Up-regulation of Fibronectin and Tissue Transglutaminase Promotes Cell Invasion Involving Increased Association with Integrin and MMP Expression in A431 Cells

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Abstract. *In human tumors, fibronectin (FN) expression is positively associated with tumor metastatic potential and matrix metalloproteinase (MMP) secretion. Additionally, tissue transglutaminase (TG2) is implicated as playing an important role in tumor progression, and acts as a co-receptor for integrin-mediated cell binding to FN. This study explored the involvement of FN and TG2 in cancer cell metastasis using the recently established highly invasive A431-III subline. A431-III cells expressed significantly higher levels of FN and TG2 as compared to the parental line (A431-P). Knockdown of endogenous FN by small interfering RNA (siRNA) resulted in dramatic suppression of the migratory and invasive activity, and the secreted MMP-9 activity (but not MMP-2) in A431-III subline. Exogenous administration of FN to A431-III cells also increased the secreted activity of MMP-9 but not MMP-2. Interestingly, knockdown of TG2 by siRNA dramatically reduced the cell attachment, migration and invasion, and the secretion of MMP-9 and MMP-1 (but not MMP-2 and MMP-3) in A431-III cells as compared to A431-P cells. Furthermore, A431-III cells exhibited increased association of integrin β1 and β3 with FN and TG2, and knockdown of TG2 markedly suppressed integrin β1 interaction with FN. Together, this study suggests that FN and TG2 facilitate the metastatic activity of A431 tumor cells, and this may be partly attributed to TG2 enhancement of the association of FN and β integrin. In addition, the combined targeting of TG2 and FN may be an effective therapeutic strategy for cancer displaying increased expression of both proteins.*

Cell invasion is critical in the formation of metastasis in cancer, and the process involves extensive remodeling of the tissue matrix. This requires the action of matrix metalloproteinases (MMPs), a family of Zn^{2+}-dependent endopeptidases that hydrolyze extracellular matrix (ECM) proteins, such as collagens, fibronectin, vitronectin, elastin, laminin and fibrin (1). Two members of the MMP family of pericellular proteinases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), have the highest enzymatic activities against type IV collagen, the main constituent of the basement membrane that underlies the epithelium and endothelium (2). As yet, the critical determinants of invasion and metastasis are not fully understood. It has been reported that cells isolated from metastases by *in vivo* selection or sub-cloning are frequently more highly metastatic than the bulk of cells from primary tumors (3, 4). Thus selecting metastatic variants from primary tumor cells and comparing their gene expression could be an effective approach for exploring the mechanisms of tumor invasion and metastasis.

Over the past two decades, a number of *in vitro* assays have been developed for investigating the process of tumor cell invasion (5). The quantitative *in vitro* invasion assays in use today are derived and modified from the Boyden chamber system (6). These assays have a common feature, the requirement of tumor cells to secrete MMPs in order to pass through the matrix gel. We used Boyden chambers to select highly invasive A431 tumor cells (A431-III) from the parental cells (A431-P), and demonstrated a clear positive

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relationship of invasiveness with metastasis-associated activities including the rate of cell migration and invasion, and secretion of MMP-9 (7).

Fibronectin (FN), a multifunctional adhesive glycoprotein, is present on many cell surfaces, and in extracellular fluids and connective tissues. FN interactions with integrins play an important role in tumor cell migration, invasion and metastasis. Many integrins, including $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_6$, recognize the RGD sequence in FN (8). Previous study showed that FN secreted from human peritoneal tissue induces MMP-9 expression and invasion in ovarian cancer cell lines (9). Binding of FN to integrin $\alpha_5\beta_1$ was found to increase expression and secretion of MMP-1, MMP-3 and MMP-9 in rabbit synovial fibroblasts (10). In addition, whole-genome analysis of metastasis has revealed association between enhanced expression of several genes including *FN*, and progression to a metastatic phenotype (3).

Recently, tissue transglutaminase (TG2) was reported to enhance the association of FN and integrin on the cell surface (11). TG2 belongs to a family of Ca^{2+}-dependent cross-linking enzymes that catalyze post-translational modification through an acyl-transfer reaction between the glutamine γ-carboxyamide group and the lysine ε-amino group (12). TG2, a unique member of the TG family, is also a GTP-binding protein that couples certain G protein-coupled receptors (α_{1b}-adrenoreceptors, thromboxane and oxytocin receptors) to phospholipase C ($PLC\delta_1$) mediating inositol phosphate signaling (13, 14). Although TG2 mostly localizes in the cytoplasm, it is also present on the cell surface and in the ECM. Extracellular TG2 is able to bind and cross-link ECM proteins, and FN interaction with TG2 on the cell surface is best characterized (15, 16). TG2 binds to the gelatin-binding domain of FN with high affinity through its *N*-terminal β-sandwich domain (17, 18). The observed association between TG2 and focal adhesion kinase (FAK) phosphorylation-facilitating integrins β1 and β3 (11, 19) does not require the cross-linking activity of TG2 (20). TG2 serves as a FN co-receptor on fibroblasts to promote cell adhesion, spreading and migration (19-22). Additionally, several studies suggest TG2 involvement in the regulation of tumor growth and metastasis (23-25). The molecular basis of TG2 function in tumor progression remains unclear.

Our laboratory has found that the highly invasive A431-III subline secretes a higher level of MMP-9 and exhibits increased adhesion, spreading, migratory and invasive activities compared to the A431-P cells (7). The causes of these enhanced metastatic features have not yet been fully elucidated. This inspires us to explore the role of TG2 in promoting cancer. There were three specific aims. The first was to investigate how MMP-9-induced acquisition of invasive phenotype in A431-III cells is associated with the increase of FN and TG2. The second aim was to explore whether the associations of FN and TG2 with integrin β1 and β3 in A431-III were up-regulated as compared to those in A431-P cells. The third aim was to determine whether knockdown of FN and TG2 by specific siRNA could lead to the decrease in migratory and invasion activity, and MMP-9 secretion in A431-III subline. Additionally, we investigate whether TG2 knockdown in A431-III subline would suppress the level of FN interaction with integrin β1. This preliminary data suggest that TG2 enhancement of FN association with integrin β1 may trigger signaling that upregulates MMP-9 (and MMP-1) expression to promote invasive potential in A431-III cells.

