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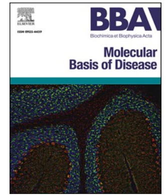

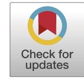

Transglutaminase 2 promotes epithelial-to-mesenchymal transition by regulating the expression of matrix metalloproteinase 7 in colorectal cancer cells via the MEK/ERK signaling pathway

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A B S T R A C T

Tissue transglutaminase 2 (TGM2) and matrix metalloproteinase 7 (MMP7) are suggested to be involved in cancer development and progression, however, their specific role in colon cancer remains elusive. The present study investigated whether TGM2 and MMP7 influence epithelial-mesenchymal-transition (EMT) processes of colon cancer cells.

TGM2 was either overexpressed or knocked down in SW480 and HCT-116 cells, and MMP7 expression and activity analyzed. Conversely, MMP7 was silenced and its correlation with TGM2 expression and activity examined. Co-immunoprecipitation served to evaluate TGM2-MMP7-interaction. TGM2 and MMP7 expression were correlated with invasion, migration, EMT marker expression (E-cadherin, N-cadherin, Slug, Snail), and ERK/MEK signaling.

TGM2 overexpression enhanced MMP7 expression and activity, promoted cell invasion, migration and EMT, characterized by increased N-cadherin and Snail/Slug expression. TGM2 knockdown resulted in the opposite effects. Knocking down MMP7 was associated with reduced TGM2 protein expression, cell invasion and migration. Down-regulation of MMP7 diminished ERK/MEK signaling, whereas its up-regulation activated this pathway. The ERK-inhibitor GDC-0994 blocked phosphorylation of MEK/ERK and suppressed TGM2 and MMP7.

TGM2 communicates with MMP7 in colon cancer cells forces cell migration and invasion by the MEK/ERK signaling pathway and triggers EMT. Inhibiting TGM2 could thus offer new therapeutic options to treat patients with colon cancer, particularly to prevent metastatic progression.

1. Introduction

Globally, colorectal cancer (CRC) ranks the fourth most common malignancy and is the third leading cause of cancer-related mortality in 2020 [1]. 20 % of patients with CRC have already developed metastases at first diagnosis, and further 25 % of patients initially presenting with localized disease will subsequently develop metastases.

Despite the implementation of multimodal systemic therapy including targeted therapy and immunotherapy, the prognosis of metastatic CRC remains unsatisfactory with a 5-year survival rate of only 15 % [2]. Innate and acquired resistance are the most prominent factors responsible for treatment failure. It is a big challenge to improve the current treatment protocols and to offer the patients a precise and individualized therapeutic option. Unfortunately, the genetic heterogeneity of the tumor and the complex interactions between intracellular signaling pathways and the tumor microenvironment make it difficult to identify and select highly specific druggable targets.

Tissue transglutaminase 2 (TGM2) is a multifunctional calcium-

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dependent protease that belongs to the transglutaminase family. TGM2 catalyzes the cross-linking reaction between protein glutamine residues and lysine residues, which are involved in a variety of physiological and pathological processes, including epithelial-to-mesenchymal transition (EMT), cancer development and progression. Studies on breast cancer cells demonstrated increased expression of TGM2 to be associated with acquisition of a mesenchymal morphology and induction of tumor proliferation [3]. TGM2 promotes EMT in hepatocellular carcinoma (HCC) cells by linking inflammatory effects and pseudohypoxia in the HCC microenvironment [4]. In CRC, the expression of TGM2 correlated with the acquisition of stem cell-like characteristics in CRC cell lines including the expression of EMT markers [5]. Furthermore, knockdown of TGM2 increased the chemosensitivity of CRC cells to 5-Fluorouracil indicating that TGM2 might also be involved in resistance development [6]. In fact, Kaplan-Meier plots and log-rank test showed that CRC patients with high TGM2 expression have significantly worse prognosis in overall and disease-free survival. Accordingly, CRC patients with metastatic disease exhibited significantly higher TGM2 expression levels in the primary tumor compared to patients with localized tumor stages [7]. A recent investigation on CRC tissue documented an elevated expression and increased enzymatic activity of TGM2, compared to matched normal colon mucosa cells. It has been assumed in this study that TGM2 may serve as an essential survival factor in CRC cells presumably by inhibition of the central tumor suppressor p53 [8]. Whether TGM2 plays a role in EMT processes in CRC, however, still remains unclear.

Aside from TGM2, the proteolytic enzyme matrix metalloproteinase 7 (MMP7) has gained high interest as an attractive target molecule, since it is closely involved in cancer development, proliferation, differentiation, and metastasis [9]. MMP7 has been demonstrated to correlate with the recurrence of CRC and is, therefore, considered to be an effective biomarker having prognostic and diagnostic value [10]. Interestingly, even urinary detection of MMP7 may allow early CRC detection [11]. Based on a transcriptome analysis, MMP7 ranked as one of the most significantly downregulated EMT genes upon TGM2 knockdown in CRC cells [8]. This finding is highly intriguing as it suggests the potential for cross-communication between TGM2 and MMP7. Therefore, targeting the TGM2-MMP7 axis might emerge as a powerful strategy to treat CRC. Here we aimed to assess whether TGM2 and MMP7 interact with each other and to investigate the underlying mechanisms modulating EMT processes in CRC.

2. Materials and methods

2.1. Cell Culture

The human colon cancer cell lines SW480 (RRID:CVCL_0546) and HCT 116 (RRID:CVCL_0291) were ordered from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Heidelberg, Germany). All cells were cultured in McCoys 5a growth medium containing L Glutamine, 10 % fetal bovine serum, 10 mM Hepes, 100 μg Gentamycin sulfate. Cells were grown at 37 °C under 5 % CO_2.

2.2. Antibodies

The antibodies used were as follows: TGM2 (Abcam Cat# ab2386, RRID:AB_2287299), MMP7 (Abcam Cat# ab205525, RRID:AB_2861279), N-Cadherin (Abcam Cat# ab76011, RRID:AB_1310479), *E*-Cadherin (Abcam Cat# ab231303, RRID:AB_2923285), Snail/Slug (Abcam Cat# ab180714, RRID:AB_2728773), phospho-p90RSK (Cell Signaling Technology Cat# 11989, RRID:AB_2687613), phospho-MEK1/2 (Cell Signaling Technology Cat# 9154, RRID:AB_2138017), β-actin (Cell Signaling Technology Cat# 3700, RRID:AB_2242334) and pERK1/2 (BD Biosciences Cat# 612358, RRID:AB_399647). Secondary horseradish peroxidase-conjugated antibodies against mouse (Abcam Cat# ab205719, RRID:AB_2755049). Detailed information is provided in Supplements (Supplement S1, antibodies).

2.3. Generation of shRNA constructs and transduction

For knockdown experiments, two targeting shRNAs against TGM2 were constructed as follows (shTGM2–1; 5′CCGGTATCACCCACACCTACAA ATACTCGAGTATTTGTAGGTGTGGGTGATATTTTG-3′, and shTGM2–2; 5′CCGGTTGTGCTGGGCCACTTCATTTCTCGAGAAATGAAGTGGCCCAGC-ACAATTTTTG-3′) and non-targeting control sequence shCtrl; (ACCGGC TGAAGAGCCTGATCAA). shRNAsequences were then cloned into a third generation self-inactivating HIV-1 based lentiviral vector system on the backbone of pLKO.1. Lentiviral packaging was carried out as previously described [12,13]. Transcription efficiency of cells was verified and monitored by detecting red fluorescent protein (tdTOMATO) via flow cytometry. $0.1*10^6$ SW480 and $0.05*10^6$ HCT-116 were transduced with lentiviral vectors at a multiplicity of infection (MOI) of 5 for 48 h.

