Molecular Cancer Research

miR-19–Mediated Inhibition of Transglutaminase-2 Leads to Enhanced Invasion and Metastasis in Colorectal Cancer

D. Cellura[1], K. Pickard[1], S. Quaratino[1], H. Parker[2], J.C. Strefford[2], G.J. Thomas[1], R. Mitter[3], A.H. Mirnezami[1,4], and N.J. Peake[1]

Abstract

Transglutaminase-2 (TG2) is a critical cross-linking enzyme in the extracellular matrix (ECM) and tumor microenvironment (TME). Although its expression has been linked to colorectal cancer, its functional role in the processes that drive disease appears to be context dependent. There is now considerable evidence of a role for microRNAs (miRNA) in the development and progression of cancer, including metastasis. A cell model of metastatic colon adenocarcinoma was used to investigate the contribution of miRNAs to the differential expression of TG2, and functional effects on inflammatory and invasive behavior. The impact of TG2 in colorectal cancer was analyzed in human colorectal tumor specimens and by manipulations in SW480 and SW620 cells. Effects on invasive behavior were measured using Transwell invasion assays, and cytokine production was assessed by ELISA. TG2 was identified as a target for miR-19 by *in silico* analysis, which was confirmed experimentally. Functional effects were evaluated by overexpression of pre-miR-19a in SW480 cells. Expression of TG2 correlated inversely with invasive behavior, with knockdown in SW480 cells leading to enhanced invasion, and overexpression in SW620 cells the opposite. TG2 expression was observed in colorectal cancer primary tumors but lost in liver metastases. Finally, miR-19 overexpression and subsequent decreased TG2 expression was linked to chromosome-13 amplification events, leading to altered invasive behavior in colorectal cancer cells.

Implications: Chromosome-13 amplification in advanced colorectal cancer contributes to invasion and metastasis by upregulating miR-19, which targets TG2. *Mol Cancer Res; 13(7); 1095–105. ©2015 AACR.*

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Introduction

Colorectal cancer is the fourth most common malignancy worldwide and the third most common malignant cause of mortality in the western world (1, 2). Although advances in screening and treatment have improved life expectancy in recent decades, prognosis remains significantly poorer in later stages when disease has spread to lymph nodes and distant metastatic sites (3). Understanding and preventing this invasive progression would therefore significantly benefit patient outcome worldwide.

[1]Molecular Mechanisms Research Unit, Cancer Research UK Centre, University of Southampton Cancer Sciences Division, Somers Cancer Research Building, Southampton University Hospital NHS Trust, Southampton, United Kingdom. [2]Cancer Genomics, Cancer Sciences, University of Southampton, Southampton, United Kingdom. [3]Bioinformatics Unit, London Research Institute, Cancer Research UK, Lincoln's Inn Fields, London, United Kingdom. [4]Department of Colorectal Surgery, Southampton University Hospital NHS Trust, Southampton, United Kingdom.

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A.H. Mirnezami and N.J. Peake contributed equally to this article.

Corresponding Authors: A.H. Mirnezami, Molecular Mechanisms Research Unit, Cancer Research UK Centre, Somers Cancer Research Building, University of Southampton Cancer Sciences Division, Southampton University Hospital NHS Trust, Tremona Road, Southampton SO166YD, United Kingdom. Phone: 44-7790518527; Fax: 44-2380795152; E-mail: ahm@soton.ac.uk; and N.J. Peake, njpeake@soton.ac.uk

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Transglutaminase-2 (TG2) activity has been linked to multiple biologic processes associated with tumor development and progression, such as cell adhesion, motility, invasion, apoptosis, chemoresistance, and epithelial–mesenchymal transition (4, 5). The most ubiquitous member of the transglutaminase family of protein cross-linking enzymes, TG2 has been observed in various cancer tissues and cell lines, with activity linked to disease progression and metastasis in tumors with a diverse range of origins (6–10). TG2 has been identified as a potential marker of colorectal cancer progression by immunohistochemical analysis, following previous work demonstrating differential expression of TG2 in colorectal cancer cell lines with different metastatic potential (11–13). However, published studies aiming to identify a definitive role for TG2 in cancer cell biology have demonstrated sometimes contradictory functional roles, such as promoting or inhibiting apoptosis. TG2 therefore appears to act in a context-dependant manner that may relate to cellular location and the availability of its many identified protein substrates (14), or to the balance between different isotypes of the enzyme that have been shown to have opposing consequences on cell behavior (15).

microRNAs (miRNA) are a family of short, noncoding, single-stranded RNAs, which inhibit the function of multiple target genes by binding to their 3′-untranslated region (UTR), leading to direct degradation of target mRNA or inhibiting translation (16). A wide body of work now links miRNA expression to colorectal cancer by altering the expression of oncogenic and tumor-suppressive genes (17, 18). Furthermore, miRNA deregulation is strongly linked to disease progression, with changes in miRNAs linked to metastasis (19, 20). These "metastaMirs" are attractive therapeutic targets for treating metastatic colorectal

cancer, as each miRNA influences the expression of multiple proteins downstream that may contribute to the development of the complex, multifactorial metastatic phenotype (21).

Because TG2 has multiple cell substrates and plays a critical role in cancer cell behavior, its expression is carefully controlled. As well as translational regulation, TG2 abundance is also controlled through the SUMO pathway (22), and enzymatic function is dependent on the presence of calcium and inhibited by GTP (23). To date, few studies have examined miRNA regulation of TG2, despite both TG2 and miRNAs being closely linked to cancer progression. In this study, we investigated the differential expression of TG2 in colon cancer cell lines and tissue sections taken from primary and metastatic tumors, examined how TG2 expression affected invasive characteristics and inflammatory mediators synthesized by these cells, and finally determined how miRNA regulation alters these functional properties.

Materials and Methods

Cell lines and reagents

The primary adenocarcinoma cell line SW480 was obtained from the European Collection of Cell Culture, along with the patient-matched lymph-node metastasis–derived line SW620. Cells were cultured and passaged according to supplied information. siRNA targeted against TG2 was obtained from Invitrogen, and transfected into cells using HiPerFect reagent (Qiagen) according to the manufacturer's recommendations. TG2 expression plasmid (pLPCX-TG2) and the active site mutant (pLPCX-C277S) plasmid were used as previously described (22), along with an empty vector control (pcDNA3.1), and transfected into cells using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. Pre-miR-19a plasmid (Genecoepia) and a corresponding scrambled plasmid control (SCC) were transfected into SW480 cells using Fugene 6 (Roche) according to the manufacturer's recommendations (SW480/miR19A and SW480/SCC). Stable transfection was achieved by selecting resistant clones using puromycin (1 μg/mL), cell sorting by FACS for the IRES-driven GFP reporter, and after expansion used at early passage (<10). Twenty-four hours prior to experiments, cells were also transfected with a miR-19A mimic or corresponding scramble control (Qiagen) using HiPerFect reagent (Qiagen) according to the manufacturer's instructions. After 24-hour incubation, cells were trypsinized and used for experimental testing.

