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Tissue transglutaminase (TG2) - a wound response enzyme

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1. ABSTRACT

Repair of tissue after injury depends on a series of concerted but overlapping events including, inflammation, re-epithelialization, neovascularization and synthesis and stabilization of a fibrous extracellular matrix (ECM) that is remodeled to emulate normal tissue over time. Particular members of the transglutaminase (TG) family are upregulated during wound healing and act as a novel class of wound-healing mediators during the repair process. This group of enzymes which crosslink proteins via epsilon(gamma-glutamyl) lysine bridges are involved in wound healing through their ability to stabilize proteins and also by regulating the behavior of a wide variety of cell types that are recruited to the damaged area in order to carry out tissue repair. In this article we discuss the function of the most widely expressed member of the TG family "tissue transglutaminase" (TG2) in wound repair. Using both early and recent evidence from the literature we demonstrate how the multifunctional TG2 affects the stability of the ECM, cell-ECM interactions and as a consequence cell behavior within the different phases of wound healing, and highlight how TG2 itself might be exploited for therapeutic use.

2. OVERVIEW OF WOUND REPAIR PROCESS

Wound healing involves a series of intricately regulated cellular and biochemical events that are orchestrated by a large number of regulatory molecules such as proinflammatory cytokines and growth factors, and matricellular proteins (1, 2). Wound healing begins immediately after injury progressing through the complex phases of blood clotting, inflammation, new tissue formation, and finally tissue remodeling (Figure 1). Following injury, especially after cutaneous damage, the body must react rapidly and repair the wound to prevent further blood loss, tissue damage and infection. Disruption of blood vessels and subsequent extravasation of blood constituents to the wound leads to platelet aggregation and blood coagulation (3, 4). The aggregated platelets together with the stabilized fibrin matrix forms the hemostatic plug, which provides the provisional matrix necessary for cell migration and attachment as well as storage of growth factors required during the following stages of the healing process (4). Within a few hours after injury, a robust inflammatory response takes place whereby neutrophils and macrophages are recruited to the injured area from the rich capillary network surrounding the wound site. Infiltrating

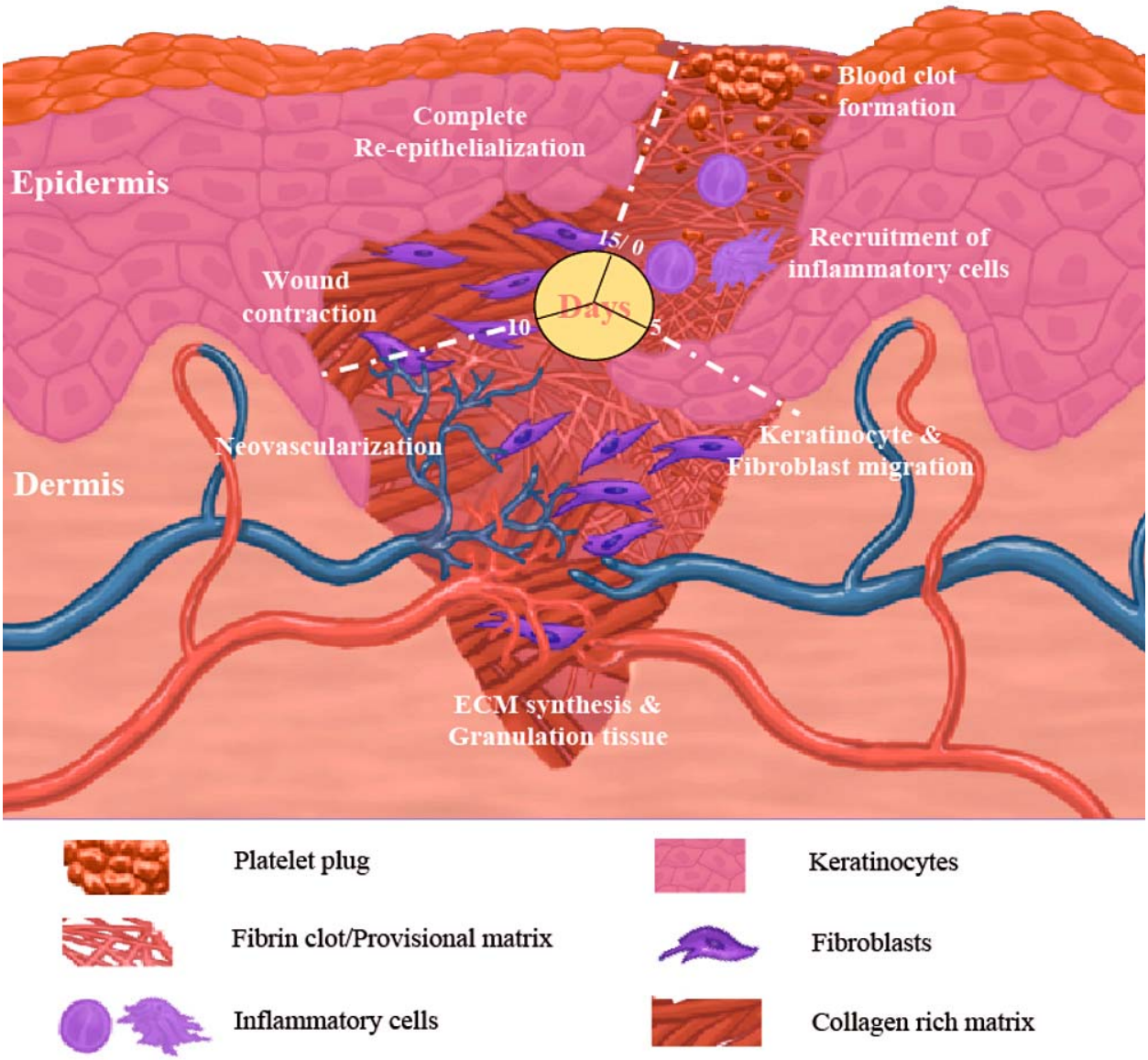

Figure 1. Wound healing around the clock. The scheme shows the main events occurring during the different stages of wound healing with a time line. The first 12- 24hours after injury is defined by the platelet plug and blood clot formation. Inflammatory cells invade the blood clot triggering acute inflammatory response. At days 5-10 re-epithelialization is accompanied by migration and proliferation of fibroblasts and endothelial cells forming the granulation tissue. Fibroblasts gradually replace the provisional matrix with collagen rich matrix and transform into myofibroblasts. Between 10 to15 days post-injury, the transition from granulation to scar tissue occurs, leaving a collagen-rich scar tissue slowly remodeled in the following months under the wound surface now completely covered with a neoepidermis.

neutrophils cleanse the wound area by solubilizing the debris, and macrophages phagocytose the debris along with invading foreign particles and bacteria. Macrophages also release growth factors and cytokines that activate subsequent events – cell migration, proliferation, matrix synthesis and remodeling– that lead to re-epithelialization, formation of granulation tissue and new blood vessels (2). In cutanous wounds, re-epithelialization begins with keratinocyte migration across the wound area over and through the original hemostatic plug to repave the epithelial barrier. The proliferation and migration of fibroblast to the wound site results in replacement of the provisional matrix with granulation tissue consisting mainly of fibronectin (FN) and collagen. While the differentiation of fibroblast to myofibroblasts helps to bring the margins of the wound together, endothelial cells migrate and proliferate to re-vascularize the wound site. Finally, a transition from granulation tissue to mature scar takes place, as the resident cells undergo apoptosis, leaving slowly remodeled scar tissue rich in large collagen bundles strengthened with intermolecular cross-links (5-7).

