Biochemical Pharmacology 80 (2010) 1921–1929

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Review

Transglutaminase 2: A multi-tasking protein in the complex circuitry of inflammation and cancer

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ARTICLE INFO

Article history:
Received 22 April 2010
Accepted 16 June 2010

Keywords:
Inflammation
Drug resistance
Metastasis
Epithelial to mesenchymal transition
Cancer stem cell

ABSTRACT

Metastasis of primary tumors to distant sites and their inherent or acquired resistance to currently available therapies pose major clinical challenge to the successful treatment of cancer. The identification of tumor-coded genes and how they contribute to the progression of cancer is required to improve patient outcomes. Recently, cells that have undergone the epithelial–mesenchymal transition (EMT), which share characteristics with cancer stem cells (CSC) have been implicated to play a role in drug resistance and metastasis of several types of cancer. In this review, we discuss the relationship among transglutaminase 2 (TG2), the EMT, and CSCs in inflammation and cancer. TG2 is a structurally and functionally complex protein implicated in such diverse processes as tissue fibrosis, wound healing, apoptosis, neurodegenerative disorders, celiac disease, atherosclerosis and cancer. Depending on the cellular context, TG2 can either promote or inhibit cell death. Increased expression of TG2 in several types of cancer cells has been associated with increased cell invasiveness, cell survival and decreased survival of patients with cancer. Down-regulation of TG2 by small interfering RNA (siRNA) or its inhibition by small molecule inhibitors has been shown to significantly enhances the therapeutic efficacy of anticancer drugs and inhibit metastatic spread. In addition, TG2-regulated pathways are involved in promoting or protecting normal and tumor cells from death-induced signaling. We discuss the contribution of TG2-regulated pathways to the development of drug resistance and progression to metastatic disease and the therapeutic potential of TG2 for treating advanced-stage cancer.

Contents

1. Introduction

Transglutaminases (TGs; EC 2.3.2.13) are a family of enzymes that catalyze posttranslational modification of proteins by cross-linking proteins via ε-(γ-glutamyl)lysine isopeptide bonds or through incorporating primary amines at selected peptide-bound glutamine residues [1]. Eight TGs have been identified in mammals and humans and they all require Ca^{2+} for catalytic activity, some require proteolytic cleavage of propeptides, and three of them (TG2, TG3 and TG5) are inhibited by GTP [2]. Tissue transglutaminase (TG2 or tTG) is the most diverse and ubiquitous member of the TG family. The entire gene of TG2 (*TGM2* on human chromosome 20q11-12) is composed of 13 exons and 12 introns [2] and encodes a monomeric protein of 687 amino acids ($MW \approx 78$ kDa) with four distinct domains: an N-terminal β-sandwich domain, a catalytic core domain, and two

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doi:10.1016/j.bcp.2010.06.029

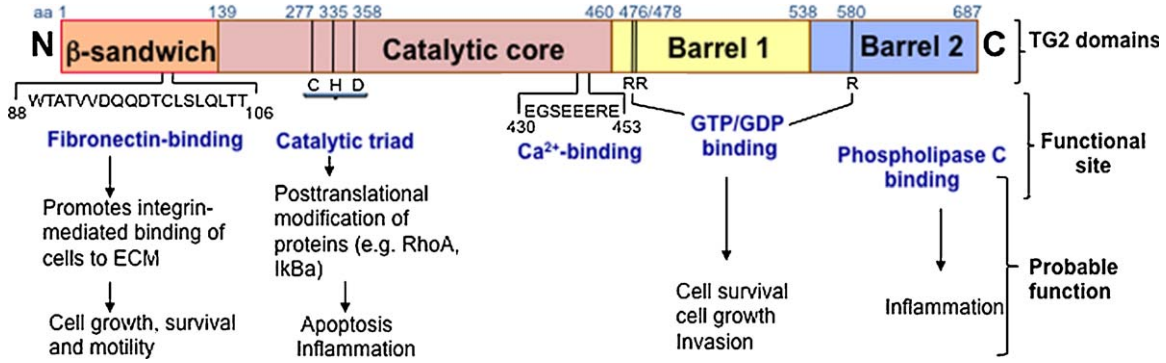

Fig. 1. Schematic representation of the structural and functional domains of transglutaminase 2 (TG2) protein. TG2 protein consists of four distinct domains and each domain has a unique function. The N-terminal β-sandwich domain contains a high-affinity binding site for fibronectin and is responsible for promoting direct or indirect attachment of cells to the ECM. In its extended form (induced by calcium), the catalytic core domain of TG2 is responsible for cross-linking various cellular proteins by establishing highly stable isopeptide bonds and generally ensures the apoptotic death of the cell. The barrel 1 domain contains a GTP/ATP-binding site that plays an important role in TG2-mediated signaling pathways. The C-terminal Barrel 2 domain under certain conditions (e.g., in GTP-bound form) can recruit and activate phospholipase C and contributes to the proinflammatory functions of the TG2 protein.

C-terminal β-barrel domains (Fig. 1). TG2 is structurally and functionally a complex protein with both intracellular and extracellular functions. In addition to catalyzing the calcium-dependent posttranslational modification of proteins, TG2 can bind and hydrolyze GTP and ATP [3]. Moreover, it can catalyze protein disulfide isomerase reaction [4] and may even function as a protein kinase [5].