Matrigel invasion assay

Invasion assays were performed using 8 μm Transwell plates (Corning). Matrigel (BD Biosciences) was diluted at 1:3 in serum-free medium and allowed to dry in the upper chamber of the wells. A total of 100,000 cells were then added to the upper chamber in serum-free medium, and complete medium was added to the lower chamber as a chemoattractant. After 24 hours, cells invading the Matrigel were released by trypsinization, and counted using a CASY TTC counter (Roche Innovatis).

Western blot analysis

Western blotting was performed to assess cellular expression of TG2, and actin expression was used to confirm equal protein loading. Cells were lysed in PBS + 1% NP-40, and briefly sonicated before centrifugation to remove insoluble material. Alternatively, in some experiments, protein extracts were prepared in 1% SDS following TRizol treatment according to the manufacturer's protocol (Ambion). Total protein content of these preparations was assessed by BCA assay (Thermo Scientific), equal quantities of protein were loaded onto SDS–PAGE gels for electrophoresis, and transferred to nitrocellulose membrane (Amersham). Membranes were blocked with 5% nonfat milk in TBS+0.5% Tween, then probed with appropriate primary antibodies; TG2 (Abcam; clone CUB7402; 1:2,000), and actin to confirm equal protein loading (Santa Cruz Biotechnology, 1:2,000). Bound proteins were detected using horseradish peroxidase (HRP)–labeled secondary antibodies (Santa Cruz Biotechnology, 1:2,000), and ECL chemiluminescent substrate (Thermo scientific).

TG2 activity assay

The assay for TG2 activity was performed as previously described (24). On the basis of incorporation of the TG2 substrate monodanslycadaverin (bio-MDC; Cambridge Bioscience), cells were incubated for 1 hour with the substrate in the presence of 200 mmol/L $CaCl_2$, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 (Sigma). Biotinylated substrate was revealed using streptavidin–FITC (BD Pharmingen; 1:150), and TG2 protein costained using the antibody clone CUB7402 (Abcam; 1:100) and detected by anti-mouse Alexa Fluor-594 antibody (BD Biosciences; 1:300). Cells were counterstained with the nuclear stain DAPI (1:1,000; Invitrogen) before mounting in Slow-fade medium (Invitrogen) and visualized under a fluorescent microscope. Mean corrected total cell fluorescence was calculated from fluorescence intensities obtained in the ImageJ, using the equation: integrated density − (area of cell × background).

Flow cytometry and immunoassay

The expression of TG2 was assessed by flow cytometry in order to compare surface expression and intracellular expression. Cells were trypsinized from culture dishes, washed three times in PBS, and suspended in flow buffer (PBS + 1% FCS, 0.05% sodium azide). Membrane permeabilization was performed where necessary using Fix-perm reagents (eBiosciences, according to the manufacturer's instructions). For both intracellular and cell-surface staining, the primary TG2 antibody CUB7402 was used (1:100; Abcam), and detection performed using anti-mouse FITC-conjugated secondary antibody (1:300; Sigma). IL8 production was measured using a commercial ELISA assay (R&D Systems).

Immunohistochemical and miRNA quantification from colorectal cancer patients

Immunohistochemical staining was performed on formalin-fixed specimens from patients undergoing resections for colorectal cancer at the University Hospital Southampton (Southampton, United Kingdom) as part of an NIHR portfolio study (UK CRN ID6067). Tumor specimens were snap-frozen in liquid nitrogen within 10 minutes of surgery and stored in a designated UK Human Tissue Act–approved tumor bank. Samples were selected from three clinically distinct groups: (i) colonic tissue from early-stage disease (stage I/II), (ii) colonic tissue from late-stage disease (lymph node involvement, stage III/IV), (iii) liver tissue from colorectal cancer metastatic disease (stage IV). Antigen retrieval was performed by microwave citrate method, and staining using the antibody clone CUB7402 (Abcam; 1:800). Semiquantitative scoring of TG2 levels on whole tissue sections was performed independently and in a blinded manner by a

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specialist pathologist (G.J. Thomas) and a further investigator (A.H. Mirnezami). A modified 3-point scoring method was used: (i) low/negative staining (<10% positivity), (ii) focal/patchy staining (10%–50% positivity), (iii) strong diffuse staining (>50% positive). All patients provided informed consent in accordance with the Helsinki protocol, and the study was approved by the regional research ethics committee.

microRNA analysis

Prediction of miRNA targets for TG2 was performed using four target prediction algorithms: TargetScan (http://www.targetscan.org; release 5.1); miRanda (http://www.microrna.org; 2010 release); miR Walk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/); and DIANA—microT (http://diana.cslab.ece.ntua.gr/microT/; v3.0). For quantification of miRNA levels in patient samples, laser-capture microdissection (LCM) was performed on frozen human tissue specimens. Sections were fixed in 75% ethanol, stained with 1% cresyl violet, and dehydrated with ethanol before air drying. Microdissection was performed on the Leica AS microdissection platform, and captured colorectal cancer tissue collected directly into lysis buffer prior to RNA isolation (RNA AQueous MicroPrep Kit; Ambion). Ten randomly selected specimens were analyzed from two clinically defined groups: (i) primary colorectal cancer tumor tissue, (ii) patient-matched liver tissue from colorectal cancer metastasis. The expression of miRNAs was performed using TaqMan assays (Applied Biosystems) according to the manufacturer's instructions, and normalized to U6 expression. Expression of miRNA was calculated relative to the endogenous reference gene U6B using the formula $2^{-\Delta\Delta C_T}$. miRNA expression in cell lines was obtained from microarray data published previously (25), which is available in the EBI database (http://www.ebi.ac.uk/arrayexpress/experiments/; accession number E-MEXP-3270).

SNP6 array hybridization, data extraction, and analysis

DNA was isolated from cell lines using the Qiagen DNeasy method prior to being purified, amplified, labeled, and hybridized to the Affymetrix SNP6 platform (Affymetrix) as previously described (26). The data were aligned (Build 36.3) and analyzed by two independent researchers using Partek Genomics Suite (Partek Inc.). Copy number alterations (CNA) were defined as a deviation of 50 consecutive probes from a normal value of 2 (±0.3), within a consecutive genomic window of 50 kb. The 270 HapMap Reference baseline (Affymetrix) was used as a control and germline copy number variants were excluded on the basis of the Database of Genomic Variants (http://projects.tcag.ca/variation/). The allele ratio was calculated for each sample using the HapMap Allele Reference baseline (Affymetrix) and in the absence of paired normal DNA; copy number neutral loss of heterozygosity (CNNLOH) was defined as a region greater than 20 Mb, extending to a telomere. We also analyzed copy number data from 437 Colon Adenocarcinoma cases from the Cancer Genome Atlas data COAD dataset (https://tcga-data.nci.nih.gov/tcga/) using the UCSC Cancer Genomics Browser (https://genome-cancer.soe.ucsc.edu/) to identify recurrent regions of copy number gain and loss that include our miRNAs of interest.

