Tissue factor signalling modifies the expression and regulation of G1/S checkpoint regulators: Implications during injury and prolonged inflammation

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Received July 11, 2024; Accepted November 1, 2024

DOI: 10.3892/mmr.2024.13404

Abstract. Tissue factor (TF) possesses additional physiological functions beyond initiating the coagulation cascade. Cellular signals initiated by cellular TF or on contact with TF-containing microvesicles, contribute to wound healing through regulating a number of cellular properties and functions. TF regulates the cell cycle checkpoints, however the underlying signalling mechanisms have not been determined. Endothelial (human dermal blood endothelial cells and human umbilical vein endothelial cells) and epithelial [human telomerase reverse transcriptase-human pancreatic nestin-expressing ductal cells (hTERT-HPNE) and AsPC-1] cells were exposed to different concentrations of recombinant TF, and the influence on G1/S checkpoint regulators examined. Short-term exposure to a lower concentration of TF promoted increased $p16^{INKa}$ and decreased $p21^{CIP1/WAF1}$ expression, together with higher early region 2 binding factor (E2F) transcriptional activity and increased phosphorylation of Thr821/826 within retinoblastoma protein, leading to cell proliferation. The increase in $p16^{INKa}$ expression was prevented following inhibition of β1-integrin, or blocking the exosite within TF with AIIB2 and 10H10 antibodies, respectively. Exposure of cells to higher concentrations of TF induced disproportionate increases in $p16^{INKa}$ and $p21^{CIP1/WAF1}$ expression, reduced retinoblastoma protein phosphorylation and E2F activity. Prolonged treatment of the immortalised hTERT-HPNE cells with recombinant TF, resulted in significant downregulation of $p16^{INKa}$ protein, which was partially due to reduced mRNA expression, together with increased E2F activity, and cyclin E mRNA expression. Although an increase in the methylation of the $p16^{INKa}$ promoter was detected, the reduction in $p16^{INKa}$ protein was concurrent with, and partly attributed to increased $p14^{ARF}$ expression. TF appears early at the site of trauma, and its concentration is an ideal gauge for determining the extent of cellular damage, initiating clearance and repair. It is hypothesised that the balance of this signal is also dependent on the ability of cells to moderate the TF, and therefore on the level of damage. However, prolonged exposure of cells for example due to inflammation, leads to the dysregulation of the G1/S checkpoint by the tumour suppressors, leading to aberrant growth.

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Key words: tissue factor, cell cycle, G1/S checkpoint, $p16^{INKa}$, $p21^{CIP1/WAF1}$, E2F, $p14^{ARF}$, retinoblastoma protein, β1-integrin, protease activated receptor 2

Introduction

Tissue factor (TF) is a 47-kDa transmembrane glycoprotein receptor regarded for its role as the initiator of the extrinsic coagulation pathway (1-3). Additionally, TF has non-haemostatic functions that arise from its ability to activate various intracellular signalling pathways including PKC, MAPK and AKT pathways (4). Cells typically come into contact with TF following injury and inflammation (3,5-7). However, prolonged TF-signalling alters the behaviour of cells impacting the progression of chronic diseases including malignancy (8-12). The cellular signals arising from TF are regulated by proteases and cell-surface receptors that interact with TF, and appear to be dependent on the concentration of TF (4,13-17). These signals also appear to be a determinant of the fate of the cell, through controlling proliferative and pro-apoptotic mediators (18). It was previously shown that exposure of cells to lower concentrations of TF, promoted the passage through the cell cycle, by upregulating proliferative mediators including Cyclin D, and downregulating pro-apoptotic factors (19). By contrast, exposure of cells to high levels of TF, or the inability to release excess TF promoted cellular apoptosis (20). This was initiated through over-activation of steroid receptor coactivator-1 (Src1) by β1-integrin, leading to prolonged p38-MAPK activation, and subsequent increase in p53 nuclear localisation and Bax expression (20,21). Consequently, the precise regulation of cell-surface TF, permits the achievement of the optimal proliferative and pro-survival signals, which may be exploited by cancer cells for maximal growth (22). Highly proliferative tumours appear to express (14,23,24) and moderate (25) cell-surface TF through different mechanisms in order to achieve the optimal cell growth.

The transit of cells through the G1/S checkpoint is mainly regulated by the inhibitors of CDK (INK) and the CDK

interacting protein (CIP)/wildtype p53-activated fragment (WAF) family of tumour suppressors (26-29). These proteins act as gatekeepers by inhibiting the kinase function of Cyclin/Cyclin dependant kinases (Cdk) complexes (29,30). The INK family of proteins ($p16^{INKa}$, $p15^{INKb}$, $p18^{INKc}$ and $p19^{INKd}$) are activated in response to different stimuli, and inhibit Cyclin D in the Cyclin D/Cdk-4 or Cdk-6 complexes (29,30). Inhibition of the Cyclin D/Cdk-4/6 complex suppresses the phosphorylation of retinoblastoma protein, which in turn suppresses the expression of the genes essential for the S-phase of the cell cycle by Early region 2 binding factor (E2F) transcription factor (29,30). This constitutes the final pro-mitogenic regulatory step in the progression of cell division (31). The second stage of the G1/S checkpoint is mitogen-independent and is promoted by the Cyclin E/Cdk-2 complex formation and regulated by $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ (28-29). Notably, at low concentrations, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ proteins act as essential promoters of the progression through the G1 phase by competing with $p16^{INKa}$, to facilitate the association between Cyclin D and Cdk-4 or Cdk-6 (32-34). As such, $p21^{CIP1/WAF1}$ has been classified as a dual-function tumour suppressor and an oncogene, simultaneously (35). Treatment of cells with TF has been shown to impact the expression of the regulatory proteins at both of the stages of the G1/S checkpoint in various cell types (19,20). For example, exposure of human umbilical vein endothelial cells (HUVECs) to TF resulted in the upregulation of a number of proliferative mediators including Cyclin D (19). TF was also reported to suppress the expression of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ in HUVECs (19). Furthermore, preventing the release of TF within microvesicles (MVs) resulting in an accumulation of the protein within human coronary artery endothelial cells, resulted in increased expression of $p21^{CIP1/WAF1}$, while enhancing the release of TF lowered the expression of $p21^{CIP1/WAF1}$ (20). It has also been shown that MVs isolated from patients with coronary artery syndrome promote the upregulation of $p16^{INKa}$ and $p21^{CIP1/WAF1}$ in cultured endothelial cells (36,37).

Although the proliferative influences of TF have been reported, the exact mechanism of signalling pathways has not yet been elucidated. TF initiates cellular signalling by both protease-dependent mechanisms involving the activation of protease-activated receptor (PAR) 2 (38-41), and protease-independent mechanisms involving the interaction of TF with β1-integrin (42-44). Activation of PAR2 has been reported to induce cell proliferation in a wide range of cell types including vascular endothelial cells, pancreatic cancer cells and colon cancer cell line SW620 (45-47). However, induction of proliferation in HUVECs following TF treatment was independent of coagulation factor VIIa (fVIIa) (19) and disrupting TF-β1-integrin interactions with a β1-integrin fragment peptide prevented TF induced proliferation in human endothelial cells (44). Furthermore, TF-containing MVs (TF + MVs) derived from different cell lines appear to have differential outcomes on endothelial cells, which was dependent on the presence of other proteins carried on the MVs including protease activated receptors and integrins (44). Therefore, to further decode the underlying mechanisms, recombinant TF, together with antibodies against TF, β_1-integrin and PAR2 were used in the present study, and the contribution of TF signals to regulation of the cell cycle was examined. The present study hypothesised that signalling from TF differentially regulates the G1/S cell cycle checkpoint, and modulates the fate of cells by inducing apoptosis and/or proliferation. In addition, the outcomes of prolonged exposure of cells to TF as an environmental contributor to disease were explored. It was hypothesised that protracted exposure to TF, such as observed during prolonged inflammation, results in adaptive alterations in these mechanisms which in turn leads to aberrations detectable during chronic conditions.

Materials and methods

Cell culture and treatment. Human dermal blood endothelial cells (HDBECs; https://promocell.com/uk_en/human-dermal-blood-endothelial-cells-hdbec.html) and HUVECs (https://promocell.com/uk_en/human-umbilical-vein-endothelial-cells-huvec.html) were purchased from PromoCell GmbH and cultured at 37˚C in MV medium (PromoCell GmbH) or M199 medium (Lonza Group, Ltd.) containing 10% (v/v) foetal calf serum (FCS) (Gibco; Invitrogen; Thermo Fisher Scientific, Inc.) until 80% confluent (19). These are primary cells which were used at ~9 divisions (cells were guaranteed for at least 15 divisions). The immortalised pancreatic epithelial cells [human telomerase reverse transcriptase (hTERT)-human pancreatic nestin-expressing ductal cells (HPNE)] were purchased from American Type Culture Collection (ATCC). The hTERT-HPNE (https://www.atcc.org/products/crl-4023) were expanded at 37˚C in DMEM (Lonza Group, Ltd.):M3 Base medium (INCELL Corporation LLC; 75:25%) containing 5% (v/v) FCS, 10 ng/ml human epidermal growth factor (hEGF; Sino Biological/Stratech Scientific Ltd.) and 100 U/ml Penicillin-100 μg/ml Streptomycin antibiotics (Lonza Group, Ltd.) until 80% confluent. The pancreatic cancer cell line, AsPC-1 (https://www.atcc.org/products/crl-1682) was purchased from ATCC and cultured at 37˚C in RPMI-1640 medium (Lonza Group, Ltd.), containing 10% (v/v) FCS until 80% confluent. The cell lines were used after 3-4 passages from the time of purchase. Sets of cells ($2x10^5$) were seeded out in 12-well plates and adapted to serum-free medium prior to treatment with combinations of agents as is described below, and is also stated for each figure in the results section, and used in experiments once the cells had reached ~85% confluence. The cells were activated by the addition of recombinant relipidated Innovin TF (stock=0.13 μg/ml=1,000 U/ml; Dade Behring, Inc.), or PAR2-agonsit peptide (PAR2-AP) SLIGKV (20 μM; Sigma-Aldrich; Merck KGaA) and incubated at 37˚C overnight, or for the durations described in the results section. The two concentrations of recombinant relipidated TF utilised throughout the present study were selected to mimic mild physiological inflammation or severe pathological disease. The lower concentration of TF (0.5 U/ml) was comparable to, but higher than that detected in healthy plasma as determined using the Quantikine ELISA kit (Human Coagulation Factor III/Tissue Factor; cat. no. DCF300; R&D Systems Europe, Ltd.) (48) and represents mild inflammation/injury. The higher concentration (2 U/ml) was in line with the amount of TF released by the U87 cell line (48) but below some of

the values reported in the plasma of patients with severe cancer (49). In some experiments the cells were incubated with the CDK4/6 inhibitor ribociclib (10 nM) in the presence or absence of TF (0.5 U/ml) at 37˚C, overnight.

