Transglutaminase Is Required for Epidermal Squamous Cell Carcinoma Stem Cell Survival

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Abstract

Cancer stem cells are thought to be responsible for rapid tumor growth, metastasis, and enhanced tumor survival following drug treatment. For this reason, there is a major emphasis on identifying proteins that can be targeted to kill cancer stem cells or control their growth, and transglutaminase type II (TGM2/TG2) is such a target in epidermal squamous cell carcinoma. TG2 was originally described as a transamidase in the extracellular matrix that cross-links proteins by catalyzing e-(γ-glutamyl)lysine bonds. However, subsequent studies have shown that TG2 is a GTP-binding protein that plays an important role in cell signaling and survival. In the present study, TG2 is shown as a target for anticancer stem cell therapy in human squamous cell carcinoma. TG2 was determined to be highly elevated in epidermal cancer stem cells (ECS cells), and TG2 knockdown or suppression of TG2 function with inhibitors reduced ECS cell survival, sphere formation, Matrigel invasion, and migration. The reduction in survival is associated with activation of apoptosis. Mechanistic studies, using TG2 mutants, revealed that the GTP-binding activity is required for maintenance of ECS cell growth and survival, and that the action of TG2 in ECS cells is not mediated by NF-kB signaling.

Implications: This study suggests that TG2 has an important role in maintaining cancer stem cell survival, invasive, and metastatic behavior and is an important therapeutic target to reduce survival of cancer stem cells in epidermal squamous cell carcinoma. Mol Cancer Res; 13(7); 1083–94. ©2015 AACR.

Introduction

Epidermal squamous cell carcinoma (SCC) is a common form of skin cancer that develops in response to UV light exposure (1). SCC can often be treated by surgical excision, but the recurrence rate ranges to 30% (1). The worldwide incidence of SCC is increasing because of population aging and because of increased exposure to UV light (2).

Increasing evidence suggests the existence of cancer stem cells that have a role in tumor formation and facilitate cancer recurrence and metastasis in epithelial-derived cancers (3–8). We recently characterized epidermal cancer stem cells (ECS cells) and showed that ECS cells express stem cell markers characteristic of normal epidermal stem cells and embryonic stem cells (9). These ECS cells are able to generate tumors in immune-compromised mice following subcutaneous injection of as few as 100 cells (9). ECS cells express pluripotent markers, which are also expressed in esophageal and head/neck cancer stem cells (6, 10, 11).

As part of a search for stem cell survival proteins, we identified transglutaminase type 2 (TG2) as highly elevated in ECS cells as compared with non-stem cancer cells. TG2 is a multifunctional protein, with both enzymatic and scaffold functions, that is involved in inflammation, tissue repair, and cancer (12, 13). TG2 catalyzes a number of reactions, including calcium-dependent protein crosslinking (TGase activity), GTP-binding activity, protein disulfide isomerase activity, serine/threonine kinase activity (14), and also serves as a scaffold protein (15). Among these activities, the best characterized and most important are the transamidase (TGase) and the GTP-binding activities.

TG2 is expressed in the basal epidermal layers where we propose, it has a survival role. We now show that TG2 is markedly elevated in epidermal SCC and is selectively and highly enriched in ECS cells, suggesting it may have a role in ECS cell survival. Indeed, knockdown and inhibitor studies show that TG2 is required for ECS cell survival, sphere formation, migration, and invasion. Moreover, inhibition of TG2 activates ECS cell apoptosis. Studies with TG2 mutants indicate that GTP-binding/G-protein–related activity is required for ECS cell survival, but that TG2 transamidase activity is not. Thus, our results suggest that agents that modulate TG2 signaling may be useful cancer prevention and treatment agents.

