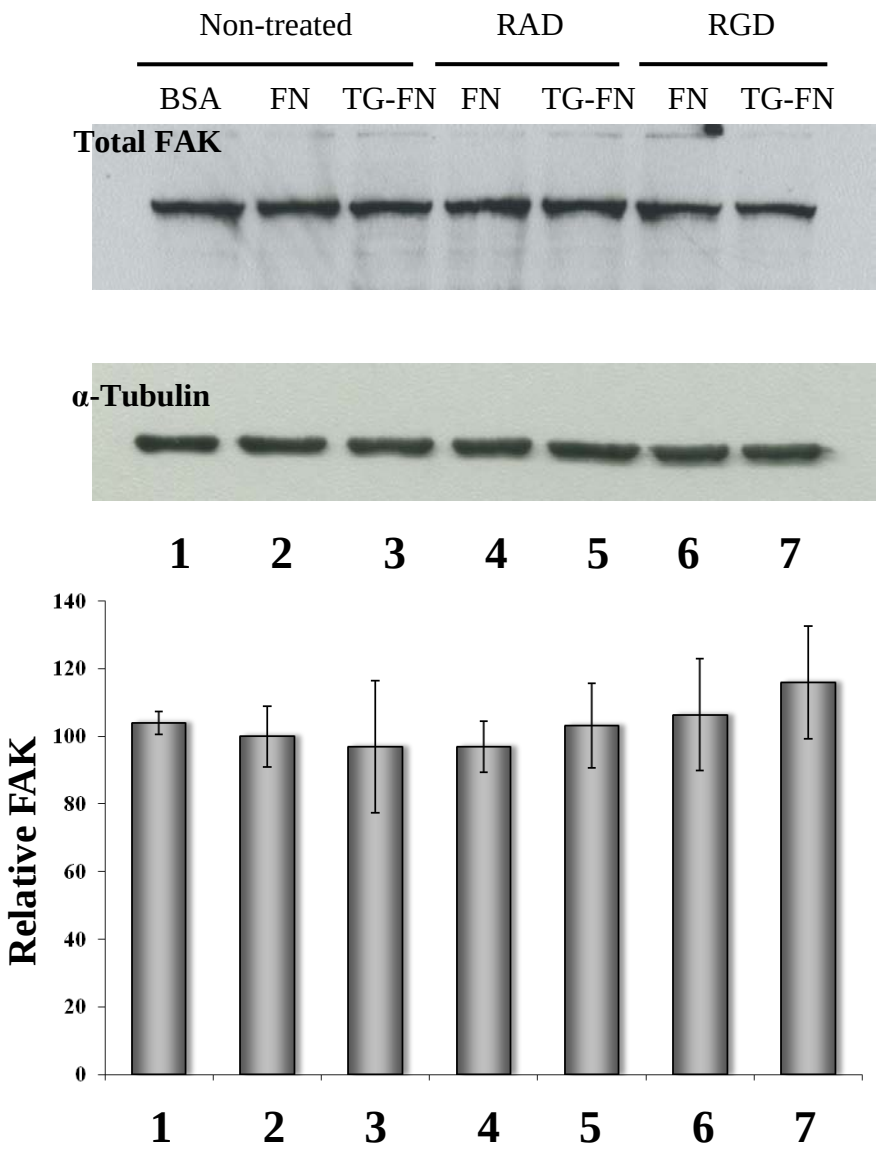

Non-treated
RAD
RGD
BSA
FN
TG-FN
FN
TG-FN
FN
TG-FN
Total FAK
α-Tubulin
1
2
3
4
5
6
7
Relative FAK
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7

**Figure 4.3.6b**

membrane, since 70% decrease of membrane PKCα was found in RGD treated cells on FN. The levels of PKCα translocation was 106% in the cells seeded on TG-FN compared to control level. Go6976 treatment resulted in a significant reduction of PKCα translocation in both RAD and RGD treated cells on FN, which could not be restored by the TG-FN complex (Figure 4.3.4). As expected, high levels of cytosol PKCα were discovered in the groups with low levels of membrane PKCα.

#### 4.3.1.5 PKCα is the potential intracellular signalling link between syndecan-2 and syndecan-4

It has been suggested that syndecan-2 can act as a downstream signalling molecule of syndecan-4 signalling transduction in actin cytoskeleton formation. In order to investigate the potential involvement of PKCα in this co-signalling pathway, syndecan-4 wild type, knockout, and Y188L syndecan-4 mutant MEF cell lysates were collected and the co-imminoprecipitation assay using anti-syndecan-2 antibody to pull down PKCα was performed to detect the interaction between PKCα and syndecan-2. As shown in Figure 4.3.5, without the presence of syndecan-4 or the binding site for PKCα within syndecan-4 intracellular domain, the interaction between PKCα and syndecan-2 was significantly weakened, compared to that in wild type MEF cells, while IgG bands indicated that there was an equal amount of protein used in this experiment. These results suggest not only the direct interaction between syndecan-2 and PKCα, but also the importance of syndecan-4 in mediating this interaction process, particularly it suggests the initiative role of the binding of PKCα to syndecan-4 in triggering the downstream interaction of PKCα to syndecan-2.

### 4.3.2 TG-FN signalling activates focal adhesion kinase

#### 4.3.2.1 The importance of p-FAK$^{397}$

Previous work indicated that FN-TG2 promotes the phosphorylation of FAK at Tyr$^{397}$ in HOB cells even in the presence of RGD peptide, which suggested the

involvement of FAK in the signalling transduction on the FN-TG2 complex. To study the role of FAK, pre-serum starved Swiss 3T3 cells (16 h) were treated with 100μg/ml of RAD or RGD peptide and then seeded on FN or FN-TG2 matrices. BSA (3%) in PBS, pH7.4 was used as negative control matrix, while cells seeded on FN matrix without peptide treatment was used as the control group. Cells were allowed to adhere for around 1 h, after washing adherent cells with PBS, pH7.4, samples were collected into cell lysis buffer. The levels of total and tyrosine phosphorylated forms of FAK in the samples were examined via Western blotting as introduced in Materials and Methods. Figure 4.3.6a presents the content of p-FAK$^{397}$, while Figure 4.4.6b shows the levels of total FAK in the samples. Tubulin was used as the internal control. The sample from the cells seeded on BSA was used as the negative control group, as shown in Figure 4.3.6, a reduction of FAK$^{397}$ phosphorylation can clearly be observed and the amount of phosphorylated FAK$^{397}$ is only 8% of that of the FN control. RAD peptide treatment did not affect the level of p-FAK$^{397}$, even though there was a slight increase of the amount of antigen in the samples from cell seeded on TG-FN, there is no statistical difference found. The RGD treatment significantly reduced the phosphorylation of FAK$^{397}$ on FN to 10%, compared to control group. However TG-FN restored the level of p-FAK$^{397}$ back to 83 % of the control level.

Total levels of FAK were also investigated via Western blotting in the samples. The blot shows a similar level of total FAK in each group and no significant differenece was found between them. The level of total protein applied in Western blotting was normalized by the internal standard tubulin. These results indicated the importance of the phosphorylation of FAK$^{397}$ in RGD-independent cell adhesion on TG-FN.

### 4.3.2.2 The importance of p-FAK$^{861}$.

As introduced before, the phosphorylation of FAK at tyrosine site 861 indicates the activation of the ligand-independent activation of the β1 integrin signalling pathway. In order to investigate the involvement of this phosphorylation site,


**Figure 4.3.7 Detection of the phosphorylation of FAK at Tyr$^{861}$ in the RGD-independent cell adhesion in Swiss 3T3 cells.**
The RGD-independent cell adhesion assay was performed as introduced before (Section 2.2.3). 50µg/sample of total protein collected in cell lysis buffer was used to detect the presence of specific antigens by using specific antibodies, including rabbit polyclonal anti-p-Tyr861-FAK (1:1000 dilution) (a, top image) and mouse monoclonal anti-FAK (0.6µg/blot) (b, top image). The membranes were stripped and re-probed with mouse monoclonal anti-α-tubulin antibody to ensure the equal loading (a and b, bottom images). The densitomertric values obtained for the bands was normalised against the values for the tubulin bands and represented as the mean net area value (relative FAK$^{861}$, or relative FAK) ± S.D. from three separate experiments. Each data point was expressed as a percentage of control FN level, which represents 100%. The * symbol indicates the statistically different values when compared to the non-treated cells seeded on FN (Lane 2). Lane 1, non-treated cells seeded on BSA, Lane 2 non-treated cell seeded on FN, and TG-FN (Lane 3), Lane 4, RAD-treated cells FN and FN-TG2 (Lane 5), Lane 6, RGD-treated cells on FN and TG-FN (Lane 7).

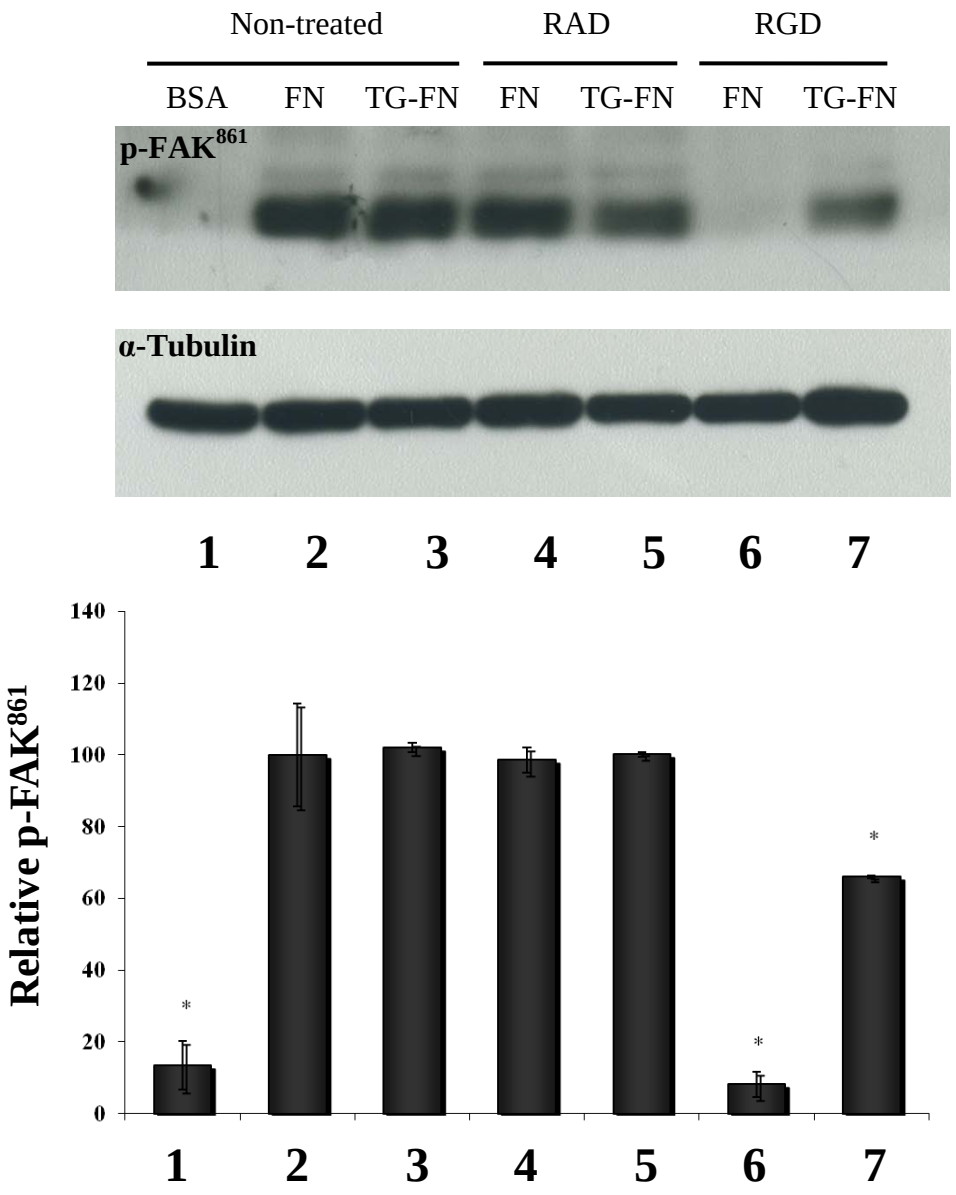

Non-treated
RAD
RGD
BSA
FN
TG-FN
FN
TG-FN
FN
TG-FN
p-FAK861
α-Tubulin
1 2 3 4 5 6 7
Relative p-FAK861
140
120
100
80
60
40
20
0
*
*
*
1 2 3 4 5 6 7

**Figure 4.3.7a**

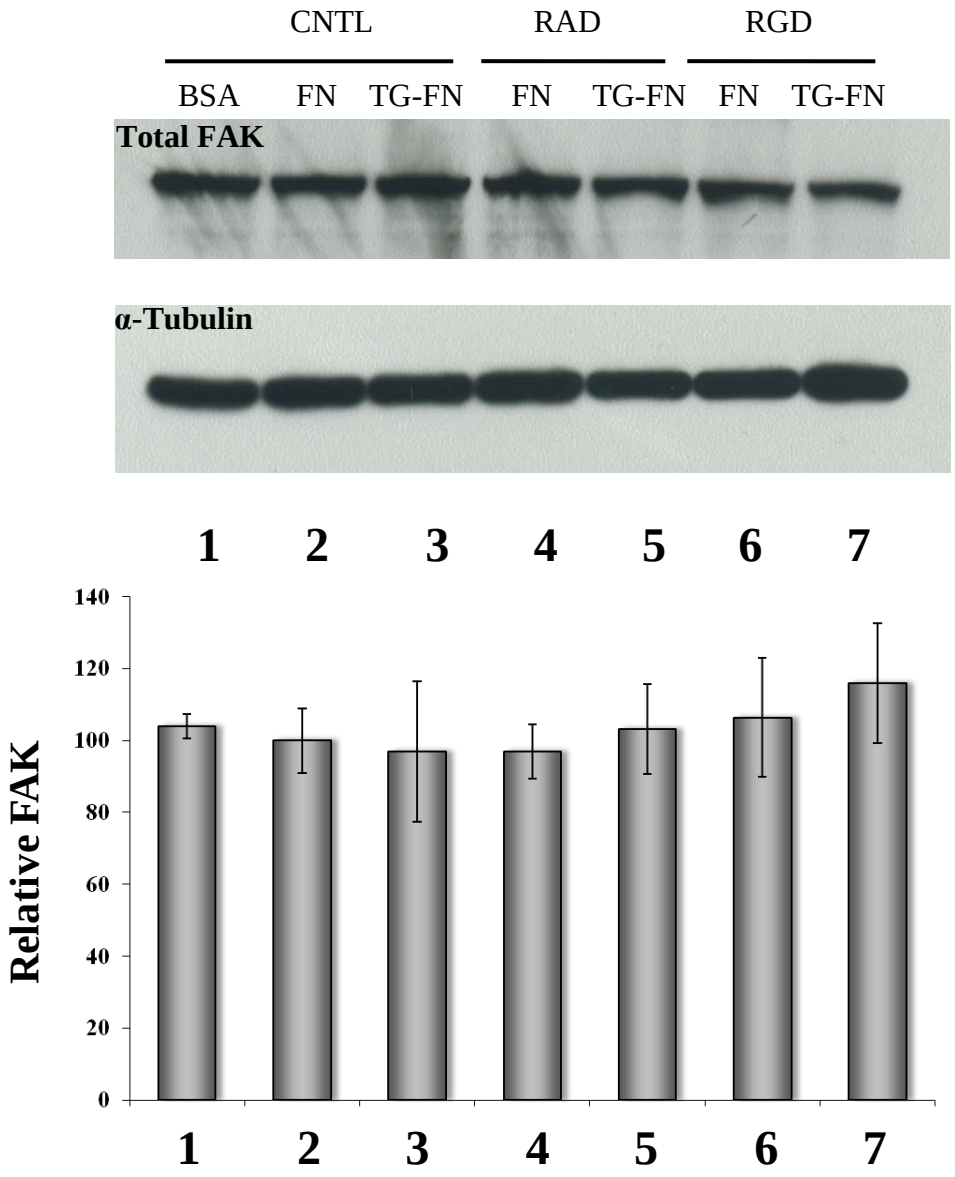

CNTL
RAD
RGD
BSA
FN
TG-FN
FN
TG-FN
FN
TG-FN
Total FAK
α-Tubulin
1 2 3 4 5 6 7
Relative FAK
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7

**Figure 4.3.7b**

pre-serum starved Swiss 3T3 fibroblasts were seeded on FN or TG-FN for around 1 hour, in the presence of RGD peptide, while RAD peptide was used as control peptide. 3% BSA in PBS, pH7.4 acted as the negative control matrix and non-treated cells on FN as control group. Western blotting was performed to detect the antigen level of p-FAK$^{861}$ and total FAK as introduced in Material and Methods. Figure 4.3.7 shows the level of p-FAK$^{861}$. As expected in the negative control group, the level of p-FAK$^{861}$ decreased to 13% of that of the control FN group, while RAD treatment did not affect the phosphorylation of FAK$^{861}$ on FN, indicating non-toxic effect of 100μg/ml of both RAD and RGD peptide on phosphorylation of FAK. The TG-FN complex increased the amount of antigen, while no significant difference was found. RGD peptide treatment significantly reduced the level of p-FAK$^{861}$ when the cells were seeded on FN to 8%, compared to control level, which was compensated by TG-FN back to 65%. No significant difference was observed in the levels of total FAK between each group via Western blotting (Figure 4.3.7). The above data suggests the involvement of the phosphorylation of FAK$^{861}$ in the signalling transduction process mediated by TG-FN and the importance of the β1 integrin inside-out signalling pathway.

