ORIGINAL ARTICLE

The role of tissue transglutaminase (TG2) in regulating the tumour progression of the mouse colon carcinoma CT26

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Abstract The multifunctional enzyme tissue transgluta-minase (TG2) is reported to both mediate and inhibit tumour progression. To elucidate these different roles of TG2, we established a series of stable-transfected mouse colon carcinoma CT26 cells expressing a catalytically active (wild type) and a transamidating-inactive TG2 (Cys277Ser) mutant. Comparison of the TG2-transfected cells with the empty vector control indicated no differences in cell proliferation, apoptosis and susceptibility to doxo-rubicin, which correlated with no detectable changes in the activation of the transcription factor NF-κB. TG2-trans-fected cells showed increased expression of integrin β3, and were more adherent and less migratory on fibronectin than control cells. Direct interaction of TG2 with β3 inte-grins was demonstrated by immunoprecipitation, suggest-ing that TG2 acts as a coreceptor for fibronectin with β3 integrins. All cells expressed the same level of TGFβ receptors I and II, but only cells transfected with active TG2 had increased levels of TGFβ1 and matrix-deposited fibronectin, which could be inhibited by TG2 site-directed inhibitors. Moreover, only cells transfected with active TG2 were capable of inhibiting tumour growth when compared to the empty vector controls. We conclude that in this colon carcinoma model increased levels of active TG2 are unfavourable to tumour growth due to their role in activation of TGFβ1 and increased matrix deposition, which in turn favours increased cell adhesion and a low-ered migratory and invasive behaviour.

Keywords Tissue transglutaminase · Colon cancer · TGFβ1 · β3 integrins · Metastasis

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Introduction

The communication between tumour cells and their microenvironment, particularly their recognition of the different extracellular matrix (ECM) ligands, is a key process during tumour progression including tumour cell proliferation, migration and matrix remodelling by these cells (Ai et al. 2008). The multifunctional enzyme tissue transglutaminase (TG2), which has important roles in cell adhesion and migration, cell survival/death, matrix depo-sition and angiogenesis, has been the focus of numerous studies in cancer biology (Kotsakis and Griffin 2007; Lentini et al. 2004). TG2 is the most ubiquitous member of the transglutaminase family which crosslink proteins through a Ca^{2+}-dependent manner via $\varepsilon(\gamma$-glutamyl) lysine bridges or via the incorporation of polyamines into peptide bound γ-glutamine residues (Griffin et al. 2002). TG2 is found in the intracellular compartment (e.g., cytosol, mitochondria, and nucleus), at the cell surface and in the ECM. Under physiological conditions, its transamidating activity normally remains latent within the cell due to low Ca^{2+} and the inhibitory effects of GTP/GDP (Smethurst and Griffin 1996). Present thinking suggests that only when Ca^{2+} homeostasis is lost and when the cell is already committed to cell death, does the crosslinking of intracellular proteins normally occur leading to apoptosis (Gundemir and Johnson 2009) and/or necrosis (Nicholas et al. 2003).

Recent evidence has suggested in breast cancer cells, TG2 may act as a non-transamidating scaffold protein in the intracellular environment by regulating cell migration, through influencing the organisation of the actin cytoskeleton (Antonyak et al. 2009).

In the Ca^{2+}-rich extracellular environment, TG2 may also function in either a transamidating or a non-transamidating role. As a novel scaffold protein, TG2 may promote cell adhesion through its ability either to act as an integrin coreceptor for fibronectin (FN) or, once deposited into the ECM and bound to FN, to function as a novel heterocomplex via binding to syndecan-4. Neither of these functions requires its transamidating activity (Akimov et al. 2000; Telci et al. 2008). In the extracellular environment, TG2 can also function through its Ca^{2+}-activated transamidating activity to increase the rate of deposition of ECM proteins such as FN and collagen I (Shweke et al. 2008; Verderio et al. 1999; Yuan et al. 2007), leading to accumulation and changes in the ECM influencing both cell adhesion and differentiation (Chau et al. 2005; Johnson et al. 2007).

Given these multifunctional roles of TG2, it is not surprising that reports have been contradictory as to whether TG2 is inhibitory to, or required for tumour progression (Grigoriev et al. 2001; Hand et al. 1988; Kotsakis and Griffin 2007; Verderio et al. 1999). In recent studies, we demonstrated that the crosslinking activity of TG2 leading to ECM deposition and the inhibition of angiogenesis was sufficient for tumour growth blockade following intratumour injection of active TG2 into mice bearing CT26 colon carcinoma tumours (Jones et al. 2006). In other studies, increased expression of TG2 in a rat fibrosarcoma model led to reduced tumour incidence (Johnson et al. 1994), while increased tumour progression of the B16-F1 and B16-F6 melanoma was observed in TG2 knockout animals when compared to their respective wild type controls (Di Giacomo et al. 2009; Jones et al. 2006). Contrary to these findings, other workers have suggested that TG2 may act as a cell survival protein in the activation of NF-κB by a mechanism involving the intracellular crosslinking of the IκB inhibitory subunit of the NF-κB complex (Mann et al. 2006; Verma and Mehta 2007b). In a recent study, it was also demonstrated that a TG inhibitor KCC009, as well as TG2 siRNA treatment, can disrupt the matrix FN assembly by glioblastoma cells, suggesting that TG2 may be a potential target in cancer chemotherapy (Yuan et al. 2007).

In order to distinguish the importance of the different functional roles of TG2 we have established a series of stable-transfected mouse colon carcinoma CT26 expressing catalytically active (wild type) and inactive TG2 (Cys277Ser mutant). Then in both in vitro and in vivo studies, we further investigated the behaviours of these cell clones with their respective controls to elucidate the different contributions that TG2 might have on the growth and progression of this mouse colon carcinoma.

Materials and methods

Cell culture

CT26 mouse colon carcinoma cells (ATCC no. 2638) were kindly provided by Prof. R. Rees (Nottingham Trent University, UK) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Transfected CT26 cells were maintained in the same medium supplemented with 400 μg/ml G418.

Isolation of CT26 (transfected with active and inactive TG2 constructs or the empty vector) from balb/c hosts was carried out by incubating 5 mm square pieces in DMEM, 100 U/ml penicillin and 100 μg/ml streptomycin, 0.2% (w/v) dispase (Gibco) at 4°C overnight. Following incubation, the tumour samples were further cut into 1–2 mm pieces using a scalpel in a rocking motion and placed into six-well plates with 0.2 ml of fully supplemented DMEM containing 10% (v/v) FBS. A further 1.5 ml of growth medium was added to the wells after 7–10 days. Following three rounds of passaging, isolated tumour cells were cultured in a standard cell growth media supplemented with 400 μg/ml G418.

Transfections

Transfection of CT26 mouse colon carcinoma cells with pSV-Neo and pSG5 expression vectors containing wild type and mutant (Cys277Ser) TG2 cDNA was achieved by co-transfecting cells with the *TG2*-containing vector (5 μg) and the selection pSV-Neo plasmid (0.5 μg) using a nucleofector system (AMAXA Biosystems) according to the manufacturer's protocol. Transfected clones resistant to 400 μg/ml G418 (Geneticin, Calbiochem) were screened for overexpression of TG2 by western blotting a high expressing TG2 clones selected.