GTPase activity has been linked to the function of TG2 as a G protein ($G_{\alpha h}$) involved in signaling from $\alpha_{1B/D}$ adrenergic receptors to downstream effectors such as phospholipase Cδ1 [3,6]. TG2 has a high-affinity fibronectin-binding site located in the N-terminal domain (Fig. 1). Although predominantly an intracellular protein (localized in the cytosol, nucleus, and cell membrane compartments), TG2 can also be secreted outside the cell, by an as-yet unknown mechanism, and it has extracellular functions. TG2 is thought to serve distinct physiological functions within different cellular compartments. Under normal conditions, TG2 in the intracellular environment exists as a latent protein due to the presence of low Ca^{2+} and the inhibitory effect of GTP/GDP (Fig. 2). However, under extreme conditions of cell stress or trauma after the disturbance or loss of Ca^{2+} homeostasis, TG2 may be activated and cause cross-linking

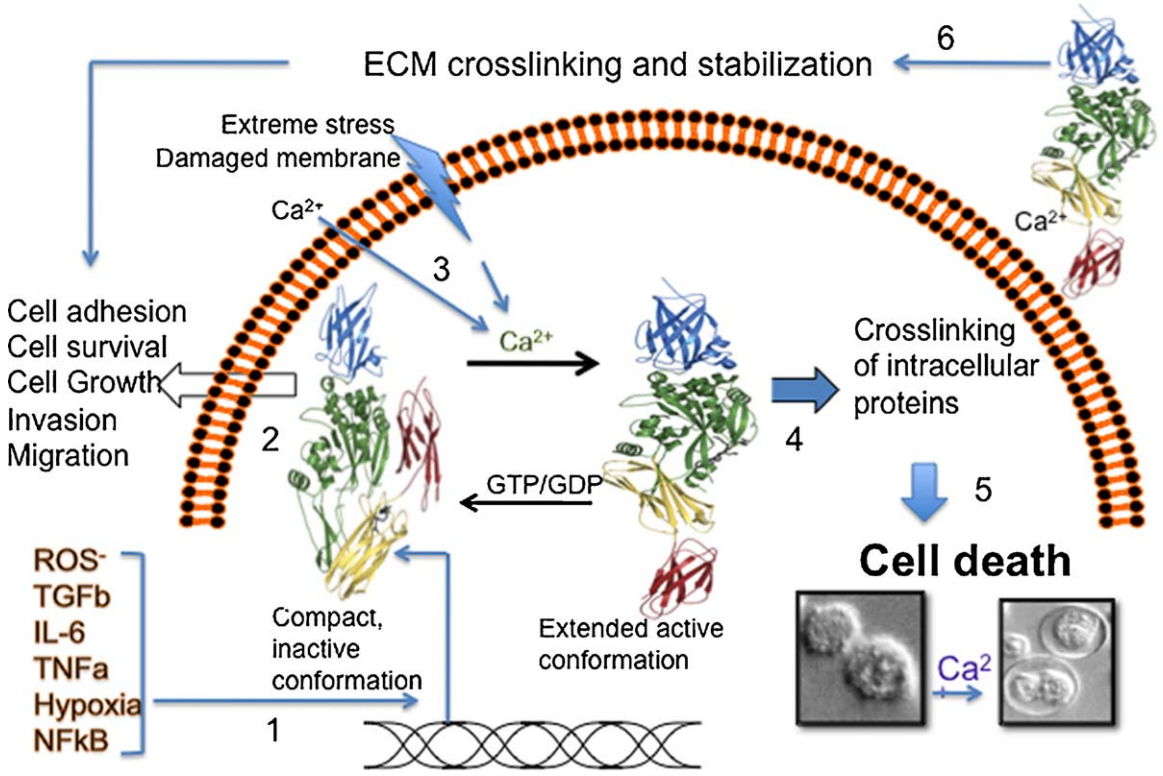

Fig. 2. TG2 expression can either inhibit or promote cell death depending on its location or other cellular contexts. Various inflammatory or stress signals upregulate TG2 expression (1). Under normal conditions, the environments inside the cell (low calcium and high GTP) are not conducive to the activation of TG2; thus, the protein remains in a compact, catalytically inactive form. As such, TG2 can directly (e.g., by associating with other proteins and altering their structure, activity, stability and function) or indirectly (by altering gene expression owing to a squelching effect) promote cell growth, survival, cell motility and invasive signaling (2). Extreme stress or trauma conditions can perturb intracellular calcium homeostasis (3), leading to alteration of TG2 from the compact form to the extended, catalytically active form (4). Once activated, TG2 can cause cross-linking of cellular proteins and result in apoptotic death (5). TG2 can be secreted outside the cell, where high calcium concentrations allows it to cross-link and stabilize the ECM proteins and promote cell attachment and motility functions (6).

of intracellular proteins, as is observed during apoptosis or necrosis [7,8].

Various important functions have, therefore, been ascribed to TG2 both in the intra- and extracellular environment, including its role in matrix stabilization, cell adhesion and migration and cell death and survival (Fig. 2). TG2 can interact with various intra- and extracellular proteins, altering their structure, function, and/or stability [9]. For example, the interaction between TG2 and IκBα is implicated in the constitutive activation of NF-κB and conferring protection against stress-induced cell damage by reactive oxygen species, inflammatory cytokines, and chemotherapeutic drugs [10,11]. Therefore, it is possible that the functions of TG2 are dictated by its cellular location, interaction with other proteins, and binding to cofactors. Interestingly, despite the variety of functions in which TG2 participates, TG2 knockout mice ($TG2^{-/-}$) are anatomically, developmentally, and reproductively normal [12]. However, studies using these animal models have indicated that TG2 plays a critical role in wound healing and that chronic expression of TG2 promotes abnormal wound healing by the accumulation of extracellular matrix (ECM) leading to fibroproliferative disorders [13].