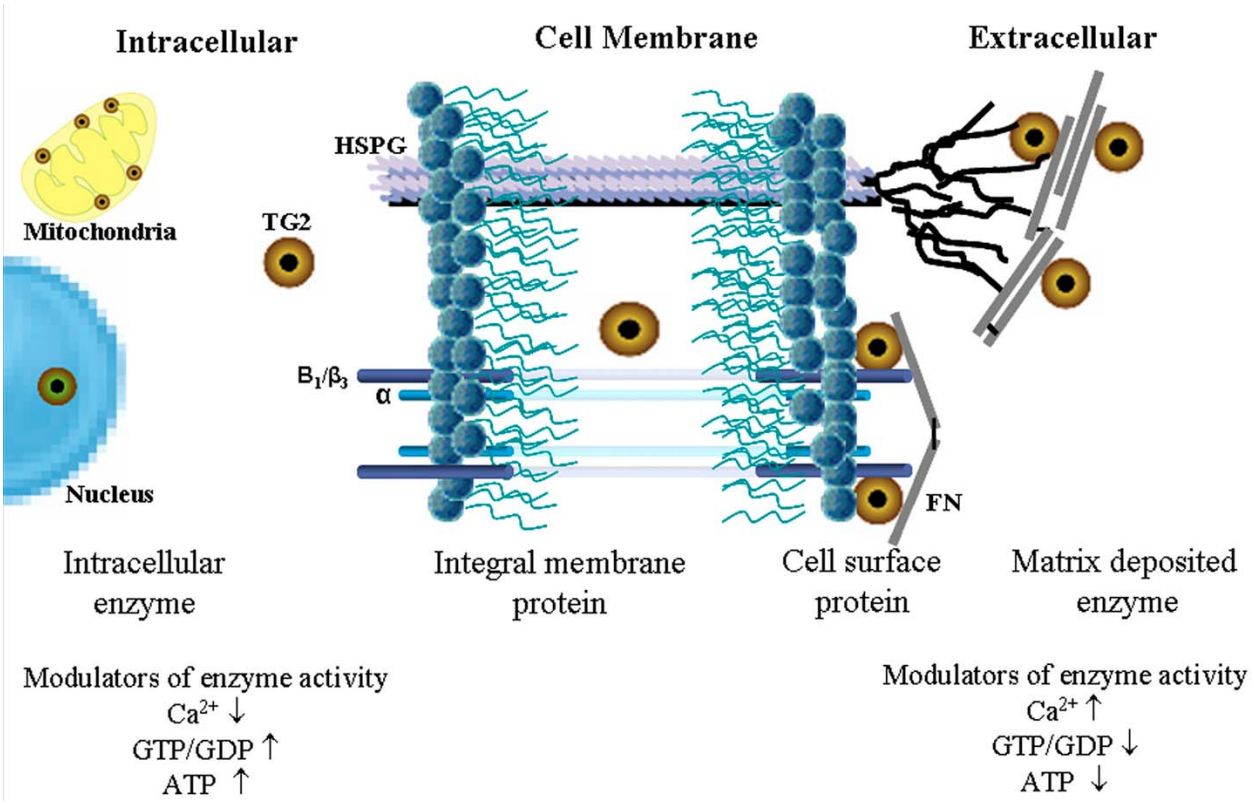

Figure 2. Cellular distribution of TG2. This figure summarizes current information and hypotheses regarding the cellular distribution of TG2. The intracellular location of TG2 is predominantly in the cytosol however the enzyme has also been reported to be present in the nucleus and associated with the mitochondria. In addition, the schematic shows the presence of TG2 in the membrane as it passes from the cytosol to the outside of the cell by a mechanism as yet not characterized. This is compatible with the ability of the enzyme to associate with phospholipid bilayers (17). At the cell surface TG2 may be associated with either beta1 or beta3 integrins in association with FN. Once deposited into the matrix and bound to FN the FN-TG2 complex may also associate with cell surface heparin sulfate proteoglycans to promote cell adhesion and survival. The modulators of TG2 activity at the bottom of the figure refer to the activity state of TG2 when present inside the cell and when present at the cell surface and in the ECM.

Numerous studies have provided significant evidence for the importance of transglutaminases during these tissue repair episodes. Transglutaminases (TGs) are a widely distributed group of protein cross-linking enzymes, of which nine members in mammals have so far been identified (8, 9). TGs catalyze the post-translational modification of proteins through a calcium dependent cross-linking reaction between peptide bound-glutamine and lysine residues of selected proteins. The reaction results in the formation of epsilon(gamma-glutamyl) lysine isopeptide bonds which are extremely resistant to proteolytic breakdown. So far TG-mediated cross-linking reactions have been reported to have important physiological functions in the general maintenance of tissue integrity through their contribution to fibrin clot stabilization (3, 10), cell-matrix interactions (11-14), hair shaft formation and stabilization of the epidermis and the structural framework of the dermo-epidermal barrier (15). This review outlines the participation of transglutaminases in wound healing, focusing in particular on the involvement of tissue transglutaminase (TG2) in the different machineries that drive tissue repair.

3. TG2 EXTERNALIZATION IN TISSUE INJURY AND STRESS

TG2 is a multifunctional enzyme with a wide distribution in mammalian tissues (16). In the cell the enzyme is found in the cytosol, in the nucleus, at the cell surface and in the extracellular matrix (ECM) (Figure 2) (8, 9, 15, 17, 18). Recent reports also suggest its presence in the mitochondria which is linked to its proposed apoptotic function (19). In addition to its calcium-regulated transamidase active site, a GTP-binding/hydrolysis site mapped to the core domain defines TG2 as a Gh protein, which has implicated its involvement in a number of signal transduction events (20). Reciprocal inhibition of the enzyme by the binding of calcium and GTP/GDP regulates the activity of TG2 by inducing conformational changes that blocks access of substrates or GTP/GDP to the reactive sites. This suggests that inside the cell when calcium levels are low, transamidating activity is likely to be dormant (8).

Following translocation to plasma membrane by a non-classical mechanism, TG2 is secreted to the cell

surface and then deposited in the ECM. Unlike other secretory proteins, TG2 lacks a signal peptide thus cannot be secreted by the classical endoplasmic reticulum/Golgi-dependent mechanism. Other proteins secreted through non-conventional routes included fibroblast growth factor-1, interleukin-1beta, thioredoxin, muscle lectin, the A subunit of Factor XIII, and the prostate TG (8). A number of hypotheses have been put forward regarding the mechanism of secretion of TG2. Site directed mutagenesis studies of TG2 has suggested that the externalization of TG2 and its deposition to the ECM is dependent on the active site confirmation and the presence of an intact N-terminal FN binding site (21, 22). Detection of integrin-TG2 complexes both inside and on the surface of the cell during integrin biosynthesis offers a further possible mechanism for TG2 secretion.