Transglutaminase activity assays

Transglutaminase activity in cell lysates, obtained after sonication of cells, was measured by the incorporation of ^{14}C-labelled putrescine into *N,N'*-dimethylcasein as described previously (Lorand et al. 1972; Jones et al. 1997). In situ cell surface-associated TG activity was measured by incorporation of biotinylated cadaverine into deoxycholate-insoluble fibronectin as previously described (Jones et al. 1997). 2×10^4 cells/well in serum-free DMEM containing 0.132 mM biotin-X-cadaverine (Molecular Probes, Oregon,

USA) were added to the FN-precoated 96-well plates and incubated for 2 h at 37°C. The reaction was stopped by 2 mM EDTA in PBS, pH 7.4, and the cells were removed by incubation with 0.1% (w/v) deoxycholate in PBS containing 2 mM EDTA, pH 7.4 for 10 min at room temperature. Biotin–cadaverine incorporation into FN was detected using an extravidin peroxidase conjugate diluted 1:1,000 in blocking buffer.

Detection of antigens in the whole cell lysates, cellular fraction, growth medium, matrix or tumour homogenates

Tumour specimens or cells were sonicated in 250 mM sucrose, 2 mM EDTA in 5 mM Tris–HCl, pH 7.4, with protease inhibitor cocktail (Sigma, UK). Homogenates solubilised in Laemmli sample buffer were separated by SDS-PAGE (8% gels), followed by Western blotting. Monoclonal antibody Cub7402 recognizing human TG2, which does not cross react with mouse, was used for the detection of TG2. In cell lysates, monoclonal antibody TG100 which has a broader specificity was also used.

TG2 secreted into the media of transfected CT26 was detected as described previously (Balklava et al. 2002). Briefly, serum-free medium was incubated with confluent cultures for 8 h. Culture supernatants were precipitated with 10% (w/v) ice cold trichloroacetic acid (TCA). Protein pellets were washed sequentially with ice cold 10% (w/v) TCA, 0.5 ml ethanol–acetone (ratio 1:1) and acetone, and air dried prior to resuspension in 2× Laemmli sample buffer. TG2 was detected by Western blotting as described above.

Deoxycholate-insoluble ECM samples were collected after seeding the cells into 60 mm dishes for 16 h. Following detaching of the cells in 2 mM EDTA in PBS, pH 7.4, the remaining matrix was washed once with 2% sodium deoxycholate in Tris–HCl, pH 8.8, containing 10 mM EDTA and 0.5 mM PMSF (Akimov and Belkin 2001) and collected in 2× Laemmli buffer. Following separation by SDS gel electrophoresis using 6% gels, the presence of FN antigen was transferred to a nitrocellulose membrane and then detected by immunoblotting using rabbit anti-FN polyclonal antibody (Sigma–Aldrich).

Cell proliferation, apoptosis and doxorubicin susceptibility assays

Viable cell number was measured by XTT according to the manufacturer's protocol (Roche Diagnostics, UK). Briefly, CT26 cells (3,000/well) were seeded in 96-well plates and the XTT assay was performed after 12, 24, 36, 48 and 72 h-incubation. Programmed cell death in TG2-transfected CT26 clones was assessed using a commercial Caspase-3

Assay system (Promega, Southampton, UK), according to the manufacturer's protocol. CT26 cells (2×10^6 cells/T25 flask) were incubated for 24 h. The apoptotic index of the cell line was measured by the ability of caspase-3 to cleave Ac-DEVD-p-nitroaniline substrate and release a chromophore, p-nitroaniline (pNA). Apoptosis in the positive controls was induced using 1 μM of the protein kinase inhibitor staurosporine, whereas irreversible and cell-permeable pan-caspase inhibitor Z-VAD-FMK was added to a final concentration of 50 μM in the negative control cultures together with 1 μM staurosporine. To investigate the susceptibility of CT26 cell clones to doxorubicin, different concentrations of doxorubicin (0.5, 1, 10 μg/ml) were used to treat the CT26 cell clones (1,000 cells/well) for 48, 96, 120 and 144 h time courses and XTT assay was performed as before.

Cell attachment assay

CT26 cell clones (2×10^5/well) in serum-free DMEM were added to a 96-well FN-coated plates (5 μg/ml, incubated at 4°C overnight) and incubated for 40 min at 37°C, 5% (v/v) CO_2. The images of the crystal violet stained cells were stained captured using an Olympus digital camera. Dessicated cells were finally solubilised by addition of 100 μl/well 30% (v/v) acetic acid, and absorbance was read at 540 nm on a Spectrafluor plate reader.

Fluorescent measurement of cellular migration

Fluoroblock nitrocellulose membranes (6 μm pore size, BD Biosciences, UK) were coated with FN as described above. 10^6 cells/ml in serum-free DMEM placed into the top chamber of the wells were allowed to migrate through the FN-coated Fluoroblock nitrocellulose member for 72 h, while the culture medium with 10% (v/v) FBS as a chemoattractant was then added to the bottom wells. Inserts were transferred to 24-well glass-bottom plates containing 4 μg/ml calcein AM (Molecular Probes, Eugene, USA) in Hanks balanced salt solution and incubated at 37°C for 90 min. The signals were detected (485/530 nm excitation/emission) from bottom-to-top using a SpectraFluor plate reader.

Immunofluorescent staining for extracellular TG2

Extracellular TG2 was measured by in situ immunofluorescent staining as described previously (Verderio et al. 1998). Live CT26 cell clones were allowed to grow for 72 h to facilitate ECM deposition and then incubated with the anti-TG2 monoclonal Cub7402. After fixation, an FITC-conjugated anti-mouse IgG secondary antibody was used to reveal ECM-associated TG2 and the fluorescence

signal was visualised by laser confocal microscopy using a Leica TCSNT system (Leica, Heidelberg, Germany).

Flow cytometry

Transfected CT26 cells seeded in 6-cm petri dishes were detached with 2 mM EDTA in PBS, pH 7.4. The suspension containing 2×10^6 live cells/ml was incubated with 3 µg/ml of either Cub7402 or non-immune mouse IgGκ (Sigma) in serum-free medium for 1.5 h at 4°C. Following washing, the cells were incubated with the FITC-conjugate secondary antibody (3 µg/ml) for 1 h at 4°C and the washed cells were then fixed in 0.5% (w/v) formaldehyde, prior to analysis for the presence of cell surface TG2 in a DAKO Galaxy flow cytometer (DAKO, UK). Resulting values represent mean fluorescence intensity. Scattering images were analysed using WinMDI software (Scripps Research Institute). Values represent percentage of peak shift compared to the isotype control gate.

