

Review

## Tissue Transglutaminase Promotes or Suppresses Tumors Depending on Cell Context

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**Abstract.** *Despite major advances in diagnosis, surgical and postsurgical techniques and adjuvant therapies, 7.5 million individuals worldwide still die of cancer every year. Most cancer deaths result because tumor cells metastasize to distant organs and/or acquire resistance to conventional therapies. Therefore, elucidation of tumor-encoded genes whose expression contribute to the development of drug resistance and metastasis can be of great significance in revealing novel therapeutic targets for the effective control and treatment of cancer. Tissue transglutaminase (TG2) is an enzyme whose expression is up-regulated in a number of cancer cell types. TG2 is a ubiquitous member of the transglutaminase family of enzymes and is implicated in such diverse processes as inflammation, wound healing, apoptosis, neurodegenerative disorders and cancer. Depending on the cell type and its localization within the cell, TG2 can serve as an antiapoptotic or a proapoptotic protein. In general, the presence of high levels ( $>1$  mM) of  $\text{Ca}^{2+}$  induces its activation, which promotes inter- and intramolecular cross-linking of proteins and results in cell death. Indeed, the use of TG2-specific antisense RNA protects cells against stress-induced cell death. In contrast, low levels ( $<1$  mM) of  $\text{Ca}^{2+}$  and a high concentration ( $>9$   $\mu\text{M}$ ) of guanosine triphosphate (the conditions that generally prevail inside cells) promotes TG2-mediated cell survival signaling. Many studies have reported increased TG2 expression in a number of cancer cell types that are known to have high resistance to chemotherapeutic drugs or are metastatic. Importantly, the increased expression of TG2 is associated with increased invasion and cell survival. Down-*

*regulation of TG2 by small interfering RNA or inhibition by small molecule inhibitors can enhance therapeutic efficacy of anticancer drugs and inhibit metastatic spread. In this review, the contextual roles of TG2 in promoting and protecting normal versus tumor cells from death-induced signaling, its contributions in promoting the drug resistance and metastasis of cancer cells and its therapeutic potential for treating advanced cancer are discussed.*

A total of 1,437,180 individuals in the United States will be diagnosed with cancer during 2008 and about 565,650 patients will die of the disease (1). For almost a century, it has been realized that cancer is a multistep process that develops over years, sometimes decades. The facts that cancer cells grow faster than normal cells and do not obey the rules of ordered growth imply that cancer is an aberration associated with cell growth (2). This observation combined with the fact that cancer is a multistep process suggest that multiple genetic events are involved with the growth deregulation seen in cancer cells. Research during the past decade has shown that these genetic events include the activation of a group of genes termed “oncogenes” and the inactivation of another group of genes termed “suppressor genes.” A fine balance between the activities of these two groups of gene products dictates normal cell growth, and a disruption of this balance appears to provide a cell with a growth advantage that ultimately leads to a neoplastic state.

In addition to genetic alterations, epigenetic changes are involved in the successful survival and progression of tumor cells. These changes primarily involve either the loss of methylation or hypermethylation of gene regulatory regions that can repress or express tissue-specific genes, respectively. The importance of such epigenetic changes in the process of tumorigenesis and metastasis is becoming apparent. Cells may acquire an epigenotype that is critical for their dissemination, successful survival and growth at distant sites. The ability of tumor cells to metastasize, proliferate and survive at distant sites is a major impediment to the

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successful treatment of cancer. More than 90% of cancer-related deaths can be attributed to metastatic disease that is resistant to conventional therapies (3). Therefore, the delineation of tumor-encoded genes whose expression contributes to the development of drug resistance and metastasis may offer immediate clinical benefits by revealing new and promising therapeutic targets. In this review, the contextual role of one such gene, *TGM2* (NM-004613), which encodes a functionally and structurally complex protein, tissue transglutaminase (TG2) whose expression plays a role in certain physiological processes (apoptosis, wound healing, inflammation, cell adhesion, extravasations), and yet its aberrant expression in tumor cells confers a deadly phenotype, is discussed.

### TG2 – A Protein of Many Traits

Tissue transglutaminase (TG2, EC 2.3.2.13) is the most diverse and ubiquitous member of the transglutaminase family of enzymes which catalyze  $\text{Ca}^{2+}$ -dependent posttranslational modification of proteins by inserting highly stable (epsilon-[gamma-glutamyl] lysine) isopeptide bonds or by conjugating polyamines at selected peptide-bound glutamine residues (4). Transglutaminase-catalyzed cross-linked products – generally a scaffold of high molecular mass proteins – are highly stable and resistant to mechanical, chemical and proteolytic degradation. The accumulation of transglutaminase-catalyzed isopeptide bonds can be observed in skin and hair and during blood clotting and wound healing.

