

T-cadherin signaling in endothelial cells

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Dedicated to my parents

ॐ भूर्भुवस्सुवः तत्सवितुर्वरेण्यं भर्गो देवस्य धीमहि
धियो यो नः प्रचोदयात् ।।

“OM (the supreme) let us meditate upon that supernal (spiritual) effulgence of the adorable Supreme Divine Reality, Which destroys all ignorance, stimulate our intellect and energize our consciousness”

Gayatri Mantra, Rigveda, 10:16:3

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“Tell me and I forget, teach me and I remember, Involve me and I learn”- Benjamin Franklin.

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1. Introduction

1.1 Cadherins

Cadherins are a large superfamily of transmembrane glycoproteins that mediate calcium-dependent homophilic cell-cell adhesion. Cadherins are expressed by most organisms from unicellular choanoflagellates to most complex vertebrates, and they are usually located at cellular adherent junctions. In addition to their pivotal role in mechanical adhesion between the cells, cadherins have multiple functions in tissue morphogenesis, cell recognition and sorting, regulated cell motility and the induction and maintenance of tissue/cell polarity (1). Cadherins play a vital role in formation and maintenance of diverse tissues and organs such as polarization of very simple epithelia, mechanical linkage of hair cell to cochlea (2), and provision of an adhesion code for neural circuit formation during wiring of the brain (3). Thus, malfunctioning of mechanisms regulating cadherin expression levels or their functionality adversely affects tissue architecture and tissue cellularity, eventually resulting in tissue diseases.

Structural diversity within the cadherin superfamily enables an assortment of cell interactions and tissue morphogenic processes. Variations in structure, along with temporal and spatial transcriptional regulation in cadherin subtype facilitate interactions that differentially modulate cadherin activity and intercellular signaling responses to adhesion (1). In spite of their structural diversity, members of the cadherin super family typically possess N-terminal tandem repeat extracellular cadherin domains (EC domains) that contain a cadherin-specific motif. Although the number of EC domains may range from 5 to 34, each EC domain comprises about 110 amino acids and connections between these domains are rigidified by specific binding of three Ca^{2+} ions (4). This Ca^{2+} binding is essential for adhesive function of cadherins, hence the derivation of their name - *Calcium dependent adherent protein*. Typically cadherins also possess a transmembrane domain and a cytoplasmic domain. Interactions of the cytoplasmic domain with intracellular molecules vary between cadherins; for example classical cadherins interact with catenins and actin cytoskeleton where as desmocollins interacts with intermediate filaments (Fig. 1). T-cadherin is an atypical GPI anchored cadherin, possessing the N-terminal EC domain structure but lacking transmembrane and cytoplasmic domains (Fig. 1).

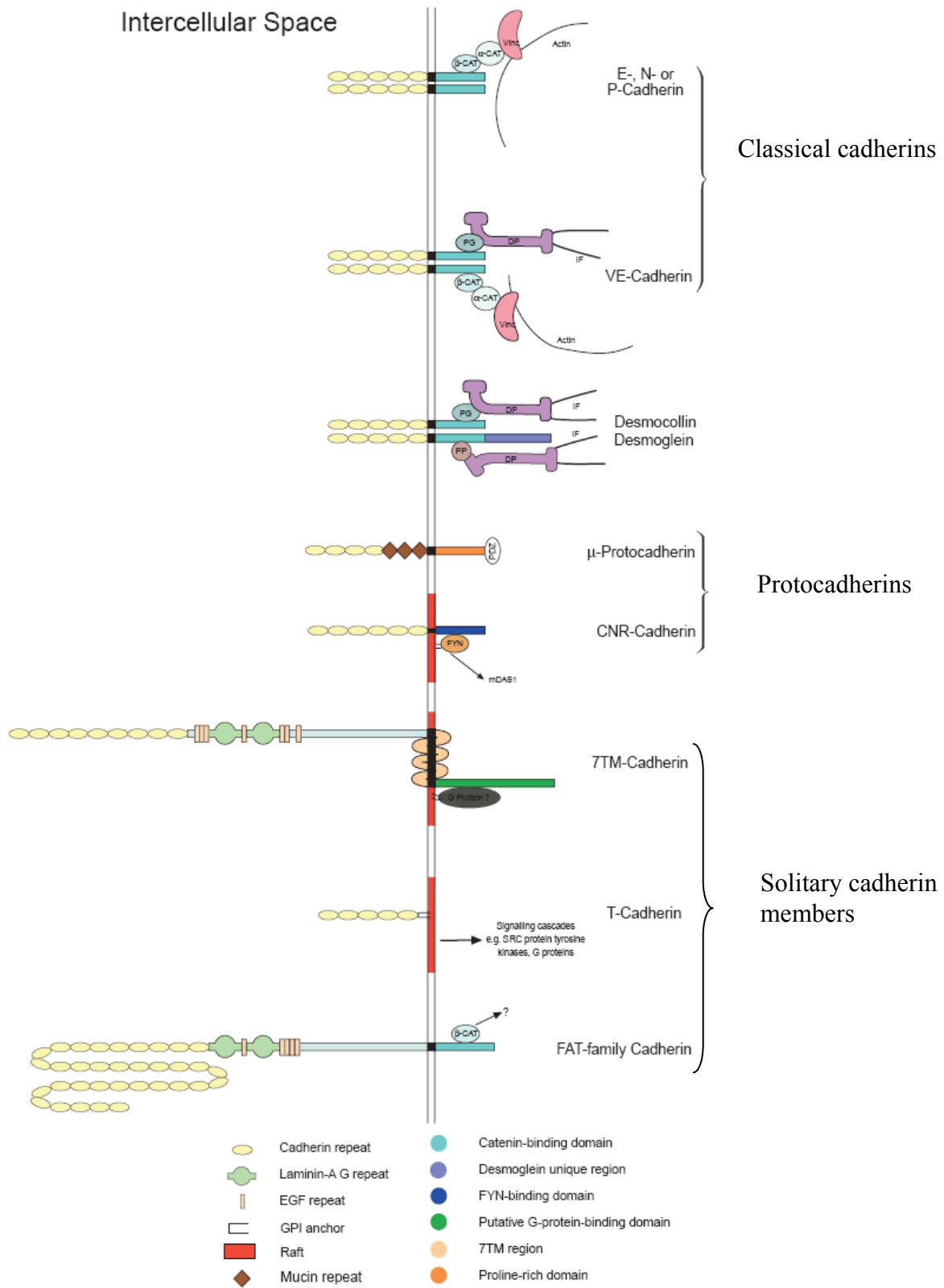


Figure 1: Schematic overview of the cadherin superfamily (5).

E-cadherin was the very first cadherin to be discovered; it was found in the early 1980s by Jacob and coworkers and was then named uvomorulin (6, 7). Soon afterwards N-cadherin was found in neural tissue (8, 9) and P-cadherin was found in placental tissue (10). Now, almost three decades on, more than 100 cadherins have been reported (11). Molecular phylogenetic analysis of cadherin family revealed six major subfamilies in addition to several solitary members (11). The six subfamilies comprise classical or type I cadherins, atypical or type II cadherins, desmocollins, desmogleins, protocadherins and flamingo cadherins; solitary members include T-cadherin, FAT and many invertebrate cadherins (Fig. 2).

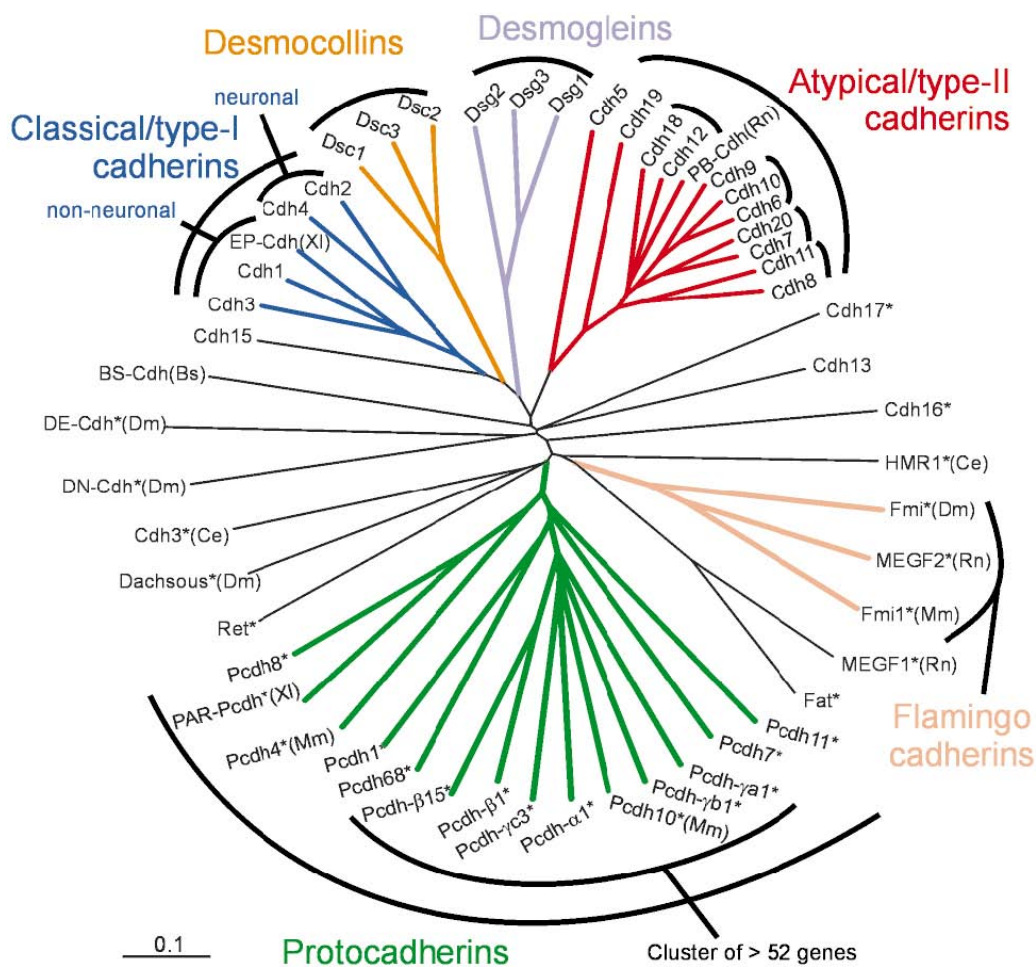


Figure 2: Phylogenetic tree of the cadherin superfamily (11).

Classical cadherins

Classical cadherins were the first cadherin subtype identified. They are expressed by almost all vertebrate tissues, are responsible for homophilic adhesion and are localized within adherent junctions. Classical cadherins have five EC domains and modulate adhesion through dynamic interactions with the cytoskeleton. The cytoplasmic domain of classical cadherins contains highly conserved unique sequences. Classical cadherins can be subdivided into type I and type II according to the presence or absence of a Histidine-Alanine-Valine (HAV) motif on the first extracellular domain (EC1) which is the domain responsible for homophilic adhesion. Type I classical cadherins possess this HAV motif; E-cadherin, N-cadherin, P-cadherin, M-cadherin and EP-cadherin belong to this family. Type II cadherins do not possess the HAV motif but contain conserved tryptophan residues in EC1; VE-cadherin, Cadherin-11, Cadherin-8 and MN-cadherin belong to this family (12). Type II classical cadherins have been reported to exhibit heterophilic adhesion (13). Additionally it is recognized that EC domains of some cadherins (e.g. N-cadherin) can bind to other proteins such as fibroblast growth factor to induce downstream signaling (14).

Conserved cytosolic domains of classical cadherins interact with either β - or γ - catenin which bind to α -catenin, which in turn interacts with the actin cytoskeleton via vinculin (Fig. 3) (5). Cadherin binding to β -catenin and the involvement of the latter in the Wnt

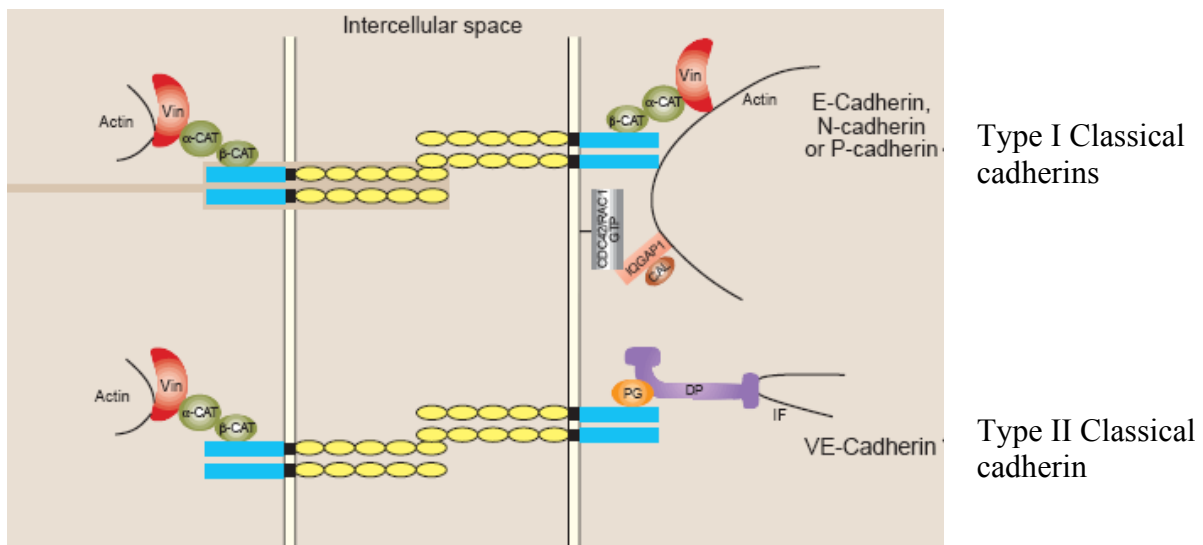


Figure 3. Homophilic adhesion between classical cadherins (5).

signaling pathway enables cross-talk between cadherins and the Wnt pathway leading to modulation of several biological activities such as proliferation, migration and cell survival (15, 16). The cytoplasmic domain of classical cadherins also has binding affinities for various other proteins responsible for modulation for interactions with actin, thereby affecting the strength of homophilic adhesion. These include small GTPases CDC42 and Rac and IQGAP1 (17-19). IQGAP1 also binds to calmodulin which plays a role in modulating calcium sensitive environment for the homophilic adhesion of cadherins (20). p120, an armadillo family protein has been demonstrated to regulate CDC42 and Rac in cadherin interactions (21).

Type II classical VE-cadherin, specific to vascular endothelial cells, has multiple cytoplasmic binding partners (Fig. 3). In addition to p120 regulated binding with α/β and γ -catenin to actin, the cytosolic domain of VE-cadherin binds to plakoglobin (PG) which in turn binds to desmoplakin (DP) and then to Intermediate Filaments (IF) (22), (23).

Classical cadherins play a pivotal role in tissue morphogenesis during development. Different roles of cadherin during development are illustrated in Fig. 4.

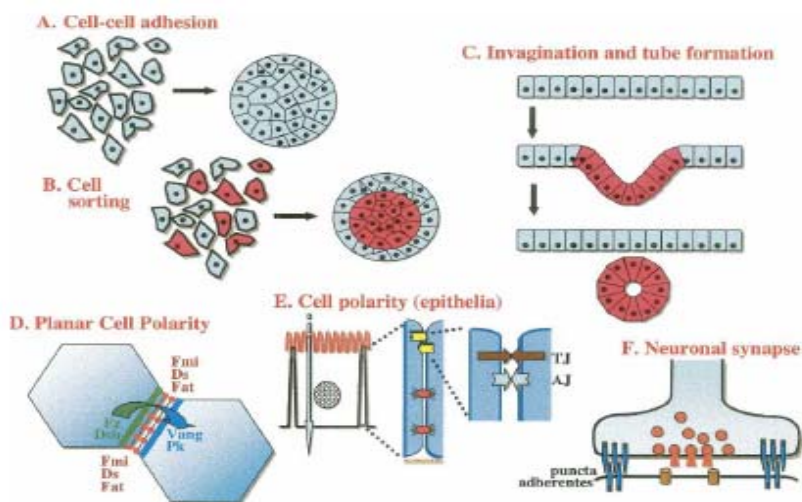


Figure 4: Developmental roles of cadherins (1).

Each subtype of cadherin tends to exhibit restricted and distinct cell type expression. E-cadherin is expressed in epithelium, N-cadherin in neural tissue and muscle, R-cadherin in forebrain, P-cadherin in placental tissue, Cadherin-6 in kidney and VE-cadherin in endothelium. Tight regulation in expression of various cadherins during different stages of development is accomplished by a prominent phenomenon known as “Cadherin switching”. A classical example of cadherin switching

is Epithelial-to-Mesenchymal Transition (EMT) during mesoderm formation in embryogenesis. During EMT, epithelial cells transit from an epithelial morphology with a stationary phenotype to a mesenchymal and fibroblastic morphology with a migratory phenotype through a concomitant loss of E-cadherin expression and gain of N-cadherin expression. The phenomenon of EMT has been well studied in the context of tumor progression and metastasis.

VE cadherin belonging to the Type II classical subtype is expressed only in the endothelium and plays a crucial role in vasculogenesis, vascular permeability and vascular remodeling. The cytoplasmic tail of VE-cadherin complexes with VEGF receptor and induces PI3K/Akt pathway signaling resulting in positive effects on endothelial cell survival, proliferation and migration.(24).

Protocadherins

The protocadherin subfamily comprises more than 80 members, representing the largest group of the cadherin superfamily. Protocadherins are expressed by all vertebrates and certain sea sponges. Protocadherins possess 4-7 EC domains and although they have calcium-dependent homophilic interaction activity, the activities are relatively weak compared with the classical cadherins. (25, 26). Further unlike classical cadherins, the cytoplasmic domains of protocadherins are highly variable, and most of them do not show any appreciable homology with the cytoplasmic domains of classic cadherins. Some protocadherins have heterophilic interaction activity and their cytoplasmic domains associate with unique cytoplasmic proteins, which confer various biological functions on the protocadherins (26).It has been proposed that the main function of protocadherins may not be the cell– cell adhesion activity, but other functions such as the specificity determination of cell– cell interaction and signal transduction (26).

Interactions between the cytoplasmic domain of protocadherins and intracellular proteins remain poorly investigated. Two of the better studied protocadherins are μ -cadherin which can bind via its PDZ domain, and CNR-cadherin which is localized in rafts which can interact with src family kinase FYN. In mammals protocadherins are expressed in the nervous system and an important function appears to involve synapses (27). The role of protocadherins in development has been well studied in xenopus and zebrafish. Paraxial protocadherin (PAPC) and axial protocadherin (APC) are very important in regulation of embryogenesis. ARCADLIN is induced by synaptic activity (28). Recently epigenetic methylation of Protocadherin 10 was observed in hematologic malignancies

(29). In humans protocadherins are regulated by a large gene cluster located in 5q31-33; interestingly, genomic sequences corresponding to the ECs of many protocadherins have essentially no introns, (30), which is in a sharp contrast to the case of classic cadherins.

Desmosomal Cadherins

Desmosomal cadherins are located at desmosomes, which are sites for cell-cell adhesion present in tissues subjected to mechanical strain such as epidermis and myocardium. Desmosomal cadherins comprise two subfamilies, desmocollin (DSC) and desmoglein (DSG), each possessing three subtypes, DSC 1, DSC2, DSC 3 and DSG 1, DSG 2, DSG 3 (31). EC domains of desmosomal cadherins possess 50% homology with classical cadherins. (11). Cytoplasmic domains of DSC and DSG interact with intermediate filaments bound to plakoglobin and plakophilins via desmoplakins (Fig. 5).

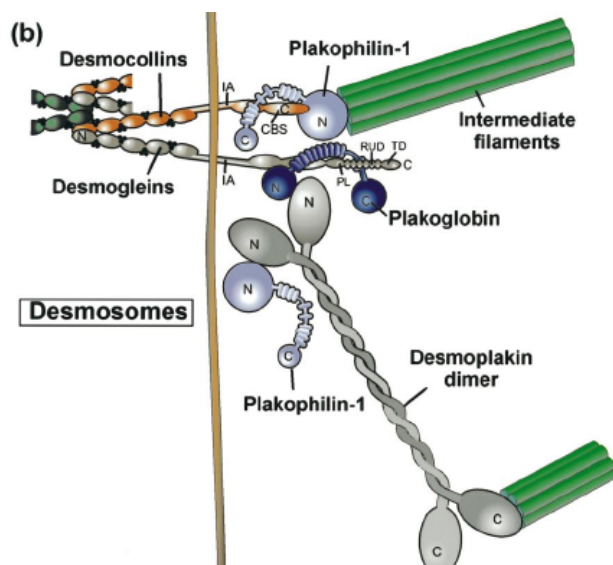


Figure 5: Desmosomal cadherins (11).

DSC 1 is expressed in differentiated uppermost epithelium and papillae of tongue. DSC 2 is widely expressed in epithelia, lymph nodes and cardiac muscle. DSC 3 is expressed in epithelia. DSG 1 and 3 are confined to stratified squamous epithelium and DSG 2 is expressed by all the tissues possessing desmosomes. (11). RNAi mediated silencing of DSC 2, expression of dominant negative DSG and knockout DSG 3 results in decline in desmosomes, increased asymmetry and detachment of desmosomes (32, 33). Desmosomes are often observed in heterophilic adhesion (34). Gene clusters of desmosomal cadherins are located on 18q12 where they are arranged in a DSC cluster and a DSG cluster.

Solitary members of cadherin superfamily

The FAT family members

Drosophila FAT is a very large cadherin protein with 34 tandem EC domains, four EGF repeats, two laminin repeats, a single transmembrane-spanning region and a cytosolic domain. Recessive lethal mutations in FAT leads to defects in larval imaginal disc overgrowth and differentiation (35). *Drosophila* daschsous protein is highly related to FAT and has 27 EC domains. Daschsous is expressed in imaginal discs and brain in larvae. FAT and daschsous have cytoplasmic domains predicted to bind β -catenin. (36). Recently it was reported that human FAT1 is involved in vascular smooth muscle cell proliferation and migration (37).

Seven transmembrane cadherins

Seven transmembrane cadherins, also known as starry night or Flamingo (Fmi), were first identified in *Drosophila*. These possess 9 EC domains, EGF like domains and a laminin motif in addition to a seven transmembrane-spanning domain homologous to GPCRs and a cytosolic domain (38). Cytoplasmic tails do not contain any catenin binding domain and putative binding partners have not yet been identified. Fmi exhibits homophilic and homotypic adhesion properties. Two mammalian paralogues have been identified, mCELSRI in mouse (39) and hMEGF2 in humans (40). Mutations in Fmi have recently been shown to lead to retinal axon malfunction (41).

T-cadherin

T-cadherin (T-cad) is an unusual member of cadherin superfamily; while possessing the N-terminal tandem cadherin repeat structure, it lacks both transmembrane and cytoplasmic domains, and is bound to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (42). T-cad was first cloned from a cDNA pool of chick embryo brain (42). The most N-terminal EC domain has 58% homology with E-cadherin but does not possess the HAV motif that is crucial to homophilic interactions mediated by the classical cadherins (11). T-cad has five EC domains and a propeptide in its precursor form (130kDa), which upon cleavage gets converted to mature form (105kDa) (Fig. 6); unlike the classical cadherins, the precursor form of T-cad is also expressed on the cell surface.

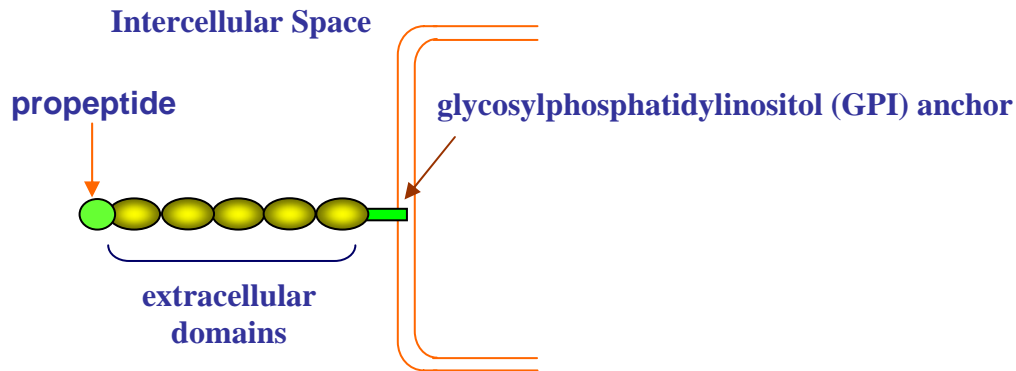


Figure 6: Schematic diagram of T-cadherin structure.

CHO cells upon transfection with T-cad cDNA exhibit only weak calcium-dependent homophilic adhesion. Adhesive properties of T-cad in transfected cells were abrogated when treated with phosphatidylinositol-specific phospholipase C, suggesting some involvement of the GPI anchor (43). However, accumulating data suggests that T-cad shares little functional similarity with classical members of the cadherin family. In particular, the global distribution of T-cad over the cell body, its absence at intercellular contacts in cell monolayers, and its localization to the apical surface of polarized transfected cells imply that participation in the formation of intercellular adherent junctions is not a major function of T-cad.

A role for T-cad in tissue organization was first demonstrated in the avian embryonic nervous system where the protein influenced the pattern of neural crest cell migration and maintained somite polarity (44). T-cad inhibited neurite outgrowth when neuron populations were grown on T-cad substrata and anti T-cad antibodies abrogated the effects, suggesting T-cad acts as negative guidance cue for axon projections (45). T-cad is also expressed in human brain where it acts as negative growth regulator of epidermal growth factor in neuroblastoma cells (46).

T-cad gene is localized on 16q24 and has been described as tumor suppressor gene since many cancer cell lines (e.g. breast, colon, lung, *inter alia*) display allelic loss of T-cad which is correlated with tumor progression (47). Frequent epigenetic modification of T-cad promoter by aberrant methylation resulting in allelic loss and diminished expression of T-cad has been reported for a variety of cancers including breast (48), lung (49, 50), colorectal (51) (52), cervical (53), and

nasopharyngeal (54), digestive tract (55, 56), skin (57, 58), pancreatic (59) cancers and also chronic myeloid leukemia (60) and malignant B cell lymphomas (61). In PC12 cells T-cad has been demonstrated to be a target gene for DNA methyltransferase 3B (Dnmt3b), but Dnmt3b-dependent suppression of T-cad expression occurs via methylation-independent mechanisms (62). T-cad has been shown to be regulated by glucocorticoids and aryl hydrocarbon agonists in osteocarcinoma cell lines and vascular smooth muscle cells, respectively (63, 64).

Investigations on T-cad in the cardiovascular system, where T-cad is highly expressed, suggest biological functions that are distinct from the tumor suppressive role described in the field of oncology, but which rather bear resemblance to the negative guidance role described in the embryonic nervous system. Current knowledge about the role of T-cad in the vasculature is discussed in detail in section 1.3.

1.2 Vascular remodeling

Normal vasculature: structure and function

The normal muscular artery comprises three layers (Fig. 7). The adventitia, the outermost layer, consists of fibroblasts, collagen, proteoglycans and *vasa vasorum*. The media comprises smooth muscle cells (SMC) and collagen fibers. Media and adventitia are separated by the external elastic lamina. The intima is composed of endothelial cells (EC) and underlying connective tissue. Intima and media are separated by the internal elastic lamina.

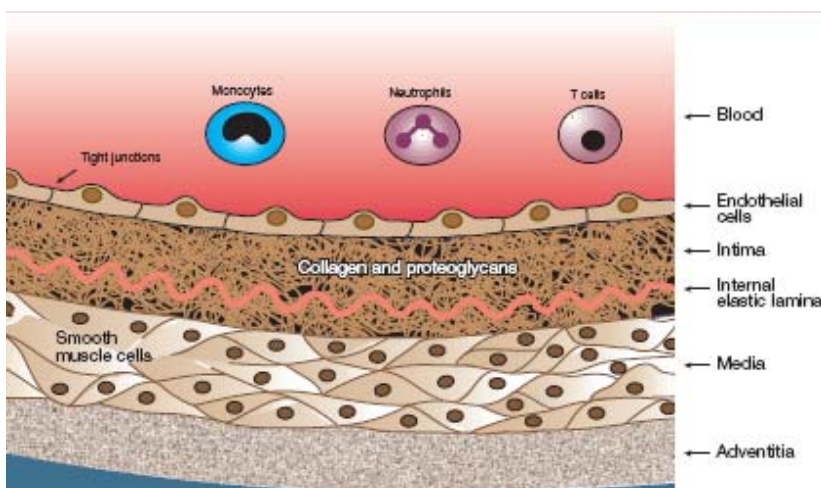


Figure 7: Schematic structure of normal large artery (65).

The vascular endothelium forms a continuous monolayer lining blood vessels and heart. In adult human the endothelium covers a surface of 1000m², displaying a cobble stone morphology with polygonal and tightly bound EC (66, 67). Functions of EC are heterogeneous in different vascular bed due to differential gene expression and the influence of surrounding tissues (68). EC regulate many biological processes: (1) serve as a semi-permeable membrane, controlling transfer of molecules into the vessel wall; (2) maintain a non-thrombogenic blood-tissue interface by regulating thrombosis, thrombolysis and platelet adherence; (3) modulate vascular tone and blood flow; (4) regulate immune inflammatory reactions, largely controlling leukocyte interactions with vessel wall; (5) modify lipoproteins in artery walls; (6) regulate growth of other cell types, particularly SMC.

SMC are spindle shaped with single elongated nuclei. SMCs perform many important functions; (1) vasoconstriction and dilation in response to physiological and pharmacological stimuli by contraction and relaxation, thereby controlling blood pressure; (2) synthesis and deposition of collagen, elastin and proteoglycan; (3) synthesis and secretion of growth factors, cytokines; (4) migration and proliferation during vessel repair and remodeling.

Vascular remodeling in proliferative vascular disorders (atherosclerosis, restenosis)

Vascular remodeling is essentially a process of redistribution of the cellular constituents of vessel wall resulting in change of their structure and function without changing volume. Vascular remodeling is a physiologically protective phenomenon during embryogenesis, wound healing and the reproductive cycle. Pathological vascular remodeling leads to various vascular disorders such as hypertension, atherosclerosis and restenosis after vessel injury, resulting in enhanced vasoconstriction and reduced blood flow. Clinical complications resulting from pathological remodeling include myocardial infarction and stroke.