Co-immunoprecipitation

CT26 clone lysates were collected in lysis buffer with 500 µM TG inhibitor R283 (Griffin et al. 2008) as described previously (Telci et al. 2008). Cell lysates (150 µg) were pre-cleared by incubating with non-specific rabbit IgG (Santa Cruz, USA) and the protein A-Sepharose bead slurry (GE Healthcaree, Sweden) at 4°C for 1 h and 90 min, respectively. They were then incubated with 0.5 µg of rabbit anti-β3 integrin antibody (Cell signalling, UK) for 90 min at 4°C, followed by 50 µl of protein A-Sepharose bead slurry for 2 h at 4°C. The β3 immunocomplex was collected in $2\times$ Laemmli sample buffer and assayed via Western blotting to detect TG2.

Detection of the TGFβ1 secretion via ELISA

CT26 cell clones were seeded into 24-well plate and allowed to adhere for 2 h in complete medium. Growth factor-free replacement medium was replaced and supplemented with 10 µg/ml of purified guinea pig liver TG (gplTG) or 500 µM of the site-directed transglutaminase inhibitors R283 or R294 (Griffin et al. 2008). The cells were treated for 16 h and the culture medium was collected for TGFβ1 detection via ELISA according to the manufacturer's introduction (eBiosciences).

Detection of the presence of NF-κB in cytosol and nuclei fractions

The cytosol and nuclei fractions from Neo1 and TG3 CT26 cells were prepared using the activation assay kit from FIVEphoton according to the manufacturer's instructions.

The presence of NF-κB-p65 within the cytosol and nuclear fractions was detected via Western blotting using an anti-NF-κB (p-65) antibody (Cell Signalling, UK), while the phosphorylated form of IκB in the cytosol fraction was detected using an anti-phosphorylated-IκB antibody (Cell Signalling, UK).

Animal studies

Animal procedures were carried out in accordance with the UK Home Office Animal Act (1986). Female balb/c mice (Harlan Olac, UK) were inoculated with 2×10^3 transfected CT26 cells in 0.1 ml DMEM by subcutaneous injection in the right flank. Subcutaneous tumour growth was measured at 2 days intervals with a calliper and animals were killed before the individual tumour diameter exceeded 1 cm^2.

Statistical analysis

Differences between datasets were determined by the Mann–Whitney or student t test using *Minitab* or *Sigma Stats* packages at a significance level of $p < 0.05$.

Results

Screening CT26 clones for total TG2 antigen and activity

Following selection by G418-resistance, the active TG2 clones TG3 and TG5, and inactive mutant (Cys277Ser) transfected clones INA13 and 14, together with control clones Neo1 and Neo2, were chosen for further study (Fig. 1a). Measurement of TG activity in cell lysates indicated that the inactive mutant TG2 construct did not confer any further activity to the CT26 cells, compared to the transfected controls. Both the transfected control cells and the inactive mutant transfected cells had no detectable activity (Fig. 1b), which was confirmed by Western blotting for the Neo1 and Neo2 control cells. All clones were characterised by a phenotype of rod-like cell bodies extended in length, with no major morphological differences observed (Fig. 1c). Cell-surface TG activity was only detectable in clones TG3 and TG5 (Fig. 1d).

Cell surface and matrix distribution of TG2 antigen

Flow cytometric analysis confirmed the presence of TG2 at the cell surface of both active and inactive mutant TG2-transfected clones (Fig. 2a). In situ fluorescence-stained matrix-associated TG2 revealed patchy fibrillar staining of the externalised enzyme in clones TG3, and TG5, whereas
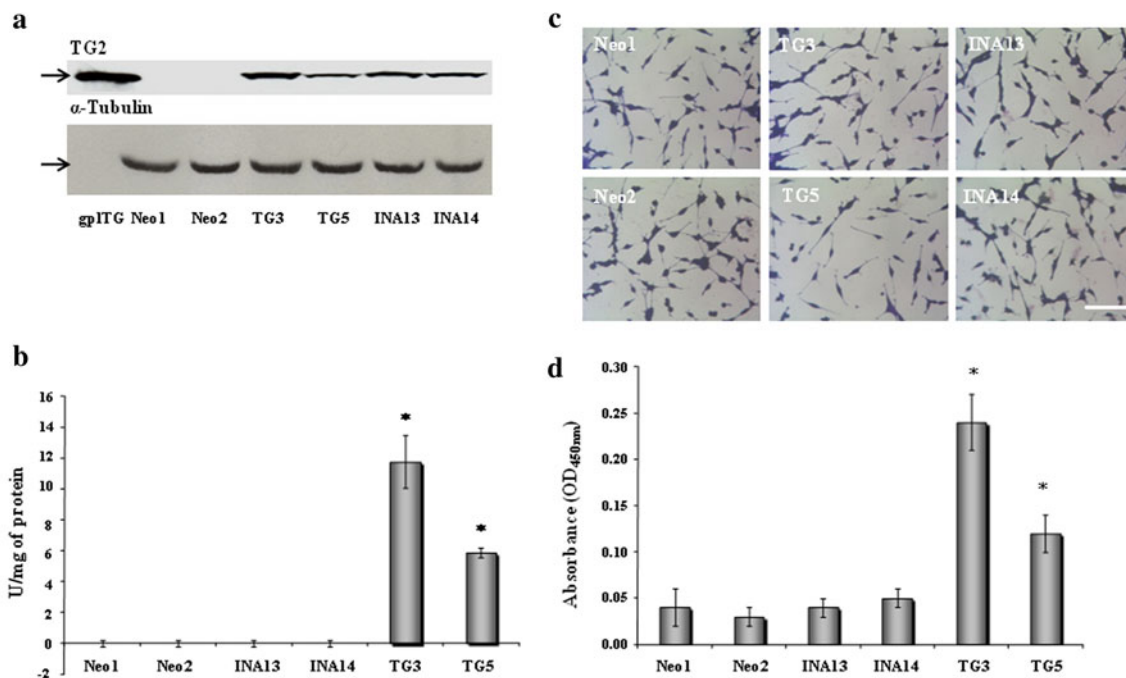
Fig. 1 The establishment of the CT26 cell clones following transfection with active and inactive TG2. **a** Western blotting was performed to detect the presence of TG2 antigen within the CT26 cell clones, Neo 1 and 2 (CT26 cell transfected with empty vector), INA13 and 14 (CT26 cells transfected with inactive TG2) and TG3 and 5 (CT26 cells transfected with active TG2) using mouse anti-TG2 monoclonal antibody Cub7402 (Neomarkers). Guinea pig liver TG (gplTG) was used as the TG2 standard, while α-tubulin was used as the equal-loading standard. **b** Total TG activity of CT26 clones was measured by the (^{14}C)-putrescine incorporation assay using the lysates from 4×10^6 cells as described in the "Materials and methods". Results represent mean values $\pm$ SD. *Asterisk* represents significant difference ($p < 0.05$) between the experimental groups and the control groups (the empty vector-transfected CT26 cells). **c** CT26 clone morphology was examined after staining with 0.5% (w/v) crystal violet. Images were captured using an Olympus digital camera attached to a light microscope (*Bar* 50 μm), which representative of nine images from each group. **d** Cell surface-associated TG activity was measured by incorporation of biotinylated cadaverine into deoxycholate-insoluble fibronectin as described in the "Materials and methods". Results represent mean values $\pm$ SD. *Asterisk* represents significant difference ($p < 0.05$) between the experimental groups and the control groups (Neo1 and Neo2)

the ECM of the inactive CT26 clones was found to be devoid of enzyme (Fig. 2b), which is in keeping with earlier studies in fibroblasts (Balklava et al. 2002). Western blotting of serum-free cell culture media from confluent cultures revealed the presence of the enzyme in the medium of the active TG2 clones only (Fig. 2c).