### 4.3.3 ERK1/2 dependent signalling on TG-FN

#### 4.3.3.1 ERK1/2 inhibitor blocks the compensatory effect of TG-FN

It has been reported that ERK1/2 is one the most important downstream molecules in the β1 integrin-relevant cell survival pathway (Yee et al., 2008). To investigate the potential importance of the interaction between ERK1/2 in RGD-independent cell adhesion mediated by TG-FN, cell adhesion was performed by using a specific ERK1/2 inhibitor, PD98059 in Swiss 3T3 cells. As shown in Figure 4.3.8, the RAD treatment did not affect etiher cell attachment or spreading in the DMSO vehicle control (0.1% (v/v)), suggesting at the concentration of 100μg/ml, no toxicity was found in both RAD and RGD-treated cells. In these fibroblasts, 35% and 29% of cell attachment and spreading was blocked,


**Figure 4.3.8 The involvement of phosphorylation of ERK1/2 in RGD-independent cell adhesion to FN and TG-FN matrices.**
The Swiss 3T3 fibroblasts were treated with ERK1/2 inhibitor PD98059 (10μM, 1 h) and the cell adhesion assay was performed as described previously (Section 4.2.1). The attachment and spreading of Swiss 3T3 cell was analysed as introduced in Methods (Section 2.2.3). Each data point represents the mean percentage of attached cells (cell attachment) or the mean percentage of spread cells (cell spreading) ± S.D. of three separate experiments performed in triplicate. The ordinates in the top graphs represent the mean cell attachment expressed as mean percentage of DMSO-treated Swiss 3T3 cells (control) attachment to FN ± S.D., presenting 100%. The mean attachment value ± S.D was 200 ± 5. The ordinates in the lower graphs designate the mean percentage of spreading cell, expressed as mean percentage of spreading on FN ± S.D. The mean percentage of control spreading on FN ± S.D was assessed as 88 ± 1, and normalized to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups.

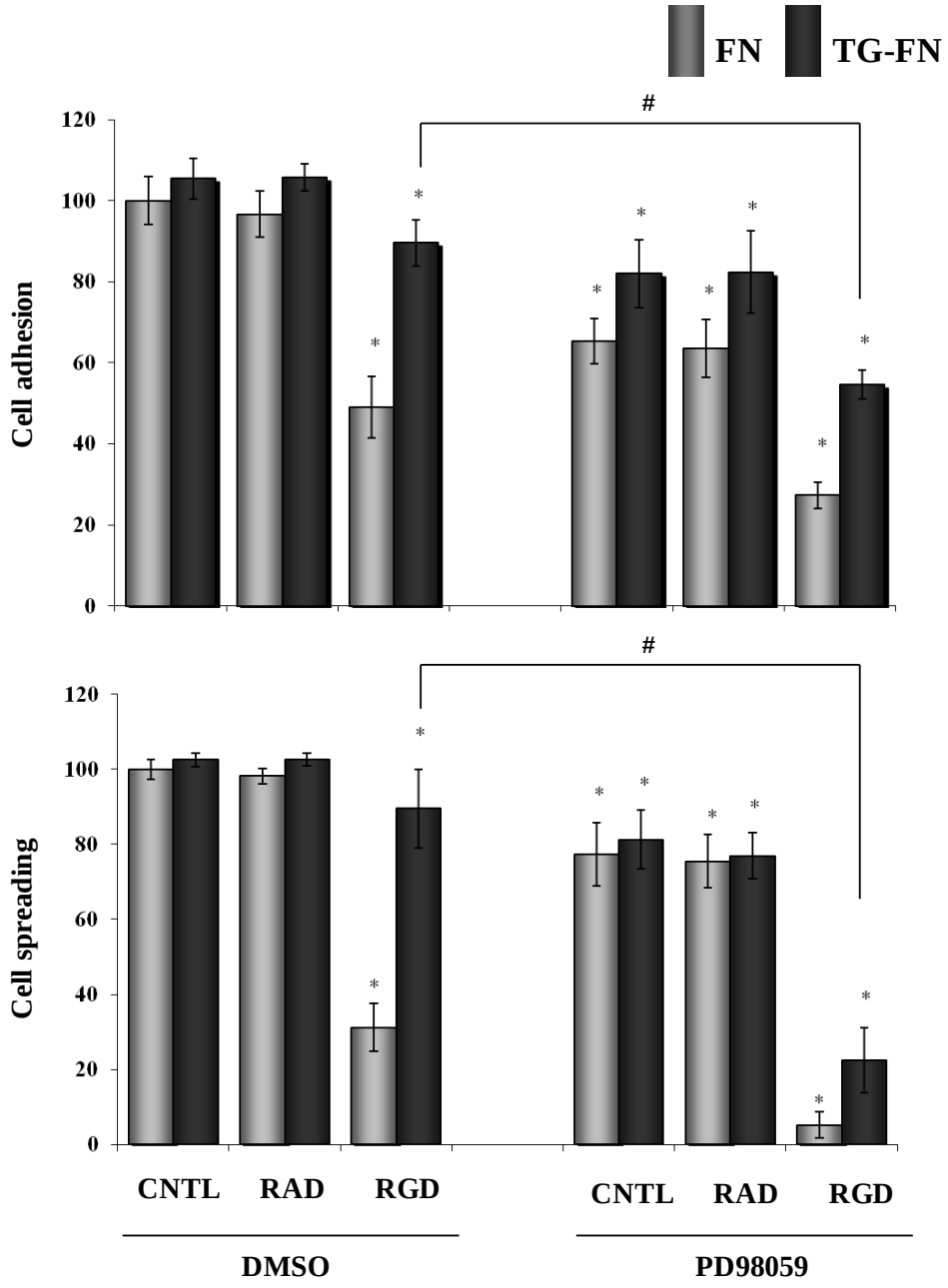

FN
TG-FN
Cell adhesion
Cell spreading
120
100
80
60
40
20
0
#
*
CNTL
RAD
RGD
DMSO
PD98059

**Figure 4.3.8**


**Figure 4.3.9 Detection of the phosphorylation of ERK1/2 in the RGD-independent cell adhesion in Swiss 3T3 cells.**
The RGD-independent cell adhesion assay was performed as introduced before (Section 2.2.3). 50µg/sample of total protein collected in cell lysis buffer was used to detect the presence of specific antigens by using specific antibodies, including rabbit polyclonal anti-p-ERK1/2 (1:1000 dilution) (a, top image) and mouse monoclonal anti-ERK1/2 (0.6µg/blot) (b, top image). The membranes were stripped and re-probed with mouse monoclonal anti-α-tubulin antibody to ensure the equal loading (a and b, bottom images). The densitometric values obtained for the bands was normalised against the values for the tubulin bands and represented as the mean net area value (relative p-ERK1/2, or relative ERK1/2) ± S.D. from three separate experiments. Each data point was expressed as a percentage of control FN level, which represents 100%. The * symbol indicates the statistically different values when compared to the FN control. Lane 1, non-treated cells seeded on BSA, Lane 2 non-treated cell seeded on FN, and TG-FN (Lane 3), Lane 4, RAD-treated cells FN and FN-TG2 (Lane 5), Lane 6, RGD-treated cells on FN and TG-FN (Lane 7).

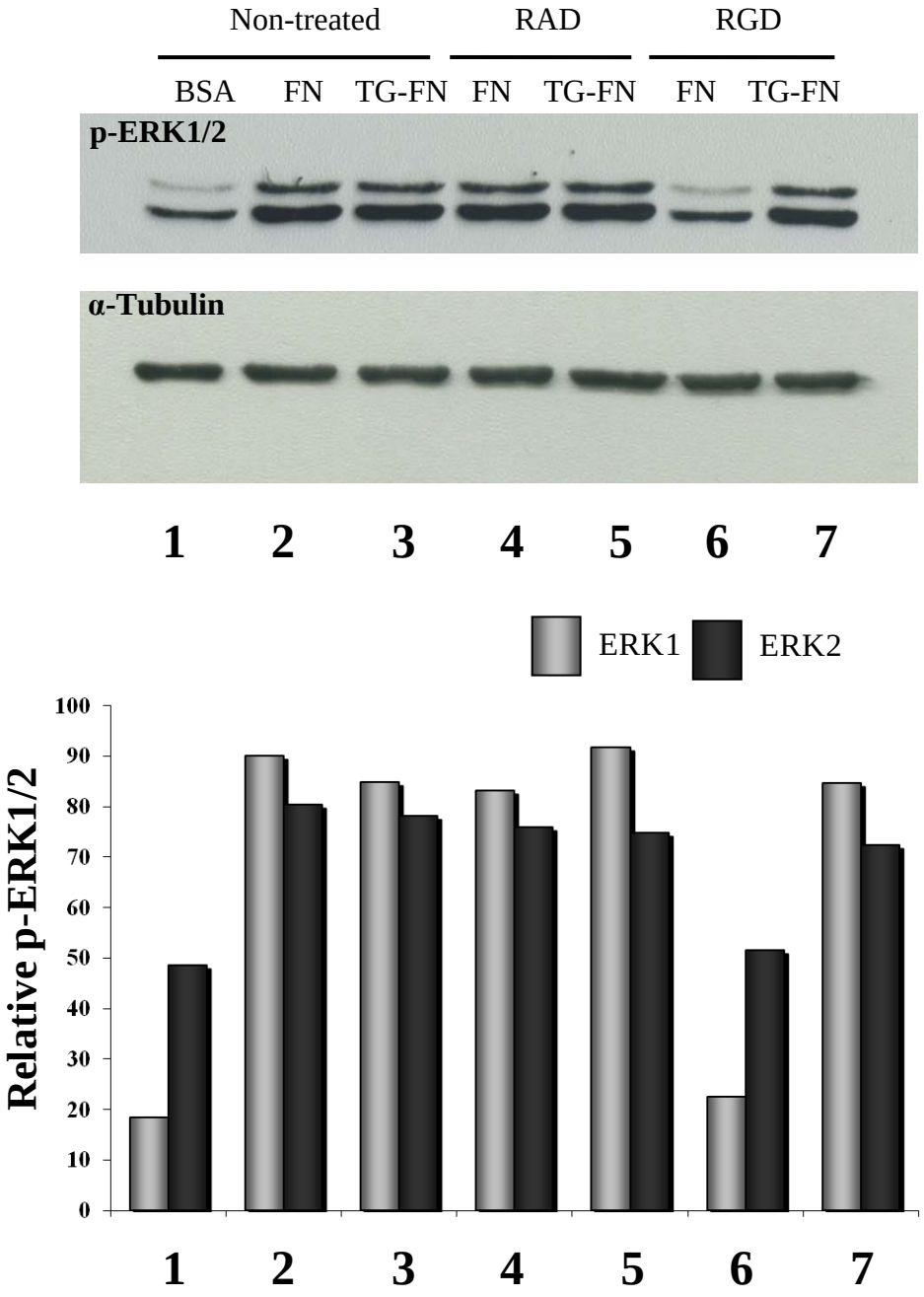

Non-treated
RAD
RGD
BSA
FN
TG-FN
FN
TG-FN
FN
TG-FN
p-ERK1/2
α-Tubulin
1 2 3 4 5 6 7
ERK1
ERK2
Relative p-ERK1/2
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7

**Figure 4.3.9a**

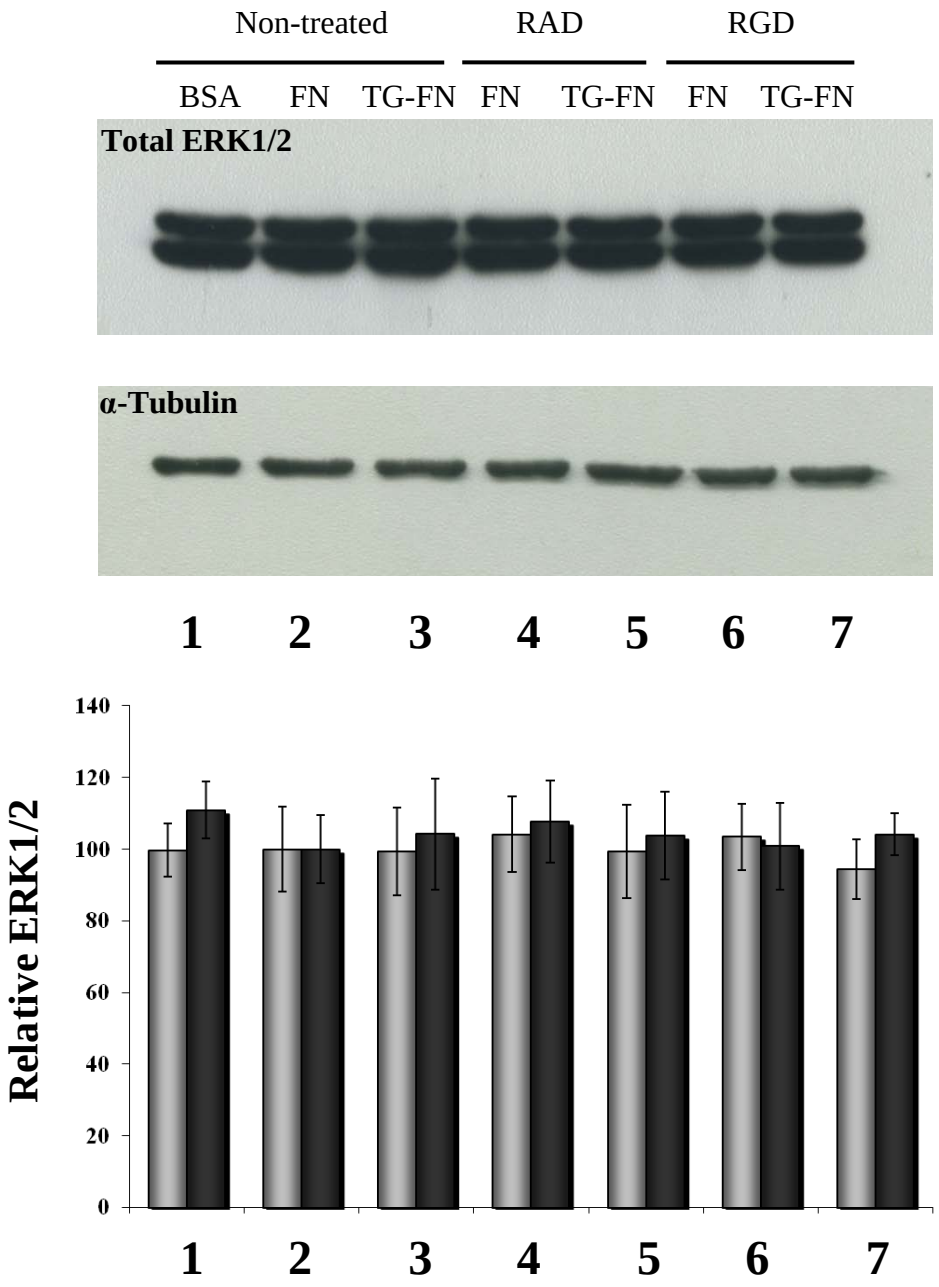

Non-treated
RAD
RGD
BSA
FN
TG-FN
FN
TG-FN
FN
TG-FN
Total ERK1/2
α-Tubulin
1 2 3 4 5 6 7
Relative ERK1/2
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7

**Figure 4.3.9b**

respectively in the presence of PD98059, suggesting the involvement of ERK1/2 in the cell adhesion process. When treated with RGD peptide, a further 38% inhibition of cell attachment and 65% of spreading took place on FN. Most importantly, the inhibitory effect of PD98059 significantly inhibited the compensation of TG-FN, compared to the DMSO-treated cells ($p<0.05$). As shown in Figure 4.3.8, in the presence of RGD peptide, the compensation dropped from 87% to 57% in cell attachment and 82% to 22% in cell spreading, suggesting that TG-FN is dependent on ERK1/2 signalling to rescue cells from RGD-independent cell adhesion.

#### 4.3.3.2 TG-FN compensates the phosphorylation of ERK1/2 in the presence of the RGD peptides

The potential influence of RGD on the phosphorylation of ERK1/2 was further investigated by detecting the phosphorylation of ERK1/2 in HOB cells seeded on FN or TG-FN matrices via Western blotting (as described in Section 2.2.8). Figure 4.3.9a represents the p-ERK1/2 levels, while figure 4.3.9b shows the total level of ERK1/2 in fibroblast cells on BSA, FN or TG-FN in the presence of RAD or RGD peptide. BSA was used as a negative control and led to 80% reduction in ERK1 and 50% in ERK2. Confirming the cell adhesion results, no effect of RAD peptide in ERK1/2 phosphorylation was obtained compared to non-treated control groups. RGD peptide treatment reduced the levels of ERK1 and ERK2 down to ~40% in both ERK1 and ERK2, compared to control cells on FN. Even though no significant difference was discovered between cells on TG-FN and FN in the presence of RAD peptide, TG-FN restored the level of p-ERK1/2 back to around 90% in the RGD-treated cells.

Western blotting was also performed in parallel to study the effect of RGD inhibition on the total levels of ERK1/2 in the same protein samples. As shown in Figure 4.3.9, RGD treatment did not affect the total levels of ERK1/2 compared to RAD treatment, and no difference was found between the FN and the TG-FN samples. The above data suggests that the compensatory effect of TG-FN is dependent on the phosphorylation of ERK1/2.

### 3.3.4 β1 integrin inside-out signalling is involved in the signal transduction mediated by TG-FN

#### 3.3.4.1 The effect of β1 integrin functional blocking antibody on the phosphorylation of FAK

FAK is the downstream signalling molecule in the $\beta_1$ integrin pathway. The phosphorylation of $Tyr^{861}$ and $Tyr^{397}$of focal adhesion kinase (FAK) indicates the activation of the signal transduction mediated by $\beta_1$ integrins. It has been proven that the phosphorylation of $Tyr^{861}$ is independent of the need for integrin ligand binding, and is a key step in the mediation of the inside-out signalling pathway of $\beta_1$ integrin unlike the phosphorylation of $Tyr^{397}$which indicates the activation of FAK in the cells adherent to their cell binding sites within the ECM (Shi and Boettiger, 2003). To investigate the potential function of $\beta_1$ integrin functional blocking antibody— HMβ1-1 (Noto, *et al*., 1995), p-$Tyr^{861}$ and p-$Tyr^{397}$ were used as two parameters to study which signalling pathway of β1 integrin is affected by this antibody. In HMβ1-1 treated Swiss 3T3 cells, the antibody inhibited the phosphorylation at $Tyr^{397}$ compared to isotype antibody treated cells on FN matrix, while the phosphorylation of $Tyr^{861}$ was not affected (Figure 4.3.10). This suggests that the effect of HMβ1-1 is to block the outside-in signalling pathway, but not the inside-out signal transduction of β1 integrin, as such it inhibits cell adhesion on FN.