TG2 3′-UTR luciferase reporter assay

TG2-3′-UTR wild-type and TG2-3′-UTR mutant vectors were generated by GenScript Inc. A 750 bp region of the TGM2 gene 3′-UTR containing the single predicted miR-19a binding site was synthesized and was subcloned into the pRL-TK plasmid vector (Promega) downstream of the *Renilla*-Luc gene at the *Xba*1 site. Insert orientation was in the same sense as the luciferase reporter in pRL-TK. The mutant vector was generated by changing the sequence TTTGCACA to TTTATTGA. Reporter genes were transfected into SW480/SCC and SW480/miR-19a lines using Fugene 6, and luciferase activity quantified using the Dual-Luciferase reporter system (Promega) to collect the activity of firefly (PGL3 vector control) and *Renilla* (TG2-3′-UTR) measured in the same sample. SW480/SCC and SW480/miR-19a cells were plated at 4,000 cells per well in 100 μL DMEM in a CulturPlate-96 microplate (PerkinElmer). Twenty-four hours after plating, cells were transfected with 30 nmol/L Pre-miRNAs, 10 ng PGL3, and 500 ng of TG2-3′-UTR (wild-type or mutant) vectors per well. Light produced was measured using a plate reader at 2-second intervals and activity was calculated as *Renilla* activity per light unit of firefly activity. 3′-UTR *Renilla* activity was normalized to firefly activity, and results presented as the difference between the wild-type and mutant vectors.

Statistical analysis

Statistically significant differences between experimental conditions were assessed using the Student *t* test, and paired *t* test where appropriate. Alternatively, where multiple comparisons were necessary, ANOVA with the Bonferroni post-hoc test was used. All analyses were performed in GraphPad Prism, and P values of <0.05 were considered statistically significant. All experiments were performed a minimum of three times.

Results

TG2 expression is decreased in SW620 cells compared with SW480 cells

It has been reported previously that differential TG2 levels are observed in the SW model of metastasis, with high levels in the primary colon adenocarcinoma SW480 line, and significantly reduced levels in the patient-matched SW620 lymph-node–derived line (11). We confirmed these observations at protein level by Western blot analysis (Supplementary Fig. S1A), showing considerable reduction of the full-length 79-kb isoform of TG2 in SW620 cells. Decreased protein expression of this full-length transcript in SW620 cells was matched by a decrease in mRNA transcript level (Supplementary Fig. S1B). Interestingly, protein expression of the 55-kD exon-10–truncated splice variant of TG2 (TG2-E10) was actually relatively higher in SW620 cells (Supplementary Fig. S1A). This is in contrast to a decrease in mRNA transcript level (Supplementary Fig. S1B), indicating differential regulation of the splice variants.

Reduced TG2 is associated with increased invasiveness in SW480/SW620 cells

Because TG2 levels were significantly reduced in SW620 cells when compared with SW480, and SW620 cells are more invasive (12, 27), we hypothesized that TG2 may be inversely correlated to invasive potential. In an invasion assay using Matrigel as a substrate, SW620 cells, as expected, displayed increased invasive behavior (Fig. 1A), with counts of invading cells reaching 20,000 compared with 5,500 for SW480 cells. This was not due to significant differences in cell proliferation within the lower chamber as differences were not observed in an MTT proliferation assay (data not shown), nor were any differences accounted for by

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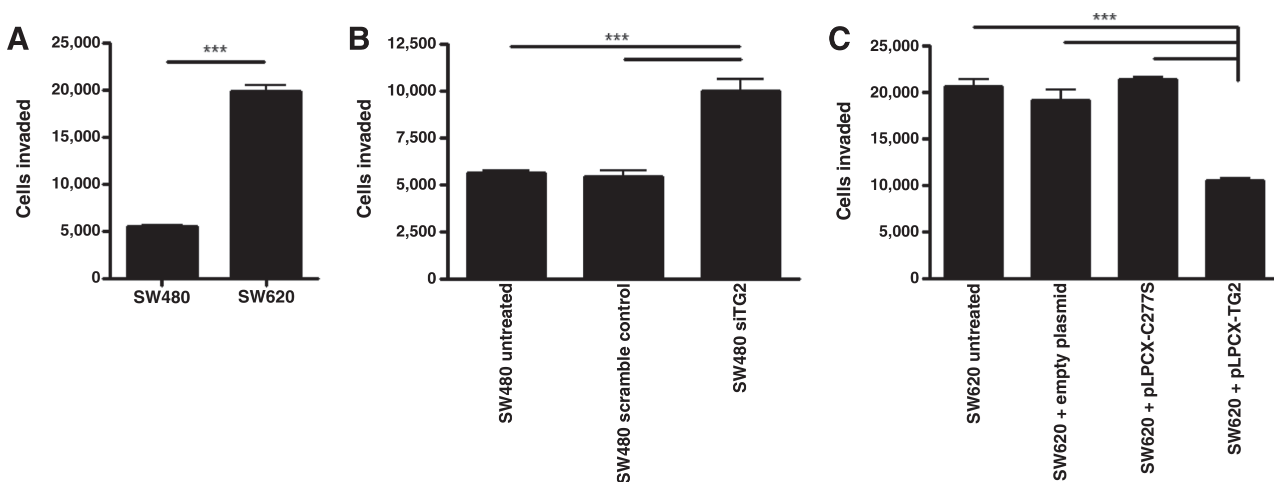

Figure 1.
TG2 is involved in the invasive ability of SW cells. The number of cells invading through Matrigel substrate toward an FCS chemoattractant in 24 hours was compared for SW480 cells and SW620 cells (A), in the presence or absence of TG2 siRNA to knock down TG2 expression, or scrambled siRNA as a control, in SW480 cells (B), and following the transfection of a TG2 expression plasmid or the catalytically inactive expression plasmid C277S into SW620 cells (C). Statistical significance is indicated by the asterisks, ***, $P < 0.0001$.

apoptosis using Annexin V staining (data not shown). When SW480 cells were treated with siRNA to TG2, the cells became significantly more invasive, showing a 100% increase in the number of invading cells when compared with cells either untreated or treated with a scrambled, control siRNA ($P < 0.0001$; Fig. 1B). In contrast, overexpression of TG2 in SW620 cells using a plasmid encoding TG2 significantly decreased the number of invading cells by around 50%, compared with untreated, empty vector, or cells transfected with the cross-linking-deficient TG2 plasmid C277S ($P < 0.0001$; Fig. 1C). Thus, TG2 cross-linking activity appears to restrict invasive behavior of SW cells, whereas loss of TG2 in SW620 cells facilitates invasion.