Although TG2, like all transglutaminases, generally acts as a cross-linking enzyme, in the absence of an appropriate acceptor peptide, it can cause deamination of protein-bound glutamine residues (5). TG2 can bind and hydrolyze guanosine triphosphate (GTP) with an affinity and rate similar to those of the  $\alpha$  subunits of large heterodimeric G proteins and small Ras-type G proteins (6). TG2 mediates the coupling of  $\alpha_{1\beta-8}$  adrenergic receptors and thromboxane and oxytocin receptors to phospholipase C, thereby inducing the production of inositol phosphate and the activation of protein kinase C (7). When bound to guanosine diphosphate (GDP) or GTP, TG2 cannot catalyze a transamination reaction. However, this inhibition is suspended in the presence of accessible  $\text{Ca}^{2+}$ , which serves as a switch between the protein cross-linking and signaling functions (8). In addition, TG2 can function as a protein disulfide isomerase, an ATPase and a protein kinase (9-11).

Although predominantly a cytosolic protein, TG2 can translocate to the plasma membrane in a 1:1 complex with  $\beta$  integrin (12, 13). In the plasma membrane, TG2's association with integrins on the cell surface and its high-affinity for fibronectin promote cell attachment to the proteins of the extracellular matrix (ECM) and the transduction of signaling pathways (14, 15). TG2 can also

be transported into the nucleus with the help of the nuclear transport protein importin- $\alpha 3$  (16). In the nucleus, TG2 can function as a G protein or as a transaminase in response to nuclear  $\text{Ca}^{2+}$  resulting in the cross-linking of proteins such as histone, retinoblastoma protein (pRb) and the SP1 transcription factor (17, 18). TG2-catalyzed cross-linking of these proteins can induce significant structural and functional changes in them.

TG2 is also involved in the modeling of the ECM (19), cell survival (20), glucose-stimulated insulin secretion (21), cell adhesion (13), neuronal differentiation (22), type 2 diabetes (23) and platelet aggregation (24). In the presence of high levels of  $\text{Ca}^{2+}$  TG2 cross-links cellular proteins and induces their polymerization and the formation of detergent-insoluble structures. The protein scaffolds thus formed stabilize the structure of a dying cell before it is cleared by phagocytosis; this prevents the release of intracellular components and a subsequent inflammatory response (25).

### TG2 as a Proapoptotic Protein

The *in vivo* expression of TG2 is a dynamically regulated process. For example, during limb morphogenesis, TG2 expression is observed only in developmentally transient structures in embryonic limbs. The major morphogenetic events of the limb associated with TG2 expression are cartilage maturation during skeletal development, interdigital apoptosis and differentiation of skeletal muscle (26). Maturation of the cartilage during endochondral ossification is characterized by intra- and extracellular TG2 accumulation in the zone of hypertrophic chondrocytes. As an intracellular enzyme, TG2 can be detected in mesenchymal cells of the prospective joints, in apoptotic cells of the interdigital web and in myoblasts of the skeletal muscle. A strong correlation exists between TG2 expression and apoptosis during interdigital web formation, implantation of an embryo in utero (27) and mammary gland regression (28). While TG2 is generally undetectable in most cells, its mRNA is transcribed at the onset of apoptosis (29). Moreover, the overexpression of TG2 in mammalian cells primes cells for suicide and the induction of apoptosis. Indeed, the transfection of an antisense TG2 construct into cells was associated with resistance to apoptosis induction, while the transfection of a sense construct into such cells enhanced spontaneous apoptosis (30).

TG2 is one of the few genes induced in response to certain hormones and stressors. To act as a proapoptotic protein, TG2 must be activated at its transamination axis, which can be achieved by exposing TG2 to mM concentrations of  $\text{Ca}^{2+}$  (Figure 1). Once activated, TG2 can irreversibly cross-link cellular proteins resulting in the formation of a detergent-insoluble protein scaffold and modification of cell organization, thus contributing to the ultrastructural changes

