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T-cadherin upregulation correlates with cell-cycle progression and promotes proliferation of vascular cells

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Abstract

Objective: In vascular tissue, T-cadherin (T-cad) levels correlate with the progression of atherosclerosis, restenosis and tumour neovascularization. This study investigates whether T-cad influences proliferation of vascular cells. **Methods and Results:** Cultures of human umbilical vein endothelial cells (HUVEC) and rat and human aortic smooth muscle cells (rSMC, hSMC) were used. T-cad was overexpressed in HUVEC and hSMC using an adenoviral expression system. In cultures released from G_1/G_0 synchrony parallel immunoblot analysis of T-cad and cell cycle phase specific markers ($p27^{Kip1}$, cyclin D1, E2F1, PCNA, cyclin B) showed increased T-cad protein levels subsequent to entry into early S-phase with sustained elevation through S-and M-phases. T-cad was increased in G_2/M-phase (colchicine) synchronized cultures. In FACS-sorted cell populations, expression of T-cad in S-and G_2/M-phase was higher than G_1/G_0-phase. Compared with empty-and LacZ-vector infected controls, HUVEC and hSMC overexpressing T-cad exhibited increased proliferation as assessed in enumeration and DNA synthesis assays. Additionally, following release from G_1/G_0 synchrony, HUVEC and hSMC overexpressing T-cad enter S-phase more rapidly. Flow cytometry after BrdU/propidium labelling confirmed increased cell cycle progression in T-cad overexpressing cells. **Conclusion:** In vascular cells, T-cad is dynamically regulated during the cell cycle and its expression functions in the promotion of proliferation. T-cad may facilitate progression of proliferative vascular disorders such as atherosclerosis, restenosis and tumour angiogenesis.

Keywords: T-cadherin; Vascular cells; Cell cycle; Proliferation; Adenoviral expression

1. Introduction

Proliferation of vascular cells plays an important role in progression of many vascular diseases. Abnormal vascular smooth muscle cell (SMC) growth and migration stimulated by endothelial cell (EC) and inflammatory cell-derived growth factors and cytokines are critical steps in formation of the atherosclerotic plaque, in neointimal hyperplasia during bypass vein graft failure or during restenosis after percutaneous transluminal angioplasty [1]. EC proliferation is necessary for restoration of blood supply to ischaemic tissues through angiogenesis [2], and through re-endothelialization, is beneficial to atherosclerotic plaque stability [3]. However, excessive EC proliferation importantly contributes to pathological tumour angiogenesis [2] and the neovascularization of atherosclerotic lesions which is a cause of plaque instability, rupture and thrombosis [3].

Cell growth, motility, differentiation and survival are greatly dependent on both cell–cell and cell–extracellular matrix adhesive interactions. Cadherins, a major constituent of cell–cell adherent junctions, are transmembrane receptors that mediate calcium-dependent intercellular adhesion [4]. During embryogenesis, cadherins play an important part in cell sorting, control of cell polarity and morphogenesis [4], while in the adult, loss of cadherin-mediated adhesion can cause cancer development and progression [5]. The participation of the cadherins in formation of blood vessels and maintenance of their structural integrity has been acknowledged only more recently. VE-cadherin, an EC-specific cadherin and the major component of endothelial adherent junctions, is involved in vasculogenesis, angiogenesis, EC

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survival and control of vascular permeability [6]. N-cadherin participates in the formation of abluminal adherent junctions between ECs and pericytes during the early phase of brain angiogenesis [7]. T-cadherin (T-cad, or H-cadherin, cadherin-13) is an atypical cadherin widely expressed in the cardiovascular system [8]. It lacks transmembrane and cytosolic domains and is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Although T-cad can mediate weak homophilic cell aggregation in suspensions of transfected cells [9], accumulating evidence suggests that T-cad might not function as a true intercellular adhesion molecule. In contrast to classical cadherins, T-cad is not concentrated at sites of cell–cell contacts, is expressed on the luminal but not the baso-lateral surface of polarized transfected cells [9,10], and undergoes re-distribution to the leading edge in migrating vascular cells [10]. T-cad has also been shown to function as a repulsive cue for motor-axon projection in the embryonic nervous system [11] and to induce cell de-adhesion and promote directed cell migration in vascular cells [12].

Similarly to classical cadherins, T-cad has a lower expression profile in sporadic breast, prostate, colorectal, and lung cancers, and tumour cells transfected with T-cad cDNA exhibit decreased proliferative and invasive potential [13]. However, in vascular tissue the progression of atherosclerosis, restenosis and tumour neovascularization is attended with marked increases in T-cad levels on the surface of EC and SMC [8,14,15], suggesting rather a positive correlation between T-cad expression on vascular cells and their motility and growth. This study examines the relationship between vascular cell expression of T-cad and proliferation.

2. Methods

2.1. Cell culture

The use of animal and human material in this study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication no. 85-23, revised 1996), and the principles outlined in the Declaration of Helsinki. Human umbilical vein EC (HUVEC) were purchased from PromoCell (Heidelberg, Germany) and normally cultured in basal EC growth medium containing low serum (2% FCS) and EC growth supplement (PromoCell). For HUVEC, all tissue culture surfaces were pre-coated with 0.1% gelatine. HUVEC were used at passages 2–6 during which expression of markers (von Willebrand factor, CD 31, VE-cadherin) for differentiated EC remained steady. Rat and human aortic smooth muscle cells (rSMC and hSMC) were isolated and phenotypically characterized in primary cultures as described previously [16]. rSMC (passages 6–13) were normally cultured in Dulbecco's modified essential medium supplemented with 10% fetal calf serum (FCS), 10 mM TES/HEPES (pH 7.3), 100 U/ml penicillin/streptomycin and 20 mM Glutamax (Invitrogen, Basel, Switzerland). The same medium with inclusion of smooth muscle growth supplement (Cascade Biologics, Portland, USA) was used for hSMC (passages 4–10). Growth-arrest culture conditions were as follows: rSMC-serum-free DMEM containing 0.1% BSA for 36 h with one medium change after 18 h. hSMC-serum-free DMEM containing 0.1% BSA for 24 h. HUVEC—in basal EC culture medium containing 0.1% serum for 18 h. Growth-arrest under these conditions was confirmed (90–95% of cells in G_1/G_0 phase) by standard flow cytometry after propidium iodide (PI) labeling and analysis using ModFit software.