In some experiments, TF was pre-incubated at 37˚C for 60 min with 10H10 antibody (20 μg/ml; cat. no. 9010-5059; Bio-Rad Laboratories, Inc.) to block TF proliferative signalling via the TF exosite (50) or HTF1 antibody (20 μg/ml; cat. no. 16-1429-85; eBioscience; Thermo Fisher Scientific, Inc.) to block the protease activity of the TF-fVIIa complex (51). In other experiments, the cells were pre-incubated at 37˚C for 60 min with AIIB2 antibody (20 μg/ml; cat. no. AIIB2-c; Merck KGaA) to block β1-integrin signalling, or with SAM11 antibody (20 μg/ml; cat. no. sc-13504; Santa Cruz Biotechnology, Inc.) to block PAR2 activation prior to the addition of TF. The cells were incubated at 37˚C for 24 h and samples were separated for mRNA and protein isolation as described below. Finally, sets of HDBECs and HUVECs were incubated with TF (in the presence and absence of the inhibitory antibodies, as aforementioned) or PAR2-AP (20 μM) for 24 h at 37˚C. The cells were then fixed with glutaraldehyde (3% v/v) at room temperature for 10 min, washed 3 times with PBS and cell numbers were assessed using crystal violet (0.02% w/v) staining (Sigma-Aldrich; Merck KGaA) as described previously at room temperature for 30 min (52). The stain was then eluted in 1% (w/v) sodium dodecyl sulphate solution at room temperature for 10 min, and absorptions measured at 590 nm as described previously (52).

For prolonged treatment, epithelial cells were cultured at 37˚C in 25 cm^2 flasks and repetitively supplemented with recombinant TF (0.5 U/ml) every 2-3 days. The cells were passaged every 7 days, at which time the cells had reach ~90% confluence. The cells were counted manually using a haemocytometer and samples collected for mRNA and protein isolation as described below.

RNA isolation and RT-PCR. Total RNA was isolated using the Monarch total RNA extraction kit (New England BioLabs, Inc.) from 1x10^5 cells. Samples of the extracted RNA (100 ng) were amplified using the primer sets shown in Table SI. The relative amount of each mRNA was determined against β-actin using QuantiTect primer set (Qiagen AB; sequence not disclosed by the company). Reverse transcription (RT) and qPCR were carried-out sequentially using the GoTaq 1-Step RT-qPCR System (cat. no. A6020; Promega Corporation). GoTaq 1-Step RT-qPCR System contained GoScript Reverse Transcriptase and RNasin Plus RNase Inhibitor. RT was performed at 48˚C for 30 min. The GoTaq 1-Step RT-qPCR System also contained GoTaq Hot Start Polymerase, BRYT Green fluorescent dye, $MgCl_2$, dNTPs and a proprietary reaction buffer. The qPCR reactions consisted of a denaturing step at 95˚C for 15 sec and an annealing and extending step at 60˚C for 1 min. The reactions were performed using an iCycler thermal cycler (Bio-Rad Laboratories, Inc.) for 40 cycles. Following amplification, the relative amounts of target mRNA were determined using the $2^{-\Delta\Delta Cq}$ method (53). In some experiments and for illustrative purposes, end-point RT-PCR amplifications were carried out using the primers shown in Table SI for the number of cycles shown and the products were analysed by 2% (w/v) agarose gel (Thermo Fisher Scientific, Inc.) electrophoresis.

Western blot analysis. Cells (1x10^5) were lysed in Cell culture lysis reagent (cat. no. E1531; Promega) at 4˚C for 30 min on a rotator. The protein content of samples was assessed using Pierce BCA protein assay (cat. no. 23227; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. Samples were added to Laemmli Buffer (cat. no. S3401-10VL; Sigma-Aldrich; Merck KGaA; solution contains 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl; pH ~6.8). Aliquots (10 μg protein) of the lysates were separated by electrophoresis carried out on a denaturing 14% (w/v) polyacrylamide gel (Flowgen). The separated proteins were then transferred to a nitrocellulose membrane (GE Healthcare) and blocked with Tris-buffered saline Tween 0.01% (v/v) (TBST; Sigma-Aldrich; Merck KGaA; pH 8) at room temperature for 60 min. The membranes were probed overnight at 4˚C with either a goat anti-human p16 antibody (1:2,000 v/v; cat. no. AF5779; R&D Systems Europe, Ltd.), a mouse anti-human p21 antibody (WA-1; cat. no. MCA2325; Bio-Rad Laboratories, Inc.), a rabbit anti-human p14 antibody (cat. no. abx013162; Abbexa, Ltd.) or a rabbit anti-human Cyclin E1 antibody (cat. no. abx012757; Abbexa, Ltd.), each diluted 1:3,000 (v/v) in TBST. Membranes were then washed and developed at room temperature for 60 min with goat anti-mouse IgG (cat. no. sc-2008), goat anti-rabbit IgG (cat. no. sc-2007) or donkey anti-goat IgG (cat. no. sc-2022) alkaline phosphatase-conjugated antibodies (Santa Cruz Biotechnology, Inc.), diluted 1:3,000 (v/v) in TBST, and visualised using the Western Blue stabilised alkaline phosphatase-substrate (Promega Corporation). All measurements were normalised against the respective GAPDH band probed using an HRP-conjugated rabbit anti-human GAPDH (W17079A; cat. no. 607901; BioLegend, Inc.), or a goat anti-human GAPDH antibody (V18; cat. no. sc-20357; Santa Cruz Biotechnology, Inc.) diluted 1:3,000 (v/v) in TBST. Band densities were analysed using the ImageJ 1.53t Software (National Institutes of Health).

Construction of E2F reporter vector and measurement of transcriptional activity. The preferred consensus sequence for binding of E2F transcription factor has previously been demonstrated to include the sequence 5'-TTTCGCGC-3' (54-58). The double-stranded consensus binding DNA for E2F-1 transcription factor (5'-ATTTAAGTTTCGCGCCCTTTCTCA-3') was synthesised with Mlu I and Bgl II restriction sites at the two ends of the underlined preferred sequence, to be unidirectionally cloned into the pGL3-promoter vector (Promega Corporation), and successful clones identified by sequencing (Eurofins Scientific). The pGL3-promoter vector contains a SV40 promoter upstream of the luciferase gene, but does not include an enhancer element required for efficient transcription. The plasmid DNA-construct (1 μg) was transfected into HDBECs (1x10^5) using TransIT-2020 (3 μl; Geneflow, Ltd.) according to the manufacturer's instructions. The cells were incubated at 37˚C for 24 h to allow the plasmid to be expressed. The cells were then treated with recombinant TF (0-2 U/ml) as described below for a further 24 h, and the luciferase activity was measured using Nano-Glo® Luciferase Assay Substrate (Promega Corporation) and a luminometer (Junior LB 9509; Titertek-Berthold). The measurements were carried out alongside appropriate positive and negative controls which were treated with 20% (v/v) FCS or were serum-starved, respectively.

In vitro measurement of retinoblastoma protein phosphorylation at Thr821/826. Sets of endothelial cells ($5x10^4$) were seeded out in 96-well plates and treated with recombinant TF (0-2 U/ml) at 37˚C, overnight. The cells were then fixed at room temperature for 10 min using glutaraldehyde (3% v/v), permeabilised and incubated at room temperature with a goat anti-phospho-Thr821/826 human retinoblastoma protein antibody (1:1,000 v/v; cat. no. sc-16669; Santa Cruz Biotechnology, Inc.) in TBST overnight. The cell samples were then washed and probed with an HRP-conjugated donkey anti-goat IgG (1:3,000 v/v; cat. no. sc-2020; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h, and developed with TMB substrate (Promega Corporation). The relative amounts of the phosphorylated protein were determined by measuring the absorptions at 450 nm (PolarStar Optima plate reader, BMG Labtech GmbH). This procedure was only used qualitatively since both dephosphorylation and hyperphosphorylation of retinoblastoma protein influence its function.