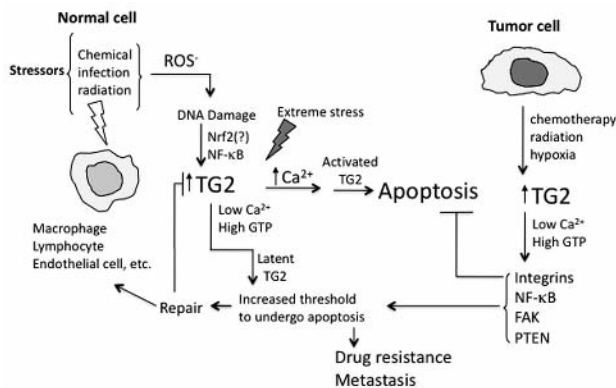


Figure 1. TG2 serves as a prosurvival and proapoptotic signaling protein in normal and cancerous cells. In normal cells, TG2 is an early-response gene that is up-regulated in response to inflammation and the presence of certain hormones or stress signals (such as ROS-). It is likely that transcription factors, such as Nrf-2 or NF-κB, that get activated during inflammation or in response to ROS- are responsible for inducing the TG2 gene. Under normal circumstances, the low level of cytosolic  $\text{Ca}^{2+}$  (nM) and high concentrations of guanosine triphosphate (>7  $\mu\text{M}$ ) keep the enzyme in its latent form. However, this catalytically inactive TG2 can perform other functions. For example, TG2 can associate with integrins and similar signaling molecules to activate signaling pathways and thereby contribute to increased cell survival, cell growth and invasion. Once the damage has been repaired, cells return to a normal state by shutting down TG2 expression. Nevertheless, if the stress or cellular damage is too severe,  $\text{Ca}^{2+}$  can escape into the cells through leaky membranes, resulting in the activation of TG2, the cross-linking of cellular proteins and the induction of apoptosis. Similar events may also occur in tumor cells; however, once TG2 expression is turned on in tumor cells (probably in response to ROS-, hypoxia, chemotherapy or irradiation) it cannot be turned off owing to a defective on-off mechanism, resulting in constitutive activation of cell survival, cell growth and invasive signaling pathways.

typical of cells undergoing apoptosis. The insoluble TG2-catalyzed protein scaffold stabilizes the integrity of dying cells before they are cleared by phagocytosis and thus prevents the nonspecific release of harmful intracellular components (*e.g.*, lysosomal enzymes and nucleic acids) and the consequent inflammatory responses and scar formation in nearby tissue (25, 31). During the late stages of apoptosis TG2 can be cleaved by caspase 3, which disables TG2's cross-linking function; this effect implies that a functional relationship exists between TG2 and other members of the apoptotic machinery (32). This notion is supported by the observation that TG2<sup>-/-</sup> knockout mice were unable to clear and have defective clearance of apoptotic cells (31).

**TG2 and Bax protein.** According to a recent report, mitochondria from cells with high expression of TG2 show constitutive hyperpolarization and produce higher amounts of reactive oxygen species (ROS) than cells with low expression of TG2 (33). Cell-permeable peptides derived

from the TG2 sequence can induce conformational change to Bax and cause it to translocate to the mitochondria and to release cytochrome c. The active site of mutant TG2 (C277S) could also promote cell death in HL-60 and U937 cancer cells, which suggests that the transamination function of TG2 is not exclusively responsible for the induction of apoptosis. Indeed, TG2's BH3 domain has also been implicated in inducing apoptosis(34). TG2 and Bax interact through their BH3 domains (33). Because of this interaction, Bax can serve as a substrate for TG2 at the mitochondrial level. Moreover, the TG2-catalyzed polymerization of Bax can occur when the  $\text{Ca}^{2+}$  level at the mitochondrial site is increased during apoptosis. Bax's covalent polymerization may stabilize its pore-forming and cytochrome c-releasing conformation. Indeed, mitochondria with TG2-polymerized Bax appeared clustered in discrete cytoplasmic regions, with few cristae and extremely electron-dense matrix during apoptosis (33). These results suggest a TG2-dependent shift of mitochondria to a higher polarized state before the loss of mitochondrial transmembrane potential. Similarly, the induction of TG2 by retinoic acid in adenocarcinoma and neuroblastoma cell lines sensitized cells to apoptosis owing to mitochondrial hyperpolarization (33). The TG2-dependent hyperpolarization of mitochondria can be considered an important early event in cell sensitization and commitment to apoptosis.

**TG2 and other apoptotic proteins.** Many genes involved in apoptosis encode proteins that may generate or respond to oxidative stress (35). In this regard, the overexpression of TG2 in neural cells determines the imbalance of the redox status of these cells and leads to the accumulation of ROS- in association with a large depletion in the levels of reduced glutathione (GSH) (36). In such an environment, glutathione-S-transferase (GST) P1-1 acts as a very efficient acyl donor as well as an acyl acceptor substrate for the TG2-catalyzed reaction (36). The TG2-dependent polymerization of GST P1-1 leads to its functional inactivation and the accumulation of ROS- (36). Massive GSH depletion characterizes the early phases of apoptosis and the fact that GST P1-1 might be functionally inactivated by TG2-catalyzed oligomerization indicates a potential proapoptotic role for TG2.

DAP-like kinase (DLK) is a nuclear serine/threonine-specific kinase, which has been implicated in apoptosis. DLK belongs to a subgroup of serine/threonine protein kinases, called mixed-lineage kinases, that act as key regulators of the stress-activated c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase signaling pathway (37). DLK-induced apoptosis requires that DLK be relocated to the cytoplasm, specifically to the actin cytoskeleton, which is achieved through interaction with the proapoptotic protein Par-4 (38). TG2-dependent DLK oligomerization occurs

early in the apoptotic response and significantly enhances the kinase activity of DLK and consequently its ability to activate the JNK pathway (39). Functional studies demonstrate that TG2-mediated oligomerization of wild-type DLK sensitizes cells to calphostin-C-induced apoptosis, while cross-linking of a kinase-inactive variant of DLK does not (39). The overexpression of DLK in neural cells and in sympathetic neurons induces apoptosis through the mitochondrial-dependent apoptotic pathway. Conversely, the overexpression of a dominant-negative form of DLK in these cells prevents apoptosis, indicating that DLK is involved in the control of TG2-induced cell death. The overexpression of TG2 induces either spontaneous apoptosis or renders neural cells highly susceptible to apoptosis induced by various stimuli in a caspase-independent fashion (40). Similarly, studies conducted on pancreatic cancer cells revealed that TG2-induced mitochondrial membrane polarization could lead to the induction of apoptosis in a caspase-independent manner (41).

