Extracellular transglutaminase 2 has a role in cell adhesion, whereas intracellular transglutaminase 2 is involved in regulation of endothelial cell proliferation and apoptosis

C. Nadalutti*, K. M. Viiri*, K. Kaukinen†, M. Mäki* and K. Lindfors*

*Paediatric Research Centre, University of Tampere and Tampere University Hospital, Tampere, Finland, and †Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital and Medical School, University of Tampere, Tampere, Finland

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Abstract

Objective: Transglutaminase 2 (TG2) is a multifunctional protein with an important role in vascular biology, where it is involved in cell–matrix interaction, cell attachment and cell population expansion. In efforts to elucidate the role of TG2 in endothelial cell biology, in this study, we measured several endothelial cell characteristics in cells where TG2 was specifically knocked down by RNAi.

Materials and methods: The effect of small interfering RNA (siRNA)-TG2 on human umbilical vein endothelial cells was studied. Adhesion and cell viability were assessed by chemical reduction of MTT, and cell proliferation was analysed by flow cytometry. Apoptosis was evaluated by annexin V/PI dual staining and protein expression level was assayed by western blotting.

Results: We found that siRNA-TG2 reduced endothelial cell number, lead to cell adhesion deficiency, cell cycle arrest in G₁ phase and induction of apoptosis. Our results show that exogenously added TG2 could reverse loss of adhesion but did not overcome the defect in cell proliferation, nor could it inhibit siRNA-TG2-induced apoptosis.

Conclusion: We conclude that TG2 loss in endothelial cells causes reduction in cell number as a result of cell cycle arrest, flaws in adhesion and induction of apoptosis. Our results imply that reduction in cell number and increased apoptosis in response to TG2 silencing is independent of the cell adhesion process. Altogether, our findings underline the significance of

TG2 in endothelial cell cycle progression and cell survival, *in vitro*.

Introduction

Transglutaminase 2 (TG2) is a member of the transglutaminase (TG) enzyme family (EC2.3.2.13), involved in a plethora of biological functions. TG2 is ubiquitously expressed in most mammalian tissues and is involved in a number of cell processes such as angiogenesis (1), apoptosis (2), cell differentiation (3) and wound healing (4,5).

TG2 is predominantly an intracellular protein and functions inside the cell as a protein disulphide isomerase, an ATPase or a protein kinase (6–9), however, its best characterized feature is its G-protein function (10,11). In addition, TG2 can be found inside the nuclear compartment in association with histones, and histone phosphorylation activity has also been reported there (12). Moreover, TG2 can be secreted to the outside of cells, where it stabilizes the extracellular matrix, making it resistant to mechanical and proteolytic degradation (13,14). Furthermore, TG2 is present on the cell membrane, where it can associate with integrins and provide a binding site for fibronectin (FN) by simultaneously binding to β -integrins and FN. TG2-mediated adhesion also involves focal adhesion kinase (FAK), whose expression, maturation and activity TG2 is known to regulate (15).

Altogether, TG2 plays a key role in a number of physiological processes and a cell survival function for TG2 is thus strongly implicated. As TG2 has an established role in angiogenesis (1,4), the molecular mechanism by which it affects proliferation, adhesion and apoptosis in endothelial cells has been examined here, using small interfering RNA (siRNA) knock-down. We have shown a central role for TG2 in cell survival in living cells, where it is needed for cell cycle progression from G₁ to S phase. In addition to this, our data demonstrate that the roles of TG2 in

Correspondence: K. Lindfors, Paediatric Research Centre, Medical School, FIN-33014 University of Tampere, Tampere, Finland. Tel.: +358 3 3551 8403; Fax: +358 3 3551 8402; E-mail: katri.lindfors@uta.fi

proliferation and apoptosis are dependent on the intracellular pool of the protein, whereas its role in adhesion is mediated by extracellular TG2 independently of its transamidase activity.

Materials and methods

Cell lines and cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Cambrex Bio Science, Walkersville, MD, USA) and maintained in HuMedia-EGM™ (EGM-1) (Clonetics®, San Diego, CA, USA). In all experiments performed, cells used were between passages 2 and 6.