2. TG2 in inflammation

Inflammation is essential for wound healing and tissue repair and involves a complex series of events such as cell migration, cell proliferation, synthesis and stabilization of the ECM, neovascularization, and apoptosis. It is a dynamic process mediated as a result of altered homotypic (cell–cell) and heterotypic (cell–ECM) interactions among multiple cell types (fibroblasts, endothelial cells, macrophages, granulocytes, immune cells, etc.). Chronic inflammation due to ageing, infection or stress (physical, chemical or hormonal) can lead to serious pathological conditions, such as degenerative fibrotic diseases (e.g., renal and pulmonary fibrosis, pancreatitis, and cirrhosis) and cancer [14].

Several studies have indicated the involvement of TG2 during initial phase of wound healing and inflammation [13–15]. Cytokines and growth factors secreted during early phases of cell injury regulate TG2 expression. For example, transforming growth factor (TGF)-β1 induces TG2 expression in keratinocytes and dermal fibroblasts via the TGF-β1 response element, which is located in the *TGM2* gene promoter [16]. Tumor necrosis factor (TNF)-α induces TG2 synthesis in liver cells via activation of IκBα phosphorylation. This causes the dissociation of IκB from the nuclear factor (NF-κB), allowing the nuclear translocation of NF-κB, which then binds to the *TGM2* promoter and induces its expression [17]. TG2 can also activate NF-κB via the non-canonical pathway by cross-linking IκBα [11,18]. TG2 expression is enhanced in cartilage tissue by IL-1 [19] and in liver cells by IL-6 [20]. In response to cutaneous injury, TG2 expression and activity is increased at sites of neovascularization and in endothelial cells, skeletal muscle cells and macrophages infiltrating wounds in the border between healthy and injured tissue [21].

A recent report demonstrated that TG2-deficient mice are better protected from lipopolysaccharide-induced septic shock than their wild-type counterparts are [22]. This effect was associated with decreased NF-κB activation, decreased neutrophil recruitment into the kidney and peritoneum, and reduced damage to renal and myocardial tissues. These observations suggest that TG2 promotes the pathogenesis of septic shock. Similarly, TG2-deficient mice have been used to determine whether TG2 plays a protective or pathologic role during liver and renal injury. Results of that study showed that more TG2-deficient mice than control mice died in response to carbon tetrachloride- and alcohol-induced liver injury, indicating a protective role of TG2 in liver injury [23,24]. This protection of wild-type mice by TG2 was associated with an increased inflammatory response and increased ECM accumulation. Studies to determine the involvement of TG2 in renal fibrosis revealed that TG2-deficient mice show far less damage to kidney damage than in their normal counterparts [25]. This effect was associated with reduced infiltration of macrophages and myofibroblasts, decreased collagen synthesis and decreased TGF-β activation in TG2-deficient mice [25].

Macrophages are among the first cell types to accumulate at sites of inflammation or tissue injury, and they contribute to the resolution of inflammation by generating TGF-β1. Macrophages at these sites also synthesize large amounts of TG2. Even in primitive organisms such as horseshoe crabs, circulating hemocytes, which act as a major defense system, express high TG2 levels. TG2 in hemocytes is immediately activated in response to lipopolysaccharide (LPS). In more complex organisms, the differentiation of monocytes into macrophages is associated with the upregulation of TG2 with a concomitant decrease in levels of blood-clotting factor XIIIa [26]. This transition is directly correlated with the phagocytic capacity of macrophages. Indeed, studies in TG2 knockout mice showed an impaired ability of macrophages to phagocytose apoptotic cells, and this resulted in autoimmunity [27]. A small fraction of TG2 (10–20%) in monocytic cells exists in 1:1 complex with beta-integrin (β1, -3 and -5) and serves as a co-receptor for integrin-mediated adhesion of cells to the ECM [28]. This ability of TG2 may be responsible for the extravasation and migration of monocytes during inflammation into tissues containing fibronectin matrices.

The interaction of cells with the ECM plays a critical role in regulating cell growth, survival, migration, and invasion signaling. Using *in vitro* cell line models, the role of TG2 in promoting the cell–ECM interaction has been well studied in both normal and transformed cells. For example, TG2 expressing fibroblasts showed increased cell attachment and spreading, whereas TG2-deficient fibroblasts exhibited decreased adhesion and spreading [29]. Available evidence suggests that TG2 promotes cell adhesion in two ways. First, TG2 enhances the formation of fibronectin matrix and stabilizes the ECM. Second, TG2 promotes the interaction between fibroblasts and fibronectin by interacting directly with integrin β1, β3 and β5 [30]; heparin sulfate chains of syndecan-4 [31]; or the orphan G-coupled cell adhesion receptor GPR56 [32]. In those studies, integrin- and syndecan-4 mediated adhesion and spreading of fibroblasts on fibronectin did not require the catalytic function of TG2, and modulated protein kinase C and focal adhesion kinase activities. Thus, in the inflammatory environment, TG2 may play a physiologic role in protecting cells from cell death and promoting their motility. Indeed, recent studies have indirectly supported this contention and suggested that fibroblasts and immune cells in the microenvironment have a more profound influence on the development and progression of carcinoma than has been previously appreciated. Therefore, understanding the significance of TG2 in tumors and tumor microenvironments may have important therapeutic implications.

