

Crosslinking and G-protein functions of transglutaminase 2 contribute differentially to fibroblast wound healing responses

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Summary

Tissue transglutaminase (TG2) affects cell-matrix interactions in cell spreading, migration and extracellular matrix (ECM) reorganisation. Using fibroblasts deficient in TG2 or overexpressing normal or crosslinking-deficient enzyme, we show that the extracellular crosslinking activity and intracellular G-protein function in signal transduction contribute differentially to regulation of cell-matrix interactions. TG2-deficient cells displayed normal attachment but delayed spreading on ECM substrata and defects in motility unrelated to crosslinking. Blocking antibodies to TG2 failed to induce similar defects in normal fibroblasts. TG2-deficient fibroblasts had defects in focal adhesion turnover and stress fibre formation, showed changes in focal adhesion kinase (FAK) phosphorylation and failed to activate protein kinase C α (PKC α). Phospholipase C (PLC) and PKC α inhibitors blocked spreading of normal fibroblasts whilst PKC activators

induced spreading in TG2-deficient cells. In contrast, ECM remodelling was not only compromised by TG2 deficiency but also by overexpression of dominant negative enzyme and TG inhibitors. TG2 activity increased matrix tension and was required for membrane type 1-MMP (MT1-MMP)-dependent activation of MMP-2. Our results demonstrate that TG2 is involved in the control of dynamic adhesion formation in cell spreading and migration via regulation of phospholipase C activity. By virtue of its crosslinking activity, the enzyme plays a central role in regulating ECM remodelling.

Movies available online

Key words: Transglutaminase, Cell adhesion, Wound healing, Signalling

Introduction

Transglutaminases (TG; EC 2.3.2.13) form a family of structurally and functionally related enzymes that stabilise protein assemblies through the formation of intra- or intermolecular N^ε(γ -glutamyl)lysine bonds (Aeschlimann and Paulsson, 1994). Several of these enzymes have specialised functions in distinct biological processes such as the role of factor XIIIa in fibrin clot stabilisation and TG1 in keratinocyte cornified envelope formation (Muszbek et al., 1996; Nemes and Steinert, 1999; Aeschlimann and Thomazy, 2000). In contrast, TG2 (tissue TG, TG_C) is a multifunctional enzyme apparently involved in disparate biological processes. TG2-mediated crosslinking plays a role in stabilisation of ECM and promotes cell-matrix interactions (Aeschlimann and Paulsson, 1991; Aeschlimann and Thomazy, 2000; Aeschlimann et al., 1995; Martinez et al., 1994; Corbett et al., 1997; Verderio et al., 1998; Gaudry et al., 1999). In addition, TG2 (called G α_h in this context) has GTPase activity and has been shown to be involved in intracellular signal transduction, mediating agonist-induced signalling from $\alpha_{1B/D}$ -adrenergic receptors to downstream effectors such as phospholipase C δ 1 (PLC δ 1)

(Feng et al., 1996; Baek et al., 2000). GTP serves as a potent allosteric inhibitor that suppresses the Ca²⁺-activated crosslinking activity of the enzyme. TG2 is the most widely distributed form of TG in tissues and accumulates in cells undergoing terminal differentiation and at sites of tissue injury and inflammation (Aeschlimann and Thomazy, 2000). In skin, TG2 is an essential component of the wound healing program, and transamidation of extracellular components is required for the de novo formation of a functional dermo-epidermal cohesion apparatus (Dolynchuk et al., 1994; Raghunath et al., 1996; Haroon et al., 1999; Mearns et al., 2002). However, excessive crosslinking by TG2 has also been implicated in the pathogenesis of diseases related to wound healing and neovascularization, most notably fibrogenic reactions in various organs (Aeschlimann and Thomazy, 2000). Overexpression of TG2 in the heart caused interstitial fibrosis in transgenic mice and showed no evidence of an alteration in adrenergic receptor signalling (Small et al., 1999). This suggests a central role for TG2 in the control of fibroblast activities. The absence of an overt developmental phenotype in TG2^{-/-} mice (De Laurenzi and Melino, 2001; Nanda et al.,

2001) may relate to its co-expression with other TGs in many biological contexts. We have recently identified TG gene clusters in the human and mouse genome which contain several novel enzymes that are closely related to TG2 (Grenard et al., 2001) and are upregulated in TG2^{-/-} mice in a tissue-dependent manner. Nevertheless, a major function for TG2 in tissue repair is suggested by the recently reported abnormal wound healing response and reduced macrophage activity in TG2 knockout mice (Mearns et al., 2002; Szondy et al., 2003).

Independent of its catalytic functions, TG2 binds to several ECM proteins, and fibronectin in particular, with high affinity (LeMosy et al., 1992). Thus, when externalised to the cell surface TG2 accumulates in the pericellular matrix where it promotes matrix assembly and/or contributes to the stabilisation of cell-matrix interactions (Gaudry et al., 1999). Whilst a number of studies have shown that alterations in TG2 expression cause changes in cell adhesion (attachment and/or spreading) and modulate the metastatic potential of tumour cells, different activities of TG2 have been implicated (Gentile et al., 1992; Martinez et al., 1994; Jones et al., 1997; Akimov et al., 2000). Support for a direct link between crosslinking of fibronectin and cell adhesion comes from a study where a series of fibronectin variants carrying point mutations were generated. The spreading of fibroblasts on fibrin clots formed with mutant fibronectin lacking the major TG crosslinking site was significantly reduced as compared to clots formed with recombinant wild-type fibronectin (Corbett et al., 1997). However, it has recently been proposed that the effect of TG2 on cell adhesion is mediated by a non-catalytic mechanism involving the formation of a trimeric complex through simultaneous interaction of the enzyme with the gelatin-binding, 42 kDa fragment of fibronectin and β 1- or β 3-integrins (Akimov et al., 2000).

The present study addresses the mechanism through which TG2 affects cell-matrix interaction in cell adhesion, migration and ECM remodelling. We show that TG2 expression is not sensitive to the mechanical environment of the cells, is highly induced in fibroblasts undergoing senescence, and that increased TG2 expression is responsible for the associated changes in fibroblast-mediated ECM reorganisation. Deficiency in TG crosslinking results in reduced collagen matrix contraction and activation of gelatinase, hallmarks of fibroblast mediated ECM remodelling. In contrast, the role of TG2 in modulating dynamic adhesion formation in cell spreading and migration is independent of its externalisation and crosslinking function. In this context, TG2 is required for intracellular signal transduction resulting in the activation of protein kinase C α (PKC α). Our results demonstrate that the protein crosslinking and G-protein functionality of TG2 independently contribute to the regulation of cell-matrix interactions in ECM remodelling.

Materials and Methods

Materials

Oligonucleotides and cell culture reagents were obtained from Life Technologies and restriction enzymes from Promega.

Isolation and culture of fibroblasts

Tissue specimens were obtained with Ethical Committee approval and informed consent from patients attending the University Hospital of

Wales, Cardiff. Cultures of primary foreskin fibroblasts were established as described previously (Stephens et al., 1996) and maintained in DME medium supplemented with 2 mM L-glutamine, non-essential amino acids, antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B) and 10% FCS at 37°C in a 5% CO₂ humidified atmosphere. Prior to seven population doubling levels (PDLs) cells were considered primary cells.

Establishment of senescent and immortalised fibroblasts

The HCA2 strain of normal diploid fibroblasts was maintained as above and in vitro life span determined (Bond et al., 1999). Senescence occurred at an estimated PDL of 66-67. For immortalisation, cells were grown to PDL 31.5 before being infected with amphotropic retrovirus pBABE-hTERT (McSharry et al., 2001) to rescue cells from senescence by expression of the catalytic subunit of human telomerase (hTERT). hTERT-transfected HCA2 cells have been grown for >280 PDL beyond the point when the parental HCA2 cells senesced.

Fibroblast-populated collagen lattices (FPCL)

Cells (1.5×10^5 and 1×10^6 for ECM reorganisation and RNA extraction, respectively) were resuspended in 0.75 ml medium and added to 3 ml of 2 \times DMEM, 0.75 ml 100 mM NaOH, 0.75 ml gelatin-A Sepharose-pretreated FCS and 2.25 ml of 1.7 mg/ml rat tail tendon collagen I in 53 mm bacteriological grade culture dishes (Cook et al., 2000). FPCLs were allowed to set for 60 minutes at 37°C and subsequently detached from the edge of the plate where indicated (floating lattices) prior to the addition of 2 ml medium (containing 10% gelatin-A Sepharose-pretreated FCS) for further culture. The degree of lattice contraction was determined from three diameter measurements performed on each of three replicate samples at the indicated time points.

PCR amplification of TGs

Cells ($1 \times T75$) or FPLCs ($4 \times$ lattices) were mechanically disrupted in 1 ml and 2 ml, respectively, of UltraspecTM RNA solution (AMS Biotech), and total RNA extracted with chloroform and precipitated with isopropanol following the manufacturer's instructions. 1 μ g total RNA was reverse transcribed into DNA using 300 U MMLV reverse transcriptase (Life Technologies) and 0.12 μ g/ μ l random hexamer primers in a total volume of 25 μ l. Primers were designed for the different mRNAs (Table 1) that yield products spanning exon-intron boundaries to ensure that products were derived from mRNA only. PCRs were performed with 1.0 μ l of the RT reaction using 0.625 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) and the respective buffer supplemented with 2 mM MgCl₂, 0.2 mM dNTPs and 12.5 pmol of each primer in a total of 25 μ l. 35 cycles were carried out in an ABI GeneAmp 9600 thermal cycler, each cycle consisting of denaturation at 94°C for 45 seconds, annealing at 60°C for 1 minute and extension at 72°C for 1.5 minutes. Products were analysed on 1% agarose gels and sequenced using the dRhodamine Terminator Cycle Sequencing kit (Applied Biosystems) and an ABI 310 Genetic Analyzer.