Overexpression of TG2 does not affect cell proliferation, apoptosis or the susceptibility to doxorubicin

Over a time course of 72 h (Fig. 3a), no difference in viable cell number was observed between the cells transfected with the active or inactive TG2 or the empty vector-transfection ruling out any influence of TG2 on cell proliferation. Similarly, apoptotic indices of the different clones when measured by caspase-3 activity were also very low and comparable throughout all clones (Fig. 3b). Previous work suggested that a high level of TG2 was discovered in drug-resistant breast cancer cell MCF-7 (Herman et al. 2006).

We, therefore, investigated the effect of doxorubicin on the CT26 cell clones. No significant difference in cell viability was found between the three clones Neo1, INA14 and TG3 at the concentrations 0.5, 1, and 10 μg/ml, suggesting the over-expression of either active or inactive mutant TG2 does not affect the susceptibility of the CT26 colon carcinoma to the anti-cancer drug doxorubicin (Fig. 3c).

Cell adhesion and migratory behaviour of CT26 clones: relationship to expression of integrins β1, β3, β5 and syndecan-4

CT26 clones overexpressing the active and inactive mutant forms of TG2 demonstrated a significant increase in cell adhesion on fibronectin-coated surfaces when compared to the transfected controls (Fig. 4a(i) and (ii)). This is consistent with their migratory behaviour on FN-coated surfaces, which showed reduced migration in both the active and mutant TG2-transfected clones, compared to the transfected controls (Fig. 4b).
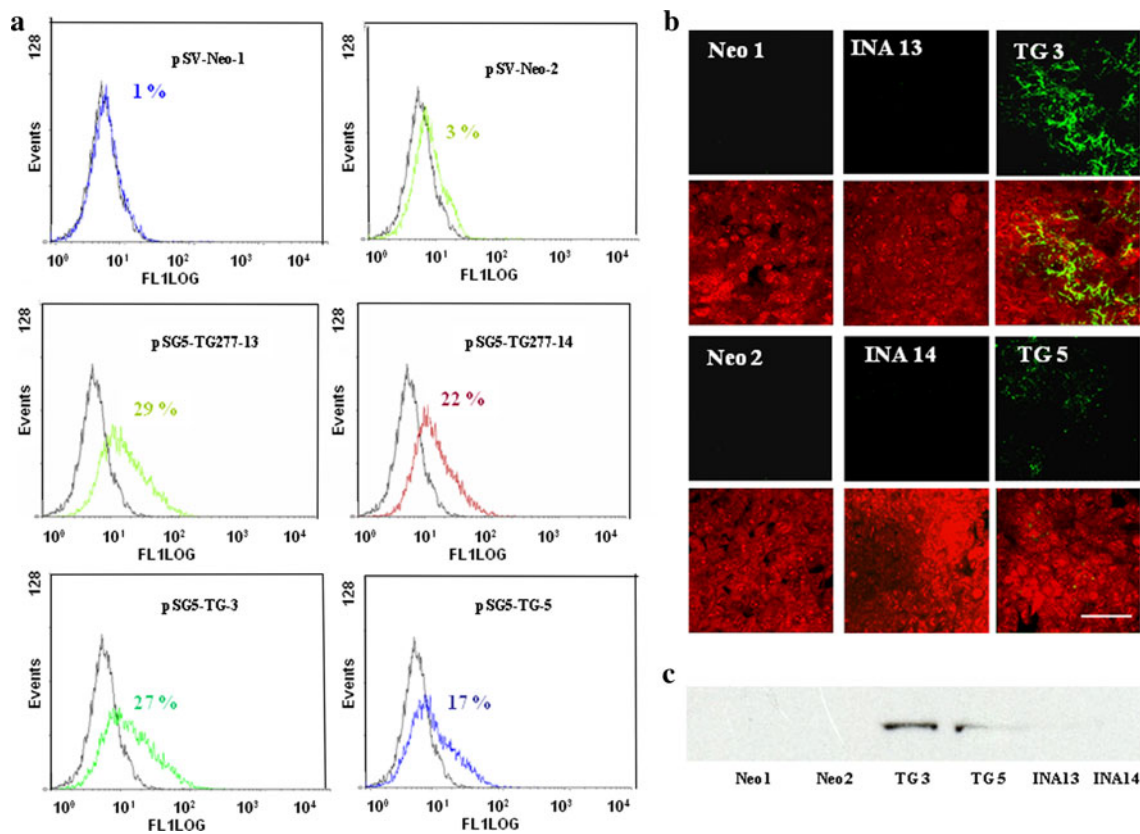
Fig. 2 The distribution of TG2 in CT26 cell clones. **a** Cell surface TG2 antigen was detected by flow cytometry using a DAKO Galaxy flow cytometer as described in the "Materials and methods". An anti-mouse IgGκ was used as the negative control antibody. Scattering images were further analysed using the WinMDI application. Values represent percentage of peak shift compared to the isotype control gate. **b** Deposition of the extracellular TG2 antigen into the ECM of the CT26 clones transfected with active (TG3 and 5) and inactive (INA13 and 14) TG2 was detected by in situ immunostaining as described in the "Materials and methods". Fixed cells were counterstained with propidium iodide (PI). Images of TG2 antigen were captured under the FITC filter (*top panels*) of a Leica TCSNT confocal microscope. Merged images of TG2 antigen with PI staining observed using the TRITC filter (*bottom panels*). *Bar* 50 μm. The image shown is representative of nine images from each group. **c** TG2 secreted in the culture media of TG2-transfected CT26 cells was detected as described in the "Materials and methods"

Analysis of the different cell surface receptors showed that TG2 might associate with indicated increased expression levels of $\beta 3$ integrin in both active (TG3 and TG5) and inactive mutant INA13 and 14 clones (Fig. 4c). Changes in the expression of $\beta 1$ and $\beta 5$ integrins and syndecan-4, which might also account for the observed changes in cell adhesion, were not detectable. Immunoprecipitation of TG2 in the Neo1, INA14, and the TG3 and TG5 cell clones using an anti-$\beta 3$ integrin antibody confirmed the direct association of the two proteins following western blotting and immunoprobing with anti-TG2 antibody (Fig. 4d).