### TG2 as an Antiapoptotic Protein

Paradoxically, TG2 expression can also protect cells from apoptosis. Several rapidly dividing cancer cells are known to overexpress TG2. Cancer cells that exhibit resistance to anticancer drugs or that are isolated from metastatic sites are known to express increased levels of TG2 (14, 42). The significance of increased TG2 expression and its implications in protecting cancer cells from apoptosis and conferring drug resistance and metastatic phenotype are discussed below.

*TG2, apoptosis, drug resistance and metastasis.* The development of drug resistance and metastasis are major impediments in the successful treatment of cancer. A common feature among drug-resistant tumors and metastases is that their cells are profoundly nonapoptotic (43). It is becoming apparent that some oncogenic mutations disrupt apoptosis, thereby initiating tumor development, progression and metastasis (44). Indeed, several genetic and epigenetic alterations that confer resistance to apoptosis have been identified in advanced cancers. For example, the activation of prosurvival signal transduction pathways such as those mediated by Ras, phosphoinositide-3-kinase (PI3K/Akt) and nuclear factor-kappa B (NF- $\kappa$ B) are frequently observed in advanced cancer (44, 45). The realization that diverse anticancer drugs kill tumor cells by activating common apoptotic pathways has raised the interesting possibility that single mutations that disable apoptosis might be the cause of multidrug resistance (45).

For tumor cells to progress and metastasize to distant sites, they must circumvent stressful conditions such as hypoxia, nutrient factor deprivation and altered cell adhesion. Tumor cells must survive in a foreign environment and overcome

and evade continual attack by the host's immune system. Each hurdle imposes selection pressure that enables certain tumor cells to survive by disabling apoptotic pathways so that by the time a tumor metastasizes its cells are highly resistant to pharmacological and physiological death-inducing signals. Thus, the ability of tumor cells to circumvent apoptosis not only helps them to exhibit resistance to cytotoxic drugs but also to grow and survive in the hostile environments of foreign tissue. This may explain, in part, why metastatic tumors are highly resistant to chemotherapeutic drugs and, conversely, why cell lines selected *in vitro* for resistance to chemotherapeutic drugs are more likely to cause metastatic disease if implanted *in vivo*.

Many recent reports have implicated TG2 expression in the growth and progression of cancer cells. Thus, cancer cells selected for resistance to chemotherapeutic drugs or that are isolated from metastatic sites typically exhibit elevated levels of TG2 (42, 46-52). Importantly, the down-regulation of endogenous TG2 by gene-specific small interfering RNA (siRNA), antisense RNA or ribozyme reversed drug-resistance in breast, pancreatic, lung and ovarian carcinoma cells, both *in vitro* and in animal models (51-56). Similarly, the inhibition of TG2 by small molecule inhibitors rendered neuroblastoma cells sensitive to chemotherapy both *in vitro* and *in vivo* (57, 58). However, treating breast cancer cells with epidermal growth factor up-regulated TG2 expression and protected the cancer cells from doxorubicin-induced apoptosis (59).

Similar to drug resistance, the development of a metastatic phenotype in cancer cells is associated with the increased expression of TG2. In an attempt to identify metastasis-associated proteins by proteomic analysis, Jiang *et al.* (60) identified TG2 as one of 11 proteins that were selectively amplified in metastatic human lung carcinoma. Similarly, comprehensive analysis of more than 30,000 genes by three different techniques revealed that TG2 is one of the most differentially expressed genes in pancreatic tumors (61). The increased expression of TG2 has also been linked to the enhanced adhesion of ovarian cancer cells to fibronectin and to the directional migration of those cells (49). Accordingly, ovarian cancer cells with knockdown TG2 showed diminished capacity to disseminate to the peritoneal surface and mesentery in an intraperitoneal ovarian xenograft mouse model.

Hwang *et al.* (51) showed that the overexpression of TG2 in tumor samples from ovarian cancer patients was associated with high tumor stage. Using univariate analysis, they found that TG2 overexpression was associated with significantly worse patient survival (median survival of 2.29 years for patients with high TG2 expression *versus* median survival not yet reached for patients with low TG2 expression). In multivariate analysis including age, stage, tumor grade, level of cytoreduction and TG2 expression, only tumor stage ( $p<0.002$ ), level of cytoreduction