Quantitative PCR

Real-time PCR was carried out on an ABI 7700 Sequence Detection System using the 5' nuclease assay. Fluorescently labelled probes (5' 6-carboxyfluorescein and 3' 6-carboxytetramethylrhodamine) were obtained from Applied Biosystems: TG1, 5'-TGCTCAATGTCTCA-GGCCACGTCAAGG; TG2, 5'-AGCTACCTGCTGGCTGAGAG-GGACCTC. PCRs were carried out as described previously (Wagener et al., 2001) using the following concentrations of forward and reverse primers (Table 1) and probe, respectively: TG1, 300 nM/300 nM/125

Table 1. Primer design

Gene product	Forward primer	Reverse primer	Amplicon size (bp)
TG1	5'-ACCGTGTGACCATGCCAGTG	5'-GCTGCTCCCAGTAACGTGAGG	172
TG2	5'-ATGAGAAATACCGTGACTGCCTTAC	5'-CAGCTTGCGTTTCTGCTTGG	167
TG3	5'-GCTGTTGGGTAACCTGAAGATCG	5'-GCTACGTCGATGGACAACATGG	164
TG4	5'-TCACGCTTTCCAGTACCCTGAGT	5'-CAGGCTTTCCAAAGAGAACCTGAC	140
TG5	5'-CAAAGAGCATCCAGAGTGACGAGCGGG	5'-GGGCTGTCCTGGCTCAGTGATGTGGGC	187
TG6	5'-GTGAAGGACTGTGCGCTGATG	5'-CGGGAAGTGAGGGCTTACAAG	171
TG7	5'-CCTCATCAATGGGCAGATAGC	5'-CTTGACCTCGTTGCTGCTGA	136
Factor XIIIa	5'-GACCAATGAAGAAGATGTTCCGT	5'-GAAGGTCGCTTTGAATCTGCAC	169
Band 4.2 protein	5'-GTGTGCCAAGTTCAGTTCACG	5'-TCAGGGGCTACCACGGTGAC	120

For ribosomal protein S26 see Wagener et al. (Wagener et al., 2001).

nM; TG2, 300 nM/300 nM/150 nM. Calculation of starting concentration was based on standard curves for each target DNA run in parallel. Ribosomal protein S26 was used as an internal reference of housekeeping gene transcription for normalisation between different cDNA samples.

TG2 expression constructs

cDNAs encoding human TG2 and a variant thereof containing a point mutation in the active site (TGC→AGC, Cys277→Ser) were kindly provided by P. J. A. Davies, Houston, TX (Gentile et al., 1992). The coding sequence was excised with *NcoI* (containing start codon) and *Bsu36I* (containing stop codon) restriction enzymes. 5'-overhangs were filled in with DNA polymerase I (Klenow fragment, Promega), and the blunt-end fragment was ligated into the expression vector pcDNA3 (Invitrogen) linearised with *EcoRV* following standard procedures. Constructs with the coding sequence inserted in sense (pcDNA3-TG2) or antisense (pcDNA3-TG2as) orientation were isolated. To generate pcDNA3-TG2(Ser), a *XbaI/FseI* fragment of pcDNA3-TG2 was replaced with the respective fragment of the cDNA harbouring the active site mutation. Plasmid DNA was prepared with the Wizard Minipreps DNA Purification System (Promega) and sequenced by the dideoxy chain termination method (Sequenase 2.0; Pharmacia) to confirm the absence of mutations.

Stable transfection of fibroblasts

Immortalised HCA2 cells were seeded into 35 mm dishes, grown to 60% confluency and transfected with the different TG2 constructs using 6.0 µl TransIT[®]-LT1 reagent (Mirus) and 2.0 µg of plasmid DNA in a final volume of 2 ml of complete medium according to the manufacturer's protocol. After 48 hours, the medium was exchanged with complete medium containing 400 µg/ml of G418 (Invitrogen) for selection. Cell clones were harvested after 10-14 days of selection and maintained on selection medium unless otherwise indicated.

Protein extraction

Transglutaminase

Cells (100 mm dish, 90% confluent) were washed twice with cold PBS and extracted by scraping into 1.0 ml of 0.25 M sucrose, 1% Triton X-100 on ice (soluble fraction). The extract was cleared from particulate material by centrifugation at 15,000 *g* for 10 minutes at 4°C, and the resulting pellet was homogenised in 20 mM Tris-HCl, pH 8.0, containing 1% SDS, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF, and incubated for 5 minutes on ice (particulate fraction).

Signalling molecules

Cells were left in suspension or seeded at a density of 1×10⁶ cells

per 100 mm dish in four parallel dishes. At the indicated time, cells were washed with PBS and scraped into 1 ml ice-cold lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1% (octylphenoxy)polyethoxyethanol, 1 mM Na₃VO₄, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 2 mM NaF, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 10% glycerol) and lysed by sonication. The extract was centrifuged at 15,000 *g* for 10 minutes at 4°C and the soluble cytosolic fraction collected. The pellet was re-extracted in lysis buffer supplemented with 0.5% Triton X-100 on ice, yielding the membrane fraction. Protein concentrations were determined using the bicinchoninic acid reagent (Pierce) as described by the supplier, with BSA as a standard.

Immunoblotting

For each cell line and time point an equal amount of protein was separated on 4-20% SDS-PAGE Tris-glycine gels (Novex) under reducing conditions (1% 2-mercaptoethanol), and transferred (100 mA for 2 hours in 12 mM Tris/96 mM glycine, pH 8.3, 20% methanol) onto nitrocellulose membranes (Protran[®], Schleicher & Schuell). After blocking with TBS containing 5% non-fat milk powder, the membrane was probed for 60 minutes with monoclonal antibody (mAb) CUB7402 to TG2 (NeoMarkers; 2 µg/ml), with affinity purified polyclonal antibodies to FAK (Santa Cruz; 4 µg/ml), Tyr³⁹⁷ (Calbiochem; 0.5 µg/ml), Tyr⁵⁷⁶ or Tyr⁹²⁵ (Santa Cruz; 1 and 2 µg/ml, respectively) phosphorylated FAK, with mAb H-7 to PKCα (Santa Cruz; 2 µg/ml) or with mAb P-Tyr-100 to phosphotyrosine (Cell Signaling; 3 µg/ml), followed by incubation with affinity purified HRP-conjugated anti-mouse or anti-rabbit antibody (Dako) for 60 minutes. Bound antibodies were finally visualised using the ECL[™] reagent kit (Amersham).

Radioactive TG assay

Cells were grown for 24 hours in the absence of G418 and proteins were extracted as described for immunoblotting. The enzyme reaction was performed by incubating samples of 160 µl containing 100 mM Tris-HCl, pH 8.3, 5 mM CaCl₂, 10 mM DTT, 8 µCi [³H]putrescine (NEN), 625 nM *N,N*-dimethyl casein (Sigma) and 20 µg of cell extract, at 37°C for 30 minutes, and label incorporation determined as described previously (Aeschlimann and Paulsson, 1991).

Analysis of MMP and TIMP activity

The relative amounts of pro-enzyme and matrix metalloproteinase (MMP) species produced by the cells in the FPCLs was analysed using gelatin zymography (Blain et al., 2001). Briefly, 5 µl of conditioned medium was electrophoresed through 7.5% SDS-PAGE gels containing 0.5 mg/ml gelatin. For visualisation of tissue inhibitor of MMP (TIMP) activity by reverse zymography, 12.5% SDS-PAGE

gels were used that contained 2 mg/ml gelatin and MMP-2 (6.7% conditioned medium from bovine secondary cell line BOVS-1) (Blain et al., 2001). SDS was removed by soaking the gels in 2.5% Triton X-100 for 60 minutes, and MMPs were activated by incubation in 50 mM Tris-HCl, pH 7.8, containing 50 mM CaCl₂ and 500 mM NaCl overnight at 37°C. MMP/TIMP activity was visualised by staining with Coomassie Brilliant Blue R and quantified by densitometry as described previously (Cook et al., 2000). Membrane type 1-MMP (MT1-MMP) activity was determined in extracts of cell layers (5×10⁵ cells seeded) 24 hours after seeding using the MMP-14 Biotrak Activity Assay (Amersham).

Cell attachment assay

96-well microtitre plate wells were coated with 40 µg/ml of tail tendon collagen I or plasma fibronectin (Sigma) overnight at 4°C, followed by blocking of non-specific binding by incubation with 1% BSA for 4 hours at 4°C. Cells were resuspended in serum free medium, seeded at 2×10⁴ per well (6 replicates), and incubated for 60 or 180 minutes at 37°C/5% CO₂. The adherent cells were washed (PBS), fixed in 70% ethanol for 15 minutes and stained with 0.1% crystal violet for 25 minutes. Excess dye was removed by washing with H₂O, the bound dye solubilised using 0.2% Triton X-100 and the absorbance at 540 nm determined.

Cell spreading

Fibroblasts were serum starved, washed in serum free medium, dissolved in DMEM or DMEM supplemented with a pharmacological inhibitor, TPA, or LPA at the indicated concentrations and seeded in uncoated or collagen I-coated 24-well plates at a density of 1×10⁴ cells/well. In some experiments cells were stimulated with 10% FCS. Cell spreading was followed by capturing images every 30 minutes up to 7 hours. Experiments were carried out in triplicate.

Scratch wound assay

Fibroblasts (2×10⁴) were seeded in duplicate in a 24-well tissue culture plate and grown for 72 hours. A single scratch was made with a pipette tip across the cell layer and after rinsing with PBS, the repopulation of the denuded area monitored by capturing images every 30 minutes for 48 hours at fixed positions. All cell lines were analysed in parallel and the experiment repeated once.

Immunocytochemistry

Cells were fixed in 4% PFA, permeabilized in TBS containing 0.5% Triton X-100 and non-specific binding sites blocked by incubation with 1% BSA/0.1% Triton X-100 in TBS. F-actin was visualised with 1 µg/ml FITC-conjugated phalloidin (Sigma) and vinculin with 6 µg/ml mAb VIN-11-5 (Sigma) (or non-specific mouse IgG as control), followed by Texas Red-conjugated secondary antibodies (Cappel).