Materials and Methods

Materials. The A431 tumor cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). RPMI-1640, streptomycin and fetal bovine serum (FBS) were sourced from GIBCO (Grand Island, NY, USA). Monoclonal antibody against TG2 (CUB7402) was acquired from NeoMarkers (Fremont, CA, USA). Rabbit polyclonal antibody against FN was obtained from Sigma (St. Louis, MO, USA). Monoclonal antibodies against human integrin β_1 (JB1A) and human integrin β_3 (B3A) were obtained from Chemicon (Temecula, CA, USA). MMP-1, MMP-3, MMP-9 antibodies were purchased from Biogenesis (Sandown, NH, USA). Anti-FAK antibody was acquired from BD Biosciences (San Diego, CA, USA). Anti-phosphotyrosine antibody (PY99) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TG2 siRNA, FN siRNA, and non-specific siRNA were purchased from Ambion (Austin, TX, USA).

Preparation of cell lysates and cell fractionation. Both A431-P and A431-III Cells were harvested and washed three times with PBS. For detection of proteins in whole cell lysates, cells were lysed in Gold lysis buffer containing protease inhibitors (20 mM Tris-HCl, pH 7.9, 1 mM EGTA, 0.8% NaCl, 0.1 mM β-glycerylphosphate, 1 mM sodium pyrophosphate, 10 mM NaF, 1 mM $NA_4P_2O_7$, 1 mM Na_3VO_4, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). For proteins in cytosolic and membrane fractions, cells were disrupted in hypotonic buffer containing protease inhibitors (10 mM Hepes, pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin) by gentle homogenization, and cytosolic fractions were obtained as supernatants by centrifugation at 100,000 × *g* for 30 min at 4˚C. The pellets were homogenized in the Gold lysis buffer at 4˚C overnight, and membrane fractions were obtained as supernatants by centrifugation at 15,000 × *g* for 20 min at 4˚C. To detect proteins secreted into the culture medium, confluent cells were grown in serum-free media for 12 h, and then cultured media were collected and centrifuged to remove any floating cells. These conditioned media were concentrated using high-performance concentrate filters according to the manufacturer's protocol (Topsfield, MA, USA). The protein content was determined by Bradford assay (26) prior to immunoblotting and co-immunoprecipitation analyses.

Immunoblotting analysis. The A431-P and A431-III cell lysate samples were mixed with 5× sample buffer (250 mM Tris-Cl, pH 6.8, 10% (w/v) SDS, 0.1% (w/v) bromophenol blue, 50% glycerol, and 0.5 M mercaptoethanol) and boiled for 5 min, separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membrane (BD

Biosciences, San Jose, CA, USA). The membrane blots were blocked in phosphate buffered saline (PBS) containing 5% bovine serum albumin (BSA) for 1 h at room temperature, and incubated with primary antibody overnight at 4˚C. After washing with tris-buffered saline Tween-20 (TBST) containing 20 mM Tris-HCl (pH 7.6), 0.8% (w/v) NaCl and 0.25% Tween-20, the blots were incubated with secondary antibody conjugated with horseradish peroxidase. Then the membranes were washed three times with TBST, and immunoreacted bands were detected with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia, Uppsala, Sweden) and exposed on Kodak BioMax X-ray film.

Reverse transcriptase-polymerase chain reaction (RT-PCR). A431-P and A431-III total RNA were isolated using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France), and reverse transcribed using the Superscript™ kit (Invitrogen, Carlsbad, CA, USA). Briefly, 5 μg of total RNA, 1 μl of 50 μM Oligo(dT) Primers, and 1 μl of 10 mM dNTP were mixed, denatured at 65˚C for 5 min, and then placed on ice for 1 min. Subsequently, 2 μl of 10× RT buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M dithiothreitol (DTT), 1 μl of RNaseOUT™ (40 U/μl) and 1 μl of SuperScript™ III RT (200 U/μl) were added, incubated at 25˚C for 10 min and 50˚C for 50 min, then heat inactivated at 85˚C for 5 min. One microliter of RNase H was added, and the mixture was then incubated at 37˚C for 20 min. The PCR reaction contained 36.5 μl of nuclease-free water, 5 μl of 10× PCR buffer (Invitrogen), 4 μl of 2.5 mM dNTP, 1 μl each of 100 μM forward and reverse primers, 2 μl of RT product, and 0.5 μl of 5 U/μl Bio Taq DNA polymerase. The PCR program used was denaturing at 94˚C for 30 s, annealing at 55˚C for 30 s, and extension at 72˚C for 60 s for 30 cycles. The respective forward and reverse primers for *TG2* were 5'CTCGTGGAGCCAGTTATCAACAGCTAC3' and 5'TCTCGAAGTTCACCACCAGCTTGTG3, for *FN* were 5'GAAGCTCTCTCTCAGACAACCA3' and 5'AGGTCTGCGGCA GTTGTCAC3', and for *β-actin* were 5'GCTCGTCGTCGACA ACGGCTC3' and 5'CAAACATGATCTGGGTCATCTTCTC3'. The PCR products were run on 1.2% agarose gels, stained with ethidium bromide, and visualized using a UV light box.

Transfection of small interference RNA (siRNA). A431-P and A431-III cells (5×10^5 cells/well) were plated into six-well plates and allowed to adhere for 12 h. Cells were then transfected with 30 nM of *TG2*, *FN* or non-specific siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 2 μl of transfection reagent were added to 100 μl of RPMI-1640 culture medium, thoroughly mixed and incubated at room temperature for 5 min; 6 μl of siRNA were added to 150 μl of culture medium, thoroughly mixed and then combined with the diluted Lipofectamine 2000. This was gently mixed and incubated at room temperature for 30 min, and then added to the cultured cells which were then incubated. Cell lysates were prepared and cell culture conditioned media were collected for further analyses.