2.4. Generation of TGM2 overexpressing cell lines

To overexpress TGM2, a coding sequence of human TGM2-isoform 1 was cloned into the lentiviral expression vector pRRL.PPT.SFFV.IRES.VENUSnucmem that coexpresses a nuclear membrane-bound fluorescent protein VENUS. All constructs were verified by Sanger sequencing. The parental vector pRRL.PPT.SFFV.IRES.VENUSnucmem was used as vector control. TGM2 overexpression was verified by western hybridization [8].

2.5. Quantitative PCR

RNA was extracted using Nucleospin RNA extraction kit from Macherey-Nagel (Düren, NRW, Germany) according to manufacturer's protocol. 250 ng of RNA was reversed transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's protocol. Validated gene specific primers were obtained from Bio-Rad: MMP7, Unique Assay ID:qHsaCED0044775;TGM2, Unique Assay ID: qHsaCID0007428 and GAPDH, Unique Assay ID: qHsaCED0038674. PCR runs were performed on MX3005p (Stratagene, San Diego, USA). To verify the specificity of the amplification, melt curve was generated. Expression levels were normalized with GAPDH (Housekeeping gene). Relative fold change of MMP7 and TGM2 gene expression were calculated as delta-delta cycle (ΔΔCT). Each experiment was performed in duplicate and repeated in three independent experiments.

2.6. Western hybridization

Cells were lysed in M-PER Mammalian extraction Reagent supplemented with 1× Halt protease inhibitor (Thermo Fisher, USA) according to manufacturer's protocol. 50 μg of protein was applied to SDS-PAGE and transferred to a PVDF membrane using the Trans-Blot Turbo-Transfer System (Bio-Rad). Primary antibodies were incubated over night at 4 °C, washed and further incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Antigen and antibody complex was visualized with ECL (Enhanced Chemiluminescence) (GE Healthcare). Membrane was analyzed and documented on VilberLourmat Fusion FX system. The band density was quantified using Bio1D software.

2.7. MMP7 activity assay

Cells were lysed in 50 mM Tris-HCL buffer pH 7.0 containing 0.1 % Triton X-100 and homogenized in Precellys homogenizer at 6500 rpm for 5 s, 2 rounds. Homogenized samples were centrifuged at 13,000 xg for 10 min to collect supernatant and immediately frozen at −80 °C. 5 μg of protein was analyzed for the presence of active MMP7 using Quickzyme MMP7 activity assay (Quickzyme) according to manufacturer's instruction. The active MMP7 present in the sample was detected by

adding detection enzyme followed by addition of chromogenic substrate and measured at 405 nM by ELISA plate reader (TECAN).

2.8. MMP7 siRNA transfection

Transient transfection of short interfering RNA (siRNA) was performed with sequences targeting MMP7 (sc-41,553, Santa Cruz Biotechnology Inc., CA, USA). Scrambled siRNA-A (sc-37,007, Santa Cruz Biotechnology) was used as control and to evaluate RNAi off target effects. siRNA transfection system (sc-45,064, Santa Cruz Biotechnology Inc.) was used for transfections. To verify the effectivity of MMP7 knockdown, protein lysates were analyzed by western hybridization.

2.9. Cell invasion assay

Cell invasion assays were conducted in 24-well using Matrigel (Corning, NY, USA) coated 8.0 μm pore ThinCert translucent PET membrane (Greiner bio-one, Frickenhausen, Germany) according to the manufacturer's instructions. 24 h siMMP7 transfected (knockdown) or overexpressing TGM2 CRC cells were dislodged, counted and resuspended at 20,000 cells in 200 μl serum-free medium. Cell suspensions were then pipetted into the upper compartment while in the lower compartment 700 μl culture medium containing 10 % FBS were added. After 24 h incubation, the cells that did not invade to the lower chamber were removed using a cotton swab and the invaded cells found underneath the membrane were fixed with 4 % paraformaldehyde for 10 min and stained with propidium iodide for 10 min at room temperature or visualized by green fluorescent protein (Venus GFP). Total invaded cells were counted per well and documented using Zeiss Axio Observer Z-1 Microscope (Carl Zeiss Microscopy, Oberkochen, Germany).

2.10. Cell migration, wound healing (Scratch) assay

24 h siMMP7 transfected or transduced TGM2 overexpressing CRC cell lines were seeded at $0.5*10^6$ cells/well (HCT-116) and $0.7*10^6$ cells/well (SW480) in a six-well plate until 100 % confluence was achieved for 24–48 h. A vertical wound was generated using a 200 μl pipette tip scratched through the cell monolayer. The medium and cell debris were then removed and freshly replaced. The gap area was assessed on Day 0 and at Day 2. Images were obtained by Zeiss AXIO Observer Z-1 at 10 × 10 magnification. Wound closure was determined by measuring the gap area using Zeiss AxioVision software (v 4.7).

2.11. TGM2 activity assay

Transamidase activity of TGM2 of 48 h lentiviral transduced SW480 and HCT-116 were determined. Protein lysates were extracted using M-Per lysis medium with protease inhibitor cocktail. TGM2 enzymatic activity was determined using Tissue Transglutaminase Microassay kit (Zedira, Darmstadt, Germany) according to the manufacturer's instructions.

2.12. 2.12. Immunofluorescence

TGM2 overexpressing cells were seeded in 8 well chamber slide (Corning) and were cultured for 24 h. Cells were fixed with 4 % formaldehyde-methanol free at room temperature for 10 min and subsequently permeabilized with ice cold methanol for 10 min at room temperature. Cells were incubated overnight at 4 °C in the dark with following primary antibodies, *E*-Cadherin, TGM2, and MMP7 were diluted at 1:100, N-Cadherin was diluted at 1:500 and Snail/Slug was diluted at 1:50. Cells were washed and stained with secondary Phycoerythrin-conjugated antibodies against mouse (Abcam Cat# ab97024, RRID:AB_10698222) and rabbit (Abcam Cat# ab7007, RRID: AB_955477) at a dilution of 1:100 and incubated at room temperature for 1 h. Cells were washed and mounted with Antifading kit with DAPI (Invitrogen by Themo Fischer scientific, USA). Slides were cured overnight at 4 °C in the dark and then imaged and analyzed in Axio observer Z1 (Zeiss Germany) on the following day.

2.13. Protein simple western

Cells were lysed in M-PER Mammalian extraction Reagent containing 1× Halt Protease Inhibitor (Thermo Fisher, USA) according to manufacturer's protocol. Lysed cells were homogenized by Precellys Homogenizer (Peqlab). 0.05 μg of Protein was applied on a ProteinSimple 12–230 KDa capillary cartridge separation module (Biotechne) according to manufacturer's instruction. Dilution of primary antibodies were as follows; TGM2 (1:200), MMP7 (1:100), Snail/Slug (1:50), N- and *E*-Cadherin (1:100) and β-actin (1:100). All antibodies specific to cell signaling pathways, p-Erk1/2, p-MEK1/2, p-p90RSK were diluted at 1:100. Separation of proteins and immunodetection were done in a fully automated capillary system (WES), Jess, Simple Western System technology (Bio-Techne, Minneapolis, MN, USA).