TG2 knock-down by siRNA

Specific oligonucleotide for siRNA-TG2 (5'-AAGGGC GAACCACCTGAACAA-3') was purchased from Invitrogen (Invitrogen Life Technologies, Carlsbad, CA, USA). To control general toxicity of siRNA, a negative oligonucleotide control sequence, not homologous to any human mRNA (5'-AATTCTCCGAACGTGTCACGT-3') (Invitrogen Life Technologies), was used. Transfections of siRNA oligos were carried out using Lipofectamine™ RNAi MAX Transfection Reagent (Invitrogen), according to manufacturer's instructions. Silencing efficiency of TG2 was determined by western blot analysis.

SDS-PAGE and immunoblotting

For western blotting, HUVECs were resuspended in Laemmli loading buffer [63 mM Tris-HCl, 10% glycerol, 2% SDS, 0.1% (w/v) bromophenol blue, 2.5–5.0% β-mercaptoethanol] and 10 µl of total lysates was separated by 10% SDS-PAGE electrophoresis under reducing conditions. After gel electrophoresis and transfer of proteins to a nitrocellulose membrane, nitrocellulose sheets were blocked at room temperature (RT) for 1 h in 5% non-fat dry milk, and incubated overnight at +4 °C using specific primary antibodies. These included mouse monoclonal antibodies against TG2, CUB7402 (NeoMarkers, Fremont, CA, USA), phospho-FAK (Chemicon, Lake Placid, NY, USA), active caspase-3 (R&D System, Minneapolis, MN, USA), α-actin (1A4; Sigma-Aldrich, St Louis, MO, USA), β-actin (AC-74, Sigma-Aldrich), γ-tubulin (GTU-88, Sigma-Aldrich), cyclin E (sc-247) (Santa Cruz Biotechnology, Delaware Avenue, CA, USA), cyclin B (sc-245) (Santa Cruz Biotechnology) and p-21 (sc-817) (Santa Cruz Biotechnology), as well as rabbit polyclonal antibody against human FAK (Upstate Cell Signaling, Danvers, MA, USA). Immunoreactivity was detected by

sequential incubation of membranes with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Glostrup, Denmark) for 1 h at RT, and visualized using a chemiluminescence detection system (Amersham, ECL Plus Western Blotting Detection System, Buckinghamshire, UK). Autoradiographical films were scanned for densitometry analysis using 1D image analysis software (Kodak, New Haven, CT, USA). Protein expression was normalized to β-actin or γ-tubulin, and HUVECs without any treatment were considered as 100% standard.

Cell number assay

Cell viability assay was performed in 96-well plates, pre-coated with 5 µg of human plasma FN (Sigma-Aldrich). Briefly, 1×10^4 cells collected after siRNA-TG2 on days 1, 3 and 5 were plated on FN pre-coated plates and grown for 24 h. Cell viability was measured by addition of Cell Titer 96 solution (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Adhesion assay

The cell adhesion assay was performed on FN-precoated plates. Briefly, after cell detachment using trypsin, HUVECs were washed in Hank's balanced salt solution (HBSS) and 1×10^4 cells were seeded in 96-well plates. Cells were allowed to attach for 1 h and attachment was quantified using Cell Titer 96 solution (Promega), according to the manufacturer's instructions.

Immunofluorescent staining

HUVEC cells transfected by siRNA oligos were cultured on FN-precoated chamber slides (BD-Falcon™, Bedford, MA, USA) for 1, 3 and 5 days. HUVECs were fixed in 4% paraformaldehyde, washed with phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich); cells were then blocked with 1% BSA. Immunolabelling was carried out using anti-FAK antibody (diluted 1:200), which was detected by Alexa Fluor® 488-conjugated secondary anti-rabbit antibody (1:3000) (Invitrogen, Molecular Probes™, Leiden, the Netherlands). Actin stress fibres were visualized using fluorescein isothiocyanate (FITC)-labelled phalloidin (Invitrogen, Molecular Probes™). All staining was visualized by confocal fluorescent microscopy (Axion Vision, Olympus IX70, Wallac, Waltham, MA, USA).