Deposition of the enzyme into the ECM is particularly important following cell damage and stress. TG2 mediated protein cross-linking has been implicated in the reconstruction and/or stabilization of the fine structure of the ECM. For example, Upchurch *et al.* (23) has shown that endogenous TG2 is localized in the ECM around the wound area following puncture wounding of a fibroblast cell monolayer. Following studies reported a dramatic increase in deposition of TG2 to the ECM in complex with FN during tissue repair and cellular stress (8). Stress factors leading to progressive deterioration of organs such as liver, kidney and lung was also reported to be associated with the redistribution of TG2 to the ECM (24-26). Recent findings from our laboratory show that treatment of mouse dermal fibroblasts with mitochondrial dysfunction agents results in an early stress-induced release of TG2 at the cell surface and associated ECM in a controlled and regulated manner (unpublished data). We have recently reported the effect of other stress factors leading to changes in cellular distribution of TG2 (27-30). For example, elevated glucose levels leads to a mRNA-dependent increase of TG2 antigen in the ECM of renal proximal tubular epithelial cells, consistent with its proposed role in diabetic nephropathy (29).

Identification of TG2-mediated epsilon(gamma-glutamyl) lysine cross-links during the different stages of the dermal tissue repair process and the presentation of impaired dermal-wound healing in TG2-deficient mice define an important role for TG2 in wound healing biology (31-33). Early studies with animal models reported increased TG2 expression and activity following cutanous wounding (23, 31, 34). In these experiments, detectable levels of TG2 antigen and activity were found in endothelial cells, macrophages, and skeletal muscle cells throughout the different stages of the healing process (32). The secretion of TG2 in response to injury has been also revealed at the cellular level. Time course analysis of *in situ* TG2 activity in scratch-wounded cell monolayers of fibroblasts and epithelial-like cells demonstrated a dramatic increase in TG2-mediated cross-links at the edge of the wound bed (35).

Research carried out to understand the specific participation of TG2 in the different stages of the healing process is still ongoing. Collective evidence demonstrating how TG2 is involved in wound healing at multiple levels is summarized in the following sections.

4. BIOLOGICAL BASIS OF THE TISSUE REPAIR PROCESS AND TG2 INVOLVEMENT

4.1 Initial response to injury; Hemostasis

Tissue injury activates the blood coagulation cascade, leading to the formation of the blood clot which rapidly fills the wound site. Upon injury, subendothelial tissue factor is exposed to the blood flow, which initiates a series of zymogen activation reactions, resulting in thrombin-mediated conversion of soluble fibrinogen into insoluble fibrin monomers. These monomers self-assemble and form double-stranded fibrils, lateral association of which results in polymer formation with increased fiber thickness (36). During these final stages of the cascade, the A subunit of Factor XIII (Factor XIIIA) is activated to Factor XIIIa by thrombin, which catalyzes a transamidation reaction initially resulting in the cross-linking of the gamma-chains on adjacent fibrin monomers. The cross-linking of alpha-chain polymers and alpha-gamma heteromers are catalyzed more slowly by Factor XIIIa, which then gives the clot additional strength (3). The cross-linking of fibrin is of utmost importance not only to stem the loss of blood but to provide a network for cell migration and new tissue formation (37). Emerging evidence suggests that the stimulation of TG2 synthesis in endothelial cells by increased local thrombin concentrations and the abundant presence of TG2 in erythrocytes which are trapped in the fibrin clot may also contribute to fibrin stabilization (38). Indeed, it has been reported that patients with Factor XIIIA deficiency may present only a 20% impairment in wound healing, suggesting a compensatory pathway for fibrin stabilization in tissues of these patients (3, 39). Erythrocyte transglutaminase has been shown to rapidly generate alpha-alpha polymers and hybrid alpha-gamma-chain fibrin cross-links (40, 41). TG2 has also been involved in the polymerization of fibrinogen and FN on the cell surface (40, 41). In addition both Factor XIIIa and TG2 are able to incorporate plasminogen activator inhibitors into the fibrin clot (42). Thus, TG2 is likely to play a supporting role to Factor XIIIa in favoring both the amplification and stabilization of the hemostatic blood clot (Figure 3).

4.1.1 Provisional matrix

Organization of the provisional matrix occurs as plasma fibronectin (pFN), vitronectin and thrombospondin are incorporated by intermolecular cross-links into the existing fibrin matrix. The plasma protein FN plays a prominent role in hemostasis both as a key component of the fibrin-rich matrices and as an important cell adhesive glycoprotein. Assembly of FN in the provisional matrix is catalyzed by Factor XIIIa through the formation of covalent bonds between reactive glutamine residues in FN and the lysine residues in the alpha-chain of fibrin (43). This covalent FN-fibrin cross-linking requires FN molecules with intact N-terminal modules and is vital to the biological role of FN in hemostasis during tissue repair. Covalent cross-linking of FN into the fibrin clot leads to enhanced elasticity, turbidity and thickness in the clot, conferring