2.14. Co-immunoprecipitation

Immunoprecipitation (IP) was performed using Dynabeads™ Protein G Immunoprecipitation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Whole cell lysates were extracted using Pierce™ IP lysis buffer containing 1× Halt protease inhibitor (Thermo Fischer, USA). 5 μg of mouse anti-TGM2 antibody (CUB7402, Abcam) was incubated with the magnetic beads to form antibody-bead complex. The Protein extracts were added to the magnetic antibody complex for 1 h to bind TGM2 and was further eluted at 70 °C for 10 min. The eluted target protein was applied on a ProteinSimple 12–230 KDa capillary cartridge separation module by Simple Western technology (WES) (Bio-Techne, Minneapolis, MN, USA) for protein detection of TGM2 and MMP7. For input control, TGM2, MMP7 and β-actin from whole cell lysates were also determined.

2.15. Correlation analysis

The correlation between TGM2, MMP7, and EMT-related signaling has been evaluated using data from the TCGA and Gepia2 database.

2.16. Statistical analysis

Statistical analysis and figures were performed using GraphPad Prism 9.0 (RRID:SCR_002798) (GraphPad Software Inc. La Jolla, CA, USA). All values are presented as means ± SD from at least three independent experiments, unless otherwise stated. For the comparison of two independent groups, either an independent *t*-test (unpaired) was performed for normally distributed data, or the non-parametric Mann-Whitney *U* test was used if the data did not meet the assumptions of normality. For comparisons involving more than two groups, a one-way ANOVA followed by Tukey's post hoc test was applied to assess the differences between group means. Spearman's correlation analysis was conducted to evaluate the correlation between expression levels, as this method does not assume normality and is appropriate for ordinal or non-normally distributed data. A *p*-value of <0.05 was considered as statistically significant.

3. Results

3.1. TGM2 regulates MMP7 expression and activity

TGM2-MMP7-interaction was explored in TGM2 knockdown and TGM2 overexpressing CRC cells. TGM2 knockdown in both SW480 and HCT-116 cells was associated with diminished MMP7 gene (Fig. 1A) and protein expression (Fig. 1B) and reduced MMP7 activity (Figure1 C). In contrast, TGM2 overexpression resulted in enhanced MMP7 gene

(Fig. 1D) and protein expression (Fig. 1E), along with up-regulated MMP7 activity (Fig. 1F), in SW480 and HCT-116 cells (all Western blot data are also shown in supplements S2, Western blots).

3.2. MMP7 knockdown decreases TGM2 expression and CRC cell invasion and migration

MMP7 knockdown by siRNA targeting in SW480 and HCT-116 cells reduced MMP7 protein expression (Fig. 2A) and MMP7 enzymatic activity (Fig. 2B). Interestingly, reduced MMP7 was also associated with decreased TGM2 protein expression as demonstrated in Fig. 2A. 24 h after MMP7 knockdown, cell invasion and migration assays were performed. Invasion of SW480 and HCT-116 cells were significantly reduced upon MMP7 knockdown in comparison to cells transfected with non-targeting siRNA (siCtrl) (Fig. 2C). Accordingly, cell migration of MMP7 knockdown cells was considerably suppressed, compared to the controls as assessed by the Scratch wound healing assay (Fig. 2D).

3.3. TGM2 overexpression enhances CRC cell invasion and migration and induces epithelial-mesenchymal transition (EMT)

Next, we assessed whether high levels of TGM2 promote CRC cell invasion, migration and EMT. Indeed, the number of invasive cancer cells overexpressing TGM2 was significantly increased, compared to the controls (Fig. 3A). Moreover, TGM2 overexpression markedly promoted migration of cancer cells, leading to faster wound closure on day 2, compared to control cells (Fig. 3B). We further tested whether TGM2-induced cell invasion depends on MMP7 activity by knocking down MMP7 protein expression in TGM2 overexpressing CRC cells. Indeed, downregulation of MMP7 protein in TGM2 overexpressing SW480 cells significantly blocked invasion (Fig. 3C) and migration (Fig. 3D), supposing a significant role of MMP7 in TGM2-mediated EMT. This process was associated with a lowered TGM2 expression level (Fig. 3E).

Subsequently, Western hybridization analysis was conducted to evaluate the link between TGM2 and EMT. The EMT markers N-Cadherin, Snail/Slug, and MMP7 were all enhanced in TGM2 overexpressing SW480 (Fig. 4A) and HCT-116 cells (Fig. 4B), while shRNA mediated TGM2 knockdown (shTGM2) was associated with suppressed expression of N-Cadherin, Snail/Slug, and MMP7, compared to the respective controls. *E*-Cadherin expression remained unchanged in both TGM2 overexpressing and TGM2 knockdown CRC cells (Fig. 4A-C). The results were verified by immunofluorescence staining, demonstrating increased levels of N-Cadherin, Snail/Slug and MMP7 in TGM2 overexpressing CRC cells (Fig. 4C).

3.4. TGM2 directly interacts with MMP7 and is linked to ERK/MEK signaling

MMP7 is known to be involved in cancer cell invasion through the ERK/MEK signaling, thus we assessed whether TGM2-mediated EMT is linked to this pathway. The expression of phosphorylated MEK/ERK signaling proteins in SW480 cells was analyzed after shRNA-mediated knockdown of TGM2. Protein analysis revealed that MMP7 was down-regulated along with diminished levels of p-ERK1/2, p-MEK1/2 and p-