TG2 activity in SW cells is localized intracellularly

Because TG2 activity decreased invasiveness of SW cells, we wished to define its cellular localization, because cell-surface TG2 may interact directly with the cell-surface and extracellular matrix (ECM) proteins (28, 29), whereas intracellular TG2 has a cell signaling function (24, 30, 31). Imaging of TG2 by immunofluorescent microscopy showed that TG2 protein was expressed throughout the cytoplasm in both SW480 and SW620 cells (Fig. 2A, red). Furthermore, cytoplasmic TG2 was catalytically active as assessed using a fluorescent TG2 substrate (Fig. 2A, green). The staining for protein and activity were colocalized (Fig. 2A, merge). Expression of both TG2 protein and cross-linking activity was higher in SW480 cells compared with SW620 cells (mean CTCF of 33.3×10^5 compared with 18.3×10^5 for protein, 19×10^5 compared with 9.3×10^5 for activity, respectively), consistent with previous data. Significant TG2 was also observed localized to the nucleus (Fig. 2A). By comparing nuclear expression to cytoplasmic expression, we observed that 17%/24% of the cellular TG2 protein/activity was localized to the nucleus of SW480 cells, and 11% of both protein/activity was localized to the nucleus of SW620 cells. The absence of cell-surface TG2 data were confirmed by flow cytometry. Cells stained for cell-surface expression of TG2 showed very limited staining, and no differences were observed between SW480 and SW620 cells (Fig. 2B). In contrast, when cells were permeabilized prior to staining, strong expression of TG2 was observed, with >99% cells expressing TG2, and with considerably stronger staining in SW480 cells compared with SW620 cells.

TG2 activity influences inflammatory profile of SW cells

Because intracellular TG2 is known to drive proinflammatory signaling (9, 23, 30), we also measured IL8 production by SW480 and SW620 cells. IL8 was not detected in SW620 supernatants, but was clearly produced by SW480 cells. Using TG2-specific siRNA, TG2 levels could be reduced from 300 to 100 pg/mL compared with control ($P < 0.05$; Fig. 2C). We next compared the expression of cytokines in the two cell lines, and observed that the production of proinflammatory cytokines by SW480 cells was generally higher when compared with SW620 cells, including IL8 and TNFα (Supplementary Fig. S2A; $P < 0.05$). However, although TG2 siRNA reduced IL8 and TNFα mRNA expression, the differences were not significantly different (Supplementary Fig. S2B), and we could not detect expression of TNFα in cell supernatants (data not shown). Because proinflammatory signaling pathways are also linked to the release of enzymes that can break down tissue, we also investigated whether matrix metalloproteinase (MMP) production was influenced by TG2. As expected, the more invasive SW620 cells expressed higher levels of MMP mRNA, notably significantly higher levels of MMP-7 ($P < 0.05$; Supplementary Fig. S3A). Interestingly, SW620 cells also expressed significantly lower mRNA of one member of this enzyme family, MMP-14 ($P < 0.05$; Supplementary Fig. S3A). However, when we compared MMP mRNA expression in SW480 cells treated with and without siRNA to TG2, no significant changes to MMP mRNA expression were observed (Supplementary Fig. S3B), indicating that TG2-linked invasion was not directly related to enhanced MMP production.

TG2 levels are downregulated in metastatic tumors compared with primary tumors

Because our data in the SW model demonstrated downregulation of TG2 in the metastatic SW620 cell line, and correlation of TG2 levels with tumor invasiveness, we investigated the

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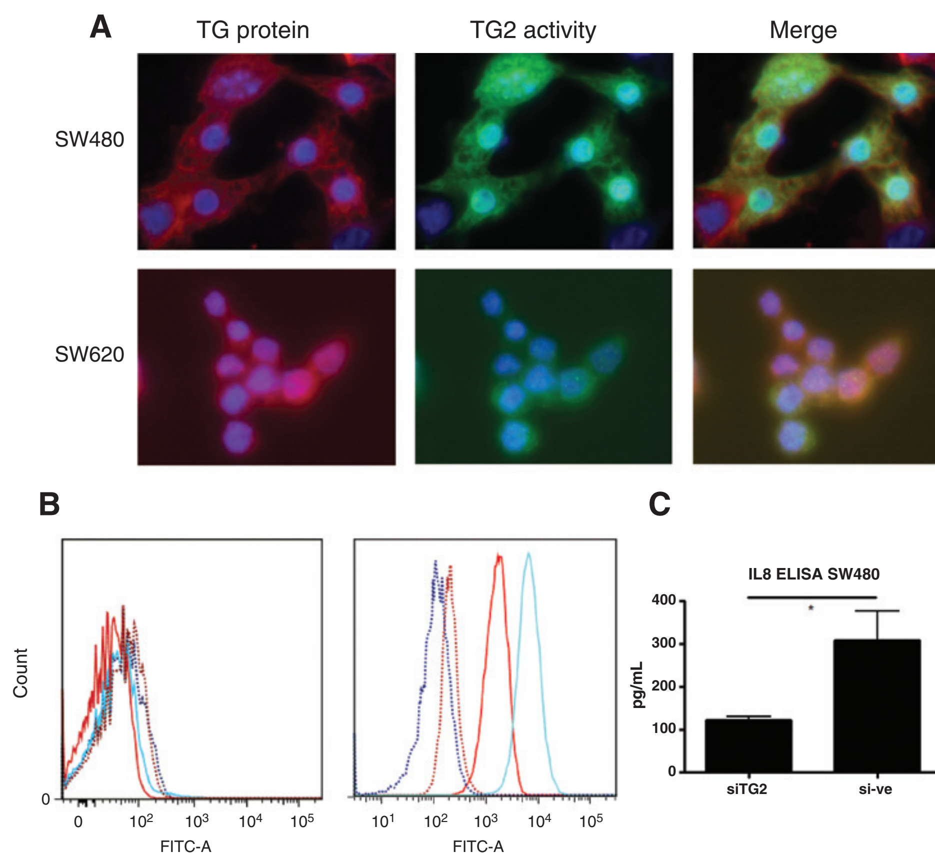

Figure 2.
TG2 is distributed throughout the cell cytoplasm, and is not localized to the cell surface in SW cells. The cellular distribution of TG2 was assessed by measuring the presence of TG2 protein in fixed cells by immunolocalization with the antibody clone 7402 coupled to Alexa Fluor 492–conjugated secondary antibody (A, red stain), and colocalizing protein to TG2 activity visualized using the biotinylated MDC-substrate assay coupled to a FITC-streptavidin secondary antibody (A, green). DAPI counterstaining (blue) indicates the cell nucleus (A). The presence of cell-surface TG2 was also determined by flow cytometry (B), using TG2 staining of whole live cells with the antibody clone 7402 (left), compared with cells permeabilized to assess intracellular levels (right panel). SW480 cells are displayed as blue lines, and SW620 cells as red lines. The dotted lines represent unstained controls. IL8 production by SW480 cells in the presence or absence of siRNA to TG2 was analyzed by ELISA (C). *, $P < 0.05$.

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expression of TG2 in human colorectal cancer sections, comparing primary tumors taken from patients grouped according to confirmed lymph node involvement, and from liver metastases. Staining for TG2 was detected in primary tumor sections (Fig. 3A and B), mainly at the invasive front (Fig. 3A), but not in liver metastases (Fig. 3C). Scoring by two independent investigators quantified this differential expression pattern (Fig. 3D), with TG2 expression found to negatively correlate with tumor stage. However, in these sections the most intense staining was detected in the tumor stroma (Fig. 3A–C), with cells surrounding the cancerous cells appearing to produce significant amounts of TG2, both at the primary and metastatic sites. No TG2 staining was observed in normal epithelia (data not shown).

