Taurine transport in human placental trophoblast is important for regulation of cell differentiation and survival

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The outermost cell layer of the human placenta, the syncytiotrophoblast, is a specialised multinucleate tissue that functions as a solute-transporting epithelium and endocrine/paracrine organ, delivering nutrients to the foetus and producing hormones that sustain pregnancy. Consequently, maintenance of the syncytiotrophoblast is vital for a successful pregnancy. The syncytiotrophoblast exists in a terminally differentiated post-mitotic state; it is generated and renewed from the underlying population of mononuclear cytotrophoblast cells, which undergo proliferation, differentiation and finally fusion with the syncytiotrophoblast, permitting the acquisition of fresh cellular components. It is hypothesised that apoptosis and subsequent shedding of syncytial knots into the maternal circulation complete the process of syncytiotrophoblast nuclear turnover.1 However, there is an alternative hypothesis proposed whereby syncytial nuclei continue to accumulate until the end of pregnancy.2

Despite the controversy surrounding the life cycle of apoptotic nuclei during normal trophoblast turnover, it is agreed that there is increased trophoblast cell death in placentas from pregnancies complicated by foetal growth restriction (FGR).3 Low birth weight is associated with an increased risk of neonatal morbidity and mortality, and development of metabolic and cardiovascular diseases in adulthood.4 Ongoing research investigating the cause(s) of altered trophoblast turnover in FGR has identified a number of factors that could trigger inappropriate apoptosis, including elevated inflammatory cytokines such as tumour necrosis factor alpha (TNFα)5.

Abnormal turnover compromises syncytiotrophoblast integrity and renewal with consequences for nutrient delivery to the foetus, and this is thought to be a major contributing factor to FGR. Consistent with this is the finding that in addition to aberrant trophoblast turnover in FGR, there is reduced activity of certain amino acid transporters in the syncytiotrophoblast including that of system bε responsible for taurine uptake into

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Abbreviations: BLAST, Basic Local Alignment Search Tool; FGR, foetal growth restriction; MAPK, mitogen-activated protein kinase; MVM, microvillous membrane; NT, non-targeting siRNA; PBS, phosphate buffered saline; PE, pre-eclampsia; PI, propidium iodide; PKC, protein kinase C; QPCR, quantitative polymerase chain reaction; RNS, reactive nitrogen species; siRNA, small interfering RNA; STAT3, signal transducer and activator of transcription 3; TBS, Tris buffered saline TNFα, tumour necrosis factor alpha

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essential because the human foetus and placenta lack the necessary synthetic enzymes. Therefore, foetal and placental demand for taurine must be met by transport of taurine from maternal plasma into the syncytiotrophoblast and across the placenta via system \( \beta \). Foetal plasma taurine concentrations are lower in FGR compared with normal pregnancies, suggesting taurine to be important for foetal growth. Unlike other amino acids, taurine is not incorporated into proteins and is largely involved in promoting development of the central nervous system, retina, kidney, and endocrine pancreas. Taurine is the most abundant free amino acid in human placenta (intracellular concentration \( \sim 10 \text{ mM} \), maternal and foetal plasma 60 and 135 \( \mu \text{M} \), respectively). The reason why intracellular taurine levels are so high in the syncytiotrophoblast is unknown, but could reflect the cytoprotective functions of taurine and also its role as an osmolyte, important for cell volume regulation, as found in other cell types. All cells have to regulate their volume in order to survive; macromolecular synthesis, cell growth, differentiation, apoptosis and hormone secretion are all influenced by the cellular hydration state.

We therefore hypothesised that as well as being directly important for foetal development, system \( \beta \)-mediated taurine transport by the placenta indirectly affects foetal growth by maintaining the normal process of trophoblast cell turnover. We further hypothesised that reduced taurine uptake by syncytiotrophoblast could compromise cytoprotection against TNF-\( \alpha \), an inflammatory cytokine that is elevated in FGR, and increase susceptibility to inappropriate apoptosis.

