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ORIGINAL ARTICLE

The role of TG2 in ECV304-related vasculogenic mimicry

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Abstract Tumour vasculogenesis can occur by a process referred to as vasculogenic mimicry, whereby the vascular structures are derived from the tumour itself. These tumours are highly aggressive and do not respond well to anti-angiogenic therapy. Here, we use the well characterised ECV304 cell line, now known as the bladder cancer epithelial cell line T24/83 which shows both epithelial and endothelial characteristics, as a model of in vitro vasculogenic mimicry. Using optimised ratios of co-cultures of ECV304 and C378 human fibroblasts, tubular structures were identifiable after 8 days. The tubular structures showed high levels of TG2 antigen and TG in situ activity. Tubular structures and in situ activity could be blocked either by site-directed irreversible inhibitors of TG2 or by silencing the ECV304 TG2 by antisense transfection. In situ activity for TG2 showed co-localisation with both fibronectin and collagen IV. Deposition of these proteins into the extracellular matrix could be reduced by inclusion of non-cell penetrating TG inhibitors when analysed by Western blotting suggesting that the contribution of TG2 to tube formation is extracellular. Incubation of ECV304 cells with these same irreversible inhibitors reduced cell migration which paralleled a loss in focal adhesion assembly, actin cytoskeleton formation and fibronectin deposition. TG2 appears essential for ECV304 tube formation, thus representing a potential novel therapeutic target in the inhibition of vasculogenic mimicry.

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Abbreviations

TG2	Tissue transglutaminase
TAA	Tumour angiogenic activity
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
HUVEC	Human umbilical vein endothelial cells
ECM	Extracellular matrix
FN	Fibronectin
DMEM	Dulbecco's modified Eagles media
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
HRP	Horseradish peroxidase
DAB	Diaminobenzidine
FITC	Fluorescein isothiocyanate
IF	Immunofluorescence
IHC	Immunohistochemistry

Introduction

Tumour angiogenic activity (TAA) refers to the ability of tumour cells to stimulate neoangiogenesis, a process crucial for the supply of nutrients and oxygen essential for tumour survival and progression (Giatromanolaki et al. 2004). During this well characterised angiogenic process, endothelial cells in response to key growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) form tubular structures, which grow towards and colonise the growing tumour. Although originally thought that the vasculogenesis of tumours could only occur via the growth of surrounding blood vessels into the tumour, it has recently been shown that the vascular

structures of some tumours has a neoplastic origin (Ricci-Vitiani et al. 2010) and share comparable genomic features with the neoplastic cells themselves. These tumour-derived vascular structures, referred to as vasculogenic mimicry (Bissell 1999), are able to join up with the normal endothelial derived surrounding blood vessels to provide a highly vascularised tumour. They are difficult to treat, since the tumour vasculature does not respond to conventional antiangiogenic therapy and are generally found in highly aggressive tumours where prognosis is poor. Once thought to be derived from human umbilical vein endothelial cells (HUVEC), and which has the ability to form vascular structures when grown on matrigel and collagen I (Hughes 1996; Fujimoto et al. 2006), is the cell line ECV304. ECV304 cells show angiogenic ability and the presence of certain endothelial cell markers including thrombomodulin, αv integrins, KDR, CD34 and VE cadherin (Kiessling et al. 1999). However more recently, genetic fingerprinting studies revealed that ECV304 cells share identical characteristics to the bladder cancer epithelial cell line T24/83, which also shows the ability to form tubular structures (Suda et al. 2001). Hence ECV304 cells are now accepted as bladder cancer derived epithelial cells. They have the ability to form mimic vasculogenic structures both in vitro where they can form collaborative networks with HUVECs and in vivo where they show various types of vessel morphology (Fujimoto et al. 2006). In this respect, they serve as a well characterised cell model for looking at the cellular mechanisms of an alternative form of tumour vasculogenesis (Fujimoto et al. 2006).

One protein shown to be linked to endothelial cell derived angiogenesis is the multifunctional Ca^{2+}-dependent protein-crosslinking enzyme tissue transglutaminase (TG2) (Griffin et al. 2002). Among the larger family of TGs, TG2 is probably the most widely expressed member in mammalian tissues and cell types (Wang et al. 2011). It is found both in the intracellular and extracellular environment although its mode of secretion is still unknown (Verderio et al. 2004). Under pathological conditions and under stress conditions, TG2 can be overexpressed, resulting in increased externalization onto the cell surface and deposition into the extracellular matrix (ECM), where it can form the hetero-complex with matrix protein fibronectin (FN) or exert its crosslinking function on a variety of ECM proteins, such as fibronectin, collagens, vitronectin and laminin (Collighan and Griffin 2009). Both cell surface heparan sulphates and integrins play a role in the non-transamidating cell adhesion functions of TG2 (Wang et al. 2010, 2011). TG2 is abundantly expressed in HUVEC cell cultures (Sane et al. 1991), where it may play an important role in basement membrane assembly (Martinez et al. 1994). In angiogenesis, Haroon et al. (1999) showed that the addition of exogenous TG2 to a rat dorsal skin flap window chamber model of wound healing had an enhanced effect on angiogenesis. In contrast, increased addition of exogenous TG2 to angiogenic cell co-culture models was shown to inhibit angiogenesis by promoting increased matrix deposition (Jones et al. 2006). Endostatin, the C-terminal of Collagen XVIII known to stimulate angiogenesis, is also shown to be one of the recently reported partners for TG2 during angiogenesis (Faye et al. 2010). Given this strong link of TG2 to tumour angiogenesis and the finding that ECV304 cells express high levels of TG2 that are thought to be important in cell adhesion (Jones et al. 1997), the aim of this work is to pursue the role of TG2 in a novel but different cell model of in vitro tumour vasculogenesis using the ECV304 cell line co-cultured with human dermal fibroblasts.

Materials and methods

TG inhibitors

Site-directed TG inhibitors R283 and R294 were synthesised in house as previously described. R283 has been found to act both intracellularly and extracellularly, while R294 are not membrane permeable (Griffin et al. 2008).

Cell lines

ECV304 cell lines ECV-S3 (a neomycin resistant stably transfected control cell line phenotypically similar to wild type cells) and ECV-B4 (a TG2 antisense stably transfected cell line) (Nicholas et al. 2003) were routinely cultured in Dulbecco's modified Eagles media (DMEM) supplemented with 2 mM glutamine and 10% (v/v) fetal bovine serum (FBS). Primary human foreskin fibroblasts (C378) were a generous gift from Professor Ed Wood (University of Leeds, UK). Cells were harvested by exposure to 0.25% (w/v) bovine trypsin in phosphate buffered saline (PBS) pH 7.4, and collected cells were washed in normal culture media, counted and seeded into the appropriate tissue culture vessel.

Epithelial and fibroblasts co-culture system

Experiments were performed in 12-well cell culture plates, and 2×10^4 ECV304 cells and 2×10^4 C378 cells were seeded into 1 ml of normal growth media. For immunochemical staining experiment, 2×10^3 cells of each cell type were seeded into 300 µl of growth medium in 8-well glass chamber slides. Co-cultures were incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO_2, and tubules were allowed to develop spontaneously over a 14-day period, with regular changes of growth media every 72 h. In some experiments, cells were seeded into media

that was pre-mixed with TG inhibitors R283 or R294 in normal growth media with a final concentration of 500 μM. Cells were cultured in the presence of the inhibitor for the duration of the experiment with regular changes in medium plus inhibitor every 72 h.

