Tissue Transglutaminase Regulates Matrix Metalloproteinase-2 in Ovarian Cancer by Modulating cAMP-response Element-binding Protein Activity*§

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Tissue transglutaminase 2 (TG2) is overexpressed in epithelial ovarian cancer (EOC) and promotes intraperitoneal metastasis. How TG2 facilitates the spread of EOC is unknown. Here, we show that TG2 regulates the expression and function of matrix metalloproteinase-2 (MMP-2), a critical mediator of tissue invasiveness. TG2 knockdown down-regulates MMP-2 protein and mRNA expression in SKOV3, IGROV-1, MDA-MB-436, and PC-3 cancer cells. TG2 knockdown or inhibition of TG2 activity using KCC009 decreases MMP-2 gelatinase activity in cancer cells. MMP-2 expression and function are regulated by TG2 at transcriptional level, as demonstrated by quantitative PCR and reporter assays. We used bioinformatics and chromatin immunoprecipitation to identify a CREB binding site in the MMP-2 promoter. Binding of CREB to the MMP-2 promoter was diminished in cells that expressed decreased TG2 levels. TG2 knockdown decreased CREB phosphorylation, and CREB knockdown decreased MMP-2 expression. The effect of TG2 on CREB activity and MMP-2 transcription is mediated by TG2-dependent degradation of protein phosphatase 2 (PP2A-α). We show that PP2A-α complexes with and is targeted for degradation by TG2. In addition to their related in vitro expression levels, TG2 and MMP-2 expression were significantly correlated in vivo, as shown by concordant immunostaining in peritoneal xenografts and in human ovarian tumors. The capacity of TG2 to regulate MMP-2 expression in vitro and in vivo identifies a mechanism that may facilitate tissue invasion and the spread of EOC. The demonstration that TG2 induced degradation of PP2A-α activates CREB, and thereby increases MMP-2 transcription, provides novel mechanistic insight into the pro-metastatic function of TG2.

Transglutaminase 2 (TG2), an enzyme overexpressed in epithelial malignancies (1, 2), cross-links proteins by acylation-dependent degradation of protein phosphatase 2 (PP2A-α). The demonstration that TG2 induces CREB phosphorylation, and CREB was diminished in cells that expressed decreased TG2 levels. MMP-2 promoter. Binding of CREB to the MMP-2 promoter was diminished in cells that expressed decreased TG2 levels. TG2 knockdown decreased CREB phosphorylation, and CREB knockdown decreased MMP-2 expression. The effect of TG2 on CREB activity and MMP-2 transcription is mediated by TG2-dependent degradation of protein phosphatase 2 (PP2A-α). We show that PP2A-α complexes with and is targeted for degradation by TG2. In addition to their related in vitro expression levels, TG2 and MMP-2 expression were significantly correlated in vivo, as shown by concordant immunostaining in peritoneal xenografts and in human ovarian tumors. The capacity of TG2 to regulate MMP-2 expression in vitro and in vivo identifies a mechanism that may facilitate tissue invasion and the spread of EOC. The demonstration that TG2 induced degradation of PP2A-α activates CREB, and thereby increases MMP-2 transcription, provides novel mechanistic insight into the pro-metastatic function of TG2.

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2 The abbreviations used are: TG2, transglutaminase 2; EOC, epithelial ovarian cancer; MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; CREB, cAMP-response element-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interference RNA; ChIP, chromatin immunoprecipitation; IHC, immunohistochemistry; PP2A-α, protein phosphatase 2.

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FAK signaling or inflammatory pathways that converge on NF-κB, the MMP-2 group of metalloproteinases is less responsive to extracellular stimuli and is expressed constitutively in tissues.

The MMPs, particularly MMP-1, -2, and -9, are expressed abundantly in EOC cells (16–20). MMP-2, also known as gelatinase A, is overexpressed in ovarian tumors and secreted in ascites fluid (20, 21), and its activity correlates with clinical outcome (22). Secretion of MMP-2 by transformed ovarian epithelial cells is a critical event in the process of peritoneal dissemination, regulating the attachment of EOC cells to the peritoneal surface through cleavage of fibronectin and vitronectin in the extracellular milieu (23). However, MMP-2 regulation in EOC remains poorly understood. Given their important roles in the extracellular matrix, TG2 as a matrix-tightening factor and MMP-2 as a remodeling factor, we hypothesized that a tightly regulated balance between TG2 and MMP-2 may exist in the tumor milieu whereby the proteins regulate each other.