Measurement of matrix FN and TGFβ1 in the CT26 transfected clones

Measurement of TGFβ1 levels in the cell culture medium from the clones Neo1, INA14 and TG3 (Fig. 5a) indicated a significant increase in the TGFβ1 levels in TG3, compared to the transfected control cells (Neo1) and the inactive mutant clone (INA14). The site-directed TG inhibitors R283 and R294 significantly ($p < 0.05$.) reduced the active levels of TGFβ1 produced by clone TG3, indicating its dependence on TG activity. Interestingly, the inhibitor R283, which unlike R294 is membrane permeable, also reduced the levels of TGFβ1 in the INA14 clone, when compared to control cells which may be due to other effects rather than its inhibition of TG2 given the ability of this inhibitor to penetrate the cell membrane. No significant reduction in TGFβ1 levels was found with the non-permeable TG2 inhibitor R294 in this particular clone. Exogenously purified gplTG added to the cell culture medium gave rise to a comparable increase in TGFβ1 in all the clones treated confirming the importance of TG2 in TGFβ1 activation. Equal expression levels of TGFβ receptors I and II were found in all of the three clones—Neo1, INA14 and TG3—when analysed by Western blotting (Fig. 5b). One important effect of TGFβ1 is related to increased deposition of ECM proteins such as collagen and FN (Rosenkranz
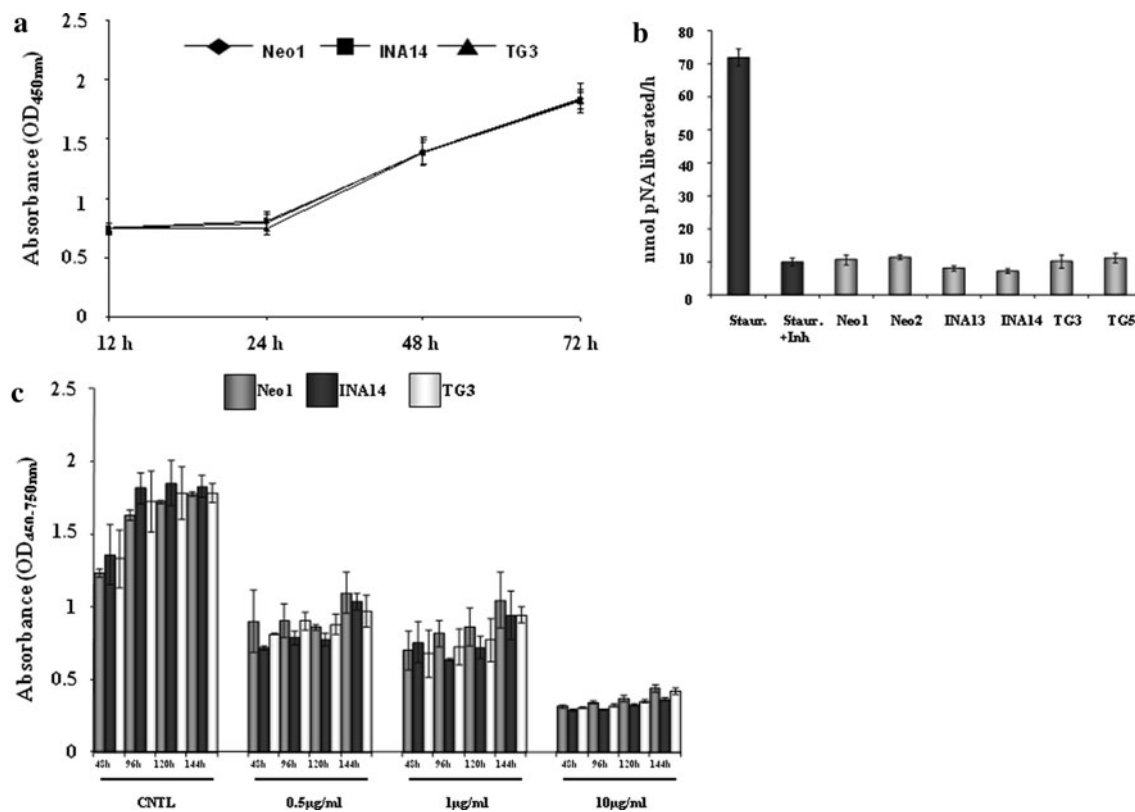
Fig. 3 The effect of TG2 on the CT26 cell proliferation, apoptosis and susceptibility to doxorubicin. **a** Viable cell numbers of the different CT26 cell clones were counted by the XTT assay after 12, 24, 48 and 72 h growth by measuring the absorbance at 490 and 750 nm as described in the "Materials and methods". Results represent mean values ± SD. **b** Programmed cell death was measured by caspase-3 activity detected in CT26 clone lysates, 16 h post-seeding, as described in the "Materials and methods", and was expressed as pmol of pNA liberated per hour by reference to a pNA standard curve. Addition of 1 μM staurosporine (staur) to cultures was used as the positive control, whereas staurosporine and an appropriate apoptosis inhibitor was used as the negative control incubation with (staur. + inh). Results represent mean values ± SD. **c** To investigate the susceptibility of CT26 cell clones to doxorubicin, CT26 cells (1,000 cells/well) were incubated with different concentrations of doxorubicin (0.5, 1, 10 μg/ml) for 48, 96, 120 and 144 h and XTT assay was performed using a microplate reader at 490 and 750 nm. Results represent mean values ± SD

2004). The levels of deoxycholate-insoluble FN matrix measured by Western blotting in the different clones indicated that both TG3 and TG5 contained a much higher level of both monomeric and polymerised FN (unable to enter the resolving gel), compared to the transfected Neo1 control and the clone transfected with the inactive mutant TG2 (INA14), which both deposited very little matrix FN. However, when cells were treated with equal amounts of exogenous gplTG, the levels of matrix-associated FN (particularly polymerised FN) increased in all the different clones (Fig. 5c), thus paralleling the increases found for TGFβ1 when TG2 was added exogenously.

Levels of NF-κB activation in the transfected CT26 clones

We next assessed whether TG2 could increase the active NF-κB levels in the CT26 clones transfected with the active and inactive mutant TG2. As shown in Fig. 5d, no significant difference of NF-κB activation, as measured by

the levels of p65 in the nuclear fraction, could be detected in the different clones studied. Moreover, the levels of p65 and the free inhibitory binding protein IκB (in its phosphorylated form) in the cytosol fraction were also comparable in the different clones. In addition, NF-κB activation, as measured by the above parameters, was not affected by the addition of the site-directed TG inhibitors R283 and R281 (Griffin et al. 2008), when added to the different clones (Fig. 5d), which rules out the involvement of TG2 in the activation of NF-κB in the TG2-transfected CT26 colon cancer cell lines.