Abnormal endothelial activation such as bacterial infection or inflammation leads to local alterations in the endothelium affecting homeostasis, control of vascular tone and increased permeability. Such malfunctions of the endothelium are collectively referred to as *endothelial dysfunction* (69). A dysfunctional endothelium is more permeable to cholesterol containing low density lipoprotein which increasingly accumulate within the intima, undergo oxidation and promote atherosclerosis. Activated EC express intercellular adhesion molecule (ICAM), vascular adhesion molecule (VCAM), platelet- endothelial cell adhesion molecule (PECAM), selectin and integrins for recruitment of monocytes (70), followed by their transendothelial migration into

intima. Within the intima monocytes differentiate into macrophages in response to macrophage colony-stimulating factor and produce reactive oxygen species which oxidize LDL (65). Oxidized LDL is engulfed by macrophages which become foam cells. T-cells (usually CD4+), mast cells, dendritic cells, B-cells and NKT cells are different immunocompetent cells that can migrate into the intima and produce cytokines like interleukins (IL-4, IL-13, IL-5, IL-10, IL-8), TGF β , IFN- γ and TNF α . The cytokine-rich extracellular milieu sustains a proinflammatory status and SMC proliferation and migration to the intima (*intimal thickening*), and also induces neovessel formation (*neovascularization*); the combination of these processes increases the mechanical vulnerability of the atherosclerotic plaque and the risk of plaque rupture and thrombosis (71, 72). A schematic representation of cellular composition of atherosclerotic plaque is shown in figure 8.

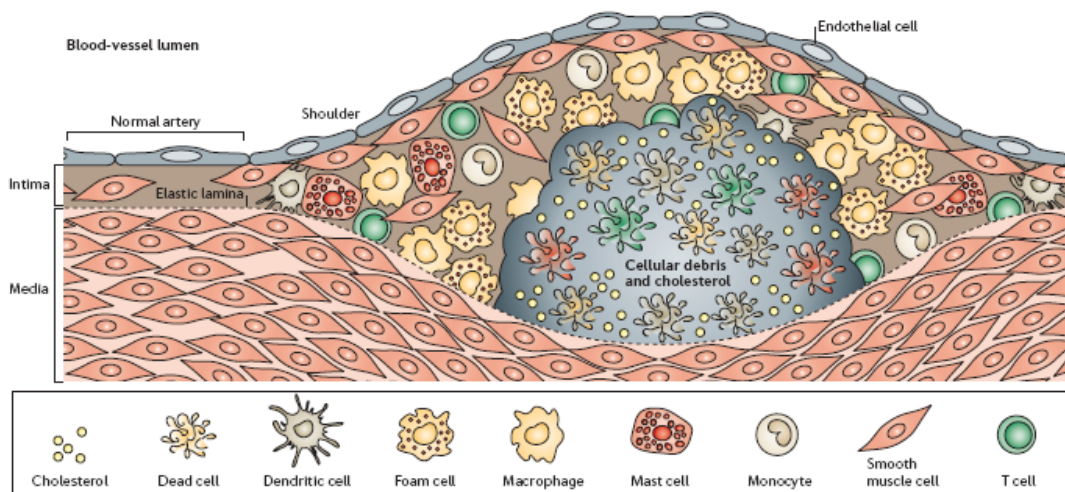


Figure 8: Cellular composition of atherosclerosis plaque(71).

Intimal thickening: Proliferation and migration of SMC from media to intima plays a key role in neointima formation and thickening. Followed by intimal injury, different cell types including EC, platelets and inflammatory cells release growth factors and cytokines which results in phenotypic change of SMC from contractile quiescent state to active synthetic state leading to SMC proliferation (65, 73). Platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor β 1 (TGF β) epidermal growth factor (EGF) and insulin growth factor (IGF) are major growth factors affecting SMC proliferation and migration (74). SMC migration and proliferation is also modulated by extracellular matrix (ECM) components such as integrins, collagen, fibronectin, thrombospondin, tenascin and vitronectin (75), and by metalloproteinases and cadherins (76).

Neovascularization: Vasa vasorum normally nurture the outer (adventitial) layer of the vessel wall. Neovascularization frequently accompanies pathological thickening of the intima; there is an increase in *vasa vasorum* which become a major source for nutrients to the thickened vessel wall (77). EC proliferation, migration and outgrowth are key cellular events for neovascularization within plaque. Intimal thickening creates conditions of hypoxia whereby hypoxia inducible factor (HIF-1) is released with subsequent induction of proangiogenic molecules such as vascular endothelial growth factor (VEGF), VEGF receptors, endothelial nitric oxide synthase (eNOS) and ephrins (78). Intra-plaque neovascularization facilitates RBC extravasation, lipid deposition and cause internal bleeding resulting in plaque instability (77).

Oxidative stress in cardiovascular disorders

Oxidative stress is a common risk factor in many cardiovascular diseases and plays a pivotal role in each step of pathophysiology of atherosclerosis and other cardiovascular disorders. Important sources of reactive oxygen species (ROS) are NADPH oxidases, Xanthine oxidase and eNOS (79). ROS produced from vascular cells oxidizes LDL leading to stimulation of macrophages and other inflammatory cells (72). Cytokines, TNF- α , angiotensin II and VEGF induce adhesion molecules such as VCAM-1, ICAM-1 and P-selectin in a ROS dependent fashion (80). As mentioned above SMC proliferation and migration are key processes in neointima formation; induction of SMC proliferation and migration by PDGF requires endogenously produced H₂O₂ (81). Metalloproteinases facilitating degradation and reorganization of extracellular matrix during vascular remodeling are activated by ROS (82). Significant induction of NADPH oxidase dependent ROS is observed in experimental restenosis (rat carotid artery balloon injury) (83). Oxidative stress is also reported to induce intraplaque angiogenesis in experimental models leading to plaque destabilization (84). Various roles of oxidative stress in cardiovascular disorders have been extensively reviewed (85-88).

1.3 T-cadherin in the vasculature

T-cadherin is widely expressed in the vasculature. EC and SMC, resident cells of the vessel wall express both precursor (130kDa) and mature (105kDa) forms of T-cad. Initially T-cad was identified in human aortic media membranes isolates as an atypical low density lipoprotein (LDL) binding protein of 105kDa (called p105) using ligand (LDL) binding techniques (89). Partial sequencing of this p105 revealed its identity as T-cad (90). Subsequent ligand binding studies

demonstrated the co-existence of the 130kDa precursor form of T-cad in membranes of SMC and its ability to bind LDL (91). A 100-fold molar excess of HDL was required to abrogate 50% of the LDL-binding activity of T-cad, suggesting lipoprotein-specificity of this activity (89). GPI-specific phospholipase C treatment of SMC and conditions of calcium-depletion inhibited the LDL-binding activity of T-cad (91). HEK293 cells transfected with T-cad exhibited increased cell aggregation and LDL binding activity in a both calcium- and GPI-dependent- fashion (92). LDL-binding to T-cad was not detected when electrophoresis was conducted under reducing conditions suggesting a requirement for intact disulfide linkages (89). GPI-less T-cad transfected in HEK293 cells and *E. coli* did not exhibit LDL binding indicating a requirement of the GPI moiety; other GPI-anchored proteins did not show any affinity towards LDL (92). Another group later demonstrated that in HEK293 cells transfected with T-cad LDL-binding induced elevation of intracellular calcium levels and increased cell migration (93).

Although T-cad is expressed in various organs and tissues, Western blotting (Fig. 7) revealed its predominant expression in cardiovascular tissues (heart, aorta, carotid, iliac and renal arteries, with the highest level in the aorta) (94). In the nervous system T-cad protein was shown to be expressed in spinal cord, but not in the cerebral cortex, cerebellum and hypothalamus. However, Northern analysis revealed the presence of T-cad mRNA in cerebral cortex, thalamus and midbrain (46). T-cad is not expressed at detectable levels in secretory organs (liver, pancreas, thyroid and adrenals), hollow organs (stomach, esophagus, intestine, and bladder) and pulmonary tissues (Fig. 7). *In vitro* cultures of epithelial cells, neuronal cells and fibroblast cells do express T-cad.

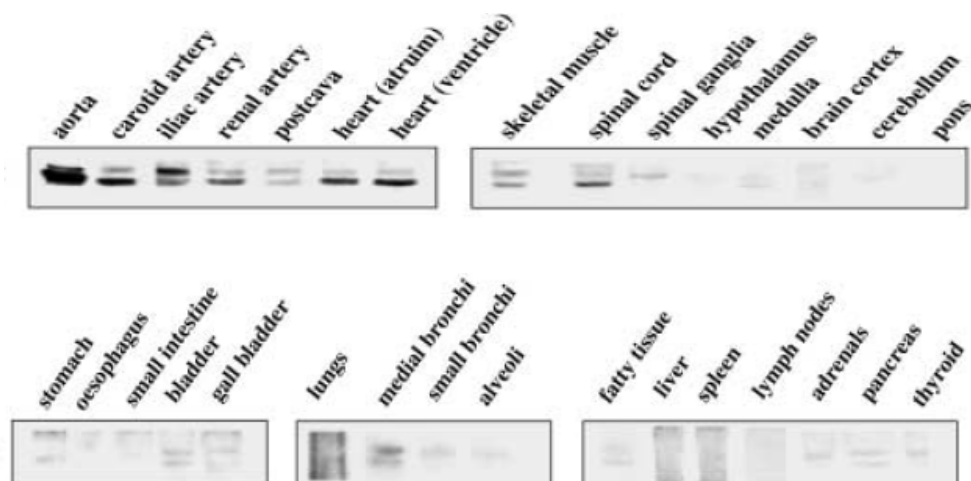


Figure 7: Expression of T-cad in human tissues/organs (94).

Immunohistochemical analysis of aorta sections revealed expression of T-cad in EC, SMC and pericytes but not in adventitial fibroblasts or in blood derived cells (Fig. 8A & B). The highest expression of T-cad was observed in intima and in its proteoglycan layer.

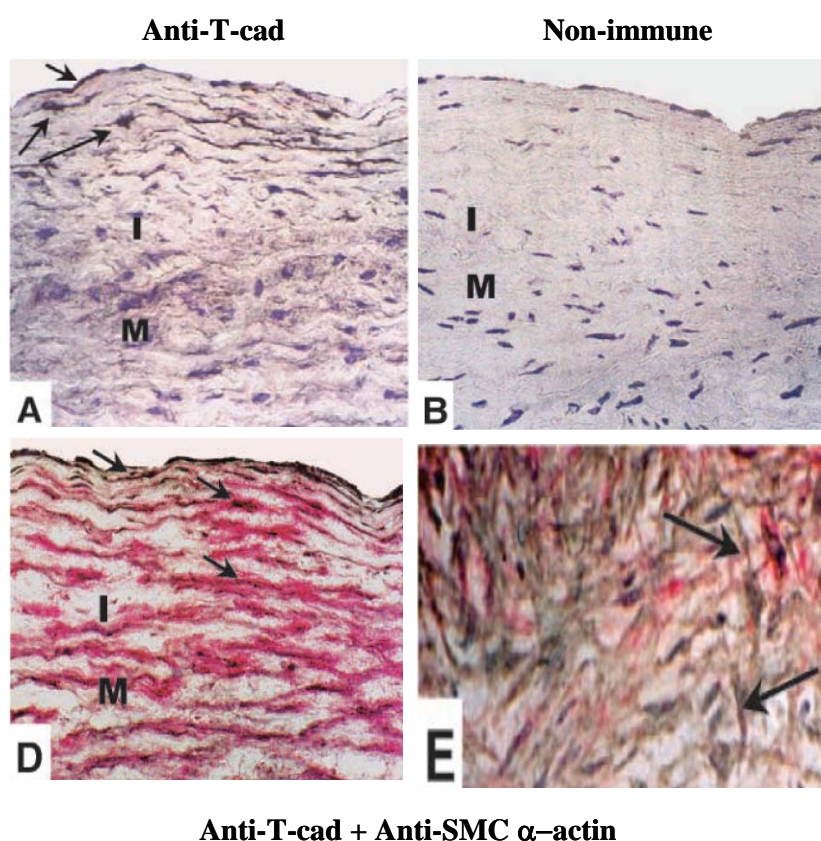


Figure 8: T-cad in human aorta sections (94).

T-cad was colocalised with smooth muscle α -actin and an inverse relationship between intensities of their expression was observed (94). Cells located to the subendothelial intimal layer expressed low levels of smooth muscle α -actin and high levels of T-cad. In contrast, cells in the deeper muscular-elastic intimal layer displayed high levels of smooth muscle α -actin and low levels of T-cad, suggesting a phenotype-associated expression of T-cad expression (Fig. 8 D&E). Although T-cad was not present in the adventitial layer, T-cad was highly expressed in adventitial *vasa vasorum* (composed of EC).

Immunohistochemical analysis of atherosclerotic lesions of varying severity indicated upregulation of T-cad in intimal SMC as compared to normal aortal tissue (94). Experimental restenosis following balloon catheterization of the rat carotid artery was associated with elevation of T-cad expression at an early time after endothelial injury, and with a further elevation during the

subsequent phase of neointimal formation that is characterized by increased SMC cell migration and proliferation (95). Taken together the data suggested that upregulation of T-cad during atherosclerosis and experimental restenosis might play some role in vascular remodeling.

GPI-anchored T-cad is not localized at adherent junctions but rather distributed globally over the cell surface. T-cad is localized within lipid rafts; these are cholesterol- and sphingolipid-rich membrane domains which are enriched with other GPI-anchored proteins (e.g. CD59 and uPA receptor) and signaling molecules (e.g. Src kinases) (96). This raft domain location of T-cad together with the outward orientation of T-cad implies some sensory function and participation in signal transduction.

In vitro data supports participation of T-cad in many cellular processes such as vascular differentiation, migration and proliferation of SMC and EC and angiogenesis. Adenoviral mediated overexpression of T-cad in EC and SMC resulted in cell cycle progression and a concomitant promotion of proliferation (97). T-cad exhibits adhesive functions upon homophilic ligation with antibody against T-cad or with recombinant immobilized soluble protein (98). Homophilic ligation of T-cad induces polarization and migration of endothelial cells in a RhoA/ROCK and Rac dependent fashion (99, 100). T-cad stimulates in-gel outgrowth of endothelial sprouts in 3-dimensional EC-spheroid and heart tissue models of angiogenesis. *In vivo*, myoblast-mediated delivery of recombinant soluble T-cad to mouse skeletal muscle facilitates VEGF-induced angiogenesis, supporting a physiological role for T-cad as a proangiogenic protein (101).

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2. Dissertation objectives

In spite of major progress toward understanding the function of T-cad in vascular cells, knowledge regarding the regulation of T-cad expression and the signaling mechanisms whereby T-cad affects vascular cell behaviour is limited. This thesis focuses on three issues.

T-cad and oxidative stress (section 3.1.):

In vascular tissue, T-cad is up-regulated in vivo under disease conditions associated with oxidative stress and concomitant cell migration, proliferation and apoptosis/survival. It is possible to hypothesize some functional relationship between oxidative stress, T-cad expression, and cell survival status. The specific objectives here were:

- 1) To investigate whether T-cad is modulated in EC by oxidative stress
- 2) To investigate whether T-cad plays a role in regulating apoptosis/cell survival of EC
- 3) To identify intracellular signaling pathways activated by T-cad in EC

T-cad and molecular mediators (section 3.2.):

T-cad influences several parameters of angiogenesis including endothelial cell (EC) differentiation, migration, proliferation and survival. This presupposes signal transduction networking via mediatory regulators and molecular adaptors since T-cad lacks transmembrane and cytosolic domains. The specific objectives here were:

- 1) to identify a membrane proximal molecule that could mediate inward signal transmission by GPI-anchored T-cad.
- 2) to further delineate proliferation and survival signal transduction mechanisms activated by T-cad in EC

Transcriptional regulation of T-cad (section 3.3.):

T-cad has been referred to as a tumor suppressor gene since T-cad expression is diminished by aberrant promoter methylation in malignant cells. The transcriptional regulation of T-cad in vascular cells has not been studied. The specific objectives here were:

- 1) to characterize the minimal promoter region of T-cad
- 2) to study the transcriptional regulation of T-cad in EC

3. Methods and Results

3.1. T-cad and oxidative stress

The results of this project have been published.

T-cadherin protects endothelial cells from oxidative stress-induced apoptosis.

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T-cadherin protects endothelial cells from oxidative stress-induced apoptosis

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ABSTRACT

In vascular tissue, T-cadherin (T-cad) is up-regulated in vivo under disease conditions associated with oxidative stress and concomitant cell migration, proliferation and apoptosis/survival. Using cultures of human umbilical vein endothelial cells (HUVEC), we examined whether there is a functional relationship between oxidative stress, T-cad expression, and cell survival status. Culture of HUVEC under conditions of oxidative stress (e.g., serum deprivation, inclusion of H₂O₂) resulted in increased T-cad expression. Oxidative stress-induced increases in T-cad were inhibited by the free radical-scavenging antioxidant, N-acetylcysteine, and the flavin-containing oxidase inhibitor, diphenyleneiodonium. Thus reactive oxygen species (ROS) contribute to stress-induced elevation of T-cad in HUVEC. Compared with control cells, HUVEC overexpressing T-cad (T-cad+HUVEC) had higher phosphorylation levels for phosphatidylinositol 3-kinase (PI3K) target Akt and mTOR target p70^{S6K} (survival pathway regulators), but lower levels for p38MAPK (death pathway regulator). T-cad+HUVEC exposed to stress (serum-deprivation, TNF- α , actinomycin D, staurosporine) exhibited reduced caspase activation together with increased cell survival. Protection against stress-induced apoptosis in T-cad+HUVEC was abrogated by either PI3K-inhibitor wortmannin or mTOR-inhibitor rapamycin. We conclude that T-cad overexpression in HUVEC protects against stress-induced apoptosis through activation of the PI3K/Akt/mTOR survival signal pathway and concomitant suppression of the p38 MAPK proapoptotic pathway. ROS-induced changes in T-cad expression may play an important role in controlling tissue cellularity during vascular remodeling.

Key words: serum-withdrawal • survival • signaling

T-cadherin (T-cad, H-cadherin, cadherin-13) is an atypical glycosylphosphatidylinositol (GPI)-anchored member of the cadherin superfamily (1). In contrast to classical cadherin family members, T-cad lacks the typical transmembrane and cytoplasmic domains, is globally distributed over the cell body, is absent at intercellular contacts in cell monolayers, and undergoes redistribution to the leading edge in migrating vascular cells (2). T-cad can mediate calcium-dependent, homophilic binding in transfected cells, but these interactions are weak because absence of linkage to the cytoskeleton does not permit the formation of stable

intercellular adhesive forces (1, 3). Moreover, in polarized transfected cells T-cad is localized to the apical but not the baso-lateral surface (4). Thus T-cad is functionally distinct from classical cadherins with respect to formation of intercellular adherent junctions.

Accumulating data indicate that T-cad may have multiple signaling functions in vascular remodeling processes. In vascular tissue, T-cad is up-regulated during atherosclerosis (5), restenosis after balloon angioplasty (6), and tumor angiogenesis (7), these being conditions associated with migration and proliferation, as well as concomitant apoptosis of resident smooth muscle cells (SMC) and endothelial cells (EC) (8–11). T-cad has been attributed to function as a guidance receptor, which would be relevant to migratory processes. Homophilic ligation of T-cad has been shown to function as a repulsive cue for motor-axon projection in the embryonic nervous system (12), to promote deadhesion in SMC and EC (13), and also to induce RhoA/ROCK- and Rac-dependent cell polarization and directed cell migration in EC (14). A positive functional relationship between levels of T-cad expression and proliferation in vascular cells also exists, although this does not depend upon homophilic ligation. T-cad levels were higher in sparsely seeded, exponentially growing cultures of SMC than at confluency (15); cell cycle analysis in SMC and EC cultures demonstrated higher expression of T-cad in S- and G2/M-phases than in G1/G0-phase (16), and overexpression of T-cad in EC or SMC facilitated cell cycle progression and proliferation (16). Paradoxically, up-regulation of T-cad was observed in rat aortic SMC cultured after serum starvation for a prolonged (48 h) period (15). Withdrawal of growth factors and nutrients from cultures of normally adherent cells can lead to weakened extracellular matrix attachment and induction of apoptosis (17, 18). However, given the positive relationship between T-cadherin expression and proliferation of vascular cells *in vivo* (5–7) and *in vitro* (16), up-regulation of T-cad may rather participate in cell survival responses to stress.

Here, we address mechanisms underlying the elevation of T-cad in serum-starved EC and the influence of elevated T-cad on EC survival status. *In vitro*, serum withdrawal is known to induce oxidative stress through excess production of reactive oxygen species (ROS) (19–21), and disruption of cell attachment to extracellular matrix leading to cell detachment is also associated with an elevation of ROS (17, 22). ROS are also elevated *in vivo* during atherosclerosis, restenosis, and angiogenesis and play a major role in control of tissue cellularity through signaling effects on diverse biological processes, including both survival and apoptosis (11, 23–26). The ultimate survival or death response to a given condition of oxidative stress reflects the balance between signaling pathways that are directly or indirectly modulated by ROS. Vascular cells possess a multitude of redox-sensitive signaling systems, including, *inter alia*, the extracellular signal-regulated kinases (ERKs), the stress-activated protein kinase (SAPK) family, which include c-Jun N-terminal kinases (JNKs) and p38 MAPK, Akt, and caspases (11, 23–27). In this study, we investigated whether oxidative stress up-regulates T-cadherin expression in EC. The functional role of the increase in T-cadherin expression in endothelial cell survival and the underlying signaling pathways were analyzed.

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell GmbH (Heidelberg, Germany) and normally cultured in basal endothelial cell growth medium

containing low serum (2% FCS) and endothelial cell growth supplement (PromoCell). All tissue culture surfaces were precoated with 0.1% gelatin, and HUVEC were used at passages 2-6, during which expression of markers (von Willebrand factor, CD 31, VE-cadherin) for differentiated EC remained steady. Some experiments were repeated using the immortalized human microvascular EC line HMEC-1 (28), for which culture conditions were as above but with FCS at 10%. Apoptosis was induced by inclusion of TNF- α (40 ng/ml), staurosporine (1 μ M), actinomycin D (100 ng/ml), high glucose (30 mM) or serum withdrawal (DMEM/0.1% BSA). For analysis of the involvement of ROS, the following compounds were used; H₂O₂ (1 mM), for generation of ROS, N-acetylcysteine (NAC; 30 mM), a ROS scavenger, diphenyleneiodonium (DPI; 10 μ M), an inhibitor of flavin-containing oxidases, mitochondrial (complex I) NADH dehydrogenase inhibitor (rotenone; 10 μ M), and inhibitor of the mitochondrial (complex III) cytochrome *b-c₁* inhibitor (antimycin A; 10 μ M), and nitric oxide synthase (NOS) inhibitor *N*-monomethyl-L-arginine (L-NAME; 1 mM). Wortmannin (10 nM) and rapamycin (10 nM) were used to inhibit PI3 kinase (PI3K) and phosphorylation of p70 S6 kinase (p70^{S6K}), respectively. HA14-1 (50 μ M) was used to inhibit Bcl-2. The above chemical compounds were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany). Experimental protocols variously applied in this study are described within the Results and figure legends.

Fluorescence microscopy

HUVEC were plated (3×10^4 cells/well) into 8-well chamber Lab-Tek Permanox slides precoated with 0.1% gelatin, cultured overnight, and then subjected to serum deprivation until the cells began to detach (4–6 h). HUVEC were fixed with 4% paraformaldehyde in PBS, preincubated with 10% FCS in PBS and then immunostained for T-cad using purified polyclonal antibody against the first extracellular domain of T-cad (or nonimmune rabbit IgG for controls) and secondary Cy3-labeled anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) (2, 13). Nuclei were counterstained with Hoechst (Molecular Probes, Leiden, Netherlands). All procedures were carried out at room temperature and with careful rinsings in order to avoid loss of all weakly adherent cells. Images were captured using an Olympus IX-50 inverted microscope equipped with a digital camera (Olympus Optical, Schwerzenbach, Switzerland) and AnalySIS 3.2 software (Soft Imaging System GmbH, Münster, Germany).

Adenoviral infection

Overexpression of T-cad in HUVEC using Adeno-X Expression System (Clontech, Palo Alto, USA), determination of viral titers and infection protocols have been detailed previously (13, 16). Briefly, HUVEC in normal growth media were seeded at a density of $1-2 \times 10^4$ cells/cm², allowed to adhere and infected overnight with empty- or T-cad-containing adenoviral particles at a final approximate concentration of 100 pfu/cell. Uninfected parental HUVEC and empty-vector- or T-cad-containing vector infected cells adhere identically onto the standard gelatin-coated substratum (13).

Immunoblotting

The method of immunoblotting has been described previously (15). Lysis buffer was normally PBS containing 1% SDS and protease inhibitor cocktail (Sigma), with inclusion of 1 mM orthovanadate and 5 mM NaF in samples analyzed for protein phosphorylation status, 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 G α s subunit. The following primary antibodies were used: rabbit antibody against the first extracellular domain of T-cad generated in our lab (5); anti-G α s (Calbiochem, Darmstadt, Germany); anti-cleaved caspase-3 (Asp¹⁷⁵), Akt, phospho-(Ser⁴⁷³)Akt, Fak, phospho-(Tyr^{576/577})Fak, ERK1/2, phospho-(Thr²⁰²/Tyr²⁰⁴)ERK1/2, p38, phospho-(Thr¹⁸⁰/Tyr¹⁸²)p38, JNK1, phospho-(Thr¹⁸³/Tyr¹⁸⁵)JNK1, p70^{S6K} and phospho-(Thr³⁸⁹)p70^{S6K}, BCL-X_L, p21 (all from Cell Signaling, New England Biolabs GmbH, Frankfurt, Germany), survivin (Abcam, Cambridgeshire, UK). For negative controls, nonimmune rabbit or mouse IgG (Sigma-Aldrich Chemie, Deisenhofen, Germany) were appropriately used as the substitute for primary antibody. Secondary HRP-conjugated anti-rabbit IgG (1:5000) and HRP-conjugated anti-mouse IgG (1:5000) were from Southern Biotechnology (BioReba AG, Reinach, Switzerland). Amersham ECL (Amersham Biosciences, Little Chalfont, UK) and the Enhanced Luminescence System or SuperSignal West Dura from Pierce (Rockford, IL) were variously used for detection of immunoreactive proteins. Scanned images of Ponceau S stains and immunoblots were analyzed using AIDA Image or Scion (NIH) Image software. Figures show representative immunoblots.

Assay for caspase activity

HUVEC were seeded at 2×10^4 cells/well in 96-well plates and allowed to adhere overnight. After apoptosis induction protocols as described in the Results, cells were assayed for caspases activity fluorimetrically ($\lambda_{\text{ex}}=499$ and $\lambda_{\text{em}}=520$) using a fluorimetric caspase assay kit (Homogeneous caspases assay as per manufacturer's instructions; Roche Diagnostics GmbH, Mannheim, Germany). Substrate solution was added to wells without prior removal of any detached cells or medium change. Unless otherwise specified, caspase activity is expressed as arbitrary fluorescence units (au.) per 2×10^4 cells.

Assay for cell survival

For these assays, HUVEC were seeded at 1.5×10^5 cells/well in 6-well plates and allowed to adhere overnight before experimentation. To estimate cell survival following treatments, detached cells were removed by medium aspiration, wells were rinsed with PBS and numbers of remaining adherent cells quantitated by enzymatic detachment (0.25% trypsin/1mM EDTA in PBS) and counting in a Coulter counter. Cell survival (presented in figures as % of serum control), expresses the number of adherent cells present after a given treatment relative to those in untreated controls (arbitrarily taken as 100%).

Statistics

Unless otherwise stated all results are given as means \pm SD, and at least 3 independent experiments for any given protocol were performed. Statistical analyses were performed by one-way ANOVA followed by post hoc Bonferroni's multiple comparison test when appropriate. A *P* value of <0.05 was considered significant.

RESULTS

T-cad is up-regulated in apoptotic cells

The first series of experiments were designed to generally assess whether there might be some relationship between T-cad expression on endothelial cells and apoptosis. HUVEC were cultured under a variety of conditions known to induce apoptosis; these included culture under normal serum-containing conditions with inclusion of high glucose (30 mM) for 72 h, inclusion of TNF- α (40 ng/ml), staurosporine (1 μ M), or actinomycin-D (100 ng/ml) for 18 h, and also culture under conditions of serum starvation for 18 h. After treatment, dishes were tapped to dislodge weakly adherent cells, and then all detached cells were collected by medium aspiration and centrifugation. Separate lysates of the detached and adherent cell populations were prepared and analyzed by Western blotting for T-cad and active caspase 3. Regardless of the apoptosis-inducing condition, there was a consistent 2-3-fold elevation of T-cad expression in detached HUVEC, as compared with adherent HUVEC or untreated control HUVEC, and active caspase 3 was detectable only in detached cell populations ([Fig. 1A](#)). Expression of G α subunit, which served as the internal control, remained steady.

The above experiments might imply up-regulation of T-cad only in apoptotic cells. To investigate this issue, immunocytochemical analysis was performed after an abbreviated (4-6 h) period of serum deprivation to enable fixation of both adherent HUVEC and those undergoing detachment. HUVEC were stained for T-cad using primary anti-T-cad/secondary Cy3-labeled antibodies and with Hoechst for nuclear counterstaining ([Fig. 1B](#)). An increased intensity of staining for T-cad was clearly evident after serum deprivation, which in some cells was also associated with nuclear chromatin condensation (i.e., late stages of apoptosis). We could only detect a few such apoptotic cells, because in spite of careful handling procedures, most of the detaching/apoptotic cells are washed off. Nevertheless, the data demonstrate that T-cad is up-regulated after apoptosis induction.

In subsequent experiments, we focused on the response of HUVEC under conditions of serum-deprivation since this is one of the most widely used and reproducible experimental techniques for apoptosis induction in nontransformed cells.

Serum withdrawal induced apoptosis: T-cad elevation precedes caspase activation

To determine the kinetics of changes in T-cad expression in response to serum withdrawal, HUVEC were serum-deprived for different time intervals, and after removal of detached cells by gentle rinsing of culture wells the adherent cell population was lysed and immunoblotted for T-cad. A transient ~2-fold elevation of T-cad in adherent cells was observed within the first 2-3 h, and thereafter levels decreased to ~25% of control levels ([Fig. 2A](#)). Alterations in levels of T-cad, as measured in whole cell lysates reflect changes in surface expression, as verified by an increased level of T-cad levels in plasma membrane preparations (29) and by an increased immunofluorescence after live cell staining for T-cad (data not shown). Analysis of caspase activity in HUVEC cultures (assay including both detached and adherent cells) showed that caspase activation began within 4 h after serum withdrawal, reached peak activation at ~6-8 h, and then returned to basal levels ([Fig. 2B](#)). Permanent loss of attachment to the substratum is characteristic of endothelial cell death *in vitro*, and therefore to obtain an index of cell survival,

the numbers of adherent cells were quantitated at different intervals after serum deprivation. Loss of attachment initially occurred after ~4 h and by 6 h, 50% of cells had detached (Fig. 2C). Thus serum withdrawal-associated elevation in T-cad levels precedes activation of caspase(s) and cell detachment. Comparable results to those described above were obtained with the microvascular EC line HMEC-1 (data not shown).