Here we report that tissue transglutaminase regulates the expression of MMP-2. Concordant expression of TG2 and MMP-2 was observed in cancer cell lines, ovarian tumors, and xenografts. We show that in ovarian cancer cells TG2 modulates CAMP-response element-binding protein (CREB) activation and the expression of MMP-2 in a CREB-dependent fashion. The effects of TG2 on CREB activity are due to its targeting the PP2A-α serine-threonine phosphatase for degradation. These data add a new dimension to our previous results implicating TG2 in metastasis and suggest a new role for TG2 in this process. These results support the conclusion that TG2 may be an important target in ovarian cancer metastasis.

MATERIALS AND METHODS

Reagents—Antibodies against TG2 and MMP-2 were from NeoMarkers (Fremont, CA), antibodies to pSer133CREB, CREB, and poly(ADP-ribose) polymerase were from Cell Signaling Technology Inc. (Beverly, MA), anti-PP2A catalytic subunit α was from BD Biosciences (San Jose, CA), and anti-GAPDH were from Biodesign International (Saco, ME). Secondary horseradish peroxidase-conjugated antibodies were from Amersham Biosciences (San Francisco, CA) and Santa Cruz Biotechnology Inc. Recombinant human MMP-2 was from R&D Systems (Minneapolis, MN), and recombinant human TG2 was from NeoMarkers. The TG2 inhibitor KCC009 was provided by Alvine Pharmaceuticals.

Cell Culture—The human cancer cell lines SKOV3, MDAMB436, OV90, A2780, and PC-3 were obtained from the American Type Culture Collection. The IGROV-1 cell line was a gift from Dr. L. Malkas (Indiana University). SKOV3 and OV90 cells were cultured in growth medium containing 1:1 MCDB 105 and M199 (Cellgro) supplemented with 10% fetal bovine serum (Cellgro) and 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). IGROV-1, MDA-MB436, A2780, and PC-3 cells were grown in minimal essential medium containing 10% fetal bovine serum, 1 mm sodium pyruvate, and 1% antibiotics. Cells were grown at 37 °C under 5% CO₂.

Transfections—To knock down TG2, an antisense construct cloned into pcDNA3.1 was transfected into SKOV3 cells using FuGENE. Control cells were transfected with pcDNA3.1. The AS-TG2 plasmid was a generous gift from Prof. Janusz Tucholski (University of Alabama) (24). Transfection efficiency was 30–40% in SKOV3 cells, as determined by green fluorescent protein expression. Stable transfected clones were established by selection with G418. Knockdown of TG2 was demonstrated by Western blot analysis. To overexpress TG2, human full-length TG2 subcloned in the retroviral vector PQCXIP was transduced into A2780 and OV90 ovarian cancer cells that do not express endogenous TG2. The control vector carrying the puromycin selection gene was transduced in parallel. Pooled, stable clones were collected after selection, and TG2 overexpression was verified by Western blotting. Transient transfection of short interfering RNA (siRNA) was performed with sequences targeting TG2 (Dharmacon), CREB (Santa Cruz Biotechnology Inc.), and PP2A-α (Upstate Cell Signaling). Scrambled siRNA (Dharmacon) was used as control. DharmaFECT (Oz Biosciences, Marseille, France) was used for siRNA transfections.

Western Blotting—Cells were lysed into a buffer containing protease inhibitors leupeptin (1 μg/ml), aprotinin (1 μg/ml), phenylmethylsulfonyl fluoride (400 μM), and sodium orthovanadate (Na₃VO₄, 1 mM). Lysates were sonicated briefly and centrifuged (14,000 rpm, 15 min, 4 °C) to sediment particulate material. Nuclear and cytosolic fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Equal amounts of protein (50 μg) were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking, membranes were probed with primary antibody overnight at 4 °C. After incubation with a horseradish peroxidase-conjugated secondary antibody, antigen-antibody complexes were visualized using enhanced chemiluminescence. Images were captured by a luminescent image analyzer with a charge-coupled device camera (LAS 3000, Fujifilm). Densitometry analysis was performed with MultiGauge 3.0 software (Fujifilm USA Inc., Valhalla, NY).

Immunoprecipitation—Cells were lysed on ice into 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, leupeptin (1 μg/ml), aprotinin (1 μg/ml), and phenylmethylsulfonyl fluoride (400 μM). After 15 min, lysates were centrifuged (13,000 rpm, 30 min) to pellet debris. 500 μg of protein from the supernatant was incubated overnight at 4 °C with 5 μg of anti-TG2, anti-PP2A-α, or IgG, then with 50 μg of Protein G Plus-Agarose beads for 90 min at 4 °C. Protein complexes were centrifuged in wash buffer, boiled for 5 min in 1× SDS protein loading dye, and used for Western blotting as described.