The effect of overexpression of TG2 in CT26 on tumour growth and animal survival

Tumour incidence was found to be comparable in all six clones but the growth of the CT26 clones TG5 and TG3 containing active TG2 was significantly decreased when compared to the Neo1 and Neo2 controls at the time points from 19 days onwards (Fig. 6a). The inactive transfected
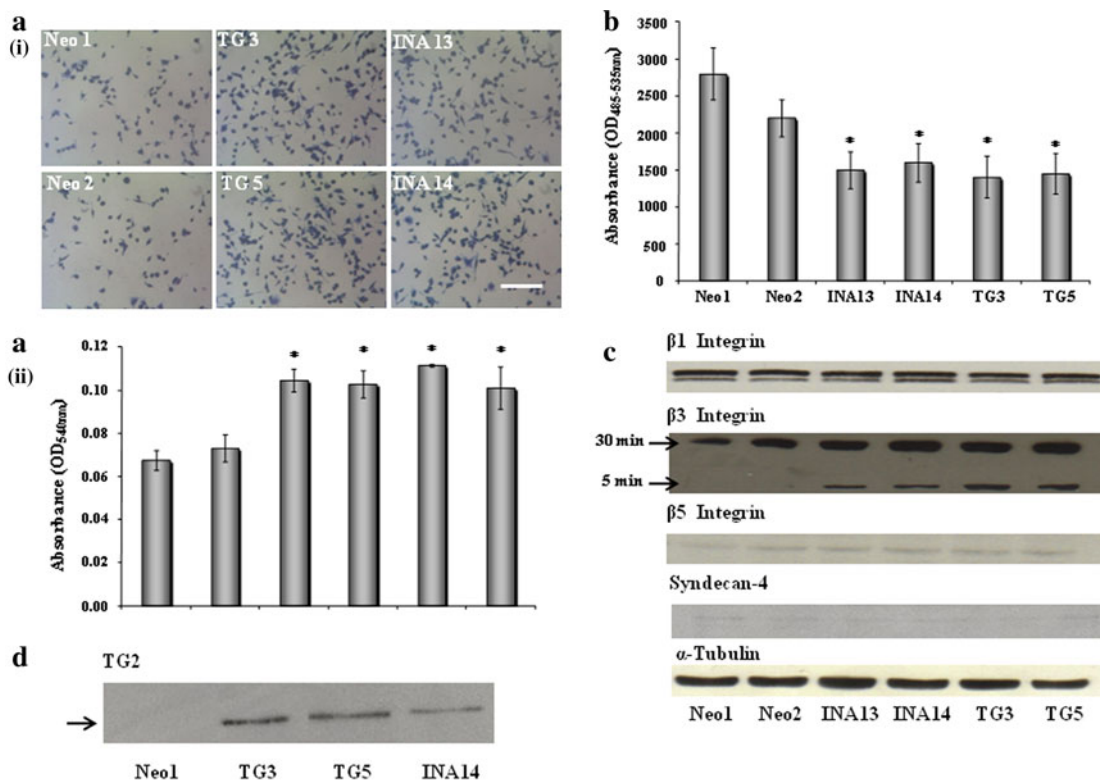
Fig. 4 The effect of TG2 on cell adhesion and migration and detection of cell surface receptors. **a** Attachment of CT26 clones on fibronectin-coated surfaces was undertaken as described in the "Materials and methods". (**i**) Images are representative of three non-overlapping fields, obtained from four separate samples (*Bar* 100 μm). (**ii**) stained cells were further solubilised in 30% (v/v) acetic acid for 30 min at room temperature and resulting absorbance was recorded at 540 nm using a SpectraFluor plate reader. Results are expressed as mean value ± SD from two independent experiments. *Asterisk* represents significant difference ($p < 0.05$) between the experimental groups and the control groups (the empty vector-transfected CT26 cells). **b** The expression of TG2 on the migration of CT26 clones was undertaken on FN-coated Fluoroblock nitrocellulose membranes as described in the "Materials and methods". Results represent mean values ± SD from three separate experiments. *Asterisk* represents significant difference ($p < 0.05$) between the experimental groups and the control groups (the Neo1 and 2 clones). **c** Western blotting was performed to detect the presence of the cell surface receptors β1, 3 and 5 integrins and syndecan-4 in CT26 clones using Western blotting as described in "Materials and methods". The membranes were re-probed for α-tubulin detection as a standard for the equal loading. **d** The interaction between β3 integrins and TG2 was investigated by co-immunoprecipitation using anti-β3 antibody to pull down the β3 immuno-complex and Western blotting was used to detect the presence of TG2 antigen within this complex using anti-TG2 antibody Cub7402

controls INA13 and 14 did not show any significant difference in growth when compared to the Neo1 and Neo2 control groups. This decrease in tumour growth in the active TG2-transfected clones was accompanied by an increase in the percentage animal survival (Fig. 6b). The TG5 clone, which when injected expressed less active TG2 than clone TG3, demonstrated both the slowest growth rate and the best percentage of animals surviving. Both clones INA13 and INA14 transfected with inactive Cys277Ser mutant showed tumour growth rates and animal survival percentages more comparable to the transfected control clones. Inspection of the liver and lungs at tumour harvest indicated no detectable signs of secondary tumours. Immuno-probing of western blots obtained from tumour tissue at 26 days growth for the presence of human TG2

indicated its presence in all the TG2-transfected clones although somewhat reduced (Fig. 6c), compared to the original cells used for inoculation. The amount of TG2 antigen present in TG3 tumours was reduced compared to that found in TG5, which may account for the difference in tumour growth rates of these two clones transfected with the active TG2. To confirm this selective reduction in TG2 in clone TG3 during tumour growth, tumours at day 26 were resected and CT26 cells were selected from the tumour mass in the presence of G418. Assay for TG2 antigen in the cultured cells by western blotting confirmed that a reduction in TG2 antigen had occurred in clone TG3, when compared to clone TG5, during tumour growth (Fig. 6d), thus confirming what was found in the tumour tissue.
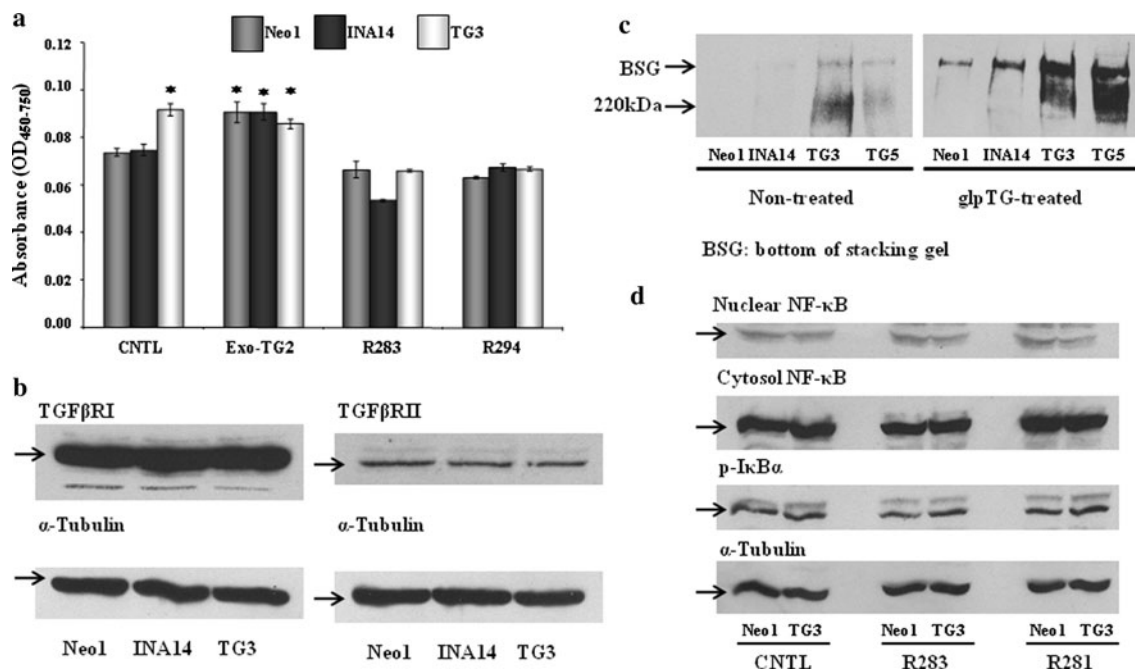
Fig. 5 The effect of TG2 on TGFβ1 release and its related matrix FN deposition and NF-κB activation. **a** The release of TGFβ1 into the medium of CT26 cell clones. CT26 cell clones were detected by ELISA as described in the "Materials and methods". Results represent mean values ± SD. *Asterisk* represents significant difference ($p < 0.05$) between the experimental groups and the control groups (the non-treated Neo1 cells). **b** Detection of the presence of TGFβRI and TGFβRII in CT26 cell clones was undertaken by Western blotting using specific antibodies as described in the "Materials and methods". The membranes were re-probed for α-tubulin detection as a standard for equal loading. **c** For detecting matrix-FN deposition, the deoxycholate-insoluble matrix samples were collected and the FN present detected by Western blotting as described in the "Materials and methods". **d** For measurement of the activation of NF-κB in CT26 cell clones the cytosol and nuclei fractions from Neo1 and TG3 CT26 cell clones were prepared using the activation assay kit as described in "Materials and methods". Western blotting was performed to detect the antigens of NF-κB and phosphorylated IκB within the samples using specific antibodies. The membrane with the cytosol fraction samples was then re-probed to detect α-tubulin as a measure of equal loading