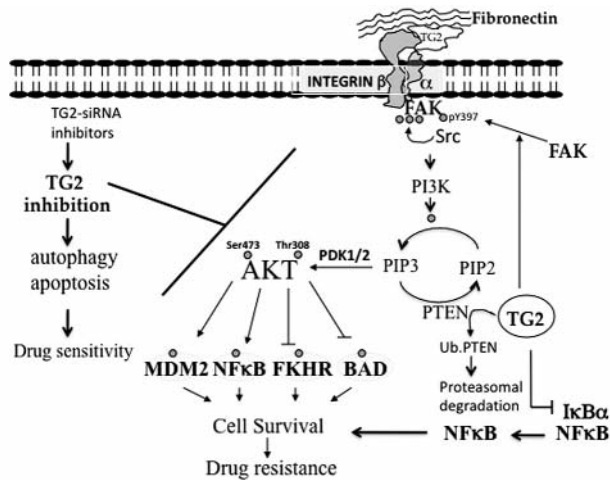


Figure 2. TG2 expression promotes drug resistance and invasion of cancer cells. The association of TG2 with integrins (e.g., integrin  $\beta 1$ ,  $\beta 3$ ,  $\beta 4$  and  $\beta 5$ ) can increase their avidity for extracellular matrix proteins such as fibronectin and result in the activation of downstream cell survival through FAK-PI3K/Akt signaling pathways. TG2 can also affect FAK phosphorylation directly by associating with FAK or indirectly by promoting PTEN degradation via the ubiquitin-proteasomal pathway. Either collectively or individually, activation of these signaling pathways in the PI3K/Akt axis leads to the phosphorylation of various downstream substrates, the activation of MDM2 and NF- $\kappa$ B and the inhibition of FKHR and BAD proteins, resulting in increased cell survival and chemoresistance. On the other hand, by inducing cross-linking of the inhibitor of kappa B alpha (I $\kappa$ B $\alpha$ ), TG2 can induce dissociation of the NF- $\kappa$ B/I $\kappa$ B $\alpha$  complex, resulting in constitutive activation of NF- $\kappa$ B and the induction of downstream target genes involved in promoting cell growth, survival and invasion.

( $p < 0.001$ ) and TG2 overexpression ( $p < 0.04$ ) were independent predictors of poor survival (51). Similarly, comparison of TG2 expression in paired samples of primary tumors and lymph node metastases from breast cancer patients revealed significantly higher TG2 expression in the metastases ( $p < 0.001$ ) (47). Notably, the high expression of TG2 in pancreatic tumor samples was significantly associated with nodal metastasis (32 out of 48 node-positive tumor samples were TG2 positive *versus* 10 out of 27 node-negative tumor samples;  $p = 0.017$ ), lymphovascular invasion (33 of 51 lymphovascular invasive tumors were TG2 positive *versus* 9 of 24 noninvasive tumors;  $p = 0.045$ ) and clinical stage (30 of 45 stage IIb pancreatic ductal adenocarcinomas were TG2 positive *versus* 10 of 26 stage IIa tumors;  $p = 0.027$ ). These data clearly suggest that TG2 expression in cancer cells promotes chemoresistance and metastasis.

**TG2-regulated cell signaling pathways.** As previously said, TG2-mediated post-translational modification of pRb is an early step in apoptosis (17). However, nuclear TG2 can interact with pRb and protect HEK293 cells from apoptotic

insults (62). For example, pRb<sup>-/-</sup> fibroblasts required the presence of both pRb and TG2 to protect cells from apoptosis induced by retinoic acid (RA) (63); the authors suggested that TG2's antiapoptotic activity results from its interaction with pRb independent of its transamination function. In a related study, Tucholski and Johnson (64) demonstrated that intracellular localization of TG2 and its level of activity differentially regulates cell death: cytosolic TG2 elicits proapoptotic behavior, nuclear TG2 elicits antiapoptotic behavior and membrane-bound TG2 exhibits neither pro- nor antiapoptotic behavior (64).

**TG2 and caspase 3.** Under certain circumstances TG2 can inhibit caspase 3 activity and attenuate apoptosis. Thus, TG2-catalyzed cross-linking reactions inactivate caspase 3 and inhibit apoptosis in Bax-deficient cells. Disruption of TG2 activity by either the TG2-inhibitor monodansylcadaverine or short-hairpin RNA inhibits its ability to catalyze protein cross-linking reactions and restores caspase 3 activation in response to thapsigargin treatment (65).

**TG2 and focal adhesion kinase.** Focal adhesion kinase (FAK) is a 125-kDa non-receptor protein tyrosine kinase that plays an important role in cell survival, migration and invasion (66). FAK is activated by integrin clustering and transmits adhesion-dependent and growth factor-dependent signals to cells to promote growth, survival and invasive functions (67, 68). Several reports have suggested that a link exists between FAK and cancer (69-72). For example, Weiner *et al.* (71) found increased levels of FAK in 17 of 20 invasive tumors and in 15 of 15 metastatic tumors of different origins, but no FAK in six normal tissue samples. Similarly, Owens *et al.* (72) found increased levels of the FAK protein in 100% of colon and 88% of breast tumor samples, with FAK expression often being associated with advanced disease.