Analysis of Gene Expression by Microarray—A tumor metastasis pathway focused microarray GE array kit was purchased from SuperArray Bioscience (Bethesda, MD). The kit determines expression of 120 genes that suppress or increase tumor metastasis and housekeeping genes as controls. The array compared gene expression in ovarian cancer cells stably transfected with pcDNA3.1 or AS-TG2. Briefly, total RNA from each cell line was reverse-transcribed, and the corresponding cDNA was biotin-labeled, per manufacturer’s instructions. The cDNA was denatured, and hybridization was carried out in GEHyb solution to nylon membranes spotted with gene-specific fragments.
Transglutaminase and MMP-2

overnight at 60 °C. After washing, blots were exposed to x-ray film. GEArray Analyzer version 1.2 was used for analysis.

Gelatin Substrate Zymography—Fresh serum-free medium was added to confluent cells. The media was conditioned for 1, 3, 6, and 24 h and centrifuged at 3000 rpm for 5 min to sediment cellular debris. Equal volumes of conditioned media were fractionated under non-reducing conditions on 9% polyacrylamide gels co-polymerized with 2 mg/ml gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100 (3 times, 20 min) to remove SDS and renature gelatinases, and then incubated overnight in developing buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM CaCl2, 0.01% Brij-35) at 37 °C to induce gelatin lysis by renatured MMP-2. Coomassie Brilliant Blue R-250 0.005% was used for staining. After de-staining in 40% methanol-10% acetic acid, MMP activity was detected as transparent bands against the Coomassie Blue-stained background. Photographs of the zymograms were taken with a charge-coupled device camera (LAS 3000 Fuji Film), and the intensity of bands was determined using the Java image densitometry processing program ImageJ (National Institutes of Health).

Reverse Transcription-PCR—Semi-quantitative and real-time PCR were used to quantify MMP-2 and PP2A-α expression in AS-TG2 or control cells. RNA extracted from the cells using RNA STAT-60 Reagent (Tel-Test Inc., Friendswood, TX) was reverse-transcribed in a final volume of 20 μl using the iScript cDNA synthesis kit (Bio-Rad). Used primers include: MMP-2, GCTCAGATC CGTGGTGAGAT (f) and GGTGCT-TGCTGA GTAGATCC (r); PP2A-α, AGCCATCCACTTTCCGAG (f) and TTGCCCAAGGTTGTAACCAG (r); and GAPDH, GATTCACCCATGGCAATTTCC (f) and CAGGTGGAAGTGGGAC (r). The reverse transcriptase product (1 μl) and primers were heated at 94 °C for 90 s followed by 28 rounds of amplification for GAPDH and 32 cycles for MMP-2 (30-s denaturing at 94 °C, 30-s annealing at 55 °C, and 30-s extension at 72 °C) followed by a final extension of 10 min at 72 °C. The reverse transcription-PCR product was visualized under UV light after fractionation on a 1.5% agarose gel. For real-time PCR we used the FastStart TaqMan Probe Master (Roxy, Roche Applied Science) on an ABI Prism 7900 platform (Applied Biosystems) according to the manufacturer’s procedures. Primers specific for MMP-2 were 5′-ATAACCTGGATGCCGTCGT (f) and 5′-AGGCCAC-CTTGAAGAGTGAGTGC (r) and Universal Probe Library probe #70. Primers specific for GAPDH were 5′-AGGCAC-CATCGCTCAGAC (f) and 5′-GCCCCATACGC-AATCC (r), and Universal Probe Library probe #60, as control. At the end of the PCR reaction a melting curve was generated and the cycle threshold (Ct) was recorded for the reference gene and for GAPDH. Relative expression of MMP-2 was calculated as ΔΔCt, measured by subtracting the Ct of the reference gene from that of the control. Results are presented as means ± S.D. of replicates. Each experiment was performed in duplicate and repeated at least three independent experiments.

Chromatin Immunoprecipitation—To detect the interaction between CREB and the MMP-2 promoter, we used a ChIP assay. In brief, SKOV3 cells stably transfected with pcDNA3.1 or AS-TG2 were serum-starved for 24 h, fixed with 1% formalde-
similar results. Each reaction was performed in duplicate and repeated with
the NR4A2 promoter (forward: GCC AAT GTG CCT TTG TTT ATG and reverse: AAC ACC AAA AAC CAC CCA AG), a
known CREB target. As a negative control, DNA immunoprecipitated with CREB antibody was amplified with primers
expected together with
—Deletion of the CREB binding
—MMP-2 activity was measured
—Results are expressed as the mean ± S.E. The Student t test was compared results of real-time PCR
—A Dual-Luciferase Assay quantified
—Tumor Collection and IHC—
—Noblotting. Additionally, a multi-
—The NR4A2 promoter (forward: GCC AAT GTG CCT TTG TTT ATG and reverse: AAC ACC AAA AAC CAC CCA AG), a known CREB target. As a negative control, DNA immunoprecipitated with CREB antibody was amplified with primers designed for the MMP-2 promoter, upstream of the CREB binding site. For real-time PCR, the primers were GCCCTAGGAGCGACAGATGTT (f) and TTGCCTCTCTCGCGATCT (r) in conjunction with probe #90 from the Universal Probe Library, and the analysis was performed as described above. Each reaction was performed in duplicate and repeated with similar results.