Materials and Methods

Antibodies and reagents

Dulbecco’s modified Eagle’s medium (11960-077), sodium pyruvate (11360-070), i-GLutamine (25030-164), and 0.25% trypsin-EDTA (25200-056) were purchased from Gibco. Heat-inactivated FCS (F4135), anti–β3–actin (A5441), A23187 ionophore (C7522), and trypan blue (T8154) were purchased from Sigma. Cell lysis buffer (9803) was purchased from Cell Signaling
Technology. Anti-TG2 (MAB3839) was purchased from EMD Millipore. Antibody for Sox2 (ab15830-100) was purchased from Abcam. Antibodies for caspase-3 (9665), Nanog (4839), and NF-kB-p65 siRNA (6261) were purchased from Cell Signaling Technologies. Anti-Oct4 (611203) was purchased from BD Transduction Laboratories. Peroxidase-conjugated anti-mouse IgG (NA934V) was purchased from Amersham Biosciences. Proteins were detected by immuno blot (17, 18).

Plasmids

Plasmids encoding wild-type TG2 and TG2(C277S) cloned in EC1214 vector were provided by Dr. Kapil Mehta. Plasmids encoding TG2(R850A), TG2(Y326F), and TG2(W241A), cloned in pcDNA3.1, were provided by Dr. Gail Johnson (14, 19, 20).

Lentivirus production

Lentiviruses were generated using lentivirus production in pcDNA3.1, were provided by Dr. Gail Johnson (14, 19, 20).}

Electroporation of nucleic acids

Cells were electroporated exactly as outlined (21). In applications using siRNA, the cells were harvested 72 hours after electroporation and electroporated a second time following the same protocol (double electroporation). This resulted in sustained knockdown of the target transcript.

In situ TG2 activity assay

Cells (40,000) were plated in 24-well attachment dishes in spheroid media and grown until 50% confluent. FC was added in 2 mL of serum-free medium at a final concentration of 20 μg/mL and incubated for 4 hours. The wells were washed twice with serum-free spheroid medium, and then 2 mL of fresh serum-free medium was added containing 0 to 20 μg/mL NC9. After 30 minutes, the wells were supplemented with 10 μg/mL A23187. Cells were incubated for an additional 90 minutes and then washed three times with Ca2+/Mg2+ free HBSS, fixed with formalin, washed with PBS, and imaged to detect fluorescein.

Trypan Blue viability assay

SCC-13 cells were grown as spheroids in 6-well cluster dishes in spheroid medium for 8 days and then treated with 0 to 20 μg/mL NC9. At 0, 24, 48, and 72 hours after NC9 treatment, spheroids were counted, and all cells in the well were collected to prepare a single-cell suspension in Hank's Balanced Salt Solution. Trypan Blue solution (0.5 mL, 0.4%) was added to a 15-mL conical tube with 0.3 mL of Hank's Balanced Salt Solution and 0.2 mL of cell suspension. After 10 minutes, 8 μL of the mixture was transferred to a hemocytometer to count viable and total cell number.

Invasion assay

Matrigel (BD Biologics) was diluted into 2 mL of 0.01 mol/L Tris-HCl/0.7% NaCl to a final concentration of 300 μg/mL, filter sterilized, and 0.1 mL was added per BD BioCoat cell insert. After 2 hours, near-confluent SCC13-TG2-shRNA2 and SCC13-Control-shRNA cells were harvested and 25,000 cells were plated in 100 μL of growth media containing 1% FCS atop the Matrigel layer. Growth medium containing 10% FCS was added to the bottom chamber followed by an overnight incubation at 37°C. The following day a cotton swab was used to remove cells from the upper side of the membrane, the membrane was rinsed with PBS, fixed with 4% paraformaldehyde for 10 minutes, washed again, and stained with 1 μg/mL DAPI for 10 minutes. The underside of the membrane was viewed with an inverted fluorescent microscope, and nuclei were counted.