Analysis of DNA methylation by bisulphite conversion. Genomic DNA (gDNA) was extracted from HPNE cells ($3x10^4$ cells) using the Monarch Genomic DNA purification kit (New England BioLabs, Inc.) according to manufacturer's instructions. Bisulphite conversion of the gDNA (750 ng) was carried out using the MethylDetector Bisulfite Modification Kit (Active Motif, Inc.) according to manufacturer's instructions. To assess the methylation state of the extracted gDNA, bisulphite modified DNA was used in nested methylation specific (MS)-PCR experiments with MS-primers to the p16 gene promoter region, provided with the MethylDetector kit. The PCR amplification was carried out using 10 ng bisulphite modified gDNA, with Taq DNA polymerase (1U) in the supplied reaction buffer (Invitrogen; Thermo Fisher Scientific, Inc.), 200 μM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 5% (v/v) DMSO (Sigma-Aldrich; Merck KGaA). Each of the nested amplification steps was carried out for 35 cycles at an annealing temperature of 60˚C. The outer reaction was carried out using primers specific for methylated DNA; 5'-TTA TTAGAGGGTGGGGCGGATCGC-3' (forward) and 5'-CCA CCTAAATCGACCTCCGACCG-3' (reverse), and also using synthesised primers, specific for unmethylated DNA; 5'-TTA TTAGAGGGTGGGGTGGATTGT-3' (forward) and 5'-CCA CCTAAATCAACCTCCAACCA-3' (reverse). Aliquots (4 μl) of the outer PCR reactions were then used as the template for the inner PCR reactions using primers specific for methylated DNA; 5'-TTATTAGAGGGTGGGGCGGATCGC-3' (forward) and 5'-GACCCCGAACCGCGACCGTAA-3' (reverse), and also synthesised primers specific for unmethylated DNA; 5'-TTATTAGAGGGTGGGGTGGATTGT-3' (forward) and 5'-CAACCCCAAACCACAACCATAA-3' (reverse). The products (149 bp) were then examined by 2% (w/v) agarose gel electrophoresis. Samples of the gDNA were also amplified by PCR using primers for β-actin (forward 5'-TGATGGTGG GCATGGGTCAGA-3' and reverse 5'-CTGTGGTGGTGA AGCTGTAG-3') and the products were examined in parallel, as loading control.

Statistical analysis. Presented data include the calculated mean values ± the calculated standard error of the mean from the number of experiments indicated in each figure. Statistical analysis was carried out using the GraphPad Prism version 9.0 (Dotmatics). Significance was determined using one-way ANOVA and Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Exposure of cells to exogenous TF modulates the expression of $p16^{INKa}$. The experiments in this part of the present study were carried out in HDBECs and HUVECs, which have previously been shown to have similar properties and respond comparably to inflammatory stimuli (59). Incubation of endothelial cells with either 0.5 or 2 U/ml recombinant TF resulted in increased expression of $p16^{INKa}$ mRNA in HDBECs (Fig. 1A) and HUVECs (Fig. S1A) when compared with the control cells which were untreated. In order to explore the mechanism of TF-mediated regulation of $p16^{INKa}$, cells were pre-incubated for 60 min with antibodies which block the signalling by β1-integrin (AIIB2) or PAR2 (SAM11). Alternatively, the recombinant TF was pre-incubated for 60 min with 10H10 antibody to block TF direct signalling (50), or HTF1 antibody to block the protease activity of the TF-fVIIa complex (51). Finally, groups of cells were incubated with the PAR2-activating peptide (SLIGKV). The TF-induced increase in $p16^{INKa}$-mRNA expression was suppressed following the inhibition of β1-integrin signalling (using AIIB2), or by blocking of the exosite on TF using 10H10 antibody when compared with cells supplemented with recombinant TF only (Fig. 1A). The blocking of PAR2 activation using SAM11 antibody, or pre-incubation of TF with HTF1, did not influence the upregulation of $p16^{INKa}$ mRNA expression by TF. Moreover, activation of PAR2 using the agonist peptide alone did not alter the $p16^{INKa}$ expression. Analysis of $p16^{INKa}$ protein by western blotting confirmed the observed alterations in mRNA expression (Fig. 1B and C). Collectively these data indicate that the expression of $p16^{INKa}$ was dependent on the interaction of TF with β1-integrin and was unaltered on prevention of the activation of PAR2. In agreement with the data obtained using the endothelial cells, supplementation of hTERT-HPNE and AsPC-1 cells with recombinant TF (0.5 and 2 U/ml) resulted in 50 and 60% increases in $p16^{INKa}$ mRNA expression respectively in both cell types (data not shown). These cells were not examined in the presence of any of the inhibitory antibodies.

Exposure of cells to exogenous TF modulates the expression of $p21^{CIP1/WAF1}$. Incubation of endothelial cells with the lower concentration of recombinant TF (0.5 U/ml) reduced the expression of $p21^{CIP1/WAF1}$ mRNA, whilst treatment with the higher concentration of TF (2 U/ml) significantly increased $p21^{CIP1/WAF1}$ mRNA expression when compared with untreated control cells (Figs. 2A and S1B and C). Inhibition of β1-integrin signalling (AIIB2) on cells prior to the addition of recombinant TF (0.5 U/ml) marginally reduced the expression of $p21^{CIP1/WAF1}$ when compared with cells supplemented with TF only (Fig. 2A). Notably, inhibiting PAR2 activation using SAM11 antibody on cells, and to a lesser extent blocking TF proteolytic activity using the HTF1 antibody, reversed the reduction in $p21^{CIP1/WAF1}$ expression. These increases in $p21^{CIP1/WAF1}$ expression were comparable with those observed

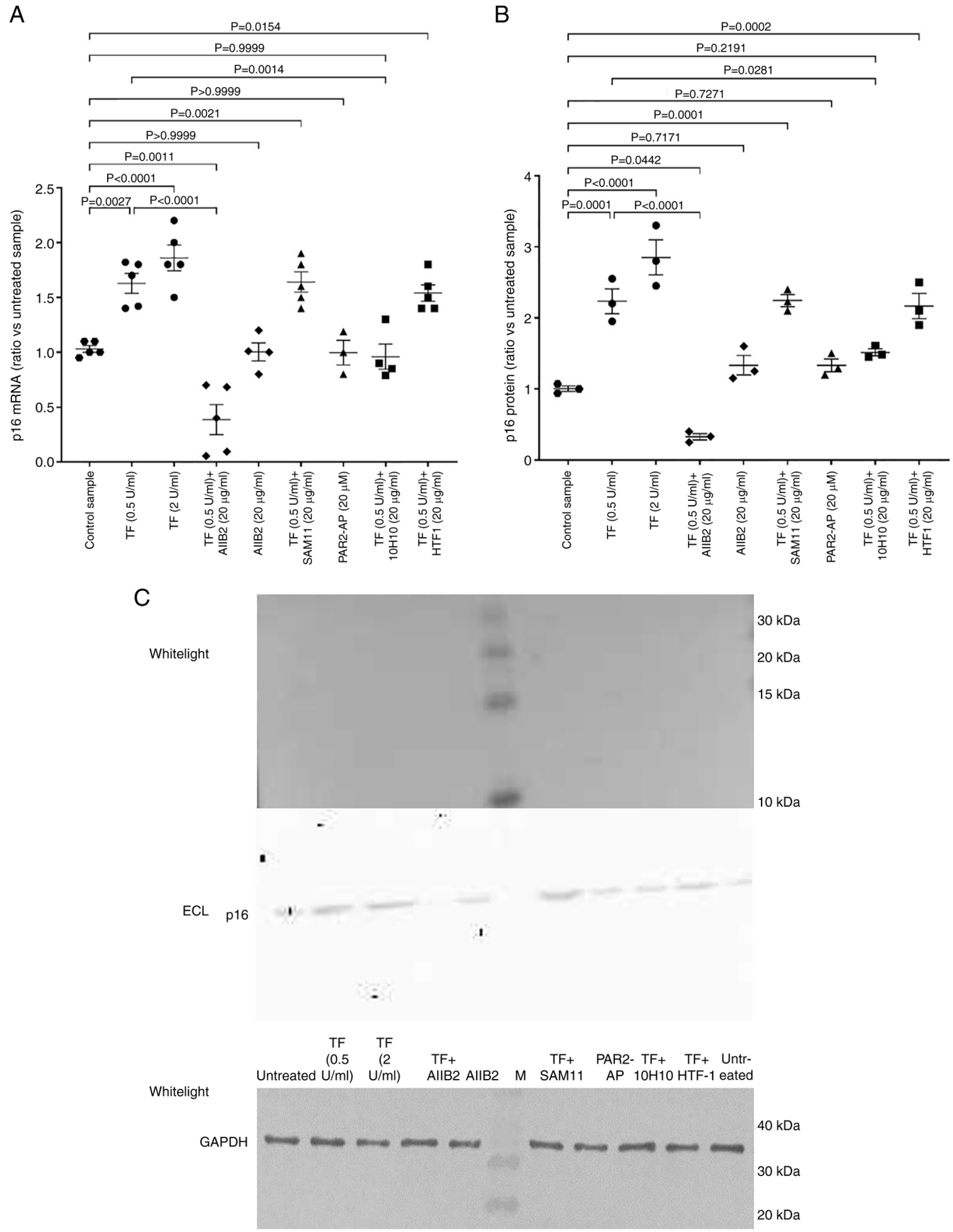

Figure 1. HDBECs (2x10^5) were incubated for 24 h with recombinant TF (0, 0.5 and 2 U/ml) or PAR2-AP (SLIGKV; 20 μM), or with recombinant TF (0.5 U/ml) that was pre-incubated for 60 min with 10H10 or HTF1 antibodies (20 μg/ml). Cells were also pre-incubated for 60 min with AIIB2 or SAM11 antibodies (20 μg/ml), prior to addition of TF. The cells were harvested after 24 h, into separate aliquots (1x10^5 cells). Total RNA was isolated from one group and the mRNA quantified by RT-qPCR, against β-actin. Other aliquots were analysed by western blotting. The data show the amounts of (A) Inhibitor of CDK p16^{INKa} mRNA (n=5), and (B) the relative amounts of p16^{INKa} protein (n=3) calculated from (C) the western blots of p16^{INKa} protein (using a goat anti-human antibody) and against GAPDH. TF, tissue factor; PAR2, protease-activated receptor 2.