2.2. Overexpression of T-cad

T-cad was overexpressed in HUVEC and hSMC using Adeno-X™ Expression System (Clontech, Palo Alto, USA) as described previously [12]. Viral titer was determined by End-Point dilution assay. For infections, HUVEC and hSMC in normal growth media were seeded at a density of $1-2 \times 10^4$ cells/cm^2, allowed to adhere and infected overnight with empty, LacZ (Clontech-Becton Dickinson) or T-cad containing adenoviral particles at a final approximate concentration of 100 pfu/cell. Trypan blue exclusion assay confirmed full viability of all infected cultures. Infected cultures were rinsed, trypsinized (0.25% trysin/1 mM EDTA in PBS) and re-seeded into culture dishes appropriate for any given functional assay. Expression levels of characteristic differentiation markers for HUVEC (von Willebrand factor, CD31, VE-cadherin) and hSMC (smooth muscle α-actin, desmin) were equivalent in parental and infected cultures (data not shown).

2.3. Cell proliferation

For cell enumeration, HUVEC and hSMC infected with empty, LacZ or T-cad containing adenoviral particles cells were growth-arrested, re-seeded (2×10^3 cells/cm^2) and cultured for up to 6 days in normal culture media with medium refreshment on days 2 and 4. Cell numbers were determined daily by trypsinization and counting in a Coulter counter. For DNA synthesis, infected HUVEC and hSMC were re-seeded (2×10^3 cells/cm^2), cultured normally for 2 days, and then growth-arrested. Thereafter cells were cultured for a further 24 h in normal culture media containing FCS, and DNA synthesis assessed by [^{3}H]-thymidine incorporation assay [17].

2.4. Cell cycle

Cells were seeded (2.5×10^3 cells/cm^2) and cultured normally for 2 days before growth-arrest. Cultures were released from growth-arrest by addition of normal culture media. For immunoblotting, at selected consecutive intervals cells were gently rinsed with PBS, lysed and analysed for specific cell-cycle markers. For flow cytometry, 22 h of

release from growth-arrest BrdU (10 μM) was added and culture continued for a further 14 h, after which cells were harvested by trypsinization, washed with PBS and resuspended in 70% ice-cold ethanol. Cells were labelled with FITC-conjugated anti-BrdU (Becton Dickinson) and PI according to protocols detailed by Becton Dickinson, and analysed using a FACScalibur cytometer (Becton Dickinson, San Jose, CA, USA) and ModFit software. For cycle blockade, cultures at 80% confluence were incubated for 18 h without additions (control), or with inclusion of 0.1 mM colchicine or 10 mM hydroxyurea, washed carefully with PBS, lysed and analysed by immunoblotting. Viability of treated cultures was proven by trypan blue exclusion and absence of active caspase 3 on immunoblots of cell lysates.

2.5. Cell sorting

rSMC at 70% confluency in 75-cm^2 flasks were collected after detachment by incubation in PBS containing 1 mM EDTA and 2 mM EGTA (PEE) for 10–15 min, at 30 °C, pelleted, resuspended in PBS and fixed by addition of ice-cold ethanol (final 70%). Before sorting, cell suspensions were washed twice with PEE, and resuspended (10^7 cells/ml) in PEE containing 0.1% Triton-X-100, PI (20 μg/ml) and DNAse-free RNAse (0.2 mg/ml). Cells were sorted using a FACS Vantage SE cytometer (Becton Dickinson). Phase integrity was verified by subjecting aliquots of sorted populations to flow cytometry and analysis using ModFit software. After lysis, equal amounts (2×10^5 cells/lane) were analysed by immunoblotting (available anti-T-cad antibodies not sufficiently sensitive for use in FACS).

2.6. Immunoblotting

The method of immunoblotting has been described previously [18]. Lysis buffer was PBS containing 1% SDS, 1 mM PMSF, 2 μg/ml pepstatin, 20 μg/ml aprotinin, 30 μg/ml bacitracin, protein concentrations were determined using the Lowry method, and electrophoresis was in 8 % or 10% SDS-polyacrylamide gels under reducing conditions. Protein loading equivalence was routinely controlled after electrotransfer to nitrocellulose membranes by staining with Ponceau S and by immunoblotting for actin and/or Gαs subunit. The following primary antibodies were used: rabbit antibody against the first extracellular domain of T-cad generated in our lab (1 μg/ml) [8]; mouse anti-VE-cadherin (clone 75; 2μg/ml), anti-N-cadherin (clone 32, 2 μg/ml), anti-$p27^{Kip1}$ (clone 57; 1:500), anti-cyclin D1 (clone DCS-6; 1:500), anti-E2F1 (clone KH95/E2F; 1:500), anti-cyclin B (clone 18; 1:1000) all from BD Biosciences, San Diego, CA, USA; mouse anti-PCNA (1:300, clone M870) and rabbit anti-von Willebrand factor (1:200) from DAKO, Glostrup, Denmark); rabbit anti-Gα (1 μg/ml, Calbiochem, Darmstadt, Germany); rabbit anti-cleaved caspase-3 (Asp175) (1:500, Cell Signaling-New England Biolabs, Frankfurt am Mein, Germany); mouse anti-actin (clone C-2; 1:200) and rabbit-anti-CD31 (1:100) from Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit anti-desmin (1:100) and mouse anti-smooth muscle α-actin (clone 1A4; 1:400) from Sigma-Aldrich Basel, Switzerland. Secondary HRP-conjugated anti-rabbit IgG (1:5000) and HRP-conjugated anti-mouse IgG (1:5000) were from Southern Biotechnology (BioReba, Reinach, Switzerland). Amersham ECL (Amersham Biosciences, Little Chalfont, UK) and the Enhanced Luminescence System or SuperSignal West Dura from Pierce (Rockford, IL, USA) were variously used for detection of immunoreactive proteins. Scanned images of Ponceau S stains and autoradiograms were analysed using AIDA Image or Scion (NIH) Image software. Figures show representative autoradiograms from any given single experiment.

2.7. Statistical analysis

Results are given as mean ± S.D. Statistical analyses were performed by One-way ANOVA followed by post hoc Bonferroni's multiple comparison when appropriate. A P value of <0.05 was considered significant.