Results

TG2 is required for ECS cell survival and spheroid formation

Our previous studies show that ECS cells, which comprise less than 0.2% of the total cancer cell population, can be isolated from bulk SCC-13 cancer cells by growth as spheroids in nonattached conditions (9). An important finding is that ECS cells (spheroids) are highly enriched for expression of TG2 as compared with non-stem cell (monolayer) cultures (Fig. 1A) and that this expression is associated with expression of stem cell markers, including Oct4, Nanog, and Sox2 (Fig. 1A). To determine whether TG2 has a role in ECS cell maintenance and spheroid formation, we treated SCC-13 cells with control- or TG2-siRNA to knockdown TG2 (Fig. 1B) and monitored ability to form spheroids. Markedly fewer spheroids are formed by TG2 knockdown cells (Fig. 1C).
Moreover, loss of spheroid formation, following TG2 knockdown, is associated with accumulation of single cells (Fig. 1D). To confirm this finding, we prepared TG2-negative cell lines by infection of SCC-13 cells with TG2-shRNA encoding lentivirus. Figure 1E confirms the SCC-13-TG2-shRNA2 cells, which have reduced TG2 expression (Fig. 1F), form spheroids less efficiently than SCC-13-control-shRNA cells. Most SCC-13-TG2-shRNA2 cells remain as single cells (Fig. 1G). However, some spheroid formation is observed in SCC-13-TG2-shRNA2 cultures, and we wondered whether this is due to TG2 re-expression. To test this, we grew SCC-13-TG2-shRNA2 cells as nonattached spheroids for 10 days and then isolated spheroids and single cells for preparation of extracts. Figure 1H reveals that the few spheroids that do form express TG2, further suggesting that TG2 is required for spheroid formation.

We next studied the impact of TG2 inhibitor on spheroid formation. Spheroids were grown for 8 days and then treated with NC9, an irreversible inhibitor of TG2 transamidase (TGase, crosslinking) activity (16). NC9 treatment reduced spheroid number (Fig. 2A), which is associated with accumulation of spheroid fragments and single cells (Fig. 2B). A key issue is whether the NC9 treatment reduces cell viability. As shown in Fig. 2C, as assessed by ability to exclude trypan blue, NC9 causes a concentration-dependent reduction in viable cell number. Figure 2D shows that the reduction in TG2 activity is not associated with reduced TG2 level. This indicates that TG2 knockdown (Fig. 1) or inhibition of activity (Fig. 2) reduces ESC cell survival. To confirm that NC9 inhibits TG2 transamidase (TGase) activity, we loaded cells with FC, a known transglutaminase substrate, added 0 or 20 μmol/L NC9, activated TG2 TGase activity by treatment with calcium ionophore, and monitored for intracellular incorporation of the fluorescent label. This experiment shows that TG2 activity is reduced by NC9 treatment (Fig. 2E). To gain some insight regarding the mechanism, we monitored for cleavage of procaspase 3. Figure 2F shows that the response to NC9 includes cleavage of procaspase 3 and that this cleavage is initiated within 1 hour after NC9 addition.
We next assessed whether NC9 can prevent spheroid formation initiated from single cells. We seeded SCC-13 single cells in spheroid growth conditions and after 12 hours added NC9, and monitored spheroid number over 14 days. We did not replenish the medium or add fresh NC9 during this time course. Spheroid number increases in all groups until day 8, but then selectively declines in the NC9-treated groups (Fig. 3A). This is associated with morphological survival of the spheroids until 8 days and progressive destruction from 8 to 14 days (Fig. 3B), indicating that spheroids can form in the presence of NC9, but that fully formed spheroids undergo NC9-associated destruction. This is consistent with NC9-related destruction of mature spheroids shown in Fig. 2.

In addition, the findings shown in Fig. 3 suggest that a single treatment with NC9 produces long-lasting effects on ECS cell survival and spheroid formation. We next monitored the impact of another transglutaminase inhibitor, FC, on spheroid formation. FC acts as a competitive substrate inhibitor of transglutaminase that prevents TG2 interaction with intracellular targets (22, 23). Spheroids were grown for 8 days and then treated with FC. FC treatment reduces spheroid number and promotes accumulation of spheroid fragments and dissociated single cells (Fig. 3C and D).