Site-directed Mutagenesis—Deletion of the CREB binding site from the MMP-2 promoter was performed with the QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). The DNA sequence GTGACGA at position –205 was deleted, and the DNA sequence of the truncated promoter was verified.

Assay of Gelatinase Activity—MMP-2 activity was measured as the ability to hydrolyze a fluorogenic substrate (7-methoxy- coumarin–4-yl-acetyl-Pro-Leu-Gly-Leu-3-(2,4-dinitrophenyl-
with a DAKO Detection Kit (DAKO, Hamburg, Germany). A pathologist blinded to the identity of the samples reviewed all slides.

Gene Reporter Assays—A Dual-Luciferase Assay quantified CREB and MMP-2 promoter activity in pcDNA3.1 and AS-TG2 SKOV3 cells. In brief, cells were transiently co-transfected with 3XCtRE (25) or an MMP-2 promoter luciferase, respectively, together with Renilla plasmids, at a ratio of 10:1 using DreamFect Gold transfection reagent (OZ Biosciences). The MMP-2 reporter plasmid was a gift from Dr. Etty Benveniste of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Renilla activity was measured using a TD-20/20 luminometer (Turner Biosystems) 24 h after transfection. Assays were performed in triplicate and repeated twice. To control for transfection efficiency, values for luminescence were normalized to Renilla activity.

Statistical Analysis—Results are expressed as the mean ± S.E. The Student t test was compared results of real-time PCR and gelatinolytic activity assays. We used kappa coefficient to relate TG2 expression to CREB phosphorylation in ascites. To relate TG2 to MMP-2 immunoreactivity in human tumor sam-

Transglutaminase and MMP-2

L-2,3-diaminopropionyl)-Ala-Arg-
NH₂, R & D Systems, Minneapolis, MN). In brief, recombinant full-length pro-MMP-2 was incubated with aminophenylmercuric acetate, a known MMP-2 activator (positive control), DMSO (negative control), or recombinant, full-length TG2 for 1 h at 37 °C in 50 mm Tris, pH 7.5, 150 mm NaCl, 10 mm CaCl₂, and 0.05% Brij-35. Upon addition of the substrate, fluorescence of the cleaved peptide was measured at an excitation wavelength of 320 nm and emission wavelength of 405 nm in a Molecular Devices SPECTRAmax Gemini XPS fluorescence microplate reader.

Tumor Collection and IHC—

Xenograft tumors harvested 8 weeks after intraperitoneal injection of pcDNA3.1 and AS-TG2 transfected SKOV3 cells into nude mice were examined histologically and by immunohistochemistry (IHC). Xenograft-derived cell cultures were characterized by immunoblotting. Additionally, a multi-tissue array from Pantomics (San Francisco, CA), including 31 human epithelial ovarian tumors arrayed in duplicate was immunostained for MMP-2 and TG2. IHC for TG2 was carried out as previously described (1). To detect MMP-2, we used a polyclonal antibody at concentration of 1:100 (NeoMarkers) and the avidin-biotin peroxidase technique
Transglutaminase and MMP-2

...the Spearman coefficient of correlation was utilized at $p = 0.05$. To analyze differences in IHC intensity between tumors from AS-TG2 and control cells, we used the Cochran-Armitage Trend Test at the 0.05 level of significance.

RESULTS

**TG2 Modulates the Expression of MMP-2 and Other Metastasis-related Proteins**—Having previously demonstrated that TG2 is overexpressed in EOC and enhances intraperitoneal tumor dissemination (1), we explored whether TG2 expression is correlated with that of other genes that function in metastasis. We used SKOV3 cells stably transfected with antisense (AS-TG2) or a control vector (pcDNA3.1) and the metastasis focused OligoGE Superarray. Results presented in Fig. 1 identify several genes with differential expression in AS-TG2 compared with control cells. In cells with decreased expression of TG2, we noted diminished expression of MMP-2, fibronectin, density-regulated protein, breast cancer metastasis suppressor 1, and metastasis-associated 1, and others. Because MMP-2 is a critical mediator of metastasis in EOC (23), we focused on TG2-mediated regulation of MMP-2.