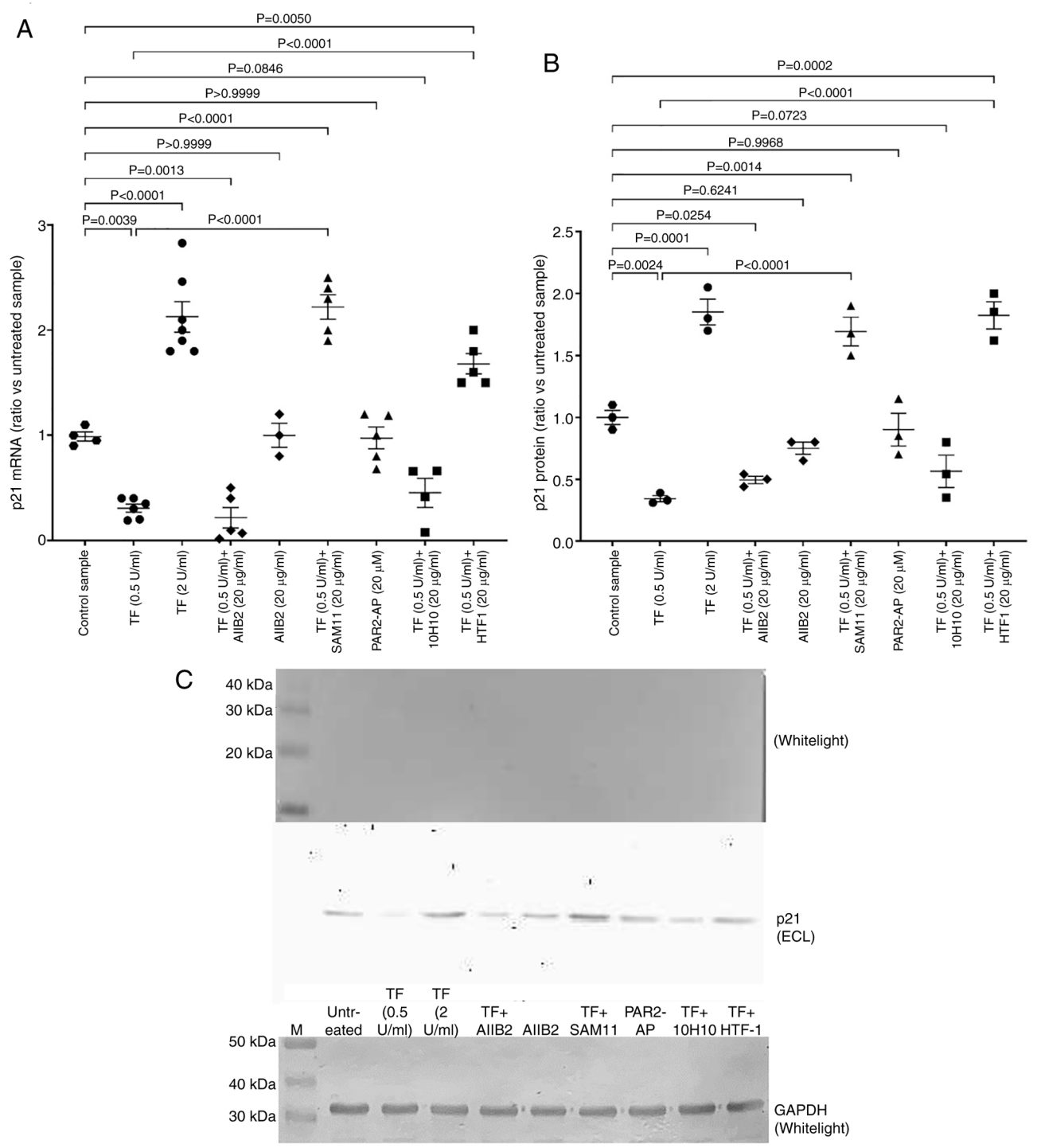

Figure 2. HDBECs ($2x10^5$) were incubated for 24 h with recombinant TF (0, 0.5 and 2 U/ml) or PAR2-AP (SLIGKV; 20 μM), or with recombinant TF (0.5 U/ml) that was pre-incubated for 60 min with 10H10 or HTF1 antibodies (20 μg/ml). Groups of cells were also pre-incubated for 60 min with AIIB2 or SAM11 antibodies (20 μg/ml), prior to the addition of TF. The cells were harvested after 24 h, into separate aliquots ($1x10^5$ cells). Total RNA was isolated from one group and the mRNA quantified by RT-qPCR, against β-actin. Other aliquots were analysed by western blotting. The data show the amounts of (A) CDK interacting protein/Wildtype p53-activated fragment $p21^{CIP1/WAF1}$ mRNA (n=5), and (B) the relative amounts of $p21^{CIP1/WAF1}$ protein (n=3) calculated from (C) the western blots of $p21^{CIP1/WAF1}$ protein, (using a mouse anti-human antibody) and against GAPDH. TF, tissue factor; PAR2, protease-activated receptor 2.

with the higher TF concentration (2 U/ml). By contrast, induction of $p21^{CIP1/WAF1}$ expression was not significantly influenced by the blocking of the TF exosite, and also appeared to be unaffected by the direct activation of PAR2 alone. Analysis of $p21^{CIP1/WAF1}$ protein by western blotting further confirmed the observed alterations in mRNA expression (Fig. 2B and C). These data indicate the possible involvement of β1-integrin signalling in TF-induced regulation of $p21^{CIP1/WAF1}$, as well as highlighting the exaggerated enhancement of $p21^{CIP1/WAF1}$ expression on prevention of PAR2 activation.

Exposure of cells to exogenous TF modulates the expression of cyclin D and E2F activity. Incubation of endothelial cells with the lower concentration of recombinant TF (0.5 U/ml) resulted