Results

Age-related changes in ECM reorganisation by fibroblasts are mirrored by changes in TG expression

Fibroblasts exert tractional forces in a matrix leading to the contraction of unrestrained, floating collagen lattices and to the development of tension in attached, restrained gels and have been shown to respond to consequential changes in mechanical stress by specific changes in protein expression (Chiquet, 1999). These activities are essential for normal tissue turnover and wound healing. We assessed the ability of neonatal, pre-

senescent and senescent fibroblasts to contract collagen lattices (Fig. 1A) and found that senescent fibroblasts produced a significantly increased initial rate of lattice contraction, i.e. contracted the lattice to 40% of its original size within 24 hours while neonatal fibroblasts required 72 hours to exert the same extent of contraction. Rescuing the cells from replicative senescence by conferring the ability to extend telomers through transfection with the hTERT gene restored the rate of lattice contraction to the level observed for neonatal cells (Fig. 1A) suggesting that this change is related to cell ageing and is not culture induced. In many cell types, terminal differentiation is associated with upregulation of TG2 (Aeschlimann and Thomazy, 2000). TG2 is by far the most abundant TG in dermal fibroblasts whilst several other TG gene products including TG1, TG5 and TG7 are expressed as well (Fig. 1B,C). To investigate whether TG2 may, in part, be responsible for the differences in lattice reorganisation by the different fibroblast populations, we determined the expression of TG2 at the mRNA and protein level using quantitative PCR and immunoblotting. TG2 expression was upregulated 5- to 10-fold in senescent cells as compared to pre-senescent or immortalised cells independent of the culture environment (average change in three independent experiments: monolayer, 10-fold increase; attached lattices, 5-fold increase; floating lattices, 8-fold increase) (Fig. 1C). In contrast, TG1 expression remained relatively constant between the cell populations (changes <2 fold) (Fig. 1C). The changes in TG2 mRNA levels translated into similar changes at the protein level as estimated by densitometry (Fig. 1D). These results suggest that TG2 upregulation is a specific change linked to the cell senescence program and is not caused by changes in the mechanical environment.

Alteration of TG2 expression results in changes in fibroblast mediated ECM reorganisation

To determine the effect of alterations in TG2 expression, hTERT immortalised cells were stably transfected with constructs for high level constitutive expression of TG2 sense RNA, antisense RNA and a TG2 mutant RNA where the catalytic Cys residue was replaced by Ser. The mutation results in a loss of TG activity but conserves other functions of the enzyme such as GTP binding and hydrolysis (Baek et al., 2001). Between 20 and 30 clones were isolated in each case and screened by immunoblotting for the desired changes in TG2 expression. Three clones were selected in each case and used for experiments. As comparable results were obtained with the different clones, results are given for a single clone in each case (same clone throughout). TG2 synthesis (Fig. 2A) and activity (Fig. 2B) was increased about 10-fold in the TG2 sense as compared to mock transfected cells, whilst TG2 was not detectable in TG2 antisense transfected cells, which also showed TG activity levels comparable to background. In contrast to large changes in the amount of soluble TG2, the insoluble membrane-associated pool of TG2 was only increased 3- and 2-fold in TG2 sense and C277S mutant cells, respectively, as compared to control (Fig. 2A) suggesting that membrane association is dependent on a limiting factor, most likely an interaction with a membrane-bound protein. No significant differences in growth rates were found between the cell lines as determined using the MTT assay (results are given

as [day 3-day 1]/day 3: mock, $65\% \pm 4$; TG2 sense, $68\% \pm 7$; TG2 antisense, $63\% \pm 4$; TG2 C277S, $60\% \pm 7$) suggesting that cell proliferation was unaffected by changes in TG2 expression.

The alteration of TG2 expression prompted large differences in collagen lattice reorganisation. Whilst the pattern of contraction in mock transfected cells was similar to the hTERT immortalised parental cells and pre-senescent fibroblasts, the TG2 sense transfected cells showed a significant increase in the initial rate of lattice contraction similar to the senescent cells as well as in the extent of lattice contraction over a 14 day period (Fig. 2C,D). The lack of cell proliferation in the senescent population explains why differences in lattice reorganisation were only seen at the early time points in this case (Fig. 1A). Overexpression of the dominant negative C277S mutant or the lack of TG2 expression prompted a significant decrease in the rate of lattice contraction suggesting that TG crosslinking has a major impact on the ability of cells to exert tension on the ECM (Fig. 2C,D). This was further confirmed by the fact that supplementation of the medium with the competitive TG inhibitor monodansylcadaverine ($100 \mu\text{M}$) similarly inhibited lattice contraction while the unreactive analogue dansylamidopentanol (Aeschlimann et al., 1995) had no effect (results not shown).

It is well established that fibroblasts cultured in a 3-dimensional collagen matrix up-regulate MMP-2 expression and produce active MMP-2 (Zigrino et al., 2001). We have shown that senescent HCA2 fibroblasts have increased levels of active MMP-2 as compared to their pre-senescent or hTERT immortalised counterparts (Stephens et al., 2003). Similarly, whilst the expression of MMP-2 at the mRNA level was not

affected by modulation of TG2 expression (results not shown), MMP-2 protein secretion into the medium and MMP-2 activation were significantly reduced in both the TG2 antisense and C277S transfectants (Fig. 3A,C). At the same time, TIMP levels remain unaffected as shown by reverse zymography (Fig. 3B). MT1-MMP has been implicated in the activation of MMP-2 (Zigrino et al., 2001) and we therefore determined the MT1-MMP activity expressed by the different cell lines using a chromogenic substrate assay based on activation of modified pro-urokinase (Verheijen et al., 1997). The mock, TG2 sense and TG2 antisense cells in monolayer culture (24 hours after seeding 5×10^5 cells) expressed 14.4 ± 0.5 , 16.0 ± 0.3 and 5.4 ± 0.3 ng MT1-MMP/mg total protein, respectively. As it has been shown that MT1-MMP expression is induced by culture in a three-dimensional matrix in comparison to monolayer culture, we have also determined the MT1-MMP activity of cells grown for 24 hours on collagen lattices. The activity was reduced by 30% in TG2-deficient cells as compared to mock or TG2-overexpressing cells; lactate dehydrogenase activity in extracts varied by $\sim 10\%$. Hence, the reduction in active MMP-2 production correlates with reduced levels of MT1-MMP activity expressed by TG2-deficient cells.

Alteration of TG2 expression modulates cell-matrix interactions resulting in an aberrant wound response

Contradictory results have been obtained with regard to whether TG2 affects cell-matrix interactions through its crosslinking activity or through non-catalytic functions (Akimov et al., 2000; Balklava et al., 2002; Verderio et al., 1998; Corbett et al., 1997), whilst our results show that TG

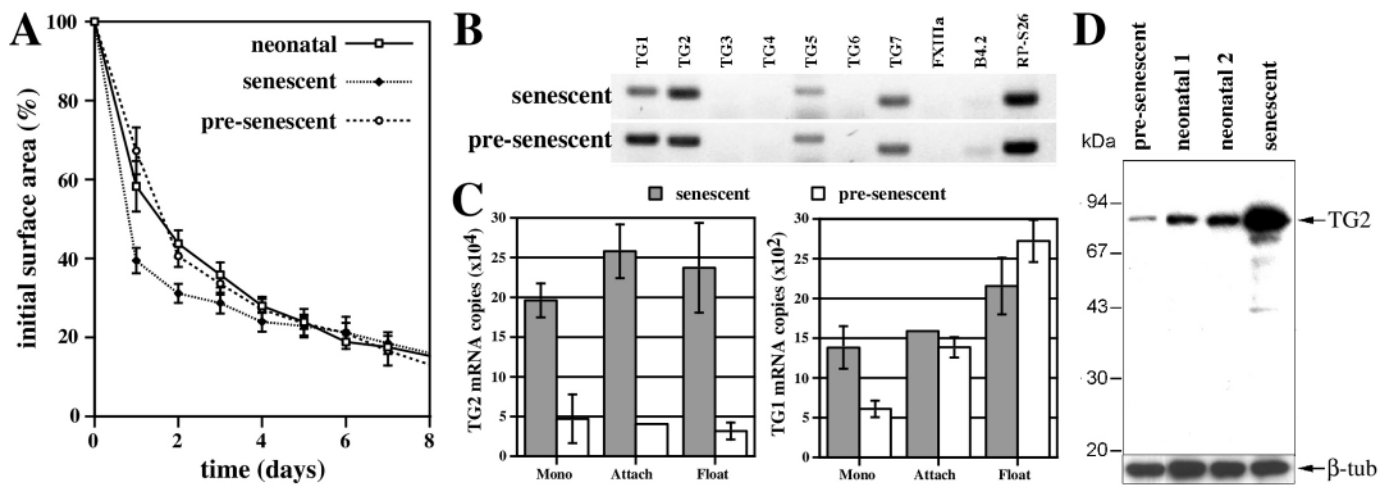
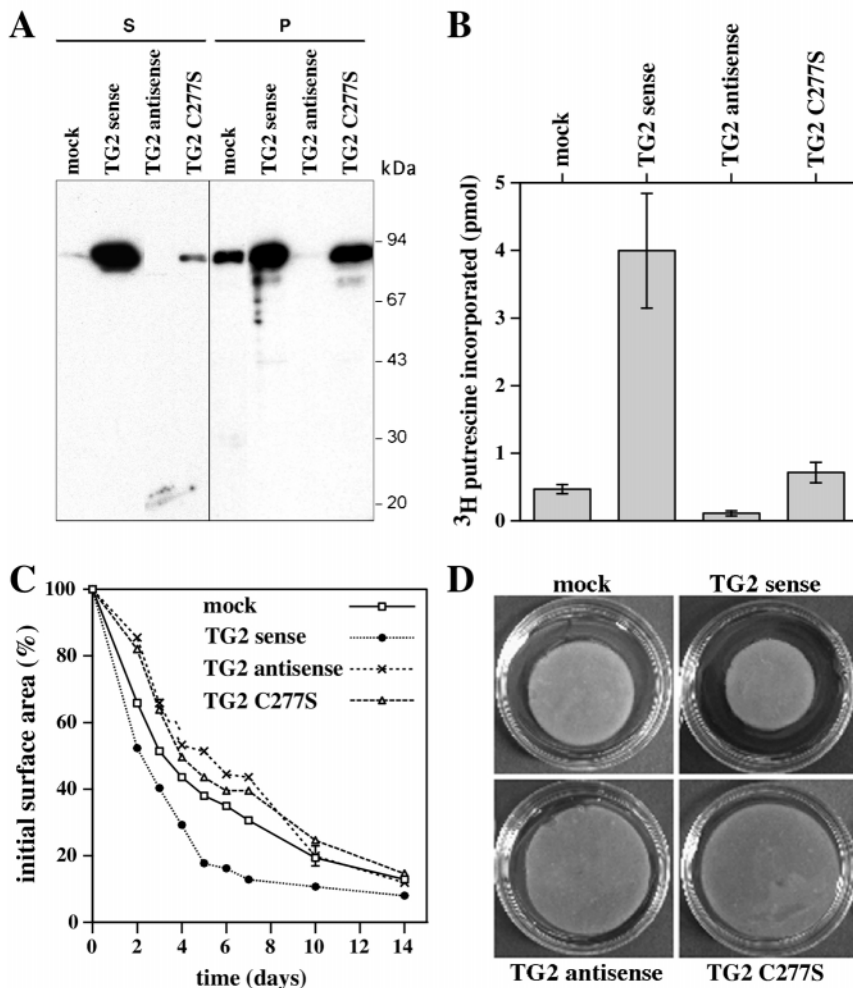