XTT assay for cell proliferation

ECV304 and C378 co-cultures incubated with TG2 inhibitors R283 and R294 (500 μM) at the time points of 24, 48, 72 and 96 h culture period were incubated with XTT reagents (Roche, UK) and signals were detected using plate reader at 450 and 750 nm (background reading, which was erased from the absorbance 450 nm).

Immunohistochemistry and fluorescence staining

Cultures in 12-well plates that demonstrated mature tubule formation were fixed in 3.7% (w/v) paraformaldehyde in PBS, pH 7.4 for 15 min and permeabilized with 0.1% Triton X-100 in PBS, pH 7.4 for 10 min. Non-specific protein binding was blocked by incubating fixed cells with 300 μl 3% (w/v) bovine serum albumin (BSA) in PBS, pH 7.4 (the blocking buffer) overnight at 4°C. Blocked plated were incubated with the primary antibodies (see Table 1) diluted in the above buffer for 2 h at 37°C with gentle shaking, then plates were washed three times and incubated with the appropriate species-specific secondary antibody conjugated to Horseradish peroxidase (HRP) (Table 1) in blocking buffer for 2 h at 37°C with gentle shaking. Following antibody treatment, plates were washed three times and developed with the chromogenic HRP substrate diaminobenzidine (DAB) and H_2O_2 with metal enhancer. The staining reaction was terminated by removal of the developer and wells were washed with PBS, pH7.4. Staining was observed by light microscopy and photographed using an Olympus digital camera.

For visualisation of in situ TG2 activity, cells were cultured in 8-well chamber slides in growth medium containing 0.5 mM fluorescein isothiocyanate (FITC)-cadaverine at the day 14 of the experiment. This time-period corresponds to the tubular development period in culture that occurs after the co-culture becomes a uniform monolayer of cells. Cultures that demonstrated mature tubule formation were fixed and permeabilized with two washes of methanol at −20°C for 20 min. Double-labelling of cells was performed as described for HRP staining with the exception that TRITC-labelled secondary antibodies were employed. Stained cultures were mounted in 70% (v/v) glycerol and viewed via confocal microscopy.

For the actin cytoskeleton or vinculin staining, the ECV304 cells (5×10^4 cells/well) were seeded into 8-well chambers and allowed to adhere for 10 h in the presence or absence of TG2 inhibitors. Cells were fixed and permeabilized as described above and then blocked with the blocking buffer for 30 min. For actin staining, the cells were incubated with FITC-labelled phalloidin (20 μg/ml) in blocking buffer at 37°C for 2 h. For the vinculin staining, the cell slides were incubated with anti-vinculin antibody and relevant secondary (Table 1) in blocking buffer

Table 1 List of antibodies used for IHC, IF and Western blotting (WB)

Antigen	Species source	Company	Comments	Dilution	Label
TG2	Mouse	Thermo, UK	–	1:50 (IHC and IF) 1:1,000 (WB)	–
Collagen I	Goat	Santa Cruz, Germany	Angiogenesis marker	1:100 (IHC and IF)	–
Collagen IV	Rabbit	Covalab, France	Extracellular matrix	1:100 (IF) 1:1,000 (WB)	–
Fibronectin	Rabbit	Sigma-Aldrich, UK	Extracellular matrix	1:100 (IF) 1:1,000 (WB)	–
Vinculin	Mouse	Sigma-Aldrich, UK	Focal adhesion	1:100 (IF)	–
ε(γ-Glutamyl) lysine crosslink	Mouse	Abcam, UK	Crosslinking products	1:1,000 (WB)	–
Mouse IgG	Goat	Santa Cruz, Germany	Secondary antibody	1:200 (IHC) 1:1,000 (WB)	HRP
Goat IgG	Rabbit	Santa Cruz, Germany	Secondary antibody	1:200 (IHC)	HRP
Rabbit IgG	Swine	Dako, UK	Secondary antibody	1:1,000 (WB)	HRP
Mouse IgG	Rabbit	Dako, UK	Secondary antibody	1:200 (IF)	TRITC
Goat IgG	Rabbit	Santa Cruz, Germany	Secondary antibody	1:50 (IF)	TRITC
Rabbit IgG	Swine	Dako, UK	Secondary antibody	1:50 (IF)	TRITC

at 37°C for 2 h, respectively. Slides were mounted with Vectashield mountant (Vector Laboratories) and examined via confocal microscopy (Wang et al. 2011).

Western blotting

To detect the presence of antigen in the ECV304 and C378 co-cultures, the cell lysates were collected at the days of culture indicated. SDS-PAGE was performed to separate the proteins in the cell lysates and Western blotting was carried out to detect the target proteins by using specific targeting antibodies as described before (Wang et al. 2010). Tubulin was used as a standard for protein loading.

For the matrix proteins, the cells were lifted using 2 mM EDTA in PBS, pH 7.4 and the remaining matrix after washing in the same buffer was collected into Laemmli buffer and Western blotting was performed as described previously (Wang et al. 2010).

Wound healing assay

ECV304 cells were pre-seeded into 12-well plates and allowed to settle down for 16 h. The wound areas were prepared by scratching the mono-cell layer with 1000 µl tip. The cells were allowed to migrate into the wound area for 10 h in 1% serum, to minimise proliferation, followed by fixation and permeabilization as introduced above and co-stained with May-Grunwald and Giemsa stains as described previously (Wang et al. 2010).

Results

The tubular formation in ECV304 and C378 dermal fibroblasts co-culture system

In order to develop a more physiological cell model to study tube formation, a coculture system of ECV 304 cells together with human dermal fibroblasts (C378) was used comparable to that previously used with HUVECs. We first measured the relative levels of TG2 present in the ECV304 and C378 cell cultures by Western blotting which showed that in the C378 cells TG2 was not detectable under these conditions, while it was found to be abundant in the ECV304 cells (Fig. 1a). To optimise the ECV304 and C378 fibroblasts co-culture system for tubular formation, different ratios of the two cell systems were used over different time periods. When ECV304 cells and primary human fibroblasts C378 were seeded at equal cell ratios into 12-well plates, tubular structures started to form at day 8 (Fig. 1b–d) and were easily visible by phase microscopy after 10–14 days incubation period (Fig. 1e).