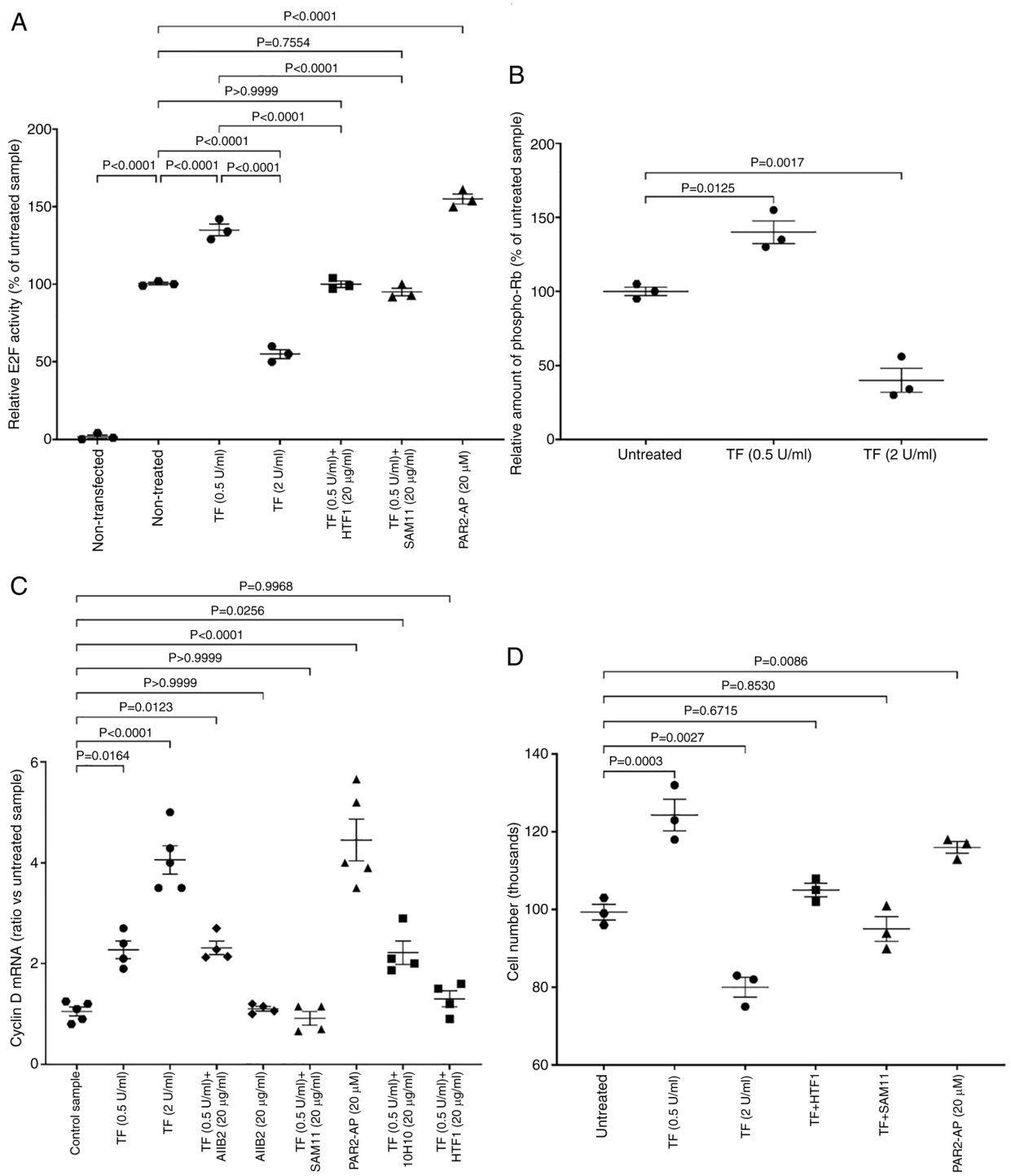

Figure 3. Examination of the influence of TF on cell cycle indicators. (A) Groups of HDBECs ($1x10^5$) were transfected with the pGL3-promoter vector containing the E2F-1 enhancer sequence. The cells were then treated for 24 h with recombinant TF (0, 0.5 and 2 U/ml), combinations of TF (0.5 U/ml) with the shown antibodies, or with PAR2-AP (SLIGKV; 20 μM) and the luciferase activity was measured within 24 h (n=3). (B) Groups of cells ($5x10^4$) were seeded in 96-well plates and treated with recombinant TF (0, 0.5 and 2 U/ml) for 24 h. The cells were then fixed and probed with an anti-phospho-Thr821/826 human retinoblastoma protein antibody (1:1,000 v/v) for 1 h. The samples were then incubated with an HRP-conjugated donkey anti-goat IgG diluted 1:3,000 v/v) for 1 h, developed with a TMB substrate and the absorptions determined at 450 nm (n=3). (C) Groups of cells ($1x10^5$) were incubated for 24 h with TF (0, 0.5 and 2 U/ml) or PAR2-AP (20 μM), or with recombinant TF (0.5 U/ml) that was pre-incubated for 60 min with 10H10 or HTF1 antibodies (20 μg/ml). Groups of cells were also pre-incubated for 60 min with AIIB2 or SAM11 antibodies (20 μg/ml), prior to addition of recombinant TF. The cells were harvested after 24 h, total RNA was isolated and the amount of cyclin D1 mRNA determined against that of β-actin (n=4). (D) Groups of cells ($1x10^5$) were treated as aforementioned and cell numbers were determined using crystal violet staining (n=3). TF, tissue factor; PAR2, protease-activated receptor 2; E2F-1; Early region 2 binding factor.

in increased transcriptional activity of E2F, as measured using the luciferase reporter when compared with control cells which were untreated (Fig. 3A). By contrast, incubation of the cells with the higher concentration of TF (2 U/ml) reduced E2F activity. These values were in line with the relative amounts of phosphorylated retinoblastoma protein (Thr821/826)

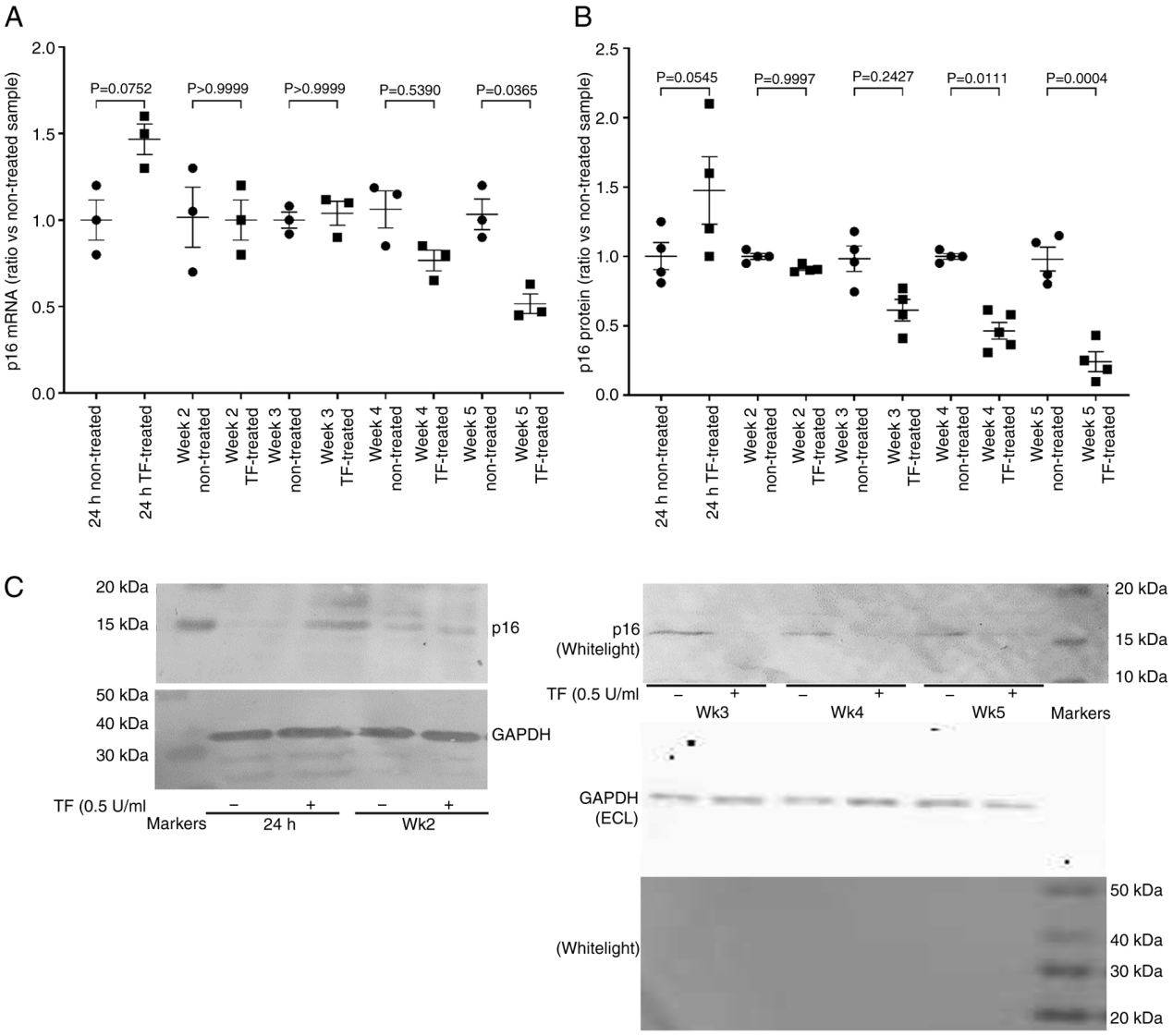

Figure 4. Immortalised human epithelial cells (human telomerase reverse transcriptase-human pancreatic nestin-expressing ductal cells) were cultured in 25 cm^2 flasks and supplemented with recombinant TF (0.5 U/ml) or were untreated. The cells were harvested on the indicated weeks, total RNA was isolated from one group and the mRNA quantified by RT-qPCR, against β-actin. Other aliquots were analysed by western blotting. The data show the amounts of (A) Inhibitor of CDK $p16^{INKa}$ mRNA (n=3), and (B) the relative amounts of $p16^{INKa}$ protein (n=4) calculated from (C) the western blots of $p16^{INKa}$ protein (using a goat anti-human antibody) and against GAPDH. TF, tissue factor; Wk, week.

(Fig. 3B). These measurements were used semi-quantitatively as an indicator of the state of the protein, since both dephosphorylation and hyperphosphorylation of retinoblastoma protein reduce its function. Treatment of cells with SAM11 to block PAR2, or pre-incubation of recombinant TF with HTF1 to inhibit the protease function prevented the change in E2F activity. Moreover, activation of PAR2 on the cells induced E2F activity. Incubation of cells with the CDK4/6 inhibitor ribociclib (10 nM) suppressed E2F activity regardless of the presence of TF (Fig. S2). Incubation of cells with recombinant TF promotes the upregulation of cyclin D1 mRNA (19), and was used as an indicator of the entry into the G1-phase of the cell cycle. In the present study, incubation of endothelial cells with recombinant TF resulted in dose-dependent increases in cyclin D1 mRNA expression (Figs. 3C and S1B and D). Entry into the G1-phase was also promoted by the direct activation of PAR2, and was reduced by either blocking of PAR2 activation with SAM11 antibody prior to addition of TF, or by inhibition of protease function of the TF-fVIIa complex using the HTF1 antibody (Fig. 3C). Neither blocking of β1-integrin signalling, nor blocking of the exosite on TF using 10H10 antibody had any significant influence on cyclin D1 mRNA expression. To explore the potential outcome on cell proliferation, the treated cells were incubated for 24 h and cell numbers were determined. In agreement with the aforementioned findings, incubation of cells with the lower concentrations of recombinant TF (0.5 /ml) or the direct activation of PAR2 promoted increases in HDBEC numbers (Figs. 3D and S3), and HUVEC numbers (Fig. S1E) as measured by crystal violet assay. By contrast, treatment of cells with the higher concentration of recombinant TF resulted in the reduction in cell numbers (Figs. 3D and S3 and S1E).