Fig. 1. Upregulation of TG2 expression in fibroblasts is linked to cell senescence and is independent of culture conditions. (A) Neonatal foreskin, senescent HCA2 and hTERT immortalised HCA2 fibroblasts were seeded into 0.5 mg/ml collagen type I lattices and diametric contraction was measured over a 14-day period. Values are the mean \pm s.d. ($n=3$). (B,C) Senescent and hTERT immortalised fibroblasts were grown in monolayer culture (Mono) or seeded into either attached (Attach) or unrestrained (Float) collagen I lattices for 2 days prior to RNA isolation. HCA2 fibroblasts were shown to express 4 iso-enzymes with TG activity (TG1, TG2, TG5 and TG7) by RT-PCR (B). mRNA levels were quantified with the 5' nuclease assay using the TaqMan system and are presented as a mean \pm s.d. (C). (D) hTERT immortalised HCA2, neonatal foreskin (from two individuals) and senescent HCA2 fibroblasts were extracted with 1% SDS/4 M urea-containing buffer. Cell extracts were separated in 4–20% SDS-PAGE gels and analysed for TG2 expression by immunoblotting with mAb CUB7402. Mol mass standards are indicated on the left. Re-probing of the membrane with antibodies to β -tubulin (β -tub) shows equal sample loading (bottom panel). Note, the comparison of pre-senescent to senescent HCA2 cells demonstrates the senescence-associated upregulation of TG2 for an individual fibroblast strain while the comparison of the pre-senescent HCA2 cells to neonatal cells from other individuals shows the level of variability in TG2 expression between individuals.

crosslinking impacts on ECM tension. We have therefore undertaken a series of experiments to investigate the contribution of its different presumptive functions to the molecular events controlling fibroblast-mediated matrix reorganisation. We compared attachment of the established fibroblast lines on different ECM substrata (Fig. 4). Whilst cell attachment of TG2 sense transfected cells was marginally increased over mock or TG2 antisense transfectants (Fig. 4A), which were not significantly different from each other, major differences were apparent in the cell morphology between the cell lines within the first 24 hours after seeding (Fig. 4B). These differences were independent of the substratum and were apparent when cells were grown on tissue culture plastic (Fig. 4B), fibronectin- or collagen I-coated dishes or on type I collagen lattices (Fig. 5A). This suggests that it is not the primary integrin-mediated attachment but the subsequent spreading process of the cells that is significantly altered by modulation of TG2 expression. TG2-overexpressing cells were fully spread within 1-2 hours and had a flattened morphology with an increased number of cellular projections when compared to mock transfected cells, whereas TG2-deficient cells remained in an early rounded stage of cell spreading with circumferential ruffled borders for up to 10 hours (depending on the substratum). In contrast to TG2-overexpressing cells, parental cells and mock transfected cells showed a clear

polarisation with lamellipodia and filopodia at the cell front (Fig. 4B, arrows). Consistent with an alteration in cytoskeletal organisation, staining with FITC-phalloidin revealed more extensive stress fibres in TG2-overexpressing cells than in mock transfected cells, whereas stress fibres were small or absent in TG2-deficient cells (Fig. 5B, Fig. 6D). Double labelling with vinculin established that in TG2-deficient cells focal complexes are formed at the base of actin filament-containing microspikes (filopodia) but these fail to mature into focal adhesions (Fig. 5B, arrowheads and lower inset) while in TG2-overexpressing cells mature focal adhesions are enlarged (Fig. 5B, arrows). In contrast to TG2-deficient cells, cells overexpressing dominant negative TG2 are competent in establishing mature focal adhesions (Fig. 5B, arrows). In addition, supplementation of the medium with up to 100 $\mu\text{g}/\text{ml}$ of mAb CUB7402 to TG2 (cells were incubated with the antibody in suspension for 30 minutes prior to plating) did not alter the spreading pattern of mock transfected or parental cells whereas plasmon resonance spectroscopy studies showed that the mAb preparation blocked binding of plasma fibronectin to immobilised TG2 (results not shown). Similarly, incubation of cells with 1 or 20 $\mu\text{g}/\text{ml}$ purified active TG2 did not promote spreading of TG2-deficient cells. These results suggest that the intracellular pool of TG2 is required for cell spreading and that its function is independent of crosslinking.



In addition to morphological changes, cells with altered TG2 expression exhibited differences in motile properties in *in vitro* wound assays consistent with a role of TG2 in regulating adhesion dynamics. Fibroblast monolayers were scratch wounded and the repopulation of the cell-free surface followed by time-lapse microscopy (Fig. 6A; see also Movies 1-4, <http://jcs.biologists.org/supplemental/>). Normal and mock transfected

Fig. 2. TG2 expression level modulates collagen lattice reorganisation by fibroblasts. hTERT immortalised HCA2 fibroblasts were stably transfected with mock, TG2 sense, TG2 antisense and TG2 C277S mutant expression constructs. (A) Transfected fibroblasts were extracted with 0.25 M sucrose/1% Triton X-100 to harvest the soluble TG fraction (S) and subsequently with 1% SDS/1 mM EDTA/1 mM DTT-containing buffer to solubilise the particulate TG fraction (P). Cell extracts were separated in 4-20% SDS-PAGE gels and analysed for TG2 expression by immunoblotting with mAb CUB7402. Mol mass standards are indicated on the right. Note, 5% and 25% of total protein extracted was applied for soluble and particulate fractions, respectively. (B) TG activity expressed by transfected fibroblasts was determined by incorporation of [^3H]putrescine into N,N-dimethylcasein for 30 minutes at 37°C. The results from four independent measurements are presented as the mean \pm s.d. (C,D) Transfected fibroblasts were seeded into 0.5 mg/ml collagen type I lattices and diametric contraction was measured over a 14 day period. Values are the mean \pm s.d. ($n=3$). (D) Macroscopic images of lattices at day 3.

fibroblasts migrated into the de-nuded area as individual cells, repopulating the wound within 20 hours. Fibroblasts lacking TG2 failed to repopulate the wound because of deficiencies in motility. Tracking of individual cells highlighted not only differences in the average speed of movement but also a lack of directional movement into the wound space (Table 2; Fig. 6B). Consistent with the alterations in cell spreading, the cells failed to form a leading edge with a lamellipodium characteristic of motile fibroblasts (Kaverina et al., 2002) (Fig. 6D) and in contrast to the other cell lines, arranged with the long axis perpendicular to the direction of movement (Fig. 6C). Staining with FITC-phalloidin highlighted the lack of reorganisation and stress fibre development in response to the wound stimulus and instead revealed a predominant cortical actin meshwork (Fig. 6D). Fibroblasts overexpressing TG2 had a hyperspread appearance with more extensive stress fibres but were polarised with a typical leading edge towards the wound (Fig. 6D, arrows). However, these cells failed to retract, elongated extensively and expanded into the wound space as an interconnected cell sheet (Fig. 6A,C) suggesting that reduced motility (Table 2) in this case is linked to a deficiency in disassembly of cell-cell or cell-matrix adhesion complexes. In contrast, cells overexpressing the TG2 C277S mutant migrated identically to mock transfected cells (Fig. 6A,C) suggesting that the phenotype in TG2-overexpressing cells relates to excessive crosslinking. However, supplementing the medium with 50 $\mu\text{g/ml}$ mAb CUB7402 or 100 μM monodansylcadaverine had no effect on the motility of normal fibroblasts (results not shown). This together with the observed differences between TG2-deficient and dominant negative TG2-expressing cells suggests that TG2 has a primary function in regulating cell-substratum interaction in an inside-out manner.

TG2 regulates cell adhesion by controlling activation of PKC α

Integrin clustering and thereby induced phosphorylation of cytoskeletal and signalling proteins regulates focal adhesion formation and turnover, and has profound effects on dynamic adhesion in cell spreading and motility. While cell attachment via integrins occurs in the absence of phosphorylation of focal adhesion components including FAK and paxillin, the subsequent spreading of cells is regulated by Tyr phosphorylation of these proteins (Wilcox-Adelman et al., 2002). Immunoblotting of extracts of the fibroblast lines at different time points during spreading with antibodies to pTyr revealed phosphorylation of a number of proteins including a ~125 kDa protein identified as FAK by stripping and re-probing of the membrane with FAK-specific antibodies (Fig. 7A). Whilst there was no difference in FAK expression in the different cell lines, Tyr phosphorylation of FAK in TG2-deficient cells was increased several-fold as compared to parental, mock transfected or TG2-overexpressing cells (Fig. 7A). (Auto)phosphorylation of FAK at Tyr³⁹⁷ is induced upon integrin clustering and has been shown to play a critical role in regulating dynamic adhesion and in integrating adhesion and growth factor signals (Owen et al., 1999; Sieg et al., 2000). It results in SH2-dependent recruitment and binding of Src-family protein Tyr kinases, FAK activation and the co-ordinated FAK-Src protein complex signals have been