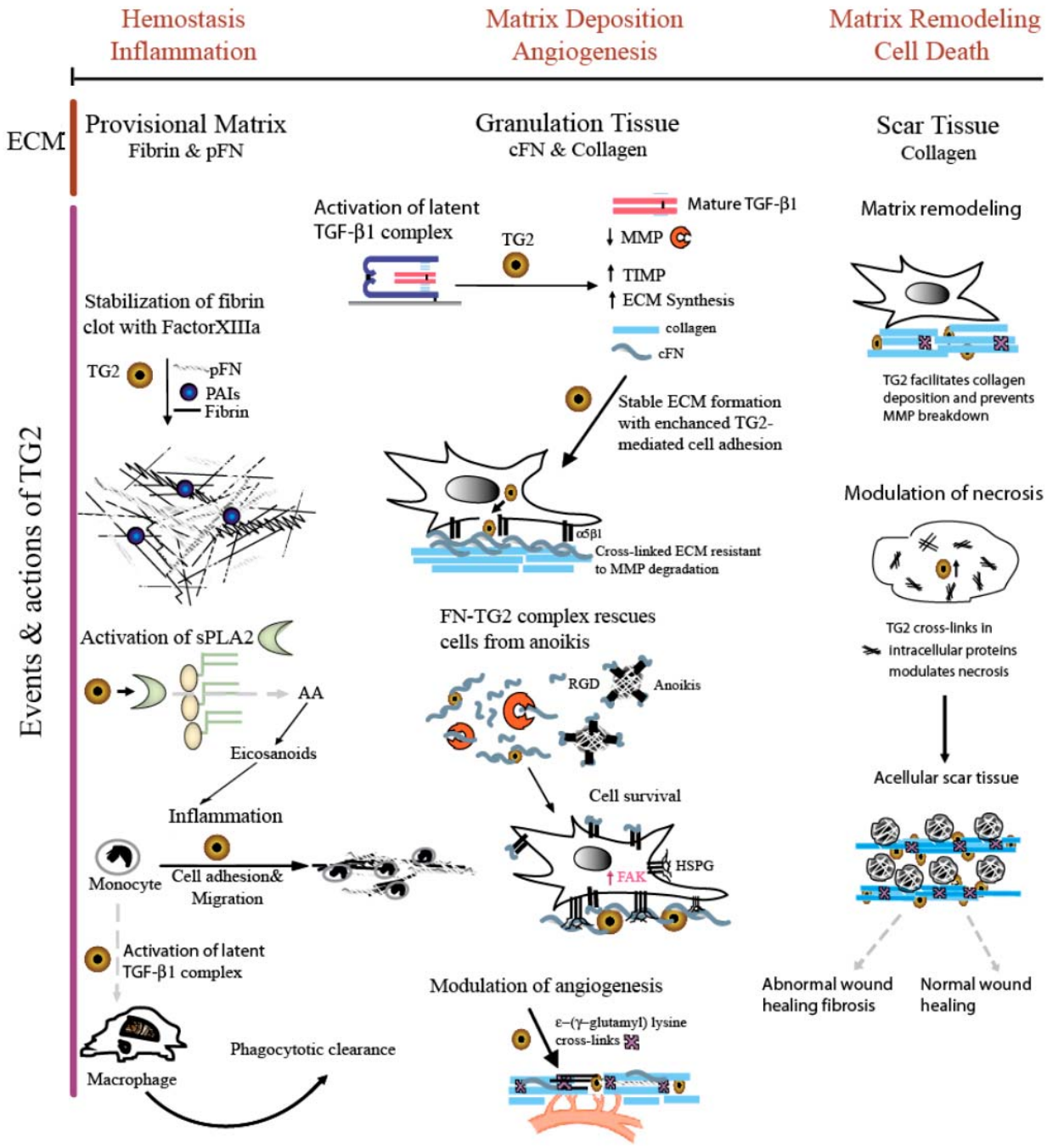

Figure 3. Roles of TG2 in the wound healing response. The scheme portrays the proposed roles of TG2 in different phases: Hemostasis/Inflammation; Matrix Deposition and Angiogenesis; Matrix Remodeling and the formation of scar tissue. Deposition of scar tissue which is essentially collagen is dependent on the severity and continuation of the initial insult. The scar tissue formed can be normal with few obvious signs of scarring or can be excessive resulting in fibrosis and massive scarring. Abbreviations: ECM, extracellular matrix; pFN, plasma fibronectin; cFN, cellular fibronectin; PAIs, plasminogen activator inhibitors; sPLA2, secretory phospholipase A_2 enzyme; TGF-beta1, transforming growth factor beta 1; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; RGD, peptide fragments containing Arg-Gly-Asp recognition sequence for integrins; HSPG, heparan sulfate proteoglycans; FAK, focal adhesion kinase.

increased stability and resistance of the clot to deformation (37). Covalent interaction of FN and fibrinogen may also be important in efficient anchoring of the blood clot to the exposed subendothelial ECM, which is required for clot retraction and wound closure.

Additionally, transglutaminase-mediated formation of the FN-fibrin matrix may enhance the functional capacity of the provisional matrix for cell adhesion, migration, and proliferation during wound healing and neovascularization. For example, the cross linking of FN to fibrin gel by Factor XIIIa enhances the adhesion and migration of fibroblasts (44). Similarly, TG2-mediated formation of fibrinogen alpha-domain polymers seems to enhance the adherence of endothelial cells, which is important in the formation of new blood vessels (45). It is quite possible that in these later stages of the hemostatic process TG2 released from either the entrapped erythrocytes or from damaged / stressed endothelial cells contributes to the assembly of the provisional matrix.

In the complex series of events during blood coagulation, platelets which are rich in Factor XIIIA are activated within the provisional matrix. Factor XIIIa is suggested to facilitate the association of platelets with the blood clot via the platelet membrane glycoproteins, GPIIb and GPIIIa, which are substrates for FXIIIa (46). The activation of platelets is a complex process, however, a recent study showed that the cross-linking of serotonin to the procoagulant proteins from granules or plasma enhanced the binding of these serotonin conjugated proteins to fibrinogen on the platelet surface leading to platelet granulation (47). Once activated, platelets not only secrete cytokines and growth factors but also release ECM and matricellular proteins. One of the matricellular proteins, thrombospondin 1, can serve as a substrate for Factor XIIIa (48), and be incorporated into the provisional matrix (49). Thrombospondin contributes to the activation of transforming factor growth factor-beta1 (TGF-beta1), the main mediator of ECM deposition during tissue repair (7). Concurrently, platelet activation transforms the blood clot from a supporting matrix, through which cells can migrate, to a reservoir of cytokines and growth factors, which kick starts the inflammatory phase and the later stages of the tissue repair process.

4.2 Inflammation

Injury or destruction of tissues triggers a localized protective inflammatory phase characterized by a vascular and cellular response, including dilatation of arterioles, capillaries, and venules, with increased permeability and blood flow, exudation of fluids, and leukocytic migration into the wound area. The release of histamine by mast cells in the injured area has been associated with the initial vasodilation. A more prolonged vascular permeability is achieved by the activation of the kinin and phospholipase pathway. Activation of phospholipase A_2 enzymes (PLA_2) are considered to regulate the generation of vasodilatory eicosanoids such as prostaglandins and leukotrines. PLA_2 liberates arachidonic acid (AA) from plasma membranes by catalyzing the hydrolysis of the sn-2 fatty-acyl bond of membrane phospholipids. This reaction was shown to be the rate limiting step in the biosynthesis of eicosanoids by cyclooxygenase (50). Thus far, the PLA_2 enzymes have been considered to be the key enzymes in inflammation-mediated eicosanoid production (50, 51). Increased catalytic activity of the secretory isoform of PLA_2 ($sPLA_2$) was reported by *in vitro* studies when TG2 treatment was found to induce conformational changes in $sPLA_2$. The enhanced $sPLA_2$ activity was brought about either by the formation of an intramolecular isopepeptide bond within $sPLA_2$ or through polyamination of the phospholipase by incorporation of radioactively labeled polyamines (52, 53). These observations suggested an *in vivo* modification of $sPLA_2$ activity by TG2 during the inflammatory process (Figure 3). In support of this hypothesis, recombinant peptides, which serve as dual TG2 and $sPLA_2$ inhibitors were able to inhibit TG2-catalyzed post-translational activation of $sPLA_2$. Moreover these chimeric peptides showed an anti-inflammatory effect in an *in vivo* experimental model of allergic conjunctivitis (54). The importance of TG2 in the eicosanoid cascade is further demonstrated in a transgenic mouse model where Gh/TG2 is overexpressed in ventricular myocytes. In these cells, expression of cyclooxygenase-2, thromboxane sythase and thromboxane receptors were all upregulated resulting in cardiac failure (55).

Local vasodilation increases regional blood flow while decreasing the velocity of the flow. Both the decrease in net flow rate and cytokines such as IFN-gamma, TNF-beta, and IL-1, activates the endothelial cells leading to changes in the expression of adhesion molecules in the capillary walls at the wound site (56). The upregulation of the selectin family of adhesion receptors facilitates the initial binding of leukocytes to endothelium thus extruding them from the rapid blood circulation. Increase in expression of intercellular adhesion molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule (VCAM)-1 and beta2 integrins mediate tighter adhesion and arrest of leukocytes. Following adhesion, leukocytes spread and squeeze through the basement membrane to the endothelial lining of the capillary to migrate interstitially into the surrounding tissue (57). This process is known as diapedesis or extravasion and mediated by alphaVbeta3 integrins and chemotactic factors including prostaglandins and leukotrines (58).

Accumulating evidence is beginning to point out the crucial involvement of cell-surface TG2 in cell migration which is independent of its transamidating activity. On the cell surface, TG2 is thought to act as an integrin-binding adhesion co-receptor for FN thus regulating cell adhesion (12, 59, 60). TG2-integrin complexes of beta1 and beta3 subfamilies have been detected both inside the cell during biosynthesis and on the cell surface acting as a bridge between the integrins and FN. Sharp increases in TG2 levels have been reported on the surface of differentiating monocytes, which was sustained throughout the later stages of differentiation. The down-regulation of TG2 by expression of antisense constructs resulted in a decrease in adhesion and migration of monocytes suggesting a role for TG2 in the regulation of

monocytic cell extravasion (61). In addition, inflammatory cytokines are reported to increase TG2 expression on the cell surface, which may promote the adhesion/motility of blood leukocytes during inflammation (62-64). In an *in vitro* cell model of transendothelial migration (diapedesis), adhesion and migration of lymphocytes across IL-6, IFN-gamma and TNF-alpha stimulated endothelium was inhibited by monoclonal antibodies directed against endothelial cell surface TG2 (64). It is thus conceivable that TG2 expression in the endothelium may have a regulatory role in blood clot formation, inflammation and in vascular permeability.