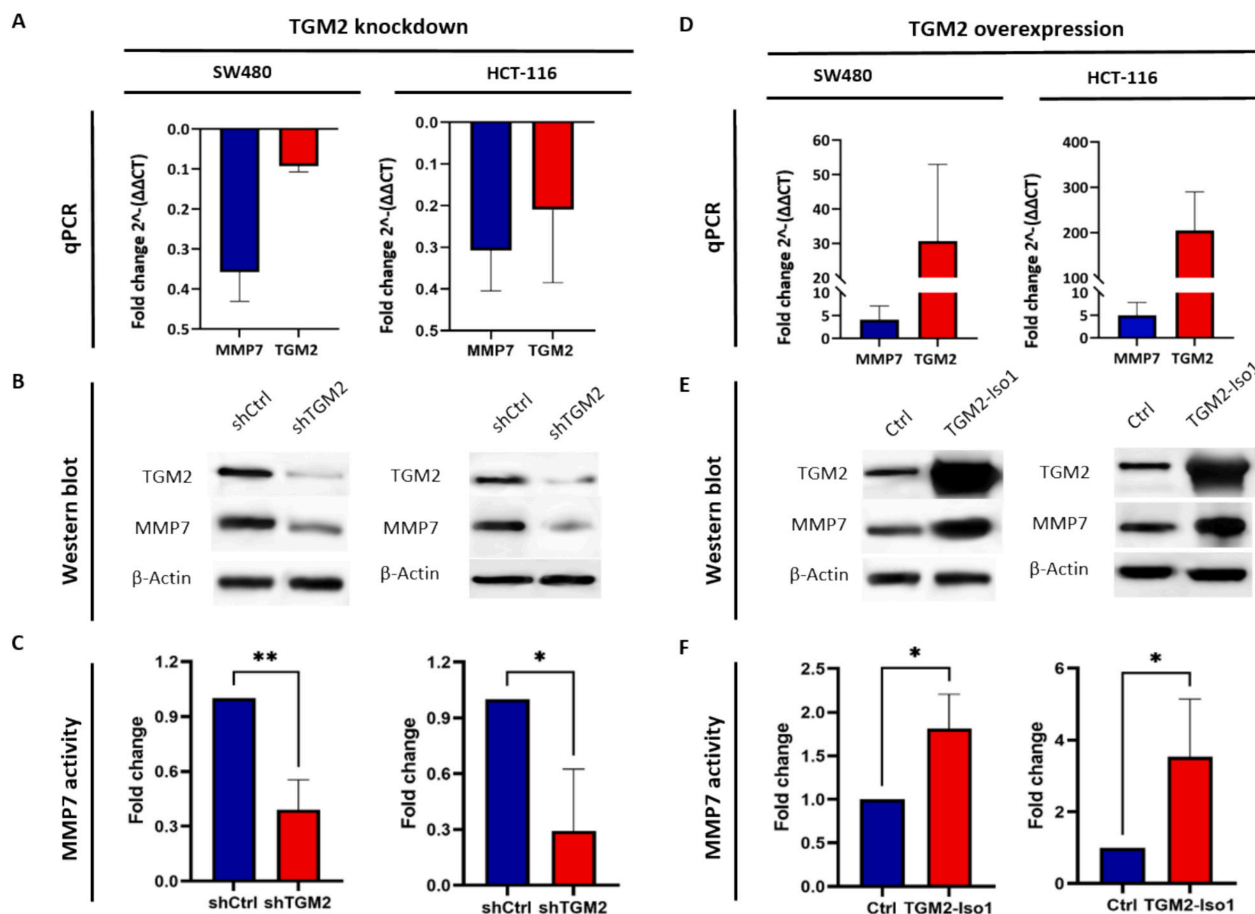

Fig. 1. Knockdown and overexpression of TGM2 regulates MMP7 expression and enzymatic activity in CRC cell lines. A-C. TGM2 and MMP7 gene (A) and protein expression (B), and MMP7 enzymatic activity (C) in TGM2 knockdown SW480 and HCT-116 cells. D-F. TGM2 and MMP7 gene (D) and protein expression (E), and MMP7 enzymatic activity (F) in TGM2 overexpressing SW480 and HCT-116 cells. *$p < 0.05$, **$p < 0.01$ Mann-Whitney *U* test. shCtrl, Short hairpin RNA control; shTGM2, Short hairpin TGM2, Ctrl, Vector control; TGM2-iso1, TGM2 isoform 1.

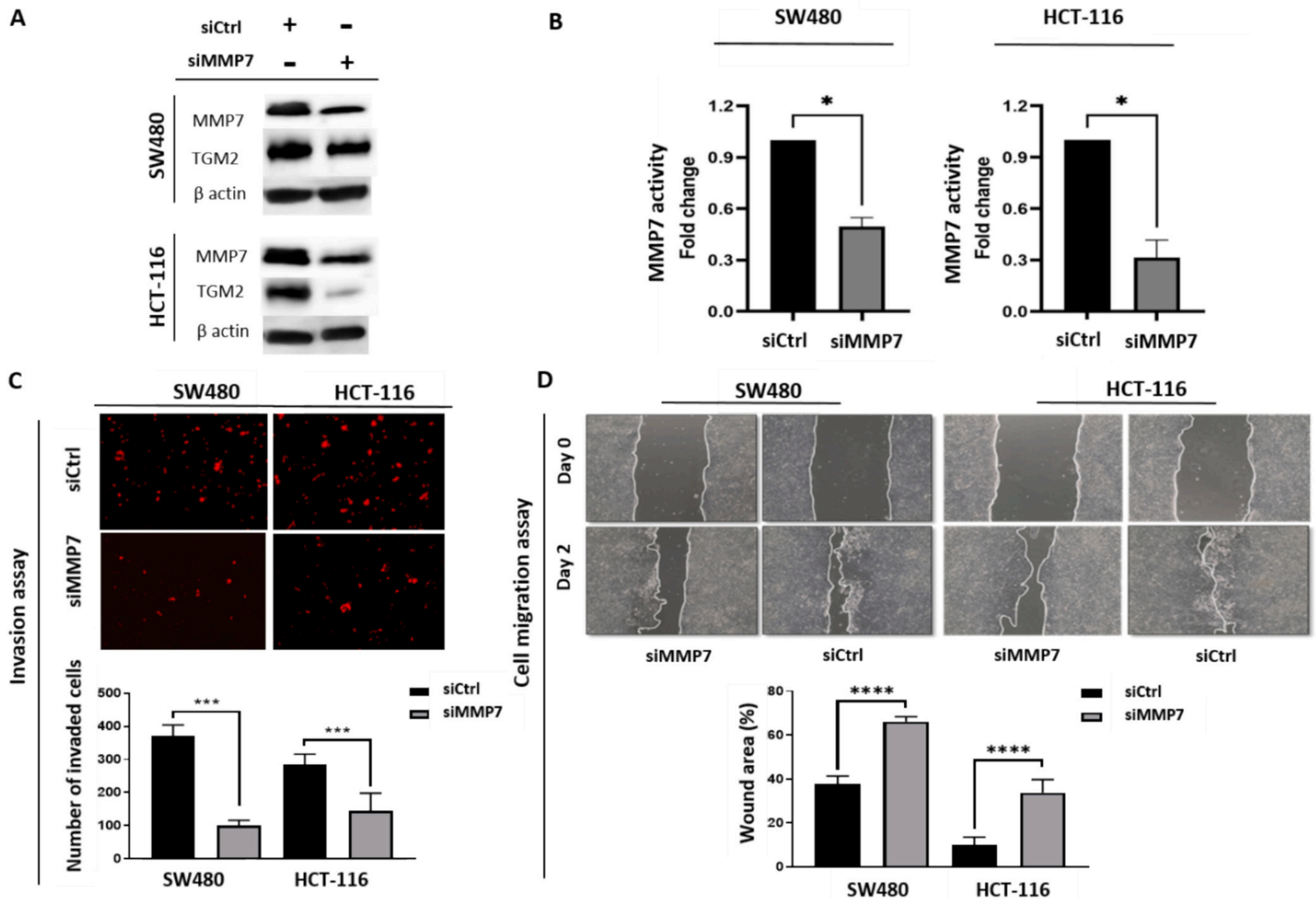

Fig. 2. MMP7 siRNA inhibits invasion and migration capability of CRC cells. A. Protein detection of MMP7 and TGM2 in SW480 and HCT-116 cells treated with siMMP7. B. MMP7 activity in CRC cell lines treated with siMMP7 or siCtrl. C. Chemotaxis of SW480 and HCT-116 cells. Invaded cells were stained with propidium iodide (PI) (red) and counted (bar chart). D. Representative photomicrographs (magnification = 50×) of cell migration (Scratch wound healing assay) of siMMP7 treated SW480 and HCT-116 cells. Mean wound areas of three independent experiments are shown in the bar chart. All assays were performed at 24 h post siMMP7 transfection ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, Mann-Whitney U test. siCtrl, Small interfering RNA control; siMMP7, Small interfering RNA MMP7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

p90RSK (Fig. 5A). Vice versa, in SW480 cells overexpressing TGM2, upregulation of MMP7 was observed with enhanced p-ERK1/2, p-MEK1/2 and p-p90RSK compared to the vector control (Fig. 5B).

To investigate the direct interaction between MMP7 and TGM2, TGM2 was immunoprecipitated in SW480 and HCT-116 cells and MMP7 measured thereafter. Fig. 5C depicts a notable amount of MMP7 detected in both SW480 and HCT-116 samples, whereas no MMP7 and TGM2 were seen at all in the mouse lgG control. Moreover, the pull-down of MMP7 was considerably enhanced in SW480 and HCT-116 cells after overexpressing TGM2 compared to the corresponding control (Fig. 5C).