#### 3.3.4.2 TG-FN compensates the RGD-independent cell adhesion through β1 integrin inside-out signalling pathway

After confirming the function of HMβ1-1 in blocking the outside-in signalling pathway of β1 integrin, cell adhesion assay was performed on FN and TG-FN matrices. At the concentration of 25μg/ml, HMβ1-1 treatment led to a 46% decrease in cell attachment and blocked 56% of cell spreading on FN compared to non-treated cells seeded on FN, while its isotype control antibody did not

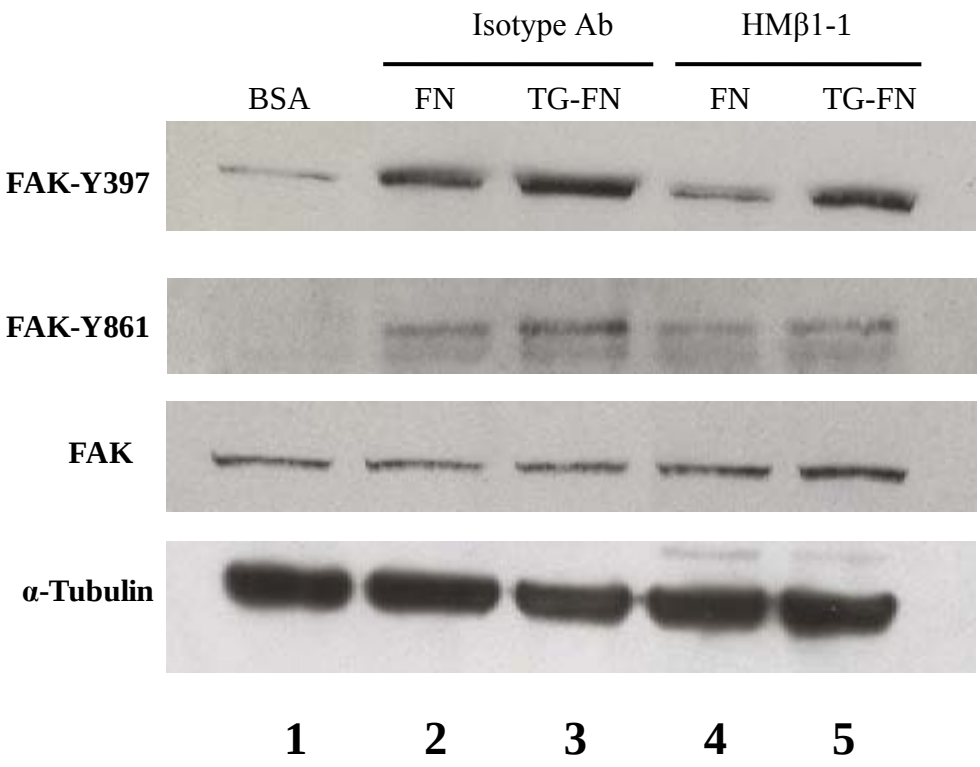



**Figure 4.3.10 Detection of the phosphorylation of FAK at Tyr$^{397}$ and Tyr$^{861}$ in the RGD-independent cell adhesion of HMβ1-1-treated Swiss 3T3 cells.**
The RGD-independent cell adhesion assay was performed as introduced before (Section 2.2.3) by using β1 integrin functional blocking antibody 25μg/ml HMβ1-1 pre-treated-Swiss 3T3 cells (1 h). 50μg/sample of total protein collected in cell lysis buffer was used to detect the presence of specific antigens by using specific antibodies, including rabbit polyclonal anti-p-Tyr397-FAK (1:1000), rabbit polyclonal anti-p-Tyr861-FAK (1:1000 dilution), and mouse monoclonal anti-FAK (0.6μg/blot). The membranes were stripped and re-probed with mouse monoclonal anti-α-tubulin antibody to ensure the equal loading. Lane 1, non-treated cells seeded on BSA, Lane 2 Isotype negative control antibody-treated cell seeded on FN, and TG-FN (Lane 3), Lane 4, HMβ1-1-treated cells FN and TG-FN (Lane 5).

**Figure 4.3.10**


**Figure 4.3.11 The involvement of inside-out signalling of β1 in RGD-independent cell adhesion to FN and TG-FN matrices.**
The Swiss 3T3 fibroblasts were treated with 25μg/ml of β1 integrin functional blocking antibody HMβ1-1 for 1 h and cell adhesion assay was performed as described previously (Section 2.2.3). The isotype control antibody was used as a negative control treatment. The attachment and spreading of Swiss 3T3 cell was analysed as introduced in the Methods (Section 2.2.3.4). Each data point represents the mean percentage of attached cells (cell attachment) or the mean percentage of spread cells (cell spreading) ± S.D. of three separate experiments performed in triplicate. The ordinates in the top graphs represent the mean cell attachment expressed as mean percentage of non-treated Swiss 3T3 cells (control) attachment to FN ± S.D., presenting 100%. The mean attachment value ± S.D. was 135 ± 5. The ordinates in the lower graphs designate the mean percentage of spreading cell, expressed as mean percentage of spreading on FN ± S.D. The mean percentage of control spreading on FN ± S.D was assessed as 80 ± 2, and normalized to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of the antibodies when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups.

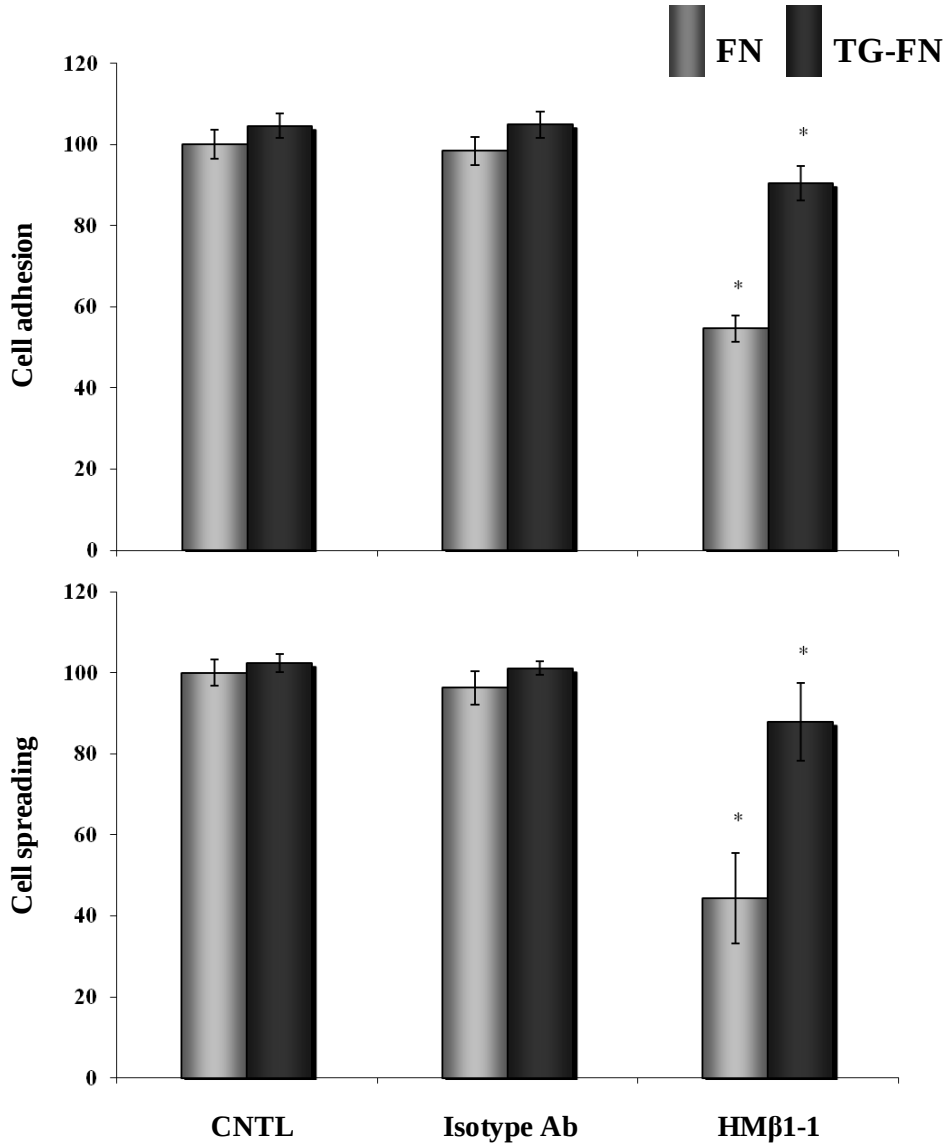

FN
TG-FN
Cell adhesion
Cell spreading
120
100
80
60
40
20
0
*
CNTL
Isotype Ab
HMβ1-1

**Figure 4.3.11**

show any effect on either cell attachment or spreading. This confirms the effect of HMβ1-1 on cell adhesion is not due to its toxicity. In contrast cells seeded on TG-FN matrix restored the loss of cell attachment and spreading back to ~90% (Figure 4.3.11). It has been suggested that HMβ1-1 exerts its blocking effect by inhibiting the signalling transduction of the β1 integrin outside-in pathway (Noto, *et al*., 1995), which has been confirmed (see above) by Western blotting of the phosphorylation of p-FAK$^{397}$ and p-FAK$^{861}$. Hence the compensatory effect of TG-FN in the presence of this antibody was understood to be dependent on the inside-out signalling of β1 integrins in an outside-in pathway-independent manner.

### 4.3.5 ROCK is a downstream molecule in the TG-FN signalling pathway

Previous data indicated that syndecan-2 is important for TG-FN to mediate RGD-independent cell adhesion. To further investigate this signalling pathway, ROCK, one of the important intracellular molecules in syndecan-2 signal transduction, was studied by using its specific inhibitor—Y27632 in the RGD-independent cell adhesion assay. Fibroblasts cells were pre-treated with 10μM Y27632 for 30 min and then cell adhesion in the presence of RGD or RAD peptide was performed as introduced in Section 2.2.3. DMSO was used as the vehicle control, while non-treated cells on FN were used as the control group. As shown in Figure 4.3.12, at the concentration of 0.1% (v/v), on FN no significant difference in cell adhesion and spreading was observed between the DMSO group and control group in Swiss 3T3 cells, suggesting no toxicity of DMSO on both cell lines, meanwhile RAD treatment did not show toxic effects on both cell lines, as shown in previous results. Y27632 treatment led to 37% and 80% loss of cell attachment and spreading, respectively, on FN in these fibroblasts, suggesting inhibition of ROCK affected cell spreading more than cell attachment on FN. In the presence of RGD peptide, Swiss 3T3 cells lost a further 40% in cell attachment and non-spreading cells were found in this group. Unlike the significant compensatory effect of this matrix complex in the non-treated and DMSO-treated cells, TG-FN could not compensate the loss of either cell attachment or spreading in Swiss 3T3 cells in the presence of ROCK inhibitor

Y27632, since no significant difference was found between the cells seeded on FN and TG-FN matrices ($p>0.05$). This data suggests that the TG-FN complex depends on the intracellular signalling molecule ROCK to exert its compensatory effect on RGD-independent cell adhesion.


**Figure 4.3.12 The involvement of ROCK in RGD-independent cell adhesion to FN and TG-FN matrices.**
The Swiss 3T3 fibroblasts were treated with ROCK inhibitor Y27632 and cell adhesion was performed as described previously (Section 4.2.1). The attachment and spreading of Swiss 3T3 cell were analysed as introduced in Methods (Section 2.2.3). Each data point represents the mean percentage of attached cells (cell attachment) or the mean percentage of spread cells (cell spreading) ± S.D. of three separate experiments performed in triplicate. The ordinates in the top graphs represent the mean cell attachment expressed as mean percentage of DMSO-treated Swiss 3T3 cells (control) attachment to FN ± S.D., presenting 100%. The mean attachment value ± S.D. was 140 ± 2. The ordinates in the lower graphs designate the mean percentage of spreading cell, expressed as mean percentage of spreading on FN ± S.D. The mean percentage of control spreading on FN ± S.D was assessed as 83 ± 3, and normalized to 100%. The * symbol represents significant difference ($p<0.05$) between FN and TG-FN matrices in the presence and/ or absence of RAD or RGD peptide when compared to the non-treated cells seeded on FN. The # symbol represents significant difference ($p<0.05$) between individual groups.

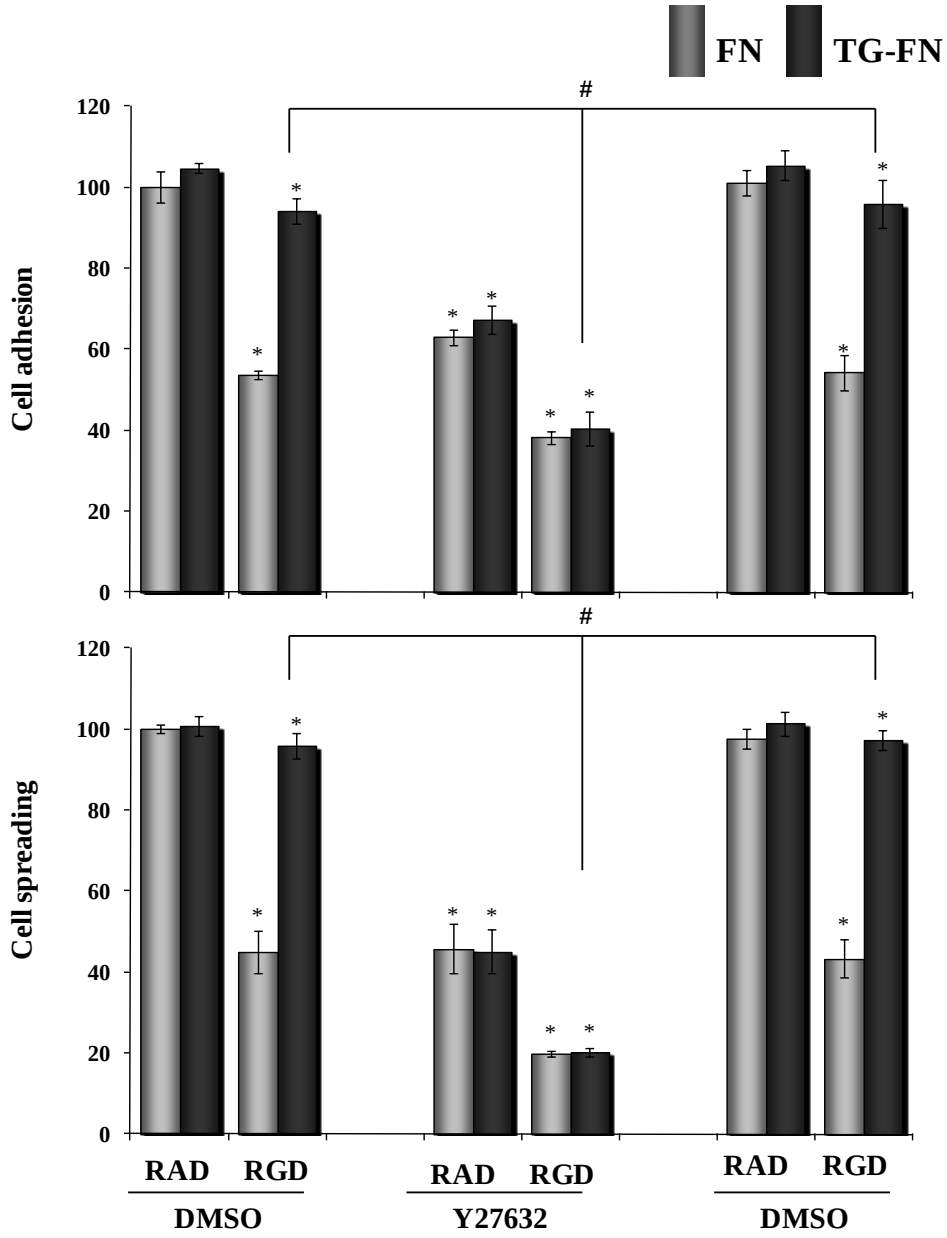

FN
TG-FN
Cell adhesion
Cell spreading
120
100
80
60
40
20
0
#
*
RAD
RGD
DMSO
Y27632

**Figure 4.3.12**

## 4.4 Discussion

The previous chapter demonstrated that cell surface receptors β1 integrin and syndecan-4/2 are crucial for the TG-FN complex to rescue the cells from RGD-dependent loss of cell adhesion. This chapter aimed to further investigate which intracellular signalling molecule or molecules are involved in this signal transduction process.

As one of the most important intracellular signalling molecules in syndecan-4 and β1 integrin co-signalling, the potential role of protein kinase C α (PKCα) was first investigated by using PKCα inhibitor Go6976 in cell adhesion assays. This inhibitor not only significantly blocked the cell adhesion process on FN, but also inhibited the compensatory effect of TG-FN in RGD-independent cell adhesion and spreading. This was the first indication that PKCα could be the intracellular signalling molecule in TG-FN mediated cell adhesion. This part of work was further explored by using special syndecan-4 mutant MEF cells, in which the syndecan-4 knockout cells were transfected with a syndecan-4 cDNA with the PKCα binding site mutated within the syndecan-4 intracellular domain (Bass et al., 2007b). As expected, without the support for the intracellular interaction between PKCα and syndecan-4, TG-FN lost its compensatory effect completely on RGD peptide-treated Y188L MEF cells, further confirming that PKCα participates in the signal transduction in cell adhesion on TG-FN. As a crucial link between syndecan-4 and β1 integrins, PKCα can bind to the cytoplasmatic domain of β1 integrins once it is activated and translocated from the cytosol to membrane. In order to prove that PKCα can directly interact with β1 integrins, which plays an important role in mediating RGD-independent cell adhesion on TG-FN, a specially designed GK21 peptide that mimics the PKCα binding site within β1 integrin cytoplasmatic domain (Parsons et al., 2002), was used in cell adhesion assays. In the peptide-treated cells, TG-FN failed to compensate the loss of cell attachment and spreading induced by GK21, suggesting not only the importance of PKCα, but also the interaction between PKCα and β1 integrins for TG-FN to exert its compensatory effect on RGD peptide induced loss of cell adhesion. As an indication of its activation, PKCα translocates from the cytosol

to membrane, where it binds to β1 integrins. In Western blotting by using PKCα specific antibody, PKCα antigen could be detected in both cytosol and membrane fractions, while in non-treated cells seeded on TG-FN, more signal was detected in membrane fractions compared to the cells on FN, suggesting that TG-FN activates PKCα signalling pathway and in turn promotes its translocation. This effect of TG-FN was inhibited by the treatment of PKCα inhibitor Go6976, which further confirmed the cell adhesion results and provided solid proof of the crucial role of PKCα. Even though it has been suggested that syndecan-2 is the downstream cell surface receptor for syndecan-4, there is no proof for this hypothesis yet. By using syndecan-4 knock out and Y188L mutant cells in co-immunoprecipitation assays, the decreased interaction between PKCα and syndecan-2 was detected in these two cells compared to wild type MEF cells, which for the first time demonstrates that syndecan-4 regulates the interaction between PKCα and syndecan-2 and suggested that PKCα is also a crucial link between syndecan-4 and syndecan-2 signalling.