TG2 is a predicted miRNA target

To better understand the differential protein expression of TG2 splice variants in SW cells (Supplementary Fig. S1A), we next examined the mRNA expression of TG2 splice variants by RT-PCR. In SW480 cells, expression of the mRNA for all of the TG2 splice variants assessed was higher in SW480 compared with SW620 cells, at differences ranging from 100- to 2,000-fold ($P < 0.0001$; Supplementary Fig. S1B). The protein and mRNA expression of full-length TG2 therefore appear to correlate, but the protein level of the 55-kD splice variant does not correlate to the mRNA expression of TG2-E10. There is an increasing evidence that miRNAs are intimately involved in the metastatic progression of colon cancer (18, 20, 25, 32). miRNAs bind to the 3′-UTR of their target genes, and the TG2 splice variants are 3′-truncated (15, 33). We therefore examined the possibility that TG2 may be a target for miRNA regulation, and explain why expression of TG2 splice variants is different at mRNA and protein level. Potential miRNA regulators of TG2 were identified by *in silico* analysis of the 3′-UTR using a panel of four target prediction algorithms. These identified only a single miRNA, miR-19a/b, predicted to bind to the 3′-UTR of TG2 across all platforms. Binding of miR-19a/b was predicted to occur at a conserved UUUGCACA sequence at position 1588–1595 of the 3′-UTR (Supplementary Fig. S4A), suggesting miR-19a/b may represent a potential regulatory miRNA for TG2.

miR-19 is upregulated in metastatic tumors compared with primary tumors

The level of miR-19 in sections taken from patients with colorectal cancer was assessed using LCM, in order to isolate epithelial and stromal expression (Supplementary Fig. S4B). miRNA microarray profiling showed that miR-19a/b expression was significantly different in tumor epithelia when compared with normal epithelia ($P < 0.05$; Supplementary Fig. S4C). miR-19a/b expression was not significantly different in tumor stroma compared with normal stroma (Supplementary Fig. S4D). Noticeably, both epithelial and stromal analyses showed several specimens with high expression of miR-19; interestingly, however, these did not correlate to the same patients for epithelial/stromal expression. Our data indicated that differences in TG2 expression were observed between primary tumor specimens and liver metastases.

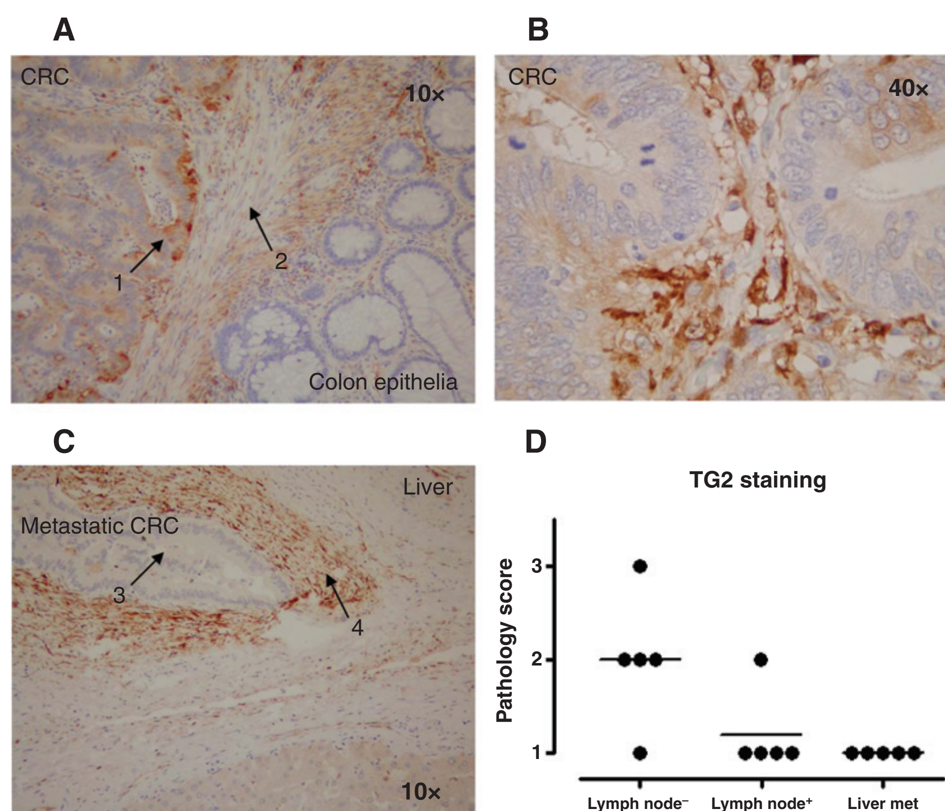

Figure 3.
TG2 is expressed in human colorectal cancer specimens, but lost in liver metastases. TG2 was stained in colorectal cancer tumor sections using antibody clone 7402. A, representative image showing staining in primary colorectal cancers, with arrows indicating tumor (1) and stroma (2). B, TG2 staining at higher magnification in primary colorectal cancer, and C illustrates a representative image of a liver metastasis, with arrows showing tumor cells (3) and stroma (4). In both cases, strong stromal expression of TG2 is observed. As illustrated following scoring by a senior pathologist, TG2 expression is lost in sections taken from liver metastases (D).

We therefore analyzed miR-19a using TaqMan in LCM samples to compare these two groups. miR-19a expression was significantly upregulated in sections taken from liver metastases compared with sections taken from primary tumors (Fig. 4A; $P < 0.01$).

miR-19 is upregulated in SW620 cells compared with SW480 cells, and its genomic locus is amplified in colorectal cancer

To test whether overexpression of miR-19 could be a mechanism for TG2 downregulation, we first established the levels of miR-19a and miR-19b in SW620 cells compared with SW480 cells. microRNA microarray profiling of SW620 and SW480 cells demonstrated a 2.6-fold increase of miR-19a and a 3-fold increase in miR-19b in SW620 cells compared with SW480 cells [normalized values of 274.49 vs. 105.45, and 3082.57 vs. 1026.25 for miR-19a and miR-19b, $P = 0.01$, and <0.0001, respectively (25)], subsequently validated by qPCR analysis, confirming observations from other groups (20, 34). Deregulation of miRNAs has been attributed to genomic copy number changes (35), and miRNAs have been noted to be over-represented in regions of genomic gain in colorectal cancer (36), consequently we next examined whether copy number changes could account for the upregulation of miR-19a and b in the SW cell lines. SW480 and SW620 cells were analyzed using a Genome-Wide Human SNP Array 6.0 with the HapMap 270.422 dataset as reference. miR-19a and miR-19b1 are located on chromosome 13 and both were found to be gained in both cell lines (Fig. 4B). In contrast, miR-19b2 showed normal copy number in both cell lines from its locus on chromosome X (data not shown). To clarify whether the miR-19a/b loci are subject to copy number change in primary human colorectal cancer, we next examined the Cancer Genome Atlas (TCGA) dataset. In the data available from 437 human colorectal cancers, both miR-19a and miR-19b were subject to amplification through recurrent chromosome 13 gains. However, this was due to recurrent gains in chromosome 13, rather than any focal CNAs at the specific miR-19 loci (Fig. 4C). The data therefore indicate that advanced colorectal cancer frequently gains an additional copy of the whole chromosome 13. Further analysis of the TCGA dataset reveals that this amplification in colorectal cancer patients occurs in later stages of disease; no significant differences are observed when comparing patients with stage I, II or III disease, but significant differences ($P < 0.05$) are seen when comparing stage III and IV disease (data not shown).