3.5. ERK1/2 blockade correlates with TGM2 and MMP7 suppression

GDC-0994, a selective ERK1/2 inhibitor, effectively blocked phosphorylation of MEK1/2, ERK1/2, and 90RSK. Importantly, it also diminished the expression of TGM2 and MMP7 in TGM2 overexpressing SW480 cells (Fig. 6).

3.6. Correlation analysis

The in vitro findings are supported by TCGA data and datasets from Gepia. TGM2 positively correlated with Snail, Slug, N-cadherin, Erk1, p90RSK, and MEK (Fig. 7A). Positive correlation was also seen between MMP7 and TGM2 (Fig. 7B).

4. Discussion

EMT is a crucial mechanism of tumor progression and resistance development. Hence, targeting and reversing EMT processes might represent an innovative approach to treat cancer and to increase the therapeutic efficacy of established drugs. The evidence presented here suggests that TGM2 and MMP7 in concert drive EMT progression, resulting in an increased invasiveness of CRC cell lines in vitro. Consequently, we postulate that TGM2 may emerge as an important drug target in the treatment of CRC. TGM2 has been linked to progression of several tumor entities, as ovarian, pancreatic, lung, and breast cancer [14]. Studies on CRC are quite limited. 15 years ago, gene expression analysis in paired CRC cases have been carried out demonstrating a higher TGM2 expression in CRC tissue than in the corresponding normal tissue [15]. TGM2 expression as a marker of poor outcome in CRC has also been documented by others, with high expression level to be associated with increased relapse risk and poor overall survival [16].

Novel data have meanwhile been published documenting an association between TGM2 expression levels and worse prognosis in CRC patients [7]. However, the underlying molecular mode of action, has not been fully elucidated and only few data are available dealing with this issue.

Based on protein-protein interaction analyses, TGM2 binds to the tumor suppressor p53 in CRC cells, thereby preventing apoptosis

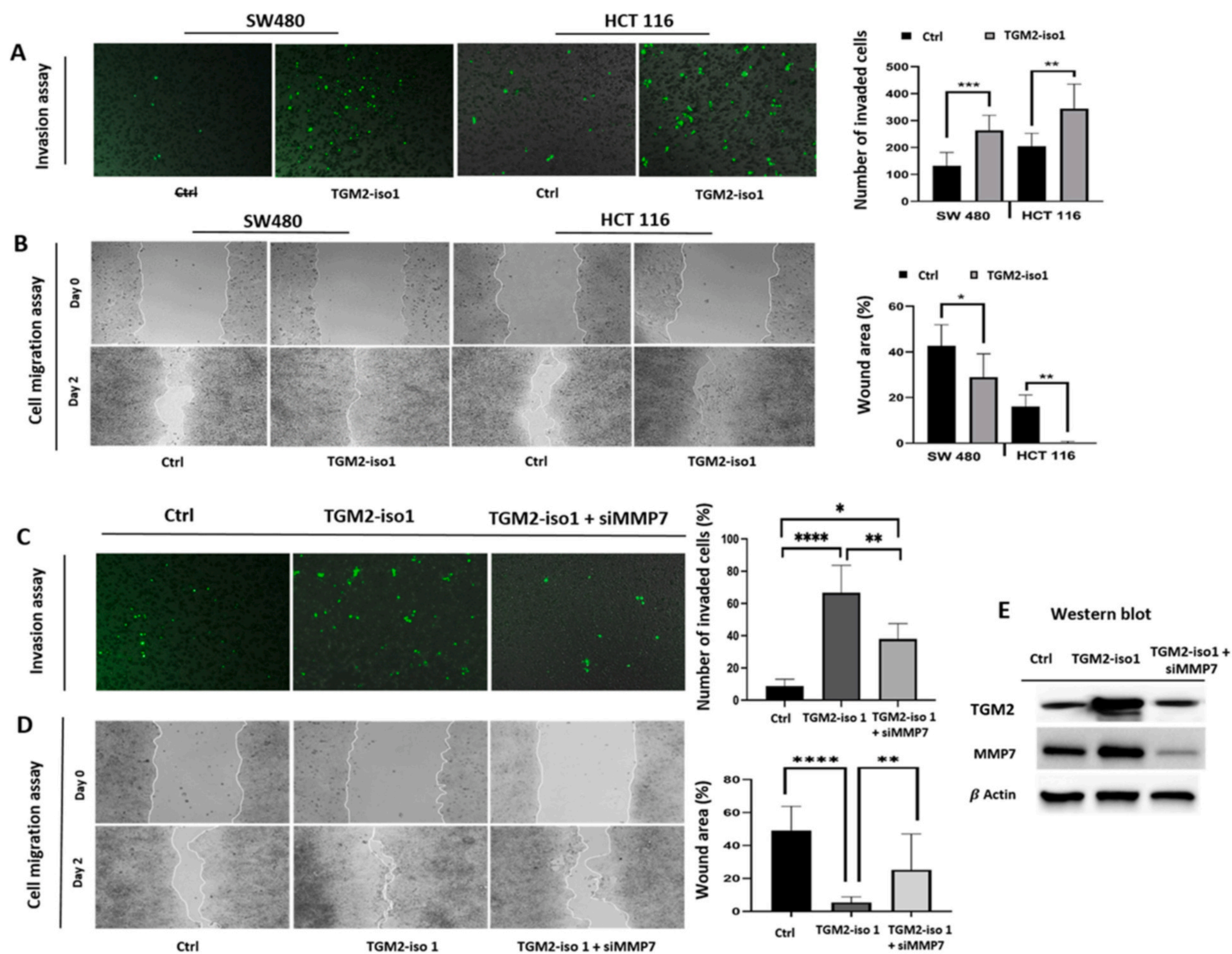

Fig. 3. TGM2 overexpression (TGM2-iso1) increases invasion and migration of CRC cells depending on MMP7 regulation. A. Representative visualization of CRC cell lines invading through matrigel coated membrane inserts. Cells that have migrated to the lower part of the membrane were visualized by green fluorescent protein (VenusGFP) and counted (bar chart). B. Representative photomicrographs (magnification: 50×) of migration activity of TGM2 overexpressing CRC cell lines assessed by the wound healing assay on day 0 and 2. Bar chart shows mean wound areas on day 2. C. Invasion assay of TGM2 overexpressing SW480 cells silenced with siMMP7. Invaded cells were visualized by VenusGFP and counted (bar chart). D. Wound healing assay of TGM2 overexpressing SW480 cells treated with siMMP7 (magnification: 50×). Wound areas at day 2 are shown in the bar chart. E. Representative Western hybridization images of TGM2 overexpressing SW480 cells treated with siMMP7. β-actin served as loading control. All assays were performed at 24 h post siMMP7 transfection. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, Mann-Whitney *U* test or One way-ANOVA (Tukey's multiple comparisons test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

induction [8]. Another investigation pointed to the interaction of the microRNA miR-532-3p with TGM2 as a prerequisite to promote apoptosis [17]. miR-214 is also discussed to target TGM2, with a negative correlation between miR-214 as well as TGM2 expression and metastatic CRC progression [18]. Finally, TGM2 has been postulated to regulate CRC viability via the Wnt/β-catenin pathway [19].