We examined MMP-2 protein expression in AS-TG2 and pcDNA3.1 SKOV3 cells (Fig. 2A, left). A lower level of MMP-2 expression was observed in lysates of AS-TG2 than control cells. MMP-2 is a secreted protein, leading us to assay its expression in conditioned media. Serum-free media conditioned by AS-TG2 cells contained significantly less MMP-2 protein than media conditioned by control cells (Fig. 2A, right). We verified this effect in another ovarian cancer cell line, IGROV-1, in which TG2 expression was knocked down using siRNA. We found decreased expression of MMP-2 in IGROV-1 cells transfected with TG2-directed siRNA compared with cells transfected with scrambled siRNA (Fig. 2B).

To examine how TG2 affects MMP-2 functionally, we compared MMP-2 activity in serum-free medium conditioned by AS-TG2 or pcDNA3.1-transfected SKOV3 cells by using gelatin zymography. Gelatinase A activity was lower in media conditioned for 24 h by AS-TG2 cells compared with control cells (Fig. 2C, left). A time course, in which medium was conditioned for various times, shows that MMP-2 gelatinase activity is substantially lower in media conditioned by AS-TG2 than control cells over a 1- to 24-h time course (Fig. 2C, right).

We next determined whether TG2 regulates MMP-2 expression in other invasive cancer cell lines, including MDA-MB 436 breast cancer cells, and PC-3 prostate cancer cells. TG2 knockdown in these cells was affected by transient transfection of TG2-targeted siRNA. Decreased MMP-2 expression (Fig. 2D, left) and activity (Fig. 2D, right) was observed in MDA-MB 436 cells in which TG2 was down-regulated compared with control. Similarly, MMP-2 expression was diminished in PC-3 cells transfected with TG2-targeted siRNA compared with control (Fig. 2E). These results show that TG2 regulates MMP-2 expression and activity in ovarian and other invasive cancer cell lines.

**TG2 Does Not Directly Activate MMP-2**—Having observed that MMP-2 expression and activity correlate with the expression level of TG2, we tested whether TG2 directly activates MMP-2 in a cell-free system. Recombinant TG2 was incubated for 1 h with recombinant MMP-2 in the presence of a gelatinase substrate peptide, and MMP-2 activity was measured based on cleavage of a fluorescein-labeled peptide. Aminophenylmercuric acetate, a known activator of MMP-2, was used as positive control for the assay. Our results show that TG2 did not activate MMP-2 in vitro (Fig. 3A) and suggest that the effects of TG2 on gelatinase are not direct.

To investigate whether the enzymatic activity of TG2 plays a role in regulating MMP-2 expression and function, we used KCC009, a dihydroisoxazole TG2 inhibitor (27). Treatment of ovarian cancer cells with KCC009 decreased the expression (Fig. 3B) and gelatinase activity (Fig. 3C) of MMP-2, suggesting that the enzymatic activity of TG2 is important in regulating the expression and activity of MMP-2.

**TG2 Regulates MMP-2 Transcription**—To understand the mechanism through which TG2 affects MMP-2 we determined whether TG2 affects the expression level of MMP-2 mRNA. Semi-quantitative PCR showed that the level of MMP-2 mRNA was substantially diminished in AS-TG2 compared with normals.
TG2 Regulates MMP-2 Expression by Activating CREB—CREB has been implicated in the transcriptional regulation of MMP-2 in melanoma cells (28). We examined the MMP-2 gene sequence using promoter motif software (TFM explorer) and identified a potential CREB response element (TGACG) at position −205 to −200 in the MMP-2 promoter (Fig. 5A). This led us to investigate whether CREB mediates the effect of TG2 on MMP-2 in ovarian cancer cells. To examine this possibility, we knocked down CREB using siRNA and found that diminished CREB expression (Fig. 5B, right panel) was associated with a concomitant decrease in the expression of MMP-2 (Fig. 5B, left panel). Using a ChIP assay we found that a CREB antibody pulled down the −280 to −200 MMP-2 promoter sequence in SKOV3 cells (Fig. 5C). This result suggests that CREB directly modulates MMP-2 transcription in ovarian cancer cells. To further evaluate the significance of CREB binding to the MMP-2 promoter in the initiation of MMP-2 transcription, the sequence GTGACGA at position −205 was deleted. We then assayed the capacity of wild-type and mutant MMP-2 to activate an MMP-2 reporter promoter construct. Transcriptional activation was diminished >3-fold by deletion of the CREB binding site from the MMP-2 promoter (Fig. 5D), suggesting that the CREB binding motif in the MMP-2 promoter is functionally important.