FAK activity can be regulated by tyrosine phosphorylation, serine/threonine phosphorylation or protein-protein interaction (70) (Figure 2). Indeed, the overexpression of TG2 in cancer cells is associated both with the constitutive activation of FAK and its downstream PI3K/Akt pathway (48). It is believed that the interaction of TG2 with FAK induces conformational changes and autophosphorylation of the FAK protein at tyrosine residue 397 (pY397). Once autophosphorylated, FAK can recruit Src kinase. With Src kinase, FAK can phosphorylate other tyrosine sites (Y407, Y576, Y577, Y861 and Y925) and activate several downstream signaling pathways (e.g., RAS/ERK, PI3K/Akt and Crk/Dock180/Rac) (69).

Furthermore, it was recently shown that TG2 can inversely regulate the expression of PTEN. PTEN is known for its ability to inhibit tumor cell growth, invasion, migration and focal adhesion (73), primarily by means of dephosphorylating and

thus deactivating signaling proteins such as Akt and FAK. Genetic deletions and mutations of PTEN are frequently observed in various human cancers. In addition to genetic changes, some post-translational modifications can also affect the stability and degradation of PTEN and have a profound effect on tumorigenesis (74). Indeed, a recent report demonstrated that the oncogenic protein NEDD4-1 acts like E3 ubiquitin ligase for PTEN and promotes its degradation *via* a ubiquitin-proteasomal pathway (75). Similarly, phosphorylation of PTEN by casein kinase 2 is important for its stability (76). Verma *et al.* (74) found that TG2 expression affected the stability of PTEN by preventing its phosphorylation and promoting its degradation *via* the ubiquitin/proteasome pathway. Thus, TG2 contributes to the activation of FAK and Akt in two complementary ways: by direct phosphorylation of FAK (48) and by inhibiting the phosphatase activity of PTEN (74).

**TG2 and NF- $\kappa$ B activation.** The transcription factor NF- $\kappa$ B plays an important role in regulating genes that are involved in controlling cell growth, apoptosis and metastatic functions (77). Constitutive activation of NF- $\kappa$ B has been observed in various cancers, and its role in drug resistance and metastasis has been envisaged (77, 78). In this context, it is interesting to note that TG2 expression has been shown to result in NF- $\kappa$ B activation. Thus, various cancer cell types with elevated TG2 expression also show increased activity of NF- $\kappa$ B (79). TG2-mediated activation of NF- $\kappa$ B was significantly inhibited by TG2 enzyme inhibitors but not by the dominant-negative inhibitor of kappa B alpha (I $\kappa$ B $\alpha$ ). Importantly, the knockdown of TG2 expression by siRNA resulted in strong inhibition of NF- $\kappa$ B activation, whereas ectopic expression of TG2 caused strong activation of NF- $\kappa$ B (79). The cross-linking activity of TG2 was essential for NF- $\kappa$ B activation. Indeed, I $\kappa$ B $\alpha$  has been shown to be a good substrate for TG2-catalyzed cross-linking reactions. Thus, *in vitro* incubation of the I $\kappa$ B $\alpha$  protein with purified TG2 and Ca<sup>2+</sup> could effectively catalyze the cross-linking of I $\kappa$ B $\alpha$  into high molecular weight polymers (80). The polymeric form of I $\kappa$ B $\alpha$  has a much lower affinity for binding with the p50:p50 complex, which suggests that TG2-mediated post-translational modification of I $\kappa$ B $\alpha$  may hamper its ability to associate with p50:p50 complex, resulting in constitutively active NF- $\kappa$ B. In a recent report, Kim *et al.* (53) observed that inhibition of TG2 activity attenuated NF- $\kappa$ B activation and reversed the sensitivity of drug-resistant breast cancer cells to doxorubicin. Cao *et al.* (54) reached a similar conclusion and suggested that TG2 prevents apoptosis induced by cisplatin by activating the NF- $\kappa$ B survival pathway in ovarian carcinoma cells.

TG2 can also associate directly with p50:p50 complex in the cytoplasm and with p50 in the nucleus. The significance of this association is not clear. It is tempting to speculate that an association between TG2 and the p50:p50 complex may

mitigate the binding of I $\kappa$ B $\alpha$  to the NF- $\kappa$ B complex in the cytoplasm, resulting in the constitutive activation of NF- $\kappa$ B. TG2 can also serve as a kinase and its serine-threonine kinase activity can phosphorylate histones and p53 (81). Because p53 undergoes phosphorylation by various kinases at S536, it is likely that p53 serves as a substrate for TG2 kinase activity.