These hypotheses have been tested by investigating cytotrophoblast differentiation and survival following exposure to TNF-\( \alpha \) in vitro after siRNA-mediated knockdown of the system \( \beta \) amino acid transporter protein, taurine transporter (TauT) (encoded by the SLC6A6 gene). Primary cytotrophoblast cells isolated from term human placenta were used as a model for investigating trophoblast cell turnover because, when maintained in culture, these mononucleate cells are mitotically inactive, and therefore unable to proliferate. Instead, the cytotrophoblast cells aggregate at 18–24 h of culture and subsequently fuse to form multinucleated cells, reminiscent of the process of differentiation into syncytiotrophoblast in vivo.

**Results**

**siRNA-mediated knockdown of SLC6A6/TauT.** Transfection of cytotrophoblast cells with 50 nM SLC6A6-specific siRNA significantly reduced target mRNA expression by 71% (median, range: 64–84%, \( n = 5 \)) when compared with untransfected control cells (Figure 1a). Expression of mRNA for the housekeeping gene, \( \beta \)-actin, was unaffected by transfection with SLC6A6-specific siRNA (Figure 1a), indicating that this siRNA does not cause off-target/non-specific effects. Transfection of cytotrophoblast cells with 50 nM non-targeting siRNA did not affect mRNA expression for SLC6A6 nor \( \beta \)-actin (Figure 1a). Exposure of cytotrophoblast cells to DharmaFECT 2 transfection reagent alone (mock transfected) caused a small but significant decrease in SLC6A6 mRNA expression when compared with untransfected controls cells (Figure 1a). Despite reduced expression of SLC6A6 mRNA in mock transfected cells, TauT protein expression and activity in these cells was comparable to untransfected controls (Figures 1b and c). SLC6A6 mRNA knockdown in cells transfected with 50 nM SLC6A6-specific siRNA was accompanied by reduced TauT protein expression, evidenced by diminished immunofluorescent detection of this target protein when compared with untransfected and mock transfected control cells, as well as cells transfected with 50 nM non-targeting siRNA (Figure 1b). System \( \beta \) amino acid transporter/TauT initial rate activity was significantly reduced by 64% (median, range: 60–82%, \( n = 5 \)), following SLC6A6/TauT knockdown (Figure 1c). Successful knockdown of TauT expression and activity in the primary cytotrophoblast cells was therefore achieved.

**TauT knockdown reduces intracellular taurine accumulation by cytotrophoblast cells.** Intracellular taurine accumulation by untransfected control cytotrophoblast cells reached steady state after 24 h (Figure 2a). As taurine is not metabolised, this accumulation reflects the stable intracellular taurine concentration when uptake via TauT and efflux out of the cell (thought to occur via volume-regulated anion channels) are at steady state. Similarly, in cells transfected with 50 nM non-targeting or SLC6A6-specific siRNA, steady state occurred after 24 h (Figure 2a). However, the ability of the cytotrophoblast cells transfected with SLC6A6-specific siRNA, and therefore with reduced TauT expression and activity, to accumulate intracellular taurine was severely compromised (~33% of that achieved by control cells at 24 h, Figure 2b). Taurine uptake was allowed to occur over a total period of 48 h but, even by this time point, the TauT-deficient cells were unable to accumulate any additional intracellular taurine (Figure 2a). Total protein content of cells transfected with SLC6A6-specific siRNA did not decline during this prolonged period of culture and was comparable to total cell protein content of untransfected controls and cells transfected with non-targeting siRNA at the 48 h time point (data not shown). This indicates that cell number was unaffected by transfection, even over a prolonged period, and a fall in the number of cells cannot underlie the reduced \( ^{33} \text{H}\) taurine accumulation. These observations demonstrate that reduced TauT activity in cytotrophoblast cells (Figure 1c: initial rate) is associated with lower intracellular taurine levels at steady state.

**Trophoblast differentiation is impaired following TauT knockdown.** Desmosomes are specialised epithelial cell–cell junctions between adjoining cells. As cytotrophoblast cells differentiate and become multinucleated, the loss of cell–cell boundaries parallels a decrease in desmosomal proteins. Untransfected cytotrophoblast cells differentiated normally during the 66 h in culture, evidenced by reduced immunofluorescent staining of desmosomal proteins (Figure 3a, 18 versus 66 h control). This morphological differentiation was accompanied by a significant increase in human chorionic gonadotrophin (hCG) secretion over time in culture from 2.2 mIU/mg protein/h at 18 h to 29 and 318 mIU/mg protein/h at 42 and 66 h, respectively (mean, \( n = 6 \), \( P < 0.001 \) Kruuk–Wallis test), consistent with concomitant biochemical differentiation.
There was significantly less multinucleation at 66 h in cells transfected with SLC6A6-specific siRNA, suggesting a role for TauT-mediated taurine transport and/or intracellular taurine in trophoblast morphological differentiation (Figures 3a and b). Transfection of cells with non-targeting siRNA did not affect multinucleation (Figures 3a and b), eliminating the possibility that impaired trophoblast morphological differentiation was an effect of the transfection procedure itself. However, reduced