Next, we tested whether the expression and activity of TG2 affect CREB. For these experiments, CREB phosphorylation at Ser133 was compared in AS-TG2 and control cells using an antibody that detects CREB phosphorylation on Ser133 and ATF-1 phosphorylation on Ser63, as these sites are homologous. Phosphorylation of CREB and ATF-1 was diminished in nuclear extracts of AS-TG2 compared with control cells (Fig. 6A, left), and to a lesser extent in lysates of AS-TG2 compared with control cells (Fig. 6A, middle). The expression of CREB was equal in AS-TG2 and control cells (Fig. 6A, left). To further evaluate the role of transglutaminase activity in CREB function, we assayed CREB phosphorylation in cells treated with the TG2 enzymatic inhibitor, KCC009. CREB Ser133 phosphorylation (Fig. 6B, left), but not expression of total CREB (Fig. 6B, right) was potently inhibited by KCC009, suggesting that TG2 is essential to CREB activation.

We tested whether the activity of CREB is affected by the expression, or activity, of TG2. A gene reporter assay in which AS-TG2, or the control cells, were transfected with a 3XCRE luciferase promoter showed that CREB transcriptional activity was decreased 11-fold in AS-TG2 cells compared with control cells (Fig. 6C, p = 0.008). Furthermore, inhibition of TG2 with KCC009 decreased CREB transcriptional activity 10-fold (Fig. 6D, p = 0.004).

To begin to define how MMP-2 transcription is affected by TG2-dependent CREB activity, we used a ChIP assay to compare binding of CREB to the MMP-2 promoter in AS-TG2 or control cells. PCR amplification of the MMP-2 promoter in the
Transglutaminase and MMP-2

**Figure 5.** TG2 regulates MMP-2 transcription by activating CREB. A, schematic of a CREB binding site at −205 bp in the human MMP-2 promoter and the positions of primers used to amplify this region in the ChIP assay (below). B, Western blot assay of MMP-2 (left panel) and total CREB (right panel) and GAPDH (control) in SKOV3 cells transfected with siRNA targeting CREB or control siRNA. C, ChIP demonstrates that CREB binds the MMP-2 promoter region. Immunoprecipitated chromatin from SKOV3 cells was used for PCR amplification. A 120-bp PCR product was detected by gel agarose electrophoresis from chromatin immunoprecipitated with an antibody against CREB (lane 4) using primers flanking the CREB-binding region of the MMP-2 promoter (−275 to −156 bp). Positive controls consist of 10% of the total chromatin in the absence of immunoprecipitation (lane 2, input) and chromatin immunoprecipitated with CREB antibody and amplified with primers for NR4A2 promoter, known to be transcriptionally regulated by CREB (lane 3). Negative controls consist of chromatin immunoprecipitated with IgG and amplified with MMP-2 promoter primers (lane 6) and chromatin immunoprecipitated with CREB antibody and amplified with primers to a region of the MMP-2 promoter upstream of the predicted CREB binding site (lane 5). D, MMP-2 promoter activity measured by a reporter assay using SKOV3 cells stably transfected with a MMP-2 promoter luciferase construct or a MMP-2 promoter luciferase construct with a deleted CREB-binding sequence. To control for variations in transfection efficiency, values for luminescence were normalized to Renilla activity. Data are the means of duplicate measurements ± S.D. (p = 0.0002).

chromatin fragment pulled down by a CREB antibody was reduced in AS-TG2 compared with control cells (Fig. 6E, lane 3 versus 4). Equal amounts of MMP-2 promoter amplicons were observed when soluble chromatin from these cells was subjected directly to PCR amplification (input, lanes 1 and 2). The specificity of CREB antibody binding to the MMP-2 promoter is shown by the inability of IgG to immunoprecipitate the PCR product (Fig. 6E, lanes 5 and 6). Furthermore, real-time PCR demonstrated a greater than 2-fold difference in amplification of the MMP-2 promoter pulled down by the CREB antibody (Fig. 6F, p value = 0.03). These results show that, in ovarian cancer cells, CREB activity is modulated by TG2 and affects MMP-2 expression and function.

**TG2-dependent CREB Activation Involves Degradation of PP2A**—We hypothesized that TG2 may act on and affect the expression of PP2A-α. Incubation of cells with the TG2 inhibitor KCC009 increased the expression of PP2A-α, suggesting that TG2 negatively affects PP2A-α expression (Fig. 7H). The major function of TG2 is to cross-link proteins that may then be targeted for degradation. To test whether PP2A-α is a target of TG2, we tested its stability in cells expressing low or high levels of TG2 treated with cycloheximide. In cells expressing low TG2 levels, PP2A-α expression remained stable during cycloheximide treatment, whereas, in control cells, PP2A-α declined (Fig. 7I). In addition, PP2A-α mRNA level was equal in AS-TG2 and control cells, supporting the concept that TG2 affects PP2A-α post-translationally (Fig. 7I). Our observations suggest that TG2 increases CREB activity by promoting degradation of PP2A-α, and affecting transcription of MMP-2 (Fig. 7K).