3. Results

3.1. T-cad expression during the cell cycle

In order to evaluate changes in T-cad expression during cell cycle, we analysed levels of T-cad in parallel with recognized markers of cell-cycle progression ($p27^{Kip1}$, cyclin D1, E2F1, PCNA, cyclin B). Subconfluent rSMC, hSMC and HUVEC were first synchronized in G_1/G_0 by serum deprivation (time 0), and after release from growth-arrest by serum addition, protein levels were analysed in cells collected at consecutive intervals. Fluctuations of the different cell-cycle markers in rSMC (Fig. 1A), hSMC (Fig. 2B) and HUVEC (Fig. 2A) subsequent to release from growth-arrest generally corresponded well to the literature data (reviewed in Refs. [19,20]). Levels of both precursor 130 kDa and mature 105 kDa T-cad proteins were found to increase subsequent to entry into early S-phase (down-regulation of p27, upregulation of cyclin D1), and remained elevated through both S-phase (E2F1, PCNA) and M-phase (cyclin B) transit. Actin and/or Gα, used as internal controls, remained steady throughout the growth period. Attestation that alterations in levels of T-cad as measured in whole cell lysates reflect changes in surface expression was obtained by determining T-cad levels in membrane preparations [21] from HUVEC and hSMC at G_1/G_0 synchrony, 12 and 24 h after release from growth-arrest (insets above T-cad panels in Figs. 1 and 2).

To confirm elevated expression of T-cad in M- and S-phase cells, T-cad expression was examined after flow

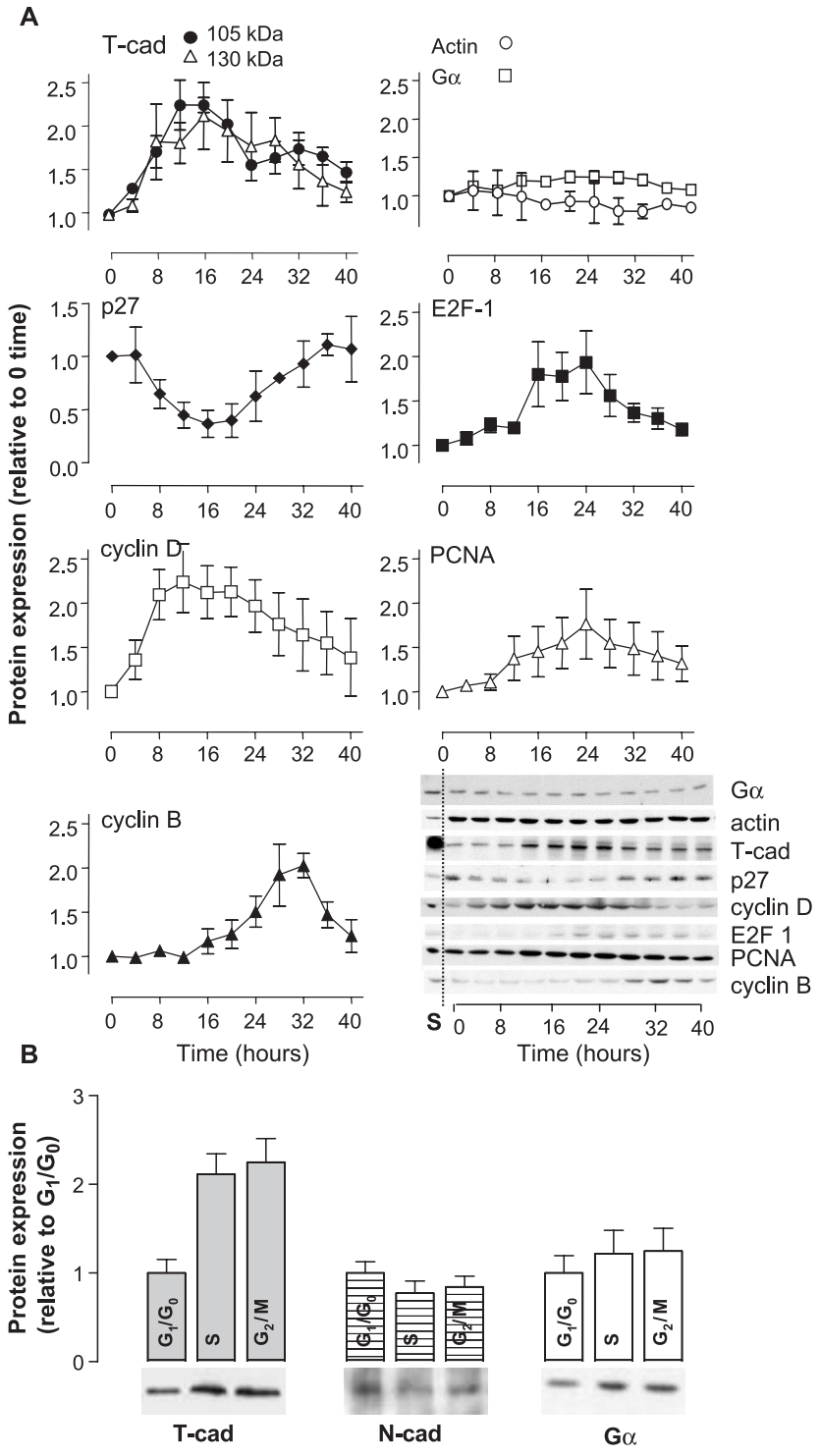

Fig. 1. Cell-cycle-associated alterations in T-cad protein expression in rSMC. Panel A. The indicated proteins were analysed by immunoblotting of rSMC cell lysates prepared at consecutive intervals after release from growth-arrest. Positive controls (lane S) included: CHAPS extract from human aortic media for T-cad (105 kDa), HUVEC cell lysate for actin (40 kDa) and Gα (45 kDa), HeLa cell lysate for E2F-1 (60 kDa) and $p27^{Kip1}$ (27 kDa), Jurkat cell lysate for cyclin B (62 kDa) and cyclin D (36 kDa). The experiment was performed on three separate occasions. Panel B. Expression of T-cad, N-cadherin and Gα in G_1/G_0-phase, S-phase, and G_2/M-phase sorted rSMC populations. Three independent experiments were performed.

cytometric sorting of cells into homogeneous G_1/G_0-phase, S-phase or G_2/M-phase populations. Due to the large starting numbers of cells required in order to finally obtain sufficient quantities of pure-phase populations for immunoblot analysis, we investigated only rSMC. The results (Fig. 1B) show a greater ($P<0.001$) expression of T-cad in S-