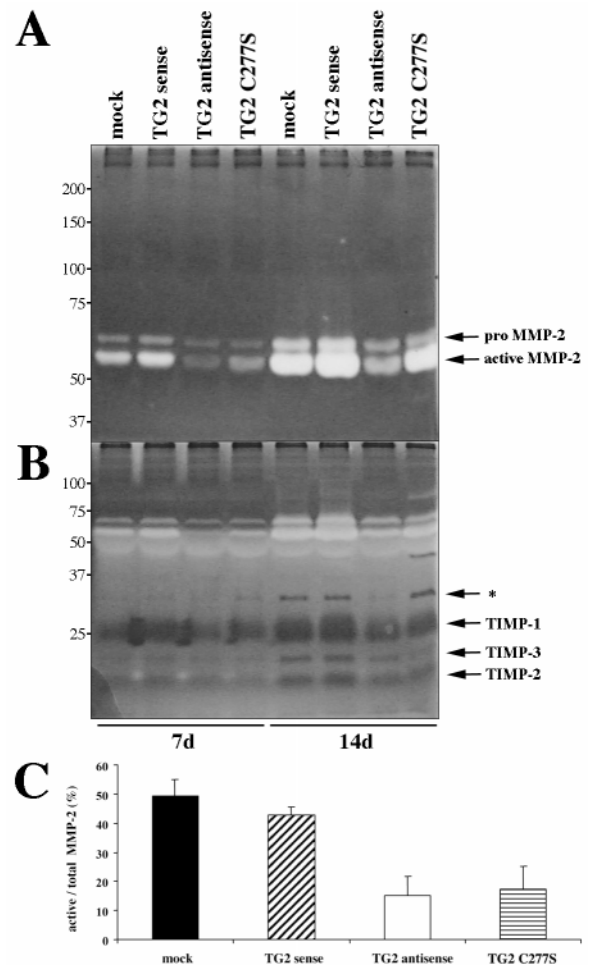


Fig. 3. ECM crosslinking by TG2 affects production of active MMP-2. Mock, TG2 sense, TG2 antisense and TG2 C277S mutant transfected HCA2 fibroblasts were cultured in 0.5 mg/ml collagen I lattices, and conditioned media from day 7 and 14 cultures were analysed for gelatinase and TIMP activity by gelatin zymography (A) and reverse zymography (B), respectively. The position of the 72 kDa proform and 62 kDa activated forms of MMP-2 as well as TIMP-1, -2 and -3 are indicated on the right. An unidentified higher molecular mass band with TIMP activity, presumably a TIMP complex, is indicated with an asterisk. Molecular mass standards (kDa) are indicated on the left. (C) Densitometric analysis of the relative levels of pro- and active MMP-2 in conditioned medium after 14 day in culture. Values are the mean \pm s.e.m. of two independent experiments.

shown to promote cell motility in fibroblasts through activation of the Ras-ERK2/MAP kinase pathway or through p130^{CAS}/Crk-dependent Rac activation (Schlaepfer et al., 1998; Klemke et al., 1998). Immunoblotting of the cell extracts with antibodies to Tyr³⁹⁷-phosphorylated FAK showed a comparable increase in phosphorylation during cell spreading in all cell lines (Fig. 7A) and immunolocalisation showed that Tyr³⁹⁷-phosphorylated FAK was associated with vinculin-containing focal complexes (results not shown). This suggests that integrin-FAK signalling was unchanged in TG2-deficient cells and that differential phosphorylation of FAK involves an alternative phosphorylation site. Immunoblotting with antibodies specific for other pTyr

sequences in FAK demonstrated that the increased phosphorylation occurred in the catalytic domain and not in the C-terminal Tyr⁹²⁵ in the Grb2 binding site that mediates signalling through the MEK-ERK pathway (Fig. 7B). Likewise, specific inhibitors of MAP kinases including MEK inhibitor PD98059 (up to 30 μ M; Fig. 8A,D) had no effect on spreading of HCA2 fibroblasts, and immunoblotting of extracts of serum starved cells seeded on collagen I with antibodies to p42/p44 and their phosphorylated forms, respectively, showed no differences in ERK activation in the different cell lines (results not shown). In contrast, plating quiescent fibroblasts under different conditions showed that the dependence of FAK phosphorylation at Tyr⁵⁷⁶ on ECM signals, serum, or activation of Rho (with LPA, 500 ng/ml) is lost in TG2-deficient cells (Fig. 7B). Unlike the autophosphorylation site (Tyr³⁹⁷), the catalytic domain of FAK remained partially phosphorylated in TG2-deficient serum-starved cells in suspension suggesting a deregulation in Src-mediated FAK activation.

In addition to specific integrins, syndecan-4 also localises to focal adhesions of fibroblasts spread on different substrata (including fibronectin and collagen I) and syndecan-4 has been shown to be essential for focal adhesion assembly and stress fibre formation (Longley et al., 1999). Moreover, disruption of the syndecan-4 gene abolishes migration of primary fibroblasts in in vitro wound healing assays (Echtermeyer et al., 2001). These syndecan-4-mediated effects involve signalling through PKC isoforms and Rho family

GTPases, and binding to PtdIns(4,5) P_2 and activation of PKC α in particular (Couchman et al., 2002). It has also been shown that in fibroblasts, a PKC-mediated pathway controls c-Src activation and can stimulate FAK catalytic activity and signalling independent of FAK phosphorylation at Tyr³⁹⁷ (Schlaepfer et al., 1998; Wilcox-Adelman et al., 2002; Cary et al., 2002). Immunoblotting of cell extracts showed that PKC α synthesis was transiently induced in HCA2 fibroblasts during spreading, with elevated levels present 30-60 minutes after cell seeding (Fig. 7C). A large increase in the membrane-bound fraction of PKC α in mock transfected and TG2-overexpressing cells after 60 minutes indicates its activation. In contrast, no change in subcellular distribution of PKC α occurred in TG2-deficient cells (Fig. 7C). The phorbol ester TPA promoted rapid spreading in TG2-deficient cells with a maximal effect at 1-16 nM (Fig. 8H,I; arrows) through induction of focal complex turnover (Fig. 8K,L; arrow). However, the PKC inhibitor bisindolylmaleimide I at a concentration of 10 nM inhibited spreading of mock transfected or TG2-overexpressing cells but its analogue bisindolylmaleimide V at the same concentration had no effect (Fig. 8B,C). Similar results were obtained with the PKC α / β I selective inhibitor Gö6976 (5 nM) (results not shown). The PLC inhibitor U-73122 (5 μ M) blocked spreading while the analogue U-73343 had no effect (Fig. 8F,G,J). Collectively, these results suggest that signalling via the PLC-PKC α pathway is required for efficient spreading of these cells and that TG2 is required for this signalling event.

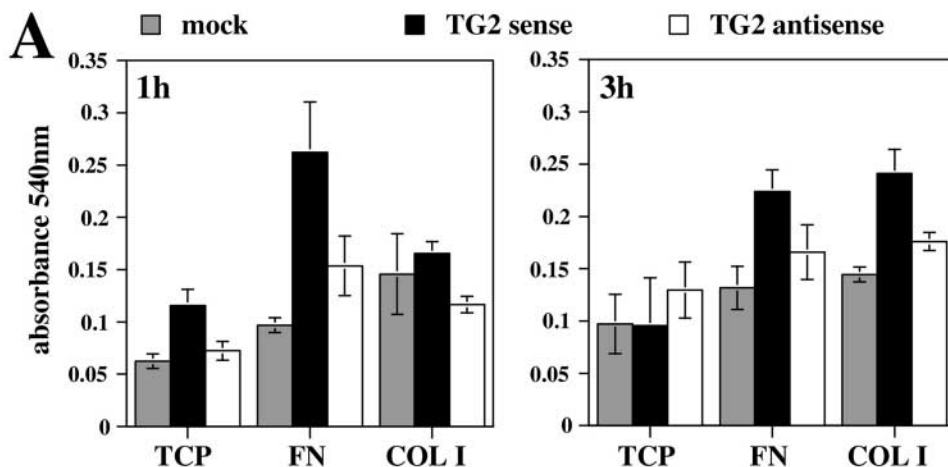
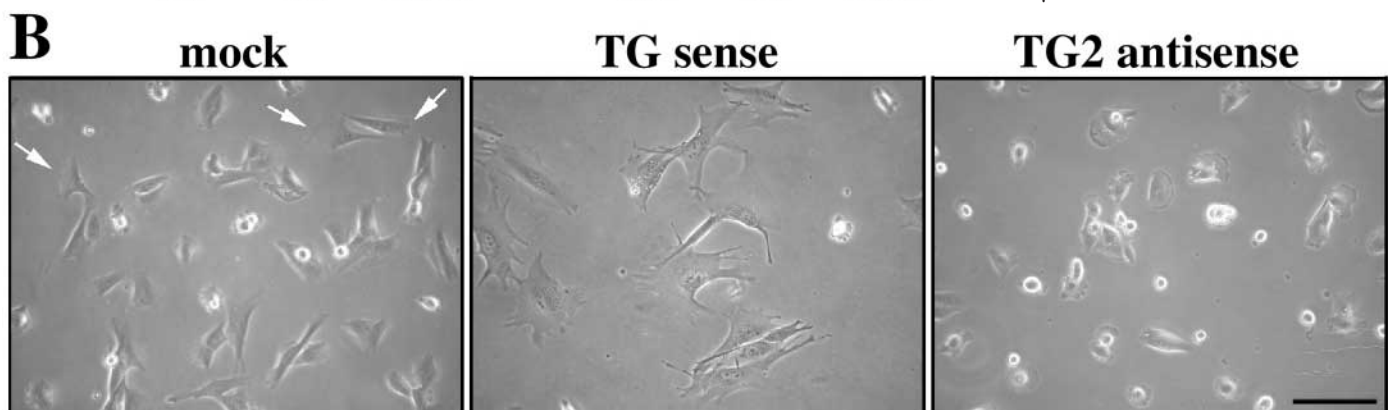


Fig. 4. Altering TG2 expression affects cell-matrix interaction. (A) Fibroblasts (1.5×10^4) were seeded in wells of a 96-well plate coated with either 40 μ g/ml collagen I (COL I) or plasma fibronectin (FN), or left uncoated (TCP). After 1 or 3 hours incubation, adherent cells were quantified by staining with crystal violet and measuring the cell-bound dye by determining the absorbance at 540 nm in Triton X-100 extracts. Values are the mean \pm s.d. of two independent experiments containing triplicate samples. (B) Phase contrast images of cells 5 hours after seeding on tissue culture plastic are shown. Scale bar: 100 μ m.