miR-19 directly targets TG2 and alters the invasive behavior of SW cells

To confirm our *in silico* prediction of miR-19 targeting TG2, we manipulated miR-19a levels in SW480 cells by establishing stable cell lines overexpressing a scrambled plasmid control (SW480/SCC), a miR-19a expression plasmid (SW480/miR-19a), and by transient transfections with molecular miRNA mimics. Downregulation of TG2 was observed by Western blot analysis in SW480 cells manipulated to overexpress miR-19a (Fig. 5A), which was statistically significant when assessed by densitometry ($P < 0.05$). The direct binding of miR-19a to the 3′-UTR of TG2 was assessed using a luciferase reporter assay, which showed a reduction of 3′-UTR activity by almost 50% in the SW480/miR-19a cells ($P < 0.01$; Fig. 5B). To evaluate the functional

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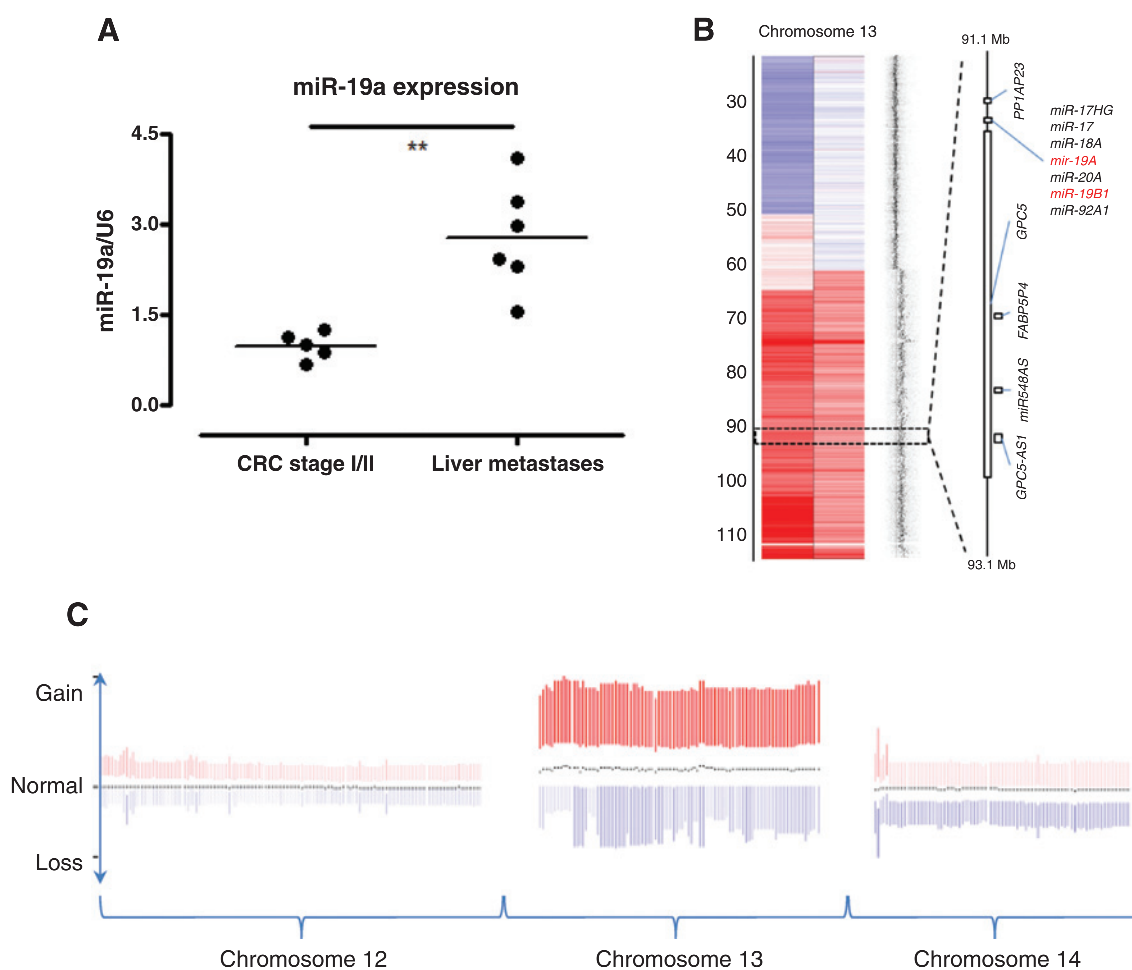

Figure 4.
miR-19a is upregulated in metastatic cells through instability of chromosome 13. The expression of miR-19a was assessed by TaqMan in RNA isolated from sections of primary colorectal cancer tissue compared with liver metastases (A). Copy number heatmap from SNP6 data of chromosome 13 forSW620 and SW480 to the left and right, respectively. Normal copy number, gain, and loss are shown in gray, red, and blue, respectively. Data show duplication/amplification of distal 13q, including the miR-19a and miR-19b1 loci (B). Relative copy number for chromosomes 12, 13, and 14 positioned from left to right from the COAD dataset (C). The mean profile for each chromosome is shown by the black dots, indicating copy number at different points along each chromosome from 437 patients with colorectal cancer. The color intensity is proportional to the deviation from the median, and the shift upward toward red indicates increased chromosome 13 copy number in relation to chromosomes 12 and 14.

consequences of this, invasion assays were performed on these stable cell lines. SW480 cells overexpressing miR-19a showed enhanced invasiveness when compared with control cells (Fig. 5C; $P < 0.01$). Thus, elevating the levels of miR-19a has a similar effect to reducing TG2 using siRNA. To confirm that TG2 was the target for miR-19a in this model, we transfected SW480/19a cells with the TG2 expression plasmid, lacking the 3′-UTR binding site for miR-19a (pLPCX-TG2). Restoring TG2 in this way reduced invasion of SW480/miR-19a cells to a level similar to that observed in 480/SCC cells in a statistically significant manner (Fig. 5C; $P < 0.05$). Finally, IL8 production from the stable SW480 cell lines was assessed by ELISA. Although slightly lower IL8 production was seen from SW480/miR-19a cells compared with SW480/SCC cells, this was not significant (Fig. 5D). However, transfection with the pLPCX-TG2 plasmid significantly increased the production of IL8 ($P < 0.05$), further illustrating the functional effect of TG2 lacking the 3′-UTR miRNA-binding site.