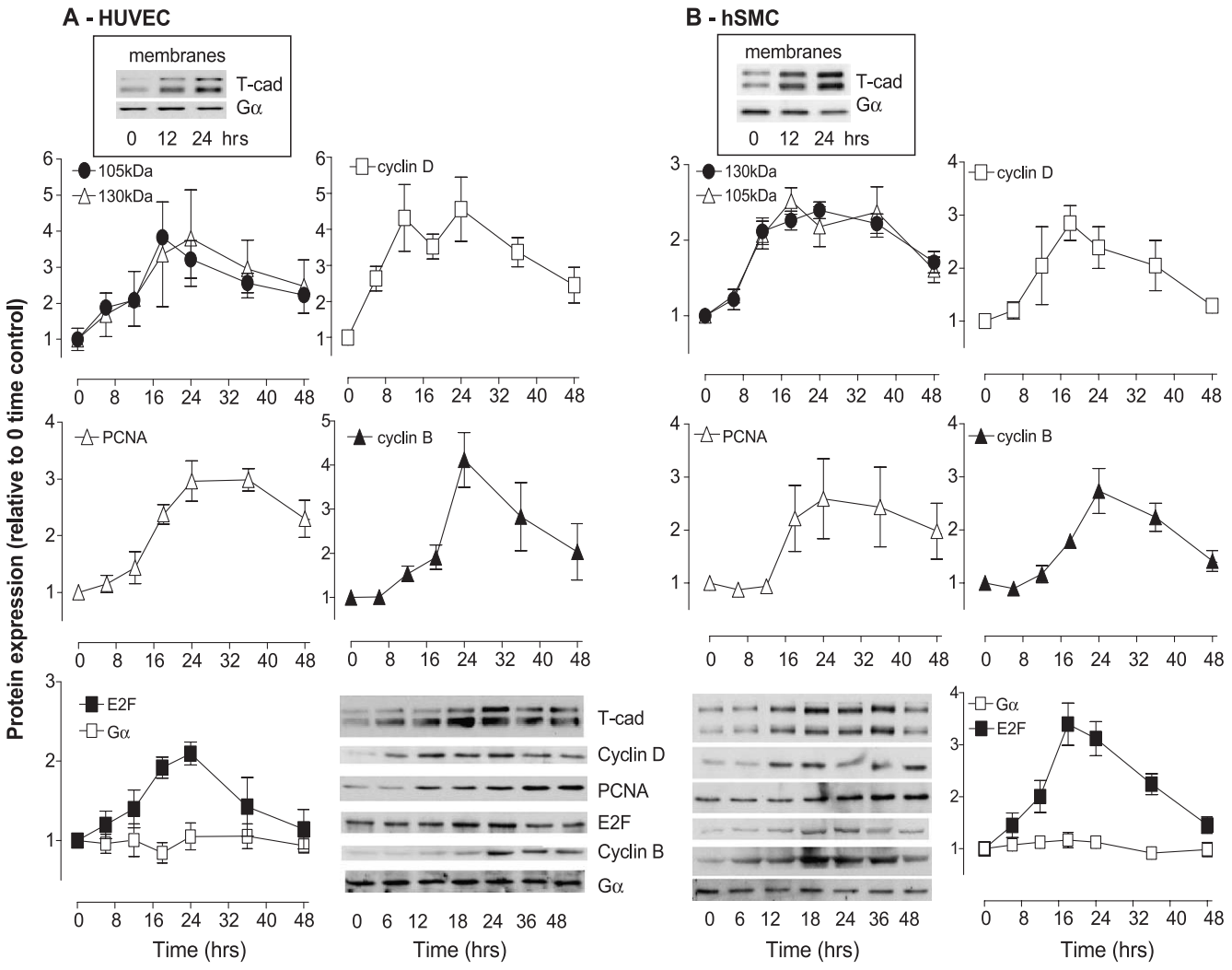

Fig. 2. Cell-cycle-associated alterations in T-cad protein expression in human vascular cells. Lysates of HUVEC (Panel A) and hSMC (Panel B) prepared at consecutive intervals after release from growth-arrest were analysed by immunoblotting for the indicated proteins. Three independent experiments were performed. The blots inset above the T-cad frames are of membrane isolates from cells collected at 0, 12 and 24 h.

and G_2/M-phase populations as compared to G_1/G_0-phase cells. Changes in N-cadherin (slight decreases vs. G_1/G_0) and Gα (slight increases vs. G_1/G_0) were not significant.

3.2. T-cad expression in cells treated with hydroxyurea and colchicine

T-cad protein expression was examined in cultures of rSMC, hSMC and HUVEC after an 18-h treatment with either 10 mM hydroxyurea (for accumulation of cells at G_1/S interface), or 0.1 mM colchicine (to induce accumulation of cells at G_2/M interface). Viability of cultures so treated was proven by complete trypan blue exclusion and absence of active caspase 3 on Western blots of cell lysates. In the case of HUVEC and rSMC where many mitosing cells were observed after colchicine treatment, the poorly attached dividing cells (Fig. 3A–D, lanes 3b) were collected (after gently tapping the dishes) and analysed separately from attached non-dividing cells (Fig. 3A–D, lanes 3a). Cultures of hSMC displayed a much lower mitotic activity, and although the amount of rounded cells in M-phase after colchicine treatment was visibly higher than in untreated cultures, we could not gather sufficient material for analysis on Westerns. Therefore, colchicine-treated hSMC cultures were very gently rinsed to minimize detachment of rounded M-phase cells and dividing and non-dividing hSMC were collectively analysed (Fig. 3A–C, lanes 3).

In hydroxyurea-treated cultures, T-cad levels remained unchanged (HUVEC and hSMC) or slightly increased (rSMC) as compared with control cultures (Fig. 3A, cf. lanes 2 vs. lanes 1). Following colchicine treatment, we observed a significant increase of T-cad protein in HUVEC (left panel), hSMC (central panel) and rSMC (right panel) that was due to a higher expression in poorly attached M-phase cells (Fig. 3A cf. lanes 3b—detached cells vs. lanes 3a—attached cells). The effect of colchicine was most prominent in rSMC which were the fastest growing of the three cell types examined (Fig. 3A, right panel). In these