![Figure 1](image-url)

**Figure 1** Confirmation of TauT knockdown in 66-h cytotrophoblast cells. (a) SLC6A6 and β actin mRNA expression. (b) Immunofluorescent detection of TauT protein (red) in cells counterstained with DAPI (blue). Scale bar represents 50 μM and refers to all images. A lack of fluorescence following pre-absorption of primary antibody with a 10 × excess of antigenic peptide (−ve) or omission of the primary antibody (−1°) confirmed specificity. (c) TauT activity. All observations were made 48 h post transfection. Key to labelling: control = untransfected, mock = transfection reagent only, NT = non-targeting siRNA, TauT = SLC6A6-specific siRNA. Error bars represent median ± interquartile range, n = 5. *P < 0.05 versus 100% (i.e., matched untransfected control), Wilcoxon-signed Rank test. #P < 0.05 versus mock and NT siRNA, Kruskal–Wallis with Dunn’s multiple comparison test.
morphological differentiation of cytotrophoblast cells following TauT knockdown was not associated with reduced biochemical differentiation, as there was no significant difference in hCG secretion by untransfected control cells and cells transfected with non-targeting (open squares) or SLC6A6-specific (open circles) siRNA. Duplicate measurements from one placenta. (b) Scatter plot of intracellular taurine accumulation after 24 h. Each point represents the mean of duplicate observations of three placentas (line represents median). NT = non-targeting siRNA, TauT = SLC6A6-specific siRNA.

Figure 3  TauT knockdown impairs cytotrophoblast morphological differentiation in vitro. (a) Immunofluorescent detection of desmosomes (green) in 18 h (inset) and 66 h cytotrophoblast cells counterstained with propidium iodide (red). The white arrows indicate multinucleated cells (defined as ≥ 3 nuclei within desmosomal boundaries). (b) Percentage of multinucleated cells at 66 h following transfection, relative to matched control cells (n = 7, error bars represent median ± interquartile range). **P < 0.01 versus 100% Wilcoxon-signed Rank test. NT = non-targeting siRNA, TauT = SLC6A6-specific siRNA.
specifically, the ability of trophoblast cells to accumulate intracellular taurine facilitates their differentiation into a multinucleated syncytiotrophoblast and protects against inappropriate cell death in response to an inflammatory cytokine.

It is well documented that in both placental and non-placental cell types, taurine is a key osmoregulator with cytoprotective functions. In order to carry out these important roles, it is essential that intracellular taurine concentration is maintained by appropriate regulation of cellular taurine uptake and efflux. In placentas from normal pregnancy, taurine is accumulated in the syncytiotrophoblast to reach levels of ~10 mM. In vitro studies have
demonstrated that TauT activity in the foetal-facing basal membrane of the syncytiotrophoblast is only 6% of that measured in the maternal-facing microvillous membrane (MVM), suggesting that the high concentration of tauin found within the syncytiotrophoblast is achieved by the uptake from maternal blood. Norberg et al. found that TauT activity in MVM of placentas from pregnancies complicated by FGR was reduced by 34% compared with normal pregnancies. In the current study, a 64% reduction in trophoblast TauT activity led to a 66% decrease in intracellular tauin accumulation. It is therefore probable that intracellular tauin levels are lower in the syncytiotrophoblast of placentas from FGR pregnancies, although this remains to be determined.