In vitro wound-healing migration assay. Cells transfected with *FN* siRNA were plated onto six-well culture plates in RPMI-1640 containing 10% FBS (2×10^6 cells/well). After 24 h, the cell monolayer was wounded by manually scratching with a pipette tip, washed with PBS, and then incubated at 37˚C for 24 h. Cells were photographed at 0 and 24 h after wound scratching under a phase contrast microscope using an Olympus IX70 camera (Tokyo, Japan). Experiments were carried out in triplicate.

In vitro invasion assay. In vitro invasiveness was investigated according to a previously described procedure (27) with modifications. In brief, the filter of 24-well transwell unit was coated with 0.1 ml of 0.4 mg/ml EHS Matrigel (BD Biosciences). The lower compartment contained RPMI-1640 with 10% FBS as a chemoattractant. A431-P and A431-III cells pre-transfected with *FN, TG2* siRNA were placed in the upper compartment (1×10^5 cells/0.5 ml of RPMI-1640 containing 0.1% BSA) and incubated at 37˚C for 48 h. After incubation, the filters were fixed with 3% glutaraldehyde in PBS and stained with crystal violet. Cells on the upper surface of the filter were gently scraped off, and those that had penetrated through the Matrigel to the lower surface of the filter were counted under a microscope. Three independent experiments were performed with triplicate measurements for each experiment.

Gelatin zymography. Gelatinases secreted from both A431-P and A431-III cultured cells were measured using gelatin zymography (28). In brief, samples of conditioned media were subjected to electrophoresis on 3-18% linear gradient SDS-polyacrylamide gels co-polymerized with 0.1% gelatin (Sigma). The volume of each medium sample analyzed was normalized according to the cell number. After electrophoresis, the gels were washed for 30 min in 2.5% Triton X-100, and incubated in reaction buffer (50 mM Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$, 0.02% NaN_3) at 37˚C for 24 h. The gels were then stained with Coomassie Blue R-250 in 10% acetic acid/20% ethanol for 1 h, and destained in the same solution without dye. A clear zone on the gel indicated the presence of gelatinase activity, which was quantified using densitometry.

Cell attachment assay. The 24-well plates were coated with FN (10 μg/ml) (BD Bioscience) for 1 h at 37˚C and then blocked with 1% BSA for 30 min. Cells pre-transfected with *TG2* or non-specific siRNA were serum starved overnight, seeded onto 24-well culture plates (10^5 cells/well in 0.5 ml of RPMI-1640 medium) and incubated at 37˚C for 15 or 30 min. After incubation, plates were washed with PBS, and adherent cells were fixed with 3% glutaldehyde/PBS, stained with crystal violet, and counted under a microscope (>500 cells counted for each treatment sample).

Co-immunoprecipitation. A431-P and A431-III cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 0.55 sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, with 0.5 mM PMSF, 0.5 mM benzamidine, 10 mg/ml leupeptin, and 10 mg/ml aprotinin). One milligram of each cell lysate was incubated with antibodies against FN, integrin β_1 or β_3, or FAK, followed by protein G-Agarose (Santa Cruz Biotechnology). The immunoprecipitated samples were then subjected to immunoblotting analyses (TG2, FN or phosphotyrosyl-protein) as described above. The membranes were then stripped and reprobed with antibody against the immunoprecipitated molecule (FN, integrin β_1 or β_3, or FAK). Additionally, immunoprecipitation assays were used to assess the degree of FAK phosphorylation.

Confocal microscopy. A431-P and A431-III cells were plated in serum-free medium on coverslips without FN coating for 6 h at 37˚C, and then fixed with 4% paraformaldehyde in PBS. After several washes with PBS, the cells were blocked with 1% BSA for 30 min, and then incubated with 10 μg/ml anti-TG2 monoclonal

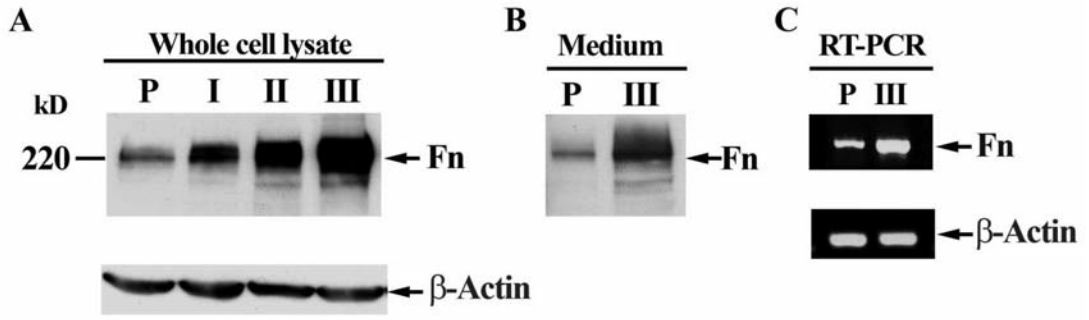

Figure 1. *FN expression is increased in the highly invasive A431-III subline. A: FN protein levels in whole–cell extracts of A431-P, A431-I, A431-II and A431-III cells were determined by immunoblotting. The membrane was stripped and reprobed with β-actin antibody serving as the internal control. B: FN secretion in A431-P and A431-III culture. Cells were grown in serum-free media for 12 h, then conditioned media were collected and concentrated for immunoblotting. C: FN mRNA levels in A431-P and A431-III cells were determined by RT-PCR.*

antibody and 10 μg/ml rabbit anti-FN antibody for 1 h without permeabilization. Cells were then immuno-stained with a combination of rhodamine-conjugated goat anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG (Chemicon), and then visualized using confocal image microscopy.

Statistical analysis. Quantitative data from three-to-six independent experiments are expressed as the mean (±SEM) and analyzed by unpaired Student's *t*-test at a significance level of 0.05.