Involvement of reactive oxygen species in T-cad elevation under conditions of serum withdrawal

Possible mechanism(s) leading to T-cad up-regulation under conditions of serum withdrawal were investigated. The ROS generated are causally involved in induction of apoptosis (21, 30), and in HUVEC, serum-withdrawal induces oxidative stress (19). Because ROS-induced apoptosis in HUVEC is related to reduction of expression of Bcl-2, a survival-associated protein (20, 31), we first tested whether the elevation of T-cad expression observed under conditions of serum withdrawal was dependent upon Bcl-2 inhibition. HUVEC under normal serum-containing culture conditions were exposed to HA14-1 (50 μ M, 4 h), an inhibitor of Bcl-2 activity (32). Induction of apoptosis by HA14-1 was confirmed by the presence of active caspase 3 in both adherent and detached cell populations, but this was not accompanied by an elevation of T-cad in either cell population (Fig. 3A). This observation suggests the stimulus for T-cad elevation occurs upstream of Bcl-2, and thus we next examined the contribution of ROS. HUVEC were incubated either under control serum-containing conditions or serum withdrawal conditions without or with inclusion of N-acetylcysteine (NAC; 30 mM), a ROS scavenger (33). Adherent cells were analyzed by immunoblotting for T-cad. NAC did not affect T-cad expression under control conditions (data only for serum+NAC shown, but not significantly different from serum only), but it completely abrogated the stimulatory effect of serum deprivation of T-cad expression (Fig. 3B). Interestingly, inclusion of NAC under serum withdrawal conditions progressively reduced T-cad expression to levels that were even lower than under control culture conditions. As a positive control for the association between T-cad expression and ROS, HUVEC were exposed to exogenous H₂O₂ (1 mM, 4 h) under normal serum-containing conditions without or with inclusion of NAC. Direct induction of oxidative stress by H₂O₂ resulted in an increased expression of T-cad in adherent HUVEC (and HEMC-1 cell line, data not shown), which could be completely inhibited by NAC (Fig. 3C).

To shed some light on the endogenous source of ROS leading to T-cad up-regulation under conditions of serum withdrawal, HUVEC were subjected to serum deprivation for 4 h in the presence of either diphenyleneiodonium (DPI), an inhibitor of flavin-containing oxidases, among them NADPH oxidase and xanthine oxidase, rotenone, the mitochondrial (complex I) NADH dehydrogenase inhibitor, antimycin A, the mitochondrial (complex III) cytochrome *b-c₁* inhibitor, and *N*-monomethyl-L-arginine (L-NAME), a nitric oxide synthase inhibitor. Western blot analysis of adherent cells showed that the serum withdrawal-induced increase in T-cad levels was sensitive to inhibition by DPI, but not by rotenone, antimycin A or L-NAME (Fig. 3C).

T-cadherin overexpression protects HUVEC from serum withdrawal-induced apoptosis

Because T-cad elevation in adherent HUVEC under conditions of serum withdrawal precedes caspase activation and T-cad levels were high in detached HUVEC, it is possible that T-cad

serves some proapoptotic function. However, ROS can elicit both apoptosis and survival signals, and our previous studies demonstrated that overexpression of T-cad in HUVEC increases their proliferative and migratory potential (13, 16). We investigated whether the up-regulation of T-cad, which occurs in HUVEC following serum withdrawal might represent a survival response. The time course of caspase activation (using fluorimetric caspase assay) in response to serum withdrawal in parental HUVEC (P) and HUVEC infected with empty (E) or T-cad (T-cad⁺)-containing adenoviral particles was compared. T-cad overexpression did not influence basal caspase activity under control serum-containing culture conditions, but following serum-deprivation, the degree of caspase activation was significantly blunted in T-cad⁺-HUVEC as compared with P- and E- HUVECs (Fig. 4A). We also examined whether overexpression of T-cad might protect HUVEC from apoptosis induced by TNF- α , staurosporine, or actinomycin-D. Caspase activity was assayed in P-, E-, and T-cad⁺- HUVEC after exposure to these compounds for 6 and 12 h. Parallel wells subjected to serum deprivation served as the positive apoptosis experimental control. Whereas a protective effect of T-cad overexpression was evident at both 6- and 12-h periods in serum-deprived cultures, in the case of TNF- α , staurosporine, or actinomycin-D protection against apoptosis was significant only at the 12-h period (Fig. 4B). Determination of the number of adherent cells after 8 h of incubation in the presence of TNF- α , staurosporine, or actinomycin-D revealed a significantly greater number for T-cad⁺-HUVEC than for P- and E-HUVEC (Fig. 4C). Taken together, the data indicate that up-regulation of T-cad can augment cell survival under different conditions of stress.

Signaling pathway(s) mediating T-cad-dependent protection against stress-induced apoptosis

The next series of experiments were aimed at determining the signal pathway(s) through which T-cad might mediate its survival effects. The PI3K/Akt/mTOR signal cascades are major effector pathways mediating survival signals downstream of oxidative stress (9, 24, 27). To assess their potential involvement in the survival/apoptosis protection effects P-, E- and T-cad⁺- HUVEC were serum-deprived in the absence or presence of either wortmannin, a PI3K inhibitor or rapamycin, a mTOR inhibitor. As expected (34), wortmannin concomitantly increased caspase activation (Fig. 5A) and decreased cell survival (Fig. 5B) in all HUVECs under both serum-containing ($P < 0.01$) serum withdrawal ($P < 0.001$) culture conditions. However, PI3-kinase inhibition abrogated the blunted apoptosis response of T-cad⁺-HUVEC to serum-deprivation; caspases activity and cell survival were comparable between T-cad⁺-, P- and E-HUVECs (Fig. 5). Inhibition of mTOR also abrogated the blunted apoptosis response of T-cad⁺-HUVEC to serum-deprivation (Fig. 5). These observations indicate that T-cad might signal through the PI3K/Akt/mTOR cascades. We next examined the phosphorylation status of PI3K target Akt in P-, E-, and T-cad⁺-HUVEC under serum- and serum deprivation (6 h) conditions. p-Akt in all cells was significantly reduced following serum deprivation, but for both culture conditions T-cad⁺ cells exhibited a significantly higher level of p-(Ser⁴⁷³)Akt than the control HUVECs (Fig. 6A). To verify the apparent “hyper”-activation of the PI3K/Akt pathway in T-cad⁺-HUVEC, we determined the phosphorylation status of p70^{S6K}, a downstream target (via mTOR) of Akt (35). Serum deprivation significantly decreased the levels of p-p70^{S6K} in P-, E-, and T-cad⁺-HUVEC. However, under both serum-containing and serum deprivation conditions, the level of p-p70^{S6K} cells in T-cad⁺-HUVEC was significantly higher compared with P- and E-HUVECs (Fig. 6B).

Because a reciprocal relationship between the PI3-kinase/Akt survival pathway and the p38 MAP kinase proapoptotic pathway has been demonstrated (34, 36, 37), we examined whether T-cad overexpression might concomitantly affect p38 MAPK phosphorylation. Serum deprivation significantly increased levels of p-p38 in all HUVEC, but in T-cad⁺-HUVEC, the levels of p-p38 under both serum-containing and serum deprivation conditions were significantly lower compared with P- and E-HUVECs ([Fig. 6C](#)).

To confirm that T-cad overexpression transmits survival signals through the PI3K/Akt/mTOR pathways, we analyzed the phosphorylation status of Akt, p38 and p70^{S6K}, as well as the levels of active caspase 3, after incubation of cells for 6 h under serum-containing and serum-free conditions and without or with inclusion of wortmannin (PI3K inhibitor) and rapamycin (mTOR inhibitor). As expected wortmannin reduces levels of p-Akt regardless of culture condition; however, whereas in the presence of serum, p-Akt remained higher in T-cad⁺-HUVEC (vs. P- and E-HUVEC), under conditions of serum withdrawal in the presence of serum, p-Akt in all HUVECs was reduced to a similar, very low level ([Fig. 7A](#)). In the case of p38, the inverse was true. Wortmannin increased p-p38 levels regardless of culture condition; however, in the presence of serum, p-p38 remained lower in T-cad⁺-HUVEC (vs. P- and E-HUVEC), whereas under conditions of serum withdrawal p-p38 in T-cad⁺-HUVEC increased to levels comparable at least with E-HUVEC ([Fig. 7A](#)). Rapamycin completely inhibited phosphorylation of p70^{S6K} in all cultures thus effectively eliminating the typical differential in p-p70^{S6K} between T-cad⁺-HUVEC and P- and E-HUVECs ([Fig. 7B](#)). However, rapamycin did not completely normalize p-p38. Levels of total Akt, p38, and p70 were not affected by culture conditions without or with inclusion of wortmannin and rapamycin ([Fig. 7](#)). Active caspase 3 was not detectable in any of the cultures under serum-containing conditions, either with or without wortmannin ([Fig. 7A](#)) or rapamycin ([Fig. 7B](#)). After serum withdrawal, active caspase 3 was present in P- and E-HUVECs, but barely detectable in T-cad⁺-HUVEC. The protective effect of T-cad overexpression on serum withdrawal-induced apoptosis was blocked by inclusion of wortmannin or rapamycin, whereby levels of active caspase 3 were comparable between T-cad⁺ and P- or E-HUVECs ([Fig. 7](#)). Inclusion of NAC did not eliminate the signaling differential between T-cad⁺-HUVEC and P- or E- HUVECs under serum or serum-withdrawal conditions (analysis performed only with respect to p-Akt/Akt, p-p38/p38, and caspase 3, data not shown). Taken together, these data indicate that overexpression of T-cad leads to a concurrent potentiation of the PI3K/Akt/mTOR survival pathways and repression of the p38 MAPK pro-apoptotic pathway.

Finally, we investigated the potential contribution of other survival/death pathways, including extracellular signal-related kinase ERK1/2, stress-activated protein kinase JNK/SAPK1 (27), FAK (38), Bcl-X_L (39), p21 (40), and survivin (41). T-cad⁺ and P- or E-HUVECs did not differ with respect to either total levels or the phosphorylation status of ERK1/2, JNK/SAPK1, and FAK under serum or serum withdrawal (6 h) conditions, and neither were any differences detectable for p21, Bcl-X_L or survivin ([Fig. 7C](#)). These pathways thus appear not to contribute to the prosurvival properties of T-cad overexpression. However, it is possible that under other experimental conditions, evidence for involvement of one or more of these pathways might emerge.

DISCUSSION

This study has examined whether there is a functional relationship between oxidative stress, T-cad expression, and cell survival status. Oxidative stress is an important determinant in the pathophysiology of atherosclerosis, restenosis, and angiogenesis, and the mechanisms by which oxidative stress mediates alterations in vascular cell function are complex. The outcome of oxidative injury varies with cell type, oxidative agent, and also with the extent and duration of injury. Furthermore, ROS are capable of eliciting both apoptosis and survival signals which can result in a wide range of responses encompassing proliferation, growth arrest, cell survival, and cell death (24, 26). Our investigation shows that ROS contribute to stress-induced elevation in T-cad on HUVEC and that overexpression of T-cad in HUVEC protects against stress-induced apoptosis through concomitant activation of the PI3K/Akt/mTOR survival signal pathways and suppression of the p38 MAPK proapoptotic pathway (Fig. 8).

Indirect evidence for a relationship between oxidative stress, T-cad expression, and apoptosis was obtained from experiments demonstrating a 2-3-fold up-regulation of T-cadherin in apoptotic HUVEC under a variety of conditions known to induce oxidative stress, including serum withdrawal (19) or exposure to TNF- α (42), staurosporine (43), actinomycin-D (44), and high glucose (45). A role for ROS in up-regulation of T-cad was directly supported by the ability of the potent antioxidant N-acetylcysteine (33) to completely abrogate both serum withdrawal- and H₂O₂-induced up-regulation of T-cad. Our observation of inhibition of the serum withdrawal-induced increase in T-cad by DPI suggests that the principle source of ROS derives from flavin-containing oxidases (which could include NAD(P)H oxidase and xanthine oxidase) (24, 33). Transcriptional mechanisms regulating T-cad expression in response to oxidative stress are not yet known.

The data showing increased T-cad in detached, apoptotic HUVEC populations and that serum withdrawal-induced T-cad up-regulation occurs within the adherent monolayer prior to caspase activation and cell detachment could be interpreted to indicate proapoptotic functions for T-cad. However, such an interpretation is in contradiction to our previous demonstration of enhanced proliferation potential in HUVEC and SMC that overexpress T-cad (13, 16). We therefore considered that elevation of T-cad in HUVEC under conditions accompanied by oxidative stress may rather represent a survival response and addressed this issue by examining the influence of T-cad overexpression on sensitivity to serum deprivation-induced apoptosis and signal transduction.

First evidence of some prosurvival function of T-cad was obtained by the experiments demonstrating that HUVEC overexpressing T-cad exhibited significantly reduced caspase activation and concomitant increased cell survival to not only serum deprivation, but also to TNF- α , staurosporine, and actinomycin-D. Further support that T-cad may function in cell survival was derived from experiments demonstrating participation of PI3K/Akt/mTOR cascade, which is a major survival signaling pathway in many cell types, including EC (24, 26, 27, 35). First, we observed higher phosphorylation levels (without a change in total protein) of both PI3K target Akt and mTOR target p70^{S6K} in T-cad+ HUVEC under both serum-containing and serum-deprived culture conditions provide that T-cad might signal through the PI3K/Akt/mTOR cascade. Second, under serum-deprived conditions, levels of active caspase 3, caspases activity, as well as cell survival were normalized (between T-cad+, - and E-HUVECs) by inclusion of

either PI3K inhibitor (wortmannin) or mTOR inhibitor (rapamycin). “Constitutive” (i.e., under normal serum-containing conditions) hyperactivation of the PI3K/Akt/mTOR pathway in T-cad⁺ HUVEC may endow these cells with an improved ability to resist induced apoptosis, as evidenced by the almost negligible presence of active caspase 3 following serum deprivation. Because the PI3K/Akt/mTOR cascade is also important for driving G1-S cell cycle progression, its hyperactivation in T-cad⁺ HUVEC might clarify our earlier finding of their increased proliferation potential (16).

Of other herein studied MAPK and SAPK pathways that can modulate cell survival and are affected by oxidative stress (i.e., ERK1/2, JNK/SAPK1, and p38 MAPK/SAPK2), T-cad overexpression affected only p38 MAPK/SAPK2, which is an important modulator of the proapoptotic pathway in various cell types, including EC (34, 36, 37). Our results showing that the phosphorylation level of p38 is reduced in T-cad⁺ HUVEC under both serum-containing and serum-deprived culture conditions, provide additional evidence for survival effects due to T-cad overexpression. The “constitutive” suppression of p38 MAPK in T-cad⁺ could confer an apoptosis resistance additional to that afforded by hyperactivation of the PI3K/Akt/mTOR cascade. Wortmannin, but not rapamycin, caused near normalization (between T-cad⁺ and control HUVECs) of p38 phosphorylation, which is consistent with previous studies demonstrating cross-talk between PI3K/Akt and p38 MAPK pathways in HUVEC (34, 36, 37).

Antiapoptotic effects involving PI3K/Akt activation have also been shown for N-, E- and VE-cadherins. Homophilic cadherin engagement-dependent survival signals mediated downstream of PI3K/Akt activation are diverse. These include stabilization of antiapoptotic Bcl-2 protein expression for N-cadherin in prostate carcinoma cells (46); either down-regulation of proapoptotic MEK/ERK1/2 in intestinal epithelial cells (47) or up-regulation of MEK/ERK1/2 and Bcl-2 in squamous carcinoma cells (48) for E-cad; and down-regulation of proapoptotic p53/p21 (49) and of survivin (50) for VE-cadherin in endothelial cells. The mechanisms of signal transmission are likely to be distinct from those used by T-cad. For N-, E- and VE-cadherins, Akt activation depends upon homophilic adhesion interactions and requires association of PI3K with the cadherin-catenin-cytoskeletal complex (46, 47, 49). The prosurvival effects demonstrated in this study were not dependent on confluency (i.e., all experiments performed on cultures at ≈60–80% confluency). Moreover, T-cad lacks the transmembrane and cytoplasmic domains necessary for catenin mediated linkage to the cytoskeleton, and does not mediate homophilic intercellular adhesion (2, 13). It can be speculated that T-cad-dependent signaling occurs because of its location within rafts (51), in which diverse signaling molecules compartmentalize and can be variously assembled to generate specific signaling platforms (52). The molecular adaptors with which T-cad must coassemble or interact in order to effect intracellular signaling have not yet been investigated.

In conclusion, we demonstrated that 1) T-cad is up-regulated in EC in response to oxidative stress (i.e., serum deprivation) via flavin-containing oxidases and 2) T-cad overexpression in EC can elicit survival signaling responses that protect against stress-induced apoptosis. T-cad may thus contribute to regulation of tissue cellularity not only by stimulating endothelial cell proliferation and migration (13, 16), but also by promoting cell survival under stress conditions.

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Fig. 1

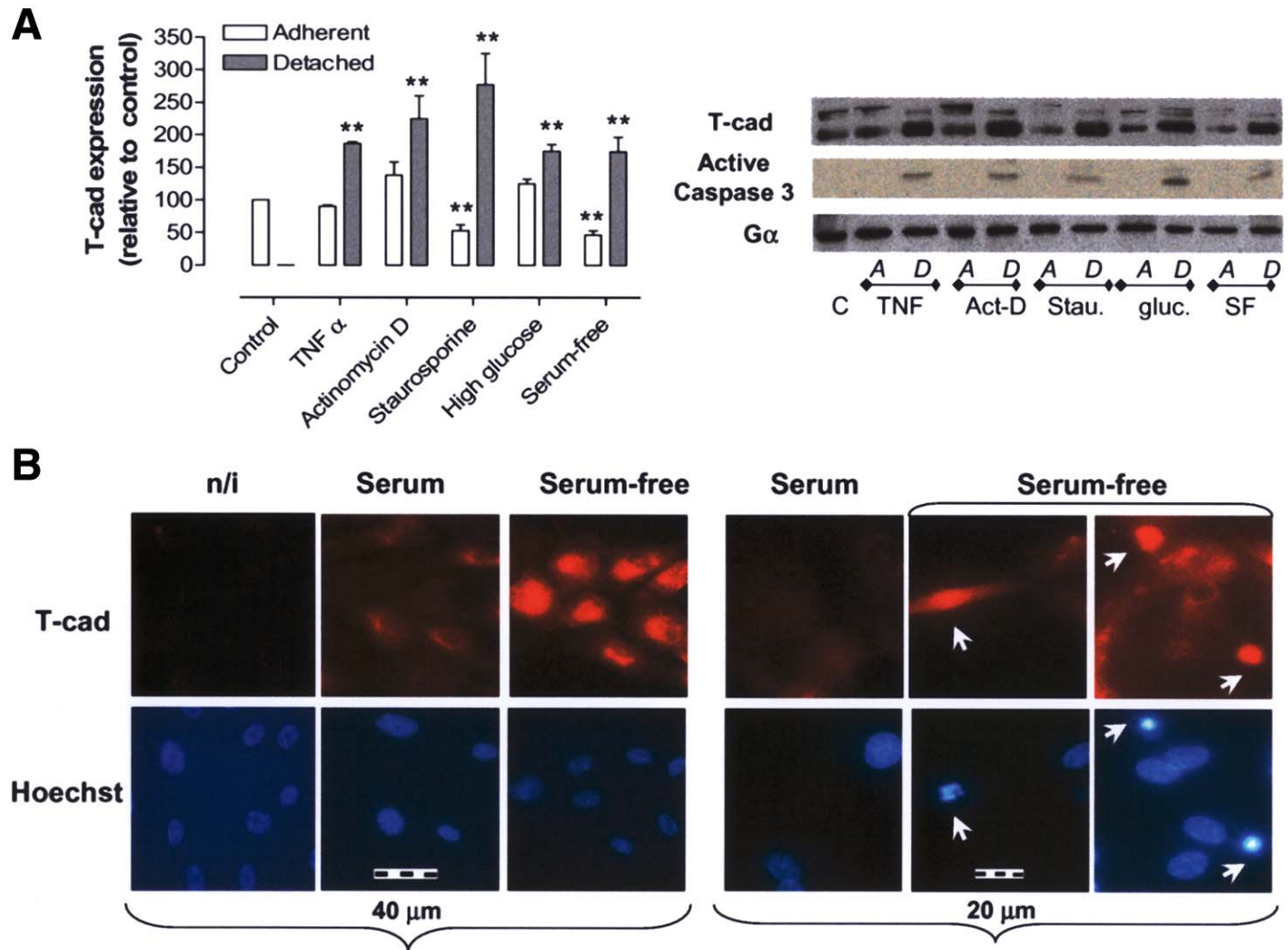


Fig. 2

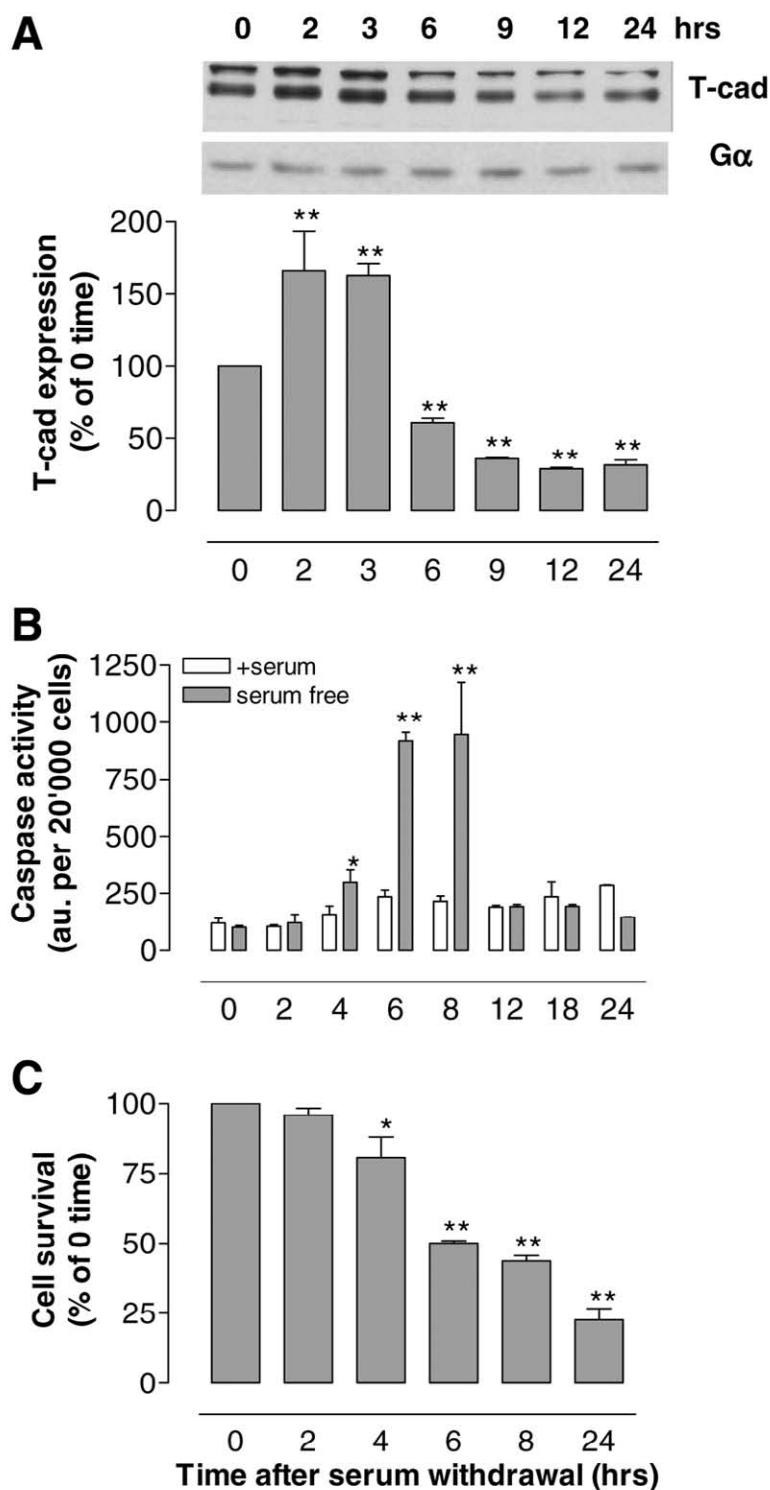


Figure 2. Serum starvation-induced up-regulation in T-cad expression precedes activation of caspase and cell detachment. **A)** Adherent HUVEC were lysed at indicated time intervals after serum withdrawal and analyzed for T-cad expression. **B)** Caspase activity was assayed fluorimetrically in HUVEC (including both adherent + detached populations) cultured in the presence and absence of serum. **C)** Cell survival was evaluated after rinsing of culture dishes by enumeration of adherent HUVEC. Asterisks indicate significant difference: * $P < 0.05$ and ** P at least < 0.01 from zero time controls.

Fig. 3

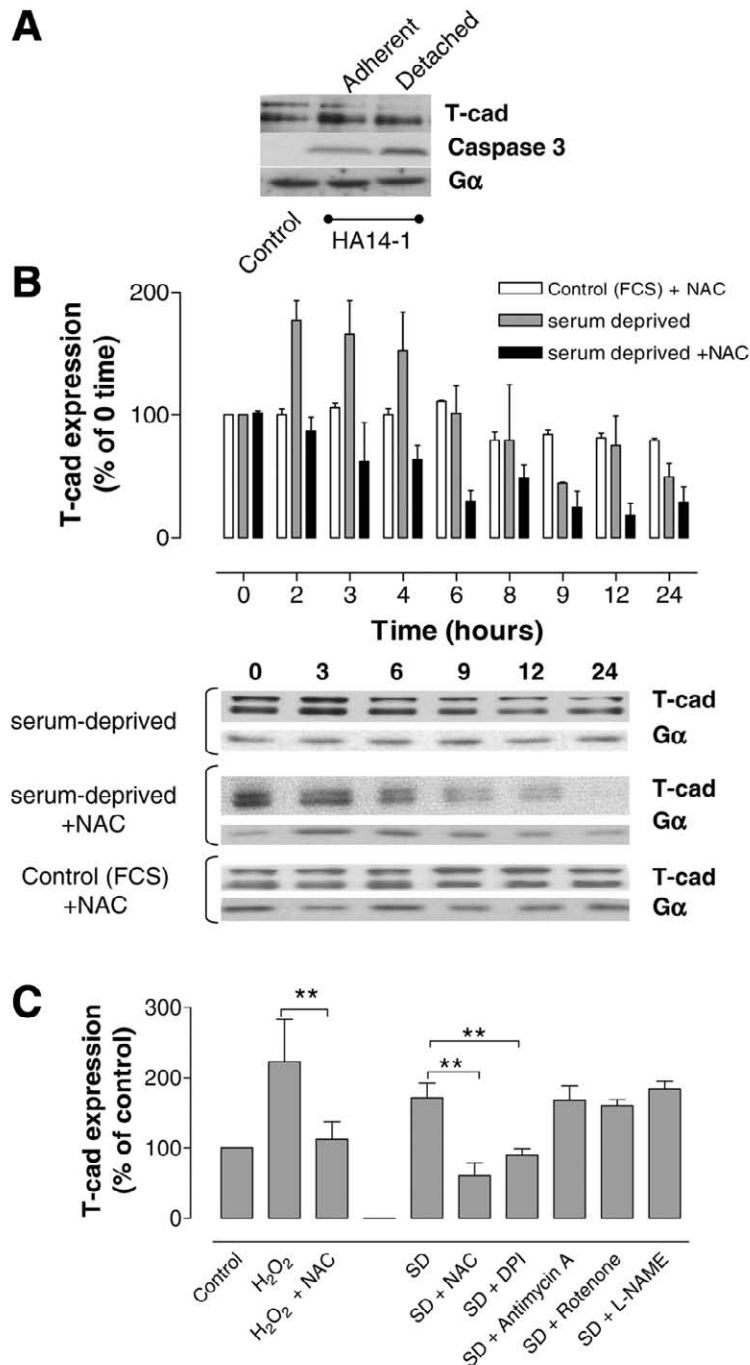


Figure 3. Serum starvation-induced changes in T-cad are independent of Bcl-2 inhibition but dependent upon ROS. **A)** HUVEC under normal serum-containing conditions were incubated for 4 h without (control) or with inclusion of 50 μ M HA14-1; separately prepared lysates of adherent and detached cell populations were immunoblotted for T-cad and active caspase 3. The experiment was performed twice with comparable results. **B)** Lysates of adherent HUVEC were immunoblotted for T-cad following culture for the indicated times under normal serum-containing and serum withdrawal conditions without or with inclusion of 30 mM NAC. The experiments were performed with interval series of either 0, 2, 4, 6, 8, 12, and 24 h or 0, 3, 6, 9, 12, and 24 h. Changes in T-cad protein expression are expressed relative to that at time 0 (arbitrarily taken as 100%), and the histogram presents combined data from both of the series. A representative blot from a single experiment is shown. **C)** HUVEC were incubated for 4 h either under serum-containing conditions in the absence (control) or presence of 1 mM H₂O₂ and without or with inclusion of 30 mM NAC, or under serum-deprived (SD) conditions without or with inclusion of 30 mM NAC, 10 μ M DPI, 10 μ M antimycin A, 10 μ M rotenone or 1 mM L-NAME. Lysates of adherent cells were immunoblotted for T-cad and G α . **Significant difference ($P < 0.01$; $n = 3$).