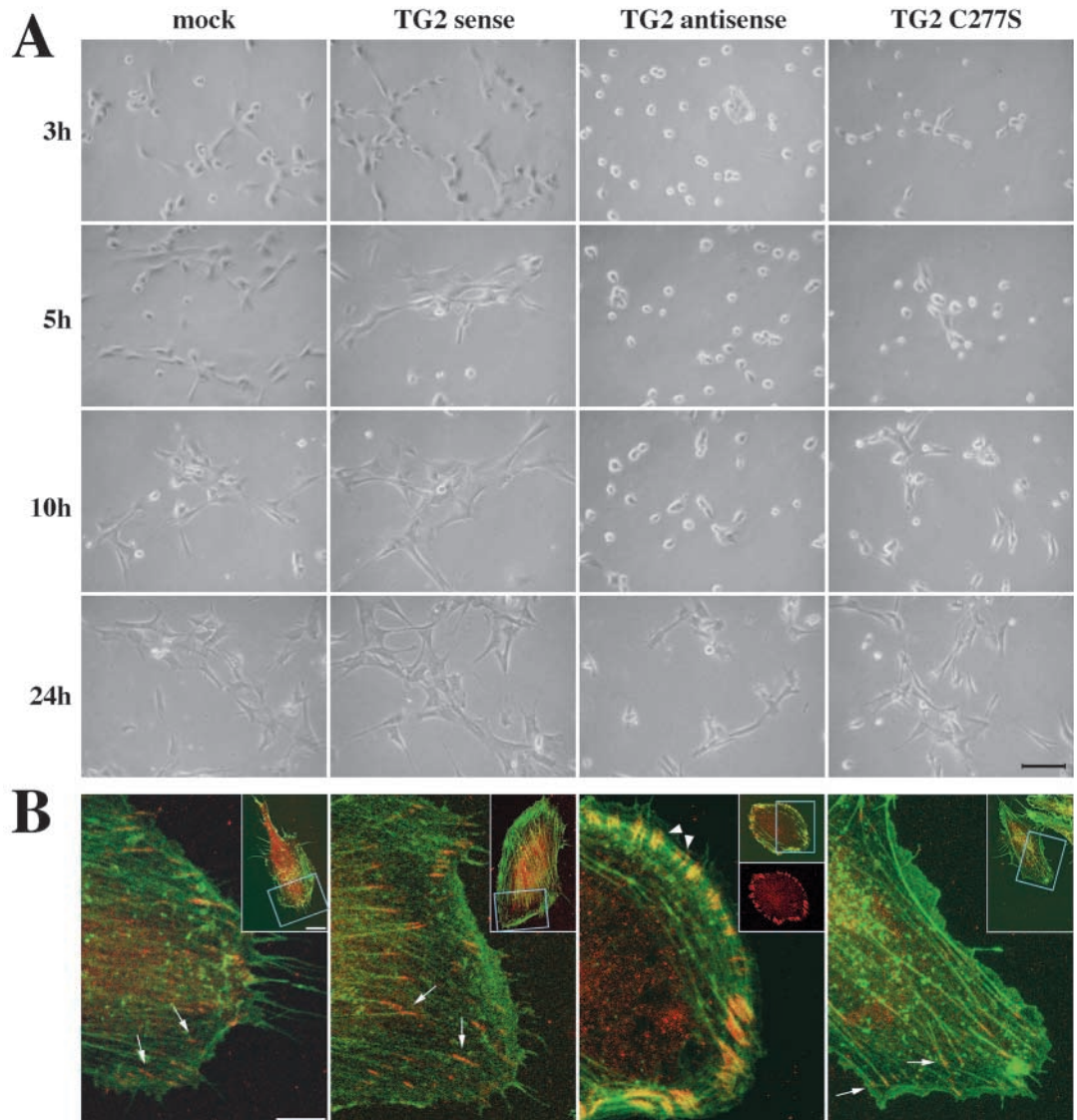


Fig. 5. TG2 is required for cell spreading. (A) Phase contrast images of fibroblasts cultured on top of collagen I lattices at different time points after seeding. (B) F-actin (FITC-phalloidin) and vinculin (TRITC immunolabelling) distribution in fibroblasts 6 hours after seeding on collagen I-coated glass coverslips. Images represent an extended focus view of five sections of a Z-series taken at 3 μm distance using confocal laser scanning microscopy. The area of the cell shown at high magnification has a blue frame in the insets. In the TG2-deficient cell, the lower inset shows a basal section with vinculin labelling only. Arrows indicate mature focal adhesions associated with stress fibres; arrowheads indicate focal complexes at cell periphery. Scale bars: 100 μm (A) and 10 μm (B).

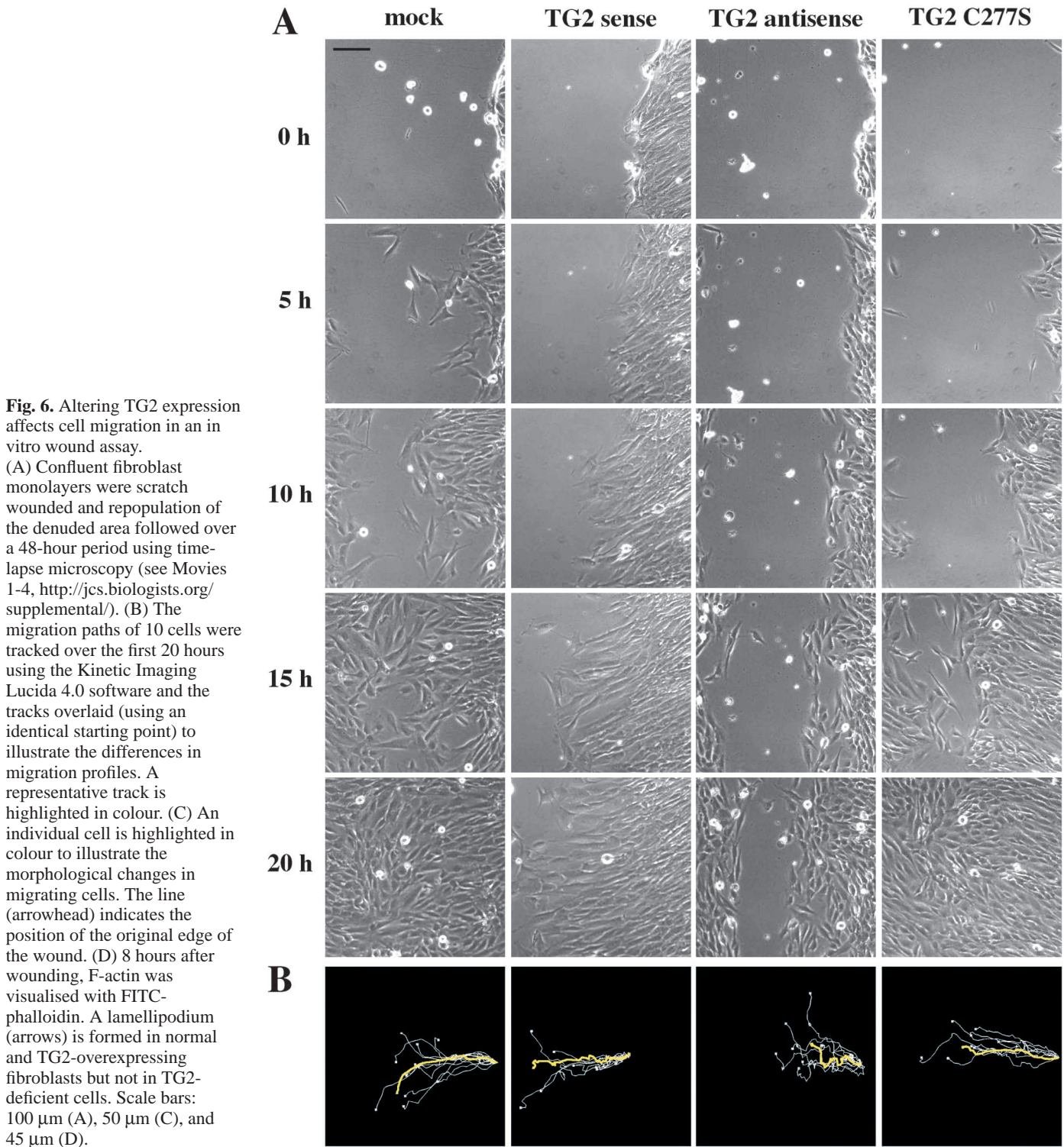
Discussion

Despite a large amount of evidence implicating TG2 in cell-matrix interactions (Gentile et al., 1992; Corbett et al., 1997; Jones et al., 1997; Akimov et al., 2000), the precise role of TG2 in cell adhesion has not been clearly established. Our results demonstrate that TG2 has a major impact on dynamic adhesion in cell motility and on ECM remodelling. We show that both extracellular crosslinking and intracellular signalling activities of TG2 differentially contribute to the regulation of cell-matrix interactions.