**TG2 and integrin-mediated signaling.** Depending on the cell type, 10-30% of  $\beta$ 1-integrins on the cell surface can exist in complex with TG2 (13, 15). Recently, two other members of the  $\beta$ -integrin family ( $\beta$ 4- and  $\beta$ 5-) were identified that could form a similar complexes with TG2 in cancer cell membranes (12, 52). Integrins are a family of cell-surface proteins that serve as receptors for ECM proteins (*e.g.*, fibronectin, vitronectin, laminin and collagen) and influence several aspects of cancer cell behavior, including motility, invasion, growth and survival, in response to the integrins binding to ECM ligands. In normal cells, the interaction of integrin with ECM ligands is a highly regulated phenomenon and ensures the cells' controlled growth, survival and migration. Alternatively, certain proteins can associate with integrins to enhance the integrins' affinity for ECM ligands. The first such interaction was identified between integrin  $\beta$ 3 and the integrin-associated protein, which is also known as CD47 (82). Signaling transmitted by integrins to inside cells can be modulated by such protein-protein interactions. In view of these observations, the ability of TG2 to form complexes with  $\beta$  integrins may have important implications for the deregulated proliferation, survival and migratory functions of cancer cells (Figure 2). Indeed, TG2 is known to promote the interaction between cell surface integrins and fibronectin to support cell adhesion, motility, invasion and survival functions (83). In addition, syndecan-4 can also interact with TG2-bound integrins, raising the possibility that TG2 contributes to cell adhesion. Thus, fibroblasts not expressing syndecan-4 showed lower levels of FAK<sub>pY397</sub> during the initial phase of cell adhesion to fibronectin than wild-type cells. A similar defect was observed in early FAK<sub>(pY397)</sub> activation in TG2-null mouse embryonic fibroblasts when compared with the fibroblasts expressing wild-type TG2; the early FAK activation was accompanied by hyperactivation of RhoA and decreased cell migration (84).

**TG2 protection from cell death.** Autophagy, or type II programmed cell death, is a highly regulated form of cell death that plays a crucial role in various physiological processes such as development, homeostasis, elimination of unwanted cells and carcinogenesis. The observation that cancer cells tend to undergo less autophagy than normal cells implies that defective autophagy can confer resistance to stresses such as hypoxia, acidity and poisoning by chemotherapeutic agents and promote tumor cell survival

and tumorigenesis. However, the mechanisms underlying the inhibition or attenuation of autophagy in cancer cells remain elusive. In a recent study, it was demonstrated that protein kinase C-delta (PKC $\delta$ ) constitutively protects cells from autophagy by up-regulating TG2 expression (85). The down-regulation of TG2 by siRNA or by PKC $\delta$  inhibition resulted in significant autophagic cell death of pancreatic cancer cells (85). Transfection of MDA-Panc28 cells with TG2 siRNA (but not control siRNA) resulted in a massive accumulation of autophagic vacuoles, as revealed by phase-contrast microscopy. Acridine orange staining showed that the vacuoles were highly acidic and were accompanied by an accumulation of microtubule-associated light chain proteins, the hallmarks of cells undergoing autophagy. Sustained inhibition of TG2 for >5 days resulted in severe cell damage and death. Transmission electron microscopy images further confirmed that the inhibition of TG2 was associated with the formation of autophagosomes containing disintegrating cellular organelles and that autophagic vesicles had merged with lysosomes and mitochondria (85). Similar effects of TG2 down-regulation have been reported in breast cancer cells (52) and malignant melanoma cells (50). Thus, the down-regulation of TG2 by siRNA rendered these cancer cells highly susceptible to apoptosis. These results support the 'oncogenic addiction' hypothesis in the sense that once TG2 is up-regulated, cancer cells become dependent on TG2-regulated signaling pathways for their survival. Based on these data, it is reasonable to believe that elevated TG2 expression in cancer cells can serve as a promising therapeutic target for reversing drug resistance.

### TG2 as a Therapeutic Target

From the preceding discussion, it is apparent that inhibiting TG2 represents a realistic approach to reversing chemotherapy resistance in cancer cells. As proof of this concept, the therapeutic potential of elevated TG2 expression to treat orthotopically growing ovarian and pancreatic tumors was determined (51, 55). Using small ( $\leq 100$  nm), neutrally charged liposomes (dioleoyl-phosphatidylcholine [DOPC]) as the delivery system, it was found that TG2 siRNA could effectively down-regulate TG2 expression in orthotopically grown tumors. TG2 siRNA-DOPC alone or in combination with docetaxel demonstrated marked antitumor activity against chemotherapy-sensitive (HeyA8) and -resistant (HeyA8-MDR) ovarian tumors in mice (51). To determine the potential mechanisms underlying the antitumor effect of TG2 siRNA-DOPC, its effect was then examined on several biological endpoints, including cell proliferation (by analysis of Ki-67), angiogenesis (by analysis of CD31) and apoptosis (using the TUNEL- terminal deoxynucleotidyl transferase dUTP nick end labeling - assay). A more than 90% reduction in Ki-67 expression was evident in tumors