Focal adhesion kinase (FAK) is an important kinase in $\beta_1$ integrin signalling in regulating focal adhesion and actin cytoskeleton formation. Once activated by β1 integrin signal, FAK can be autophosphorylated at $Tyr^{397}$, which will leads to the further phosphorylation at $Tyr^{861}$, $Tyr^{925}$, etc. RGD peptide treatment led to significant loss of p-$Tyr^{397}$ in cells seeded on FN compared to non- or RAD-treated cells, which could be restored in the cells on TG-FN matrix, suggesting this complex compensates RGD-independent cell adhesion in a FAK-dependent manner. The compensated phosphorylation of $Tyr^{861}$ can also be detected in RGD-treated cells on the TG-FN matrix. The interesting discovery of RGD-independent p-$Tyr^{861}$ in TG-FN matrix offered more information, considering that p-$Tyr^{861}$ indicates the activation of the ligand-independent cell adhesion process mediated by β1 integrins (Shi and Boettiger, 2003), suggesting the activation of $\beta_1$ inside-out signalling pathway by TG-FN mediated cell adhesion.

The improvement of cell adhesion and spreading mediated by TG-FN in an RGD-independent manner suggests the possibility that this matrix can protect the cell from cell adhesion- related apoptosis (named anoikis). In order to investigate

the cell survival pathway mediated by β1 integrins, the role of its downstream signalling molecule ERK1/2 was studied by using its specific inhibitor PD98059. As expected, PD98059 significantly inhibited the compensatory effect of TG-FN in RGD-treated fibroblasts, which offered the first indication of the involvement of ERK1/2 in the TG-FN signalling pathway. The importance of ERK1/2 was further proven by detecting the phosphorylation of ERK1/2 in RGD-treated and control cells on FN or TG-FN matrices via Western blotting. The p-ERK1/2 signal was significantly decreased in RGD-treated cells on FN, which was restored by the TG-FN complex, indication that ERK1/2 regulates RGD-independent cell survival pathway mediated by the TG-FN matrix complex.

The role of p-FAK$^{861}$, as an indicator of the activation of the inside-out signalling in $\beta_1$ integrin signalling pathway, has been introduced above. The compensation of p-FAK$^{861}$ by TG-FN suggested that this complex could exert its function in mediating cell adhesion through the inside-out signal transduction of β1 integrins activated by syndecan-4. In order to further confirm this hypothesis, a β1 integrin functional blocking antibody-HMβ1-1, which has been suggested to block the β1 integrin outside-in signalling (Noto et al., 1995), was used in cell adhesion assays on FN and TG-FN matrices. In order to confirm the effect of HMβ1-1 on the β1 integrin signalling, the phosphorylation of Tyr$^{861}$ and Tyr$^{397}$ were investigated via Western blotting. This functional blocking antibody inhibited p-Tyr$^{397}$, while the p-Tyr$^{861}$ was not significantly affected, which further confirmed that HMβ1-1 blocked cell adhesion via inhibiting the β1 integrin outside-in signalling, but not the inside-out one. Unlike the cells on FN, the cell adhesion and spreading process on TG-FN was not affected by the HMβ1-1 treatment, suggesting that TG-FN compensates RGD-independent cell adhesion via activating β1 integrin inside-out signalling triggered by its interaction with syndecan-4.

As a well-known downstream signalling molecule in syndecan-2 signal transduction, the potential involvement of ROCK was investigated by using its specific inhibitor Y27632 in the RGD-independent cell adhesion assay. Y27632 completely blocked the compensation of TG-FN in RGD-treated fibroblasts, suggesting ROCK is involved in cell adhesion on TG-FN. Interestingly, this

inhibitor affected cell spreading process more significantly than cell adhesion on both FN and TG-FN, further confirming the importance of syndecan-2 in regulating cell spreading and actin cytoskeleton formation.

To sum up the above observation, this chapter's work demonstrates that the intracellular signalling molecules PKCα, FAK, ERK1/2, and ROCK are crucial for TG-FN to exert its compensatory effect on RGD-induced loss of cell adhesion. Importantly PKCα is likely to be the essential link between syndecan-4 and syndecan-2 in activating $\beta_1$ integrin inside-out signalling pathway mediated by TG-FN.

# Chapter 5:
# Fibronectin deposition mediated by TG-FN matrix

# Chapter 5: Fibronectin deposition mediated by TG-FN matrix

## 5.1 Introduction

The importance of FN matrix for the survival and functioning of adhesion-dependent cells, including cell adhesion, growth, development, differentiation and migration, has been very well documented (Kaspar et al., 2006). Cells adherent to FN matrix can in turn moderate the structure and deposition of FN molecules, also known as FN fibril assembly. This is initiated by the interaction between cell surface receptors integrins and matrix FN. Cell surface integrin-bound FN is deposited into matrix with the association of actin skeleton formation. It has been reported that the above process of FN deposition depends on the involvement of different cell surface receptors and intracellular signalling molecules. α5β1 integrin is one of major cell surface receptors that has been proven to participate in FN assembly. Interacting with soluble or secreted FN on the cell surface, clustered α5β1 integrin can promote formation of short FN fibrils and link FN to the actin skeleton network through its intracellular domain. Other cell surface receptors (e.g. syndecan-2) are also suggested to be involved in FN deposition via association with α5β1 integrin, meanwhile intracellular molecules, PKC (Lin et al., 2002) and RhoA (Zhong, *et al*., 1998) also participate in this process.

The involvement of cell surface TG2 in fibronectin fibril formation and deposition has been previously documented (Akimov, et al, 2001), where it was shown that the enzyme cooperates with but cannot substitute for α5β1 integrin in fibronectin assembly. Transamidating activity was not thought to be involved in this mechanism. In contrast in other reports the crosslinking activity of TG2 was reported to be important in FN assembly and deposition (Verderio et al., 1999; Yuan et al., 2007). Previous work demonstrated the importance of TG-FN in restoring RGD-independent cell adhesion, while the long-term effect of TG-FN in rescuing RGD-induced anoikis has not been demonstrated yet. In this chapter the potential role of TG-FN in regulating FN fibril assembly process is investigated.

The aim of this chapter is to further study the influence of TG-FN matrix in regulating the matrix turnover process, such as FN fibril formation in the presence of RGD peptides. By using fluorescence staining of biotin-labelled exogenous FN, confocal microscopy was used to detect the deposited FN matrix by the cells seed on FN or TG-FN matrix. Certain type of cell surface knockout MEF cells, binding site mutant cells and the siRNA treatment were used in the experiments to investigate the involvement of these receptors in the FN deposition by the cells seeded on FN-TG matrix.

## 5.2 Methods

### 5.2.1 Vinculin staining

Sub-confluent cells were serum-starved for 16 h and harvested. After 20 min treatment with RAD or RGD peptide, cells were seeded in 8-well glass chamber slides ($8\times10^4$ cells/well) previously coated with FN or TG-FN and allowed to attach and spread for 1 h. Cells were fixed and permeablized as described previously (Section 2.2.3.2). For staining of vinculin, cells were blocked in PBS buffer supplemented with 3% (w/v) heat-inactivated BSA and then incubated with mouse anti-vinculin antibody (Sigma-Aldrich) in blocking buffer for 2 h, followed by 2 h incubation with FITC-conjugated anti-mouse secondary antibody (Dako). Coverslips were mounted with Vectashield mounting medium and using constant PMT and section depth settings 9 random fields/ samples were captured by a Zeiss LSM510 laser confocal microscopy using the Zeiss LSM Image Browser.

### 5.2.2 Biotin-labelled FN

Biotinylated FN prepared according to manufacturer's protocol. 1 mg/ml of human plasma fibronectin (2.2μM (Merck, UK)) was incubated with 22μM Sulfo-NHS-LC-Biotin (Peirce, UK) (ratio 1:10) at room temperature for 30 min. Biotin-FN solution was removed by dialysis in dialysis buffer (PBS, pH7.4) at 4ºC for 24 h. The concentration of biotinylated FN solution was determined by the Lowry Method (Section 2.2.7). Meanwhile the labelling effect was studied via Western blotting by using Extr-avidin conjugated antibody and anti-FN antibody to detect biotinylated FN and FN protein, respectively. Untreated human plasma FN was used as the internal standard (Section 2.2.8-2.2.10).

### 5.2.3 FN staining

A monolayer of cells on FN or TG-FN with RAD or RGD treatment was prepared as introduced in Section 2.2.3.1. Following a 1 h incubation, 50nM

exogenous biotinylated-FN was added into fresh serum free medium. After different time courses of incubation, fixed cells (Section 2.2.3.2) were blocked with 3% (w/v) heat-inactivated BSA in PBS pH7.4 and then incubated with 1μg/ml Cy5-streptavidin (Jackson Immno Research) for 2 h at 37ºC. After washing 5 times in PBS, pH7.4, the slides were mounted with Vectashield mountant (Vector Laboratories) and examined by confocal fluorescence microscopy (Zeiss).

## 5.3 Results

### 5.3.1 TG-FN promotes the loss of cell adhesion formation caused by the RGD peptides

It has been well documented that vinculin is one of the major proteins in focal adhesion. In order to investigate the role of TG-FN in focal adhesion formation, immunofluorescence staining of vinculin was performed by using wild type MEF cells. After RAD or RGD treatment, MEF cells were seeded onto FN or TG-FN matrices for 1 h. After blocking with 3% (w/v) BSA in PBS, pH7.4 for 30 min, the cells were incubated with anti-vinculin antibody and then FITC-conjugated secondary antibody. Immunofluorescence signals were detected using the confocal microscope, as described in Section 2.2.4. As shown in Figure 5.1, more vinculin is present at focal contacts detected in the cells on TG-FN complex compared to the FN matrix in the presence of RAD peptide. On the FN matrix, RGD peptide reduced the presence of vinculin in focal adhesions and the cells remained roundish morphologically. TG-FN restored the formation of focal adhesions inhibited by the RGD peptide treatment. This data indicates TG-FN can compensate the loss of focal adhesions induced by RGD peptides.

### 5.3.2 Detection of biotinylated-FN

After biotinylation and dialysis, Western blotting was performed to detect biotin-labelled FN protein by using both anti-human FN and Extra-avidin conjugated antibody. The Western blots displayed in Figure 5.3.2 shows an SDS-PAGE (3% (w/v) polyacrylamide stacking gel and a 5% (w/v) resolving gel) separation. Human plasma FN was used as standard marker. Biotinylated FN was detected by using Extr-avidin conjugated antibody at molecular weight of 220kDa, meanwhile FN protein was detected by using anti-human FN antibody, no difference was observed between biotin-labelled FN and standard FN molecule, suggesting that biotinylation does not affect the molecular weight of FN molecules.

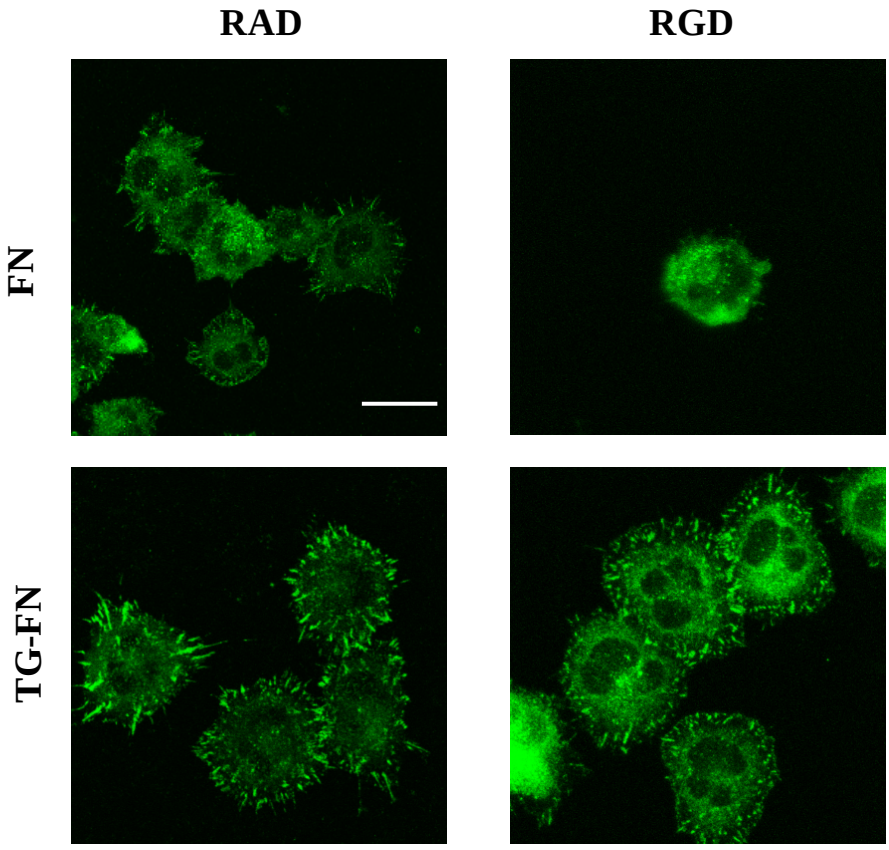



**Figure 5.3.1 TG-FN compensates the loss of focal adhesion formation caused by RGD peptide.**
The focal adhesion was stained as described in Section 5.2.1. $4\times10^4$ cells/ well of wild type MEF cells were seeded onto FN or TG-FN matrices for 1 h in the presence of RAD or RGD peptide. After fixation and blocking, the cells were incubated with anti-vinculin antibody (1:200 dilution) for 2 h and FITC-conjugated anti-mouse secondary antibody (1:200 dilution) for 2 h at 37ºC. The slides were mounted with Vectashield mounting medium and using constant PMT and section depth settings 9 random fields/sample were captured by Zeiss LSM510 laser confocal microscopy using Zeiss LSM Image Browser.

**Figure 5.3.1**

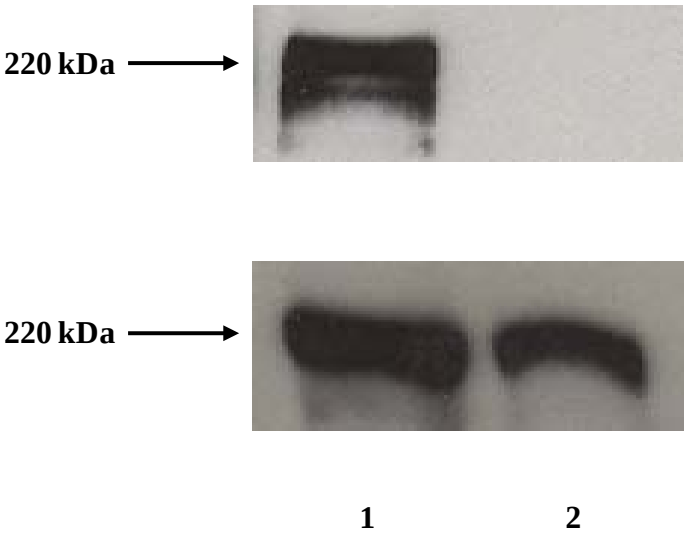



**Figure 5.3.2 Detection of biotinylated FN molecule via Western blotting**
Biotinylated FN solution was prepared as described in Section. Briefly, 2.2μMof FN solution in PBS was incubated with 22μM Sulfo-NHS-LC-Biotin for 30 min at room temperature. The solution was dialysed in PBS, pH7.4 for 24 h and protein concentration was determined via Lowry Method. Western blotting was performed to detect biotinlylated FN and FN antigens. In figure 5.3.2, the upper blot shows biotinylated FN (by using Extr-evidin conjugated antibody, 1:2000 dilution), while the bottom blot represents the FN protein antigen by using anti-FN antibody (1:2000 dilution and secondary antibody 1:2000 dilution). Lane 1, biotinylated FN and Lane 2, human plasma FN standard.

**Figure 5.3.2**

### 5.3.3 TG-FN restores the loss of FN deposition induced by the RGD peptide treatment

To study the potential involvement of TG-FN in FN matrix deposition, biotin-labelled FN was used for incubation with MEF cells treated with RAD or RGD peptide on FN or TG-FN matrices, while different time courses were applied to detect the time-dependent (1-6 h) effect of TG-FN on FN assembly as introduced in Section 5.2.3. In Figure 5.3.3, the biotin-labelled FN was detected. In RAD peptide-treated cells, TG-FN started to promote FN deposition within 1 h incubation, which increased the FN fibril formation in a time-dependent manner, compared to FN alone. With increasing time, more well-organized FN fibrils around and over the cells were observed in the TG-FN groups. Unlike the control cells with RAD treatment, RGD peptide inhibited the formation of FN fibrils and biotin-labelled FN which remained scattered around the cell surface. In longer-term incubations, even though there was FN deposition, the FN matrix was randomly deposited with short and non-organized fibrils. Unlike on the FN matrix, cells seeded on TG-FN complex restored the FN fibril assembly in the presence of RGD peptide, and the FN deposition increased in a time-dependent manner, which agrees with the results obtained from cells treated with RAD peptide. And well-formed FN fibril network was observed in these groups on TG-FN at 6 h incubation with the cells, suggesting the importance of TG-FN in regulating FN deposition in an RGD-independent manner.