Prolonged exposure of cells to exogenous TF alters the regulations of G1/S checkpoint proteins. In order to assess the influence of prolonged exposure to TF, hTERT-HPNE cells

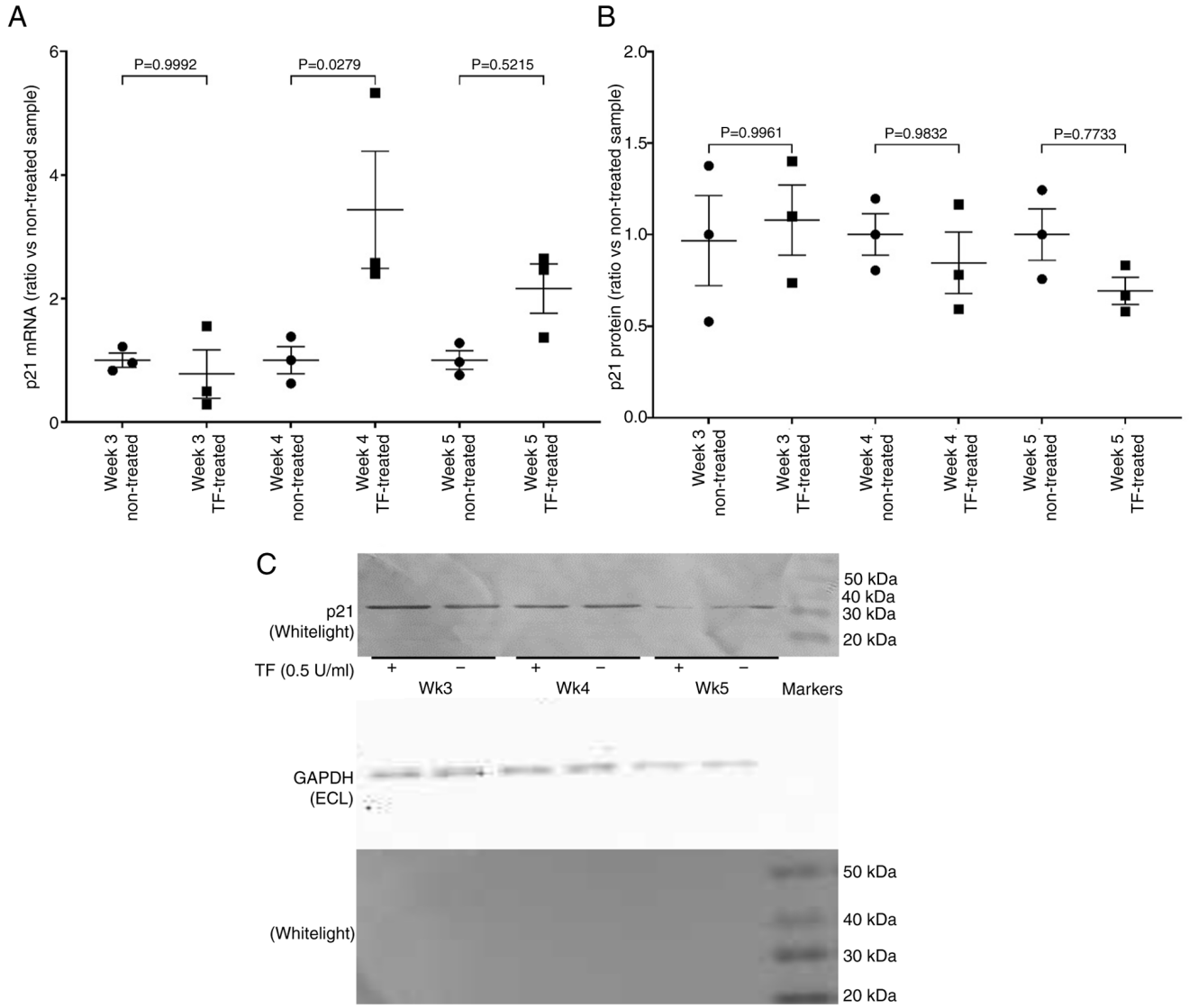

Figure 5. Immortalised human epithelial cells (human telomerase reverse transcriptase-human pancreatic nestin-expressing ductal cells) were cultured in 25 cm^2 flasks and supplemented with recombinant TF (0.5 U/ml) or were untreated. The cells were harvested on the indicated wks, total RNA was isolated from one group and the mRNA quantified by RT-qPCR, against β-actin. Other aliquots were analysed by western blotting. The data show the amounts of (A) CDK interacting protein/Wildtype p53-activated fragment $p21^{CIP1/WAF1}$ mRNA (n=3), and (B) the relative amounts of $p21^{CIP1/WAF1}$ protein (n=3) calculated from (C) the western blots of $p21^{CIP1/WAF1}$ protein (using a mouse anti-human antibody) and against GAPDH. TF, tissue factor; Wk, week.

were supplemented with recombinant TF (0.5 U/ml) every 2-3 days for up to 5 weeks, and the outcome on $p16^{INKa}$ mRNA and protein levels examined. Similar studies in primary endothelial cells were not feasible due to the durations involved. As aforementioned, supplementation of hTERT-HPNE with recombinant TF resulted in a 50% increase in $p16^{INKa}$ mRNA expression within 24 h when compared with untreated control cells (Fig. 4A). These values were normalised by week 2, but further decline only became significant on week 5 when compared with untreated control cells (Fig. 4A). Short-term treatment (24 h) of AsPC-1 cells with 0.5 U/ml of TF also resulted in a 50% increase in $p16^{INKa}$ mRNA, but was not analysed further. In HPNE cells the level of cellular $p16^{INKa}$ protein began to decline by week 3 of TF treatment when compared with control cells grown for the same number of weeks in untreated media (Fig. 4B and C). Examination of the levels of $p21^{CIP1/WAF1}$ mRNA over the same period, indicated increased levels of mRNA expression on weeks 4 and 5 (Fig. 5A), but was not reflected in increased cellular $p21^{CIP1/WAF1}$ antigen (Fig. 5B and C).

In order to assess the ability of the cells to progress past the G1/S checkpoint, the expression of cyclin E mRNA and the transcriptional activity of E2F were examined. Prolonged treatment of cells with recombinant TF (0.5 U/ml) resulted in the upregulation of cyclin E mRNA (Fig. 6A) and protein (Fig. 6B and C). Additionally, measurement of E2F activity using the luciferase reporter, indicated 3-fold increased activity on weeks 4, but the increases were not significant on week 5 (data not shown). Collectively, these data indicate the permissive progress of cells through the G1/S checkpoint.

Prolonged exposure to TF leads to $p16^{INKa}$ promoter methylation and $p14^{ARF}$ upregulation. To investigate the mechanism of the reduction in $p16^{INKa}$, gDNA was extracted from TF-treated and untreated cells, converted with bisulphite, and the status of the $p16^{INKa}$ promoter was analysed by MS-PCR. Analysis

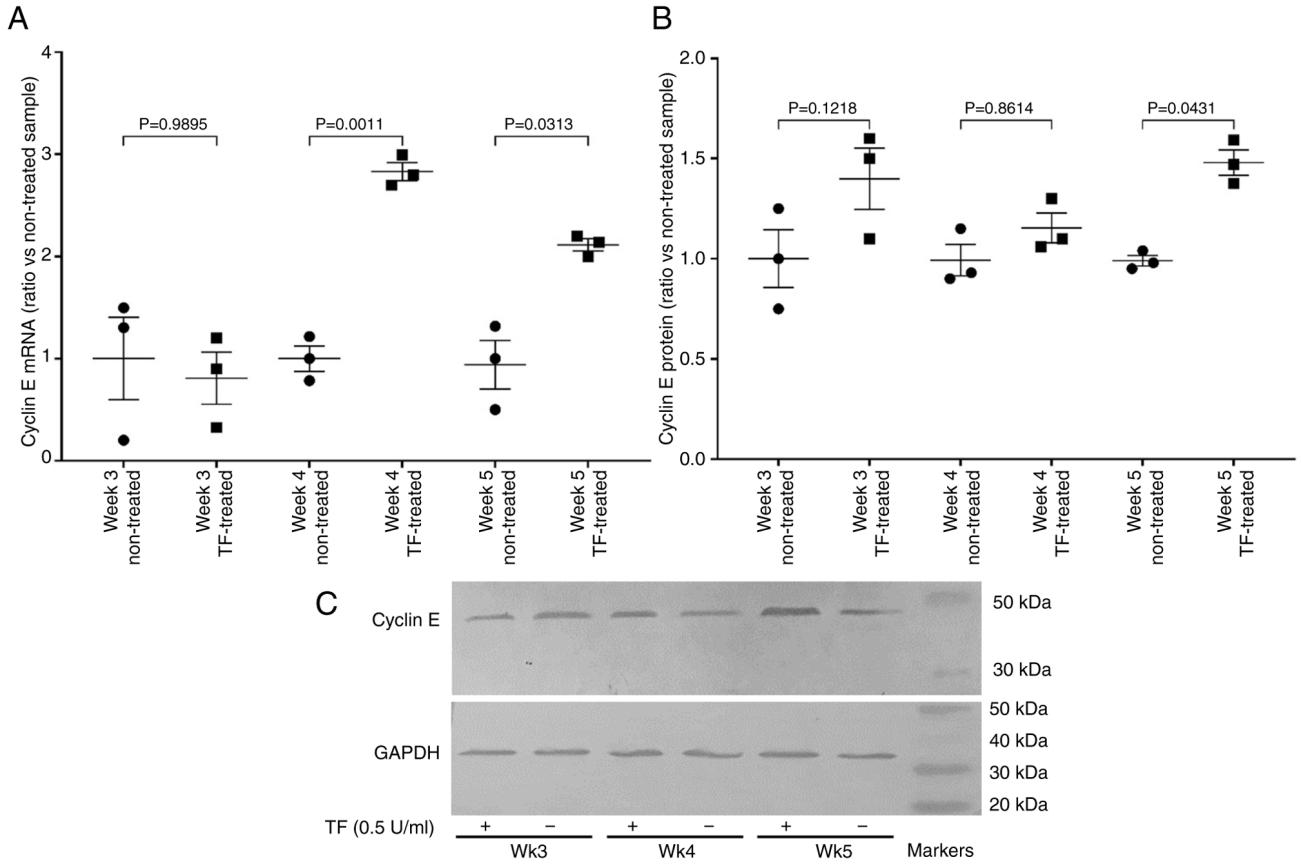

Figure 6. Immortalised human epithelial cells (human telomerase reverse transcriptase-human pancreatic nestin-expressing ductal cells) were cultured in 25 cm^2 flasks and supplemented with recombinant TF (0.5 U/ml) or were untreated. The cells were harvested on the indicated weeks, total RNA was isolated from one set and the mRNA quantified by RT-qPCR, against β-actin. Other aliquots were analysed by western blotting. The data show the amounts of (A) cyclin E mRNA (n=3), and (B) the relative amounts of Cyclin E protein (n=3) calculated from (C) the western blots of Cyclin E protein (using a rabbit anti-human antibody) and against GAPDH. TF, tissue factor.

of the gDNA indicated the presence of both methylated and non-methylated promoter sequences in the hTERT-HPNE cells. Moreover, the ratio of methylated:unmethylated DNA increased significantly by week 4 of supplementation with recombinant TF when compared with control cells grown for 4 weeks in untreated media (Fig. 7A and B). It should be noted that the untreated control hTERT-HPNE cells showed partial methylation of the $p16^{INKa}$ promoter, which was expected since the immortalisation of some cells by constitutive hTERT expression has an impact on the regulation of $p16^{INKa}$ expression, but not the function of this protein (60,61).