Characterization of the expression of TG1 and TG2 and in situ activity in the tubular structures

Given the epithelial origin of the ECV304 cells, the presence of both TG2 and TG1 was investigated in the co-culture system during tubule development. Figure 2Aa and Ba show the detection by immunohistochemistry (IHC) staining of TG2 after a 14-day culture period, in which the presence of TG2 in the tubular structure covered area can be clearly identified. Moreover like TG2, these tubes were also found to be rich in collagen I (Fig. 2Ca and b). Using Western blotting, the presence of TG2 and TG1 antigens in the co-cultures during days 1–14 cultures were detected. As shown in Fig. 2d, only trace levels of TG1 could be detected, when compared with that shown for TG2 with comparable protein loadings. Interestingly, there was not a dramatic increase in TG2 levels during the culture period, but only an increase in smaller Mr enzymatic degradation fragments in the late culture periods. To gain a deeper insight into the presence and locality of TG2 in tubular structures, both IHC and immunofluorescence (IF) staining were undertaken, using the specific anti-TG2 antibody Cub7402 as shown in Fig. 2Ab and 2B. The presence of TG2 was found in large amounts with a pattern of staining that resembled the tubular structures. Importantly measurement of in situ TG activity using FITC-cadaverine (a well characterised fluorescent primary amine TG substrate) incorporation into endogenous proteins indicated the active TG to be co-localized with the TG2 antigen staining (Fig. 2Ab–d), but also found in fibrous structures surrounding the tubes (Fig. 2Bc). The finding of a strong TG2 presence and activity with the tubes strongly suggests it is the major enzyme contributing to the actual TG activity during tubular formation.

Downregulation of TG2 expression in ECV304 cells by antisense or inhibition of crosslinking activity abolished the formation of tubes by ECV304 cells

To confirm that the TG2 found in the tubular structures is derived from the TG 2 rich ECV 304 cells and to further investigate if TG2, but not other members of the TG family, is the effector enzyme in regulating tubule formation, the bal-1 TG2 antisense cDNA was stably transfected into ECV304 cells to knockdown the expression of the enzyme (ECV304-B4) resulting in a 90% knockdown of TG2 (Nicholas et al. 2003), while the negative control antisense was used in establishing the ECV304-S3 cell line. These antisense transfected ECV304 cells were then used in the co-culture system. Figure 3b, d and f show that the downregulation of TG2 expression in ECV304-B4 cells abolished the formation of the tubular structures, compared to the well-formed tubes found in the control ECV304-S3

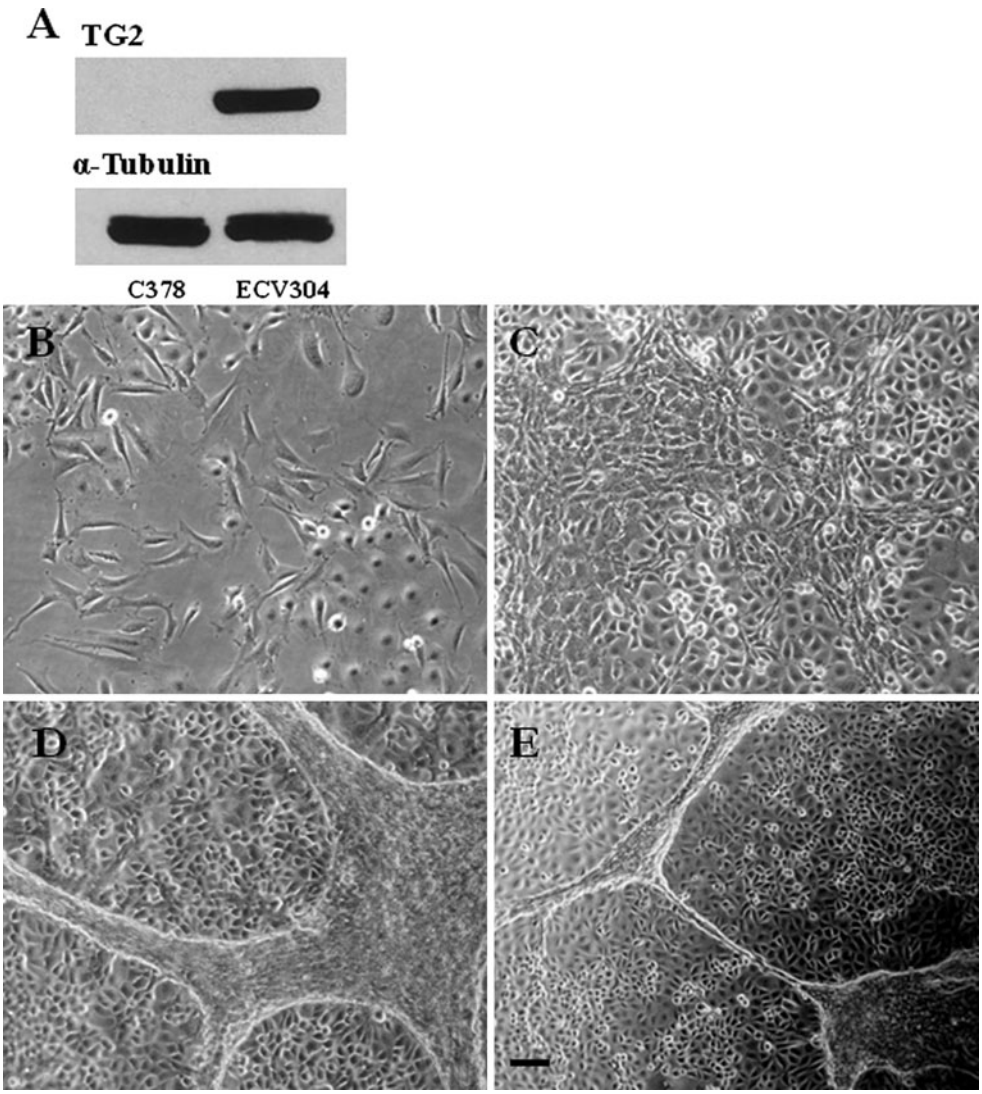

Fig. 1 Co-culture of ECV-304 with primary human C378 dermal fibroblasts promotes tubulogenesis. **a** The presence of TG2 in C378 and ECV304 cells. Western blotting was performed to detect the presence of TG2 antigen in either the C378 or the ECV304 cells by using specific anti-TG2 antibody Cub7402. Tubulin was used for normalisation of equal protein loadings. **b–e** ECV304 and C378 were co-cultured in 12-well tissue culture plates as described in the "Materials and methods" for up to 14 days. **b–e** Live cells viewed by phase contrast microscopy at various stages of development (**b**, day 3; **c**, day 5; **d**, day 8; **e**, day 10). *Bar* on panels equals 100 μm

cells (Fig. 3a, c and e). Measurement of in situ activity by FITC-cadaverine incorporation in the co-cultures with the antisense transfected cells further confirmed that the ECV304 cells are the major source of active TG2 in the tubular structures (Fig. 3c, d). Unlike the ECV304-S3 cells which showed similar patterns of tube formation, in situ activity and collagen I staining to that of wild type ECV304 cells (Fig. 3e), ECV304-B4 cells failed to lay down highly organized tubular structures (Fig. 3f).