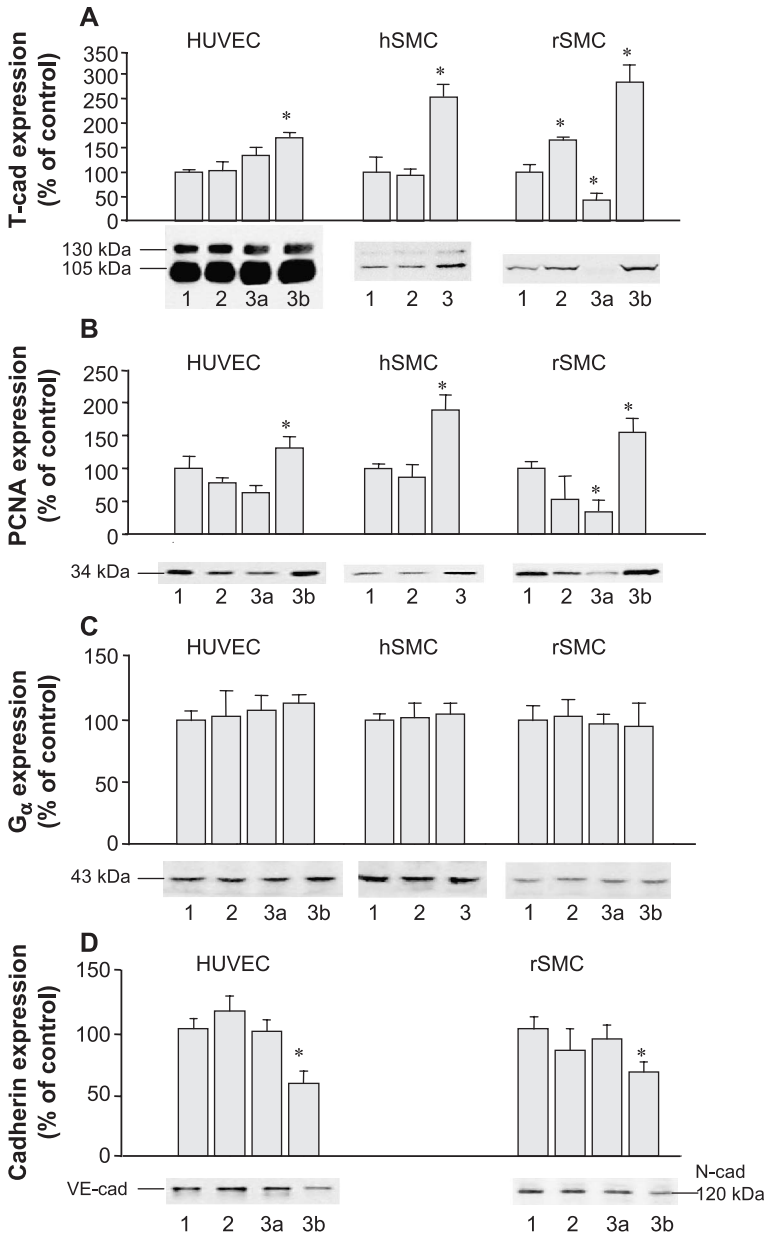

Fig. 3. T-cad expression is increased in M-phase of the cell cycle. Cells were incubated for 18 h in normal growth medium without additions (control, lanes 1), or with 10 mM hydroxyurea (lanes 2) or 0.1 mM colchicine (lanes 3, 3a, 3b). In colchicine-treated HUVEC and rSMC (A–D, left and right panels) rounded detaching cells in M-phase (lane 3b) were collected and analysed separately from attached cells (lane 3a). In colchicine-treated hSMC (A–C, central panels), all cells were collectively analysed. T-cad (Panel A), PCNA (Panel B), Gα (Panel C) and VE- and N-cadherins (Panel D) were analysed by immunoblotting. Expression is calculated relative to that (arbitrarily taken as 100%) under control conditions. Four independent experiments were performed. Statistical analysis was performed by One-way ANOVA. Asterisks indicate where treated and control cultures differ significantly (P at least <0.01).

cultures, expression of T-cad in poorly attached M-phase cells (lane 3b) increased by approximately three-fold as compared to control (lane 1), while in attached non-dividing cells (lane 3a) expression was two-fold less than in control (the latter obviously comprising a mixture of cells at different stages of the cell cycle). As in the foregoing experiments, changes in T-cad were measurable in membrane preparations (data not shown), confirming that alterations in T-cad protein reflect changes in surface expression.

Changes in proliferating cell nuclear antigen PCNA (Fig. 3B) generally correlated with those of T-cad (Fig. 3A). Gα expression remained constant (Fig. 3C). Compared with control cells, expression of classical VE- and N-cadherins (Fig. 3D) was unchanged in hydroxurea-treated cells (lanes 2) but decreased by approximately 30–40% after colchicine treatment in the detached M-phase cells (cf. lanes 3b—detached cells vs. lanes 3a—attached cells and lanes 1—untreated cells).

3.3. Influence of T-cad overexpression on cell proliferation

To further investigate the apparent influence of T-cad on cell proliferation, T-cad was overexpressed in cultured HUVEC and hSMC using an adenoviral expression system. The high level of surface T-cad expression (vs. controls) and its stability throughout the experimental period was proven by immunoblotting of membrane isolates (Fig. 4). In cell enumeration assays, we found that T-cad overexpression increased proliferation rates of both HUVEC and hSMC as compared to their respective empty vector and LacZ controls (Fig. 4). Although moderate (approximately 1.5-fold increase above controls), the effect was reproducible and significant (P at least <0.05). DNA synthesis assay confirmed this effect whereby, compared with empty vector and LacZ controls, HUVEC and hSMC overexpressing T-cad incorporated significantly more [^{3}H]-thymidine (P at least <0.01 at all tested FCS concentrations; Fig. 4).

3.4. Influence of T-cad overexpression on cell-cycle progression

Infected cells were synchronized in G_1/G_0, released from growth-arrest and analysed for progression through the cell cycle both by immunoblotting of cell-cycle-phase specific markers and flow cytometry. In both HUVEC (Fig. 5) and hSMC (Fig. 6) overexpressing T-cad, the induction of cyclin D occurred more rapidly indicating a more rapid entry into early S-phase. Induction of the S-phase markers, PCNA and E2F1, as well as the M-phase marker cyclin B also occurred earlier and reached higher levels in T-cad overexpressing cells. Flow cytometry confirmed the more rapid cycle progression in T-cad-overexpressing cells whereby both the % of BrdU-positive cells, and % of cells in S-phase and G2/M phase was significantly greater (P at least <0.01) than in control cells (Table 1).