Cell cycle analysis by flow cytometry

For cell cycle analyses, HUVECs were grown to 60–70% confluence, detached using 0.25% (w/v) trypsin in

Figure 1. Evaluation of transglutaminase 2 (TG2) knock-down efficiency in endothelial cells. Relative amount of TG2 expression in untransfected endothelial cells (HUVEC) as well as those transfected with control siRNA (negative control) or siRNA-TG2 1, 3 and 5 days post-transfection. Bars represent mean TG2 expression as percentage of control and error bars indicate standard error of the mean. P -value < 0.05 was considered significant. Protein expression was normalized to β -actin and control was considered as 100%. Data derived from at least three independent experiments, repeated in duplicate are shown. In the lower part, representative Western blots show amounts of TG2 and β -actin used as loading control for protein extracts.

5 mM EDTA (Invitrogen) on days 1, 3 and 5 after transfection. Cells were washed with PBS and incubated with FACS hypotonic staining buffer (100 mM sodium citrate, 0.2% Triton X-100, 1 mg propidium iodide (PI) and 700 U/ml RNase), as described in Sigma-Aldrich protocols, and analysed by flow cytometry (Expo32 ADC, Epics[®]XL-MCL; Beckman Coulter, Harbor Boulevard, CA, USA).

Detection of cell death and apoptosis

The number of cells undergoing apoptosis was quantified using an Annexin V-FITC kit (Calbiochem, Beckman Coulter, Harbor Boulevard, CA, USA), according to the manufacturer's instructions. Briefly, 1×10^4 cells in different culture conditions were collected, washed, resuspended in binding buffer, and mixed with Annexin V-FITC and PI. After 5 min dark incubation at room temperature, cells were analysed using the flow cytometer (Expo32 ADC, Epics[®]XL-MCL).

Rescue siRNA-TG2 phenotype assays

To establish whether proliferation defects observed in siRNA-TG2 HUVECs were due to extracellular TG2, we

designed rescue assays for all cell parameters analysed for silenced TG2 cells. Cell number and adhesion rescue assays were performed in 96-well plates precoated with 5 μ g of human plasma FN and with 20 μ g purified guinea pig TG2 (Sigma-Aldrich) (FN-TG2), as described elsewhere (16). Further experiments were performed using human recombinant TG2. Briefly, for cell number assay, 1×10^4 cells collected after siRNA-TG2 on days 1, 3 and 5 were plated on FN-TG2-precoated plates and grown for 24 h. Cell viability was measured by addition of Cell Titer 96 solution (Promega), according to the manufacturer's instructions. Cell adhesion rescue assay was performed as described previously (16) on FN-TG2-precoated plates. Briefly, after cell detachment with trypsin, HUVECs were washed in HBSS then 1×10^4 cells were seeded on 96-well FN-TG2-precoated plates. Cells were allowed to attach for 1 h and attachment was quantified using the same kit used as for the cell viability assay, according to the manufacturer's instructions. Apoptosis rescue assay was performed on FN-TG2-precoated plates. Briefly, cells with siRNA-TG2 were seeded in six-well FN-TG2-precoated plates. HUVECs were allowed to expand in number for 24 h and proportion of apoptotic cells was quantified as described above, according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test and data are presented as mean values. *P*-value <0.05 was considered statistically significant.

Results

Down-regulation of TG2 by siRNA led to decrease in number of endothelial cells

To gain new insights into the role of TG2 in endothelial cell biology, effects of its down-regulation by siRNA on HUVECs were investigated. Conventional western blotting showed that expression of TG2 after siRNA decreased in a time-dependent manner. Maximum down-regulation of TG2 was observed after 5 days silencing, when expression level of the protein was reduced to 40% of that in cells transfected with control siRNA (Fig. 1). The immediate early effect of TG2 knock-down was significant reduction in total number of cells already after only 1 day of siRNA-TG2 (Fig. 2). Decrease in cell number was progressive and subsequently, after

Figure 2. Assessment of endothelial cell number after transglutaminase 2 (TG2) silencing at different time-points. HUVECs were transfected with Scrambled siRNA (100 nM) and siRNA-TG2 (100 nM), as described in the Materials and methods section and cell viability was assessed by chemical reduction of MTT after different time points, as indicated. Data are expressed as percentage of control values on fibronectin, which represents 100%. Error bars indicate standard error of mean. 'HUVEC' indicates cells without any treatment; negative control represents cells transfected with non-silencing and siRNA-TG2 with TG2-specific siRNA-oligonucleotides respectively. Data are derived from at least three independent experiments, repeated in duplicate with initial cell number of 1×10^4 . A *P*-value <0.05 was considered significant.