Our experiments conducted on two different CRC cell lines document that TGM2 is directly linked to MMP7, highlighting their collaborative role in driving EMT and EMT-related processes in CRC. In fact, TGM2 interacted with MMP7 as shown in the immunoprecipitation assay, and the expression levels of TGM2 and MMP7 correlated well with the migration and invasion activity of the applied CRC cell lines. Our data point to an important role of TGM2 as a driver of metastatic events, which coincide with previous research findings. Specifically, CD133+ colon cancer stem-like cells exhibiting high TGM2 expression acquired migratory properties, whereas CD133- cells characterized by low TGM2 levels did not [20]. The authors of the referenced study concluded that migration may be closely associated with TGM2. miR-214 and miR-532-3p have recently been shown to be involved in CRC cell migration and invasion.

Interestingly, down-regulation of miR-532-3p activated CRC cell proliferation, migration and invasion, associated with elevated expression of MMP-2 and MMP-9 [21]. This is notable, since both, miR-532-3p and miR-214, functionally target TGM2, further indicating the connection between TGM2 expression and metastatic events [17,18]. Interestingly, MMP7 protein expression and MMP7 activity were closely correlated with TGM2 expression in the CRC cell lines. Integrative bioinformatic analysis identified MMP7 as one of the 10 most prominent genes involved in CRC pathogenesis, EMT, and metastasis [22]. Additionally, MMP7 ranked among the top four differentially expressed genes in CRC as verified by single-cell RNA sequencing [23]. Our results point to decreased invasion and migration potential of CRC cells following MMP7 knockdown. Further, TGM2 expression levels correlated with MMP7 expression and enzymatic activity. Hereby, the increased invasive potential of TGM2 overexpressing CRC cells depends on MMP7 activity. Thus, it is inferred that TGM2 promotes the invasion and metastasis of CRC cells by regulating MMP7 activity. Based on the immunoprecipitation assay, an interaction between TGM2 and MMP7 has been documented. Whether TGM2 stabilizes MMP7 activity or influences MMP7 expression requires further investigation. However, previous studies have demonstrated that silencing TGM2 in HCT-116 cells suppressed the expressions of MMP2 and MMP9, which are also

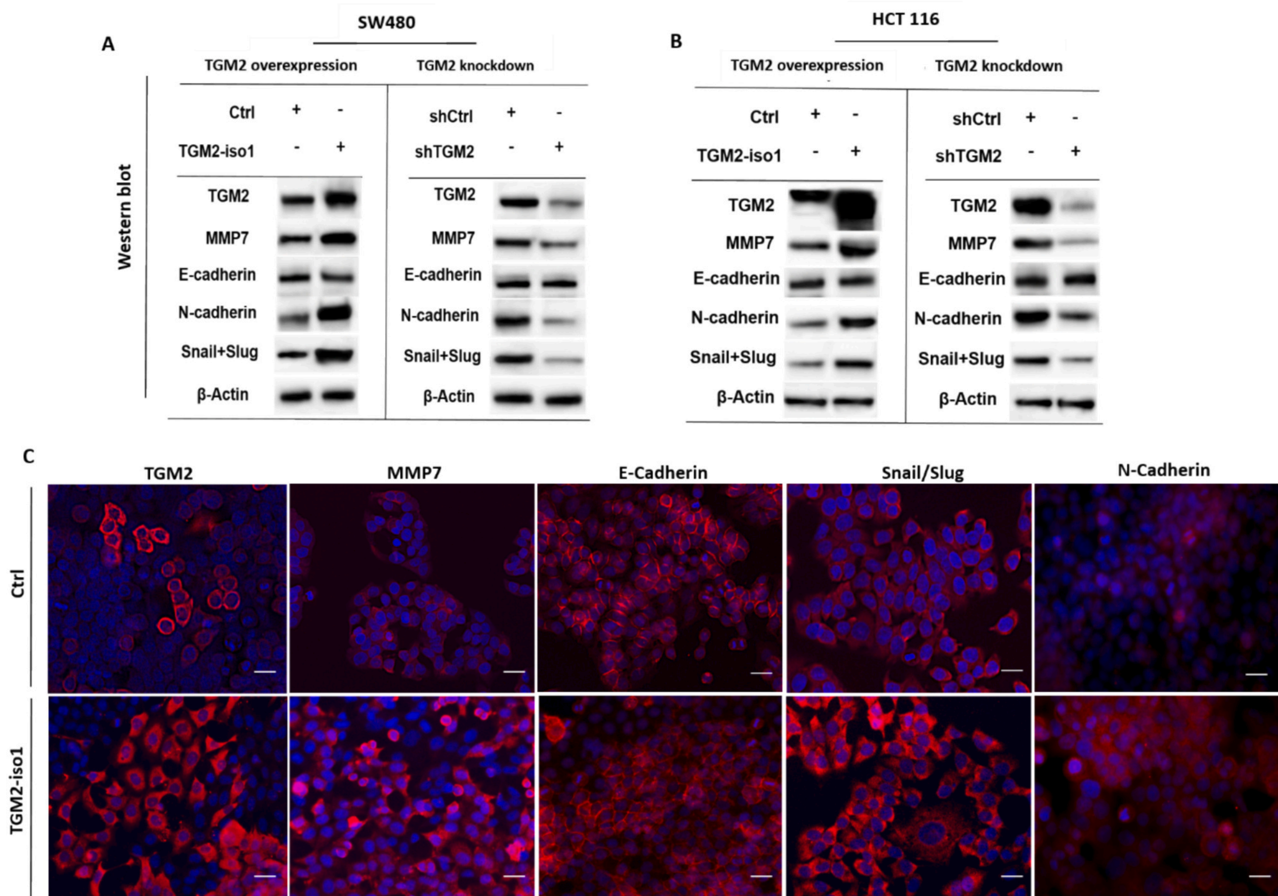

Fig. 4. TGM2 overexpression induces EMT in CRC cell lines. A. and B. Detection of TGM2, MMP7, E-cadherin, N-cadherin, Snail/Slug protein expression in TGM2 overexpressing and TGM2 knockdown CRC cell lines assessed by Western hybridization analysis. β-actin served as loading control. C. Immunofluorescence staining of TGM2, MMP7, E-Cadherin, Snail/Slug, N-Cadherin in TGM2 overexpressing and Ctrl-transduced HCT-116 cells. Scale bar = 20 μm.

involved in motile spreading of CRC cells [19].

Considering the significant role of MMP7 in cancer, this molecule has recently gained attention for drug development [24,25]. As TGM2 is shown here to be bound to MMP7, targeting TGM2 might be a promising strategy to block CRC progression by simultaneously suppressing both TGM2 and MMP7. E-cadherin and N-cadherin, including transcription factors of the Snail family (e.g. Slug), serve as the pivotal drivers of the EMT program [26]. In our study, TGM2 expression in SW480 and HCT-116 cells did not only correlate with MMP7 but also with N-Cadherin and Snail/Slug. Sufficient evidence has been provided that both N-Cadherin and Snail/Slug promote malignancy, advance disease progression and trigger resistance to chemotherapy [27]. A clinical trial involving patients with CRC revealed that increased levels of N-cadherin and Snail were associated with earlier tumor recurrence [28]. High Snail expression was also detected in 76 % and 70 % of primary tumors and lymph node metastases of patients with stage III colonic adenocarcinoma, which significantly correlated with poor overall and relapse-free survival rates [29]. We postulate that TGM2, through MMP7, is linked to N-cadherin and Snail/Slug, suggesting the intriguing option that therapeutic blockade of TGM2 might represent an elegant strategy to, at the very least, delay CRC progression and resistance development. However, further investigation is warranted to elucidate the direct mechanisms involved. Surprisingly, E-cadherin was not influenced by TGM2. This finding was unexpected, since E-cadherin is also closely associated with EMT and tumor metastasis. An intriguing experiment on starvation-induced EMT in CRC cells showed that increased migratory potential and chemoresistance was associated with an upregulation and cytosolic internalization of E-cadherin [30]. This suggests that EMT might not always entail a loss of E-cadherin but rather involve protein translocation processes. However, this result may not allow to conclude to a similar process in our setting. Recent observations by Chen and colleagues indicated that EMT in Caco2 colon cancer cells was driven by N-cadherin and Snail, without any changes of E-cadherin expression [31]. Hence, it seems plausible that TGM2 does not directly influence E-cadherin in our experimental system. Supporting this interpretation, E-cadherin mRNA and protein levels were not downregulated in correlation with TGM2 expression in primary hepatocellular carcinoma cells [32].