### 5.3.4 The crucial role of PKCα in FN deposition mediated by TG-FN

Previous work suggested that PKC is involved in the FN deposition process (Lin et al., 2002). In order to investigate the potential involvement of PKCα in FN deposition mediated by TG-FN, GK21 peptide (which blocks the interaction between PKCα and the intracellular domain of β1 integrins) and the syndecan-4 Y188L mutant MEF cells (the PKCα binding site mutant in syndecan-4 intracellular domain) were used in the FN fibril formation assay on FN or TG-FN matrices. After 1 h incubation on FN or TG-FN, the GK21 peptide pre-treated wild type MEF,

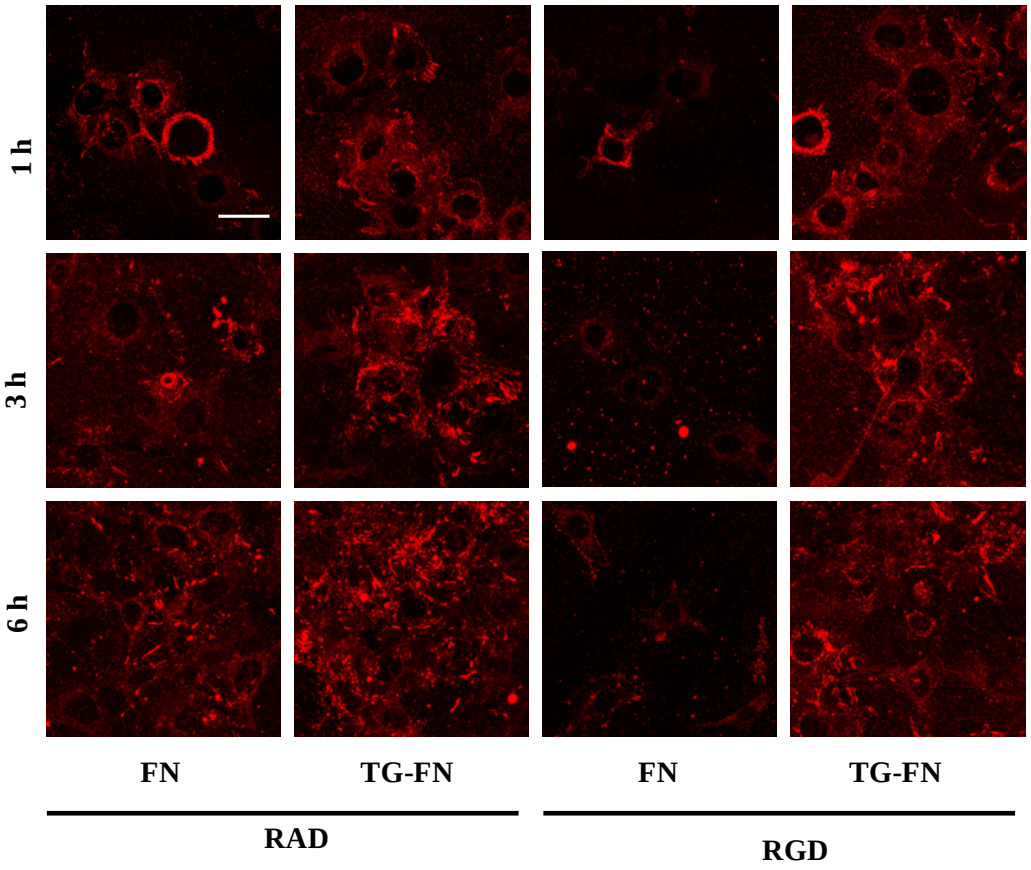



**Figure 5.3.3 TG-FN restores the loss of FN deposition induced by RGD peptide.**
$6\times10^4$ cells/ well wild type MEF cells were seeded into FN or TG-FN coated chamber slides in the presence of RAD or RGD peptide. After 1 h incubation, the monolayer of cells were washed once with serum free medium and then incubated with 50nM biotinylated FN for 1 h, 3 h, and 6 h, respectively. At the end of each incubation and fixation of the cells with 3.7% paraformaldehyde in PBS, pH 7.4, following 30 min blocking in 3% BSA in PBS, pH 7.4, biotinylated FN was stained with 1µg/ml Cy5-streptavidin in 3% heat-inactivated BSA in PBS, pH 7.4. Slides were mounted with Vectashield mountant (Vector Laboratories) and examined by confocal fluorescence microscopy (Leica Lasertechnik).

**Figure 5.3.3**

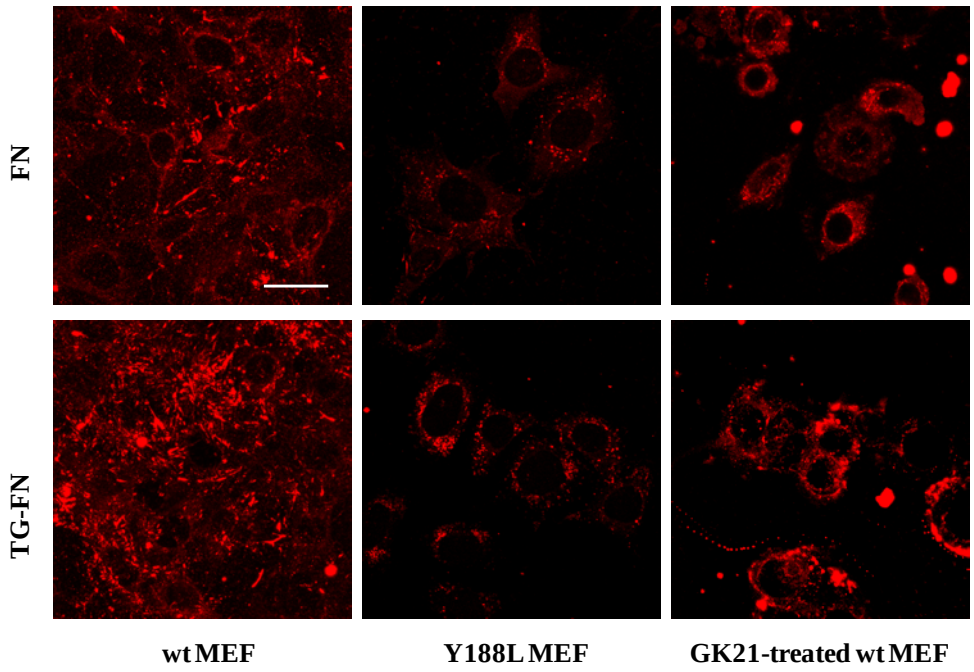



**Figure 5.3.4 TG-FN mediates FN deposition via PKCα- dependent signalling pathway.**
Wild type MEF cells were pre-treated with 8μM GK21 peptide for 1 h to block the interaction of PKCα and the β1 integrin intracellular domains. Wild type MEF, Y188L (PKCα-binding domain mutant syndecan-4 MEF cells) and GK21 peptide-treated wild type MEF cells were seeded onto FN or TG-FN matrices at the density of $6\times10^4$ cells/ well and allowed to adhere for 1 h. 50nM biotinylated FN was added to monolayer of cells in serum free medium and incubated for 6 h. Following washing, fixation, and blocking procedures, deposited FN was stained by using Cy5-streptavidin (1μg/ml) for 1 h and the mounted slides with Vectashield mountant were visualized by confocal microscopy.

**Figure 5.3.4**

Y188L MEF and wild type MEF cells were incubated with 50nM exogenous biotinylated-FN for 6 h and then FN deposition was detected via fluorescence staining. As shown in Figure 5.3.4, wild type MEF cells on FN matrix started to organize the FN matrix after 6 h incubation, while more well-organized FN fibrils were observed in the group of cells seeded on the TG-FN complex. Unlike the wild type cells, GK21 peptide treatment and Y188L mutant cells could not deposit a FN matrix and biotin-labelled FN was in the main deposited around the cell surface, suggesting the importance of PKCα in the FN deposition process. Most importantly, TG-FN could not compensate the FN fibril formation in the GK21-treated or the Y188L syndecan-4 mutant cells. The above data suggests that PKCα is the crucial link in FN deposition process. The loss of increased FN deposition in cells seeded on TG-FN suggested that the TG-FN complex promotes FN deposition process through a PKCα-dependent pathway. Meanwhile the potential involvement of β1 integrins and syndecan-4 was suggested since it has been proved in previous work that PKCα links the signalling pathway of these two cell surface receptors—β1 integrin and syndecan-4.

### 5.3.5 The importance of α5β1 integrin in FN deposition regulated by TG-FN

By using GK21 peptide-treated MEF cells, it is suggested that TG-FN promotes FN deposition via a PKCα signalling pathway mediated by interaction between PKCα and β1 integrin. To investigate the roles of α5 and β1 integrins, the FN deposition assay was performed by using α5 and β1 integrin null MEF cells. Monolayers of cells on FN or TG-FN matrices were prepared following a 1 h incubation of above cells on these matrices. 50nM exogenous biotinylated FN was then added into each group and incubated with the cells for 16 h, immunofluorescence was detected by confocal microscopy. Figure 5.3.5 shows that in wild type cells, well-organized FN fibrils were deposited by the cells seeded on both FN and TG-FN matrices. Without the presence of α5 or β1 integrins, even after long-term incubation (16 h), the cells still could not deposit well-organized FN fibrils, compared to the wild type cells, only randomly

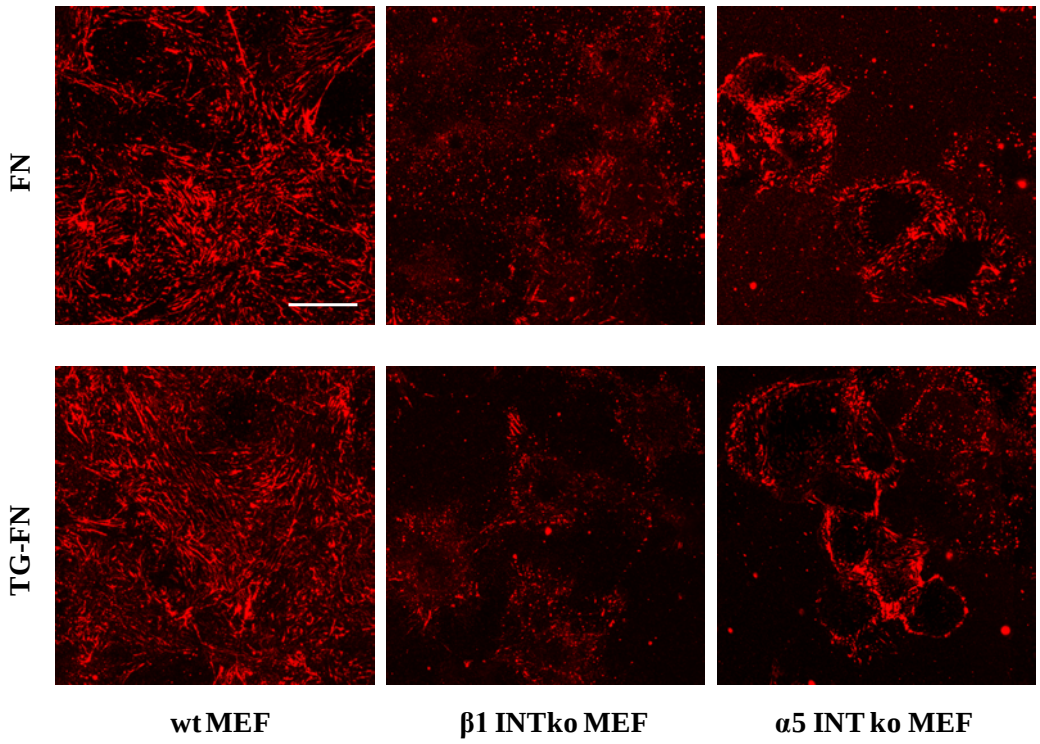



**Figure 5.3.5 α5 and β1 integrins are crucial for TG-FN to compensate RGD-independent FN deposition.**
Wild type, β1 integrin null MEF and α5 null EA5 fibroblasts were seeded onto FN or TG-FN matrices at the density of $6\times10^4$ cells/ well for 1 h to obtain the monolayer of above cells. 50nM biotin-labelled FN in serum free medium was incubated with cells for 16 h. The slides were prepared as introduced in Section 5.2.3 and visualized by confocal microscopy.

**Figure 5.3.5**

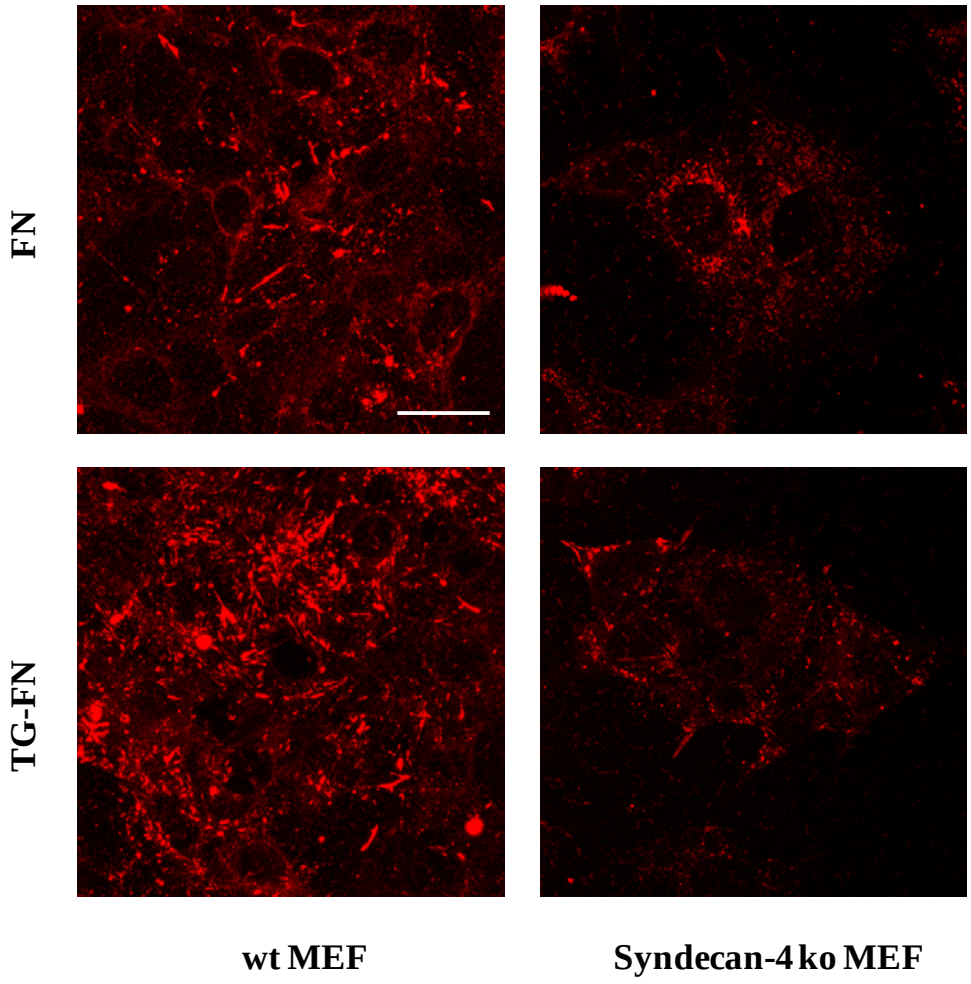



**Figure 5.3.6 The importance of syndecan-4 in FN deposition mediated by TG-FN complex.**
Monolayers of wild type and syndecan-4 null MEF cells were prepared as introduced before (Section 5.2.3). 50nM biotinylated FN was used to incubate with the cells on FN or TG-FN matrices. After 6 h incubation, the deposited biotin-labelled FN was staining by using Cy5-streptavidin (1μg/ml) and visualized by confocal microscopy as introduced in Section 5.2.3.

**Figure 5.3.6**

deposited FN matrices was observed in those groups, while in EA5 α5 null MEF cells, biotin-labelled FN was discovered only around the periphery of cells . No difference was observed between the null cells seeded on FN and TG-FN matrices, indicating TG-FN could not compensate the loss of FN deposition caused by the absence of cell surface receptors α5 and β1 integrins. These results suggest that the FN deposition process mediated by TG-FN is dependent on cell surface receptor α5β1 integrin.

### 5.3.6 TG-FN mediates FN deposition in a syndecan-4-dependent manner

Previous data suggested that PKCα, as a downstream signalling molecule in syndecan-4 pathway, is important for TG-FN to restore the RGD-independent cell adhesion and FN deposition process. To investigate the potential role of syndecan-4 in the above process, syndecan-4 null MEF cells were incubated with biotinylated FN and after 6 h incubation, FN deposited in ECM was visualized via immunofluorescence staining. As shown in Figure 5.3.6, after a 6 h incubation, wild type MEF cells deposited well-organized FN fibrils on the FN matrix, but cells on TG-FN produced more FN. Unlike in the wild type cells, no well-organized FN fibrils were found in syndecan-4 null MEF cells, randomly deposited FN was found and the majority of biotin-labelled FN still found around the periphery of cells. This result indicates the involvement of syndecan-4 in mediating cell-related FN fibril formation on TG-FN matrix.