**TG2 is required for ECS cell migration**

We recently demonstrated that epidermal cancer–derived ECS cells migrate more efficiently than non-stem cancer cells (9). To determine whether this requires TG2, we monitored the ability of TG2 knockdown cells to invade Matrigel and close a scratch wound. Figure 3E and F shows that TG2 loss reduces cell migration through Matrigel. Figure 3G shows that the ability of cells to close a scratch wound is also reduced in the absence of TG2 (Fig. 3G). Moreover, Fig. 3H shows that pretreating the cells with NC9 reduces Matrigel invasion.

**TG2 GTP-binding function is required for activity**

The two most important enzymatic functions of TG2 are the TGase and GTP-binding activities (14). We therefore examined which activity is required for ECS cell spheroid formation. To accomplish this, we measured the ability of wild-type and mutant TG2 to restore SCC13-TG2-shRNA2 cell spheroid formation. Plasmids encoding wild-type and mutant TG2 forms were delivered by electroporation. The mutants include C277S (no TGase activity, partial GTP-binding activity), R580A (wild-type TGase activity, no GTP-binding activity), W241A (no TGase activity, wild-type GTP-binding activity), and Y516F (partial TGase activity, partial GTP-binding activity; Fig. 4A; ref. 19). Figure 4B shows that each electroporated mutant is expressed at a comparable level. We also show (Fig. 4C) that each mutant localizes in a pattern that resembles that of wild-type endogenous TG2 (SCC13-Control-shRNA, EV) and that SCC13-TG2-shRNA2 cells express reduced levels of TG2.
We next assessed the ability of wild-type and mutant TG2 to drive spheroid formation. The time course in Fig. 4D shows that SCC13-TG2-shRNA2 cells (TG2 knockdown) form 80% fewer spheroids than empty vector (EV)–electroporated SCC13-Control-shRNA cells when measured at 5 day. We next examined the ability of wild-type TG2 and TG2 mutants to restore SCC13-TG2-shRNA2 cell spheroid formation (Fig. 4D). The general trend is the same at all times. Considering the 5-day time point, expression of wild-type TG2 restores spheroid number to approximately 80% of control. Mutants C277S and W241A, which retain partial and full GTP-binding function, respectively, also largely restore spheroid formation. In contrast, Y516F, which retains partial GTP-binding activity, slightly restores spheroid formation, and R580A, which lacks GTP-binding function, does not restore spheroid formation.
The fact that C277S and W241A, which lack TGase activity, restore spheroid formation suggests that TGase activity is not required. We also examined the impact of wild-type TG2 and mutants on cell migration and wound closure. SCC13-TG2-shRNA2 cells (TG2 knockdown) have reduced efficiency of wound closure compared with SCC13-Control-shRNA cells that express normal endogenous levels of TG2 (Fig. 5A). Mutants C277S and W241A, which retain partial and full GTP-binding function, respectively, largely restore wound closure. Y516F, which retains partial GTP-binding activity, is also effective, but R580A, which lacks GTP-binding function, is largely ineffective (Fig. 5A). The fact that C277S and W241A, which lack TGase activity, enhance closure suggests that TGase activity is not required. Figure 5B shows the wound images at the 20-hour time point for a representative experiment. Figure 5C shows that these cell lines show no difference in cell number over 3 days of growth, suggesting that differences in cell proliferation rate cannot explain the marked difference in wound closure and invasions rates.