Fig. 4

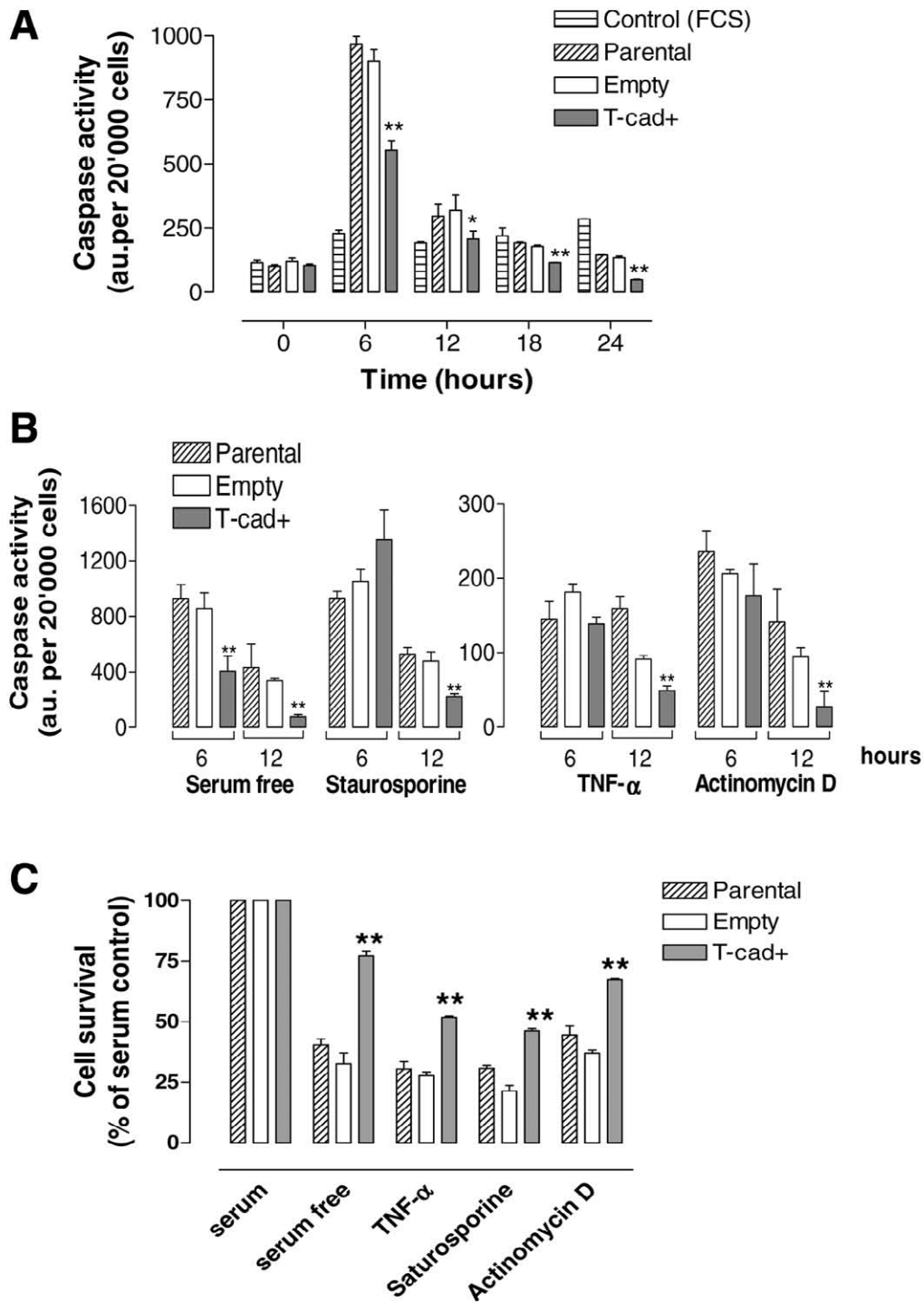


Figure 4. T-cad overexpression in HUVEC protects against apoptosis. **A)** Parental, empty-vector and T-cad+ HUVECs were incubated under serum-free conditions and fluorimetrically assayed for caspases activity at the indicated times. Parental HUVEC maintained under normal serum-containing culture conditions served as the negative assay control. **B)** Caspases activity was measured following incubation for 6 and 12 h either under serum-free conditions, or under serum-containing conditions in the presence of staurosporine (1 μ M), TNF- α (40 ng/ml), or actinomycin-D (100 ng/ml). **C)** Cell survival of following 8 h incubation under the same conditions as in **B)** was evaluated by enumeration of the adherent cells; cell numbers are expressed relative to those in serum-containing controls (arbitrarily taken as 100%). Asterisks indicate where T-cad+ differ significantly from parental and empty-vector cultures (* P <0.05, ** P at least <0.01). There were no significant differences between parental and empty-vector HUVECs following serum deprivation or treatment with the apoptosis-inducing compounds.

Fig. 5

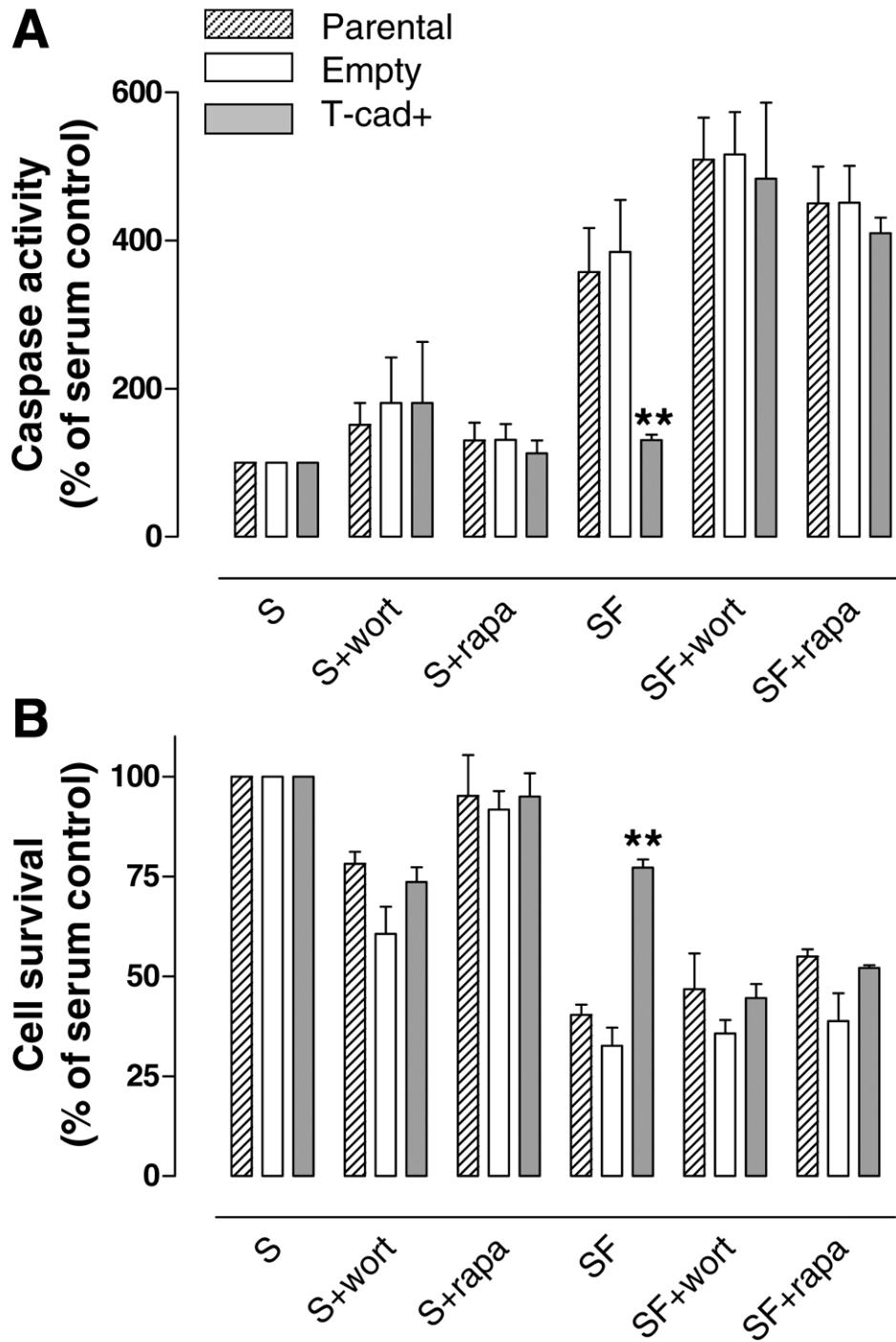


Figure 5. Effect of PI3K inhibition on caspases activation in control and T-cad overexpressing HUVEC. Caspases activity (A) and cell survival (B) was determined in parental, empty- and T-cad overexpressing (T-cad+) HUVEC following incubation for 6 h either in the presence (S) or absence of serum (SF), and without or with inclusion of 10 nM wortmannin (wort) or 10 nM rapamycin (rapa). For each cell type, changes in caspases activity and cell survival are expressed relative to their respective serum controls. **T-cad+ differs significantly ($P < 0.01$) from parental and empty. There were no significant differences between parental and empty.

Fig. 6

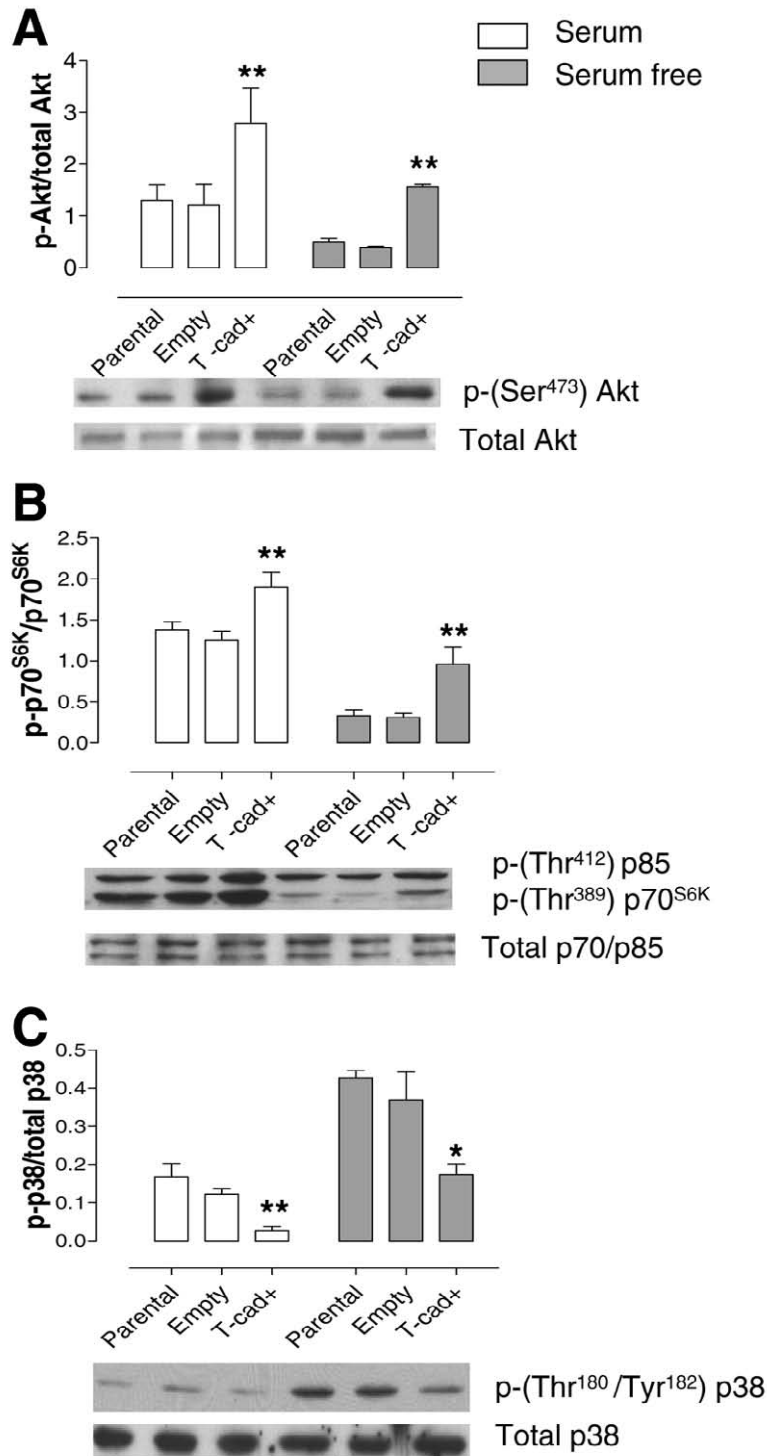


Figure 6. Overexpression of T-cad in HUVEC leads to hyperactivation of Akt signaling and reduced activation of p38. Lysates of parental, empty-vector, and T-cad+ HUVEC incubated for 6 h under normal serum-containing and serum-free conditions were immunoblotted for total Akt/phospho-Akt (60kDa; **A**), total p70^{S6K}/phospho-p70^{S6K} (70 kDa; **B**), and p38/phospho-p38 (43 kDa; **C**). Parental, empty-vector, and T-cad+ HUVEC did not differ with respect to total Akt, p70^{S6K} and p38 levels under either serum-containing or serum-withdrawal conditions. Ratios of total protein/phosphorylated protein are given. Representative blots are presented. Asterisks indicate where T-cad+ HUVEC differ significantly (* $P < 0.05$, ** P at least < 0.01) from parental and empty-vector HUVECs. There were no significant differences between parental and empty-vector HUVECs under either of the culture conditions.

Fig. 7

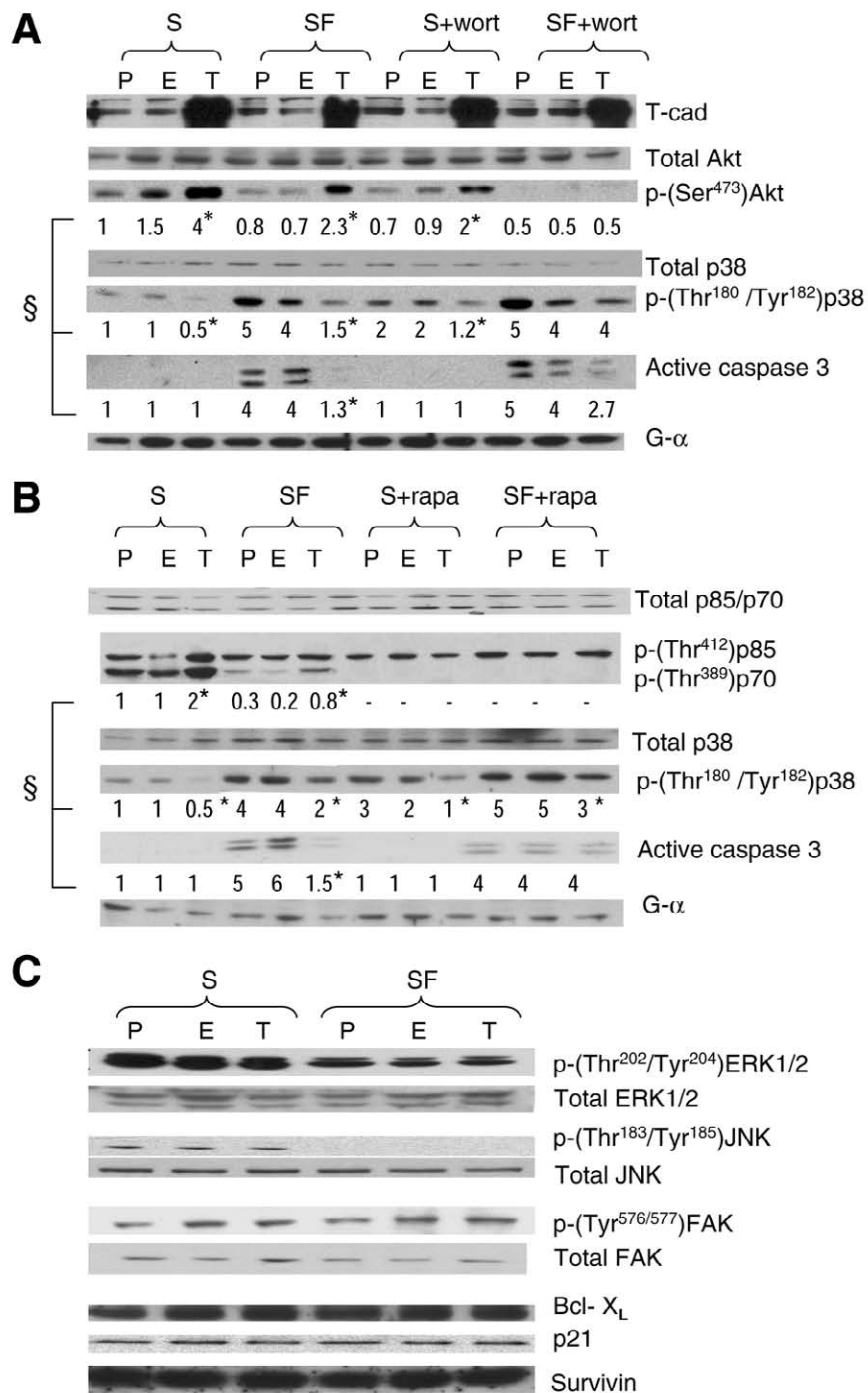


Figure 7. T-cad overexpression rescues HUVEC from serum deprivation-induced apoptosis via PI3 kinase/Akt/mTOR pathway. Parental (P), empty (E), and T-cad overexpressing (T) HUVEC were incubated for 6 h either in the presence or absence of serum (A–C), and without or with inclusion of 10 nM wortmannin (A) or 10 nM rapamycin (B). Cell lysates were prepared and immunoblotted for T-cad, active caspase 3, Akt/phospho-Akt, p38/phospho-p38, p70^{S6K}/phospho-p70^{S6K}, Erk1/2/phospho-ERK1/2 (42/44 kDa), JNK1/phospho-JNK1 (46/56 kDa), FAK/phospho-FAK (125 kDa), BCL-X_L (30 kDa), p21 (21 kDa), and survivin (16 kDa). G- α served as the internal loading control. P, E and T did not differ with respect to total Akt, p70^{S6K}, p38, Erk1/2, JNK1, or FAK levels under any of the experimental conditions. §, numbers below the blots (mean values) indicate band intensities relative to that (arbitrarily taken as 1) in parental HUVEC under normal serum-containing culture conditions. Quantitative data for C are not shown as there were no differences between P, E, and T. Asterisks indicate where expression level in T differed significantly (P at least <0.01) from P and E. There were no significant differences between P and E under any of the experimental conditions.

Fig. 8

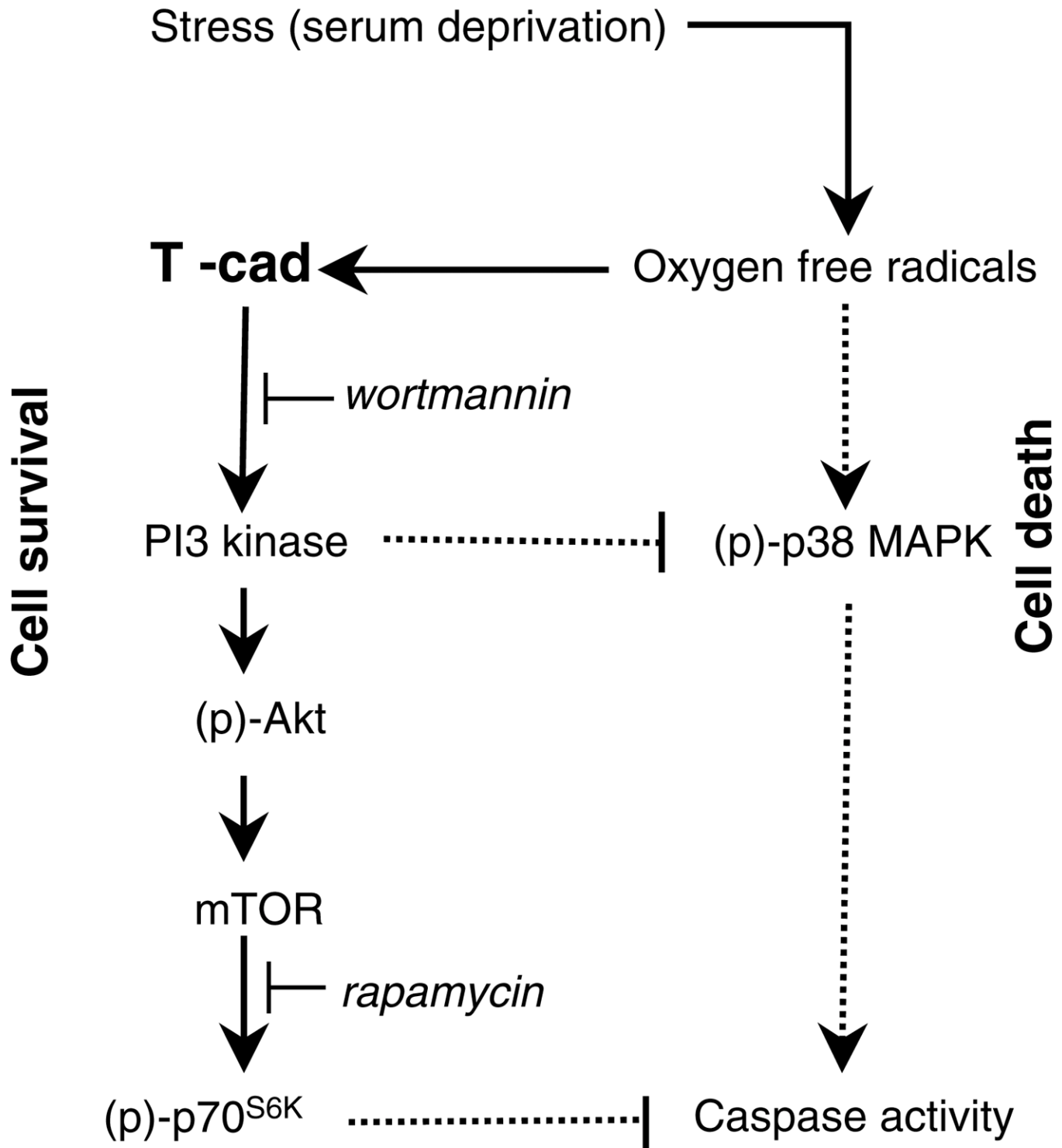


Figure 8. Proposed role of T-cad as a survival protein. Expression of T-cad in HUVEC is increased in response to reactive oxygen species generated during stress (e.g., serum-deprivation) and protects against apoptosis via activation of the PI3K/Akt/mTOR survival pathways and concomitant suppression of the p38 MAPK proapoptotic pathway.

3.2. T-cad and molecular mediators

The results of this project have been recently accepted by FASEB J for publication.

Integrin-linked kinase is an essential mediator for T-cadherin-dependent signaling via Akt and GSK3 β in endothelial cells.

(The accepted manuscript is appended)

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Integrin-linked kinase is an essential mediator for T-cadherin-dependent signaling via Akt and GSK3 β in endothelial cells

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Running title: T-cadherin utilizes ILK as a signaling mediator

ABSTRACT

Glycosylphosphatidylinositol-anchored T-cadherin (T-cad) influences several parameters of angiogenesis including endothelial cell (EC) differentiation, migration, proliferation and survival. This presupposes signal transduction networking via mediatory regulators and molecular adaptors since T-cad lacks transmembrane and cytosolic domains. Here, using pharmacological inhibition of PI3K, adenoviral-mediated T-cad-overexpression, siRNA-mediated T-cad-depletion and agonistic antibody-mediated ligation we demonstrate signaling by T-cad through PI3K-Akt-GSK3 β pathways in EC. T-cad-overexpressing EC exhibited increased levels and nuclear accumulation of active β -catenin, which was transcriptionally active as shown by increased Lef/Tcf reporter activity and elevated levels of cyclin D1 mRNA and protein. Co-transduction of EC with constitutively active GSK3 β (S9A-GSK3 β) abrogated stimulatory effects of T-cad active β -catenin accumulation, proliferation and survival. Integrin-linked kinase (ILK), a membrane proximal upstream regulator of Akt and GSK3 β , was considered a candidate signaling mediator for T-cad. T-cad was present in anti-ILK immunoprecipitates and confocal microscopy revealed co-localization of T-cad and ILK within lamellipodia of migrating cells. ILK-siRNA abolished T-cad-dependent effects on ^{ser473}Akt/^{ser9}GSK3 β phosphorylation, active β -catenin accumulation and survival. We conclude ILK is an essential mediator for T-cad signaling via Akt and GSK3 β in EC. This is the first demonstration that ILK can regulate inward-signaling by GPI-anchored proteins. Further, ILK-GSK3 β -dependent modulation of active β -catenin levels by GPI-anchored T-cad represents a novel mechanism for controlling cellular β -catenin activity.

Key words: GPI-anchored protein, signal transduction, active β -catenin accumulation, proliferation, survival.

INTRODUCTION

T-cadherin (T-cad), an atypical member of the cadherin superfamily, is widely expressed in the cardiovascular system. Expression of T-cad in vascular cells is markedly increased during atherosclerosis (1), restenosis after balloon angioplasty (2) and tumour neovascularisation (3). Accumulating data supports that T-cad participates in several processes (e.g. differentiation, migration, proliferation and survival) that occur during vascular remodelling and angiogenesis. *In vitro*, homophilic ligation of T-cad on the endothelial cell (EC) surface with soluble recombinant T-cad or with agonistic antibodies induces the motile phenotype via activation of Rho and Rac pathways (4, 5), facilitates cell migration (5) and stimulates in-gel outgrowth of endothelial sprouts in 3-dimensional EC-spheroid and heart tissue models of angiogenesis (6). *In vivo*, myoblast-mediated delivery of recombinant soluble T-cad to mouse skeletal muscle facilitates VEGF-induced angiogenesis (6). Moreover, T-cad expression in EC and aortic smooth muscle cells (SMC) is dynamically regulated during the cell cycle, and adenoviral-mediated overexpression of T-cad in EC and SMC promotes cell cycle progression and proliferation (7). Upregulation of T-cad in EC occurs *in vitro* in response to oxidative stress and adenoviral-mediated overexpression protects against oxidative stress-induced apoptosis (8).

Signal transduction pathways mediating the effects of T-cad on vascular cell differentiation, migration, proliferation and survival are not well delineated. T-cad overexpression is accompanied by activation of the PI3-kinase/Akt/mTOR/p70S6 kinase axis (8). T-cad ligation, which in EC induces cell polarization and angiogenic switching and increases cell motility (5) also leads to activation of Akt (6). Ligation-dependent effects of T-cad on EC motility require activation of RhoA/ROCK and Rac (4). T-cad-promoted survival was found to be associated with concomitant PI3K-dependent activation of the PI3K/Akt/mTOR survival signal pathway and suppression of the p38 MAPK proapoptotic pathway (8); this might reflect a cross-talk between p38 MAPK and Akt (9) or involvement of other upstream regulators of p38 MAPK (e.g. small GTPases (10), integrin-linked kinase (ILK) (11)).

The multiple effects of T-cad on EC behavior presuppose signal pathway intersection and networking. GSK3 β is a multifunctional serine/threonine kinase that serves a pivotal point of convergent signaling pathways in EC to control angiogenic responses, including proliferation, migration and survival (12, 13). Akt-catalyzed phosphorylation of ser-9 on GSK3 β leads to its inhibition and in a cascade affects the functionality of diverse GSK3 β substrates including metabolic and signaling proteins, structural proteins and transcription factors (14, 15). Several other kinases including protein kinase A, protein kinase C and p70S6 kinase can modulate activity of GSK3 β by phosphorylation on ser-9 (14, 15). ILK, although lacking critical consensus sequences of Ser/Thr kinases in the ILK kinase domain, has also been shown to have PI3K-dependent kinase activity toward GSK3 β on ser-9 (16, 17). ILK can associate with some components of the Wnt signaling pathway (18, 19). This association can modulate acute canonical Wnt signaling and β -catenin stabilization in a PI3K-GSK3 β -independent manner (19). On the other hand prolonged Wnt signaling was proposed to lead to growth factor activation of the PI3K-ILK-GSK3 β signaling axis (19).

How T-cad might transduce its signals to intracellular effectors is currently unknown. T-cad shares the general molecular organization of cadherin extracellular domains, but lacks transmembrane and cytoplasmic domains and is attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (20). Signaling by GPI-anchored proteins is thought to

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require their interaction with adaptor molecules within plasma membrane lipid rafts (21, 22), where GPI-anchored proteins, including T-cad (23) are preferentially localized.

In this study we aimed to further delineate proliferation and survival signal transduction mechanisms activated by T-cad in EC, and to identify a membrane proximal molecule that could mediate inward signal transmission by GPI-anchored T-cad.

MATERIALS AND METHODS

Cell culture: Human umbilical vein endothelial cells (HUVEC) (PromoCell GmbH, Heidelberg, Germany) were cultured in low serum (2%)-containing EC growth medium (PromoCell). All tissue culture surfaces were pre-coated with 0.1% gelatine, and HUVEC were used at passages 2-4 during which expression of markers (von Willebrand factor, CD 31, VE-cadherin) for differentiated EC remained steady. Human microvascular EC line HMEC-1 (24) was cultured in the same medium supplemented with 10% FCS.

Reagents and antibodies: PI3K inhibitor LY294002 was from Sigma-Aldrich (Buchs, Switzerland). GSK3 fusion protein was from Cell Signaling Technology Inc. (Danvers, MA., USA). Transfection of siRNA was carried out using siPORT Lipid (Ambion (Europe), Cambridgeshire UK). Protein G Sepharose beads were from Amersham Biosciences (Little Chalfont, UK). The following antibodies were used: polyclonal antibody against the first extracellular domain of T-cad generated in our laboratory (25), anti-Akt, anti-phospho-(Ser⁴⁷³)Akt, anti-GSK3 β and anti-phospho-(Ser⁹)GSK3 β (Cell Signaling, New England Biolabs GMBH, Frankfurt, Germany), anti- β -catenin, anti-active β -catenin (ABC) (Upstate Cell Signaling Solutions, Lake Placid, NY, USA), anti-ILK (clone 65.1, Sigma-Aldrich and clone 65.1.9, Upstate), anti-cyclin D1 (BD Biosciences), anti- β -actin (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Anti-rabbit and anti-mouse secondary antibodies coupled to horseradish peroxidase were from Southern Biotechnologies (BioReba AG, Reinach, Switzerland). Secondary anti-species Cy-2- and Cy3-labelled IgG were from Jackson ImmunoResearch Laboratories (West Grove, USA). Amersham ECL, Pierce (Rockford, Ill., USA) Enhanced Luminescence System or Pierce SuperSignal West Dura were variously used for detection of immunoreactive proteins.

Adenoviral infection: Adenoviral vectors encoding wild type GSK3 β (wt-GSK3 β) was a kind gift of Dr. Morris Birnbaum (University of Pennsylvania, Philadelphia, USA). Catalytically inactive GSK3 β (KM-GSK3 β) and non-phosphorylatable constitutively active mutant of GSK3 β (S9A-GSK3 β) were a kind gift of Dr. Kenneth Walsh (Tufts University, Boston, MA, USA). Adenoviral vector encoding T-cad has been described previously (5, 7). All constructs were amplified in HEK293 cells and purified by ultracentrifugation. Viral titers were determined as PFUs (5, 7). For infection, HUVEC were typically incubated with adenovirus at a multiplicity of infection (MOI) 50-100 for 12h. Under these conditions, the transfection efficiency was greater than 90%.

siRNA transfection: Short interfering RNAs (siRNA) for T-cad (5'-GGACCAGUCAAUUCUAAACtt (26)) and ILK ((5'-CCCGGCUCAGGAUUUUCUCtt (27)) were purchased from Microsynth (Balgach, Switzerland). Equal amounts of complementary RNA

oligonucleotides were combined to a final concentration of 20 mM and annealed according to the protocol supplied by the manufacturer. RNA oligos 5'CUCUGUUCGUCCAUGCACGga and 5'AAGGUGGUUGUUUUGUUCAtt were used as negative controls for T-cad and ILK siRNA, respectively; neither of these siRNAs affected expression of either T-cad or ILK at both transcriptional and protein levels. Transfection of HUVEC with siRNA's was performed using Si-PORT Lipid (Ambion (Europe) Ltd., Huntingdon, Cambridgeshire, UK) according to manufacturer's recommendations, and unless otherwise stated, cells were analysed 48-72h post-transfection. The efficiency of ILK and T-cad silencing, routinely determined by immunoblotting, ranged between 70-80%.