Given that there is high level of the active TG2 present in the tubule structures, which appears dominated by active extracellular TG2, we next investigated the role of the transamidating activity of TG2 in regulating tubule formation. To achieve this, the site-directed irreversible TG2 inhibitors R294 [which acts extracellularly (Griffin et al. 2008)] and the extracellular and intracellular-acting TG inhibitor R283 were added to the co-culture system at a final concentration of 500 μM, a concentration which shows no toxicity on either of the cells found in the co-culture system (Fig. 4a). Figure 4 shows the collagen I staining as a marker of tubular formation after a 14-day incubation period. Both inhibitors significantly reduced the formation of tubes (Fig. 4b–d), confirming that the TG2 activity observed is acting extracellularly. To investigate the effect of the TG2 inhibitors on the deposition of TG2 into the ECM, the ECM fractions from ECV304 and C378 co-cultures were collected

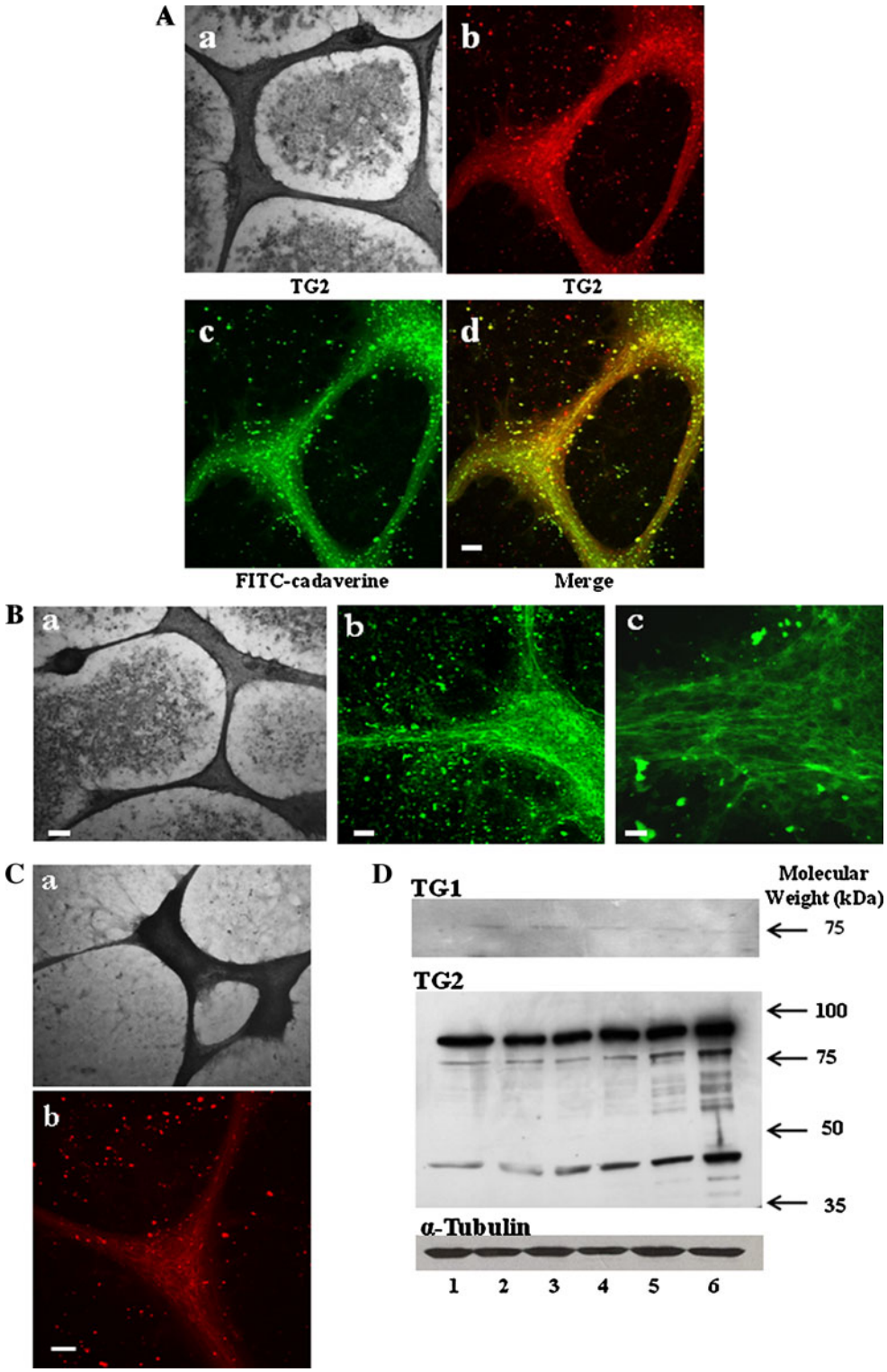

Fig. 2 Identification of TG antigens and collagen I in ECV304/C378 co-cultures. **A**–**C** Fixed cells at day 14 where tubules were revealed by immunoperoxidase and IF staining for TG2 and TG activity as described in the "Materials and methods". *Bars* on **A**, **B***a* and **C** equal 100 μm, *bar* on **B***b* equals 50 μm and *bar* on **B***c* equals 25 μm. **D** Western blotting was performed to detect the presence of TG2 or TG1 in the ECV304 and C378 co-cultures as introduced in "Materials and methods". *Lane 1*,1–3 days; *lane 2*,1–5 days; *lane 3*, 1–7 days; *lane 4*, 1–9 days; *lane 5*, 1–11 days; *lane 6*, 1–13 days. Tubulin was used for normalisation of equal protein loadings