The first passengers of diapedesis are neutrophils whose primary role is phagocytosis and wound debridement. Neuotrophils are essential for cleaning the initial rush of contaminating bacteria and cell debris. Other than decreasing the likelihood of infection in the wound, neutorophils might facilitate the re-epithelialization process by secreting pro-inflammatory cytokines. Neutrophil infiltration is followed by macrophage migration into the wound site. Macrophages play an integral role in wound healing as they conclude the inflammatory and debridement process by phagocytosing the expended neutrophils and any remaining cell/matrix debris (6). The activation of macrophages has been characterized with increased TG2 levels, which is reported to be associated with enhanced phagocytotic capacity (65-67). Comparative studies in TG2 deficient mice confirmed the direct correlation between TG2 levels and phagocytosis. Upon induction of thymic apoptosis, a defect in the clearance of apoptotic cells by macrophages was observed in the thymus of TG2 knock out mice (68).

In addition to these defense functions macrophages once activated, also release a repertoire of cytokines and growth factors, which initiate the proliferative phase of the wound healing. Acting in a paracrine manner, these cytokines recruit other cells involved in wound healing and regulate fibroblast chemotaxis, proliferation, and collagen synthesis. The major macrophage-secreted cytokine involved at different stages of tissue repair is TGF-beta1. Phagocytosis of apoptotic cells by macrophages induces TGF-beta1 secretion, which would then act as a down-regulator of the pro-inflammatory cytokines (69).

The cytokine TGF-beta1 is synthesized from cells as a biological inactive precursor protein consisting of the mature TGF-beta1 associated with a propeptide called latency associated peptide (LAP), and a latent TGF-beta1 binding protein (LTBP). This physiologically inactive complex is referred to as large latent TGF-beta1 complex and it is unable to bind to TGF-beta1 receptors. The dissociation of the mature enzyme from LAP is required for the release of the activated form of TGF-beta1. The LTBP is not necessary to render the growth factor latent; however, it plays an important role in targeting TGF-beta1 into the ECM (70). The molecular mechanism of TGF-beta1 activation remains somewhat controversial; however accumulating evidence indicates TG2 participation in this process. *In vitro* studies demonstrated that both the large latent complex and LTBP-1 are substrates for TG2 suggesting an important role in targeting the latent TGF-beta1 complex to the ECM (71-73). In addition, co-localization of TG2, FN and LTBP-1 are found at the cell surface where TG2 will be activated by its association with calcium (73). Hence targeted inactivation of TG2 should in theory reduce the activation of TGF-beta1 resulting in defective macrophage activity and regulation of pro-inflammatory cytokines. In support of this hypothesis, Szondy *et al.* (68) reported that TG2 -/- mice show a deficiency in TGF-beta1 activation leading to impaired phagocytosis and prolonged inflammation.

In summary, TG2 appears to have two major functions in inflammation: At the early stages, TG2 is involved in the recruitment of inflammatory cells into the wound area, triggering the acute inflammation. In the later stages, TG2 acts on macrophages to promote phagocytosis and TGF-beta1 activation, thereby curbing the inflammatory response and accelerating the onset of re-epithelialization (Figure 3). The interrelationship between TG2 and inflammation are further shown in various inflammatory conditions (e.g. celiac disease, Crohn's disease, ulcerative colites) characterized by abnormal TG2 activity (74).

4.3 Re-epithelialization

In unwounded skin, keratinocytes of the basal layer differentiate to corneocytes, which are embedded in a matrix of lipid-enriched membranes, and form the protective barrier, substratum corneum. The corneocyte cytoplasm is surrounded by a cross-linked protein shell "the cornified cell envelope" conferring the skin with chemical resistance and flexibility. The organization and regulation of epidermal barrier function is guarded by the four different members of TG family, namely keratinocyte TG (TG1), epidermal TG (TG3), TG2 and the TGX (TG5). The physical connection between keratinocytes is provided by intercellular structures called desmosomes, while hemidesmosomes serve as the primary anchor contacts of keratinocytes to the basal lamina (5, 75).

Rebuilding the epithelial tissue during wound healing involves a series of exquisite episodes starting with the dissection of the clot and damaged stroma from the wound space by cells from residual epithelial structures. Re-epithelialization is driven by the marriage of regulated phenotypic changes that includes retraction of intracellular tonofilaments, dissolution of desmosomes and hemidesmosomal links, with a series of precisely orchestrated migratory and proliferative events in coordination with protease expression at the wound margin (5, 76). The re-epithelialization must be harmonized with fibrinogenesis and angiogenesis occurring in the underlying neodermis, called granulation tissue. Formation of peripheral cyoplasmic actin filaments and dissolution of desmosomes and hemidesmosomes allow keratinocytes to gain lateral movement. Migration through the provisional matrix and underlying wound dermis involves the interaction of integrin receptors expressed on epidermal cells with a variety of ECM proteins and desiccation of eschar from viable tissue. The leading-edge keratinocytes

degrade the ECM and open up a path along the collagenous dermis and the fibrin eschar by the activation of the fibrinolytic enzyme plasmin and production of matrix metalloproteinases (MMPs). The MMPs are a family of enzymes that cleave various components of the ECM in the pericellular space (77). Upregulation of MMPs during re-epithelialization contributes the dissolution of collagenous basal lamina and subsequent migration of keratinocytes off the dermal substratum (77).

Following the onset of the migration, epidermal cells behind the actively migrating cells undergo a proliferative burst, replacing cells lost during the injury. The network of biological stimuli for the migration and proliferation of epidermal cells during re-epithelialization is very extensive and discussed in detail in several recent outstanding reviews (2, 6).

End point of re-epithelialization is reached when the wound surface has been repaved by a monolayer of keratinocytes. The proliferation and migration of epidermal cells ceases and the basement-membrane proteins reappear in a highly organized membrane-structure reestablishing the basal lamina. Epidermal cells reassume their normal phenotype, forming new desmosomal and hemidesmosomal adhesions to the basal lamina. A new stratified epidermis reforms as keratinocytes undergo the standard differentiation program to form the cornified envelope and dermo-epidermal junctions are reassembled to anchor the epidermis to underlying to dermis (5, 76).

In a full-thickness incision wound model in mice, increased expression of TG1 and its cross-linking substrate involucrin on the plasma membrane of suprabasal keratinocytes was detected near the wound edge at 2-hour post-wounding and subsequent hours, casting a role for TG1 in the formation of premature cornified envelope and remodeling of the stratum corneum during re-epithelialization (78). In partnership with TG1, TG3 might be involved in maturation of epithelial layer by catalyzing the specific cross-linking of a number of structural proteins in the cornified cell envelope (79).

The expression of TG2 in the epithelial layer was restricted to the proliferative basal layer and somewhat transient as it was reduced at the end of re-epithelialization. In contrast, TG2 antigen was persistently detected at the developing dermo-epidermal junctions (32). Early studies using antibodies against the epsilon(gamma-glutamyl) lysine cross-link suggested a correlation between the stability of dermo-epidermal junctions and TG2 (80). In keratinocyte autographs presented to burned patients, TG2 immunoreactivity was detected in the dermal connective tissue along with the basement membrane of the regenerated epidermis. Furthermore, clinical stability of the skin graft observed in the burned victims was associated with TG2-mediated cross-linking of anchoring fibrils of dermo-epidermal junctions, providing direct evidence for the role of TG2 in reestablishment and stability of these structures (34).