Results

Increased endogenous FN expression correlates with metastasis-associated activities in highly invasive A431-III cells. We have successfully selected a highly invasive A431-III subline from the A431-P cell line using Boyden chamber assay, and found that A431-III cells secreted much higher levels of MMP-9 and displayed greater migratory and invasion activities as compared with the parental cells (7). However, the cause of these changes in A431-III cells has not been elucidated. Earlier findings have shown that FN treatment induces MMP-9 secretion and invasion in ovarian cancer cell lines (9). This prompted our interest to investigate the relative expression level of endogenous FN and its relation to MMP-9 secretion and invasive behavior in A431-P and A431-III cells.

Immunoblotting analysis shows that the cellular FN protein level progressively increased in A431-I, A431-II and A431-III cells (7) as compared with A431-P (1.81-, 2.65- and 3.87-fold, respectively) (Figure 1A). The level of secreted FN also increased in A431-III cells relative to that of A431-P (Figure 1B). Additionally, the *FN* mRNA level was elevated in A431-III cells relative to that of A431-P as determined by RT-PCR (Figure 1C). We next investigated the relationship of increased endogenous FN expression and metastasis-associated activities including cell migration, invasive behavior and MMP activity using siRNA knockdown approach. Transfection of *FN* siRNA significantly reduced FN levels in A431-P and A431-III cells, and with greater effect in A431-III cells (Figure 2A). The *in vitro* wound healing assay demonstrated that control A431-III cells repopulated the wound area faster than did A431-P cells, and delayed migration was much evident in *FN* siRNA-transfected A431-III cells (Figure 2B). Furthermore, *in vitro* invasion assay by enumerating the cells that penetrated through the matrigel-coated transwell inserts demonstrated that knockdown of endogenous FN dramatically suppressed the invasive activity of A431-III and A431-P cells in a dose-dependent manner (Figure 2C). We next determined whether the increased FN level contributing to the increased invasiveness of A431-III cells was related to increased MMP activity. Knockdown of endogenous FN by siRNA greatly reduced the secreted MMP-9 gelatinase activity in A431-III cells, but not MMP-2 activity (Figure 2D). To further confirm the positive relationship between FN level and MMP-9 secretion, A431-III cells were treated with exogenous FN of different concentrations. We found that exogenous FN increased the secreted MMP-9 activity in A431-III cells (Figure 2E). These results suggest that elevated invasiveness of A431-III cells is partly attributed to the increase in FN-induced MMP-9 secretion.

Taken together, these data suggest that the increased migratory activity and invasiveness of A431-III cells are strongly associated with the up-regulation of FN expression.

Increased endogenous TG2 expression correlates with metastasis-associated activities in highly invasive A431-III cells. Recent findings indicate that TG2 can act as a co-receptor for integrin-mediated cell binding to FN in fibroblasts, stimulating FAK phosphorylation that in turn triggers several downstream signaling pathways related to cell migration and invasion (11). It was of great interest to investigate the relative expression level of endogenous TG2 and its relation to MMP-9 secretion and invasive behavior in A431-P and its sublines. Interestingly, cellular TG2 protein levels progressively increased in A431-I, A431-II, and A431-III cells as compared with A431-P (2.11-, 3.45- and 3.95-