3. TG2 in cancer

Cancer progression shares many similarities with the inflammatory response and tissue injury and remodeling [33,34]. Increased TG2 expression and transamidation activity is a common feature of many inflammatory diseases [13,15,18]. Hence, various cytokines and growth factors (such as, TGF-β1, TNF-α, and IL-6) secreted during tissue injury or wound healing are potent inducers of *TG2* gene expression [16,17,19]. It is also becoming evident that inflammatory responses play a critical role during tumor initiation, promotion, invasion and metastasis. Immune cells that infiltrate tumors can engage in cross-talk with cancer cells and modulate their growth, survival, and progression patterns [33,34]. Similarly,

many reports have demonstrated down-regulated TG2 expression in primary tumors and upregulated expression in secondary metastatic tumors or those resistant to chemotherapy and its expression has been implicated in disease progression [35,36]. Thus, the recent observation that silencing of *TG2* gene expression due to hypermethylation of the CpG island that overlaps the transcriptional and translational start site of *TG2* may explain relative sensitive of primary tumors for chemotherapeutic drugs [37]. Similarly, increased expression of TG2 in cancer cells has been liked to increased drug resistance, metastasis and poor patient survival [35,36,38–43]. These findings suggest an interconnecting link between chronic inflammation, TG2, and progression of metastatic and drug-resistant cancer.

Although studies of drug resistance and metastasis have generally proceeded independently along separate pathways, there are several reasons to believe that they share many common features. For example, advanced-stage cancers, which harbor a number of genetic alterations such as overexpression of *Bcl-2* and inactivation of *p53*, are also resistant to apoptosis. This resistance not only endows tumor cells with an increased ability to grow and survive in hostile environments of foreign tissues (metastasis) but also allows them to express a drug-resistant phenotype. Indeed, tumor cells selected for drug resistance *in vitro* are more malignant *in vivo*. Conversely, metastatic tumors generally exhibit higher resistance to drug and radiation therapies than do their primary counterparts.

Multiple studies have shown elevated TG2 expression in many types of cancer cells, including pancreatic carcinoma [38], breast carcinoma [39], malignant melanoma [40], ovarian carcinoma [41,42], lung carcinoma [43], and glioblastoma [44]. For example, analysis of more than 30,000 genes from tumor samples revealed that *TG2* is one of the more highly expressed genes in pancreatic adenocarcinoma [45]. Similarly, in an attempt to identify metastasis-associated proteins by proteomic analysis, Jiang et al. observed that TG2 was 1 of the 11 proteins that were selectively amplified in metastatic human lung carcinoma [46]. Treatment of cancer cells with epidermal growth factor (EGF) induced the expression of TG2 and protected cells from doxorubicin-induced apoptosis [47]. These observations strongly support that aberrant expression of TG2 confers resistance to chemotherapeutic drugs and promotes invasive potential of cells. Table 1 summarizes various cancer cell lines, which show elevated levels of TG2 expression when selected for resistance to chemotherapy drugs. Similarly, metastatic tumors from patients with breast cancer [39], malignant melanoma [40], and ovarian carcinoma [41,42] show substantial increases in TG2 expression compared with that in their primary counterparts. In contrast, down-regulation or inhibition of TG2 by small interfering RNA (siRNA), antisense RNA, ribozyme, or small molecule inhibitors in various cancer cell types has been shown to increase their sensitivity to chemotherapy-induced cell death and inhibition of invasion, both *in vitro* and in animal models [35,41,48]. In ovarian cancer, increased expression of TG2 enhanced their adhesion to fibronectin and promoted directional cell migration whereas ovarian cancer cells with knockdown TG2 showed diminished tumor dissemination on the peritoneal surface and in mesentery in an intraperitoneal ovarian xenograft mouse model [42]. Furthermore, in patients with ovarian cancer, overexpression of TG2 in tumor cells was associated with significantly worse overall survival [41]. Similarly, overexpression of TG2 in pancreatic tumor samples was strongly associated with nodal metastasis, lymphovascular invasion and poor overall patient survival [48]. Together, these observations strongly support that overexpression of TG2 confers resistance to chemotherapeutic drugs and promotes the invasive potential of malignant cells.

Table 1
Aberrant TG2 expression is associated with increased drug resistance in multiple cancer cell types.

Cancer	Model system	Resistance	Reference
Breast	Cell lines	Doxorubicin	[10,36,37,39,44,47]
Ovarian	Cell lines Orthotopic mouse Patients	Paclitaxel Cisplatin	[42,73,89]
Lung	Cell lines	Cisplatin	[43]
Pancreatic	Cell lines Orthotopic mouse Patients	Gemcitabine	[35,48,76]
Neuroblastama	Cell lines	BCNU	[44,90]
Melanoma	Cell lines	Cisplatin Carbamazine	[40]

It is generally believed that the mechanisms responsible for promoting growth, survival, and invasive ability of cancer cells also operate in normal cells. The major difference is that, in normal cells, these pathways are under tight regulation; once the stimulus or event that triggered these pathways dies down, cells either revert back to a quiescent state or undergo cell death. In contrast, as a result of genetic or epigenetic changes, cancer cells generally become independent of exogenous factors for their growth and survival [49]. In this context, it is worth mentioning few facts about the significance of TG2 expression in normal cells. For example, macrophages collected from inflammatory sites accumulate large amounts of TG2 protein [50]. In view of our current understanding of TG2 in cancer cells, it is conceivable that, in normal cells, induction of TG2 in inflammatory macrophages is related to their ability to migrate to inflammatory sites and to protect against cytotoxic mediators produced by or in response to infectious agents. Recent reports have documented a direct role of TG2 in promoting the migratory functions of normal cells. Expression of TG2 in T lymphocytes has been shown to promote their transmigration across endothelial cells [51]. TGF-β-induced expression of TG2 in retinal pigment epithelial cells has been linked to their increased migration on fibronectin-coated matrices [52]. Retinoic acid induced TG2 in neuroblastoma cells was shown to augment their migration and invasion functions [53]. Similarly, ectopic expression of TG2 in mesenchymal stem cells (MSCs) was shown to promote their adhesion on fibronectin and to activate FAK, Src, and PI3K [54]. Furthermore, TG2-transfected MSCs could effectively restore the cardiac functions of infarcted myocardium [54].