TGM2 is also implicated in the ERK-MEK signaling pathway, where upregulation of TGM2 enhances and its downregulation diminishes the phosphorylation of MEK, ERK and 90RSK. MEK1/2 are "gatekeepers" in the MAPK signaling pathway and the only specific regulators that activate ERK by phosphorylating the tyrosine and threonine regulatory sites [33]. Further, MMP7 is known to be involved in cancer cell invasion through ERK-MEK signaling [34]. This led us to investigate the interaction between TGM2/MMP7 and MEK1/2, which was subsequently confirmed by Western hybridization analyses. The results indicate that TGM2 may play a key role in promoting the activation of ERK signaling. Our observation has been validated by studies using the ERK-inhibitor GDC-0994. In fact, GDC-0994 not only attenuated ERK activity but also reduced TGM2 levels in TGM2 overexpressing cells. It should be mentioned in this context that GDC-0994 serves as a specific ERK-inhibitor but also inhibits ERK-dependent p90RSK phosphorylation, although with a lower potency. Therefore, we cannot explain in detail how TGM2 and MMP7 are involved in EMT regulation. Regardless of this and consistent with our data, the ERK1/2 inhibitor U0126 inhibited

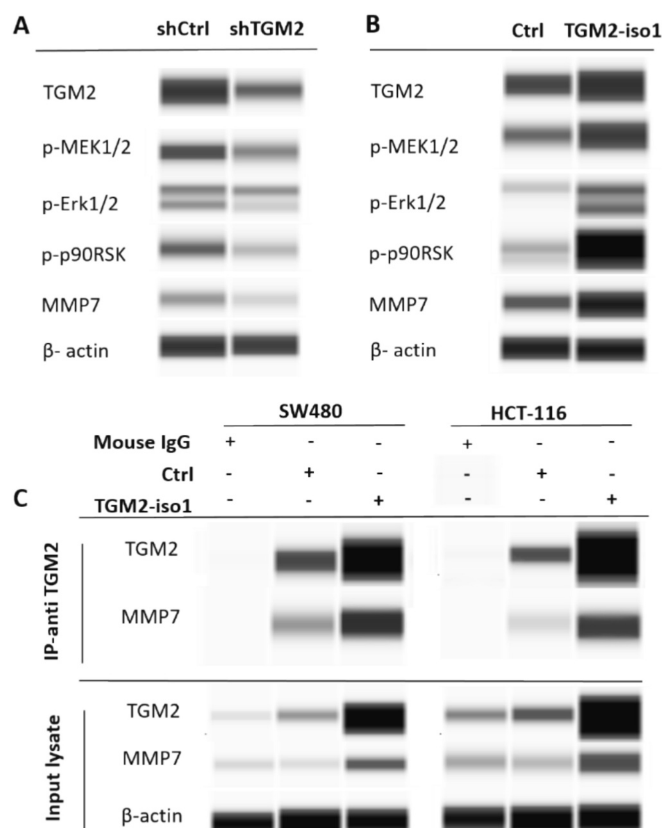

Fig. 5. A. Protein expression of TGM2, p-MEK1/2, p-Erk1/2, p-p90RSK, and MMP7 in SW480 cells after shRNA mediated knockdown of TGM2 (shTGM2) compared to the control (shCtrl). B. Protein expression of TGM2, p-MEK1/2, p-pErk1/2, p-p90RSK, and MMP7 expression in TGM2 overexpressing SW480 cells compared to the control (Ctrl). β-actin served as loading control. C. TGM2 protein was immunoprecipitated using anti-human TGM2 in SW480 and HCT-116 cells. Lysates were analyzed using Protein Simple Wes and probed with anti-TGM2 and anti-MMP7. For input control, TGM2, MMP7 and β-actin from whole cell lysate prior to IP (Immunoprecipitation) were assessed.

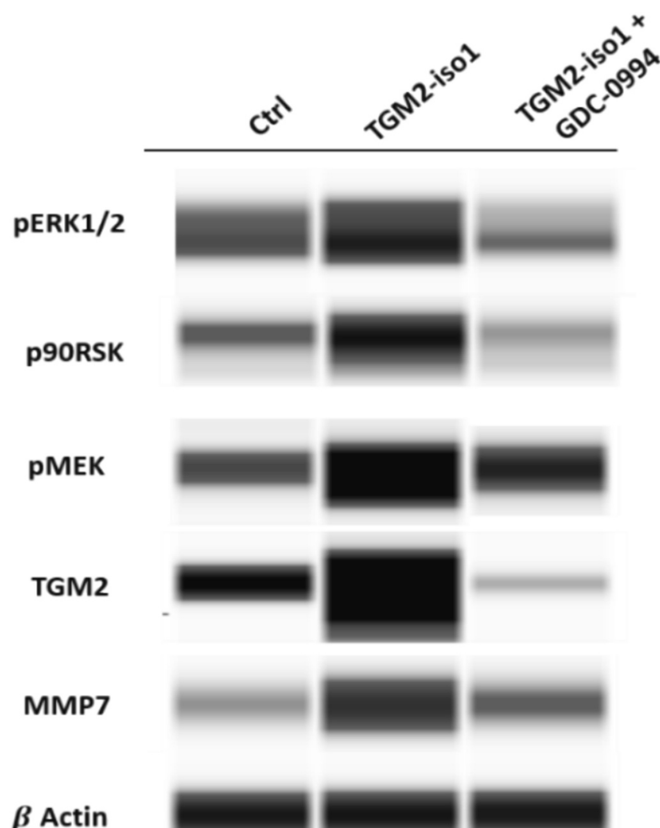

Fig. 6. ERK/MEK inhibition regulates TGM2 and MMP7 in SW480 cells. pERK, p90RSK, pMEK, TGM2 and MMP7 were detected from protein lysate of TGM2 (TGM2-iso1) overexpressing SW480 cells treated with 0.5 μM GDC-0994 for 72 h, compared to Ctrl-transduced and untreated TGM2 (TGM2-iso1) overexpressing SW480 cells. β-actin served as loading control.

TGM2-induced cell proliferation, migration, and invasion of the gastric carcinoma cells MKN-45 and NCI-N87 [35], although MMP7 was not investigated. Given that MMP7 has also been shown to interact with TGM2 in our experiments, we postulate that TGM2-MMP7 cross-communication triggers the downstream activation of the MEK/ERK pathway. MEK signaling plays a pivotal role in the development of various types of cancers. Consequently, it is not astonishing that numerous MEK inhibitors are currently undergoing validation in both clinical and preclinical settings. However, dose-limiting toxicities and the possibility for resistance development may impede their efficacy [36]. Interestingly, Ram et al. recommended to target EMT rather than MEK to achieve greater effectiveness in cancer treatment [36]. We have presented evidence that TGM2 promotes EMT by regulating the expression of MMP7 via the MEK/ERK signaling pathway. Therefore, implementing TGM2 inhibitors into clinical practice could overcome current obstacles in the treatment of CRC. Indeed, there is a growing recognition of the significant therapeutic potential of targeted TGM2 inhibitors in clinical settings [37,38].