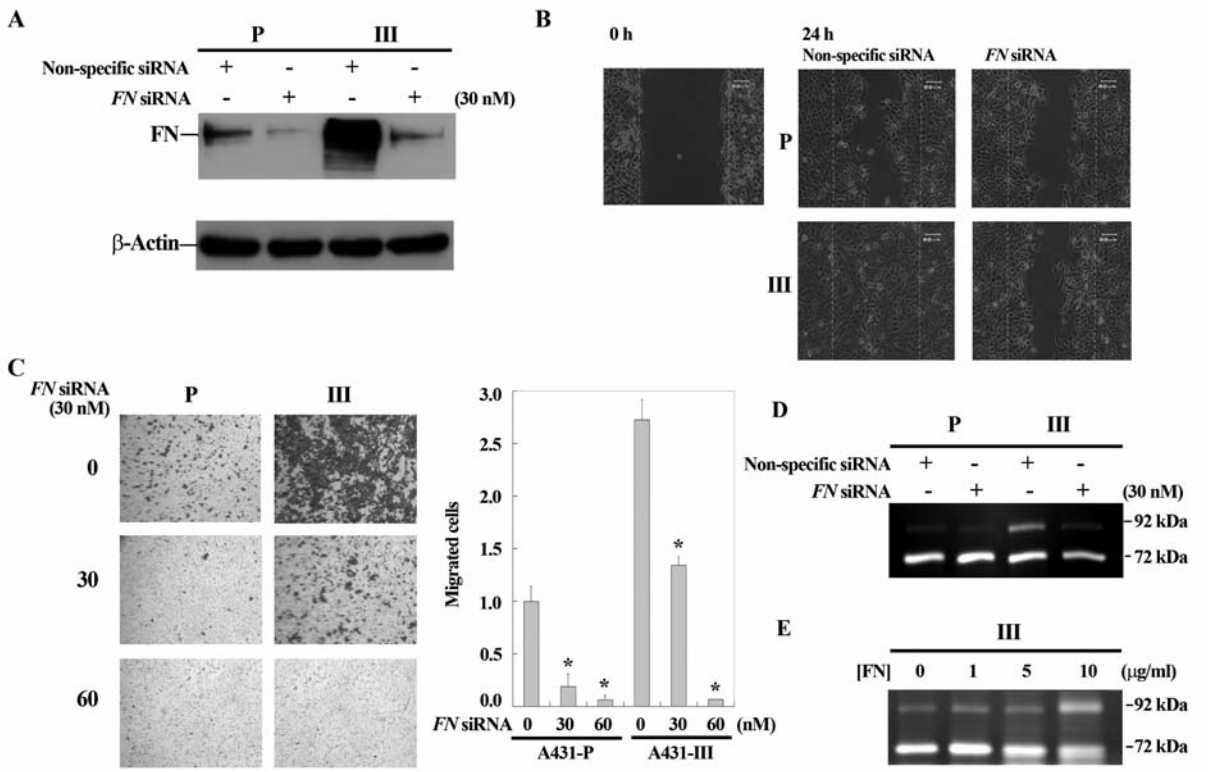

Figure 2. *Endogenous FN expression correlates with invasiveness and MMP9 activity in A431-III cells. A-D: A431-P and A431-III cells were transfected for 48 h with 30 nM FN siRNA or nonspecific siRNA prior to further analyses. A: Cells were then plated onto six-well plates and allowed to grow in the presence of 10% FBS for 20 h. FN expression was determined by immunoblotting. This figure is a representative of three independent experiments. B: In vitro wound healing migration assays were performed, and cell images at 0 and 20 h were taken under a phase-contrast microscope. C: In vitro invasion assays were performed. Cells that penetrated through the Matrigel to the lower filter surface after 48 h were stained, and then visualized and counted by microscopy. Each bar represents the mean (±SEM) of triplicate wells compared to the A431-P control value (expressed as 1.0). The asterisk designates a significant difference between FN siRNA-transfected and control cells ($p<0.05$). This figure is representative of three independent experiments. D: Effect of endogenous FN on the secreted gelatinase activities in A431-P and A431-III cells. Cell culture-conditioned media were collected and normalized according to cell numbers prior to gelatin zymography analysis. E: Effect of exogenous FN on gelatinase activities in A431-III cells. Cells were treated with different concentrations of FN for 12 h, and conditioned media were analyzed by gelatin zymography.*

fold, respectively) (Figure 3A). The increased expression of TG2 in A431-III cells relative to that of A431-P was confirmed by RT-PCR (Figure 3B). In addition, increased TG2 protein levels in A431-III cells were also observed in the cytosol and membrane fractions, and the conditioned media as compared with A431-P cells (Figure 3C).

We next investigated the relationship of increased endogenous TG2 expression and metastasis-associated activities including cell attachment, migration, invasion behavior and MMP activity using an siRNA knockdown approach. Transfection of *TG2* siRNA greatly suppressed TG2 levels in A431-P and A431-III cells (Figure 4A). We previously observed that A431-III cells have greater specific adherence to FN compared with A431-P cells (7); it is reasonable to hypothesize that increased surface TG2 level in A431-III cells could enhance cell attachment. Here, we show that *TG2* knockdown reduced cell attachment to FN substratum, but not BSA, in A431-P and A431-III cells (Figure 4B). This suggests that TG2 is an important factor in promoting FN-dependent attachment in A431 cells. The *in vitro* wound healing assay demonstrated that control A431-III cells repopulated the wound area faster than did A431-P cells, and delayed migration was much evident in *TG2* siRNA-transfected A431-III cells (Figure 4C). Additionally, the *in vitro* invasion assay demonstrated that knockdown of endogenous TG2 significantly suppressed the invasive activity of A431-III cells (Figure 4D). We next determined whether the increased TG2 level contributing to increased invasiveness of A431-III cells was related to increased MMP activity. Knockdown of endogenous TG2 by siRNA reduced the secreted MMP-9, but not MMP-2 gelatinase activity in A431-III cells (Figure 4E). This is further confirmed by immunoblotting analysis demonstrating that *TG2* knockdown decreased secreted protein levels of MMP-9 and MMP-1, but not MMP-2 and MMP-3 in A431-III cells (Figure

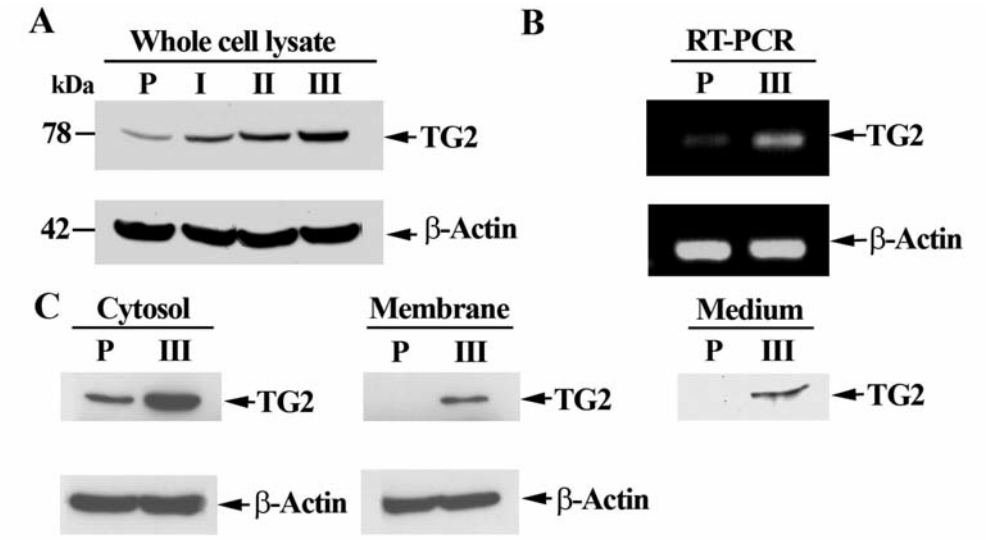

Figure 3. *TG2 expression is increased in the highly invasive A431-III cells. A: TG2 protein levels in whole–cell lysates of A431-P, A431-I, A431-II and A431-III cells were determined by immunoblotting. The membrane was stripped and reprobed with β-actin antibody serving as the internal control. B: TG2 mRNA levels in A431-P and A431-III cells were determined by RT-PCR. C: TG2 presence in the cytosol, membrane and conditioned media in A431-P and A431-III cells were further analyzed by immunoblotting.*

4F). These results suggest that elevated invasiveness of A431-III cells is partly attributed to an increase in TG2-induced secretion of MMP-9 and MMP-1.

Taken together, these results suggest that elevated cellular attachment, migration and invasiveness of A431-III cells are strongly associated with the up-regulation of TG2 expression and TG2-induced secreted activity of MMP-9 and MMP-1.