5 days, number of cells had declined to 35% when compared with that of the non-transfected control group (Fig. 2).

As reduction in cell number could be due to adhesion or proliferation defects or increased apoptosis, we next analysed these cell parameters after transient post-transcriptional silencing of TG2. Knocking down TG2 by siRNA caused marked loss of cell adhesion on FN already after 1 day silencing of TG2 (Fig. 3a). In addition to this, cells appeared rounded, showed shortcomings in spreading and detached easily (data not shown). As it is generally accepted that FAK and its activity promote cell adhesion (17), we investigated whether siRNA-TG2 led to changes in FAK total protein and pFAK (pY397) expression. Interestingly, down-regulation of TG2 by silencing resulted in reduced amount of FAK protein level and subsequent decrease in pFAK (pY397) levels after 3 and 5 days siRNA-TG2, in comparison with control groups (Fig. 3b).

Further to this, immunofluorescence analyses of TG2-silenced cells revealed that colocalization of FAK and F-actin was disrupted (Fig. 3c, white arrows) and focal adhesions were disorganized after 5 days of siRNA-TG2, compared with control groups (Fig. 3c, white arrow-heads).

Furthermore, down-regulation of endogenous TG2 caused extensive actin reorganization and reduced stress fibre formation (Fig. 3c, white arrows); presumably as a consequence of this, cells adopted an aberrant shape when compared to both control groups. Moreover, we also observed that in siRNA-TG2 cells, there was marked decrease in α -smooth muscle actin expression (data not shown).

Down-regulation of TG2 arrested cell cycle progression at G₁ phase and induced apoptosis

To address the question of whether reduced number of TG2-siRNA cells was due to defects in cell cycle progression, cell cycle analysis was carried out by flow cytometry. These experiments revealed a statistically higher fraction of siRNA-TG2 cells in G₀/G₁ already at day 3, compared to control groups, as shown in Fig. 4a. As the cell cycle is tightly controlled by regulatory molecules such as cyclins, we investigated protein expression level of cyclin E and cyclin B, which are known to be essential for control of cell cycle progression in G₁/S and G₂/M transition phases respectively. Our results showed that the G₀/G₁ block observed in cells with down-regulation of endogenous TG2 was accompanied by significant and progressive up-regulation of cyclin E (Fig. 4b) and down-regulation of cyclin B (Fig. 4c). Moreover, cell cycle analysis revealed an increased number of apoptotic cells,

Figure 3. Analyses of adhesion and focal adhesion kinase (FAK) expression after transglutaminase 2 (TG2) silencing. (a) Endothelial cells were transfected with scrambled siRNA (100 nM) and siRNA-TG2 (100 nM), as described in the Materials and methods section and cell adhesion was determined by chemical reduction of MTT. Data are expressed as percentage of control values, which represented 100%. Experiments were performed with 1×10^4 of cells. Error bars indicate standard error of the mean. All data are derived from at least three different experiments, repeated in duplicate. P -value < 0.05 was considered significant. (b) Western blot analysis was performed to determine constitutive level of total FAK and pFAK (pY397) expression after siRNA-TG2 at different time points, as indicated. Equal protein loading was confirmed by γ -tubulin. (c) Immunofluorescence microscopy analysis detecting disruption of focal adhesions (white arrows), after down-regulation of TG2 at day 5, and loss of FAK colocalization with stress fibres (white arrowheads) in comparison with control cells. 'HUVEC' indicates cells without any treatment; negative control represents cells transfected with control non-silencing siRNA and siRNA-TG2 cells treated with specific siRNA-TG2 oligonucleotides.

clearly identifiable after 5 days silencing (Fig. 4a). This was confirmed by staining with annexin V-FITC. Analyses of annexin V binding showed that the down-regulation of TG2 induced apoptosis dramatically in a time-dependent manner (Fig. 5a). It is well known that p53 and its downstream target gene *p21* are involved in cell cycle arrest preceding apoptosis (18,19). We therefore investigated whether increased apoptosis of TG2-siRNA cells could be coupled to increased expression of p53, p21 and downstream effector caspase 3, and found increased apop-

tosis to coincide with activation of caspase 3 and up-regulation of p53 along with its downstream target gene *p21*, as shown by western blotting (Fig. 5b).