Since $p16^{INKa}$ protein levels may be reduced by the expression of $p14^{ARF}$, the expression of $p14^{ARF}$ was also analysed. Examination of the hTERT-HPNE cells showed increases in the levels of $p14^{ARF}$ mRNA and protein expression in TF-treated cells compared with untreated cells (Fig. 8A-C).

Discussion

TF is considered to have additional functions beyond the initiation of coagulation within the body, and contributes to the healing process by regulating cellular responses including apoptosis of severely damage cells and proliferation of viable cells (4,8-20). The precise regulation of these processes and the distinction between viable and damaged cells is imperative in order to prevent the survival of aberrant cells, without unnecessary cell loss. The rapid exposure of TF at the site of injury makes it an ideal first-on the scene regulator, to initiate these clearance and repair processes, as well as the immediate initiation of blood coagulation (5-7). Additionally, as a protein present in the sub-endothelial vasculature, it is hypothesized that the level of TF exposure is likely to be determined by the amount of injury. One of the mechanisms by which TF impacts clearance and repair processes is likely to be by regulating cell cycle progression within cells at the site of injury (19). In support of this, the ability of TF to promote the entry of cells into cell cycle was previously shown (19). However, progress through to the end of the G1/S checkpoint was regulated by the amount of TF, with either high levels of TF (19,22) or inability of cells to dissipate any excess TF resulting in cellular apoptosis (20,21).

The regulation of the transition through the G1/S checkpoint is controlled at two stages. The earlier mitogen-responsive stage is promoted by Cyclin D/Cdk 4/6 complex formation and may be inhibited by $p16^{INKa}$ (30). $p16^{INKa}$ inhibits the Cyclin D/Cdk 4/6 mediated phosphorylation of the retinoblastoma protein, permitting E2F activity and the progression through the G1 phase (29,29). In the present study it was shown that in HDBECs TF induces an upregulation in $p16^{INKa}$ expression which appears to be mediated by TF-β1-integrin signalling in a saturable manner, and independent of PAR2 activation. The

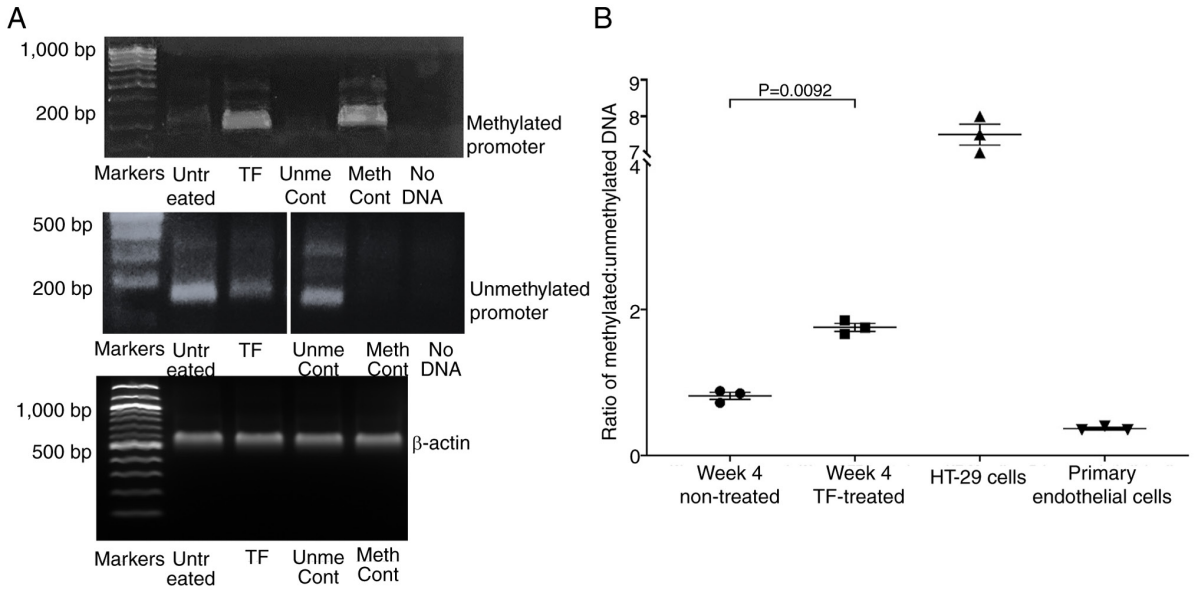

Figure 7. Immortalised human epithelial cells (human telomerase reverse transcriptase-human pancreatic nestin-expressing ductal cells) were supplemented with recombinant TF (0.5 U/ml) or were untreated. The cells were harvested on week 4, gDNA was extracted from the cells ($3x10^4$ cells) and bisulphite conversion of the gDNA (750 ng) was carried out using the MethylDetector Bisulfite Modification Kit. DNA was also extracted from HT-29 cells (methylated control) and HDBEC (unmethylated control) and processed as aforementioned. The modified DNA (10 ng/reaction) was amplified with methylation-specific, and unmethylated-specific sets primers to the p16 gene promoter region. Each of the nested amplification steps was carried out for 35 cycles at an annealing temperature of 60˚C. Aliquots (4 μl) from the outer reactions were then used as the template for the inner PCR reactions using primers specific for methylated and unmethylated DNA. Both amplicons generated bands of 149 bp. A control β-actin sample was also amplified and examined alongside. (A) The products were examined by 2% (w/v) agarose gel electrophoresis and (B) the band intensities determined and the ratios of the methylated:unmethylated DNA calculated (n=3). TF, tissue factor; gDNA, genomic DNA.

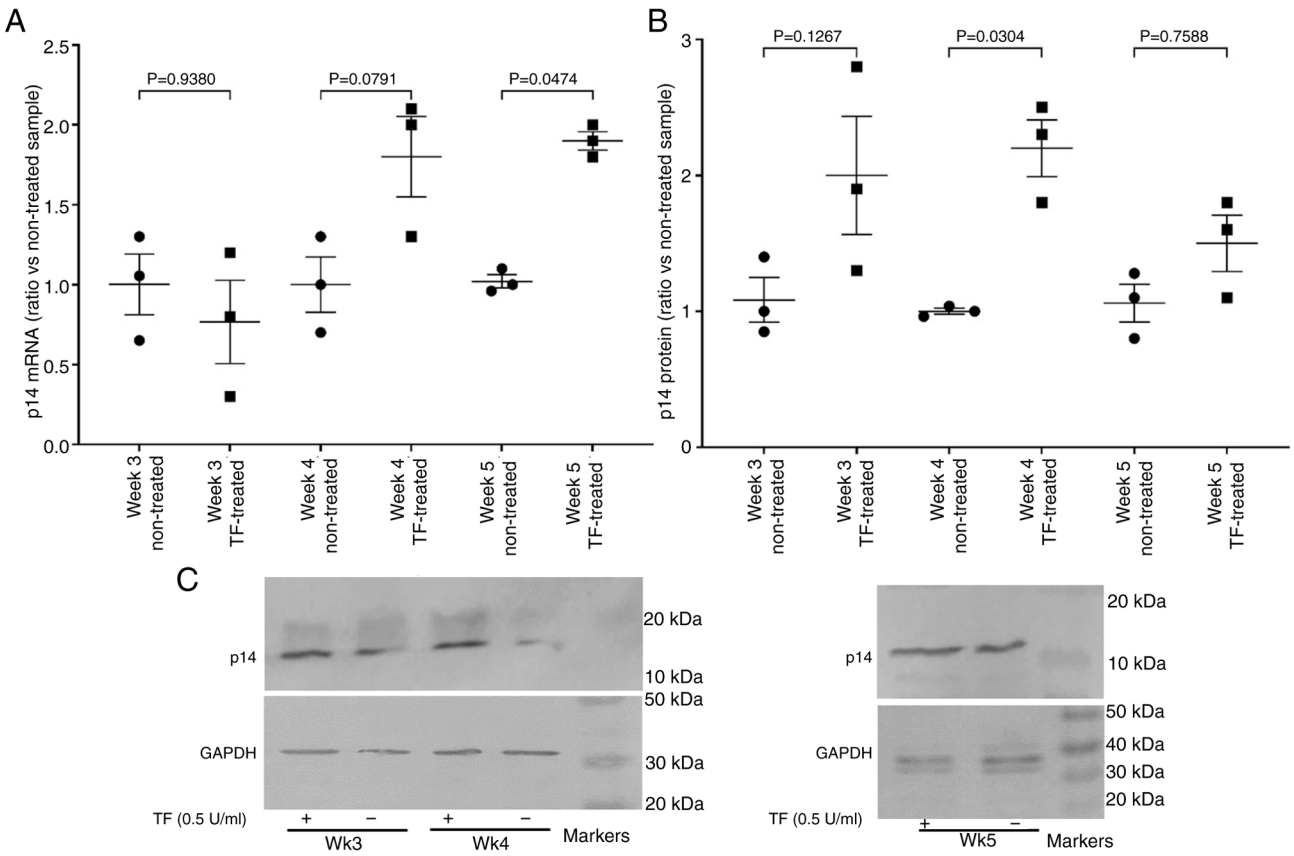

Figure 8. Immortalised human epithelial cells (human telomerase reverse transcriptase-human pancreatic nestin-expressing ductal cells) were cultured in 25 cm^2 flasks and supplemented with recombinant TF (0.5 U/ml) or were untreated. The cells were harvested on the indicated wks, total RNA was isolated from one set and the mRNA quantified by RT-qPCR, against β-actin. Other aliquots were analysed by western blotting. The data show the amounts of (A) Alternative reading frame $p14^{ARF}$ mRNA (n=3), and (B) the relative amounts of $p14^{ARF}$ protein (n=3) calculated from (C) the western blots of $p14^{ARF}$ protein (using a rabbit anti-human antibody) and against GAPDH. TF, tissue factor; Wk, week.