Our data demonstrate that TauT-mediated tauin transport in trophoblast cells has a role in their fusion/differentiation and multinucleation, but does not influence hCG secretion. This observation suggests that intracellular tauin has selective effects on these two well-characterized events in trophoblast differentiation, with maintenance of normal intracellular tauin being necessary for syncytialisation, but not for hormone secretion. This is consistent with previous reports that biochemical and morphological differentiation of trophoblast cells in vitro can be independent events. Reduced placental TauT activity in FGR could, therefore, lead to impaired syncytial formation and renewal. Indeed, there is evidence that cytotrophoblast cells isolated from pregnancies with placental insufficiency and FGR have a significantly lower cell–cell fusion index compared with those isolated from normal placentas. Trophoblast fusion events and their regulation are poorly understood. Exactly how TauT-mediated tauin transport influences trophoblast cell fusion/multinucleation requires investigation but two mechanisms are proposed. Intracellular tauin could be important for intracellular signalling events, which facilitate morphological differentiation. In non-placental cells, intracellular tauin modulates the expression and phosphorylation of proteins involved in the MAPK, STAT3 and PKC signalling pathways. Each of these signalling molecules are involved in cell differentiation, therefore, alterations in intracellular tauin could affect their role in this process. Alternatively, tauin could help maintain gap junctional intercellular communication required for cell fusion, as has been shown in liver. Indeed, molecular exchanges through gap junctions preceding cellular fusion are essential for trophoblast differentiation and generation of the multinucleated syncytiotrophoblast.

In addition to an impaired ability to differentiate morphologically, we have demonstrated that TauT-deficient trophoblast cells are more susceptible to TNFα-induced apoptosis. Elevated levels of TNFα have been reported in FGR and this has been suggested to contribute to the increase in trophoblast apoptosis associated with these pregnancy complications. However, we have demonstrated that TNFα induces significant apoptosis in the cytotrophoblast cells only when their ability to accumulate intracellular tauin has been reduced through SLC6A6-specific knockdown. This observation is in agreement with studies in other cell types that have revealed cytoprotective functions of tauin. Interestingly, an increased ability to accumulate tauin via overexpression of SLC6A6 attenuated cisplatin-induced apoptosis in a renal cell line, supporting the hypothesis proposed by Han et al. that SLC6A6 acts as an antiapoptotic gene by facilitating TauT-mediated tauin accumulation. It is therefore proposed that it is the reduced placental TauT activity, together with elevated levels of TNFα, that contribute to increased trophoblast cell death in cases of FGR.

The data presented, which highlight the importance of TauT-mediated tauin transport for trophoblast fusion/differentiation and survival, provide good reason for identifying the cause(s) of reduced placental TauT activity in FGR. Placental expression of TauT is comparable between normal pregnancies and those complicated with FGR. Therefore, one possible explanation for reduced placental TauT activity in this pregnancy condition is post-translational modification of the TauT protein, causing a conformational change that reduces the affinity for ligands. Molecular cloning and characterisation of TauT has revealed the presence of several phosphorylation sites. Phosphorylation of TauT by protein kinase C decreases its affinity for tauin, and activation of this kinase in placental villous fragments and trophoblast cells leads to reduced TauT activity. TauT can also be nitrated, a modification of tyrosine residues by reactive nitrogen species (RNS), and this could underlie the decreased syncytiotrophoblast TauT activity in FGR. Tyrosine residues are essential for the activity of TauT in human placenta and in vitro experiments suggest that nitrative stress impairs placental TauT activity. Levels of RNS are elevated in FGR and TauT nitration could be increased as a consequence, but this remains to be investigated. There is evidence that an increased proportion of TauT is nitrated in placentas from pregnancies complicated with pre-eclampsia (V Roberts and L Myatt, personal communication). Pre-eclampsia is a serious disease of pregnancy sometimes accompanied by FGR that is also associated with abnormal trophoblast turnover. It will be interesting to determine whether syncytiotrophoblast TauT activity is also reduced in this pregnancy complication.

In summary, we have demonstrated that reduced TauT activity in cytotrophoblast cells leads to impaired morphological differentiation and an increased susceptibility to apoptotic cell death. Such alterations in utero would have implications for syncytiotrophoblast renewal, compromising the transfer of nutrients from the maternal circulation towards the foetus via other transporters present on the MVM, and also cause disruption to the endocrine function of the syncytiotrophoblast, leading to placental insufficiency with consequences for foetal growth and wellbeing. The ability of trophoblast cells to accumulate sufficient intracellular tauin for their differentiation and survival is therefore crucial for a healthy pregnancy.