FN-TG2 restored adhesion in siRNA-TG2 endothelial cells but did not reverse proliferation and apoptosis

To establish whether lower proliferation and higher apoptosis of siRNA-TG2 cells were solely due to adhesion defects, we applied rescue assays adopted from Verderio

Figure 4. Effect of transglutaminase 2 (TG2) knock-down on cell cycle. (a) Cells were transfected with scrambled siRNA (100 nM), as described in the Materials and methods section and cell cycle analysis was studied by flow cytometry, showing G₁ cell cycle arrest with down-regulation of TG2. Expression and quantification of cyclin E (b) and cyclin B (c) after siRNA-TG2 by western blot analyses, at different time-points; γ -tubulin was used as a loading control for protein extracts. Bars represent cyclin E and cyclin B as percentage of the control with error bars indicating standard error of the mean. Data derived from at least three different experiments, repeated in duplicate are shown. P -value < 0.05 was considered significant. 'HUVEC' indicates cells without any treatment, negative control represents cells transfected with control non-silencing siRNA and siRNA-TG2 cells treated with specific siRNA-TG2 oligonucleotides.

et al. (16) with minor modifications, to see if addition of exogenous TG2 could rescue phenotypes observed after down-regulation of TG2 by small interfering RNAs. Adhesion defects were completely abolished in siRNA-TG2-treated cells, plated in 96-well plates precoated with FN and commercial purified guinea pig TG2

(Fig. 6a). On the other hand, exogenously added guinea pig TG2 bound to FN was not able to circumvent reduction in cell number of siRNA-TG2 cells (Fig. 6b). Comparable results were achieved by human recombinant TG2 concerning proliferation (data not shown). Similarly, exogenous addition of purified guinea pig TG2 was not

Figure 5. Effect of transglutaminase 2 (TG2) silencing on apoptosis. (a) HUVECs were transfected with Scrambled siRNA (100 nM) and siRNA-TG2 (100 nM), as described in the Materials and methods section, and apoptosis was assessed by AnnexinV-FITC labelling at different time points. Bar represent proportion of apoptotic cells expressed as percentage of total cells. In each experiment and time point, 1×10^4 cells were analysed. (b) Expression of pro-apoptotic protein caspase 3 as well as p53 and p21 on day 5 as shown by western blot analyses. Quantification of bands was performed using Kodak 1D image analysis software (Kodak, New Haven, CT, USA). Bars represent relative amounts of each protein indicated as percentage of control. Inserts below show representative western blots from experiments where γ -tubulin was used as a loading control for protein extracts. Error bars indicate standard error of mean. Data derived from at least three different experiments repeated in duplicate are shown. P -value <0.05 was considered significant. 'HUVEC' indicates cells without any treatment; negative control represents cells transfected with control non-silencing siRNA and siRNA-TG2 cells treated with specific siRNA-TG2 oligonucleotides.

able to normalize proportion of apoptotic cells where endogenous TG2 had been down-regulated, after 1 day of silencing (Fig. 6c). In this regard, we were not able to perform a rescue apoptosis assay with siRNA-TG2 at days 3 and 5 after transfection, as cells were non-viable for completion of detection of cell death by Annexin V-FITC and PI staining.

Discussion

In the present study we found that down-regulation of endogenous TG2 in living HUVECs by siRNA, reduced cell number, by loss of adhesion, inhibition of proliferation and increasing apoptosis, in a time-dependent manner. Furthermore, our results show that exogenous

addition of TG2 could reverse loss of adhesion, whereas it did not overcome loss of cell proliferation nor could it inhibit TG2-siRNA-induced apoptosis.