Increased FN and TG2 interaction is associated with integrin and FAK activation in highly invasive A431-III cells. FN interaction with integrins, particularly β1 and β3, is important in tumor cell migration, invasion and metastasis (8). Therefore, we investigated the degree of FN association with integrin β1 and β3 in A431-P and A431-III cells using co-immunoprecipitation. We noted TG2 association with FN in A431-P and A431-III cells (Figure 5A). There were great increases in FN and TG2 interactions with integrin β1 and β3 in the highly invasive A431-III cells as compared with A431-P cells, and both A431-III and A431-P cells appeared to have similar levels of integrin β1 and β3 (Figure 5B). We next determined the cellular localization of TG2 and FN in A431-P and A431-III cells using confocal microscopy. A431-III cells displayed increased surface TG2 (red fluorescence) and FN (green fluorescence) level and their co-localization as compared with A431-P (Figure 5C and D).

TG2 knockdown greatly suppressed the invasiveness of A431-III cells (Figure 4D), we therefore speculate that the positive correlation between TG2 and cell invasiveness is partly dependent on TG2-enhanced association of integrin with FN. To test this hypothesis, we determined β1 integrin association with FN when endogenous *TG2* was knocked down by siRNA. *TG2* knockdown had no apparent influence on the level of integrin β1 in A431-P and A431-III cells, yet it dramatically reduced the degree of FN association with integrin β1 in A431-III cells to the level of that in A431-P cells (Figure 6). This supports the notion that TG2 facilitates the binding of integrin β1 and FN in highly invasive A431-III cells.

Taken together, these results suggest that TG2 exerts crucial role in mediating cell adhesion, migration, and invasion of A431 cancer cells through FN-mediated integrin signaling.

Discussion

Tumor invasion and metastasis are the major causes of death in patients suffering from cancerous diseases. Therefore, understanding the molecular mechanisms that underlie the development of metastasis is imperative to the development of effective anticancer therapies. Comparison of primary tumor cells with metastatic analogs on the basis of the differences at gene or protein levels will facilitate a gradual understanding of the metastatic process of cancer. FN is frequently used as a substratum to promote cell adhesion *in vitro*. Previous studies have also found that signal transduction through the FN receptor induces the expression of MMPs such as collagenase and stromelysin (10). Additionally, FN treatment activates MMP-9 secretion and invasion in ovarian cancer cells (9). Clark and co-workers selected highly metastatic melanoma cells from mouse tumors and found enhanced expression of several genes including FN using DNA array analysis (3). The relationship between the increased expression of FN and the degree of

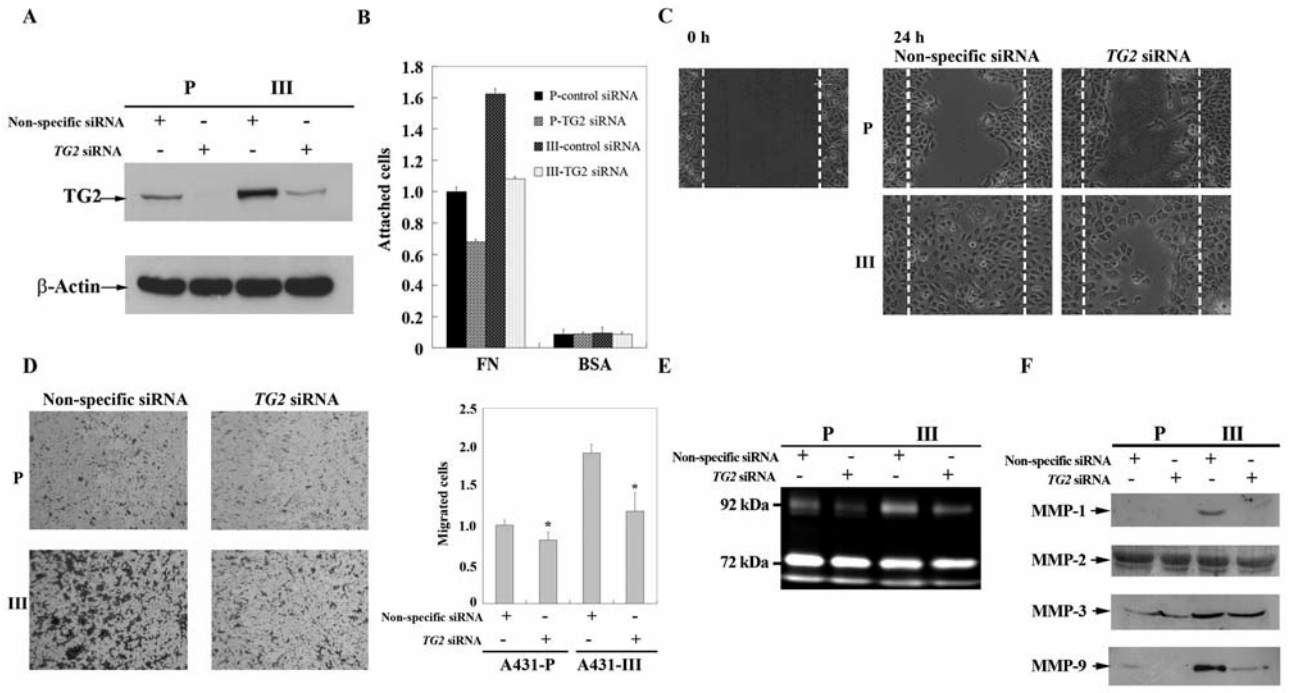

Figure 4. *Endogenous TG2 expression correlates with invasiveness and MMP-9 and MMP-1 secretion in A431-III cells. A431-P and A431-III cells were transfected for 48 h with 30 nM TG2 siRNA or nonspecific siRNA prior to further analyses. A: Cells were then plated onto six-well plates and allowed to grow in the presence of 10% FBS for 20 h. TG2 levels were determined by immunoblotting. This figure is a representative of three independent experiments. B: Cell attachment assays were performed. Cells were plated on FN- or BSA-coated wells for 30 min. Adherent cells were counted by visual microscopy examination. Each bar represents the mean (±SEM) of triplicate wells relative to the A431-P control value (expressed as 1.0). This figure is a representative of three independent experiments. C: In vitro wound healing migration assays were performed, and cell images at 0 and 24 h after wound scratch were taken under a phase contrast microscope. D: In vitro invasion assays were performed. The cells penetrating the Matrigel to the lower filter surface after 48 h were stained and visualized by microscopy. Each bar represents the mean (±SEM) of triplicate wells relative to the A431-P control value (expressed as 1.0). The asterisk designates a significant difference between siRNA-transfected group and the respective control ($p<0.05$). E: Effect of endogenous FN on the secreted gelatinase activities in A431-P and A431-III cells. Cell culture-conditioned media were collected and normalized by cell number prior to gelatin zymography analysis. F: Effect of endogenous TG2 on the secretion of MMP-1, -2, -3 and -9 in A431-P and A431-III cells. Cell culture-conditioned media were collected and concentrated for immunoblotting analysis.*