Reporter gene analysis: Reporter plasmids for Lef/Tcf transcription factors (Bat-Lux) were a kind gift from Dr. Stefano Piccolo (University of Padua, Italy). Bat-Lux plasmid constitutes seven repeats of Tcf binding element and siamois minimal, cloned upstream of Luciferase gene in pGL3 backbone. 16h after infection of HMECs with empty and T-cad-containing adenovirus cells were co- transfected with either pGL3 or Bat-Lux constructs and pRLTK vector as an internal transfection control using Lipofectamine 2000TM. Luciferase activity was determined after 48h using Dual Luciferase Assay Kit (Promega, Madison, WI, USA).

RNA extraction and reverse transcription polymerase chain reaction: Isolation of mRNA, reverse transcription, and real-time PCR analysis were performed as described previously (6). Cyclin expression was normalized to the expression of GAPDH gene. Primer sequences were as follows: 5'-CGTGGCCTCTAAGATGAAGGA-3' forward and 5'-CGGTGTAGATGCACAGCTTCTC-3' reverse for cyclin D1; 5'-TCATGACCACAGTCCATGCC-3' forward and 5'-GCCATCCACAGTCTTCTGGGT-3' reverse for GAPDH.

Cell proliferation and survival assays: Proliferation was determined by cell enumeration as described previously (7). Survival response to serum deprivation (6h culture in medium containing 0.1%BSA instead of 2%FCS) was assayed using a fluorimetric caspase assay kit (Homogeneous caspases assay; Roche Diagnostics GmbH, Mannheim, Germany) as detailed previously (8). Caspases activity, measured as arbitrary fluorescence units (au.) per 2×10^4 cells, is expressed relative to activity in respectively control-transduced cells.

Immunofluorescence microscopy: Immunofluorescence techniques have been described in detail previously (4, 28). Briefly, HUVEC were plated at a density of 10^5 cells/well onto 0.5% gelatine-precoated round 12-mm glass coverslips in 24-well plates. Unless otherwise specified stainings were performed on fixed (4% paraformaldehyde) and permeabilized (0.1% Triton X-100) cells. Cells were sequentially incubated with primary antibodies (anti-ABC, anti-ILK or anti-T-cad) and secondary anti-species Cy-2- and Cy3-labelled IgG. For non-specific controls, non-immune species IgG substituted primary antibodies. Coverslips were mounted upside-down on slides using FluorSaveTM reagent (Calbiochem, Darmstadt, Germany). Single-stained samples were studied under a Zeiss Axiophot fluorescent microscope (Zeiss, Feldbach, Switzerland), and photos were taken using a digital camera and AnalySIS software (Soft Imaging System GmbH, Munich, Germany). Double-stained samples were examined using a laser scanning confocal microscope LSM 5410 (Zeiss, Feldbach, Switzerland). Images were processed and analysed for colocalization on an O² Workstation (Silicon Graphics Computer Systems, Mountain View, CA, USA) using Imaris 3.0 and Colocalization Bitplanes Software (Bit plane AG, Zurich, Switzerland). Micrographs present typical images.

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Co-immunoprecipitation: Subconfluent HUVEC in 10cm dishes were infected with empty or T-cad-containing adenovirus. After 48h cells were washed twice with PBS and lysed by incubation for 2h at 4°C in buffer (0.7ml/dish) containing 100mM NaCl, 50mM Tris-HCl, pH 8.0, 1% Triton X-114, 0.2% SDS, 5mM CaCl₂, Complete Mini protease inhibitor cocktail (Roche). Lysates were centrifuged (16000g, 10min, 4°C) and supernatants precleared with mouse non-immune IgG (50µg/ml) overnight at 4°C. Non-immune IgG were precipitated by mixing with protein G-Sepharose 4 FastFlow beads (30µl/sample) for 2h at 4°C and centrifuging for 30s at 16000g. Supernatants were incubated with anti-ILK or non-immune IgG (as control) (15µg/ml) overnight at 4°C following precipitation with protein G-Sepharose beads and centrifugation as described above. Supernatants were discarded, agarose beads were washed thrice with 1ml of PBS and 80µl of 2xLaemmli sample buffer was added to the pelleted beads. Samples were analysed for T-cad by immunoblotting.

In vitro ILK assay: ILK pull-down was performed as described (16, 17) with minor modifications. Cell lysis was performed directly on the plates with lysis buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 50mM HEPES, pH 7.5, and proteases mixture inhibitor (Roche). Equivalent lysate concentrations (determined using the Lowry method) were precleared with nonspecific IgG as above, and supernatants immunoprecipitated with anti-ILK antibody overnight at 4°C. Protein G Sepharose was added and after 4h ILK immune complexes were collected, washed twice with lysis buffer and twice with kinase wash buffer (10mM MgCl₂, 10mM MnCl₂, 50mM HEPES, pH 7.5, 0.1mM sodium orthovanadate, 1mM dithiothreitol). The washed pellet was suspended in 25µl kinase assay buffer (10mM MgCl₂, 10mM MnCl₂, 5mM HEPES, pH 7.5, 1mM sodium orthovanadate, 2mM NaF, 200µM ATP), containing 2.5µg of GSK-3 fusion protein as substrate (29), and incubated for 30min at 30°C. The reaction was terminated by adding an equal volume of 2x SDS loading buffer and phosphorylation of the substrate was detected by immunoblotting using anti-phospho-Ser⁹-GSK3β.

Immunoblotting: The method of immunoblotting has been described previously (8, 25). Lysis buffer was PBS containing 1% SDS, 1mM PMSF, 2µg/ml pepstatin, 20µg/ml aprotinin, 30µg/ml bacitracin, 1mM orthovanadate and 5mM NaF. Protein concentrations were determined using the Lowry method and after appropriate dilution into Laemmli buffer, equal amounts of protein/lane were loaded for resolution by SDS-PAGE and subsequent transfer onto nitrocellulose. Proteins were immunoblotted with specific primary antibodies and detected using horseradish peroxidase–conjugated secondary antibodies and chemiluminescence. Equivalence of protein loading was routinely controlled by both Ponceau S staining and immunoblotting with anti-β-actin. Scanned images of immunoblots were analyzed using AIDA Image or Scion (NIH) Image software. Representative images of immunoblots are shown.

RESULTS

PI3K-GSK3β axis signaling by T-cad in HUVEC

T-cad upregulation exerts stimulatory effects on PI3K/Akt pathway (8), one of the main signaling cascades modulating GSK3β activity in mammalian cells (14, 15). To examine participation of GSK3β in T-cad-mediated signaling in human umbilical vein endothelial cells (HUVEC) we first determined the effect of T-cad overexpression on phosphorylation of GSK3β on ser-9 (p^{ser9}GSK3β). Adenoviral mediated overexpression of T-cad in HUVEC (T-cad⁺) results in

hyperphosphorylation of GSK3 β (Fig. 1A); this was true for both HUVEC cultured under normal growth conditions and following serum-deprivation, although in the latter case levels of p^{ser9}GSK3 β were markedly lower (Fig. 1A). As reported for Akt ((8); and included herein as experimental control), the effects of T-cad on p^{ser9}GSK3 β were dependent upon PI3K (Fig. 1B). Inclusion of PI3K inhibitor LY294002 under normal culture conditions reduced p^{ser9}GSK3 β to comparably low levels in Empty-vector (E) - and T-cad (T⁺) - transduced cells. Sensitivity of T-cad-dependent p^{ser9}GSK3 β phosphorylation to PI3K inhibition indicates that the ability of T-cad to modulate GSK3 β activity is independent of canonical Wnt signaling.

T-cad expression level affects accumulation of active β -catenin

Since T-cad overexpression increases cell cycle progression and proliferation of HUVEC (7) and because β -catenin has been shown to be one of the characteristic mediators of GSK3 β actions in the nucleus, we hypothesized that T-cad might affect the activity of β -catenin. We performed immunoblotting using anti-catenin antibodies that distinguish between total cellular β -catenin or active (unphosphorylated) β -catenin (30). Whereas total β -catenin levels were similar between T-cad⁺- and E-HUVECs, levels of active β -catenin were elevated in T-cad⁺-HUVEC (Fig. 2A). Immunofluorescence microscopy using anti-active β -catenin (ABC) revealed a higher degree of cells positive for nuclear β -catenin in T-cad⁺-HUVEC as compared to E-HUVEC (35% vs. 6%, P<0.001, Fig. 2B).

To confirm the specificity of T-cad effects on GSK3 β phosphorylation and nuclear accumulation of active β -catenin we examined HUVEC in which T-cad expression was either depleted (siRNA-mediated) or overexpressed (adenoviral-mediated). Akt phosphorylation on ser-473 (p^{ser473}Akt) in T-cad-depleted HUVEC was additionally evaluated since we previously examined only consequences of T-cad overexpression (8). In T-cad-siRNA-transduced HUVEC, levels of p^{ser473}Akt, p^{ser9}GSK3 β and active β -catenin were significantly reduced compared with control siRNA-transduced HUVEC (Fig. 3A). This profile is reciprocal to that observed in T-cad overexpressing HUVEC in which levels of p^{ser9}GSK3 β , active β -catenin and p^{ser473}Akt were significantly higher than in control empty-vector transduced HUVEC (Fig. 3B). Incubation of HUVEC with agonistic anti-T-cad antibodies against the first domain, mimicking homophilic ligation (5), also increased levels of p^{ser473}Akt, p^{ser9}GSK3 β and active β -catenin (Fig. 3C). Levels of total β -catenin, GSK3 β and Akt were not affected by depletion or overexpression of T-cad or by inclusion of antibodies. Taken together these data unambiguously demonstrate modulation of Akt and GSK3 β axis signaling and levels of active β -catenin by T-cad.

To examine the dependence of T-cad-induced active β -catenin accumulation upon GSK3 β inactivation we compared HUVEC after transduction with T-cad-containing (T-cad⁺) or empty adenovectors (E) and/or with adenovectors carrying kinase mutant GSK3 β (KM), constitutively active GSK3 β (S9A) or wild type GSK3 β (wt). Cell lysates were immunoblotted for total and active β -catenin and for total and p^{ser9}GSK3 β (Fig. 4A). Equivalent signal intensities for total GSK3 β in KM-, S9A- or wt-transduced HUVEC indicated equivalent expression of GSK3 β protein. Compared with E-HUVEC, and as expected (13, 31), levels of active β -catenin were elevated in KM- and wt-transduced HUVEC and reduced in S9A-transduced HUVEC. Levels of active β -catenin in T-cad⁺-, KM- and wt-transduced HUVEC were comparable. In T-cad⁺+KM and T-cad⁺+wt co-transduced HUVEC the levels of active β -catenin and p^{ser9}GSK3 β were higher than those in singly-transduced HUVEC (Fig. 4B C). These additive effects were not present in E+KM or E+wt co-transduced HUVEC. Most importantly, co-transduction of T-cad⁺-HUVEC

with S9A abrogated the stimulatory effects of T-cad on both p^{ser9}GSK3 β and active β -catenin and (c.f. T-cad⁺+S9A vs. T-cad⁺, Fig. 4B C). These data support that GSK3 β underlies accumulation of active β -catenin induced by T-cad.

To determine whether the effect of T-cad on nuclear accumulation of active β -catenin is functionally relevant we performed reporter gene analysis using a BAT-lux plasmid, a β -catenin-specifically driven lymphocyte enhancer-binding protein/T cell factor (Lef/Tcf) reporter construct (32). T-cad- and empty- adenovector-infected HUVEC were co-transfected with BAT-lux or empty plasmids and with pRLTK plasmid constitutively expressing Renilla luciferase as internal control. Lef/Tcf promoter activity in T-cad⁺-HUVEC was greater than in control E-HUVEC (Fig. 5A). To obtain additional evidence that T-cad increases β -catenin activation of Lef/Tcf transcription factors we investigated expression of cyclin D1, a key transcriptional target of β -catenin/Lef/Tcf (33). Immunoblotting and quantitative RT-PCR experiments demonstrated elevated levels of cyclin D1 protein (Fig. 5B) and cyclin D1 mRNA (Fig. 5C), respectively, in T-cad⁺-HUVEC.

T-cad effects on proliferation and survival are dependent upon GSK3 β inhibition

We have previously reported that T-cad upregulation increases cell cycle progression and proliferation under normal culture conditions (7) and also enhances survival under conditions of stress induced by serum-deprivation (8). Here the possible role of GSK3 β in these functional responses was investigated using HUVEC transduced with T-cad and/or the various GSK3 β vectors. Figure 6A shows proliferation profiles of singly transduced HUVEC; whereas T-cad transduction increased proliferation (vs. Empty) GSK3 β -KM or GSK3 β -wt had no effect and constitutively active GSK3 β -S9A was inhibitory. Co-transduction with both T-cad and constitutively active GSK3 β -S9A genes completely abrogated stimulatory effects of T-cad on cell proliferation (Fig. 6B; c.f. T-cad⁺+S9A vs. T-cad⁺). Apoptosis induced by serum deprivation was similarly reduced in GSK3 β -KM-, GSK3 β -wt- and T-cad⁺-HUVEC (Fig. 6C; vs. Empty). Co-transduction with both T-cad and constitutively active GSK3 β -S9A abolished the ability of T-cad to protect cells from apoptosis induced by serum deprivation (Fig. 6C; c.f. T-cad⁺+S9A vs. T-cad⁺). Proliferation and survival parameters in Empty+GSK3 β -KM, Empty+GSK3 β -wt or Empty+GSK3 β -S9A co-transduced HUVEC were not different than those in HUVEC singly transfected with GSK3 β -KM, GSK3 β -wt or GSK3 β -S9A, respectively (data not shown). Taken together these data indicate that the ability of T-cad to induce inhibition of GSK3 β is requisite for its ability to stimulate proliferation and protect from apoptosis

ILK is a mediator of T-cad signaling

In vitro, ILK can serve as a membrane-proximal upstream PI3K-dependent modulator of key signaling molecules crucial for cell survival, cell proliferation and the process of angiogenesis, including Akt and GSK3 β (17, 34-36). Since T-cad expression affects both Akt and GSK3 β activities, and in a PI3K-dependent manner, we considered the possible involvement of ILK in T-cad-induced effects.

We first examined ILK-activity in E-HUVEC and T-cad⁺-HUVEC by pull-down (anti-ILK) kinase assays with GSK3 β fusion protein as substrate and detection of p^{ser9}GSK3 β by Western blotting. Phosphorylation of GSK3 β on ser-9 was markedly greater with ILK-immunoprecipitates from T-cad⁺-HUVEC than E-HUVEC; p^{ser9}GSK3 β was not detected when ATP was omitted from the assay buffer, thus excluding possible contribution from endogenous p^{ser9}GSK3 β (Fig.

7A). The differential in ILK activity between T-cad⁺-HUVEC and E-HUVEC is also unlikely to be explained by any difference in levels of ILK protein since ILK immunoreactivity in whole cell lysates was similar. These experiments indicate that T-cad can “activate” ILK.

We next examined the consequences of ILK knock-down on downstream signaling pathways affected by T-cad. Western blot analysis revealed that transfection of HUVEC with ILK siRNA suppressed ILK expression up to 90% after 72 hrs compared to control siRNA transfected cells (Fig. 7B). Silencing of ILK expression resulted in a striking reduction in levels of p^{ser473}Akt, p^{ser9}GSK3 β and active β -catenin in both E- and T-cad⁺-HUVEC, and a loss of any differential in these parameters between E- and T-cad⁺-HUVEC (Fig. 7B). Additionally, silencing of ILK expression abolished the ability of T-cad to protect cells from apoptosis induced by serum deprivation (Fig. 7C). These data show that ILK mediates T-cad induced signaling and is an essential component of the T-cad signaling cascade.

T-cad associates with ILK

Since both T-cad (23) and ILK (37) are located within raft membrane domains the ILK-dependent activation of signaling by T-cad may involve some association or co-localization. To investigate this possibility we performed co-immunoprecipitation studies on E- and T-cad⁺-HUVEC. T-cad was found to be specifically present in anti-ILK immune complexes, and at higher levels in T-cad⁺-HUVEC than in E-HUVEC (Fig. 8A). Confirmation of the putative association between ILK and T-cad by reverse immunoprecipitation with anti-T-cad antibodies was not possible since neither our own affinity purified anti-T-cad antibodies nor commercially available anti-T-cad antibodies were efficient in immunoprecipitating T-cad. Confocal microscopy was applied to further examine the association between ILK and T-cad. Immunofluorescence was performed after wounding of subconfluent monolayers in order to be able to examine protein localization in both spread and migrating cells. Two different anti-ILK antibodies (clone 65.1, Sigma-Aldrich and clone 65.1.9, Upstate Biotechnologies) were tested. The typical staining pattern for T-cad in HUVEC, namely a global and punctuate localization over the entire cell body and an enrichment within lamellipodia of migrating cells (Fig. 8B, middle panels) has been described previously (28). In HUVEC the staining pattern for ILK using either of the anti-ILK antibodies was mostly fibrillar over the cell body and with punctuate localization at sites of focal adhesion (Fig. 8B, left panels). In our analyses we additionally observed a pronounced enrichment of ILK within lamellipodia of migrating cells where it co-localized with T-cad (Fig. 8B, right panels).

DISCUSSION

This study has identified T-cad as a novel ILK-activating protein in HUVEC and demonstrated that ILK is an essential proximal mediator for GPI-anchored T-cad-dependent signal transduction via Akt and GSK3 β in HUVEC. To our knowledge, this is the first demonstration that ILK can function as a regulator for signaling by GPI-anchored proteins. We also demonstrate for the first time that alterations in T-cad expression can affect levels of active β -catenin and that T-cad can induce an ILK-GSK3 β -dependent increase and nuclear accumulation of β -catenin which interacts with Lef/Tcf transcription factors to activate target gene expression. This represents a novel mechanism (i.e. independent of both canonical Wnt and classical cadherin pathways) for controlling cellular β -catenin activity by GPI-anchored proteins. Signaling by T-cad via p^{ser9}GSK3 β phosphorylation is pivotal to its stimulatory effects on EC proliferation and survival.

Unequivocal signaling of T-cad through Akt, GSK3 β and subsequently β -catenin in HUVEC was demonstrated using a variety of experimental approaches including pharmacological inhibition of PI3K, adenoviral mediated overexpression of T-cad and inhibition of T-cad expression by RNA-interference. Collectively the experiments show that alterations (increase or decrease) in T-cad expression in HUVEC modify (increase or decrease, respectively) the phosphorylation status of Akt and GSK3 β and the level of active β -catenin, and that the effect of T-cad on these signaling effectors is dependent upon PI3K activity. Complete abolition of T-cad induced active β -catenin accumulation following co-transduction with constitutively active GSK3 β mutant (GSK3 β -S9A) demonstrates that the stimulatory effect of T-cad on active β -catenin accumulation in HUVEC was dependent upon GSK3 β inhibition.

Phosphorylation of Akt and stabilization and nuclear translocation of β -catenin as a consequence of GSK3 β phosphorylation are the two main pathways that regulate cell survival and proliferation (14, 15, 38-42). We have previously proposed that upregulation of T-cad under conditions of oxidative stress functions to protect HUVEC from apoptosis via enhanced survival signaling through Akt/mTOR (8). Here we show that transduction of HUVEC with constitutively active GSK3 β mutant (GSK3 β -S9A) completely abrogated pro-proliferative and anti-apoptotic effects of T-cad indicating that signal transmission via GSK3 β inactivation is also important for T-cad modulation of EC growth and survival functions.

Interestingly when wtGSK3 β was co-transduced with T-cad the measured parameters for active β -catenin and proliferation resembled those found in HUVEC co-transduced with T-cad and GSK3 β kinase mutant KM, indicating a similar decrease in kinase activity. This would be consistent with the high ratio of ^{pser9}GSK3 β /total level of phosphorylated GSK3 β following overexpression of wt GSK3 β . Furthermore, since parameters in the case of wtGSK3 β /empty vector-co-transduced HUVEC were not different from HUVEC transduced with only wtGSK3 β it is possible to speculate on either an additive effect of endogenous and transduced GSK3 β phosphorylation or a higher recruitment and inactivation GSK3 β via the T-cad/ILK/Akt signal relay in T-cad overexpressing cells. Reported effects of overexpression of wt GSK3 β are variable and it is unclear whether one can predict increased enzymatic activity (like constitutively active mutant S9A) or decreased activity (like dominant negative mutant KM) on the basis of changes in GSK3 β phosphorylation status. It has been shown that overexpression of leads to increased GSK3 β phosphorylation but an increase in parameters of GSK3 β enzymatic activity (31, 43, 44). On the other hand it has also been shown that overexpression of wt GSK3 β is accompanied by a decrease in GSK3 β phosphorylation and an increase of parameters of GSK3 β enzymatic activity (45). A further study did not examine phosphorylation but found functional equivalence between control and wt-GSK3 β –transfected cells (43). Additional discrepancies also evidently exist with respect to levels of total GSK3 β (vs. Control empty vector) whereby Rossig et al., (44) and Gong et al.,(43) reported increased levels (as expected) whereas Hashimoto et al.,(31) and Salas et al., (45) reported no change.

That T-cad expression could affect levels of active β -catenin was unexpected. Classical cadherins, including VE- and N-cadherin in endothelial cells, are recognized to function as modulators of β -catenin signaling, by regulating the availability of free β -catenin for nuclear translocation (40-42, 46). The intracellular domains of classical cadherins such as N- and VE-cadherin interact with β -catenin, γ -catenin and p120ctn to assemble the cytoplasmic cell adhesion complex that is critical for the formation of extracellular cell-cell adhesion. β -catenin and γ -

catenin bind directly to α -catenin, which links the cytoplasmic cell adhesion complex to the actin cytoskeleton. Disruption (via loss of cadherin) or alterations (via phosphorylation) of interactions between β -catenin and cadherins not only alters intercellular adhesions but can also regulate levels of the availability of β -catenin for nuclear translocation (40-42, 46). GPI-anchored protein T-cad does not possess the necessary cytoplasmic domain to effect a direct physical association with either β -catenin or other intracellularly located molecules (e.g. GSK3 β) that may regulate β -catenin availability (20). Thus, T-cad effects on accumulation and nuclear translocation of active β -catenin must necessarily proceed via some proximal molecular mediator that couples T-cad induced signal transmission to intracellular effectors.

ILK is a ubiquitously expressed protein composed of four ankyrin repeats, a pleckstrin homology phospholipid-binding motif, and a C-terminal Ser/Thr kinase domain. A key function of ILK is to provide a molecular scaffold for the assembly of proteins involved in connecting integrins and growth factor receptors to the actin cytoskeleton (47-52). Furthermore, and although controversy exists as to whether ILK is a *bona fide* kinase, it can also regulate the activities of key signaling components (e.g. Akt and GSK3 β) involved in control of cell survival, cell cycle progression, cell adhesion, and ECM modification (37, 47-49, 53-55). We considered ILK as a candidate molecular mediator for T-cad for a number of reasons. ILK activity is regulated in a PI3K manner (17), it is an upstream regulator of Akt phosphorylation on ser473 (17, 35, 36, 55-57) and GSK3 β phosphorylation on ser9 (17, 58, 59), and it has been shown to regulate nuclear β -catenin and cyclin D1 levels (58), all these properties being common to T-cad induced signaling in HUVEC. Functionally, ILK has been demonstrated to be an important regulator of EC phenotype and differentiation, adhesion, survival, proliferation and angiogenesis (27, 34, 35, 57, 60-62), processes also affected by T-cad in HUVEC (4-8, 28). Furthermore, both ILK and T-cad are located within plasma membrane raft domains (23, 37), which would facilitate the proximity necessary for ILK to serve as a mediator for T-cad. Raft domains function as assembly platforms for the dynamic formation of complexes between surface receptors, signal transduction regulators and scaffolding proteins, and many of the molecular components regulating cell polarization, motility, adhesion, proliferation and survival are associated with plasma membrane rafts (21, 22).

Several lines of evidence in our study support that T-cad indeed utilizes ILK to elicit signaling in HUVEC. Firstly, *in vitro* pull-down assay for ILK (with exogenous GSK3 β fusion protein as substrate) showed increased activity in immunoprecipitates from HUVEC overexpressing T-cad. Secondly, and more importantly, siRNA-mediated ILK depletion completely abolished stimulatory effects of T-cad on Akt and GSK3 β phosphorylation and accumulation of active β -catenin. Thirdly, T-cad was present in anti-ILK immune complexes. Fourthly, confocal microscopy demonstrated colocalization of T-cad and ILK within lamellipodial extensions of migrating cells.

A number of studies have examined the role of ILK in modulating EC function (27, 34, 35, 57, 60-62). EC-specific deletion in mice and antisense-mediated silencing in zebra fish were both shown to confer lethal vascular morphogenic insufficiency *in vivo*; *in vitro* analysis of ILK-deleted murine EC demonstrated a disruption of integrin-matrix interactions, increased apoptosis and a reduction of phosphorylation of Akt on ser473 without a change in phosphorylation of GSK3 β on ser9 (34). An *in vitro* study using bovine aortic EC showed that siRNA-mediated silencing of ILK increased cell adhesivity to a variety of specific matrix substratum components (collagen I, vitronectin, fibronectin, fibrinogen), but impaired cell spreading, migration and capillary morphogenesis; these effects were attributed to destabilization of cell-matrix through

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alterations in surface distribution of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins and were not accompanied by changes in ser473 Akt or ser9 GSK3 β phosphorylation (27). In HUVEC ILK was shown to associate with VEGF receptor (57), while transduction with dominant negative, kinase deficient ILK or pharmacological inhibition of ILK resulted in inhibition of a number of VEGF-induced responses including adherence to collagen 1, phosphorylation of ser473 Akt, migration, survival, proliferation and capillary morphogenesis (35, 57, 60). In both HUVEC and endothelial progenitor cells ILK plays a key role in cell survival signaling responses (ser473 Akt or ser9 GSK3 β phosphorylation) to anchorage deprivation, nutrient deprivation or hypoxia; furthermore using a model of mouse hindlimb ischemia ILK was demonstrated to promote endothelial progenitor cell recruitment and neovascularization of ischemic tissue (61, 62). Thus, from the above there is a growing consensus that ILK plays a key role in the complex process of vascular morphogenesis by influencing different mechanisms.

The data herein support a model (Fig. 9) for T-cad-induced signal transduction whereby ILK serves as an essential proximal downstream regulator for T-cad to enable inward-directed signals via intracellular effectors Akt/GSK β and subsequent modulation of proliferation and survival responses. “Activation” of ILK by T-cad through proximal association is an appealing mediator paradigm for PI3K/Akt/GSK3 β axis signaling by other GPI-anchored proteins. Whether T-cad and ILK constitute part of a larger signaling complex including other transmembrane adapter proteins and cytoplasmic signaling molecules requires further investigations.

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Abbreviations list

T-cad, T-cadherin; ILK, integrin-linked kinase; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; SMC, smooth muscle cell; GPI, glycosylphosphatidylinositol; PI3K, phosphatidylinositol-3-kinase

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FIGURE LEGENDS

Figure 1: Hyperphosphorylation of GSK3 β in T-cad overexpressing HUVEC is PI3K-dependent. Subconfluent cultures of HUVEC were transduced overnight with empty (Empty or E)- or T-cad (T-cad⁺ or T⁺)- containing adenovirus and then rinsed with PBS before further incubations. Panel A: HUVEC were incubated for 6h in normal serum-containing growth medium or serum-free medium (DMEM+0.1% BSA), rinsed with PBS and lysed. Whole cell lysates were immunoblotted for p^{ser9}GSK3 β /total GSK3 β (46kDa). Values are mean \pm SD (n=4); ** indicates significant difference (P<0.001) between Empty- and T-cad⁺- HUVEC. Panel B: E- and T⁺-HUVEC were incubated with PI3K inhibitor LY294002 (10mM; Ly) for 6h under serum-containing (S) and serum- free (SF) conditions. After rinsing whole cell lysates were prepared and immunoblotted for T-cad (105 and 130kDa), total/p^{ser473}Akt (60kDa) and total/p^{ser9} GSK3 β . Blots from a single given experiment are presented; comparable results were obtained in at least 3 other separate experiments.

Figure 2: T-cad overexpressing HUVEC exhibit increased nuclear accumulation of β -catenin. Panel A: Whole cell lysates of subconfluent cultures of HUVEC transduced overnight with Empty and T-cad- (T-cad⁺)-containing adenovectors were immunoblotted for total and active β -catenin (92kDa). Values are mean \pm SD (n=5); ** indicates significant difference (P<0.001) between Empty- and T-cad⁺- HUVEC. Panel B: For immunofluorescence Empty- and T-cad⁺-HUVEC were seeded onto gelatine-coated coverslips 12h post-infection and allowed to adhere. After 6h, cells were rinsed, fixed with 4% paraformaldehyde and sequentially incubated with anti-active β -catenin and secondary anti-mouse Cy3-conjugated antibodies. Samples were analysed under a Zeiss Axiophot fluorescent microscope. E1/T⁺1 and E2/T⁺2 are representative fluorescence micrographs of Empty/T-cad⁺-HUVEC from 2 independent experiments. Scale bar, 50 μ m. Values below the micrographs represent the degree of nuclear β -catenin translocation calculated as the ratio of those cells with nuclei positive for β -catenin to the total number of cells per field. 10 fields per sample were randomly selected for analysis.

Figure 3: T-cad expression levels and homophilic ligation modulate PI3K/Akt/GSK3 axis signaling in endothelial cells.

Panel A: Subconfluent HUVEC were transduced with Control siRNA or T-cad specific siRNA and after 72h cells were lysed and immunoblotted for T-cad (105 and 130kDa), total/p^{ser473}Akt (60kDa), total/p^{ser9}GSK3 β (46kDa), total/active β -catenin (92kDa) and β -actin (43kDa) as internal protein loading control. Panel B: Subconfluent HUVEC were transduced overnight with T-cad-containing (T-cad⁺) and Empty adenovirus. Cells were washed once with PBS and lysed. Cell lysates were immunoblotted for the same proteins as above. Panel C: Subconfluent HUVEC were incubated with purified anti-T-cad antibodies or purified non-immune rabbit IgG (both 10 μ g/ml) for 6 hours, washed once with PBS, lysed and then immunoblotted as above. Each panel presents image sets from a single given experiment; given values are levels of p^{ser473}Akt, p^{ser9}GSK3 β and active β -catenin expressed relative to levels in the respective controls (mean \pm SD, n= at least 3).