4. Discussion

The role for T-cad in regulation of cell proliferation has mostly been studied in cancer. Similarly to classical cadherins, T-cad expression was found to be decreased or absent in some tumour samples and cancer cell lines, including breast, lung, colon, neuroblastoma, prostate, bladder, melanoma carcinomas (reviewed in Ref. [13]). Overexpression of T-cad in neuroblastoma cells inhibited cell invasion and growth responses to EGF [22], while overexpression in C6 glioma cells was associated with decreased cell motility and a reduction of proliferation that involved G_2 phase arrest

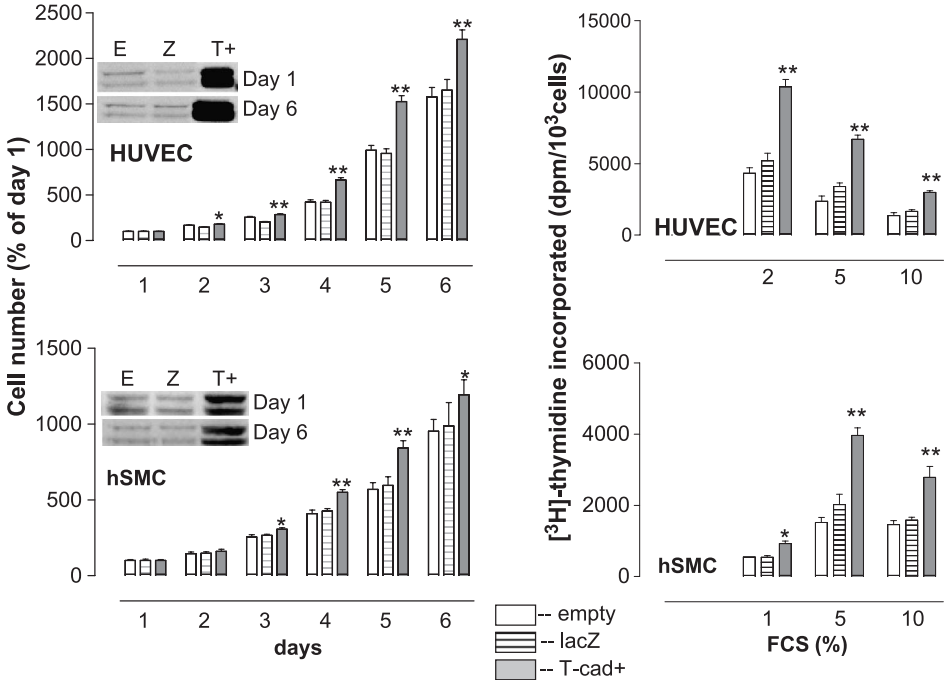

Fig. 4. Cell growth is increased in T-cad overexpressing hSMC and HUVEC. Cells infected with empty-vector (open bars, E), vector carrying LacZ (striped bars, Z) or T-cad cDNA (filled bars, T+) were compared with respect to their proliferation rates by cell enumeration (left panels) and DNA synthesis (right panels). Blots inset illustrate stability of T-cad overexpression in T+ cells during the course of growth analysis. FCS concentrations used in the cell enumeration experiments were 2% and 10% for HUVEC and hSMC, respectively. Six and three independent experiments were performed for cell enumeration and DNA synthesis, respectively. Statistical analysis was performed by One-way ANOVA followed by post hoc Bonferroni's multiple comparison. Asterisks indicate significant differences (*$P<0.05$, **P at least <0.01) between cells overexpressing T-cad and either empty-vector or LacZ controls. Empty and LacZ controls did not differ.