Discussion

In this study, we have undertaken both in vitro and in vivo studies on a mouse CT26 colon carcinoma cell line with the objective of elucidating the different contributions of the multifunctional enzyme TG2 on the growth and progression of this mouse colon carcinoma model. Studies on cells transfected with both the active wild type enzyme and the inactive Cys277Ser mutant indicated very little difference in cell morphology, cell proliferation and endogenous rates of apoptosis, when compared to the empty vector-transfected controls. However, when cells transfected with the active wild type TG2 were subcutaneously injected into mice, the tumour growth rates were significantly slower and percentage animal survival was increased, in comparison to animals injected with the other transfected CT26 cells. Examination of tumour tissue for the presence of TG2 indicated that it was still detectable in all the tumours, where the original cells contained the transfected enzyme although slightly lower in the tumours derived from the active clone TG3. Moreover, when cells were cultured from resected tumour tissue and the CT26 cells selected for in G418, assay of TG2 antigen in these cells confirmed a reduced amount of TG2 antigen in TG3 cells compared to TG5 cells. This reduction in TG2 in TG3 clones may explain why this clone grew faster than the other active TG2-transfected clone TG5, which originally had less TG2. These data are consistent with much earlier studies investigating the effects of TG2 expression on tumour growth where the active TG2 was transfected into a highly metastatic fibrosarcoma cell line (Johnson et al. 1994) and into a neuroblastoma cell line (Piacentini et al. 1996). However, in both the latter studies, cells carrying inactive TG2 Cys277Ser mutant were not studied and no detailed mechanisms for the observations were given. They are also consistent with the observation of increased tumour growth and progression of the B16 melanoma (F1and F6) in a mouse TG2 knockout model (Di Giacomo et al. 2009; Jones et al. 2006). Taking on board the proposed physiological functions for TG2 (Collighan and Griffin 2009; Griffin et al. 2002) and those proposed for its roles in cancer progression (Kotsakis and Griffin 2007; Mehta 2009; Verma and Mehta 2007a), our next step was to
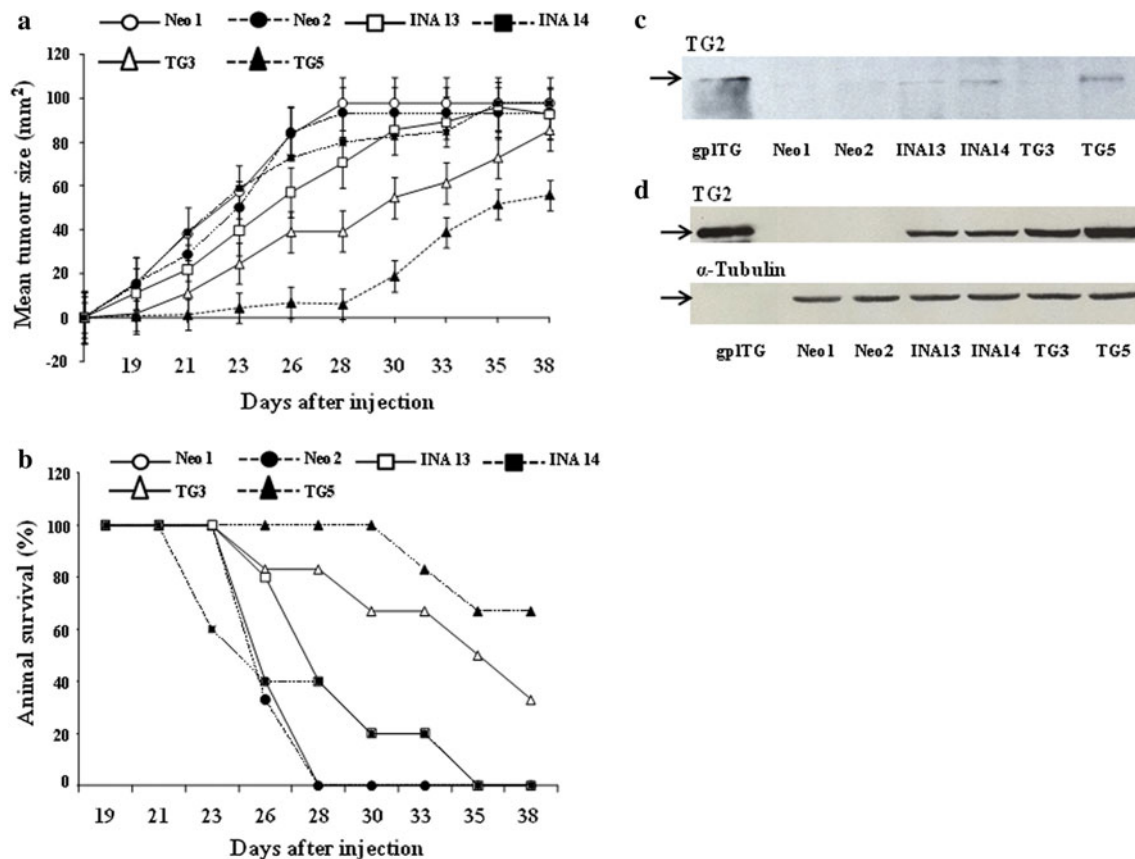
Fig. 6 The effect of TG2 on CT26 tumour growth. Exponentially growing CT26 clones were harvested and resuspended to a final concentration of 2×10^4 cells/ml in DMEM, prior to subcutaneous implantation of 100 µl cell suspensions into 8-week-old balb/c mice. Following injections, animals were checked every 2 days for tumour development. The results shown are from a representative experiment undertaken in duplicate and indicate mean values ($n = 6$). **a** Average tumour growth rate expressed in mm^2 was measured up to 38 days post-injection. **b** Percentage animal survival, following injection of the malignant CT26 clones. **c** For detection of TG2 expression in tumour samples, tumour specimens were homogenised in 250 mM sucrose, 2 mM EDTA, 5 mM Tris–HCl in the presence of protease inhibitors to yield a 20% (w/v) final homogenate concentration. 20 µg of total protein was analysed for TG2 antigen by Western blotting, gplTG was used as a positive control. **d** CT26 tumour cells from Balb/c animals were isolated as introduced in "Materials and methods" and Western blotting was performed to detect the presence of TG2 antigen. Western blots were undertaken three times and used tubulin as a marker for equal protein loading, while gplTG was used as a positive control