Figure 4: T-cad induced accumulation of active β -catenin in HUVEC is GSK3 β -dependent. Subconfluent HUVEC were transduced overnight with the following adenovectors either alone or in combination, as indicated: Empty (E) and T-cad (T-cad⁺), GSK3 β Kinase Mutant (KM), GSK3 β Constitutively Active (S9A) and GSK3 β Wild Type (wt) adenoviruses. Cell lysates were immunoblotted for T-cad, total/p^{ser9}GSK3 β , total/active β -catenin and β -actin. Representative immunoblots from one of 3 independent experiments are shown in panel A. Changes in the ratios

active β -catenin/total β -catenin (Panel B) and $p^{\text{ser}9}$ GSK3 β /total GSK3 β are expressed relative to the ratios in E-transduced HUVEC. Values are mean \pm SD (n=3). * indicates significant difference (P<0.01) between T-cad⁺/wt cotransduced HUVEC and either T-cad⁺- or wt-transduced HUVEC.

Figure 5: T-cad induced elevation in nuclear β catenin in HUVEC is functionally relevant.

Panel A: Empty and T-cad transduced HUVEC were co-transfected with either pGL3-Empty or pGL3-BAT-Lux plasmid and with pRLTK plasmid as internal control. Promoter activity was assessed after 48h by dual luciferase assay. Panels B and C: Subconfluent cultures of HUVEC were transduced overnight with Empty or T-cad adenovectors, and then processed for either immunoblot analysis of cyclin D1 protein (36kDa) expression (Panel B) or RT-PCR analysis of cyclin D1 mRNA expression (Panel C). Values are mean \pm SD (n=3) and ** indicates significant difference (P<0.001) between Empty and T-cad infected HUVEC.

Figure 6: T-cad effects on proliferation and survival in HUVEC are dependent upon GSK3 β inactivation.

Subconfluent HUVEC were singly transduced with Empty, T-cad, GSK3 β -wt, GSK3 β -S9A or GSK3 β -KM adenovectors (Panel A) or co-transduced with Empty or T-cad vectors and GSK3 β adenovectors as indicated (Panel B). After overnight infection, HUVEC were passaged into 96-well plates at 2×10^3 cells/well. Cell enumeration was performed using a Coulter Counter after completed adherence (day 0) and thereafter every 24 h for up to 5 days. Data are given as mean \pm SD (n=3; SD's in range of 5-10%). ξ , Growth of HUVEC transduced with Empty vector alone was comparable to Empty/KM and Empty/wt co-transduced HUVEC (individual profiles not shown; --X-- in Panel B). For caspase assay (Panel C) subconfluent HUVEC were passaged into 96-well plates at 2×10^4 cells/well after overnight transduction. After 24h, apoptosis was induced in HUVEC by culture for 6h under conditions of serum-deprivation; caspase activity was measured using Homogenous caspases assay kit (Roche Diagnostics). Data are mean \pm SD (n=3).

Figure 7: ILK is a necessary mediator of T-cad signalling.

Panel A: ILK activity in subconfluent cultures of Empty and T-cad-transduced HUVEC was measured after pull-down from equivalent concentrations of cell lysates using anti-ILK (or non-immune IgG as control) as described in Methods. Cell lysates were immunoblotted for T-cad, ILK (59kDa) and β -actin. Immune-complexes were assayed for ILK activity by incubation in the absence and presence of ATP with GSK3 β fusion protein as substrate, and subsequent immunoblot analysis of levels of $p^{\text{ser}9}$ GSK3 β . Blots from one of two independent experiments are presented. Panels B and C: After overnight transduction of subconfluent with control Empty (E) or T-cad (T⁺) adenovector HUVEC were transfected with control and ILK specific siRNAs using siPORT Lipid. Growth medium was replaced after 4h and HUVEC cultured for 72 h. Thereafter HUVEC were either lysed for immunoblot analysis of the indicated proteins (Panel B), or assayed for survival using homogenous caspases assay kit following a 6h period of culture under conditions of serum deprivation (Panel C). In panel B levels of $p^{\text{ser}473}$ Akt, $p^{\text{ser}9}$ GSK3 β and active β -catenin in T⁺ are expressed relative to respective levels in E; representative blots from one of three independent experiments are presented. Data are mean \pm SD (n = 3) and significant differences between E-and T-cad⁺ - HUVEC are indicated with asterisks (P<0.001).

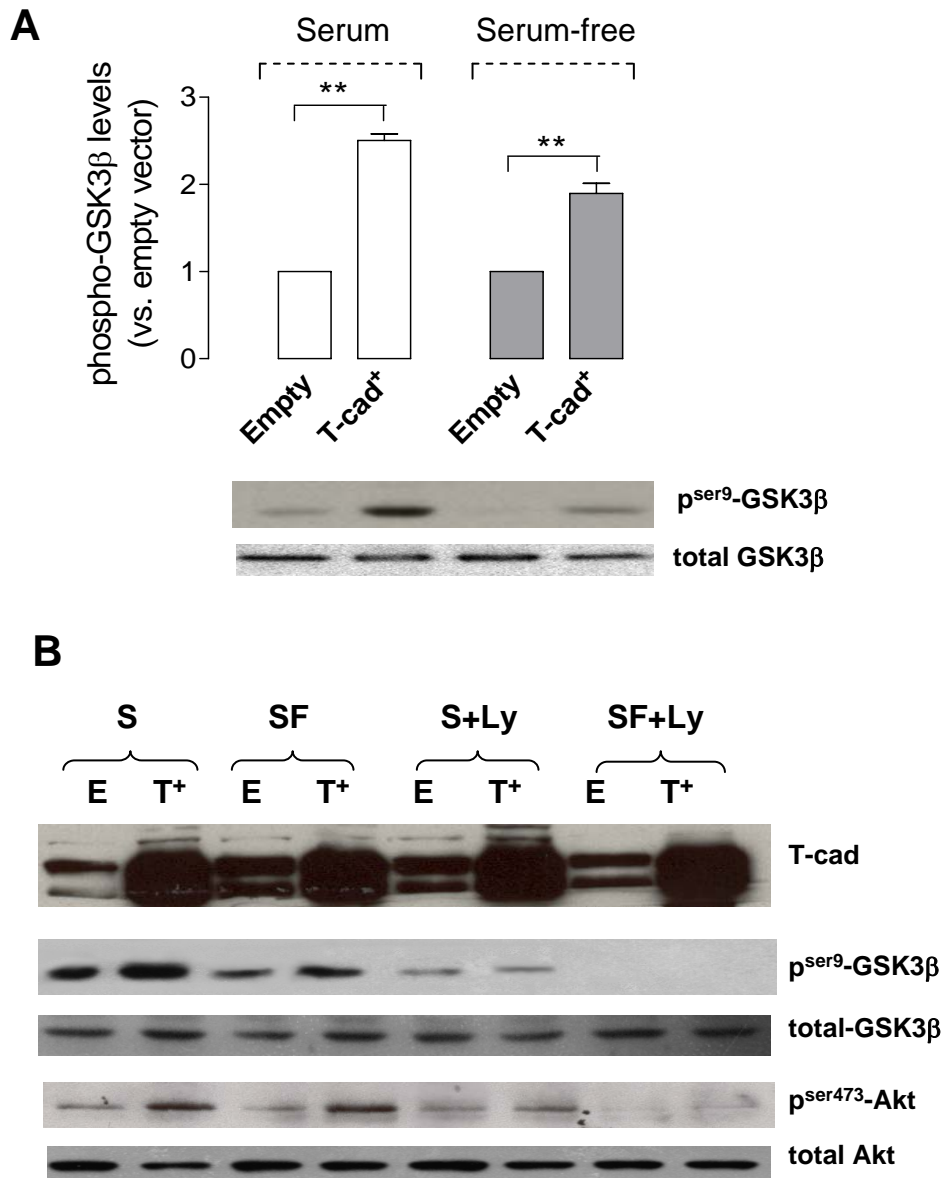
Figure 8: T-cad co-associates with ILK in HUVEC.

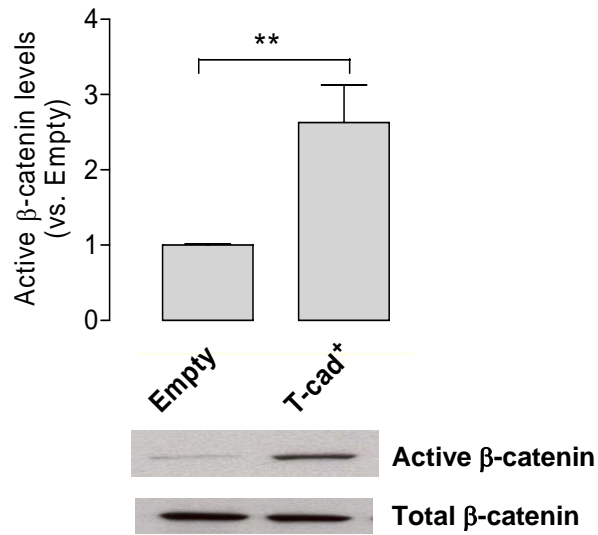
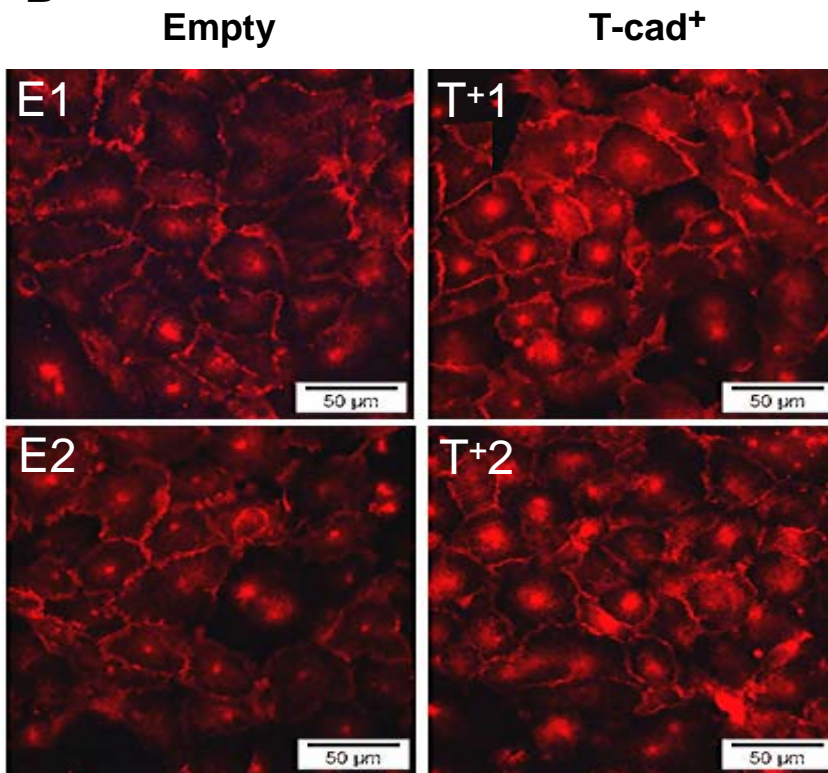
Panel A: Subconfluent HUVEC were transduced overnight with Empty or T-cad adenovectors virus, and cell lysates processed for co-immunoprecipitation with anti-ILK (or non-immune IgG as control). Immunoprecipitates were analysed by Western blotting for T-cad. Panel B: For immunofluorescence HUVEC were seeded

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onto 0.5% gelatine-coated coverslips and allowed to adhere overnight. Cell layers were scraped-wounded to permit analysis of migrating cells. After 6h cells were washed with PBS, fixed with 4% paraformaldehyde, 0.1% Triton X-100 and 1% sucrose for 20 mins, followed by a rinse with PBS. Cells were sequentially stained with anti-ILK antibodies (either clone 65.1, Sigma (a) or clone 65.1.9, Upstate (b)) and anti-mouse Cy2-labelled IgG, followed by a second sequential staining with anti-T-cad antibodies and anti-rabbit Cy3-labelled IgG. Samples were analysed under a laser scanning confocal microscope. Representative micrographs of single channel fluorescence for ILK (green) and T-cad (red) and co-localization fluorescence channel are presented. Note co-localization at leading edges of migrating cells (arrows). Scale bar, 20 μ m.

Figure 9. ILK mediates signal transduction by GPI-anchored T-cad. The schematic proposes a model for T-cad-induced signal transduction whereby ILK can serve as a proximal mediator enabling inwardly-directed signals to intracellular effectors Akt/GSK β and subsequent downstream modulation of proliferation and survival responses. Dashed lines indicate uncertainty as to whether ILK directly regulates phosphorylation of Akt//GSK3 β and/or whether T-cad and ILK constitute part of a larger signaling complex including other transmembrane adapter proteins and cytoplasmic signaling molecules.

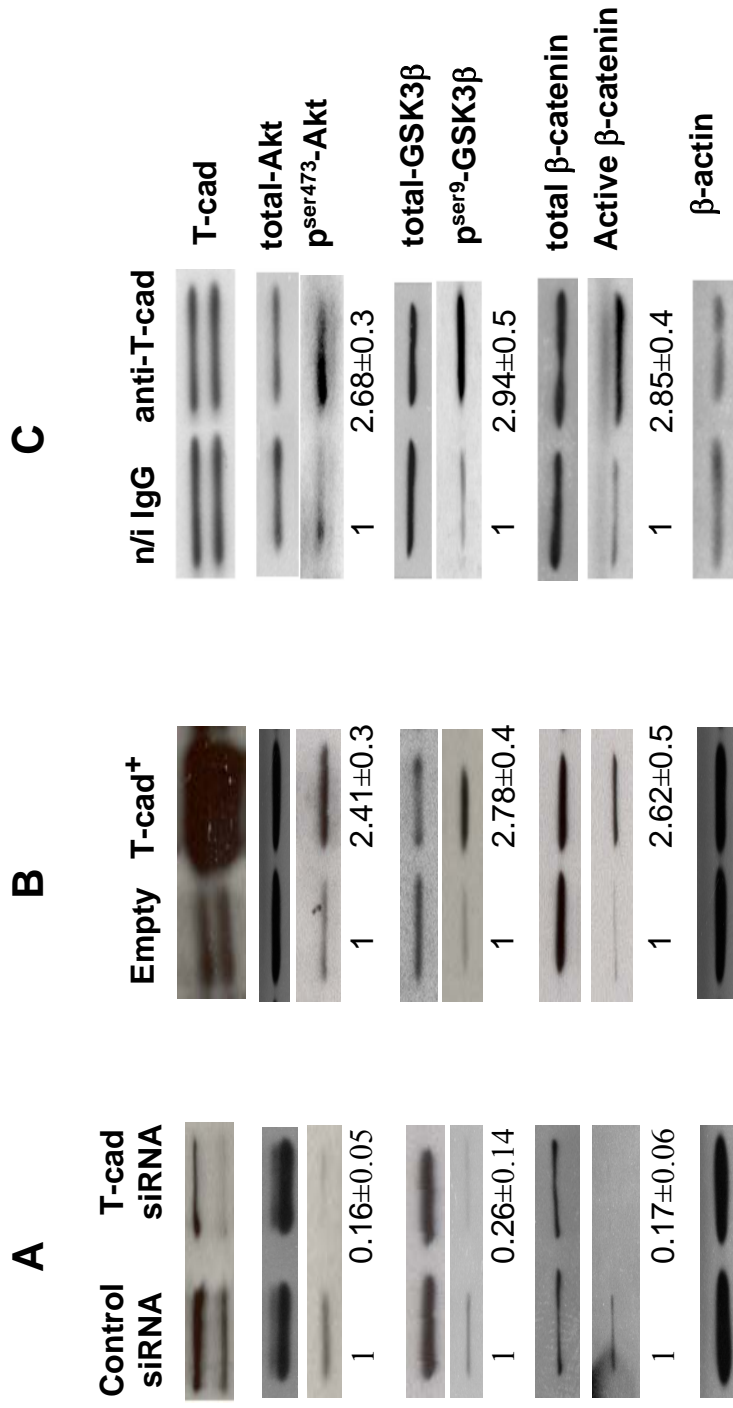


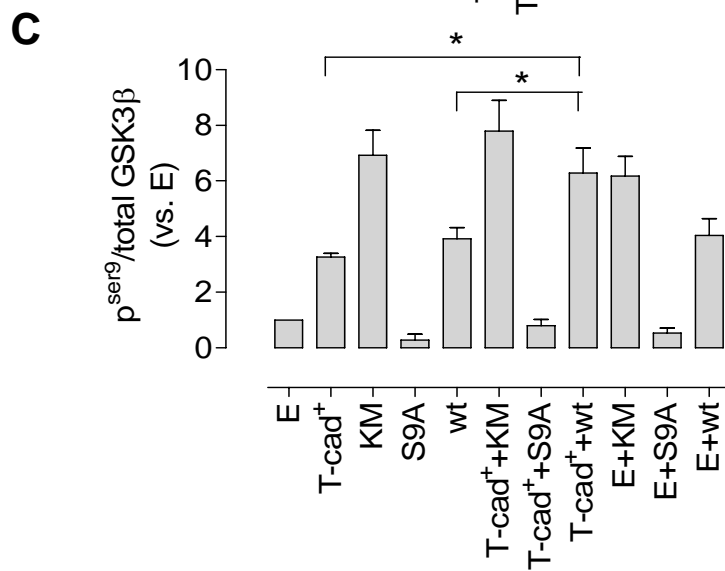
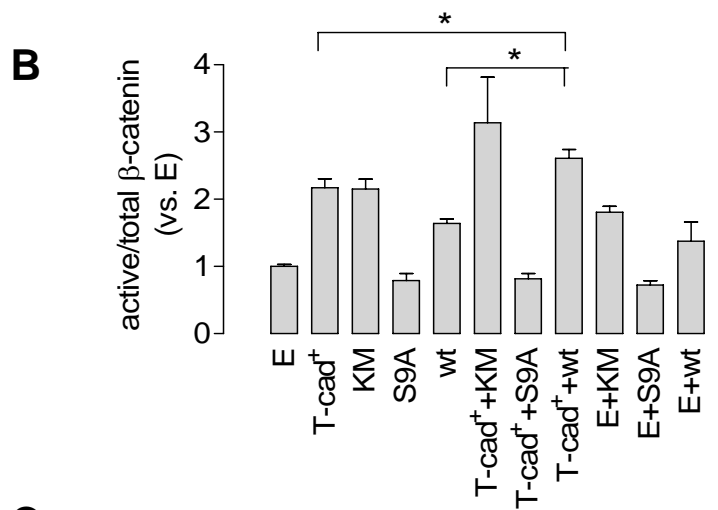
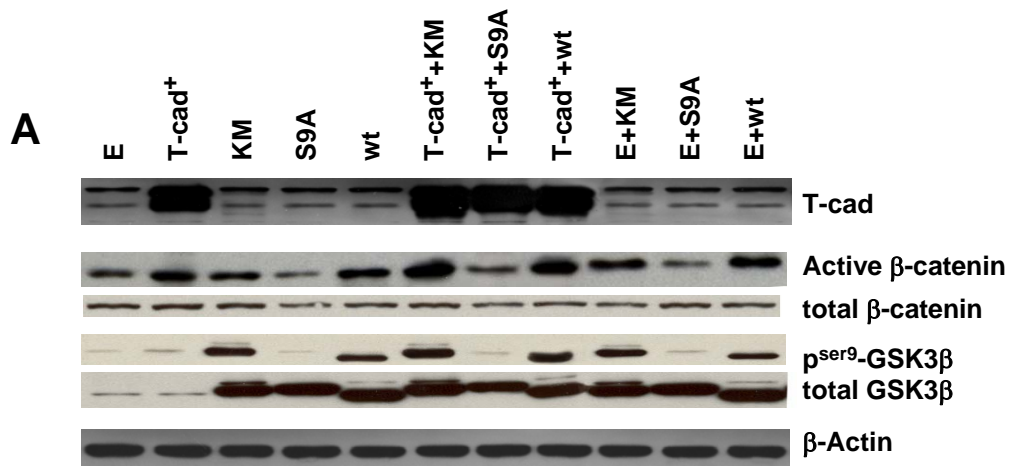
A**B**

% cells positive for
nuclear β -catenin

6 \pm 3

35 \pm 9 **





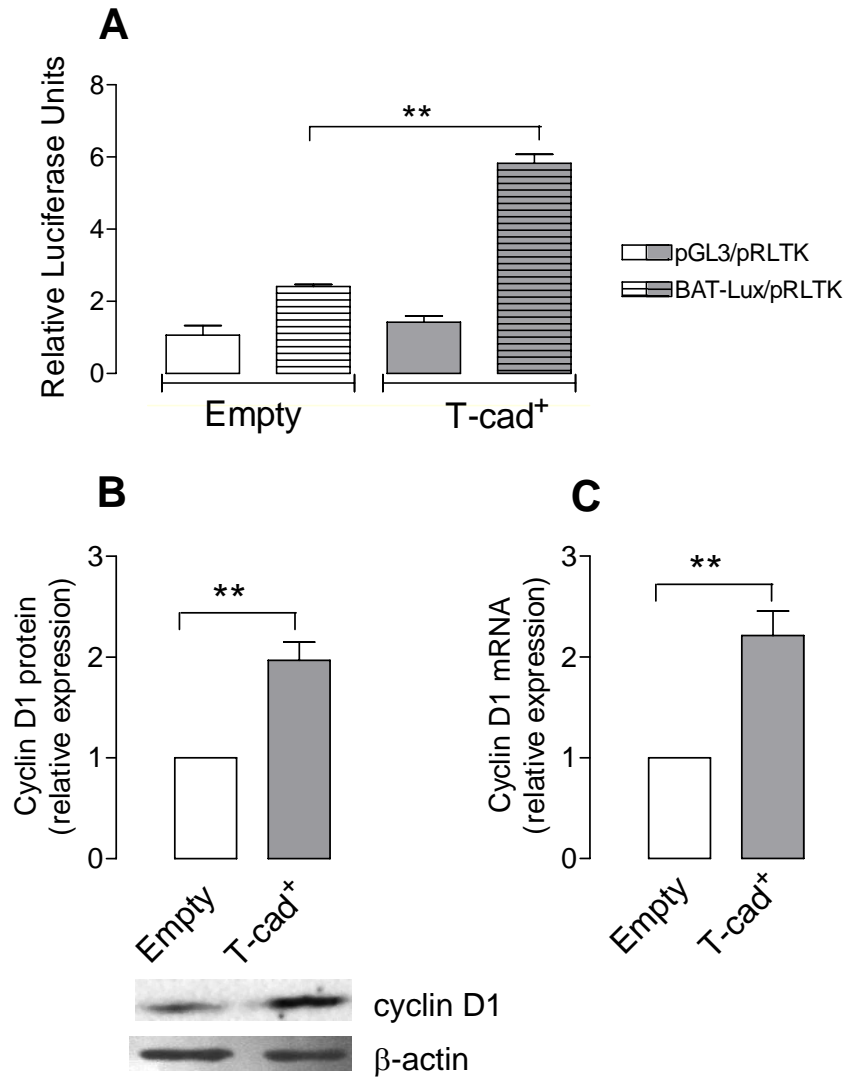
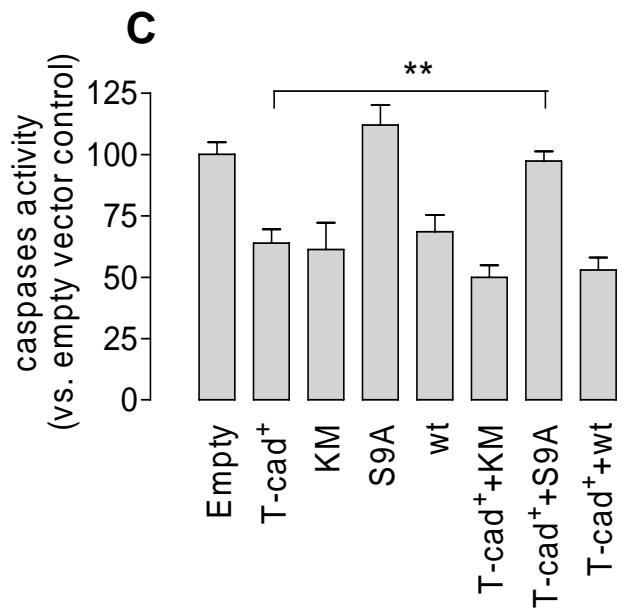
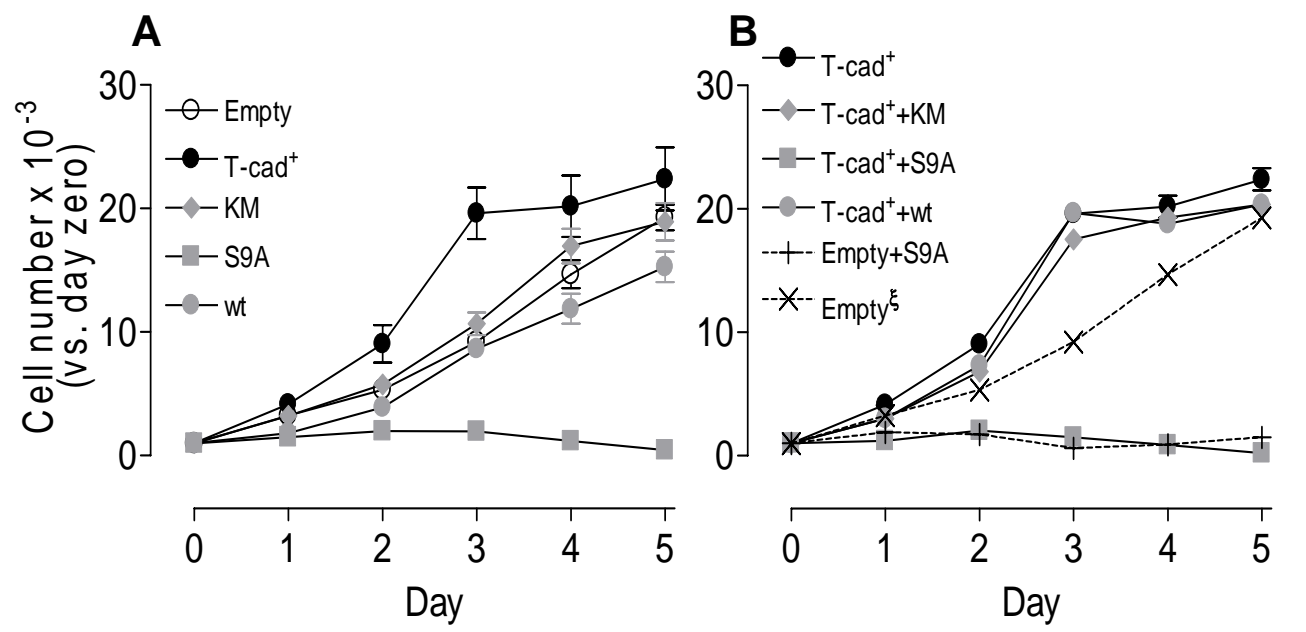
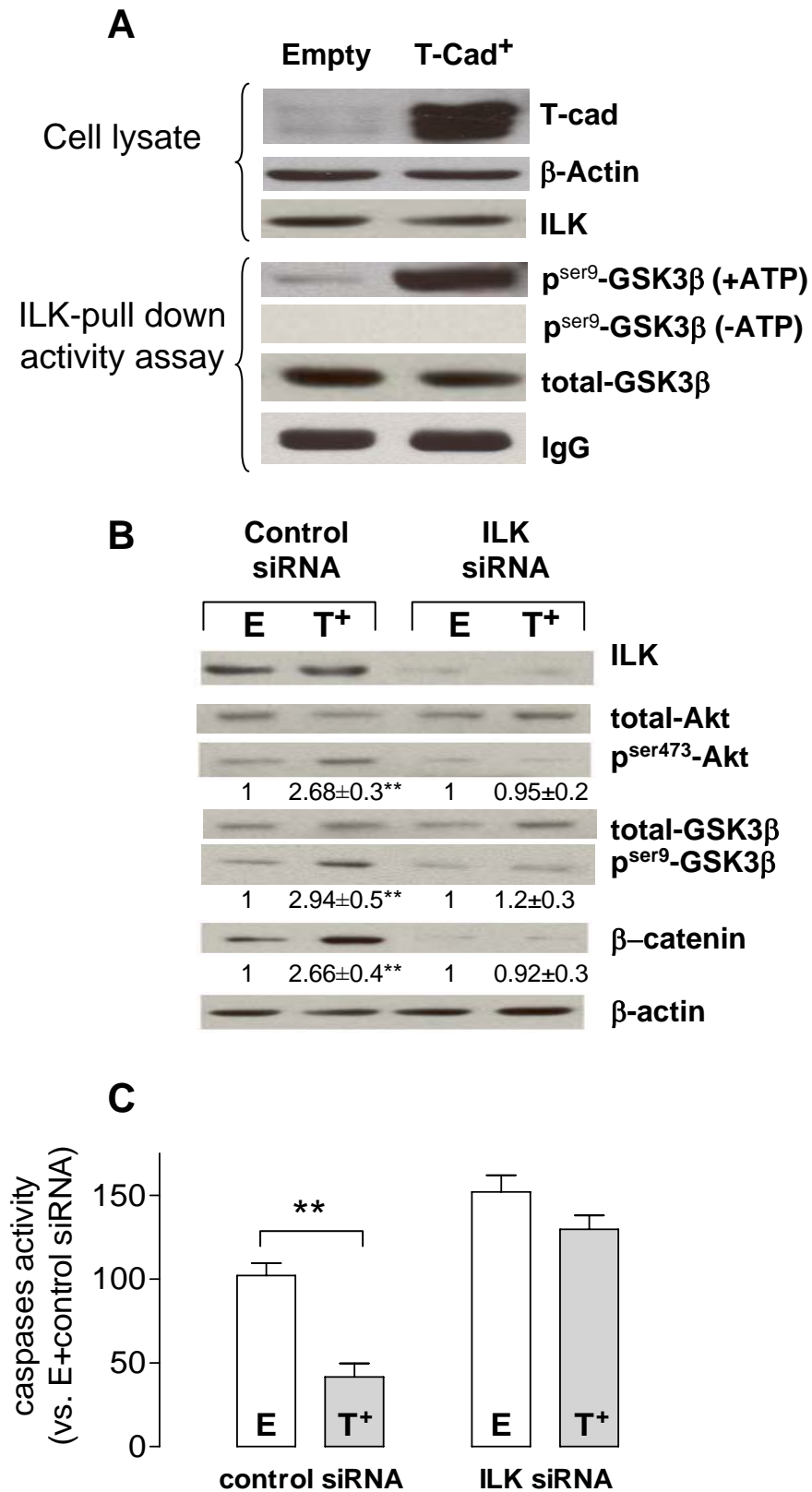
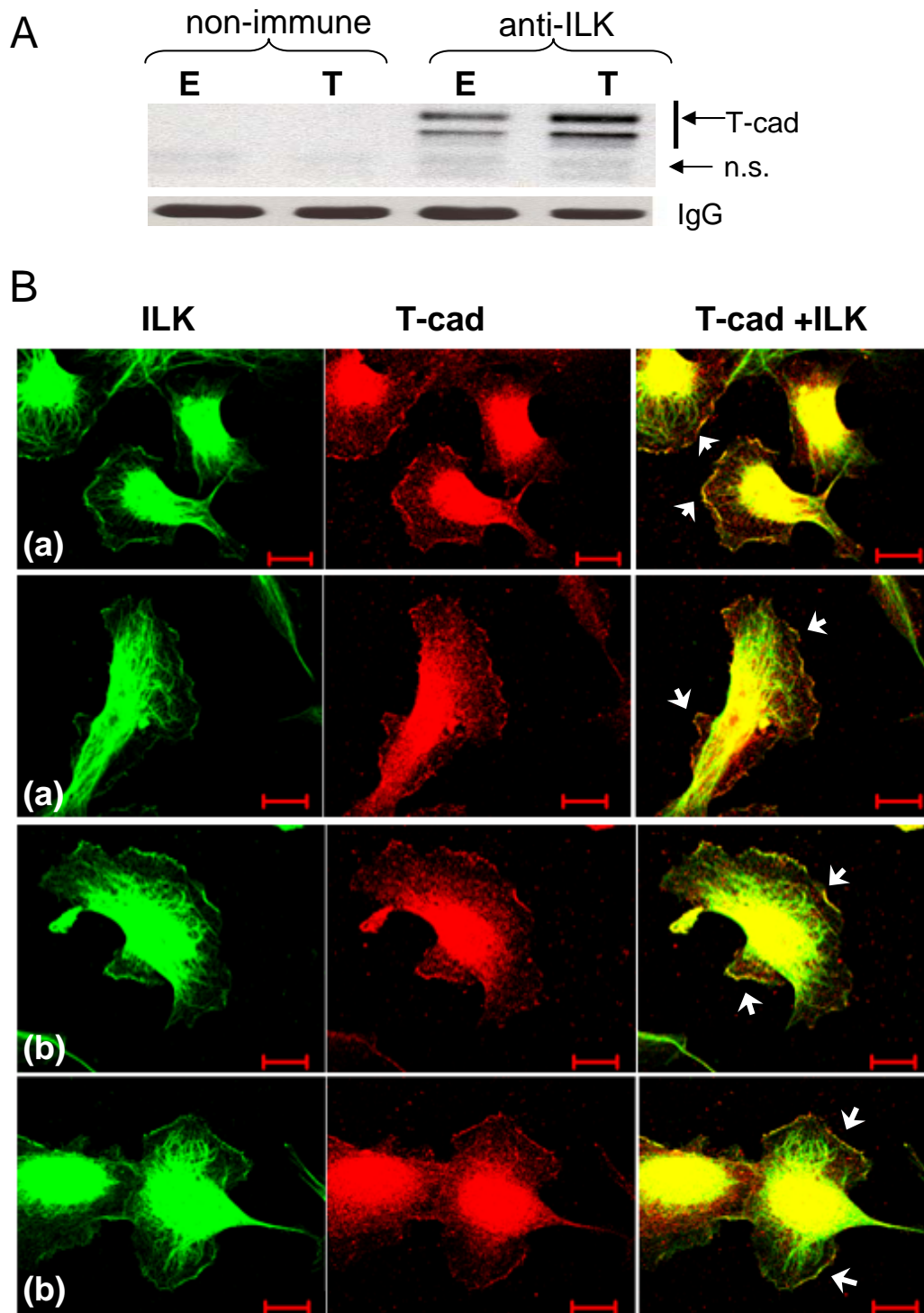
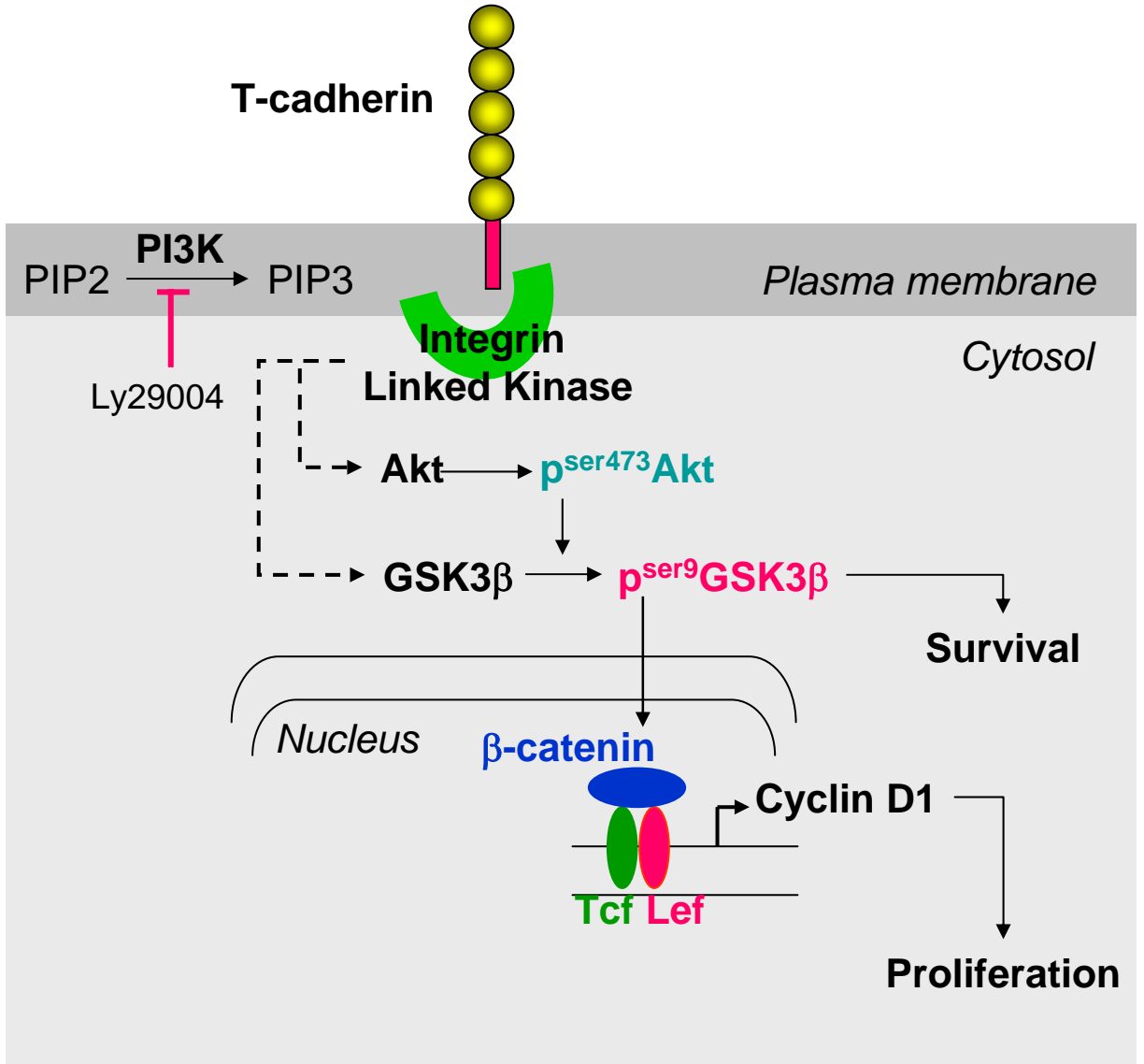