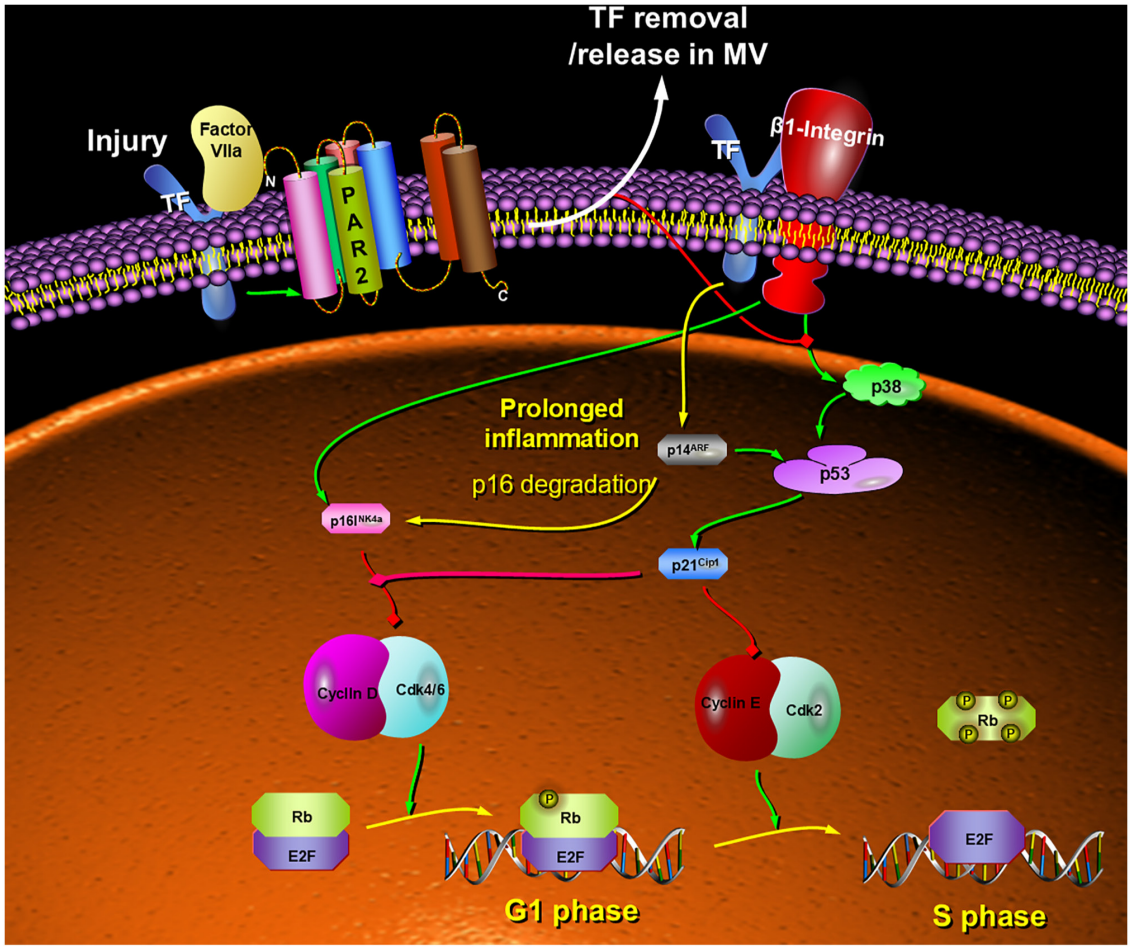

Figure 9. Proposed model for the mechanism by which the level of TF on the cell surface may have differential outcomes on G1/S checkpoint regulation. The presence of TF on the cell surface differentially upregulates the expression of Inhibitor of CDK p16^{INKa}, CDK interacting protein/Wildtype p53-activated fragment p21$^{CIP1/WAF1}$ and Alternative reading frame p14^{ARF}, which is dependent on the concentration of TF and the ability of the cell to dissipate excess TF. Therefore, alterations in p21$^{CIP1/WAF1}$ are highly effective in the regulation of the cellular response to acute stress. The interplay between these proteins modulates the signal permitting passage through the cell cycle, or alternatively its arrest. Consequently, the concentration of TF may be an ideal gauge for determining the level of cellular damage. However, the adaptive loss of p16^{INK4a} function may be promoted by prolonged inflammation leading to permissive transition through the G1/S checkpoint, even in the absence of mutational loss of p16^{INKa}. TF, tissue factor; PAR2, protease-activated receptor 2, Rb, retinoblastoma protein; E2F, early region 2 binding factor; p21$^{CIP1/WAF1}$, CDK interacting protein/wildtype p53-activated fragment; p16^{INK4a}, inhibitor of CDK; p14^{ARF}, alternative reading frame.

second step in the G1/S checkpoint is mitogen-independent and is promoted by the Cyclin E/Cdk 2 complex formation and inhibited by p21$^{CIP1/WAF1}$ and p27^{KIP1} (31). TF has a concentration dependant influence on p21$^{CIP1/WAF1}$ expression in HDBECs, with low concentrations causing a decrease in expression and high concentrations causing an increase. These data agree with previous observations which suggest opposite outcomes on p21$^{CIP1/WAF1}$ expression, depending on the retention of TF by the cell (18,20). However, in the present study inhibition of the protease activity of TF-fVIIa partially augmented the p21$^{CIP1/WAF1}$ expression. Since PAR2 is a robust promoter of the incorporation of TF and its release within cell-derived MVs (62), it was hypothesised that inhibition of TF-fVIIa prevents the PAR2-induced release of TF + MVs, resulting in the accumulation of TF on the surface of the cells, which amplifies the subsequent TF signalling (Fig. 9). At lower TF concentrations, p21$^{CIP1/WAF1}$ facilitates progression through the checkpoint by competing with p16^{INKa} (32-34) and explains the rapid alterations in E2F activity, which is in turn responsible for the expression of genes required for the S-phase. Finally, it should be noted that the processes involved in clearance and healing include a substantial number of proteins and mechanisms. A large amount of further study is required to determine how the signalling arising from TF interacts and synergises with mechanisms induced by other mediators of healing and repair. Furthermore, how the culmination of all of these processes over the long-term are likely to give rise to chronic diseases requires extensive and diverse investigations.

It has been proposed that the disruption of the CDKN2A gene (encoding p16^{INKa}) is one of the major events in cancer development and progression to malignant phenotype (63,64), as well as the onset of chemoresistance (65). In the present study, prolonged incubation of hTERT-HPNE cells with recombinant TF increased methylation of the p16^{INKa} promoter region, together with a late-onset reduction in mRNA expression which were comparable to previous observations reporting the methylation of the proximal region of the oestrogen receptor on prolonged exposure of cells to TF (66). However, the more substantial reduction in p16^{INKa} protein following long-term TF treatment was

also attributed to the expression of $p14^{ARF}$. The CDKN2A locus encodes for two separate proteins, $p16^{INKa}$ and $p14^{ARF}$, which are expressed via separate first exons and by usage of alternative reading frames (67). $p14^{ARF}$ inhibits murine double minute 2 (mdm2)-mediated removal of the tumour suppressor protein p53. However, $p14^{ARF}$ may also induce the degradation of $p16^{INKa}$ leading to the reduction of the latter protein, without altering its mRNA expression (68). Overall, the data from the present study suggest that prolonged exposure of cells to TF may be an environmental factor that can confer an advantage to early cancerous cells, even in the absence of mutational loss of $p16^{INKa}$ expression by allowing accelerated growth and transformation to the malignant phenotype.

In conclusion, the present study indicates that upon injury or trauma, TF concentration acts as a cellular gauge for the proximity/magnitude of injury sustained by cells. The interaction between TF and β1-integrin differentially upregulates the expression of $p16^{INKa}$, $p21^{CIP1/WAF1}$ and $p14^{ARF}$. Subsequently, the interplay between these proteins translates the signal which depends on the concentration of TF present on the cell surface. The alterations in $p21^{CIP1/WAF1}$ are highly effective in the regulation of the response to acute stress and therefore, ideal for gauging the level of cellular damage. However, the data also allude to an adaptive mechanism by which the loss of $p16^{INKa}$ function may be promoted by inflammatory factors, even in the absence of loss of $p16^{INKa}$ expression.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SJF and CE designed the study, carried out the experimental work. SJF, ECF and CE evaluated the data, confirmed the authenticity of all the raw data and prepared the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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