invasiveness in cancer cells has been investigated in only a few reports. Our present study demonstrates that the endogenous *FN* gene is up-regulated in highly invasive A431-III subline compared to the parental A431-P cells (Figure 1), and A431-III cells also secreted elevated levels of MMP-9 and MMP-1 (Figure 4). Knockdown of *FN* expression by siRNA reduced the migratory and invasion activity of A431-III cells (Figure 2B and C), indicating that the increased FN expression contributes to the increase of migratory and invasive potential in A431 sublines selected by their ability to penetrate through ECM-coated filter chamber. Additionally, *FN* knockdown reduced the secreted MMP-9 gelatinase activity (Figure 2D), while treatment with exogenous FN elevated MMP-9 activity in A431-III cells (Figure 2E). *MMP-9* gene expression is regulated by transcription factors such as SNAIL, TWIST, AP-1, NF-κB, SP1 and ETS-1 (29). FN is known to trigger the RAS-MAPK pathway, which in turn activates these transcription factors (30, 31). Additionally, a recent study showed that interleukin-induced human muscle cell migration is dependent on NF-κB and AP-1-mediated MMP-9 expression (32). It thus appears reasonable to speculate that *FN* up-regulation in A431-III cells may act as an upstream stimulator that activates MMP-9 expression resulting in their elevated invasiveness.

The association between TG2 expression and tumor progression has been the subject of several recent studies. It has also been shown that decreased TG2 expression and activity were accompanied by increasing cancer metastasis. Johnson and co-workers demonstrated that transfection of *TG2* into a highly malignant hamster fibrosarcoma led to reduced incidence of primary tumor growth (33). In addition, Haroon and colleagues reported that TG2 is expressed as a host response to tumor invasion and inhibits tumor growth in rats (34). In contrast, several studies indicate a positive correlation of TG2 expression and cancer invasiveness/metastatic activity. A previous report showed that *TG2* mRNA and protein expression were elevated in highly metastatic human melanoma cell lines compared to

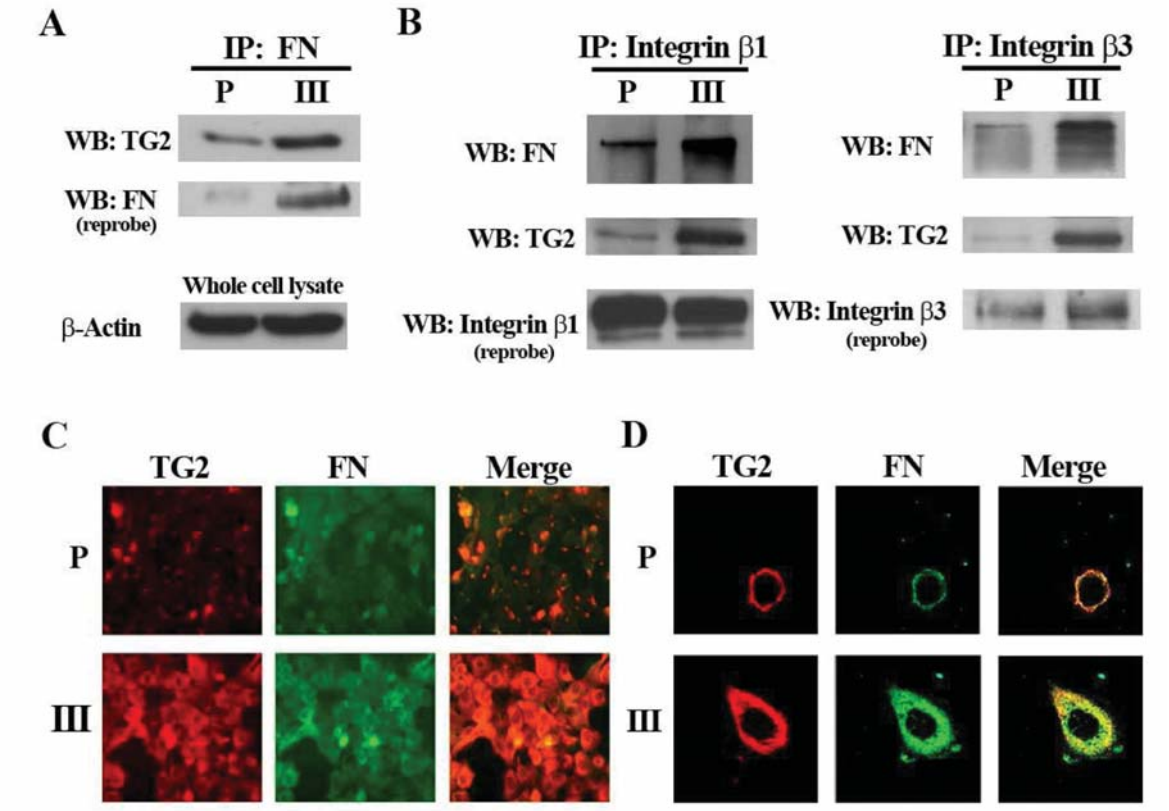

Figure 5. *Increased FN and TG2 interaction with integrin β_1 and β_3 in highly invasive A431-III cells. A: Cell lysates from A431-P and A431-III cells were immunoprecipitated with FN antibody, and then subjected to immunoblotting and probed with TG2 antibody. The membrane was stripped and reprobed with FN antibody. β-Actin served as a loading control. B: Cell lysates were immunoprecipitated with integrin β_1 or integrin β_3 antibody, and then subjected to immunoblotting and sequentially probed with FN and TG2 antibodies. The membrane was stripped and reprobed with integrin β_1 or integrin β_3 antibody. C, D: A431-P and A431-III cells were plated in a serum-free medium on non-FN-coated coverslips for 6 h. Non-permeabilized cells were double labeled for surface TG2 (red) and FN (green). Yellow color indicates co-localization of green and red fluorescence visualized by confocal microscopy, and images are presented as wide angle (C), and single-cell (D) views.*