### 5.3.7 Cell surface syndecan-2 is required by TG-FN in regulating FN deposition

The role of syndecan-2 in FN deposition has been demonstrated. Previous work also suggested that the inhibition of syndecan-2 expression significantly reduced the compensatory effect of TG-FN on the RGD-independent cell adhesion. In order to investigate the role of syndecan-2 in the FN fibril formation in the fibroblasts seeded on TG-FN, syndecan-2 specific targeting siRNA was used to silence syndecan-2 in MEF cells, while the universal negative control siRNA was

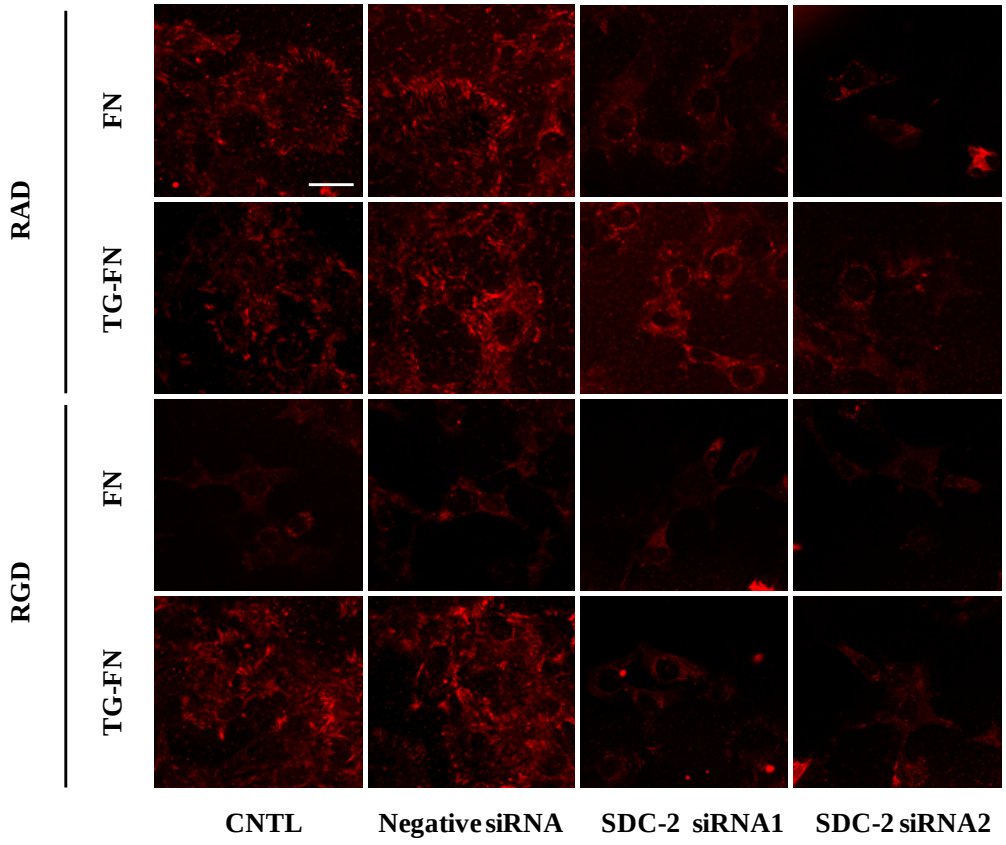



**Figure 5.3.7 Syndecan-2 is required by TG-FN to regulate FN fibril formation by fibroblasts.**
Monolayers of wild type and syndecan-2 siRNA or universal negative control siRNA-treated MEF cells were prepared as introduced before (Section 5.2.3). 50nM biotinylated FN was used to incubate with the cells on FN or TG-FN matrices. After 6 h incubation, the deposited biotin-labelled FN was staining by using Cy5-streptavidin (1μg/ml) and visualized by confocal microscopy as introduced in Section 5.2.3.

**Figure 5.3.7**

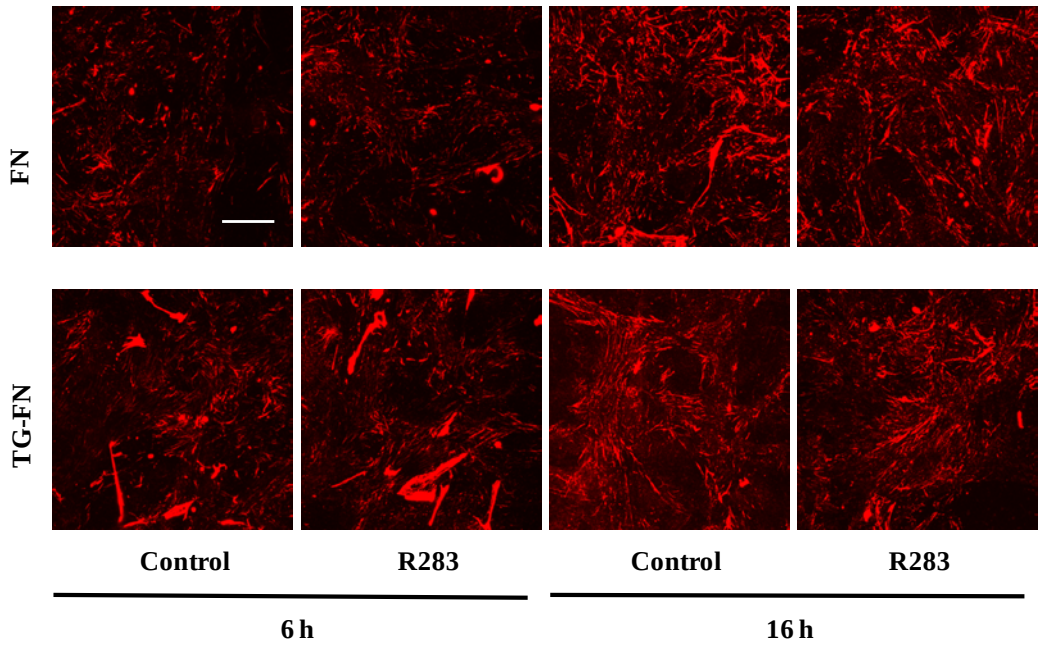



**Figure 5.3.8 The involvement of TG2 activity in mediating FN fibril assembly.**
Monolayers of wild type MEF cells were prepared as introduced before (Section5.2.3). 50nM biotinylated FN was used to incubate with the cells in the presence of 500μM TG2 inhibitor R283 on FN or TG-FN matrices. After 6 h and 16 h incubation, the deposited biotin-labelled FN was staining by using Cy5-streptavidin (1μg/ml) and visualized by confocal microscopy (Section 5.2.3).

**Figure 5.3.8**

used as the negative control treatment. The 50nM biotinlyated FN was incubated with these cells for 6 h and the FN deposited in ECM was detected via confocal microscopy. As shown in Figure 5.3.7, well-organized FN fibrils were observed in the matrix laid down by the RAD-treated wild type MEF cells, while only randomly deposited FN was deposited by syndecan-2 siRNA treated-MEF cells. No difference of the biotinylated FN was discovered between the non-treated or the universal negative control siRNA-treated cells, ruling out the toxicity of the siRNA treatments with the RAD peptides. Unlike the wild type MEF cells, TG-FN could not enhance the FN fibril formation with the reduced expression of syndecan-2. In the presence of RGD peptide, when seeded on the TG-FN matrix, wild type or the control siRNA-treated MEF cells restored the FN fibril formation induced by RGD peptide, while the abolishment of the cell surface syndecan-2 resulted in the loss of the compensatory effect of TG-FN. The above data suggest that syndecan-2 is important in regulating FN fibril formation and crucial for TG-FN to compensate the RGD-induced loss of matrix FN deposition by fibroblasts.

### 5.3.8 The effect of TG-FN on FN deposition is independent of the transamidating activity of TG2.

Previous work suggested that cell surface TG2, as an enzyme with the transamidating activity, can mediate FN deposition. To investigate whether the effect of matrix TG2 on FN fibril formation is dependent on its transglutaminase activity, TG2 inhibitor R283 (Balklava et al., 2002; Freund et al., 1994) was incubated with wild type MEF cells and the biotin-labelled FN was monitored after a 6 h and 16 h incubation. Meanwhile as shown in previous result, there is no detectable cell surface TG2 present in MEF cells, which provides an ideal model to investigate the matrix TG2 activity in regulating FN deposition. Figure 5.3.8 presents the matrix biotinylated FN staining. After 6 h incubation, cells started to lay down matrix FN fibrils, and a well-organized FN matrix was found after 16 h incubation. Most importantly, the TG2 inhibitor R283 at the concentration of 500μM, which has been published to completely inhibit TG2 activity, did not affect the FN deposition process compared to the non-treated

cells. These results suggest that matrix TG2 within the TG-FN complex mediates the FN deposition process in a transamidating activity-independent manner.

## 5.4 Discussion

The involvement of TG2 in fibronectin fibril formation and deposition has been previously documented (Akimov and Belkin, 2001) where it was shown that the enzyme cooperates with but cannot substitute for α5β1 integrin in fibronectin assembly. Transamidating activity was not thought to be involved in this mechanism. In contrast in other reports the crosslinking activity of TG2 was reported to be important in FN assembly and deposition (Verderio et al., 1999; Yuan et al., 2007). In order to investigate whether the FN bound TG2 via its interaction with syndecan-4 is also involved in FN fibril assembly, FN matrix deposition was studied by using biotin-labelled FN in mouse embryo fibroblasts. Importantly cell surface TG2 was not detectable in the MEF cells used in these experiments, thus providing an ideal model to answer the above question, since the potential interference of cell surface TG2 is ruled out. The TG-FN complex increased the rate of fibril formation by wild type MEF cells when compared to cells plated on FN alone in the early time periods (1-6 h) which appeared to equalise in both sets of cells after 16 h when saturation of the system had probably occurred. Importantly even in the presence of RGD peptide the deposition of FN was still maintained in the presence of TG2. However as expected (Mao and Schwarzbauer, 2005), RGD peptide treatment of cells seeded on FN alone led to rounded cells with lack of focal adhesion assembly and a marked inhibition of FN matrix deposition with most exogenous biotin-labelled FN shown to accumulate on the cell surface. This fits with previous observations in that focal adhesion point assembly, actin cytoskeleton formation and intracellular signalling via RhoA is required for FN fibril formation to occur (Fernandez-Sauze et al., 2009; Zhong et al., 1998). Of particular interest is the observation that even in the presence of RGD peptide which may be expected to block a large proportion of α5β1 integrin sites fibril formation still occurs at an effective level. However this is not surprising when considering that the TG-FN matrix when bound to syndecan-4 even in the presence of RGD peptides still leads to focal adhesion assembly, resulting in a signalling cascade leading to activation of syndecan-2, ROCK, Rho and formation of a rigid actin cytoskeleton. Previous studies have suggested cell surface heparan sulfate chains

particularly those of syndecan-2 in FN assembly (Klass et al., 2000; Mao and Schwarzbauer, 2005), a process mediated by activation of PKCα and RhoA (Xian et al., 2009). The importance of syndecan-4 in supporting cell spreading, a crucial process in cell-mediated FN fibril assembly, is well demonstrated (Okina et al., 2009). However in long-term incubations (20 h), no difference in FN fibril formation has been observed in syndecan-4 siRNA treated and control cells (Huveneers et al., 2008). Given the potential importance of syndecan-4 in the TG-FN mediated fibronectin deposition at earlier time periods we similarly investigated the importance of syndecan 4 using the early time stages (6 h) in FN fibril formation. In syndecan-4 null and syndecan-4 mutant Y188L (a mutant for PKCα binding) (Bass et al., 2007) MEF cells, and cells where the GK21 peptide was used to block the interaction between PKCα and β1 integrin obvious differences in FN fibril formation, when compared to wild type control cells, were discernible. In syndecan-4 null, Y188L mutant and GK21 peptide-treated cells, biotin-labelled FN was located mainly on the cell surface and when present the biotin labelled FN was distributed randomly in the cell attached area. This suggests for the first time, the importance of syndecan-4 in the initiating stages of FN fibril formation in a PKCα and β1 integrin-dependent manner. However it should be noted that in previous findings using longer time periods and where syndecan-4 was silenced using siRNA-treatment (Huveneers et al., 2008; Mao and Schwarzbauer, 2005) the loss of syndecan-4 in FN deposition could be compensated by other cell surface receptors e.g. syndecan-2 or α5β1 integrin (Huveneers et al., 2008; Klass et al., 2000). Unlike in the RGD-treated wt MEF cells, TG-FN could not restore the loss of FN fibril assembly in cells lacking syndecan 4, α5 or β1 integrin (Huveneers et al., 2008), indicating the dependence of TG-FN on these cell receptors in mediating FN fibril formation. Use of the TG2 inhibitor R283 (Balklava et al., 2002; Freund et al., 1994) ruled out any involvement of transamidating activity in fibril formation in this process.

# Chapter 6:

# Discussion

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The interaction between cells and the ECM plays a crucial role in regulating cell adhesion, migration, proliferation, differentiation and the relevant signalling transduction process (Raines, 2000). Initiated by the interaction between cell surface receptors and its substratum, cell adhesion is regulated by different matrix proteins, receptors and intracellular signalling molecules. According to the type of the matrix protein(s), receptors and signalling molecules involved, a cells destiny is determined. Among the matrix proteins, fibronectin is one of the most important matrix proteins in mediating fibroblast cell adhesion (Pankov and Yamada, 2002). As a high-molecular-mass glycoprotein, FN can exist in a soluble form synthesized by hepatocytes and secreted into the in blood or as an insoluble fibril extracellular or basement membrane protein produced by fibroblasts or other cells (Mosher, 1984).

The molecular structure of FN molecule has been well investigated and reported, especially the domains that are related to cell adhesion, which is one of its essential functions as an ECM proteins. Located at the $III_{9\text{-}10}$ domain of FN, the presence of the Arg-Gly-Asp (RGD) motif was first reported by Rierschbacher and Ruoslahti (1984), which was identified as the major integrin binding site in charge of the interaction of FN with about half of the integrin family members, such as α5β1, α8β1, αvβ1, αvβ3, αvβ5 and αvβ6 integrins etc. (Ruoslahti, 1996). It has been wildly accepted that $\beta_1$ integrins are the major cell surface receptors that mediate cell adhesion on FN (Takagi, et al., 2003). Once bound to the RGD cell-binding domain, integrins can activate their intracellular signalling molecule focal adhesion kinase (FAK) and trigger the autophosphorylation of FAK at $Tyr^{397}$, which can in turn promote the phosphorylation of other Tyrosine sites including $Try^{925}$ and $Try^{576}$. This activation of β1 integrins has been known as the outside-in signalling pathway, since the direct interaction between integrins and its matrix ligand are required (Luo et al., 2007). As one of the essential components of focal adhesion (FA), the activation of FAK is crucial for both the

actin skeleton and the focal adhesion formation. Apart from its role in regulating cell adhesion and spreading, integrins are also important in the cell survival pathway mediated by ERK1/2 to prevent the cells from undergoing apoptosis and to promote their survival, with activation of FAK acting as an upstream signalling molecule of the cell survival pathway (van Nimwegen and van de Water, 2007). Other members of the integrin family, such as β3 integrins, are also involved in cell adhesion on FN via their interaction with the RGD cell-binding domain (Switala-Jelen et al., 2004). It has been reported that increased cell surface β3 integrin expression can enhance cell adhesion on FN (Timar et al., 1998), while the importance of these integrins as a major component of FA has also been widely reported and studied to investigate the effect of FA on cell behaviour (Wehrle-Haller and Imhof, 2002).

The involvement of another cell surface receptor—syndecan-4 and its interaction with FN in cell adhesion was revealed by the finding that even in the presence of the RGD-cell binding domain, the cells still could not fully accomplish cell spreading, until the presence of the heparin binding domains was provided to the cells (Bloom et al., 1999; Woods et al., 1984). By binding to the heparin-binding sites within FN (present at the domains $III_{12\text{-}14}$ within FN), syndecan-4 can further support cell adhesion by activating its intracellular signalling molecule protein kinase C α (PKCα) to support the FA formation (Woods et al., 2000). Further studies by different groups were devoted to pursuing the role of PKCα in regulating syndecan-4 signalling transduction. As a crucial step of PKCα activation, the translocation of the kinase from the cytosol to the membrane is required by the downstream signal transduction (Nakashima, 2002), where it interacts with the cytoplasmatic domain of β1 integrins to activate these receptors intracellularly (Parsons et al., 2002). The β1 integrin and syndecan-4 signalling pathways are linked by PKCα, known as the β1 integrin and syndecan-4 co-signalling pathway which is crucial in regulating FN-mediated cell adhesion. Most recent evidence demonstrated that syndecan-4 can also function as a co-receptor for β3 integrins in regulating RGD-dependent astrocytes cell adhesion via promoting FA and actin stress fibre formation in the cells through RhoA and

PKCα signalling (Avalos et al., 2009).The third cell surface receptor binding site is the CS-1 sites residing in repeats III4-5 of FN, also known as the HepIII domain. CS-1 interacts in the main with two members of integrin family, $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins (Moyano et al., 1997). It has been reported that $\alpha_4\beta_1$ integrins can mediate cell adhesion through the association of chondroitin sulfate proteoglycans (CSPGs) (Moyano et al., 1999). Other studies suggest that the effect of $\alpha_4\beta_1$ integrins on FA organization and actin skeleton formation is influenced through inhibiting the activation of RhoA (Moyano et al., 2003).