Figure 6









3.3. Transcriptional regulation of T-cadherin in endothelial cells

The results of this project have been drafted as a preliminary manuscript.

Characterization of minimal promoter region of T-cadherin in endothelial cells.

(The manuscript is appended)

Characterization of minimal promoter region of T-cadherin in endothelial cells.

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ABSTRACT

T-cadherin (T-cad) an unusual glycosylphosphatidylinositol (GPI)-anchored member of the cadherin superfamily is expressed widely in the brain and cardiovascular system, and absent, decreased or even increased in cancers. Little is known about mechanisms that control of T-cad expression. This study aimed to investigate transcriptional regulation of T-cad in endothelial cells (EC), using human microvascular EC line (HMEC-1) as the cell model. Treatment of HMEC-1 with methylase inhibitor 5'-azacytidine did not affect T-cad protein expression indicating epigenetic modification by methylation does not regulate T-cad expression in endothelial cells. Conditions of oxidative stress (serum-deprivation or presence of H₂O₂) elevate T-cad protein levels in HMEC-1. Reporter analysis using serially deleted T-cad promoter stretches ranging from -99bps to -2304bps located the minimal promoter region of T-cad within -285bps from the translation start site. Serum-deprivation or exposure to H₂O₂ increased reporter activity in HMEC-1 transfected with -285bps construct, and this could be normalized by inclusion of antioxidant N-acetylcysteine. Gel shift assay using seven different oligos starting from -1 to -284bps revealed specific nucleoprotein complex particular to -156 to -203bps. This was increased using nuclear extracts from oxidatively stressed HMEC-1, suggesting -156 to -203bps oligo contains redox-sensitive binding element(s) responsible for oxidative stress-induced T-cad upregulation is present between -156 to -206bps from the translation start site. Although relevant transcription factors could not be successfully identified, the study supports transcriptional regulation of T-cad in endothelial cells by redox-dependent mechanisms.

INTRODUCTION

T-cadherin (T-cad, cadherin-13, H-cadherin) is an unusual glycosylphosphatidylinositol (GPI)-anchored member of the cadherin super family. Diverse functions have been described for T-cad. In the embryonic nervous system T-cad functions as negative guidance cue regulating motor-axon outgrowth (1). T-cad has been attributed tumor suppressor functions since in several cancers (e.g. breast, lung, colon) loss of T-cad expression correlates with tumour progression, and upregulation of T-cad *in vitro* negatively modulates tumour cell growth and invasion (reviewed in (2)). However, in other cancers (e.g. liver, metastatic lung cancer) T-cad expression is elevated (3, 4), and *in vitro* studies suggest a function for T-cad in epithelial-to-mesenchymal transition and promotion of invasion (5)). In retinoblastomas T-cad expression is unchanged (6). In the vasculature T-cad is upregulated in proliferative disorders such as atherosclerosis and restenosis (7, 8), and *in vitro* studies a function for T-cad in promotion of cell cycle progression and proliferation of smooth muscle cells has been demonstrated (9). T-cad is upregulated in tumor-penetrating blood vessels (5, 10, 11) and stimulates angiogenesis *in vitro* and *in vivo* (12). *In vitro* studies on endothelial cells demonstrate T-cad functions in promotion of proliferation and migration, induction of an angiogenic phenotype and protection against oxidative stress-induced apoptosis (12-15). T-cad has also been shown to be a low density lipoprotein binding protein (16, 17) and a receptor for adiponectin (11).

Transcriptional regulation of T-cad is complex and poorly understood. T-cad gene has been localized on chromosome 16q24 along with a few other cadherins (18). Frequent epigenetic modification of T-cad promoter by aberrant methylation resulting in allelic loss and diminished expression of T-cad has been reported for a variety of cancers including breast (19), lung (20, 21), colorectal (22) (23), cervical (24), and nasopharyngeal (25), digestive tract (26, 27), skin (28, 29), pancreatic (30) cancers and also chronic myeloid leukemia (31) and malignant B cell lymphomas (32). In PC12 cells T-cad has been demonstrated to be a target gene for DNA methyltransferase 3B (Dnmt3b), but Dnmt3b-dependent suppression of T-cad expression occurs via methylation-independent mechanisms (33). Other studies have investigated external factors regulating expression of T-cad. *In silico* analysis of 5' upstream promoter region of T-cad gene revealed potential binding sites for the glucocorticoid, progesterone and estradiol receptors, and in osteosarcoma cells glucocorticoids and sex steroids were found to variously influence T-cad transcription and/or protein expression (34).

Arylhydrocarbon (AHR) response elements can also be identified (34, 35) and polycyclic aromatic hydrocarbon AHR agonists were found to suppress T-cad transcript and protein expression in vascular smooth muscle cells (35). In a study on prostate development, potential androgen responsive elements in the 5' upstream promoter region were suggested as a possible mechanism mediating suppression of T-cad gene expression in response to androgen (36). In endothelial cells FGF (11) and oxidative stress (15) upregulate T-cad protein expression; mechanisms regulating T-cad gene expression have not yet been investigated in this cell type.

Here we report that T-cad expression in endothelial cells is not regulated by promoter methylation. We characterize the minimal promoter region of T-cadherin in endothelial cells by reporter analysis, and using gel shift assay we demonstrate the putative presence of redox-sensitive regulatory elements within -156 to -203bps stretch.

MATERIALS AND METHODS

Cell lines and cell culture: Human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell GmbH (Heidelberg, Germany) and cultured in EC growth medium containing low serum (2 %) EC growth supplement (PromoCell). All tissue culture surfaces were pre-coated with 0.1% gelatine, and HUVEC were used at passages 2-4 during which expression of markers (von Willebrand factor, CD 31, VE-cadherin) for differentiated EC remained steady. Human microvascular EC line (HMEC-1) was cultured in the same medium supplemented with 10 % FCS. Oxidative stress was induced by incubating cells under serum-free conditions (DMEM+0.1 %BSA) or by exposure to 1mM H₂O₂. N-acetyl cysteine (30 mM) was used as antioxidant. 5'-Azacytidine (10 μ M (Sigma Aldrich) was used as DNA methylase inhibitor.

***In silico* analysis of T-cad promoter:** T-cad promoter sequence from human chromosome16 (16q24) was downloaded from NCBI database. Upstream of start site (-1 to -2304 bps) was analysed using online software to identify putative binding elements for transcription factors based on GENOMATIX (www.genomatix.de), TRANSFAC (www.cbrc.jp) and TESS (www.cibl.uppen.edu) databases.

Preparation of constructs: T-cad promoter sequence from -1 to -2304 was cloned into pGL3 plasmid (Promega) in between BglIII and MluI restriction sites. Various size deletions of promoter were generated by PCR with different 5' primers and a fixed 3' primer, using genomic DNA of endothelial cells as template. The sequence of single reverse primer was 5' TTT GTC CGA CTA GAA GCG CCC 3' (-1 to -21) and various forward primers were, 5' GGC AGA GCC TCT CCT CAA 3' (-81 to -99), 5' CCT GGT CAT CAG CCT CTA CC3' (-165 to -183), CAA ATG GGA TGC CAC CTC 3' (-267 to -285), 5' CGC CAG TCC CCC GTG CAA TTC 3' (-352 to -373), 5' GCC CCT CCC TGC CTT CTG CTG 3' (-1149 to -1170), 5' TGT GGG AAA CGT GAG GCT AGA TC 3' (-1561 to -1584), 5' GCC AGC AGA ACA GCC CAG GAA AA 3' (-2281 to -2304).

Transient transfection and reporter gene assay: HMEC-1 cells were transfected with the various constructs using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. 10^5 cells were transfected with 1400ng of either empty pGL3 plasmid or pGL3 with promoter inserts and with 100ng of pRLTK plasmid expressing renilla luciferase as internal control. Firefly and Renilla luciferase activity were measured 48 hours after transfection using Dual luciferase assay system (Promega) in a Luminometer (Berthold Inc).

Preparation of Nuclear Extract: Nuclear extract was prepared by modified protocol formulated by Dignam et al (37). Subconfluent HMEC-1 cells were briefly washed with PBS and cells were collected by trypsinisation. Cells were washed with PBS followed by incubation with Buffer A (25mM HEPES pH7.9, 1.5mM MgCl₂, 10mM KCl, 0.5mM DTT, 0.1mM EDTA, 0.1mM EGTA, 0.1mM Na₃VO₄ and protease inhibitor cocktail (Roche)) for 30min. on ice. NP-40 was added to cell suspensions (final 10%), samples were vortexed for 10sec. and then centrifuged at 12000rpm for 1min. at 4°C. Nuclear pellets were suspended in cold Buffer C (20mM HEPES pH7.9, 1.5mM MgCl₂, 420mM NaCl, 0.2mM EDTA, 25% (v/v) glycerol, 0.1mM Na₃VO₄ and protease inhibitor cocktail). Suspensions were subjected to vigorous shaking in a rotary shaker for 1h at 4°C and then centrifuged at 12000rpm for 10min. Supernatants were collected, aliquoted and stored at -70°C.

Electrophoretic Mobility Shift Assay: EMSA reactions were performed with following oligonucleotides. Oligo -1 to -52

GTGCAGCCGCGTGCATGAATGAAAACGCCGCCGGGCGCTTCTAGTCGGACAAAATG,
 Oligo-34to-83 AAAGCCTGGCTCCCACGGAAAATATGCTCAGTGCAGCCGCGTGCATGAAT
 Oligo -84 to -139
 TGCTGATCTATTTGGGAAGTTGGCTGGCTGGCGAGGCAGAGCCTCTCCTC,
 Oligo -110 to -
 163AGCCTCTACCCAATGCTTTCGTGATGCTGCTGCTGATCTATTTGGGAAGTTGGC,
 Oligo -156 to -203
 GGAAGGAATCCGTCTTGTAAGCCATTGGTCCTGGTCATCAGCCTCTACCCAATGC,
 Oligo -194 to -243
 GGTGCTACCCCGTATCTGCCATGCAAAACGAGGGAGCGTTAGGAAGGAATC,
 Oligo -224 to -284,
 5'CAAATGGGATGCCACCTCCGCGGGGCTCGCTCCTCGCGAGGTGCTACCCCGTATCTG
 CCAT.

Oligos were radiolabeled with [α^{32} P]-dCTP using standard techniques. Binding reactions were performed in a 15 μ l volume reaction mixture containing 1X binding buffer (25mM HEPES pH7.9, 50mM KCl, 1mM DTT, 1mM EDTA, 10% glycerol), 1 μ g salmon sperm DNA, 1 μ g poly dIdC, 1ng radiolabeled probe and 15 μ g of nuclear extract for 30min. at RT°C. For competition assays, cold oligos or non specific DNA (100X unless otherwise specified) were added 15min. prior to the labeled oligos. Samples were loaded onto 4% non-denaturing polyacrylamide gels. Gels were dried onto Whatmann paper and visualized by Phosphorimaging.

RESULTS

Regulation of T-cad in endothelial cells is not epigenetically controlled

Upstream of the translation start site of T-cad gene is rich in CpG islands which are frequently methylated in cancer cells. The occurrence of partial or complete methylation patterns leading to partial or complete inhibition of gene expression has been described (26, 38). We evaluated whether epigenetic modification of T-cad promoter by methylation in endothelial cells might play a role in regulating their constitutive level of T-cad protein expression. HUVEC, HMEC-1, Melanoma Cells

(NA8 cell line) and Jurkat cells were cultured for 72 hours in the absence and presence of 5'-azacytidine (AzaC), a potent inhibitor of DNA methylase, and whole cell lysates subsequently analysed for T-cad protein by immunoblot analysis (Fig. 1). AzaC treatment did not alter expression of precursor/mature (130kDa/105kDa) T-cad protein in either HUVEC or HMEC-1, suggesting the promoter of T-cad is not methylated in endothelial cells since In contrast, in melanoma cells, which expressed only the 130kDa precursor form, treatment with AzaC induced a 3-fold increase in expression of T-cad. Jurkat cells, transformed T lymphocytes, which did not express T-cad under normal culture conditions, synthesized 130kDa precursor form following treatment with methylase inhibitor.

Promoter structure of T-cad

T-cad gene encodes for 2.3kb cDNA and is located on chromosome 16q24. The sequence of T-cad promoter with database-identified putative transcription factor binding elements is presented in Figure 2. Previous studies have demonstrated that T-cad promoter is TATA-less and a GC-like box sequence is located at -266bps (20, 34). Additionally three possible transcription start sites at different locations, -73bps, -120bps and -473bps in malignant cells were identified(20, 34). Various online databases (GENOMATIX, TRANSFAC and TESS) were utilized to perform pattern searches within T-cad promoter for putative transcription factor binding sites. In addition to the previously identified Aryl Hydrocarbon Receptor element (AHR) (34, 35), we found Hypoxia Responsive Element (HRE) overlapping AHR element at -45bps. Various GGAA/T elements were detected starting from -67bps,-119bps, -198bps, -202bps and -277bps. Transcription factors of Ets family members, Egr-1, Pea3 and Nuclear Factor of Activated T-cells family members (NFAT) bind to GGAA/T element. Software depicted a CAAT box at -154bps and another in reverse strand as an inverted CAAT Box at -175bps. CEBP and NF-Y transcription factor family members bind to CCAAT element. GATA-1 and Lyf-1 sites were predicted at -227bps and - 115bps, respectively. Consensus sequences for Nkx-2, CREB, Cdx, AML-1a, SP1, AP1, Stat5 and MZF1 are located in the distal 5' flanking region of T-cad.

Minimal promoter region of T-cad is localized within -285bps

Serially deleted T-cad promoter stretches were cloned into luciferase reporter plasmid pGL3 basic and co-transfected with constitutively active renilla luciferase into HMEC-1. Seven such constructs

ranging from -99bps to -2304bps were analysed (Fig. 3). The first series of reporter assays were conducted under normal serum-containing culture conditions (Fig. 3). The shortest construct from the translational start site (-99bps) showed insignificant levels of reporter activity, ruling out the presence of any functional regulatory elements within -99bps. Transfection with -173bps construct resulted in a \approx 2-fold increase in reporter activity compared with negative control (empty pGL3+pRLTK). Maximum reporter activity, which was \approx 4 fold above negative control, was observed in cells transfected with -285bps construct, thus suggesting the presence of minimal regulatory elements within -285bps. Luciferase activity in all further lower deletion constructs up to -2304bps remained steady at \approx 2-3-fold.

Since T-cad protein expression in HUVEC is upregulated under conditions of serum-deprivation and is mediated by reactive oxygen species (15), we next assessed whether oxidative stress affects the above-described pattern of reporter activity in HMEC-1. For these experiments luciferase activity in HMEC-1 transfected with each of the seven constructs was determined following a 3h period of serum-deprivation. Compared with serum-conditions, reporter activity for -99bps was unchanged, there was a small increase (from 2-fold to 3-fold) for -173bps, and a significant elevation (from 4-fold to 7-fold, $p < 0.01$) of reporter activity was observed for -285bps (Fig. 3). Interestingly, for all further lower deletion constructs (-373bps, -1170bps, -1584bps, and -2304bps) there was no upregulation of reporter activity under serum-free conditions.

Transcription factor (s) binding element is present on -156 to -203 bps

To search for physical interactions between regulatory elements and transcription factors, we performed gel shift assays with nuclear extracts prepared from subconfluent, proliferating HMEC-1 and oligos designed from T-cad promoter. Since reporter activity was observed within -173bps and elevated in -285bps, we designed seven different oligos starting from -1 to -284bps; oligos were of 50-60bps length with minimal overlap in order maintain integrity of putative regulatory elements. The first series of gel shift assays aimed to detect interactions between the different oligos and nuclear extract. Apart from a number of complexes (presumed non-specific) common to all oligos we detected a nucleoprotein complex particular to -156 to -203bps oligo (Fig. 4A), which falls within the minimal promoter region detected by reporter gene analysis.

To validate the specificity of this nucleoprotein complex, competition assay was performed with different concentrations of cold -156 to -203bps oligo. We found a concentration-dependent displacement only of the complex particular to -156 to -203bps (Fig. 4B). A further competition assay compared displacement of -156 to -203bps oligo by a non-specific oligo within the promoter (oligo -34 to -83bps) or by cold -156 to -203bps oligo (Fig. 4C). Displacement was observed only with its own cold and not with the non-specific cold oligo, further validating specificity of the identified nucleoprotein complex particular to -156 to -203bps.

-285 bps region is modulated by oxidative stress

We have previously demonstrated that serum-deprivation-induced increase of T-cad protein in HUVEC is due to oxidative stress involving NADPH oxidase (15). Thus having demonstrated a serum-deprivation-induced elevation of reporter activity in HMEC-1 transfected with -285bps construct (Fig. 3), we next investigated whether -156 to -203bps oligo might contain a redox-sensitive regulatory element responsible for T-cad upregulation following serum deprivation.

First, whole cell lysates (for Western's) and nuclear extracts (for EMSA's) were prepared from subconfluent HMEC-1 cultured under serum-free conditions for different times (0-4 hrs). Western blot analysis showed an increase in T-cad protein expression in HMEC-1 within 2hrs (Fig. 5A), confirming our finding in HUVEC (15). Gel shift analysis using -156 to -203bps oligo revealed an increase in formation of the putative specific nucleoprotein complex within 2hrs without a change in non-specific complexes (Fig. 5B), suggesting -156 to -203bps oligo contains relevant binding element(s).

Next, to validate the oxidative stress-modulated minimal promoter region of T-cad, we examined the influence of an external source of reactive oxygen species (ROS) and antioxidants on reporter activity of -285bps. HMEC-1 transfected with -285bps construct were cultured for 2hrs either in the absence of serum or in the presence of H₂O₂ and without or with inclusion of N-acetyl cysteine (NAC); whole cell lysates and nuclear extracts were prepared. Immunoblot analysis showed an NAC-sensitive increase in T-cad protein in HMEC-1 following serum-deprivation or exposure to an external source of ROS (Fig. 6A), confirming our observations in HUVEC (15). Both serum-deprivation and treatment with H₂O₂ increased reporter activity 2-fold above that under control

serum-containing conditions, and these oxidative-stress inductions of reporter activity were abrogated in the presence of NAC (Fig. 6B). These data suggest regulatory element(s) located in -285bps are responsible for redox-sensitive modulation of T-cad.

Hypoxia Response Element is located at -45, overlapping with AHR element. To investigate the possible functionality of HRE we induced hypoxia in HMEC-1 and HUVEC by treatment with 3% oxygen, 150 μ M cobalt chloride, 100 μ M deferoxamine and 10 μ M cyclopiroxolamine. T-cad protein levels in both HMEC-1 and HUVEC remained unchanged (data not shown), and therefore we did not pursue gel shift or reporter assays under conditions of hypoxia.

DISCUSSION

This study aimed to investigate transcriptional regulation of T-cad in endothelial cells and is the first to identify the minimal promoter region of T-cad gene in endothelial cells. The main findings are: (1) epigenetic modification by methylation does not regulate T-cad expression in endothelial cells; (2) the minimal promoter region of T-cad is located within -285bps from the translation start site; (3) transcription factor(s) binding regulatory element responsible for oxidative stress-induced T-cad upregulation is present between -156 to -206bps from the translation start site.

Epigenetic modifications including promoter methylation and histone (de)acetylation are common mechanisms for tissue specific gene expression. Some genes (e.g. human tissue transglutaminase gene) are regulated by differential methylation whereby the promoter is hypermethylated and silenced in neoplastic cells but hypomethylated in normal cells leading to its constitutive expression (39). Other genes (e.g. endothelial nitric oxide synthase gene) are regulated by transcription factor(s) for tissue/cell specific constitutive expression whereas the promoter is methylated in all other cell types (40). Yet other genes (e.g. E-cadherin) are regulated in a tissue specific manner by either promoter methylation or by co-repressors.(41) We found that AzaC did not affect expression of T-cad in endothelial cells but induced expression in melanoma and Jurkat cells. This suggests T-cad belongs to the eNOS-like regulated gene family where it is regulated by transcription factor(s) for constitutive expression in EC but suppressed or modulated by promoter methylation in other cell types. Expression of T-cad is variable in different in malignant cells. This study shows that

melanoma cell line expressed only precursor T-cad and this was upregulated upon treatment with AzaC, whereas Jurkat cells expressed precursor T-cad only after methylase inhibition. Additionally, in a study on hepatocarcinoma (5), of six tested hepatoma cell lines only one (Mahlavu) expressed T-cad (both precursor and mature) protein, and the rest were differentially (between 4-100%) methylated; the effect of methylase inhibition was not investigated.

In contrast to CArG boxes as muscle specific regulatory elements (42), endothelial cell specific regulatory elements have not been reported. Spatial and temporal differences in endothelial gene expression have been proposed to account for endothelial heterogeneity and vascular diversity (43, 44). Similarities exist between promoter of T-cad and those of other cadherins and endothelial-specific genes. For example, T-cad promoter is TATA-less (20, 34) as are E-cadherin (45), P-cad (46), and endothelial markers such as eNOS (47) and VEGF(48). Transcription start sites at 3 different positions at -73bps, -12bps and -473bps have been located in osteosarcoma cells (34) and in lung cancer cell lines (20). We observed minimal promoter activity within -285bps, but not in -99bps indicating that the -120bps transcription start site might be functional in endothelial cells. A previous study putatively correlated location of AHR element at -45bps with Ahr ligand-dependent repression of T-cad in rat smooth muscle cells (35). Since the -99 bps construct did not exhibit any reporter activity in endothelial cells, it is possible that differences in regulation of T-cad within different constituent cells of the vasculature exist. Support for this assumption derives from observations that vasoactive substances such as angiotensin II and thrombin induce T-cad expression in human smooth muscle cells but not in endothelial cells (unpublished data).

Endothelial cells transfected with -285bps exhibited a 4-fold increase of minimal reporter activity, whereas a 9-fold increase in reporter activity was demonstrated in osteosarcoma cells transfected with -434bps (34). Additionally the minimal reporter activity observed within -285bps in endothelial cells remained steady with all further deletion fragments tested. An abundance of transcription factor(s), different regulatory proteins or malignancy might explain the higher reporter activity in osteosarcoma cells. Serum-deprivation- and H₂O₂- induced reporter activity in cells transfected with -285bps could be normalized by antioxidant N-acetylcysteine. Additionally formation of specific nucleoprotein complex formation (using -156 to -203bps oligo) was increased under serum-free conditions. These data support that the regulatory element present within -285bps/-156 to -203bps in

endothelial cells is modulated by reactive oxygen species. Under conditions of oxidative stress induction of reporter activity was observed only for -285bps fragment but not for further deletion fragments, suggesting a possible presence of negative regulatory elements between -285bps to -373bps that might be functional under oxidative stress.

Specific nucleoprotein complex formation was observed with -156 to -206bps oligo where two copies of GGAA (Ets binding element), one GATA and two copies of CAAT are located. The presence of GGAA/T elements and their regulation by Ets transcription factor family members have been reported upstream of many endothelial cell specific markers such as eNOS (40)(49) VE-cadherin (50), vWF (51) , Tie1/2 (52) and VEGF receptors (Flk1) (53). The regulation of GGAA/T elements by NFAT has also been demonstrated in endothelial cells (54). Endothelin and PECAM gene expression are controlled by GATA transcription factors (40). We were unable to determine any supershift in gel shift assays using antibodies against Ets1/2, NFAT or GATA (data not shown).