Limitations of our study should finally be considered. The in vitro results have not been validated in vivo. This is important, since the in vitro model does not fully capture the complexity of the tumor microenvironment found in living organisms. Indeed, concerted interaction of the tumor cells with cancer-associated fibroblasts, endothelial cells, immune cells as well as with matrix proteins may evoke signaling events different from the ones observed in tumor mono-cultures. Finally, the restricted use of few colorectal cancer cell lines (SW480 and HCT-116) may limit the generalizability of the findings. Different cell lines may have varying genetic backgrounds and phenotypic characteristics, which might influence their response to TGM2 and MMP7.

In summary, our data reveal that TGM2 is correlated with MMP7 expression and activity. The TGM2-MMP7 cross-communication promotes migration and invasion of CRC cells by regulating the MEK/ERK signaling pathway. Additionally, the interaction between TGM2 and MMP7 contributes to an elevated expression of EMT markers, namely N-cadherin and Snail/Slug. Whether the interaction between TGM2 and MMP7 is of direct nature or due to an interaction with further intracellular proteins is not clear yet. In fact, TGM2 plays a prominent role in several cancer-related pathways. TGM2 activates MEK/ERK and elevates β-catenin expression [39]. TGM2 is also involved in AKT/mTOR phosphorylation [40]. In turn, β-catenin [41], AKT/mTOR [42], and MEK/ERK signaling [43] have all been reported to be associated with expression and secretion of MMP7 in CRC cells. It might be worthwhile to investigate how these pathways might be involved in TGM2 triggered MMP7 activation.

Ongoing investigations are required to verify our in vitro findings in an in vivo model. Given the potential role of TGM2 inhibitors as antitumor drugs, TGM2 inhibition might open new therapeutic options, particularly for patients with metastasized CRC, offering promising avenues for future investigations. Actually, there are no clinical trials reported for colorectal cancer with TGM2 or MMP7 inhibitors. However, treatment with a selective oral TGM2 inhibitor attenuated gluten-induced duodenal mucosal damage in patients with celiac disease in a preliminary trial [44] highlighting the role of TGM2 as a therapeutic

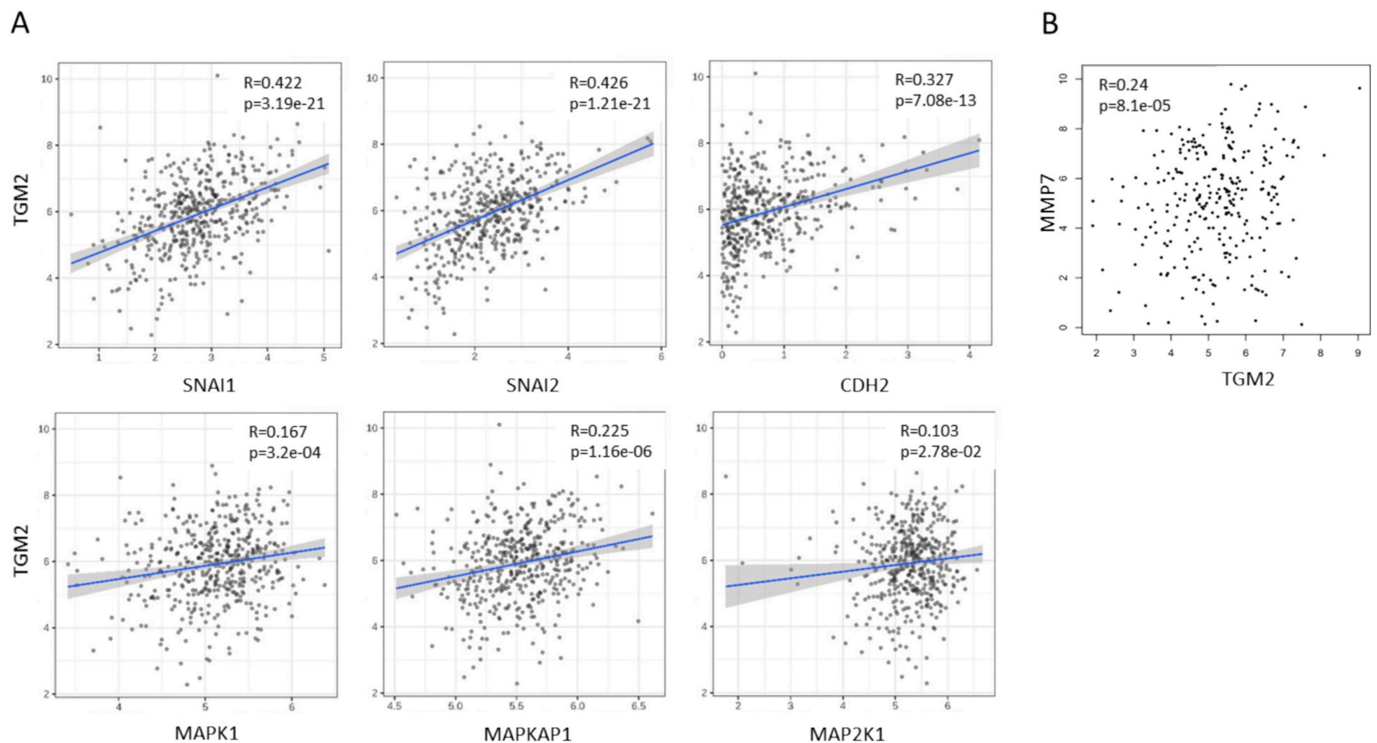

Fig. 7. A) Correlations between TGM2 and TGM2 related signaling, taken from TCGA, $n = 458$. B) Correlation between MMP7 and TGM2 expression, taken from Gepia2, $n = 275$. All values are given as expression level measured as log2 TPM. SNAI1 = Snail, SNAI2 = Slug, CDH2 = N-cadherin, MAPK1 = Erk1, MAPKAP1 = p90RSK, MAP2K1 = MEK. Shown are individual tumors, the linear regression line with 95 % confidence interval, the Spearman r correlation coefficient, and p value.

target. Recently, Wang and colleagues have presented evidence of a TGM2-dependent filamentous coating of CXCL12–KRT19 heterodimers which prevents intratumoral accumulation of T cells [45]. It is intriguing to evaluate in this context whether TGM2 may also be involved in immune escape processes. Still, our results presented here may not be transferred to further tumor entities. In fact, an update given by Zaltron et al. points to a tissue-specific role of TGM2 including both oncogenic as well as tumor-suppressive properties [40]. This note should also be taken care of in ongoing studies.

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CRediT authorship contribution statement

Roman A. Blaheta: Writing – review & editing, Writing – original draft, Project administration, Methodology, Conceptualization. **Jiaoyan Han:** Investigation. **Elsie Oppermann:** Writing – original draft, Investigation. **Wolf Otto Bechstein:** Writing – review & editing, Supervision. **Katrin Burkhard:** Investigation. **Axel Haferkamp:** Writing – review & editing, Supervision. **Michael A. Rieger:** Writing – review & editing, Supervision, Project administration. **Patrizia Malkomes:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

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Declaration of competing interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data availability

Data will be made available on request.

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