Tissue transglutaminase is a multi-functional protein with transamidating, GTPase, protein disulphide isomerase (PDI) activities, while recent research also suggested TG2 may function as a serine threonine protein kinase. Even though TG2 lacks the characteristic features for externalization, the enzyme can be secreted through the non-classical Golgi/ER pathway onto cell surface and is then deposited into ECM (Balklava et al., 2002). Due to the different locations of this enzyme, TG2 can regulate cell behaviour through various mechanisms. Intracellularly, the functions of TG2 are probably more relevant to cell survival and differentiation. For example, the cytoplasmatic TG2 acts as a pro-apoptotic protein in the HEK293 cells (Milakovic et al., 2004), as well as regulating the differentiation of neutrophil granulocytes (Balajthy et al., 2006). On the cell surface, TG2 has been identified as a co-receptor for β integrins to enhance cell adhesion on FN (Akimov and Belkin, 2001b; Akimov et al., 2000) by promoting integrin clustering (Janiak et al., 2006) and further to support FN fibril formation (Akimov and Belkin, 2000, 2001a). It has also been reported that TG2 at the leading edge of the migrating cells can mediate EGF-related cell migration (Antonyak et al., 2009). It has been reported that the crosslinking of matrix protein by TG2, e.g. FN and collagen, can protect them from degradation by cell surface enzymes including MMPs, which can be beneficial to cell adhesion and proliferation (Chau et al., 2005; Collighan and Griffin, 2009). Previous work by Griffin and colleagues (2003) suggested that in both osteoblast and fibroblast cell models the TG-FN complex, in which TG2 acts as a matrix protein without the transamidating activity, can compensate the RGD-independent cell adhesion in a

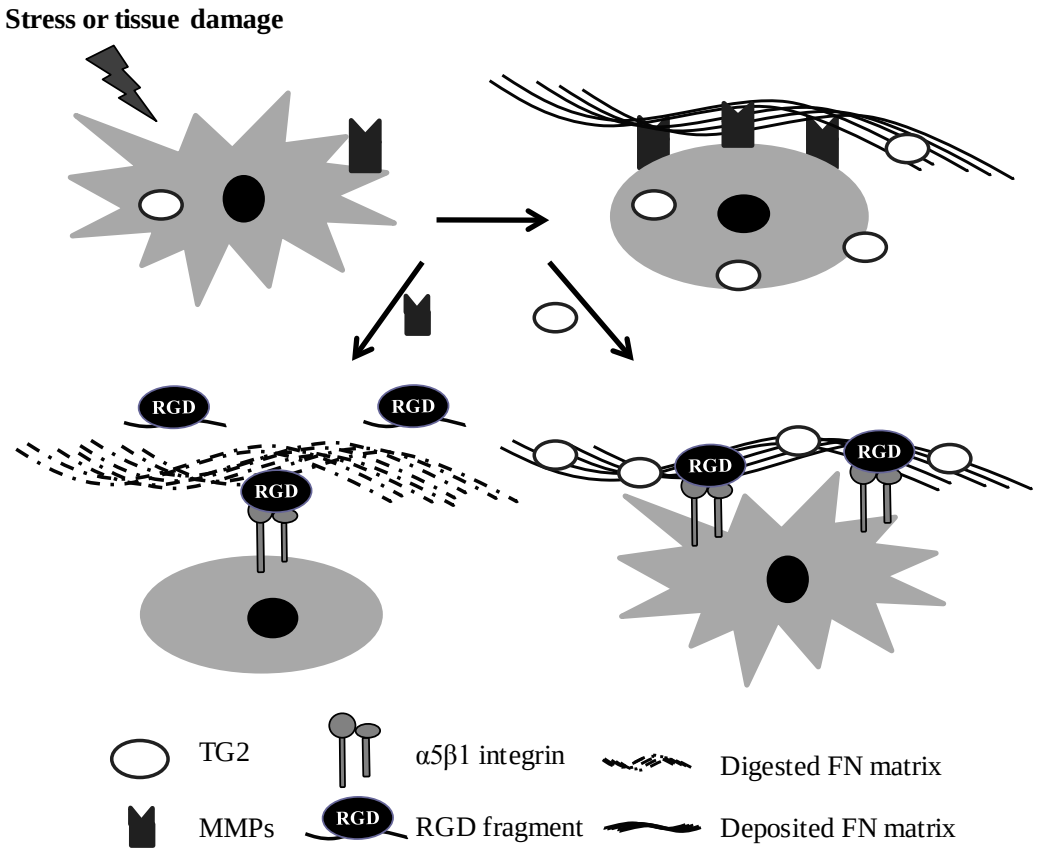


**Figure 6.1 Schematic showing the effect of stress and tissue damage on cell adhesion and matrix remodelling.**

During stress or tissue injury, cells increase the expression of TG2, which leads to its secretion and deposition into the ECM, leading to the formation of a TG2 and FN hetero-complex— TG-FN. MMPs can digest the matrix proteins and result in the release of the RGD motif containing matrix fragments, which can inhibit the interaction of the cell with the ECM, leading to masking the cell surface integrin receptors, ultimately Anoikis. The TG-FN complex can rescue the cells from the RGD peptide induced loss of cell adhesion, thus maintaining cell survival.

**Figure 6.1**

cell surface heparan sulphate-dependent manner (Verderio et al., 2003). It has been shown that the release of the RGD peptides in a stress-induced process e.g. during wound healing following tissue trauma, due to proteolytic digestion by matrix metalloproteinases (MMPs), can compete for integrin binding sites and block the integrin-mediated cell-ECM interaction and in turn induce the loss of cell adhesion and the induction of anoikis (Buckley et al., 1999). Regarding the fact that increased expression and matrix deposition of TG2 has been found in the cells under stress conditions (Kawai et al., 2008; Upchurch et al., 1991). It was therefore felt essential to investigate the mechanism of the formed TG-FN complex in regulating RGD-independent cell adhesion, which may provide a further role for TG2 in the wound healing process, but in addition may also provide a promising application of this TG-FN complex in the tissue engineering field (Figure 6.1).

The aim of this project was to study the mechanism and physiological importance of the TG-FN-mediated cell adhesion process which can occur in the presence of integrin blocking RGD peptides

In order to distinguish the roles played by cell surface TG2 ,where it acts as an integrin coreceptor ,and matrix bound TG2 in RGD-independent cell adhesion, tg2-MEF cells with stable over-expression of human TG2and their wt controls were used. Since there is no detectable TG2 antigen in whole cell lysates, at the cell surface and in the matrix in the wild type MEF cells, these cells provide an ideal model to investigate the role of matrix TG2 in RGD-independent cell adhesion without the interference from the exogenous TG2. In the transfected tg2-MEF cells, the wild type TG2 can be detected in cell lysates, on the cell surface and in the ECM, suggesting the successful transfection of this cell line. In the cell adhesion assay, even with the increased expression of cell surface TG2, no enhanced increase in cell adhesion was obtained in these cells in the presence of the RGD peptides, only when seeded onto the TG-FN matrix was cell attachment and spreading restored in both the wild type and the tg2-MEF cells

thus ruling out the involvement of cell surface TG2 in the RGD-independent cell adhesion and further confirming that, only when deposited into ECM and bound to FN, can TG-FN mediate RGD-independent cell adhesion.

In order to specify which component of this TG-FN complex is the functional part in mediating RGD-independent cell adhesion on TG-FN, heparin, the natural binding partner with high affinity for both TG2 and FN (Bennett et al., 1997; Gambetti et al., 2005), was used to block the binding sites within these two molecules. ELISA studies (Verderio et al., 1999) indicated that pre-treatment of FN with heparin did not influence the immobilization of TG2 on FN. Blocking the heparin-binding sites within FN did not affect the compensatory effect of TG-FN in RGD-treated cells, however when heparin treatment was applied after the formation of TG-FN matrix, thus blocking the heparin-binding ability of TG2, this TG-FN complex lost its compensatory effect on the RGD-independent cell adhesion completely. This suggested that TG2 is the functional component within the TG-FN heterocomplex that mediates the RGD-independent cell adhesion. Previous use of heparinase to digest the cell surface heparan sulfates demonstrated the importance of heparan sulfate chains for TG-FN to regulate RGD-independent cell adhesion (Verderio et al., 2003). In order to further confirm this result, heparan sulfate mutant CHO cells was used in the cell adhesion assay on the FN and TG-FN matrices. The significant inhibition of cell spreading in the mutant CHO cells on FN, confirmed the role of heparan sulfate chains in regulating cell adhesion on TG-FN. Unlike the wild type cells, TG-FN failed to restore the cell attachment and spreading in the RGD peptide-treated cells, further supporting the direct involvement of heparan sulfates in mediating the cell adhesion on TG-FN.

Among the heparan sulfate proteoglycan family, syndecan-4 is the most widely expressed member (Oh and Couchman, 2004) and has been reported to be crucial in associating in the β1 integrin-related actin cytoskeleton formation (Beauvais and Rapraeger, 2004). Since it is also the major resource of heparan sulfate

chains in fibroblasts, syndecan-4 was first investigated as the target receptor in this RGD-independent cell adhesion on TG-FN. In syndecan-4 knockout MEF cells (Bass et al., 2007), the cell adhesion assay revealed that these cells failed to response to the TG-FN matrix in the presence of RGD peptide, however once this receptor was introduced back into the knockout cells, MEF cells with stably transfected human syndecan-4 cDNA re-gained their response to TG-FN even in the presence of the RGD peptides. This offered the first hint of the involvement of syndecan-4 in regulating RGD-independent cell adhesion on TG-FN. To investigate the role of syndecan-4 in the organization of the actin skeleton (Woods et al., 2000), actin stress fibre formation was visualized by using fluorescence staining. In the wild type and syndecan-4 addback cells a well-organized actin skeleton was observed in the cells on FN or TG-FN matrices, however once treated with the RGD peptide, the cells lost their actin cytoskeletal architecture, which could be restored by the TG-FN complex. Unlike the wild type and addback cells, syndecan-4 null MEF cells presented a reduction in longitudinal stress fibres and only stress fibre bundles were found at the cell periphery. Most importantly, this disruption of the actin architecture could not be compensated by the TG-FN complex, further confirming the essential role of syndecan-4 in mediating the RGD-independent cell adhesion on TG-FN. The direct interaction between matrix TG2 and cell surface syndecan-4 was detected via co-immunoprecipitaion by using a specific anti-syndecan-4 antibody against the intracellular domain of the syndecan-4 molecule to pull down the immuno-complex, while the syndecan-4 knockout MEF cells were used as the negative control cell line. By using the RGD peptide treated MEF cells seeded on the heparin pre-treated TG-FN matrix, the heparin binding domain on TG2 was blocked and cell adhesion by FN-TG matrix was completely inhibited indicating TG2 to be the matrix protein that cells to bind to in the heterocomplex. Since TG2 antigen, either in the cell lysate or at the cell surface was not detected in syndecan-4 null MEF cells, which was comparable to the observation in syndecan-4 wild type and add-back cells, this further supports that syndecan-4 and the exogenous matrix TG2 undergo a direct interaction. Previous work demonstrated that the core proteins of syndecan-4 can also mediate cell adhesion process (Echtermeyer et al., 1999). Thereby, in order to distinguish which part(s)

of syndecan-4 molecule, i.e. the heparan sulfate chains or the core proteins, are involved in the interaction between TG2 and syndecan-4, heparan sulfate mutant CHO cells and its wild type control cells were used in the co-immunoprecipitation assay using the syndecan-4 targeting antibody. This revealed that without the presence of heparan sulfate chains, there was no detectable interaction between TG2 and syndecan-4 demonstrating that the heparan sulfate chains are essential in mediating the binding of syndecan-4 to matrix bound TG2.

The presence of syndecan-4 and β1 integrin co-signalling pathway has been well reported (Humphries et al., 2005). Even though the previous work from Griffin and colleagues demonstrated that the β1 integrin functional blocking antibody did not affect the compensatory effect of TG-FN in the RGD-independent cell adhesion (Verderio et al., 2003), this did not rule out the involvement of the inside-out signalling of β1 integrins. Based on the discovery that, in β1 integrin knockout MEF cells (Retta et al., 1998), the TG-FN matrix lost its compensation effect when cells were treated with RGD peptides (which could be restored by introducing human β1 integrin cDNA into the knockout cells), the crucial role of β1 integrins in RGD-independent cell adhesion was suggested. By using syndecan-4 wild type, knockout and addback MEF cells seeded on the heparin-treated TG-FN matrix in co-immunoprecipitation assays, the possibility of the direct interaction between syndecan-4 and β1 integrins was ruled out (Xian et al., 2010). This suggested that the intracellular link between syndecan-4 and β1 integrins is not as by a direct interaction when involved in mediating the RGD-independent cell adhesion on TG-FN. As one of the major partner of β1 integrins in regulating cell adhesion on FN, the potential role of α5 integrins (Cukierman et al., 2001; Johansson et al., 1997) in the cell adhesion process was also studied by using α5 integrin knockout mouse embryo cells and the control cells transfected with human α5 integrin cDNA (Huveneers et al., 2008). The failure of the TG-FN complex in restoring the cell attachment and spreading in the presence of RGD peptides, suggesting that the α5β1 integrin complex is crucial for TG-FN to exert its compensatory effect on the RGD peptides, This

was further confirmed by the cell adhesion assay by using a specific α5β1 integrin targeting peptide A5-1 (Kim et al., 2008) and another α5β1 integrin targeting inhibitor c34 (Heckmann et al., 2008), which have been reported to block the cell adhesion process mediated by the α5β1 integrins.

Since the involvement of another two cell surface receptors— α4β1(Isobe et al., 1999; Takahashi et al., 2000) integrins and β3 integrins (Akimov et al., 2000) has been reported in TG2-related cell adhesion , the potential roles of these two integrins were investigated by using a specific blocking antibody and a knockout MEF cell line, respectively. No effect of the α4β1 integrin blocking antibody on FN or TG-FN-mediated cell adhesion was discovered in the presence of the RGD peptides in short-term cell adhesion assays (around 40 min), suggesting that α4β1 integrins are not required by this TG-FN complex in short-term RGD-independent cell adhesion. This supports the previous report in that, unlike the α5β1 integrin, although α4β1 integrin is involved in actin skeleton organization in certain cell types, the involvement of syndecan-4 signalling pathway is not required (Peterson et al., 2005). Even though slight inhibition in cell spreading on FN was observed in the β3 integrin knockout MEF cells, which confirmed the role of β3 integrins in regulating FA formation (Switala-Jelen et al., 2004), it did not influence the compensation of TG-FN in RGD peptide-treated MEF cells, suggesting that TG-FN mediates the RGD-independent cell adhesion in a β3 integrin-independent manner.

Recent work demonstrated that syndecan-2, another member of the syndecan family can be involved in actin cytoskeleton formation, as a downstream signalling molecule of syndecan-4 signalling pathway, although the link between these two Syndecans has not been fully elucidated (Oh and Couchman, 2004; Whiteford et al., 2007). It is also suggested that syndecan-2 can be involved in the $\alpha_5\beta_1$ integrin-regulated actin stress fibre formation (Kusano et al., 2000). A subsequent report from the same group demonstrated the relevant independent role of this receptor in actin cytoskeleton organization with the regard to the fact

that in the cells with a comparable expression of α5β1 of overexpression of syndecan-2 could enhance actin stress fibre formation (Munesue et al., 2002). Considering the presence of syndecan-2 in fibroblasts (Whiteford et al., 2008), the hypothesis of the involvement of syndecan-2 in regulating cell adhesion by TG-FN was investigated by using syndecan-2 targeting siRNAs to silence the syndecan-2 in MEF cells. Unlike the non-treated or the negative control siRNAs treated cells, the syndecan-2 siRNA treatment abolished the compensatory effect of TG-FN cell adhesion completely in the presence of the RGD peptides, indicating that syndecan-2 is also required by TG-FN to exert its compensatory effect on the RGD peptides. This was further confirmed by actin stress fibre staining which showed that the reduction of the syndecan-2 expression resulted in a poorly organized actin cytoskeleton even in the presence of the TG-FN complex. Most interesting, was the finding that no direct interaction between syndecan-2 and matrix TG2 could be detected via co-immunoprecipitation, suggesting the indirect involvement of syndecan-2, probably as a downstream effector, in the RGD-independent cell adhesion on TG-FN.

The above work demonstrated that at the cell surface receptor level, TG-FN requires the presence of syndecan-4, syndecan-2 and α5β1 integrins to exert its compensatory effect on RGD-independent cell adhesion. These three receptors could work as a network linked by their intracellular signalling molecules.

As introduced above, PKCα has been reported as the crucial link for the syndecan-4 and β1 integrin signalling pathway (Humphries et al., 2005). The involvement of these two receptors in TG-FN mediated RGD-independent cell adhesion was confirmed by previous results. Thereby, PKCα was the first intracellular signalling molecule to be investigated in our model. First of all, PKCα activation was blocked by a specific PKCα inhibitor Go6976 (Jensen et al., 2009), which abolished the compensatory effect of TG-FN on the RGD-independent cell adhesion, suggesting the potential dependence on PKCα by TG-FN to exert its compensatory effect. The activation of PKCα by syndecan-4 is

triggered by the binding of PKCα with the cytoplasmatic domain of syndecan -4 (Woods et al., 2000). Since the PKCα binding domain within syndecan-4 intracellular core protein is known it provides the possibility of the development of the syndecan-4 mutant Y188L, which is unable to bind PKC alpha and which can be transfected into Syndecan 4 null MEF cells (Bass et al., 2007). The loss of compensation of TG-FN in the RGD-induced cell adhesion assay when using the Y188L MEF cells suggested the importance of the interaction between PKCα and syndecan-4 in regulating the RGD-independent cell adhesion by TG-FN. The translocation of PKCα from cytosol to membrane is a crucial step during its activation (Nakashima, 2002). By detecting the presence of PKCα antigens within the cytosol and membrane fractions in the RGD peptide-treated fibroblast in the presence or absence of the PKCα inhibitor Go6976, it was demonstrated that both the RGD peptides and Go6976 inhibited the PKCα translocation. Importantly, TG-FN can promote PKCα translocation in the RGD peptide-treated fibroblasts, but not in the Go6976-treated cells, suggesting that TG-FN requires PKCα activation to exert its function in mediating cell adhesion in the RGD-independent manner. On the inner membrane surface, PKCα binds to the cytoplasmatic domain of β1 integrins and in turn triggers the inside-out signalling of β1 integrins. A specific cell-penetrating peptide GK21 (which mimics the PKCα binding domain within β1 integrins) was used to block the binding between PKCα and β1 integrins (Parsons et al., 2002). The loss of interaction between PKCα and β1 integrins completely abolished the compensatory effect of TG-FN, which not only confirmed the importance of PKCα as a downstream signalling molecule for syndecan-4 in TG-FN signalling pathway, but also indicated the involvement of the inside-out signalling of β1 integrins in the RGD-independent cell adhesion mediated by this matrix complex. Even though accumulating research suggests that syndecan-2 can associate with syndecan-4 during cell spreading and actin cytoskeleton formation, the actual mechanism of this co-signalling pathway has not been reported (Whiteford et al., 2007). Considering the importance of PKCα in the crosstalk between syndecan-4 and β1 integrins (Humphries et al., 2005), the hypothesis that PKCα could also be involved in the signal transduction between syndecan-4 and syndecan-2 was tested in syndecan-4 null and Y188L mutant

cells and in co-immunoprecipitation assays by using syndecan-2 specific antibody to pull down the syndecan-2 binding proteins. The reduced PKCα signals in the knockout and mutant cells for the first time demonstrated that PKCα could be the essential link between syndecan-4 and syndecan-2. To sum up, the above findings, PKCα is the important regulator in syndecan-4/2 and β1 integrin co-signalling pathway, which is crucial for TG-FN to exert its compensatory effect on the RGD-independent cell adhesion.