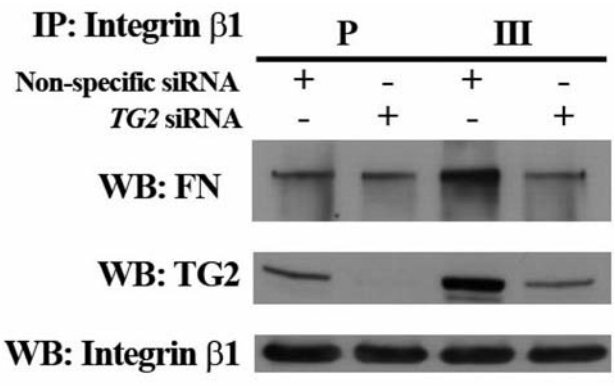

Figure 6. *Knockdown of endogenous TG2 decreases integrin–FN interaction in A431-III cells. A431-P and A431-III cells were transfected for 48 h with 30 nM TG2 siRNA or nonspecific siRNA. Cell lysates were then immunoprecipitated with integrin β_1 antibody, and then subjected to immunoblotting and sequentially probed with FN and TG2 antibodies. The membrane was stripped and reprobed with integrin β_1 antibody.*

weakly metastatic analogs (23). In addition, TG2 was identified as one of the 11 proteins selectively amplified in metastatic human lung carcinoma by proteomic analysis (24). Mehta and colleagues also demonstrated *TG2* up-regulation in drug-resistant and metastatic breast cancer cells (25). Our present study clearly shows that TG2 expression in highly invasive A431-III cells is 3-4 times higher than that of the less invasive A431-P cells (Figure 3). It has been demonstrated that the extracellular catalytic domain of TG2 is not required for cell adhesion and migration (11), and that the domain can inhibit motility by either increasing matrix tension that enhances focal adhesion, or by linking cell to ECM components (22). The *N*-terminal β-sandwich domain of TG2 mediates FN-integrin association to activate FAK (11), which triggers downstream pathways that promote cell adhesion and migration, the imperative process in tumor progression. Thus, it remains to be identified whether various TG2 domains indeed play opposite roles in tumor formation.

Recent studies have found that cell-surface TG2 can serve as a FN co-receptor (11). TG2 exerts this function by associating with integrin β1 and β3 while simultaneously binding to FN through the gelatin-binding domain. The integrin-mediated interaction of TG2 and FN can promote fibroblast adhesion, spreading and migration, and it is independent of TG2 catalytic activity (11, 19). Our previous study found that A431-III cells displayed higher adhesive, spreading, migratory and invasive activity than their A431-P counterparts (7). Here, we further demonstrate that when up-regulated TG2 expression in A431-III cells was silenced, attachment, migratory and invasive activity of these cells were reduced to the level of A431-P cells (Figure 4B, C and D). In addition, elevated MMP-9 and MMP-1 levels in highly invasive A431-III cells were greatly decreased when their TG2 expression was silenced (Figure 4E and F). These results indicate that increased TG2 expression is closely associated with the invasive potential in A431-III cells.

A recent study showed that cell surface TG2-induced integrin clustering may contribute to the increased capacity of TG2-expressing metastatic cells to adhere and migrate (35). We also demonstrate increased association of integrin β1 and β3 with FN and TG2 in A431-III cells compared to A431-P cells (Figure 5B), and knockdown of *TG2* expression reduced integrin β1 interaction with FN (Figure 6). These studies support the previously raised concept of Akimov and co-workers that the association of TG2 with integrin β1 and β3 is functionally adaptive, which strengthens the interaction between low-affinity integrin and FN (11). A recent study showed that TG2 expression also promotes invasion and survival in breast cancer cells, and TG2 is closely associated with integrin β1, β4 and β5 (36). In addition, TG2-positive breast cancer cells displayed increased adherence to FN and induced strong FAK activation (37). Increased FAK activation is associated with the progression to an invasive cell phenotype (38). It has also been demonstrated that FAK functions to coordinate a Src-p130Cas signaling complex, promoting RAC and JNK activation to alter the transcriptional regulation of cell invasion-associated gene targets such as MMP-9 (39). We therefore propose that increased TG2 and FN expression in A431-III cells may in part trigger integrin-mediated signaling that up-regulates MMP-9 and MMP-1 expression, and leads to enhanced invasiveness in A431-III cells. Alternatively, TG2 was reported to be involved in the PI3K/AKT signaling pathway (40). MMP gene expression is primarily regulated through AP-1 or NF-κB *via* MAPK or PI3K-AKT pathways (41). In addition, a recent report showed that overexpression of TG2 results in constitutive activation of NF-κB in breast cancer cell lines (42). Therefore, it is possible that TG2 may activate NF-κB and up-regulate MMP-1 and MMP-9 expression to enhance cancer cell migration and invasion. This awaits further study.

In conclusion, this study indicates that simultaneous up-regulation of TG2 and FN critically enhance cancer cell adhesion, migration and invasiveness during tumor progression, and this may partly attributed to facilitation of integrin activation and MMP expression. Therefore, combined targeting of TG2 and FN may be an effective therapeutic strategy for tumors displaying increased expression of both proteins. Additionally, this highly invasive cancer subline A431-III would be an excellent *in vitro* model for investigating the mechanisms of cancer cell invasion through FN and TG2-related events.

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