**TG2 Expression and MMP-2 Expression Are Correlated in Vivo**—We previously showed that intraperitoneal inoculation of nude mice with SKOV3 cells with diminished TG2 expression led to decreased peritoneal dissemination (1). Now we...
examined the effects of TG2 on MMP-2 in vivo by using IHC, postulating that the effects of TG2 on MMP-2 expression correlate with metastasis. We found decreased expression of MMP-2 in xenografts derived from AS-TG2-transfected cells compared with control cells (supplemental Fig. S1A). A higher proportion of AS-TG2-derived tumors displayed low intensity MMP-2 staining, whereas more tumors from vector-transfected cells displayed high intensity (3+) MMP-2 staining (supplemental Fig. S1B, p = 0.04). Coordinate diminished expression of TG2 and MMP-2 was also observed in lysates of cells cultured from AS-TG2 compared with control xenografts (supplemental Fig. S1C). We also immunostained human ovarian tumors on a multi-tissue array. Moderate to strong (2+ to 3+) TG2 and MMP-2 staining was observed in 92 and 96% of the tumors, respectively (supplemental Fig. S2) and the Spearman coefficient of correlation was r = 0.41 (p = 0.01), pointing to a significant correlation in vivo between TG2 and MMP-2.

**DISCUSSION**

We previously showed that TG2 is overexpressed in ovarian tumors and plays a role in peritoneal dissemination (1). The present study provides insight into how TG2 facilitates the spread of ovarian cancer. We show that TG2 regulates MMP-2 expression in cancer cells and in human ovarian tumors. We demonstrate that TG2 induces the expression of MMP-2 in cancer cells, in human ovarian tumors, and in xenografts derived from cancer cells. The effect of TG2 on MMP-2 expression, and consequently MMP-2 activity, is mediated by the capacity of TG2 to activate CREB. We identify PP2A-α as a phosphatase that suppresses CREB activity. We also show that PP2A-α is a component of a complex that contains TG2 and that the association of TG2 and PP2A-α is important as TG2 induces degradation of PP2A-α. This is the first report implicating TG2 in the regulation of MMP-2 expression and activity. This is also the first study to show that TG2 regulates CREB phosphorylation, through an effect on a CREB-associated phosphatase, in ovarian cancer cells. A consequence of these events is that TG2 affects the transcription and expression of proteins important to the spread of cancer, including MMP-2.

The correlation of TG2 and MMP-2 expression levels is not surprising, because the proteins exert complementary functions in the extracellular milieu. TG2 cross-links proteins in the extracellular matrix, while MMPs break down the matrix through their peptidase activities. Interestingly, membrane-type MMPs degrade cell surface transglutaminase, thereby suppressing cell adhesion and motility on fibronectin (34). Further-
Transglutaminase and MMP-2

more, MMP-2, in concert with membrane-type MMPs, degrades cell surface TG2 in fibrosarcoma cells and inhibits its enzymatic function (35). The proteins appear to interact directly, with the predicted docking site located in proximity to the TG2 catalytic domain. This interaction may explain the inhibitory effect of MMP-2 on the function of transglutaminase (35). In the present study, we demonstrate the reverse mechanism. We show that TG2 regulates the expression and function of MMP-2 in cancer cells and tumors. Our data support the existence of a complex interplay between forces that strengthen those that loosen (i.e. MMPs) the extracellular matrix.

Regulation of MMP-2 by TG2 is transcriptional and mediated by CREB. CREB is an ubiquitously expressed transcription factor belonging to the leucine zipper superfamily that regulates the expression of many genes in response to cAMP (36, 37). CREB is inactive when dephosphorylated and is phosphorylated at Ser133 by cAMP-dependent protein kinase A, calcium2+-calmodulin, Akt, p38 MAPK, and other kinases activated by growth factors or stress (38–40). CREB phosphorylation at Ser133 increases its transcriptional activity (41), by altering its association with adaptor proteins that engage the transcriptional complex. TG2 activates CREB in neurons, contributing to their differentiation, however the exact mechanism was not discovered (42). It was speculated that TG2 modulates the activity of adenylyl cyclase, by affecting its conformation, and thus contributes to increased cAMP production and enhanced CREB activity (42). Here we show that TG2 expression and function affect CREB phosphorylation and transcription in ovarian cancer cells and that the TG2 inhibitor, KCC009, inactivates the transcription factor.