An important property of the highly malignant tumor cell is its ability to survive in hostile host environments as it passes through the lymphatic system or the bloodstream in its attempt to colonize at distant sites. The survival of tumor cells that have escaped into the vasculature depends on their ability to dock with and adhere to tissues where they are able to metastasize [55]. Such selection pressures may explain why the expression of TG2 is upregulated in secondary tumors than in primary tumors. TG2 expression has been shown to correlate positively with the propensity of human tumors to metastasize. The results of one proteomic analysis indicated that TG2 expression was significantly higher in highly metastatic lung cancer cell lines than in those with lower metastatic potential [56]. One possible mechanism of action for TG2 in the metastatic cascade was proposed by Kong and Korthuis [57] in their study of free-floating melanoma cells in isolated arterioles, which demonstrated that TG2 stabilized contact points of tumor cells with the subendothelial matrix. In contrasting report Xu and Hynes [58] observed that down-regulation of TG2 expression in melanoma cancer cells promoted their ability to metastasize [58]. These conflicting results of TG2 expression in cancer cells suggest that the relevance of TG2 to cancer biology

may depend on the type and stage of the cancer cell and more importantly on the localization of TG2 within or outside the cell. Thus, nuclear, cytosolic, membranous, or extracellular TG2 may impact cell growth, survival or invasion in completely different ways as depicted in Fig. 2. Therefore, a precise understanding of the expression and function of TG2 in the context of cancer stage and type is important for implementation of TG2-based interventions to disrupt malignant invasion, growth, and survival.

4. Epithelial to mesenchymal transition (EMT), inflammation, and cancer

Cancer progression shares many similarities with inflammatory responses and tissue injury and remodeling. As early as 1863, Rudolph Virchow provided the first indication of a possible link between inflammation and cancer. He hypothesized that cancer originates at the sites of chronic inflammation and suggested that some classes of irritants together with the tissue injury and ensuing inflammation that they cause, may enhance cell proliferation and cancer progression [59]. This idea remained quiescent for many years until several lines of evidence, from epidemiological studies to molecular studies of genetically modified mice, led to a general acceptance that inflammation and cancer are closely linked. Epidemiological studies have shown that chronic inflammation predisposes individuals to various types of cancer. For example, ulcerative colitis, chronic gastritis, hepatitis, and chronic pancreatitis and their respective associations with colon, gastric, liver, and pancreatic carcinomas represent few examples of the relationship between inflammation and tumor progression. Moreover, anti-inflammatory drugs are known to reduce the risk of cancer development. It is estimated that underlying infections and inflammatory responses are linked to 15–20% of all deaths from cancer worldwide [60]. Thus, it is becoming clear that the proliferation of cells alone is not enough to cause cancer. Sustained cell proliferation of oncogenically transformed cells in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA-damage promoting agents is needed for successful neoplastic growth and spread. The molecular mechanisms that link inflammation and cancer have remained elusive until recently. Recent advances in understanding of molecular pathways that govern the association of inflammation with organ fibrosis and cancer, point to the EMT as a common link in the progression of these pathological conditions [61,62].

The EMT marks the conversion of polarized epithelial cells into highly motile fibroblastoid-like cells. Thus, it involves the loss of intracellular cohesion, disruption of the ECM, modifications of the cytoskeleton, and increased cell motility and invasiveness. The reduction in intracellular cohesion is mainly the result of alterations in the intracellular junctions composed of desmosomes, adherens junctions, and tight junctions. Epithelial cells use E-cadherin (encoded by the *CDH1* gene) as a major protein in adherens junctions and promote its interaction with the extracellular domain of another E-cadherin molecule from a neighboring cell [63]. The intracellular domain of E-cadherin is connected to a protein complex containing beta-, alpha, and p120 catenins, which interact with the intracellular actin filaments network. This creates a communication pathway among cell contact, regulation of the cytoskeleton and the cell shape, which is necessary to keep the epithelial cells immobile and physically linked to each other. During the EMT, the activity of the adherens junctions is substantially modified, predominantly owing to the replacement of E-cadherin by N-cadherin, a process called "cadherin switching" [64]. Aberrant expression of N-cadherin has a dominant effect on interactions between cells, given that, even in the presence of E-cadherin, N-cadherin can augment the motility of tumor cells [65].

During embryogenesis, the EMT plays a pivotal role in tissue development [61]. Its reactivation in adults can be a physiologic attempt to control inflammatory responses and heal damaged tissues [62]. However, in pathological context, such as in tumors or during organ fibrosis, this healing response may act in a harmful manner, and result in metastasis and organ failure. Indeed, the evidence is accumulating in support of the EMT playing an important role in cancer progression through which epithelial cancers invade and metastasize [61,62,66]. The EMT is characterized by a loss of cell adhesion and apical basal cell polarity and increased cell motility.

Cells in the EMT acquire the ability to degrade the basement membrane and migrate through the ECM to populate different areas during embryogenesis or cancer progression, or to behave like profibrotic myofibroblast in the interstitial spaces between tissues. Certain elements, such as TGF-β1 and hypoxia that are known for their role in control of inflammation and induction of tumor cell death, can act as inducers of the EMT in the inflammatory microenvironment. TGF-β1 acts through a complex of type I and type II transmembrane serine-threonine kinase receptors. In response to TGF-β1 binding, the type II receptor kinases phosphorylate the type I receptors, which then leads to the activation of Smad2 and Smad3 through their direct C-terminal phosphorylation by the type I receptor. Activated Smad2 and -3 then form trimers with Smad4 and translocate into the nucleus, where they associate and cooperate with other transcription factors to activate or repress the transcriptional regulation of target gene(s). Several studies have investigated the role of TGF-β1 activated Smads in the EMT [67–69]. Increased expression of Smad2 or Smad3 with Smad4 induces the EMT or enhances the induction of EMT by the activated form of TGF-β type I receptors, whereas expression of dominant-negative versions of Smad2 or Smad3 blocks the TGF-β induced EMT in NMuMG cells. Consistent with the essential role of Smad3 in the EMT, renal tubular epithelial cells deficient in Smad3, did not undergo EMT in response to TGF-β stimulation. Similarly, keratinocytes derived from Smad3−/− mice showed reduced migration in response to TGF-β stimulation.