The next step was to investigate whether the inside-out signalling pathway of β1 integrins which could be activated by PKCα and is required by TG-FN to mediate the cell adhesion process. A functional blocking antibody against mouse β1 integrins, HMβ1-1 (Noto et al., 1995), was used in the cell adhesion on the FN and TG-FN matrices, meanwhile the phosphorylation of $Tyr^{397}$ and $Tyr^{861}$ in FAK was detected in the antibody-treated cells to investigate the function of this antibody. Previous reports suggested that the phosphorylation on $Tyr^{861}$ in FAK is independent of the ligand binding of β1 integrins and is the indicator of the activation of the β1 integrin inside-out signalling pathway (Shi and Boettiger, 2003). The cell adhesion and the Western blotting results revealed that the loss of cell adhesion induced by HMβ1-1 was due to its blocking effect of the β1 outside-in signalling (showing by the reduced signal in p-$Tyr^{397}$) but not its inside-out signalling (since the p-$Tyr^{861}$ signals were not significantly affected by this antibody). The restored cell attachment and spreading by TG-FN in the antibody-treated cells suggested that this complex mediates the cell adhesion process through the inside-out signalling pathway in an outside-in signal transduction-independent manner. By further detecting the phosphorylation of p-FAK at $Tyr^{397}$ and $Tyr^{861}$ in RGD-treated cells on the FN or TG-FN matrices, it was showed that the RGD peptides blocked the phosphorylation at both sites of FAK, which can be compensated in the cells seeded on the TG-FN matrix, indicating that FAK is the downstream molecule in TG-FN signalling pathway through its activation at $Tyr^{397}$ and $Tyr^{861}$.

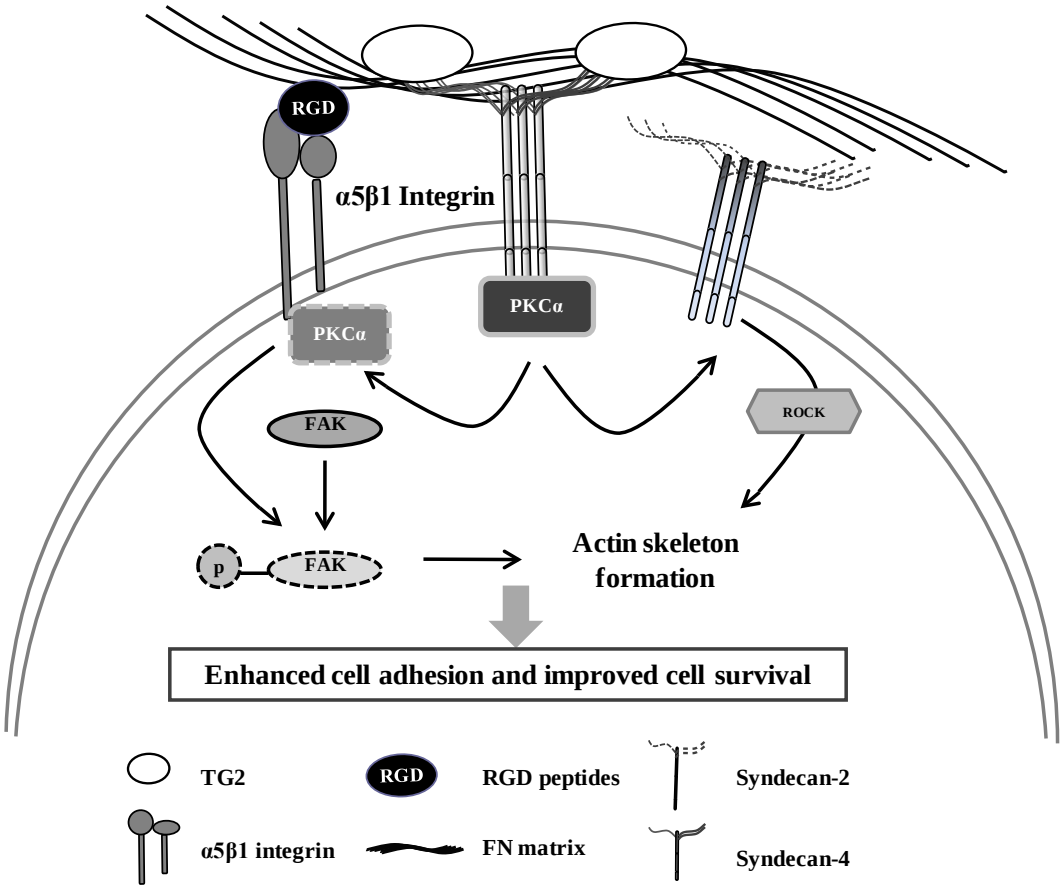


**Figure 6.2 Schematic showing the α5β1 integrin and syndecan-4/2 co-signalling pathway in the mediation of the RGD-independent cell adhesion on TG-FN.**

Via its direct interaction with the cell surface heparan sulphate proteoglycan syndecan-4, TG2 within the TG-FN heterocomplex activates syndecan-4 mediated signalling via PKCα which in turn triggers the inside-out signal transduction of α5β1 integrins and actin cytoskeleton formation by syndecan-2, thus maintaining cell adhesion and homeostasis.

**Figure 6.2**

The importance of another molecule in β1 integrin signalling—ERK1/2 (Saleem et al., 2009) was investigated firstly by using its specific inhibitor PD98059 (Fujita et al., 2007). The inhibitor treatment significantly inhibited the compensatory effect of TG-FN in the presence of the RGD peptides, indicating the involvement of ERK1/2. The phosphorylation of ERK1/2 (p-ERK1/2) indicates the activation of these kinases, which was significantly inhibited by the RGD peptide treatment in the cells seeded on FN matrix, while the p-ERK1/2 was restored by the TG-FN complex, suggesting that this matrix compensates the effect of RGD peptide to protect cells from anoikis through the ERK1/2 signalling. The importance of syndecan-2 in mediating the RGD-independent cell adhesion by TG-FN was confirmed by using a specific inhibitor of Rho kinase (ROCK) Y27632, the proposed downstream signalling molecule in syndecan-2 pathway (Whiteford et al., 2007). The Y27632-treated cells failed to response to the TG-FN complex, which suggests that ROCK is involved in the RGD-independent cell adhesion and further supports the importance of syndecan-2 in the process.

To conclude, by using specific inhibitors and detecting the phosphorylation of the various kinases, the requirement by TG-FN for the involvement of intracellular signalling molecules, including PKCα (the crucial link between syndecan-4/2 and β1 integrin co-signalling pathway), FAK, ERK1/2 and ROCK, was demonstrated in the RGD-independent cell adhesion. In addition the activation of the inside-out signalling of β1 integrin is essential for the TG-FN complex to exert its function (Figure 6.2).

Fibronectin (FN) mediates the cell adhesion process by interacting with cell surface receptors through its different cell binding sites. It is essential to embryogenesis and found associated with the ECM in large quantities during wound healing and angiogenesis. Cell surface TG2 involvement in fibronectin deposition in an integrin dependent but transamidating independent manner has been reported previously (Akimov and Belkin, 2001a). In contrast in other

reports the crosslinking activity of TG2 was also reported to be required in FN assembly and deposition (Verderio et al., 1999; Yuan et al., 2007). As a stress response protein, the deposition and binding of matrix TG2 with FN can rescue the cells from the loss of cell adhesion induced by the blocking of the interaction between integrins and the FN cell binding domains by RGD containing peptides (Verderio et al., 2003). By monitoring FN fibril formation through addition of soluble biotinylated FN, the involvement of TG2 transamidating activity was ruled out in this event by addition of a site directed TG irreversible inhibitor R283 (Balklava et al., 2002; Beck et al., 2006). We show that the TG-FN complex can increase the rate of fibril formation by wild type MEF cells when compared to equal numbers of cells adhered on FN alone in the early stages of FN deposition (1-6 h). Importantly in keeping with the role of this stress induced heterocomplex in wound healing and matrix turnover it was demonstrated that even in the presence of the RGD containing peptides FN fibril formation was still maintained. This was in direct contrast to cells seeded on FN alone where RGD peptide treatment led to rounded cells with lack of focal adhesion assembly and a marked inhibition of FN fibril assembly. This fits with previous observations in that focal adhesion point assembly, actin cytoskeleton formation and intracellular signalling via RhoA is required for FN fibril formation to occur all of which are present in cells seeded on TG-FN in the presence of RGD peptides (Mao and Schwarzbauer, 2005; Zhong et al., 1998).

Interestingly it has been reported in longer-term incubations (20 h) that no difference in FN fibril formation was observed between syndecan-4 siRNA-treated cells and control cells (Huveneers et al., 2008). Given our observation that syndecan-4 may be important in the early stages of fibril formation following cell spreading, a further investigation of fibril formation was performed in syndecan-4 knockout cells, the syndecan-4 Y188L mutant (which is unable to bind PKCα) (Bass and Humphries, 2002) and GK21 peptide (Parsons et al., 2002) treated (which blocks binding of PKC alpha to syndecan-4) cells. The results of these experiments clearly indicated that syndecan-4 and its downstream signalling molecule PKCα is crucial for FN bound TG2 to mediate

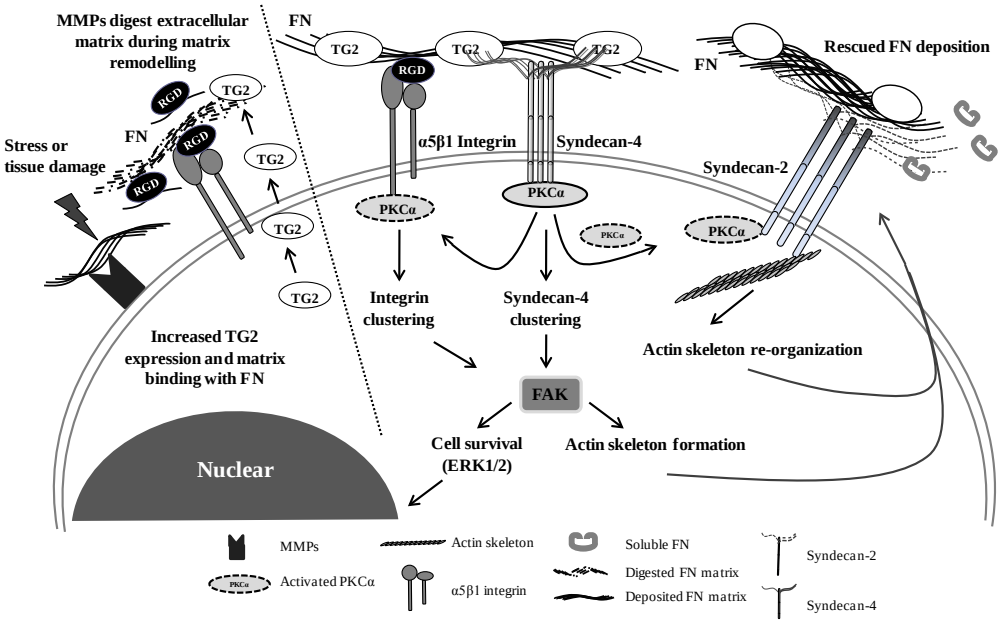


**Figure 6.3 Schematic summarizing the TG-FN-mediated cell adhesion and FN fibril formation pathway.**

As a response to stress induction/ tissue injury, the increased expression of TG2 leads to its externalization and deposition into ECM and formation of TG-FN complex, meanwhile the upregualtion and secretion of proteases, such as the MMPs, start to degrade the matrix protein during matrix turnover, leading to release of the RGD motif containing fragments which block integrin mediated cell adhesion inducing the loss of cell adhesion and cell death. The direct interaction between matrix FN bound TG2 and the heparan sulfate chains of syndecan-4 lead to activation of the inside-out signalling of β1 integrins by the binding between PKCα and the intracellular domain of β1 integrins, promoting α5β1 integrin-related cell adhesion and focal adhesion formation. As a downstream molecule of syndecan-4, syndecan-2 is activated by intracellular PKCα, without the direct interaction with the matrix TG2, this mediates cytoskeletal formation and cell contraction via activation of ROCK, while its extracellular domain together with any α5β1 integrins not blocked by RGD peptides capture the soluble FN molecules and mediate the FN fibril deposition process.

**Figure 6.3**

FN fibril formation. The poorly formed FN fibrils by β1 or α5 integrin knockout fibroblasts also confirmed that these two receptors are crucial for TG-FN to enhance the FN fibril formation by fibroblasts seeded on it. Previous reports have demonstrated a role for RGD-independent mechanisms involving heparan sulphate proteoglycans in FN polymerisation and syndecan-2 has been implicated has having a regulatory role in this mechanism (Klass et al., 2000). The observation that siRNA silencing of syndecan-2 considerably reduced FN fibril formation when cells were bound to FN–TG in the presence of RGD peptides strongly suggested the importance of this syndecan-2 in TG-FN mediated cell adhesion, cell contractility and FN fibril formation.

To conclude the above findings, the stress induced secretion and deposition of TG2 and its binding with matrix FN can rescue the RGD-induced loss of cell adhesion through syndecan-4/2 and β1 integrin co-signalling pathway mediated by their intracellular signalling molecules PKCα, FAK, ERK1/2 and ROCK. The role of this TG-FN complex and its relevant signalling pathway in the RGD-independent cell adhesion/survival is also crucial in regulating FN fibril formation, which is crucial to both wound healing and angiogenesis (Mao and Schwarzbauer, 2005), while it could also be pathological during situations of tissue fibrosis where the presence of large amounts of both TG2 and fibronectin are found (Verderio et al., 2005) (Figure 6.3).

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# Appendix 1: List of Abbreviations

| | |
|---|---|
| **Aβ** | Amyloid β- protein |
| **ADP** | Adenosine-5'-diphosphate |
| **ANT1** | Adenine nucleotide translocator 1 |
| **APC** | Antigen presenting cells |
| **AR** | α1B- adrenoceptor |
| **ATCC** | American Type Culture Collection |
| **ATP** | Adenosine-5'-triphosphate |
| **BSA** | Bovine serum albumin |
| **$Ca^{2+}$** | Free calcium ion |
| **CBD** | Cell binding domain |
| **$CCl_4$** | Carbon tetrachloride |
| **CD** | Celiac disease |
| **cDNA** | Complementary deoxyribonucleic acid |
| **CE** | Cornified envelope |
| **CHO** | Chinese hamster ovary cells |
| **CNS** | Central nervous system |
| **DFMO** | Difluoromethylornithine |
| **DH** | Dermatitis herpetiformis |
| **DMEM** | Dulbecco's modified Eagle's medium |
| **DMSO** | Dinethyl sulphoxide |
| **DNA** | Deoxyribonucleic acid |
| **DTT** | Dithiothreitol |
| **FAK** | Focal adhesion kinase |
| **ECL** | Enhanced chemiluminescence |
| **ECM** | Extracellular matrix |
| **EDTA** | Ethylene diamine tetraacetic acid |
| **ELISA** | Enzyme linked immunoabsorbant assay |
| **ERK** | Extracellular signal-regulated kinase |
| **FITC** | Fluorescein isothiocyanate |
| **FXIII** | Factor XIII |
| **FXIII A** | Factor XIII A subunit |
| **FXIII B** | Factor XIII B subunit |
| **FN** | Fibronectin |
| **γ-IFN** | γ-interferon |
| **GA** | Gluten ataxia |
| **GAG** | Glycosaminoglycan |
| **GDP** | Guanosine-5'-diphosphate |
| **gplTG** | Guinea pig liver transglutaminase |
| **GTP** | Guanosine-5'-triphosphate |
| **GRGDTP** | H-Gly-Arg-Gly-Asp-Thr-Pro-OH |
| **GTPase** | Gunoaine-5'-triphosphatase |
| **HCV** | Hepatitis C virus |
| **HSPGs** | Heparan sulfate proteoglycans |
| **HRP** | Horseraish peroxidise |
| **HS** | Heparan sulfate |
| **IFN- γ** | Intereferon- γ |
| **IgG** | Immunoglobulin |

| | |
|---|---|
| **IGFBP-3** | Insulin-like growth factor-binding protein-3 |
| **IRS-2** | Insulin receptor substrate 2 |
| **JMD** | Juxtamembrane domain |
| **kDa** | Kilodaltons |
| **LTPB-1** | Latent TGF-β1 binding protein-1 |
| **M** | Molar |
| **MAPK** | Mitogen-activated protein kinase |
| **MEF** | Mouse embryo fibroblast |
| **MEK** | MAPK kinase 1 |
| **ml** | Milliliters |
| **Mm** | Millimolar |
| **µ** | Micro |
| **µl** | Microlitres |
| **Mm** | Micromolar |
| **Mrna** | Messsenger ribonucleic acid |
| **MT1-MMP** | Membrane type 1- metalloproteinase |
| **Nm** | Nanomolar |
| **NF-κB** | Buclear factor kappa-light-chain-enhancer of activated B cells |
| **NO** | Nitric oxide |
| **OA** | Osteoarthritis |
| **PAGE** | Polyacrylamide gel electrophorsis |
| **PBS** | Phophate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PDI** | Protein disulphide isomerise |
| **PGs** | Proteoglycans |
| **pH** | Negative log of hydrogen ion concentration |
| **PHSRN** | Pro-His-Ser-Arg-Asn |
| **PI** | Propidium iodide |
| **PI3K** | Phosphoinositide 3-kinases |
| **PKC α** | Protein kinase C α |
| **PKC δ** | Protein kinase C δ |
| **PLC** | Phospholipase C |
| **PLCδ** | Phospholipase C δ |
| **PMSF** | Phenylmethylsulfonyl fluoride |
| **RA** | Retinoic acid |
| **RBC** | Red blood cell |
| **RREs** | Retinoid-responsive elements |
| **SDS** | Sodiumdodecyl sulphate |
| **SDS-PAGE** | Sodium dodecyl sulphate- polyacrylamide gel electrophoresis |
| **siRNA** | Small interfering RNA |
| **ROCK** | Rho kinase |
| **SPRs** | Small proline-rich proteins |
| **TEMED** | N,N,N',N'-tetramethylene diamine |
| **TG** | Transglutaminase |
| **TGFβ** | Transforming growthe factor β 1 |
| **THG** | Thapsgargin |
| **TIG3** | Tazarotene-induced gent 3 |
| **TG2** | Tissue transglutaminase |

| | |
|---|---|
| **TGF-β1** | Transforming growth factor β 1 |
| **TMB** | 3,3',5,5'-tetramethylbenzidine |
| **TNF** | Tumour necrosis factor |
| **Tris** | Tris (Hydroxymethyl)-aminoethane |
| **Triton X-100** | t-Ocylphenoxypolyethoxyethanol |
| **VEGFR** | Vascular endothelial growth factor receptor |
| **XTT** | Sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitor) benzeme sulfonic acid hydrate |