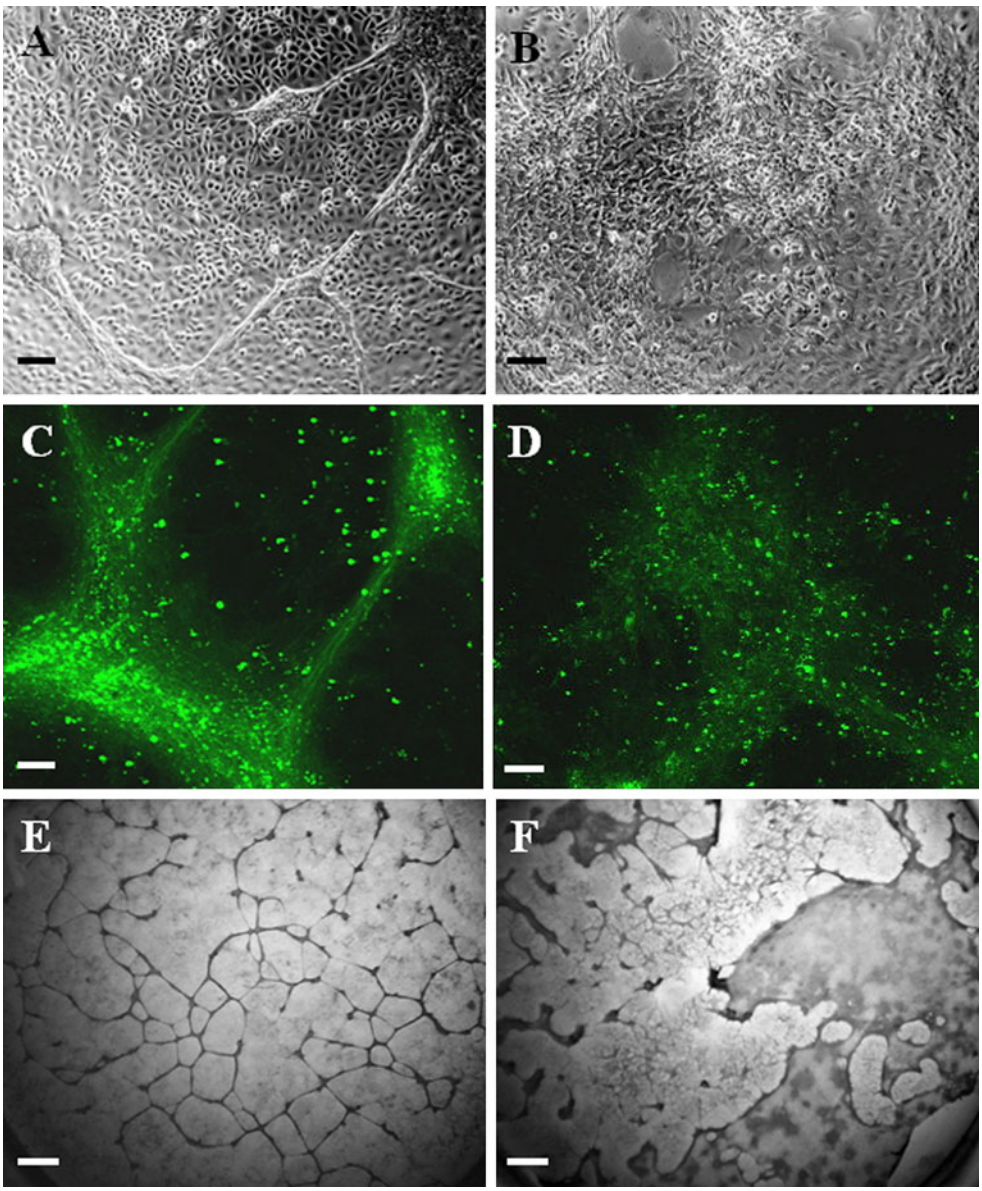

Fig. 3 TG2 antisense transfected ECV-B4 cells fail to form organised tubules when co-cultured with C378. Tubular development in ECV-S3 (transfected control)/C378 (**a**, **c** and **e**) and ECV-B4 (TG2 antisense-transfected)/C378 (**b**, **d** and **f**) co-cultures. **a**, **b** Live cells viewed by phase contrast microscopy at day 10 and **e**, **f** show whole wells stained for collagen type I, while **c** and **d** show in situ TG activity by FITC-cadaverine incorporation. *Bars* in **a** and **b** are 100 μm, and *bars* in **c** and **d** are 50 μm, and in **e** and **f** 1 mm

after 14-day culture and Western blotting was performed, which showed reduced deposition of TG2 into ECM in the presence of both TG2 inhibitors R283 and R294.

The cellular distribution of TG2 and its binding partners in the tubular structures

TG activity was revealed by in situ staining using FITC-cadaverine incorporation into endogenous proteins and visualized under confocal microscopy. Figure 5a shows by confocal microscopy the abundance of in situ incorporation of FITC-cadaverine incorporation into the ECM laid down by the cells, which is present in tight cord-like patterns in the lumen of the tubules and in the angled fibrillar matrices at the tubular junctions. To investigate if there is any relationship between the active enzyme and its known binding partners—FN and β1 integrin, further confocal microscopy was performed. Figure 5b and c show the double staining of the active enzyme (green) and its ECM binding partner protein fibronectin (FN, red) in mature tubules and in different sections of the tubes from top to bottom (shown in Fig. 5d, e and f, respectively). The

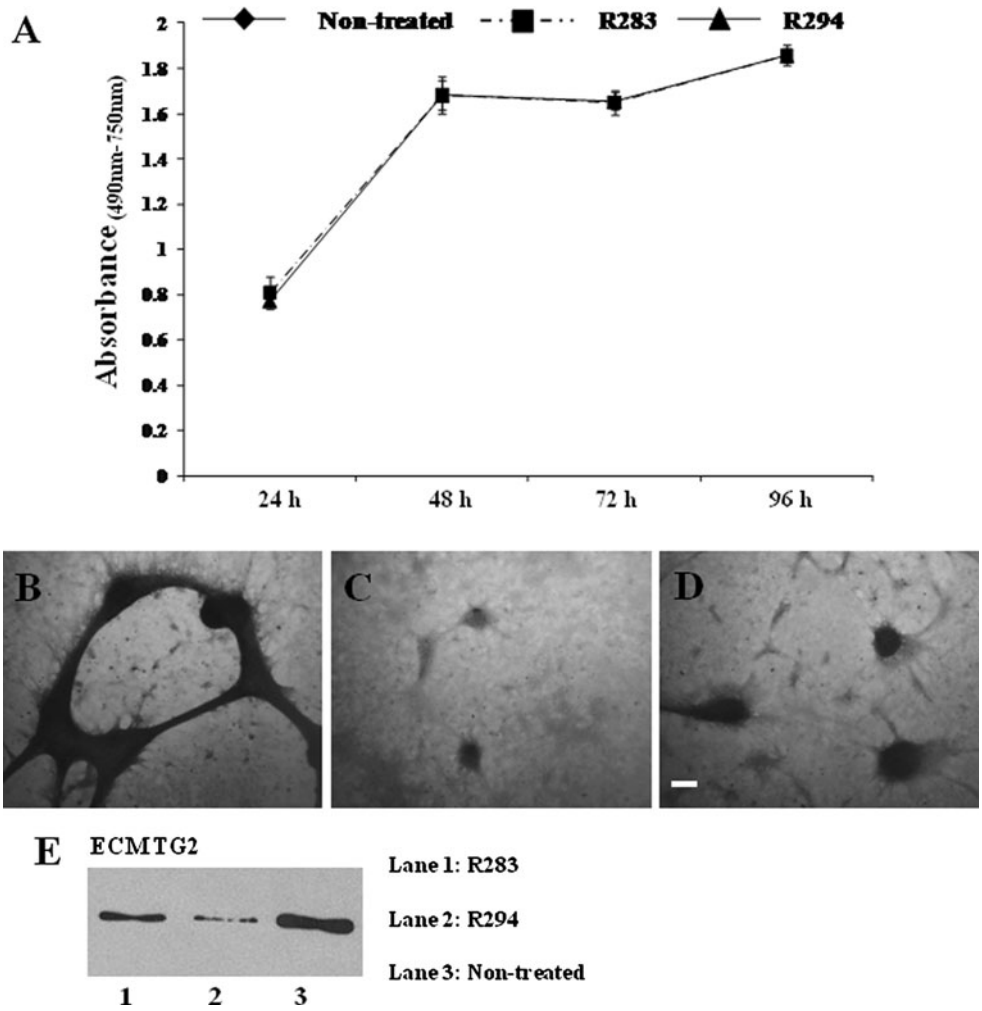

Fig. 4 Inhibition of TG2 activity inhibits tubulogenesis. **a** Effects of TG inhibitors R283 and R294 (500 uM) on cell viability of ECV304 and C378 co-cultures. For **b–d** ECV-304/C378 were co-cultured in the presence of TG active-site directed inhibitor R294 for 14 days (**c**) or R283 (**d**) at a concentration of 500 μM as described in "Materials and methods". **b** Untreated cells. Tubules were revealed by immunoperoxidase staining for collagen type I. *Bar* equals 100 μm. **e** The presence of TG2 in ECV304 and C378 co-culture matrix fractions in the presence of TG2 inhibitor R283 and R294 after 14-day culture shown by Western blotting as described in "Materials and methods"

obvious co-localization of these two signals at the centre of the tubes was detected in the different sections of the samples studied through the whole sections of the tubular structure (from top of the tube to bottom). Interestingly, the proposed TG2 binding partner β1 integrin (Fig. 5h), also known as a co-receptor for TG2 in regulating FN mediated cell adhesion and migration, was found to co-localize (Fig. 5i) with the TG in situ activity (Fig. 5g). Western blotting was performed to study the effect of TG2 inhibitors on the deposition of the FN matrix in ECM, which showed that both inhibitors R294 and R283 significantly blocked the FN deposition into the ECM (Fig. 5j), which matches with the previous discovery of the reduced TG2 in the ECM of the inhibitor- treated co-culture samples (Fig. 4e).