obtained from mice treated with TG2 siRNA-DOPC alone or in combination with docetaxel. Tumors obtained from mice treated with docetaxel alone or with control siRNA-DOPC consistently showed high levels of Ki-67 staining (51). The blood vessel density was also evaluated in tumors obtained from mice treated with control siRNA-DOPC, docetaxel alone, TG2 siRNA-DOPC alone or TG2 siRNA-DOPC plus docetaxel. The results revealed a significant decrease in the mean blood vessel density in tumors recovered from mice that received TG2 siRNA-DOPC or docetaxel alone. A densitometric analysis showed a marked difference in the mean microvascular density in mice that received TG2 siRNA-DOPC plus docetaxel. Finally, apoptosis was evaluated in orthotopic tumors using TUNEL staining. Minimal cell apoptosis was apparent in ovarian tumors from mice treated with empty liposomes, control siRNA-DOPC or control siRNA-DOPC with docetaxel (51). However, tumors from mice treated with TG2 siRNA-DOPC alone or TG2 siRNA-DOPC plus docetaxel showed a marked increase in apoptosis. Interestingly, the increase in apoptosis in tumors from mice treated with TG2 siRNA-DOPC plus docetaxel was greater than that in the tumors from mice treated with TG2 siRNA-DOPC alone (51).

Similarly, treating mice with TG2 siRNA-DOPC caused a significant reduction in the growth of orthotopic pancreatic tumors (55). Treatment with TG2 siRNA rendered the tumors sensitive to gemcitabine. Gemcitabine is currently used as standard chemotherapy for patients with pancreatic cancer, but it has minimal impact on prognosis and survival (median survival with treatment remains at 4-6 months). Importantly, the number of metastatic foci recovered from mice treated with TG2 siRNA-DOPC alone or in combination with gemcitabine was more dramatically reduced (>80%) than that for mice treated with control siRNA-DOPC or gemcitabine alone (55). Given the clinical relationship between TG2 expression and the poor prognosis of patients with cancer, particularly those with chemotherapy-resistant tumors, these findings support the possibility that targeted TG2 silencing combined with chemotherapy is a promising therapeutic option.

In this regard, the use of siRNA specifically to silence TG2 expression holds great promise for the development of therapeutic agents directed against drug-resistant and metastatic cancers. Already, the use of siRNA to silence genes has become a powerful tool for determining protein function and gene discovery. Moreover, siRNA-based therapeutic agents have the potential to inhibit molecular targets that are not treatable with conventional small molecules, antibodies or other biological agents. A major challenge in using siRNA-based drugs in humans will be the effective delivery to the target tissue because naked siRNA is unstable in plasma. In contrast, chemically modified siRNA is thought to be relatively stable when administered

systemically in mice, but this siRNA must be administered in large doses (30-50 mg/kg) because its stability actually reduces drug bioavailability. Such large doses of siRNA may not be practical for therapeutic applications in humans because of costs and safety issues.

An alternative delivery system may be to encapsulate siRNA into lipid-based nanoparticles. The encapsulation of siRNA into such nanoparticles ( $\leq 100$  nm in diameter) has enabled an effective dosing in mice and monkeys at a low dosage (0.2-2 mg/kg) (86, 87). As further proof, it was shown that DOPC nanoliposomes successfully delivered TG2 siRNA to orthotopically grown pancreatic (55) and ovarian (51) tumors in mice, accomplishing the goal of effectively silencing the target gene and inhibiting tumor growth and metastasis. In addition, two recent reports further documented the effectiveness of DOPC nanoliposome in delivering siRNA and silencing the oncoprotein EphA2 (88) and FAK (89) in tumors growing in mice. Similarly, DOPC nanoliposomes were highly effective in delivering siRNA and silencing neuropilin protein and thus inhibiting metastasis of colorectal cancer in mice (90). The latter researchers also demonstrated that this delivery system was equally effective in silencing the expression of interleukin-8 in orthotopically growing ovarian tumors (91). From all these studies, it is apparent that the effectiveness of DOPC liposomes for delivering TG2 siRNA can be rapidly translated into the clinical setting for the treatment of chemoresistant cancers.

## Conclusion

TG2, which binds GTP and catalyzes  $\text{Ca}^{2+}$ -dependent cross-linking of proteins (transamination activity), has been implicated both in the promotion of cell death and in the protection of cells from apoptotic insults. The switching between the two opposite functions is dictated, in part, by the nature of the cell type, localization of TG2 within the cell (cytosolic *versus* nuclear *versus* membrane) and intra- and extracellular environments. In general, TG2's switching from the antiapoptotic to the proapoptotic function mainly depends on the levels of intracellular  $\text{Ca}^{2+}$  and the ability of TG2 to bind to GTP (Figure 1). TG2's selective overexpression in chemoresistant and metastatic cancer cells and its ability to promote cell survival and invasion functions (Figure 2) makes it a promising therapeutic target. Because of its dual role as a proapoptotic and an antiapoptotic protein, either activation of TG2 to its protein cross-linking configuration or its inhibition and down-regulation can be used as the therapeutic approach. For example, use of small molecules that can bind and activate TG2 or that can elevate intracellular  $\text{Ca}^{2+}$  and thus activate TG2 can be effective in driving tumor cells to apoptosis. Alternatively, the inhibition of TG2 either by inhibiting the

transcription factor that drives its expression or by using siRNA can deprive tumor cells of critical survival pathways and result in cell death. To accomplish this, investigators should expend the effort to find the small molecules that can either enhance or inhibit TG2 functions.

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