Oxidative stress is a major stimulus for proliferative vascular disorders such as atherosclerosis (55) and restenosis (56) and for intraplaque angiogenesis (57). T-cad is upregulated during restenosis (8), atherosclerosis (7) and angiogenesis (10) and *in vitro* studies have demonstrated T-cad to be a redox-sensitive protein (15). The present study supports transcriptional regulation of T-cad in endothelial cells by oxidative mechanisms. While we have been unable to identify a relevant transcription factor responsible for T-cad gene regulation in response to oxidative stress, we have located the minimal promoter region (-285bps) and revealed a specific redox-sensitive binding element within -156 to -203bps.

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Figure Legends

Figure 1: T-cad is not regulated by promoter methylation in endothelial cells.

HUVEC, HMEC-1, Melanoma and Jurkat cells were cultured in presence (5-AzaC) or absence (C) of 10 μ M 5'-azacytidine for 72 hours. Cells were lysed and processed for Western blotting with anti-T-cad antibodies and with anti-G α as internal control. Representative blots from at least 3 independent experiments are shown.

Figure 2: 5' flanking region of T-cad gene.

Promoter region of T-cad gene was analyzed using various databases (described in Material and Methods). Putative regulatory elements are indicated in bold font. GC like box is located at -266. Ets transcription factors binding to GGAA/T elements were found at 67bps, -119bps, -198bps, -202bps and -277bps. CCAAT elements were detected at -154 and -175. AHR/HRE elements were detected at 45bps. Arrowhead denotes translation start site. Asterisks denote previously reported transcription start sites. -156 to -203 bps responsible for formation of specific nucleoprotein complex (see Figure 5) is boxed

Figure 3: Reporter gene analysis for T-cad promoter.

HMEC-1 were co-transfected with 1400 ng of various deletion fragments cloned in pGL3 and 100ng of pRLTK (internal control) using Lipofectamine 2000. Luciferase activity was measured 72 hours after transfection. Experiments were performed in HMEC-1 under normal serum-containing culture conditions (upper panel) and following 3 hours serum-deprivation (DMEM+0.1% BSA). Data express luciferase activity relative to negative control (empty pGL3 + pRLTK) and represent mean \pm SD of at least 4 independent experiments. The asterisk indicates significant difference ($p < 0.01$) between serum and serum-deprivation conditions.

Figure 4: Electrophoretic mobility shift assay reveals formation of specific nucleoprotein complex within -156 to -203 bps.

Panel A: Gel shift assays were performed with radiolabeled oligos designed from T-cad promoter from -1 to -284bps and nuclear extracts of proliferating, subconfluent HMEC-1. Samples were resolved on 4% acrylamide gels, and nucleoprotein complexes were detected by Phosphorimaging. A representative image of 3 separate experiments is presented. Apart from complexes common to all oligos (ns), a complex particular to -156 to -203bps oligo was observed (indicated by arrow and ?). Differences in intensity of assumed nonspecific complexes (ns) are due to non-uniform labeling.

Competition assay was performed with radiolabelled -156 to -203bps oligo and various concentrations (denoted by X) of cold -156 to -203bps oligo (Panel B), or with either cold -156 to -203bps oligo or non-

specific -34 to-83bps oligo (each at 100X). Double headed arrow indicated specificity of nucleoprotein complex.

Figure 5: Oxidative stress induces T-cad protein expression and formation of nucleoprotein complex.

Cell lysates and nuclear extracts were prepared from HMEC-1 at the indicated intervals after serum deprivation. Panel A: Cell lysates were immunoblotted for T-cad and $G\alpha$ as internal control Panel B: Equivalent amounts (15 μ g) of nuclear extracts were processed for gel shift assay with -156 to -203bps oligo. The experiment was performed twice.

Figure 6: Minimal promoter region of -285bps of T-cad is modulated by oxidative stress

Panel A: Subconfluent HMEC-1 were cultured for 2 hours under serum-free conditions (SF) or in the presence of 1mM H_2O_2 without or with inclusion of 30mM N-acetylcysteine (NAC). Cell lysates were immunoblotted for T-cad and $G\alpha$. A representative blot of at least 3 independent experiments is shown. Panel B: HMEC-1 were transfected with -285bps construct. 72 hours post-transfection cells were cultured as for Panel A followed by measurement of luciferase activity. Data express luciferase activity relative to activity under control serum-containing conditions and represent mean \pm SD of 3 independent experiments. The asterisk indicates where reporter activity is significantly different ($p < 0.01$) from control serum conditions.

Figure 1

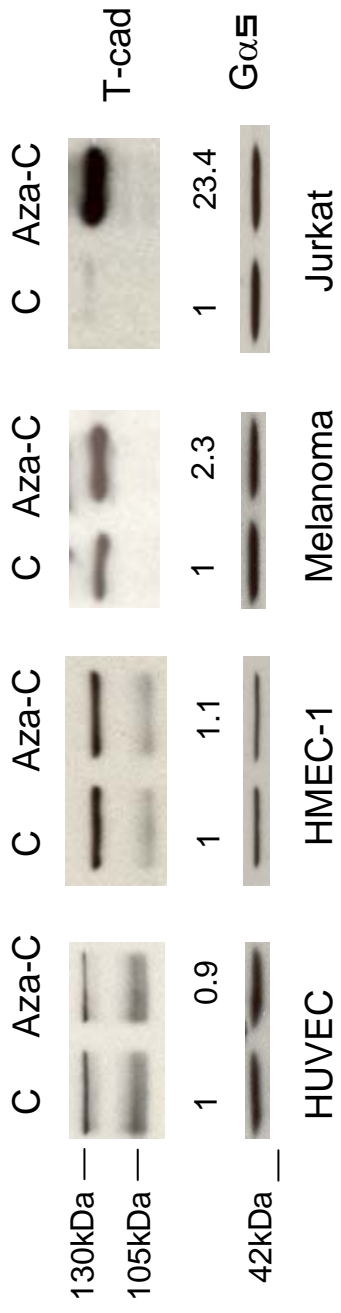


Figure 3

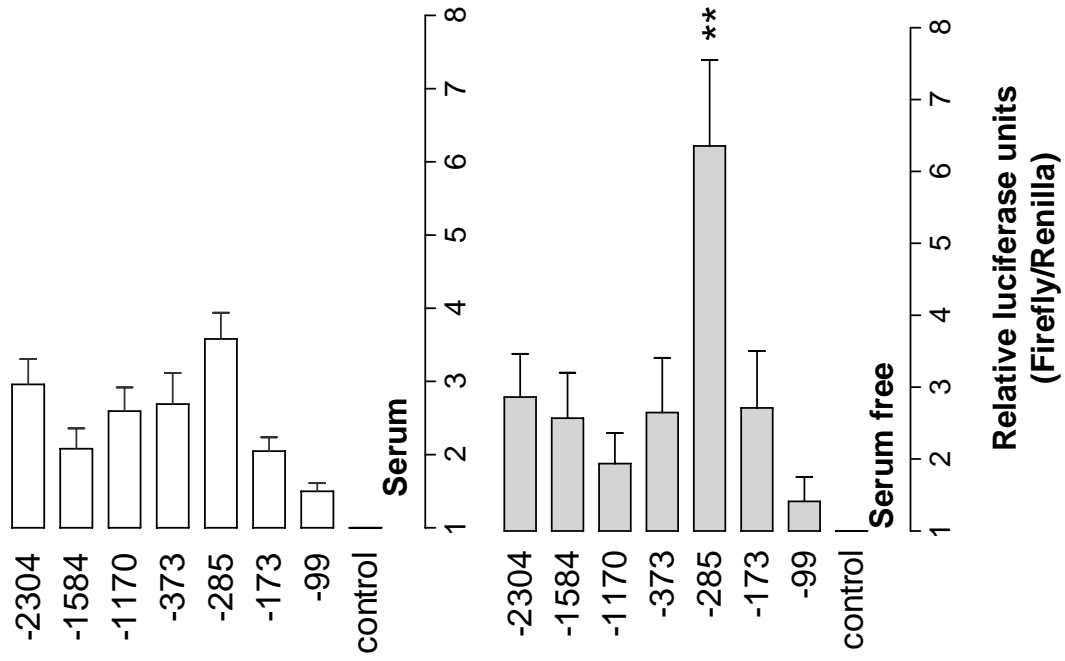


Figure 4

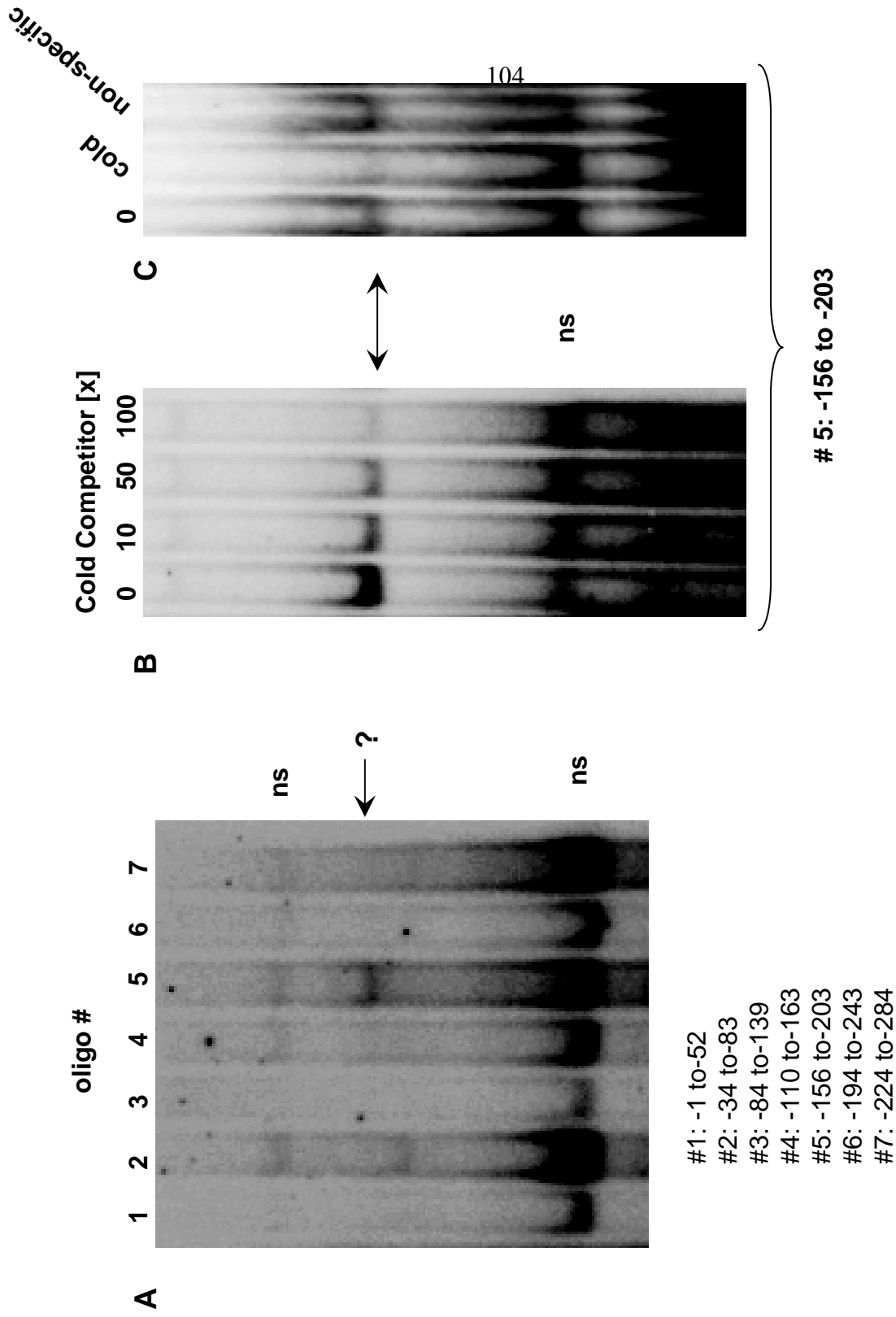


Figure 5

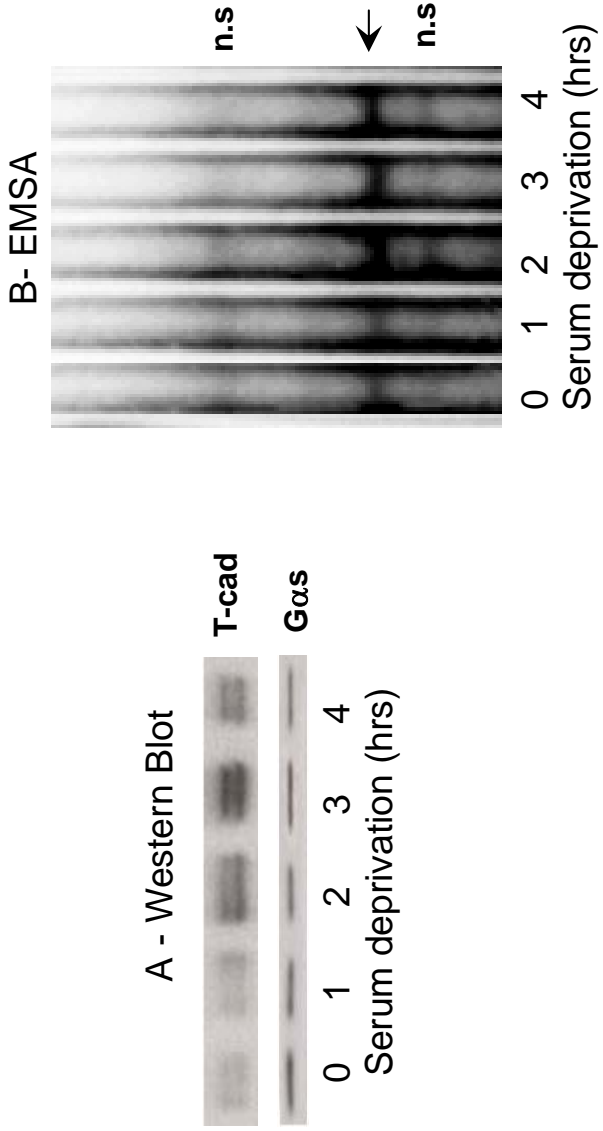
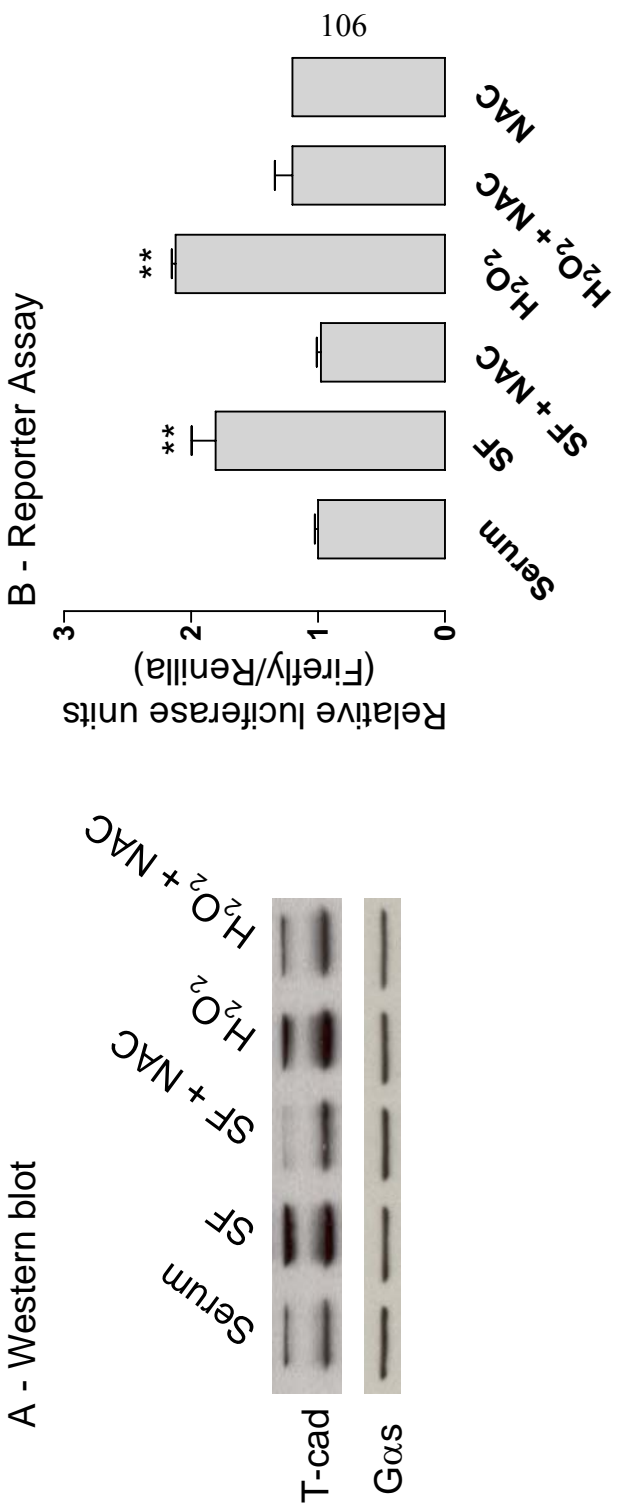


Figure 6



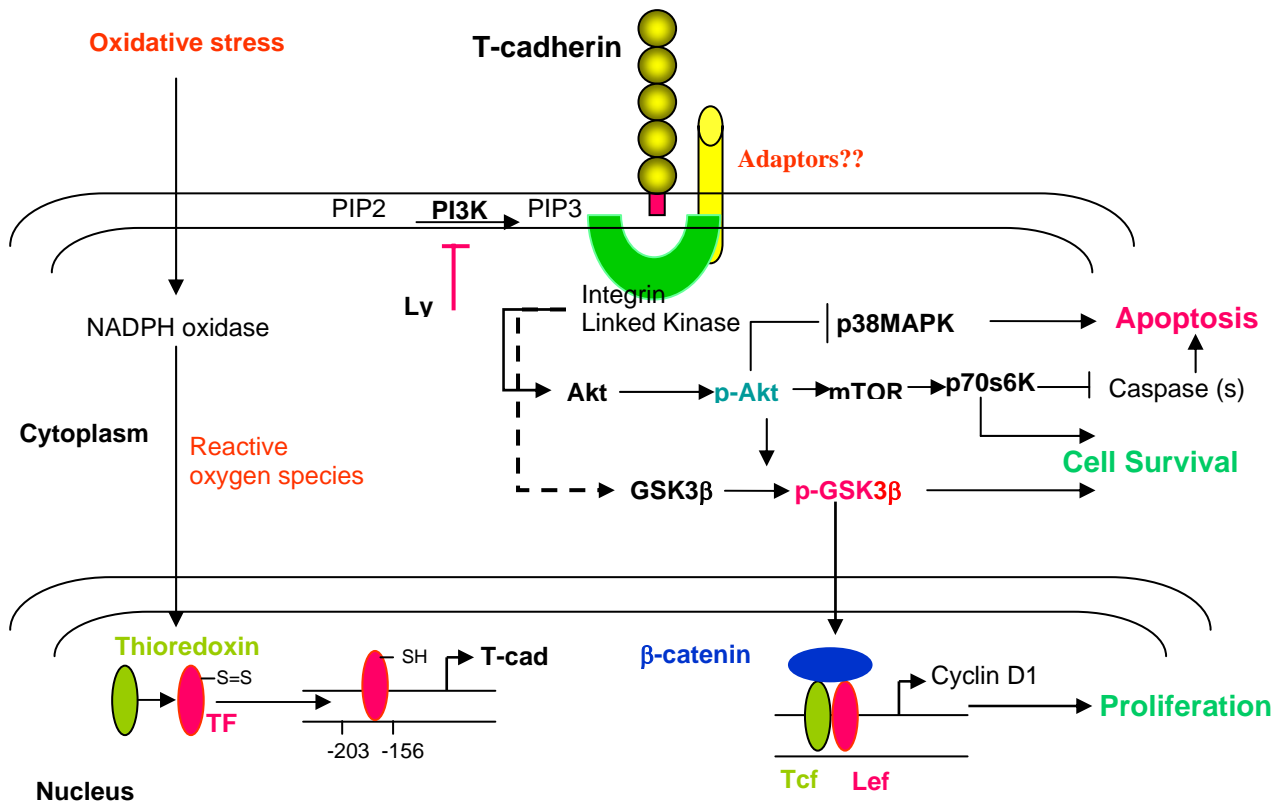
4. Key findings and perspectives

T-cad is an unusual member of cadherin superfamily, is widely expressed in cardiovascular system and is upregulated during proliferative vascular disorders such as atherosclerosis and restenosis. *In vitro* T-cad facilitates important biological processes such as differentiation, proliferation and migration of vascular cells (EC and SMC). T-cad has been proven to possess pro-angiogenic properties *in vitro* and *in vivo*. All of the above biological processes are associated with oxidative stress and cell death/survival. This dissertation has addressed a number of issues including:

- (1) whether T-cad is modulated under oxidative stress,
- (2) whether T-cad regulates endothelial apoptosis/survival,
- (3) which signaling pathways are activated by T-cad,
- (4) which molecular mediators does T-cad use to effect outside-in signaling,
- (5) characterization of the minimal promoter region of T-cad,
- (6) transcriptional regulation of T-cad.

4.1. Key Findings

The key findings of this dissertation are schematically summarized in Figure below.



We observed an elevation in T-cad levels under condition of oxidative stress induced by serum-deprivation and H₂O₂; this response was normalized upon inclusion of an antioxidant, N-acetyl cysteine or NADPH oxidase inhibitor diphenyleneiodonium, suggesting T-cad induction by reactive oxygen species is NADPH oxidase dependent. Adenoviral mediated overexpression of T-cad facilitated EC survival upon induction of apoptosis by serum-deprivation and various apoptosis-inducing pharmacologicals. Western blot analysis of lysates infected with Empty –adenovirus (E) and T-cad adenovirus (T-cad⁺) resulted in hyperactivity of anti-apoptotic proteins (Akt and mTOR target p70S6 kinase) and diminished activity of pro-apoptotic proteins (p38 MAPK and active caspase3). PI3 kinase inhibitor, wortmannin, and mTOR inhibitor, rapamycin, normalized anti-apoptotic effects of T-cad; these data suggest that upregulation of T-cad in response to oxidative stress functions to protect EC by concomitant induction of PI3K/Akt/mTOR pathway and suppression of p38/caspase3 pathways.

Subsequently we focused on identifying downstream targets of Akt and candidate proximal molecular mediators for T-cad. T-cad⁺-EC exhibited hyperphosphorylation of glycogen synthase β (GSK3 β) and concomitant nuclear accumulation of active β -catenin, a transcription factor regulating cell cycle proteins. Use of various GSK3 β -carrying adenovectors (kinase mutant, dominant negative or wild type) we demonstrated that T-cad induced nuclear accumulation of β -catenin is GSK3 β -dependent. siRNA mediated knockdown of T-cad resulted in decreased phosphorylation of Akt and GSK3 β and also in reduced nuclear accumulation of β -catenin. T cell factor (TCF) and Leukocyte enhancer factor (LEF) are co-factors for β -catenin; we found that luciferase (reporter) activity of TCF/LEF elements in T-cad⁺-EC was markedly increased as compared to E-EC. Cyclin D1, one of the important regulators of the cell cycle is a target of β -catenin/TCF/LEF transcription machinery; T-cad⁺-EC showed increased mRNA and protein levels of cyclin D1 and increased cell proliferation. In searching for molecular mediators of T-cad we considered Integrin linked kinase (ILK) as a putative candidate, because both the proteins are located in rafts and ILK acts upstream of Akt and GSK3 β in a PI3K-dependent fashion. T-cad⁺-EC exhibited increased ILK “kinase” activity in a pull-down assay. RNAi-mediated knockdown of ILK abrogated effects of T-cad on both phosphorylation of Akt and GSK3 β and the nuclear accumulation β -catenin, suggesting involvement of ILK in T-cad signaling. Confocal microscopy studies revealed colocalisation of T-cad and ILK in EC which was most prominent within leading edges of migratory cells and at focal adhesions. Anti-ILK immunoprecipitates contained T-cad

indicating the existence of T-cad/ILK complexes, and supporting our hypothesis that ILK can function as a proximal molecular mediator for T-cad-elicited PI3K/Akt/GSK3 β signaling.

Transcriptional regulation of T-cad in endothelial cells is poorly understood. To characterize the minimal promoter region of T-cad, we cloned serially deleted fragments of T-cad promoter stretches into luciferase reporter vector (pGL3). Reporter gene analysis exhibited basal levels of luciferase activity within -285bps suggesting existence of minimal promoter region within -285bps from translational start site. Oxidative stress elevated reporter activity of -285 bps construct, suggesting the minimal promoter region might be responsible for the redox sensitivity of T-cad expression. To identify regulatory elements (transcription factors) responsible for T-cad regulation gel shift assays were performed using nuclear extracts of EC and various oligos designed from T-cad promoter region from -1 to -284 bps. We identified specific binding of regulatory protein(s) between -156 to -203 bps. Nuclear extracts from serum-deprived EC exhibited increased binding to -156 to -203 bps oligo, suggesting that the identified nucleoprotein complex could function to induce T-cad expression under conditions of oxidative stress. To identify transcription factor(s) within the identified nucleoprotein complex we performed pull-down assay using nuclear extracts of EC, biotinylated -156 to -203 bps and streptavidin agarose beads. Proteins pulled down were subjected to microsequencing by mass spectrometry. Interestingly thioredoxin was found to be present. Thioredoxin is a 12kDa protein induced by NADPH oxidase under stress and it acts as an antioxidant by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Following translocation to the nucleus reduces transcription factors enabling their binding to regulatory elements. Preliminary data using RNAi technology showed that knockdown of thioredoxin abrogates oxidative stress-induced upregulation of T-cad in EC, suggesting that NADPH dependent-induction of T-cad involves nuclear translocation of thioredoxin.

4.2. Perspectives

In the present dissertation we successfully delineated T-cad-induced signaling pathways leading to EC survival and proliferation, and studied transcriptional regulation under oxidative stress conditions. There are still many issues to resolve.

Identification of Transcription factor(s)

Biotin-streptavidin pull down assay and microsequencing revealed thioredoxin as a putative “indirect” regulator of T-cad transcription. This needs to be confirmed. Further it is necessary to identify thioredoxin-sensitive transcription factors which would directly modulate T-cad transcript expression. The following experimental approaches may be helpful.

- 1) Immunoprecipitation of thioredoxin from cytosol and incubating with nuclear extracts and radiolabelled oligo -156 to -203 followed by performance of gel shift assays to assess whether thioredoxin increases binding of unknown TF to regulatory elements located in -156 to -203 oligo. This would further confirm role of thioredoxin in formation of nucleoprotein complex.
- 2) Precipitation of thioredoxin from nucleoprotein complex (i.e oligo -156 to -203 and nuclear extracts) and detection of co-precipitated TF using mass spectrometry after comparison of differentials between non-immune and thioredoxin pull-down precipitates by silver staining. This could identify relevant TF(s).
- 3) If any TF is detected by mass spectrometry, supershift assays can be conducted to validate the results.
- 4) A siRNA approach or use of dominant negative mutants of detected TF(s) with the goal of demonstrating suppression of T-cad expression would confirm the functional relevance of the identified TF.

Involvement of different signaling pathways/crosstalk in T-cad signaling

Thus far we have established that T-cad induces PI3K/Akt/GSK3 β axis signaling and that this participates in the effects of T-cad on EC proliferation and survival. Because T-cad also influences other functions of EC (e.g. differentiation, adhesion, migration) it is likely that further signaling pathways are activated. Gene expression profile using microarray technology could shed light on different pathways involved. An experimental approach to address the issue is as follows.

- 1) Extraction of total RNA from ECs either overexpressing T-cad or depleted of T-cad and processing for microarray analysis. Modulation of genes due to overexpression or depletion of T-cad would be analysed with different softwares classified for different signal transduction pathways known to participate in physiological processes such as angiogenesis, proliferation, migration and survival.
- 2) Validation of microarray data by RT-PCR or by western blotting for particular gene products.
- 3) After validation of modulated genes, T-cad-dependent activation/inhibition of the candidate signaling pathways can be monitored.

- 4) Ideally, the experiments above should be performed on EC under different conditions, such as at quiescence, during active proliferation, active migration or oxidative stress, *inter alia*.

Signaling pathways induced by T-cad upon LDL binding

Past studies (using both ligand-blotting cell binding assays) of my laboratory have shown that T-cad is an LDL binding protein. Signaling pathways induced by T-cad upon LDL binding are poorly understood. My laboratory has further shown that overexpression of T-cad or homophilic ligation with antibody against T-cad induces PI3K/Akt/GSK3 β pathway signaling. It would be interesting to investigate whether LDL binding to T-cad also induces this pathway and/or whether physiological processes such as angiogenesis are affected. Experimental approaches to answer the above questions would be as follows:

- 1) Investigate time- and dose- dependent effects of isolated LDL on signal pathways in ECs that either overexpress T-cad or are depleted of T-cad.
- 2) ECs that either overexpress T-cad or are depleted of T-cad can be examined for functional responses to LDL (e.g. growth, migration, cell survival/apoptosis, differentiation, *in vitro* angiogenesis).

Conditional knockdown of T-cad in endothelium in mice

Knock-out mouse models are considered a good tool to study physiological significance of a gene. The T-cad knock-out mouse does exist (Barbara Ranscht et al., unpublished), but it was not made available to us. Since we focus on the role of T-cad in the endothelium and have demonstrated proangiogenic properties for T-cad, it would be perhaps more interesting and useful for us create a conditional knock-out of T-cad in the endothelium. Such a knock-out model could be created on the background of knock-out mice models (e.g. apo E^{-/-}) used to investigate atherosclerotic disease; this would be useful for investigating intraplaque angiogenesis. A conditional knock-out of T-cad in the endothelium might also be useful for investigating the role of T-cad in tumor angiogenesis, in which case tumor cells could be subcutaneously implanted. The approach of conditional knock-out in endothelium has been made successfully for genes such as Tubedown-1 protein, VEGF, Focal adhesion kinase, β -catenin, *inter alia*.

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Publications

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7. **Manjunath B. Joshi**, Danila Ivanov, Maria Philippova, Emmanouil Kyriakakis, Paul Erne, Thérèse Resink. Characterization of minimal promoter region of T-cadherin in endothelial cells (Manuscript drafted).

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13. **Joshi M B**, Philippova M, Ivanov D, Erne P, Resink.T. Integrin linked kinase is a molecular adaptor for T-cadherin-induced Akt/GSK3 β -axis signaling in endothelial cells. *Targeting –the-Kinome Symposium, Basel, December 4-6, 2006*

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