Our findings indicating that TG2 is needed for complete adhesion are in line with earlier reports describing involvement of TG2 in adhesion of various cell types (20–26) in a manner independent of its transamidase activity (data not shown), (16,27). Moreover, our study is in agreement with previous studies showing diminished activation and autophosphorylation of FAK (pY397) following TG2 silencing, in pancreatic cancer cells (28). Notably, it has been recently shown by Lim *et al.* (29), that in primary human cells, FAK knock-down raised p53/p21 levels. In line with this, we have observed that endogenous down-regulation of TG2 induced decrease in FAK and

Figure 6. Exogenous addition of transglutaminase 2 (TG2) to rescue siRNA-TG2 endothelial cell phenotype. Endothelial cells were transfected with Scrambled siRNA (100 nM) and siRNA-TG2 (100 nM), as described in the Materials and methods section, and (a) adhesion, (b) cell number and (c) apoptosis of untransfected HUVECs as well as those transfected with control non-silencing siRNA (negative control) and siRNA targeting TG2 (siRNA-TG2) on fibronectin (FN) and to FN-bound TG2, were evaluated. Data expressed as percentage of control values, of 100%. Experiments were performed with 1×10^4 of cells. In (a) and (b), bars represent percentage of cells compared with control. In (c), bar represents proportion of apoptotic cells expressed as percentage of the total cells. Error bars indicate standard error of mean. Data are derived from at least three different experiments, repeated in duplicate. P -value < 0.05 was considered significant.

pFAK expression was coupled with increase in p53/p21 and enhancement of apoptosis.

Although adhesion defects in the siRNA-TG2 of our cells could have explained the initial result of reduced cell number after TG2 down-regulation, we have shown that addition of exogenous TG2 was able to reverse the adhesion defect only, as also reported previously (16), without overcoming proliferation failure. This would suggest that reduction in cell number in response to TG2 silencing is independent of the adhesion process.

To our knowledge, the present study is the first to demonstrate that down-regulation of TG2 expression in endothelial cells leads to cell cycle arrest and this is coupled with elevated expression of cyclin E and decreased expression of cyclin B, known to play an essential role in cell cycle progression through G_1 to S and from G_2 to M phase respectively. It is important to note that Mangala *et al.* (30) have shown that knock-down of TG2 in breast cancer cells with siRNA markedly reduced their viability, even when they were cultured on FN-coated surfaces. Our results are in line with these findings but in our system, the proliferation rescue assay performed on FN-bound TG2 provided additional insight that any survival function of TG2 was due to its presence in an intracellular pool.

Noteworthy, earlier reports have shown that overexpression of TG2 in malignant hamster fibrosarcoma cells resulted in impairment of the cell cycle (31). However, Mian *et al.* (31) have demonstrated that increased expression of TG2 protein can affect progression through the cell cycle from S phase to G_2 /M, and this depends on GTP-binding activity of TG2. Hence, it is interesting that both overexpression and down-regulation of TG2 induce impairment of cell cycle progression, thus supporting a direct involvement of TG2 in regulation of cell survival and cell division.

It is well established that growth arrest may lead to either cell survival but permanently arrested, or to death (32). Apoptotic cell death is activated if cells are not able to overcome the injury causing cell cycle arrest.

According to our results, this would seem to be the case for our endothelial cells, which, due to loss of intracellular TG2, pursue G₁ cell cycle arrest and subsequently commit apoptosis *via* activation of the caspase cascade. Furthermore, we found that increased apoptosis coupled with increased p53/p21 levels could not be prevented by addition of exogenous TG2, thus implying that in our experimental settings, the crucial cell survival role is played by intracellular TG2.

Notably, it has been reported that depending on cell context, TG2 can either promote or inhibit cell death. Yet, increased expression of TG2 in several types of cancer cell has been associated with augmented cell invasiveness and cell survival (28–31,33,34). Conversely, down-regulation of TG2 by siRNA or TG2 inhibition by small molecule inhibitors has been shown to significantly sensitize cancer cells to apoptosis (28,30,33). Additionally, TG2-regulated pathways are involved in promoting or protecting normal and tumour cells from death-induced signalling (33,34). Consequently, different outcomes related to level of TG2 expression and apoptosis, might be explained by different cell lines being used in investigations or that primary cells such as HUVECs are more vulnerable to loss of TG2 than cancer cells, where expression level of TG2 is increased. Even if TG2 knock-out mice did not exhibit growth defects *in vivo*, this may be due to different regulatory signals or compensatory survival mechanisms by other transglutaminase family members.

In conclusion, our results demonstrate that knocking down TG2 in endothelial cells leads to cell cycle arrest at G₁ phase, to adhesion defects and to increased apoptosis. Our findings indicate that the extra- and intracellular functions of TG2 are clearly distinct from each other with regard to cell adhesion and regulation of cell cycle and apoptosis respectively.

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