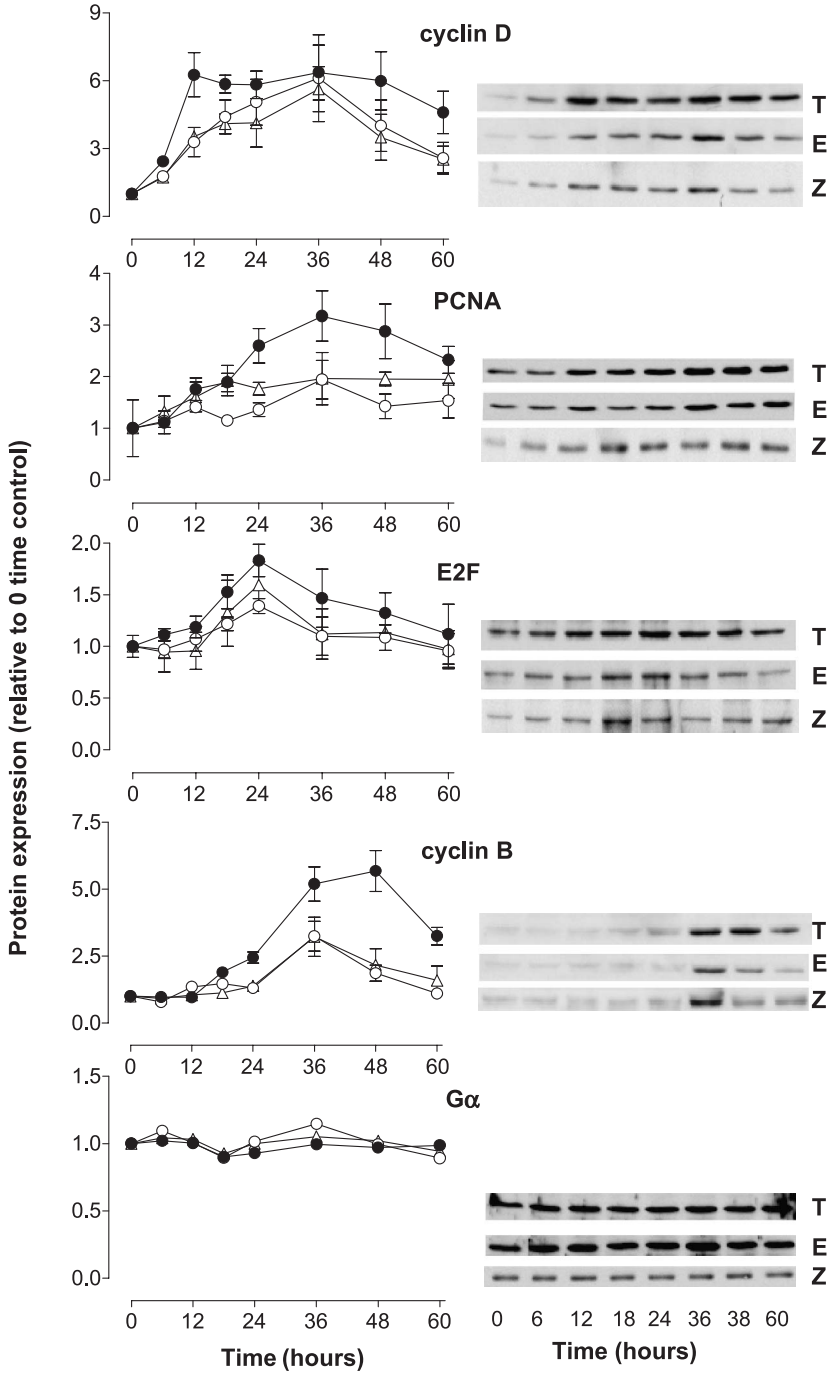

Fig. 5. Cell-cycle progression in control and T-cad overexpressing HUVEC. Lysates of HUVEC infected with empty vector (E, open circles) and vectors carrying LacZ (Z, open triangles) or T-cad cDNA (T, filled circles) prepared at consecutive intervals after release from growth-arrest were analysed by immunoblotting. Data for changes in expression (relative to 0 time) are given as mean ± S.D. ($n = 3$), except for Gα (mean values only) for which there was sufficient lysate for analysis in only two of the experiments.

[23]. In contrast, overexpression of T-cad was demonstrated in astrocytoma tumours with heterozygosity or inactivation of the neurofibromatosis 1 tumour suppressor gene [24] and in tumorigenic liver tissue [25]. High expression of T-cad was also found in the MAHLAVU hepatocellular carcinoma cell line, whereas it was absent in HepG2, PLC/PRF/C, TONG, HA22TNGH hepatocellular carcinoma cell lines [25]. T-cad is also expressed in osteosarcoma cell lines [26]. Thus, the influence of T-cad on cell proliferation may vary between cell types.

A positive relationship between T-cad upregulation and stimulation of vascular cell growth and motility first emerged through immunohistochemical demonstration of increased T-cad levels on SMC and EC in proliferative

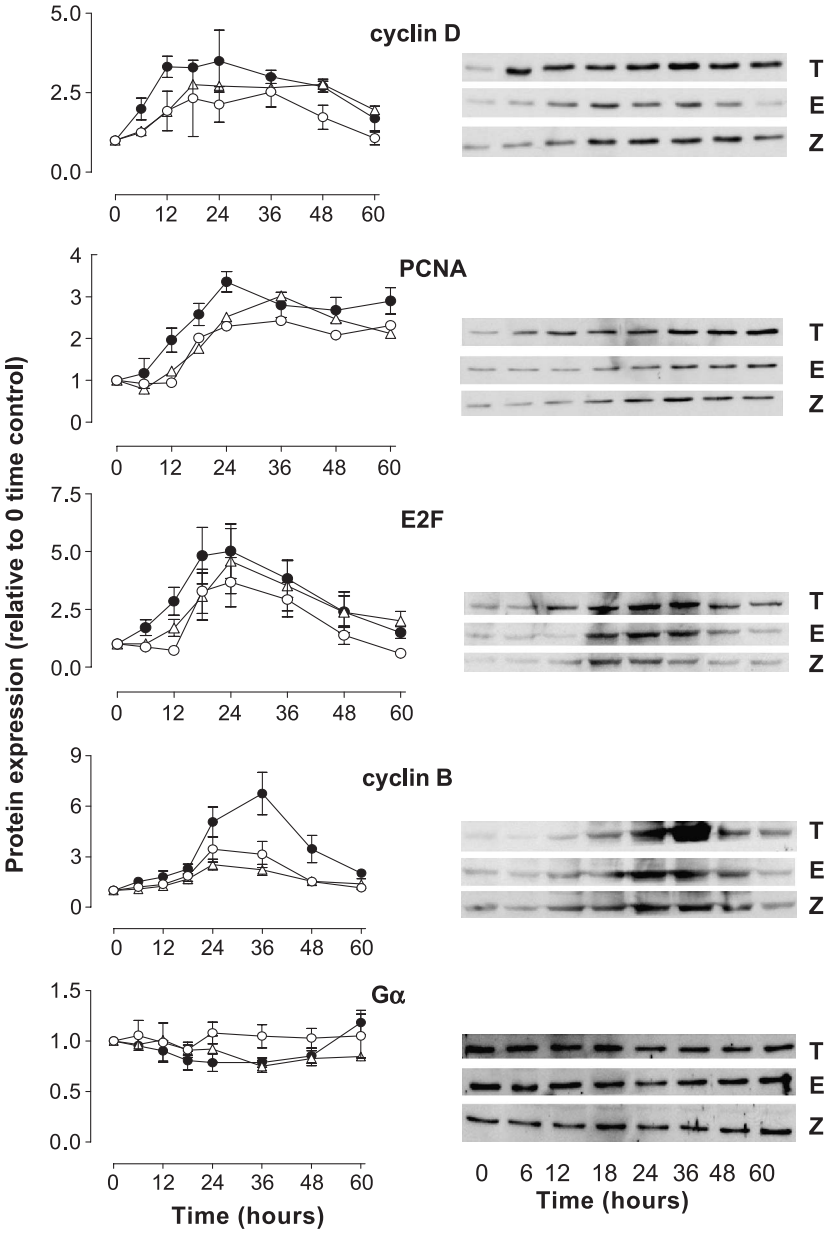

Fig. 6. Analysis of cell-cycle markers in control and T-cad overexpressing hSMC. Lysates of hSMC infected with empty vector (E, open circles) and vectors carrying LacZ (Z, open triangles) or T-cad cDNA (T, filled circles) prepared at consecutive intervals after release from growth-arrest were analysed by immunoblotting. Three independent experiments were performed and data reflect changes in expression relative to 0 time.

vascular diseases such as atherosclerosis and restenosis [8,14,15]. This hypothesis was later supported both by observations of elevated T-cad expression during tumour angiogenesis [15], and in vitro studies showing that T-cad induces cell detachment, promotes phenotypic modulation of EC to a polarized pro-migratory phenotype and facilitates directed cell migration [12]. These studies strongly support a positive relationship between T-cad and vascular cell motility/migration. However, the relationship between T-cad and cell proliferation was unclear. T-cad levels were higher in sparsely seeded rSMC and decreased when cultures reached confluency suggesting that T-cad is upregulated in actively growing cells [18]. Paradoxically, serum-starvation of rSMC for 48 h also caused T-cad upregulation on the cell surface [18]. Interpretation here is confounded by the induction of apoptosis (after 48-h serum-starvation in rSMC), a condition where T-cad expression is also altered and possibly contributes to cell survival (unpublished observations, manuscript in preparation). To clarify the relationship between T-cad and cell proliferation, the present study specifically examined how T-cad levels change in proliferating cells during the cell cycle, and whether overexpression of T-cad may in its turn influence vascular cell growth rates.