4.4 Formation of Granulation Tissue

4.4.1 Matrix Deposition

The migration and proliferation of keratinocytes at the wound edge is followed by migration of macrophages, fibroblasts and endothelial cells into the wound space at the same time. The macrophages serve as the battery for growth factors necessary to stimulate fibroblast and endothelial proliferation. Fibroblasts migrate to the provisional matrix and enrich the matrix to support cell growth by depositing large amounts of ECM. Endothelial morphogenesis leads to the formation of new blood vessels providing oxygen and nutrients necessary to sustain cell metabolism (5, 76).

It is certain that the provisional matrix stability is important for cell migration and cell adhesion as it presents a scaffold by interacting with transmembrane receptors. The provisional matrix component FN plays a major role in the regulation of these processes. For example, provisional matrix maturation by FN assembly and expression of FN-receptors on the fibroblast cell surface has proven to be the rate-limiting step for granulation tissue formation (37, 81). As a high affinity FN binding protein, it is not surprising that TG2 has been implicated in the stabilization and assembly of the FN matrix throughout several studies. Initial *in vitro* studies indicated FN-TG2 binding to be independent from the calcium-dependent transamidating activity and assigned the N-terminal gelatin binding domain of FN as the putative TG2 binding site (14, 82). The binding of TG2 to FN was later consolidated by reports visualizing FN-TG2 complex in the ECM of cultured cells (12, 59, 60, 83, 84). *In situ* activity assays in which cultured cells were incubated with a fluorescent primary amine substrate clearly showed that TG2 is in a catalytically active state at the cell surface and can modulate the FN matrix by forming non-reducible cross-linked FN polymers leading to its stabilization (83). In support of a role for TG2 in matrix stabilization, TG2-mediated cross-linking was detected in the both provisional matrix and new granulation tissue of punch biopsy wounds (32). Nicholas *et al.* (35) reported a dramatic increase in TG2 transamidating activity in the ECM of mechanically wounded monolayers of fibroblasts induced to over-express TG2. In addition to the ECM, increases in intracellular TG2 levels and TG2 cross-linking activity at the edge of the wound bed suggested the stabilization of the wound area by enhancing matrix strength and/or valency. The importance of TG2 has also been demonstrated in UV damage to the dermis. Following the exposure of human dermal fibroblast to UV, TG2 activity was increased in the ECM leading to rapid stabilization of the matrix by inducing the multimerization of FN into SDS-insoluble high molecular weight polymers (85). The general picture that emerges from these studies is that TG2-mediated cross-linking reactions results in the rapid stabilization and modulation of the matrix in the immediate wound area, which further promotes subsequent wound healing by promoting matrix accumulation and regulating cell proliferation and migration.

Growing evidence suggests that TG2 can also influence cell-matrix interactions by serving as a novel cell adhesion protein, independent of its cross-linking activity

(35, 61). Once in complex with FN, TG2 transamidating activity is downregulated (13, 14); however its functionality as a cell adhesion co-receptor is protected from proteolytic degradation by MMPs involved in cleaving a path for cell migration (13, 14, 84, 86). We have recently shown that a TG2 rich FN matrix complex which mimics the *in vivo* physiological and pathological conditions could restore loss of cell adhesion following the inhibition of the classical FN RGD (ArgGlyAsp)-dependent cell adhesion pathway mediated by alpha5beta1 integrin receptors. In contrast, TG2 matrix alone or TG2 immobilized on the gelatin binding fragment of FN was not sufficient to support the RGD-independent cell adhesion, indicating the requirement for the full-length FN. Cell adhesion to the FN-TG2 complex induces the formation of focal adhesion contacts, the assembly of associated actin stress fibers, and FAK phosphorylation in the presence of RGD peptide. Treatment with a PKC-alpha inhibitor significantly reduced the cell adhesion to FN-TG2 after RGD inhibition, suggesting the involvement of the PKC-alpha (13).

The observation that the complex of FN-TG2 rescued primary fibroblasts from the apoptosis induced by RGD inhibition of adhesion (anoikis) might be relevant in situations of matrix breakdown during wound healing, where proteolytic fragments of FN and other matrix proteins competing for integrin binding sites would lead to detachment-induced apoptosis. TG2 protected from MMPs in a complex with FN would thus ensure an adhesion-mediated cell survival mechanism during these traumatic conditions (Figure 3). Although it is not clear how this matrix complex generates intracellular signals, experiments point out to the cell-surface heparan sulfate proteoglycans (HSPGs) as candidate receptors, since the proteolytic degradation of cell surface HSPGs of HOB cells and mouse embryonic fibroblasts diminished the RGD-independent cell adhesion in response to FN-TG2 (1, 13).

4.4.2 Fibroblast Migration and Neovascularization

Within the perspective of wound healing, recent studies demonstrated the intracellular, cell surface and ECM role of TG2 during fibroblastic cell migration (1, 21, 87). Fibroblast migration is a multistep process involving cytoskeletal organisation, receptor activation and cell motility allowing the disruption or severing of matrix attachments at the rear of the cell and retraction of the trailing edge of the cell. *In vivo*, fibroblasts, stimulated by growth factors and chemo-attractants released during inflammation, concentrate at the edge of the damaged matrix to synthesize the first set of matrix proteins and to concurrently migrate and proliferate along these proteins until the wound site is filled with granulation tissue. Comparably, *in vitro*, upon scratch wounding of the confluent monolayer, cells start to migrate and proliferate at the edge of the rupture until the gap is filled and contact inhibition reduces the cell proliferation. Using this model with TG2-sense and antisense-transfected cells, it has been shown that cell migration on tissue culture plastic depends on the expression of active TG2 (1, 21, 61, 87). Overexpression of active TG2 resulted in reduced cell mobility, suggesting a failure of detachment of cell-cell or cell-matrix contacts at the trailing end (1, 21, 87). Alternatively, downregulation or targeted inactivation of TG2 expression greatly reduced cell migration on a plastic substratum because of a failure in the establishment of coordination in the actin filament cytoskeleton at the leading edge causing a loss in front–rear polarity. This suggests the importance of the intracellular TG2 G-protein or the TG2 cell surface adhesion function in the migration process (87). In a different experimental set-up measuring the area of peripheral outgrowth from a cell-seeded agarose droplet, it was shown that cells overexpressing catalytically active and inactive TG2 demonstrated a slower rate of migration on FN (21). Thus, the increased adhesive and reduced motility effect of TG2 on FN unlike plastic does not seem to require the cross-linking activity of the enzyme. However, the interaction of cell surface TG2 with FN was necessary for cell migration, since antibodies against TG2 inhibit the migration on FN in a dose-dependent manner.