Discussion

There is a widespread body of literature spanning the past two decades illustrating that TG2 is involved with many cellular processes linked to tumor development and progression including chemoresistance, adhesion, migration, invasion and EMT, and TG2 has been found in tumor cells from a variety of origins. The primary role of TG2 is as a protein cross-linking enzyme, linking glutamine and lysine residues, and this can eventually lead to the formation of protein aggregates (37). A wide range of TG2 substrates has been reported, which includes both intra- and extracellular proteins, implying a role for TG2 both inside and outside of the cell. The range of cell behaviors linked to TG2 and sometimes contradictory results in studies of TG2 activity suggest a role that is isoform-, context- and cell-type dependant (14).

It is interesting that we observe an inverse relationship between TG2 and invasion *in vitro* in the SW cell model. This finding

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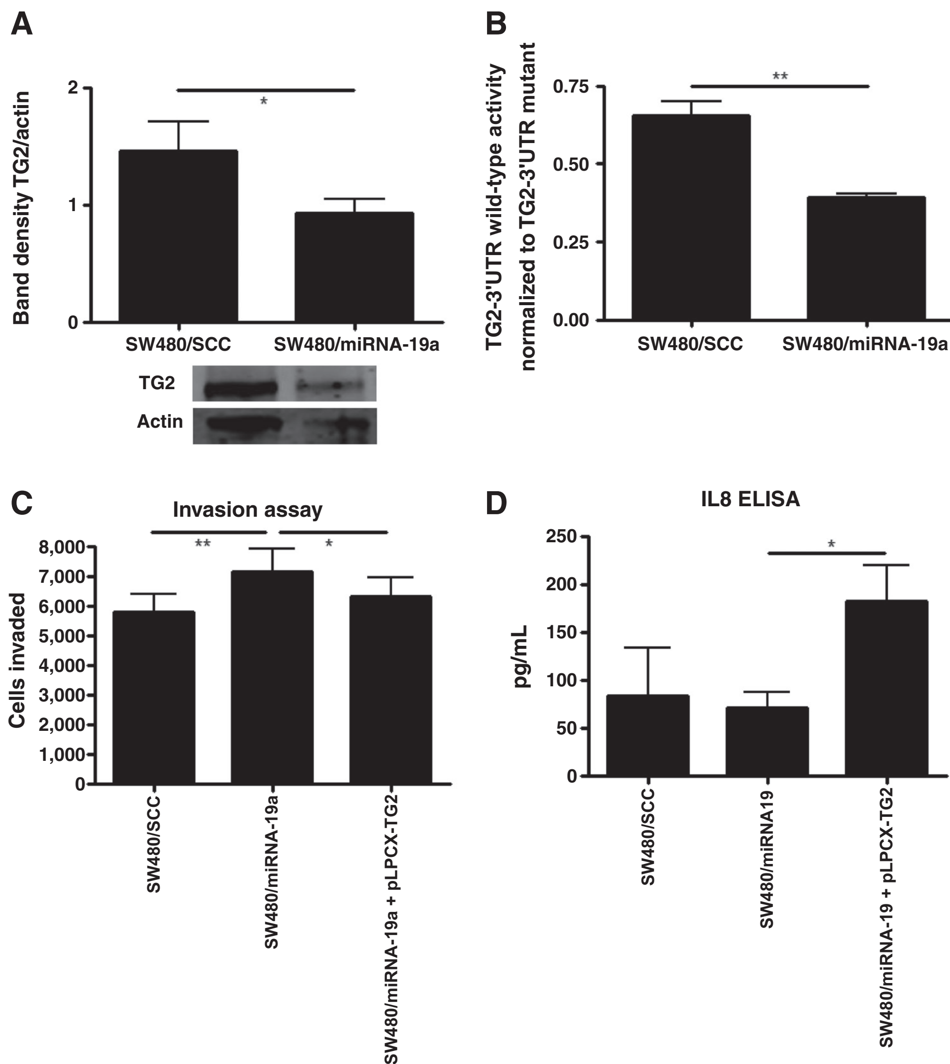

Figure 5.
miR-19 promotes changes in colorectal cancer cell behavior through TG2. The effect of miR-19a overexpression in SW480 cells was assessed following stable transfection with a miR-19a expression plasmid (480/miR-19a), compared with a control plasmid (SCC). Western blotting was performed to observe the effect of miR-19a on TG2 protein levels (A) and quantified by densitometry, and direct targeting of TG2 by miR-19a was assessed using a 3′-UTR luciferase reporter gene (B). The effect of miR-19a overexpression on invasion was assessed in the SW480 stable lines using the Matrigel invasion assay (C). The effects of miR-19a on invasion were established to be through TG2 by cotransfecting the cells with a TG2 expression plasmid lacking the 3′-UTR miRNA binding sequence. The effect of miR-19a on inflammatory signaling was assessed using IL8 ELISA on supernatants from SW480 stable cell lines (D).
*, $P < 0.05$; ** = $P < 0.01$.

supports studies describing a correlation between metastatic potential and TG2 expression in colorectal cancer cells (12), but runs contrary to reports in several other cell models (8, 27, 38, 39). In a similar manner to studies showing that TG2 can function in a pro- or antiapoptotic manner (14), the cell-, isoform-, and context dependence of TG2 makes interpretation of such apparently contradictory results difficult. In an important study, it was demonstrated that the truncated form of TG2 promoted cell death, in contrast to the full-length protein which promoted cell survival (32). Although we observe a downregulation of full-length protein in SW620 cells compared with SW480 cells, we actually observe a small upregulation of the truncated protein (Supplementary Fig. S1). If cell behavior is linked to the balance between TG2 isoforms, this shift could well be critical—it is notable that the smaller form is truncated at the 3′-end, making it likely that miR-19 will not inhibit transcription of the truncated protein. Because this truncation also removes the GTP-binding site that inhibits cross-linking function, this isoform is highly active and may compensate for the miR-19–induced reduction of the full-length protein in the metastatic cell line, maintaining critical functionality such as adhesive and migratory behavior.

Interestingly, in several previous studies where TG2 has been shown to promote invasive behavior, this activity was not dependent on cross-linking activity, and active site mutated TG2 was also able to promote invasion (8, 39). In contrast, when we transfected the active site mutated TG2 into SW620 cells, no effect on cell invasion was observed. Given the clear inhibition of invasive behavior we observe when we transfect active TG2, we conclude that in the SW model system, TG2 inhibits invasive behavior in a cross-linking dependent manner. Because promotion of invasion by TG2 in other model systems is not dependent on cross-linking it likely involves signal transduction mediated by interaction with integrins/FAK at the cell membrane/ECM boundary (8, 27–29, 40). Our observation that SW cells lack cell-surface expression of TG2 supports a role for TG2 in restricting invasion in early-stage colorectal cancer through this separate mechanism. We did not specifically examine secretion of TG2 in this study, but experiments to examine whether TG2 released directly into the matrix would be informative, because modification of ECM by TG2 is known to restrict invasion (41). In early colorectal cancer, it may be the case that cancer progression is driven by cross-linked, stiffened ECM (42), linking TG2 expression to poor prognosis as

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proposed by previous reports (13), but at the expense of rapid invasion, a feature that is reversed as the tumor progresses.