TG2 crosslinking increases tension in the ECM

We show that TG2 expression in fibroblasts is not sensitive to changes in the culture environment (monolayer versus three-dimensional matrix) and does not alter in response to mechanical stress. However, the crosslinking activity of TG2 plays an essential role in generating tension in the matrix as suggested by the fact that TG2-deficient cells and cells overexpressing inactive TG2 display comparably reduced

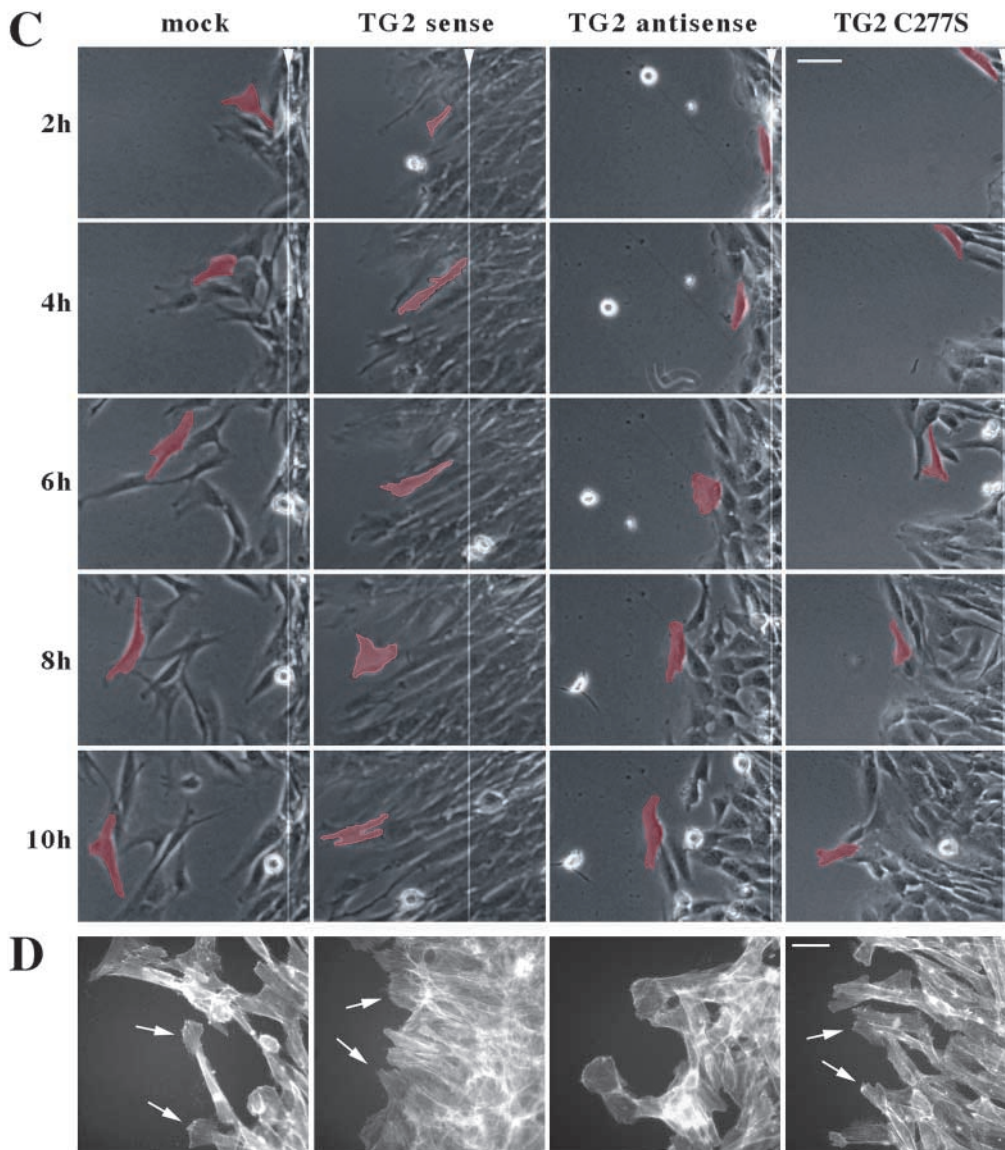
ability for collagen lattice contraction. Furthermore, lattice contraction was sensitive to TG inhibitors. Our data obtained with TG2-deficient and dominant negative TG2-expressing fibroblasts further show for the first time a direct link between TG crosslinking and the control of MMP activity. The crosslinking deficiency leads to reduced MMP-2 activation. Activation of pro-MMP-2 by MT1-MMP involves the formation of a ternary complex with TIMP-2 and subsequent cleavage of the complexed proMMP-2 by uncomplexed MT1-MMP (Zigrino et al., 2001). Our results show that reduced crosslinking leads to decreased MT1-MMP activity while TIMP levels remain unchanged thereby altering the stoichiometry of the components and inhibiting zymogen activation. Integrin clustering, which is induced by aggregation of binding sites, can induce MT1-MMP (Ellerbroek et al., 2001) and could be promoted by crosslinking of ECM proteins. Alternatively, TG2 crosslinking could regulate the activity of MT1-MMP activating proteases. It is not known whether crosslinking can affect furin activity, which has been implicated in MT1-MMP activation in this system (Zigrino et



al., 2001). However, MT1-MMP can also be activated by plasmin and crosslinking of either plasminogen or components involved in regulating its activity is known to modulate plasmin activity (Aeschlimann and Thomazy, 2000). The presence of a protein complex with TIMP activity on reverse zymograms that is absent in TG2-deficient cells suggests that TG2 can also directly interact with a TIMP family member.

TG2 is involved in regulation of cell adhesion in cell migration

Fibroblast migration is a multistep process involving (1) extension of actin-rich cell processes at the leading edge, (2) formation and stabilisation of focal adhesions at the newly established cell periphery, (3) generation of contractile forces in the cytoskeleton and (4) detachment of the trailing end



brought about by the forces acting on disassembling adhesions at the rear of the cell. Hence, fibroblast morphology and motility relies upon forces generated by the cell and the regulation of the interaction of cell surface receptors including $\beta 1$ - and $\beta 3$ -integrins and syndecan-4 with the ECM. TG2-deficient fibroblasts showed significantly reduced motility because of a failure in the establishment of a leading edge and a loss in the coordination of migration in space suggesting a failure in establishing tension in the actin filament cytoskeleton (Benigno et al., 2001). Whilst in fibroblasts overexpressing the inactive TG2 C277S mutant migration was indistinguishable from that of normal fibroblasts, cells overexpressing active TG2 displayed reduced motility because of a failure of detachment of cell-cell or cell-matrix contacts at the trailing end. This could either be related to a defect in the regulation of focal adhesion disassembly or increased ECM stabilisation.

It has recently been shown that TG2 overexpression in Swiss 3T3 cells reduces the area of peripheral outgrowth from a cell-seeded agarose droplet (Balklava et al., 2002) and that downregulation of TG2 in monocytes reduces migration on

fibronectin in a transwell assay (Akimov and Belkin, 2001). Both studies suggested that the effect of TG2 on cell motility relates to the interaction of cell-surface-associated TG2 with fibronectin. In contrast to their findings, we have found that antibodies to TG2 do not inhibit the migration of normal fibroblasts in a scratch wound assay. This difference may relate to the fact that fibronectin was used as a substratum in both previous studies. As these antibodies block the interaction of TG2 with fibronectin (unpublished results) (Akimov et al., 2000), the induced differences in fibronectin assembly may affect cell motility independently of intracellular actions of the enzyme.

The molecular mechanisms underlying the events at the leading edge of a migrating cell and substratum-mediated cell spreading have a large degree of overlap and the latter served as a model for detailed analysis of the molecular changes. We show that TG2 is required for efficient spreading of fibroblasts on ECM ligands employing different integrins. In contrast to studies focusing on fibronectin or its 42 kDa fragment (Akimov et al., 2000), we found that spreading of HCA2 fibroblasts on type I collagen was not

affected by antibodies to TG2 and that incubation with exogenous TG2 did not promote spreading of TG2-deficient cells. These results suggest that TG2 can promote cell adhesion through a mechanism independent of externalisation and fibronectin binding. Cell spreading is driven by the assembly of filamentous actin structures, whereby the GTPase RhoA regulates the formation of focal adhesions associated with actin stress fibre bundles and Rac1 and Cdc42 the formation of focal complexes in association with lamellipodia and filopodia (Kaverina et al., 2002). Although TG2-deficient cells formed filopodia, they were deficient in the formation of a lamellipodium, mature focal adhesions and stress fibres suggesting a deregulation of Rac1 and/or RhoA (Nobes and Hall, 1999). The FAK/Src-Cas/Crk signalling pathway links Rac activation to integrin engagement and regulates focal adhesion formation and directional cell migration. Autophosphorylation of FAK at Tyr³⁹⁷ in response to integrin activation mediates c-Src recruitment and the downstream signalling events (Owen et al., 1999). FAK null murine fibroblasts show reduced and delayed cell spreading and a

Table 2. Migration speed of normal and transfected fibroblasts

Time	Mock	TG2 sense	TG2 antisense	TG2 C277S
3-5 hours	35.3±10.8	17.3±6.1*	18.6±3.5†	26.8±10.4
5-7 hours	34.3±12.5	18.8±7.5*	19.5±7.0†	30.8±8.6
7-9 hours	31.6±9.4	20.5±7.2*	18.8±5.6†	23.9±6.1

Average migration speeds (mean±s.d. in $\mu\text{m}/\text{hour}$) for individually traced fibroblasts ($n=10$) from 3 to 9 hours after wounding.

*Mock versus TG2 sense ($P<0.01$).

†Mock versus TG2 antisense ($P<0.01$).

diminished rate of migration due to inhibition of focal adhesion turnover, a phenotype reminiscent of that of TG2-deficient cells (Ren et al., 2000). We found no differences in FAK expression in the different cell lines but increased levels of Tyr phosphorylation of FAK in TG2-deficient cells. However, there were no differences in the timing or extent of phosphorylation

of FAK at Tyr³⁹⁷ suggesting that integrin-mediated signalling to FAK is unaltered in the different cell lines. Dynamic adhesion formation is controlled by a regulatory loop between RhoA and FAK (Ren et al., 2000). c-Src is part of this link and its kinase activity is required for focal adhesion turnover and for cells to polarize and form a leading edge (Timpson et al., 2001). Adhesion-independent activation of FAK (phosphorylation at Tyr⁵⁷⁶) as we have observed in TG2-deficient cells will disrupt the Rho GTPase cycle and thereby the turnover of focal adhesions. Recent data suggests that c-Src can phosphorylate and activate FAK in the absence of binding to Tyr³⁹⁷-phosphorylated FAK via its SH2 domain (Cary et al., 2002). Hence, it is possible that FAK phosphorylation at Tyr⁵⁷⁶ in the TG2-deficient cells is due to a loss in the regulation of Src activity. Alternatively, TG2-dependent signalling may regulate the activity of one of the cellular Tyr phosphatases (PTEN, PTP-PEST and Shp-2) which are known to dephosphorylate FAK.