The formation of new blood vessels, also known as neovascularization or angiogenesis, requires the stimulation of endothelial cells to migrate and proliferate by angiogenic factors, which are mostly represented by serum born cytokines (VEGF, FGF, TGF-beta) and the surrounding ECM (7). Based on the fact that the migration of endothelial cells and the formation of new vessels are affected by the microcomposition of the ECM, TG2 was expected to play an important role during subsequent angiogenesis in the colonization of the granulation tissue. Haroon *et al.* (32), showed TG2 antigen to be expressed predominantly in blood vessels at the sites of neovascularization within 24hour of wounding. *In vivo* administration of recombinant TG2 to dorsal skin flap window chambers on the days 1 and 2 of the post-surgery led to an increase in vessel length and density (32). This suggests that prior to endothelial cell migration and morphogenesis, TG2 might play a structural role in establishment of initial cell adhesion contacts and ECM assembly thus preparing the extracellular milieu for angiogenesis. In contrast, if TG2 levels are increased at the later stages of angiogenesis, it might lead to extensive cross-linking of the basement membrane and surrounding ECM thus making the invasion of the surrounding ECM impossible by proliferating and migrating endothelial cells. Such an example of this is seen when increased scar tissue leads to tissue fibrosis (8, 9, 73). In support of this hypothesis, analysis of differential gene expression during capillary morphogenesis in 3D collagen matrices showed a clear downregulation of TG2 gene expression during a period of extensive branching morphogenesis (88). Interestingly, increased expression of TG2 in the ECM found at the tumor interface causes increased matrix stability, which can inhibit tumor growth by blocking tumour invasion and angiogenesis (89). Similarly, administration of active but not inactive TG2 to an *in vitro* co-culture angiogenesis model retarded endothelial tube formation due to increased matrix stability, which could be detected by the formation of a dense fibrotic matrix that blocked the capillary vessel growth (1). Such opposing effects of TG2 on angiogenesis during the early and late stages of repair are probably reflected by the changes in

ECM composition, the cell surface receptor repertoire and the transamidating dependent and independent functions of TG2.

4.5 Matrix remodeling and wound closure

Wound contraction commences almost concurrently with collagen synthesis. Migratory fibroblasts now present throughout the wound, secrete and replace the transient provisional matrix with a collagen-rich matrix. A proportion of wound fibroblasts at the periphery of the wound differentiate to myofibroblasts, the specialized "contractile fibroblast", which can generate strong contractile forces due to the presence of large bundles of actin stress fibers and expression of alpha-smooth muscle actin. Myofibroblasts also display a synthetic phenotype with increased expression of genes encoding for collagens I, III, VI, XII, matricellular proteins, TGF-betas and other cytokines (90). Based on the recent findings it is likely that TG2 can also be included in the list of the proteins secreted by myofibroblasts as an increase in TG2 mRNA and protein levels have been detected during *in vitro* trans-differentiation of hepatic cells to collagen producing myofibroblasts (91). The majority of secreted TG2 was found to be associated with the cell surface, whereas the remaining portion was cytosol- and ECM-associated. This increase in TG2 levels might be important in collagen remodeling during the maturation phase. During remodeling, collagen becomes organized into large bundles facilitating intramolecular and intermolecular collagen cross-links. TG2 actively participates in cross-linking a number of different types of collagen including II, III V, VII and XI and more recently collagen type I (1, 8, 15). In rhabdomyosarcoma cells, TG2 can catalyse lysyl oxidase independent cross-linking within collagen V and XI, leading to covalent stabilization of the collagen matrix (92). Interestingly, these TG2-mediated epsilon(gamma-glutamyl) lysine cross-links occur at the same regions targeted by interstitial collagenases such as MMP 1 and MMP 8, possibly making these sites less accessible to these proteases and the collagen fibrils less susceptible to proteolysis. Purified collagen type I when cross-linked with TG2 increases its resistance to activated MMP-1 (24). The cross-linked collagen fibrils became 3 times less susceptible to MMP-1 digestion in comparison to self assembled collagen fibrils. Further cross-linking of FN to collagen fibril by TG2 formed a matrix increasingly stable to the action of MMP-1. In a more recent study, we have demonstrated that collagen I when modified by TG2 leads to the formation of an improved substratum for cell adhesion and proliferation of human osteoblasts and dermal fibroblasts, suggesting that exposure of cryptic binding sites within the cross-linked collagen may induce outside-in signaling via integrin-dependent mechanisms (Figure 4) (93). Changes in the mechanical characteristics of cross-linked collagen may also support cell migration by promoting mechanotransduction cell signaling (94).

The modulation of collagen synthesis and catabolism is likely to be of great importance in the maintenance of connective tissue homeostasis. The degradation of the ECM and subsequent tissue remodeling is controlled by the combined effects of MMPs and tissue inhibitor metalloproteinases (TIMPs) (77). Recent work has shown that the degree of collagen lattice contraction was significantly reduced in TG2-deficient and dominant negative TG2-expressing fibroblasts (87). This cross-linking deficiency led to decreased levels of membrane type-1 matrix metalloproteinase (MT1-MMP) activity, a membrane activator of the secretory soluble MMP-2, thereby reducing the activation of MMP-2. In a different study, it was demonstrated that fibroblasts cultured on TG2-cross-linked collagen express reduced levels of active MMP-1, but increased levels of active MMP-2, when compared to cells cultured on untreated collagen (93). This may account for the increased resistance of the TG-2 cross-linked collagen to cell-mediated degradation by osteoblasts and dermal fibroblasts.

Wound contraction is modulated by TGF-beta1 and platelet-growth factor stimulation, integrin-collagen matrix interaction and the cross-linking of collagen bundles. TGF-beta1 promotes the expression of ECM genes including collagen and FN and suppresses the activity of genes such as MMPs, which promote ECM degradation (95). As already outlined, TG2 may modulate matrix deposition and activation of TGF-beta1. In turn, TGF-beta1 can increase the expression of TG2 via the BMP4 response element within the TG2 gene promoter, resulting in a positive amplification loop (96). Excess TGF-beta1 and increased expression of TG2 will shift the balance of matrix turn-over towards collagen synthesis and accumulation with increased matrix strength, which following chronic or continuous insult to the wound would lead to excessive scarring and progressive fibrosis. There are examples of disease conditions that strongly argue for the causative role of TGF-beta1 and TG2 in the accumulation of ECM and the onset of tissue fibrosis (26, 28, 38, 97). Thus far, examples of fibrotic diseases related to increased expression of TG2 include diabetic nephropathy, liver cirrhosis, idiopathic pulmonary fibrosis, rheumatoid arthritis, and atherosclerosis. In an experimental model of diabetic nephropathy, we have demonstrated that exposure of kidney proximal tubule cells to high glucose leads to an increase in ECM-associated TG2 which was paralleled with an elevation in total deposited ECM and matrix associated epsilon(gamma-glutamyl) lysine protein cross-links. Inhibition of TG activity in these proximal tubule cells reduced both deposited collagen, FN and ECM-associated epsilon(gamma-glutamyl) lysine, suggesting that the observed increase in the deposition of ECM proteins during diabetic nephropathy can be directly linked to their covalent cross-linking by elevated TG activity (28, 29). Similarly, mRNA-dependent increase in TG2 activity and enzyme-mediated cross-linking were detected in the liver ECM during the early and late stages of liver fibrosis (25). These observations give further support to the role of TG2 in the fibrotic response and the importance of the enzyme in the formation and stability of atherosclerotic plaques where increases in enzyme levels were correlated with the cross-linking of the collagen III-lipoprotein core, making the plaque less prone to rupture (38).