Cell cycle associated T-cad expression in HUVEC and SMC was assessed using different experimental protocols:

Table 1
T-cad overexpression promotes cell-cycle progression

	Cell-cycle-phase distribution of cells (%)			
	G_1/G_0	S	G_2/M	Total BrdU-positive
HUVEC-empty	70.5 ± 2.3	6.7 ± 3.4	19.6 ± 1.2	26.1 ± 2.8
HUVEC-T-cad	56.9 ± 7.1*	13.0 ± 2.4*	30.0 ± 4.5*	41.0 ± 5.6*
hSMC-empty	80.2 ± 1.9	7.6 ± 2.9	12.1 ± 0.6	18.3 ± 2.1
hSMC-T-cad	70.4 ± 1.1*	11.4 ± 1.7*	19.8 ± 2.6*	29.8 ± 3.6*

95–98% of cells were in G_1/G_0 and 1–3% were BrdU-positive at growth-arrest. FACS analysis after double PI and BrdU staining was at 36 h after release from growth-arrest.

*$P < 0.01$ for T-cad vs. empty.

(1) cells were synchronized in G_1/G_0 phase and then released from growth-arrest; (2) cells were synchronized in early S-phase or M-phase by treatment with hydroxyurea or colchicine, respectively; (3) cells from exponentially growing cultures were sorted into different stages of the cycle by FACS. All protocols yielded similar results, namely T-cad protein levels are increased subsequent to entry into early S-phase and remain elevated through S-phase and M-phase. Comparison of proliferation rates and cell-cycle characteristics of EC and SMC overexpressing T-cad with empty vector or LacZ controls shows that T-cad stimulates proliferation. Together, the data identify T-cad as a growth-promotory molecule for vascular cells. Although increases in cell number in T-cad-overexpressing cells never exceeded two-fold as compared to controls, this mild stimulation may have important consequences for cell behaviour in vivo where vascular cells, even after vessel injury leading to intimal thickening [27], have low proliferation rates.

Although the mechanisms of T-cad-dependent stimulation of vascular cell growth have yet to be elucidated, one may speculate on some relationship to the anti-adhesive properties of T-cad [12]. Repetitive attachment and detachment during the cell cycle is critical to proliferation of normal adherent cells. Among molecular mechanisms, mediating cell-cycle-dependent decrease in adhesiveness is dephosphorylation of focal adhesion kinase on tyrosine residues and phosphorylation on serine/threonine which leads to disassembly of the FAK/p130Cas/c-Src signalling complex and inhibition of focal adhesion formation until the post-mitotic spreading [28]. The marked decrease in expression of classical VE- and N-cadherins shown herein for colchicine-treated cells, which contrasts with the increase in T-cad, could reflect a need for the cell to lose contacts with its neighbours before cytokinesis. A similar observation has been made for classical E-cadherin, which was lost from cell–cell adhesion sites during mitosis [29]. It is plausible to attribute a distinct function to T-cad, namely to facilitate transition of the cell from an attached to a detached state during cell division. The anti-adhesive influence of T-cad may also contribute to pathological vascular tissue remodelling, since decreased cell adhesion to the substratum favours cell phenotypic modulation and improves the ability of a cell to respond to growth stimuli at the site of injury.

Promotion of vascular cell proliferation by T-cad may seem paradoxical in terms of the traditional concept of inhibitory influences of cadherin-dependent cell–cell interactions on cell growth. This concept emerged largely through studies on classical E-cadherin showing that its downregulation and/or impaired function allow malignant cells to become unresponsive to contact inhibition, acquire a motile and proliferative phenotype, escape from their site of origin, and finally to invade and metastasize [30]. The growth inhibitory effect is believed to relate to the capacity of cadherins to link β-catenin at the membrane thus limiting its nuclear translocation and abolishing its action as oncogene [31]. In vascular tissue, similar stabilizing functions are attributed to VE-cadherin. VE-cadherin expression and clustering at intercellular junctions can block the proliferative response of EC to VEGF via recruitment of VEGFR-2 to VE-cadherin–β-catenin complex and resultant inhibition of VEGFR-2 phosphorylation [32]. Disruption of VE-cadherin junctions in EC results in increased VEGF production [33]. Growth factor treatment, in its turn, may inhibit cell–cell adhesion by downregulation of VE-cadherin and catenins [34] or tyrosine phosphorylation of VE-cadherin complex [35].

However, a growing body of data suggests that some cadherins, including N- and P-cadherins, do not conform to traditional concepts of growth inhibitory functions for cadherins. For example, a high P-cadherin level correlates with higher proliferation rates in breast cancer [36] and a splice variant of cad-11 promotes invasion of breast cancer cells [37]. The role for N-cadherin in regulation of cell growth is controversial. N-cadherin induces contact inhibition in CHO cells [38], is downregulated in adrenocortical carcinoma [39], in haemangioma-derived cell line EOMA [40] and in SMC after stimulation of cell proliferation [41]. However, other studies demonstrate upregulation of N-cadherin in malignant pheochromocytoma [39] and in SMC during neointima formation in experimental restenosis, as well as an ability of anti-N-cadherin blocking antibody to reduce migration rates in vitro [42]. During melanoma development, loss of functional E-cadherin accompanies gain of N-cadherin expression, and N-cadherin promotes migration of melanocytic cells [43]. In breast cancer cell lines, N-cadherin stimulates motility and growth even in the presence of the normally growth-suppressive E-cadherin [44].

The present study identifies T-cad as another member of cadherin family which can stimulate proliferation in some cell types. T-cad-promoted proliferation in vascular cells T-cad may contribute to progression of vascular disorders such as atherosclerosis, restenosis and tumour angiogenesis. Functional differences between T-cad and the growth-inhibitory E- or VE-cadherins may be partly attributed to the atypical structure of T-cad molecule. The absence of transmembrane and cytoplasmic domains excludes direct interaction of T-cad with the catenin pathway and implies

existence of other mediatory molecular partners. T-cad is enriched in lipid raft domains of plasma membrane [45] that also contain other GPI-anchored proteins, Src family kinases, Ras-protein, protein kinase C, MAP-kinase and GTP-binding proteins [46]. Conceivably some of these molecules may serve as adaptors coupling extracellular T-cad to intracellular signal transduction systems involved in stimulation of vascular cell proliferation.

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