To investigate if TG2 activity might also influence basement membrane assembly, the ECM protein collagen IV was immunostained (Fig. 6a, b) and was found to co-localize with the active enzyme (Fig. 6c, d), as shown in Fig. 6e and f. The presence of collagen IV was also detectable by Western blotting of cell extracts at day 14 culture, which was found to be severely diminished when co-cultures were incubated with the TG inhibitors R283 and R294 (Fig. 6g). Evidence for the occurrence of protein crossslinking in the growing tubes was obtained by Western blotting of cell extracts at different time periods with the anti $\varepsilon(\gamma$-glutamyl) lysine crosslink antibody. This showed the presence of crosslinks in proteins ranging from 60 kDa to polymers greater than 250 kDa with increased polymer formation as the tube formation increased (Fig. 6h).

The inhibition of TG2 activity blocked the ECV304 cell migration and abolished the organization of actin cytoskeleton and formation of focal adhesions

To further investigate the potential mechanism of TG2 crosslinking in regulating the migratory ability of ECV304 cells, a function required for tube formation, cell migration was measured using the wound healing scratch assay in the presence and absence of the site directed TG inhibitors R294 (which acts extracellularly) and R283 (which acts

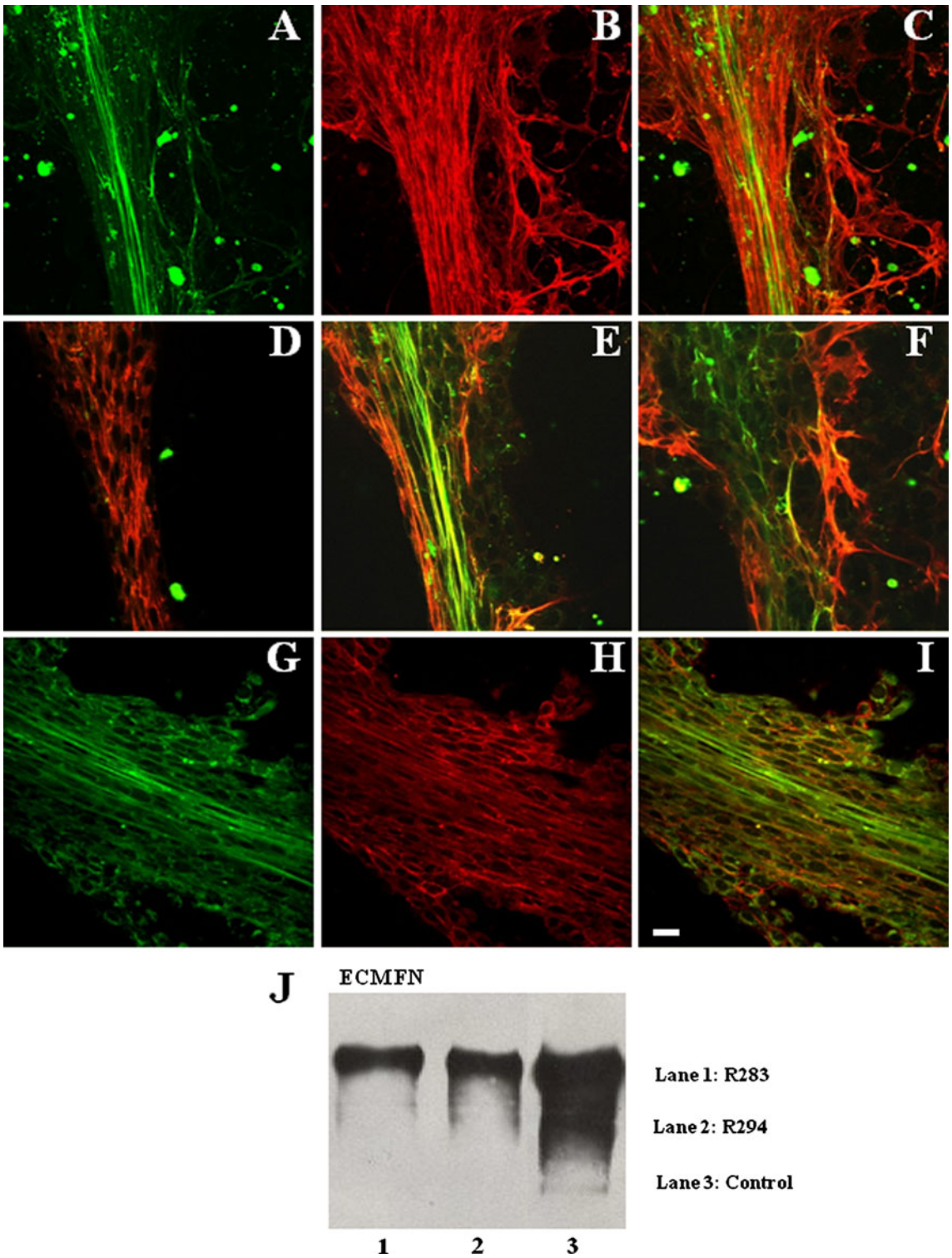

Fig. 5 Distribution of TG2 in situ activity, fibronectin and β1-integrin in tubular structures. Cocultures of ECV304 and C378 were grown in the presence of the fluorescent TG2 substrate FITC-cadaverine (*green*) to measure in situ activity (**a**, **c**, **e**, **f**, **g** and **i**) and immunostained for fibronectin (*red*, **b**–**f**) or β1 integrin (*red*, **h** and **i**). **a**–**c** show combined confocal sections revealing in situ TG activity (**a**), fibronectin (**b**) and merged image (**c**) of the edge of a tubule adjacent to a branching point. **d**–**f** show individual sections from the same merged image of the top (**d**), middle (**e**) and bottom (**f**) of the tubule. **g** shows in situ TG activity, β1-integrin (**h**) and merged image (**i**) from a larger vessel. *Bar* 25 µm. **j** the presence of FN antigen in the ECV304 and C378 co-culture matrix fractions in the presence of TG2 inhibitors R283 and R294 after a 14-day culture period detected by Western blotting as described in "Materials and methods" (colour figure online)