elucidate the mechanism(s) for this effect of the active TG2 on tumour growth.

One of the important functions of TG2 pertains to its role in the extracellular environment where it may influence both cell adhesion (Akimov et al. 2000; Telci et al. 2008; Verderio et al. 2003) and matrix accumulation/stability (Akimov and Belkin 2001; Chau et al. 2005). In all TG2-transfected clones, TG2 was found at the cell surface, but in keeping with our earlier studies (Balklava et al. 2002; Gaudry et al. 1999), only in the clones transfected with the active wild type enzyme was it found deposited in the extracellular matrix (ECM). The presence of the enzyme at the cell surface either in its active or inactive mutant form led to an increase in cell adhesion on fibronectin (FN) by a mechanism probably linked to its ability to act as an integrin coreceptor for FN (Akimov et al. 2000), since overexpression of TG2 in the CT26 cells is accompanied by an increase in the β3 integrin, not present in the controls. Moreover, immunoprecipitation studies revealed the direct association of TG2 with β3 integrin. No change was found in other TG2-related cell adhesion cell surface receptors, i.e. β1 and β5 integrins and syndecan-4. β3 integrins can mediate cell adhesion and migration on FN via their binding to the RGD cell binding-domain. Interestingly, like TG2, it plays important roles in both wound healing and angiogenesis (Switala-Jelen et al. 2004; Tadokoro et al. 2003) and has been linked to TG2 function in macrophage phagocytosis (Toth et al. 2009). The increased cell adhesion on FN exhibited by the TG2-transfected cells probably explains their reduced migration on FN surfaces. However, given that both clones transfected with either active wild type or the inactive mutant display a similar adhesive/migratory phenotype, it does not account for the differences in tumour growth observed

between these clones. Since TG2 may influence cell survival of tumours via activation of NF-κB (Mann et al. 2006; Verma and Mehta 2007b), we investigated whether the level of NF-κB was different in each of the transfected clones to rule out its influence on tumour growth. All representative clones tested showed no differences in the activation of NF-κB, which probably explains their comparable levels of endogenous apoptosis and also their comparable susceptibility to doxorubicin.

The importance of TG2 in ECM deposition and stabilization is now well established (Chau et al. 2005; Collighan and Griffin 2009; Fisher et al. 2009). TG2 activity can influence this process either directly by crosslinking (Chau et al. 2005) or indirectly by the crosslinking and activation of matrix-associated latent TGFβ (Verderio et al. 1999). Accumulating reports indicate that TG2-mediated matrix changes can inhibit tumour invasion (Di Giacomo et al. 2009; Jones et al. 2006; Mangala et al. 2005), while a TG2-crosslinked extracellular matrix can also inhibit angiogenesis (Jones et al. 2006; Myrsky et al. 2009). We recently demonstrated that intratumour injection of TG2 into mice bearing the CT26 tumours led to induction of fibrosis and tumour regression (Jones et al. 2006). Turning our attention to assess whether the levels of the fibrotic cytokine TGFβ1 were different between the CT26 clones, we found increased levels of TGFβ1 in the medium of the active TG2 transfected clone, which could be inhibited by the inclusion of site-directed TG inhibitors in the culture medium. Moreover, addition of exogenous TG2 to the cell cultures of the inactive mutant or the empty vector cells raised the level of active TGFβ1 to the level of the clone carrying the active enzyme, thus supporting earlier findings for the involvement of TG2 in TGFβ1 activation (Huang et al. 2009; Telci et al. 2009).

Importantly, we found that all the CT26 clones investigated carried the TGFβ receptor I (TGFβRI) and TGFβ receptor II (TGFβRII). Hence any increase in the levels of active TGFβ1 such as those found in the active TG2 transfected clone should lead to autocrine stimulation of the CT26 clone resulting in increased matrix protein deposition. Analysis of the transfected clones clearly indicated an increase of deoxycholate-insoluble matrix FN found in the two clones transfected with the active TG2. Moreover, the increased levels of TGFβ1 produced by the active TG2 transfected clone could also lead to paracrine stimulation of stromal cells in the tumour environment. Evidence, that this increase in FN deposition, was linked to increased levels of extracellular TG2 was demonstrated by the increased levels of FN found in the clones carrying the inactive mutant TG2 and the empty vector, when TG2 was added to the medium of these cells. Our data therefore indicate that the CT26 cells transfected with the active TG2

secrete this enzyme into the ECM, where it is able to activate latent matrix bound TGFβ1. The consequence is the increased deposition and stabilization of matrix proteins such as FN either by the tumour cells themselves or by surrounding stromal cells, leading to increased cell adhesion, decreased migratory ability and inhibited invasion of the surrounding matrix, thus slowing down tumour growth and progression.

Over the last two decades, various reports have looked at the relationship between cancer development and TG2 expression (Jones et al. 2006; Kotsakis and Griffin 2007; Mehta 2009). The take home message has been that TG2 expression and activity is generally altered when the tumour cells are compared to normal cells (Ientile et al. 2007; Kotsakis and Griffin 2007). Whether this is an increase or a reduction in TG2 activity either through reduced expression or the expression of altered forms (Di Giacomo et al. 2009) seems to be dependent on tumour type and stage of tumour progression. In the early stages of tumour growth and progression decreased TG2 at the cell surface and in the matrix would facilitate increased invasion and migratory ability. In some cancer cells, cell surface TG2 may be reduced through degradation by MMPs (Belkin et al. 2004), or can be down-regulated by activated H-Ras (Akimov and Belkin 2003). However, in the later stages of tumour progression, following increased selection for the metastatic phenotype, TG2 expression may be important in cell mechanisms facilitating cell survival. In this report, we validate earlier findings (Barnes et al. 1985; Hand et al. 1988; Jones et al. 2006; Knight et al. 1990) that reduced TG2 expression is required during tumour growth and progression, and provides a mechanism relating to the importance of the enzyme in matrix synthesis and deposition explaining why this reduction should occur.

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