TG2 is also required for survival and migration of A431 ECS cells

We wanted to compare these findings in another SCC cell type. We chose A431 cells, as we have previously shown that spheroid-forming ECS cells comprise 0.03% of this cell population (9). These cells are derived from human vulvar skin. Figure 6A reveals that TG2 level is substantially elevated in A431-derived ECS cells. We next examined the impact of TG2 knockdown or treatment with NC9 on spheroid formation. Electroporation of A431 ECS cells with TG2-siRNA reduced TG2 level (Fig. 6B). As shown in Fig. 6C, TG2 loss reduces A431 cell spheroid formation by 70%. Moreover, the ability of A431 ECS cells to invade Matrigel is reduced by 50% in the presence of NC9, as is ability to close a scratch wound (Fig. 6E and F). Figure 6G will be explained in the Discussion section.

Role of NF-κB

Experiments in Fig. 3E and F show that TG2 is required for ECS cell invasion through Matrigel. Previous studies have implicated...
NF-κB as a mediator of TG2-dependent epithelial–mesenchymal transition (EMT)–related processes in several cancer cell types (24–29). We therefore examined whether NF-κB plays a role in mediating these processes in SCC-13 cells. ECS cells were electroporated with control- or NF-κB-siRNA before plating on Matrigel. Figure 7A and B shows that NF-κB knockdown does not reduce ECS cell invasion, and Fig. 7C confirms the knockdown. We next determined whether NF-κB knockdown attenuates TG2 stimulation of invasion. SCC13-TG2-shRNA2 cells were electroporated with plasmid encoding wild-type TG2 in the presence or absence of NF-κB-siRNA. Figure 7D and E shows that expression of TG2 enhances invasion and that TG2-simulated invasion is not reduced by NF-κB knockdown. Figure 7F confirms the elevation of TG2 and the knockdown of NF-κB. We also examined the impact of NF-κB knockdown on ECS cell migration. Figure 7G shows that NF-κB knockdown does not alter the rate of ECS cell wound repair (migration).

Discussion

The cancer stem cell model was predicted over 150 years ago, but only recently have technologies evolved to test this model. This concept predicts that tumors originate from tissue stem cells and that tumors retain a subpopulation of cancer stem cells that undergo aberrant differentiation that generates cellular heterogeneity in the tumor (30). These tumor stem cells display specific properties, including the capacity for self-renewal, ability to differentiate, active telomerase expression, activation of antiapoptotic pathways, increased membrane transporter activity, ability to migrate and metastasize, and expression of stem cell markers (30). This hypothesis has important implications for cancer risk assessment, detection, prevention, and treatment. It is also important to consider that the present cancer therapies appear to kill differentiated cancer cells but spare the cancer stem cell population (31, 32). This suggests that developing cancer treatment strategies that target the cancer stem cell population will be beneficial.
We have characterized putative epidermal cancer stem cells established from epidermal SCC cells. These ECS cells can be selected by growth in nonattached conditions (9). Under these conditions, <0.2% of the cells form spheroids and express a host of epidermal and embryonic stem cell markers (9). When injected into immune compromised mice, these cells form large, rapidly-growing, aggressive, invasive, and highly-vascularized tumors (9). This is in contrast with the small and nonvascularized tumors that form upon injection of cell population comprised largely of non-stem cells. Moreover, injection of as few as 100 ECS cells can drive formation of large vascularized tumors. These cells also display enhanced mobility and invasion of Matrigel (9). The fact that these cells are readily able to form aggressive tumors, as compared with non-stem cells, confirms that they are important targets for cancer prevention and therapy.

An important strategy for understanding ECS cell properties, and designing anti-ECS cell therapy, is identifying survival proteins that are highly enriched in ECS cells as compared with non-stem cells. Based on the demonstrated importance of TG2 in several epithelial cancers (33–37), we examined expression of TG2 in skin cancer. Our studies show that TG2 levels are markedly elevated in ECS cells as compared with bulk cultures of cancer cells.
Moreover, knockdown of TG2 using siRNA or by creating stable TG2 knockdown cell lines results in a marked reduction in ECS cell survival and spheroid formation. Inhibition of TG2 results in destruction of pre-established ECS cell spheroids and suppression of spheroid growth. Moreover, TG2 knockdown cells display a reduced ability to form spheroids.