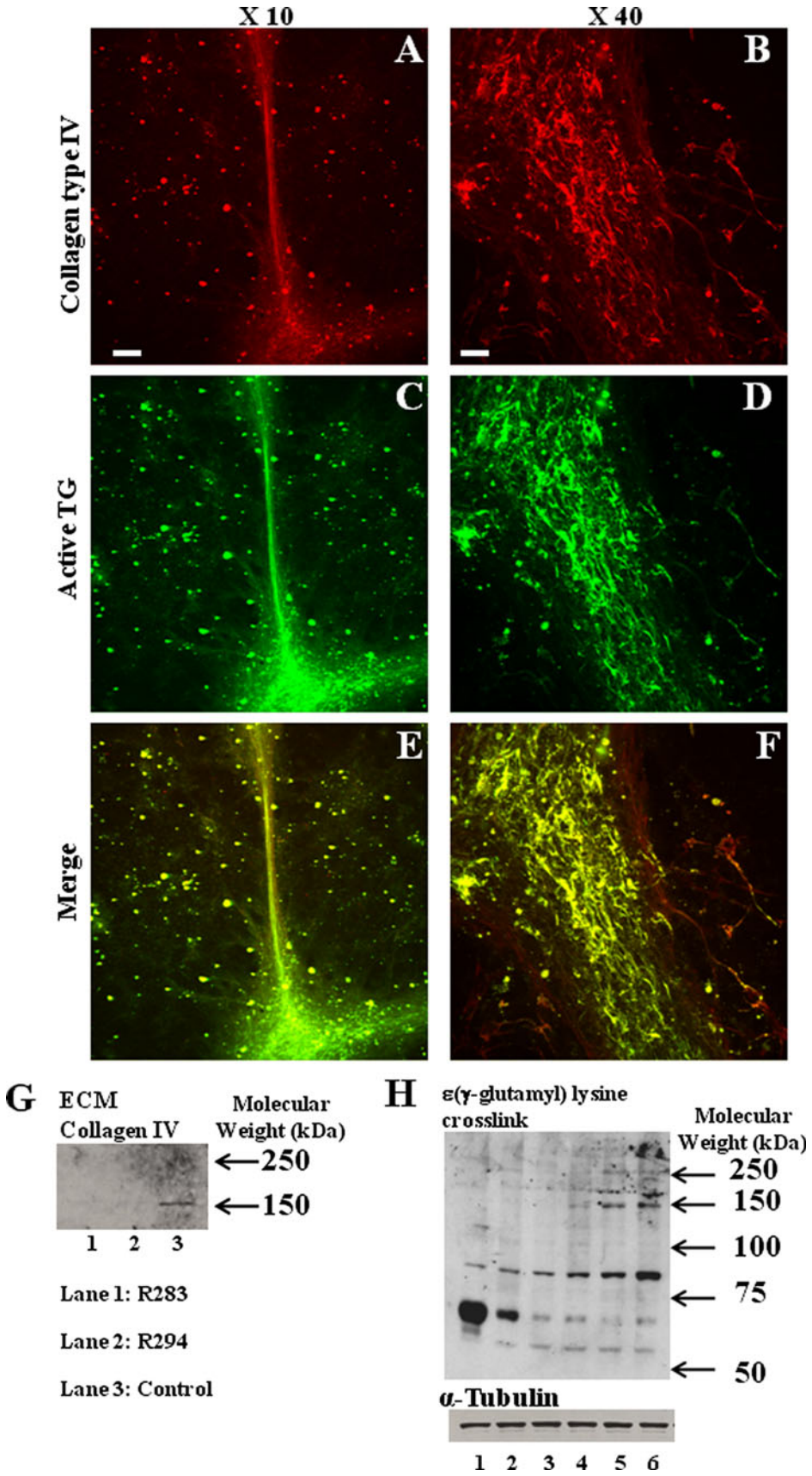

Fig. 6 The IF staining for Collagen IV and in situ TG2 activity. **a**, **b** Immmunofluorescence staining of collagen IV in the tubule from a co-culture of ECV304 and C378 at 14 days (*red*). **c**, **d** The same tubule indicating the presence of in situ TG activity. **e**, **f** Merged panels of **a**, **c** and **b**, **d**. *Bar* in **a**, 100 μm and in **b**, 25 μm. **g** Westerns blots for matrix Collagen IV at 13th day in cell lysates of 13-day cultures (*lane 3*) and with TG inhibitors 283 (*lane 1*) and R294 (*lane 2*). **h** Western blot for the ε(γ-glutamyl) lysine crosslink in cell lysates. For **h**, *lane 1*, 1–3 days; *lane 2*, 1–5 days; *lane 3*, 1–7 days; *lane 4*, 1–9 days; *lane 5*, 1–11 days; *lane 6*, 1–13 days culture. Tubulin was used to normalise equal protein loadings (colour figure online)

both at the intracellular and extracellular level). Both inhibitors significantly blocked the migration of ECV304 cells (Fig. 7a) after a 10 h incubation, indicating the importance of TG2 activity in regulating ECV304 cell migration. As shown in Fig. 7b and c, in the control cells well-organized actin cytoskeletons and focal adhesions were observed, while in the presence of the inhibitors the structure of actin cytoskeleton was destroyed and the formation of focal adhesions detected by vinculin staining was severely reduced, indicating a function for extracellular TG2 activity in maintaining the structure of the actin cytoskeleton and the focal adhesion assembly.

Discussion

In tumour development, new blood vessel formation is essential for tumour growth and progression, however, it is now recognised that tumour vasculogenesis may also occur by alterative means to that involving endothelial cells where surrounding blood vessels grow into and colonise the growing tumour (Ricci-Vitiani et al. 2010). This alternative mechanism is often referred to as vasculogenic mimicry (Fujimoto et al. 2006), whereby vascular tubules are formed from the tumour cells themselves. ECV304 cells, which are the same as the bladder cancer epithelial cell line T24/83 (Brown et al. 2000), are an example where cells have both endothelial and epithelial like characteristics (Kiessling et al. 1999) and are capable of forming tubular structures both in vitro, where they are capable of forming collaborative networks with HUVECs and in vivo (Fujimoto et al. 2006). Importantly like endothelial cells, ECV304 cells are rich in TG2 (Jones et al. 1997). Hence ECV304 cells are a well established in vitro model to study the importance of TG2 in vasculogenic mimicry (Fujimoto et al. 2006), a process initiated by tumour cells themselves.