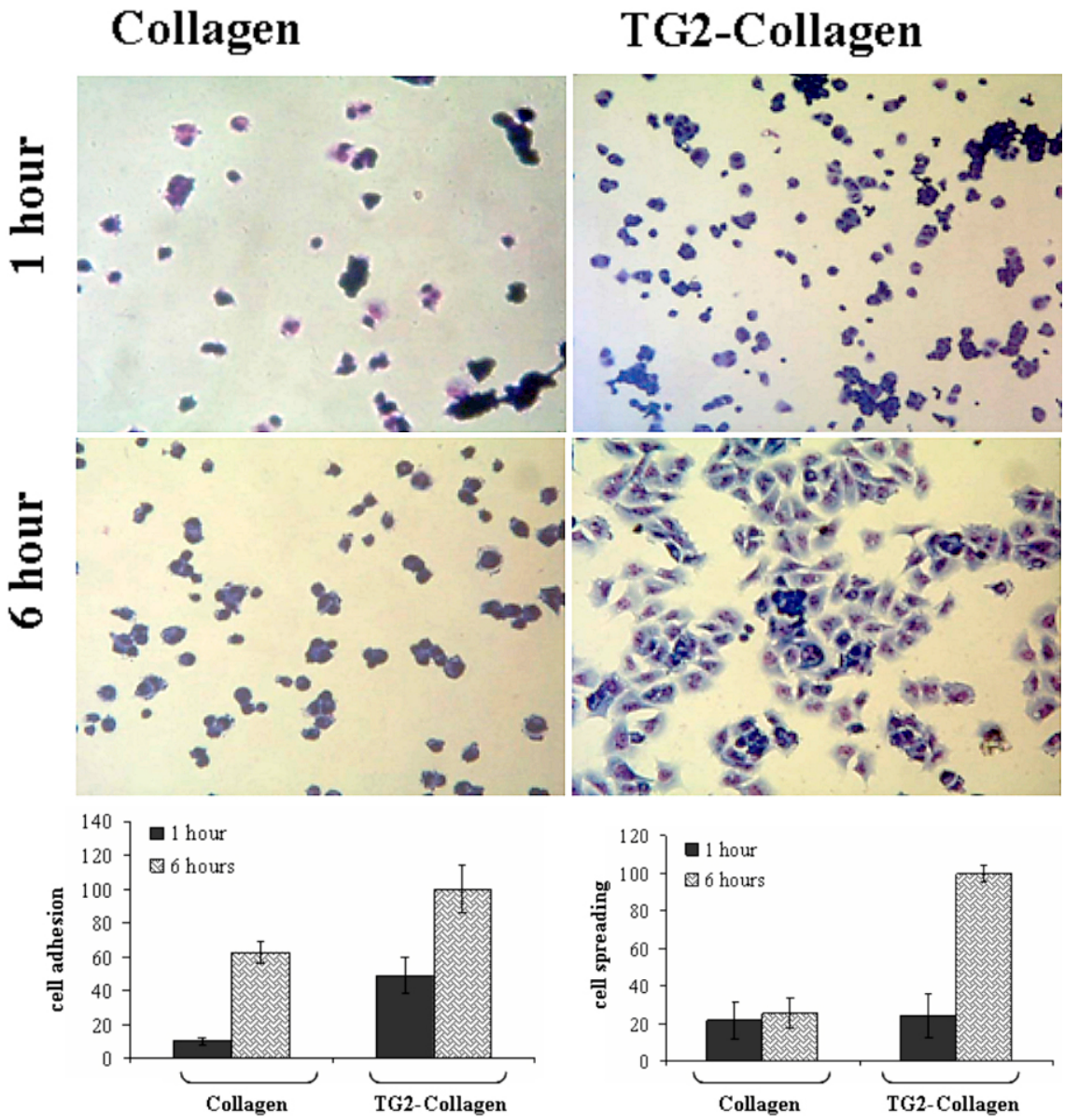

Figure 4. Cell adhesion analysis of human dermal fibroblasts on native collagen and TG-2 treated collagen gels. Cells were plated onto collagen or TG2-treated collagen gels at 2 x 10^4 cells/well for 1 and 6 hours. Following fixation and staining of cells, the number of attached and spread cells was assessed as described in (13). Each data point corresponds to the mean percentage of attached cells (cell attachment) or the mean percentage of spread cells (cell spreading) ± S.D. of three separate experiments. Data are expressed as percentage of cell attachment on TG2-treated collagen at 6 hours, which represents 100%. Quantifications showed that at the end of 6 hour incubation, cell attachment and spreading on TG2-treated collagen substratum was significantly more ($p<0.001$) compared with that on native collagen substratum.

In the final stages of wound healing, when the epithelial architecture has been reconstituted and the ECM fibrils are maximally remodeled, myofibroblasts and many new blood vessels which make up the granulation tissue disappear through cell death via apoptotis, leaving the acellular scar tissue behind. Evidence suggests that the extent of the scarring that occurs is due to the resilience of this scar tissue to further remodeling which may in turn depend on the presence and amount of epsilon(gamma-glutamyl) lysine cross-links (1, 8, 95).

5. Future directions in TG2-based wound repair

This short review has attempted to portray TGs, in particular TG2 as novel wound healing mediators. By doing so it has highlighted the importance of TGs as strategic targets for therapeutic intervention in wound repair and associated diseases.

A number of new healing strategies involving TGs are presently under development with some already in phase 2 clinical trials (98, 99). Such examples include the

use of recombinant Factor XIIIA for severe bleeding complications and for the impairment of wound healing in chronic situations such as treatment of diabetic ulcers and inflammatory bowel disease (99-101). Factor XIIIA has also been used with fibrin as a fibrin glue with applications ranging from the use as a tissue sealant to that of promoting nerve regeneration (99, 102).

TG (TG2 or microbial TG) cross-linked collagen and gelatin gels are under investigation for applications as wound dressings in soft tissue repair. Modification of these gels by TG facilitates cell colonization while providing increased mechanical strength and resistance to proteolysis by wound fluids without the toxic disadvantages of chemical cross-linkers (93).

Factor XIIIA and TG2 also present themselves as suitable therapeutic targets for modulating inflammation, and preventing thrombosis and excessive scarring (75, 102). Inhibition of TG mediated fibrin stabilization provides an alternative strategy to promoting increased fibrinolysis of circulating emboli, particularly after major surgery when intervention is normally fairly short. A comparable strategy may also be effective in preventing blood bone metastasis which often manipulate the thrombolytic system (103).

Inhibitors of TG would also be effective in the treatment of hypertrophic scarring, diabetic nephropathy and other forms of tissue fibrosis (104). The application of 1,4 diaminobutane in a suitable emollient has already shown effective results in the treatment of hypertropic scarring while animal models have shown the efficacy of using site-directed inhibitors of TG in the treatment of renal scarring (75, 104). The importance of TG2 in fibrotic disease can also be turned to advantage. Recent evidence has demonstrated that TG2 is often found upregulated around tumors possibly in an attempt to restrict growth of the tumor and/or as a stress response by the invaded host tissue. Intra-tumor injection of TG2 in animal models has demonstrated that the natural response of the body can be augmented to generate fibrosis in the tumor leading to inhibition of angiogenesis and tumor progression (75).

Other diseases states where TG2 has been implicated, possibly as a consequence of acting in its stress/wound healing capacity is celiac disease and neurodegenerative diseases such as Huntington and Alzheimer's (105).

Given the now established importance of TG in health and disease it seems there is an urgent need to find suitable specific modulators of TG activity, which have application in the many disease states involving TG2 mentioned in this review. Importantly these compounds should be potent, specific to a particular isoform and should not suffer some of the disadvantages of those previously synthesized in the past.

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