We also examined the impact of TG2 inhibitors on ECS cell survival and spheroid formation. NC9 is an active site-directed irreversible inhibitor that inactivates TG2 TGase activity and in the process locks TG2 in an extended conformation (38–40). Treating ECS cells with NC9 inhibits spheroid formation and also promotes destruction of pre-existing spheroids. This is associated with inhibition of TGase activity. We also performed structural studies that suggest that NC9 produces an open TG2 structure in which the GTP-binding site is moved to an inactive conformation (not shown). Thus, we propose that NC9 inhibits both GTP-binding and TGase activities. Ultimately, NC9 causes ECS cell apoptosis and reduced viability. This is particularly interesting, as stem cells are known to suppress apoptotic signaling pathways to enhance survival, and it is possible that TG2 has an important role...
strate that GTP binding is required for ECS cell function and cell spheroid formation, invasion, and migration. Studies show that mutants (C277A and W241A), which lack GTP binding, do not restore activity. Conversely, these same (W241A) GTP-binding function, can partially or near-fully spheroid formation, invasion, and migration, in TG2 knock-understand the role of these activities in maintaining ECS cell processes. In particular, TG2 appears to be required for ECS cell spheroid formation, survival, migration, and invasion. Survival, migration, and invasion are properties known to be required for tumor cell extravasation during metastasis, suggesting that TG2 may be required for in vivo metastasis (43–47). Indeed, such a role has been documented in other cancer types (48–50).

Recent studies suggest that in some cancer cell types, TG2 activates NF-kB to promote cancer cell survival (24–29). We therefore tested whether NF-kB mediates TG2 action in ECS cells. It is interesting that knockdown of TG2 does not impair TG2 regulation of invasion or migration (Fig. 7) or spheroid formation or EMT (not shown). NF-kB has been described as having a unique role in epidermal cells where it actually inhibits cell proliferation (51). This difference in properties may explain the lack of a role for NF-kB as a TG2 mediator in ECS cells.

TG2 is a multifunctional enzyme expressed in many tissues (52). In addition to transamidase (TGase) activity, which is activated by calcium (14), TG2 binds and hydrolyzes GTP (53). GTP bound TG2 functions in G-protein signaling (54, 55). TG2 also functions as a protein disulfide isomerase (56, 57), protein kinase (58, 59), protein scaffold (60, 61), and as a DNA hydrolase (62). The TG2 TGase and GTP-binding activities are the best studied and appear to be the most important (14). To understand the role of these activities in maintaining ECS cell function, we studied the ability of TG2 mutants to restore spheroid formation, invasion, and migration, in TG2 knockdown cells. These studies show that wild-type TG2, and mutants (Fig. 4A) that retain partial (C277S, Y526F) or full (W241A) GTP-binding function, can partially or near-fully restore spheroid formation. In contrast, R580A, which lacks GTP binding, does not restore activity. Conversely, these same studies show that mutants (C277A and W241A), which lack TGase activity, are able to form spheroids. This genetic evidence confirms a role for the TG2 GTP-binding activity in driving ECS cell spheroid formation, invasion, and migration.

We propose that the TG2 mutant data unequivocally demonstrate that GTP binding is required for ECS cell function and that the inhibitor data also support this hypothesis (Fig. 6G). NC9 is an irreversible inhibitor that covalently binds to TG2 to inactivate TGase activity (16). However, NC9 also locks TG2 into an extended conformation (38), which is associated with inactivation of GTP binding (63), as TG2 GTP binding requires a closed configuration (63). In silico structural modeling studies indicate that TG2 GTP activity inactivates when bound to NC9 (not shown). Thus, we propose that NC9 treatment inhibits both TG2 TGase and TG2 GTP-binding/G-protein function in ECS cells. Based on these findings, we conclude that TG2 is essential for cancer stem cell survival in epidermal SCC and is likely to contribute to tumor and metastasis formation in SCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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