Fibroblasts seeded onto the RGD-containing cell-binding domain fragment of fibronectin attach but do not fully spread or form focal adhesions without co-stimulation inducing either the activation of Rho (lysophosphatidic acid or antibodies clustering syndecan-4) or PKC (with TPA). Phosphorylation of Tyr³⁹⁷ is associated with activation of the Rho pathway but not the PKC pathway (Wilcox-Adelman et al., 2002). Activation of PKC α in conjunction with integrin engagement induces the recruitment of cytoskeletal components to adhesion sites and results in the establishment of mature focal adhesions and stress fibres (Woods and Couchman, 1992). Cell-substratum interactions induced rapid upregulation and subsequent activation of PKC α in HCA2 fibroblasts as indicated by translocation of the enzyme to the membrane. In contrast to the other cell lines, TG2-deficient cells failed to activate PKC α . Cell spreading was sensitive to inhibitors of Ca²⁺-dependent PKC isozymes and TPA promoted spreading in TG2-deficient cells through induction of focal adhesion turnover suggesting that the lack of TG2 expression affects PKC α activation. The inhibitor studies further demonstrated that spreading requires PLC activity but proceeds in the absence of signalling through the MAPK pathway. TG2 has been shown to specifically bind to, and regulate, the activity of PLC- δ 1 (Feng et al., 1996; Baek et al., 2000). The pleckstrin homology domain of PLC- δ 1 binds strongly to PtdIns(4,5)P₂ and thereby targets the enzyme to the plasma membrane. Therefore the interaction of TG2 with PLC- δ 1 could be responsible for the membrane-associated

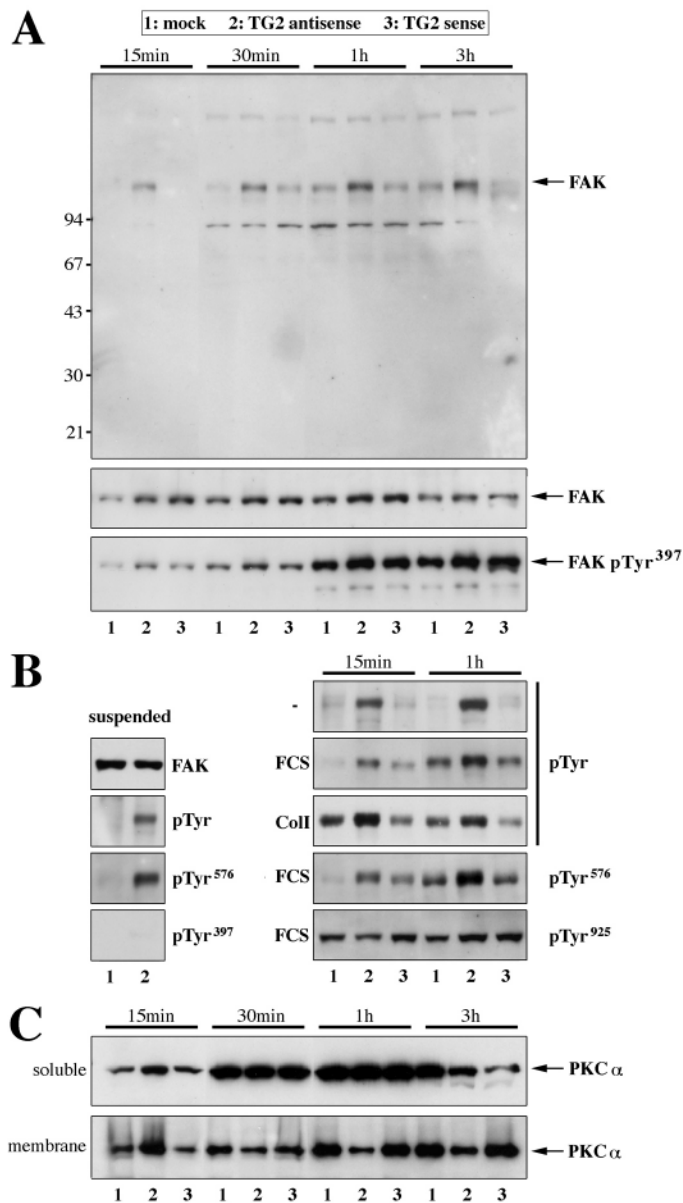


Fig. 7. TG2-deficient cells hyper-phosphorylate FAK but fail to activate PKC α . Mock (lane 1), TG2 antisense (lane 2) and TG2 sense (lane 3) transfected HCA2 fibroblasts were extracted and equal amounts of protein were separated in 4-20% SDS-PAGE gels. (A) At the indicated timepoints after seeding, cells were analysed for pTyr (top), FAK (middle), and pTyr³⁹⁷ FAK (bottom) by immunoblotting with specific antibodies. Molecular mass standards (kDa) are indicated on the left. (B) Serum starved fibroblasts were extracted after 30 minutes in suspension or at the indicated timepoints after seeding on uncoated or collagen I-coated (ColI) dishes or uncoated dishes in the presence of serum (FCS) and analysed for FAK, pTyr, and Tyr³⁹⁷, Tyr⁵⁷⁶ or Tyr⁹²⁵ phosphorylated FAK. (C) Cytosolic (top) and membrane (bottom) fractions were probed with antibodies to PKC α .

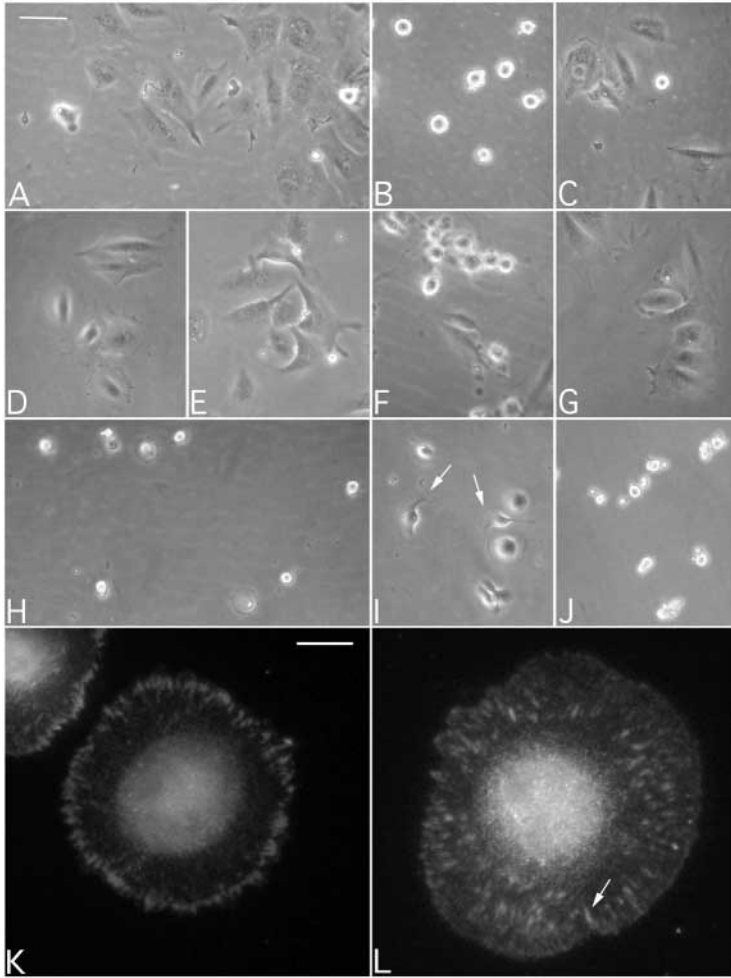


Fig. 8. PKC activity is required for cell spreading. Phase contrast images (A–J) or vinculin immunolabelling (K,L) of fibroblasts 1 hour after seeding: mock transfected cells under normal culture conditions (A) or after treatment with 10 nM bisindolylmaleimide I (B), 10 nM bisindolylmaleimide V (C), 30 μ M PD98059 (D), 1 nM TPA (E), 5 μ M U-73122 (F) or 5 μ M U-73343 (G); TG2-deficient cells under normal conditions (H,K) or after treatment with 1 nM TPA (I,L) or 5 μ M U-73122 (J). Arrows indicate spread and polarized cells (I) and mature focal adhesions (L), respectively. Scale bars: 50 μ m (A) and 9.5 μ m (K).

is influenced both by their interactions with the ECM and by their response to growth factors. Alterations in the locally resident fibroblast populations and/or their proliferative capacities have a major impact on wound healing responses and are likely to play a significant role in age-related wound healing deficiencies (Campisi et al., 1998; Mendez et al., 1998). We show that TG2 is a key factor controlling fibroblast activities relevant to the wound healing response, including migration, generation of tension in the ECM and regulation of MMP activity. TG2 expression is responsive to acute-phase injury cytokines including IL-6 and TNF- α (Aeschlimann and Tomazy, 2000), is involved in the production of active TGF- β in various biological contexts (Szondy et al., 2003) and is rapidly upregulated in skin wound healing with the highest levels of TG2 found in the early phase of the wound healing response (Haroon et al., 1999). Mearns and co-workers (Mearns et al., 2002) showed recently that healing of punch biopsy skin wounds is delayed in TG2^{-/-} mice and that in vitro, mouse embryonic fibroblasts from TG2^{-/-} mice migrated slower than fibroblasts derived from wild-type counterparts. Similar to TG2 null mice, syndecan-4 null mice develop normally but reveal defects

in wound healing in the adults (Echtermeyer et al., 2001). Taken together with the results of this study, the data suggest that TG2 and syndecan-4 have overlapping roles in integrating growth factor and ECM signals to link focal adhesion formation, fibronectin assembly and signalling through PKC α to promote fibroblast migration and angiogenesis in wound healing.

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‘particulate’ pool of TG2 (Aeschlimann and Thomazy, 2000). Such a model is consistent with the observation that overexpression of TG2 results in a disproportionate increase in the cytoplasmic pool of the enzyme and the reported colocalisation of membrane-associated TG2 with β 1-integrins in focal adhesions (Akimov et al., 2000).

Previous studies have not investigated the dynamic changes in migration and hence, we show for the first time that the different activities of TG2 impact on different aspects of cell motility. The intracellular pool of TG2 is involved in the establishment of mature focal adhesions associated with stress fibres by regulation of PKC α activation, a process which appears to relate to cytokine/growth factor signals as opposed to adhesion signals. Our finding that inhibition of crosslinking has no effect on migration of normal fibroblasts while overexpression of active enzyme as opposed to inactive enzyme is associated with reduced disassembly/detachment of the established cell-matrix interactions indicates that while the extracellular crosslinking function of TG2 is not required for migration it can inhibit motility by either increasing matrix tension, which enhances focal adhesions, or by crosslinking cell surface and ECM components.

Function of TG2 in wound healing

Wound healing is a dynamic process in which cell behaviour

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