Materials and Methods
Materials. Unless stated otherwise, all materials used were obtained from Sigma-Aldrich (Poole, UK).

Primary cytotrophoblast cell isolation and culture. Term placentas (38–40 weeks gestation) were collected with written informed consent and in accordance with the Local Ethics Committee’s approval, following caesarean section or vaginal delivery from uncomplicated singleton pregnancies. Cytotrophoblast cells were isolated using an adaptation of the method used by Kliman et al., as previously described. Cells destined for amino acid transporter activity measurements were plated onto 35-mm culture dishes (Nunc) at a density...
of 2.2-5 x 10^6. Cells destined for mRNA analysis and detection of target proteins were plated into 12-well culture plates (Nunc) at a density of 1-1.5 x 10^5. Cytotrophoblast cells were maintained for 66 h in culture medium (Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 1:1; 10% FCS (heat-inactivated), 1% gentamicin, 0.6% glutamine, 0.2% penicillin, 0.2% streptomycin) at 37 °C in a humidified incubator (95% air/5% CO2) supplemented with 100 μM taurine to mimic physiological levels in maternal blood.12

Transfection of primary cytotrophoblast cells with siRNA. Previous studies have demonstrated that at 18 h of culture, isolated cytotrophoblast cells are mononucleate and by 42 h they aggregate and fuse. At 66 h, the cells have differentiated to form large polarised multinucleate cells that resemble syncytiotrophoblast in vivo.15,38 At 18 h of culture, cytotrophoblast cells were transfected with 50 nM SLC6A6-specific siRNA (Qiagen, West Sussex, UK) using DharmaFECT2 transfection reagent (Dharmacon, Fisher Scientific UK, Loughborough, UK), as described previously.39,40 Cytotrophoblast cells transfected with non-targeting siRNA (Invitrogen, Life Technologies, Paisley, UK) and cells exposed to DharmaFECT2 only (i.e., mock transfected) were included as controls. Initially, four different SLC6A6-specific siRNAs were tested and here we present data using the construct, which most efficiently silenced SLC6A6 (target sequence: 5′-CTGGCTGTATTACCTAACTGAGA-3′). In validation experiments, this construct reduced SLC6A6 mRNA expression by 64% compared with untransfected controls, whereas mRNA silencing by the other three constructs tested were 60, 50 and 27% (mean, n = 2).

Confirmation of target-specific mRNA knockdown using QPCR. 48 h post transfection (i.e., at 66 h of culture), cells were lysed and total RNA extracted using an Absolutely RNA microprep kit (Stratagene, Agilent Technologies UK Ltd, Stockport, UK). RNA was quantified using a Quant-iT Ribogreen kit (Molecular Probes, Life Technologies), and 100 ng of total RNA from each sample was reverse transcribed using AffinityScript cDNA synthesis kit with modified Eagle's medium (DMEM) and Ham's F-12 1:1, 10% FCS (heat-inactivated). Data were analysed by Wilcoxon-signed Rank test, following normal distribution of mRNA expression in the transfected cells to expression in the corresponding untransfected control cells for each experiment.

Immunofluorescent staining. Cytotrophoblast cells that had been plated onto 16-mm glass coverslips in 12-well culture plates were fixed in methanol at −20 °C for 25 min and then stored at 4°C in PBS prior to immunofluorescent staining for either TaTu or desmosomal proteins (allowing visualisation of multinucleation).37 Following a 30-min incubation at 37°C with blocking solution (2% FCS, 2% BSA and 0.1% Tween20 in PBS) to reduce nonspecific binding, cells were incubated for 1 h at room temperature with primary antibody (1:100 dilution in blocking solution). Primary antibodies were rabbit polyclonal anti-Taurine Transporter (Alpha Diagnostics, Source BioScience LifeSciences, Nottingham, UK) and monoclonal mouse anti-desmosomal protein (D1286, mouse IgG1 isotype). Cells were then washed with PBS and the secondary antibody, AlexaFluor 568 goat anti-rabbit IgG (1:600 in block solution; Molecular Probes) and goat anti-mouse IgG FITC (P2012, 1:100 dilution), respectively, applied for 1 h at 37°C in the dark. After washing with PBS, coverslips were mounted onto glass slides using VECTASHIELD mounting medium containing DAPI (Vector Laboratories, Peterborough, UK). Immunofluorescent images were captured using a Zeiss AxioObserver Inverted Microscope.