Although our data indicated a lack of cell surface expression of TG2, we observed extensive staining in the cytoplasm and nucleus. In these cell compartments, TG2 cross-linking activity is limited under physiologically normal conditions due to high nucleotide and low calcium conditions. However, intracellular cross-linking is known to occur as a consequence of cell stress—for example, activity is upregulated by ROS (23, 43). One of the consequences of this response is the upregulation of proinflammatory signaling pathways such as NF-κβ that has been reported in both inflammatory and tumor cell models (24, 30, 31), and we identified that IL8 secretion from SW480 cells is inhibited by silencing TG2. IL8 is known to play a significant role in colorectal cancer, and has been proposed as a marker of disease progression, but to our knowledge this is the first study linking IL8 to TG2 in colorectal cancer models (44, 45). Because SW620 cells are derived from an advanced-stage, invasive, metastatic tumor, and IL8 secretion was undetectable from these cells, it may be that stress-linked proinflammatory signaling through pathways like NF-κβ promotes progression of early colorectal cancer, whereas inhibition of this signaling in advanced disease promotes evasion of the immune system in advanced colorectal cancer. Further work would to clarify this would be extremely informative.

High TG2 levels inhibited invasive behavior of SW cells, but silencing of TG2 did not significantly alter MMP expression (Supplementary Fig. S3B), although MMP expression tended to be higher in SW620 cells. This may be a consequence of the experimental system; culturing cells in plates—as we did in the present study when analyzing MMP gene expression—induces significantly different responses in tumor cells when compared with the 3D environment cells experience *in vivo*, or within the Matrigel layer of the invasion assay (46, 47). Actin is reported to be an intracellular TG2 substrate (48), so it is also feasible that intracellular TG2 could play a role in colorectal cancer cell invasion in the cytoskeletal remodeling involved during cell mobility and invasion. Further experiments to examine TG2 secretion, MMP expression, and cytoskeletal changes by SW cells in a 3D model will be extremely informative.

The downregulation of TG2 in metastatic SW620 cells and in sections taken from liver metastases illustrates the context dependence of using TG2 as either a marker of disease or as a therapeutic target in colorectal cancer. The negative relationship between TG2 expression and invasive potential has been reported previously in the SW model (12), and the inverse relationship between TG2 and metastasis observed in other studies (49–51). It will therefore be interesting to examine whether this biphasic model of TG2 involvement in cancer progression is a general phenomenon, as it would have significant implications for the targeting of TG2 therapeutically. Our observation of significant TG2 expression in the stroma of both primary and metastatic colorectal cancer, despite downregulation of TG2 in metastatic cells, suggests that the majority of TG2 is produced as a defensive response rather than by the tumor (41, 52, 53). Further clarification of the cellular source of TG2 in colorectal cancer is important, as the differential impact of TG2 activity in cancer cells and in the surrounding tissue complicates the use of TG2 as a therapeutic target. Identifying pathways that specifically regulate TG2 expressed in cancer cells may therefore offer a promising alternative approach.

Examining the role of miRNAs in regulating TG2 was a consequence of our data showing differential expression of two splice variants of TG2 at transcript and protein level; indeed, over the course of the study TG2 protein levels were observed to vary significantly. Examining putative miRNA binding sites revealed that TG2 is a predicted target for miR-19, which has two closely related members miR-19a and miR-19b within the miRNA17-92 cluster. This adds to previous studies identifying regulatory roles for miR-1285, miR-181a, and miR-218 in regulating TG2 (54, 55), and because TG2 plays an important role in inflammatory disease (22, 24), multiple miRNA pathways may therefore have an important role in regulating innate immune responses as well as cancer cell behavior linked to TG2 activity. It is interesting that we did not see a significant change in IL8 in SW480/miR-19a cells compared with SW480/SCC control cells. This would be expected given that we observed that IL8 production is inhibited by silencing TG2 in SW480 cells, TG2 is suppressed by miR-19, and we also observe that transfection of the TG2 plasmid into SW480/miR-19a cells upregulates IL8. This could be the consequence of the multiple pathways that converge on NF-κB in cancer cells, for example NF-κB activation can be inhibited by blocking K-Ras activity in SW620 cells (56). However, these data may also simply represent technical differences in manipulating TG2 using siRNA—which is highly efficient in our model—compared with manipulating TG2 using miR-19, which alters expression by less than 50% (Fig. 5A).

Multiple studies have demonstrated that miR-19 is upregulated in colorectal cancer patients, notably at the invasive front of the tumor, and also in SW620 cells when compared with SW480 cells (25, 32, 34). (32, 34), strongly implicating these miRNAs in disease progression. We focused on miR-19a, demonstrating upregulation in sections taken from liver metastases when compared with primary colorectal cancer sections, and in metastatic SW620 cells compared with primary SW480 cells. Overexpressing miR-19a led to a reduction in TG2 expression in SW480 cells, with consequent increased invasive behavior. We therefore propose that the miR-19–TG2 axis can be added to the growing list of miRNA-regulated pathways that are linked to metastasis. Moreover, we identified that both in the SW cell model and in patients with colorectal cancer, overexpression of miR-19a/b is linked to chromosomal instability on chromosome 13, at the locus encoding a series of miRNAs, including miR-19a and miR-b1. Amplification of chromosome 13 is observed frequently in colorectal cancer, despite encoding relatively few genes linked to oncogenic pathways (57), and these observations may provide a mechanistic link between colorectal cancer and instability at this locus, via miRNAs and TG2.

Identifying miRNA regulation of TG2 in colorectal cancer cells may provide a more targeted pathway to therapeutic intervention, given the presence of TG2 in both tumor and stroma. Further work is required to establish the precise mechanisms by which TG2 acts to influence cell invasion, and how invasion, inflammation, and metastasis interact to drive the disease process. If TG2 is indeed a stress response, the role of ROS and calcium may be critical as they promote TG2 activity. We were not able to alter TG2 expression using ROS inhibitors, and defective calcium signaling is an established feature of colorectal cancer (58). Continuing to investigate a pathway linking stress, inflammatory signaling and invasion has the potential to provide useful insights into the mechanisms driving an increasing burden on the world's health.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors' Contributions

Conception and design: D. Cellura, S. Quaratino, A.H. Mirnezami, N.J. Peake
Development of methodology: D. Cellura, J.C. Strefford, G.J. Thomas, A.H. Mirnezami, N.J. Peake
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Cellura, K. Pickard, H. Parker, G.J. Thomas, A.H. Mirnezami
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Cellura, K. Pickard, S. Quaratino, H. Parker, J.C. Strefford, R. Mitter, A.H. Mirnezami, N.J. Peake
Writing, review, and/or revision of the manuscript: D. Cellura, K. Pickard, S. Quaratino, J.C. Strefford, A.H. Mirnezami, N.J. Peake
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Cellura, K. Pickard, A.H. Mirnezami, N.J. Peake
Study supervision: S. Quaratino, A.H. Mirnezami, N.J. Peake
Other (funding support): A.H. Mirnezami

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