Compared with Smad3, Smad2 may play an antagonistic role in the EMT process. Loss of Smad2 is frequently noted in patients with skin cancer and Smad2 deficiency in keratinocytes has been shown to promote the EMT and accelerate skin tumor formation. This tumor promotion has been explained by increased binding of the Smad3/4 complex to the promoter of the *Snail* gene and by increased *Snail* expression in the absence of Smad2, which enhances the progression of the EMT. Similarly, Smad2−/− hepatocytes appear to be mesenchymal in nature and to migrate faster than wild-type cells do, whereas Smad3−/− hepatocytes retain their epithelial characteristics. Similar to Smad3, Smad4 plays an indispensable role in the EMT. RNA interference-mediated knockdown of Smad4 expression or expression of a dominant-negative mutant of Smad4has been shown to result in preserved E-cadherin expression, suppression of fibrotic type I collagen synthesis, and decreased bone metastasis *in vivo*. Furthermore, genetic ablation of Smad4 was shown to preserve epithelial markers and decrease occurrences of the EMT in adenocarcinoma. TGF-β was also shown to elicit signaling responses through pathways generally considered to be important effector pathways for tyrosine kinase receptors in response to ligands that do not belong to the TGF-β family. The rapid activation of these non-Smad signaling pathways by TGF-β often follows kinetics similar to those of Smad signaling, and attenuation of Smad signaling does not generally affect the activation of these non-Smad pathways. In addition, non-Smad signaling responses to TGF-β can also occur with delayed and often indirect kinetics, presumably as a result of Smad-mediated changes in gene expression. Direct activation of non-Smad signaling pathways by TGF-β occurs through interac-

tions of signaling mediators either directly with the type II and/or type I receptors or through adaptor proteins. Among the non-Smad signaling responses, activation of Erk MAP kinases, Rho GTPases NF-κB, and the PI3 kinase/Akt pathway have been linked to the EMT. Treatment of cells with chemical inhibitors that selectively or specifically block one or several of these pathways have been shown to dramatically affect the induction of the EMT phenotype and downstream transcriptional responses [70].

TGF-β can also induce the EMT in resident fibroblasts and convert them into myofibroblasts. For example, the transformation of tubular and endothelial cells into mesenchymal cells contributes to more than 60% of the myofibrobalst population in obstructed kidneys and this process plays a major role in tubulo-interstitial fibrosis [71]. The induction of the EMT by TGF-β in renal cells takes several days and involves the interplay among many pathways, including Smad proteins, the integrin-linked kinase, and Rho-family GTPases. Beta-catenin also plays a role in the transition to myofibroblasts: it synergizes with TGF-β to activate in activating the α-smooth muscle actin (*αSMA*) gene and protein expression. All of the processes that result in the transition of fibroblasts into myofibrobalst-like cells converge in the so-called EMT inducers, which are the transcription factors capable of eliciting the dramatic changes associated with this transition. Similar to their role in the EMT process of cancer cells, Snail factors have been associated with renal fibrosis, and TGF-β1 is the most potent inducer of Snail transcription. Snail expression is sufficient to induce the EMT or expression of all the hallmarks of renal fibrosis in adult mice; however, Snail factors have been shown to be strongly upregulated in fibrotic kidney from patients who have undergone nephrectomy due to urinary obstruction and kidney failure [71]. Snail directly represses E-cadherin transcription; activates, albeit indirectly, the transcription of mesenchymal genes such as *vimentin* and *aSMA*; and promotes collagen synthesis and deposition. Altogether, these findings imply that TGF-β1 induced signaling is crucial for the EMT, both in cancer cells and in chronic inflammatory diseases.

5. TG2-induced EMT in cancer cells

As discussed earlier (Section 3), multiple cancer cell types with inherent or acquired resistance to drugs or from metastatic sites exhibit increased expression of TG2. TG2 expression in cancer cells is associated with increased cell survival and invasive signaling functions. TG2 expression induces the activation of FAK, Akt, cyclic AMP response element binding protein, and NF-κB and down-regulates the tumor suppressor protein PTEN. Activation of these TG2-induced oncogenic signaling pathways promotes invasive functions and confers resistance to stress-induced apoptosis in cancer cells. Indeed, the inhibition of TG2 by small interfering RNA (siRNA) has been shown to attenuate the ability of pancreatic cancer cells to invade and metastasize both *in vitro* and in a mouse model [48]. Similar effects of TG2 inhibition on drug sensitivity and metastasis of ovarian cancer cells have been observed [41]. However, the TG2-regulated pathways that contribute to increased cell survival and metastatic potential have remained largely unknown until recently.