Amino acid transporter activity measurements and ability to accumulate intracellular taurine. 48 h post transfection (i.e., at 66 h of culture), cytotrophoblast cells were washed free of cell culture medium using Tyrode’s buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 5.6 mM glucose, pH 7.4). Na+-dependent uptake of radiolabelled taurine (3H-taurine, 1 μCi/ml, 50 nM) by control and transfected cytotrophoblast cells was then measured in duplicate as follows: uptake of 3H-taurine was carried out at 37°C in either control or Na+-free Tyrode’s buffer (135 mM choline chloride replaced NaCl, pH 7.4). Uptake was terminated after 20 min, determined in pilot experiments to represent initial rate, by washing cells in 25-ml ice-cold Tyrode’s buffer over 1 min. Cells were then lysed in 1 ml 0.3 M NaOH and the lysate counted for 3H radiation. Cell lysate protein content (mg) was determined using a commercial kit (Bio-Rad Laboratories Ltd., Hemel Hampstead, UK). Uptake of radiolabelled taurine is expressed as pmol per mg protein over 20 min.

Assessment of cytotrophoblast cell morphological differentiation. Immunofluorescent images of cytotrophoblast cells transfected for desmosomes and nuclei (see above) were used to assess multinucleation as a measure of morphological differentiation. Using a previously published method,3 three observers, blinded to the identity of the images, counted the total number of nuclei per field of view and the number of multinucleated cells (defined as ≥3 nuclei within desmosomal boundaries) by using Image Pro Plus software (MediaCybertechns, Buckinghamshire, UK). The number of multinucleated cells was then expressed as a percentage of the total number of nuclei within a given field of view. The number of nuclei per field of view was 114 (range 57–354). For each experiment, three fields of view were analysed per treatment and each treatment was performed in duplicate. The mean of these observations was then calculated to provide a value of multinucleation in transfected and untransfected control cells for each experiment. Multinucleation of transfected cells was expressed as a per cent of multinucleation of matched control cells for the corresponding experiment and analysed by Wilcoxon-signed Rank test.

Assessment of cytotrophoblast cell biochemical differentiation. Immunofluorescent images of cytotrophoblast cells transfected for desmosomes and nuclei (see above) were used to assess multinucleation as a measure of morphological differentiation. Using a previously published method,3 three observers, blinded to the identity of the images, counted the total number of nuclei per field of view and the number of multinucleated cells (defined as ≥3 nuclei within desmosomal boundaries) by using Image Pro Plus software (MediaCybertechns, Buckinghamshire, UK). The number of multinucleated cells was then expressed as a percentage of the total number of nuclei within a given field of view. The number of nuclei per field of view was 114 (range 57–354). For each experiment, three fields of view were analysed per treatment and each treatment was performed in duplicate. The mean of these observations was then calculated to provide a value of multinucleation in transfected and untransfected control cells for each experiment. Multinucleation of transfected cells was expressed as a per cent of multinucleation of matched control cells for the corresponding experiment and analysed by Wilcoxon-signed Rank test.

Detection of apoptosis. Prior to methanol fixation at 66 h, transfected and non-transfected control cells were cultured overnight in ± 100 ng TNFα. Apoptosis was determined by the presence of cleaved cytokeatin 18, detected by M30 CytopATH mouse monoclonal antibody (Roche Diagnostics Ltd, West Sussex, UK) using Ventana Benchmark immunohistochemistry as follows: cells were washed with PBS then incubated for 10 min with 3% (v/v) H2O2 in distilled water to quench endogenous peroxidase. Following a second wash step, cells were incubated at room temperature for 30 min with blocking solution (10% normal goat serum, 2% human serum and 0.1% Tween in TBS). Primary antibody (1:100 dilution in blocking solution) or non-immune mouse IgG, included as a negative control, was then applied for 1 h at 37°C. Bound primary antibody was then removed by washing with TBS. Cells were incubated for 30 min at room temperature with biotinylated goat anti-mouse secondary antibody (Dako UK Ltd, Cambridge, UK, diluted 1:200 in blocking solution), washed again with TBS and then incubated for a further...
Conflict of Interest

The authors declare no conflict of interest.

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