By optimizing the ECV304 and human fibroblasts C378 culture conditions, we first demonstrate that like HUVEC

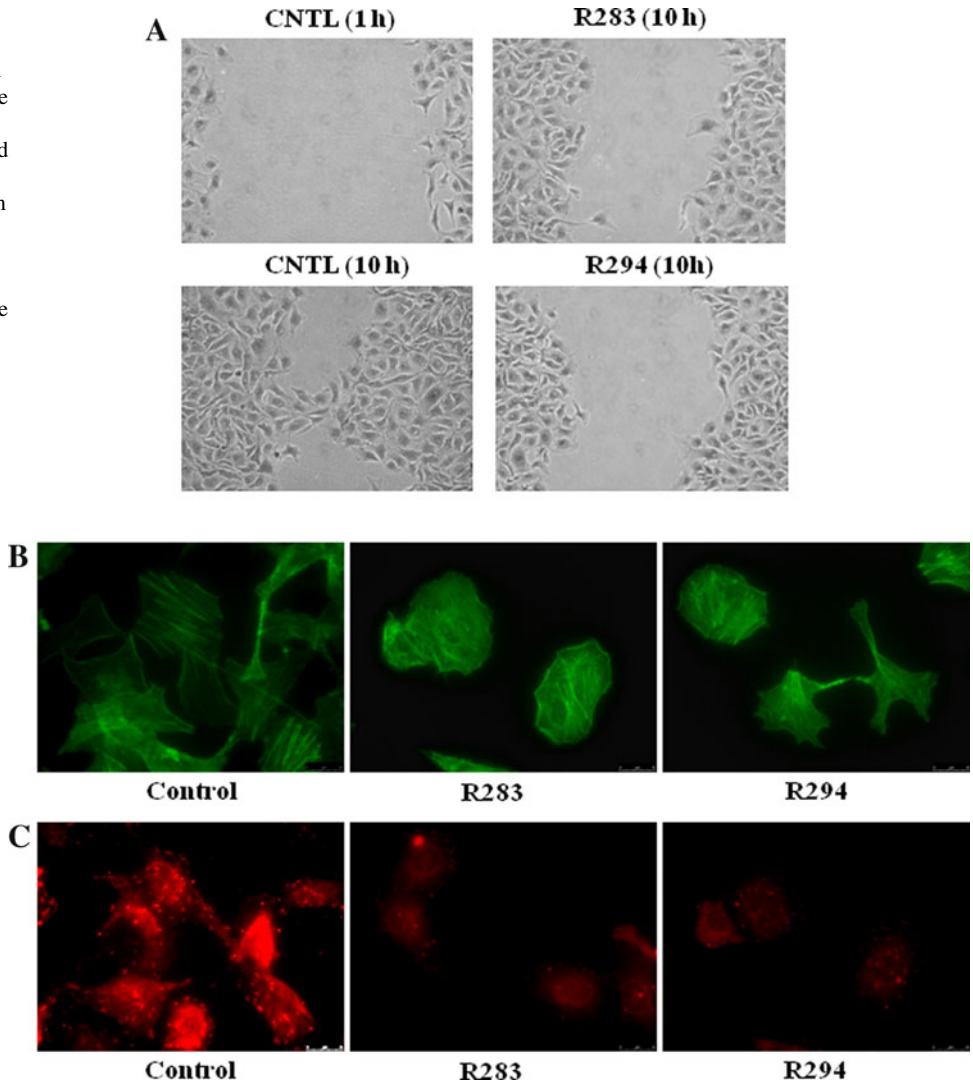

Fig. 7 The inhibitory effect of TG2 inhibitors on ECV304 cell migration, actin cytoskeleton and focal adhesion organization. **a** The wound areas were prepared by scratching ECV304 cell monolayers and the cells were allowed to migrate into the wound for around 10 h in medium containing 1% serum as described in "Materials and methods". **b**, **c** ECV304 cells were seeded into 8-well chambers for around 10 h in the presence or absence of the marked TG inhibitors. For the actin cytoskeleton and vinculin cells were fixed permeabilized and actin visualised using FITC-conjugated phalloidin (**b**) and vinculin immunostained using anti-vinculin antibody (**c**), and visualized via confocal microscopy as described in "Materials and methods"

cells, ECV304 cells are capable of forming well-developed branching tubular structures when cultured with human dermal fibroblasts. These tubular structures as well as being rich in the content of TG2 are also rich in collagen I, which is not surprising given these cells are reputed to form good tubular networks when seeded onto collagen I (Fujimoto et al. 2006). However, given the epithelial origin of ECV304, only trace levels of TG1 were found in these cells. Negligible staining for TG2 was found in the non-tubular surrounding cells which are predominantly dermal fibroblasts, which from Western blotting experiments contain very little TG2 (Fig. 1a). Moreover, in situ activity staining using FITC-cadaverine incorporation and its co-localization with TG2 antigen by IF staining further confirmed that TG2 is the major resource of active TG in the growing tubular structures.

The importance of ECV 304 cells and ECV 304 derived TG2 in tubular development was confirmed, first by the silencing of TG2 in the ECV304 cells, whereby down-regulation of TG2 expression by anti-sense transfection into ECV304 cells (Nicholas et al. 2003) practically destroyed the ability of these cells to form tubes and reduced the previously well organised in situ incorporation of FITC-cadaverine. Confirmation that it is the transamidating activity of TG2 rather than its role as cell adhesion protein (Telci et al. 2008; Wang et al. 2010) was shown by using the site-directed irreversible inhibitor R294 against TG2. This inhibitor, which acts extracellularly since it cannot penetrate cells, blocked the development of tubular formation, including the length, branches and closures of the tubes. Importantly, the ability of R294 to block tube formation strongly suggests that the role of TG2 is extracellular in tube formation.

To further investigate the role of TG2 and its binding partners/known substrates in tube formation (Collighan and Griffin 2009), fluorescence staining for in situ TG2 activity via FITC-cadaverine incorporation and IF staining for FN, collagen IV and β1 integrins was undertaken. Intense staining of TG activity could be localised with both FN and collagen IV at the centre of the tubes, suggesting these tubes may be lined with these proteins as previously noted in other studies on vasculogenesis mimicry (Fujimoto et al. 2006), with a more diverse outer localization of active enzyme with β1 integrins. Moreover, as tube formation progressed, the increased presence of crosslinked products by TG2 could also be detected in the co-cultures, as determined by Western blotting. Using Western blotting, we also demonstrated that the presence of TG2 inhibitors within the co-culture decreased the deposition of both FN and TG2 in the matrix fractions. This suggests that the main mechanism for the inhibitors in blocking tube formation is that of preventing matrix deposition, which is essential for cell adhesion and migration and tubular development. These results agree with previous findings, showing that disrupting matrix deposition by TG2 inhibitors can significantly increase the susceptibility of brain glioblastoma cells to chemotherapy (Yuan et al. 2007).

In support of our theory, we show that TG2 inhibitors block the migration of ECV304 cells, accompanied by a disruption of focal adhesion points and the formation of a poorly formed actin cytoskeleton network, which are both dependent on fibronectin deposition and required for cell migration (Antonyak et al. 2009).

In conclusion our data show that TG2 is a key player in tube formation in the ECV304 in vitro model of vasculogenic mimicry. Our evidence suggests that the roles of TG2 in tube formation are directed towards the outside of the cell and are dependent on its transamidating ability in both matrix deposition and cell migration (Antonyak et al. 2009). Inhibition of TG2 activity may therefore offer itself as an alternative therapeutic target in blocking aggressive tumour growth and progression where vasculogenic mimicry is found (Paulis et al. 2010).

Acknowledgments This work is dedicated to Richard Jones who in his prime sadly died of brain cancer. He was both a dedicated scientist and talented artist.

Conflict of interest The authors declare that they have no conflict of interest.

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