As discussed earlier (Section 4), the EMT is a known pathologic event during the progression of various diseases, including inflammation, fibrosis, and cancer [66,70,72]. The EMT is believed to be important in conferring drug-resistance characteristics to cancer cells and in promoting the conversion of early stage tumors into invasive cancer [61]. Based on these observations and the finding that TG2 expression is upregulated in drug-resistant and metastatic tumor cells (Section 3) and that TG2 plays a role during inflammation and fibrosis (Section 2), it is tempting to speculate that TG2 expression participates in these events by modulating the EMT. Indeed, in a recent publication, Shao et al. reported that TG2 expression in ovarian cancer cells could modulate the EMT and that this modulation contributed to increased invasiveness and metastasis of the cancer cells [73]. Using the loss- and gain-of-function approach, the authors concluded that TG2 induces a mesenchymal phenotype (characterized by a cadherin switch) to promote invasive behavior in ovarian cancer cells. This transition was associated with altered expression of the transcriptional repressor *Zeb1*. Recent data from our own laboratory revealed a similar phenomenon in mammary epithelial cells. In that study, neoexpression of TG2 induced the EMT in MCF10A cells as evidenced by a loss of epithelial markers (E-cadherin) and gain of mesenchymal markers (vimentin, fibronectin, N-cadherin, etc.). Moreover, TG2 expression increased the invasiveness of MCF10A cells through the Matrigel matrix, and this was associated with a loss of cell polarity in 3D culture, increased cell survival, and anchorage-independent growth (Kumar and Mehta, unpublished results).

In order to metastasize to distant sites, carcinoma cells in primary tumors must transform from immobile epithelial cells to motile mesenchymal cells and detach from the neighboring environment. Once detached from the original niche, transformed epithelial cells must acquire autonomy from growth factors and gain survival advantage. For example, normal epithelial cells, when detached form neighboring cells, die owing to anoikis. However, when these cells undergo the EMT, they are protected from anoikis and grow in an anchorage-independent manner just like their transformed counterparts [74]. Indeed, some evidence suggests that TG2 expression could rescue normal fibroblasts from anoikis [75]. This ability of TG2 may be responsible for promoting the autonomy of cancer cells from growth-regulatory mechanisms and supporting anchorage-independent growth.

After successful extravasation, transformed cells must colonize the hostile environment of foreign tissue. This process involves the growth of microscopic metastases into macroscopic metastases. Recently, it has been proposed that the EMT can enable cancer cells not only to disseminate but also to acquire the ability of self-renewal by inducing a stem cell state [74]. It has also been proposed that cancer stem cells are responsible for the formation of macroscopic metastases. In line with these observations, our preliminary data suggested that the TG2-induced EMT in mammary epithelial cells could confer stem cell phenotype ($CD44^{high}/CD24^{low}$) (data not shown). Further studies are needed to characterize the complex molecular network through which TG2 modulates the EMT and stem cell characteristics in epithelial cells. Previous reports have demonstrated that aberrant expression of TG2 in epithelial cells results in constitutive activation of FAK, Akt, and NF-κB [10,11,76,77]. These pathways are known to be intimately involved in the regulation of EMT, conferring drug resistance, and promoting metastasis [61,78,79]. For example, activated NF-κB is considered to be a hallmark of many advanced-stage tumors [80]. Thus, constitutive active NF-κB is known to confer resistance to death-inducing stimuli, including chemotherapeutic agents [81] and to promote metastasis by inducing EMT [79]. The NF-κB-induced EMT has been attributed to the increased stability of Snail due to increased synthesis of ICOP9 signolosome 2, which blocks the ubiqutination and subsequent degradation of Snail [82]. In another report, constitutive activation of NF-κB in MCF10A cells was found to induce the EMT as a result of increased expression of Zeb1 and Zeb2 [83]. Based on these observations it is reasonable to believe that TG2-induced EMT may result from constitutive activation of NF-κB and subsequent increase in Snail, Zeb1, and Zeb2 expression, as observed in ovarian and mammary epithelial cells [73] and [Kumar and Mehta, unpublished]. Indeed, TG2 was recently shown to associate with NF-κB for its recruitment to the promoter sequence of Snail and leading to its transcriptional regulation [84]. Similarly TGF-β, which is consid-