To our knowledge, this is the first evidence that a transglutaminase inhibitor affects CREB phosphorylation in cancer cells. As CREB regulates the transcription of many oncogenic proteins, this mechanism may be implicated in the anti-cancer properties of TG2 inhibitors (27, 43).

Our findings implicating CREB in the regulation of MMP-2 in ovarian cancer cells are consistent with those in a report on melanoma. That study identified CREB as a modulator of MMP-2 expression in response to platelet activating factor (28). However, our study significantly adds to those observations by showing that TG2 activates CREB by inducing the degradation of PP2A-a. We assayed for, but did not observe, an effect of TG2 on expression of the PP1 phosphatase, and were not able to co-immunoprecipitate TG2 and PP1 (data not shown). This shows that TG2 does not act promiscuously but rather targets specific proteins. A recent study identified PTEN as a TG2 target in pancreatic cancer cells (44). This

![FIGURE 7. PP2A-a is a target of TG2. A, AS-TG2 and pcDNA3.1 cells were incubated in the absence or presence of 100 nM okadaic acid (OA) for 3 h in serum-free media. A Western blot prepared from nuclear extracts of the cells was probed with antibodies to pSer133CREB and poly(ADP-ribose) polymerase (PARP). B, SKOV3 cells were transfected with control siRNA or siRNAi targeting PP2A-a. A Western blot prepared from lysates of the cells was probed with antibodies to PP2A-a and GAPDH. C, Western blot analysis for pSer133CREB in nuclear extracts of SKOV3 cells transfected with PP2A-a targeted siRNA or control siRNA. Poly(ADP-ribose) polymerase was used as loading control. D, a Western blot prepared from lysates of AS-TG2 or pcDNA3.1 SKOV3 cells was probed with antibodies to PP2A-a, TG2, and GAPDH. E, a Western blot prepared from lysates of OV90 cells transfused with human TG2 or a control vector was probed with antibodies to PP2A-a, TG2, and GAPDH. F, a Western blot prepared from lysates of A2780 cells transfused with human TG2 or a control vector was probed with antibodies to PP2A-a, TG2, and GAPDH. G, Western blots prepared from TG2 (left) or PP2A-a (right) immunoprecipitates of SKOV3 cells were probed with anti-TG2 and anti-PP2A-a. Immunoprecipitations with IgG served as controls for each experiment. H, SKOV3 cells were incubated in the absence or presence of KCC009 for 24 h. A Western blot prepared from lysates of the cells was probed with antibodies to PP2A-a and GAPDH. Densitometry quantifies PP2A-a expression. I, AS-TG2- and pcDNA3.1-transfected cells were treated with 20 μg/ml cycloheximide for up to 6 h. A Western blot prepared from cell lysates was probed with antibodies to PP2A-a and GAPDH, a loading control. Densitometry quantifies PP2A-a expression. J, semiquantitative reverse transcription-PCR for PP2A-a and GAPDH in SKOV3 cells stably transfected with AS-TG2 and pcDNA3.1. K, proposed model for regulation of MMP-2 by TG2 in ovarian cancer cells.](Image 49x358 to 407x733)
observation, together with our report, suggests that TG2 targets proteins with tumor suppressor activity, particularly phosphatases, for degradation.

Our observations identify a mechanism through which TG2 modulates gene expression and promotes the spread of ovarian cancer. By promoting the degradation of PP2A-α, and thereby activating CREB, TG2 contributes to the transcriptional regulation of MMP-2. Delineation of the consequences of the capacity of TG2 to act through CREB by targeting PP2A-α and perhaps other tumor suppressor proteins to induce changes of gene expression is likely to provide insight into genetic changes that promote cancer progression. Identifying and characterizing molecular events such as those described here is a step toward the development of targeted cancer therapies.

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Transglutaminase and MMP-2

The role of transglutaminase (TG2) in cancer progression and invasion has been extensively studied. TG2 is a Ca2+ dependent enzyme that catalyzes the formation of covalent bonds between amino groups of lysine or arginine residues. This reaction results in the crosslinking of extracellular matrix proteins and thus stabilizes the tumor microenvironment. Additionally, TG2 has been shown to regulate gene expression by targeting the transcription factor CREB. This regulation is mediated through the PP2A-α phosphatase, which dephosphorylates CREB, thereby promoting its transcriptional activity. The ability of TG2 to modulate gene expression and promote the spread of ovarian cancer suggests that targeting this enzyme may represent a potential therapeutic strategy for the treatment of this disease.