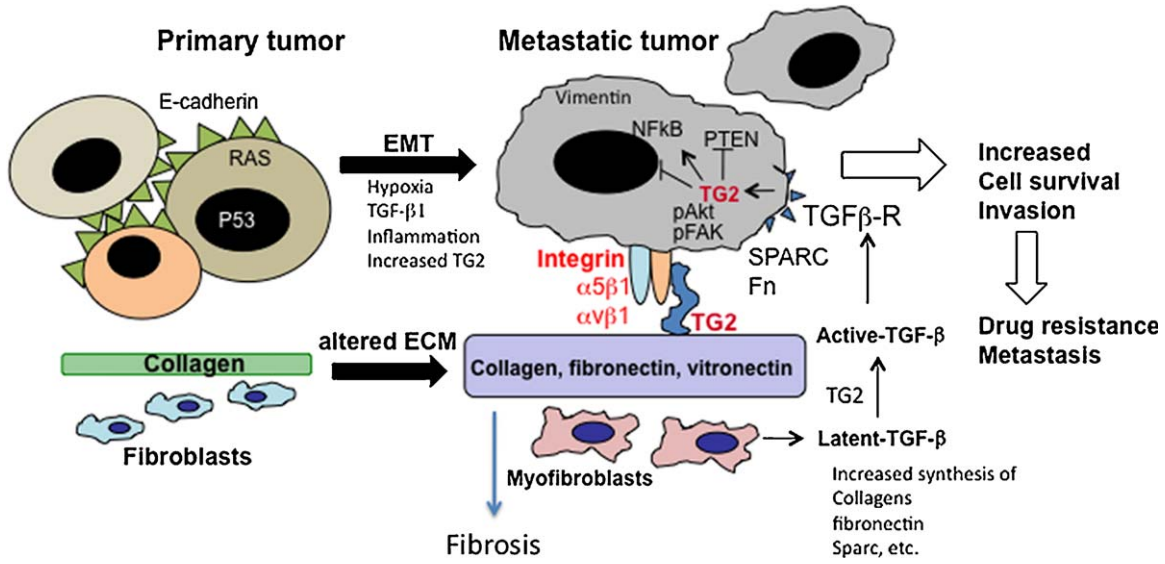

Fig. 3. Progression of primary tumor cells to invasive carcinoma results from increased TG2 expression (due to hypoxia or other inflammatory signals). TG2 expression induces the EMT and activates oncogenic signaling (Akt, FAK, NF-κB, down-regulation of PTEN, etc.). The EMT, which shares many molecular characteristics with CSCs further promotes drug resistance and metastasis of primary tumor cells. In addition, the upregulation of TG2 in tumor-resident fibrobalsts (e.g., in response to TGF-β1 production by tumor associated macrophages) induces their differentiation into myofibroblasts with increased collagen and other matrix proteins synthesis. Increased synthesis and cross-linking of ECM proteins by TG2 in extracellular environments renders the ECM stiff, which has been suggested to promote focal adhesions, increased PI3K activation, and increased invasion of epithelial cells [88].

ered to be a potent inducer of EMT both in normal and pathological conditions [61,67], can cross-talk with TG2. Thus, TGF-β can induce TG2 expression [16,85] and TG2 can activate TGF-β [86]. Although, in our preliminary studies, we did not observe any activation of TGF-β signaling in TG2-transfected MCF10A cells, nevertheless, TGF-β failed to induce EMT in absence of TG2, suggesting that TG2 may serve as an important downstream mediator of TGF-β-induced EMT. It is conceivable that TG2 represents a converging point for TGF-β induced non-canonical signaling that is considered critical in induction of the EMT and supports TG2 as a common link between inflammation and cancer. Based on these observations we hypothesize a model (Fig. 3) supporting that continuous production of mediators, such as ROS-, TGF-β, and IL-6 at smoldering inflammatory sites due to chronic infection, tissue injury, or tumor growth, results in constitutive expression of TG2, which orchestrates multiple downstream signals to affect such critical processes as EMT and acquisition of stem cell like characteristics. Altered homotypic (cell–cell) and heterotypic (cell–ECM) interactions that ensue the loss- and gain- of EMT/CSC-related genes confer cell growth and cell survival advantages, increased synthesis of ECM proteins [87], and increased ability of cells to migrate and invade the surrounding tissue. These changes result in fibrotic response, desmoplastic response and transformation of primary tumor into metastatic tumor [88]. These observations also support our belief that TG2 is a promising therapeutic target for reversing drug resistance and inhibiting metastatic spread of tumor cells. Indeed, down-regulation of TG2 by gene-specific siRNA resulted in sensitization of orthotopically growing ovarian [41] and pancreatic [48] tumors to chemotherapeutic drugs and attenuated their dissemination in a nude mouse model.

6. TG2 as a therapeutic target

About 11 million new cases of cancer are diagnosed annually worldwide and 6.7 million people die of the disease. Virtually, all the cancer-related deaths can be said to have occurred because the chemotherapy failed or the disease has metastasized. Therefore, the discovery that aberrant expression of TG2 in cancer cells contributes to chemoresistance and metastasis in a wide spectrum of cancer types, offers a unique opportunity to treat/manage cancer during early and advanced stages. The development of specific and potent TG2 inhibitors can change the way cancer is treated and thus offers a huge market potential. TG2 represents a single target that can modulate multiple pathways and functions in cancer cells and thus its inhibition can simultaneously deprive cancer cells of multiple pathways that are critical for their growth and survival. Our data strongly supported that transamidation activity of TG2 is not involved in promoting the EMT, chemoresistance or metastasis. Therefore, alternate approaches to downregulate TG2 expression in tumor cells holds great promise in reversing chemoresistance and inhibiting the metastasis.

The application of small interfering RNA (siRNA) oligonucleotides to silence gene expression has profound implications for intervention of human diseases. The extraordinary sequence specificity of siRNA makes it an attractive tool for selective silencing of the gene for cancer therapy. To this end, several groups in the academia as well as in industry are attempting to develop effective vehicle for delivering siRNA to *in vivo* growing tumors. We have successfully employed this approach to deliver TG2 siRNA to orthoptopically growing pancreatic [48] and ovarian [48] tumors in an attempt to render them sensitive to chemotherapeutic drugs and inhibit their metastasis. This approach may provide a novel method for treating drug-resistant and metastatic tumors, which together account for >90% cancer-related deaths.

Similarly, the four structural domains of TG2 are unique and have specific function(s) (Fig. 1). For example, the catalytic domain contains the catalytic triad (Cys-His-Asp) and can induce posttranslational modification of some critical signaling proteins. The N-terminal β-sandwich is responsible for binding to fibronectin and promotes cell–ECM interaction and integrin/platelet growth factor receptor (PDGFR)-mediated signaling [91]. The two C-terminal β-barrels contain sequences that are responsible for binding to GTP/GDP and serve as signaling molecules. In view of TG2's complex structure and multifunctional nature, it is important to determine the domains/motifs of TG2 that is critical in promoting functional and phenotypic alterations (EMT, stemness,

adhesion, motility, invasion, etc.) in cancer cells. Such information will provide important leads for future design of small molecule inhibitors for intervention therapy.

Acknowledgements

The work in authors' laboratory was supported in part by a grant from Susan G. Komen for the Cure Foundation and by National Institutes of Health grant CA131062 (to KM). The authors wish to acknowledge important contributions by various investigators in the field, which we are unable to cite due to space limitations. We wish to thank Ms. Virginia M. Mohlere for editorial help of this manuscript.

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